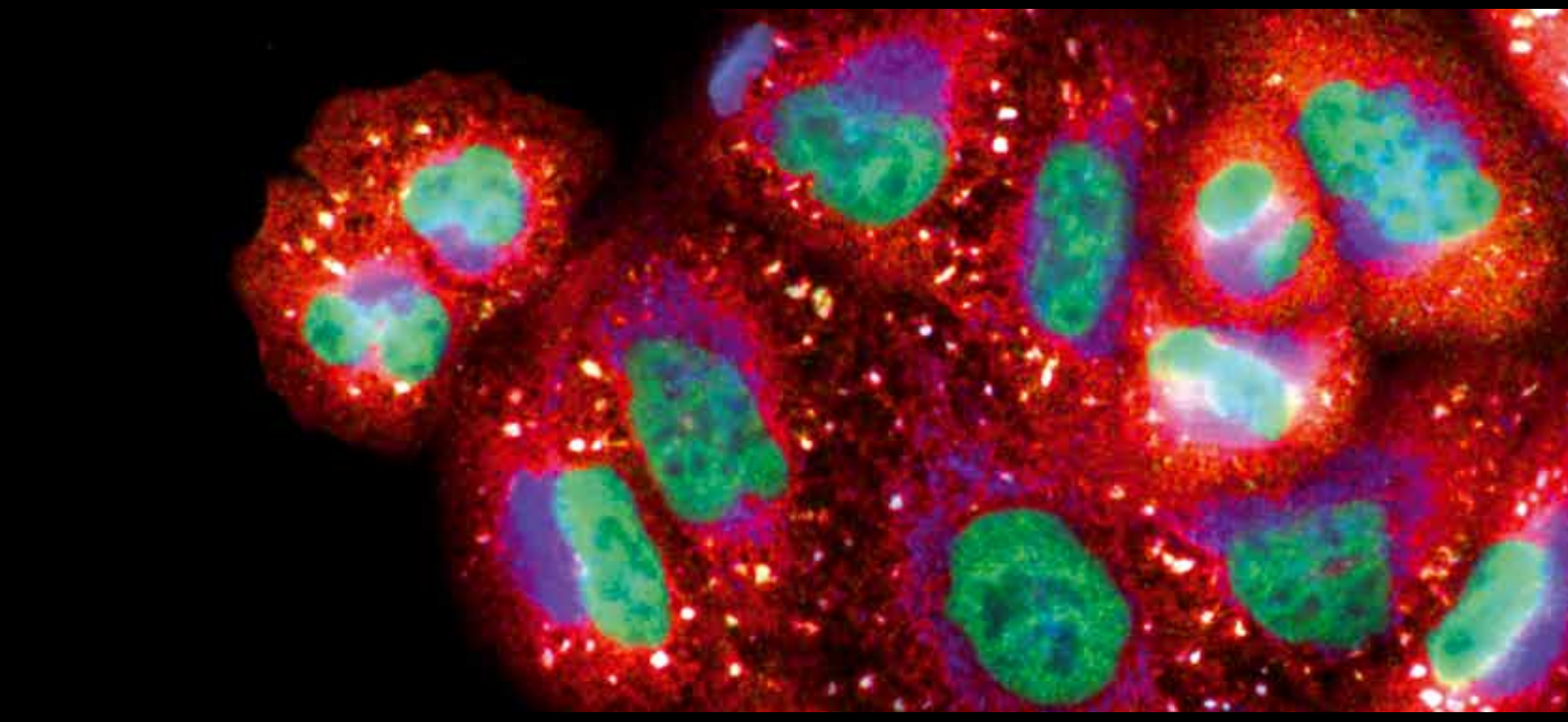
Oxidative Medicine and Cellular Longevity

The Janus of Oxidative Stress Signaling in Different Pathophysiological Conditions

Guest Editors: Sumitra Miriyala, Manikandan Panchatcharam, Aimee Landar, Meera Ramanujam, Saurabh Chatterjee, and Anantharaman Muthuswamy



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Editorial

The Janus of Oxidative Stress Signaling in Different Pathophysiological Conditions

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While reactive oxygen species (ROS) are important for normal cellular activities, deviant production of ROS, or diminished capacity to scavenge excessive ROS, leads to an imbalance in the redox environment of the cell. Because of the dual role of ROS in cells in the production and removal of cellular ROS, a greater understanding of oxidative stress, under both normal and disease-causing conditions, and the involvement of cell organelle (mitochondrial, endoplasmic reticulum) ROS in global regulation of gene expression can illuminate the contribution of mitochondria and other cell organelles in the development of disease and may lead to the advancement of new and novel therapeutic modalities that exploit oxidative stress in treating many diseases.

Understanding the role of ROS signaling and redox biology in pathophysiological conditions is reflected by the wised range of topics covered in this special issue.

T. Peng et al. underscore dual phase of mitochondrial respiratory chain defective cells harboring less mitochondrial stress due to low mitochondrial respiratory chain activity during mitochondrial ROS-mediated mitochondrial Ca^{2+} stress during severe oxidative insult.

N. V. Gorbunov et al. propose that the cell survival mechanisms activated in lipopolysaccharide-treated mesenchymal stromal cells in vitro could be a part of adaptive responses employed by stromal cells under septic conditions.

F. Tseng et al. provided a platform for an in vitro assay to characterize the effects of bone marrow mesenchymal stem cells on lipopolysaccharide-stimulated microglia. A powerful cell culture tool for investigating the molecular and cellular changes in microglia are bone marrow mesenchymal stem cells cocultures.

R. Dumitrascu et al. have shown that obstructive sleep apnea is an independent risk factor for cardiovascular disease such as arterial hypertension, heart failure, and stroke. The results clearly show that radical flux exerts direct cytotoxic effects, decreases NO bioavailability, enhances lipid peroxidation, increases sympathetic activity, and activates the proinflammatory transcription factor NF-κB leading to the well-known clinical manifestations of obstructive sleep apnea in the cardiovascular disease system.

C. Tronel et al. demonstrated the involvement of Fe^{2+} in brain ROS production and the deleterious effects of heme-oxygenase-1 expression in vivo neuroinflammatory model linked to a hyperproduction of ROS, itself promoted by Fe^{2+} liberation.

M. Godínez-Rubí et al. have reviewed on the role of nitric oxide donors as possible neuroprotective therapeutic agents for ischemia/reperfusion treatment.

S. Whelan and B. S. Zuckerbraun manuscript reviews on the mitochondria signaling to other components of

stress response via ROS, the unfolded protein response, mitochondrial autophagy, and biogenesis. The avenues of mitochondrial signaling were discussed in this review.

Y. Zhou et al. discuss how ROS regulates different steps in vascular development, including smooth muscle cell differentiation, angiogenesis, endothelial progenitor cells recruitment, and vascular cell migration, while Y. J. H. J. Taverne et al. review focuses on the function of ROS in cardiovascular pathology and on the effects of antioxidants on cardiovascular outcomes with emphasis on the so-called oxidative paradox.

A. J. Lepedda et al. suggest the presence of a more pronounced oxidative environment in unstable plaques. Identifying specific oxidative modifications and understanding their effects on protein function could provide further insight into the relevance of oxidative stress in atherosclerosis.

E. Menshchikova et al. data appear to indicate a possible role of hydrogen peroxide in intercellular communication during organization, maturation, and "dissociation" of granulomas in the dynamics of the process.

X. Zhan et al. study eventually addresses the mechanisms and biological functions of tyrosine nitration in pituitary tumorigenesis and will discover nitro protein biomarkers for pituitary adenomas and targets for drug design for pituitary adenoma therapy.

A. V. Ermakov et al. provided in their in vitro data suggesting that the oxidized DNA is a stress signal released in response to oxidative stress in the cultured cells, and, possibly, in the human body; in particular it might contribute to systemic abscopal effects of localized irradiation treatments.

M. Tsai et al. study shows that enhanced prostacyclin synthesis reduced glial activation and ameliorated motor dysfunction in hemiparkinsonian rats. Prostacyclin may have a neuroprotective role in modulating the inflammatory response in degenerating nigrastriatal pathway.

J. Espino et al. underlie the antioxidant and immune enhancing actions displayed by melatonin, thereby providing evidence for the potential application of this indoleamine as a "replacement therapy" to limit or reverse some of the effects of the changes that occur during immunosenescence.

M. Jerkic et al. indicate that eNOS-derived ROS contributes to endothelial dysfunction and likely predisposes to disease manifestations in several organs of hereditary hemorrhagic telangiectasia patients.

B. Song et al. review aims are to briefly describe the mechanisms, functional consequences, and detection methods of mitochondrial dysfunction. They describe the advantages and limitations of the Cys-targeted redox proteomics method with alternative approaches. Finally, they discuss various applications of this method in studying oxidatively modified mitochondrial proteins in extrahepatic tissues or different subcellular organelles and translational research.

K. E. Al-Otaibiet et al. results suggest a significant role of oxidative stress, proinflammatory myeloperoxidase, and vasoregulatory nitric oxide in the pathogenesis of contrast-induced nephropathy.

G. Aliev et al. provide a review discussing the link between cancer and Alzheimer disease via oxidative stress induced by nitric oxide-dependent mitochondrial DNA over proliferation and deletion.

S. Miriyala et al. provided a therapeutic approach showing that a novel tetra peptide derivative exhibits in vitro inhibition of neutrophil-derived reactive oxygen species and lysosomal enzymes release.

A. S. Kunt and M. H. Andac have shown a clinical study proving that persistent oxidative stress during reperfusion may lead to depressed myocardial function resulting in low cardiac output syndrome necessitating inotropic or intra-aortic balloon counterpulsation support. Besides total antioxidant capacity decreases during operation in a significant proportion of patients undergoing isolated coronary artery bypass which is more prominent and serious.

These manuscripts represent an exciting and insightful snapshot of current oxidative stress biology. State of the art, existing challenges and emerging future topics are highlighted in this special issue, which may inspire the reader and help advance the present redox biology.

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We would like to thank all the authors, reviewers and the guest editors for making this special issue possible.

Sumitra Miriyala
Manikandan Panchatcharam
Aimee Landar
Meera Ramanujam
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Research Article

A Novel Tetrapeptide Derivative Exhibits In Vitro Inhibition of Neutrophil-Derived Reactive Oxygen Species and Lysosomal Enzymes Release

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Neutrophil infiltration plays a major role in the pathogenesis of myocardial injury. Oxidative injury is suggested to be a central mechanism of the cellular damage after acute myocardial infarction. This study is pertained to the prognostic role of a tetrapeptide derivative PEP1261 (BOC-Lys(BOC)-Arg-Asp-Ser(tBu)-OtBU), a peptide sequence (39–42) of lactoferrin, studied in the modulation of neutrophil functions in vitro by measuring the reactive oxygen species (ROS) generation, lysosomal enzymes release, and enhanced expression of C proteins. The groundwork experimentation was concerned with the isolation of neutrophils from the normal and acute myocardial infarct rats to find out the efficacy of PEP1261 in the presence of a powerful neutrophil stimulant, phorbol 12-myristate 13 acetate (PMA). Stimulation of neutrophils with PMA resulted in an oxidative burst of superoxide anion and enhanced release of lysosomal enzymes and expression of complement proteins. The present study further demonstrated that the free radicals increase the complement factors in the neutrophils confirming the role of ROS. PEP1261 treatment significantly reduced the levels of superoxide anion and inhibited the release of lysosomal enzymes in the stimulated control and infarct rat neutrophils. This study demonstrated that PEP1261 significantly inhibited the effect on the ROS generation as well as the mRNA synthesis and expression of the complement factors in neutrophils isolated from infarct heart.

1. Introduction

Reactive oxygen species (ROSs) have been shown to exert a direct inhibitory effect on myocardial function in vivo and have a critical role in the pathogenesis of myocardial stunning [1, 2]. Oxidative stress and formation of ROS could set off a cascade of biochemical and molecular squeal such as the xanthine dehydrogenase/xanthine oxidase conversion, leading to over production of ROS [3, 4]. Oxidative ischemic injury is suggested to be a central mechanism of the cellular damage affecting all organs and tissues after ischemia; however, the mechanisms, which trigger and modulate this damage, have not been fully characterized.

Polymorphonuclear leukocytes (PMNLs) are short-lived, terminally differentiated cells that act against all infections and they are one of the most important cellular components involved in host defense. Circulating PMNLs participate in host defense by margination and extravasations at the site of inflammation [5]. Although neutrophils are essential to host defense, they have also been implicated in the pathology of ischemia [6, 7] and in many chronic inflammatory conditions [8, 9]. Neutrophil levels are activated in myocardial infarction [10], and subsequently, activated neutrophils produce reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and possibly hydroxy radical ($OH^{\bullet}$) [11, 12].

Therefore, the accumulation of oxygen free radicals and activation of neutrophils are strongly implicated as important pathophysiological mechanisms mediating myocardial ischemia [2, 13]. Thus, the site of inflammation is characterized by a high concentration of stimulated neutrophils, which secrete ROS and proteolytic enzymes [14–16].

Complement activation constitutes facet of inflammation, which occurs during ischemia [17–19]. A variety of entities activate complement, including antibodies, membranes of microorganisms, and free radicals [20]. Although it is known that free radicals activate the complement system, the effect of free radicals on complement transcription remains unexplained.

In the present work, we identified that PEP1261 [21] could inhibit the ROS and lysosomal enzymes release from activated neutrophils isolated from acute myocardial infarct rats [1].

2. Materials and Methods

This study conforms to the guiding principles of Institutional Animal Ethics Committee (IAEC), Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication no. 85–23, revised 1996).

2.1. Chemicals. Phorbol 12-myristate 13 acetate (PMA), cytochrome C, superoxide dismutase, phenol red, dextran, O-dianisidine hydrochloride, ficoll-histopaque (Sigma 1077), glycerol, hexadecyltrimethylammonium bromide, Hank's balanced salt solution (HBSS), horse radish peroxidase, and Triton X100 were purchased from Sigma (St. Louis, MO, USA). Folin-Ciocalteau reagent, hemoglobin, hydrogen peroxide, and p-nitrophenol phosphate were obtained from Sisco Research Laboratories (Bombay, India). All other chemicals used were of analytical grade.

2.2. Experimental Animals. Female rats (Wistar) weighing 180–200 g were inbred in a pathogen free facility, and they were maintained in environmentally controlled rooms with 12 h light/dark cycle. The animals received commercial rat diet and water ad libitum.

2.3. Synthesis of PEP1261. PEP 1261 used in this study was synthesized by solution phase methodology as represented schematically in Figure 1 and purified by column chromatography. The homogeneity of the peptide was established by thin layer chromatography. Proton NMR spectra and IR spectra were recorded using Brucker 300 MHz FT-NMR spectrometer and Nicolet DX-20 FT-IR spectrometer, respectively. Amino acid analysis of the peptide derivative was performed by precolumn derivatization with phenylisothiocyanate (PITC) using HPLC. Reverse phase HPLC separation of PTC amino acids was performed with Pharmacia LKB LCC 2252/LKB VWM 2141 unit with fixed or variable wavelength detector at 254 nm. DuPont Zorbax PTH column, 25 cm in length, 0.46 cm internal diameter, and 5 μm particle size, was used. The FAB mass spectra were recorded on a JEOL SX 102/DA-6000 Mass Spectrometer Data system [21].

2.4. Development of the Infarct Model. Myocardial infarct model was induced in rats as described earlier [1] with modifications to minimize the early mortality rate. Briefly, the animals were given a mucus secretor blocker glycopyrrolate [2 μg/kg.b.wt., i.m) and were anaesthetized with ketamine [50 mg/kg.b.wt., i.p] and diazepam (2.5 mg/kg.b.wt., i.p]. After anesthesia, endotracheal intubation was performed and a tube connected to a positive pressure respirator was introduced. After establishing positive pressure respiration, left intercostal thoracotomy was performed using aseptic technique, and the third and fourth intercostal ribs were separated with a small retractor to expose the heart. The pericardium was opened carefully avoiding any injury to the heart. The left coronary artery (LCA) and its branches could be seen easily without any amplification or use of a surgical microscope. The pattern of the LCA was carefully examined in order to ligate the left anterior descending coronary artery. A 6–0 atraumatic proline silk suture was passed through the epicardial layer around the midway of the left anterior descending coronary artery. Following coronary occlusion, the thorax was closed in layers, the endotracheal tube was removed, and the animals were brought back to normal respiration.

2.5. Isolation of Neutrophils from Rats. Blood was drawn from sham-operated and infarct rats, and neutrophils were separated according to Newman et al. [22]. The neutrophils were suspended in HBSS, and the cell concentration was determined using a haemocytometer.

2.6. Stimulation Studies. To ascertain the optimal concentration of PEP1261 on neutrophils isolated from rats, myeloperoxidase (MPO) was used as a sensitive marker and the results showed an inhibitory concentration (IC_{50}) of 94.10 ± 8.38 μM (data not shown). Hence, further studies were carried out with an optimal concentration of 120 μM for PEP1261 and KRDS. Neutrophils (1×10^6 cells/well) were left for adherence for 1 h, and they were stimulated by the addition of PMA (100 ng/mL) [23] for 1 h at 37°C. In the case of sham-operated and infarct rat, neutrophils were isolated and after 1 h adherence and PEP1261 was added. The culture was terminated after 1 h of PEP1261 treatment.

MPO was assayed in the cell lysate after extracting the enzyme in phosphate buffer containing hexadecyl trimethylammonium bromide [24].

For H_2O_2 assay [25], the medium was removed and to the adherent neutrophils, 100 μL (1 μg/mL) of PMA was added followed by the addition of 1000 μL of HBSS containing horse radish peroxidase (19 units/mL) and phenol red (0.02%). After 1 h of incubation, 100 μL of 1 M NaOH was added and the color developed was read at 605 nm.

For the assay of $O_2^{\bullet -}$ [26], the medium was removed and the adherent neutrophils were incubated for 1 h in HBSS containing 80 μM cytochrome C and 10 μM PMA. The color developed in the supernatant was read at 580 nm. The amount

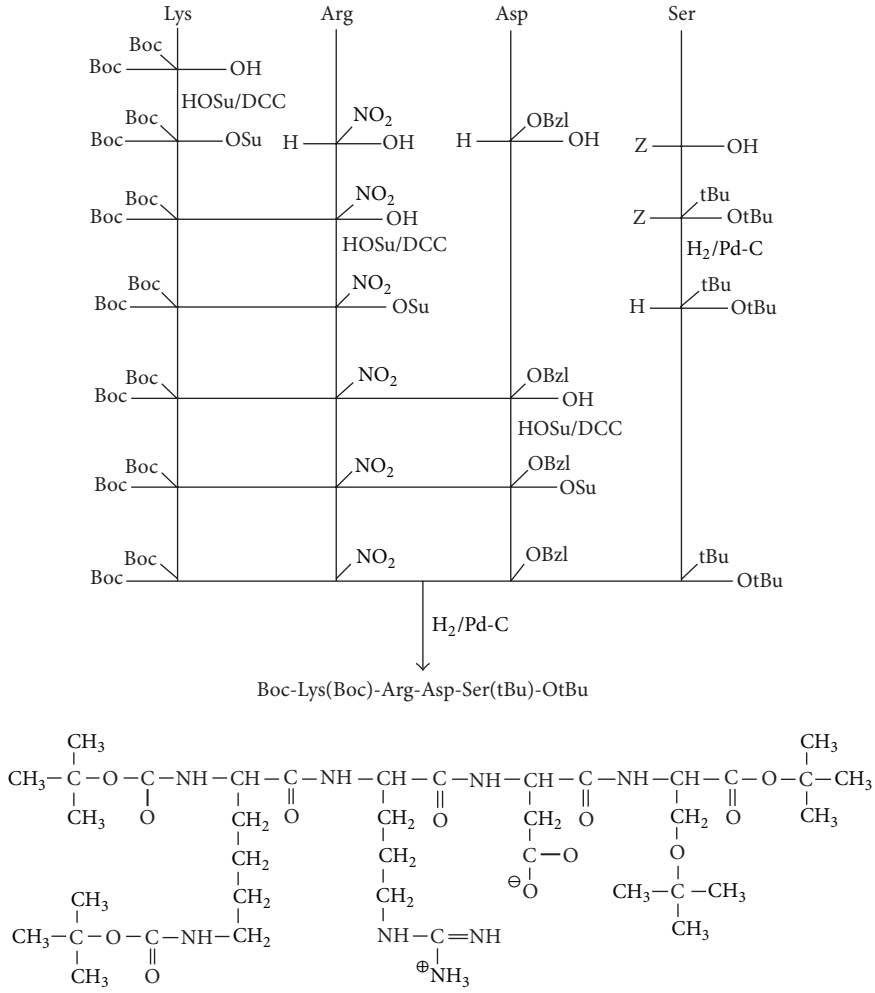

Figure 1: Scheme of synthesis of PEP1261 with its structure.

of $O_2^{\bullet-}$ released was measured by the amount of cytochrome C using a molar extinction coefficient of $21 \times 10^3\ M^{-1}\ cm^{-1}$.

For the lysosomal enzyme assays, after removing the cell free medium, the cells were lysed by adding 0.5 mL of ice cold 0.1% Triton X100 in 0.25 M sucrose, subjected to repeated freezing and thawing and assayed for acid phosphatase [27] and cathepsin D [28].

2.7. Complement Expression Study. Total RNA from neutrophils isolated from sham operated and infarct tissue was extracted using Trizol reagent (kit supplied by Gibco-BRL (Life technologies) (US Patent no. 5,346,94)) a monophasic solution of phenol and guanidine isothiocyanate developed by Chomczynski and Sacchi [29] (Table 1).

2.8. Statistical Studies. Univariate analysis was carried out for all the parameters, and the results were analyzed by nonparametric statistics Mann-Whitney "*U*" test.

3. Results

Studies on PMA stimulated rat neutrophils showed the beneficial effect of PEP1261 on the ROS generation and lysosomal enzymes release, and, hence, experiments were performed on neutrophils isolated from myocardial ischemic rats. While the levels of H_2O_2 and $O_2^{\bullet-}$ were significantly increased in both PMA stimulated and in the neutrophils isolated from myocardial infarct rats, PEP1261 showed a protective effect towards free radical generation, irrespective of the nature of the stimulant ($P < 0.01$) (Figure 2).

In comparison with PMA treated cells, PEP1261 cells demonstrated a significant decrease in PMA-induced MPO activation ($P < 0.01$). Consistent with these observation, neutrophils derived from myocardial infarct rat were significantly inhibited upon PEP1261 treatment ($P < 0.01$).

Lysosomal enzymes release in response to PMA was also enhanced in neutrophils and was corrected by treatment with PEP1261 as shown in Figure 3. PMA stimulation resulted in a moderate increase ($P < 0.05$) in the levels of cathepsin D and acid phosphatase, and the levels of these enzymes were noticed to decrease upon treatment with PEP1261 ($P < 0.05$).

To ensure that the results obtained are from the neutrophils derived from the acute myocardial infarcted rats, RT-PCR amplification from total RNA extracts was used to establish the presence and relative values of the mRNAs for

Table 1

Gene	Sequence of primer (Sense)	Sequence of primer (Antisense)	Product length (base pair)
C5	CAGCATAATTCAGGGTGAACG	CAGCTTGTCATTTGAGCCAC	315
C6	TGCAGTGACAAAACGGAACAACCTC	TGCAGTCTTCCTCTTGTCGCTTCTC	338
C7	GGAACAGAGTCAATACCAAAAG	ACTGCGTGAAGAAGATGATGAAGAT	248
C8	GACTGCGACCCTCTTGACTCTGCTC	TTTCGGAAGGTACTGACAGCCATGG	258
C9	GAATGAGCCCCTGGAGTGAATGGTC	CATTTCCGCAGTCATCCTCAGCATC	316

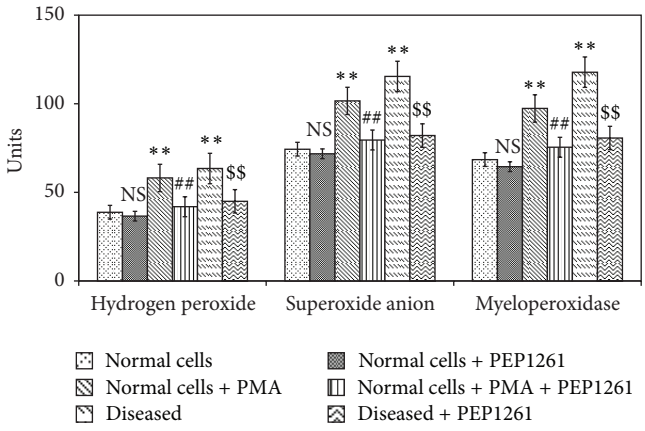

Figure 2: Effect of PEP1261 on myocardial infarct rat neutrophils ROS generation. All values are mean ± SD ($n = 20$). H_2O_2 level was expressed as μmoles of H_2O_2 liberated/0.5×10^6 cells. $O_2^{\bullet -}$ level was expressed as nmoles of O_2^- liberated/min/mg protein. MPO level was expressed as μmoles of H_2O_2 utilized/min/mg protein. NS = nonsignificant as compared to control; $^{**}P < 0.01$ as compared to control; $^{\#\#}P < 0.01$ as compared to PMA stimulated cells; $^{\$\$}P < 0.01$ as compared to rat myocardial infarct cells.

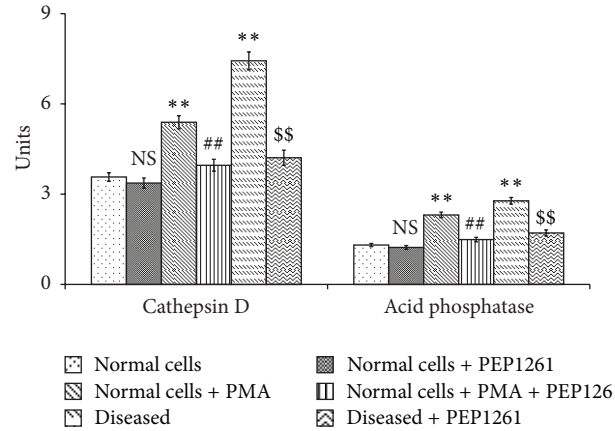

Figure 3: Effect of PEP1261 on myocardial infarct rat neutrophils lysosomal release. All values are mean ± SD ($n = 20$). Acid phosphatase activity was expressed as μmoles of p-nitrophenol liberated/h/mg protein. Cathepsin D activity was expressed as μmoles of tyrosine liberated/h/mg protein. NS = nonsignificant as compared to control; $^{*}P < 0.05$ as compared to control; $^{\#}P < 0.05$ as compared to PMA stimulated cells; $^{\$}P < 0.05$ as compared to rat myocardial infarct cells.

the functional terminal C proteins, namely, C5, C6, C7, C8, C9, and GAPDH (Figure 4).

4. Discussion

Neutrophils represent the first line of host defense against all types of infection, and they are also involved in the pathology of various inflammatory conditions [30]. The ability to survive an infection challenge might depend on the appropriate modulation of these neutrophil functions [31].

Oxidative stress, defined as an increase in the production of ROS, namely, $O_2^{\bullet -}$, H_2O_2, and $OH^{\bullet}$ has been related to reperfusion injury in heart and other organs [32, 33]. Neutrophils recruitment depends on the presence of inflammatory mediator. These cells, therefore, may exacerbate tissue injury through the release of free radicals and proteolytic enzymes. The oxygen metabolites are produced by a membrane bound enzyme complex, the NADPH oxidase. Thus, in ischemic conditions, there is always the adhesion and activation of neutrophils with the generation of free radicals. Hence, the development of tissue injury depends upon the balance between the generation of ROS and tissue antioxidant status [34]. Any disturbance in this equilibrium in favor of free radicals causes an increase in oxidative stress and initiates subcellular changes leading to cardiomyopathy and heart failure [10].

Several drugs were found to curtail these deleterious effects, and of recent interest is the role of sequence specific peptides as therapeutic agents in many disorders. The sequence H-Lys-Arg-Asp-Ser-OH (KRDS) is an analog of RGDS and corresponds to residues 39–42 of human lactoferrin. It is well known that human lactoferrin exhibits anti-inflammatory and antimicrobial properties, and it has a role in regulating various components of the immune system, growth factor activity, and in inhibiting platelet aggregation [35]. This sequence, situated near the N-terminal region of the protein and known to inhibit platelet aggregation in vitro conditions, was suitably modified with hydrophobic groups (PEP1261) to enhance the permeability across the membrane and also to increase the stability.

Preliminary studies were carried out using PMA stimulated human neutrophils, and PEP1261 was found to significantly inhibit ROS species generation and lysosomal enzymes release at a concentration of 120 μM. The concentration of 120 μM did not seem to be very high since earlier experiments showed that the peptide inhibited platelet aggregation only after critical micellar concentration (CMC) (60 μM) was

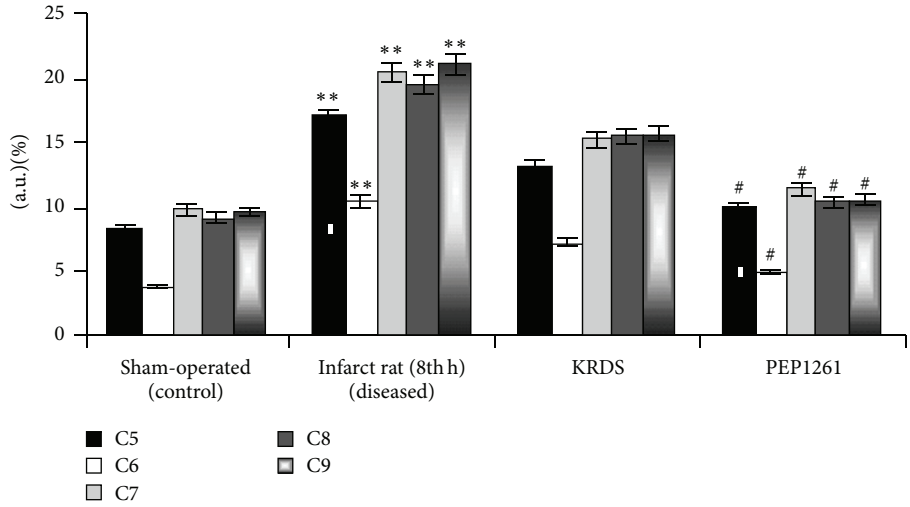

Figure 4: Densitometry analysis of the functional complement factor proteins. All values are mean ± SD ($n = 6$). $^{**}P < 0.01$ as compared to control and $^{\#}P < 0.05$ as compared to diseased (8th hour infarct myocardium) using post hoc tukey's test.

achieved and a concentration above 153 μM was found to effectively inhibit platelet aggregation [21].

MPO activity, a marker of leukocyte accumulation, was markedly elevated in heart injury. It was shown recently that the neutrophils characteristically invaded the myocardial tissue during ischemia and they were observed to be the major source of free radicals [2]. The result of this study showing PEP1261 significantly reducing $O_2^{\bullet-}$, H_2O_2, and MPO levels both in the case of stimulated and myocardial rat infarct neutrophils was also in agreement with that of Manikandan et al. [36].

The extensive release of $O_2^{\bullet-}$ derivatives was probably involved in the pathogenesis of tissue damage following ischemia in stroke and myocardial infarction and in respiratory distress syndrome [35]. Ischemia induced an acute inflammatory response in myocardial tissue with an early phase of neutrophil accumulation, which was accelerated by reperfusion. The rise in H_2O_2 level during myocardial ischemia indicated that intensification of oxygen free radical production occurred. This might be the result of hypoxanthine conversion by xanthine oxidase, catecholamine autooxidation, polymorphonuclear neutrophil activation, and/or derangement within mitochondrial electron transfer [37]. In experimental models, interventions that depleted neutrophils or inhibited their function caused a significant reduction in myocardial infarct size [36].

Lysosomal enzymes could completely degrade the components of connective tissue such as collagen, protein-mucopolysaccharide complexes, glycoproteins, and elastin [38]. These observations concurred with our findings that there was an increased activity of lysosomal enzymes such as acid phosphatase and cathepsin D in myocardial ischemic neutrophils which were well brought under the control levels upon PEP1261 treatment.

In summary, neutrophil-mediated ROS, as well as lysosomal enzyme leak in particular, increases after injury. Treatment with PEP1261 attenuates ROS generation and may attenuate cardiac inflammation. We propose that these observations reflect a role for complement mediated activation of neutrophil in acute myocardial infarction. Our observation may suggest novel strategies to attenuate inflammation during cardiac injury and suggest that a better understanding of inflammatory cells and environmental stimuli that alter ROS levels is warranted.

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Review Article

Mitochondrial Signaling: Forwards, Backwards, and In Between

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Mitochondria are semiautonomous organelles that are a defining characteristic of almost all eukaryotic cells. They are vital for energy production, but increasing evidence shows that they play important roles in a wide range of cellular signaling and homeostasis. Our understanding of nuclear control of mitochondrial function has expanded over the past half century with the discovery of multiple transcription factors and cofactors governing mitochondrial biogenesis. More recently, nuclear changes in response to mitochondrial messaging have led to characterization of retrograde mitochondrial signaling, in which mitochondria have the ability to alter nuclear gene expression. Mitochondria are also integral to other components of stress response or quality control including ROS signaling, unfolded protein response, mitochondrial autophagy, and biogenesis. These avenues of mitochondrial signaling are discussed in this review.

1. Introduction

Since the first observations of mitochondria in the mid to late 1800s, our understanding of their structure and function has evolved significantly. The first half of the 20th century saw the characterization of the mitochondrion as the major source of energy leading to its epithet, "the powerhouse of the cell." This paved the way for localization of the respiratory chain and TCA cycle components, as well as the confirmation of the oxidative phosphorylation hypothesis in the following years. Mitochondria were found to have DNA, RNA, and protein synthesis capabilities, and seminal investigations into mitochondrial function in yeast led to an improved understanding of mammalian mitochondrial biogenesis [1].

Nuclear factors governing mitochondrial biogenesis and function have been extensively studied over the past several decades leading to the discovery of an array of nuclear respiratory factors, hormone receptors, and important transcription factor coactivators that collectively influence mitochondrial biogenesis, oxidative phosphorylation, fatty acid β-oxidation, and reactive oxygen species production among a myriad of other effects [2].

In 1987, Parikh et al. investigated changes in nuclear gene expression in response to mutations in mitochondrial DNA (mtDNA) in yeast [3]. This and subsequent studies utilizing genome wide transcriptomic analyses identified target genes likely involved in a signal transduction pathway from mitochondria to the nucleus termed the retrograde pathway, which includes the retrograde response genes: RTG1, RTG2, and RTG3. Though this retrograde signaling pathway is centered on glutamate homeostasis, it has since been implicated in a number of other processes such as mitochondrial DNA maintenance, autophagy, and cellular longevity.

Concurrently, a progressive appreciation of mitochondrial function (and dysfunction) in metazoans has implicated the mitochondrion in pivotal roles in bioenergetic homeostasis, metabolic regulation, innate immunity, and aging to name a few. Rho^0 cell models (cells that are devoid of mitochondrial DNA) have shed light on the role of mtDNA and its products in cellular feedback mechanisms, and several mutations in human mtDNA have been identified that are responsible for a number of neuromuscular disorders, mostly involving defective mitochondrial t-RNA; however, mammalian homologues of the retrograde response genes are yet to be identified. As opposed to yeast, though, the heterogeneous tissues of metazoans also have a heterogeneous population of mitochondria, and energy metabolism is not

uniform throughout [4, 5]. It may be, then, that a cohort of signals from mitochondria as well as a wide array of cellular responses to mitochondrial dysfunction represent a complex evolution of the collective mitochondrial retrograde signal.

This review discusses antegrade signaling from nucleus to mitochondria as well as the retrograde response in yeast. Retrograde signaling in mammalian cells along with similar stress signaling including the unfolded protein response and intermitochondrial signaling is reviewed as well.

2. The Antegrade Pathway: Nuclear Contribution to Mitochondrial Biogenesis

Mitochondria are double-membraned organelles present in almost all eukaryotic cells. Endosymbiotic theory postulates that they, along with other organelles such as chloroplasts in plants, originated from free-living bacteria that were taken into cells and developed a symbiotic relationship. The evolution of this complex relationship hypothesizes that eukaryotic cells with glycolytic energy production via the nuclear genome and cytosolic machinery merged with the oxidative mitochondrion. Most of the mitochondrial genome was then transferred to the nuclear DNA. In this new complex relationship, the nuclear DNA not only encodes the genes for determining cellular and organismal structure but also the genes for glycolysis and most of the genes for oxidative metabolism [6]. The mitochondrial genome, which is maternally inherited, retains the core genes for generating, maintaining, and using the mitochondrial inner membrane potential. The epigenome then coordinates nuclear DNA gene expression based upon the environmental calories available. Therefore, the mitochondria are semiautonomous in that they depend on nuclear contribution for much of their functionality, and in fact, many of the catalytic regions of the complexes are controlled by the nucleus [7]. Furthermore, nuclear genes are responsible for the transcription factors and the transcriptional machinery required for the expression of the mitochondrial genome. Each mitochondria carries varying copy numbers—usually 2–10 copies per organelle—of their own double-stranded DNA plasmids, and due to a high mutation rate, mitochondrial DNA can vary within a single cell, and populations in organs vary based upon regional energy environments. The human mitochondrial genome consists of 37 genes: 13 for protein subunits, 22 for mitochondrial t-RNA, and two for ribosomal RNA [2].

Advances in the understanding of the molecular basis of mtDNA transcription were made with the characterization of the transcription factor, Tfam (formerly known as mtTFA) [8, 9]. It was identified as a high-mobility group (HMG) box protein involved in specific binding to enhancers upstream from bidirectional promoters in the D-loop of the mitochondrial chromosome. Tfam has also been shown to bind randomly at nonspecific sites of mtDNA prompting the suggestion that it functions to stabilize mtDNA as well. Mitochondrial polymerase has been purified in yeast and consists of a single subunit RNA polymerase (RPO41p) coupled to a specificity factor [10]. While human polymerase has not been purified, cDNA database screening has identified proteins with homology to lower eukaryotes as well as similarity to the bacteriophage polymerases T7 and T3 [11, 12]. This has been shown to act in concert with mtTFB, a protein with significant homology to prokaryotic sigma factors involved in promoter recognition. While this is also analogous to the yeast specificity factor Mtf1p, it does not confer specificity in higher eukaryotes. Both RPO41p as well as other eukaryotic polymerases initiate transcription in a nonspecific manner and require specificity factors in order to associate with the appropriate promoter region. As mentioned, Mtf1p serves this function in yeast, whereas Tfam assumes the responsibility in humans [11].

A new set of nuclear-encoded transcription factors were discovered when Evans and Scarpulla identified recognition sites on the cytochrome c promoter with no homology to those found in yeast [13]. A transcription factor termed as nuclear respiratory factor 1 (NRF-1) was then found to have specific biding sites in the promoters of cytochrome c as well as other genes involved in the electron transport chain [14]. The scope of influence of NRF-1 along with a second nuclear respiratory factor, NRF-2, has since been expanded tremendously to include vital components of oxidative phosphorylation, the mitochondrial transcription factors Tfam and mtTFB, rate-limiting steps in heme synthesis, ion channel synthesis, and mitochondrial protein import, assembly, and shuttling [15, 16]. Other nuclear transcription factors are shown to have recognition sites in the promoters; genes encoding respiratory proteins are the estrogen-related receptor ERRα, which also regulates fatty acid β-oxidation, and the general transcription factor Sp1, which appears to be unique in its ability to both positively and negatively regulate respiratory component transcription [17]. Regulation of fatty acid β-oxidation represents another important layer of nuclear control. While not technically part of the respiratory apparatus, this pathway is used by mitochondria to generate acetyl CoA as a carbon source for the TCA cycle and ultimately provide substrate for the electron transport chain. In addition to regulation by ERRα, peroxisome proliferator-activated receptors (PPARα and PPARδ) perform this function, though in contrast to ERRα, they do not seem to influence transcription of respiratory proteins [2] (Figure 1).

Finally, a family of coactivators has been shown to interact broadly with these distinct nuclear respiratory transcription factors, potentially providing a means of coordination or fine-tuning. The most prominent of these is the peroxisome proliferator-activated receptor gamma, coactivator 1-alpha (PGC1α) [18]. First recognized for its interaction with PPARγ in adipocyte differentiation, PGC1α responds to a complex set of physiologic signals to activate NRF1, NRF2, Tfam, mtTFB, ERRα, PPARα, and all the attendant sequelae culminating in mitochondrial biogenesis. This coactivator, in particular, seems to represent an important link between the products of mitochondrial function or dysfunction and the subsequent alterations in nuclear gene expression [19].

3. The Retrograde Pathway

3.1. Retrograde Signaling in Yeast. Nuclear regulation of the mitochondrial network is extensive and complex. However,

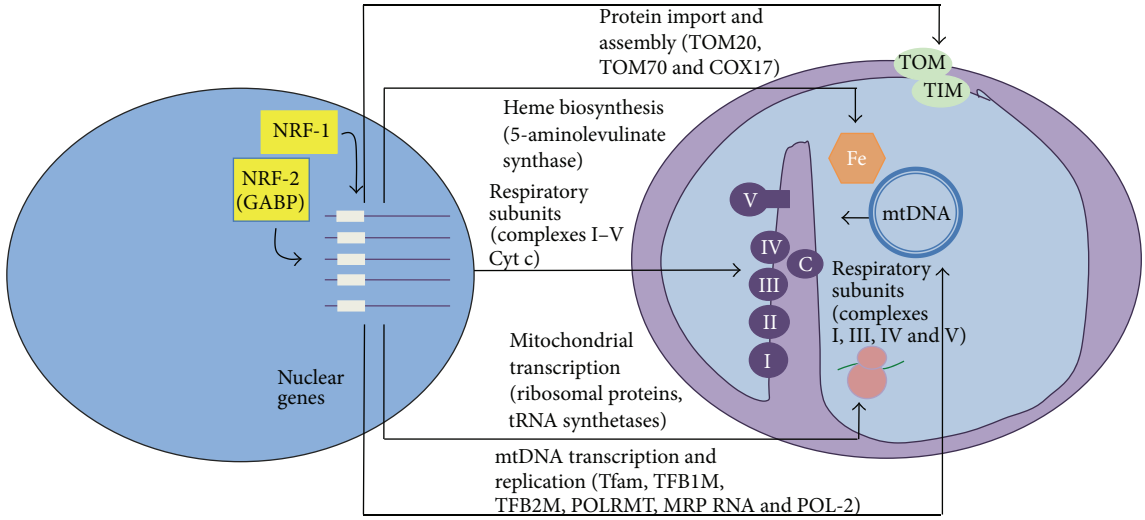

Figure 1: Diagrammatic summary of the nuclear control of mitochondrial functions by NRF-1 and NRF-2 (GABP). NRFs contribute both directly and indirectly to the expression of many genes required for the maintenance and function of the mitochondrial respiratory apparatus. NRFs act on genes encoding cytochrome c, the majority of nuclear subunits of respiratory complexes I–V, and the rate-limiting heme biosynthetic enzyme 5-aminolevulinate synthase. In addition, NRFs promote the expression of key components of the mitochondrial transcription and translation machinery that are necessary for the production of respiratory subunits encoded by mtDNA. These include Tfam, TFB1M, and TFB2M as well as a number of mitochondrial ribosomal proteins and tRNA synthetases. Recent findings suggest that NRFs are also involved in the expression of key components of the protein import and assembly machinery. Adapted with permission from [2].

the paradigm of mitochondrial signaling leading to changes in nuclear gene expression is relatively novel and is considered mitochondrial retrograde signaling. Many pathologic conditions, as well as some physiologic ones, are associated with mitochondrial dysfunction, which has become increasingly correlated with subsequent changes in nuclear gene expression. The first studies done in yeast deficient in mtDNA identified the accumulation of several RNA transcripts in the nucleus [3]. Later, transcriptomic analyses in rho^0 cells identified a variety of nuclear-encoded transcripts that were increased [20, 21].

Liao et al. recognized that the CIT2 gene, encoding the peroxisomal citrate synthase (CIT2), was consistently and dramatically upregulated in rho^0 cells and became regarded as the prototypical target of the retrograde pathway in yeast [22]. CIT2 plays an important role in citrate synthesis as part of the glyoxylate pathway in peroxisomes as opposed to the mitochondrial citrate synthase enzyme, Cit1. Glutamate is the only nitrogen source for biogenesis in yeast and is derived primarily from the α-ketoglutarate generated in the TCA cycle. The TCA cycle is disrupted in respiratory-deficient cells, and peroxisomal anaplerotic contributions become critical to maintenance of an adequate pool of α-ketoglutarate. Identification of regulators of the CIT2 gene led to discovery of several retrograde response (RTG) genes. Four positive and at least four negative regulators of CIT2 have been identified [23, 24]. Subsequent microarray analyses on rho^0 cells have suggested many areas along the TCA and glyoxylate cycles that are controlled in a similar fashion [25]. Additionally, Freije et al. used RNAi knockdown of glycolytic enzymes in *Drosophila* followed by microarray analyses to show a shift from oxidative phosphorylation to aerobic glycolysis [26].

Rtg2p is a cytoplasmic phosphohydrolase central to the induction of the retrograde response. Activation of Rtg2 leads to disinhibition of the downstream transcription factors, Rtg1p and Rtg3p. This is achieved by dephosphorylation of the inhibitory factor Mks1p. Partially, dephosphorylated Mks1p is also targeted for degradation by the E3 ubiquitin ligase, Grr1p. While this would seem to designate Grr1p as a positive regulator of the retrograde response, it has been suggested that its primary role involves degradation of a free pool of Mks1p making the Rtg2p-mediated regulation more efficient. Conversely, two 14-3-3 proteins, Bmh1p and Bmh2p, have been shown to interact with Mks1p preventing Grr1p-dependent degradation and, therefore, inhibiting activation of the Rtg1/3p heterodimer [27, 28].

The TOR kinase complexes have also been shown to inhibit the retrograde response, perhaps consistent with their other functions in nutrient sensing [29]. Integral to this inhibitory effect is the Lst8p protein that is a component of the TOR1/2p complex. Glutamate has been proposed to exert a negative feedback effect on the pathway either directly or through the membrane-bound SPS amino acid-sensing complex, though absence of glutamate alone is not sufficient to inhibit retrograde target genes. An additional level of control is implicated in the observation that the Rtg2p protein contains an ATP binding domain that is required for its function. This suggests that Rtg2p may act as an ATP sensor, activating retrograde signaling in response to low ATP levels [30] (Figure 2).

Given the role of the mitochondria in producing ATP, it is not surprising that the relative metabolic state of the

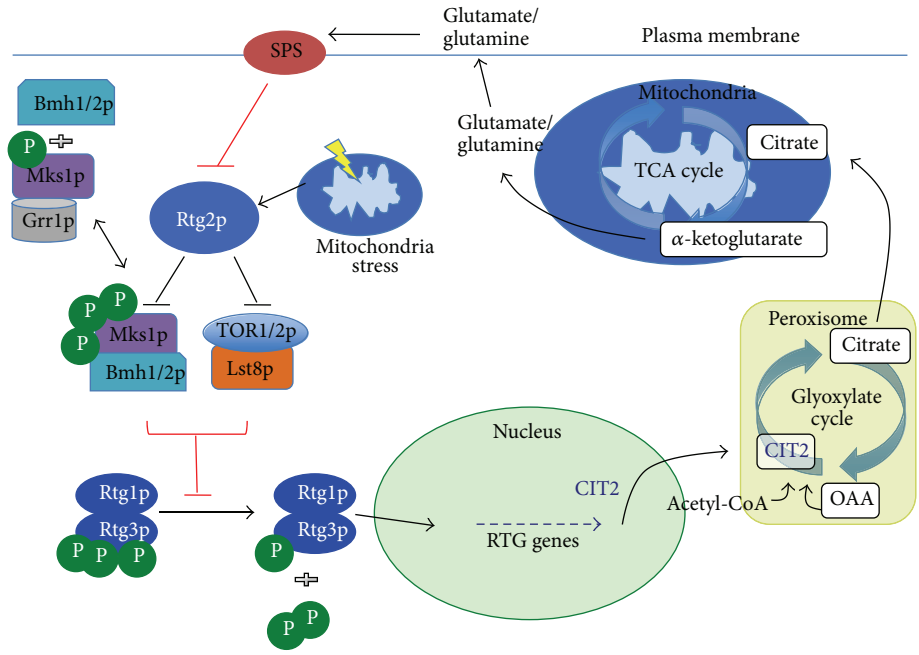

Figure 2: Positive and negative regulators of the retrograde pathway. The retrograde pathway is constitutively inhibited by Mks1p as well as TOR1/2p/Lst8p which hyperphosphorylates (P) the Rtg1/3p heterodimer. Bmh1/2p stabilizes the phosphorylated Mks1p contributing to its activity and preventing its degradation. Mitochondrial stress activates Rtg2p which dephosphorylates Mks1p. Mks1p then dissociates from Bmh1/2p and is degraded by Grr1p. Rtg2p also inhibits the inhibitory factor Lst8p. Additionally, Lst8p is part of the TOR1/2p complex and is also controlled by canonical regulators of TOR. The disinhibition of the Rtg1/3p heterodimer allows dephosphorylation and translocation to the nucleus where it activates the RTG genes. The prototypical RTG gene CIT2 encodes peroxisomal citrate synthase (CIT2) which converts Acetyl-CoA and oxaloacetic acid (OAA) to citrate. This contributes nitrogen to the TCA cycle in order to maintain an adequate supply of α-ketoglutarate. Ultimately, this leads to production of glutamate which is the ultimate source of biosynthetic reactions in yeast. The plasma membrane amino acid sensor SPS inhibits Rtg2p in a negative feedback mechanism in the presence of excess glutamate.

cell with regard to ATP concentration would have an impact on mitochondrial activity. Amiott and Jaehning showed that each mitochondrial promoter has a unique sensitivity to mitochondrial ATP concentration and that levels of ATP have a direct relationship on the activity of mitochondrial RNA polymerase (mtRNAP) [10]. Furthermore, they suggested a role of AMP/ATP concentration in coordinating mitochondrial and nuclear gene expression via Snf1 kinase, the ortholog of mammalian AMP kinase. Overall, energy status as measured by AMP can influence retrograde signaling as well. After disruption of the electron transport chain, increases in AMP can modulate cell cycle progression [31].

3.2. Calcium Homeostasis and Retrograde Signaling. Calcium signaling and homeostasis is critical to normal cell function. It is responsible for initiation of life after fertilization of the oocyte, differentiation of cells during development, intracellular and intercellular signaling, and ultimately for initiation of cell death [32]. Mitochondria have long been recognized to have important roles in calcium signaling and homeostasis and are often separated into two groups with relation to their primary function in this respect: those in excitable cells and those in nonexcitable cells [33]. Transient oscillations in calcium concentration are organized by location and amplitude and are important in transmitting intra- and intercellular signals. Mitochondrial calcium concentration is generally low due to a set point generated by the Na+/Ca++ antiporter and the Ca++ uniporter on the inner mitochondrial membrane, while most intracellular calcium is sequestered in the endoplasmic or sarcoplasmic reticulum [34]. In excitable cells, such as brain dendritic cells, cardiac myocytes, smooth muscle cells, and others, mitochondria can influence cytosolic calcium in a variety of different ways. With increasing concentrations of calcium, they can store calcium in the matrix in the form of hydroxyapatite (the main building block of bone), tricalcium phosphate, or other calcium phosphate precipitates. Therefore, mitochondria can propagate calcium-driven signals in two ways: acting as a calcium sink in order to prevent feedback inhibition or acting as a calcium reservoir releasing more calcium to the cytosol to amplify signals. Interestingly, calcium can also activate plasma membrane potassium channels to hyperpolarize a cell depressing excitability. The difference between the ultimate consequences of intracellular calcium concentration and its handling by mitochondria seems to be mostly related to the spatial relationships, length of calcium transients, and to a smaller extent to the amplitude of the oscillations.

A sustained elevation of intracellular calcium is associated with initiation of either necrotic cell death or the initiation of apoptotic machinery [35]. One example is glutamate excitotoxicity in neurons with overstimulation of

the NMDA receptor followed by prolonged cytosolic calcium elevation. Mitochondria undergo the membrane permeability transition with swelling of the matrix and rupture of the outer membrane. If all mitochondria are affected, cell necrosis ensues; however, when enough mitochondria are functional to sustain ATP production after caspase activation and cytochrome c release, apoptotic cell death follows.

Given the importance of calcium in cell processes and the function of the mitochondrion in calcium signaling, it is not surprising that calcium would play a key role in mitochondrial retrograde signaling (also called mitochondrial stress signaling). This can be inferred by the well-described membrane permeability transition in response to mitochondrial stress associated with $\Delta\Psi m$ depolarization, the appearance of the permeability transition pore, and calcium efflux. It has also been seen in experiments analogous to those done in yeast in which nuclear transcriptional analysis of mammalian rho^0 cells is investigated [20].

3.3. Retrograde Signaling in Mammalian Cells. Though mammalian orthologues of the Rtg proteins have not been identified, some target genes of mammalian mitochondrial retrograde signaling have been described [36]. Alteration of mtDNA in several cell lines generally resulted in mitochondrial membrane depolarization and increased cytosolic calcium leading to increased transcription of genes regulating calcium homeostasis. Amuthan et al. cultured human pulmonary adenocarcinoma A549 cells in the presence of ethidium bromide to selectively and partially inhibit mtDNA replication in a similar fashion to their prior work in C2C12 rhabdomyoblasts [37]. They were able to show mtDNA depletion results in 2-3-fold increase in steady state cytosolic calcium. Calcineurin, ERK1, and ERK2 were increased resulting in nuclear translocation of transcription factors such as NFATc- and JNK-activated ATF2. Nuclear targets involved in calcium transport and storage were also induced including the ryanodine receptors (RyR1/RyR2), calreticulin, and calsequestrin. Additionally, antiapoptotic proteins Bcl2 and Bcl-X_L were elevated, and proapoptotic proteins Bid and Bax were decreased. Though a number of nuclear-encoded mitochondrial proteins containing CRE sequences have been found to be elevated in mitochondrial dysfunction, Arnould et al. identified CaMKIV-induced CREB phosphorylation as a new component of the retrograde pathway with Vankoningsloo et al. later adding C/EBP homologous protein (CHOP) as well [38, 39]. These results were reversed with restoration of mtDNA and were inhibited in these and other models when calcium was removed from the system. Biswas et al. provided an important link between mitochondrial stress and NFκB activation in a manner distinct from canonical regulation by TNFα, IKKα/β, and IκBα [40]. They showed that genetic (mtDNA depletion) or metabolic (CCCP addition) mitochondrial stress results in calcineurin-dependent inactivation of IκBβ, allowing NFκB/Rel translocation to the nucleus. It has been suggested that as an organism increases in complexity, NFκB takes over more of the responsibilities of stress signaling. Though NFκB has no close homology to the Rtg proteins, Srinivasan et al. showed strong homologies in pathways common to both [41].

In addition to increasing cytosolic calcium concentration, mitochondrial stress has been shown to produce excess reactive oxygen species (ROS). When this stimulus overwhelms the resident antioxidant defense consisting of the superoxide dismutases, catalases, and glutathione peroxidases among others, it can result in lipid peroxidation, activation of the permeability transition pore, and apoptosis [42]. In contrast to the catastrophic consequences of overwhelming oxidant stress, though, ROS have been shown to be important second messengers in physiologic and pathologic conditions. Mitochondria can communicate among themselves via ROS second messengers as described below, and ROS can be part of an important retrograde signal by stimulating the antioxidant response element (ARE) of cytoprotective genes. One notable example is nuclear factor- (erythroid-derived 2-) related factor 2 (Nrf2). Nrf2 resides in the cytoplasm and is constitutively degraded by Keap1. In the presence of ROS, Keap1 undergoes a conformational change releasing Nrf2 that is then translocated to the nucleus [43]. There, it binds the ARE of genes involved in the antioxidant response like heme oxygenase and inducers of mitochondrial biogenesis such as NRF-1. Formentini et al. recently showed that overexpression of ATPase inhibitory factor 1 (IF1) in a colon cancer cell line was associated with mitochondrial-induced ROS-mediated retrograde signaling [44]. The elaboration of ROS was required for activation of the canonical NFκB pathway and resulted in cell proliferation- and Bcl-X_L-mediated resistance to drug-induced cell death.

Much of the work dedicated to deciphering the mammalian retrograde response has utilized cancer cell lines reflecting the fact that the mitochondrial defects have been associated with many types of cancers since the initial description of "aerobic glycolysis" in cancer cells by Warburg. A number of mitochondrial and nuclear DNA defects affecting genes involved in mitochondrial metabolism are associated with prosurvival or invasive properties [45, 46]. Correia et al. showed that infiltrating astrocytomas had a marked decrease in mtDNA copy number that was associated with increased levels of mitochondrial polymerase catalytic subunit and the mitochondrial transcription factors Tfam and mtTFB1/2 [47]. Wallace provides an excellent review [48], outlining multiple examples of mitochondrial genetic and metabolic defects leading to altered nuclear gene expression and tumorigenic progression.

4. Additional Mechanisms of Mitochondrial Retrograde/Stress Signaling

4.1. Mitochondrial Unfolded Protein Response (mtUPR). The complex compartmentalization of mitochondrial networks and different sources of protein synthesis require coordination of protein import/export, folding, and proper integration. Prior identification of the roles of the cytosolic heat shock response as well as the endoplasmic reticulum unfolded protein response (erUPR) in intracellular protein homeostasis led to the characterization of the mitochondrial unfolded protein response in a similar function. To that end, several chaperone proteins were identified to play an

important role in mitochondrial protein homeostasis [49]. These included the HSP-60 and HSP-70 family proteins, which in *C. elegans* are represented by hSP-60 and hSP-6, respectively. By using *C. elegans* reporter constructs in which green fluorescent protein expression was coupled to the promoter elements of hSP-60 and hSP-6, Haynes et al. identified nuclear genes important to the mtUPR. CLPP-1 is a protein homologous to the *E. coli* protease ClPP [50]. It localizes to the mitochondria and is important for initiation of the UPR (Figure 3). Additionally, the transcription factor DVE-1 was seen to interact with the promoters of the chaperone genes as well as with the ubiquitin-like protein UBL-5, which could potentially act as an amplification signal similar to the amplifying signals seen in the erUPR. While CLPP-1 is necessary for DVE-1 localization to the nucleus, the messengers between the two compartments have yet to be elucidated [51].

Although mammalian homologues of these components have not yet been identified, mammalian mtUPR target genes have been identified and include chaperonin 60, chaperonin 10, mtDNAJ, and ClPP. Additionally, these targets are upregulated in absence of induction of stress proteins involved in canonical erUPR or the cytosolic heat shock response implying some specificity to the pathway. One target common to the UPRs is the transcription factor C/EBP homologous protein (CHOP) [52]. While in the erUPR, one result of CHOP is the induction of apoptosis; its function in mtUPR is not yet clear, though there is evidence to suggest that it is involved in adaptive, prosurvival pathways [53]. For example, mtUPR in mammalian tumor cells has been associated with protective roles promoting cancer cell survival. Siegelin et al. showed that mtUPR in murine glioblastoma cells was dependent on HSP-90 and associated with tumor cell survival and adaptation [54]. Inhibition of the mtUPR by antagonizing HSP-90 resulted in apoptosis and prevention of tumor growth. In breast cancer cells, the mtUPR is activated in response to protein accumulation in the intermembrane space, and ROS production activates estrogen receptor alpha (ERα) [55]. This results in activation of NRF1 and other cytoprotective responses to overcome the mitochondrial stress. Additionally, disruptions in unfolded protein responses in both ER and mitochondria have been implicated in the development of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases among others [56, 57].

4.2. Intermitochondrial Signaling. If the mitochondrion has been shown to be a dynamic organelle, the mitochondrial network is anything but static. Spatial and temporal organization of mitochondria has been shown to be varied and complex. Most studies involving mitochondrial network dynamics have been done in cardiac myocytes, which typically have a tightly packed, lattice-like arrangement. Mitochondria are the primary source of intracellular ROS, and progressive oxidative stress leads may potentially lead to depolarization of the mitochondrial membrane potential ($\Delta\Psi$m). The mitochondrial permeability transition (MPT), mediated by the permeability transition pore (PTP), is a central event in bioenergetic failure and mitochondria-initiated apoptosis and is regulated by the redox state of the mitochondrion among several other factors including calcium flux as mentioned above [58]. Perturbations in the physiologic oscillations $\Delta\Psi$m can lead to progressive mitochondrial dysfunction. Zorov et al. described a method of intermitochondrial communication during oxidative stress termed ROS-induced ROS release (RIRR) in which local oxidative stress leads to release of the superoxide radical (O_2^-) [59]. Superoxide acts as a messenger between mitochondria leading to a wave of membrane depolarization and further ROS release. Neighboring chains of mitochondria appeared to cooperate in reversible waves of depolarization. Zhou et al. used live cardiac myocytes coupled with a mathematical model of RIRR to show that O_2^- is the specific mediator of the wave of depolarization and that a reversible change in $\Delta\Psi$m spread progressively in a spatiotemporal diffusion until a critical threshold was reached leading to global depolarization [60]. Park and Choi further suggested that differing spatial relationships between mitochondria in differing tissues potentially lead to alterations in primary messenger (O_2^- versus H_2O_2) of RIRR as well as the effectiveness of different antioxidants on propagation [61].

Multiple different mechanisms of RIRR have since been described ranging from direct mitochondrial-generated ROS to complex ROS generation secondary to antioxidant inhibition or ROS-induced injury [62]. As a consequence, intermitochondrial ROS signaling is a system by which mitochondrial network dynamics can be coordinated in response to a complex system involving the myriad stimuli leading to oxidative stress and the antioxidant response system.

4.3. Mitochondrial Autophagy, Mitoptosis, and Biogenesis. As a corollary to retrograde and intermitochondrial signaling, autophagy is a form of quality control through interorganellar signaling. Autophagy is an evolutionarily conserved process of removing or recycling damaged organelles by engulfing them in a double-membraned autophagosome that is then taken to a lysosome for degradation [63]. Cells can maintain quality control of organellar function through a baseline level of autophagic activity [64]. In times of stress, however, the autophagic machinery can be upregulated in order to maintain cellular function by preventing the accumulation of nonfunctioning, potentially toxic organelles. Mitophagy, or macroautophagy that specifically involves mitochondria, is an important component of this process since nonfunctioning mitochondria can be particularly toxic through their generation and release of ROS and reactive nitrogen species (RNS) [65]. Unmitigated oxidative stress can lead to cell death through necrosis or apoptosis. Mitophagy is an adaptive process that is initiated through complex and incompletely understood signaling in order to prevent persistent cell damage and ultimate cell death [63, 66].

Another mechanism by which cells can eliminate damaged mitochondria is mitoptosis [67]. During cellular energy crises in which mitochondrial stress leads to impaired oxygen utilization, ROS production increases resulting in fragmentation of the mitochondrial network, clustering of damaged

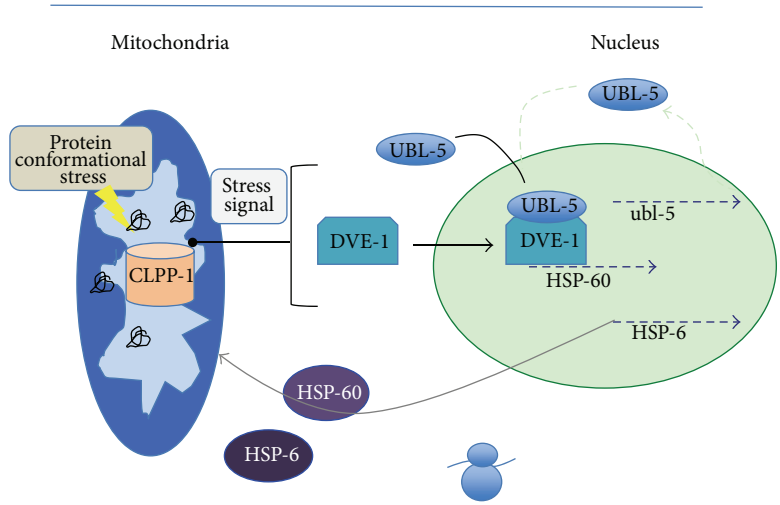

FIGURE 3: Hypothetical model of the *C. elegans* UPR^{mt} pathway. Protein conformational stress in the mitochondrial matrix triggers CLPP-1 proteolysis of an unknown substrate, producing a stress signal (blue line). The stress signal is conveyed to the cytoplasm and induces nuclear translocation and complex formation of UBL-5 and DVE-1, as well as binding of DVE-1 to the promoter of the chaperone target gene, HSP-60. This stress-signaling pathway results in the induction of mitochondrial chaperone genes, HSP-60 and HSP-6. ubl-5 expression is also upregulated, which in turn amplifies the UPR^{mt} signal (green-dotted line). HSP-60 and HSP-6 are imported into the mitochondria, where they help to restore protein homeostasis by refolding rogue proteins. Adapted with permission from [51].

mitochondria in the perinuclear region, incorporation into a single-membraned mitoptotic body, and finally extrusion of the mitoptotic body via exocytosis or blebbing [68]. It is not clear whether this mechanism acts independently or in concert with autophagy. Lyamzaev et al. found that mature mitoptotic bodies were not associated with autophagosome, and suggested that in the setting of whole-cell energy catastrophe, mitoptosis may be a faster mechanism of mitochondrial clearing than mitophagy [68].

Once damaged organs have been cleared, a new population of mitochondria will need to be generated. The physical basis for this is, in part, the raw materials harvested through the autophagic recycling of damaged organelles. The physiological basis for the new population is mitochondrial biogenesis. Biogenesis may be initiated at the same time as autophagy or secondary to subsequent autophagic signaling. A number of signals such as ROS, calcium, energy status, and others influence the activation of the aforementioned nuclear coactivator PGC1α and the associated nuclear respiratory factors [69–71]. In this way, mitochondrial homeostasis is restored, and the cell is able to avoid bioenergetic failure and death.

4.4. Mitochondria and the Innate Immune System. The innate immune response relies heavily on ROS production in phagocytes by NADPH oxidase for bactericidal capability [72]. Recently, mitochondrial-generated ROS have been shown to contribute to macrophage bactericidal activity in response to activation of cell surface Toll-like receptors (TLR1, TLR2, and TLR4) [73]. These TLRs activate the signaling adaptor and tumor necrosis factor-associated factor 6 (TRAF6) that translocates to the mitochondria. It then ubiquitinates the protein evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) that is localized to the mitochondria and is involved in complex I assembly. This leads to interruption of the respiratory chain, migration of mitochondria to the phagosomes, and increased production of mitochondrial ROS.

4.5. Mitochondria and Longevity. Reactive oxygen species have long been implicated in aging, senescence, and cancer, and since mitochondria are a main source of ROS, they are often regarded as prime targets for modulation of aging [74, 75]. Use of traditional antioxidants to this end has been largely unsuccessful, perhaps because they are not reaching the appropriate compartment. More recently, however, mitochondrial-targeted antioxidants have shown some promises in this regard [76, 77]. Murphy et al. developed a system of targeting antioxidants such as ubiquinone to lipophilic cations that would preferentially migrate to the relatively negatively charged mitochondrial matrix [78–80]. Additionally, this is a rechargeable antioxidant in that it can regenerate a reduced form by accepting electrons from the respiratory chain.

Skulachev et al. have expanded on this idea by creating SkQ-type antioxidants [81]. One drawback of these constructs is a tendency to act as prooxidants at higher concentrations. SkQ antioxidants are composed of a penetrating ion ("Skulachev ion"-Sk) and a plastoquinone (Q), which is used in place of ubiquinone, and have shown higher efficiency and lower prooxidant activity than previous compounds. In an expansive project, Skulachev et al. were able to show beneficial effects of SkQ antioxidants in multiple areas associated with tumorigenesis and aging [77, 81]. SkQ reduced cancer development in p53-deficient mice; it prevented age-related changes of retinopathy and cataracts in multiple

mammalian models, and it increased life span in a number of models including the fungus *Podospora*, the crustacean *Ceriodaphnia*, *Drosophila*, and mice.

The retrograde response has also been implicated in aging and longevity [82]. Senescent mitochondria develop progressive genetic instability. In yeast, this is manifested by accumulation of extrachromosomal ribosomal DNA circles (ERC) [83]. Though activation of the retrograde response has been shown to induce ERC formation in some cases, continued activity of the retrograde response appears to contribute to longevity and prevent further genomic instability. Additionally, yeast replicative life span is dependent on a concept of age asymmetry in which mitochondrial dysfunction is not inherited in the daughter cells during division [84]. Damaged mitochondria are segregated in the mother cell with the daughter cells receiving a complement of normal mitochondria thereby conferring the capacity for a normal life span [85].

5. Conclusion

Mitochondria require nuclear input in addition to their own genetic information and are aptly considered semi-autonomous structures. One could also argue the reverse. Increasingly, mitochondria are seen to control nuclear gene expression as well as function and even fate of the cell. Improved understanding of mitochondrial signaling and metabolism provides significant potential to impact future of diagnosis and therapy in a wide array of physiology and pathophysiology.

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Research Article

Oxidative Stress and Free-Radical Oxidation in BCG Granulomatosis Development

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Background. Little is known about the role of free-radical and oxidative stress signaling in granuloma maturation and resolution. We aimed to study the activity of free-radical oxidation processes in the dynamics of BCG-induced generalized granulomatosis in mice. *Methods*. Chronic granulomatous inflammation was induced in male BALB/c mice by intravenously injecting the BCG vaccine, and the production of oxidative stress (activity of free-radical oxidation processes) and histological changes in the lungs, liver, and peritoneal exudate were measured 3, 30, 60, and 90 days after infection. *Results*. The tuberculous granuloma numerical density and diameter continuously increased from day 30 to day 90, and the macrophage content within the granulomas progressively diminished with a concomitant elevation in the number of epithelioid cells. The activity of the free-radical oxidation processes in the liver (i.e., the intensity of the homogenate chemiluminescence) reached a maximum at postinfection day 60 and subsequently began to decrease. The peak generation of reactive oxygen species by phagocytes in the peritoneal exudate (measured using flow cytometry) was also shifted in time and fell on day 30. *Conclusions*. The rise in the steady-state concentration of H_2O_2 in the liver of mice with BCG-induced granulomatosis is not related to local H_2O_2 production by phagocytes, and a decrease in the severity of generalized inflammation precedes the resolution of local inflammation.

1. Introduction

The generation of inflammatory granulomas that result from the proliferation and transformation of phagocytic cells is a hallmark of many infectious (e.g., tuberculosis (TB) and tularemia) and noninfectious (e.g., silicosis, asbestosis, and granulomatous hepatitis) diseases (more than 70 disease entities) [1, 2]. When it is necessary to isolate foreign objects, including microorganisms, granulomatous inflammation is generally believed to appear; these objects cannot be removed by the normal process of phagocytosis (with subsequent degradation). However, researchers face several complicated problems when studying this phenomenon. For instance, the appearance of idiopathic granulomas with an inducer of unknown nature is a distinguishing feature of sarcoidosis, Wegener's disease, and several other diseases [2, 3]. Another problem is the almost complete lack of effective tools and techniques to influence granulomatous processes; anything offered by modern medicine is essential either the therapeutic removal of an inducer (for infectious granulomas) or the surgical removal of the affected organ. Another problem is the high unpredictability in the development and resolution of inflammatory granulomas; although these processes depend on inducing factors, they are notably individual. If we consider the problem of granulomogenesis in general, the least studied topic is the participation of reactive oxygen and nitrogen species (RONS) as an evolutionarily ancient mechanism of intra- and intercellular regulation; this regulation orchestrates (by means of redox signaling) cell migration, cooperation, functional activity, life cycle, proliferation, and death [4].

A classic example of infectious granulomatosis is TB, the most common infectious disease in the world. At the heart

of its development and manifestation is the persistence of the pathogen *Mycobacterium tuberculosis,* mainly in the vacuolar apparatus of macrophages cells that are specialized in antibacterial protection, 90% of which are determined by oxygen-dependent mechanisms [3]. There are many researches that consider both the mechanisms of free-radical antimycobacterial host phagocyte protection and the methods by which *M. tuberculosis* avoids RONS attack [3, 5–7], as well as oxidative stress development. However, up to now, no effective redox-dependent methods to treat TB or control an *M. tuberculosis* infection have been proposed. The high incidence of TB in people with NAD(P)H oxidase genetic defects [7] and the prospects for targeted delivery of nitric oxide using inhalable microparticles containing NO donors [8] indicate the need for better understanding of the role of free-radical processes involving RONS in *M. tuberculosis* persistence during the dynamics of TB granulomatosis.

For the first phase of studying the roles of oxidative stress and reactive oxygen species (ROS) as effectors and regulators of granulomatous inflammation, we attempted to examine the change in the activity of free-radical oxidation processes during the dynamics of chronic BCG-induced generalized granulomatosis in mice and compared this change with the morphological changes.

2. Materials and Methods

2.1. Animal Model. The Animal Care Committee of the Research Center of Clinical and Experimental Medicine approved the experimental protocol. Male BALB/c mice (weight: 18–22 g, age: 2 months) were purchased from the Research Institute of Clinical Immunology SB RAMS (Novosibirsk, Russia). The mice were housed in an environment with controlled temperature and controlled light and divided into eight groups ($n = 5$ in each group) as follows: four groups with a model of generalized tuberculous granulomatosis (3, 30, 60, and 90 days after a single injection of 0.5 mL of BCG vaccine (Microgen, Russia) in 1 mL of saline into the tail vein) and four control groups (3, 30, 60, and 90 days after a single injection of 1 mL of saline into the tail vein) [1, 2]. The animals were weighed and sacrificed by cervical dislocation. Peritoneal leukocyte samples were obtained to evaluate the oxidative metabolism of these cells. The livers and lungs were quickly removed, weighed, and processed for histological examination and preparation of liver homogenates. These organs were selected because they are the most often affected in generalized tuberculosis and they also contain the largest compartment of cells of the mononuclear phagocyte system, which form the basis of granulomas.

2.2. Histological Examination. Liver and lung fragments were fixed in 10% neutral formalin, dehydrated in ascending alcohol solutions, and embedded in paraffin. The sections (4-5 μm thick) were stained using the hematoxylin/eosin and Van Gieson/Elastin techniques and studied using light microscopy (AxioImager A1, Carl Zeiss, Germany). Specific histochemical staining by the Ziehl-Neelsen stain was used to visualize *Mycobacterium bovis* in the tissues. Using the morphometry method (AxioVision software, rel. 4.8), the numerical density of the granules and their diameters were determined; these parameters were used as the morphological criteria for the tuberculosis activity. This activity is caused by the chemoattractant gradient, which is created by alive mycobacteria (free and persistent in macrophages). The granule size showed the value of the chemoattractant potential [1, 2].

2.3. Activity of Free-Radical Oxidation Processes

2.3.1. Chemiluminescence (CL). The livers were rinsed with saline, minced with scissors, and homogenized on ice in a Potter-Elvehjem tissue grinder with 5 vol (w/v) of Hanks' balanced salt solution without phenol red (HBSS) (200 mg/mL). After recording the background CL of the measuring cuvette at 37°C in a chemiluminometer (Photon, Russia) for 2 minutes, 2 mL of liver homogenate was placed in the cuvette and then incubated for 2 minutes, after which the spontaneous CL was measured for 2 minutes. Afterward, 0.1 mL of 100 nM luminol (Serva, Germany) solution was injected, the luminol-amplified CL (LACL) was measured for 2 minutes, 0.1 mL of H_2O_2 solution was then added (final concentration 39.5 mM), and the H_2O_2-induced luminol-amplified CL (H_2O_2-LACL) was measured. The CL intensity was expressed in arbitrary units (1 a.u. =5 impulses/s) with each value representing an average. The averaged background CL intensity of the measuring cuvette was subtracted from the averaged values for the spontaneous and luminol-amplified chemiluminescence.

2.3.2. Flow Cytometry. Peritoneal exudate cells were obtained by peritoneal lavage with cold RPMI 1640 medium (Biolot, Russia) supplemented with 1% (v/v) fetal bovine serum (Biolot), and kept on ice until measurement. To measure the total ROS production, isolated cells were incubated for 15 min in 1 mL of HBSS containing 10 mM 2′,7′-dichlorodihydrofluorescein diacetate (Sigma, USA) or 50 mM dihydroethidium bromide (Sigma). The former is deacylated intracellularly and rapidly oxidized by ROS to yield the highly fluorescent product 2′,7′-dichlorofluorescein (DCF), and oxidation of the latter molecule, which is not fluorescent, in cells by superoxide anion radicals results in the formation of 2-hydroxyethidium (2OH-E), whose fluorescence is in the red.

We investigated both spontaneous ROS and the ROS stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma). Using the FACSCalibur (Becton-Dickinson, USA) flow cytometer, we measured the intensity of the DCF-dependent fluorescence ($\lambda_{Em} = 488$ nm, $\lambda_{Ex} = 520$ nm), which is predominantly an indicator of H_2O_2 generation by cells, and the 2OH-E fluorescence ($\lambda_{Em} = 488$ nm, $\lambda_{Ex} = 630$ nm), which is mainly sensitive to the superoxide anion. The gating of the viable macrophages and granulocytes was based on light scattering (forward and side scatter) properties. The results of the cell fluorescence intensity were normalized to the spontaneous fluorescence in control mice, taken as 100%.

Table 1: Numerical density and cellular composition of BCG granulomas in the liver and lungs of BCG-infected mice.

Parameter	Liver			Lungs		
	Time after infection (days)					
	30	60	90	30	60	90
Numerical density of granulomas (Nai), 3.64×10^5 μm^2	3.08 ± 0.26	7.84 ± 0.64#	8.36 ± 0.54	7.40 ± 0.57	8.24 ± 0.51	11.24 ± 0.46#
Diameter of granulomas, μm	47.25 ± 1.26	53.18 ± 1.54#	58.92 ± 1.32#	49.69 ± 1.24	68.71 ± 0.34#	71.12 ± 1.82
Macrophage number, %$	65.12 ± 0.69	33.85 ± 0.87#	14.72 ± 1.33#	47.94 ± 0.53	27.11 ± 0.86#	23.13 ± 0.59#
Epithelioid cell number, %$	22.56 ± 0.87	57.05 ± 0.89#	77.35 ± 1.42#	26.37 ± 0.23	52.21 ± 1.04#	58.21 ± 0.23#
Neutrophil number, %$	2.47 ± 0.18	0.69 ± 0.14#	0.21 ± 0.09#	1.83 ± 0.23	2.53 ± 0.49	2.09 ± 0.42
Lymphocyte number, %$	7.51 ± 0.30	5.47 ± 0.20#	1.64 ± 0.27#	12.48 ± 1.21	7.19 ± 0.36#	3.92 ± 0.31#
Fibroblast number, %$	2.34 ± 0.20	2.94 ± 0.18#	6.08 ± 0.24#	11.38 ± 0.31	10.96 ± 0.36	12.65 ± 0.81#

The results represent the mean ± SEM.
$100%: the total number of cells in granuloma. #The value significantly differs from the value for the previous observation period ($P < 0.05$).

2.4. Statistical Analysis. The Kolmogorov-Smirnov test was used to check whether the variables were normally distributed. For variables with a normal distribution, the parametric t-test was used for two independent samples, and the data are represented as the mean ± SEM. Variables that were not distributed normally were evaluated using the Mann-Whitney nonparametric test, and the data are represented as the median and the lower (Q_1) and upper (Q_3) quartiles. The relationships between the variables were assessed by Spearman's rank correlation coefficient (r). P values less than 0.05 were considered significant.

3. Results

3.1. Histological Examination. Histological examination revealed that 30 days after infection the mice developed disseminated tuberculous inflammation, which was manifested morphologically by BCG granuloma formation in the internal organs and visceral membranes. *M. bovis* bacteria were detected in the foci of the granulomatous inflammation. However, necrotic changes in the granulomas of the lungs and liver of the mice were not found in any experimental group. This result was most likely caused by the weakened virulence of *M. bovis* in BCG (used to vaccinate the animals), and, therefore, by the decrease in the chemotactic capacity and the direct effect of the mycobacterial cell wall lipid components on granuloma cells [1, 2].

The numerical density of granulomas in the liver and lungs increased by 2.7 and 1.5 times, respectively, throughout the 30–90th days of infection, and simultaneously, the granuloma diameter increased by 24.6% in the liver and 43.1% in the lungs (Table 1). The study of the granuloma cellular composition revealed that the macrophage, neutrophil, and lymphocyte numbers consistently declined (the lung neutrophil count did not change), but the numbers of epithelioid cells and fibroblast increased. This finding indicates a stable course of tuberculosis with a tendency toward progression and no propensity toward a spontaneous cure. The numerical density and diameter of the granulomas in the lungs were higher than those in the liver at all stages of observation

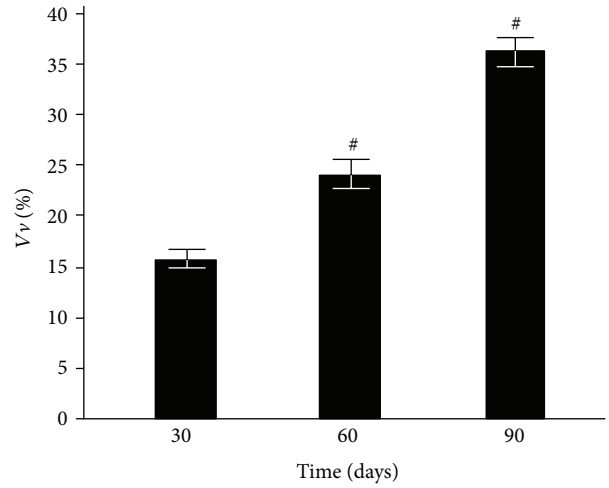

Figure 1: Volume density of mononuclear infiltrates in the lung interstitium of control and BCG-infected mice. The results represent the mean ± SEM. #The value differs significantly from the value for the previous observation period ($P < 0.05$).

(Table 1). The number of epithelioid cells in the liver and lung granulomas did not differ significantly at day 30 after infection and increased by 3.5 and 2.2 times, respectively, at day 90. Simultaneously, an elevation of lung mononuclear infiltrates in the interstitium was observed; this volume density was enhanced 2.3-fold from the 30th to the 90th day (Figure 1).

Such differences in the morphogenesis of the tuberculous granulomatous inflammation in the liver and lungs of experimental animals can be related to the structural features of alveolar macrophages, which contain a large number of lysosome-like structures in the cytoplasm, and the close topographical interrelation of all alveolus wall components, both among themselves and with blood elements [9]. In addition, because pulmonary macrophages function in an aerobic environment with increased oxygen tension, the major bactericidal mechanism in the lungs is free radicals [9, 10]. Thus, excess RONS generation leads to both destabilization of cell membranes and lung tissue damage. In turn, degradation products

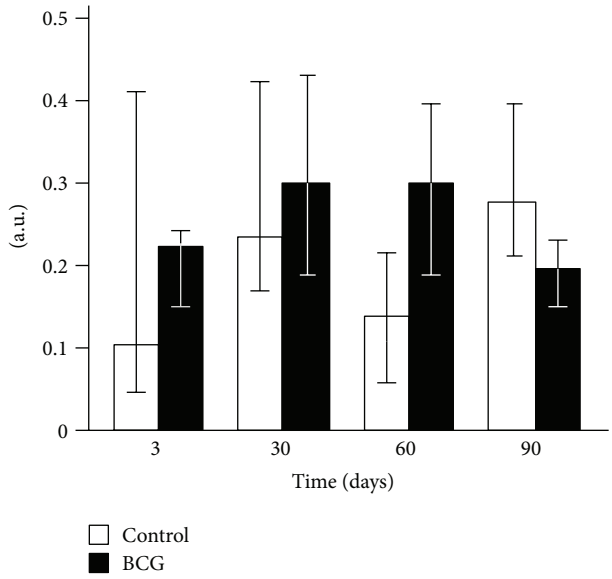

Figure 2: Spontaneous chemiluminescence of liver homogenates of control and BCG-infected mice. The bar represents the median value, and the error bar indicates the lower and upper quartiles.

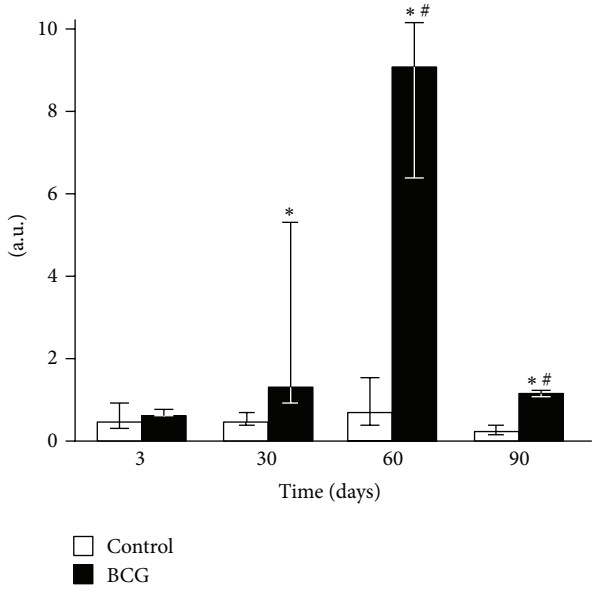

Figure 3: Luminol-amplified chemiluminescence of liver homogenates of control and BCG-infected mice. The bar represents the median value, and the error bar indicates the lower and upper quartiles. #The value differs significantly from the value for the previous observation period ($P < 0.05$). *Significant difference between the BCG and control groups ($P < 0.05$).

can attract new populations of macrophages and T cells to the lesion area. Thus, the granuloma diameter increases via an expansion of the peripheral zone, which is represented by mononuclear cells (macrophages and lymphocytes), and via diffuse infiltrative alterations of the interstitium similar to mononuclear alveolitis [9].

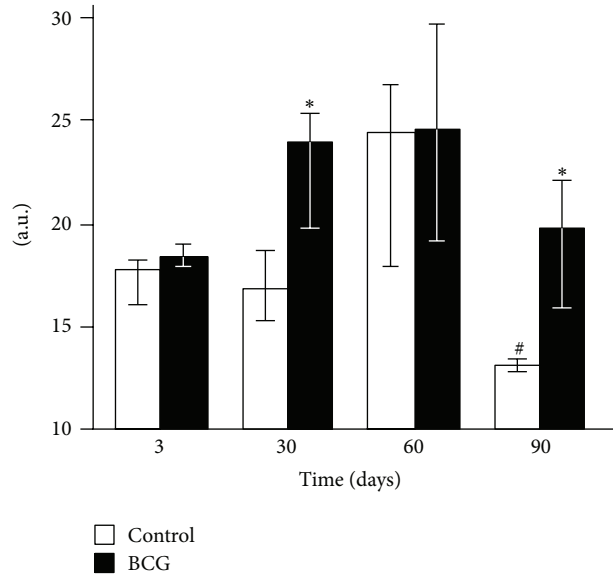

Figure 4: Luminol-amplified H_2O_2-induced chemiluminescence of liver homogenates of control and BCG-infected mice. The bar represents the median value, and the error bar indicates the lower and upper quartiles. #The value differs significantly from the value for the previous observation period ($P < 0.05$). *Significant difference between the BCG and control groups ($P < 0.05$).

3.2. Activity of Free-Radical Oxidation Processes

3.2.1. Chemiluminescence (CL). None of the liver homogenate CL parameters changed 3 days after the BCG administration to mice (Figures 2–4). After 30 days of infection, the spontaneous CL did not differ significantly from the control values, but when luminol was introduced into a registration system, we observed a significant increase in the light intensity in the BCG-injected group, which was even more pronounced for the CL induction by hydrogen peroxide (Figures 2–4). The LACL value enhancement was even more pronounced 60 days after-infection and was not only 8.7 times higher than that in control but also significantly greater than the LACL intensity of the liver homogenates of mice 30 days after the BCG vaccine injection (Figure 3). The H_2O_2-LACL values in the experimental and control groups were similar for the same observation period (Figure 4). After 90 days of the experiment, the control and experimental mice differed in both the LACL and the H_2O_2-LACL intensities (Figures 3 and 4), and the latter was paradoxically low in the control group.

When analyzing the relationships between various CL parameters and the body and organ weights, interesting patterns were revealed. For instance, the body weight of the control animals was correlated to a significant extent with the H_2O_2-LACL and the liver and lung weights. After intravenous administration of the BCG vaccine, the liver weight was also positively correlated with the homogenate LACL and the lung weight. Furthermore, there was a negative correlation between the lung and H_2O_2-LACL, whereas the inverse relationship between H_2O_2-LACL and body weight was no longer relevant (Table 2).

Table 2: Relationship (r) between body and organ weights and CL intensity of the liver homogenates of control and BCG-infected mice for the entire period of observation.

	Spontaneous CL	LACL	H_2O_2-LACL	Body weight	Liver weight	Lung weight
Spontaneous CL		*0.12*	*−0.36*	*−0.18*	*0.25*	*−0.06*
LACL	**0.07**		*0.22*	*−0.19*	*0.32*	*−0.27*
H_2O_2-LACL	**0.06**	**0.46**		*−0.62**	*0.09*	*−0.44*
Body weight	**0.24**	**0.22**	**−0.08**		*0.51**	*0.65**
Liver weight	**−0.06**	**0.62***	**−0.07**	**0.67***		*0.27*
Lung weight	**−0.09**	**0.12**	**−0.57***	**0.49***	**0.63***	

Upper right part of table: control (in italics); lower left: BCG injection (in bold). * Significant r values.

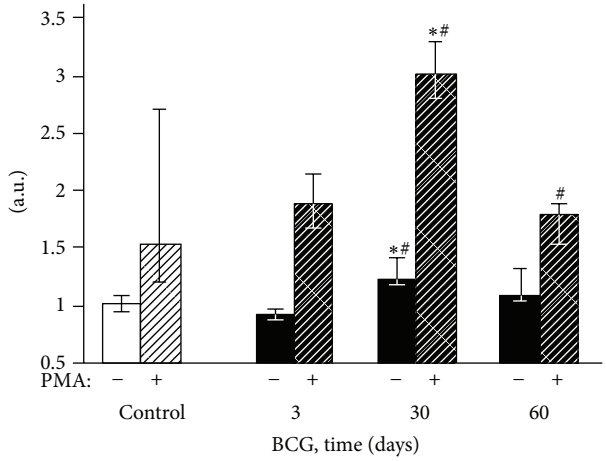

Figure 5: DCF-dependent fluorescence of exudate polymorphonuclear neutrophils of control and BCG-infected mice. The bar represents the median value, and the error bar indicates the lower and upper quartiles. #The value differs significantly from the value for the previous observation period ($P < 0.05$). *Significant difference between the BCG and control groups ($P < 0.05$).

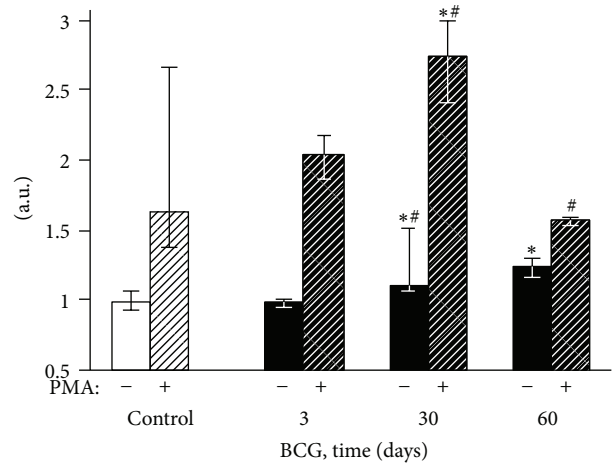

Figure 6: DCF-dependent fluorescence of exudate macrophages of control and BCG-infected mice. The bar represents the median value, and the error bar indicates the lower and upper quartiles. #The value differs significantly from the value for the previous observation period ($P < 0.05$). *Significant difference between the BCG and control groups ($P < 0.05$).

3.2.2. Flow Cytometry. Three days after the intravenous BCG vaccine injection, the fluorescence intensity of the peritoneal exudate cells did not differ from the control group values (Figures 5–8). After 30 postinfection days, the spontaneous and PMA-stimulated ROS generation by the exudate granulocytes increased, indicating their metabolic activation (Figures 5 and 7). Moreover, during the same period, both activation (increase of the spontaneous DCF fluorescence intensity) and priming (enhancement of phorbol ester-stimulated DCF fluorescence) of peritoneal macrophages were revealed (Figures 6 and 8). The intensity of H_2O_2 and O_2^- generation by the phagocytes in the mouse peritoneal cavities decreased significantly 60 days after the intravenous BCG vaccine injection, mainly returning to the original values. However, the 2OH-ET-dependent granulocyte fluorescence (both spontaneous and after induction of respiratory burst by PMA) and the unstimulated DCF-dependent macrophage fluorescence remained somewhat elevated (Figures 5–8).

4. Discussion

Currently, the study of granulomatous inflammation focuses on cytokine regulation despite the nosological identification of hereditary chronic granulomatous disease, which is based on a variety of defects in the membrane NAD(P)H oxidase complex, resulting in phagocytic cells that cannot generate superoxide anion radicals [11]. This fact is interpreted to indicate that ROS interferes with granuloma formation, and the genesis of the latter is mainly due to decreased phagocyte microbicidal activity [12]. However, this interpretation appears superficial because intercellular redox regulation occurs via neutral oxidative stress effectors, primarily nitric oxide ($NO^\bullet$) and hydrogen peroxide (H_2O_2). Superoxide anion radicals are the most effective scavengers of nitric oxide radicals; thus cells with NAD(P)H oxidase defects are characterized by more active NO-mediated cell-cell communication, which may contribute to granuloma formation.

NO participation in the formation of both infectious and noninfectious granulomas in humans and animals has long been known [13]. The main producer of NO radicals in macrophages and within a granuloma is inducible NO synthase, whose expression is controlled by the transcription factor NF-κB and increases in response to endotoxin, proinflammatory cytokines, and other factors. The main sources of $NO^\bullet$ in granulomas are macrophages and giant Pirogov-Langhans cells and, to a lesser extent, epithelioid

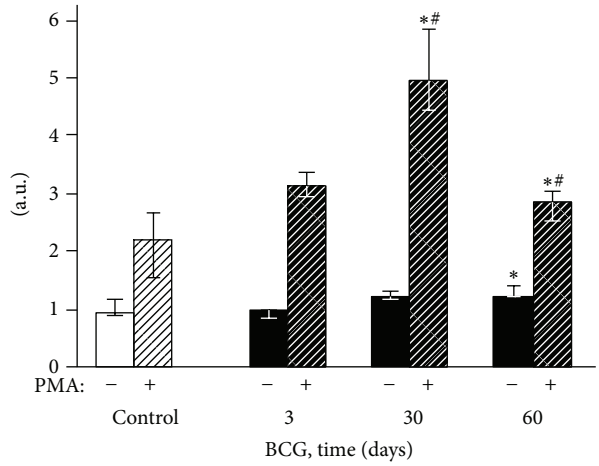

Figure 7: 2OH-E-dependent fluorescence of exudate polymorphonuclear neutrophils of control and BCG-infected mice. The bar represents the median value, and the error bar indicates the lower and upper quartiles. #The value differs significantly from the value for the previous observation period ($P < 0.05$). *Significant difference between the BCG and control groups ($P < 0.05$).

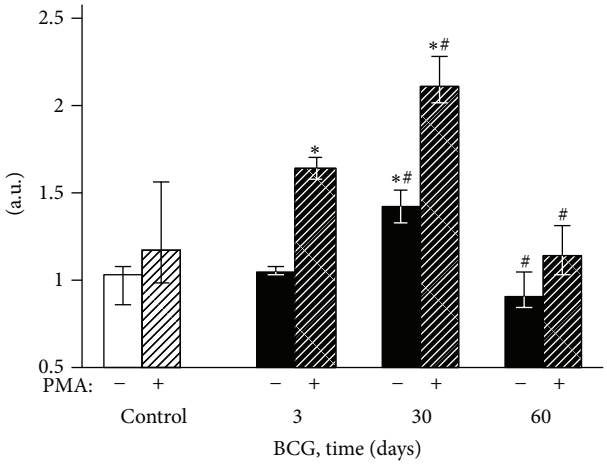

Figure 8: 2OH-E-dependent fluorescence of exudate macrophages of control and BCG-infected mice. The bar represents the median value, and the error bar indicates the lower and upper quartiles. #The value differs significantly from the value for the previous observation period ($P < 0.05$). *Significant difference between the BCG and control groups ($P < 0.05$).

cells [14]. NO radicals are believed to be a major factor that kills mycobacteria and limits the growth of pathogens in tuberculosis [3]. Tumor necrosis factor α and interferon γ participate in the host defense against mycobacteria, also by increasing NO production [15]. The inducible NO synthase content in the granulomas of BCG-infected cattle increases, reaching a maximum at day 42 (10 times more than on the 15th day), and then decreases after fibrosis development [16]. Some authors claim that NO synthesis by macrophages determines granuloma organization and development [17]. The molecule serves as a chemoattractant and regulates the differentiation and activation of epithelioid cells [18]. In turn, the expression of inducible NO synthase by granuloma cells is regulated by the NRAMP1 protein [14]. One of the functions of NRAMP1 is to transport bivalent metals (including Fe^{2+} ions) into the phagosome. In the presence of hydrogen peroxide, this transport results in the generation of the hydroxyl radical, $^{\bullet}OH$, which is fatal for mycobacteria.

The participation of hydrogen peroxide in the formation and development of granulomatous inflammation has been studied to a lesser extent; however, investigators research not only the cytotoxic potential of H_2O_2 as an oxidative stress mediator but also the regulatory potential of the molecule. The H_2O_2/eosinophil peroxidase system has been shown to be directly involved in the destruction of *Schistosoma mansoni* eggs in a granuloma [19]. Furthermore, the microbicidal activity of guinea pig alveolar macrophages toward various strains of *M. tuberculosis* and *M. bovis* was not related to the intensity of the respiratory burst and H_2O_2 generation, although the efficiency of phagocytosis of different bacterial strains was inversely correlated with their virulence [20]. Both the administration of the compounds that inhibit hydrogen peroxide and neutrophil depletion with a specific antiserum reduced the formation of noninfectious granulomas in a dose-dependent manner [21, 22], particularly when chemokine expression on the surface of endothelial cells (decrease of monocyte chemotactic protein-1 expression) was inhibited [22].

Our study shows that BCG granulomatosis in the liver is not accompanied by a change in free-radical lipid peroxidation activity in the whole organ. This activity was measured by the intensity of spontaneous CL in the liver homogenate, which directly depends on the steady-state concentration of lipid alkoxyl and peroxyl radicals [3, 23] (Figure 2). The activities of free-radical oxidation processes and oxidative stress, measured by the luminol-dependent CL of the homogenates, increased simultaneously with a peak at day 60 after infection (Figure 3). This increase, along with the appearance of a correlation between the liver weight and the intensity of the liver homogenate LACL in BCG-infected animals (Table 2, the lower left part), suggests an increase in the steady-state concentration of H_2O_2 in the liver of infected animals. We believe that this H_2O_2 increase occurs mainly in the granulomas, although we do not rule out the contribution of adjacent hepatocytes. Abdallahi et al. [24] also revealed that the maturation of *Schistosoma mansoni*-induced granulomas in mouse livers is accompanied by a gradual increase in liver H_2O_2 generation. Moreover, the steady-state concentration of hydrogen peroxide also increases outside of the granuloma in neighboring hepatocytes (although to a lesser extent).

The intensity of the H_2O_2-induced luminol-dependent chemiluminescence of biological substrates directly depends on their oxidizability [3]. The fact that on day 60 (in contrast to postinfection days 30 and 90) the addition of exogenous hydrogen peroxide to the liver homogenates did not result in an increase of the luminol-amplified chemiluminescence over the control values (Figure 4) suggests that the H_2O_2 production reached maximal values at this time point.

The absence of peritoneal granulocyte activation at the early stages of the experiment (day 3), both spontaneous and PMA-induced (Figures 5 and 7), is consistent with the data indicating that polymorphonuclear leukocytes do not

directly capture BCG in the liver (in contrast to Kupffer cells and newly recruited monocytes/macrophages) [25]. In addition, during this period, granulomas are still not formed and are present as an immature small infiltrate. However, granulocytes are involved in the liver and lungs as cells that affect inflammation in mature granulomas (30 days) [1], and this involvement is followed by the activation of oxygen-dependent metabolism. The decrease of ROS generation by peritoneal exudate neutrophils and macrophages begins earlier (at day 60, Figures 5–8) than the decline of activity of free-radical oxidation in the liver (Figure 2). This timing indicates that the mitigation of the severity of generalized inflammation precedes the resolution of local inflammation. We consider the resolution of inflammation as the transition into the production phase, the prevalence of fibrosis over phagocyte migration in the area, and the resulting oxygen-mediated degradation. After postinfection day 60 in the granulomas, the percentage of neutrophils and macrophages, the most active RONS producers, also consistently decreases. This decrease occurs despite the successive enhancement of the number and diameter of granulomas in the liver and lung and the volume density of mononuclear infiltrates in the lungs; these enhancements are mainly due to the increase in the number of epithelioid cells (Table 1 and Figure 1). Thus, it is not clear whether the observed increase in the steady-state concentration of hydrogen peroxide in the liver of infected animals, especially 60 days after BCG vaccination, is due to the direct generation of H_2O_2 by phagocytes.

5. Conclusions

The study of oxidative stress (produced by the enhancement of endogenous free-radical oxidation processes) and of morphological changes during BCG vaccine-induced chronic inflammation allowed us to conclude the following. In the lungs and livers of mice up to postinfection day 90, the numerical density and diameter of the granulomas sequentially increase mainly due to the enhancement of the epithelioid cell number, whereas the number of phagocytic cells reduces. In contrast, the activity of free-radical oxidation processes in the liver and peritoneal exudate enhances at days 60 and 30 after BCG vaccine injection, respectively, and gradually decreases thereafter. Thus, the rise in the steady-state H_2O_2 concentration in the liver of infected animals is not related to its local production by phagocytes, and a decrease in the severity of generalized inflammation precedes the resolution of local inflammation. The data obtained on the uncoupling of H_2O_2- and progenitor O_2^--related processes on the systemic (phagocytes in the peritoneal cavity) and local (liver homogenates) levels at different stages of BCG-induced granulomatosis appear to indicate a possible role of hydrogen peroxide in intercellular communication during organization, maturation, and "dissociation" of granulomas in the dynamics of the process.

Conflict of Interests

The authors declare that there are no conflict of interests for their paper.

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Review Article

Reactive Oxygen Species and the Cardiovascular System

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Ever since the discovery of free radicals, many hypotheses on the deleterious actions of reactive oxygen species (ROS) have been proposed. However, increasing evidence advocates the necessity of ROS for cellular homeostasis. ROS are generated as inherent by-products of aerobic metabolism and are tightly controlled by antioxidants. Conversely, when produced in excess or when antioxidants are depleted, ROS can inflict damage to lipids, proteins, and DNA. Such a state of oxidative stress is associated with many pathological conditions and closely correlated to oxygen consumption. Although the deleterious effects of ROS can potentially be reduced by restoring the imbalance between production and clearance of ROS through administration of antioxidants (AOs), the dosage and type of AOs should be tailored to the location and nature of oxidative stress. This paper describes several pathways of ROS signaling in cellular homeostasis. Further, we review the function of ROS in cardiovascular pathology and the effects of AOs on cardiovascular outcomes with emphasis on the so-called oxidative paradox.

1. Introduction

In the mid 1950s, free radicals were first proposed to be involved in the pathophysiology of a number of diseases [1]. However, due to their short life span and the technical difficulty of detecting them, it took till the 1980s to recognize the importance of reactive oxygen species (ROS) as important players in biological systems [2]. Nowadays, it is widely accepted that ROS play a crucial physiological role, not only in various diseases, but also in cellular homeostasis [3].

ROS are chemically reactive molecules derived from molecular oxygen and formed as a natural by-product of aerobic metabolism. During energy conversion, ROS are produced as a by-product of oxidative phosphorylation, which is presumed to be the major source of superoxide ($O_2^{\bullet -}$) production [4, 5]. ROS can also be produced through a variety of enzymes including xanthine oxidase and NAD(P)H oxidase [3].

Under normal circumstances, ROS concentrations are tightly controlled by antioxidants, keeping them in the picomolar range [3]. These low concentrations of ROS enable their role as second messengers in signal transduction for vascular homeostasis and cell signaling. When excessively produced, or when antioxidants are depleted, ROS can inflict damage onto lipids, proteins, and DNA. This intracellular reduction-oxidation imbalance, called oxidative stress, can subsequently contribute to the development and/or progression of cardiovascular diseases such as atherosclerosis, ischemia-reperfusion injury, chronic ischemic heart disease, cardiomyopathy, congestive heart failure, and even ensuing arrhythmias [2, 6–8].

Apparently, within cellular physiology there is a paradoxical role for ROS, which is temporally and spatially defined. In this paper we will discuss this dual role by summarizing the aspects of ROS generation and metabolization in the cardiovascular system, with focus on the role of ROS in cardiovascular cell signaling, in particular hydrogen peroxide (H_2O_2). In addition, we will discuss the role of ROS in ischemic heart disease.

2. Molecular Basis of ROS

ROS encompass free radicals, oxygen ions, and peroxides, both organic and inorganic, but all derived from molecular oxygen. They are formed as necessary intermediates in

a variety of normal biochemical reactions [3]. Only when produced in excess or not appropriately controlled, they can inflict damage within the body.

A division can be made into two groups: free radicals and other ROS. Free radicals have an extremely high chemical reactivity due to the unpaired free electron (i.e., superoxide anion $O_2^{\bullet-}$ and hydroxyl radical $OH^{\bullet}$). Other ROS like H_2O_2 and peroxynitrite ($ONOO^-$) are not considered free radicals as they lack the free unpaired electron and thus have oxidizing rather than reactive effects [3, 9].

2.1. Formation of $O_2^{\bullet-}$. Within living cells, $O_2^{\bullet-}$ is produced through two distinct pathways, namely, enzymatically (Figure 1) and nonenzymatically. The latter occurs when a single electron is directly transferred to oxygen by reduced coenzymes or prosthetic groups (e.g., flavins or iron sulfur clusters).

For most tissues, the primary source of $O_2^{\bullet-}$ is situated in the mitochondrial electron transport chain. It contains several redox centers that may leak approximately 1%-2% of the electrons to oxygen [5, 10]. Enzymatically, $O_2^{\bullet-}$ is produced from a variety of substrates, through different enzymes, most importantly NAD(P)H (nicotinamide adenine dinucleotide phosphate) oxidases, xanthine oxidases, and endothelial nitric oxide synthase (eNOS), as will be discussed below [11].

NADPH/NADH oxidases, located on the cell membrane of polymorphonuclear cells, macrophages, and endothelial cells, play an important role in generation of $O_2^{\bullet-}$ [3, 9]. Under normal circumstances, NAD(P)H oxidases catalyze the reaction of NAD(P)H, H^+, and oxygen to form $NAD(P)^+$and H_2O_2. These oxidases are mainly present in adventitial fibroblasts but in different vascular pathologies, such as atherosclerosis and hypertension; upregulation of NAD(P)H expression has been shown in endothelial and vascular smooth muscle cells [12].

The conversion of xanthine dehydrogenase to xanthine oxidase (XO) provides another enzymatic source of $O_2^{\bullet-}$ and H_2O_2, which in turn constitutes a source of $OH^{\bullet}$. The relative amounts of $O_2^{\bullet-}$ and H_2O_2 formed by XO depend on the conditions. At physiological oxygen concentrations between 10%, and 21%, H_2O_2 constitutes about 75% of ROS formed, whereas at lower oxygen concentrations, H_2O_2 formation from XO approaches 95% [13].

The oxidase itself converts hypoxanthine into xanthine and xanthine into uric acid. Normally this process accounts for a small part of the ROS production but under pathological conditions, it has been proposed to mediate deleterious processes in vivo. For example, after reperfusion, large quantities of XO are released into the circulation possibly reacting with plasma purine substrates and molecular oxygen to produce ROS [14]. Chemical reactions forming molecular oxygen through NAD(P)H and xanthine oxidases are summarized in (1)–(4), respectively.

NAD(P)H oxidase reaction :

$$\mathrm{NAD(P)H} + 2\mathrm{O_2} \longrightarrow \mathrm{NAD(P)^+} + 2\mathrm{O_2^{\bullet-}} + 2\mathrm{H^-} \quad (1)$$

Xanthine oxidase (XO) reaction :

$$\mathrm{Hypoxanthine} + \mathrm{H_2O} + 2\mathrm{O_2} \longrightarrow \mathrm{Xanthine} + 2\mathrm{O_2^{\bullet-}} + 2\mathrm{H^-} \quad (2)$$

$$\mathrm{Xanthine} + \mathrm{H_2O} + 2\mathrm{O_2} \longrightarrow \mathrm{Uric\ acid} + 2\mathrm{O_2^{\bullet-}} + 2\mathrm{H^-} \quad (3)$$

$$(\mathrm{Hypo})\,\mathrm{Xanthine} + \mathrm{H_2O} + \mathrm{O_2} \longrightarrow \mathrm{Urate} + \mathrm{H_2O_2}. \quad (4)$$

Within vascular pathology, a potential important source of $O_2^{\bullet-}$ can be attributed to endothelial nitric oxide synthase [15]. There are three NOS isoforms, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). In most cardiovascular tissues, nNOS and eNOS are constitutively present [3]. NOS enzymes normally catalyze the conversion of L-arginine to L-citrulline and produce nitric oxide (NO). eNOS is a heterodimer with both reductase and oxygenase domains on each monomer. In order to produce NO via eNOS, electrons must be transferred from the cofactor NAD(P)H to flavin adenine dinucleotide and flavin adenine mononucleotide to heme. The electron flow through eNOS to L-arginine, resulting in the production of NO, is dependent on the availability of its cofactors [16].

The balance between nitric oxide (NO) and $O_2^{\bullet-}$ production is regulated by the availability of tetrahydrobiopterin (BH_4). BH_4 is involved in the catalytic process of L-arginine oxidation [17]. With impaired bioavailability of BH_4, $O_2^{\bullet-}$ is released rather than NO, a condition referred to as "eNOS uncoupling" [15] (Figure 2), where electrons that normally flow from the reductase domain to the heme group, now divert towards molecular oxygen rather than L-arginine [15, 18]. In vascular disease, a major part of this catalytic enzyme is uncoupled due to BH_4 deficiency. The consequent increase in $O_2^{\bullet-}$ rapidly reacts with NO to form peroxinitrite ($ONOO^-$). $ONOO^-$ oxidizes BH_4 leading to "eNOS uncoupling," and more production of $O_2^{\bullet-}$, thereby creating a vicious circle of ROS induced ROS production [15]. As will be discussed later on, the resulting endothelial dysfunction disturbs normal vascular responses and is associated with the development of atherosclerosis [11]. Importantly, endothelial dysfunction has been shown to be a prognostic factor for progression of atherosclerotic disease as well as cardiovascular event rate [19].

2.2. Reduction of $O_2^{\bullet-}$. Oxidation-reduction reactions are highly similar to acid-base reactions and concern the transfer of electrons. The key in these reactions is that electrons are exchanged between reaction partners and not shared as with covalent bindings. Oxidation-reduction reactions are matched set, meaning that for every oxidation reaction there is simultaneous reduction reaction and are therefore called "half-reactions."

Oxidation refers to loss of electrons, while reduction denotes the gain of electrons. The change of electrons between partners can be predicted by using the oxidation number, which is the algebraic difference between the number of protons and electrons in a specific ion. The produced intermediates are able to oxidize (by donating electrons) several molecules. When oxygen is scarce, cells move to a more

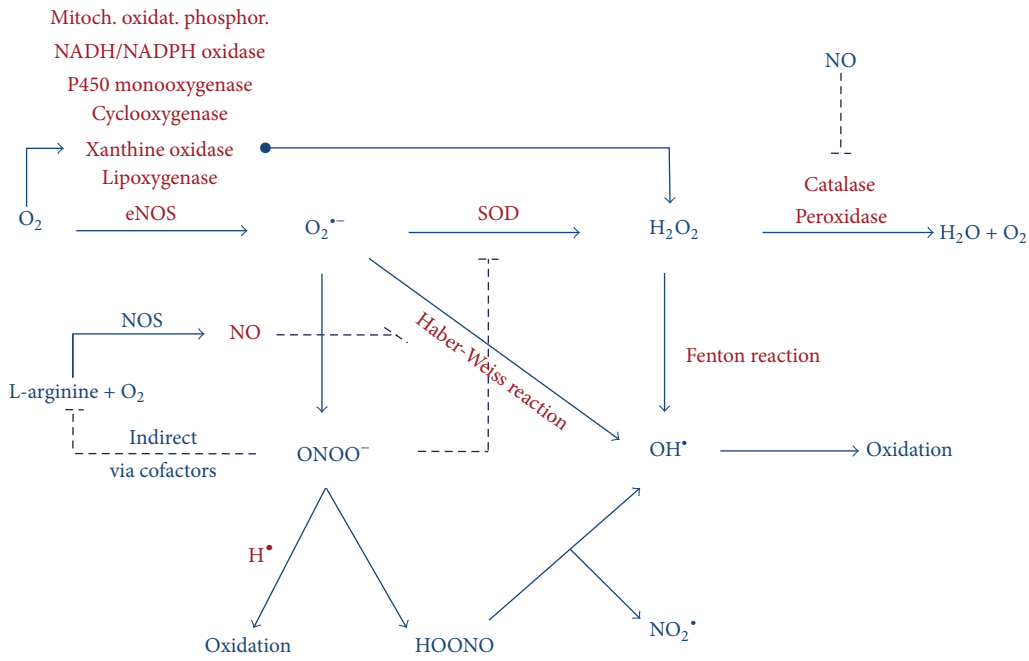

Figure 1: Summary of production and removal of various reactive oxygen species. Superoxide ($O_2^{\bullet -}$) can dismutate in several ways, either spontaneously through a reaction with superoxide dismutase (SOD), through the Haber-Weiss reaction, or in reaction with nitric oxide (NO) and its radical ($NO^\bullet$). Through SOD, hydrogen peroxide (H_2O_2) is formed and further reduced by catalase and peroxidase to form water and oxygen. Also, H_2O_2 can be formed directly from xanthine oxidase. The hydroxyl radical ($OH^\bullet$) is formed through the Haber-Weiss reaction, through the Fenton reaction, and from peroxynitrous acid (HOONO). $O_2^{\bullet -}$ can also scavenge NO to form peroxynitrite ($ONOO^-$) leading to nitroso-redox imbalance. NADH/NADPH oxidase: nicotinamide adenine dinucleotide (phosphate); (e)NOS: (endothelial) derived nitric oxide synthetase; NO: nitric oxide, NO_2: nitric dioxide; mitoch. Oxidat. phosphor, mitochondrial oxidative phosphorylation; dotted lines: inhibition.

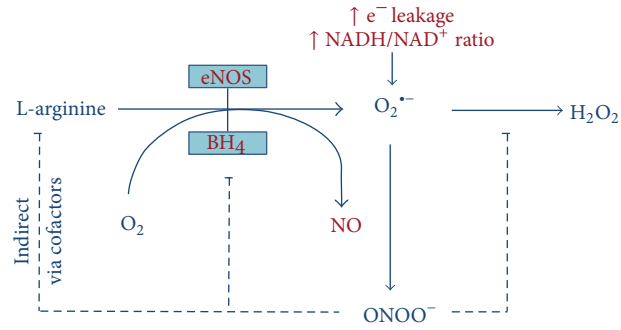

Figure 2: eNOS uncoupling. Excess superoxide ($O_2^{\bullet -}$) production, for example, after myocardial infarction, results in scavenging of nitric oxide (NO) to form peroxinitrite. The latter inhibits coupling of not only endothelial derived nitric oxide synthetase (eNOS) and tetrahydrobiopterin (BH_4), but also L-arginine and superoxide dismutase (SOD), which creates a downward spiral of enhanced $O_2^{\bullet -}$ production. Finally, eNOS gets uncoupled and produces $O_2^{\bullet -}$ rather than NO, sustaining the loop of nitroso-redox imbalance.

reduced state resulting in altered function of biomolecules. This redox signaling comprises oxireductive chemical reactions that alter proteins posttranslationally, thereby creating a coupling between redox state and cell function [6].

$O_2^{\bullet -}$ can dismutate (be reduced) in several ways (Figure 1), either spontaneously through a reaction with superoxide dismutase (SOD), through the Haber-Weiss reaction, or in reaction with NO. $O_2^{\bullet -}$ has a half-life of 10^{-9} to 10^{-11} s while in the presence of SOD, the half-life decreases to 10^{-15} s. The reaction catalyzed by SOD reduces two $O_2^{\bullet -}$ radicals to form oxygen and H_2O_2 which in turn can be fully reduced to H_2O and oxygen:

$$2O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2. \quad (5)$$

H_2O_2 is oxidized by peroxidase and catalase. It has a half-life of 10^{-3} s in the absence of catalase and 10^{-8} s in its presence. Alternatively, H_2O_2 can react with reduced transition metals—called the Fenton reaction—to form $OH^\bullet$ and OH^- or $OOH^\bullet$ and H^+, when combined with Fe^{2+} or Fe^{3+}, respectively [3]:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^\bullet + OH^- \quad (6)$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + OOH^\bullet + H^+. \quad (7)$$

The typical range for the iron dose is 1 part of Fe per 5–25 parts of H_2O_2. The optimal pH for the Fenton reaction is between 3 and 6. When the pH is too high, iron precipitates in $Fe(OH)_3$ and will decompose H_2O_2 into oxygen. The Fenton reaction occurs predominantly at the endoplasmatic

reticulum but not at mitochondria or other intracellular compartments [20]. Liu and coworkers showed that the Fenton reaction is involved in oxygen sensing, through regulation of genetic expression of hypoxia inducible factor-1 (HIF-1) [20]. An important role of HIF-1 is to establish the optimal balance between glycolytic and oxidative metabolism at any oxygen concentration to maximize ATP production without increasing ROS levels. Thus, HIF-1 induces metabolic reprogramming in cells that are oxygen deprived, thereby reducing mitochondrial respiration, minimizing $O_2^{\bullet-}$ production [21], and contributing to a fast responding oxygen-sensing system.

The reaction of $O_2^{\bullet-}$ with $NO^{\bullet}$, controlled by the rate of diffusion of both radicals, forms the very potent oxidant $ONOO^-$. $ONOO^-$ in turn is oxidized or reacts with a hydrogen radical ($H^{\bullet}$) to form the stable HOONO. The latter dismutates quickly into $OH^{\bullet}$ and free nitrogen species ($NO_2^{\bullet}$). Thus concentrations of $OH^{\bullet}$ increase by means of H_2O_2 and HOONO dismutation (metal independent pathway). Alternatively, $OH^{\bullet}$ can be generated through the Haber-Weiss reaction, when superoxide radicals and H_2O_2 molecules spontaneously combine to form molecular oxygen and hydroxyl radicals:

$$O_2^{\bullet-} + H_2O_2 \longrightarrow OH^{\bullet} + OH^- + O_2. \quad (8)$$

3. ROS and Cell Signaling

3.1. Signal Transduction Pathways of Cellular Responses to ROS. Most cells have been shown to generate a small burst of ROS when stimulated by, for example, cytokines, angiotensin II (Ang II), endothelin-1 (ET-1), and platelet derived growth factor (PDGF) [22], leading to the hypothesis that ROS play an important role in cellular homeostasis and communication [3] (Figure 3). ROS signaling involves alterations in the intracellular redox state and oxidative modification of regulatory and contractile proteins (Figures 4–5).

The intracellular redox state within cellular homeostasis is primarily balanced by the glutathione/glutathione disulfide couple which functions as a major redox buffer, indicating/determining the redox state of the cell [23]. Glutathione (GSH) is abundantly present in the cytosol, nucleus, and mitochondria. It is synthesized in the cytosol and transported to the mitochondria and the nucleus [24]. GSH exhibits protection against ROS by (a) participating in amino acid transport through the plasma membrane, (b) scavenging $OH^{\bullet}$, H_2O_2, and lipid peroxidases via glutathione peroxidase (GPx) (catalytic reaction), (c) being a cofactor in numerous detoxifying enzymes (e.g., GPx), and (d) regeneration of the most important AOs back to their active form [25]. The latter function is linked with the redox balance of GSH with its oxidized form GSSG [26].

Glutathione exists in reduced (GSH) and oxidized (GSSG) states. When reduced, the thiol group of cysteine can donate a reducing equivalent to other unstable molecules such as ROS. By doing so, glutathione itself becomes reactive and quickly reacts with another reactive glutathione to form glutathione disulfide (GSSG). Once oxidized, glutathione can be reduced back by glutathione reductase, thereby using NAD(P)H as an electron donor. Under normal physiological conditions, more than 90% of the glutathione in the cell is in the reduced form (GSH) and less than 10% exists in the disulfide form. An increase in GSSG/GSH ratio, for example due to inactivation of glutathione reductase by $ONOO^-$, is considered to be indicative of oxidative stress [27].

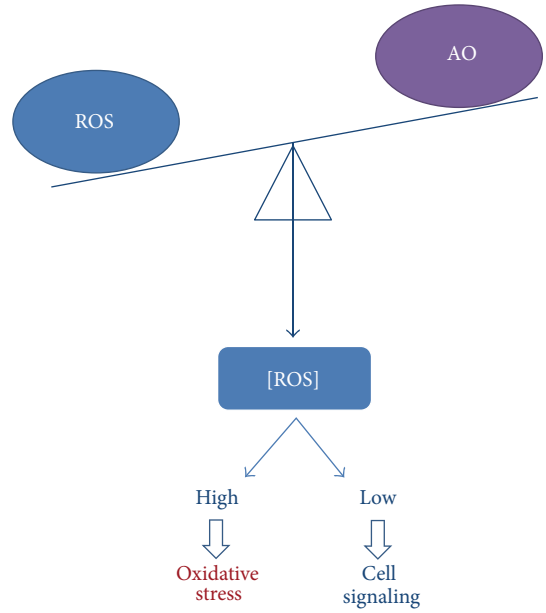

Figure 3: Redox balance. The production of reactive oxygen species (ROS) is tightly controlled by antioxidants (AOs) keeping the concentration of ROS ([ROS]) in the picomolar range. This low [ROS] is necessary for adequate cell physiology. When ROS is excessively produced or AOs are depleted, there is a high intracellular [ROS] leading to oxidative stress and resulting in cellular damage.

ROS can posttranslationally modify proteins. Redox signaling typically involves amino acid oxidation, hydroxylation, or nitration. Targets usually are redox sensitive cysteine residues within the proteins, which have a low ionization pKa of 4-5 compared to a pKa of 8.5 of nonreactive cysteines in other proteins [28, 29]. The modifications in these redox sensitive proteins alter their conformation, stability, activity and/or ability to interact with other proteins, resulting in modulation of cellular function. Redox sensitive proteins include proteins involved in calcium handling as well as contractile proteins, proteins involved in various signaling pathways and proteins involved in transcriptional activities.

Redox modulation of calcium handling proteins directly affects cardiac contraction by altering intracellular calcium. Examples of redox sensitive calcium handling proteins are calcium calmodulin kinase II (CaMKII), the ryanodine receptor on the sarcoplasmic reticulum, sarcoplasmic reticulum ATPase (SERCA), and phospholamban (for review see [29, 30]). Moreover, the contractile proteins can also be oxidatively modified by oxidation or nitrosylation [30]. Typically, oxidation of contractile proteins is assumed to depress cardiac function, although recently some modifications have been identified that actually increase contractility. The current understanding on how oxidative stress modulates cardiac function is limited mostly because many studies have

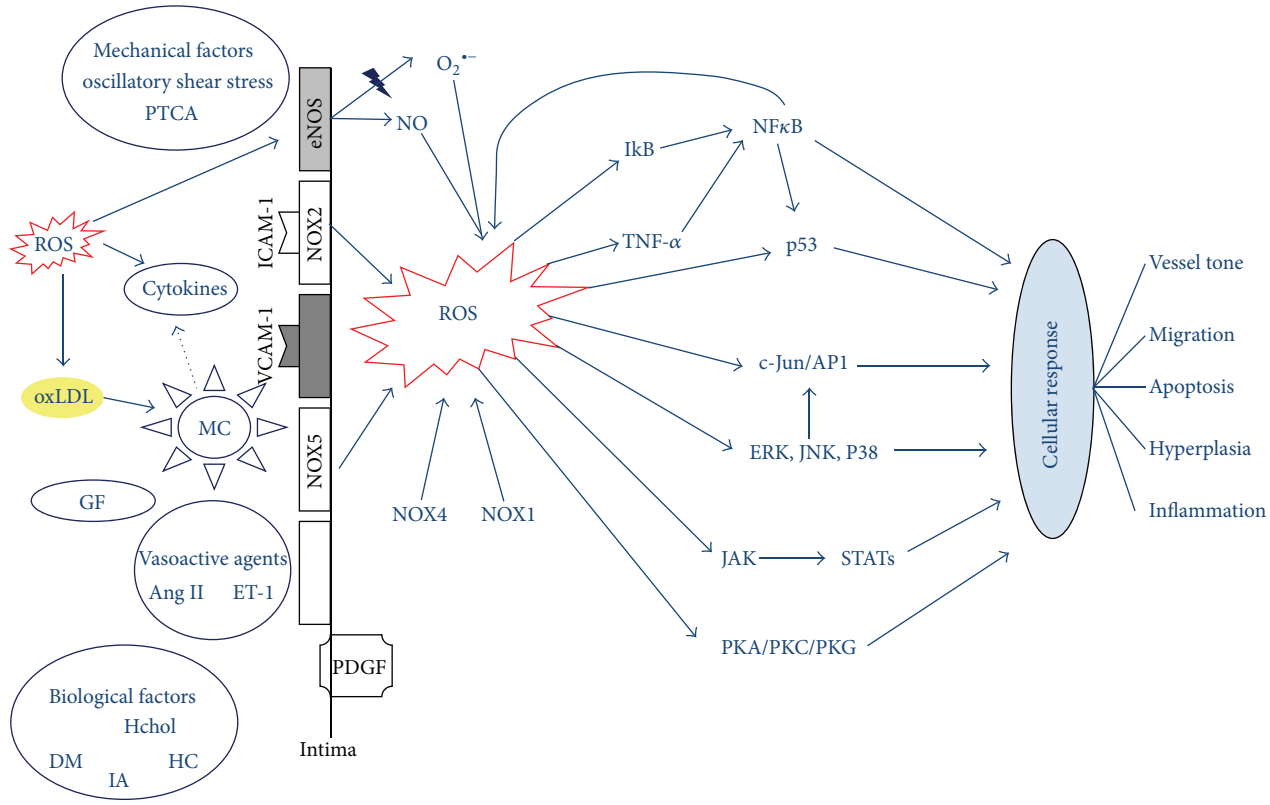

FIGURE 4: ROS production and vascular signaling. Several mechanical as well as circulating factors can increase ROS concentrations by acting on the tunica intima. The increased amounts of ROS activate specific second messenger systems which finally convey a cellular response. Hchol: hypercholesterolemia; DM: diabetes mellitus; IA: infectious agents; HC: homocysteine; MC: monocyte; GF: growth factors (PDGF, IGF-1, EGF, etc.); Cytokines (IL-1, TNF-α, etc.); oxLDL: oxidized low-density lipoprotein; eNOS: endothelial derived nitric oxide synthetase; PK: protein kinase A/C/G.

focused on isolated myofilament proteins whereas oxidative modifications of different contractile proteins occur simultaneously in vivo and act in concert. Hence, the contributions of the individual oxidative modification are difficult to establish [30].

The second group of proteins affected by direct redox modification are protein kinases and phosphatases. Since tyrosine phosphorylation is an early signaling event in many signal transduction pathways, alterations in activity through redox modification of protein kinases upstream in the signaling cascade result in indirect modulation of protein kinases more downstream in the cascade. Tyrosine phosphorylation in vascular smooth muscle cells is important in the control of vascular tone. Thus, tyrosine phosphatase inhibitors generally constrict smooth muscle, whereas tyrosine kinase inhibitors cause relaxation [28]. Oxidative modification results in inhibition of phosphotyrosine phosphatases (PTP 1A, PTP1B, and PTEN), while the protein kinase Src is activated by oxidation. Src has many targets in the cell. Interestingly, Src activates receptor tyrosine kinases such as the EGF-receptor in vascular smooth muscle cells. This activation occurs independent of EGF, and the activated receptor then acts as a signaling platform for the stimulation of phospholipase enzymes, production of lipid mediators, and activation of downstream kinases such as PI3K, Akt, ERK, and PKC [28].

PKC is directly activated by oxidation of the cysteine residues in its regulatory site, which occurs at low concentrations of oxidants. Conversly, PKC is inhibited by oxidation of cysteine residues in its catalytic domain, which occurs at higher concentrations of oxidants. Alterations in PKC activity affect many signaling cascades in the cell, including modulation of calcium sensitivity of the myofilaments and receptor tyrosine kinase signaling [28, 30]. The cAMP-dependent protein kinase A (PKA) and the cGMP-dependent protein kinase G (PKG) are also susceptible to redox modification. Both PKA and PKG are involved in regulation of vascular tone as well as cardiomyocyte contraction. When PKA oxidation occurs in its regulatory domain, it promotes dissociation of the catalytic and regulatory subunits resulting in cAMP independent PKA activation [29, 30]. However, similar to PKC, oxidation of cysteine residues in the catalytic subunit inhibits PKA activity [29]. Oxidation of PKG in its dimerization domain results in activation of the enzyme independently of the NO-cGMP pathway [29]. Oxidative modification of PKA, PKC, and PKG results in altered phosphorylation of the myofilaments, thereby modulating cardiac as well as vascular function.

The small monomeric G-proteins ras, rac-1, and RhoA are also activated by ROS. Activation of RhoA results in its translocation to the plasma membrane and activation of

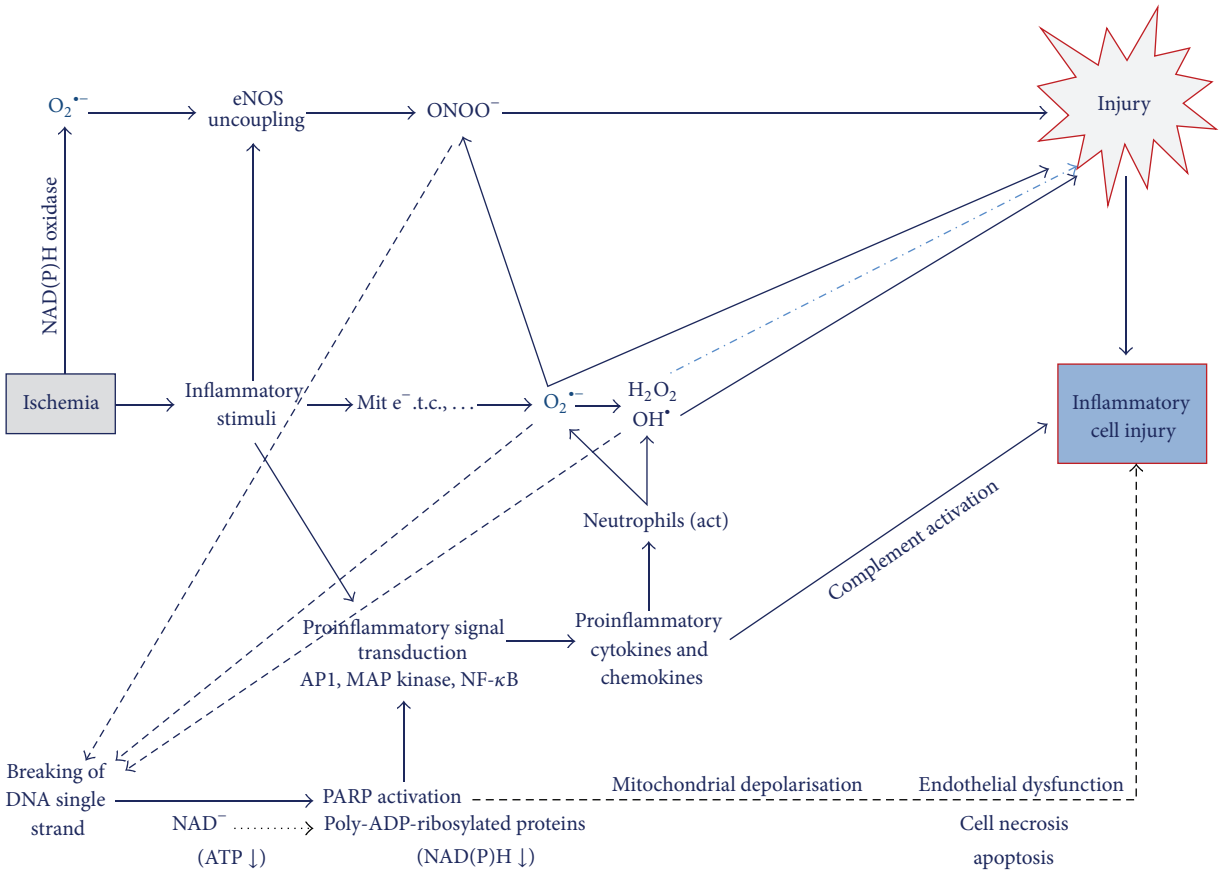

Figure 5: Different pathways leading to cell injury after ischemia. Ischemia due to atherosclerotic obstruction leads to an inflammatory process which provides the starting point of many other pathways of cellular injury via ROS production. Three main paths distinguished, being endothelial derived nitric oxide synthase (eNOS) uncoupling, mitochondrial electron transport, and proinflammatory signal molecules. Further, the produced ROS interact leading to DNA breaking and thus protein modification, with further cellular injury and dysfunction. Mit e^-.t.c.: mitochondrial electron transport chain; ATP: adenosine triphosphate; NAD(P)H: nicotinamide adenine dinucleotide phosphate oxidase; PARP: poly-ADP-ribosylated proteins; $O_2^{\bullet-}$: superoxide; H_2O_2: hydrogen peroxide.

Rho-kinase. Rho-kinase appears to be a key player in cardiovascular function and cardiovascular pathology. Thus, in vascular smooth muscle cells, Rho-kinase regulates calcium sensitivity of the myofilaments via inhibition of myosin light chain phosphatase [31]. Moreover, activation of Rho-kinase contributes to smooth muscle proliferation, hypertrophy and motility [32]. Rho-kinase activation is responsible for upregulation of NAD(P)H oxidases, thereby contributing to a vicious circle of ROS, leading to Rho-kinase activation resulting in more ROS production. In endothelial cells, Rho-kinase negatively regulates NO-production both by destabilizing eNOS mRNA and through impairment of eNOS activity [31, 32], thereby also contributing to augmentation of oxidative stress. In cardiomyocytes the role of Rho-kinase is less well understood although it is thought to function in a similar way to its role in vascular smooth muscle. In addition, Rho-kinase is thought to be involved in cardiomyocyte hypertrophy and apoptosis [31]. Yet, the precise role of Rho-kinase, as well as its modulation by redox regulation in cardiac myocytes, remains to be determined.

Another group of kinases that are not directly redox sensitive but very important in cardiovascular cell signaling are mitogen-activated protein kinases (MAPKs) (Figure 4). MAPKs are indirectly activated by ROS via the ROS sensitive kinases Src, PKC, ras, and the MAPK kinase kinase ASK-1 [33]. MAPKs are divided into three subgroups: extracellular signal regulated kinases (ERKs): ERK1 and ERK2; c-Jun N-terminal kinases (JNKs): JNK1, JNK2, and JNK3; and p38 kinases: p38 α, β, γ, and δ [3, 34, 35]. In addition to being indirectly activated by ROS, MAPKs are activated by environmental stresses and inflammatory cytokines, which are also known to induce oxidative stress.

The third important group of redox sensitive proteins in the cardiovascular system are the proteins involved in transcriptional activity, not only including transcription factors but also histone deacetylases (HDAC). ROS can both inhibit and stimulate cellular NF-κB signaling [36], while certain NF-κB regulated genes play a major role in regulating the amount of ROS in the cell. Also, recently ROS have been shown to directly connect the important redox sensitive transcription factors NF-κB and HIF-1, implicating a novel signaling pathway in cardiovascular pathology (Figure 5) [37].

Histone acetylation by histone acetylases promotes gene expression, while histone deacetylation by HDACs inhibits gene expression. Oxidation of HDAC4 and HDAC5 that are expressed in cardiac myocytes results in export of these HDACs from the nucleus, thereby inhibiting their activity. As these HDACs normally inhibit the transcription of prohypertrophic genese their oxidation may be involved in induction of hypertrophy [29].

The long-term consequences of ROS for cardiovascular (dys)function depend on the balance between signals promoting proliferation or growth inhibition and/or cell death. ROS can alter this balance leading to either excessive angiogenesis or loss of endothelial cells [2]. The dual role of ROS in "fine-tuning" the balance between apoptosis and excessive cell growth is illustrated by observations that, during ischemia-reperfusion injury, ROS trigger apoptosis, while ROS generated during ischemic preconditioning prevent apoptosis [38–41]. ROS generated during ischemic preconditioning are capable of upregulating expression of the Bcl-2 gene [42], which regulates the intrinsic pathway of apoptosis [43, 44]. This gene is also activated via the nuclear transcription factor NF-κB and its activation has been shown to reduce apoptosis [42, 45].

Conversely, endothelial apoptosis initiated by tumor necrosis factor-α (TNF-α) and mediated by activation of JNK can be attenuated by ROS scavenging [46]. TNF-α has been implicated in inflammatory responses of the heart and vasculature. Thus, TNF-α is one of the inflammatory cytokines that are produced in the ischemic region and surrounding myocardium following myocardial infarction. Also, vessels from subjects with diabetes are characterized by an increased TNF-α production, increased ROS production, and endothelial dysfunction [47]. Similarly, endothelial dysfunction induced by advanced glycation end products (AGEs) is mediated through elevated TNF-α expression and induction of ROS production with NF-κB functioning as the link between TNF-α and AGEs/RAGE signaling [48, 49]. TNF-α and NF-κB are interrelated in that translocation of NF-κB into the nucleus has been proposed to be pro-inflammatory and, either directly or indirectly, leads to a significant increase in TNF-α production while TNF-α activates NF-κB, which then regulates genes involved in inflammation, oxidative stress, and endothelial dysfunction [49, 50]. Interestingly, ROS produced in response to TNF-α can further activate NF-κB which again activates TNF-α creating a vicious circle [51]. Hence, ROS play a key role in the induction of vascular dysfunction in response to TNF-α [47].

3.2. The Role of H_2O_2 in Signal Transduction. H_2O_2 is an interesting molecule within the ROS family. It is a waste product of mitochondrial electron transfer and, hence to be created, no additional energy is required. The chemical properties of H_2O_2, such as a short half-life, rapid metabolization by catalase, and rapid reaction with thiols, are ideal properties for H_2O_2 to act as a signaling molecule.

H_2O_2 mediates diverse physiological responses including cell differentiation, proliferation, and migration, and has been proposed to be involved in metabolic vasodilation [3, 70]. In cells stimulated with growth factors and cytokines (PDGF, EGF, insulin, TNF-α, and interleukins), the NAD(P)H oxidase gp91 Phox and homologues form the major source of H_2O_2 [34, 70–72]. However, the coupling between receptor activation to the NAD(P)H oxidase complex (Nox) is still poorly understood [73]. In order to mediate different responses, H_2O_2 modifies the activity of key signaling proteins. It catalyzes redox reactions, oxidizing primarily cysteine residues of proteins thereby altering their function. For example, the activity of tyrosine phosphatases is H_2O_2 dependent. The chemical configuration of these phosphatases contains a cysteine and arginine site resulting in a low PK_a and existing as a thiolate anion. The latter is more susceptible to H_2O_2 oxidation which abolishes its activity and is reversed by cellular thiols [74]. Not only tyrosine phosphatase but also tyrosine kinase (Src) is oxidized by H_2O_2 [75]. An overall regulation must exist, that is, both temporal and spatial, ensuring process activation (only when and where needed) and termination after exerting its effects.

In order to induce protein alterations, H_2O_2 must increase rapidly above a certain threshold, but each cell contains natural AO enzymes. Therefore H_2O_2 has to be protected from destruction. H_2O_2 is inactivated by peroxiredoxin, which is part of a family of antioxidant enzymes whose thioredoxin peroxidase activity plays an important role in protecting against oxidative stress [76]. Interestingly, H_2O_2 causes hyperoxidation, and thereby inactivation, of peroxiredoxin, prolonging H_2O_2 bioavailability. The inactive peroxiredoxin can be reactivated by the adenosine triphosphate-dependent enzyme sulfiredoxin [77].

How H_2O_2 is actually delivered to the cytosol remains incompletely understood. H_2O_2 must cross the lipid bilayer towards the target molecules in the cytosol. Although it is generally assumed that H_2O_2 crosses the membrane freely, recent research indicates some membranes to be poorly permeable to H_2O_2 [78]. A shift in membrane lipid composition or a transport through aquaporins was presented as an alternative pathway to transfer H_2O_2 molecules to the cytosol [79]. Alternatively, H_2O_2 may also pass through gap junctions to exert its effect [80–82].

There is clearly a duality present in the role for H_2O_2 in cell proliferation. On the one hand, low concentrations of H_2O_2 play an important role in regulating cell growth, although the question remains if this effect is exerted merely through second messengers (JAK/STAT) or if H_2O_2 also has a direct effect on growth. On the other hand, high concentrations of H_2O_2 are responsible for cell apoptosis while moderate doses cause the cell to arrest in the G1 phase [83]. Recently, H_2O_2-induced apoptosis was shown to be mediated through a PKC-dependent pathway, antagonized by Akt and heme oxygenase-1 [84]. Also, recent data support the hypothesis that H_2O_2 can function as a transmitter of the apoptotic signal from the region of programmed cell death to neighboring healthy cells [85]. Apoptotic cells produce H_2O_2, thereby possibly contributing to the pathogenesis of, for example myocardial infarction and ageing. More specifically, mitochondrial produced ROS are needed for the generation of the apoptotic signal since specifically designed mitochondrial antioxidants (such as SkQs) inhibit this pathway [86].

H_2O_2 may also be involved in the response to vascular injury. Vascular smooth muscle cell (VSMC) death has been shown to occur after mechanical trauma like stenting. In response to vascular injury, potent chemotactic factors, such as bFGF, PDGF, TGF-β, and Ang II, are released [2]. These chemotactic factors in turn regulate VSM proliferation and migration. Two independent research groups have shown that this release of chemotactic factors is H_2O_2 dependent and thus reduced when administrating a scavenger like catalase [87, 88]. The hypothesis for ROS involvement in VSMC proliferation is further supported by a study showing that catecholamine induced VSCM proliferation can be blocked by N-acetylcysteine, tiron (superoxide scavenger), and diphenylene iodonium [89]. To allow VSMC migration and vascular remodeling, degradation of the extracellular matrix is required, which is partially accomplished by matrix metalloproteinases (MMPs). Both activity and expression of MMP-2 and MMP-9, the two MMPs thought to be most important in vascular remodeling, have been shown to be regulated by the nitroso-redox balance. Thus, H_2O_2 and $OONO^-$ increase MMP-2 activity while reduction of MMP-2 and MMP-9 can be obtained by overexpressing eNOS [90, 91]. Not only the activity of the MMP family is modulated by ROS but also their expression, thereby providing a dual mechanism for ROS to regulate vascular remodeling.

3.3. ROS and Pathophysiology (Figures 4 and 5). The mitochondrial respiratory chain is one of the most prominent cellular sources of ROS. Hence, ROS production is related to oxygen consumption, making cells with high oxygen consumption more prone to oxidative stress. Oxygen consumption is particularly high in cardiac myocytes that are therefore equipped with a high number of mitochondria and a high level of respiratory chain components per milligram of mitochondrial protein. To prevent oxidative damage, these cells contain enzymatic scavengers of ROS such as SOD, glutathione peroxidase, catalase, and coenzyme Q10. Possibly together with nonenzymatic AO, they neutralize the deleterious effects of ROS. Mitochondria also possess the ability to repair themselves after oxidative damage using enzyme systems like phospholipid hydroperoxide glutathione peroxidase (PHGP). PHGP is a selenium containing enzyme directly reducing peroxidized acyl groups in phospholipids [92]. However, under pathological conditions, these protective mechanisms may fall short and make the cardiac myocytes vulnerable to oxidative damage.

With increased production of ROS, damage can be inflicted directly via oxidative modification of redox sensitive proteins [3, 10]. Also, inflammation, which in turn stimulates the release of $O_2^{\bullet-}$, leads to cell injury, either directly or by depleting the natural AOs. An overview of the systems leading to cell damage via $NO^{\bullet}$, $ONOO^-$, $O_2^{\bullet-}$, $H_2O_2/OH^{\bullet}$, complement activation, and PARP activation is presented in Figures 3, 4, and 5.

Some examples of how oxidative stress is involved in cardiac and vascular pathologies are described in the following sections.

3.4. Hypoxia, Ischemia, and Reperfusion. Medical strategies treating acute myocardial infarction require restoration of blood flow to the ischemic region. Unfortunately, this reperfusion is associated with a burst of ROS, which may continue for hours [93], and recruitment of inflammatory cells [94, 95]. These high levels of ROS cause structural damage of the heart, capillary leak, and influence cardiomyocyte metabolism thereby impairing both systolic and diastolic function [96]. Furthermore, not only ischemic damage, but also reperfusion, can produce dysfunction of the cardiac conduction system leading to arrhythmias [7, 97]. Besides increased ROS production, hypoxia, ischemia, and reperfusion have also been found to reduce levels of SOD, GSH, glutathione peroxidase, and ascorbate [98]. Hence, reduced scavenging further contributes to development of oxidative stress.

Reperfusion also inflicts damage on the vascular endothelium, with alterations in blood cells and microembolization, as well as vascular compression due to myocyte swelling, leading to changes in endothelial structure and alignment. The duration of ischemia is an important determinant of the extent of reperfusion damage. Ischemia-reperfusion damage can be reduced by ischemic preconditioning [99]. The mechanisms underlying this protection by ischemic preconditioning are incompletely understood [100]. Yet, a role for ROS as triggers for and mediators of this protective phenomenon has been consistently demonstrated. ROS can trigger preconditioning by causing activation of the mitochondrial K_{ATP} channel, which then induces generation of ROS and NO that are both required for preconditioning induced protection [101–104]. Importantly, ischemic preconditioning can be mimicked by administration of free radical donors S-morpholinosydnonimine [105] and even $ONOO^-$ [106] while preconditioning can be blocked by free radical scavengers.

Ischemic preconditioning is therefore a clear example of the so-called oxidative paradox: AOs not only reduce deleterious ROS accumulation but also molecules necessary for cardioprotection.

3.5. Atherosclerosis. The majority of cardiovascular disease is a direct consequence of atherosclerosis. The transfer of oxidized low-density lipoprotein (ox-LDL) from the vessel lumen into the tunica media is regarded as the initiator for atherosclerosis at sites with endothelial damage [107]. Mechanical factors like fluid shear stress patterns play an important role in maintenance of endothelial function and initiation of endothelial dysfunction [3]. Thus, laminar shear stress induces expression of AO genes and production of $NO^{\bullet}$, preventing apoptosis and monocyte adhesion [108]. Branched arteries exposed to oscillatory shear stress are prone to atherosclerosis. This type of flow leads to continuous NAPDH-dependent production of $O_2^{\bullet-}$ [109, 110]. Increased $O_2^{\bullet-}$ can subsequently uncouple eNOS, thus creating an extra source of $O_2^{\bullet-}$ production and leading to a vicious circle of ROS-induced ROS production. Upregulation of adhesion molecules (VCAM-1, E-selectin, P-selectin, and ICAM-1) [111] at locations with disrupted flow patterns is also ROS

Table 1: Major studies with possible beneficial effects of AOs on cardiovascular outcomes in humans.

Author/study	Journal	Design/FU	Population*	Agents (dosage/day)	Results
Stephens et al./ CHAOS [52]	The Lancet	DB, PC 1.4 y (3 d–3 y)	$N = 2002$; M and F; mean 61.8 y; ischemic heart disease patients; secondary prevention	E (800 mg or 400 IU)	↓↓ I nonfatal MI, trend ↑ CV death
Duffy et al. [53]	The Lancet	DB, PC 30 d	$N = 45$; M and F; mean 48.5 y; HT patients	C (500 mg)	↓ BP in otherwise healthy HT
Boaz et al./ SPACE [54]	The Lancet	DB, PC 2 y	$N = 196$; M and F; 40–75 y; haemodialysis patients	E (800 IU)	↓↓ combined endpoint of AMI = CV death + stroke
Neri et al. [55]	Clinical Therapeutics	DB, PC 15 d	$N = 46$; M and F; mean 40 y; DM/glucose intolerance patients	NAC (600 g) + C (250 mg) + E (300 mg)	↓ OS and inflammation
Accini et al. [56]	Nutrition, Metabolism and Cardiovascular Diseases	DB, PC 4 m	$N = 57$; M and F; 23–65 y; dyslipidemic patients	E (4 mg); PUFAn-3 (6602 mg EPA + 440 DHA); niacin (18 mg); γOZ (40.2 mg)	↓ OS and inflammation markers

The CHAOS study is the largest study to report a strong decrease in nonfatal MI but, conversely, a slight increase in cardiovascular death. Other studies were performed in smaller groups. Overall, no overwhelming positive effects could be found in the studies. Population*: N: number of patients; M: male; F: female; y: age in years. DB: double blind; PC: placebo controlled; d: days; m: months; E: vitamin E; C: vitamin C; NAC: N-acetylcysteine; PUFAn-3: polyunsaturated fatty acids n-3; EPA: eicosapentaenoic; DHA: docosahexaenoic; γOZ: γ-oryzanol; I: incidence; BP: blood pressure; CV: cardiovascular; MI: myocardial infarction; HT: hypertension; AMI: acute myocardial infarction; OS: oxidative stress.

dependent and is further enhanced by cytokines like interleukins, TNF-α, Ang II, and vascular endothelial growth factor (VEGF) [3, 109–111]. Upregulation of adhesion molecules facilitates adherence and transmigration of leucocytes (Figures 4 and 5). Once converted to macrophages, they are capable of producing much higher amounts of ROS. ROS convert ox-LDL into highly oxidized LDL which itself is engulfed by these macrophages, forming foam cells and initiating the formation of the fatty streak.

Overall, the amount and pattern of blood flow are very important for endothelial function where aberrant flow patterns predispose to ROS production and atherosclerosis.

3.6. Clinical Evidence for Therapeutic Use of Antioxidants? The deleterious effects of ROS can be reduced by restoring the imbalance between production and clearance of ROS [112]. Gey and colleagues found low rates of cardiovascular disease (CVD) in people consuming AO rich diets [113]. This second line of defense includes nonenzymatic antioxidant substances from dietary intake such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), GSH, flavonoids, carotenoids, and others. In vitro studies indicated oxidation inhibition of low-density lipoproteins (LDL) by a number of these nonenzymatic AOs. Exogenous therapeutic administration of antioxidants has therefore been proposed as therapy for oxidative stress and cardiovascular disease. Despite some promising effects of such AO administration (Table 1), particularly in animal studies, caution should be warranted as these results could usually not be reproduced in clinical trials. Thus, conflicting results on the use of dietary supplementation of AOs, especially vitamin C, vitamin E, β-carotene, and selenium, have been presented (Table 2). AO supplementation is potentially deleterious for normal "redox homeostasis." Not only is the redox balance very delicate, but also ROS play important roles in cell signaling and are therefore essential for survival of the organism [3, 9, 114]. The most relevant AOs used in dietary supplementation are flavonoids and vitamins C and E. The beneficial cardiovascular effects of these substances may not be limited to their AO effect, as they also include anti-inflammatory, platelet inhibitory, and antithrombotic effects.

One of the most studied AO supplements in prevention of cardiovascular disease is vitamin E. Many studies suggest a protective role for vitamin E, which has led to a massive marketing of vitamin E supplements. Conversely, large randomized studies (Table 2) could not substantiate this role for vitamin E. Indeed, meta-analysis failed to find a cardio protective effect nor did it find a reduction of clinical events in high risk patients or in patients with established disease [115–117]. Interestingly, a recent analysis even reports an increased mortality after using supplements of β-carotene and vitamin E [117].

When comparing Table 1 with Table 2, clinical trials fail to show a protective effect of AOs in humans. Not only are the positive outcome studies largely outnumbered by trials with no effects, they are also, in most cases, lacking the statistical power to be conclusive. The reason for failure of these AOs in clinical practice is most likely multifactorial.

First of all, it is very difficult to detect subjects with a comparable imbalance between ROS production and AO defenses. Second, biochemical aspects inherent to each substance used, posology, and intake ratio must be taken into account. The lack of knowledge of the optimal dosage and route of administration for the various AOs is a serious limitation. For example, the bioavailability of vitamin C is determined by the availability of its transporter in the small intestine, and an increase of oral administration of vitamin C can actually decrease bioavailability [118]. This example illustrates that pharmacodynamical and pharmacokinetical

Table 2: Major studies with no beneficial effects of AOs on cardiovascular outcomes.

Author/study	Journal	Design/FU	Population*	Agents (dosage/day)	Results
Hennekens et al./ physician health [57]	The New England Journal of Medicine	DB, PC, 2 × 2 12 y (βC)	N = 22.071; all M, 40–84 y; former or current smokers	βC (50 mg) on alt days	No effect on CV death, AMI, or all-cause mortality
Rapola et al. [58]	The Lancet	DB, PC 5.3 y	N = 1862; all M; 50–69 y; smokers with previous MI	E (50 mg) + βC (20 mg)	No ↓ of MCE, ↑ risk FCHD
Virtamo et al. [59]	Archives of Internal Medicine	DB, PC 6.1 y	N = 27.271; all M; 50–69 y; smokers, no MI history; primary prevention	E (50 mg) + βC (20 mg)	E: ± ↓ I fatal CHD, no ↓ I nonfatal CHD; βC no effect
Italiano/GISSI Prevenzione Investigators [60]	The Lancet	OL, PC, 2 × 2 3.5 y	N = 11.234, M and F; stratified for all age groups; AMI within 3 months; secondary prevention	E (600 mg) + fish oil (10 mg)	E: no effect AMI + death + stroke, fish oil: ↓ AMI + death + stroke
Yusuf/HOPE [61]	The New England Journal of Medicine	DB, PC, 2 × 2 4.5 y	N = 9451; M and F; ≥55 y; high risk CD patients; primary and secondary prevention	E (800 mg or 400 IU) Ramipril	E: no effect AMI + CV death + stroke; Ramipril: ↓ AMI + CV death + stroke
De Gaetano/PPP [62]	The Lancet	OL, PC, 2 × 2 3.6 y	N = 4495; M and F; mean 64.4 y; high risk CD patients; primary prevention	E (300 mg) Aspirin(100 mg)	E: no effect Aspirin: ↓ AMI + CV death + stroke
Collins et al./ HPSCG [63]	The Lancet	DB, PC 5 y	N = 20.563; M and F; 40–80 y; CD, other OAD, DM patients	C (250 mg) + E (600 mg) + βC (20 mg)	No ↓ 5 y mortality
Törnwall et al. [64]	European Heart Journal	DB, PC 5–8 y	N = 29.133; all M; 50–69 y; smokers with risk on MCE or MI history	E (50 mg) or βC (20 mg) or both	βC: ↑ risk nonfatal MI; E: no effect
Armitage et al./ HPS [65]	BMC Medicine	DB, PC, 2 × 2 5.5 y	N = 20.536; M and F; 40–80 y; high risk CD patients; primary and secondary prevention	Simvastatin (40 mg) C (250 mg) + E (600 mg) + βC (20 mg)	AO: no effect
Cook et al./WACS [66]	Archives of Internal Medicine	DB,PC, 2 × 2 9.4 y	N = 8171; all F; ≥1 CVE in history, secondary prevention	C (500 mg) + E(600 IU) on alt days + βC (50 mg) on alt days	No effect AMI + CV death + stroke + morbidity
Lee et al. [67]	Journal of The American Medical Association	DB, PC, 2 × 2 10.1 y	N = 39.876; all F; >45 y, healthy.	E (600 IU) Asiprin (100 mg)	No benefit for major CV events. No effect on total mortality
Lonn/HOPE II [68]	Journal of The American Medical Association	DB, PC 7.0 y	N = 3994, >55 y with vascular disease or DM; extension of HOPE I trial.	E (400 IU)	No prevention of major CV events. No prevention of cancer. Risk of HF may be ↑.
Kataja-Tuomola et al. [69]	Annals Medicine	DB, PC, 2 × 2 6.1 y	N = 29.133, all M smokers, some with DM.	E (50 mg/d) βC (20 mg/d)	No protective effect on macrovascular outcomes or total mortality.

Large multicenter studies all presented the same result that oral AOs had no beneficial effect on cardiovascular outcomes. Some studies even showed an increased risk of coronary heart disease. Population*: N: number of patients; M: male; F: female; y: age in years; 2 × 2: 2 × 2 factorial design comparing placebo, agent A, agent B, and combination of agent A and B; DB: double blind; PC: placebo controlled; E: vitamin E; C: vitamin C; βC: beta-carotene; FCHD: fatal coronary heart disease; MCE: myocardial event; CHD: coronary heart disease; AMI: acute myocardial infarction; CV: cardiovascular; OS: oxidative stress; IU: international units; HF: heart failure.

properties of each AO should be known to optimize their application. To be effective, it is imperative that AOs reach the specific compartments of the cell where ROS are generated. For many vascular cells, this requires uptake into the cytoplasm (or vesicles) [2]. Importantly, AOs can become oxidants in some cellular compartments.

Third, lack of clinical improvement may be attributed to the selection of the population. Most patients enrolled in the

clinical trials have already established CVD and in these cases AO therapy may be too late to be effective since, in animal studies, most protocols describe administration of AOs prior to the initiation of the disease. Finally, and perhaps most important, the choice of AOs should be based on the identity and location of ROS responsible for pathology. So instead of experimenting with cocktails of AOs in human trials, basic research should focus on targeting the specific pathways of different ROS responsible for the given pathology [112, 119].

The lack of cardiovascular benefit of AOs that are presently available has initiated research on new and more effective compounds. Clinical studies show that while these novel compounds do not reduce endpoints related to atherosclerosis, they improve endothelial function by increasing local NO bioavailability and therefore endothelium-dependent vasodilatation. Promising new agents are NO-donor phenols [120] and AGI-1067 that inhibits pro-inflammatory gene expression [121]. Other potentially promising AOs act through targeting NAPDH oxidases, Nox (VAS2870), and Nox2 peptide (gp91-dstat), preventing eNOS uncoupling or inhibiting xanthine oxidase (allopurinol) [2, 72, 119]. Technological developments also allow discovery of new functions for existing AOs. For example, oxidative damage of DNA was shown to be repaired in cells by naturally occurring phenols independent of known DNA repair enzymes thereby entailing possible new approaches [122].

Moreover, targeting AO therapy at specific sites of ROS production may be more effective in treatment of cardiovascular disease than global AO therapy [123]. Mitochondrial ROS scavenging is effective in treating hypertensive rats [124] and led to the realization that mitochondria play a central role in the pathogenesis of cardiovascular disease. New pharmacological approaches enable targeting of therapeutic substances at the mitochondria [125]. In particular, AOs conjugated with triphenylphosphonium cation such as mitoquinone (MitoQ), mitovitamin E, and mitophenyltertbutyline achieve much higher concentrations in the mitochondrial membrane, than those in the cytosol, due to the negative membrane potential of the inner mitochondrial membrane [126]. A new type of compounds, named SkQs, consisting of an antioxidant moiety (plastoquinone) and a penetrating cation, has been synthetized. This group of AOs specifically prevented oxidation of mitochondrial cardiolipin, arrested H_2O_2-induced apoptosis, and blocked necrosis initiated by ROS [86, 127]. Furthermore, SkQs also appear very promising in inhibiting the development of age-related diseases [86, 128]. However, so far, no studies have been performed that target mitochondria in cardiovascular disease in humans.

4. Conclusion

ROS play a dual role in cardiovascular (patho)physiology. ROS signaling plays an important part in endothelial function, vascular tone, and cardiac function. Conversely, when excessively produced, ROS can disrupt cellular signaling and inflict cellular damage. It thus appears that concentration and location of ROS are the main determinants of their function.

Due to their very short half-life and technical difficulties of measuring ROS in vivo, little is known about the "safe margins" of ROS concentrations in cell signaling. Therefore, it is difficult to estimate which part of ROS production contributes to cellular homeostasis and normal physiological functioning and when ROS production becomes excessive and thereby detrimental.

Although the deleterious effects of ROS can potentially be reduced by restoring the imbalance between production and clearance of ROS through administration of AOs, the dosage and type of AOs should be tailored to the location and nature of oxidative stress. Continuous administration of AOs in vivo can be unfavorable for normal cell signaling which, at least partially, explains the lack of clinical evidence on the beneficial actions of AO administration.

New research should focus on matching AO therapy to oxidant stress present in the cardiovascular system. In vitro studies are extremely important to obtain knowledge on the mechanisms of oxidative damage as well as potential repair mechanisms, and when extrapolated to the in vivo setting with caution, they are likely to contribute to improve the therapeutic strategies for cardiovascular disease.

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Research Article

Adaptive Redox Response of Mesenchymal Stromal Cells to Stimulation with Lipopolysaccharide Inflammagen: Mechanisms of Remodeling of Tissue Barriers in Sepsis

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Acute bacterial inflammation is accompanied by excessive release of bacterial toxins and production of reactive oxygen and nitrogen species (ROS and RNS), which ultimately results in redox stress. These factors can induce damage to components of tissue barriers, including damage to ubiquitous mesenchymal stromal cells (MSCs), and thus can exacerbate the septic multiple organ dysfunctions. The mechanisms employed by MSCs in order to survive these stress conditions are still poorly understood and require clarification. In this report, we demonstrated that *in vitro* treatment of MSCs with lipopolysaccharide (LPS) induced inflammatory responses, which included, but not limited to, upregulation of iNOS and release of RNS and ROS. These events triggered in MSCs a cascade of responses driving adaptive remodeling and resistance to a "self-inflicted" oxidative stress. Thus, while MSCs displayed high levels of constitutively present adaptogens, for example, HSP70 and mitochondrial Sirt3, treatment with LPS induced a number of adaptive responses that included induction and nuclear translocation of redox response elements such as NFkB, TRX1, Ref1, Nrf2, FoxO3a, HO1, and activation of autophagy and mitochondrial remodeling. We propose that the above prosurvival pathways activated in MSCs *in vitro* could be a part of adaptive responses employed by stromal cells under septic conditions.

1. Introduction

It is well documented that common complications of traumatic injury and acute irradiation syndrome are bacterial infection and associated sepsis, which are considered as the major factors of high morbidity and mortality of the illnesses [1–4]. Sepsis has been defined as the acute systemic inflammatory response syndrome that occurs during infection and toxicosis [2]. Therefore, work in the field of septic shock has long focused on inflammation as the leading pathogenic mechanism. However, a variety of therapeutic approaches, mainly anti-inflammatory in nature, have failed to cure human sepsis (e.g., studies involving IL-1α, TNF-β, prostaglandins, leukotrienes, etc.) [2, 5]. Due to the failure of anti-inflammatory strategies, the physician community faces the question of whether inflammation or immunosuppression is the driving factor of death from sepsis [2, 5]. This problem leads to the search for other potential mechanisms that could produce adverse effects on host metabolome resulting in septic toxicosis. The elucidation of other major (vital) pathways affected by the oxidative stress (redox stress) from the acute bacterial inflammation is critical to solving this problem. Indeed, cumulative oxidative effects of reactive oxygen and nitrogen species (ROS and RNS, resp.) generated

in overreactive responses of the reticuloendothelial, endothelial, and lymphoepithelial cells to bacteria and bacterial factors can eventually alter integrity of tissue barriers, which sustains immunochemical homeostatic interactions of tissues with internal and external environments.

It has been well determined that one of the essential constituents of tissue barriers is mesenchymal stromal cells (MSCs) [6–9]. Although MSCs are considered to be ubiquitously integrated into conjunctive, vascular, skin, lung, intestinal, and other tissues, their major source in the body is the bone marrow, which releases MSCs upon injury and inflammation [6–9]. The data obtained recently from the research conducted on bone marrow MSCs show that these cells display antibacterial and immunomodulatory properties, can moderate septic toxicosis and improve survival in experimental sepsis [10–14]. Moreover, the effector system which mediates MSCs response to inflammatory stimuli, such as LPS, is composed of a network of toll-like receptors and pattern-recognition receptors [14], for example, the molecular machinery that can also promote inflammatory redox stress [15–18]. In conjunction with these phenomena, there are numerous data gathered from various models indicating that, paradoxically, the inflammagens directly and indirectly can also induce the cellular prosurvival adaptive mechanisms mediated by the redox-response elements and autophagy [18–27]. So far, there is only limited information on the adaptive mechanisms enabled in MSCs under inflammatory conditions [14, 28]. In part, it could be due to complicity of architecture of mesenchymal network in tissues. Therefore, in the current work we explored primary cultures of mouse bone marrow MSCs challenged with lipopolysaccharide (LPS) inflammagen.

We hypothesized that (i) a challenge of MSCs with LPS could result in the redox stress; (ii) the adaptive response of MSCs to the redox stress was accompanied by upregulation of the redox-response factors such as thioredoxin-1 (Trx1), apurinic apyrimidinic endonuclease redox effector factor 1 (Ref1), nuclear factors NFκB, forkhead box O3a (FoxO3a), and NF-E2-related factor 2 (Nrf2), heme oxygenase 1 (HO1), and autophagy; (iii) activation of autophagy in the LPS-challenged MSCs enabled remodeling of the damaged cellular constituents including mitochondria. The objective of this communication is to provide experimental evidence of a potential role of MSCs in sustaining redox homeostasis of tissue barriers under the septic oxidative stress.

2. Materials and Methods

Mouse bone marrow mesenchymal stromal cells (MSCs) phenotype and features are well defined in recent reviews [14]. The establishment of MSC cultures used in the present research was described previously [11] when they were determined to be the bone marrow colony-forming unit fibroblasts [11, 29]. They lack hematopoietic and endothelial lineage markers (CD45, CD34, CD4, and CD117) but are positive for a wide variety of other cell surface molecules (CD44, CD105, and Sca1). The cells expressed collagen type III and matrix metalloproteinases types 3, 9, and 13 and responded to stimulation with the platelet-derived growth factor. These cells were expanded and cultivated in hypoxic conditions (5% O_2, 10% CO_2, 85% N_2) in Mesencult medium (Stemcell Technologies Inc.).

MSC cultures were grown to approximately 80% confluency prior to being used in the experiments. LPS (Sigma-Aldrich Co., catalog number L4391) from *E. coli* 0111:B4 was used in concentrations of 0.05–2.5 μg/mL. Challenge of MSCs with LPS was conducted in a "pulse" mode for 1–3 h, and then incubation medium was replaced with a fresh one. Pyrrolidine dithiocarbamate (PDTC, 10 μM) was used to inhibit NFκB-mediated response to the LPS challenge as reported recently [30].

The challenged cells were either fixed or harvested and, then lysed at different time points following LPS challenge (1–24 h). The obtained cell lysates were kept frozen at −80°C until further analyses. The LPS-induced gene and protein expressions were determined by qRT-PCR and immunoblotting techniques. Fluorescence imaging techniques were used for (i) assessment of nuclear translocation of p65 subunit of NFκB, that is, (p65) NFκB, thioredoxin 1 (TRX1), Ref1, and nuclear factor (erythroid-derived 2)-like 2, (Nrf2), (ii) expression and activity of iNOS, (iii) assessment of formation of ROS and RNS with dihydrorhodamine 123 assay, (iv) assessment of apoptotic transformations with annexin V assay, (v) formation of LC3-containing autophagosomes and mitochondrial fusion, and (vi) estimation of proliferative activity with Ki67 marker. LPS-induced mitochondrial remodeling and mitophagy, that is, autophagy of mitochondria, were demonstrated through transmission electron microscopy (TEM).

For qRT-PCR analyses, total cellular RNA was isolated from MSCs using the Qiagen RNeasy Miniprep kit, quantified by measuring the absorbance at 260 nm and qualified by electrophoresis on a 1.2% agarose gel. cDNA was synthesized using SuperScript II (Invitrogen) and qRT-PCR was performed using SYBR Green iQ Supermix (Bio-Rad), each according to the manufacturers' instructions. The following primer sequences were used for qRT-PCR: iNOS Forward 5′ CAGCTGGGCTGTACAAACCTT 3′; iNOS Reverse 5′ CATTGGAAGTGAAGCGTTTCG 3′; IL-1a Forward 5′ CGGGTGACAGTATCAGCAAC 3′; IL-1a Reverse 5′ GACAAACTTCTGCCTGACGA 3′; IL-1b Forward 5′ CCCAACTGGTACATCAGCAC 3′; IL-1b Reverse 5′ TCTGCTCATTCACGAAAAGG 3′; IL-6 Forward 5′ AGTCGGAGGCTTAATTACACATGTT 3′; Il-6 Reverse 5′ AAGTGCATCATCGTTGTTCATACA 3′; IL-8 Forward 5′ GCGCCTATCGCCAATGAG 3′; IL-8 Reverse 5′ AGGGCAACACCTTCAAGCTCT 3′. The quality of qRT-PCR data was verified by melt curve analysis, efficiency determination, agarose gel electrophoresis, and sequencing. Relative gene expression was calculated by the method of Pfaffl using the formula $2^{-\Delta\Delta Ct}$.

For protein analyses, MSCs were lysed and total proteins were extracted in accordance with the protocol described previously [11]. Aliquots of proteins were resolved on SDS-polyacrylamide slab gels (NuPAGE 4%–12% Bis-Tris; Invitrogen, Carlsbad, CA, USA). After electrophoresis, proteins were blotted onto a PDVF membrane and the blots were incubated with antibodies (1 μg/mL) raised against MAP LC3,

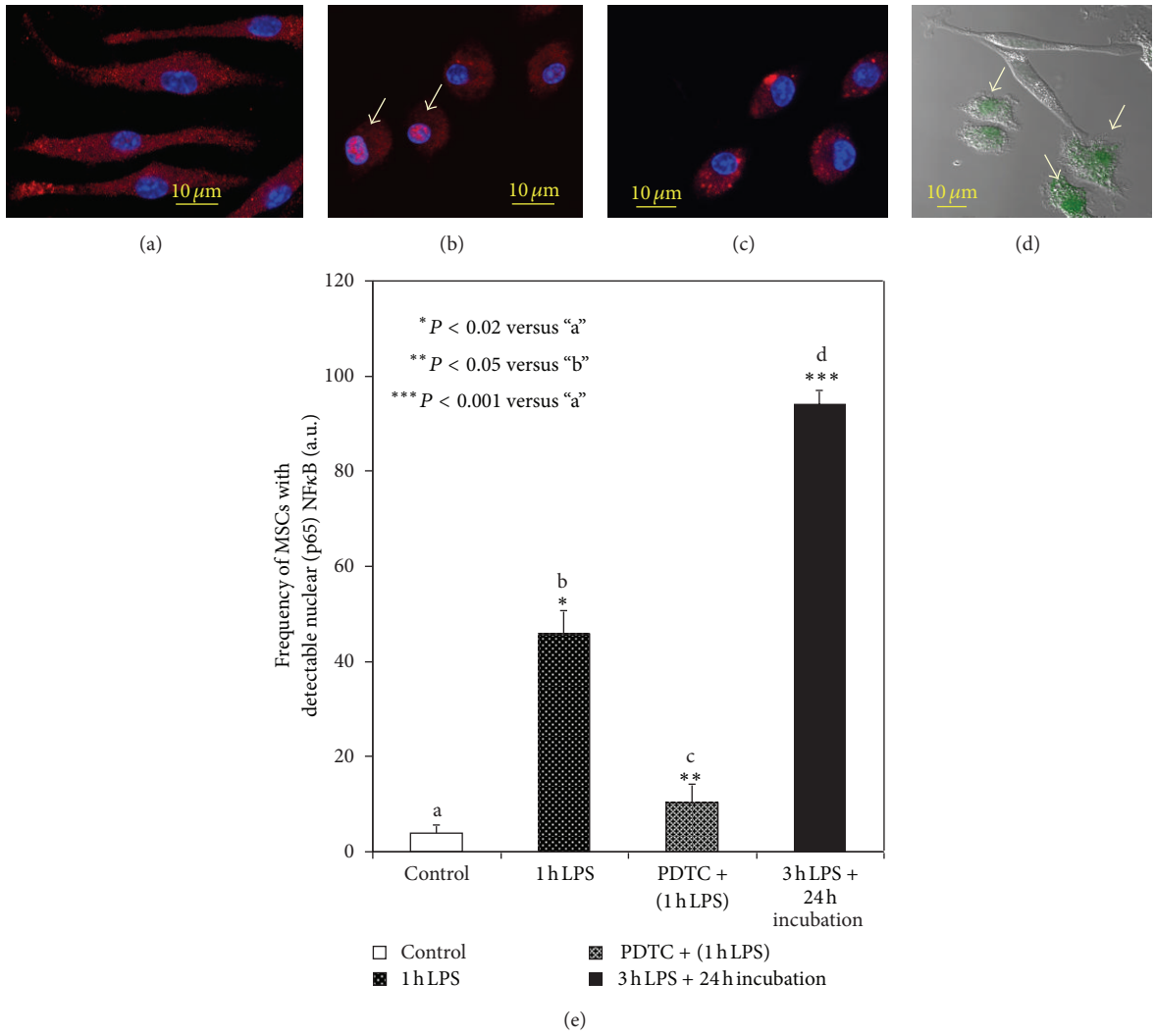

Figure 1: Confocal immunofluorescence assessment of nuclear translocation of (p65) NFκB in MSCs following stimulation with LPS. ((a)–(c)) Overlay of projections of nuclei (blue channel) and (p65) NFκB (red channel) in MSCs. (a) Control cells. (b) MSCs challenged with 500 ng/mL LPS for 1 h. Activation of (p65) NFκB nuclear translocation was defined by an increase in immunofluorescence of (p65) NFκB in the nuclear regions. Nuclear regions of MSCs were determined by counterstaining of nuclear DNA with Hoechst 33342 (blue channel). Nuclear localization of (p65) NFκB appears in purple (indicated with arrows). (c) is the same as (b) except that MSCs were preincubated with 10 μM PDTC (pyrrolidine dithiocarbamate), an inhibitor of NFκB. (d) The treatments were the same as in (c) but the presented image is overlay of projections of Annexin V (green channel), a marker of proapoptotic transformations, and a respective DIC image of MSCs. Apoptotic events are indicated with arrows. The confocal images were taken with pinhole setup to obtain 0.5 μm Z-sections. Conditions: MSCs were incubated with 500 ng/mL LPS in the medium either 1 h or 3 h (see Section 2). (e) Histogram depicting increase in frequency (per 100 cells) of occurrence (p65) NFκB nuclear translocation in MSCs treated with LPS. Spatial appearance of (p65) NFκB in MSCs was determined with immunofluorescence confocal microscopy as indicated in Section 2. Confocal projections of nuclear fractions of (p65) NFκB are shown in ((a)–(c)).

Nrf2, (p65) NFκB, HSP70, Sirt3, p62/SQSTM1, HO1, iNOS, and actin (Abcam, Santa Cruz Biotechnology Inc., EMD Millipore, and Sigma-Aldrich, Co.) followed by incubation with species-specific IgG peroxidase conjugate.

For expression and spatial localization of proteins in MSCs, the cells (5 specimens per group) were fixed in 2% paraformaldehyde, processed for immunestaining, and analyzed with fluorescence confocal microscopy [11]. Normal donkey serum and antibody were diluted in phosphate-buffered saline (PBS) containing 0.5% BSA and 0.15% glycine. Any nonspecific binding was blocked by incubating the samples with purified normal donkey serum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1 : 20. Primary antibodies were raised against MAP LC3, iNOS, Ref1, Trx1, (p65) NFκB, Nrf2, FoxO3a, p53, and Tom 20 (a mitochondrial marker). That was followed by incubation with secondary fluorochrome-conjugated antibody and/or streptavidin-Alexa Fluor 610 conjugate (Molecular Probes, Inc., Eugene, OR, USA), and with Heochst 33342 (Molecular Probes, Inc., Eugene, OR, USA) diluted 1 : 3000. Secondary

Table 1: qRT-PCR assessment of gene transcription in MSCs challenged with LPS.

LPS ng/mL	IL1A Expression, a.u.	IL1β Expression, a.u.	IL6 Expression, a.u.	IL8 Expression, a.u.	iNOS Expression, a.u.
0	1 ± 3.25	1 ± 1.82	1 ± 1.54	1 ± 1.41	1 ± 3.14
50	194 ± 2.77*	317 ± 1.63*	99 ± 1.48*	26 ± 1.8*	882 ± 2.46*
100	265 ± 2.33*	465 ± 1.78*	155 ± 1.4*	46 ± 1.36*	1128 ± 2.3*
500	315 ± 2.35*	755 ± 1.68*	47 ± 2.67*	18 ± 1.98*	1596 ± 2.46*
1000	247 ± 2.38*	498 ± 1.78*	98 ± 1.6*	11 ± 1.61*	1489 ± 2.37*
2500	338 ± 2.35*	729 ± 1.57*	224 ± 2.32*	20 ± 3.33*	1438 ± 2.25*

Conditions: MSCs were incubated with 50 ng/mL–2500 ng/mL LPS for 3 h and then were lysed for mRNA extraction and qRT-PCR analysis. The presented data are statistically significant at the confidence level $^*P < 0.005$, ($n = 3$).

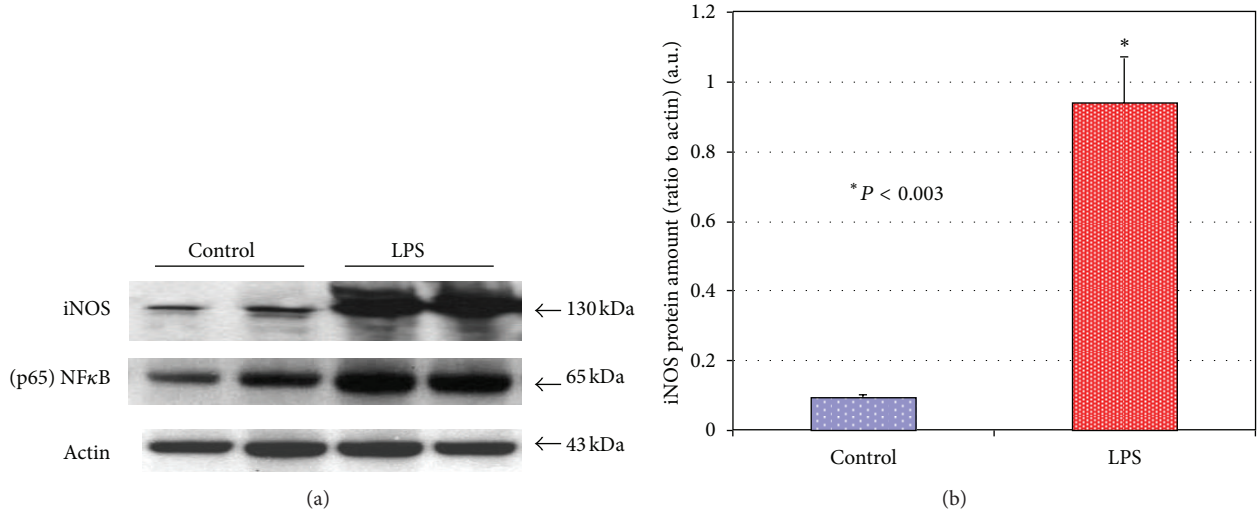

Figure 2: Immunoblot analysis of expression of NFκB and iNOS proteins in MSCs subjected to LPS challenge. (a) Immunoblotting bands of NFκB and iNOS. (b) Densitometry histograms of iNOS bands in MSCs subjected to challenge with LPS. The presented bars indicate the relative density of iNOS protein (normalized to density of actin bands). The statistical significance was determined by Student's t-test ($n = 3$). Conditions: MSCs were incubated with 500 ng/mL LPS for 3 h. The cells were harvested at 24 h following challenge with LPS.

antibodies used were Alexa Fluor 488 and Alexa Fluor 594 conjugated donkey IgG (Molecular Probes Inc., Eugene, OR, USA). Negative controls for nonspecific binding included normal goat serum without primary antibody or with secondary antibody alone. Five confocal fluorescence and DIC images of crypts (per specimen) were captured with a Zeiss LSM 710 microscope. The immunofluorescence image analysis was conducted as described previously [12].

Analysis of nitric oxide (NO) formation in LPS-challenged MSC was as follows. DAF-FM diacetate (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate, Life Technologies Corp) was utilized for detection of NO formation in living cells 24 h after challenge with LPS (500 ng/mL). DAF-FM is essentially nonfluorescent until it reacts with NO to form a fluorescent benzotriazole. The reagent solution (5 μM in PBS) was applied to the cells and a formation of the fluorescent adduct was monitored with a confocal Zeiss LSM 710 microscope. L-N^6-(1-iminoethyl)lysine (LNIL, Sigma-Aldrich Co.), a selective inhibitor of iNOS, was used for suppression of NO production in the cells.

Dihydrorhodamine 123 (DhRho 123, Life Technologies Corp) was utilized for detection of formation of ROS and RNS (i.e., peroxynitrite) in the cells 24 h after challenge with LPS (500 ng/mL). Dihydrorhodamine 123 is an uncharged and nonfluorescent reactive oxygen species (ROS) indicator that can passively diffuse across membranes where it is oxidized to cationic rhodamine 123 which localizes in the mitochondria and the cytoplasm and exhibits green fluorescence. The reagent solution (10 μM in PBS) was applied to the cells and a formation of the fluorescent product was monitored with a confocal Zeiss LSM 710 microscope. L-N^6-(1-iminoethyl)lysine (LNIL, Sigma-Aldrich Co), a selective inhibitor of iNOS, was used for suppression of NO release and the consequent RNS-dependent oxidation of DhRho 123 in the cells.

For transmission electron microscopy (TEM), MSCs in culture were fixed in 2% formaldehyde and 2% glutaraldehyde in PBS overnight, postfixed in 2% osmium tetroxide in PBS, dehydrated in a graduated series of ethanol solutions, and embedded in Spurr's epoxy resin. Blocks were processed

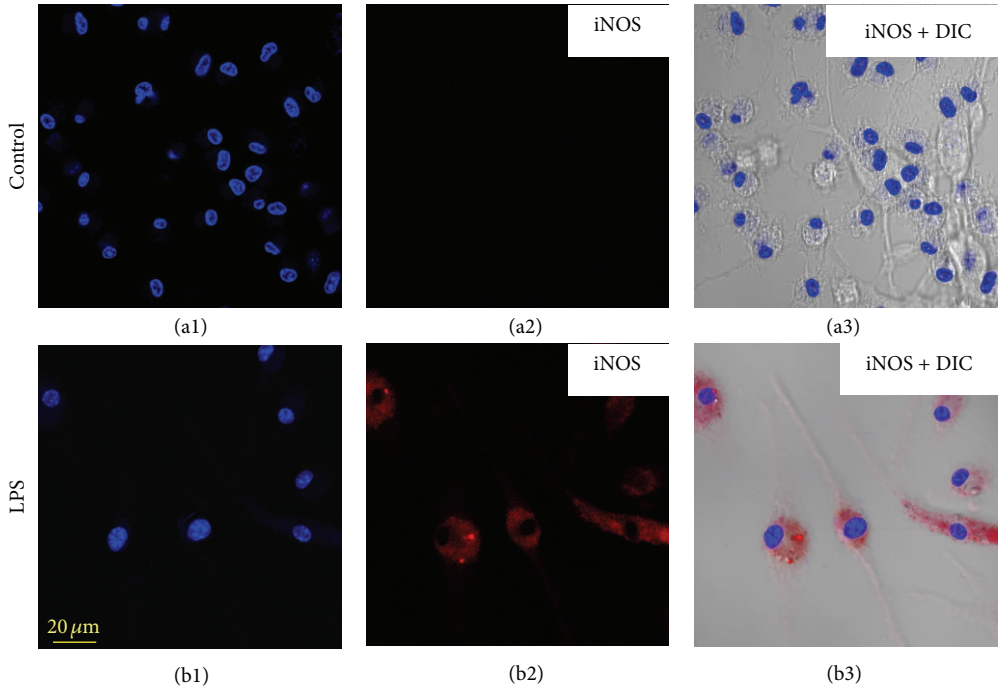

Figure 3: Confocal immunofluorescence imaging of iNOS protein in MSCs challenged with LPS. (a1) Projections of nuclei (blue channel) in control MSCs. (a2) Projections of iNOS (red channel), (a3) overlay of projections presented in (a1) and (a2) and a respective DIC image. (b1) Projections of nuclei (blue channel) in LPS-challenged MSCs. (b2) Projections of iNOS (red channel) and (b3) overlay of projections presented in (b1) and (b2) and a respective DIC image. A massive accumulation of iNOS occurred in the LPS-challenged MSCs. Counterstaining of nuclei was with Hoechst 33342 (blue channel). The confocal images were taken with pinhole setup to obtain 0.5 μm Z-sections. Conditions were the same as indicated in Figure 2.

as described previously [11]. The sections of embedded specimens were analyzed with a Philips CM100 electron microscope.

Statistical significance was determined using Student's t-test for independent samples. Significance was reported at a level of $P < 0.05$.

3. Results

In the first set of experiments, we assessed alterations in the MSC stress-response proteins following LPS challenge. LPS, a major component of the outer membrane of gram-negative bacteria, is considered to be a strong inflammagen. In stromal cells, LPS-induced activation of Toll-like receptor type 4 triggers a danger signal leading to nuclear translocation of NFκB and subsequent upregulation of several known inflammatory mediators including iNOS producing NO [14, 26, 30]. Ultimately, LPS-induced effects can result in redox stress.

As previously published, control MSCs have relatively high amounts of constitutive NFκB [11]. Confocal immunofluorescence imaging of (p65) NFκB revealed in controlled MSCs that the protein immunoreactivity was predominantly present in the cytoplasm (Figure 1(a)). The (p65) NFκB projections in LPS-challenged cells are shown in Figure 1(b). These data suggest that the challenge of the cells with LPS promoted a prompt (within 1 h) increase in the nuclear fraction of NFκB (Figure 1(b), indicated with arrows). The nuclear translocation of (p50)(p65) NFκB is considered to be a part of antiapoptotic response to stress-induced factors [14, 19]. Therefore, preincubation of MSCs with 10 μM PDTC, an inhibitor of the NFκB pathway, suppressed nuclear translocation of (p65) NFκB (Figure 1(c)) that was accompanied by proapoptotic transformations and a loss of cell confluency after application of LPS (Figure 1(d)). A summary of quantitative assessment of these LPS-induced effects is presented in a histogram in Figure 1(e). As shown in Figures 1(e) and 1(d), an increase in nuclear translocation of (p65) NFκB was observed in almost all MSCs treated with 500 ng/mL LPS (3 h pulse) 24 h after-treatment. The observed LPS-induced transactivation of NFκB in MSCs was accompanied by a drastic expression of proinflammatory mediators including IL-1α, IL-1β, IL-6, and iNOS that occurred in a dose-dependent manner (Table 1, qRT-PCR analysis).

A maximum expression of iNOS was observed at a dose of 500 ng/mL LPS (Table 1); therefore, our further experiments on LPS-induced MSC toxicity were conducted using this dose. It should be noted that while MSCs displayed resistance to substantially higher doses of LPS (up to 5000 ng/mL), they experienced inhibition of proliferative activity under those conditions (data not shown). Moreover, there is evidence (Dr. Elliott TB, unpublished data) that the LPS dose 500 ng/mL in blood can induce in mice a severe septic syndrome with a predicted mortality rate 80%–90%.

LPS-pulse challenge for 3 h resulted in prolong changes in redox-status of MSCs. Thus, 24 h after the LPS challenge we observed increases in (p65) NFκB translocation in ≈90%

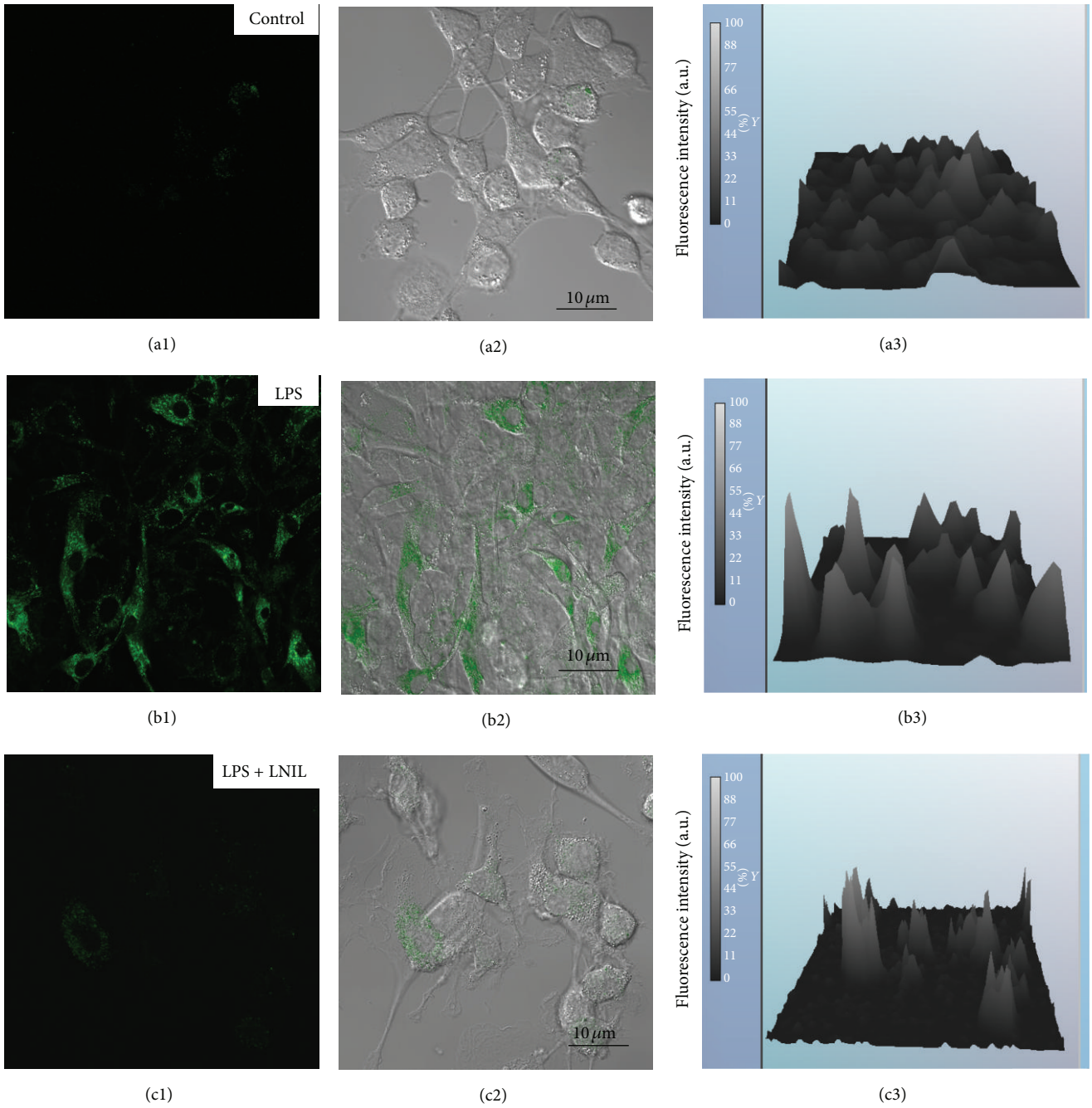

Figure 4: Confocal immunofluorescence imaging of the DAF-FM—detectable nitric oxide in MSCs challenged with LPS. (a1) Projection of adduct of DAF-FM with NO (DAF-FM-NO) (green channel) in control MSCs. (a2) Overlay of projection of DAF-FM-NO shown in (a1) and a respective DIC image. (a3) Histogram of relative fluorescence of DAF-FM-NO shown in (a1). (b1) Projection of adduct of DAF-FM-NO (green channel) in LPS-challenged MSCs. (b2) Overlay of projection of DAF-FM-NO shown in (b1) and a respective DIC image. A dramatic increase in DAF-FM-NO fluorescence occurred in the LPS-challenged MSCs. ((c1)–(c3)) Same as (b1–b3) except that LPS-challenged MSCs were treated with LNIL, an iNOS inhibitor. Suppression of DAF-FM-NO fluorescence occurred in the LPS-challenged MSCs.

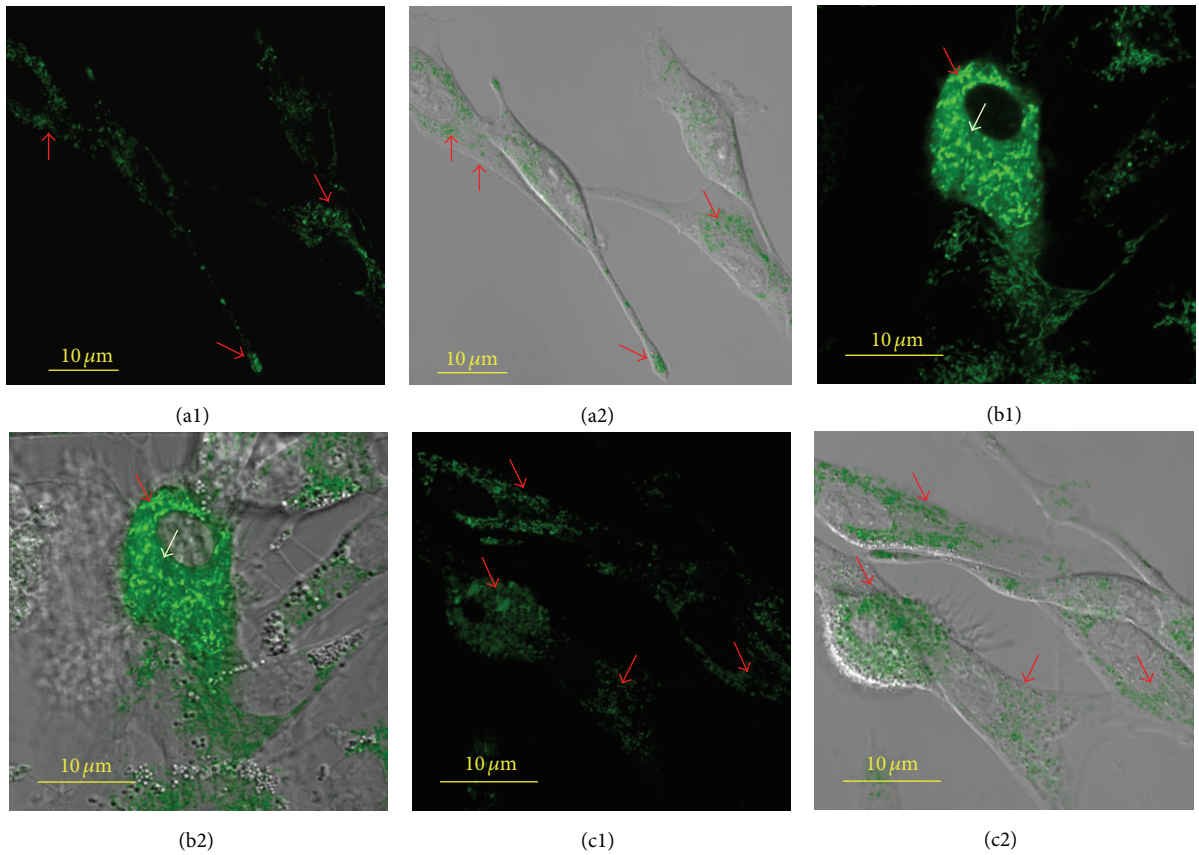

Figure 5: Confocal immunofluorescence imaging of the DhRho 123 detectable ROS/RNS products in MSCs challenged with LPS. (a1) Projection of oxidized form of DhRho 123 (Rho 123) (green channel) in control MSCs. (a2) Overlay of projection of Rho 123 shown in (a1) and a respective DIC image. (b1) Projection of Rho 123 (green channel) in LPS-challenged MSCs. (b2) Overlay of projection of Rho 123 shown in (b1) and a respective DIC image. A dramatic increase in Rho 123 fluorescence occurred in the LPS-challenged MSCs. (c1) Projection of Rho 123 (green channel) in LPS-challenged MSCs and treated with LNIL, an iNOS inhibitor. (c2) Overlay of projection of Rho 123 shown in (c1) and a respective DIC image. Suppression of Rho 123 fluorescence occurred in the LPS-challenged MSCs. Bright green fluorescence of the ROS-activated Rho 123 shown in mitochondria is shown with red arrows. Diffused green fluorescence of the ROS/RNS-activated Rho 123 in the cytoplasm is shown with white arrows in (b). The confocal images were taken with pinhole setup to obtain 0.5 μm Z-sections. The experimental conditions were the same as indicated in Figure 2.

of the treated MSCs (Figure 1(e)) that was accompanied by a dramatic accumulation of iNOS protein in the cells (Figure 2). The immunoblotting data presented in Figure 2 were confirmed by immunofluorescence imaging of iNOS protein in the cells (Figure 3). Accumulation of iNOS resulted in excessive production of NO in the cells determined by increase in fluorescence intensity of the fluorescent adduct of DAF-FM with NO in the LPS-treated cells (Figures 2 and 2). This effect was suppressed in the presence of LNIL, a specific iNOS inhibitor (Figure 2). An increase in the formation of ROS and RNS associated with the LPS stimulation was monitored with DhRho 123, another molecular probe, which is subjected to oxidation in the presence of ROS and peroxynitrite and is thus converted to fluorescent Rho 123. The results of DhRho imaging in the cells are presented in Figure 5. As shown in the figure, in control cells a moderate Rho 123 fluorescence appeared only in mitochondria, a major generator of ROS under normal condition (Figure 5(a)). Dramatic changes in Rho 123 fluorescence were observed in MSCs after treatment with LPS (Figure 5(b)). Indeed, compare to control group these MSCs were characterized by substantially increased fluorescence of Rho 123 in mitochondria (Figures 5(c) and 5(d)). Moreover, green fluorescence of Rho 123 also occurred in the entire cytoplasm (Figures 5(c) and 5(d)). This observation indicated that in LPS-treatment of MSCs also caused induction of the ROS/RNS generating pathways outside the mitochondrial bodies and resulted in redox stress. Interestingly, elongation of mitochondria due to activation of mitochondrial fusion was observed under these conditions (Figure 5(b)). This increase in Rho 123 fluorescence was suppressed in the presence of LNIL, a specific iNOS inhibitor (Figure 5(c)). Overall, the presented data suggested that the LPS-challenged cells experienced the redox stress due to increase in iNOS-dependent production of NO. Therefore, we expected upregulation of the redox-response elements mediating cell adaptation to long-lasting stress conditions.

It is well accepted that numerous vitagenes are evolutionarily adapted by cells to manage oxidative stress; they include but are not limited to redox-sensitive transcriptional

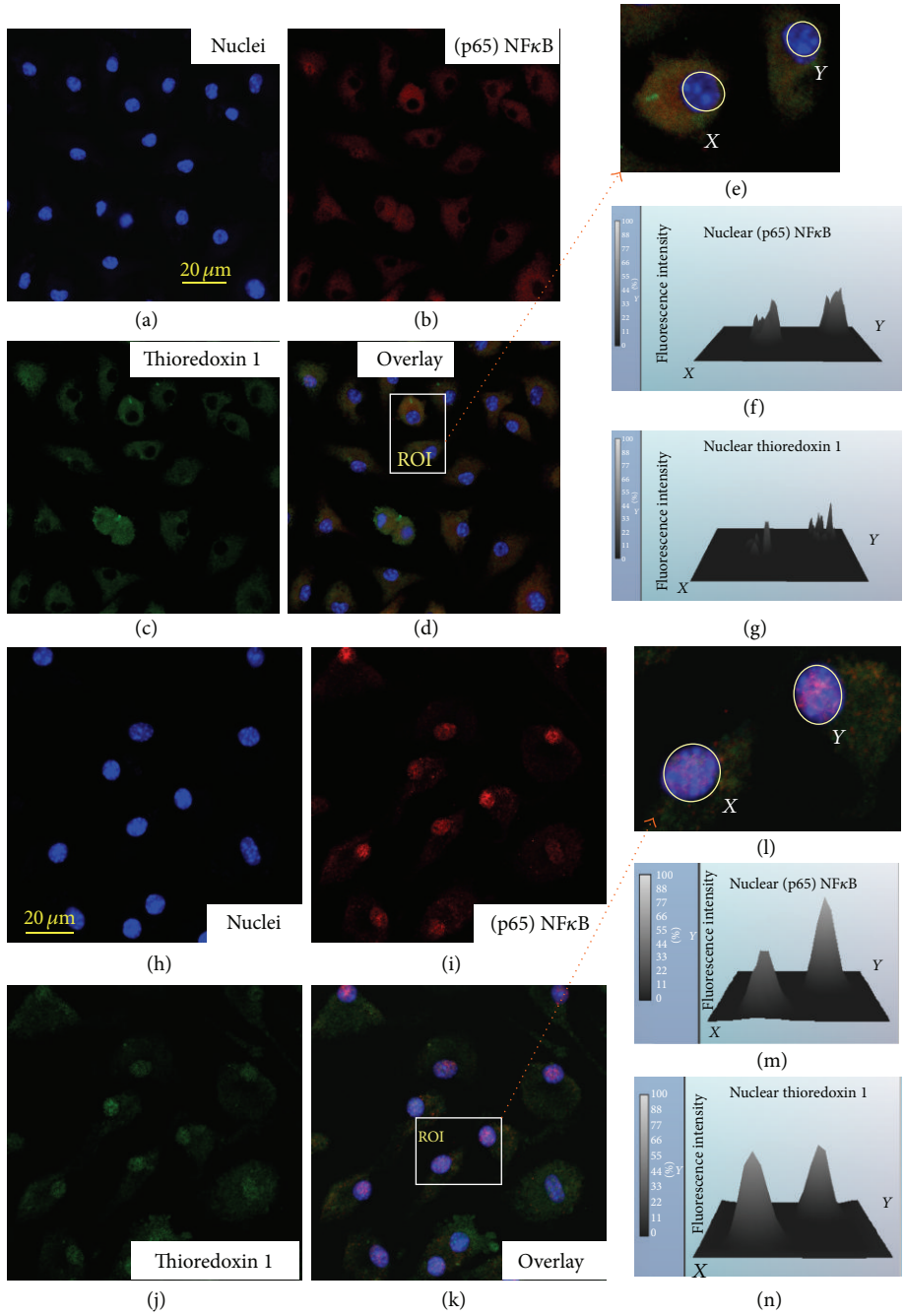

Figure 6: Confocal immunofluorescence imaging of nuclear translocation of NFκB and thioredoxin 1 (TRX1) in MSCs challenged with LPS. ((a)–(c)) Projections of (p65) NFκB (red channel) and TRX1 (green channel) in control MSCs. (d) Overlay of projections of (p65) NFκB, TRX1, and nuclei presented in ((a)–(c)). (e) ROI selected in (d). (f) Histogram of immunofluorescence of (p65) NFκB in nuclei shown in (e). (g) Histogram of immunofluorescence of TRX1 in nuclei shown in (e). ((h)–(j)) Projections of (p65) NFκB (red channel) and TRX1 (green channel) in LPS-challenged MSCs. (k) Overlay of projections of (p65) NFκB, TRX1, and nuclei presented in ((h)–(j)). (l) ROI selected in (k). ((m)-(n)) Histograms of immunofluorescence of (p65) NFκB and TRX1 in nuclei shown in (l). Counterstaining of nuclei was with Hoechst 33342 (blue channel). The confocal images were taken with pinhole setup to obtain 0.5 μm Z-sections. The experimental conditions were the same as indicated in Figure 2.

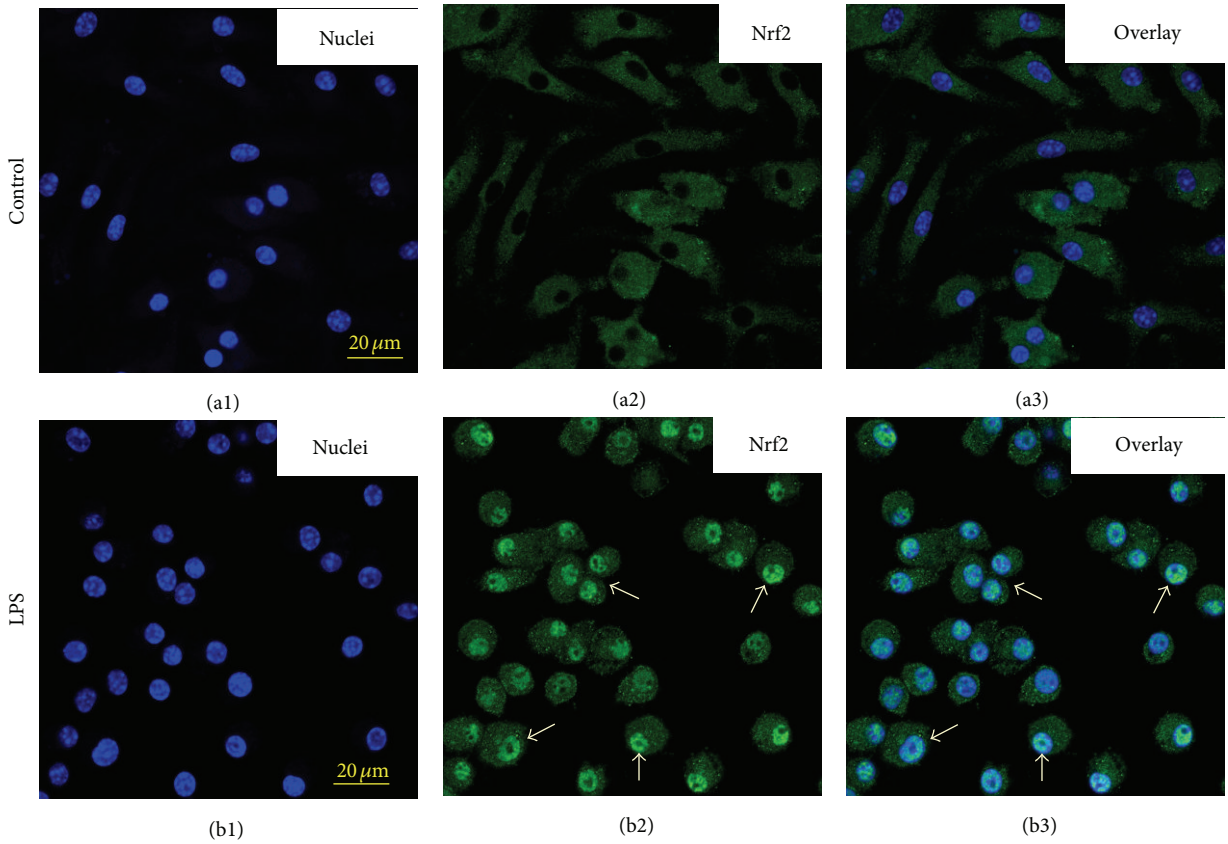

FIGURE 7: Assessment of nuclear translocation of Nrf2 redox-response element in MSCs challenged with LPS. Counterstaining of nuclear DNA was with Hoechst 33342 (blue channel). Nrf2 staining is in green. Nrf2 localized in nuclei appears in turquoise/green color due to interference of "green" and "blue" (indicated with arrows). Experimental conditions were the same as indicated in Figure 2.

factors, antioxidants, heat shock proteins, and regulators of autophagy and mitochondrial functions [22, 24, 25, 27, 31–37]. From our results presented in Figure 2, a 3 h pulse challenge of MSCs with LPS resulted in a substantial increase in NFκB in the cell protein fraction. NFκB is known as a redox-sensitive transcription factor that contains a critical cysteine residue (Cys-62) in the p50 subunit that is involved in DNA binding [24, 36]. NFκB is normally present in the cytoplasm in a complex with the inhibitory subunit IkB but under oxidative conditions, IkB is phosphorylated by I-kB kinase (IKK), ubiquitinated, and subsequently degraded. Excessive oxidative stress can lead to the oxidation of Cys-62 which does not affect its translocation to the nucleus but rather interferes with DNA binding and decreases gene transactivation [24, 38]. Therefore, in the presence of oxidative stress, nuclear translocation of activated (p50)(p65) NFκB has to be synchronized with increase in nuclear fraction of reductants TRX1 and Ref1 [24, 38]. Overall, while the NFκB system has been recognized to be primarily activated by inflammagens (such as LPS) via Toll-like and other receptors, it was the first mammalian transcription factor determined to be redox regulated and suggested to be directly activated by ROS and RNS [24, 36, 38].

Confocal projections of cellular NFκB shown in Figures 6(a)–6(f) indicate a relatively low level of nuclear fraction of NFκB in control cells. This balance dramatically changed after challenge with LPS (Figures 6(h)–6(n)). That was associated with increases in nuclear fractions of TRX1, a reducing factor essential for activation of oxidized nuclear NFκB; note that the localization of nuclear TRX1 appeared in close proximity with nuclear (p65) NFκB (Figure 6).

One of the most crucial cellular defense mechanisms against oxidative stress and nitrosative stress is mediated by the transcription factor Nrf2 [23, 24, 33, 38, 39]. Under the basal condition, Nrf2 is compartmentalized in the cytoplasm and Nrf2-dependent transcription is repressed by a negative regulator Keap1. In the presence of ROS and RNS, Nrf2 is released from a complex with Keap1 and translocated to the nucleus where it activates antioxidant responsive element-(ARE-) dependent gene expression to maintain cellular redox homeostasis [23, 24, 40]. In this respect, mechanisms of "sensing" the redox stress by Nrf2 seem similar to those demonstrated for NFκB. Therefore, it was not surprising that the patterns of upregulation of Nrf2 and NFκB in the LPS-challenged MSCs were similar (Figures 2, 6, and 7), with exception that in contrast to NFκB we did not observe a significant increase in nuclear fraction of Nrf2 at 1 h pulse LPS challenge of the cells (data not shown) as we did with NFκB.

At this stage, it was reasonable to assume that deep metabolic changes essential for long-term survival of the cells

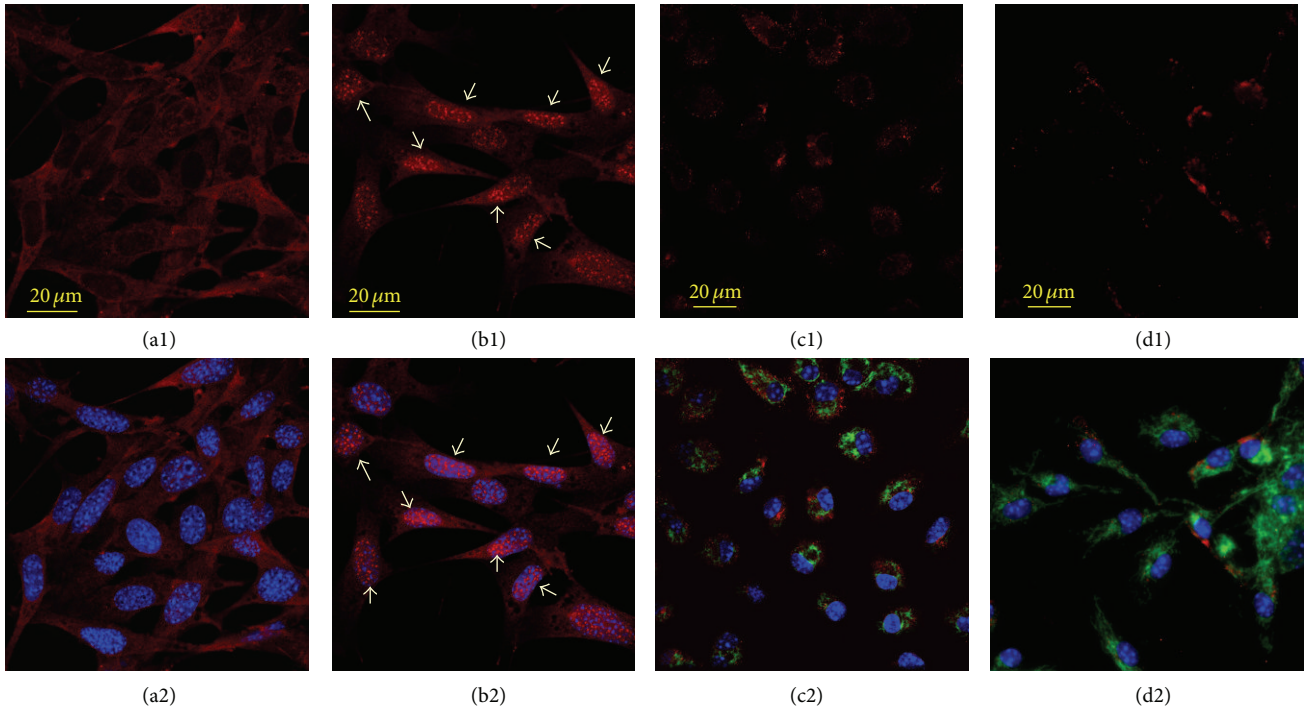

Figure 8: Assessment of nuclear translocation of FoxO3a and p53 redox-response element in MSCs challenged with LPS. Counterstaining of nuclear DNA was with Hoechst 33342 (blue channel). FoxO3a staining is in red (control (a1) and (a2) versus LPS treatment (b1) and (b2)). ((a2)-(b2)) overlay of confocal projections of FoxO3a protein (red channel) and nuclear DNA (blue channel). FoxO3a nuclear translocation is indicated with arrows in (b1) and (b2), where nuclear fraction of FoxO3a appears in pink color due to interference of "red" and "blue". ((c1), (c2), (d1), (d2)) Confocal projections of p53 protein (red channel) in MSCs (control (c1) and (c2) versus LPS (d1) and (d2)). Counterstainings were done with Hoechst 33342 (for nuclear DNA, blue channel) and anti-TOM20 IgG (for mitochondria, green cannel). (c2) and (d2) are overlay of confocal projections of p53 protein (red channel), TOM20 protein (green channel), and nuclear DNA (blue channel). Note that there was no detectable increase in the p53-immunofluorescence in nuclear areas of LPS-challenged MSCs. The confocal images were taken with pinhole setup to obtain 0.5 μm Z-sections. Experimental conditions were the same as indicated in Figure 2.

under redox stress conditions may proceed via a cascade of events which are driven by synchronous activation (or suppression) of different signaling mechanisms. For example, we tested LPS-induced nuclear translocation of two other transcriptional factors, namely, FoxO3a and p53. FoxO3a, a member of a family of mammalian forkhead transcription factors of the class O, has been recently proposed as mediator of diverse physiologic processes, including regulation of resistance to redox stress and increase in longevity [31, 32]. Opposite to FoxO3a, p53 transcriptional factor is the well-discussed master regulator of apoptotic cell death, which can be activated in stromal cells by redox genotoxic stress [41]. The effects of LPS challenge on nuclear translocation of FoxO3a and p53 are shown in Figure 8. Indeed, as expected, a massive increase in nuclear fractions of FoxO3a occurred in the LPS-treated cells (Figure 8; control (a1) and (a2) versus LPS treatment (b1) and (b2)) that corroborated with effects observed for Nrf2 and NFκB (Figures 6 and 7). Meanwhile, there were no significant changes in nuclear immunofluorescence of p53 protein (Figure 8; control (c1) and (c2) versus LPS (d1) and (d2)).

The above analyzed transcriptional factors, that is, Nrf2, NFκB, and FoxO3a, are implicated in regulation of a variety of adaptogens, antioxidants, and mediators of autophagy and mitochondrial remodeling including HSP70, HO1, p62, Sirt3, and LC3 [23, 24, 33, 35, 42]. Moreover, a growing body of evidence suggests involvement of chaperone heat-shock proteins and adaptor proteins in autophagy events [42, 43]. The results of immunoblot analyses of these proteins in the LPS-treated MSCs are shown in Figure 9(a). LPS-induced expression of HSP70 and Sirt3 was insignificant (Figure 9(a)) and was apparently due to high levels of the constitutively present proteins. Meanwhile, we observed a substantial increase in HO1 protein (Figure 9(b)) that was in concord with the LPS-induced response of Nrf2 (Figures 7 and 9(a)), a transactivator of HO1 [35].

Remarkable responses occurred in the ubiquitin-associated target adaptor p62/SQSTM1 and LC3 type I and type II proteins (Figure 9(a)), which are mediators of macroautophagy (ATPhG) [11, 22, 37, 43]. A key step in the autophagosome biogenesis is the conversion of light-chain protein 3 type I (LC3-I, also known as ubiquitin-like protein, Atg8) to type II (LC3-II). The conversion occurs via the cleavage of the LC3-I carboxyl terminus by a redox-sensitive Atg4 cysteine protease. The subsequent binding of the modified LC3-I to phosphatidylethanolamine, that is, the

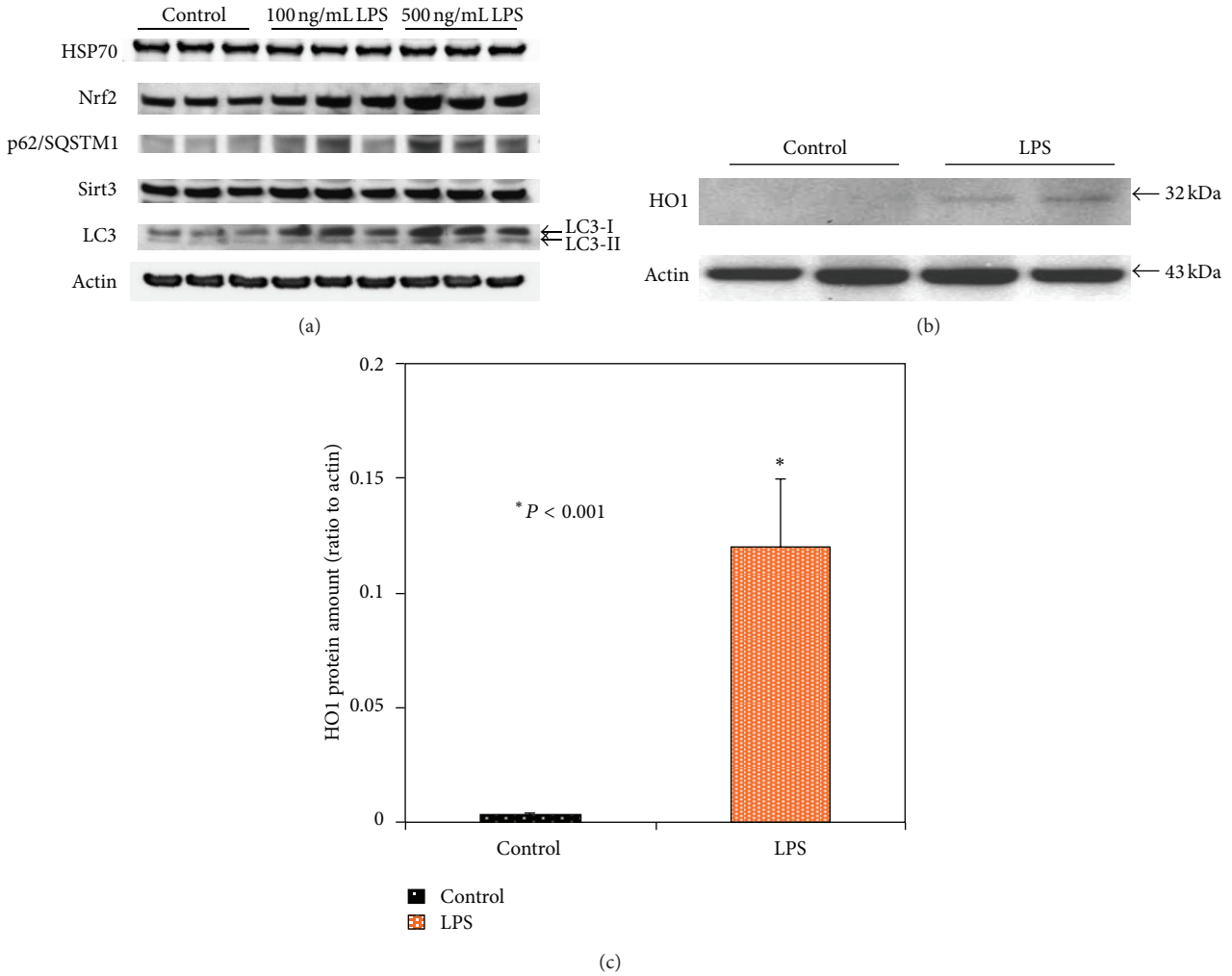

Figure 9: Western immunoblot analysis of redox-response and autophagy-mediated proteins in MSCs challenged with LPS. (a) Representative immunoblotting bands of HSP70, Nrf2, p62/SQSTM1, Sirt3, and LC3 proteins. The protein extracts were obtained from MSC cultures 24 h after challenge with LPS. ((b)-(c)) Representative immunoblotting bands of Hemeoxygenase 1 (HO1) protein (b) and respective densitometry histograms of HO1 bands (c) in MSCs stimulated with LPS. The presented bars indicate the relative density of HO1 protein (normalized to density of actin bands). The statistical significance was determined by Student's t-test ($n = 3$). Conditions: MSCs were incubated with 500 ng/mL LPS for 3 h. The cells were harvested at 24 h following challenge with LPS.

process of lipidation of LC3-1, on the isolation membrane as it forms, is mediated by E-1- and E-2-like enzymes Atg7 and Atg3 [11, 22, 37]. Thus, conversion of LC3-I to LC3-II and formation of LC3-positive vesicles are considered to be a marker of activation of ATPhG [11, 22, 37]. As shown in Figure 9(a), a challenge of MSC with LPS resulted in increases in both LC3-I and LC3-II expression as determined by immunoblotting and indicated upregulation of the LC3-I to LC3-II transition. At this stage, our further investigation was focused on immunofluorescence confocal imaging and TEM analysis of ATPhG-mediated remodeling in the LPS-challenged cells.

In the second set of experiments, we analyzed autophagy/autolysosomal response and mitochondrial remodeling in MSCs subjected to LPS challenge. The ATPhG pathway is considered to be an evolutionarily developed prosurvival mechanism, which removes and processes damaged and misfolded proteins, and compromised organelles in response to redox stress [21, 25, 27, 37, 44]. Activation of ATPhG is associated with formation of autophagic/autolysosomal vacuoles in the cytoplasm which mediate proteolytic processes [11, 22, 25, 27].

The images presented in Figure 10 indicate that upregulation of LC3-I/LC3-II proteins in the LPS-challenged cells was associated with massive formation of the LC3-positive vesicles featuring autophagosomes and autolysosomes.

The further assessment of autophagy events with TEM revealed in the LPS-challenged cells the presence of characteristic multiple vacuoles, which were formed by double-layer membranes and sequestered constituents of different densities (Figure 10). Some of these vacuoles can be identified as secretory autolysosomes by the presence of multilamellar structures (most likely fibers of collagen) released extracellularly, while others contained fractured organelles including compromised mitochondria (Figure 11).

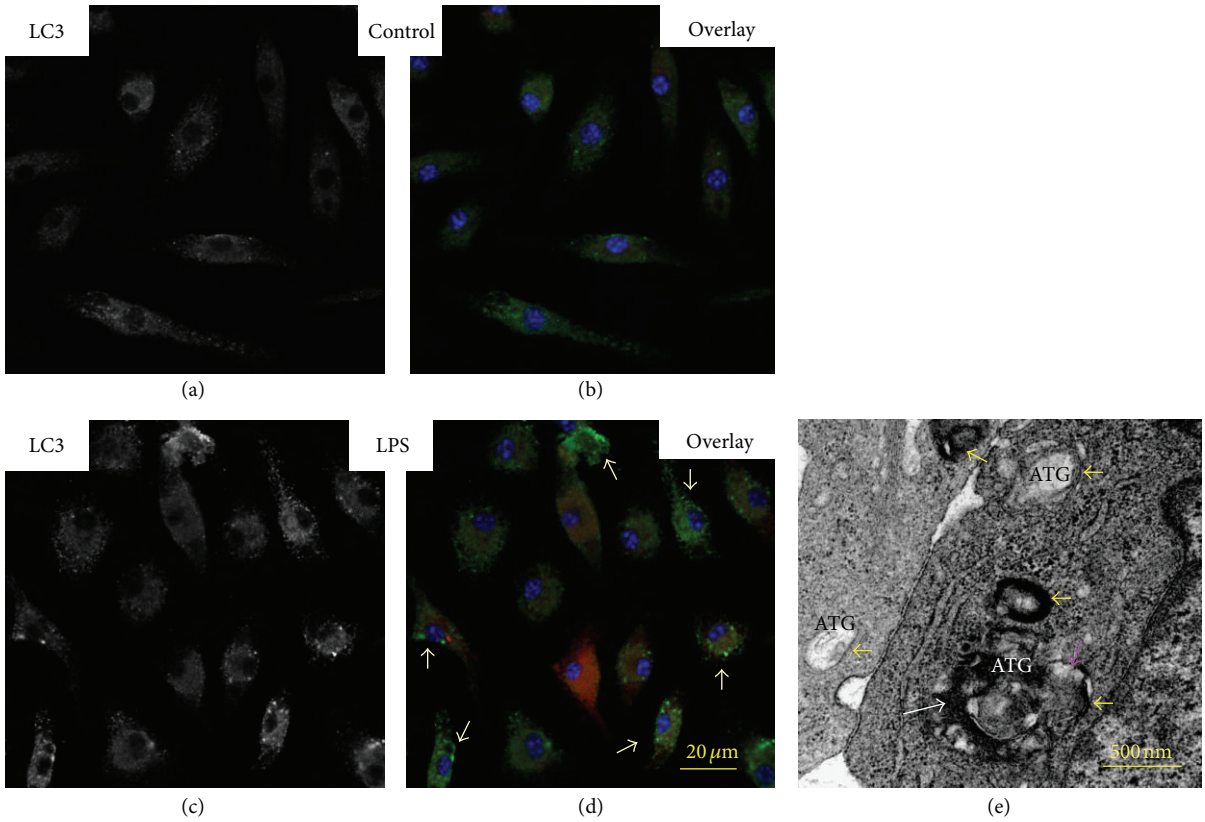

Figure 10: Assessment of autophagosome formation in MSCs challenged with LPS. ((a)–(d)) Confocal immunofluorescence imaging. (a) Green channel. LC3 projection in control MSCs. (b) Overlay of projections of LC3 (green channel), iNOS (red channel), and nuclei (blue channel) in control MSCs. (c) Green channel. LC3 projection in MSCs challenged with LPS. (d) Overlay of projections of LC3 (green channel), iNOS (red channel), and nuclei (blue channel) in MSCs challenged with LPS. Spatial localization of LC3 is indicated with white arrows. Conditions: MSCs were incubated with 500 ng/mL LPS for 3 h. The cells were analyzed 24 h after challenge with LPS. Counterstaining of nuclei was with Hoechst 33342 (blue channel). The confocal images were taken with pinhole setup to obtain 0.5 μm Z-sections. (e) TEM image of MSCs challenged with LPS. Autophagosome (ATG) membranes are indicated with yellow arrows; fragments of mitochondrion in ATG are indicated with white arrow; fusion of lysosomes with ATG is indicated with pink arrow.

Recent observations suggest that autophagosomes do not form randomly in the cytoplasm but rather sequester mitochondria selectively [45, 46]. Selection of compromised mitochondria for mitophagy requires activation of the PINK1/PARKIN pathway and implication of adaptor proteins, for example, p62/SQSTM1, and ubiquitin-like modifiers, which target mitochondria and ultimately mediate fusion of the processed mitochondria with autophagosome [45, 46].

The images presented in Figures 10(e) and 11(b)–11(d) indicate that mitochondria can be fused with autophagosomes in similar sizes while further degradation occurred in large-size autolysosomes.

The observed mitophagy was accompanied by extensive mitochondrial fusion resulting in the remodeling and expansion of the mitochondrial network (Figures 11 and 12). The events of mitochondrial fusion and formation of elongated mitochondria (over 10 μm length) were captured with TEM and confocal immunofluorescence microscopy and are presented in Figures 11(e), 11(f), and 12(d). All the above data suggest that a short-term challenge with LPS triggered in MSCs a battery of complex adaptive responses leading to an increase in resistance to redox stress and damage to cellular constituents, and also to the remodeling of the entire mitochondrial network.

4. Discussion

The redox stress occurs in the pathogeneses of a variety of injury types, including radiation combined injury and the associated sepsis, and there are multiple original papers and reviews that were addressed recently to this phenomenon [4, 14, 47–49]. The associated secondary oxidative injury to sensitive cellular constituents and ER stress can affect homeostasis of tissue barriers and thus exacerbate the process of healing. With this respect, development of new regiments for treatment of complicated injuries can be made more efficient with better understanding of the basic cellular mechanisms implicated in redox adaptive responses in tissue barriers. This particular area of the molecular redox pathophysiology is poorly developed despite the fact that the general concept of cellular stress responses is broadly

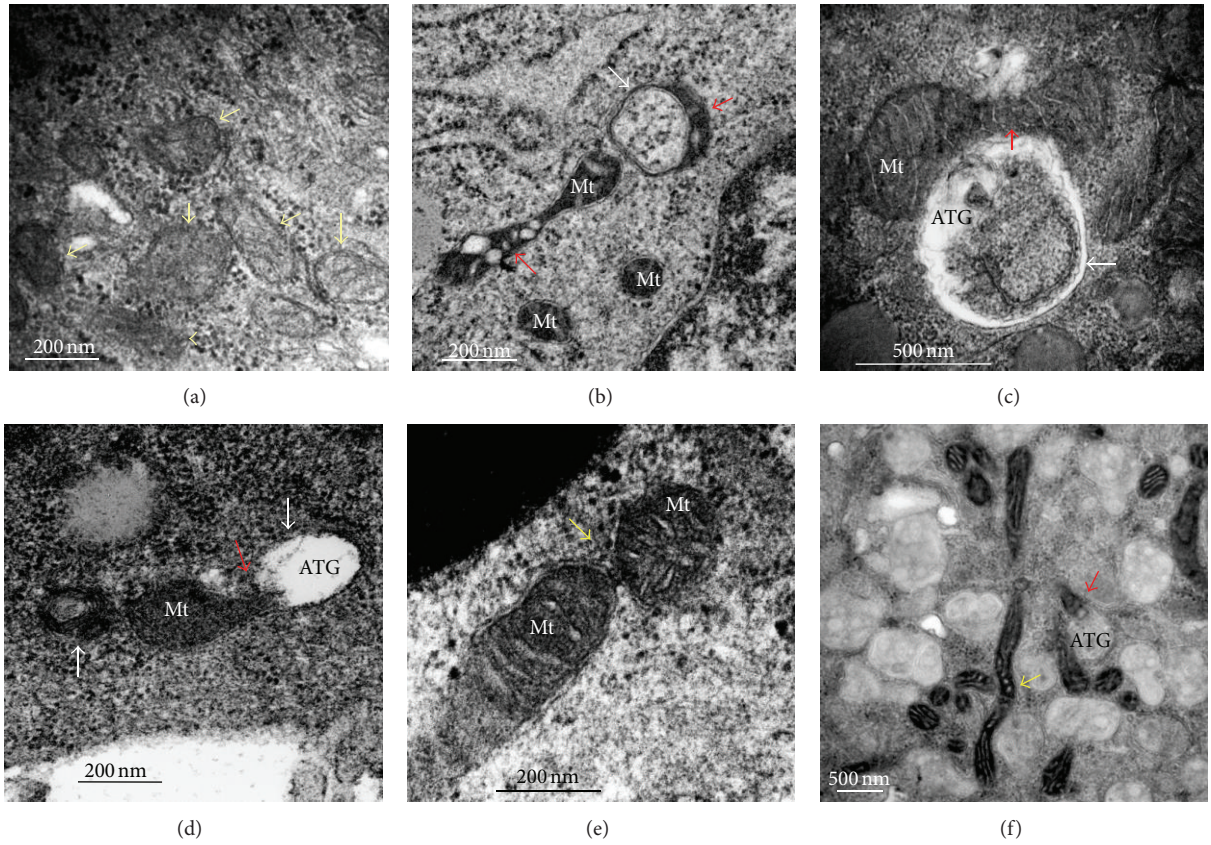

Figure 11: Transmission electron (TEM) analysis of mitochondrial remodeling inMSCs challenged with LPS. (a) Image of a control MSC. Mitochondria are indicated with yellow arrows. ((b)–(f)) Images of MSCs challenged with LPS. (b) Damaged mitochondria subjected to remodeling and mitophagy are shown with red arrows. Double-layer membrane of an autolysosome is indicated with white arrow. Mt, mitochondria. ((c)-(d)) Fusion of damaged mitochondria (Mt) with autophagosomes (ATG) is indicated with red arrows. ATG membranes are indicated with white arrows. (d) Formation of secretory autolysosomes containing multilamellar structures (indicated with red arrows) in an irradiated MSC. (e) Fusion of mitochondria (Mt) is indicated with yellow arrow. (f) Formation of elongated mitochondria is indicated with white arrow. Mitophagy is indicated with red arrow. Conditions: MSCs were incubated with 500 ng/mL LPS for 3 h. The cells were analyzed 24 h after challenge with LPS.

discussed in the literature [35, 38, 50]. Our communication is the first report demonstrating a potential role of MSCs in sustaining redox resistance of barrier functions under septic conditions.

According to the current paradigm, general stress responses involve conserved signaling modules that, in turn, are interconnected to the cellular adaptive mechanisms [21, 33, 50]. It has been shown recently that bacterial infections trigger specific sensitive mechanisms mediating inflammation, redox stress, adaptation, and remodeling [2, 3, 17, 20–22]. Redox stress *per se* stimulates signaling cascades mediated by transcription factors and pathways that are believed to play a central role in cell survival. These include, but are not limited to, a battery of thiol-containing redox-response elements, redox-sensitive transcription factors such as nuclear factor-kappa B (NFκB), Nrf2, FoxO3a, and stress-response adaptors such as the chaperone heat-shock protein 70 (HSP70) and NAD^+-dependent deacetylase sirtuin-3 (Sirt3), and activators of the autolysosomal degradation and mitochondrial remodeling. Overall, these effector systems are crucial in maintaining homeostasis, which is altered due to oxidative damage to cell constituents [35–46]. It should be noted that, while the role of the redox-induced NFκB and Nrf2 responses in cell survival is well documented, transcriptional factor FoxO3a, the autophagy/autolysosomal pathway, and mitochondrial remodeling are relatively newly determined players implicated into adaptive mechanisms [21, 30, 31, 36, 37, 42–46].

Recently, we demonstrated *in vitro* that MSCs could employ ATPhG in phagocytosis of *Escherichia coli* [11]. However, a mechanism which allowed the cells to avoid the adverse effects of the products of bacterial biodegradations, such as LPS, remained unclear. The data presented in this report indicate that *in vitro* challenge of MSCs with LPS inflammagen triggered a cascade of responses that we believe orchestrate adaptive remodeling of the cell and increase resistance to a "self-inflicted" LPS-induced oxidative stress. A pattern of these adaptive responses include induction of redox-response elements such as NFκB, TRX1, Ref1, Nrf2, FoxO3a, and activation of ATPhG and mitochondrial remodeling.

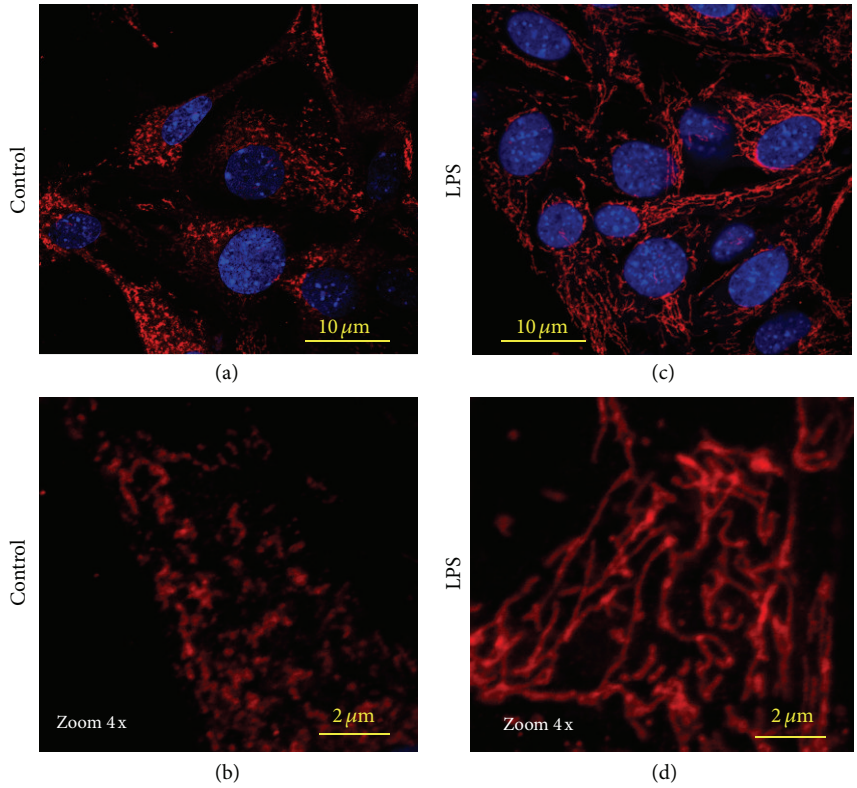

FIGURE 12: Confocal immunofluorescence imaging of mitochondrial remodeling in MSCs challenged with LPS. Mitochondrial networks were visualized using projections of TOM20 (red channel), a mitochondrial marker. ((a)-(b)) Control MSCs: mitochondrial network is presented by small-size dots. ((c)-(d)) MSCs challenged with LPS: formation of long-length mitochondrial network occurred due to mitochondrial fusion. Conditions: MSCs were incubated with 500 ng/mL LPS for 3 h. The cells were analyzed 24 h after challenge with LPS. Counterstaining of nuclei was with Hoechst 33342 (blue channel). The confocal images were taken with pinhole setup to obtain 0.5 μm Z-sections.

It should be noted that despite the presence of mitophagy and mitochondrial remodeling in the LPS-challenged MSCs, there were no significant alterations in levels of Sirt3 protein, which was a major player in mitochondrial response to the redox stress [35, 37, 46]. We assumed that it was likely due to a high constitutive expression of this protein in MSCs. Meanwhile, unlike Sirt3, there occurred a significant expression of HO1, an antioxidant protein, which utilized mitochondrial heme, which *per se* is a catalyst of the Fenton type of reactions as well as being essential for the *de novo* formation of active form of iNOS [35, 51]. Overall, our observations support a general concept of the presence of a network of a variety of signaling pathways that enable mediating cellular adaptation to the oxidative stress [33, 35, 37, 40, 43, 50].

Disclaimer

The views expressed in this paper do not necessarily represent the Armed Forces Radiobiology Research Institute, the Uniformed Services University of the Health Sciences, NIH, or the US Department of Defense.

Conflict of Interests

There is no ethical and financial conflict in the presented work.

Authors' Contribution

N. V. Gorbunov and J. G. Kiang contributed equally to the presented work.

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Review Article

Nitric Oxide Donors as Neuroprotective Agents after an Ischemic Stroke-Related Inflammatory Reaction

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Cerebral ischemia initiates a cascade of detrimental events including glutamate-associated excitotoxicity, intracellular calcium accumulation, formation of Reactive oxygen species (ROS), membrane lipid degradation, and DNA damage, which lead to the disruption of cellular homeostasis and structural damage of ischemic brain tissue. Cerebral ischemia also triggers acute inflammation, which exacerbates primary brain damage. Therefore, reducing oxidative stress (OS) and downregulating the inflammatory response are options that merit consideration as potential therapeutic targets for ischemic stroke. Consequently, agents capable of modulating both elements will constitute promising therapeutic solutions because clinically effective neuroprotectants have not yet been discovered and no specific therapy for stroke is available to date. Because of their ability to modulate both oxidative stress and the inflammatory response, much attention has been focused on the role of nitric oxide donors (NOD) as neuroprotective agents in the pathophysiology of cerebral ischemia-reperfusion injury. Given their short therapeutic window, NOD appears to be appropriate for use during neurosurgical procedures involving transient arterial occlusions, or in very early treatment of acute ischemic stroke, and also possibly as complementary treatment for neurodegenerative diseases such as Parkinson or Alzheimer, where oxidative stress is an important promoter of damage. In the present paper, we focus on the role of NOD as possible neuroprotective therapeutic agents for ischemia/reperfusion treatment.

1. Introduction

When the brain blood flow is interrupted, it results in deprivation of oxygen and nutrients to the cells; this situation constitutes an ischemic stroke. Restoration of the flux, or reperfusion, can reduce the damage, but only when this is performed very early after the onset of ischemia, and its efficacy is restricted by secondary injuries, mainly by oxidative stress (OS) and an inflammatory reaction, which lead to cell death by apoptosis [1].

It is noteworthy that ischemic damage not only affects neurons. Thus, in recent years the concept of the neurovascular unit has been highlighted, emphasizing the need to protect not only the neurons, but also all cells in the brain [2–5]. In contrast to the known vulnerability of neurons and astrocytes, it is thought that endothelial cells tend to be more resistant to ischemic or oxidative injury [6]. Hence, to be successful, stroke therapies should be widely effective and must protect all neuronal, glial, and endothelial components in the brain [7].

After focal ischemia, primary neuronal death appears rapidly in the core area and is followed by secondary death in the ischemic penumbra, which evolves from the delayed activation of multiple cellular death pathways. At the core of the ischemic lesion, one of the first events is the rapid decline of adenosine triphosphate (ATP) reserves [8]. Consequently, all energy-dependent processes gradually cease their activity, leading to changes in transmembrane potential.

The consequent depolarization (denominated anoxic depolarization) produces massive influx of Na^+, Cl^-, and Ca^{2+} inside the cell with K^+ efflux [9].

Core anoxic ischemic depolarizations induce release of neurotransmitters such as glutamate. Once released, glutamate generates a phenomenon of peri-infarct depolarization, which increases energy consumption and promotes Ca^{2+} influx into the cells [10].

The increase in intracellular Ca^{2+} in neurons and glial cells initiates a set of nuclear and cytoplasmic events that produce deep brain tissue damage that includes the following: Ca^{2+} mitochondrial overload (which compromises the already affected ATP production and promotes the opening of the mitochondrial transition pore); the increase in OS, and the activation of a number of Ca^{2+}-dependent enzymes. Such enzymes include proteases, kinases, phospholipases, and endonucleases, which destroy biomolecules [10]. Additionally, increased intracellular Ca^{2+} also promotes the production of NO from constitutive synthases that, together with acidosis and peri-infarct depolarization, contribute to the initiation of damage; later, inflammation and activation of apoptotic phenomena contribute to increased injury [2].

OS is a major mechanism implicated in stroke and in a variety of neurodegenerative diseases, mainly in Alzheimer and Parkinson (reviewed in [11, 12]). The most accepted theory regarding neurodegeneration in Parkinson disease refers to OS as the main cause of damage to neurons in the substantia nigra. In addition, in Alzheimer disease, the OS generated by the action of β-amyloid, which causes massive entry of Ca^{2+} and caspase activation, leads to neuronal death [13, 14].

During ischemia, reactive oxygen (ROS) and nitrogen species can be generated in the ischemic penumbra but can also be produced during reperfusion injury [15, 16]. Indeed, it is now established that albeit maintenance of partial or complete blood flow is essential for preserving cerebral tissue, it is during reperfusion when it paradoxically induces excessive generation of ROS, such as superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), and nitric oxide (NO), which contribute to increased neuronal death by oxidizing proteins, damaging DNA, and inducing lipid peroxidation [17].

Reperfusion-induced ROS contribute to a decrease of the NO availability responsible for postischemic endothelial dysfunction [18, 19]. During the ischemic period, reduction in O_2 availability reduces the activity of NO synthase, producing O^{2-} instead of NO; later, during reperfusion, the arrival of O_2 increases NO synthase activity. These can exert a deleterious effect by promoting nitrosative stress and diminishing the availability of NO for preserving endothelial integrity.

Over the past decade, remarkable advances have been made in understanding the basic molecular mechanisms underlying neuronal death. However, clinically effective neuroprotectants have not yet been discovered and no specific therapy for stroke is available at present. The body of experimental data supports the view that reducing OS should continue to be a potentially viable target for stroke therapy [20]. In addition, the inflammatory response requires consideration as a potential target of therapy for ischemic stroke [21]. Therefore, agents capable of modulating both elements will constitute promising therapeutic solutions [22–25].

2. Ischemic Lesion and Immune Response: Brain Inflammation

It has now been established that the Central nervous system (CNS) is able to raise an immune response to the majority of threatening stimuli, whereby resident cells generate inflammatory mediators including cytokines, prostaglandins, free radicals, complementary chemokines, and adhesion molecules that recruit immune cells and activate glia and microglia (reviewed in [21, 26–28]). The role of microglia and proinflammatory cytokines in the CNS has been characterized in models of brain insults, such as experimental stroke, the most common form of ischemic injury [26]. As mentioned previously, cerebral ischemia triggers acute inflammation, which exacerbates primary brain damage. Although inflammation should be adaptive, the release of proinflammatory cytokines has often been associated with harmful consequences to neurons and myelin [29].

The control of early CNS inflammation is a careful balancing act, as both too much and too little inflammation will lead to decreased or delayed recovery. Whether the inflammation is neurotoxic or protective may depend upon the context and the location of the inflammatory mediator in relation to an injury, and the timing of inflammatory response may determine the outcome (see Table 1 in [27]).

For example, tumor necrosis factor alpha (TNF-α) upregulated in the proximity of an evolving lesion contributes to secondary infarct growth, whereas cytokine induction remote from the ischemic lesion confers neuroprotection [30]. TNF-α could enhance apoptotic processes through its action on its tumor necrosis factor type 1 receptor (TNFR1) in models of acute (ischemia, excitotoxicity) and chronic (Alzheimer disease, multiple sclerosis) neurodegeneration [31]. TNF-α and interleukin 1beta (IL-1β) exert neurotoxicity in cerebral ischemia in the presence of elevated inducible nitric oxide synthase (iNOS), while in the absence of iNOS, both cytokines appear to contribute to neuroprotection and plasticity, highlighting the role of the context [32].

There is important recognition that protection of endothelial function and downregulation of vascular inflammation comprise part of neuroprotection phenomena and may possess added therapeutic benefit against stroke injury [33]. However, research on clinically effective neurovascular protective therapies for brain damage remains at an early phase [34]. Much attention has been focused on the role of NO in vessel protection from OS and inflammation [35]. Because OS coexists with inflammation and endothelial dysfunction, determining antioxidant status may be helpful in monitoring the progress of Nitric oxide donors (NOD) treatment. A variety of structurally different NOD, which release NO either as a free radical ($NO^{\bullet}$) or as an NO ion (NO^+/NO^-), have shown to reduce OS/inflammation and to increase cerebral blood flow [35–38]; thus, these can be considered attractive candidates for therapeutic agents in experimental models of stroke.

3. Nitric Oxide Donors (NOD) as Neuroprotective Agents in Ischemic Stroke

3.1. Nitric Oxide in Ischemic Stroke. Nitric oxide (NO) plays a dual role, that is, neuroprotection and neurotoxicity, in the pathophysiology of cerebral ischemia-reperfusion injury [39]. NO is synthesized by NOS, of which there are three known isoforms: nNOS; eNOS, and inducible or immunological NOS (iNOS). The first two are constitutively expressed and their activity is dependent on changes at the intracellular Ca^{2+} level, while iNOS acts in a Ca^{2+}-independent manner [40]. Baseline concentration of NO in the brain is mainly due to nNOS activity, and secondarily to eNOS. iNOS is not expressed under physiological conditions [41, 42].

In the brain, eNOS is mainly produced by the vascular endothelium and the choroid plexus [43]. Although eNOS-NO production is a minor part of total brain NOS activity, this enzyme is critical for the regulation of cerebrovascular hemodynamics and for the protection of endothelium integrity from inflammatory, oxidative, and procoagulant stimuli. It has been demonstrated that eNOS-derived NO scavenges ROS [44] and inhibits the expression of cellular adhesion molecules [45], platelet aggregation [46], and leukocyte adhesion [47].

During ischemia, NO concentration decreases because of oxygen deficiency [41]. However, immediately after reperfusion, biosynthesis of this molecule is triggered mainly by overactivation of nNOS, as evidenced in nNOS (−/−) mice [48] or with specific inhibitors such as 7-NI [41, 49]. Glutamate-induced Ca^{2+} overload in ischemic neurons is responsible for the rise of nNOS-derived NO [50]. Concentration of NO returns to physiological levels approximately 1 h after reperfusion [48, 49, 51] and increases again due to iNOS expression between 12 h and up to 8 days later [52, 53]. iNOS sources at this stage comprise microglia [53], astrocytes [54], endothelial cells [55], and infiltrated leukocytes [56]. The amount of iNOS-NO derived is 100–1,000 times than that produced by nNOS [57].

Therefore, NO deriving from different sources (neurons, brain vessels, glia, and neutrophils) may exert an influence on the evolution of brain damage at different time-points after an ischemic insult [42]. Thus, the use of relatively selective inhibitors of NOS isoforms and genetically modified mice has contributed to clarifying the role of NO in cerebral ischemia-reperfusion injury as follows.

Total suppression of eNOS activity in knockout mice (eNOS −/−) renders them hypertensive [48] and more susceptible to ischemia-reperfusion injury, with larger infarcts compared with those of controls and a more severe reduction in cerebral blood flow (CBF) [48, 51, 58]. Conversely, overexpression of eNOS by flavonoids induces a protective effect [59]. In contrast to eNOS, infarct volume and neuronal death are consistently decreased by *nNOS* gene deficiencies or by nNOS inhibition [48, 60–64]. nNOS abolition also reduces excitotoxicity [65], nitrosative stress [63, 66], and O^{2-} production [67] and downregulates calpain and caspase-3 in ischemic lesion [61, 64]. Additionally, during reperfusion, iNOS-produced NO contributes to brain injury [42, 68]. iNOS expression is transcriptionally regulated by nuclear factor kappa B (NF-κB) secondary to moderate inflammatory stimuli such as TNF-α [69] and IL-1β [70], and also by oxidative radicals [71]. Due to the large amount of NO produced by iNOS, this enzyme is related with high peroxynitrite production and significant nitrosative damage of biological molecules [72].

Consequently, nNOS mediates early neuronal injury, while iNOS contributes to late neuronal injury, whereas eNOS activity might be protective [41, 42, 73]. Whether the effects of this molecule are beneficial or harmful depends on the cellular compartment in which NO is generated, on its concentration, on the environment's redox state, and on the evolutive stage of ischemic brain injury [42, 48, 73].

In addition to their involvement in ischemia, the expression of iNOS in astrocytes and microglia and the production of large amounts of NO contribute to dopaminergic neuronal death in the neuropathology of experimental Parkinson disease [74, 75]. In the brains of patients with Alzheimer disease, nitrosylation of proteins is a hallmark of tissue damage [76] and is particularly high in hippocampus and cerebral cortex. The presence of beta amyloid is sufficient for triggering iNOS activation in astrocytes and microglia [77]. In addition, nNOS activity in reactive astrocytes surrounds beta-amyloid plaques in entorhinal cortex and is related with DNA fragmentation in CA1 and CA4 fields [78], while there is a correlation between neurofibrillary tangles and senile plaques with a reduction in eNOS capillary levels [79].

According to the dual role of NO in brain ischemia, there is a rationale for the use of NO for promoting treatments shortly after the occurrence of focal cerebral ischemia as neuroprotective strategies [42, 53, 80, 81]. The neurovascular protective mechanism of eNOS-NO suggests that intervention with NO may be most effective when delivered at an optimal amount by a suitable source at the correct time in an appropriate environment [82].

3.2. Nitric Oxide Donors (NOD). Nitric oxide donors are a heterogeneous group of drugs whose common feature is the ability to release NO or an NO-related species, such as the Nitrosonium ion (NO^+) or the Nitroxyl anion (NO^-), *in vitro* or *in vivo*, independently on its endogenous sources [83]. The following are the NOD most frequently employed in clinical and basic research: organic nitrates (e.g., nitroglycerin, isosorbide-5-mononitrate, nicorandil, pentaerythritol tetranitrate); S-nitrosothiols (e.g., S-nitroso-N-acetylpenicillamine and S-nitroso-glutathione); sydnonimines (e.g., molsidomine, SIN-1); NONOates (JS-K, SPERMINE-NONOate, and PROLI-NONOate), and sodium nitroprusside [84]. Table 1 illustrates NOD effects on experimental cerebral ischemia.

Despite the fact that all of these are considered NOD, they have different pharmacokinetic and dynamic profiles that determine the type and extent of their biological effects. The main determinant of these effects is the manner in which NO is released, the amount of NO generated, and the time during which it is released. Moreover, some of these generate alternative products that may arise during their metabolism or

Table 1: Nitric oxide donors in experimental cerebral ischemia.

Species	Model	Time of ischemia/reperfusion	Nitric oxide donor	Doses and administration	Effect	Reference
Rat	MCAO	20′/24 h	GSNO	1 mg/kg at the onset of reperfusion	Reduction in infarct size Increase in CBF Decrease in cortical expression of TNF-α and IL-1 in penumbral region Attenuation of iNOs expression in microglia, astrocytes and vessels of penumbral region Inhibition of monocyte/macrophage infiltration Downregulates adhesion molecules (ICAM-1, LFA-1) Reduction in TUNEL-positive cells and caspase-3 activity Blocks NF-κB (p65/p50 complex) and is able to bind to DNA in astrocytes and microglial cells *in vitro*	[53]
Rat	MCAO	20′/24 h	SNP GSNO SNAP MAHMA/NONO-ate PAPA/NONOate SIN-1	2 and 3 μmol/kg per 10 min IV at onset of reperfusion	Increase in CBF (except MAHMA and PAPA) Reduction in infarct size (GSNO, SNP, and SNAP) Improvement in neurological score (GSNO, SNP, and SNAP) Reduction in lipid peroxidation in plasma (all of them) Decrease in plasma levels of nitrotyrosine (GSNO, SNP and SNAP) Increase in NO plasma level (except SNAP) Reduction in mRNA expression of ICAM-1 (GSNO, SNAP, SNP) and E-selectin (except SIN-1)	[38]
Rat	MCAO	90′/24 h	ZJM-289	0.1 and 0.2 mmol/kg IV 1 h prior to ischemia	Improvement in neurological deficit (motor function) Reduction in infarct size Reduction in brain water content Decrease of neuronal degeneration Inhibition of nNOS expression Increase of NO level ipsilateral to ischemia Increase in cGMP level	[85]
Rat	MCAO	90′/1.5, 3, 4.5, 6 and 12 h	Sodium nitrite	480 nmol per 1 min at 1.5, 3, 4.5, and 6 h postischemia, IV	Reduction in infarct size (1.5, 3, 4.5 and 6 h) Improvement in motor function (4.5 h) Decrease of microhypoxic areas (12 h) Reduction in free reactive oxygen and nitrogen species (12 h)	[86]
Rat	MCAO	2 h/7 days	SNP Sperm-ine/NONO-ate	SNP: 0.11 mg/kg per 120 min, trans-ischemia, IV Spermine: 0.36 mg/kg per 120 min, trans-ischemia, IV	Reduction in infarct size Increase in cerebral perfusion	[87]

Table 1: Continued.

Species	Model	Time of ischemia/reperfusion	Nitric oxide donor	Doses and administration	Effect	Reference
Rat	MCAO	2 h/3 days	SIN-1	0.1 and 1 mg/kg 30 min before ischemia, IV	Reduction in infarct size in normo- and hyperglycemic rats	[88]
Rat	Permanent MCAO	24 h/no reperfusion	SNP SIN-1	SNP: 3 mg/kg/h trans-ischemia, IA SIN-1: 1.5, 3, and 6 mg/kg/h trans-ischemia, IA	Both produced an increase in CBF and a reduction in infarct size SNP decreased platelet aggregation *in vitro* but not *in vivo* at the same doses	[80]
Rat	Permanent MCAO	24 h/no reperfusion	SIN-1	3 mg/kg/h per 60 min at 3, 15, 30, 60, and 120 min after ischemia, IA	Reduction in infarct size Increase in CBF	[89]
Rat	4-VO	15′/30′, 6 h, 12 h, 3 and 5 days	SNP	5 mg/kg, 3 doses: 30 min prior to ischemia, 1 h postischemia, and 2.5 h postischemia, IP	Suppression of JNK3 downstream pathway (30′, 3 h) Increase in Akt and Bad phosphorylation (12h) Inhibition of Cytochrome c release from mitochondria (6 h) Reduction in TUNEL-positive cells and caspase-3 activity (3 h) Augmentation of neuronal survival in CA1 pyramidal layer (3–5 d)	[61]
Rat	4-VO	15′/6 h, 3 and 5 days	SNP	5 mg/kg, 3 doses: 30′ prior to ischemia, 1 h postischemia and 2.5 h postischemia, IP	Decreased hippocampal activation of nNOS by nitrosylation and phosphorylation (6 h) Suppression of JNK3 downstream pathway (6 h) Inhibition of release of Cytochrome C into cytoplasm (6 h) Attenuation of caspase-3 activity (6 h) Reduction in neuronal degeneration (5 d) and TUNEL-positive cells (3 d) in CA1 pyramidal layer	[65]
Rabbit and rat	MCAO	60′/2, 4 h respectively	ProliNO/ NONO-ate	Rabbit: 10^{-6} mol/L Rat: 10^{-5} mol/L At the onset of reperfusion per 60 min, IA	Reduction in free reactive oxygen species Reduction in infarct size	[90]
Goat	MCAO	20′/7 days	SNP DEA/NONOate DETA/NONOate	SNP: 10^{-9}–3×10^{-4} mol/L, IV DEA: 10^{-9}–3×10^{-4} mol/L, IV DETA: 10^{-7}–3×10^{-4} mol/L, IV	MCA relaxation	[91]

Lines of evidence are ordered first by animal model and then by surgical procedure and severity of the ischemia. In cases in which the effects were different at different reperfusion times, this is indicated after each effect by the corresponding time as a superscript between parentheses. MAP: Mean arterial pressure; CA1: Cornu Ammonis; CBF: Cerebral blood flow; GSNO: S-nitrosoGlutathione; IA: IntraArterial; ICAM-1: Intercellular adhesion molecule-1; IL1: Interleukin 1; iNOS: inducible Nitric oxide synthase; IP: Intraperitoneal; IV: Intravenous; JNK3: c-Jun N-terminal kinase-3; LFA: Lymphocyte function-associated antigen-1; MAHMA: Methylamine hexamethylene methylamine NONOate; MCA: Middle cerebral artery; MCAO: Middle cerebral artery occlusion; nNOS: neuronal Nitric oxide synthase; NO: Nitric oxide; PAPA: Propylamine propylamine NONOate; SAP: Systolic arterial pressure; SIN-1: 3-morpholinoSydnonimine; SNAP: S-nitroso-N-acetyl-penicillamine; SNP: Sodium nitroprusside; TNF: Tumor necrosis factor; TUNEL: Terminal dUTP nick end labeling; 4-VO: four Vessel occlusion model.

decomposition. These products may even be present in quantities exceeding NO with independent or side effects [92].

For example, organic nitrates, the most common NOD utilized in coronary artery disease, require enzymatic bioactivation in order to deliver NO [93]. Their principal effect is at the vascular level, by increasing venous capacitance and coronary vasodilation [84]. S-nitrosothiols are a heterogeneous group characterized by a nitroso group attached by a single chemical bond to the sulfur atom of a thiol [83]. S-nitrosoGlutathione (GSNO) is found *in vivo* and is

an important intermediary in organic nitrate metabolism. The remaining nitrosothiols are synthetic. These compounds act as NO-carriers, NO-reservoirs, and intermediates in protein nitrosylation. They also possess the ability to transfer the different NO species through chains of thiols, without releasing the NO molecule itself. This feature decreases the possibility of NO reacting with O^{2-} to form $ONOO^{-}$, or that of reacting with other molecules to nitrosylate these [94, 95]. Sydnonimines release NO spontaneously, without enzymatic activity. Superoxide is generated concomitantly and may combine with NO to generate $ONOO^{-}$. This process also releases significant quantities of hydroxyl radical, which increases its prooxidant potential [96]. Therefore, these compounds are considered peroxynitrite donors more than NOD and are utilized as nitrosative stress inducers in experimental models. NONOates decompose spontaneously in solution, at physiological pH and temperature, without interaction with biological molecules and in a concentration-dependent manner; thus, they usually are employed as NO-release models [90].

Sodium nitroprusside (SNP) is a compound consisting of an iron core surrounded by five Cyanide ion (CN^{-}) molecules and one molecule of the Nitrosonium ion (NO^{+}). SNP does not liberate NO spontaneously *in vitro*, but does require partial reduction (one-electron transfer) by a variety of reducing agents present in membrane cells. It is also possible to release NO from SNP by photolysis. In addition to NO, SNP can release, in aqueous solution, a range of oxidant and free radical species, such as iron, cyanide, superoxide, H_2O_2, and hydroxyl radical in direct proportion to its concentration [97–99]. Because of the nitrosative and prooxidant potential inherent in the different NOD, these have been widely used as models of neuronal damage (for a more detailed review, see [83, 84, 94]).

3.3. Sodium Nitroprusside-Induced Neurotoxicity. The potentially adverse effects of SNP on cells and tissues have been widely used *in vivo* and *in vitro* to study the mechanisms involved in nitrosative and OS injury. While many of the pharmacological effects elicited by SNP are attributed to the NO molecule, several *in vitro* studies [100, 101] revealed other biological SNP properties that are independent of the NO moiety, due to the huge number of by-products released during its decomposition (e.g., cyanide, iron, and ROS).

In vitro, SNP is usually neurotoxic. This compound is able to cause cytotoxicity in the human neuroblastoma cell line SH-SY5Y by means of OS. In addition, SNP treatment activates the (ERK1/2) pathway and inactivates the Akt pathway, leading to cell death [102]. Additionally, inhibition of ERK activation or exogenous Superoxide dismutase (SOD) treatment protects human melanoma from SNP toxicity [103]. Furthermore, in hippocampal neurons, SNP and SIN-1 are capable of decreasing Bcl-2 and increasing Bax expression along with caspase-3 activation, leading to neuronal apoptosis [104]. Concentration-dependent neuronal death was induced in cerebellar granular cells after exposure to SNP by hydroxyl radical generation, as well as by increasing the level of iron and lipid peroxidation [105]. In cultured cholinergic cells, SNP impairs oxidative metabolism of Acetyl Co-A by suppression of choline acetyltransferase and pyruvate dehydrogenase activities in mitochondria and cytoplasm. This effect triggers OS and a reduction in neuronal viability [106].

In addition to SNP, other NOD are able to elicit cytotoxicity *in vitro*. In cortical neuronal cultures, SIN-1 induced neurotoxicity by ATP depletion and protein nitration, which was counteracted by the addition of hemoglobin (a NO scavenger), SOD, and an ONOO scavenger, demonstrating that the main mediator of damage in this case is $ONOO^{-}$ [107]. In the same cell culture type, neuronal viability was significantly reduced when compared with that of controls after DETANONOate exposure. This effect was associated with a decrease in catalase activity and expression [108].

Likewise, SNP has been used to induce neurotoxicity *in vivo*. While not mimicking a specific neuropathology, the rapid and localized neurodegeneration and demyelination caused by the SNP, when injected into the brain, provides a very practical tool for studying the role of the individual molecular players that can be involved in the immediate and consequent damage implicit in neurodegenerative processes. In animal models, SNP causes acute and localized excitotoxic cell death when infused within the brain parenchyma. This damage is also associated with a transient inflammatory response [109]. The neurodegeneration caused by SNP is accompanied by microglial activation and the induction of the proinflammatory cytokines TNF-α and IL-1β. Injection of exogenous TNF-α was shown to exacerbate the damage and inflammation caused by SNP through specific and transient activation of resident microglia [109]; in contrast, the abolition of the endogenous production of TNF-α genetically is also detrimental, because it delays microglial activation, which is later expressed in an excessive manner. However, these effects do not extend to the IL-1B. Thus, this suggests that the source, timing, and dose of TNF-α are preponderant in determining the fate of neurons and myelin during SNP-induced neurotoxicity [29].

Additionally, when infused into the substantia nigra, SNP induces an acute increase in lipid peroxidation, which is blocked by NO, oxyhemoglobin, and deferoxamine (an iron chelator), suggesting that OS is elicited, at least in part, by the iron moiety of SNP [110, 111]. Thus, the addition of SNP and other NOD to neuronal cultures or into brain parenchyma causes damage through the establishment of OS, nitrosative stress, and the disturbance of cellular oxidative metabolism. The death pathway activated through these mechanisms is mainly apoptotic.

However, it should be considered that the cytotoxic effects of NOD are not necessarily due to the presence of NO, because its addition to culture media alone does not cause neurotoxicity [112]; therefore, other compounds that are also part of the NOD molecules can be delivered with different effects. Such is the case of SNP, in which its toxicity lies more in its content of iron [110, 112, 113] and cyanide ions [114] rather than in its NO content or, as the case of SIN-1, whose decomposition releases superoxide anion and hydroxyl radicals along with NO, leading to the production of large amounts of $ONOO^{-}$ [96].

Finally, it should be taken into account that the cytoprotective and physiological effects of NO described (e.g., vasodilatation, neurotransmission, endothelial protection) require extremely small concentrations (pico- to nanomolar), while harmful effects take place at higher concentrations, particularly under OS [93]. In culture and in intracerebral application, the NOD concentrations usually administered fall within the micro- to millimolar range. Thus, direct contact of NOD with neurons in culture or intraparenchymally coupled with the high concentration of these could be mimicking overactivation of nNOS or iNOS during the postischemic reperfusion period or in other neurodegenerative disorders [83]. Figure 1 depicts the signaling pathways involved in the neurotoxic effects of NO.

3.4. Nitric Oxide as an Anti-Inflammatory and Neuroprotective Agent. In contrast to the evidence presented in the previous section based on the reactivity of NO with iron and ROS, in 1994 Chiueh proposed that NO and related donor compounds may protect against the OS induced by small-molecular-weight iron complexes in the dopaminergic nigrostriatal system [115]. Since then, a growing number of reports have confirmed the potent neuroprotective and antioxidant actions of NO in the brain in experimental models of Parkinson disease [116–120]. In addition, NO has shown to inhibit lipid peroxidation of low-density lipoprotein oxidation [121, 122] in order to protect against neurotoxin-induced dopaminergic neurotoxicity [112, 118, 123, 124] and to shield cells from OS [125–127], protecting these *in vivo* through both antioxidative and -apoptotic mechanisms [118].

In the hippocampus, NO mediates cellular transduction mechanisms, regulates neuronal plasticity [128], and suppresses neuronal apoptotic cell death [129]. Thus, NO may be neuroprotective or restorative after a stroke [130–132], after traumatic brain injury [133, 134], and during Alzheimer disease [135] and depression [136].

3.5. NO Donors Exerted a Neuroprotective Effect against Cerebral Ischemia-Reperfusion Injury at Different Levels by Influencing Cellular Oxidative Status. SNP and SPERMINE-NONOate are able to reduce infarct size after transient focal cerebral ischemia when administered early [87]. Likewise, S-nitrosothiols (GSNO and SNAP) and SIN-1 additionally reduced infarct volume and improved neurological performance [38, 88, 89]. Hemodynamically, SNP, GSNO, and SNAP increase CBF in the penumbral region when administered at the onset of reperfusion [38, 80, 89].

In addition, pre- and postischemic administration of SNP attenuates the ischemia-induced increase of caspase-3 at 6 h of reperfusion and downregulates neuronal apoptosis by inhibiting increased phosphorylation of JNK, c-Jun, and Bcl-2 [61, 65]. This effect is achieved through nitrosylation of nNOS, which decreases its NO production. This means that SNP can regulate NO metabolism in the target cells. In focal ischemia, SNP and S-nitrosothiols decrease lipid peroxidation and nitrotyrosine formation in plasma, which is associated with less oxidative and nitrosative stress, neuroprotection, and fewer anti-inflammatory effects [38].

The effects of NOD on ischemia-reperfusion injury are also related with modulation of the inflammatory response, and these effects are probably the neuroprotective effects with the greatest impact after cerebral ischemia and reperfusion of this drug type. To shield nerve tissue from ischemia-reperfusion injury is not sufficient to protect the brain parenchyma, but also must preserve the integrity of the Blood brain barrier (BBB). Thus, the cerebral vascular endothelium is essential in the control of vascular inflammatory and oxidative responses, leukocyte migration, and the production of inflammatory mediators capable of spreading to nerve tissue [26]. Under physiological conditions, eNOS-NO is responsible for maintaining the integrity of the vascular endothelium. But under ischemic conditions, endothelial dysfunction could be offset by mimicking eNOS-derived NO neuroprotective functions by intravascular administration of a NOD [137]. Control of endothelial inflammatory and oxidative responses in turn allows restriction of their impact on resident brain cells, particularly on those with an inflammatory phenotype, such as microglia and astrocytes. Therefore, effective neuroprotection should include protection of the BBB and of the elements within it [138, 139].

In vivo, high expression of TNF-α, IL-1β, and iNOS in microglia and astrocytes after focal cerebral ischemia is reduced by GSNO. Likewise, GSNO induces a reduction in microglial and macrophage cells in the penumbral region, which is associated with less expression of cellular adhesion molecules such as ICAM-1 in endothelial cells [56]. Decreased expression of adhesion molecules (ICAM-1 and E-selectin) was also demonstrated with SNP and SNAP in the same model [38].

Anti-inflammatory effects are also demonstrated in other neuronal-damaged models, such as experimental autoimmune encephalomyelitis [140] and traumatic brain injury [134]. Under both conditions, NOD inhibited the expression of cell adhesion molecules and infiltration of vascular immune cells into the CNS, which subsequently led to reduction in the expression of proinflammatory cytokines at the site of injury. This suggests less damage to BBB integrity, which is an indicator of neuroprotection.

Beyond the CNS, SNP protects other organs from inflammatory damage. In cardiac surgery, SNP decreases cardiac cytokine release [141–143] and improves postischemic cardiac function [143]. In experimental models of ischemia-reperfusion injury, such as those in kidney [144] and lung [145], SNP attenuates the expression of proinflammatory cytokines and reduces leukocyte-endothelium adhesion, respectively.

3.6. Anti-Inflammatory Mechanisms of Nitric Oxide Donors. *In vitro* studies have elucidated some of the mechanisms involved in the anti-inflammatory effect of NOD. It is well documented that cerebral ischemia, and particularly reperfusion, leads to nuclear translocation of NF-κB into the core and ischemic penumbra [146, 147], as well as into the microvessels of the affected region [148, 149]. NF-κB is a key regulator of innate immunity, inflammation, and of cell survival and proliferation [150]. This inducible transcription factor is comprised of two subunits. There are five subunits that can

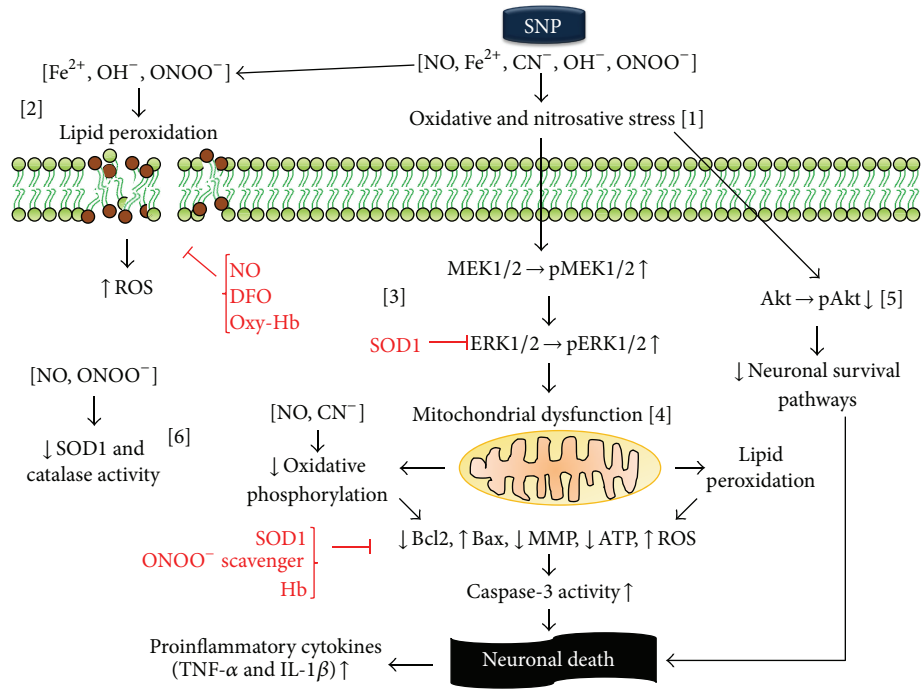

Figure 1: *In vitro and intracerebral effects of sodium nitroprusside and other nitric oxide donors (NOD) on neuronal survival.* SNP is capable of releasing or producing diverse byproducts, such as nitric oxide (NO), iron, cyanide anions, hydroxyl radicals, and peroxynitrite. Collectively, these are all capable of inducing oxidative and nitrosative stress [1], with the possibility of modifying the structure and function of proteins, nucleic acids, and lipids by means of oxidation and nitrosylation. Iron, via the Fenton reaction, generates OH^- that, together with $ONOO^-$ and other reactive species, damage membranes by lipid peroxidation [2] with decreased cellular viability. This effect is blocked by the addition of NO, oxyhemoglobin, and deferoxamine, which suggests the important role of iron and NO in this reaction. The oxidative stress (OS) produced by SNP increases the activation of MEK1/2 and its substrate ERK1/2 by phosphorylation [3]. Both effects are blocked by SOD, suggesting the participation of (O^{2-}) in this reaction, probably in the form of $ONOO^-$. Activation of ERK1/2 is associated with a reduction of Bcl2 and an increase in (Bax), and both conditions are associated with an activation of mitochondrial apoptotic pathways. Mitochondria are a target of SNP at different levels: SNP induces lipid peroxidation of its membrane with the subsequent activation of proapoptotic pathways via caspases. In addition, NO and CN^- affect the functioning of the mitochondrial respiratory chain, thereby altering mitochondrial membrane potential, reducing ATP production and the generation of large amounts of reactive oxygen species [4]. The addition of $ONOO^-$ scavengers and SOD1 counteracts this effect. Also, SNP decreases Akt phosphorylation [5] and reduces the expression and function of SOD1 and catalase [6]. These actions decrease antioxidant responsiveness and the activation of neuronal survival pathways. OH^-, hydroxyl radical; $ONOO^-$, peroxynitrite; Akt, protein kinase B (PKB); Bax, Bcl-2-associated X protein; Bcl2, B-cell lymphoma 2; CN^-, cyanide anion; ERK1/2, extracellular signal-regulated kinase 1/2; IL-1β, interleukin 1 beta; MEK1/2, mitogen-activated protein kinase kinase 1/2; MMP, mitochondrial membrane potential; NO, nitric oxide; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD1, superoxide dismutase (Cu-Zn); TNF-α, tumor necrosis factor alpha.

be combined to yield homo- or heterodimers of NF-κB as follows: p50, p52, c-Rel, p65 (RelA), and RelB [151]. C-Rel-containing dimer activation increases neuron resistance to ischemia [152]. Moreover, the prevalent heterodimer during cerebral ischemia and reperfusion is formed by p50- and p65-inducible subunits, and its activation contributes to the pathogenesis of postischemic injury [146, 152, 153]. NF-κB is maintained in latent form in the cytoplasm of cells bound to inhibitory IκB proteins. Phosphorylation of IκB releases NF-κB by permitting its translocation into the nucleus, its binding with NF-κB motifs, and the subsequent activation of its target genes. There is, in turn, an enzymatic complex responsible for IκB phosphorylation in specific serine residues, the so-called IκB kinases (IKK). Activation of IKK is essential to induce NF-κB activity [154].

In the ischemic brain, a wide range of stimuli may trigger activation of NF-κB including, among others, the following: hypoxia [155]; IL-1, and TNF-α [156]; OS [157]; glutamate [158], and NOS activity, such as nNOS and iNOS [159]. Overactivation of NF-κB after ischemia has been documented in neurons [147], astrocytes [53], microglia [160], and in endothelial cells [149]. Although in some hippocampal neurons NF-κB have a constitutive action related with neuronal survival [150], overactivation of the p50/p65 heterodimer in neurons, glial, and endothelial cells due to ischemia, appears to contribute to acute neurodegeneration. In neurons, NF-κB translocation has been associated with apoptosis [146, 147], while in glia and in vascular endothelium, NF-κB activates a proinflammatory phenotype [53, 149, 160].

Therefore, blocking inflammatory phenotype activation of NF-κB could disrupt the cascade of events that culminate in proinflammatory brain tissue destruction. In human endothelial cells, the addition of exogenous NO through GSNO limits TNF-α activation of NF-κB in a time- and

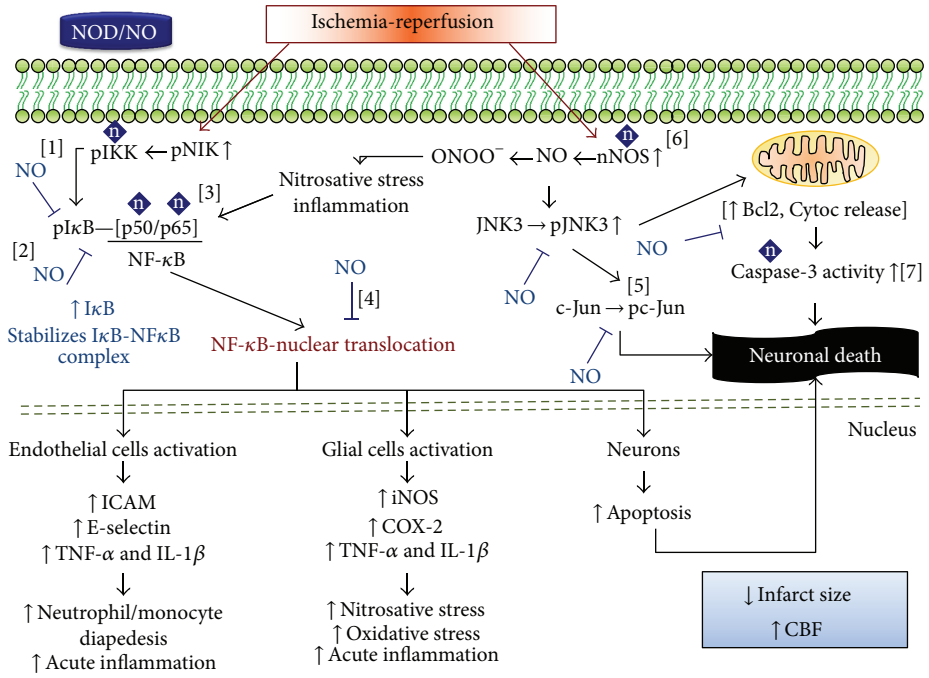

Figure 2: *Cerebral ischemia-reperfusion activates two major signaling pathways which exert an effect on NOD. (A) NF-κB pathway*. Oxidative stress (OS) and inflammatory stimuli phosphorylate NIK. Subsequently, IKK phosphorylates NIK, which in turn phosphorylates IkB, resulting in Iκb degradation and NF-κB translocation and activation. This action exerts different effects depending on the cell line. In endothelial cells, NF-κB promotes a proinflammatory phenotype, with the expression of cellular adhesion molecules and proinflammatory cytokines that induce leukocyte migration to the ischemic territory and promote acute inflammation. In glial cells, NF-κB leads to the expression of iNOS, COX-2, and proinflammatory cytokines. These effects contribute to nitrosative, oxidative, and inflammatory damage. Finally, in neurons, NF-κB induces the expression of apoptosis pathways. NOD can act at different levels in this pathway: NOD-derived NO diffuses across target cell membranes, where it is able to nitrosylate kinases located upstream of NF-κB, such as IKK, thereby suppressing their ability to phosphorylate [1]. This inhibition prevents Iκb phosphorylation and its degradation; thus the release of NF-κB. NO can also increase Iκb gene transcription and stabilize the complex formed by Iκb and NF-κB [2]. Furthermore, NOD-derived NO is capable of nitrosylating directly into the p50 and p65 subunits of NF-κB, which blocks their ability to migrate to the nucleus [3]. All of these actions prevent the nuclear translocation of NF-κB, therefore the expression of their target genes [4]. (B) *Cerebral ischemia-reperfusion increases nNOS activity, which enhances its NO production*. This NO can react with free radicals to produce $ONOO^-$ and also activates the JNK3 pathway. The result is c-Jun phosphorylation and mitochondrial dysfunction, with an increase in Bcl2 phosphorylation and cytochrome C release into the cytoplasm. In addition, this activates caspase-3 and leads to neuronal apoptosis. NOD-derived NO downregulates neuronal apoptosis by inhibiting increased phosphorylation of JNK, c-Jun, and Bcl-2 [5]. This is achieved by S-nitrosylation of nNOS, which interferes with its NO production [6]. NO is also capable of nitrosylating caspase-3 directly [7]. All of these effects, along with an increase in CBF, reduce brain damage after the ischemia-reperfusion event. Bcl2, B-cell lymphoma 2; CBF, cerebral blood flow; COX-2, cyclooxygenase 2; Cytoc, cytochrome; ICAM, intercellular adhesion molecule; IkB, inhibitors of kB; IKK, Iκb kinase; IL-1β, interleukin 1 beta; iNOS, inducible nitric oxide synthase; JNK3, c-Jun N-terminal kinases 3; NF-κB, nuclear factor kappa B; NIK, NF-κB-inducing kinase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOD, nitric oxide donors; $ONOO^-$, peroxynitrite; TNF-α, tumor necrosis factor alpha; [n] S-nitrosylation.

concentration-dependent manner [161]. This is also achieved by SNP upon stimulation with IL-1α, IL-1β, IL-4, and LPS [162]. NF-κB inhibition is in fact sustained by the constitutive activity of eNOS, because its inhibition, without an inflammatory stimulus, triggers nuclear translocation of NF-κB [156, 161, 162]. By inhibiting activation of the transcription factor, NO effectively blocks monocyte adhesion, as well as the expression of the proinflammatory target genes of NF-κB, such as TNF-α, IL-6, iNOS, V-CAM, ICAM-1, E-selectin, and COX-2 [70, 156, 162–164].

In astrocytes and microglial cells, NOD also exhibits an anti-inflammatory profile through downregulation of NF-κB. In primary rat astrocytes and in a BV2 microglial cell line, GSNO mitigates iNOS production by inhibiting the ability of NF-κB to bind to DNA [53]. Therefore, NOD are capable not only of regulating NF-κB at the vascular level, but also they possess the capability of influencing glial cell reactivity and limiting their production of iNOS, proinflammatory cytokines, and other molecules and prooxidant enzymes that are potentially harmful to neuronal cells.

Inhibition of NF-κB by exogenous NO has been documented at different levels. In the brain ischemic environment, activation of NF-κB occurs, at least in part, via ROS [165]. Former researches have shown that one of the most significant sources of ROS in the ischemic brain is through the metabolism of arachidonic acid by Cyclooxygenase (COX) [166, 167]. COX-2 expression is increased in brain tissue after global [168] and focal [167, 169] cerebral ischemia. ROS are

produced by the peroxidase step of the COX reaction in which prostaglandin G2 is converted into prostaglandin H2 [170, 171]. Hence, reducing COX-2 activity reduces oxidative damage of the ischemic brain [167, 169]. The NO donors GSNO and SNP are able to downregulate LPS-induced COX-2 protein expression via inhibition of NF-κB DNA binding activity in murine monocytes [172]. Therefore, both drugs may be candidates for neuroprotective antioxidants in cerebral ischemia. In addition, NO is a superoxide scavenger; hence, NO may inhibit NF-κB by scavenging superoxide anion [162].

Furthermore, SNP is capable of interfering directly with the ability of NF-κB to translocate into the nucleus. Specifically, SNP inactivates NF-κB by nitration of the p65 subunit at Tyr-66 and Tyr-152. This protein modification suppresses iNOS mRNA expression and prevents the activation of NF-κB target genes by TNF-α stimulation [164]. S-nitrosylation of the p50 subunit at Cys-62 has also been demonstrated as a major mechanism of NO regulation by inhibition of the p50 binding to its consensus DNA target sequence [173].

With respect to IκB-α, exogenous NO increases mRNA IκB levels and stabilizes the complex formed with NF-κB [161, 172]. This stabilization is related with S-nitrosylation of the Cys-179 of IKKß, which decreases its ability to phosphorylate IκB [174]. Additionally, NO interferes with the transient degradation of IκB-α induced by cytokines [70]. These three actions induce negative regulation of NF-κB DNA-binding activity by NOD. Figure 2 sums up the neuroprotective actions of NOD.

4. Concluding Remarks

Understanding the interaction between the CNS and the immune system will provide greater insight into several different pathologies that involve CNS inflammation and the increase in the number of potential pharmacological targets.

The great variability in the observed effects elicited by NOD, from neuroprotection to toxicity, could be due to the great diversity in doses used in the experiments, which in fact are mainly distant from the existing physiological concentrations. Clarity about the NO concentrations that exists physiologically is essential for developing a quantitative understanding of NO signaling, for performing experiments with NO that emulate reality, and for knowing whether or not NO concentrations become abnormal in disease states [175]. Several independent lines of evidence suggest that NO operates physiologically at concentrations that are orders of magnitude lower than the near-micromolar order once considered correct.

Accordingly, physiological NO concentrations range from 100 pM (or below) up to 5 nM (reviewed in [175]). Therefore, the establishment of reliable methods for directing microelectrode measurement of NO concentrations and the (most foreseeable) progression of newly developed NO biosensors for quantitative imaging of NO signaling in subcellular dimensions and in real time in tissues *in vivo* will facilitate advances in this fundamental, but yet unsettled, area.

In addition to NOD concentration, it is relevant to consider the administration pathway (intravascular, intraperitoneal, or directly into the culture media *in vitro*), as well as the cell type in which the donor exerts its action, together with the cell redox state (reduction-oxidation), because these factors are determining ones in selecting the signaling pathway that will be affected or modified by the action of these.

Therefore, therapeutic use of these molecules must be performed carefully, because they can be beneficial for one tissue or cell type and harmful for others. Given their short therapeutic window, NOD appear appropriate for use during neurosurgical procedures involving transient arterial occlusions or in very early treatment of acute ischemic stroke [87].

At present, translation from *in vitro* to *in vivo* preclinical stroke models requires further research, as clearly as that required for the case for translation from *in vivo* animal models to the clinical condition of drugs for treatment of acute ischemic stroke, which requires overcoming phase III trials in patients.

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Review Article

Link between Cancer and Alzheimer Disease via Oxidative Stress Induced by Nitric Oxide-Dependent Mitochondrial DNA Overproliferation and Deletion

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Nitric oxide- (NO-) dependent oxidative stress results in mitochondrial ultrastructural alterations and DNA damage in cases of Alzheimer disease (AD). However, little is known about these pathways in human cancers, especially during the development as well as the progression of primary brain tumors and metastatic colorectal cancer. One of the key features of tumors is the deficiency in tissue energy that accompanies mitochondrial lesions and formation of the hypoxic smaller sized mitochondria with ultrastructural abnormalities. We speculate that mitochondrial involvement may play a significant role in the etiopathogenesis of cancer. Recent studies also demonstrate a potential link between AD and cancer, and anticancer drugs are being explored for the inhibition of AD-like pathology in transgenic mice. Severity of the cancer growth, metastasis, and brain pathology in AD (in animal models that mimic human AD) correlate with the degree of mitochondrial ultrastructural abnormalities. Recent advances in the cell-cycle reentry of the terminally differentiated neuronal cells indicate that NO-dependent mitochondrial abnormal activities and mitotic cell division are not the only important pathogenic factors in pathogenesis of cancer and AD, but open a new window for the development of novel treatment strategies for these devastating diseases.

1. Introduction

Mitochondrial decay has been postulated to be a significant feature underlying aging and age-related disease processes [1]. Mitochondrial dysfunction and free radical-induced damage play a significant role in the pathogenesis of tumors, tumor-growth, metastasis, and cellular and tissue aging [2]. Decline in mitochondrial function most likely leads to

cellular energy deficits, especially during situations known to require increased energy demand and in organs or tissues where the energy needs and metabolic demand are particularly high, such as in the brain or fast-growing tumors. These deficits can compromise vital adenosine triphosphate- (ATP-) dependent cellular functions, such as detoxification, system repair, DNA replication, ATP-dependent protein degradation, and osmotic balance. As a result of this increased energy demand coupled with hypoxia and oxidative stress, some tumors switch to glycolysis to meet energy demands. Similarly, defective ATP production and increased generation of reactive oxygen and nitrogen species (ROS and RNS) may induce mitochondrial-dependent cell death as the damaged mitochondria are unable to maintain the energy demands of the cells [1].

2. Physiological Roles of NO and NO Synthase (NOS)

NO, a free radical species, is a well-known physiological signaling agent, and a pleiotropic regulator in various pathologies including tumor growth and AD [2, 3]. It is synthesized by nitric oxide synthase (NOS) enzymes by transforming L-arginine to L-citrulline. NOS enzymes comprise inducible NOS (iNOS or NOS2), endothelial NOS (eNOS or NOS3), and neuronal NOS (nNOS or NOS1) [2–6]. Various studies have shown that each of the three isoforms may be implicated in either promotion or inhibition of human cancer development. High amounts of iNOS expression, caused by activated macrophages, may be cytostatic or cytotoxic for tumor cells; in contrast, low activity may have an opposite effect and promote tumor growth [2, 6]. In fact, nitric oxide may play a crucial role in mitochondrial respiration [4–6], since even low (nanomolar) concentrations of NO were found to reversibly inhibit the mitochondrial respiratory chain enzyme cytochrome oxidase (complex IV) and compete with molecular oxygen. Inhibition of cytochrome oxidase by NO results in the reduction of the electron-transport chain, and favors the formation of the superoxide radical anions (O_2^-). NO upon reaction with superoxide radical anion forms peroxynitrite ($ONOO^-$), which is more cytotoxic than NO itself [2, 3, 7]. Peroxynitrite has been identified as a potent oxidant and potential mediator of vascular tissue injury [3] and cell death [3, 7]. Several laboratories have investigated the cellular consequences of endogenously generated and exogenously applied NO [4–6]. Accumulating evidence demonstrates that endogenous NO (using endothelial cells (EC)), basally produced or generated in response to stimulation with bradykinin, reduces the rate of oxygen consumption by the cells [8]. This finding suggests that endogenous NO modulates oxygen consumption under basal and stimulated conditions and leads to the formation of reactive oxygen species, $O_2^{\bullet-}$ [6]. Moreover, an extended study by the Moncada's group has found that prolonged exposure to exogenous NO results in persistent inhibition of mitochondrial respiration, which is localized mainly at complex I [4–6]. This persistent inhibition seems to be the result of oxidative stress generated from mitochondrial free-radical generation and involves S-nitrosylation of mitochondrial complex I. Indeed, inhibition of the respiratory chain causes its reduction and the subsequent generation of superoxide anions (vide supra). It is likely that these anions are initially converted by superoxide dismutase to hydrogen peroxide, which is known to be a transcription factor of several defense genes. If this inhibition is prolonged, it may result in the generation of peroxynitrite at the site of superoxide anion production [6]. Thus, persistent inhibition of cytochrome oxidase could elicit a two-stage response, an early one in which the main consequence is the release of small amounts of hydrogen peroxide (H_2O_2), and a later one that involves higher concentrations of H_2O_2 and formation of peroxynitrite. However, the mitochondrial DNA overproliferation under these conditions is unknown [3]. Nevertheless, many of the biological effects attributed to NO can be mediated by peroxynitrite [3]. Moreover, superoxide and NO can be produced simultaneously in close proximity, which leads to increased peroxynitrite formation [7]. Even modest increases in both superoxide and NO formation at a 10-fold greater rate increase peroxynitrite formation by 100-fold. Under proinflammatory conditions, simultaneous production of superoxide and NO is rapidly activated thereby increasing the production rates by 1,000-fold, which consequently increase the formation of peroxynitrite by up to 1,000,000-fold. The role of NO-induced mitochondrial failure in the pathogenesis of tumors, particularly tumor angiogenesis, now has been widely accepted. Recently, an aging rat model of chronic brain hypoperfusion (CBH) that mimics human mild cognitive impairment (MCI) was used to examine the role of NOS isoforms on spatial memory function. In one of our studies, rats with CBH underwent bilateral common carotid artery occlusion (2-vessel occlusion (2-VO) were compared with nonoccluded sham controls (S-VO)) [9]. After the administration of neuronal and endothelial (nNOS/eNOS) constitutive inhibitor nitro-L-arginine methyl ester (L-NAME) only 2-VO rats worsened the ability of their spatial memory [9]. Our findings indicate that vascular NO derived from eNOS plays a critical role in spatial memory function during CBH, possibly by keeping cerebral perfusion optimal through its regulation of microvessel tone and cerebral blood flow. This study could lead to the identification of therapeutic targets for preventing MCI and treatment of AD [9].

The role for NO-dependent process is quite clear in AD pathogenesis and remodeling of cortical cholinergic system through degradation of mature nerve growth factor (NGF) in AD. It is also well established that the cortical cholinergic system plays a crucial role in cognitive processing and memory formation [10, 11]. Pharmacological evidence of cholinergic atrophy and metastasis depends on matrix metalloproteinases in both diseases. In an activity-dependent manner NGF precursor forms proNGF, along with the convertases and proteases necessary to form mature NGF (mNGF) and to degrade the free, unbound mNGF by serine protease involved the matrix metalloproteinase 9 (MMP-9) [12]. However, the exact cellular mechanisms behind tumor vascular growth and the relationship to NO oxidation

products, such as nitrotyrosine products, lipid peroxidation, as well as mitochondrial DNA (mtDNA) deletion remains unknown [3].

3. The Role of Endothelin Signaling

During conditions favoring hypoxia, hypoxia-induced transcription factor (HIF-1) binds to the hypoxia response element (HRE) in the endothelin-1 (ET-1) promoter region to induce ET-1 transcription [13]. In response to hypoxia, oxidized low-density lipoprotein (LDL), cytokines, and ET-1 levels are upregulated in EC [14]. The most extensively studied member of the endothelin system or the so-called endothelin axis and its expression is induced by various cytokines and stimuli [14, 15], such as TNF stimulated ET-1 secretion in cultured bovine airway smooth-muscle cells (SMC) and human airway epithelial cells [16, 17]. The TNF superfamily of cytokines are particularly important in cancer progression and apoptosis.

The endothelin axis includes three endothelins (ET-1, ET-2, and ET-3), which are widely expressed in various human tissues including brain, skeleton muscle, testis, pancreas and have similar structure [14] (along with two G-protein-coupled endothelin receptors (ET-RA and ET-RB), two proteinases) as endothelin converting enzymes (ECE-1 and ECE-2) [18]. ET-1 and ET-2 bind to ET-RA more avidly than ET-3, while all three endothelins have similar affinity for ET-RB. When the primary physiological function of endothelins was tested in arteries and veins, ET-1 and ET-2 induce equal maximum contraction and potent responses, whereas ET-3 induces lower maximum contractions and overall a less potent response [19]. Likewise, ET-3 is a factor that attenuates ET-1 signaling through ET-RA. ET-RA is found predominantly in smooth and cardiac muscle cells, whereas ET-RB is highly expressed in EC. ET-RA primarily mediates vasoconstriction, contraction, and proliferation induced by ET-1 [20] and is found predominantly in EC, where it mediates endothelium-dependent vasodilation through NO and prostacyclin. ET-RB is believed to have multiple effects, including EC survival, NO production, and ET-1 clearance [20]. Both ET-RA and ET-RB mediate antiapoptotic effects in human SMC [21].

In many cases, increased levels of ET-1 and the receptors (ET-RA, ET-RB) are detected in tumor tissues [14]. It has been reported that ET-RA mediates ET-1 induced cancer cell proliferation [22] and promotes epithelial-to-mesenchymal transition [23]. ET-RB may mediate antiapoptosis effect induced by ET-1 [24]. The loss of ET-3 expression due to epigenetic inactivation has been reported in human breast cancer by measuring mRNA levels [25]. This is consistent with the speculation that ET-3 may attenuate ET-1 signaling through ET-RA. It favors ET-1 signaling through ET-RA when ET-3 is decreased in cancer cells. In order to understand the detailed functions of endothelins in cancer, signaling pathways in tumor cells initiated by the two different receptors need to be further explored. Diverse signaling pathways from the two different receptors can be the reason why endothelins and the receptors are regulated differently in tumor tissues.

Endothelin signaling is speculated to be involved in cell differentiation, proliferation, migration, and angiogenesis in tumors [25]. It has been demonstrated that ET-1 is overexpressed in various tumor tissues [22], including prostate tumor and high grade prostatic intraepithelial neoplasia [26], breast cancer [27], and lung tumor [28]. Expression of mRNA of ET-1, ET-RA, and ET-RB was detected in ovarian carcinoma cell lines HEY and OVCA 433 by RT-PCR, and secreted ET-1 was detected in the culture media by ELISA [22]. ET-1 and ET-RA are overexpressed in canine ovary tumors [29], which is consistent with the function of ET-RA signaling induced by ET-1 that is involved in cell proliferation. However, transfection often results in a supraphysiological level of expression of target genes, which may induce some artifacts such as increased formation of heterogeneous dimers between ETA and ETB receptors [30].

Although antagonists of endothelin receptors for the treatment of cancer are not in clinical development, specific peptide-based antagonists of ET-RA and ET-RB have been used in *in vitro* and *in vivo* cancer studies. Cancer cell proliferation was reported to be inhibited when ET-RA was specifically blocked in colorectal cancer cell lines [31]. When orally active high affinity ET-RA antagonist ZD4054, which has no detectable affinity for ET-RB, was applied *in vitro*, it inhibited ET-1 induced proliferation of human pre-osteoblast cells [21], human ovarian carcinoma cell lines HEY and OVCA 433 [22] and further demonstrated ET-RA was involved in signaling in cancer cell proliferation.

ET-1 is a potent vasoconstrictor that induces contraction at nanomolar concentrations in several vascular beds. Aliev and coworkers and others have identified multiple inducers of cell cycle reentry, ectopic cell cycle marker and ET-1 overexpression, as a hallmark of cancer, which is also involved in AD [2, 3, 32]. The complex neurodegeneration mechanism underlying AD, although incompletely understood, is characterized by an aberrant neuronal cell cycle reentry. While cell cycle is not the focus of the present paper, the oncogenic parallel between AD and cancer, especially in the context of vascular content, is one the focus of this communication. The pathological evidence of ectopic cell cycle marker and cell cycle regulatory proteins expression in AD suggests that cell cycle reentry is an earlier event, which occurs at prodromal stages, that is, those stages which show formation of either amyloid-beta (Aβ) plaques or neurofibrillary tangles (NFTs) as a hallmark for human AD and/or AD-like pathology in transgenic mice [3, 32]. In this regard, Aliev and coworkers have already demonstrated mitochondrial DNA deletion as well as mitochondrial structural abnormalities in the vascular walls of the human AD, yeast artificial chromosome (YAC), and C67B6/SJL transgenic positive (Tg+) mice overexpressing amyloid amyloid-β precursor protein (AβPP) [33]. We expect similar findings in case of cancer as well, which most likely could have an even more important role in cytotoxicity and hypoxia adaptation by primary and metastatic cancerous cells, as well as within the aging tissues including brain itself. In this regard, recent advances in understanding the pathogenesis of cell cycle reentry which relates the pathogenesis in AD as well as cancer deserves special attention. Key in the AD pathogenesis is NO formation and release

from vascular and immunologic cells and its conversion to peroxynitrite, which nitrates tyrosine residues of enzymes, and causes mitochondrial DNA damage [2, 3, 32]. Moreover, Aliev and coworkers' findings present a strong case for the role of NOSs, ET-1, and their oxidation products such as nitrotyrosine activity in the development of human colorectal cancer metastasis to liver and in malignant brain cancers [2].

4. GRK2 is Upstream in Endothelin Signaling Cascades

NO production by EC seems to be regulated via Akt/PKB signal transduction pathway, which activates eNOS. Akt physically interacts with GRK2 and inhibits Akt activity and its phosphorylation and thus production of NO [34]. In the aforementioned study, GRK2 expression increased in sinusoidal endothelial cells from portal hypertensive rats and knockdown of GRK2 restored Akt phosphorylation and NO production, and normalized portal pressure. Thus, an important mechanism underlying impaired activity of eNOS in injured sinusoidal EC was found to be defective phosphorylation of Akt caused by overexpression of GRK2 after injury (Figure 1).

Some of us and others [35–38] have also found a critical role of GRK2 in the endothelin signaling cascade and many of the effects of ET-1 on cancer may be mediated by GRK2 (Figure 2). In this regard, it is important to note the importance of GRK2 on ET-1 receptors, downstream events, and its relationship with cancer. The importance of the strong immunologic relationship to most cancers is illustrated in part by high expression of GRK2 in different cellular types of the immune system. This emerges as an important regulator of cell responses during inflammation, such as leukocyte trafficking to the inflammatory foci, T-cell egression from lymphoid organs, leukocyte activation, or proliferation [39]. GRK2 is known to phosphorylate chemokines and chemotactic receptors for CCR5, CCR2b, CXCR4, CXCR2, and substance P, S1P or formyl-peptide, respectively [39].

Aberrant epithelial cell motility plays a key role in cancer progression and metastasis. GRK2 expression levels might alter migratory responses in pathological conditions (Figures 1 and 2). A potential role for GRK2 in epithelial cell migration was investigated by Penela and colleagues, where GRK2 was found to promote actin cytoskeletal changes and paxillin localization consistent with enhanced focal adhesion turnover and higher cell motility [40]. These authors further found that GRK2 promotes increased migration towards fibronectin in different epithelial cell lines and in fibroblasts, where these effects were independent of GRK2 kinase activity. The contrary has been described in immune cells, where increased GRK2 expression facilitates migration towards fibronectin and GRK2 downregulation impairs migration of the epithelial cells [40]. GRKs seem to be a new target for therapeutic intervention. In addition to a currently available ET receptor antagonist, overexpression of GRK2 attenuated ET-induced SMC proliferation and ETA receptor desensitization mechanisms in vascular SMCs [35]. Guo and colleagues [41] showed TGFβ-induced GRK2 expression attenuates Angiotensin II-regulated vascular smooth muscle cell proliferation and migration. GRK2 acts through a negative feed-back loop mechanism to terminate TGF-induced SMAD signaling. Activation of the TGFβ signaling cascade in VSMCs results in increased GRK2 levels and inhibits Angiotensin II-induced ERK phosphorylation, and antagonizes Angiotensin II-induced VSMC proliferation and migration at Mek-Erk interface [42]. Although ET-1 can elicit prolonged physiologic responses, GRKs most likely initiate ET-R desensitization. Moreover, endothelin A and B receptors (ETA-R and ETB-R, resp.) can be regulated indistinguishably by GRK-initiated desensitization. Furthermore, GRK2 and platelet-derived growth factor (PDGF) was reported to attenuate SMC proliferation [43].

Finally, emerging evidence points a role of GRK2 as both an extrinsic and intrinsic cell-cycle regulator. GRK2 expression is reported to have distinct impact on cell proliferation and mitogenic signaling depending on both the cell type and the mitogenic stimuli (Figures 1 and 2). It also has diverse regulatory roles directly related to cancer. The complex functional interaction networks during cell cycle progression that are critical at particular stages of the cell cycle and in cell cycle progression plays a critical role in driving timely progression through G1/S and G2/M transitions in a kinase-dependent and -independent manner through interaction with CDK2/cyclinA and Pin1 [40]. In this regard, GRK2 levels are controlled normally by cell-cycle machinery and in response to DNA damage and differentially contribute either to cell cycle progression or cell arrest in a receptor-independent manner. When DNA is damaged, the pathways can be disrupted and in this case GRK2 can promote increased cell survival as a proarresting factor (see Figures 1 and 2). GRK2 protein levels are transiently downregulated during the G2/M transition through CDK2-mediated phosphorylation of GRK2, and preventing GRK2 phosphorylation impedes normal GRK2 downregulation and markedly delays cell cycle progression [40]. Of importance is GRK2 protein decay in G2, which is prevented in the presence of DNA damaging agents that trigger cell cycle arrest. Moreover, in cells with higher steady-state levels of the kinases, increased stabilized GRK2 levels inversely correlate with the p53 response and the induction of apoptosis [40]. Conversely, GRK2 is reported to cooperate with known oncogenes in transformation assays [44] and GRK2 has regulatory roles, which depend on extrinsic cues promoting cell division, as the GRK2-mediated phosphorylation of Hedgehog/Smoothened pathway triggers control of cell proliferation to promote Smo activity and relieve the Patched-dependent inhibition of cyclin B through Hedgehog ligand [45].

Certain signaling pathways instrumental in many cancers cause the upregulation of GRK2 protein levels in malignant cell lines [46, 47]. It is known that altered GRK2 expression levels modulate chemokines-mediated induction of MEK/ERK activity through both kinase-dependent and -independent function [48] and its aberrant epithelial cell motility that plays a key role in cancer progression and metastasis (Figure 1). GRK2 protein levels have been differentially upregulated in tissue samples of patients with

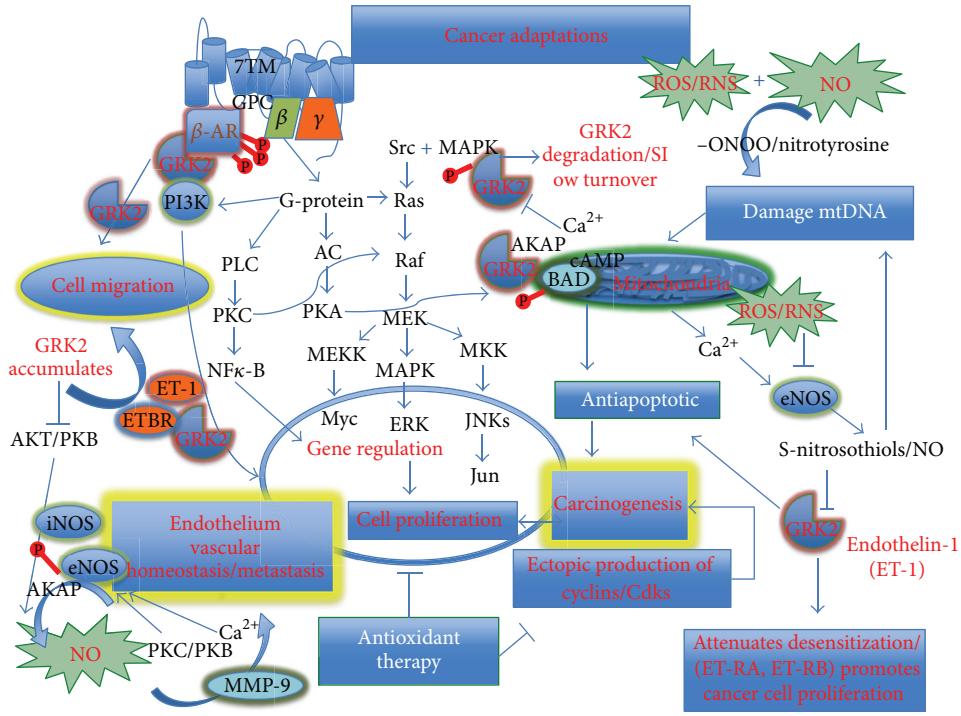


Figure 1: The hypothetical schematic drawing the potential role of GRK, MAPK, JNK, and p38 in the adaptive response during the development and metastasis of the tumorigenesis.

granulose cell tumors, with differentiated thyroid carcinoma [49, 50] or downregulated in a subgroup of prostate tumors [51], which suggests that altered GRK2 expression in specific tumor cells may affect migration in response to particular stimuli and plays a critical role in basic cellular functions such as cell proliferation, differentiation or migration during development. Further, GRK2 inhibits TGF-mediated cell growth arrest and apoptosis in human hepatocarcinoma cells [46]. On the other hand, GRK2 attenuates serum- or PDGF-induced proliferation of thyroid cancer cell lines [49] and smooth muscle cells [43], whereas its expression increases MAPK signaling in response to EGF in HEK-293 cells [52] and GRK2 kinase activity is required for IGF-1-triggered proliferation and mitogenic signaling in osteoblasts [53] (Figures 1 and 2).

5. The Role of NOSs and ET in Liver Colorectal Metastatic Tumors

The absence of perivascular nerves in tumor vessels suggests that endothelial derived vasoactive substance NO and ET-1 may be the key factors in controlling tumor blood flow during tumor growth and metastasis [2]. In our earlier study, the ultrastructural distribution of different NOS isoforms and ET-1 immunoreactivity in human colorectal metastatic tumor liver was identified to know the role of NOSs and ET-1 in the pathophysiology of colorectal metastatic tumors by using preembedding peroxidase-anti-peroxidase (PAP) and postembedding immunoelectron microscopic triple gold labeling techniques [2].

Electron Microscopic PAP techniques determination of the distribution of NOS1 immunolabeling features in control (Figure 3(a)) and metastatic colorectal cancer liver tumor tissues (Figures 3(b)–3(d) showed that NOS1 immunopositive EC were seen in control liver microvessels. In contrary to these observations, tumor vessel endothelium showed no staining for NOS1 antibody (Figure 3(b)). However, presence of the NOS1 immunopositive white blood cells was attached to vessel endothelium in tumor growth regions often observed (Figure 3(c)). In addition, NOS1 immunopositive myofibroblast (smooth muscle cell) was also seen in metastatic liver tumor tissues (Figure 3(d)).

Ultrastructural labeling of inducible NOS (NOS2) immunoreactivity in metastatic liver tumor tissues determined by using electron microscopy and PAP immunocytochemical techniques showed that almost all of tumor vessel EC was positively stained with NOS2 (Figure 4(a)). Very often a high intensity of NOS2 immunopositive precipitate accumulated close to the luminal plasmalemma of the vascular EC in the tumor growth region (Figure 4(b)), indicating the elevated tissue levels of NO and ET-1 [2, 54]. The presence of NOS2 immunopositive hepatocytes and myofibroblast-like cells is also seen throughout the tumor growth area (Figure 4(c)). However, the lipid-contained areas of the cells were free from PAP immunopositive reaction (Figures 4(c)-4(d)).

Ultrastructural features of endothelial specific NOS (eNOS or NOS3) labeling in control (Figure 5(a)) and metastatic liver tumors tissues (Figures 5(b)–5(d)) determined by using PAP method shows the presence a large number of NOS3 immunopositive EC in control liver

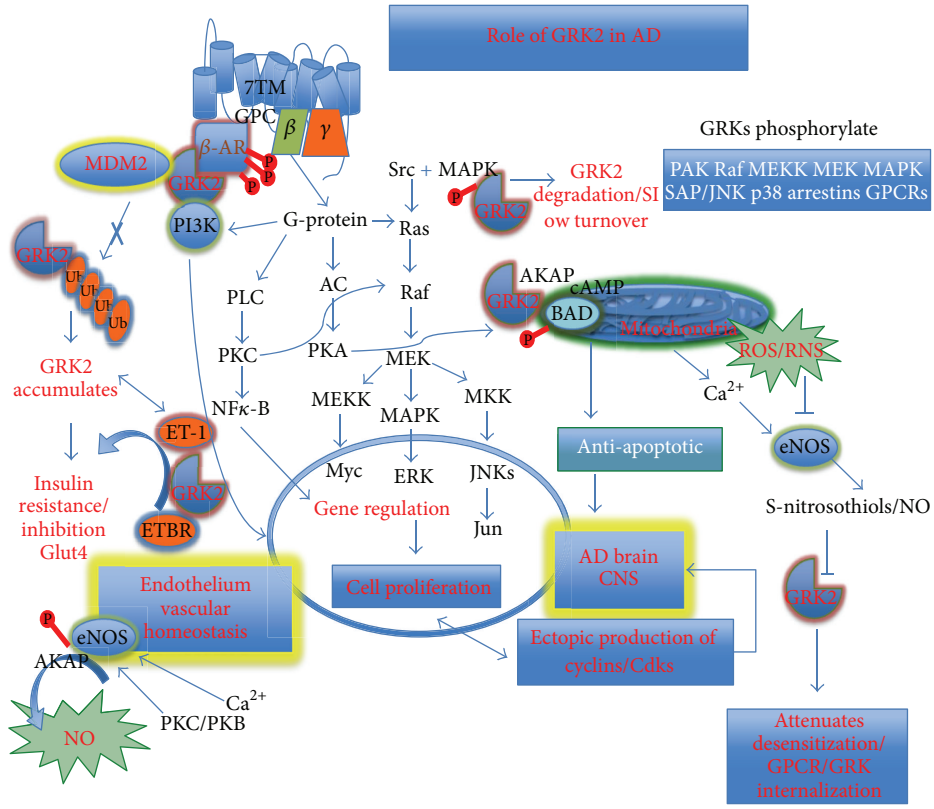


Figure 2: The schematic drawing pattern of the GRK2 overexpression that most likely appeared to be as a compensatory to the hypoxia and hypoperfusion induced oxidative stress that initiates the development and maturation of AD. Modified and reprinted with permission of CNS Neurol Disord Drug Targets [92].

microvessels. NOS3 immunostaining was absent in EC in tumor vessels (Figures 5(b)-5(c)). However, the presence of NOS3 immunopositive hepatocytes in metastatic liver tumors was seen. Lipid-enriched areas were free from NOS3 immunopositive precipitate (Figure 5(d)).

Our extended study by using postembedding triple immunogold labeling techniques showed that the clusters of NOS2 positive, but no NOS3 and ET-1 immunopositive containing gold particles were seen in tumor vessel endothelium (Figure 6(a)). The expression of NOS1 containing positive gold particles was seen in the matrix of lipid laden hepatocytes in tumor growth area (Figure 6(b)). Very often the clusters of ET-1 but not NOS1 and NOS3 positive gold particles in the cytoplasmic matrix of hepatocytes were seen (Figure 6(c)). EC from metastatic liver microvessels prepared as negative controls (through omission of the primary antibody) showed only the presence of single gold particles (Figure 6(d)). Our study highlights mitochondria as a critical constituent responsible for cell viability, which can be considered as a new research focus and of new diagnostic criteria for the earlier detection of tumors as well as treatment strategies at least in some tumors. However, further study needs to be carried out in order to clarify the exact nature of these relationships during the metastases and growth of primary and/or metastatic tumors.

6. Mitochondrial Lesions and Oncogenic Signaling Cascades

Aliev and coworkers' ongoing studies suggest that the mitochondrial lesions are the hallmarks of the primary glioblastoma (Aliev et al., unpublished observation). Vessel endothelium from tumor tissues shows the damage of mitochondrial cristae. The mitochondria-derived lysosomes appear to be a permanent feature of the glioma-derived tumor cells. Lipid laden tumor cells and surrounding cells often display varying degrees of mitochondrial abnormalities (such as mitochondria with broken cristae, presence of edema in their matrix, disruption of inner, and external mitochondrial membrane). Moreover, giant mitochondria also appear to be permanent features of tumor growth and metastases [2]. Comparatively characteristics of marginal and central portion of tumor tissues obtained from patients undergoing surgery with diagnosis of the primary glioblastoma shows that distance area of tumor tissue characterized heterogeneous distribution of damage in the structure of the mitochondria. Central regions of tumor tissues almost in all areas show astrocytes with clusters of mitochondria-derived lysosomes (Aliev et al., unpublished data). The same patterns of cellular and subcellular damage were also seen in spinal cord tumor (Aliev et al., unpublished data).

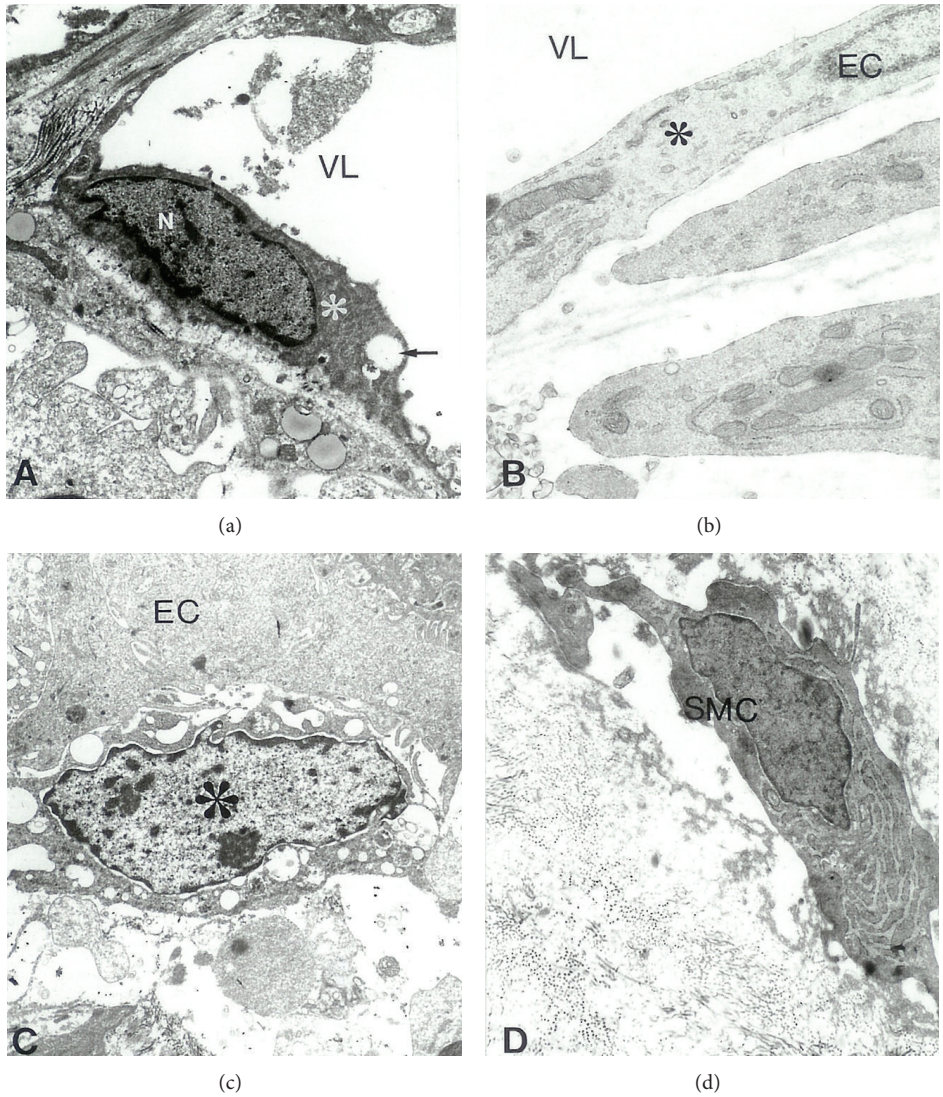


Figure 3: Electron Microscopic Peroxidase-anti-Peroxidase immunocytochemical determination of the distribution of NOS1 immunolabeling features in control (a) and metastatic colorectal cancer liver tumor tissues (b)–(d). (a) NOS1 immunopositive EC (indicated by asterisk) were seen in control liver microvessels. Vacuoles are indicated by single arrow X4 000. (b) Tumor vessel endothelium (indicated by asterisk) showed no staining for NOS1 antibody. X20,000. (c) NOS1 immunopositive white blood cells (indicated by asterisk) were attached to vessel endothelium in tumor growth regions. X6,000. (d) NOS1 immunopositive myofibroblast (smooth muscle cell) were seen in metastatic liver tumor tissues. X10,000. Reprinted with permission of J Submicrosc Cytol Pathol [2].

For the detection of mitochondrial DNA over-proliferation and deletion in tumor cells in AD tissues, Aliev and coworkers performed *in situ* hybridization [1, 32]. These studies demonstrated that successful dysregulation of cell cycle, and that early cell-cycle pathophysiology in AD may recruit oncogenic signal transduction mechanisms, which may be viewed as an abortive neoplastic transformation prominent during tumorigenesis and AD. These results also demonstrated that abnormal mitochondria and lipofuscin is a feature of hippocampal damaged neurons in human AD and aged AD transgenic (Tg+) mice that mimics human AD, and suggest a direct relationship between vascular abnormalities, BBB breakdown, neuronal loss, and amyloid depositions [1, 32]. The giant and electron dense mitochondria were reported to be a permanent feature of neuronal abnormality [1, 32]. *In situ* hybridization analysis with mouse and human mtDNA probes showed a large amount of mtDNA deletion in YAC-AβPP mice hippocampus compared with aged controls. The majority of these mtDNA deletions were found in mitochondrial-derived lysosomes in regions closely associated with lipofuscin, which suggests that proliferation, deletion, and duplication of mtDNA occurs in mitochondria, many of which have been fused with lysosomes in human AD [55–58], and transgenic mice as a model for neurodegeneration [1, 32, 57, 58]. Moreover, biopsy samples of AD patients were dominated by abnormal mitochondria

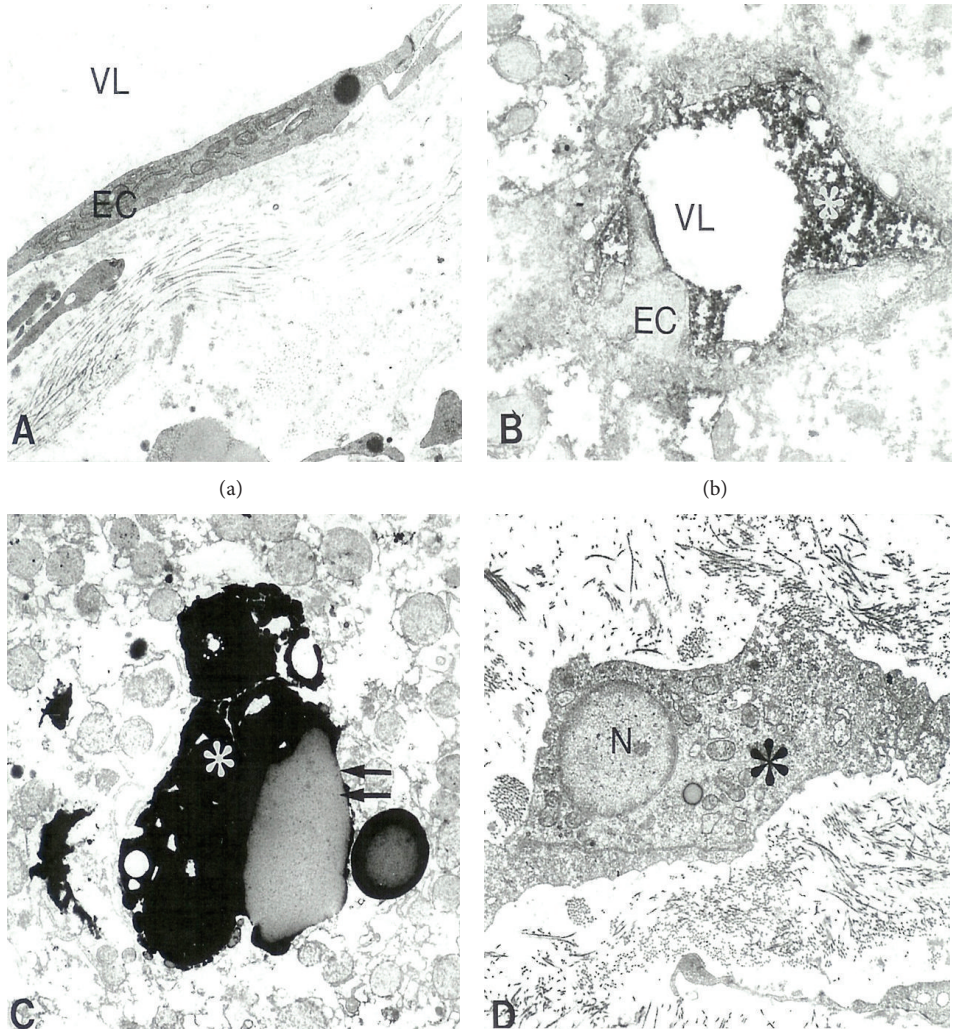
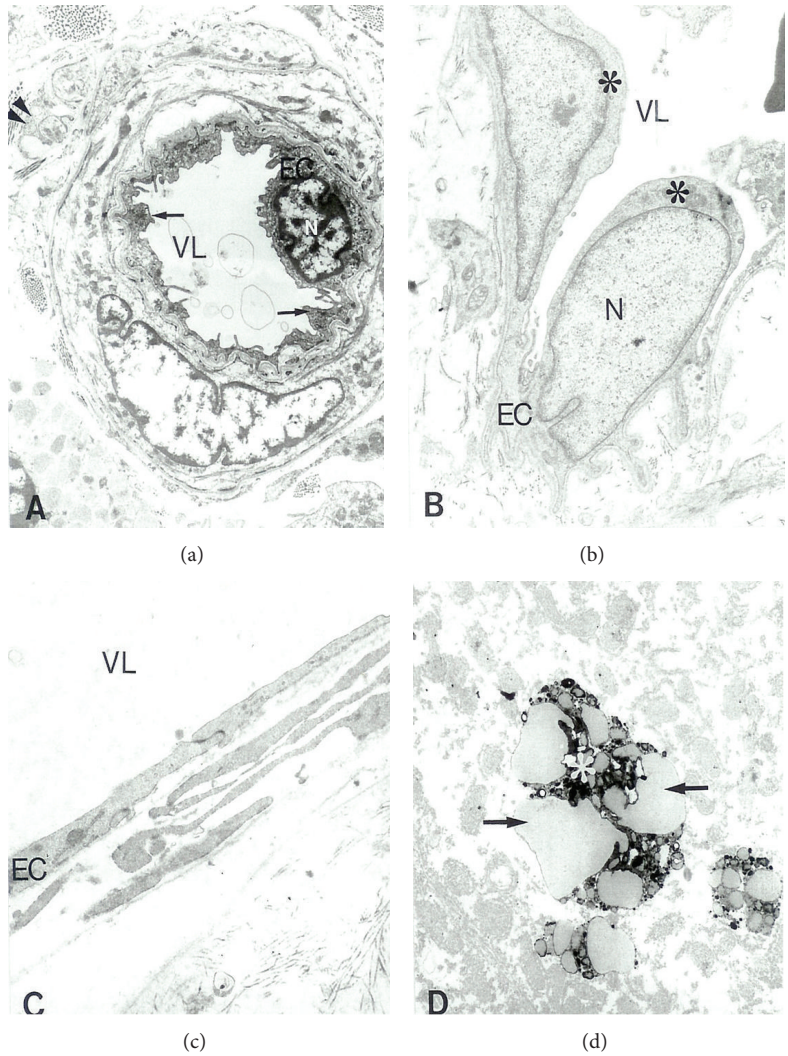


Figure 4: Ultrastructural labeling of inducible NOS (NOS2) immunoreactivity in metastatic liver tumor tissues that determined by using Preembedding Peroxidase-anti-Peroxidase Electron Microscopy Immunocytochemical techniques. (a) Tumor vessel EC was positively stained with NOS2. X8,000. (b) A high intensity of NOS2 immunopositive precipitate accumulated close to the luminal plasmalemma of the vascular EC in the tumor growth region. X8,000. (c) NOS2 immunopositive hepatocytes (asterisk). Lipid-contained areas of the hepatocytes (double arrow) were free from immunopositive reaction. X6,000. (d) NOS2 immunopositive myofibroblast-like cells (asterisk). X8,000. Reprinted with permission of J Submicrosc Cytol Pathol [2].

as compared to control group. In one study, ultrastructural localization of mtDNA by *in situ* hybridization with colloidal gold showed that deleted mtDNA is mainly found in abnormal mitochondria [55]. The common features of the mitochondrial abnormality were seen in the brain during the tumorigenesis and in AD, indicating that most likely mitochondrial DNA overproliferation/deletion appeared to be key initiating factors for tumor growth/metastases [1, 32, 57, 58]. Therefore, investigating mitochondrial abnormality may open new windows not only for the better understanding of tumor pathogenesis but also for developing new treatment strategies.

Of particular importance, the effect of mitochondrial failure during tumor growth and metastases is dependent on the following factors: oxygen deficient tissue, NOSs enzymes activity, oxidative stress, cellular changes (hepatocytes, vascular, neuronal, and glial changes), and on the concomitant mitochondrial lesions and decline in normal organ function [1, 32, 57]. Upregulation of NOSs' enzyme activity induces formation of a large amount of reactive oxygen species (ROS). This may be a key factor in mitochondrial damage and energy failure occurring during carcinogenesis. Chronic hypoxia, a predominant characteristic of tumors, initiates the mitochondrial DNA overproliferation/deletion that then induces formation of large quantities of unwanted free radicals with concomitant energy deficiency (Aliev unpublished observations).

Some of the mitochondria mechanisms, which are incidentally heavily involved in the generation of ROS, result in oxidative damage to the vascular endothelium, as well as to



(a) (b)

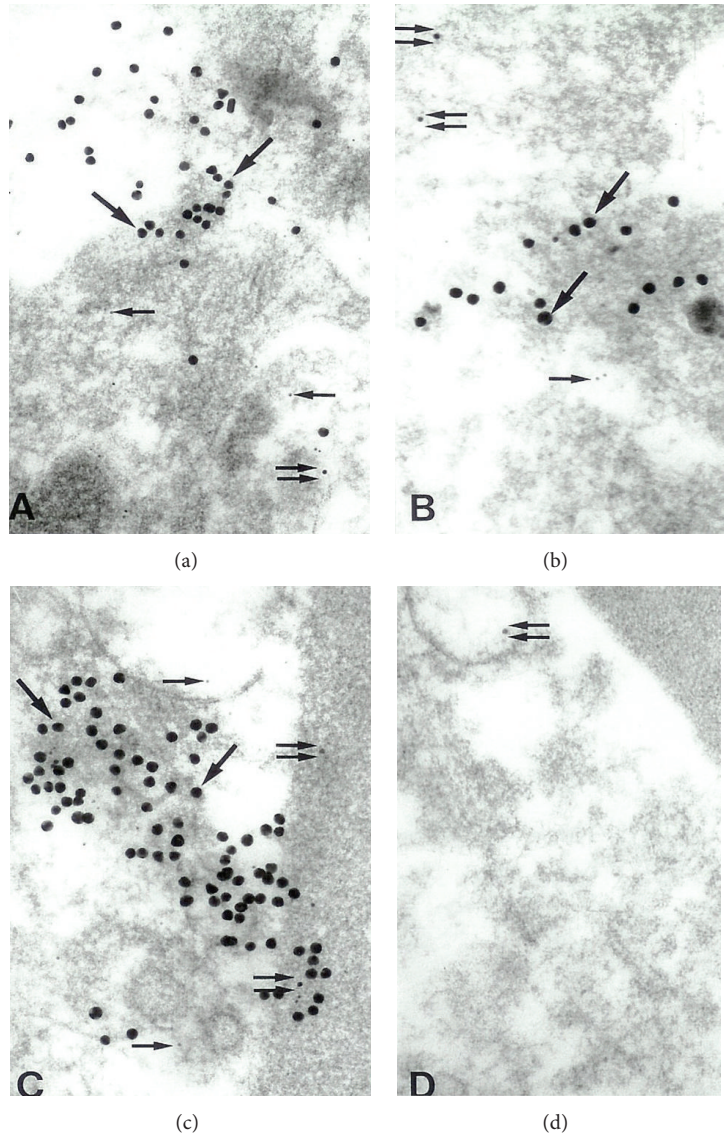
(c) (d)

Figure 5: Ultrastructural features of endothelial specific NOS (eNOS or NOS3) labeling in control (a) and metastatic liver tumors tissues (b–d) determined by using Peroxidase-anti-Peroxidase Immunocytochemistry. (a) A large number of NOS3 immunopositive EC (indicated by arrows) were seen in control liver microvessels. X5,000. (b) NOS3 immunostaining was absent in EC (asterisk) in tumor vessels. X8,000. (c) EC from tumor microvessels did not show the presence of any NOS3 immunopositive reaction. X10,000. (d) NOS3 immunopositive hepatocytes (white asterisk) in metastatic liver tumors were seen. Lipid-enriched areas were free from NOS3 positive precipitate (arrows). X8,000. Reprinted with permission of J Submicrosc Cytol Pathol [2].

other cellular constitutes in tumor tissues. Such changes also accompany tumor pathology. Previous studies demonstrated how age affects mitochondrial DNA mutations and over-proliferation in liver and brain. Brain disorders that involve chronic hypoperfusion may be responsible for concomitant energy failure and the pathogenesis that underlies both disease processes, as hypoperfusion appears to induce oxidative stress, which is largely from ROS as well as NO [3].

However, these underlying processes also play a role not only in aging and age-associated diseases, but in tumor growth and metastases. Over the time, these processes initiate mitochondrial failure, a known factor in early AD pathogenesis [1, 32, 58]. In addition, NO can be produced for 80 years by neurons in human brain without any toxicity. Paradoxically, the production of the same molecule can become highly damaging to the same neurons within a few minutes during pathological challenges as occur after cerebral ischemia. The reaction of NO with superoxide (O_2^-) to form the much more powerful oxidant peroxynitrite ($ONOO^-$) is a key element in resolving the contrasting roles of NO in physiology and pathology (vide supra). Future studies comparing the spectrum of mitochondrial damage and the relationship to NO-dependent oxidative stress-induced damage during the aging process [1, 32] and more importantly, during tumor development and metastasis are warranted [2].

Various studies demonstrated the involvement of NO in apoptosis and show that inhibition of mitochondrial respiration by NO results in a relative degree of mitochondrial hyperpolarization, an occurrence that requires the production of glycolytic ATP [6]. This observation indicates that



(a) (b)

(c) (d)

Figure 6: The distribution of NOS1-3 and ET-1 immunopositive gold particles in metastatic tumor vessel endothelium and hepatocytes that determined by using Post-embedding triple immunogold labeling techniques. (a) Clusters of NOS2 positive gold particles (20 nm, thick arrows) but not NOS3 (5 nm, single thin arrow) and ET-1 (10 nm, double arrow) were seen in tumor vessel endothelium. X100,000. (b) The expression of NOS1 (20 nm single arrow) was seen in the matrix of lipid laden hepatocytes in tumor growth area. NOS3 (10 nm) and ET-1 (5 nm) positive gold particles indicated by double and thin single arrow. X100,000. (c) Clusters of ET-1 (20 nm, single arrows) but not NOS1 (10 nm, double arrow) and NOS3 (5 nm, single thin arrow) positive gold particles in the cytoplasmic matrix of hepatocytes were seen. X100,000. (d) EC from metastatic liver microvessels prepared as negative controls (through omission of the primary antibody) showed only the presence of single gold particles (10 nm, double arrow). X100,000. Reprinted with permission of J Submicrosc Cytol Pathol [2].

the hyperpolarization may be a protective mechanism since neurons, and perhaps, other cells which do not utilize the glycolytic pathway and do not respond to NO by mitochondrial hyperpolarization, are more susceptible to NO-induced apoptosis than are glycolytically-active astrocytes (for a review, see [6]). Persistent inhibition of respiration by NO over a prolonged time eventually result in the collapse of membrane potential, ATP depletion and, ultimately, cell death (for a review, see [6]). NO may reversibly inhibit enzymes with transition metals or with free radical intermediates in their catalytic cycle. NO in micromolar concentrations reversibly inhibit catalase and cytochrome P-450 (for a review see [7]), which may transiently increase the leakage of superoxide from the electron transport chain. The superoxide so formed could then react with NO to generate peroxynitrite, which would cause irreversible injury to the mitochondria (for a review, see [7]). It can also inhibit ribonucleotide reductase, the enzyme responsible for DNA synthesis that contains a tyrosine radical. Large continuous fluxes of NO are necessary to inhibit ribonucleotide reductase, which would occur only under major inflammatory conditions or in the neighborhood of an activated macrophage. Indeed,

activated macrophages produce both NO and superoxides, so the inactivation of mitochondria in tumor cells could well have been mediated by peroxynitrite (for a review, see [7]).

An increase in the release of NO from the vascular endothelium and other tumor tissue cells, including the natural killer cells (NKH-lymphocytes), can promote antitumor growth activity. High NO concentrations are generally tumoricidal as it inhibits DNA synthesis [59, 60]. NO, through reactions from the products of mitochondrial electron transport chain, produces ROS and RNS, which in sufficiently high concentrations cause DNA damage and apoptosis. Whereas DNA damage in cancer cells helps prevent cancer metastasis, it results in neuronal loss in AD.

Our report demonstrated that metastatic colorectal cancer to liver and malignant brain cancer are characterized by overexpression of several NOS enzymes, which coexist with mitochondrial ultrastructural alterations in tumor cells. Moreover, the degree of tumor growth and metastasis is linearly correlated with the overexpression of iNOS and increased level of ET-1 immunoreactivity [2]. The role of ET-1 as a mitogen in the pathogenesis of tumor growth and metastasis has been studied extensively [2, 59, 60]. Aliev and coworkers recorded expression of ET-1 immunoreactivity not only in vascular endothelium, but also in tumor cells, activated lymphocytes, SMC, and in liver hepatocytes [2, 54]. ET-1 has been reported as a mitogenic factor against a variety of cell types including the human hepatocellular carcinoma [61–64]. Studies by Nelson and coworkers [59, 60] found that circulating plasma ET-1 was elevated in more than half of men with advanced metastatic prostate cancer (PCA). The elevation of plasma ET-1 levels has been reported in hepatocellular carcinoma [64], but all patients in that study also had cirrhosis, which is independently associated with elevations in plasma ET-1 [65]. In many tissues, cellular overexpressions of ET-1 mRNA transcripts are in close proximity with those possessing ET-1 receptors [66, 67]. It has been suggested that the increased ET-1 immunoreactivity can be used as a marker for tumor growth and metastases [2, 59, 60]. However, the exact cellular mechanisms behind tumor vascular growth and the relation to NO oxidation products identified as nitrotyrosine formation, lipid peroxidation, ET-1 activity or mtDNA deletion remain to be unknown [2, 54]. Future studies comparing the spectrum of mitochondrial damage and the relationship to NO-dependent oxidative stress-induced damage during the aging process [1, 32, 57] and more importantly, during development and metastasis of tumor are in need of the hour. In addition, it has been also suggested that NO influences cellular differentiation through induction of gene expression [67]. This is interesting because a constitutively expressed NOS2 has been described in a colorectal adenocarcinoma cell line [68]. NO produced by stimulated macrophages [69] or released by NO donor drugs [70] inhibits tumor cell growth. Earlier, Aliev and coworkers have highlighted rise of NOS2 immunoreactivity as a hallmark in human metastatic colon cancer [2]. However exact role of NOS2 activity on the mitochondrial lesions and/or mitochondrial DNA overproliferation and/or deletion in these conditions are unknown. The increased understanding of the relationship between the degree of mitochondrial lesions, NOS and nitrotyrosine protein overexpression, and mitochondrial DNA overproliferation/deletion, could give us a better understanding of tumor pathogenesis. This may eventually lead to new and effective treatments strategies. For example, if the degree of pathology can be correlated with the quantity of the NOS enzymes, immunoreactivities expressed and mtDNA overproliferation/deletion, then manipulating the systems metabolically may be sought which can lead to early death of the injured cancer cell mitochondria. Moreover, mitochondria appear to be primary targets for apoptotic cell death. Moreover, involvement of NO has already been demonstrated in apoptosis and it has been shown that inhibition of mitochondrial respiration by NO results in a relative degree of mitochondrial hyperpolarization, an occurrence that requires the production of glycolytic ATP [6].

Cytokines increase NOS2 mRNA levels in macrophages, hepatocytes, and vascular SMC's in a dose- and time-dependent manner [71]. An alternative explanation for the increased NOS2 expression in vascular endothelium and all NOS isoforms immunoreactivity in other cells in metastatic liver tissue is that a large number of noncontractile types of fibroblasts and/or myofibroblast-like cells are present in tumor tissues. The marginal increase in NOS2 immunoreactivity in tumor vessel endothelium, along with the increased expression of all three NOS isoforms in other cells in liver, were associated with a significant overexpression of ET-1 immunoreactivity in all tumor tissue cells. Our report indicated that NOS2 and ET-1 expressions are linearly correlated with the degree and nature of tumor growth [2, 54]. It is interesting that the total number of NOSs (NOS 1–3) immunopositive EC is nearly equal to the total number of ET-1 immunopositive EC. We speculate that most likely this positive feedback appears to be a compensatory action of tumor invaded organs during the tumor growth and metastases.

7. Oncogenic Parallelism between Cancer and AD: Potential Common Treatment Strategies

When cells receive growth stimuli, or mitotic drive, they upregulate cyclin-dependent kinases (CDKs) and their cognate activating cyclins to orchestrate DNA replication, cytoskeletal reorganization and cellular metabolism required for proliferation (Table 1). Hormonal signals from luteinizing hormone and other hormones can contribute to this mitotic drive [72]. Mitotic drive and the orderly progression through cell cycle, involve cyclins and CDKs which form complexes that are able to phosphoregulate a wide variety of substrates [73]. However, extrinsic mitotic pressures and proper cell cycle progression can also involve resensitization. Association with AD and AD-related cytoskeletal pathology [74] may be involved in aberrant neuronal sprouting response [75–78]. Mitotic drive may arise from inflammatory processes, oxidative stress and other excitatory stressors [79–81]. Strong support exists in literature for an AD-cell cycle-associated emergence from a quiescent state and researchers have looked at it as a recapitulation or vestige of an evolutionarily

Table 1

Marker	Role	Association with Alzheimer disease
Cyclin A	S to G2/M	[95, 96]
Cyclin B	G2/M	[97–99]
Cyclin C	No known role	
Cyclin D (D1, D3)	G0/G1/lateG1/S	[98–101]
Cyclin E	G1 to G1/S	[97, 102]
p34cdc2/cdk 1	Late G2/M	[95, 96, 103, 104]
Cdk4/Cdk6	G1/G1/S	[105–108]
Cdk5/p25/p35	G2 D1, D3 G1 Cyclins	[109–114]
Nclk cdc2-like kinase	Cyclin A kinase	[109, 115, 116]
Cdk7/MPM2	CDK activated kinase	[101, 117]
Cdc42/rac	GTPase/cell division	
p21ras	G protein/MAPK	[118, 119]
MRG 15	M phase regulator	[120]
Ki-67	LateG1,S,G2,M	[97, 120]
p105/pRb	G2/M TF	[105, 120]
PCNA	non cell-cycle specific	[99]
p107/pRb	Cdk2/4/6, check pt	[77, 105] (negative association)
c-myc	S to G2 checkpoint	[77] (negative association)
p53/MDM2	Repressor complex	[118, 120, 121]
ATM	Check-point	[77]
Raf/Raf-1	Check point kinase	[117]
p16INK4a p18p15p19	CyclinD/cdk4/6 inhibitors of M phase	[101, 122]
p27/Kip1	Cyclin D and E/cdk7 inhibitor	[77] (negative association), [101]
WAF-1/p21/Cip1	Multi-Cyclin/cdk-inhibitor (G1 and S)	[118]
Plk1/cdc5 Polo-like kinase	G2/M M check point	[123]
PP2A or PP2B	Phosphatase (Cdk5, cdc2)	[124–126]
PP-1		[112, 124]
Cdc25 Cdc25A	Phosphatase G2/M	[127, 128]
PKC/Wnt path	Translation control	[129–132]
PKA	Kinase	[133, 134]
PKN	Kinase	[135]
PI3K	Kinase	[136, 137]
AKT/PKB/RAC	Kinase	[135, 138, 139]
TGFBeta/TAK	Kinase	[140, 141]
p44/p42 MAPK (ERK1/2)	MAP kinase	[95, 142–146]
CamK	Kinase Ca/calmodulin regulated	[147]
p38 MAPK	Kinase	[146, 148, 149]
JNK/(SAPK-2/3)-alpha gamma	Kinase (stress activated)	[146, 150]
MEK	MAPK kinase	[122, 151]
GSK-3 and beta Catenin	Proline-dependent protein kinase (PDPK)	[111, 112, 131, 138, 152–157]
P120/E-cadherin	Adhesion complex	[158]
c-fos	TF/regulator	[159]
14-3-3/14-3-3zeta	Adaptor protein	[160, 161]
c-jun/p38, AP-1	TF component	[159, 162, 163]
Fyn	Transcription factor	[164, 165]
p53	TF/DNA damage	[97, 166]

Table 1: Continued.

Marker	Role	Association with Alzheimer disease
Rho	G-protein	[135]
Rap Rab	G-protein	[117]
Sos-1	Guanine nucleotide exchange factor	[167]
Grb-2	Adaptor	[167]

Modified and reprinted with permission of CNS Neurol Disord Drug Targets [92].

conserved process [82, 83] (Table 1). Recently reversion in AD pathology has been demonstrated by anticancer drugs [84]. Furthermore, AD-associated proteins and the cell cycle activation from mitotic drive are intimately linked to tau proteins as well as to Aβ, the extracellular lesion associated with the disease [85–90]. NO has both genotoxic and angiogenic properties and has been reported to inhibit the release of mitogen from platelets. Another strategy for tumor treatment has focused on the inhibition of tumor angiogenesis. It has been well established that angiogenesis is a critical event in tumor growth and metastasis [91]. Increased NO production may selectively support mutant p53 cells and may also contribute to tumor angiogenesis by upregulation of vascular endothelial growth factor [92]. There is a growing scientific agreement that antioxidants, particularly the polyphenolic forms, may help lower the incidence of disease, such as certain cancers, cardiovascular, and neurodegenerative diseases, DNA damage, or even have antiaging properties. On the other hand, questions remain as to whether some antioxidants or phytochemicals potentially could do more harm than good, as an increase in glycation-mediated protein damage (carbonyl stress) and some risk has been reported. A recent review highlights both anti- and prooxidant properties associated with polyphenolic compounds [93]. Nevertheless, the quest for healthy aging has led to the use of antioxidants as a means to disrupt age-associated deterioration in physiological function, dysregulated metabolic processes, or prevention of many age-related diseases. Although a diet rich in polyphenolic forms of antioxidants does seem to offer hope in delaying the onset of age-related disorders, it is still too early to define their exact clinical benefit for treating age-related diseases. Regardless of where the debate will end, it is clear that any deficiency in antioxidant vitamins or adequate enzymatic antioxidant defenses can shift the redox balance in some diseases [94].

8. Conclusion and Future Remarks

The absence of neuronal control (e.g., perivascular nerves) in tumor vessels suggests that endothelial-derived vasoactive substance, namely NO and ET-1, may be key factors in controlling tumor blood flow during tumor growth and metastasis. An imbalance between endothelial-derived vasoconstrictors and vasodilators, along with deficiency of antioxidant systems may result in mitochondria lesions in tumors. NO-induced mitochondrial failure is a causative factor in the pathogenesis of tumors, especially tumor angiogenesis. Conversely, recent studies have shown that apart from vasodilator and antiplatelet activities, there are other actions of NO that might be regarded as "*antiatherogenic*" (probably also "*antiangiogenic*"). NO has both genotoxic and angiogenic properties and has been reported to inhibit the release of mitogen from platelets. Another strategy for tumor treatment has focused on the inhibition of tumor angiogenesis. Increased NO production may also contribute to tumor angiogenesis by upregulation of vascular endothelial growth factor. We hypothesize that mitochondrial involvement in this cascade may be a major factor that controls tumor growth and metastasis. Future studies of mitochondrial pathophysiology in various benign and malignant tumors, including colorectal, liver, and brain cancer may provide new insights in carcinogenesis and may lead to rational targets and strategies for better and more effective cancer treatments.

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Review Article

Increased Nitroxidative Stress Promotes Mitochondrial Dysfunction in Alcoholic and Nonalcoholic Fatty Liver Disease

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Increased nitroxidative stress causes mitochondrial dysfunctions through oxidative modifications of mitochondrial DNA, lipids, and proteins. Persistent mitochondrial dysfunction sensitizes the target cells/organs to other pathological risk factors and thus ultimately contributes to the development of more severe disease states in alcoholic and nonalcoholic fatty liver disease. The incidences of nonalcoholic fatty liver disease continuously increase due to high prevalence of metabolic syndrome including hyperlipidemia, hypercholesterolemia, obesity, insulin resistance, and diabetes. Many mitochondrial proteins including the enzymes involved in fat oxidation and energy supply could be oxidatively modified (including *S*-nitrosylation/nitration) under increased nitroxidative stress and thus inactivated, leading to increased fat accumulation and ATP depletion. To demonstrate the underlying mechanism(s) of mitochondrial dysfunction, we employed a redox proteomics approach using biotin-*N*-maleimide (biotin-NM) as a sensitive biotin-switch probe to identify oxidized Cys residues of mitochondrial proteins in the experimental models of alcoholic and acute liver disease. The aims of this paper are to briefly describe the mechanisms, functional consequences, and detection methods of mitochondrial dysfunction. We also describe advantages and limitations of the Cys-targeted redox proteomics method with alternative approaches. Finally, we discuss various applications of this method in studying oxidatively modified mitochondrial proteins in extrahepatic tissues or different subcellular organelles and translational research.

1. Introduction

Mitochondria are responsible for the production of energy in the form of ATP which is used by every cell for its survival and function. In addition, mitochondria play a critical role in fatty acid oxidation, antioxidant defense, apoptosis, intermediary metabolism (including ammonia, urea, heme, steroid, pyrimidine, one carbon transfer, and glutamine metabolism), and so forth, [1–3]. The mitochondrial fat oxidation pathway is very important in providing alternative energy (e.g., ketone bodies) when glucose is supplied in limited amounts or not utilized for maximal energy production through the mitochondrial tricarboxylic acid cycle under various disease states [1]. It is known that heavy and chronic alcohol (ethanol) intake causes alcoholic fatty liver, steatohepatitis (inflammation), fibrosis, cirrhosis, and carcinogenesis in humans and experimental animal models [4, 5]. Because of the high solubility of alcohol, it is distributed in most tissues; therefore, excessive alcohol intake (e.g., binge or chronic heavy alcohol drinking) can damage virtually all tissues including liver, heart, brain, lung, pancreas, and testis [6–8]. Continuous consumption of calorie-enriched high-fat diets or administration of a choline-deficient diet in experimental animals can also cause

significant fatty liver disease (i.e., nonalcoholic fatty liver disease) [9, 10], which are clinically similar to those of the aforementioned alcoholic fatty liver disease. In addition, acute and chronic infection from hepatitis viruses can increase oxidative stress and cause various liver diseases including fibrosis and cirrhosis depending on the degree of host-viral interactions [11]. Certain drugs such as the antibreast cancer agent tamoxifen and antiretroviral drugs including AZT (zidovudine) can promote fatty liver disease ([12], and references herein). Likewise, abused substances such as marijuana (cannabinoids), nicotine (a major component of tobacco smoke), and 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) can lead to hepatic steatosis and inflammation (steatohepatitis) [13–15] as well as tissue injury in extrahepatic organs including brain and heart [16–19].

Regardless of the etiological factors, both alcoholic and nonalcoholic fatty liver diseases can result from impaired mitochondrial functions (i.e., mitochondrial dysfunction) with suppressed β-oxidation of fatty acids [2, 12, 20]. Furthermore, some exogenous agents (e.g., alcohol or high-fat diet) alone or in combination with other gene-related comorbidity risk factors can damage the liver in a synergistic/additive manner to rapidly promote or worsen the preexisting conditions, as observed in rats [21]. It is also known that other extrahepatic tissues can be negatively affected or damaged by the combination of environmental factors (e.g., excessive amounts of alcohol consumption, smoking, drugs, and abused substances) and genetic factors (e.g., variations in disease susceptible genes such as a dominant negative mutation of mitochondrial aldehyde dehydrogenase (ALDH2) gene, frequently found in many East Asians [22–25], diabetes, obesity, and neurodegenerative diseases) (Figure 1). In fact, mitochondrial dysfunction could serve as a major contributor in many disease states such as alcohol- or drug-mediated tissue injury, aging, cancer, diabetes, and various neurodegenerative diseases [20, 26–31], even though the etiological factor for each disease state is different. Despite the well-established role of mitochondrial dysfunction in many disease states, it is poorly understood how mitochondrial dysfunction occurs in these pathological conditions. In this paper, we briefly describe the role of nitroxidative stress in promoting mitochondrial dysfunction and its pathophysiological consequences. We also describe the detection of oxidatively modified mitochondrial proteins in experimental models of alcoholic and nonalcoholic fatty liver disease with redox-based proteomics approaches. Finally, we discuss potential limitations and applications of redox proteomics approaches in studying oxidatively modified proteins in different subcellular fractions in various tissues as well as future translational research.

2. Role of Oxidative Stress in Promoting Mitochondrial Dysfunction

Under normal conditions, approximately 1%-2% of oxygen leaks out as reactive oxygen species (ROS) from the mitochondrial electron transport chain (ETC) [32]. These amounts of ROS, adequately handled by the cellular defense systems under normal conditions, can regulate various cellular signaling pathways, as recently discussed [33]. However, under pathological conditions or after exposure to certain toxic agents including large quantities of alcohol, abused substances, or other therapeutic drugs [17–20, 30], greater amounts of ROS are leaked from the mitochondrial ETC, possibly at the sites of Complex I (NADH ubiquinone oxidoreductase) and Complex III (ubiquinone cytochrome c oxidoreductase), as shown in alcohol-exposed hepatocytes [34]. Ironically, mitochondria, a major source of cellular ROS, become a main target of oxidative damage because of the relatively low levels of antioxidants, such as reduced glutathione (GSH), in mitochondria compared to cytosol [35]. Consistent with a notion of ROS-mediated damage, mitochondria from tissues in animal disease models and/or human diseases show abnormal and irregular shapes and decreased functions [36, 37].

Besides the ROS generation from the mitochondrial ETC, other cellular enzymes are also known to produce ROS and reactive nitrogen species (RNS) including nitric oxide (NO). These enzymes include NADPH oxidase and myeloperoxidase in phagocytic immune cells, ethanol-inducible cytochrome P450 2E1 (CYP2E1) and CYP4A isozymes in endoplasmic reticulum (ER), cytosolic xanthine oxidase, and nitric oxide synthase isozymes including the inducible form (iNOS) in activated Kupffer cells and/or recruited neutrophils [38–45]. Many of these prooxidant enzymes are induced or activated after exposure to potentially toxic agents such as alcohol, MDMA, and high-fat diets. Elevated ROS leaked from the mitochondrial ETC and produced by these enzymes lead to increased production of a potently toxic peroxynitrite ($ONOO^-$) in the presence of NO. Peroxynitrite can covalently modify various proteins through nitration of Tyr residues [46, 47] and *S*-nitrosylation of Cys residues [48]. In fact, elevated ROS/RNS under pathological conditions suppress the activities of various antioxidant enzymes including mitochondrial superoxide dismutase (SOD2), catalase, glutathione peroxidase, and glutathione reductase while they can also decrease the levels of cellular antioxidants such as GSH and vitamins, causing increased nitroxidative stress.

Under the conditions of elevated nitroxidative stress, mitochondrial DNA, proteins, and lipids become covalently modified by oxidation, nitrosation, and/or nitration. Increased nitrosative stress can lead to various reactions, including *N*-, *O*-, and *S*-nitrosations, that modify structure of various proteins, as reviewed [49–52]. Nitrative stress represents a condition where excessive ROS reacts with NO to produce potently toxic peroxynitrite, which can nitrate Tyr residues of various proteins to produce 3-nitroTyr, frequently used as a stable marker for nitrative stress [48–52]. These types of modifications of cellular macromolecules likely contribute to alteration of their normal functions [53–55]. Deletion and/or mutation through oxidative modifications of mitochondrial DNA are particularly important, since they encode 13 polypeptides, all of which are subunits of the 4 mitochondrial ETC proteins (i.e., Complexes I, III, IV, and V) [30, 31]. Mitochondrial DNA and proteins are more susceptible to oxidative/nitrative damage due to the absence of protective antioxidant protein catalase, histones,

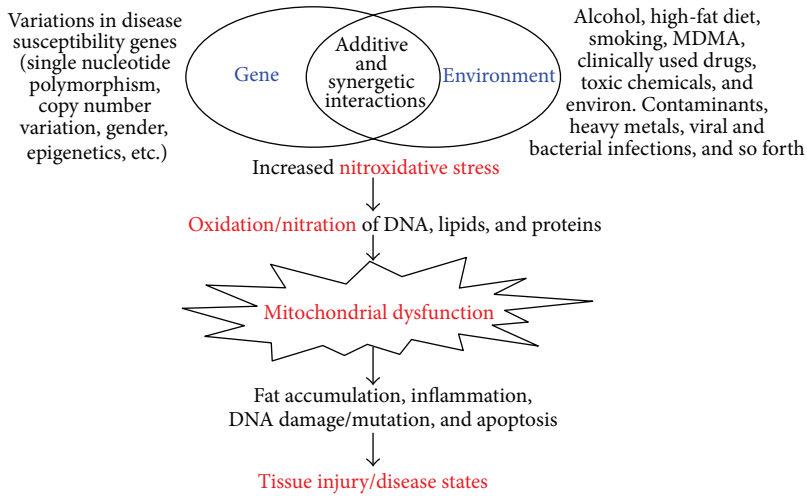

Figure 1: Synergistic interaction between gene and environment. Many toxic agents alone or in combination with other comorbidity factors including genetic elements synergistically interact and produce ROS/RNS, which decrease the levels of antioxidants and inhibit protective defensive enzymes, resulting in increased nitroxidative stress. Consequently, mitochondrial DNA, lipids, and proteins are oxidized and/or nitrated, leading to mitochondrial dysfunction, accompanied with fat accumulation, inflammation, ATP depletion, necrosis/apoptosis, and DNA damage. All these changes likely contribute to tissue injury, as observed in many disease states.

or polyamines and a relatively low activity of DNA repair enzyme in mitochondria compared to nuclei ([56], and references herein). One report suggested that the rate of mutation in mitochondrial DNA is 10-fold higher than that in the nuclear DNA [29]. In addition, the level of mitochondrial GSH is relatively low compared to that in cytosol because it has to be imported into mitochondria through the specific GSH transporter protein due to the absence of its synthesis in mitochondria. Furthermore, chronic alcohol exposure impairs the GSH transporter protein, leading to a selective deficiency of GSH in mitochondria ([35], and references herein). It is thus reasonable to assume that oxidative damage and/or deletion of mitochondrial DNA [57, 58] may lead to reduced expression and function of mitochondrial ETC proteins, contributing to greater ROS production, as shown in alcohol-exposed rats [59].

Oxidation of lipids also produces potently cytotoxic lipid peroxides such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). These lipid peroxides can suppress the activities of many mitochondrial proteins such as ALDH2 [60], involved in the metabolism of reactive acetaldehyde and 4-HNE, and Sirt3, NAD^+-dependent deacetylase [61], through covalent modifications (mostly via adduct formation with many amino acid residues including Cys, His, and Lys) [54, 55]. These lipid peroxides can alter the cell membrane functions and promote fibrosis through activation of stellate cells with elevated production of collagen and proinflammatory cytokines/chemokines that lead to recruitment of neutrophils and activation of macrophage Kupffer cells. All these events contribute to profound mitochondrial dysfunction with increased fat accumulation and tissue injury in liver and various extrahepatic organs [4, 5, 28, 29].

Numerous investigators have reported the mechanisms and consequences of increased levels of oxidized mitochondrial DNA and lipid peroxides in various human disease states as well as experimental models for human disease [12, 53, 57, 62, 63]. Compared with the numerous reports about oxidative modifications of (mitochondrial) DNA and lipids, a much smaller number of reports systematically dealt with the oxidatively modified (mitochondrial) proteins under increased nitroxidative stress associated with many disease states. We believe that part of the reason for the relatively fewer reports on protein oxidation, nitrosation, and nitration might be due to the requirement for specific reagents, the lack of suitable methods to systematically identify and purify oxidatively modified proteins, and the relatively late development of highly sensitive mass spectral instruments. Because of the delayed development of specific methods to systematically study oxidatively modified proteins, the following questions have been poorly answered: (1) do we have a suitable method to systematically identify the oxidatively modified proteins and study the causes of mitochondrial dysfunction compared to global analysis of the changes in mitochondrial proteins? (2) what are the sources of ROS/RNS in enhancing oxidative protein modifications (including oxidation and nitration)? (3) which (mitochondrial) proteins are oxidatively modified? (4) are their activities/functions altered following oxidative modifications? (5) what are the functional implications of oxidized proteins in mitochondrial dysfunction and certain disease states such as various models of fatty liver disease? and (6) can the oxidative protein modifications and subsequent mitochondrial dysfunction be prevented with a potential therapeutic agent? and (7) how do current treatment modalities of fatty liver disease impact the function and oxidation of mitochondria proteins?

3. Consequences of Mitochondrial Dysfunction

Although both genetic and environmental factors synergistically promote mitochondrial dysfunction in various pathophysiological conditions, increased nitroxidative stress represents one important common factor (Figure 1). Despite

the well-established pathological role of elevated nitroxidative stress, it has been poorly understood which mitochondrial proteins are oxidatively modified and whether their functional alterations cause mitochondrial dysfunction prior to full-blown tissue damage determined by histological and biochemical assessments. Therefore, we hypothesized that mitochondrial dysfunction is mediated by covalent modifications (e.g., oxidation, nitrosation, nitration, phosphorylation, acetylation, etc.) of various mitochondrial proteins, leading to their inactivation or loss of their biological functions. In case of mitochondrial dysfunction, we expect to observe increased levels of energy depletion, lipid peroxidation, and fat accumulation possibly due to suppressed activities of the enzymes involved in ATP synthesis, ALDH2-mediated metabolism, and fatty acid β-oxidation, respectively. In this paper, we briefly describe the functional changes of oxidatively modified ATP synthase, ALDH2, and 3-ketoacyl-CoA thiolase (thiolase) as examples.

Cederbaum et al. [64] and Chen et al. [65, 66] reported that alcohol administration directly or indirectly suppressed the activities of mitochondrial Complex enzymes through increased oxidative stress. It is also likely that decreased amounts of Complex I (NADH ubiquinone oxidoreductase), and Complex IV (cytochrome c oxidase) observed in alcohol-exposed rats [59] could have contributed to suppression of their catalytic activities. Inhibition of these Complex activities could cause more ROS leakage from the mitochondrial ETC, as observed in alcohol-exposed hepatocytes [34]. In addition, ROS can be produced at the site of mitochondrial Complex II (succinate dehydrogenase) [33].

By performing redox proteomics analysis, we sought to identify oxidatively modified mitochondrial Complex proteins in rat livers exposed to chronic or binge alcohol compared to control rats [67]. Oxidation of many mitochondrial proteins including Complexes I, III, and V protein subunits was detected. Activity measurement showed that ATP synthase (Complex V) was significantly inhibited in alcohol-exposed rats possibly through oxidative modifications (including nitration) of the enzyme [67]. Immunoblot analysis with the anti-3-nitroTyr antibody verified the presence of a 3-nitroTyr-reactive band in the immunoprecipitated ATP synthase protein only from alcohol-exposed livers, suggesting its nitration. Mass spectral analysis confirmed the nitration of Tyr residues in the catalytic β subunit, which does not have a Cys residue but must have been copurified with the Cys-containing α subunit of ATP synthase [67]. Nitration of the catalytic subunit of ATP synthase could have contributed to its inactivation. The markedly suppressed ATP synthase likely resulted in significantly decreased ATP levels, as observed in many pathological conditions [68, 69].

Systematic redox proteomics analysis of oxidatively modified mitochondrial proteins in experimental models of acute liver disease caused by alcohol, MDMA, or hepatic I/R injury revealed the detection of all 4 enzymes (medium-chain fatty acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and thiolase), involved in the mitochondrial β-oxidation of fatty acids [15, 67, 70]. Activity measurement of thiolase, the last enzyme in the mitochondrial β-oxidation pathway of fatty acids, showed inhibition of this enzyme in the animal models of alcoholic fatty liver disease, possibly through oxidative modification of the active site Cys residues (Cys^{92} and Cys^{382}) of thiolase [67]. It is likely that the active site Cys residues can undergo oxidation including *S*-nitrosylation as well as formation of adducts with 4-HNE or MDA, since their levels can be increased through lipid peroxidation under oxidative stress. Because of the oxidative modifications of 3 other enzymes in the β-oxidation pathway, as mentioned above, we expect that their activities could be altered in alcohol-exposed rats. In addition, it is likely that the activity of the NAD^+-dependent 3-hydroxyacyl-CoA dehydrogenase could be compromised due to significant changes in the NAD^+/NADH levels following alcohol intake [4, 39]. At any rate, oxidative modifications and subsequent inhibition of at least one of the mitochondrial fat oxidation pathway enzymes correlated with hepatic inflammation and fat accumulation, as assessed by biochemical measurements of triglycerides as well as histological evaluations [67].

From the redox proteomics analysis, we also identified a few oxidatively modified mitochondrial ALDH isozymes in the mitochondria from alcohol-exposed rats [67]. The ALDH gene family [71, 72] represents a large number of $NAD(P)^+$-dependent dehydrogenases (defensive enzymes) [73–75] involved in the cellular metabolism of reactive and cytotoxic aldehyde carbonyl compounds such as acetaldehyde, MDA, 4-HNE, and other lipid aldehydes that are produced during the lipid peroxidation process [54, 76]. The ALDH isozymes including mitochondrial ALDH2 are known to be inactivated by genetic/environmental factors [22–25, 71, 72, 77–79] and in many disease states [80–83]. Many isozymes of the *ALDH* gene family members including retinal aldehyde dehydrogenase (ALDH1A1/2/3) and 10-formyltetrahydrofolate dehydrogenase (ALDH1L1) contain the highly conserved active site Cys residue [84]. Oxidative modifications of the active site and other critical Cys residues of a cytosolic high-Km ALDH1A1 and mitochondrial low-Km ALDH2 (Km for acetaldehyde $\leq 0.2\,\mu$M) [75], an important enzyme in the metabolism of toxic acetaldehyde produced during ethanol oxidation, contribute to inhibition of their activities [67, 84]. Under our experimental conditions, we did not observe any significant changes in the ALDH1A1 or ALDH2 protein content, strongly suggesting that inhibition of these ALDH enzymes could be due to covalent modifications of critical Cys residues by *S*-nitrosylation and other oxidative modifications, as discussed [85, 86]. Our results are consistent with those of Venkatraman et al. who reported inhibition of ALDH2 activity without change in its content in alcohol-exposed rats, although the suppressed ALDH2 activity was not restored after incubation with 0.3 mM β-mercaptoethanol, suggesting an irreversible ALDH2 inactivation in their model [87]. Although we have not measured the specific activities of other mitochondrial ALDH isozymes such as ALDH5A1 (NAD^+-dependent succinic semialdehyde dehydrogenase involved in the catabolism of the neurotransmitter gamma-aminobutyric acid), ALDH6A1 (methylmalonate semialdehyde dehydrogenase), and ALDH7A1 (α-aminoadipic semialdehyde dehydrogenase) [71, 88], their activities are likely suppressed due to the highly conserved

active site Cys residue by a similar mechanism, as recently demonstrated with mitochondrial ALDH2 [67, 70, 85] and cytosolic ALDH1A1 [84]. Because of the inactivation of ALDH1A1, ALDH2, and other ALDH isozymes through covalent modifications such as *S*-nitrosylation [67, 85], phosphorylation [89], and other modifications [86] including adduct formations with MDA or 4-HNE [60, 90] or reactive drug metabolites [78, 79], we expect increased levels of highly reactive and cytotoxic carbonyl compounds including acetaldehyde, 4-HNE, and MDA.

In addition to ALDH isozymes, many other mitochondrial proteins were oxidatively modified and inactivated in alcohol-exposed rat livers [67] (Figure 2). We also observed similar patterns of oxidatively modified mitochondrial proteins in the animal models of acute liver disease from hepatic I/R injury [70] or MDMA exposure [14, 91], or during fasting-related oxidative stress [92]. We expect that similar patterns of oxidative modifications of many mitochondrial proteins would be identified in experimental models of nonalcoholic fatty liver disease caused by high fat diets [76] or methionine/choline-deficient diets [10, 45], based on increased oxidative stress and similar courses of disease progress between alcoholic and nonalcoholic fatty liver diseases [9].

4. Mitochondrial Dysfunction and Fatty Liver Disease Studied with Redox Proteomics Approaches

It would be ideal if the activity change of each oxidatively modified mitochondrial protein can be measured to accurately assess the degree and functional implications of mitochondrial dysfunction in different pathological conditions or before and after exposure to a potentially toxic agent including alcohol or other abused substances such as MDMA and nicotine. However, this task may be impractical due to a relatively small yield of mitochondrial proteins from whole tissue extracts and the requirement for a large amount of mitochondrial proteins for direct activity measurement. As alternative approaches, other methods including global gene expression DNA microarrays and proteomics analyses have been used to indirectly assess the relative degree of mitochondrial dysfunction [68, 93]. Although DNA microarrays allow global analysis of gene expression changes in disease states versus presumably normal control conditions, the results do not always accurately reflect the changes in the final protein amounts or activities of (mitochondrial) proteins [68, 94, 95]. Therefore, a few proteomics methods with or without gel-based tools have recently been developed to quantitatively evaluate the changes in the expressed levels of many proteins in two different specimens (e.g., disease states and apparently normal controls). The recent methods include fluorescence 2D difference in gel electrophoresis (2D DIGE) [96], cleavable isotope-coded affinity tag (cICAT) [97], isobaric tags for relative and absolute quantitation (iTRAQ) [98], multidimensional protein identification technology (MudPIT) [99], and so forth. These global proteomics methods can also be used to investigate the quantitative changes in mitochondrial proteins. In addition, a conventional proteomics approach consisting of comparative 2D gel analysis was successfully used for analyzing the protein changes in the whole tissue extracts [83, 100] or the enriched mitochondrial fractions from alcohol-exposed rats compared to corresponding controls [59]. However, despite the advantages and merits, all these global proteomics approaches may not necessarily provide valuable information about functional changes of the target proteins, since the activities of many (mitochondrial) proteins can be suppressed without significant quantitative changes of their contents, as demonstrated with ALDH2 [67, 70, 87]. These results rather suggest that the functions/activities of many (mitochondrial) proteins could be altered through posttranslational modifications such as oxidation of many amino acids including Cys residues, nitration of Tyr residues, phosphorylation of Ser, Thr and Tyr residues, acetylation of Lys residues, glycosylation of Asn residues, and adducts formation with 4-HNE, or reactive drug metabolites [65–67, 77–79, 86, 101–103]. Instead of studying covalent modifications of various amino acid residues (e.g., Cys, Trp, His, Met, Pro, Tyr, and Lys) that can be differently modified [55], we and other scientists focused on targeted proteomics approaches aiming for oxidatively modified Cys residues of many proteins that can be purified by affinity matrix, displayed on 2D PAGE gels, stained with silver, and identified by mass-spectral analysis. Identification of the oxidatively modified proteins detected with the Cys-targeted redox proteomics approach and a literature search for active site Cys residues of each oxidized protein allow us to predict functional changes (e.g., potential inhibition) of the oxidized proteins/enzymes even in the absence of any changes in protein content.

In order to study oxidative inactivation of many mitochondrial proteins responsible for causing mitochondrial dysfunction, a few redox proteomics approaches have been used to identify oxidized Cys residue(s) of many proteins by using ICAT [97], biotin-labeled iodoacetamide (BIAM) [104], 4-iodobutyltriphenyl-phosphonium [87], or biotin-*N*-maleimide (biotin-NM) [105], being used as a major sulfhydryl-detecting agent in each approach. Although each detection method has its own merit, our approach using biotin-NM as a specific probe for oxidized Cys residues revealed a positive correlation between the levels of oxidative stress in pathological conditions and the number of oxidized proteins [105]. Therefore, we believe that the redox proteomics method using biotin-NM as a sensitive probe for detecting oxidized Cys residues may have a significant advantage over the other redox proteomics methods, where the number of oxidized proteins could be inversely correlated with the increased oxidative stress and oxidized Cys residues do not efficiently react with iodoacetamide-based sulfhydryl reagents BIAM and ICAT [87, 97, 104]. The specific procedures, advantages, limitations, and alternative approaches of our method and its comparison with other redox proteomics methods have been recently described in detail [106]. Excellent review articles on the theories, benefits, and limitations of various redox proteomics approaches from other laboratories are also available [107–112].

To avoid redundancy with some of the previous reviews [106–112], we briefly describe the procedure of our simple

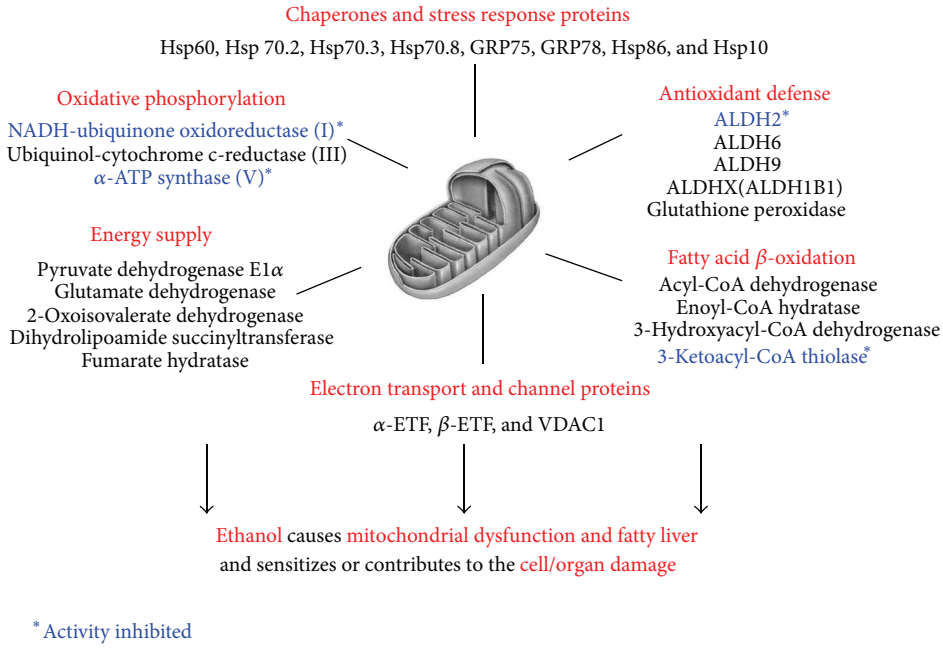

Figure 2: Summary of oxidatively modified mitochondrial proteins in alcohol-exposed rat livers. Oxidized mitochondrial proteins were purified from alcohol-exposed rats and dextrose-exposed pair-fed controls, identified by mass spectral analysis, and then grouped under different functions, as adapted from [67].

redox proteomics method as outlined in Figure 3. Many sulfhydryl groups of various proteins can be oxidatively modified (e.g., sulfenic acid, disulfide, sulfinic acid, sulfonic acid, and mixed disulfides including *S*-nitrosylation) under increased nitroxidative states. The remaining free Cys thiols of various proteins are initially reacted with *N*-ethylmaleimide (NEM), which irreversibly blocks the free thiols. After removing excess NEM by the first gel filtration step, the oxidized Cys residues including mixed disulfides are reduced to free Cys thiols with DTT. The newly reduced free Cys thiols are then switched with biotin-NM. After removing excess biotin-NM with the second gel filtration step, biotin-labeled oxidized proteins are detected by immunoblot analysis or affinity-purified with streptavidin-agarose beads for further characterizations. After washing the nonspecifically bound proteins, agarose-bound biotin-NM-labeled oxidized proteins are dissolved and analyzed by 1-D PAGE for detection with anti-biotin monoclonal antibody or 2D PAGE for protein display followed by identification by mass spectrometric analysis. By using a mild reducing agent ascorbate or GSH [113, 114] instead of DTT in the reduction step of NEM-modified proteins, we can specifically detect proteins with mixed disulfides (e.g., *S*-cysteinylation, *S*-nitrosylation, *S*-glutathionylation, and *S*-succinylation), although the ascorbate-mediated biotin-switch method can produce false-positive artifacts [115, 116].

In fact, we used this redox proteomics approach to identify many oxidized mitochondrial proteins in ethanol-exposed human hepatoma E47-HepG2 cells with stably transfected human CYP2E1, which can produce ROS during ethanol metabolism [38, 39]. The number of oxidized proteins positively correlated with the ethanol concentration and ethanol exposure time as well as the presence of CYP2E1.

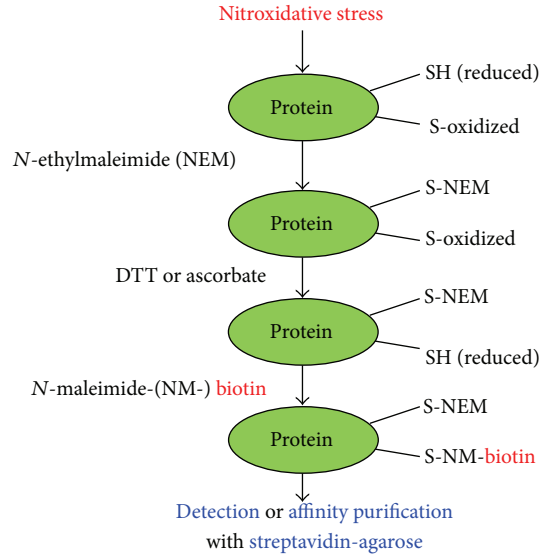

Figure 3: A schematic diagram describing a redox proteomics approach using biotin-NM as a specific probe for detecting or purifying oxidatively modified proteins, as adapted from [106].

By contrast, we observed a very limited number of oxidized mitochondrial proteins in control samples from E47-HepG2 cells that were not treated with ethanol or HepG2 cells without transfected CYP2E1 [105]. This biotin-switch redox proteomics approach was subsequently applied in analyzing oxidatively modified mitochondrial proteins in experimental animal models of alcoholic fatty liver [67] and other models of acute liver disease [15, 70]. The results from these studies revealed that many mitochondrial proteins were oxidatively

modified and some of their activities we that measured were inhibited. Temporal analyses of oxidatively modified proteins and liver histology indicated that mitochondrial dysfunction takes place long before appearance of full-blown liver damage including necroinflammation [70]. Based on these results, oxidative modification and inactivation of many mitochondrial proteins cause mitochondrial dysfunction, which then contributes to tissue injury observed at later time points. Furthermore, we expect that this redox proteomics approach can be successfully used to identify and study functional alterations of oxidatively modified proteins in mitochondrial dysfunction and tissue injury in various organs of human disease specimens.

Although these redox proteomics approaches can be used as surrogate methods to estimate the degree of mitochondrial dysfunction, they do have a few limitations. For instance, we believe that the actual number of oxidized proteins could be much greater than we observed in our studies because of relatively poor sensitivities of gel-based redox-proteomics methods in detecting proteins expressed in low quantities. In fact, our systematic analysis using the Cys-targeted biotin-switch method was unable to detect oxidative modifications of DNA repair enzymes such as O^6-methylguanine-DNA-methyltransferase, although this enzyme contains Cys in its active site and can be inactivated through *S*-nitrosylation [117]. By the same token, we also expect that some key enzymes involved in the cell metabolism/signaling pathways like mitochondrial Sirt3 [118–120] or certain transcription factors that contain critically important Cys residues could not be detected by the redox proteomics approaches although they could be oxidatively modified and thus inactivated by increased oxidative/nitrative stress under pathological conditions. In addition, some oxidized or nitrated proteins could be rapidly degraded through ubiquitin-dependent and -independent proteolysis [55, 121–124] and thus could not be detected by the current redox proteomics methods.

We believe that these limitations associated with various redox proteomics approaches can be overcome by functional analysis (including enzyme activity measurement) supplemented with immunoprecipitation of a target protein, albeit a low level of expression, followed by immunoblot analysis with anti-Cys-*S*-NO, anti-glutathione, and anti-3-nitroTyr antibody for detecting *S*-nitrosylation, *S*-glutathionylation, and nitration, respectively. A few examples of other critical proteins, that were expressed in small quantities and not detected by the systematic redox proteomics analyses but reported to be suppressed through oxidative modifications, were recently discussed [106]. It is of interest whether other critical mitochondrial proteins expressed in small quantities (e.g., sirtuin 3) can be inactivated through oxidative modifications, thus directly contributing to mitochondrial dysfunction with imbalanced energy supply [118], intolerance to cold exposure with decreased fat oxidation during fasting [119], and decreased mitochondrial complex activities [120], as observed in mice deficient of mitochondrial *sirtuin 3* gene. A recent report revealed that Cys^{280}, a critical zinc binding residue, of Sirt3 is modified by 4-HNE, resulting in its allosteric inactivation [61]. It would also be of interest to study the potential mechanisms of oxidative inactivation or degradation of some transcription factors such as NFkB as observed in alcohol-exposed genetically obese mice [125] and PPARα, a key regulator of the enzymes involved in the fat metabolism [126] and shown to be decreased in alcohol-fed mice [127], in mice with nonalcoholic steatohepatitis [128], or in acetaminophen-mediated acute liver damage [129]. Finally, the study of ER-associated drug metabolizing proteins such as cytochromes P450, that have Cys residues at their catalytic sites, may provide important insights in uncoupling of the catalytic cycle during adverse drug reactions [130].

Another limitation of the redox proteomics could be reasoned that Cys residues of many proteins can undergo various types of covalent modifications such as conjugation with carbonyl compounds such as 4-HNE and MDA elevated during lipid peroxidation under oxidative stress [54, 90, 131] or reactive metabolites of acetaminophen, produced during the metabolism of potentially toxic compounds [77–79, 124, 129]. In fact, the number of oxidatively modified proteins in acetaminophen-exposed liver tissues appears relatively small ([132], and Abdelmegeed et al., unpublished observation) despite increased nitroxidative stress [124]. These data likely reflect the fact that oxidation of Cys residues in many proteins in acetaminophen-exposed tissues could be suppressed because of their prior interactions with the reactive metabolite *N*-acetyl-*p*-benzoquinone imine and thus cannot be detected by redox proteomics approaches. However, these types of irreversible adduct formations of critical Cys residues of target proteins can be evaluated by the recovery of the functional activities after incubation with a strong reducing agent such as DTT. If the activities are restored by DTT, protein Cys residues could be modified through formation of reversible sulfenic acids or disulfides including mixed disulfides. If the activities are not recovered, Cys residues are likely modified through irreversible adducts formation [54, 90, 133] or hyperoxidation of Cys residues to sulfinic (–SOOH) and sulfonic (–SOOOH) acids ([17], and references herein). The possibility of these types of irreversible modification can be further confirmed by immunoprecipitation of the target protein followed by immunoblot analysis with anti-4-HNE or anti-acetaminophen antibody.

5. Applications of Redox Proteomics Approaches to Detect Oxidized Proteins in Other Subcellular Organelles, Many Other Tissues, and Different Disease States

We have thus far described oxidative modifications of mitochondrial proteins and their functional consequences in experimental animal models of fatty liver disease. However, it is quite logical to predict that proteins located in other subcellular organelles (e.g., cytoplasm, ER, and nuclear fractions) can also be oxidatively modified and thus contribute to tissue injury. For instance, oxidative inactivation of ER-resident chaperone proteins (e.g., protein disulfide isomerase and other heat shock proteins) can cause misfolding or unfolding of their client proteins, resulting in the unfolded protein response and ER stress. Oxidative modifications and potential inactivation of nuclear proteins

such as DNA repair enzymes including O^6-methylguanine-DNA-methyltransferase [117] or Ogg1 [56] could explain the increased levels of oxidatively modified DNA after exposure to potentially toxic compounds or under pathological conditions.

To understand the mechanism of ER stress and its pathological role, we also applied the simple biotin-switch redox proteomics method to systematically characterize oxidatively modified hepatic proteins in cytoplasm and ER from experimental animals of alcoholic and nonalcoholic fatty liver disease [134–136]. Our redox proteomics data showed that many ER-located chaperone proteins including protein disulfide isomerase, heat shock proteins, and other antioxidant enzymes including cytosolic SOD (SOD1) and peroxiredoxin are oxidized and inactivated [134, 135]. Consistent with these results, we observed increased unfolded protein responses in alcohol-exposed E47-HepG2 hepatoma cells and experimental animals (unpublished observations). It is also possible that these proteins can be inhibited through adduct formation with MDA or 4-HNE, as recently reported [133]. All these results suggest that increased ER stress, possibly originated from mitochondrial dysfunction [95] or uncoupled cytochrome P450 catalytic cycle [130], may also contribute to tissue damage.

Under specific pathological conditions or disease states, certain selected tissues are negatively affected. For instance, brain tissues are selectively distressed in neurodegenerative disorders [50] or after exposure to neurotoxic agents, while heart and blood vessels can be compromised in cardiovascular disorders. It is known that nitroxidative stress is a common factor in these pathological conditions in various tissues (Figure 1). However, it is poorly understood which proteins are oxidatively modified under different disease states. By analyzing oxidatively modified proteins in different tissues, we can also predict functional alterations of each target protein. In addition, it is of interest whether similar sets of mitochondrial proteins are oxidatively modified in different organs/tissues (e.g., liver versus other extrahepatic tissues such as brain, heart, lung, kidney, pancreas, and intestine) or different species (e.g., rodents versus humans) when analyzed by the redox proteomics approach. Our unpublished preliminary results indicate that the overall patterns of oxidized proteins in MDMA-exposed brain tissues are similar to those of the MDMA-exposed liver tissues, except for the liver-specific proteins including the enzymes involved in the mitochondrial fat oxidation pathway. By comparing the patterns of oxidative protein modifications in different tissues and species, we can estimate the role of specific proteins in mitochondrial dysfunction and disease progression of each organ or disease state.

6. Potential Translational Applications of Redox Proteomics Approaches to Evaluate Beneficial Agents to Prevent or Treat Mitochondrial Dysfunction

Once we understand the mechanism of mitochondrial dysfunction and ER stress, contributing to tissue injury, it is desirable to develop an effective strategy of prevention or therapy against mitochondrial dysfunction and organ damage based on our knowledge. We believe that the redox-based proteomics method can be used in translational research by evaluating the effectiveness or progress of treatment with a certain beneficial agent (e.g., antioxidants or cell protective agents from natural and synthetic origins). This task can be achieved by monitoring the levels of oxidatively modified mitochondrial proteins in the biological specimens before and after treatment with a beneficial agent. For instance, we have recently demonstrated a beneficial effect of a diet containing polyunsaturated fatty acids (PUFA) with physiological levels of arachidonic and docosahexaenoic acids on effectively preventing protein oxidation, mitochondrial dysfunction, and ultimately alcoholic fatty liver [137]. These results observed in rats are consistent with the beneficial effects of PUFA diets against alcoholic fatty liver in monkeys [138] and nonalcoholic fatty liver in rats fed a choline-deficient high fat diet [139]. Our results also provide the underlying mechanisms by which physiologically relevant levels of PUFA exert beneficial effects against alcoholic fatty liver in both rats [137] and monkeys [138].

As shown in Figure 4, the number and levels of oxidatively modified mitochondrial proteins were increased in alcohol-fed control rats (Base ethanol) compared to pair-fed control rats (Base control). Our results [137] showed that increased production of hydrogen peroxide and peroxynitrite in alcohol-exposed rats (Base ethanol) compared to pair-fed control group (Base control). These results are consistent with elevated levels of CYP2E1 and iNOS in ethanol-fed rats. Immunoblot analyses of oxidized proteins from each group revealed the presence of oxidatively modified thiolase and α-ATP synthase only in the Base-ethanol group. However, the increased levels of oxidized proteins in the Base-ethanol group were markedly decreased in rats fed the same amounts of alcohol in the presence of PUFA (PUFA-ethanol). Addition of PUFA to ethanol-fed rats (PUFA-ethanol) improved histological data (i.e., disappearance of fat vacuoles) with the absence of the oxidized protein bands of both thiolase and α-ATP synthase detected only in the Base-ethanol group. Furthermore, the respective activities of thiolase, ATP synthase, and ALDH2, all suppressed in the Base-ethanol group, were restored in the PUFA-ethanol group. Further mechanistic studies revealed that the PUFA diet significantly prevented activation/induction of CYP2E1 and iNOS, which produce ROS and RNS, respectively, observed in alcohol-exposed tissues (Base ethanol). Consequently, the elevated levels of a potently toxic peroxynitrite, which can *S*-nitrosylate Cys residues and/or nitrate Tyr residues of various proteins [48], were significantly decreased in the PUFA-ethanol group compared to those in alcohol-fed control rats (Base ethanol).

During our mechanistic study on I/R-related mitochondrial dysfunction, we were also able to evaluate the beneficial effect of a peroxynitrite scavenger metalloporphyrin MnTMPyP against mitochondrial dysfunction and acute hepatic I/R injury [70]. MnTMPyP pretreatment markedly suppressed the I/R-related elevation of serum transaminase levels, histological damage, iNOS expression, and oxidative modifications of key mitochondrial proteins assessed

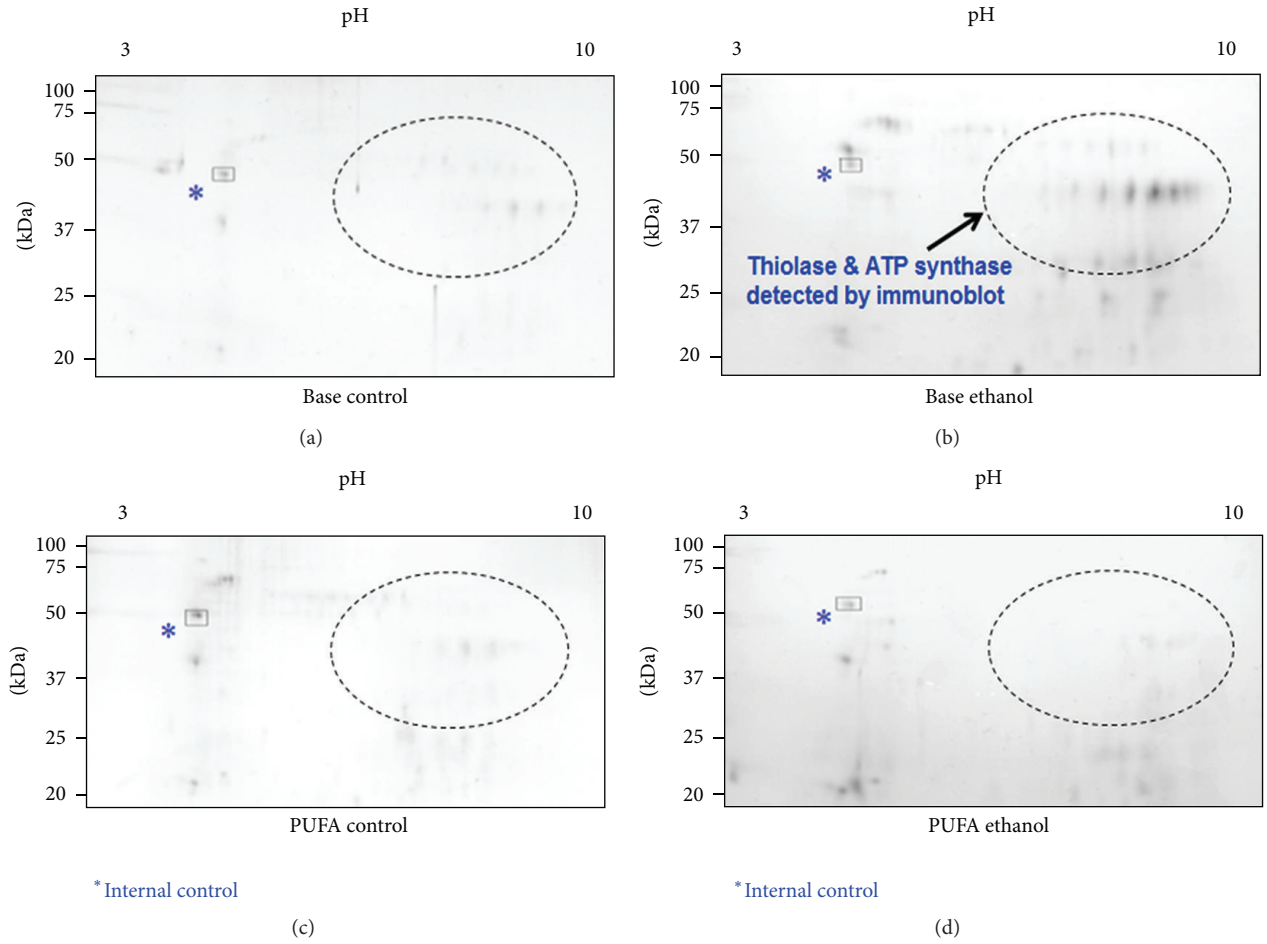

Figure 4: Translational research application of a redox proteomics approach by analyzing the oxidatively modified proteins in control and alcohol-exposed rats in the absence or presence of PUFA. Oxidatively modified mitochondrial proteins from each group were identified with a redox proteomics method using biotin-NM as a probe [106, 107], purified with streptavidin-agarose beads, then displayed on 2D gel, and stained with silver. The images of all gels were adjusted by optimizing the similar density of an endogenous, internal protein (*) in each gel. Protein spots in encircled areas in indicated samples reflect the appearance or disappearance of oxidized proteins depending on the treatment in each group, as described in and adapted from [137].

by comparative 2D gel analysis for each sample. These changes were further supported by the activity measurements of mitochondrial ALDH2, thiolase, and ATP synthase as well as histopathological evaluation. These studies provide supporting evidence for a translational research application of the redox proteomics approach against mitochondrial dysfunction and organ damage in numerous disease states. We expect that beneficial effects of many natural antioxidant agents such as polyenephosphatidylcholine [140, 141], *S*-adenosylmethionine [142], resveratrol [143], curcumin [144, 145], and silymarin [144, 145] or various synthetic agents such as vitamin E analogs and carvedilol [146] against mitochondrial dysfunction and oxidative tissue injury could be demonstrated by using the redox-based proteomics approaches. Furthermore, the redox proteomics approach may also be used in finding potential biomarkers of disease states in other extrahepatic tissues including brain, heart, lung, and kidney from experimental models as well as human tissue specimens.

Abbreviations

ALDH2:	Mitochondrial low-Km aldehyde dehydrogenase 2
BIAM:	Biotin-conjugated iodoacetamide
Biotin-NM:	Biotin-*N*-maleimide
Complex I:	NADH-dependent ubiquinone oxidoreductase
Complex III:	Cytochrome c reductase
Complex IV:	Cytochrome c oxidase
CYP2E1:	Ethanol-inducible cytochrome P450 2E1 isozyme
DIGE:	Difference in gel electrophoresis
ER:	Endoplasmic reticulum
ETC:	Electron transport chain
4-HNE:	4-Hydroxynonenal
iNOS:	Inducible nitric oxide synthase
I/R:	Ischemia-reperfusion
MDA:	Malondialdehyde

MDMA: 3,4-methylenedioxymethamphetamine
NEM: *N*-ethylmaleimide
NO: Nitric oxide
PUFA: Polyunsaturated fatty acids
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
S-NO-Cys: *S*-nitrosylated Cys
SOD: Superoxide dismutase
Thiolase: 3-Ketoacyl-CoA thiolase.

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Research Article

Enhanced Prostacyclin Synthesis by Adenoviral Gene Transfer Reduced Glial Activation and Ameliorated Dopaminergic Dysfunction in Hemiparkinsonian Rats

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Prostacyclin (PGI_2), a potent vasodilator and platelet antiaggregatory eicosanoid, is cytoprotective in cerebral circulation. It is synthesized from arachidonic acid (AA) by the sequential action of cyclooxygenase- (COX-) 1 or 2 and prostacyclin synthase (PGIS). Because prostacyclin is unstable *in vivo*, PGI_2 analogs have been developed and demonstrated to protect against brain ischemia. This work attempts to selectively augment PGI_2 synthesis in mixed glial culture or in a model of Parkinson's disease (PD) by direct adenoviral gene transfer of prostacyclin biosynthetic enzymes and examines whether it confers protection in cultures or *in vivo*. Confluent mixed glial cultures actively metabolized exogenous AA into PGE_2 and PGD_2. These PGs were largely NS398 sensitive and considered as COX-2 products. Gene transfer of AdPGIS to the cultures effectively shunted the AA catabolism to prostacyclin synthesis and concurrently reduced cell proliferation. Furthermore, PGIS overexpression significantly reduced LPS stimulation in cultures. *In vivo*, adenoviral gene transfer of bicistronic COX-1/PGIS to substantia nigra protected 6-OHDA- induced dopamine depletion and ameliorated behavioral deficits. Taken together, this study shows that enhanced prostacyclin synthesis reduced glial activation and ameliorated motor dysfunction in hemiparkinsonian rats. Prostacyclin may have a neuroprotective role in modulating the inflammatory response in degenerating nigra-striatal pathway.

1. Introduction

Parkinson's disease (PD) is characterized by the progressive degeneration of nigrostriatal dopaminergic (DA) accompanied with inflammatory changes leading to activation of microglia and astrocytes [1]. The substantia nigra (SN) of the brain is particularly rich in microglia [2, 3]. In addition, dopaminergic neurons in the SN have a reduced antioxidant capacity, rendering them vulnerable to a variety of insult. Inflammatory responses are also associated with the effects of dopaminergic neurotoxins, 6-hydroxydopamine (6-OHDA) or 1-methyl-4-pheny-1,2,3,6-tetrahydropyridine (MPTP). Activated microglia and DA cell loss were found in the primate SN years after MPTP treatment [4]. In the striatum and SN of 6-OHDA-lesioned rat brains, prominent microglial activation was detectable weeks after the lesion [5]. Intranigral injection of LPS in rats also resulted in microglial activation and degeneration of the DA system [6, 7]. Whether

microglial activation protects or exacerbates neuronal loss is presently debated, though most studies suggest that activated microglia exerts a toxic effect on neurons.

The prostanoids, a naturally occurring subclass of eicosanoids, are lipid mediators generated through the oxidative metabolism of 20-carbon fatty acids (eicosa is Greek for 20), primarily arachidonic acid (AA). Prostaglandins (PGs), synthesized from AA by cyclooxygenases (COXs), have diverse biological actions by working as local mediators. In the central nervous system (CNS), PGs maintain important functions as retrograde synaptic messengers and as early mediators of neuronal injury. The levels of PG production are mediated by the expression and activity of COX. COX exists in two distinct isoforms, constitutive COX-1 and inducible COX-2. COX-2 is responsible for the increased production of prostanoids during inflammation and stress [8, 9]. In the brain, COX-2 is constitutively expressed and is also the dominating COX isoform [10, 11] that mainly produces PGE_2 and PGD_2 [12, 13]. Prostacyclin (PGI_2), synthesized by sequential action of COX and prostacyclin synthase (PGIS), is a potent endogenous inhibitor of platelet aggregation. It inhibits platelet secretory activity and aggregation, maintains vasorelaxation, blocks monocyte-vascular wall interactions and is vasoprotective [14]. Because of its instability *in vivo*, several PGI_2 analogs have been developed and demonstrated to reduce ischemic brain damage [15–17].

Our previous results have shown that overexpression of COX-1 and PGIS was able to generate large quantity of PGI_2 in human endothelial cells [18]. Using adenovirus-mediated transfer of COX1 or COX1/PGIS, Lin et al., [19] and our coworkers [20] have demonstrated that enhanced PGI_2 synthesis in neuronal cultures or in ischemic brain was neuroprotective and had prominent influence on microglia. This work attempts to selectively augment PGI_2 synthesis in mixed glial culture and in hemiparkinsonian rats by direct adenoviral gene transfer of PGIS or bicistronic COX-1/PGIS and examines whether it confers protection or induces cell damage in Parkinson's disease (PD). Hemiparkinsonian rats were induced by injection of 6-OHDA to middle forebrain bundle (MFB), the ascending pathway of nigrostriatal system. Our results demonstrated that enhanced prostacyclin synthesis inhibited glial activation and was beneficial in hemiparkinsonian rats.

2. Materials and Methods

2.1. Materials. Lipopolysaccharide (LPS; *E. coli* 0111:B4), OHDA, dopamine, apomorphine and methylthiazol tetrazolium (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). CAY10449 was purchased from Cayman Chemical (Ann Arbor, MI). Cultured media and antibiotics were purchased from Invitrogen (Carlsbad, CA, USA). [6-^{3}H] thymidine, [1-^{14}C] arachidonic acid (AA) and radioactive prostanoids were purchased from Amersham Biosciences (Buckinghamshire, UK). Acetonitrile and other organic solvents were obtained from Merck (Darmstadt, Germany). Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich Co.

2.2. Recombinant Adenovirus (Ad). Replication-defective first generation E1-deleted adenoviral vectors were used. Adenovirus encoding GFP (expressing the green fluorescence protein of jelly fish), PGI_2 synthase (PGIS), or bicistronic COX-1/PGIS used phosphoglycerate kinase (PGK) as a driving promoter. The preparation, *ex vivo* expansion, and purification of these Ads followed methods described previously [18, 20, 21]. The viral titers of the purified Ads were determined by a plaque-forming assay and were in the range of ~10^{10} pfu/mL.

2.3. Cell Cultures and In Vitro Transduction. Mixed neuronal/glial cell cultures were prepared from the mesencephalic region of embryonic Sprague-Dawley (SD) rat fetus at gestation of 14–16 days as described in Tsai et al. [22, 23]. Briefly, cells were dissociated with mixtures of papain/protease/deoxyribonuclease I (0.1% : 0.1% : 0.03%) and plated onto poly-D-lysine coated dishes at a density of 1~2 × 10^5 cells/cm^2 in DMEM supplemented with 10% FBS. Second day after cell seeding, cultures were infected with AdGFP, AdCOX-1 or AdCOX-1/PGIS. Neuronal cells in the mixed neuron/glial cultures were identified by immunostaining against anti-βIII tubulin (dilution 1/300; Covance, CA, USA). Mixed glial cells were prepared from neonatal rat brains as described previously [24, 25]. Briefly, triturated cortexes, free of vessels and meninges, were passed through nylon cloths and plated in 75 cm^2 flasks in DMEM supplemented with 10% FBS. The cells were incubated at 37°C in a water-saturated atmosphere of 5% CO_2/95% air. When cell reached confluence, cells in the flasks were subcultured and replated into multiwell plates. Cultures showed greater than 90% positive staining for glial fibrillary acidic protein (rabbit or mouse anti-GFAP, Chemi-Con, USA), an astroglial marker. Subconfluent cultures were used for measure of proliferative activity (tritiated thymidine incorporation or MTT reduction; see below), while confluent cultures were used for assay of metabolic activity in response to ^{14}C-arachidonic acid (AA). For *in vitro* transduction, cells were fed with growth medium. Ad-GFP, Ad-PGIS, or Ad-COX-1/PGIS was added to cultured cells with a multiplicity of infection (MOI, pfu/cell) of 20. Recombinant Ad-GFP was used as a vector control and for optimizing the infection conditions. Three days after Ads transduction, cells were processed for eicosanoid measurement or treated with LPS (600 ng/mL) for 2 days. The culture medium was then assayed for nitric oxide (NO), and the cells were processed for western blot analysis. CAY10449 at 500 nM was added to mixed glial cells at 2 hr after AdPGIS transduction. Cultures were incubated for 5 days with medium and drug refilled once. Cultures were then processed for MTT reduction.

2.4. Extraction and Analysis of Arachidonic Acid (AA) Metabolites in Mixed Glial Culture and in Mixed Neuron/Glial Cultures. Confluent glial cultures or neuron/glial cultures after AdGFP, AdPGIS AdCOX-1, or AdCOX-1/PGIS transduction were measured for AA metabolic activity. Briefly, cultured cells were incubated in DMEM (serum-free) containing 10 μM [1-^{14}C] AA at 37°C for 10 min. The cells were saved for

western blot analysis, while the released fractions, containing radioactive eicosanoids, were extracted by a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) as described [20, 21]. The resulted extracts of ^{14}C-labeled AA metabolites (eicosanoids) were analyzed by reverse phase high performance liquid chromatography (HPLC; Waters model 2690) equipped with an online radioisotope detector (Packard 150-TP) as previously described [18]. Briefly, the stationary phase was Inertsil 7 ODS-3 (4.6 × 150 mm; Vercopak, Taiwan). The mobile phase consisted of programmed gradient elution between solvent A (acetonitrile) and solvent B (0.1% acetic acid, pH 3.7) at a flow rate of 1 mL/min as follows: 34% B for 10 min, 34–40% B within 4 min, 40–50% B within 1 min, 50% B for 5 min, 50–75% B within 10 min, 75–100% B within 10 min, and 100% B for 10 min. The eicosanoids were identified by their retention times with the authentic radioisotope standards.

2.5. [^{3}H]-Thymidine Incorporation Assay. The proliferative activity of mixed glial cultures after Ad transduction was investigated in subconfluent cultures by pulse of cultures with 0.5 μCi/mL [^{3}H]-thymidine for 10 hrs according to our previous methods [26, 27]. After [^{3}H]-thymidine pulse, cultured media were carefully removed and cells were washed twice with PBS. Aliquot of ice-cold 10% trichloroacetic acid (250 μL/well) was added to cells. The radioactivities in the cell lysate were measured in a scintillation counter.

2.6. 6-OHDA Lesion. Adult SD rats weighing 250–300 g were used. The animals were anesthetized by isoflurane (1-chloro-2,3,4-trifluoroethyl ether, Aerrane) with oxygen during surgery. Operations were carried out using an operating microscope under aseptic conditions. All procedures involving animals were approved by the Animals Committee of Taipei Veterans General Hospital. Surgical procedures, postoperative care, and monitoring have been described previously [28–30]. Unilateral 6-OHDA lesion of nigrostriatal pathway was performed with the rats under isoflurane anesthesia by stereotaxic injection of 6-OHDA HBr [20 μg/rat; dissolved in ascorbate (0.02% in PBS as vehicle)]. Five microliters of 6-OHDA (2 μg/μL) were injected into two sites (5 μL/site) in the ascending nigrostriatal pathway near the MFB of adult SD rats as described [31]. The coordinates of two MFB injections were AP-4.2 mm (posterior to bregma), ML-1.1 mm (lateral to the midline), DV-7.8 mm (7.8 mm below the dura), AP-4.4 mm (posterior to bregma), ML-0.9 mm, and DV-7.8 mm. The needle was allowed to remain in the brain for 5 min before being retracted at the end of the 6-OHDA infusion.

2.7. Intranigral Injection of Ad Vectors. After the rat was placed in the stereotaxic frame (Kopf Instruments, Tujunga, CA) infused with saline or 6-OHDA, injection of Ad vector to brain regions was conducted. 1 μL Ad vector suspended in PBS was injected into the vicinity of the SN at [coordinate AP −5.3 mm, ML −2.1 mm, and DV −7.2 mm from bregma] or into striatum at [AP+0.5 mm; ML+2.0 mm and DV−5.0 mm from bregma]. Ad injection was through a 5 μL Hamilton syringe fitted with a 30-gauge beveled hypodermic needle for 5 min at a rate of 0.2 μL/min. After the cessation of the injection, the needle was left in place for 5 min before being slowly withdrawn from the brain. Ad vector injection was conducted within 30 min after infusion of 6-OHDA to MFB.One microliter of storage buffer, Ad-GFP, or AdCOX-1/PGIS containing approximately 2×10^4 plaque-forming units (pfu) was injected. AdGFP was used as a mock control and for examining infective tropism.

2.8. Apomorphine-Induced Circling Behavior. One to four weeks after the infusion of 6-OHDA and Ads, a behavioral test was conducted to identify the efficacy of treatment. Rats from all groups were administered with the DA agonist apomorphine (0.5 mg/kg, s.c.) and immediately separated into individual acrylic box cages. Ten minutes later, the number of contralateral rotations to the lesioned side was recorded in each rat every 5 min for a total time of 60 min. Each rotation was defined as a complete 360° turn. Results were expressed as the total number of turns that rats completed in 60 min. In the present study, rats with 6-OHDA lesion only would have a net contralateral rotational asymmetry of >500 turns/hr.

2.9. Biochemical Assays. The production of nitric oxide (NO), as nitrite accumulation, was assayed in the medium using colorimetric reaction with Griess reagents (1% sulfanilamide/0.1% naphthyl ethylene diamine dihydrochloride/2% phosphoric acid) as described [23]. The degree of MTT reduction was used to measure cell viability or proliferative activity. Following treatment, MTT was added to cultured cells at final concentration of 0.1 mg/mL and reacted with the surviving cells for 4 hr at 37°C. The resulted blue formazan product was solubilized and measured at an absorbance of 570 nm [26].

2.10. Immunohistochemistry. Rats were perfused intracardially with 4% paraformaldehyde in PBS under deep anesthesia with sodium pentobarbital at the end of the experiments. The brains were removed, postfixed in 4% paraformaldehyde overnight, and then cryoprotected in PBS containing 30% (w/v) sucrose for 3 days. The tissues were excised and embedded in Tissue Tek OCT (Sakura Fine Technical, Tokyo, Japan) and then cross-sectioned at 20 μm thickness with a cryostat. Tissue sections were collected onto glass slides and dried at 37°C. The tissue sections were incubated with primary antibodies, followed by respective 2nd antibodies for histological evaluation as described [30, 32]. To examine the infective tropism of AdGFP, rats were processed for histological analysis at three days after nigral AdGFP infection. Double immunostaining of GFP with cell markers was conducted in coronal brain sections. Antibodies for cell markers included anti-tyrosine hydroxylase (TH) (mouse, dilution 1/150, Chemicon, Temecula, CA) for dopamine neurons, anti-GFAP (rabbit, dilution 1/1000; Chemicon, Temecula, CA) for astrocytes, Biotinylated *Griffonia simplicifolia* lectin I isolectin B4 (biotinylated GSLI-IB4, dilution 1/100; vector b1205), or anti-ED-1 (mouse, dilution 1/250; Serotec, Oxford, UK) for microglia.

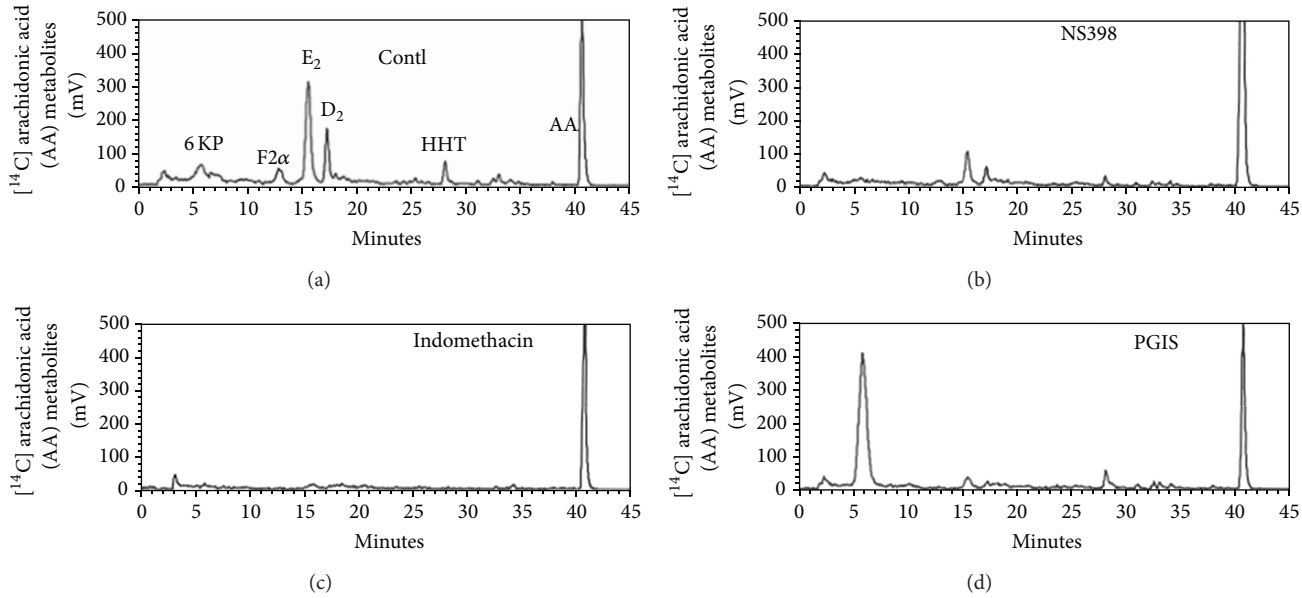

Figure 1: Analysis of ^{14}C-labelled eicosanoids generated in control or Ad-PGIS-transduced mixed glial cultures in response to [1-^{14}C] AA and COX inhibitors. Measurement of eicosanoid biosynthesis in cultures was conducted at 3 days after Ad-PGIS transduction. NS398 is a COX-2 specific inhibitor, and indomethacin is an inhibitor for both COX-1 and COX-2. Inhibitor was added to cultures 30 min before and during ^{14}C-AA pulse. 6-KP denotes 6-keto-$PGF_{1\alpha}$, the product of PGI_2 hydrolysis. Peaks of first 5-minute fractions are nonspecific. Each prostanoid peak was verified by coelution with an authentic radiolabelled prostanoid.

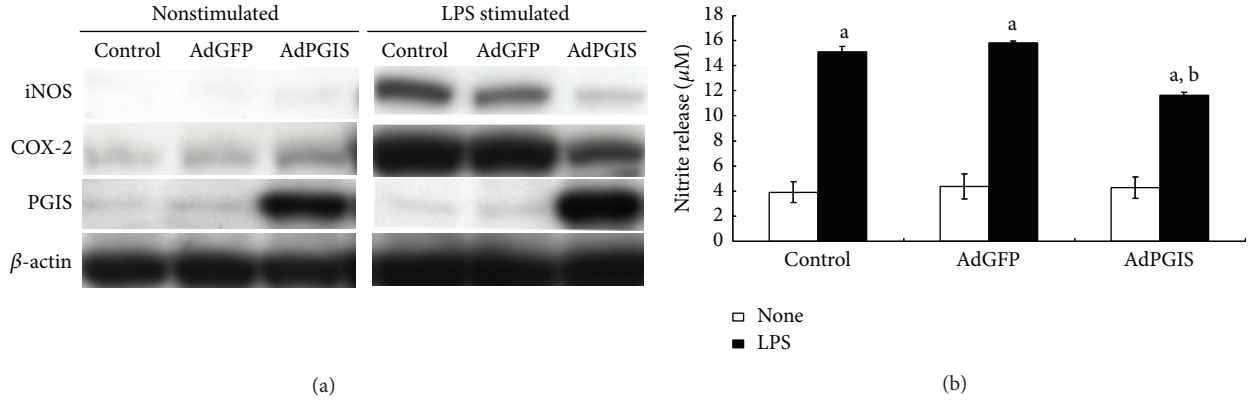

Figure 2: NO synthesis and expressions of iNOS, COX-2, and PGIS in AdGFP- and AdPGIS-transduced mixed glia cultures before and after LPS challenge. (a) Western blot analysis of protein levels of PGIS, COX-2, and iNOS in cultures receiving AdGFP or AdPGIS infection. After 3 days of Ad infection, cultures were treated with LPS (600 ng/mL) for 2 days and harvested. Equal amounts of protein were analyzed by western blot using anti-PGIS, anti-COX-2, and anti-iNOS antibodies. Protein bands were visualized using horseradish peroxidase-conjugated secondary antibodies and electrochemiluminescence (ECL). (b) Inhibition of LPS-stimulated NO production, as nitrite release, in the medium by AdGFP and AdPGIS transduction in cultures. Control on x-axis, no viral infection of naive cultures; open bar in chart indicates nonstimulated cultures. Closed bar indicates LPS-stimulated cultures. Data were expressed as means ± SEM from four independent experiments done in triplicate. $^{a}P < 0.01$ indicates significant differences between nonstimulated and LPS-stimulated cultures within each Ad-transduced cells; $^{b}P < 0.05$ AdPGIS + LPS compared with AdGFP + LPS.

2.11. Western Blot Analysis. Brain tissues or cultured cells were solubilized in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris buffer, pH7.5, protease inhibitors (Roche, Mannheim, Germany), 1 mM PMSF, 1 mM Na_3VO_4, and 1 mM DTT. Protein concentration of the resultant lysate was determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA). Equal amounts of proteins were loaded and separated using 8%–12% gels (SDS-PAGE) as described [33]. After electrophoresis, proteins in the gels were transferred to PVDF membranes (Millipore Corp., USA) and incubated overnight at 4°C with antibodies against PGIS (rabbit, dilution 1/3000), COX-2 (rabbit, dilution 1/5000, Cayman), inducible nitric oxide synthetase (iNOS, mouse, dilution 1/5000, BD Bioscience, USA), or β-actin (goat, dilution

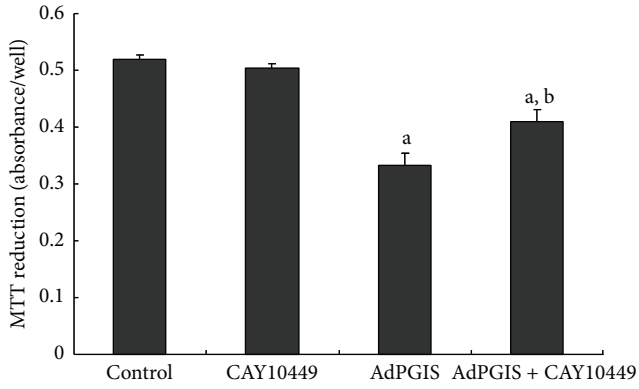

Figure 3: CAY10449 and AdPGIS transduction on MTT reduction in mixed glial cultures. CAY10449 is a high affinity ligand and functional antagonist for the human IP (prostacyclin) receptor. CAY10449 (500 nM) was added to cultured cells at 2 hr after AdPGIS transduction. Data were means ± SEM from 4 independent cultures done in duplicate. [a]$P < 0.01$ AdPGIS versus Control; [b]$P < 0.05$ AdPGIS + CAY10449 versus AdPGIS.

1/5000, Santa Cruz Biotech, USA) followed by a horseradish peroxidase-conjugated secondary antibody (dilution 1/2000, Jackson Lab) for 1 hr at room temperature. Immunoreactivity was visualized by enhanced chemiluminescent detection (Perkin Elmer Co., USA).

3. Results

3.1. Analysis of Eicosanoids Produced by Cultured Glial Cells in Response to [1-^{14}C] AA Treatment. Purified Ads were successfully expanded and purified before conducting the *in vitro* and *in vivo* experiments. Highly purified adenoviruses encoding GFP, PGIS, and bicistronic COX-1/PGIS, ranging from 10^{10}~10^{11} pfu/mL, were used in the present study. Previously, we have demonstrated high permissivity of mixed glial cultures to AdGFP infection [22]. Almost all cells expressed GFP at 2 days after 20 MOIs of Ad-GFP transduction (Similar results are shown in Figure 4(f)). In the present study, we directly examined the activities of eicosanoid biosynthetic activity in Ad-transduced mixed glial cells in response to ^{14}C-AA pulse. Because mixed glial cultures are depleted of neuronal cells, studying AA metabolic activity in glia cultures would give some clue to the relative roles of neurons versus glial cells. We incubated cultures with [1-^{14}C] AA for 10 min, extracted eicosanoids from the medium by a C_{18} cartridge, and analyzed the eicosanoids by HPLC. Two predominant peaks, prostaglandin (PG) E_2 and PGD_2, were detected in nontreated cultures (Figure 1(a)). Very little or none of PGI_2 (prostacyclin), shown as its hydrolysis product 6-keto-$PGF_{1\alpha}$ (6KP), was found. The transduction of AdGFP did not alter the metabolic profile (data not shown). The 6-keto-$PGF_{1\alpha}$ peak was mostly reduced (>80%) when cells were pretreated with NS398, a selective COX-2 inhibitor (Figure 1(b)). Furthermore, no 6-keto-$PGF_{1\alpha}$ peak was detected when cells were pretreated with indomethacin, an inhibitor for both COX-1 and COX-2. This indicates COX-2 as the major enzyme of eicosanoid synthesis in mixed glial cultures in response to ^{14}C-AA. Interestingly, AA metabolites were shunted through prostacyclin synthesis on AdPGIS transduction. Very little of PGE_2 and PGD_2 remained in AdPGIS-transduced cultures. This indicates that the overexpressing enzyme was functionally active in producing prostacyclin from AA. By contrast, AdPGIS-infected neuron/glial cultures did not augment 6-keto-$PGF_{1\alpha}$ synthesis (see Supplementary Figure 1 available online at http://dx.doi.org/10.1155/2013/649809 and Tsai et al., [20]). AdCOX-1-infected neuron/glial cultures produced predominant PGE_2 and PGD_2 peaks. Only bicistronic AdCOX-1/PGIS-infected neuron/glial cultures prominently enhanced 6-keto-$PGF_{1\alpha}$ synthesis.

3.2. Enhanced Prostacyclin Synthesis Reduced Cell Proliferation and LPS Stimulation in Mixed Glial Cells. To examine the effects of enhanced prostacyclin synthesis on cell proliferation, subconfluent glial cells were transduced with Ad-PGIS for 2 days. Tritiated thymidine was added to cultures 10 hr before cell harvest. Table 1 shows that overexpression of PGIS in cultured glial cells concurrently enhanced prostacyclin production (indicated by level of 6-keto-$PGF_{1\alpha}$, the product of PGI_2 hydrolysis) and inhibited astroglial proliferation (indicated by thymidine incorporation). To examine whether the effect of overexpressing PGIS on cell proliferation was mediated via IP (prostacyclin) receptor, CAY10449 was added to mixed glial cells at 2 hr after AdPGIS transduction. As shown in Figure 2, AdPGIS transduction reduced degree of MTT reduction in mixed glial cultures. The high-affinity IP antagonist, CAY10449, at 500 nM partially but significantly abrogated prostacyclin effect on cell proliferation. LPS is a powerful immune challenge. LPS treatment has been shown to induce release of cytokines, NO, and proinflammatory factors from macrophages [34, 35]. Effect of overexpressing PGIS on LPS stimulation was examined in mixed glial cultures. Confluent glial cultures were transduced with AdGFP or AdPGIS. Three days later, cells were further treated with LPS at a dose of 600 ng/mL for 2 days. In our previous paper [20], we used 100 ng/mL LPS to stimulate neuron/glial cultures. However, LPS failed to affect mixed glial cells at 100 ng/mL and required higher concentration of LPS for effective cell stimulation. As shown in Figure 3, Ad-PGIS transduction enhanced the expression of PGIS whereas iNOS and COX-2 levels were barely detectable. By contrast, LPS treatment induced increase of iNOS and COX-2 expression in control and AdGFP-infected cells. AdPGIS transduction effectively reduced LPS-induced COX-2 and iNOS levels. Concurrently, LPS-stimulated nitrite releases were significantly inhibited in AdPGIS-transduced cells (Figure 3(b)) compared to control and Ad-PGK-infected cells. This indicates that enhanced prostacyclin synthesis in PGIS-overexpressing cells significantly inhibited iNOS expression and NO production.

3.3. Infective Tropism of AdGFP Transduction in Mesencephalic Neuron/Glial Cultures and in Rat Substantia Nigra (SN). First, we examined the infective tropism of AdGFP in mesencephalic neuron/glial cultures which were enriched

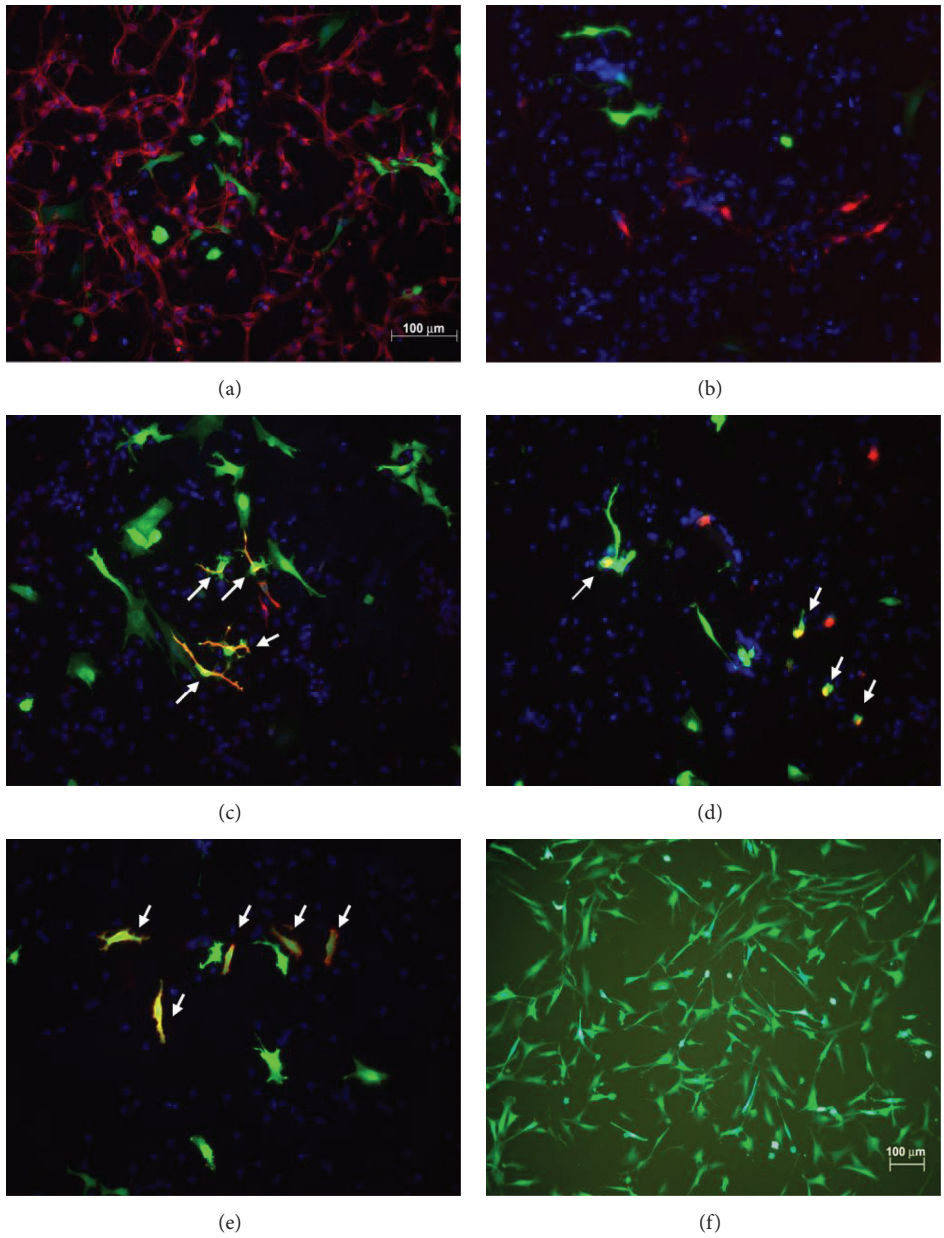

Figure 4: Infective tropism of adenovirus encoding GFP (AdGFP) in mesencephalic neuron/glial cultures and GFP expression in AdGFP-infected mixed glial cells. (a)~(e) are representative micrographs of double labeling staining of cultures maintained in serum-free condition; Green color: GFP-immunoreactivity (IR). Red color in (a) βIII tubulin-IR for neurons, (b) tyrosine hydroxylase (TH)-IR for dopamine neurons, (c) GFAP-IR for astroglia, (d) ED1-IR for microglia, (e) NG2-IR, Magnification ×200, (f) mixed glial cells, magnification ×100. Arrows or arrow head in the figure indicates double-staining IR. AdGFP (10^6 pfu/well each) was added to cultured cells in serum or serum-free medium. The infective tropism of AdGFP in serum-free condition was 19.6 ± 2.9% GFAP(+) cells, 24.4 ± 6.1% ED1(+) cells, and 43.9 ± 4% NG2(+) cells. GFP(+) cells in neuron/glial cultures in serum-free and serum-containing conditions during infection were 31.83 ± 3.12 and 19.89 ± 2.19 cells/mm^2, respectively.

with DA neurons. AdGFP (~10^6 pfu/well each) was added to cultured cells in serum or serum-free medium. As shown in Figure 4, AdGFP predominantly transduced nonneuronal cells. In serum-free condition, the AdGFP infective cells were 19.6 ± 2.9% astroglial (GFAP-positive) cells, 24.4 ± 6.1% microglia (ED1-positive) cells, and 43.9 ± 4% NG2-positive cells. No βIII tubulin-positive neuron or TH-positive DA neurons showed GFP immunoreactivity. Furthermore, the AdGFP infective efficiency in serum-containing medium (10% FCS) was reduced compared to that in serum-free condition. Using same titer of AdGFP to neuron/glial culture, GFP-positive cells in cultures maintained in serum-free and serum-containing conditions were 31.83 ± 3.12 and 19.89 ± 2.19 cells/mm^2, respectively. We further performed

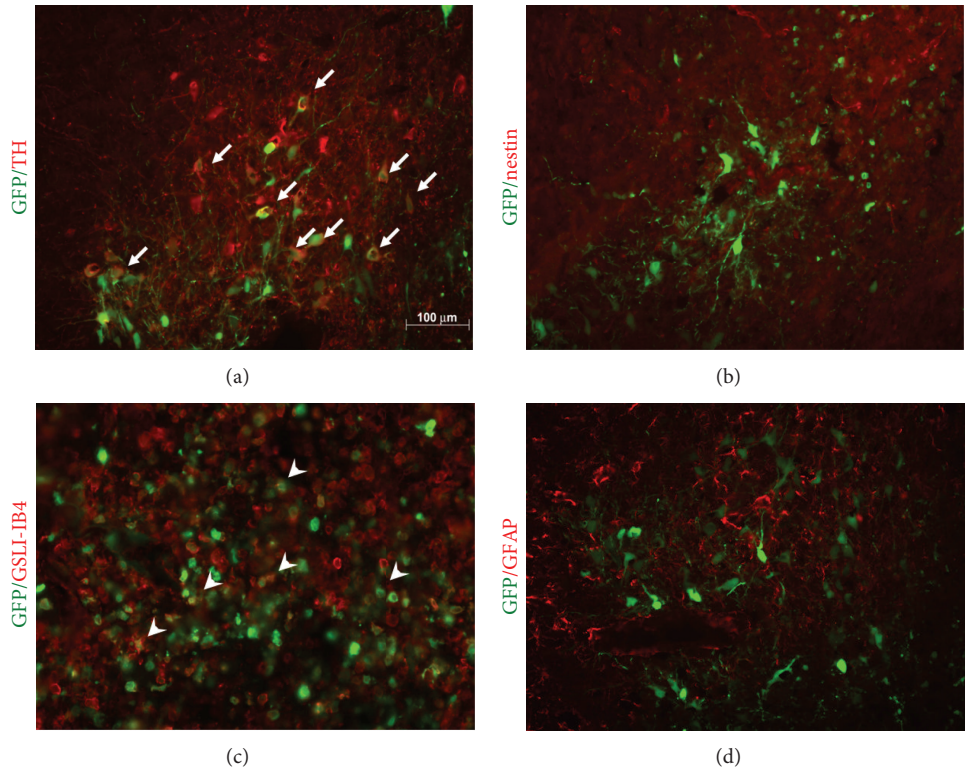

Figure 5: Infective tropism of adenovirus encoding GFP (AdGFP), which was injected into the substantia nigra (SN) 3 days ago. Representative micrographs of double labeling staining of coronal sections in the injection site. (a)~(d) Photos (magnification ×200) of each double staining; green color: GFP immunoreactivity (IR); red color: TH-IR (panel a), nestin-IR (panel b), GSLI-IB4-IR (panel c) and GFAP (panels d). Arrows or arrow heads in the figures indicate double-IR. AdGFP could transduce dopaminergic (TH-IR) neurons and GSLI-IB4-IR microglia in the SN.

Table 1: Effect of Ad-PGIS gene transfer on the proliferative activity in mixed glial cells.

	Thymidine incorporation (cpm/well)	[^{14}C] 6-keto-PGF$_{1\alpha}$ (control %)
Control	378 ± 19	100 ± 4
Ad-PGK	372 ± 24	103 ± 2
Ad-PGIS	277 ± 16*	750 ± 5**

Subconfluent rat glial cells in 24-well plates or 6 cm dishes were infected with Ad (5×10^5 pfu/w) for 2 days. [^{3}H]-thymidine (0.5 μCi) was added to culture (in 24-well plate) 10 hrs before cell harvest. Trichloroacetic-acid-(TCA-) insoluble fraction (radioactivity) was collected and counted. For glial cells grown in 6 cm dishes, cells were incubated with 10 μM [1-^{14}C] AA for 10 min. [1-^{14}C] AA metabolites in the medium were then processed for HPLC analysis. Data are expressed as means ± SEM from 4 independent repeats. $^{*}P < 0.05$; $^{**}P < 0.01$ by one way ANOVA.

double-labelled staining for AdGFP-infected coronal sections containing SN. After injection of AdGFP into the normal SN, the expression of GFP was observed along the site of injection. Figure 5 shows the representative micrographs of double-labelled immunostaining results. Some GFP-positive cells in the SN were also positively stained for TH, which denote dopaminergic neurons (Figure 5(a), arrow). Some GSLI-IB4-positive microglia (in Figure 5(b), arrow head) seemed to be immunoreactive to GFP. By contrast, almost no double-labelled staining cells were found in GFP with GFAP or nestin immunoreactivity (Figures 5(c) and 5(d)). These results showed that transgene expression could be achieved in neurons or microglia after injection of AdGFP into the SN *in vivo*. The results of AdGFP tropism in cultures and in SN were not consistent. We also infected AdGFP to the striatum and examined the infective tropism (shown in Supplementary Figure 2). Consistent with the results observed in AdGFP-infected SN *in vivo*, TH-positive, GFAP-positive, ED1-positive and nestin-positive cells were found to be double labelled with GFP in rat striatum.

3.4. Effect of Nigral Infusion of Ad-COX-1/PGIS in Hemiparkinsonian Rats. Our previous study showed that bicistronic AdCOX1/PGIS infection to neuron/glial cultures produced prominent prostacyclin synthesis, whereas AdPGIS infection did not [20]. We thus directly infused AdCOX-1/PGIS to rat SN for ensured prostacyclin production. 6-OHDA at a dose of 20 μg/rat was infused to the right MFB in rats. Within 30 minutes after 6-OHDA infusion, AdGFP or AdCOX1/PGIS (2×10^4 pfu) was subsequently infused to right SN. Behavioral test was examined weekly after surgery by apomorphine-induced turning. Rats were sacrificed 4 weeks after treatment. Regions of substantia nigra and striatum were microdissected for dopamine level measurement. Figures 6(a) and 6(b) demonstrate 6-OHDA treatment

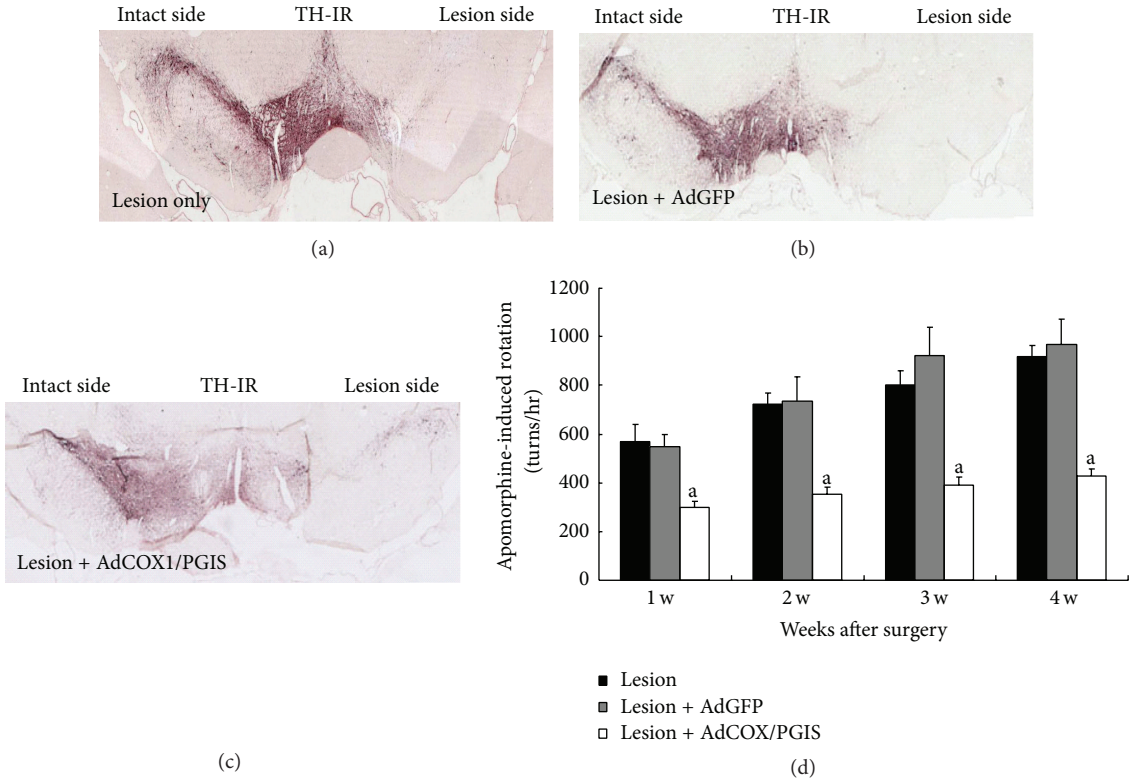

Figure 6: Nigral Ad-COX-1/PGIS infusion ameliorated 6-OHDA-induced dopamine neuronal loss and behavioral deficit. (a)~(c) Representative micrographs of TH immunoreactivity in rat substantia nigra (SNpc) of each group. 20 μm thick coronal brain sections, In (a) 6-OHDA-lesioned brain section, (b) 6-OHDA-lesioned + AdGFP-transduced brain section and (c) 6-OHDA-lesioned + AdCOX1/PGIS-transduced brain section. (d) Apomorphine-induced rotational behaviors in 6-OHDA-treated rats. TH-positive neurons in (c) were rescued by AdV-COX1/PGIS adenoviral gene transfer. 6-OHDA treatment severely depletes TH-positive neurons in right SN compared with untreated left site. Adenoviral vectors were injected into SN within 30 min after 6-OHDA treatment. Treatment rescued TH-positive neurons ipsilateral to gene delivery. In all cases, Ad treatment had no effect on the contralateral SNpc. Data are means ± SEM from 8, 5, and 13 rats for Lesion, Lesion + AdGFP, and Lesion + AdCOX-1/PGIS groups, respectively. $^{a}P < 0.01$ compared to lesion only at the same time.

severely depleted TH-positive neurons in right SN compared with untreated left site. Some TH-positive neurons were rescued by AdCOX1/PGIS gene transfer (Figure 6(c)). Treatment rescued TH-positive neurons ipsilateral to gene delivery. In all cases, Ad treatment had no effect on the contralateral SN. Apomorphine-induced turning behaviors, as a marker of motor impairment, in 6-OHDA-lesioned rats were shown in Figure 6(d). 6-OHDA-treated rats displayed a considerable number of rotations. And this effect was significantly reduced in rats transduced with AdCOX1/PGIS ($P < 0.01$). The group treated with AdGFP transduction produced no significant changes in rotation behavior when compared with 6-OHDA lesion only. Taken together, nigral Ad-COX-1/PGIS infection protected against 6-OHDA-induced dopaminergic damage and behavioral deficits.

4. Discussion

Adenovirus-mediated gene transfer is a promising tool for the treatment of neurodegenerative diseases. Recombinant adenoviral vectors target gene expression to the nervous system and offer prolonged expression of foreign proteins [23, 33, 36]. Here, we present evidence that glial cultures, mesencephalic neuron/glial cultures, or nigral dopaminergic neurons (SNpc) could effectively be infected by recombinant adenoviruses and thereby expressing transgenes. Our data with glial cultures demonstrated that unstimulated cells actively metabolized exogenous AA into PGE_2 and PGD_2. These PGs were largely NS398 sensitive and therefore considered as COX-2-derived products. This was in contrast to low AA metabolic activity in neuronal cells, which released barely detected eicosanoids [20]. Interestingly, gene transfer of Ad-PGIS to glial cultures effectively shunted the AA catabolism through synthesis of 6-keto-$PGF_{1\alpha}$, a hydrolyzed product of prostacyclin. This indicates that the overexpressing enzyme was functionally active in cooperation with endogenous COX to produce prostacyclin. Accordingly, enhanced prostacyclin production effectively inhibited glial proliferation, in part, through prostacyclin IP receptor. Furthermore, enhanced prostacyclin synthesis significantly inhibited LPS stimulation through inhibition of COX-2 and iNOS expression and NO production. Cell viabilities, measured as LDH release in medium, were not affected by Ad infection or LPS treatment (data not shown).

It is well known that LPS, a major component of the outer membrane of Gram-negative bacteria, induces inflammatory response through cytokine production [37]. We found that mixed glial cells were less sensitive to LPS stimulation in comparison to mixed neuron-glial cultures. LPS is a powerful tool for the activation of microglia. Although LPS has no known direct toxic effect on neurons, it activates microglia to release neurotoxic factors [38, 39]. Neurons, through mechanisms of cell-cell interaction, also modulate the reactivity of microglia [40, 41]. In the present study, AA metabolic activities in neuron/glial culture and in glial cultures were quite different, highlighting the involvement of cell-cell interaction. This work aims to examine the effect of PGI_2 in nervous system. Our previous results have shown that overexpression of COX-1 and PGIS is able to generate large quantity of PGI_2 [18]. AdCOX-1 infected cells could overexpress COX-1 with consistent high activity (mainly PGE_2), as shown in Supplementary Figure 1 in which ^{14}C-labelled eicosanoids were generated by Ad COX-1-infected neuron/glial cultures in response to [1-^{14}C] AA treatment. Therefore, we used the Ad-COX-1/PGIS to investigate the effect of enhanced PGI_2 synthesis in substantia nigra *in vivo*. Both COX-1 and COX-2 catalyze conversion of AA to PGG_2 and further convert to PGH_2. It is possible that overexpression of COX-2/PGIS will generate large quantity of PGI_2 as well.

Our data with adenoviral gene transfer in rat SN indicate that recombinant adenovirus could infect both dopaminergic neurons and microglia efficiently. Among GFP-expressing cells in SN, 58.7 ± 4.8% were TH-positive dopamine neurons and 24.3 ± 6.4% were microglia. The transgene expression was not found in GFAP- or nestin-positive cells, in contrast to that seen in mesencephalic neuron/glial cultures. Figure 4 shows that AdGFP preferentially infected NG2(+) cells, ED1(+) and GFAP(+) cells, while no beta III tubulin (+) neurons or TH(+) dopamine neurons were infected in mixed neuron/glial cultures. High permissivity to AdGFP transduction in mixed glial cells was also shown. Because mixed glial or neuron/glial cultures were maintained as monolayer cultures, nonneuronal cells in these cultures extended and flattened, allowing high probability of getting AdGFP infection. While SN is enriched with DAergic cell body and particularly rich in microglia [2, 3], targeting AdGFP injection to SN *in vivo* was possible to be taken up by microglia and by large area of DA dendrites *in situ*. Thus, rare GFAP/GFP double-labelled cells could be found. Our previous study showed that bicistronic AdCOX1/PGIS infection to neuronal cultures produced prominent prostacyclin synthesis [20]. This was also true in endothelial cells that the overexpressed PGIS and COX-1 were colocalized, leading to monophasic overexpression of prostacyclin [21]. We thus directly infused AdCOX-1/PGIS to rat SN for ensured prostacyclin production. Figure 6 showed that overexpression of COX1/PGIS reduced dopaminergic neuronal death induced by 6-OHDA. We provide evidence that a decrease of the number of TH-positive cells at 4 weeks after 6-OHDA lesion corresponds well with rotational behavior. 6-OHDA-treated rats displayed a considerable number of rotations. This effect was significantly attenuated in rats transduced with AdCOX1/PGIS ($P < 0.01$). The 6-OHDA lesion group with AdGFP transduction produced no significant changes in rotation behavior when compared with 6-OHDA lesion only. Taken together, nigral Ad-COX-1/PGIS infection ameliorated 6-OHDA-induced dopaminergic damage and behavioral deficits.

We used first-generation E1-deleted Ad vectors in the present study. Possible neurotoxic effects induced by Ad expression should be considered. Although we did not evaluate immune response in this study, there were no significant side effects in rats with Ad injection. Furthermore, AdPGIS, AdCOX-1/PGIS or AdGFP used in this study had been constructed with a PGK promoter that could drive prolonged (days to weeks) transgene expression. The mechanisms by which AdPGIS-transduction reduced glial activation or AdCOX1/PGIS-transduction reduced Parkinsonian dysfunction have not yet been clarified. In the brain, at least two distinct prostacyclin receptors, designated as IP1 and IP2, have been shown [42]. The IP2 receptor was found only in the CNS and thus named as central-type prostacyclin receptor [42]. The IP1 receptor is mainly coupled to G-protein-coupled receptor. Stimulation of the receptor results in cAMP production [43]. It remains to be determined which receptor subtype (IP1 or IP2) is involved in this effect. Further studies are needed to elucidate the role played by downstream molecules in the inhibition of cell proliferation and LPS stimulation and in protection to hemiparkinsonism.

In conclusion, this study shows that enhanced prostacyclin synthesis by adenovirus-mediated gene transfer of AdPGIS or AdCOX1/PGIS reduced glial activation and ameliorated motor dysfunction in hemiparkinsonian rats. We suggest that prostacyclin may have a neuroprotective role in modulating the inflammatory response in degenerating nigra-striatal pathway.

Authors' Contribution

S.-K. Shyue and H. Cheng contributed equally to this work.

Acknowledgments

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Research Article

Comparative Effects of Bone Marrow Mesenchymal Stem Cells on Lipopolysaccharide-Induced Microglial Activation

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After injury to the CNS, microglia are rapidly activated and concentrated and trigger inflammatory reaction at the sites of injury. Bone marrow mesenchymal stem cells (BMMSC) represent attractive cell sources for treating CNS injury. Although anti-inflammatory and paracrine effects of grafted BMMSC have been shown, direct modulation of BMMSC on microglia *in situ* remains unclear. The present work employs *in vitro* transwell assay to characterize the effects of BMMSC on LPS-stimulated microglia. BMMSC are cultivated in serum and serum-free (sf) conditions, namely, BMMSC and BMMSC-sf. Both cultures express major surface markers specific for mesenchymal stem cells. However, the BMMSC-sf exhibit sphere-like structure with reduced expression of two adherent cell markers, CD29 and CD90. Compared to BMMSC-sf, BMMSC are fibroblast like and have faster differentiation potential into neural-like cells. Furthermore, BMMSC release significant levels of TIMP-1 and VEGF, regardless of being alone or in coculture. The downregulated MMP-9 mRNA may be caused by TIMP-1 secretion from BMMSC. Our cell culture system provides a powerful tool for investigating the molecular and cellular changes in microglia-BMMSC cocultures.

1. Introduction

Microglia, CNS-resident macrophages, play important roles in the physiological and pathological conditions of the central nervous system (CNS). After injury to the CNS, microglia are rapidly activated and concentrated and trigger inflammatory reaction at the sites of injury [1, 2]. Ample evidence has shown that activated microglia contribute to destructive processes leading to secondary neuronal degeneration. The responses in activated microglia include morphological changes, migration [3], proliferation [4], nitric oxide (NO) production, phagocytosis, antigen presentation, and secretion of diffusion factors. Activated microglia also released excess of toxic factors (such as TNF-α, IL-1β, superoxide, and NO) [5, 6].

Neurorestorative therapy with BMMSC is a promising treatment for CNS injury. BMMSC are found to exhibit low immunogenicity and can escape recognition by lymphocytes and natural killer cells. This distinguishing feature makes the match of BMMSC between donors and recipients less restricted than other cells. In addition, BMMSC can be isolated from bone marrow with relative ease. These multipotent cells also have the ability to differentiate into other types of cells. Several beneficial effects of BMMSC have been proved, including neuron protection [7], differentiation in the lesion site [8], and increased proliferation of endogenous neuron stem cells [9]. These findings support the potential utility of BMMSC for cell-based therapeutic applications. Given that BMMSC behave as biomolecular factories, the approaches

for CNS injury treatment shall be further developed. However, there has been much controversy regarding therapeutic mechanisms and potential risks of various BMMSC activities in the injury sites. The culture media supplements are important issues to discuss. Fetal bovine serum (FBS) is generally supplemented in a complete media for *ex vivo* expansion of BMMSC. However, FBS might have contamination risks with unknown factors or prion which cause Creutzfeldt-Jakob disease (CJD) in humans. In order to accomplish successful cell therapies for CNS injury patients, it is critical to consider and avoid animal serum contaminations.

Several lines of evidence have shown that BMMSC therapeutic plasticity relies greatly on the paracrine release of molecules. However, the direct modulation of BMMSC to the endogenous immune cells of CNS, microglia, is not yet clear. In the present study, BMMSC, cultivated in serum and serum-free (sf) conditions, and an *in vitro* model of microglia-BMMSC cocultures are employed. The effectiveness of BMMSC on LPS-induced microglial activation and cytokine expression is examined and compared. Our results suggest that BMMSC release factors and exert modulation on microglia in a cell contact-independent communication.

2. Material and Methods

2.1. Materials. Culture multiwells and pipettes were obtained from Orange Scientific (Graignette, Belgium). Cultured media, fetal bovine serum (FBS), and antibiotics were purchased from Gibco (Invitrogen Corporation, USA). A rat cytokine array was purchased from R&D (ARY008). Cell surface antibodies for cytometric analysis were from BD Bioscience (USA). Lipopolysaccharide (LPS; *Escherichia coli* O111:B4) was purchased from Sigma-Aldrich (St. Louis, MO,USA). Other reagents were purchased from Sigma-Aldrich unless stated otherwise.

2.2. Microglia Culture. Microglial cells were isolated from confluent mixed glial cell cultures as described previously [10, 11]. Briefly, floating cells and weakly attached cells on the confluent mixed glial cell layer [12, 13] were isolated by shaking the flasks for 2 hrs at 180 rpm. The resulted cell suspension was transferred to culture dish and allowed to adhere at 37°C. Unattached cells were removed after 30 min. Microglia were isolated as strongly adhering cells. The enriched microglial cultures were 99% positive for OX-42 (Serotec' MCA275R) as assessed by immunostaining. Microglia were nonstimulated or stimulated by LPS (100 ng/mL) for 24 hours before coculture with BMMSC. After microglial activation, the cells were fixed for immunostaining and the medium was collected for NO release assay.

2.3. BMMSC and BMMSC-sf Culture. Bone marrow cells were isolated and prepared from femurs of young adult male Sprague-Dawley (SD) rats. Briefly, four-week-old SD rats were sacrificed by injection of sodium pentobarbital. Femurs were dissected from the attached musculature and connective tissues. Bone marrow cells were flushed out from femurs with phosphate buffered saline (PBS; GIBCO) and filtered through 70 μm pore sieve. The filtered cells were collected by centrifugation (326 ×g for 10 minutes), resuspended, and maintained as monolayer cultures in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Invitrogen, Carlsbad, CA, USA), supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO_2/95% air. Cells growing in such condition throughout 0–5 passages were designated as BMMSC. When BMMSC grew to 80% confluence at passage 0, cells were washed twice and switched to serum-free media containing DMEM/F12 supplemented with 2% B27 (Invitrogen), bFGF (20 ng/mL), and EGF (20 ng/mL). Numerous cells would suspend in serum-free medium and aggregate to form neurosphere-like mass within 7–10 days. The cellular mass was collected and replated to new culture flasks under the same condition for cell expansion, designated as BMMSC-sf. Both BMMSC and BMMSC-sf were expanded and subcultured at least for 5 passages. Phenotypic characterizations of cultures were examined by immunostaining and by flow cytometric analysis against cell surface markers. Furthermore, both cultures were processed for multilineage differentiation assays, including adipogenesis and neuronal differentiation.

2.4. Coculture Assay. Microglia were harvested from flasks and seeded 1×10^6 cells/well in 6-well plate in low glucose DMEM/F12 medium containing 10% FBS and 1% penicillin/streptomycin in the presence of absence of 100 ng/mL LPS. Approximate 3×10^5 cells/well of BMMSC or BMMSC-sf were seeded to the transwell inserts (1 μm Millicell PET membrane, Millipore) for 24 hours. The transwell inserts were then replanted on top of microglia and further cocultured for 6 or 24 hours. Conditioned media of cultures or cocultures were then collected for the analysis of ELISA, protein array, and western blot. Furthermore, the microglia after coculture with BMMSC or treatment were harvested for total RNA extraction and processed for Q-PCR analysis.

2.5. Flow Cytometric Analysis of Expressed Antigens on Cell Surface. The specific surface markers of isolated and expanded cells were detected at passages 0 to 5 by flow cytometric analysis. Briefly, BMMSC and BMMSC-sf were harvested by treatment of 5 mM EDTA in PBS solution. The cells were stained for 1 hour on ice with fluorescein isothiocyanate- (FITC-) or phycoerythrin- (PE-) conjugated antibodies for cell surface markers, including CD34 (hematopoietic lineage early marker), CD90 (Thy-1), CD44, CD54, and CD29 (integrins) (BD bioscience). The stained cells are analyzed by fluorescence-activated cell sorter (FACS Calibur flow cytometer; BD bioscience) using a 525 nm bandpass filter for green FITC fluorescence and a 575 nm bandpass filter for red PE fluorescence.

2.6. Cell Differentiation. A commercial kit (Mesenchymal Stem cell Adipogenesis kit, Chemicon SCR020) was employed for *adipocyte differentiation* of our cultured BMMSC and BMMSC-sf. Following the instructions of the kit, cultures were grown in adipogenic induction medium for 72 hrs and then replaced with adipogenic maintenance

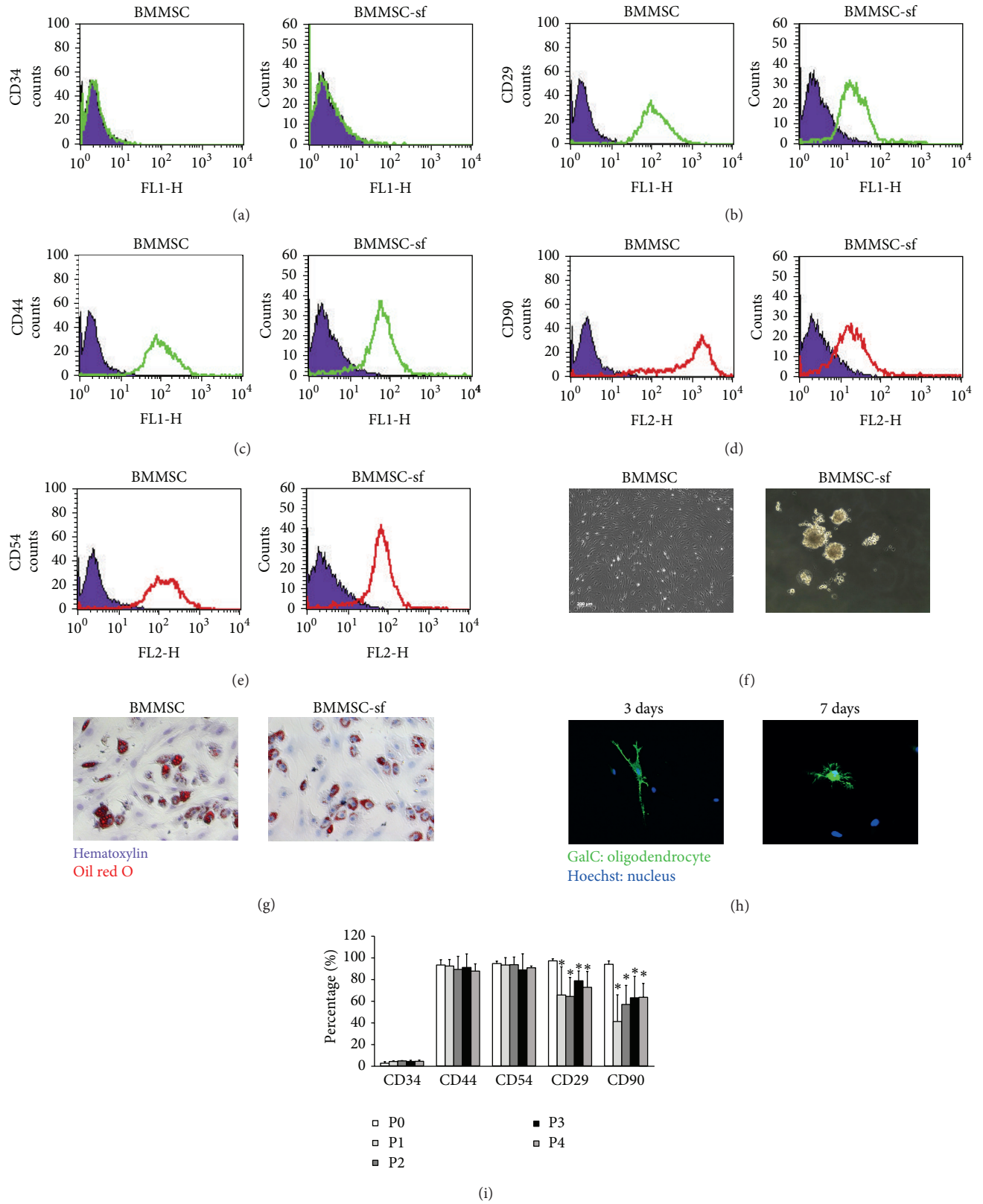

Figure 1: Characterization and flow cytometry of bone marrow mesenchymal stem cells (BMMSC) during expansion in serum-free (sf) or serum-containing medium. ((a)–(e)) Comparative flow cytometry analysis of cell surface markers in BMMSC or BMMSC-sf. (f) Phase-contrast micrograph of BMMSC and BMMSC-sf. (g) Adipocyte differentiation of BMMSC and BMMSC-sf. (h) Oligodendroglial (GalC-immunoreactive) differentiation of BMMSC for 3 or 7 days. (i) Cytometric analysis of cell surface markers in BMMSC-sf of the first four passages (P). $^{*}P < 0.05$ compared with P0.

medium for 24 hrs. The replacement of the media was repeated for three times. Finally, the cells were cultured for one additional week with adipogenic maintenance medium. The adipocytes were identified by staining with oil red solution for oil drops and counterstaining with hematoxylin solution. For *neural differentiation,* BMMSC and BMMSC-sf were harvested from cultured flasks. Equal cell numbers of BMMSC and BMMSC-sf were seeded onto poly-L-lysine-coated 24-well plate in DMEM/F-12-based differentiation medium containing 2 mM L-glutamine, 20 ng/mL bFGF, 30 ng/mL BDNF, and 5% FBS. The medium was refilled every 3 days. Cells were fixed at days 3, 7, and 14 of differentiation for immunocytochemistry.

2.7. Immunocytochemistry Analysis. Cultured cells were fixed with 4% paraformaldehyde for 30 min. Cells were further permeabilized with 0.1% Triton X-100, blocked with 1% bovine serum albumin, and immunostained with primary antibodies and with the respective fluorescently tagged secondary antibodies (Jackson ImmunoResearch Inc.). Primary antibodies included mouse anti-GalC (Chemicon, USA), mouse anti-ED-1 (1 : 200), mouse anti-inducible nitric oxide synthase (iNOS) (BD Bioscience, USA), and rabbit anti-βIII tubulin (Covance, USA). Images of cultured cells were obtained with a fluorescent microscope equipped with fluorescence optics and with a CCD camera. Micrography was performed using a 10X and 20X objective, and images were processed with imaging software (MetaMorph Imaging System, Universal Imaging Corp, Downingtown, PA, USA).

2.8. Biochemical Assays. The production of nitric oxide (NO) was assayed as the accumulation of nitrite in the medium using colorimetric reaction with Griess reagents (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/2% phosphoric acid) as described by Tsai et al. [14]. After LPS treatment, the culture medium was collected, mixed with Griess reagents, and incubated at room temperature for 10 min. The absorbance of the resultant products was measured at 540 nm. Sodium nitrite ($NaNO_2$) was used as the standard to calculate nitrogen dioxide (NO_2) concentrations. A rat protein cytokine kit (R&D, ARY008) was used to screen the expression of 29 rat cytokines in the released fractions (media) of coculture. The levels of cytokine expression were determined by the intensity of immunoreactivity, relative to that of the standard controls, following the manufacturer's instructions. TIMP-1 level in the coculture medium was further identified by using a TIMP-1 ELISA Kit (RayBio ELR-TIMP1-001). The level of TIMP-1 expression was determined by the intensity of optical density 450 nm, following the manufacturer's instructions.

2.9. Real-Time PCR. Total RNA was extracted using TRIzol kit (Invitrogen). RNA was reverse transcribed in a final volume of 20 ul using 0.5 ug of oligo-dT and 200 U Superscript III RT (Invitrogen) for 30 minutes at 50°C, followed by 2 minutes at 94°C to inactivate the reverse transcriptase. Polymerase chain reaction (PCR) amplification of the resulting cDNAs was performed under the following conditions: 35 cycles of 94°C for 30 seconds, 58°C for 45 seconds, and 68°C for 45 seconds, in which the 68°C step was increased by 5 seconds every cycle after 10 cycles. For real-time PCR, the amplification was carried out in a total volume of 10 ul containing 0.5 uM of each primer, 4 mM $MgCl_2$, 1 ul of LightCycler FastStart DNA Master SYBR green I (Roche Molecular Systems), and 5 ul of 1 : 20-diluted cDNA. The primers and sequences were iNOS (forward: AAG, AGA, CGC, ACA, GGC, AGA, G; reverse: CAG, GCA, CAC, GCA, ATG, ATG, G), IL-1β (forward: TCA, AAT, CTC, ACA, GCA, GCA, TCT, CG; reverse: ACA, CTA, GCA, GGT, CGT, CAT, CAT, CC), TNF-α (forward: GCC, GAT, TTG, CCA, CTT, CAT, AC; reverse: GGA, CTC, CGT, GAT, GTC, TAA, GTA, C), Arg-1 (forward: TTG, ATG, TTG, ATG, GAC, TGG, AC; reverse: TCT, CTG, GCT, TAT, GAT, TAC, CTC, C), and IL-4 (forward: CGT, CAC, TGA, CTG, TAG, AGA, GC; reverse: GGG, CTG, TCG, TTA, CAT, CCG), IL-10 (forward: CAC, TGC, TAT, GTT, GCC, TGC, TCT, TAC; reverse: GGG, TCT, GGC, TGA, CTG, GGA, AG), MMP-9 (forward: TGT, ATG, GTC, GTG, GCT, CTA, AAC; reverse: AAG, GAT, TGT, CTA, CTG, GAG, TCG), and RPL-13 (forward: AGG, TGG, TGG, TTG, TAC, GCT, GTG; reverse: GGT, TGG, TGT, TCA, TCC, GCT, TTC, G). PCR reactions were prepared in duplicate and heated to 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 1 minute and extending at 72°C for 20 seconds. Standard curves (cycle threshold values versus template concentration) were prepared for each target gene and for the endogenous reference (ribosomal protein L13A (RPL13)) in each sample. The quantification of the unknown samples was performed using the $_{\Delta\Delta}$Ct converting formula.

2.10. Western Blot Analysis. The following antibodies were used for western blot analysis: goat anti-TIMP-1 (1 : 1000; Santa Cruz SC-6832) and rabbit anti-VEGF (1 : 1000; Abcam). The membranes were blocked with 5% nonfat milk in PBS-T for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C. The membranes were then processed with HRP-conjugated secondary antibodies. Immunoreactive bands were visualized using chemiluminescence ECL western blotting detection reagents (Amersham, Piscataway, NJ, USA). Experiments are performed in duplicate to ensure reproducibility. Ponceau-S staining was used for internal control.

2.11. Statistical Analysis. Experimental data were expressed as the mean of independent values ± SEM and were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's t-test. Values of $P < 0.05$ were considered to show statistical significance.

3. Results

Cultured BMMSC and BMMSC-sf, expanded in serum-containing and serum-free conditions, respectively, were first compared and characterized by flow cytometry. Figures 1(a), 1(b), 1(c), 1(d), and 1(e) showed that both cells were >90% immunoreactive to CD29, CD44, CD90, and CD54, major

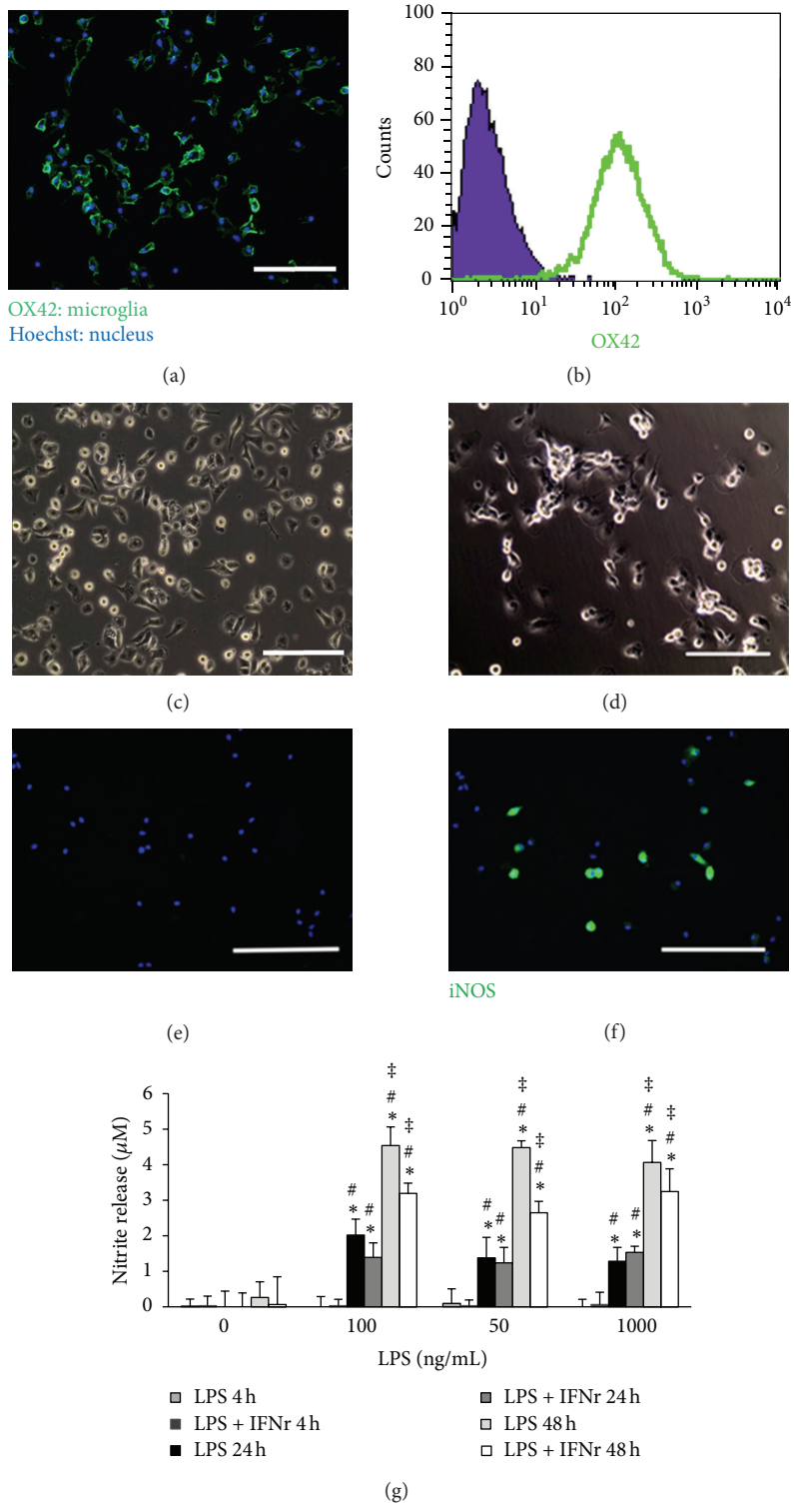

FIGURE 2: Identification of nonstimulated and LPS-stimulated microglia. (a) OX42-IR microglia counterstained with Hoechst 33258; (b) >98% cells were OX42-IR microglia analyzed by flow cytometry; (c) and (e) nonstimulated cultures and (d) and (f) LPS-stimulated cultures; (f) denotes iNOS-IR cells; (g) nitrite release to medium in LPS- or combined LPS and IFNγ-treated microglial cultures. LPS doses were ranging from 100 ng/mL to 1 μg/mL. Aliquot of media was collected at 4, 24, or 48 hrs after treatment. Data were means ± SEM, $^{*}P < 0.05$ compared with 0 ng/mL LPS (control) group. $^{\#}P < 0.05$ compared with 4 hrs treatment. $^{\ddagger}P < 0.05$ compared with 24 hrs treatment.

surface markers specific for BMMSC. By contrast, both cultures presented lower immunoreactivity (IR) to CD34, a surface marker for early hematopoietic stem cells. Phase-contrast microscope of cultured BMMSC and BMMSC-sf was shown in Figure 1(f). BMMSC exhibited healthy and fibroblast-like morphology, consistent with previous findings of BMMSC characteristics. By contrast, sphere formation could be observed in BMMSC-sf. These neurosphere-like structures were expanded for an additional 2–10 weeks (2–5 passages). Immunoreactivity to CD44, CD54, and CD34 (low) in BMMSC-sf was kept constant (>98%) regardless of cell passages (Figure 1(i)). CD29- and CD90-IR were decreased with later passages. Figure 1(g) showed that both cultures showed potentials to differentiate oil-drops-containing adipocytes. Red color denoted oil drops in the cells. No significant difference of adipocyte differentiation was detected in both BMMSC and BMMSC-sf. By contrast, the differentiation of GalC-positive cells could be detected only in cultured BMMSC at day 3 and day 7 but not in cultured BMMSC-sf (Figure 1(h)).

Microglial cultures were purified from mixed glial cultures which were prepared from neonatal rat brains. Characterization of resting and activated microglia was determined by both immunocytochemistry and flow cytometry. Almost all cells were OX42-IR microglia (Figure 2(a)). Flow cytometric results further demonstrated that these enriched cultures were >98% OX42-IR microglia (Figure 2(b)). Figures 2(c), 2(d), 2(e), and 2(f) showed the phase-contrast or iNOS-IR micrographs of nonstimulated (resting) or endotoxin LPS-stimulated microglia. Resting microglia did not express iNOS-IR (Figure 2). At 4 hours after LPS treatment, iNOS or nitrite was not detected in all treated groups. Not until 24 hours after LPS (or plus interferon γ) treatment, microglia were induced to express iNOS and release NO (in a form of nitrite) to cultured medium (Figures 2(f) and 2(g)). The nitrite levels in 100 ng/mL LPS and 100 ng/mL LPS-combined IFN-γ groups were $2.01 \pm 0.44\,\mu$M and $1.39 \pm 0.4\,\mu$M, respectively. Levels of NO release in 48 hours treatment groups were almost 2-fold higher than those of 24 hours treatment. However, similar levels of NO release were observed across LPS dosages (100 ng-1 μg/mL). Furthermore, combined treatment of LPS with IFN-γ was not as powerful as LPS alone to activate microglia. Therefore, a dose of LPS 100 ng/mL and treatment for 24 hours were mainly employed for following microglia-BMMSC coculture studies.

The transwell culture system was utilized for indirect coculture of microglia and BMMSC. Microglia were seeded in transwell and pretreated with vehicle or 100 ng/mL LPS for 24 hours before being cocultured with BMMSC or BMMSC-sf. After further incubation for 24 hours in the presence or absence of LPS, microglial cells were harvested and processed for real-time PCR analysis for immune-related cytokines and proteins. As shown in Figure 3, the quantitative expression levels of iNOS, IL1β, TNFα, IL-10, and MMP-9 were induced in LPS-treated microglia or LPS-treated cocultures of microglia with BMMSC or with BMMSC-sf at 6 hours or 24 hours after treatment. By contrast, the expression levels of arginase I, IL-4, and MMP-2 did not change among treatments. iNOS was upregulated in LPS-stimulated coculture at 24 hours. The levels of IL-1β were upregulated in all cultures by LPS stimulation at both 6 and 24 hours. Interestingly, the expression levels of IL-1β, TNF-α, and IL-10 tended to be higher at 6 hrs than at 24 hours after LPS stimulation (Figures 3(c), 3(e), and 3(f)). BMMSC cocultured with microglia for 6 hours reduced LPS-stimulated IL-10 level. MMP-9 levels were induced by LPS in all cultures. On BMMSC coculture with microglia for 24 hours, MMP-9 level was significantly attenuated (Figure 3(h)).

To evaluate the soluble factors released by microglia, BMMSC, or coculture, the media were harvested for cytokine array, western blot, and ELISA assays. Figure 4(a) showed the array identification of 29 rat cytokines released to conditioned media by microglia, BMMSC, and cocultures. All spots were quantified by Image J densitometry software (Version 1.6, National Institutes of Health, Bethesda, MD). Interestingly, spots of TIMP-1 (tissue inhibitor of metalloproteinase-1; red square) and VEGF (vascular endothelial growth factor; green square) were found to change among treatments. Histogram in Figure 4(b) showed the fold changes of TIMP-1 and VEGF among treatments. Levels of TIMP-1 and VEGF release in the 24 hr conditioned media were further identified by western blot analysis. The Ponceau-S staining (red) of IgG was used as an internal control. Positive bands, corresponding to TIMP-1 and VEGF, were observed in the conditioned media of BMMSC and in microglia-BMMSC cocultures, but not in the media of BMMSC-sf or its coculture. Because TIMP-1 levels in the conditioned medium were not consistent between array and western blot analysis, we employed ELISA assay for it. Histogram in Figure 4(d) shows the amounts, in μg/mL, of the TIMP-1 release, which was consistent with the results of western blot analysis. Therefore, TIMP-1 expression in the MLBsf group was low, not detected, or below detecting level.

4. Discussion

The central aim of the present study is to examine the effectiveness of bone marrow mesenchymal stem cells on microglial activation in a culture platform. BMMSC expanded in serum-containing and in serum-free medium were characterized and compared. BMMSC-sf were cultured in neurosphere-like structures according to methods similar to those propagation of neural stem cells [15, 16]. The phenotypes of these cells were similar to mesenchymal stem cells. Because BMMSC-sf exhibited sphere-like morphology, two adherent cell surface markers, CD29 and CD90, were reduced in BMMSC-sf when compared with BMMSC. Both cells maintained the capacity of mesodermal characteristics and multilineage differentiation. However, BMMSC differentiated faster into neural-like cells, such as GalC-positive cells.

The modulation of BMMSC on resting or activated microglia was revealed using a transwell coculture system that permits cell-contact-independent communication through diffusible soluble factors. Cellular gene expression of microglia and the released factors of cocultures were analyzed. BMMSC or BMMSC-sf cocultured with microglia did not alter the mRNA expression of iNOS, Arginase-1, IL-1β,

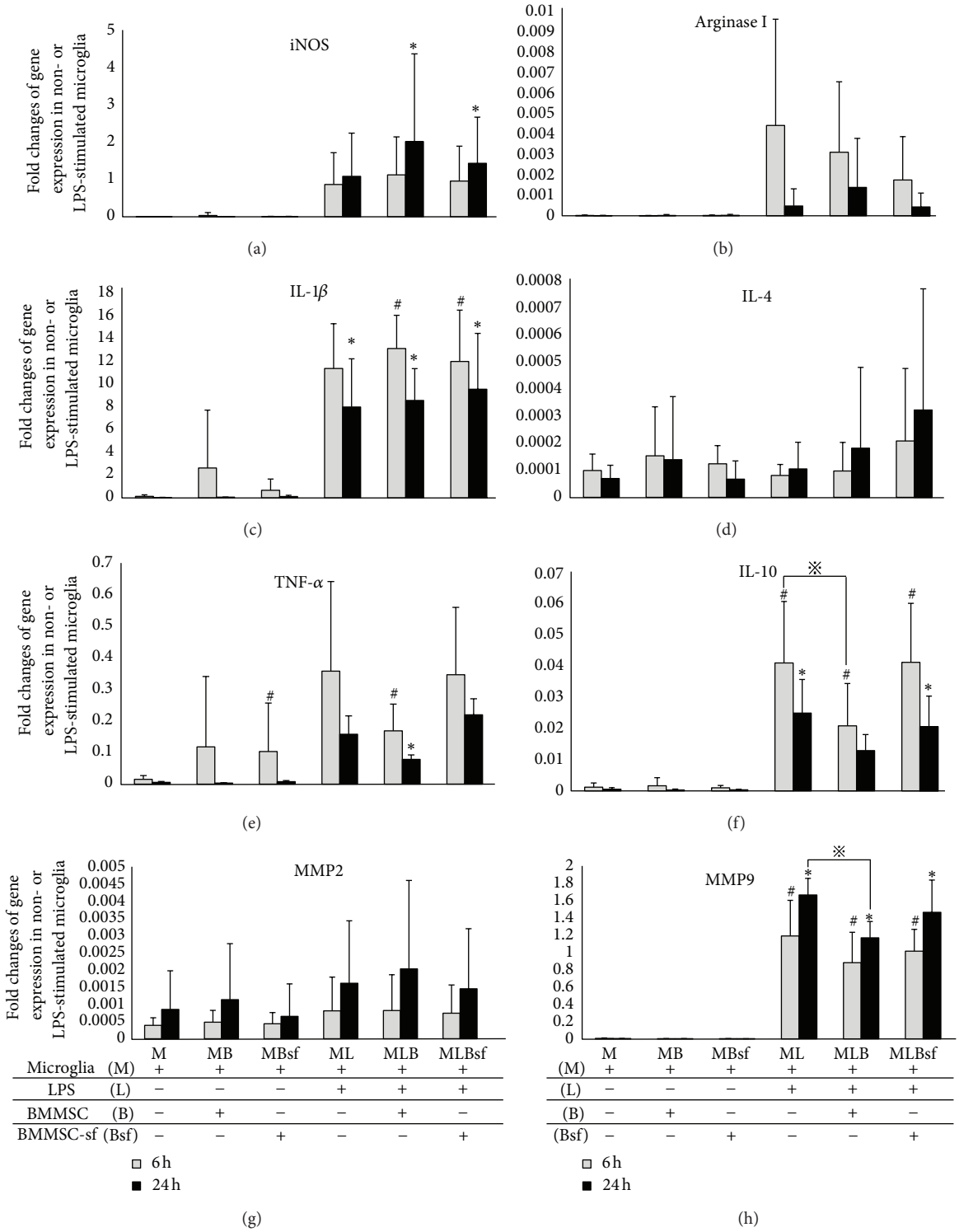

Figure 3: Quantitative changes of protein or cytokine expression in microglia cocultured with BMMSC or BMMSC-sf. Q-PCR results revealed that the expression of iNOS (a), IL1β (c), TNFα (e), IL-10 (f), and MMP-9 (h) was induced in LPS- (L-) treated microglia (M) or LPS-treated cocultures of microglia with BMMSC (b) or with BMMSC-sf (Bsf). By contrast, levels of arginase I, IL-4, and MMP-2 did not change among treatments. (a) iNOS was upregulated in LPS-stimulated MB or MBsf coculture for 24 hrs. The levels of IL-1β in (c) were upregulated by LPS stimulation at both 6 and 24 hrs. Higher expression levels of TNF-α in (e) and IL-10 in (f) were found at 6 hrs than at 24 hrs after LPS stimulation. BMMSC cocultured with microglia for 6 hr reduced LPS-stimulated IL-10 level. (h) MMP-9 levels were induced by LPS in all cultures. BMMSC cocultured with microglia for 24 hrs reduced LPS-stimulated upregulation. y-axis in (a)–(h) was the fold changes of gene expression in non- or LPS-stimulated microglia. Determinations are means ± SEM from RT-QPCR experiments. $^{\#}P < 0.05$ LPS LPS stimulation versus no LPS stimulation at 6 hrs treatment. $^{*}P < 0.05$ LPS stimulation versus no LPS stimulation at 24 hrs.

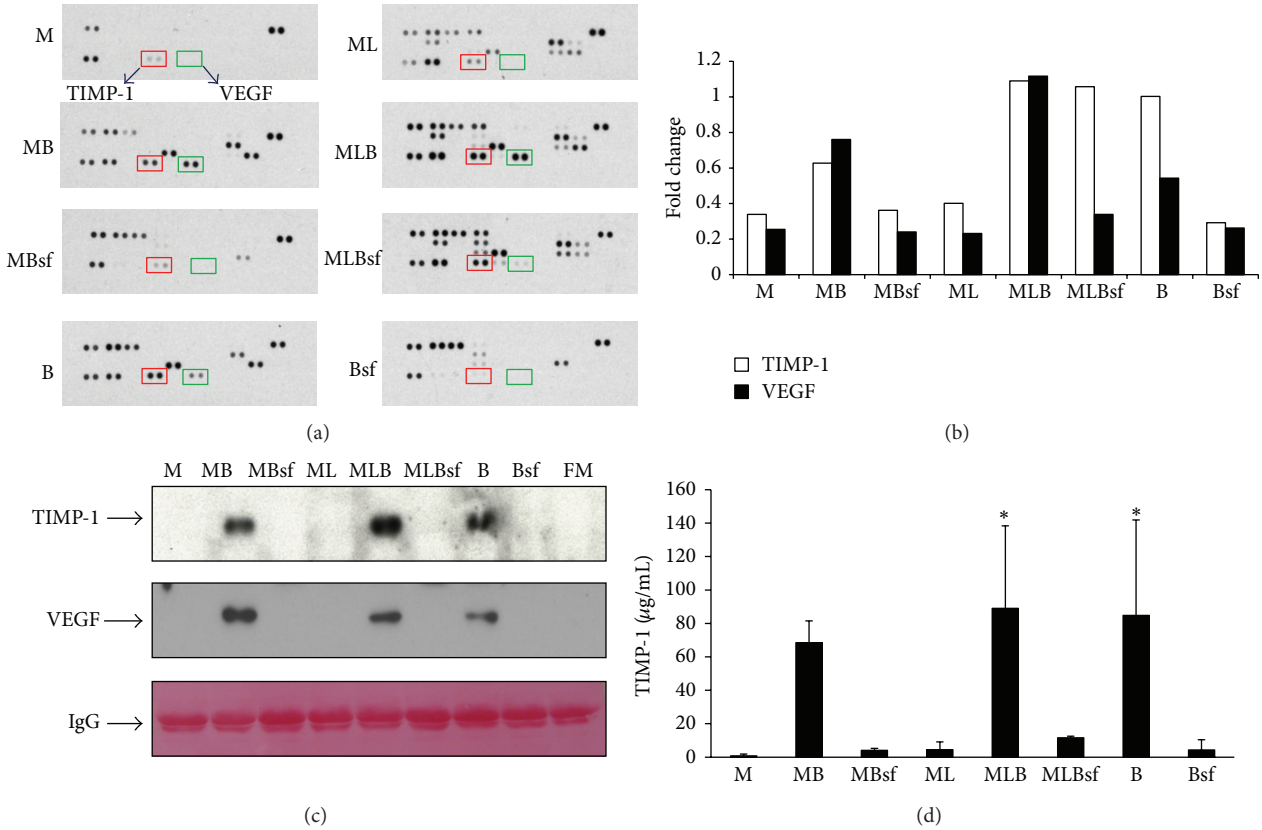

Figure 4: Identification of soluble factors released to conditioned media by microglia, BMMSC, and cocultures. (a) Expressions of 29 rat cytokines in the conditioned media. Spots of TIMP-1 (red square) and VEGF (green square) were found to change among treatments. (b) Histogram showing the fold changes of TIMP-1 and VEGF among treatments. (c) Western blot analysis of TIMP-1 and VEGF released in the 24 hrs conditioned medium of all cultures. The Ponceau-S staining (red) of IgG was used as an internal control. (d) Histogram showing the amounts, in μg/mL, of the TIMP-1 as quantified by ELISA. Determinations were means ± SEM from coculture media subjected to ELISA experiments. $^{*}P < 0.05$, compared to M group.

IL-4, TNF-α, and MMP-2. However, the mRNA expression levels of IL-10 and MMP-9 in microglia were downregulated after being cocultured with BMMSC. The reduction of MMP-9 mRNA after cocultures might be due to TIMP-1 secretion from BMMSC.

TIMP-1 was present in the conditioned medium of BMMSC or BMMSC-microglia cocultures (Figure 4), analyzed by cytokine array and western blot. ELISA assay of TIMP-1 in the media further showed significant higher TIMP concentration in MB, MLB, and B groups than others.

Microglia were activated by LPS, a cell wall component of gram-negative bacteria and a powerful immune challenge associated with an increase of numerous cytokines. The LPS-induced generation of free radicals in microglia was an upstream event serving to regulate the production of other proinflammatory factors [17, 18]. The anti-inflammatory role of grafted BMMSC as a protective mechanism has been shown in *in vitro* and *in vivo* studies [19, 20]. Although the mechanism of the inhibitory effect of BMMSC on microglial activation was not clear, evidence suggests that microglial activation can be modulated by various cytokines and neurotrophic factors [7, 21]. In the present study, BMMSC decreased mRNA expressions of IL-10 and MMP-9 from microglia stimulated by LPS in a contact-independent manner. Furthermore, significant levels of TIMP-1 and VEGF were released from BMMSC, regardless of being in coculture or not. Therefore, it was speculated that soluble factors released from BMMSC might regulate the microglia response to LPS in our experiment. Reducing microglial activation by BMMSC would lend to neuronal protection against LPS stimulating cascade.

MMP-9 belonged to the family of extracellular calcium- and zinc-dependent proteinase that degraded the extracellular matrix and other extracellular proteins [22]. MMP-9 expression had been shown in the injured spinal cord [23], and BMMSC could reduce the MMP-9 expression in injured tissues [24]. Ample evidence shows that TIMPs regulate the activities of MMPs through protein-protein interaction. Both TIMPs and MMPs had the dynamic balance in activity [25]. Among at least four TIMP subgroups, TIMP-1 exhibits higher affinity for MMP-9 and thus effectively inhibits MMP activity. Consistent with this, the present results show that BMMSC released TIMP-1, thus reducing MMP-9 expression in microglia of coculture.

BMMSC have the abilities of self-renewing and differentiation into different types of cells. These properties make

BMMSC suitable for tissue regeneration and cell therapy. In this study, we employed serum-containing and serum-free cultivation to expand adult bone marrow stem cells *in vitro*. Both cells expressed major surface markers specific for mesenchymal stem cells. In the beginning, we used fetal bovine serum for initial cell seeding and then switched to serum-free condition for BMMSC-sf expansion. This method was convenient to generate BMMSC-sf for further study. To avoid contamination of animal serum for BMMSCsf cultivation, patients' own serum might be applicable. Thus, BMMSC-sf might be promising materials for cell therapy in clinic.

In conclusion, we provided a platform for an *in vitro* assay to characterize the effects of BMMSC on LPS-stimulated microglia. Two different cultivated BMMSC were employed throughout, that is, BMMSC and BMMSC-sf. The BMMSC-sf had sphere morphology, having reduced expression of two adherent cell markers, CD29 and CD90. BMMSC exhibited typical fibroblast-like structure. BMMSC had faster differentiation potential into neural-like cells. BMMSC released significant levels of TIMP-1 and VEGF. Furthermore, it reduced mRNA expression of IL-10 and MMP-9 in microglia. This downregulated MMP-9 mRNA might be caused by TIMP-1 secretion from BMMSC in cocultures. Our cell culture system provided a powerful tool for investigating the molecular and cellular changes in microglia-BMMSC cocultures.

Authors' Contribution

Y.-S. Fu, W.-C. Huang, and H. Cheng contributed equally to this work.

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Review Article

Pituitary Adenoma Nitroproteomics: Current Status and Perspectives

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Oxidative stress is extensively associated with tumorigenesis. A series of studies on stable tyrosine nitration as a marker of oxidative damage were performed in human pituitary and adenoma. This paper reviews published research on the mass spectrometry characteristics of nitropeptides and nitroproteomics of pituitary controls and adenomas. The methodology used for nitroproteomics, the current status of human pituitary nitroproteomics studies, and the future perspectives are reviewed. Enrichment of those low-abundance endogenous nitroproteins from human tissues or body fluid samples is the first important step for nitroproteomics studies. Mass spectrometry is the essential approach to determine the amino acid sequence and locate the nitrotyrosine sites. Bioinformatics analyses, including protein domain and motif analyses, are needed to locate the nitrotyrosine site within the corresponding protein domains/motifs. Systems biology techniques, including pathway analysis, are necessary to discover signaling pathway networks involving nitroproteins from the systematically global point of view. Future quantitative nitroproteomics will discover pituitary adenoma-specific nitroprotein(s). Structural biology techniques such as X-ray crystallography analysis will solidly clarify the effects of tyrosine nitration on structure and functions of a protein. Those studies will eventually address the mechanisms and biological functions of tyrosine nitration in pituitary tumorigenesis and will discover nitroprotein biomarkers for pituitary adenomas and targets for drug design for pituitary adenoma therapy.

1. Introduction

Nitration of a tyrosine residue (NO_2-Tyr-Prot) in a protein generated from the primary *in vivo* peroxynitrite pathway, as well as the secondary myeloperoxidase and other metalloperoxidase reaction pathways [1, 2], is a potential marker of oxidative/nitrosative injuries [3, 4] and might play important molecular roles in human pituitary physiology and pathology. The pituitary plays central regulation roles in the hypothalamic-pituitary-target organ axis systems. Studies have indicated that nitric oxide (NO) [3] and nitric oxide synthase (NOS), which are the upstream molecules that promote formation of tyrosine nitration, participate in multiple axis systems [5–7]: growth hormone (GH) [8–10], prolactin (PRL) [11], adrenocorticotropin (ACTH) [12], follicle-stimulating hormone (FSH) [13], and luteinizing hormone (LH) [7, 13–15]. A global proteomics approach was used to investigate protein tyrosine nitration in human pituitary and adenoma tissues, namely, nitroproteomics. A total of eight nitrotyrosine-containing proteins (nitroproteins) in a human pituitary postmortem tissue and nine nitroproteins and three nitroprotein-interacting proteins in a human nonfunctional adenoma tissue [3, 4, 16] were identified with tandem mass spectrometry (MS/MS). Nitrotyrosine sites located within the

important protein domains or motifs [4] were involved in the tumor biological characteristics [4].

The detection and identification of endogenous nitroproteins is very challenging because of its very low level (one nitroprotein per ~10^6 proteins) in a proteome [17, 18]. Anti-nitrotyrosine antibody-based enzyme-linked immunosorbent assay (ELISA) [2, 19], immunoprecipitation [4], and one/two-dimensional-gel-electrophoresis- (1DGE/2DGE-) based Western blot analyses [3, 16, 20, 21] are effective approaches to detect and preferentially enrich endogenous nitroproteins. ELISA can measure the nitrotyrosine content, and an ELISA assay kit is commercially available (Upstate Catalog no. 17-136). 1DGE/2DGE-based Western blots can separate and preferentially enrich endogenous nitroproteins and also determine the relative level of nitrotyrosine. Immunoprecipitation can preferentially enrich endogenous nitroproteins from a complex proteome for mass spectrometry analysis. Tandem mass spectrometry (MS/MS) can identify a nitrotyrosine site in a nitroprotein [3, 4, 16, 22]. However, the sensitivity (generally high femtomole to low picomole) of mass spectrometry is the bottleneck due to the low abundance of nitroproteins in a complex pituitary proteome. Thus, preferential enrichment of endogenous nitroproteins or nitropeptides is an essential step prior to MS/MS analysis [22]. For human pituitary nitroproteomics studies, 2DGE-based nitrotyrosine Western blot analysis [3, 16] and nitrotyrosine immunoaffinity enrichment [4] were used to separate and preferentially enrich endogenous nitroproteins from a complex human pituitary control and adenoma tissue. Enriched nitroproteins were subject to trypsin digestion, followed by the amino acid sequence analysis with MS/MS to identify nitroprotein and nitrotyrosine sites. Bioinformatics was used to determine structural/functional domains and motifs of a nitroprotein and to locate the nitrotyrosine site within a protein domain/motif to clarify the roles of tyrosine nitration in a protein [4]. Pathway analysis-based systems biology was used to discover the pathway networks that involved endogenous nitroproteins from a systematical and comprehensive angle [23]. In addition, mass spectrometry characteristics of the standard nitropeptides [24] were analyzed to obtain the fragmentation to assist in the interpretation of the mass spectrometry spectrum of a tryptic peptide derived from an endogenous nitroprotein in a proteome.

2. Biological Roles of Oxidative/Nitrative Stresses in Pituitary Adenoma Pathophysiology

Reactive-oxygen/nitrogen-species- (ROS/RNS-) mediated oxidative/nitrative stresses play important roles in cellular, physiological and pathological processes [24–27]. ROS are formed by several mechanisms [1, 25, 28], including (i) synthesis through dedicated enzymes such as NADPH oxidase and myeloperoxidase, (ii) interaction of ionizing radiation with biological molecules, and (iii) an unavoidable byproduct of cellular respiration. Electrons from the electron transport chain leak away from the main path such as ubiquinone to reduce oxygen molecules to the superoxide anion. RNS are a family of antimicrobial molecules generated from the nitric oxide radical ($^{\bullet}NO$) and superoxide anion ($O_2^{-\bullet}$) produced through the enzymatic activity of inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS) neuronal nitric oxide synthase (nNOS), and NADPH oxidase, respectively [3, 4, 22, 29]. iNOS is expressed primarily in many cell types such as macrophages after induction by cytokines and microbial products, notably interferon-gamma (IFN-γ) and lipopolysaccharide (LPS), or during pathology and disease [28, 30]. RNS act together with ROS to damage cells to cause nitrosative stress. Thus, these two species are often collectively called as ROS/RNS. RNS are produced in animals from the reaction of nitric oxide ($^{\bullet}NO$) and superoxide anion ($O_2^{-\bullet}$) to form the more toxic peroxynitrite ($ONOO^-$) [17, 31].

Specific amino acid residues in a protein are sensitive targets that can be modified by ROS/RNS [24]. Tyrosine nitration in a protein is an important redox-related modification that derives from, not only the main *in vivo* peroxynitrite pathway, but also myeloperoxidase and other metalloperoxidase reaction pathways [1, 2, 22]. Protein tyrosine nitration involves addition of an electron-withdrawing group, $-NO_2$, to the phenolic ring of the tyrosine residue [4] to decrease the electron density of the phenolic ring of a tyrosine residue in a protein to change the phenolic pKa value (from ~10 for tyrosine) into the physiological pH range (~7.1 for 3-nitrotyrosine) to affect chemical properties of a tyrosine residue [4, 32, 33]. If the nitration occurred within the interacting region between an enzyme and its substrate and/or between a receptor and its ligand, then the decreased electron density could impact on the interaction intensity (enzyme-substrate, receptor-ligand) to affect the functions of that protein [4]. Furthermore, some studies demonstrated that biological protein nitration might be a dynamic and reversible process between nitration and denitration due to a discovery of a putative denitrase [33–35]. Thus, protein tyrosine nitration might have biological consequences such as redox signaling and neurotransmission in addition to its pathological consequences. Also, tyrosine nitration would compete with phosphorylation of a tyrosine residue because tyrosine nitration occurs within a tyrosine phosphorylation motif ([R or K]-x2(3)-[D or E]-x3(2)-[Y]) [36–38]. Therefore, protein tyrosine nitration occurs under physiological conditions, is enhanced under pathological conditions, and might be reversed by enzymatic or nonenzymatic mechanisms [22]. This modification can alter the functions of a protein and is associated with many physiological/pathological processes such as inflammatory diseases, neurodegenerative diseases, and tumors [3, 4, 17, 21, 27, 31].

ROS/RNS are involved in multiple hypothalamic-pituitary-target organ axis systems and are elevated in pituitary tumors [3, 4, 16, 22]. NOS is extensively expressed in the rat and human pituitary, and has an elevated activity in pituitary adenomas [3, 5–7, 39, 40]. Three types of NOS (eNOS, nNOS, and iNOS) are expressed in the pituitary gland and in pituitary adenomas, and an elevated activity of eNOS was found in the endothelial cells of pituitary adenomas [3]. nNOS and its mRNA were found to be increasing in human pituitary adenomas, and were located to

the secretory and folliculostellate cells [3]. iNOS was found in rat pituitary cells that were induced by interferon-gamma (IFN-γ) that significantly increased NO production [41]. NO activates release of luteinizing hormone-releasing hormone (LHRH) and follicle-stimulating hormone-releasing hormone (FSHRH) from the hypothalamus and of LH and FSH from the pituitary [3, 4, 13–16, 42]. NO either participates in LH secretion in gonadotrophs or requires the participation of gonadotrophs [7]. NO might stimulate or inhibit secretion of prolactin; circulating NO changed in dopamine-treated hyperprolactinaemia patients [3, 11, 43–45]. NO regulates section of growth hormone (GH) in the normal human pituitary and in acromegaly and modulates GH secretion in a dose-dependent manner in GH adenoma cells [3, 5, 46–48]. NO plays important roles in the hypothalamic-pituitary-adrenocortical axis inhibition of ACTH release [12]. Therefore, upstream molecules (NO and NOS) that form tyrosine nitration in a protein are extensively associated with physiological and pathological processes of pituitary and are especially elevated in the pituitary adenomas [3, 4, 16]. Our global nitroproteomics data confirmed that protein tyrosine nitration existed in human pituitary postmortem tissues [3, 16] and nonfunctional pituitary adenoma tissues [4]. Those nitroproteins played important roles in the physiological and pathological processes of a human pituitary [3, 4, 16]. Therefore, ROS/RNS might be important in normal human pituitary function and relevant to dysfunction in human pituitary adenomas.

3. Mass Spectrometric Characteristics of Nitropeptide

The mass spectrometry behavior of a nitrotyrosine-containing peptide (nitropeptide) greatly differs between matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) [24, 49, 50]. The MALDI UV laser can induce photochemical decompositions of the nitro group ($-NO_2$) to decrease the precursor-ion intensity of a nitropeptide to complicate the MS spectrum [24, 49–51]. ESI does not induce those decompositions [24, 27, 49–53]. In order to assist in the interpretation of MS identification of endogenous nitroproteins in human tissues and fluids, MALDI MS and MS/MS were used to study the fragmentation pattern of *in vitro* nitrotyrosine-containing peptides [24], including synthetic leucine enkephalin (LE1: Y-G-G-F-L, 555.1818 Da), nitro-Tyr-leucine enkephalin [LE2: (3-NO_2)Y-G-G-F-L, 600.0909 Da], and d5-Phe-nitro-Tyr-leucine enkephalin [LE3: (3-NO_2)Y-G-G-(d5)F-L, 605.1818 Da], with a vacuum MALDI-linear ion-trap mass spectrometer (vMALDI-LTQ).

The UV laser-induced photochemical decomposition (loss of one or two oxygen atoms of the nitro group to form the unique decomposition pattern of ions ($[M + H]^+ - 16$ and $[M + H]^+ - 32$) occurred in LE2 and LE3 compared to LE1 (Figure 1) [24]. A similar decomposition pattern ($[M + H]^+ + Na - 16$ and $[M + H]^+ + Na - 32$) for loss of one or two oxygen atoms occurred for the sodium adduct ($[M + H]^+ + Na$) of LE2 and LE3 compared to the sodium adduct of LE1 [24]. A product ion ($[M + H]^+ - 30$) was also observed in the LE2 and LE3 spectra, which could result from the reduction of the nitro group ($-NO_2$) to an amino group ($-NH_2$) [50]. Moreover, the base-peak intensity of the $[M + H]^+$ ion of LE1 (NL = 1.01E5) was much higher than that of LE2 (NL = 3.25E4) and LE3 (NL = 9.09E4), demonstrating that photochemical decomposition decreased ion intensity and complicated the MS spectrum [24]. However, recognition of this unique decomposition pattern unambiguously identified a nitrotyrosine. For vMALDI-MS/MS analysis, b- and a-ions were the most intense fragment ions compared to the y-ions (Figure 2) [24]. Compared to the unmodified peptides (LE1), more collision energy optimized fragmentation of the nitropeptide (Figure 3(a)) but increased the intensity of the a_4-ion and decreased the intensity of the b_4-ion (a-ion = the loss of CO from a b-ion) (Figure 3(b)). Furthermore, optimized laser fluence maximized fragmentation of the nitropeptide. Although MS^3 analysis confirmed the MS^2-derived amino acid sequence, MS^3 analysis requires a higher amount of peptides relative to MS^2 [24]. Thus, MS^3 analysis might not be suitable for routine analysis of endogenous low-abundance nitroproteins. Only when a target is determined, can MS^3 be used for confirmation. To detect a nitropeptide, the amount of peptides must reach the sensitivity of a mass spectrometer; for our synthetic nitropeptides, the sensitivity of vMALDI-LTQ was 1 fmol for MS detection and 10 fmol for MS^2 detection [24].

4. Enrichment of Endogenous Nitroproteins in Human Pituitary Adenomas

Nitrotyrosine formed from reaction of free or protein-bound tyrosine with RNS, such as free-radical nitrogen dioxide [54] and peroxynitrite [55], has a low-abundance (1 in $\sim 10^6$ tyrosines) oxidative protein modification in an *in vivo* proteome [17, 18]. Moreover, mass spectrometry is the crucial approach to identify nitroproteins/nitropeptides and modified sites [3, 4, 16]; however, mass spectrometry is limited by its sensitivity, generally at the levels of high femtomole to low picomole [22]. Therefore, isolation and preferential enrichment of the nitroproteins/nitropeptides are essential prior to mass spectrometry analysis [22, 27, 52, 53]. For human pituitary adenoma nitroproteomics studies, two methods were used to isolate and preferentially enrich nitroproteins from a pituitary proteome prior to mass spectrometry, including 2DGE-based nitrotyrosine Western blotting analysis [3, 16] and nitrotyrosine-affinity-column-(NTAC-) based enrichment [4].

2DGE-based nitrotyrosine Western blotting [3, 16] involved proteins extracted from a postmortem control pituitary separated by isoelectric point (pI) and relative molecular weight (Mr); separated proteins transferred to a polyvinylidene fluoride (PVDF) membrane; incubation with antinitrotyrosine antibody; and visualization (Figure 4). In the silver-stained 2D gel image (pI 3–10; Mr 10–100 kDa) that contained ca. 1000 protein spots (Figure 4(a)), a total of 32 nitrotyrosine-positive Western blot spots were detected

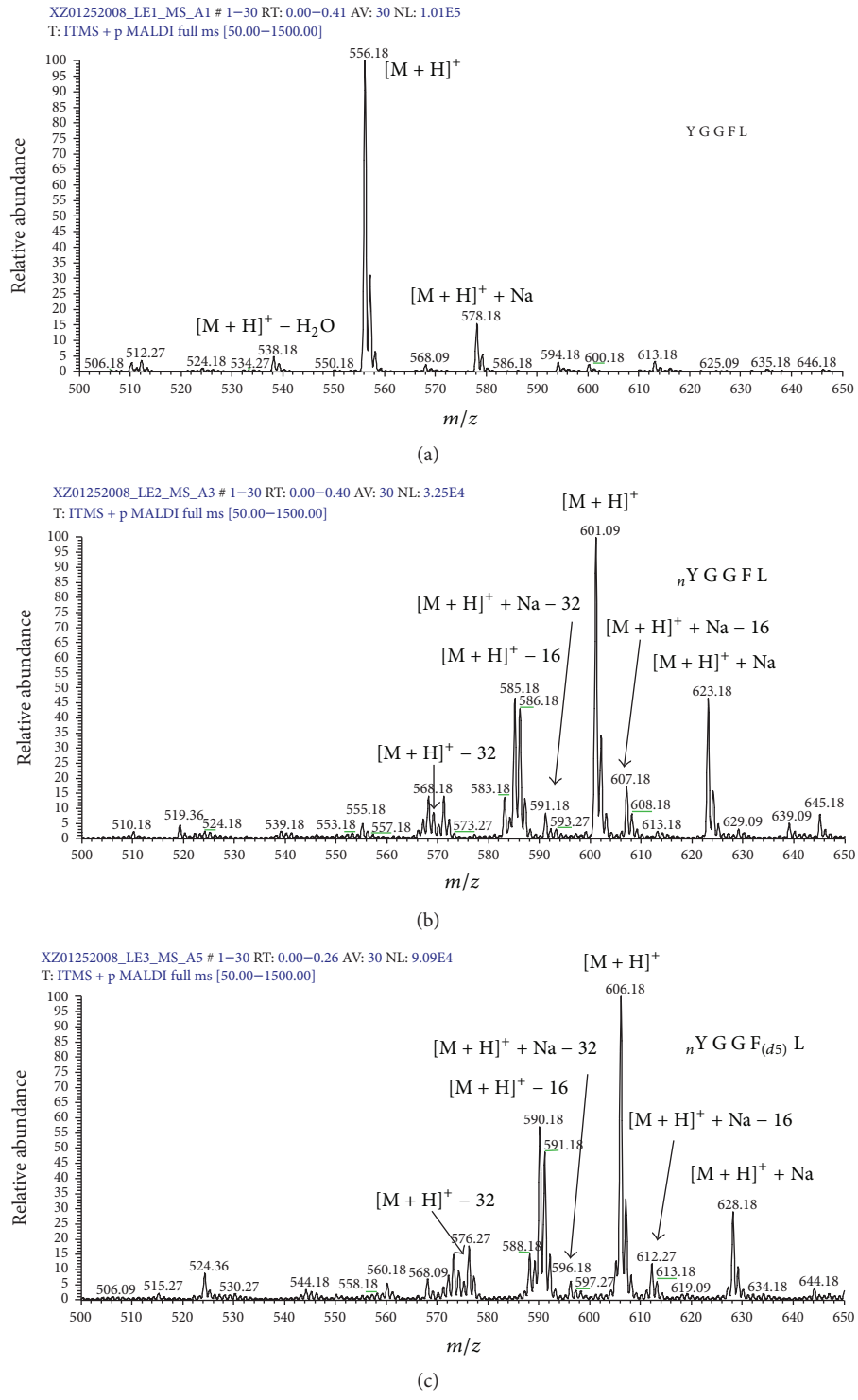

Figure 1: MALDI MS spectra of LE1 (a), LE2 (b), and LE3 (c). nY = nitro-Tyr. F(d5) = Phe residue with five 2H (d) atoms. Reproduced from Zhan and Desiderio (2009) [24], with permission from Elsevier Science, copyright 2009.

(Figure 4(c)) with 2D gel image analysis software by comparing the digitized Western blot image (Figure 4(c)) to the negative control (Figure 4(d)). Also, each nitrotyrosine-positive Western blot spot (Figure 4(c)) was matched to corresponding silver-stained 2D gel spots (Figure 4(a)) so that the silver-stained gel spots were excised for mass spectrometry analysis. Therefore, even though the abundance of a nitroprotein in a human tissue proteome is very low,

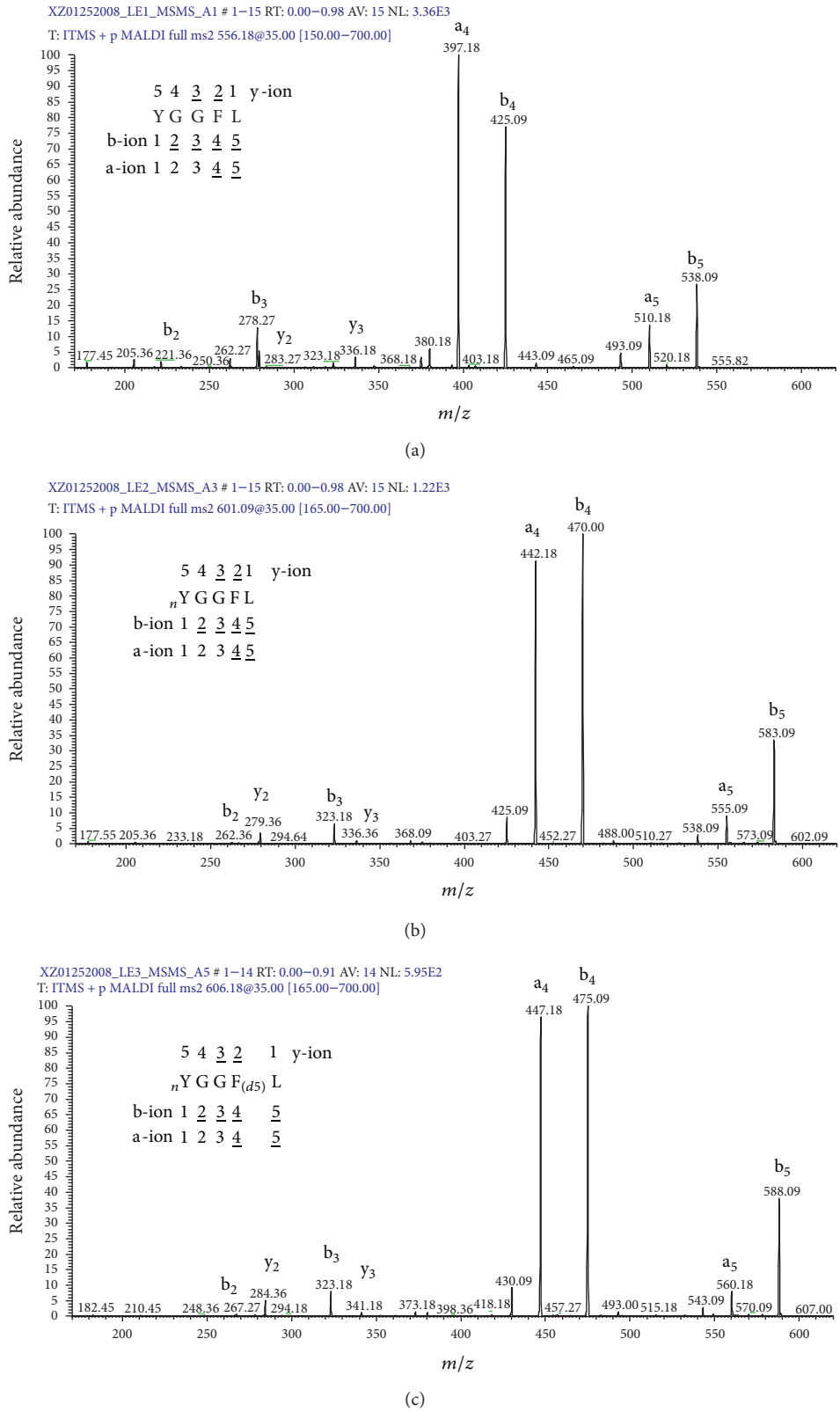

FIGURE 2: MS^2 spectra of LE1 (a), LE2 (b), and LE3 (c). nY = nitro-Tyr. F(d5) = Phe residue with five 2H (d) atoms. Reproduced from Zhan and Desiderio (2009) [24], with permission from Elsevier Science, copyright 2009.

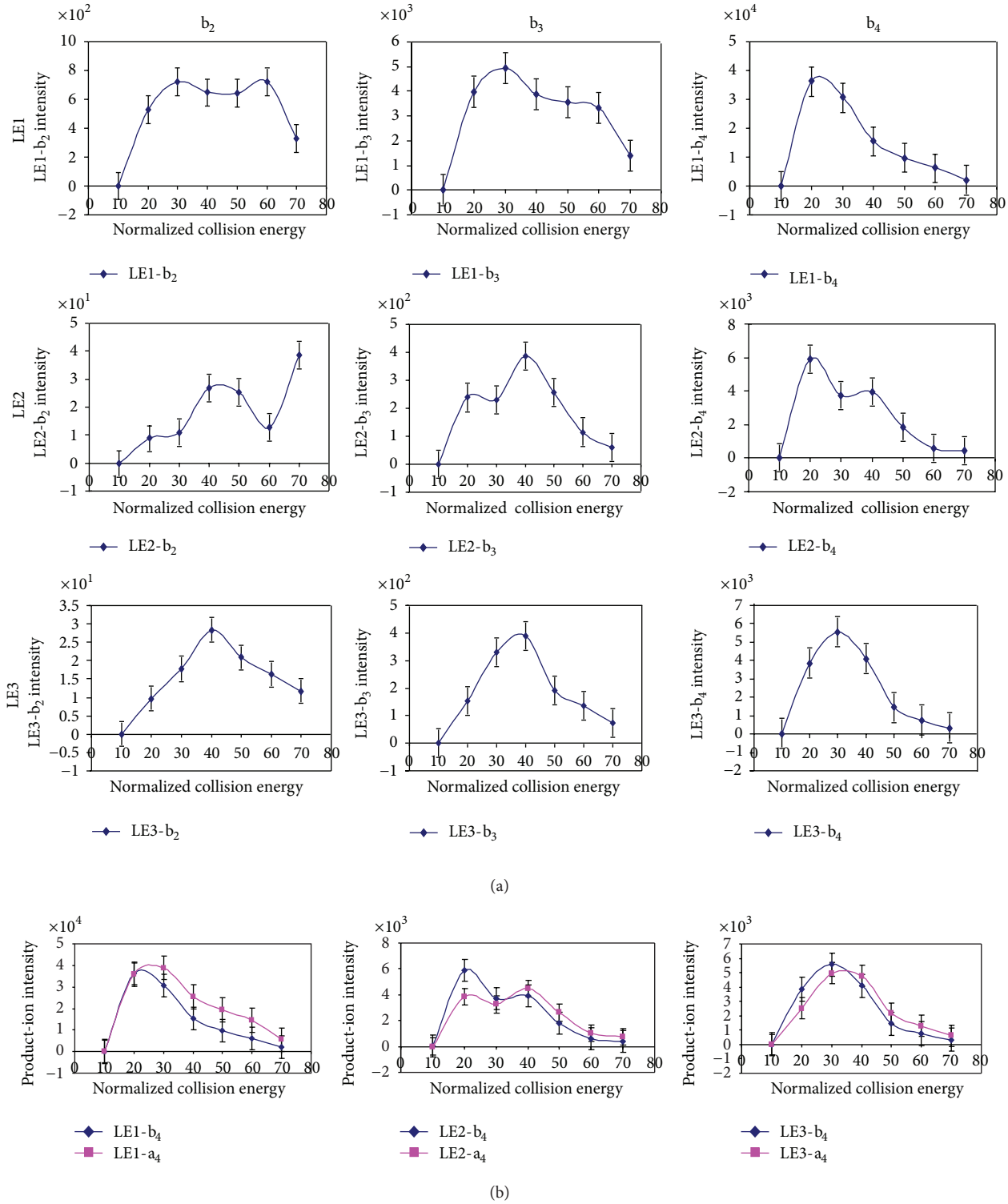

Figure 3: The effect of collision energy on the fragmentation of nitropeptides. (a) Relationship between collision energy and the product-ion intensity ($n = 3$). (b) Relationship between collision energy and the product-ion b_4 and a_4 intensities ($n = 3$). Reproduced from Zhan and Desiderio (2009) [24], with permission from Elsevier Science, copyright 2009.

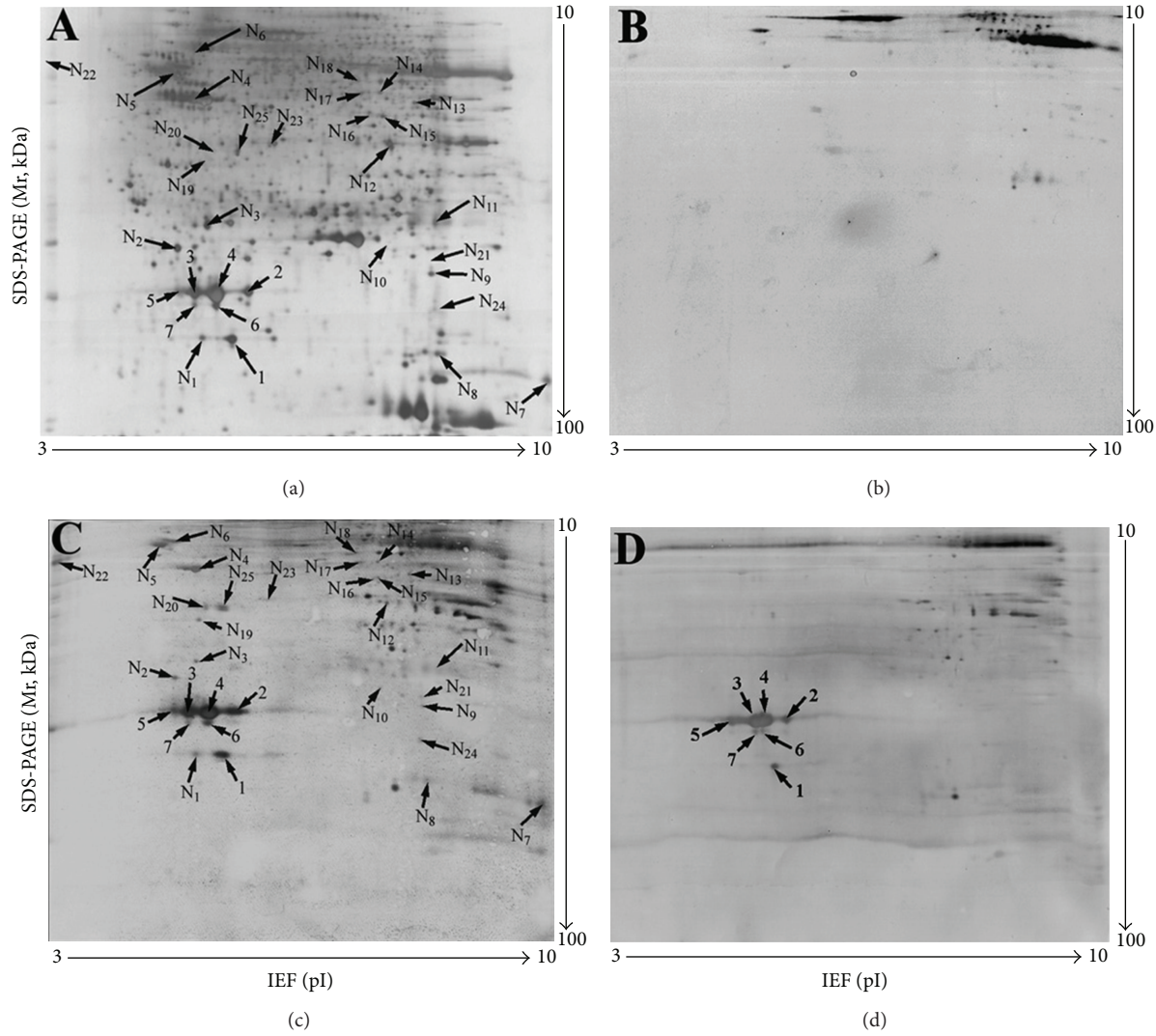

Figure 4: Two-dimensional Western blotting analysis of anti-3-nitrotyrosine-positive proteins in a human pituitary (70 ug protein per 2D gel). (a) Silver-stained image on a 2D gel before the transfer of proteins onto a PVDF membrane. (b) Silver-stained image on a 2D gel after the transfer of proteins onto a PVDF membrane. (c) Western blot image of anti-3-nitrotyrosine-positive proteins (anti-3-nitrotyrosine antibodies + secondary antibody). (d) Negative control of a Western blot to show the cross reaction of the secondary antibody (only the secondary antibody; no anti-3-nitrotyrosine antibody). Reproduced from Zhan and Desiderio (2007) [16], with permission from Elsevier Science, copyright 2007.

2DGE separates and enriches each nitroprotein to improve its immunodetection and MS characterization.

The NTAC method [4] (Figure 5) was used to preferentially enrich nitroproteins from a human pituitary adenoma proteome. Antinitrotyrosine antibodies were cross-linked to protein G beads and incubated with a pituitary adenoma protein sample. Nitroproteins and nitroprotein-protein complexes (interactomes) were bound to the cross-linked anti-nitrotyrosine antibodies. Bound nitroproteins and nitroprotein-protein complexes were eluted to provide an enriched nitroprotein sample, followed by trypsin digestion and mass spectrometry analysis. The detailed NTAC procedure was described [4]. NTAC is an effective method to isolate and enrich nitroproteins from a complicated human pituitary adenoma proteome to improve MS/MS identification of very low-abundance nitroproteins.

5. Tandem Mass Spectrometry Identification of Nitroproteins and Nitrotyrosine Sites in Human Pituitary and Adenoma

Tandem mass spectrometry is the essential method to obtain the amino acid sequence of a tryptic peptide or nitropeptide enzymatically digested from a nitroprotein and to determine nitrotyrosine sites [3, 4, 16]. A total of 32 2D gel spots corresponding to nitrotyrosine immunopositivity from a postmortem pituitary control tissue were excised,

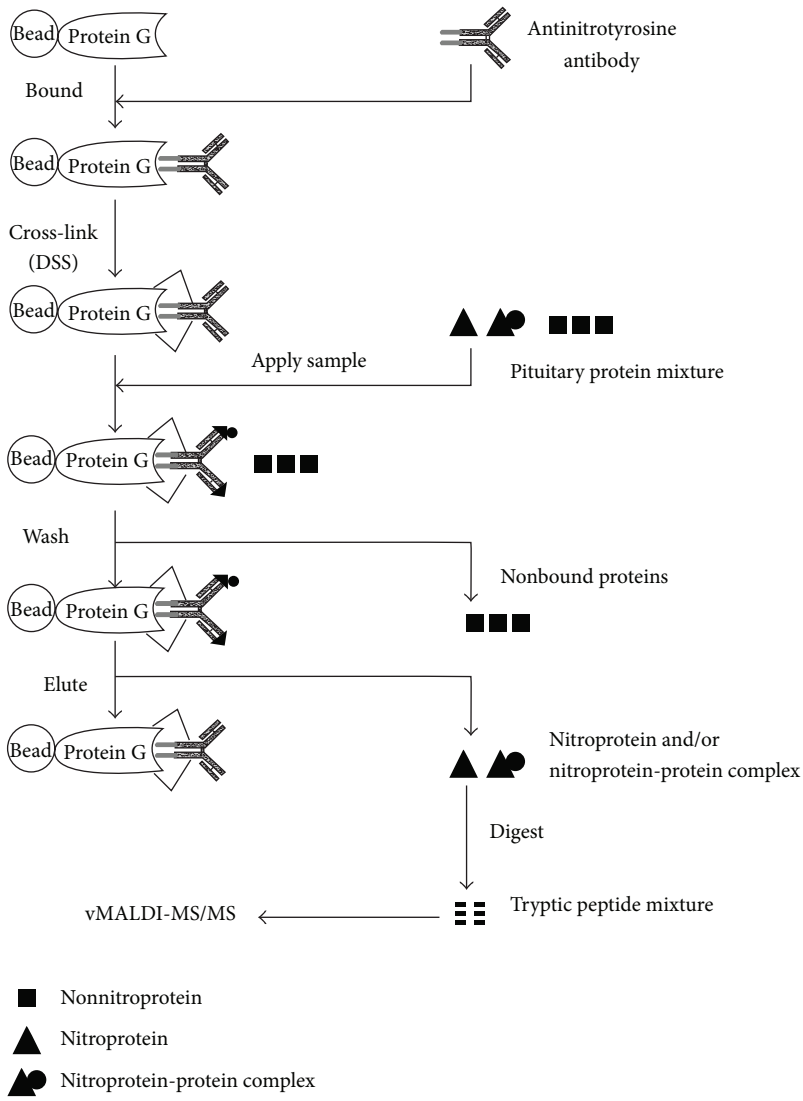

Figure 5: Experimental flow chart to identify nitroprotein and nitroprotein-protein complexes with NTAC-based vMALDI-LTQ MS/MS. The control experiment (without any anti-3-nitrotyrosine antibody) was performed in parallel with the NTAC-based experiments. Reproduced from Zhan and Desiderio (2006) [4], with permission from Elsevier Science, copyright 2006.

and proteins were extracted to identify nitroprotein and nitrotyrosine sites. Eight nitroproteins and eight nitrotyrosine sites were identified in the postmortem control pituitary (Table 1) [3, 16], including synaptosomal-associated protein, actin, immunoglobulin alpha Fc receptor, cGMP-dependent protein kinase 2, stanniocalcin 1, mitochondrial cochaperone protein HscB, progestin and adipoQ receptor family member III, and proteasome subunit alpha type 2. Those nitroproteins participate in multiple functions, including neurotransmission, cellular immunity, cellular structure and mobility, calcium and phosphate metabolism, cochaperone in iron-sulfur cluster assembly in mitochondria, membrane receptor, and the ATP/ubiquitin-dependent nonlysosomal proteolytic pathway.

The NTAC-based enriched nitroprotein samples from a pituitary adenoma were subjected to digestion with trypsin, followed by MS/MS analysis [4]. A total of nine nitroproteins and ten nitrotyrosine sites were identified from a pituitary adenoma tissue (Table 1; Figure 6), including sphingosine-1-phosphate lyase 1, zinc finger protein 432, cAMP-dependent protein kinase type I-beta regulatory subunit, Rho-GTPase-activating protein 5, leukocyte immunoglobulin-like receptor subfamily A member 4, centaurin-beta 1, proteasome subunit alpha type 2, interleukin 1 family member 6, and rhophilin 2. Also, three proteins including glutamate receptor-interacting protein 2, ubiquitin, and interleukin 1 receptor-associated kinase-like 2 were discovered to interact with nitroproteins (Table 1) to form three nitroprotein-protein complexes, including nitrated proteasome-ubiquitin complex, nitrated beta-subunit of cAMP-dependent protein kinase (PKA) complex, and nitrated interleukin 1 family member 6-interleukin 1 receptor-interleukin 1 receptor-associated kinase-like 2 (IL1F6-IL1R-IRAK2) [4].

Those nine nitroproteins and three nitroprotein-protein complexes were rationalized into a corresponding functional system (Figure 7) [4]. The nitrated proteasome-ubiquitin

Table 1: Nitroprotein and unnitrated protein identified from pituitary adenoma [4] and control tissue [3, 16].

Protein name	nY site
Pituitary adenoma	
Nitrated protein	
Rho-GTPase-activating 5 [Q13017] (ARHGAP5)	Y^{550}
Leukocyte immunoglobulin-like receptor A4 [P59901]	Y^{404}
Zinc finger protein 432 [O94892]	Y^{41}
PKA-beta regulatory subunit [P31321] (PRKAR1B)	Y^{20}
Sphingosine-1-phosphate lyase 1 [O95470]	Y^{356} Y^{366}
Centaurin-beta 1 [Q15027]	Y^{485}
Proteasome subunit alpha type 2 [P25787] (PSMA2)	Y^{228}
Interleukin 1 family member 6 [Q9UHA7] (IL1F6)	Y^{96}
Rhophilin 2 [Q8IUC4] (RHPN2)	Y^{258}
Nitroprotein-interacted protein	
Interleukin-1 receptor-associated kinase-like 2 (IRAK-2) [O43187] (IRAK2)	
Glutamate receptor-interacting protein 2 [Q9C0E4] (GRIP2)	
Ubiquitin [P629881] (UBB or UBC)	
Pituitary control	
Nitrated protein	
Synaptosomal-associated protein (SNAP91)	Y^{237}
Ig alpha Fc receptor [P24071] (FCAR)	Y^{223}
Actin [P03996] (ACTA2, ACTG2, and ACTC1)	Y^{296}
PKG 2 [Q13237] (PRKG2)	Y^{354}
Mitochondrial cochaperone protein HscB [Q8IVVL3]	Y^{128}
Stanniocalcin 1 [P52823] (STC1)	Y^{159}
Proteasome subunit alpha type 2 (PSMA2)	Y^{228}
Progestin and adipoQ receptor family member III [Q6TCH7] (PAQR3)	Y^{33}

nY: nitrotyrosine. Modified from Zhan and Desiderio [3, 4, 16], with permission from Elsevier Science, copyright 2004, 2006, and 2007.

complex is an important enzymatic complex that is involved in the intracellular nonlysosomal proteolytic pathway [4, 46, 47]. The nitrated LIRA4 might be involved in the immune system. The nitrated S1P lyase 1 participates in sphingolipid metabolism to regulate cell proliferation, survival, and cell death as well as the immune system [4, 56–58]. The nitrated CENT-beta 1 and the nitrated PKAR1-beta are involved in the PKA signal pathway. IRAK-2 in the IL1-R complex and the nitrated IL1-F6 are involved in the cytokine system. The nitrated ZFP432 is involved in transcription regulatory systems. The nitrated RHOGAP5 and the nitrated rhophilin 2 are involved in the GTPase signal pathway [4].

6. Bioinformatics Recognition of Nitrotyrosine-Containing Protein Domain/Motif of Nitroprotein

The specific domains or motifs in a protein would sustain corresponding intracellular biological functions. Location of nitrotyrosine sites into a protein domain or motif would benefit the accurate elucidation of the biological activities of tyrosine nitration. Protein domain analysis softwares, including ScanProsite (http://us.expasy.org/tools/scanprosite/), Motifscan (http://myhits.isb-sib.ch/cgi-bin/motif_scan), Inter-ProScan (http://www.ebi.ac.uk/InterProScan/), ProDom (http://prodom.prabi.fr/prodom/current/html/form.php), and Pfam (http://www.sanger.ac.uk/Software/Pfam/), were used to detect the statistically significant domains of each nitroprotein and to locate the identified nitrotyrosine site within a protein domain to gain insight into the effect of tyrosine nitration on the protein functions [4]. More information can be obtained on the Swiss-Prot annotation page of each protein.

This method was used to analyze the protein domain and motif of each nitroprotein identified from human pituitary adenoma tissue [4]. An exciting result showed most nitrotyrosine sites occur within important protein domains and motifs. For example, sphingosine-1-phosphate lyase 1 (S1P lyase 1), nitrated in human pituitary adenoma [4] (Figure 6(a)), is a key enzyme to catalyze the decomposition of S1P. Two nitrations (NO_2-^{356}Y and NO_2-^{366}Y) within the enzyme activity region could decrease the interaction intensity of enzyme:substrate (S1P lyase 1:S1P) to decrease the decomposition of S1P because the nitro group (-NO_2) is an

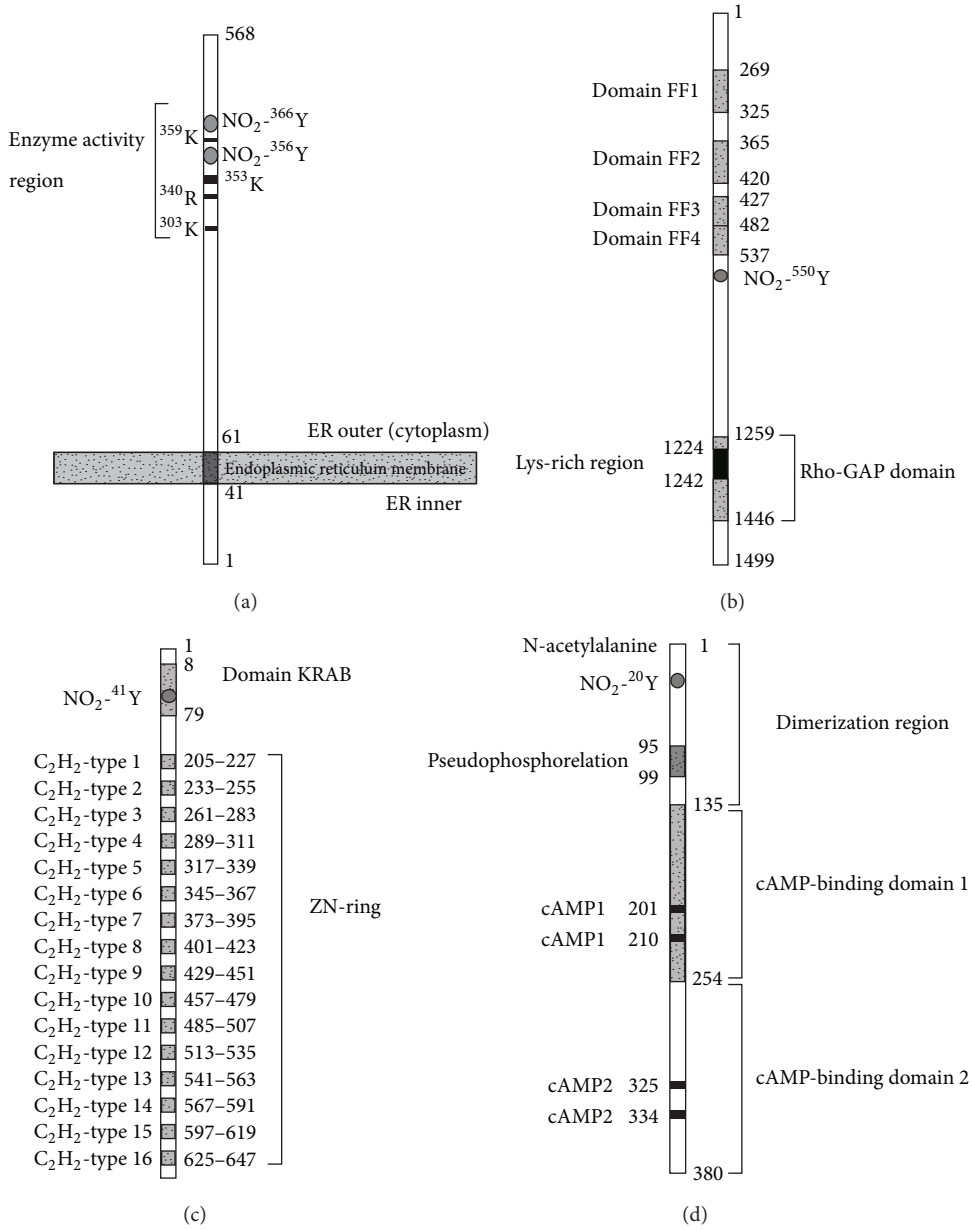

Figure 6: Nitration site and functional domains of four nitroproteins. (a) Sphingosine-1-phosphate lyase 1. The site 353 K is a pyridoxal phosphate-binding motif. (b) Rho-GTPase-activating protein 5. (c) Zinc finger protein 432. The KRAB domain is a transcriptional suppressor. The ZN-RING is a DNA-binding region. (d) cAMP-dependent protein kinase type I-beta regulatory subunit. Modified from Zhan and Desiderio (2006) [4] with permission from Elsevier Science and reproduced from Zhan and Deiderio (2009) [22] with permission from Springer Science.

electron-withdrawing group that could decrease the level of enzyme-substrate binding. Studies [56] have demonstrated that S1P, ceramide (Cer), and sphingosine (Sph), which are the sphingolipid metabolites, play an important role to regulate cell proliferation, survival, and cell death. Cer and Sph usually inhibit proliferation and promote apoptosis, whereas S1P stimulates growth and suppresses apoptosis. Because these metabolites are interconvertible, their relative levels determine cell fate. The nitration of S1P lyase 1 could increase the level of S1P relative to Cer and Sph, to stimulate the tumor cell proliferation and inhibit the apoptosis. Rho-GTPase-activating protein 5 (Figure 6(b)) contains four FF domains [59] and one Rho-GAP domain, and nitration (NO_2-^{550}Y) occurred within the region between two domains (FF4 and Rho-GAP) could affect Rho-GTPase signal transduction [4]. Zinc finger protein 432 (Figure 6(c)) is a transcript factor that includes 16 C_2H_2-type zinc fingers that bind DNA and one Kruppel-associated box (KRAB) domain that functions as a transcriptional suppressor [60–62]; nitration (NO_2-^{41}Y) within the KRAB domain could impair transcriptional

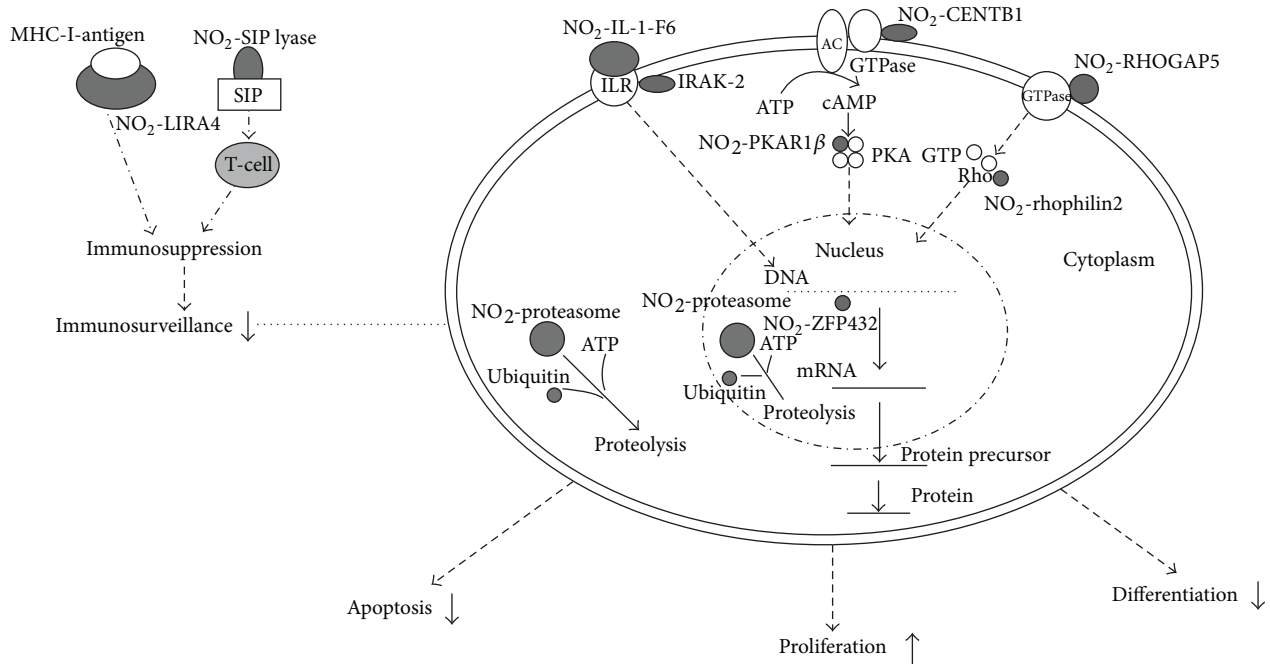

Figure 7: Experimental data-based model of nitroproteins and their functions in human nonfunctional pituitary adenomas. NO_2^-: nitroprotein. Reproduced from Zhan and Desiderio (2006) [4] with permission form Elsevier Science.

suppression [4]. The cAMP-dependent protein kinase type I-beta regulatory subunit (PKAR1-beta) (Figure 6(d)) contains one N-terminal dimerization domain, one inhibitory region (pseudophosphorylation), and two cAMP-binding domains. Each cAMP-binding domain contains two cAMP-binding sites. Nitration (NO_2-^{20}Y) within the dimerization region could affect dimerization of two regulatory chains [4].

7. Systems Biological Analysis of Signaling Pathway Networks That Involve Nitroproteins

Systems biology is a comprehensive quantitative analysis of the manner in which all components of a biological system interact functionally over time [63–65]. Relative to the traditional molecular biology methods that had been used to study the role of a single gene, single protein, or single small-molecule model, high-throughput "-omic" technologies such as genomics, transcriptomics, proteomics, and metabolomics have driven the rapid development of systems biology to study a multiple-factor model of disease and to address the network of interaction and regulatory events that contribute to a disease [23]. Pathway biology is one important component of systems biology and is used to extensively analyze "-omic" data to mine significantly signaling pathway networks and to address the biological significance of those "-omic" data.

The Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com/) and the MetaCore Pathway Analysis programs (http://www.genego.com/metacore.php/) were used to reveal signaling networks that involve nitroproteins. This method was used to analyze signaling pathway networks that involve nitroproteins identified from human pituitary adenoma [4] and control tissues [3, 16]. For eight nitroproteins from a pituitary control [3, 16] (Table 1), and nine nitroproteins and three non-nitrated proteins that interact with nitroproteins (interactomes) from a pituitary adenoma tissue [4] (Table 1), IPA pathway analyses [23] clearly indicated that those pituitary adenoma nitroproteins and their complexes are involved in the tumor necrosis factor (TNF) and interleukin 1 (IL1) signaling networks (Figure 8(a)), which function in cancer, cell cycle, and reproductive system disease. The nitroproteins in that network include ARHGAP5, PRKAR1B, PSMA2, IL1F6, and RHPN2. The nonnitrated proteins that interact with nitroproteins include IRAK2, GRIP2, and ubiquitin. Three nitroprotein-protein complexes were identified: nitrated proteasome-ubiquitin complex, nitrated beta-subunit of PKA complex, and nitrated IL1F6-IL1 receptor-IL1 receptor-associated kinase-like 2 (IL1F6-IL1R-IRAK2) complex. Those control pituitary nitroproteins are involved in the transforming growth factor beta 1 (TFGB1) and actin cellular skeleton signaling networks (Figure 8(b)), which function in gene expression, cellular development, and connective tissue development. Nitroproteins in that network include SNAP91, FCAR, actin, PRKG2, STC1, PAQR3, and PSMA2. Both networks (Figures 7(a) and 7(b)) include a beta-estradiol signal pathway, which reveals that hormone metabolism is involved in a normal pituitary and pituitary adenoma. It is consistent with the fact that NO participates in pituitary hormone metabolism in normal physiology and tumor interferes with hormone metabolism.

Moreover, among those pituitary adenoma nitroprotein data, twelve statistically significant canonical pathways

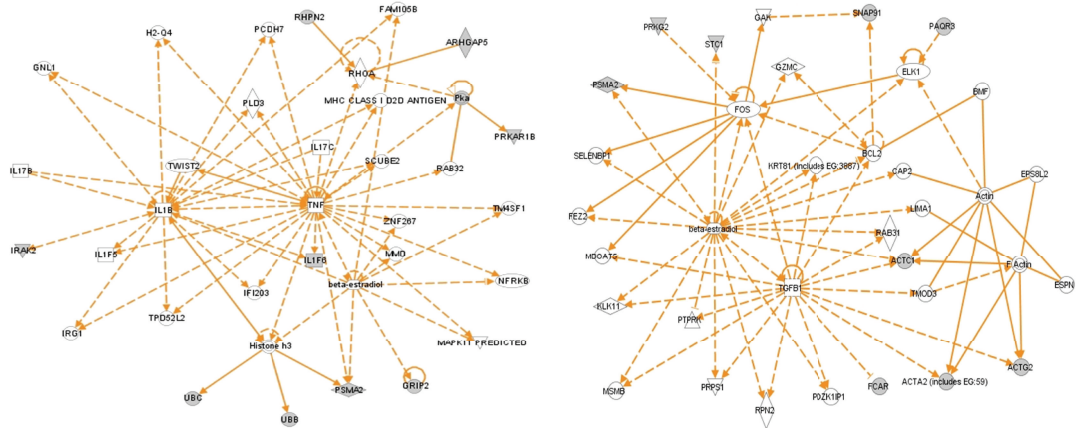

FIGURE 8: Significant signaling pathway networks mined from nitroproteomic dataset. (a) Network is derived from adenoma nitroproteomic data and function in cancer, cell cycle, reproductive system disease. A gray node denotes an identified nitroprotein or protein that interact with nitroproteins in our study. (b) Network is derived from control nitroproteomic data and function in gene expression, cellular development, and connective tissue development and function. A gray node denotes an identified nitroprotein in our studies. An orange solid edge denotes a direct relationship between two nodes (molecules: proteins, genes). An orange unsolid edge denotes an indirect relationship between two nodes (molecules: proteins, genes). The various shapes of nodes denote the different functions. A curved line means intracellular translocation; a curved arrow means extracellular translocation. Reproduced from Zhan and Desiderio [23], with permission from BioMed Central open access journal, copyright remains with the authors.

were identified that involve nitroproteins [23]. The top canonical pathways include p38 MAPK signaling, cell-cycle G2/M DNA damage-checkpoint regulation, the protein-ubiquitination pathway, sonic-hedgehog signaling, GABA-receptor signaling, Toll-like receptor signaling, amyloid processing, the phototransduction pathway, sphingolipid metabolism, IL-10 signaling, hypoxia signaling, LXR/RXR activation, and PXR/RXR activation [23]. Three statistically significant toxicity pathways were mined, including hepatic cholestasis, PXR/RXR activation, and LXR/RXR activation. Among those control pituitary nitroprotein data, twelve statistically significant canonical pathways were identified that involve nitroproteins [23], including clathrin-mediated endocytosis, caveolar-mediated endocytosis, VEGF signaling, regulation of actin-based motility by Rho, Fcy receptor-mediated phagocytosis in NRF2-mediated oxidative-stress response, macrophages and monocytes, tight-junction signaling, leukocyte extravasation signaling, integrin signaling, actin-cytoskeleton signaling, and calcium signaling. No statistically significant toxicity pathways were mined.

Among four signaling pathway network systems (mitochondrial dysfunction, oxidative stress, cell-cycle dysregulation, and MAPK-signaling abnormality) that were discovered from pituitary adenoma mapping data, comparative proteomic data, and nitroproteomic data [23], three signaling pathway network systems (oxidative stress, cell-cycle dysregulation, and MAPK-signaling abnormality) are involved in protein nitration; for example, the NRF2-mediated oxidative-stress response, cell-cycle G2/M DNA damage checkpoint regulation, and p38 MAPK signaling were discovered from pituitary adenoma nitroproteomic data [23]. Therefore, pathway systems analysis revealed that tyrosine nitration plays important roles in the pituitary tumorigenesis.

8. Future Perspectives

To date, the qualitative nitroproteomics based on 2DGE-based nitrotyrosine Western blotting [3, 16] and NTAC enrichment [4] has been used to analyze the presence of nitroproteins in a human pituitary postmortem tissues and in a human pituitary adenoma tissue, respectively. Protein domain and motif analyses [4] have been used to identify the structural or functional domain and motif and to locate nitrotyrosine site into the corresponding domain and motif in a nitroprotein to clarify the functional roles of tyrosine nitration. IPA pathway analysis [23] has been used to identify important pathway networks that are involved in nitroproteins and to address functional roles of nitroproteins from a systematic angle. However, much more research is needed to elucidate the real functional roles of tyrosine nitration in pituitary tumorigenesis.

First, nitroproteomics of single-cell types of a pituitary adenoma will be necessary. The pituitary contains multiple cell types, including GH, PRL, TSH, LH/FSH, and ACTH [66]. Those different cell types of pituitary adenomas could have not only a common mechanism in their formation but also some differences among different cell types of pituitary adenoma. Therefore, it is important to study the same and different differentially expressed nitroproteins among the

different cell types of pituitary adenomas and to discover specific nitroprotein biomarkers for pituitary adenomas. Laser capture microdissection (LCM) [67, 68] is a promising and powerful technique to enrich and isolate pure pituitary-cell populations from pituitary adenoma and control pituitary tissue, including somatotrophs, lactotrophs, thyrotrophs, gonadotrophs, and corticotrophs. LCM technique has been optimized [69] to enrich and isolate prolactin cells from postmortem pituitary tissues [70] and prolactinoma tissues [71] for proteomics analysis.

Second, quantitative nitroproteomics among different cell types of pituitary adenoma and controls are needed to determine nitroproteins that are unique to each cell type of pituitary adenoma. With precious LCM-enriched cell types, isobaric tags for relative and absolute quantification- (iTRAQ-) based quantitative proteomics and two-dimensional difference in-gel electrophoresis- (2D-DIGE-) based quantitative proteomics would be the first method to analyze differentially expressed nitroproteins (DENPs) among different cell types of pituitary adenomas. 2D-DIGE in combination with fluorescent dye stains would separate identical proteins from two samples tagged with different fluorescent dyes (Cy3, Cy5) in a single 2D gel and comigration to the same spot position [66, 72]. Quantification of identical proteins from two samples would be determined from the difference in signal intensity of two fluorescent dyes. Compared to classical 2DGE-based comparative proteomics, 2D-DIGE eliminates between-gel variations, improves reproducibility, provides a higher throughput, has a wider dynamic range, and requires only half of the protein-loading amount of conventional 2DGE [66, 73]. Therefore, 2D-DIGE-based quantitative proteomics would especially be suitable for those rare sample sources such as LCM pituitary cells from pituitary adenomas and controls [66].

iTRAQ-based quantitative proteomics involves different samples labeled with different iTRAQ reagents, equally mixed for strong cation exchange (SCX), liquid chromatography (LC), and tandem mass spectrometry (MS/MS) analyses. The intensities of reporter ions are used to quantify DENPs. Because iTRAQ equally mixes labeled samples, it will require less amount of each sample, similar to 2D-DIGE. iTRAQ would be suitable for rare samples, especially LCM-enriched samples. Moreover, iTRAQ can overcome the drawbacks of 2D-DIGE, including the limited range of 2D-DIGE to separate proteins (e.g., for a pH 3–10 NL gel, distribution of detected protein in the area of pH 4–8 and mass 15–100 kDa) [66] and the difficulty to detect low-abundance and hydrophobic proteins. However, iTRAQ cannot detect protein isoforms, whereas 2D-DIGE can [66]. Therefore, 2D-DIGE and iTRAQ would be combined to analyze DENPs unique to each cell type of pituitary adenoma to maximize nitroproteome coverage.

Third, tyrosine nitration decreases electron density of the phenolic ring of a tyrosine residue to diminish the interaction intensity between enzyme and substrate or between receptor and ligand. The three-dimensional spatial structure of a protein determines its biological functions. For a pituitary adenoma-related nitroprotein, if its three-dimensional spatial structure can be reconstructed from X-ray crystallography, then it will be very easy to interpret the effect of tyrosine nitration on the 3D structure of a nitroprotein. Meanwhile, based on the 3D structure and tyrosine nitration site and domain, it is possible for one to design a small drug towards the 3D structure and domain that contains tyrosine nitration. For example, sphingosine-1-phosphate lyase 1 (Figure 6(a)) is an enzyme, and two nitrotyrosines were found within the enzymatic activity region. If the 3D structure of sphingosine-1-phosphate lyase 1 could be reconstructed from X-ray crystallography data, then one could clearly interpret the effect of two tyrosine nitrations on its structure and functions.

Finally, a pituitary gland participates in several different hypothalamic-pituitary-target organ axes. A pituitary adenoma would impact those axes systems in a whole-body disease. Therefore, instead of pituitary adenoma tissue, cerebrospinal fluid (CSF) and blood plasma must be studied because some secreted proteins and peptides enter into the CSF and blood circulation in a pituitary adenoma patient [74]. Also, CSF and blood specimens are much more accessible from patients and controls than pituitary tissues and overcome the limitations of pituitary tissues [74]. Quantitative nitroproteomics can detect those nitroproteins and nitropeptides in a patient's CSF and blood plasma. Those CSF and blood plasma nitroproteomic and nitropeptidomic variations would lead to the development of accurate biomarkers for predictive diagnosis, early-stage diagnosis, and measurement of prevention and therapy responses.

9. Conclusions

Tyrosine nitration is an important molecular event in pituitary adenoma and is extensively associated with pituitary physiological and pathological processes. 2DGE-based nitrotyrosine Western blot coupled with MS/MS was used to detect 32 nitrotyrosine positive gel spots and eight nitroproteins and modified sites from a pituitary postmortem tissue [3, 16]. NTAC-based enrichment coupled with MS/MS was used to identify nine nitroproteins and modified sites and three nitroprotein-interacting proteins (interactomes) from a pituitary adenoma tissue [4], and most of nitrotyrosine sites were located within important protein domains/motifs. Tyrosine nitration was involved in three pathway network systems (oxidative stress, cell-cycle dysregulation, and the MAPK-signaling abnormality) that are significantly associated with pituitary adenomas [23]. Moreover, MALDI UV laser causes photodecomposition (loss of one or two oxygen atoms) of a nitro group in a nitropeptide. Recognition of the photodecomposition pattern would assist in the interpretation of an MS spectrum of an endogenous nitropeptides. In the future, one needs to perform quantitative nitroproteomics of each cell type of a pituitary adenoma to discover the nitroprotein biomarker unique to each cell type of a pituitary adenoma and further analysis of the 3D structure of that nitroprotein. In addition, it is important to develop quantitative nitroproteomics of body fluids (CSF and blood plasma) of pituitary adenoma patients to recognize body-fluid nitroproteins or nitropeptide patterns.

Abbreviations

ACTH: Adrenocorticotropin
CSF: Cerebrospinal fluid
DENP: Differentially expressed nitroprotein
ELISA: Enzyme-linked immunosorbent assay
eNOS: Endothelial nitric oxide synthase
ESI: Electrospray ionization
FSH: Follicle-stimulating hormone
GH: Growth hormone
IFN: Interferon
IL1: Interleukin 1
iNOS: Inducible nitric oxide synthase
IPA: Ingenuity pathway analysis
iTRAQ: Isobaric tags for relative and absolute quantification
LC: Liquid chromatography
LCM: Lasercapture microdissection
LH: Luteinizing hormone
LPS: Lipopolysaccharide
MALDI: Matrix-assisted laser desorption ionization
Mr: Relative molecular weight
MS: Mass spectrometry
MS/MS: Tandem mass spectrometry
nNOS: Neuronal nitric oxide synthase
NO: Nitric oxide
NOS: Nitric oxide synthase
NTAC: Nitrotyrosine affinity column
pI: Isoelectric point
PKA: cAMP-dependent protein kinase
PRL: Prolactin
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SCX: Strong cation exchange
TGFB1: Transforming growth factor beta 1
TNF: Tumor necrosis factor
1DGE: One-dimensional gel electrophoresis
2DGE: Two-dimensional gel electrophoresis
2D-DIGE: Two-dimensional differential in-gel electrophoresis.

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Review Article

Oxidized Extracellular DNA as a Stress Signal in Human Cells

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The term "cell-free DNA" (cfDNA) was recently coined for DNA fragments from plasma/serum, while DNA present in *in vitro* cell culture media is known as extracellular DNA (ecDNA). Under oxidative stress conditions, the levels of oxidative modification of cellular DNA and the rate of cell death increase. Dying cells release their damaged DNA, thus, contributing oxidized DNA fragments to the pool of cfDNA/ecDNA. Oxidized cell-free DNA could serve as a stress signal that promotes irradiation-induced bystander effect. Evidence points to TLR9 as a possible candidate for oxidized DNA sensor. An exposure to oxidized ecDNA stimulates a synthesis of reactive oxygen species (ROS) that evokes an adaptive response that includes transposition of the homologous loci within the nucleus, polymerization and the formation of the stress fibers of the actin, as well as activation of the ribosomal gene expression, and nuclear translocation of NF-E2 related factor-2 (NRF2) that, in turn, mediates induction of phase II detoxifying and antioxidant enzymes. In conclusion, the oxidized DNA is a stress signal released in response to oxidative stress in the cultured cells and, possibly, in the human body; in particular, it might contribute to systemic abscopal effects of localized irradiation treatments.

1. Introduction

The effect of information transfer from the irradiated cells (target cells) to adjacent, nonirradiated ones is known as the bystander effect (BE). The BE was shown for a number of damaging agents of both physical and chemical nature, in many types of eukaryotic cells, and covers a variety of physiological effects including the genomic instability, the cell death, and/or the adaptive response (AR) [1]. As a result of adaptive response brought about by low-dose ionizing radiation, the cells develop resistance to further irradiation at higher (damaging) doses. Both reactions (AR and BE) are closely interconnected biologically and have many similarities and characteristic features [2–5]. Interestingly, both AR and BE may be transferred to intact cells through their exposure to the media conditioned by exposed cells [6, 7]. Importantly, the development of particular variant of cellular response depends on the amount of irradiation, amount of cells, their tissue origin, and the stage of the cell cycle. In some experimental studies, the response of bystander cells might not be adaptive [1–7].

For the first time, the intercellular signaling was experimentally demonstrated on Chinese hamster cell culture [8]. Following irradiation of not more than 1% of cellular nuclei, the authors observed increased frequency of sister chromatids exchanges in 20–40% of the cultured cells. It is generally accepted that there are three possible pathways of signal transfer from the irradiated cell to the "bystander" cell: through the direct cellular contact with the formation of common membranous structures, through interaction involving the gap junctions or via the signals released to the culture medium of the irradiated cells. The third pathway is typical for the BE induced by radiation with low Linear Energy Transfer (LET) [9]. Many candidate molecules, mainly the soluble proteins, have been proposed as mediators of the bystander signaling between treated cells and bystander cells. All these data had been reviewed in details previously [10–17].

In course of our studies, we thoroughly evaluated an idea of existence of certain intrinsic cellular factor that is released from the dying cells, thus, causing the development of the bystander effect. The present work is a brief overview of our

recent findings concerning the possible role of extracellular DNA oxidation in the development of the adaptive response and bystander effect, as triggered in human cells by exposure to oxidative stress [18–35].

2. Oxidative Stress Induces the Oxidation of Cellular DNA

Many chronic diseases are accompanied by an increase in overall oxidation of genomic DNA. Under oxidative stress, the DNA bases are prone to oxidation, with the most common products being the thymidine glycol and 8-hydroxy-2′-deoxyguanosine (8-oxodG). In fact, the 8-oxodG is the most widely used "marker" for oxidative DNA damage. The 8-oxodG is formed in DNA either via direct oxidation or can be incorporated into DNA by DNA polymerase as a modified base drawn from the nucleotide pool [36, 37].

Previously published studies have reported the frequency of 8-oxodG in genomic DNA (gDNA) samples. For example, gDNA extracted from cultured cells [38, 39] contains approximately from 0.1 to 0.5 8-oxodG per 10^6 nucleotides, while normal breast tissue from cancer patients has significantly higher levels of oxidative DNA damage—up to 25 8-oxodG per 10^6 nucleotides [40]. Most of the results clearly indicate higher steady-state levels of modified DNA bases in cancerous tissues than in their cancer-free surrounding tissues. The level of oxidative modification of cellular DNA may serve as a predictive marker of cancer development [41–43]. For example, in breast carcinomas, 8-oxodG levels have been reported as being 8 to 17 times higher as compared with nonmalignant breast tissue [44–46]. Additionally, it was shown that an exposure of the MCF-10A cells to doxorubicin leads to a significant increase in the levels of eleven different oxidized forms of DNA bases [47].

The genomes of prostatic carcinoma cell lines LNCaP, DU145, and PC3 contain between 3 and 4.5 8-oxodG/10^6 nucleotides, while genomic DNA extracted from the prostatic tissue of young men contains approximately ~50 8-oxodG/10^6 nucleotides [48, 49]. With age, these levels increase to up to ~75 8-oxodG/10^6 nucleotides. Noncancerous prostatic tissue of prostatic carcinoma patients contains ~90 8-oxodG/10^6 nucleotides, while in prostatic carcinoma cells these amounts increase up to ~120 8-oxodG/10^6 nucleotides [48, 49]. Interestingly, in prostatic carcinoma cells, the levels of 8-oxodG could be induced by exposure to supraphysiological concentrations of dihydrotestosterone [50].

Human ovarian tissue contains approximately 1.3 8-oxodG per 10^6 nucleotides, while in advanced epithelial ovarian carcinoma these levels increase to 2.2 8-oxodG/10^6 nucleotides [51]. In uterine myomas, the levels of 8-oxodG are higher than those in underlying myometrium (~3-4 8-oxodG and 2 8-oxodG/10^6 nucleotides, resp.) and correlate with the size of the tumor [52].

Two-three-fold increases in levels of 8-oxodG were observed in lung carcinoma as compared to cancer-free surrounding lung tissues [53]. Among Noncancerous lung samples, the lung tissue removed from smokers had the highest increases of modified bases above the control levels and the highest overall amounts of 8-oxodG [54].

DNA extracted from PBMCs of healthy donors contains between 1 and 1.3 8-oxodG bases per 10^6 nucleotides [55–60]. In PBMCs of cancer patients, the content of 8-oxodG increases to 1.5–1.8 modified bases per 10^6 nucleotides [45, 55, 56, 59, 60]. Lymphocyte DNA from lung cancer patients had 1.7-fold higher levels of 8-oxodG compared to the controls. The difference was especially evident in current smokers [61]. The levels of 8-oxodG in DNA extracted from leukocytes of patients with Leber's hereditary optic neuropathy were 3.3 bases per 10^6 nucleotides [62]. Similar increases were observed in DNA of Parkinson's disease and multiple sclerosis patients [63, 64]. Increased levels of 8-hydroxyadenine, 8-oxodG, thymine glycol, Fapy-guanine, 5-hydroxymethyl-2′-deoxyuridine, and Fapy-adenine were observed in brains of patients with Alzheimer's disease [65]. The mean levels of 8-oxodG in neurons were 10 times higher in elderly "poor-outcome" schizophrenia than in control DNA samples [66]. A marked elevation of 8-oxodG in leukocyte DNA samples obtained from patients with chronic renal failure [67–69] and Fanconi anaemia [70] was also reported. In germ-line cells of men with type 1 diabetes, the content of 8-oxodG is increased up to 9 modified bases per 10^6 [71]. In patients with coronary artery disease, the levels of oxidative DNA damage correlate with the severity of the disease [72, 73]. Moreover, both in rats and in humans, the 8-oxodG content in DNA positively correlates with the age [74–79].

In the majority of studies, the levels of 8-oxodG were experimentally measured in total DNA extracted from the lysed cells or tissue samples. However, it is likely that some DNA molecules within the cell are substantially more prone to oxidative damage, for example, the mitochondrial DNA. As compared to the genomic DNA, the mtDNA shows substantial enrichment in GC nucleotide pairs; therefore, oxidized mtDNA may be disproportionate contributor to evaluated levels of 8-oxodG content in total DNA samples [80–82]. It should be noted that GC-rich fragment within genomic DNA tends to accumulate oxidative damage as well. One example of the preferentially oxidized DNA locus is the transcribed area of the ribosomal repeats [83].

All of this indicates that oxidation of DNA takes place in human cells as it is commonly observed both in health and disease. In cells undergoing oxidative stress and in chronic diseases, the levels of oxidative modifications of DNA increase substantially.

3. Extracellular DNA Is Enriched with the Oxidized Genomic DNA

The term "cell-free circulating DNA" (cfDNA) was coined for DNA fragments that could be collected from plasma, serum, or other bodily fluids. CfDNA circulates throughout the bloodstream of both healthy people and patients with various diseases. DNA isolated from cell-free supernatants of cells cultivated *in vitro* is known as extracellular DNA (ecDNA) [84]. EcDNA is found in the culture medium of both intact cells and cells exposed to various types of oxidative stress.

The most widely accepted hypothesis is that the main sources of cfDNA/ecDNA are the dead cells [85]. Another hypothesis suggests that cfDNA/ecDNA could be actively excreted into the medium by living cells [86]. Recently, cfDNA got recognition as a promising biomarker for noninvasive diagnostics and monitoring of various diseases [84]. However, the biological role of cfDNA in normal or pathological conditions remains unclear. The functionality of these circulating DNA fragments is determined by cfDNA properties, for example, its concentration in the blood plasma and the level of oxidative modification that can be approximated by its average content of 8-oxodG.

In plasma of healthy individuals, total cfDNA concentrations vary from 1 to ~100 ng/mL. These concentrations increase with age or in presence of various stressful conditions, for example, pregnancy, intensive exercise, or strong emotions as well as when malignancy or other chronic pathology is diagnosed. In plasma samples of patients with cancer or critical cardiovascular conditions, the concentrations of cfDNA increase up to 1000 ng/mL [84–92].

Oxidative stress is known to cause the DNA damage. The cells with the most damaged DNA die either by necrosis or by apoptosis. The oxidized DNA released from the dying cells is likely the most prominent contributor to cfDNA/ecDNA pool. Therefore, it is likely that cfDNA/ecDNA would contain larger amounts of 8-oxodG as compared to that in cellular DNA.

The comparative data describing 8-oxodG levels in cfDNA/ecDNA and in cellular DNA extracted from the same organism or cell culture are sparse. Some authors point that the levels of 8-oxodG in serum samples profiled by ELISA and by HPLC differ substantially [93, 94]. In our opinion, most likely explanation of these discrepancies is the fact that, in serum, 8-oxodG circulates both as free nucleoside and as part of oxidized cfDNA fragments. ELISA quantifies total concentrations of 8-oxodG that, in healthy donors, is around 0.3–5.9 ng/mL of plasma/serum [95–99] while HPLC-based techniques detect only free 8-oxodG that is present in plasma/serum samples at concentrations of 0.013–0.022 ng/mL [93, 94, 100–103]. One may calculate the minimal background content of 8-oxodG embedded in cfDNA fragments by subtracting bound 8-oxodG concentrations from total 8-oxodG concentrations ($0.3–0.022 \approx 0.28$ ng/mL). Given that maximal observed concentration of cfDNA is at ~1000 ng/mL, the minimal content of 8-oxodG in cfDNA could be estimated as at least 280 8-oxodG bases per 10^6 nucleotides, the figure that substantially exceeds estimated 8-oxodG content in DNA of living cells.

In our study we demonstrated that cfDNA/ecDNA is substantially enriched in 8-oxodG as compared to cellular DNA, up to 220–3000 8-oxodG per 10^6 nucleotides. The degrees of enrichment were significant when cfDNA/ecDNA and cellular DNA samples were evaluated for cancer or myocardial infarction patients, for primary tumor cells, as well as for endotheliocytes cultures that were irradiated or treated with peroxide [30, 33].

In addition to preferential enrichment of cfDNA pools with oxidized DNA of dying cells, the contents of 8-oxodG in cfDNA may depend on well-known phenomenon of somewhat slowed down degradation of GC-rich DNA fragments in human serum as compared to AT-rich fragments [21, 92, 104–106]. Moreover, under the condition of oxidative stress, an increase in proportions of mitochondrial DNA within cfDNA was documented [80–82]. This process is relevant as mitochondrial DNA, on average, contains larger amounts of 8-oxodG as compared to genomic DNA [107].

4. Oxidized cfDNA/ecDNA Is a Stress Signal

The cfDNA extracted from blood plasma of patients with high oxidative stress levels can significantly influence the physiological activity of intact cells. For example, when primary endotheliocytes (HUVECs) were exposed to cfDNA samples obtained from patients with hypertension and atherosclerosis, their NO contents substantially decreased, while the DNA samples obtained from healthy donors have no effect of NO release [28, 29]. In electrically paced cultures of ventricular neonatal rat myocytes, an exposure to the cfDNA of patients with acute myocardial infarction has produced a decrease in the frequency of contraction [108]. The cfDNA from ischemic rats decreased the levels of ROS production in neuronal cultures [32]. Both ecDNA collected from the media of primary tumor cells cultures and cfDNA extracted from plasma of cancer patients have influenced ROS production in mesenchymal stem cells (MSCs) [33]. Importantly, cfDNAs extracted from blood of myocardial infarction and rheumatoid arthritis patients stimulate the expression of DNA sensor toll-like receptor 9 (TLR9) in MSCs, while an exposure to gDNA did not influence TLR9 levels [35].

As observed both in endothelial cells and in MSCs, the samples of the genomic DNA that were oxidized *in vitro* with either H_2O_2 or Methylene Blue ($gDNA^{OX}$) evoke responses that are similar to those of cfDNA/ecDNA. In endothelial cells, exposure to $gDNA^{OX}$ stimulated an expression of *NOX4* and suppresses *eNOS*, therefore, augmenting net production of ROS and decreasing the levels of NO [34]. In mesenchymal stem cells, increased concentrations of $gDNA^{OX}$ and oxidized cfDNA/ecDNA stimulated a rapid increase in ROS synthesis and upregulated expression levels of the NF-E2-related factor-2 (NRF2), that plays a central role in antioxidant-response-element- (ARE-) mediated induction of phase II detoxifying and antioxidant enzymes along with a number of antioxidant response genes [33].

In murine macrophages, the treatment with GC-rich DNA fragments that are also enriched in 8-oxodG stimulates secretion of TNF-α [109]. The treatment of experimental animals with $gDNA^{OX}$ produced inflammation and induced production of DNA^{OX}-specific antibodies [110, 111].

An analysis of the data concerning cfDNA/ecDNA properties and the effects it produces on mammalian cells allowed us to suppose that ecDNA of irradiated cells ($ecDNA^{R}$) may somehow influence the other nonirradiated cells within the cell cultures thus acting as a soluble stress-signalization factor in a radiation-induced BE. Our further studies confirmed this assumption, having for the first time demonstrated the significance of the bystander signaling with participation of

oxidized extracellular DNA for human cells exposed to low-dose irradiation [22–27, 30, 31, 34].

5. Oxidized DNA-Dependent Signaling in Radiation-Induced Bystander Effect

5.1. $EcDNA^R$ from the Irradiated Cells Is the Signaling Factor in BE. The main source of the ecDNA is the dead or dying cells. In a number of recent studies we demonstrated that ionizing low-LET irradiation increases the rate of apoptosis in various cell cultures. It seems that some subpopulations of cultured cells possess an increased sensitivity to apoptosis that may be evoked by irradiation at low doses. To pursue this hypothesis, we isolated and characterized the population of irradiation-sensitive human lymphocytes. This subpopulation was rich in large-size activated cells, could spontaneously incorporate (3H)-thymidine, had increased radiosensitivity, and decreased activity of the excision repair, as well as a high level of spontaneous chromosomal aberrations and apoptosis, all these increasing after irradiation [112].

In our study, the apoptosis levels were assessed by evaluating the number of double-strand breaks (DSBs) in the genomic DNA by using a technique based on visualization of phosphorylated protein H2AX (γ-foci) in the site of rupture. Accumulation of γ-foci in large amounts is indicative of the apoptosis [113]. Both in HUVECs and MSCs [26, 30], an irradiation is followed by accumulation of γ-foci. In peripheral blood lymphocytes, an irradiation leads to an increase in the activity of caspase-3, one of the main cysteine proteases activated in apoptosis [20, 22, 23]. After irradiation, the dying cells release the fragments of chromatin, thus, contributing to the pool of ecDNA/cfDNA.

The electrophoretic analysis shows that the size of ecDNA fragments produced by cultured cells varies from 180 to 20,000 bp, with a predominance of the fragments 180 and 360 bp in size that corresponds to mono- and dinucleosomes, respectively [20, 22, 30]. After irradiation, the concentration of longer fragments decreases and that of the short ones increases. EcDNA of irradiated cells contained significantly larger amounts of oxidation marker 8-oxodG than ecDNA of control (nonirradiated) cells or cellular DNA of irradiated cells [30].

The studies of the bystander effect were performed in various cell types, including G0-lymphocytes of peripheral blood [22–24, 27], HUVECs [30, 34], and MSCs of adipose tissue [26, 31]. Ionizing radiation is known to render both a direct effect on cellular structures via hitting with an energy quantum or particle and an indirect effect mediated by free radicals [114]. The cellular response to irradiation depends on many factors, but the most important of them is a substantial increase in the levels of ROS. Ionization results in synthesis of ROS. The process of ROS formation after exposure to radiation takes place within the time frame of several seconds to 2–5 minutes [115]. In turn, ROS induces multiple lesions in cellular DNA, including the ruptures of desoxyribose rings, the appearance of apurinic and apyrimidinic sites, single- and double-strand breaks, DNA protein cross-links, and formation of oxidized bases [116–122].

Importantly, in control (nonirradiated) cells, the ecDNA collected from the media conditioned by irradiated cells stimulates an increase on the ROS production to approximately the same degree as DNA oxidized *in vitro* or small doses of irradiation [34]. This indicates that ecDNA released from dying irradiated cells may serve as a stress signal that conveys a bystander effect, while ecDNA of nonirradiated cells is not a stress signal as it does not induce ROS synthesis in control cells.

Various parameters of the target cell and bystander cells are being analyzed in regards to irradiation and its effects. The most commonly studied group of such parameters includes a number of cytological characteristics of cellular nuclei, including the shape of the nucleus as well as FISH-defined descriptors of chromosomal territories, that is, positions of chromosome loci as they relate to the centre of the nucleus and to each other [18]. One of the known markers for irradiation-induced chromatin rearrangement is a position of pericentromeric loci of chromosome 1 (1q12). When effects of irradiation at a dose of 10cGy were compared to those of direct oxidative stress causing agent H_2O_2, of exposure to ecDNA extracted from the media conditioned by irradiated cells ($ecDNA^R$) or of exposure to ecDNA extracted from H_2O_2-treated cells ($ecDNA^{H_2O_2}$), similar structural rearrangements of chromatin were observed. Particularly, there was a decrease of the proportion of cells with the perimembranous location of loci at 1q12 a nd an increase in the proportion of the cells with central nuclear localization of these loci. It was also shown that exposure induces approximation of the loci 1q12 of homologous chromosomes 1 within the space of the cellular nucleus [20, 22, 23, 26, 27, 30, 31]. Additionally, the nuclei of both HUVECs and adipose-derived MSCs acquired a compacted, more spherical shape [26, 30, 31]. All these effects were primarily dependent on an increase in the production of ROS that was approximately to the same degree stimulated by irradiation, H_2O_2, $ecDNA^R$, or $ecDNA^{H_2O_2}$. When an inhibitor of ROS, α-tocopherol, was added to the media, all these effects were blocked.

Irradiation-dependent chromosomal loci relocation effects were confirmed by other researchers. The transposition of chromatin regions within the nucleus is accompanied by DNA repair [123]. Moreover, within the nuclei of irradiated cells, the convergence of homologous chromosomes in the sites of DSB emergency repair has been observed [124]. The structural rearrangement of chromatin promotes the launch of gene expression program that is necessary for the development of the adaptive response, with the approximation of chromosomes themselves being an event favorable for further elimination of DSBs through the repair associated with chromosomal homologous recombination (HR) or non-homologous end joining (NHEJ). The irradiation-dependent chromosomal loci transposition had been demonstrated in lymphocytes [18–20, 22–24, 27], human endothelial cells [30], mesenchymal stem cells [26, 31], and in cancer stem cells of the mammary gland [25], thus, strongly suggesting that these effects are widespread. It should be mentioned that when irradiated cells fail to transpose the marker loci following

irradiation, elevated levels of cell death are observed already at very low doses of ionizing radiation [25].

The structural transformations of chromatin mentioned above are accompanied by activation of ribosomal gene transcription which may be evidenced by staining the cellular preparations with silver nitrate or assessing rRNA levels by quantitative PCR. ROS-dependent induction of adaptive response implies an increase in the synthesis of proteins, primarily of repair proteins and those necessary for reorganization of the genome. Therefore, an enhanced transcription of ribosomal genes and an elevated amount of rRNA in ROS stimulated cell is to be expected [19, 23, 27, 30].

An increase in F-actin polymerization was observed both in irradiated cells and in ecDNAR-treated bystander cells. Alterations in the architecture of the cellular cytoskeleton observed after exposure to X-ray radiation and ecDNAR are similar as well [30]. Our findings suggest that alterations in the architectonics of the cellular cytoskeleton appear both after exposure to X-ray radiation and ecDNA^R [30].

The study also showed that addition of ecDNA^R into the growth medium of intact endotheliocytes leads to a decrease in the number of cells with single γ-foci and to a considerable increase in the number of apoptotic cells in the population [30]. Similar effects were observed when cells were irradiated in low doses. This study supports the findings of other authors having shown that an incubation medium of irradiated cells induces the initial stages of the apoptotic cascade in bystander cells. In these experiments, an induction of apoptosis in bystander cells was also accompanied by an elevation in the content of ROS within 6-hour time frame [125].

The mirror-like patterns of the effects described above and seen in both treated and bystander cells point to the transfer of a stimulus from irradiated to bystander cells. An addition of the ecDNA produced by control (nonirradiated) cells to the medium of bystander cells does not produce any of the effects described above, and no adaptive response is observed. Interestingly, after the hydrolysis by DNAse I, the ecDNA^R produced by irradiated cells loses its stress signal properties and its ability to evoke an adaptive response [20, 22].

It is also important to note that BE is not cell-type specific. The media conditioned by irradiated cells of one cellular type conveys the bystander effect to other kinds of bystander cells exposed to this media [24]. Similarly, ecDNA^R extracted from the growth medium of irradiated endotheliocytes conveys an adaptive response in the bystander MSCs and *vice versa* (data not published).

5.2. EcDNA Signal Propagates with Aid of Oxidative Stress. The data described above indicate that the cascade of sequential events in ecDNA-signaling may be as follows:

Irradiation $\rightarrow$ [*primary oxidative stress* $\rightarrow$ oxidation of gDNA $\rightarrow$ apoptosis of some portion of irradiated cells $\rightarrow$ release of oxidized ecDNA^R $\rightarrow$ reception of the ecDNA^R signal by the bystander cells $\rightarrow$ *secondary oxidative stress*] $\rightarrow$ oxidation of gDNA in the bystander cells $\rightarrow$ apoptosis of some portion of bystander cells $\rightarrow$ release of oxidized ecDNA, and so forth.

In this cascade, the oxidative stress propagates from irradiated cells to bystander cells (Figure 1). The secondary oxidative stress that is evoked in intact bystander cells occurs after an interaction of the oxidized ecDNA^R with its receptors, or oxidized DNA sensors, that must be present on the surface or inside the bystander cells. The possible candidates for these sensors are the transmembrane proteins of the toll-like receptor family, namely, TLR9 [126]. Being transmembrane receptors, they contain a repetitive LRR domain capable of binding the ligand and a highly conservative intracellular region that ensures the interaction between the receptors and the molecules of the downstream signaling pathway, for example, an adapter protein MyD88. It is well known that the DNA fragments with unmethylated CpG motifs may serve as TLR9 ligands. In this cascade, the formation of the "DNA-TLR9" complex initiates the cellular signaling pathway that, in turn, leads to an activation of the transcription factor NF-κB, which in many different ways augments the biosynthesis of ROS. For example, TLR9 ligation may be followed by an increase in intranuclear production of $NO^\bullet$ [127, 128] or O_2^- radical [129]. In human monocytes, the binding of CpG-DNA to TLR9 is accompanied by secretion of both $NO^\bullet$ and ROS [130], while in neutrophils it leads to the production of peroxynitrite [127]. The slow-acting oxidants $O_2^{\bullet-}$, NO, and H_2O_2 are produced by sequence of metal ion-dependent enzymatic reactions that, in turn, may give rise to highly reactive compounds: $OH^\bullet$ and hypohalogenous acids, as well as $^1O_2$, $NO^\bullet$, and $NO_2^\bullet$. During bystander effect, possible participation of the Fenton reaction is evidenced by the studies that showed that the radiation-induced adaptive response depends on the production of the signal molecule NO [11, 131]. Interestingly, in macrophages, the substitution of dG with 8-oxodG in the DNA ligand for TLR9 is accompanied by a significant increase in TNF-α cytokine [109]. In other words, an oxidized DNA seems to be a stronger TLR9-stimulating ligand than nonoxidized DNA.

In our opinion, oxidized DNA is one of the components of damage-associated molecular pattern molecules (DAMPs). Its effects can potentially increase when exposure to oxidized DNA is concomitant with the presence of other DAMPs. It might be that effects of oxidized DNA are at least in part mediated by high mobility group box 1 (HMGB1) protein whose expression is enhanced after irradiation. HMGB1 functions as an extracellular damage-associated molecular pattern molecule that promotes inflammation, cellular differentiation, survival, and migration [132–136]. HMGB1 was shown as an essential component of DNA-containing complexes that stimulated cytokine production through a TLR9-MyD88 pathway. Extracellular HMGB1 accelerates the delivery of CpG-DNAs to its receptor, leading to a TLR9-dependent augmentation of IL-6, IL-12, and TNFα secretion [137–143]. There is evidence that HMGB1 protein binds preferentially to damaged DNA [144]. It was also shown that extracellular histones directly interact with TLR9 and enhance DNA-mediated TLR9 activation in immune cells [145].

In the populations of irradiated lymphocytes, the expression of *TLR9* gene and the main adaptor of its signaling pathway MyD88 increase severalfold [27]. In order to confirm

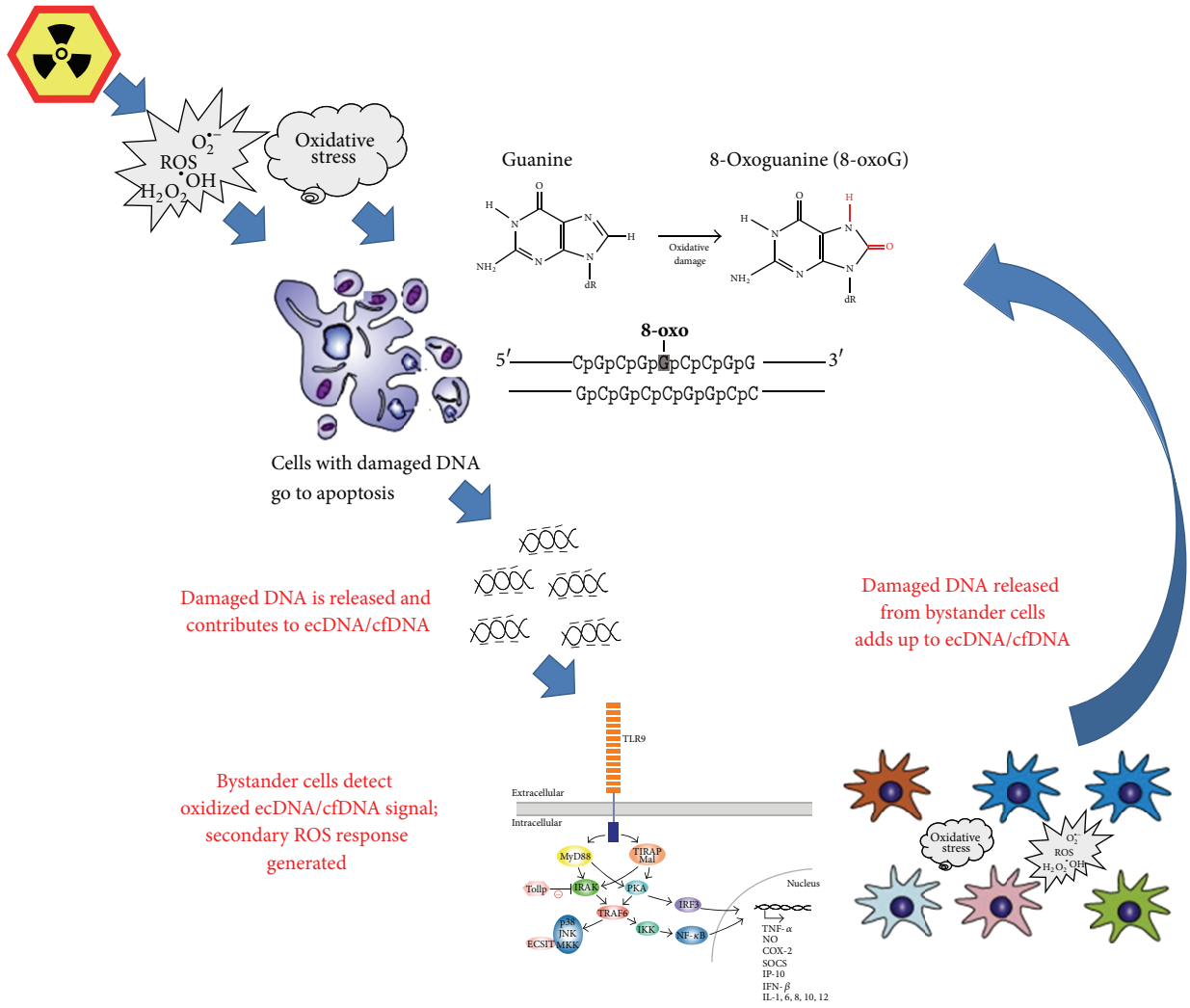

Figure 1: The proposed mechanisms for the propagation of the stress signal from irradiated cells to bystander cells. In this scheme, the 8-oxo-dG serves as a model example of DNA lesion that turns DNA fragments into the stress signal; it should be noted that other types of DNA lesions may be recognized as well. The central player that ensures amplification of the signal in this cascade is the oxidative stress. The secondary oxidative stress evoked in intact bystander cells occurs after an interaction of the oxidized ecDNA with the receptors, or oxidized DNA sensors, that must be present on the surface or inside the bystander cells. One possible candidate for oxidized DNA sensor is toll-like receptor TLR9.

participation of TLR9 in bystander DNA-signaling during the development of BE, we blocked these receptors by two inhibitors, a specific oligonucleotide suppressor that provides considerable competition in binding the ligand with the receptor or nonspecific inhibitor chloroquine that changes the pH value in endosome and makes the formation of the DNA-receptor complexes unlikely. When TLR9 pathway was blocked, there were no substantial changes in the localization of 1q12 loci and in the level of NO in bystander cell exposed to $ecDNA^R$ from irradiated cells. However, both an increase in the levels of ROS production and an activation of ribosomal genes still took place [23, 30, 34]. These findings suggest that radiation-induced bystander effect may be propagated through more than one molecular pathway. In addition to oxidized DNA-stimulated TLR9 receptors, other sensors, whose activation leads to the changes in ROS and rRNA expression levels but does not lead to transposition of the analyzed chromosomal loci in the nucleus, may be involved. Evidence pointing at existence of toll-like-receptor-independent stress signal transfer pathways was previously demonstrated by other authors [146, 147], including cytoplasmic DNA-dependent STING, AIM2, RIG-1, and DAI sensor pathways [148]. Some of these pathways are directly linked to apoptosis induction by ecDNA fragments (AIM2); others stimulate pro- and anti-inflammatory cytokine synthesis. It is possible that $ecDNA^R$ may be uptaken to penetrate into the cytoplasm and activate those pathways. It is also very well might be that eukaryotic cells contain a variety of the molecules that sense the damage in cell-free DNA, and that these cells may differentially respond to a variety of oxidized or otherwise

modified DNA bases. The reception of ecDNA produced by irradiated cells warrants further investigations.

After exposure of human lymphocytes to X-ray radiation at a low dose, adaptive reaction develops within 4–6 hours. It is known that such response takes a few cell cycles [149, 150] even longer [125, 151, 152]. Although the persistence of irradiation effects has been described in the literature about 50 years ago, its mechanisms are still to be determined. However, it is very likely that one of the main components of the long-term process of irradiation response is the generation of ROS that may remain elevated for many cellular generations [153, 154]. In our opinion, the oxidative stress may be maintained in cells after the initial irradiation event as a result of sustained activation of oxidized ecDNA-signaling pathway. This activation may be maintained by the fragments of oxidized ecDNA released from irradiated cells that die by apoptosis and release their damaged DNA into circulation or cell media. When the fragments of oxidized ecDNA interact with recipient "bystander" cells, it evokes secondary oxidative stress in some bystander cells. In turn, these bystander cells initiate apoptotic cascades that lead to further release of oxidized ecDNA. The mechanism described above takes into account previous observations that the daughter populations derived from irradiated cells retain an elevated level of ROS that play a substantial role in maintaining the adaptive response throughout cell generations [155].

6. Conclusion

Irradiation, chronic diseases, or other prooxidative stimuli and conditions lead to an increase in oxidative stress and in oxidation of cellular DNA. In case of apoptotic death of stressed cells, oxidized DNA ends up released in cell culture medium (ecDNA) or in circulation (cfDNA). In cultured cells, oxidized ecDNA serves as a stress signal that is transmitted from stressed (i.e., irradiated) cells to bystander cells. It is tempting to speculate that a similar process takes place in human body challenged with focused irradiation or suffering from chronic disease. In human cells, oxidized DNA induces additional synthesis of ROS. When ecDNA/cfDNA = dependent increase in ROS levels remains moderate, the bystander cells develop an adaptive response, that is, at least in part, due to an activation of the transcription factor NRF2, which is capable of inducing antioxidant expression program.

So far, no studies demonstrating that oxidized cfDNA may play a role in bystander effect *in vivo* were published. Effects of exposure to oxidized cfDNA should be taken into account when treating tumors with various ROS-producing agents and irradiation. As oxidized cfDNA released from the dying tumor cells enters the circulation, it is being carried to the distant organs, with its effects expected to be systemic. For example, the damaged DNA released from irradiated cells may be responsible for abscopal effects that are suspected to be depended on actions of immune system, in particular, the ones mediated by TLRs. It is possible that artificial modulation of concentration, GC-content, and the level of oxidation of cfDNA may improve clinical outcomes in patients with various chronic diseases accompanied by extensive cell death. The data summarized above indicate the necessity for further study of the effects of oxidized DNA in both *in vitro* and *in vivo* systems.

Abbreviations

AR: Adaptive responses
BE: Bystander effect
DSBs: DNA double-strand breaks
EcDNA: Extracellular DNA
OS: Oxidative stress
ROS: Reactive oxygen species
HUVEC: Human umbilical vein endothelial cells
MSC: Mesenchymal stem cells.

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Research Article

Oxidative Stress Is Related to the Deleterious Effects of Heme Oxygenase-1 in an *In Vivo* Neuroinflammatory Rat Model

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Heme oxygenase-1 (HO-1) induction is associated with beneficial or deleterious effects depending on the experimental conditions adopted and the neurodegenerative rodent models used. The present study aimed first to evaluate the effects of cerebral HO-1 induction in an *in vivo* rat model of neuroinflammation by intrastriatal injection of quinolinic acid (QA) and secondly to explore the role played by reactive oxygen species (ROS) and free iron (Fe^{2+}) derived from heme catabolism promoted by HO-1. Chronic I.P. treatment with the HO-1 inductor and substrate hemin was responsible for a significant dose-related increase of cerebral HO-1 production. Brain tissue loss, microglial activation, and neuronal death were significantly higher in rats receiving QA plus hemin (H-QA) versus QA and controls. Significant increase of ROS production in H-QA rat brain was inhibited by the specific HO-1 inhibitor ZnPP which supports the idea that ROS level augmentation in hemin-treated animals is a direct consequence of HO-1 induction. The cerebral tissue loss and ROS level in hemin-treated rats receiving the iron chelator deferoxamine were significantly decreased, demonstrating the involvement of Fe^{2+} in brain ROS production. Therefore, the deleterious effects of HO-1 expression in this *in vivo* neuroinflammatory model were linked to a hyperproduction of ROS, itself promoted by free iron liberation.

1. Introduction

Neuroinflammation is well known as an important element of brain disorders and in particular neurodegenerative diseases [1]. Microglial cells play a role often compared to the macrophage function in the central nervous system [2, 3]. Quiescent microglia are activated in the occurrence of brain damage such as oxidative stress, leading to an inflammatory cascade response. This activation is characterized by morphologic modifications and mostly secretion of proinflammatory factors such as cytokines or reactive oxygen species (ROS) [4, 5]. Without appropriate regulation, those proinflammatory agents cause brain damage to worsen. Therefore neuroinflammation is a relevant target on purpose to treat neurodegenerative diseases. Many experimental studies have been carried out to explore the effects of anti-inflammatory treatments against neurodegeneration especially in animal models, demonstrating that treatment with nonsteroidal anti-inflammatory (NSAIDs) drugs [6–8] or nitric-oxide-releasing NSAIDs [9] could have a beneficial effect on neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease, or multiple sclerosis. However, prospective anti-inflammatory strategies against disease progression in human subjects with established AD have failed to show significant positive results [10].

Heme oxygenase (HO) is the final enzyme involved in the degradation of heme [11]. The inducible isoform HO-1 has been implicated in the regulation of inflammation and this enzyme is overexpressed in response to different stimuli such as oxidative and nitrosative stresses [12]. The induction of HO-1 increases the heme catabolism into biliverdin and bilirubin, potent antioxidant scavenging peroxy radicals, and inhibits lipid peroxidation [13].

Previous studies have reported controversial effects of HO-1 induction, either deleterious or beneficial depending on the different neuroinflammatory models and various

drug exposure methods. For example, in microglial cell cultures, HO-1 induction has been shown as protective in a model of neurotoxicity induced by glutamate [14]. In experimental autoimmune encephalomyelitis, HO-1 induction demonstrated a protective role by inhibiting major histocompatibility complex II expression and lymphocyte proliferation [15]. Conversely, HO-1 induction was associated with a deleterious iron accumulation in cultured astrocytes [16] as well as in activated microglia in a rodent stroke model [17]. The anti-inflammatory role of the products of heme degradation and the potential activation of HO-1 in the brain support the potential interest of this enzyme in neuroinflammation treatment. We hypothesized that deleterious HO-1 activation effects are linked to products resulting from heme degradation during brain inflammation.

In order to test this hypothesis, we experimentally increased the expression of HO-1 using the specific inducer hemin, in a rat model of neuroinflammation obtained by unilateral striatal injection of quinolinic acid (QA). This well-known model of Huntington's diseases [18] has recently been shown to be useful to study the overexpression of the translocator protein (TSPO) as a relevant marker of neuroinflammation [19]. QA is a strong agonist of glutamate NMDA (*N*-methyl-*D*-aspartate) receptors. Overactivation of NMDA receptors causes a massive intracellular influx of calcium that leads to neuronal death by activation of various enzymes (lipases, proteases, endonucleases) triggering different cell components then leading to neuronal death [20]. Factors released during the death of neurons rapidly lead to an important microglial activation.

Therefore, the purpose of the present study was first to assess *in vivo* the effects of the HO-1 inducer hemin on both neuronal survival and microglial activation in the neuroinflammatory excitotoxic rat model induced using QA intrastriatal injection and secondly to investigate the underlying mechanisms, especially focusing on ROS production in brain structures and the hypothetic role of iron derived from HO-1 enzymatic activity.

2. Materials and Methods

2.1. Animals. Sixty-six male Wistar rats (Janvier, l'Arbresle, France) weighing ~340 ±10 g were used. The experiments were performed in accordance with the Guideline for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and with European Directives (86/609/CEE) and approved by local ethical committee (Agreement no. 2012-03-1). Rats were kept in a temperature (23 ± 0.5°C)—and humidity (43 ± 8%)—controlled environment under a 12 h light-dark cycle with food and water available ad libitum. All efforts were made to minimize animal suffering and discomfort.

2.2. Determination of the Relevant Hemin Dose. We first investigated the potential dose-related effect of chronic intraperitoneal (I.P.) treatment with hemin on cerebral expression levels of HO-1. Based on a previously published study [21], rats received a hemin solution in a daily dose of 10 mg/kg ($n = 3$) or 50 mg/kg ($n = 3$) over a 4-day period (final volume of 100 μL by injection, in DMSO). Hemin-treated rats were compared to a control group receiving its vehicle DMSO (100 μL; daily I.P.; $n = 3$). The DMSO solvent is known to be a strong antioxidant compound [22] but the volume (100 μL) necessary to solubilize hemin for I.P. injection has been already used in previous studies without influence on biological parameters and the anti-inflammatory properties of DMSO reported in the literature considered large amounts of DMSO up to 6 mL/kg [23]. On day 5, the animals were euthanized by decapitation and their brains were removed to analyze HO-1 expression by western blotting (WB).

The entire right hemispheres were rapidly crushed by Turrax, homogenized with lysis buffer 1% sodium deoxycholate, 0.1% SDS, 1% tritonX-100, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and an inhibitor protease cocktail (chymotrypsin, thermolysin papain, pronase, pancreatic extract and trypsin inhibitor; Roche), and centrifuged at 20,000 g for 1 hour. After denaturation by boiling (100°C, 5 min), beta mercaptoethanol and bromophenol blue were added to samples. Protein quantification was performed according to the Bradford method and then 25 mg of protein were separated on a 10% polyacrylamide gel and were transferred (30 min, 110 V) onto a nitrocellulose membrane (Amersham). Blots were blocked for 2 hours at room temperature with 5% (v/v) nonfat dried milk in Tris-buffered saline [10 mM Tris-HCl (pH 8.0) and 150 mM NaCl] containing 0.05% Tween 20. The membrane was incubated overnight at 4°C with rabbit polyclonal antibody against HO-1 (1/400, Bio-Rad, Marnes-la-Coquette, France) or with a mouse polyclonal antibody against rat beta-3 tubulin used as housekeeping protein (1/400, Tubb3, Santa Cruz Biotechnologies, CA, USA). The blot was then incubated with the conjugated goat antirabbit or antimouse horseradish peroxidase (1/1000, BioSource) for 2 h at room temperature. Immunoreactive proteins were detected with the ECL western blotting detection system (Amersham) using an imager (Gel doc XRS +, Biorad, Marnes-la-Coquette, France). The results were analyzed with Quantity One (Biorad, Marnes-la-Coquette, France).

2.3. Excitotoxic Neuroinflammation Model. One day after the first I.P. injection of hemin or its vehicle, the rats were anesthetized with isoflurane (4% for anesthesia induction and thereafter 2% for its maintenance) and placed in a stereotaxic David Kopf apparatus (tooth bar: −3.3 mm). The animals were unilaterally injected with 150 nmol of quinolinic acid (QA) (Sigma-Aldrich, Lyon, France) or its vehicle (0.1 M PBS, pH 7.4) into the right striatum (injection rate: 0.5 μL/min) using a 25-μL microsyringe (Hamilton, Bonaduz, Switzerland) and a micropump (KD Scientific, Holliston, MA, USA). Two microliters of QA were injected at the following coordinates: AP: +0.7 mm; ML: −3 mm; DV: −5.5 mm from bregma, according to Paxinos and Watson [24]. Body temperature (36.9 ± 0.6°C) was monitored during the surgery with a thermal probe. The injection syringe was left in place for additional 4 min to avoid QA backflow and

then slowly removed. The scalp was sutured and animals replaced in their cages and examined daily until sacrifice.

2.4. Experimental Procedure and Drug Treatment. Drugs or vehicles for control were administered I.P. the day before surgery, 2 h before surgery, and daily for 2 days. Consecutively to the above study (see Section 3), rats treated by hemin received 50 mg/kg I.P.

2.4.1. Effect of Hemin Treatment on Neuron Survival and Microglia Activation. The animals were randomly divided as follows: control group (DMSO I.P.; PBS intrastriatal; $n = 6$); QA (DMSO I.P.; QA 150 nmol/2 μL intrastriatal; $n = 6$); H (hemin 50 mg/kg; PBS intrastriatal; $n = 6$); H-QA (hemin 50 mg/kg I.P.; QA 150 nmol/2 μL intrastriatal; $n = 6$). At day 3 after surgery, the rats were euthanized for immunohistochemistry (IHC) processing. The rats were deeply anesthetized by I.P. injection of pentobarbital (Céva Santé Animale, Paris, France), perfused through the heart with 250 mL of heparinized (Héparine Choay, Sanofi-Aventis, Vitry-sur-Seine, France) saline (1 UI/mL of saline), and then followed by 400 mL of 4% paraformaldehyde (PFA, Sigma-Aldrich, Lyon, France). The brains were removed and fixed in 4% PFA for 2 h then stored 48 h in 30% sucrose and frozen at −80°C.

Five transversal sections 40 μm thick of both the striatum and hippocampus regions were used for IHC staining of neurons or activated microglia. Endogenous peroxidase was blocked using 3% H_2O_2 in 10% methanol and distilled water for 15 min. Slices were incubated overnight at room temperature with primary antibodies 1 : 500 diluted NeuN (Millipore, Molsheim, France) or 1 : 500-diluted Ox-42 (AbD Serotec, Düsseldorf, Germany), 0.1 M PBS supplemented with 0.2% v/v of Tween and 2% v/v of normal horse serum. As we used horse secondary antibodies, nonspecific binding sites were blocked by adding 2% horse serum and then incubated simultaneously with primary antibodies overnight at room temperature. Sections were then incubated with biotinylated horse anti-mouse IgG secondary antibodies (AbCys, Paris, France) for 90 min at room temperature. Neurons or activated microglia were visualized by staining with streptavidin-biotin-conjugated horseradish peroxidase (AbCys, Paris, France) for 60 min at room temperature. Peroxidase was developed for 3 min with diaminobenzidine at room temperature. Slices were analyzed under a light binocular microscope (Leica, Wetzlar, Germany) and histological analysis was performed with Histolab imagery software (US Histology Laboratories, Rockville, USA). Neuron survival and microglia activation were analyzed in 2 different areas for each brain: the area of QA injection (bregma +0.7 mm) and the cortical area at a distance from the site of injury (bregma −3 mm). The neurons were visually counted and the percentage of neuronal loss in the ipsi- versus contra-lateral hemisphere was calculated in matching areas of analysis. For Ox-42, the total surface area occupied by activated microglia was automatically measured by the imagery software and the percentage of increase in ipsi- versus contralateral hemisphere was measured for each section. Five striatal slices were made within the QA injection site (from Bregma +0.6 to Bregma +0.8). Three areas per hemisphere were then randomly selected and neurons and surface occupied by activated microglia were counted by 2 independent operators. The surface area of tissue destruction in striatum was measured using Beta-Vision Plus software (Biospace Lab, Paris, France) and the percentage of tissue loss in ipsi- versus contra-lateral hemisphere was calculated on 5 slices per animal.

2.4.2. Effect of Hemin Treatment on Cerebral Reactive Oxygen Species Production. To understand the results observed in hemin-treated rats (see Section 3), a complementary study was performed focusing on the effect of HO-1 activity on ROS production in neurons of the striatum. The specificity of HO-1 influence on ROS was tested by adding a group of animals exposed to the inhibitor of HO-1 activity zinc protoporphyrin IX (ZnPP, Sigma-Aldrich) in a concentration of 50 mg/kg 24 h and 2 h before surgery [25].

Twenty rats were separated into 4 groups as follows: control group (DMSO I.P.; PBS intrastriatal; $n = 5$); QA (DMSO I.P.; QA 150 nmol/2 μL intrastriatal; $n = 5$); H-QA (hemin 50 mg/kg I.P.; QA 150 nmol/2 μL intrastriatal; $n = 5$); ZnPP-H-QA (ZnPP 50 mg/kg I.P.; hemin 50 mg/kg I.P.; QA 150 nmol/2 μL intrastriatal; $n = 5$). Bearing in mind the short life span of ROS [26], the influence of QA and hemin on ROS expression levels was determined in the striatum at 1 h after QA injection in both QA and H-QA groups. ROS production was quantified by measuring the fluorescence of dihydroethidium (DHE, Sigma-Aldrich, Lyon, France,) which is oxidized into ethidium and 2-hydroxy ethidium by intracellular ROS [27]. After intercalation into DNA, both products derived from DHE oxidation emit a red fluorescence proportional to ROS production. Therefore, neuron immunofluorescence detection and ROS quantification were performed simultaneously to normalize ROS fluorescence measurements to the number of preserved neurons after QA injection. One hour after surgery, the animals were deeply anesthetized by I.P. injection of pentobarbital and perfused through the heart with 250 mL of heparinized saline (1 UI/mL of saline) to limit noise floor caused by blood autofluorescence [28]. ROS quantification in frozen tissue has already been described in the literature especially with brain material [29–31]. After perfusion, the brains were removed and snap-frozen at −80°C. Sections (20 μm) were fixed in PFA 4% (15 min) for neuron immunostaining, before saturation of nonspecific sites with normal horse serum (1 : 200) for 30 min at room temperature and incubation with 1 : 500 diluted primary antibody NeuN (Millipore, Molsheim, France) overnight at room temperature. Revelation was made using a secondary antibody fluorescein isothiocyanate (FITC) conjugated (1 : 200, Rockland Immunochemicals, Gilbertsville, USA) for 3 h at room temperature. ROS analyzing was performed using DHE (4 μM, Sigma-Aldrich, Lyon, France) applied to slides then cover-slipped before incubation (37°C, 30 min) in a dark humidified chamber. Preliminary study was performed to check out that the tissue fixation did not alter

the DHE signal. We compared fluorescence in frozen versus fixed/frozen brain slices and then confirmed that the DHE signal was unchanged regardless of the fixation (unpublished data). FITC and DHE fluorescence were measured using an Olympus BX51 microscope and analyzed with Cell D (Olympus, Hamburg, Germany) to measure the intensity of fluorescence and Image J (Rasband, WS, Image J, US National Institute of Health, Bethesda, MD, USA) to manually perform neuron counting. Then, the intensity of ROS fluorescence in surviving neurons population and the ratio augmentation rate (%) in ipsi- versus the contra-lateral hemisphere was calculated.

2.4.3. Influence of Ferrous Iron (Fe^{2+}) Chelator Treatment on Hemin Effects. Given that ferrous iron (Fe^{2+}) is a product of heme, we investigated the influence of the Fe^{2+} chelator deferoxamine (DFX) on neuron survival, microglia activation, tissue destruction, and ROS production in QA and hemin-treated rats. The compound was administered chronically according to the same schedule as hemin in 2 additional groups of animals as follows: DFX-QA (DFX, 150 mg/kg I.P.; QA 150 nmol/2 μL intrastriatal; $n = 5$ for IHC) and DFX-H-QA (DFX, 150 mg/kg I.P.; hemin 50 mg/kg I.P.; QA 150 nmol/2 μL intrastriatal; $n = 5$ for IHC; $n = 3$ for ROS analysis). IHC and ROS measurement were performed according to the methodology described above.

2.5. Statistical Analysis. Results are expressed as means ± SEM. Data for multiple variable comparisons were analyzed by a one-way ANOVA followed by a Newman-Keul's test as a posthoc test using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). The level of significance was $P < 0.05$.

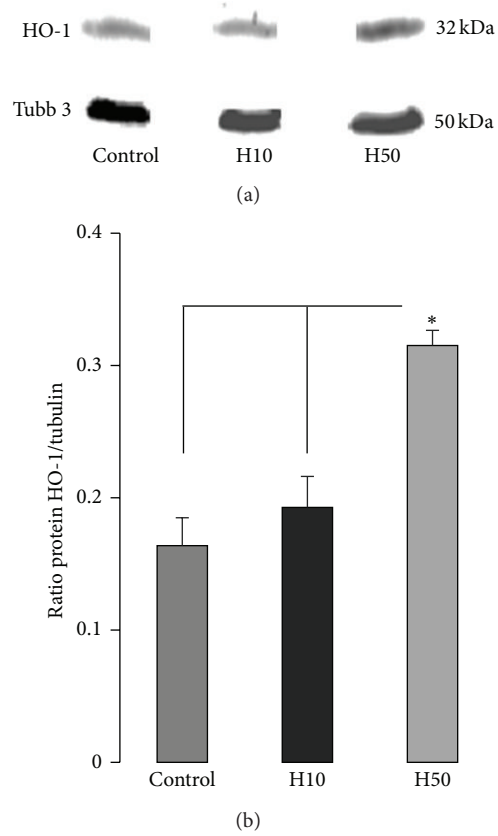

Figure 1: Dose effect of systemic hemin treatment on HO-1 protein expression in the brain (a) HO-1 and tubuline beta 3 (Tubb 3) western blotting bands for control ($n = 3$), hemin 10 ($n = 3$), and hemin 50 mg/kg ($n = 3$) groups. (b) DO of HO-1 normalized with Tubb-3. $^*P < 0.05$.

3. Results

3.1. Influence of Chronic Hemin Treatment on HO-1 Expression in the Brain. This preliminary study aimed to evaluate the effect of 4-day chronic treatment with either 10 or 50 mg/kg of the HO-1 inducer hemin on HO-1 protein expression levels in the rat brain. Western blotting measurements showed a significant ($P < 0.05$) increase of HO-1 protein expression in rats exposed to 50 mg/kg hemin versus 10 mg/kg and control (Figures 1(a) and 1(b)). Results normalized with tubulin protein are illustrated in Figure 1(b). Therefore, the highest dose of hemin (50 mg/kg) was used to evaluate the influence of HO-1 induction in the neuroinflammatory *in vivo* model.

3.2. Effect of Hemin Treatment and Influence of Ferrous Iron Chelator DFX on Neuron Survival, Cerebral Macroscopic Integrity, and Microglial Activation

3.2.1. In the Area of Quinolinic Acid Injection: Bregma +0.7 mm. Results are illustrated as a percentage of tissue loss in the ipsilateral hemisphere for all groups in Figure 2. In QA ($n = 6$), H-QA ($n = 5$), DFX-QA, and DFX-H-QA ($n = 5$) groups, major tissue destruction within the striatum did not allow neuron survival or microglia activation analysis. We observed in the ipsilateral cerebral hemisphere exposed to QA injection a major tissue loss (17 ± 6.5%) significantly ($P < 0.01$) worsened by hemin treatment (51 ± 9.0% in H-QA animals). DFX treatment significantly ($P < 0.05$) decreased tissue loss caused by hemin (33 ± 4.6% in DFX-H-QA group) but had no effect on QA alone (20.0 ± 4.7% in DFX-QA group, $n = 5$). No cerebral tissue loss was observed, except mechanical lesions consequent to the needle injection, in control and H groups.

NeuN IHC was performed by 2 independent operators. For each brain, 5 slices were processed and 3 areas in the striatum per hemisphere were used for the measurement. Neuronal loss in the ipsilateral hemisphere did not show any significant difference between H and control groups (Figure 3).

Microglial activation was analyzed using Ox-42, an antibody specific to CD11b expressed in activated microglia. The relative area of brain slices occupied by activated microglial cells was measured in 3 areas per hemisphere (5 slices) of control and H groups and the overall level in the ipsi- and contralateral hemispheres was compared. No significant difference was observed between the animals.

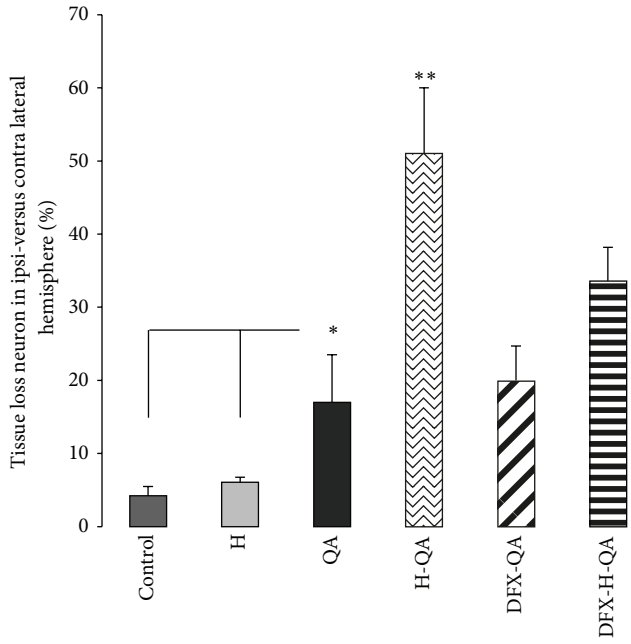

Figure 2: Relative cerebral tissue loss in the ipsilateral hemisphere versus the contralateral hemisphere 3 days after injury in control ($n = 6$), H ($n = 5$), QA ($n = 6$), H-QA ($n = 5$), DFX-QA ($n = 5$) and DFX-H-QA ($n = 5$) groups. $^{*}P < 0.05$; $^{**}P < 0.01$.

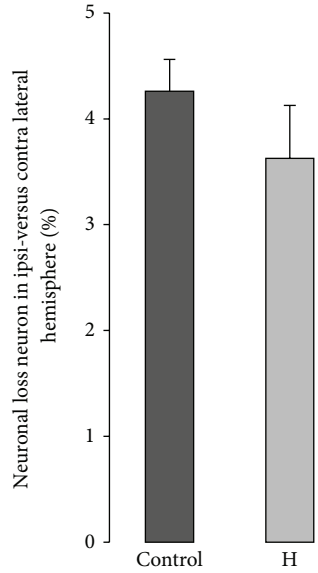

Figure 3: Relative neuronal loss in the ipsi- versus contra-lateral striatum in control and H groups 3 days after striatal injection.

3.2.2. In a Cortical Area Remote from the Site of Injury: Bregma −3 mm. In this area remote from the site of injury (Figure 4(a)), no difference in the number of neurons was found between contra- and ipsilateral cortex, regardless of the rats treatments (data not shown). However, the augmentation rate of activated microglia, evaluated by Ox-42 IHC, in the injured hemisphere was significantly ($P < 0.01$) more important in QA (71.0 ± 9.8%), DFX-QA (48.7 ± 16.8%), DFX-H-QA (105.2 ± 11.5%), and in H-QA (542.8 ± 32.3%) than in both control and H groups (Figures 4(b) and 4(c)). The microglial activation measured in the H-QA group was significantly higher ($P < 0.01$) than in the QA, DFX-QA, and DFX-H-QA groups. Interestingly, the microglia activation level in DFX-QA, and QA groups was similar but DFX significantly decreased this activation in rats exposed to hemin treatment ($P < 0.01$ between DFX-QA and DFX-H-QA).

3.3. Effect of Hemin Treatment and Influence of Ferrous Iron Chelator DFX on Neuronal Loss and ROS Production in the Striatum 1 h after Surgery. Intracellular ROS production was measured 1 h after QA injection with the DHE fluorometric method coupled with immunofluorescence NeuN-FITC neuron survival quantification to calculate ROS expression per neuron. Both measurements were performed on the same 3 areas for each hemisphere (3 slices per brain). Neuronal loss and ROS activity level were measured in control ($n = 5$), in QA ($n = 5$), in H-QA ($n = 5$), in DFX-H-QA ($n = 3$), and in ZnPP-H-QA ($n = 5$) rats exposed to chronic treatment with the inhibitor of HO-1 activity zinc protoporphyrin IX (Figures 5(a) and 5(b), resp.). Merge of neurons and ROS expression is represented in Figure 5(c).

After visual counting, the percentage of neuronal loss in the ipsilateral versus the contralateral hemispheres was analyzed. Neuronal loss in the ipsilateral hemisphere 1 h after surgery was significantly different between the control (14.0 ± 3.1%) and all other groups. Hemin significantly ($P < 0.05$) enhanced neuronal loss (60.0 ± 6.6% in H-QA group) when compared to QA alone (36.3 ± 4.6%). Both ZnPP and DFX limited the enhancement of this neuronal loss: 27.0 ± 3.9% and 23.1 ± 4.8% for ZnPP-H-QA and DFX-H-QA groups, respectively. These results are presented in Figure 5(d).

The percentage of augmentation of ROS level in neurons in the injured versus the contralateral striatum was significantly ($P < 0.05$) increased in the H-QA group (49 ± 6.2%) versus all the other groups. However, it is noteworthy that no significant difference was observed between control (21.7 ± 9.4%), QA (23.1 ± 5.5%), ZnPP-H-QA (27.3 ± 5.5%), and DFX-H-QA (39.7 ± 2.0%), although ROS increasing is greater in the latter group that in the others. Results of ROS production are summarized in Figure 5(e).

4. Discussion

We report here the effects of a chronic treatment with the direct HO-1 inductor and substrata hemin in an *in vivo* rodent model of excitotoxic neuroinflammation based on striatal quinolinic acid (QA) injection previously validated by our team [19]. Moreover, 3 days after QA injection was the time at which neuroinflammation was found to be at its greatest by our team using TSPO measurement (unpublished data) and has been chosen to analyze neuroinflammation in this study. The first step was to determine the optimal concentration of hemin that was able to significantly induce the expression of the HO-1 protein. Results obtained by WB showed that 4-day I.P. of hemin injections at a dose of 50 mg/kg were able

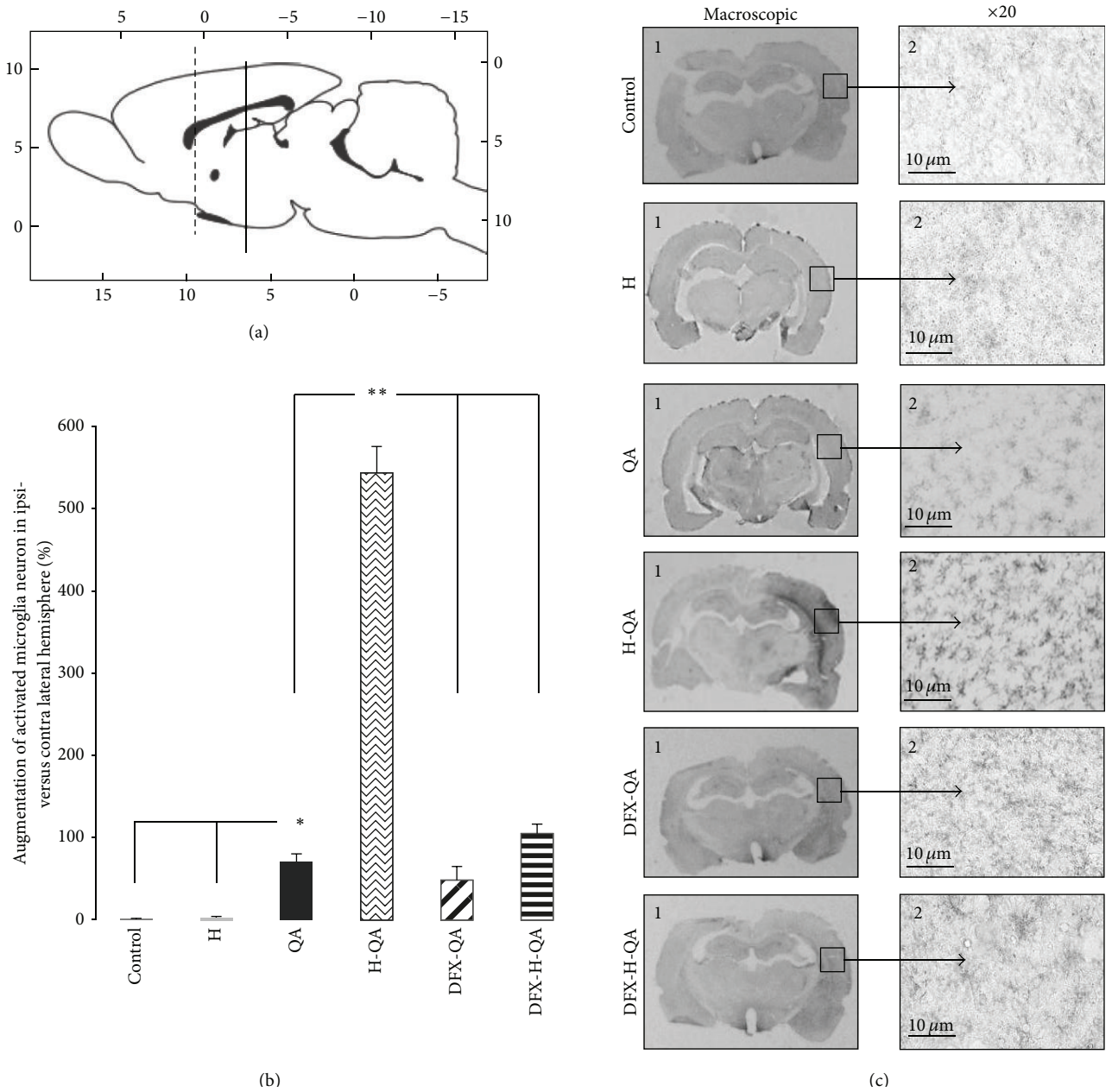

FIGURE 4: Microglial activation in the cortex (bregma −3 mm) 3 days after intrastriatal injection. (a) Sagittal rat brain representation [24]. The dotted and full lines symbolize, respectively, the site of injury (bregma +0.7 mm) and the area where Ox-42 immunohistochemistry was performed (bregma −3 mm). (b) Increasing of microglia activation in the ipsi- versus the contra-lateral hemisphere for each brain. Measurements were performed in the cortex (3 areas per hemisphere) at a distance of 3.7 mm posterior from the site of injury (bregma −3 mm). $^{*}P < 0.05$; $^{**}P < 0.01$. (c) Ox-42 immunochemistry in control, H, QA, H-QA, DFX-QA, and DFX-H-QA brains. 1: macroscopic view (bregma −3 mm). 2: microscopic view (×20) of activated microglia in the cortex of the ipsilateral hemisphere.

to induce cerebral HO-1 expression significantly higher than in the control group or at a dose of 10 mg/kg of hemin.

In the past decade, many studies have reported that HO-1 induction could be either beneficial or deleterious in the case of neuroinflammation, according to the *in vivo* or *in vitro* models and to the HO-1 induction procedure [32–34]. In our experimental conditions, the IHC method demonstrated the deleterious impact of the HO-1 induction, characterized by a significant lesion worsening in animals receiving hemin (H-QA group) versus QA rats. Therefore, we focused on the potential mechanism that could explain the harmful effect of HO-1 induction on neuroinflammation.

Microglial cells are the sensors of brain integrity through surface molecules, such as Toll-like or scavenger receptors, sensitive to background modifications that induce microglia activation [35]. In chronic neuroinflammation, unregulated microglia activity is responsible for neurotoxic factor production like ROS [5] that can lead to neuronal death. This

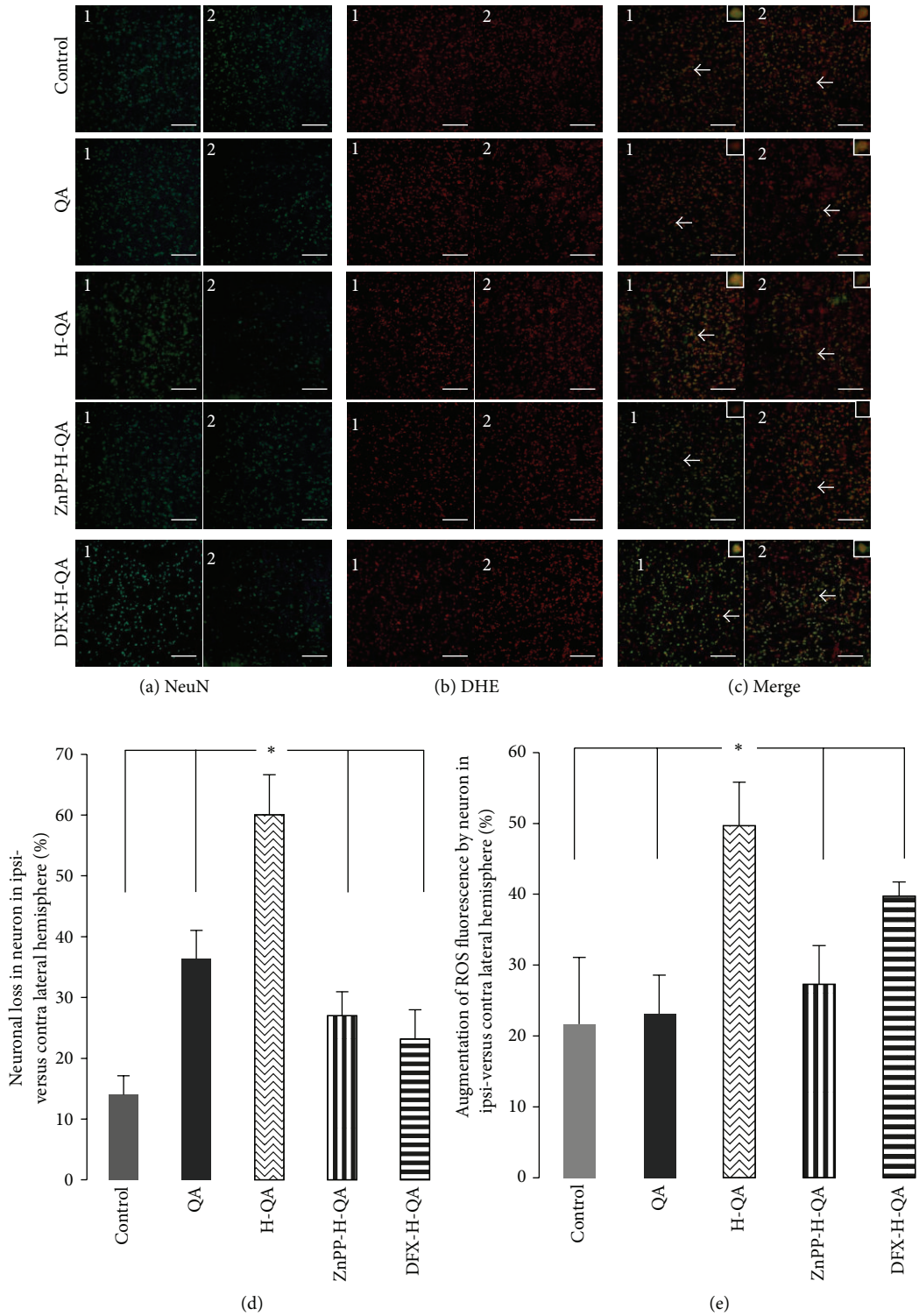

Figure 5: Immunofluorescence of NeuN and ethidium in rat striatum 1 h after QA injection. (a), (b), and (c) represent the same areas of contra- and ipsi-lateral striatum (1 and 2, resp.) of control ($n = 5$), QA ($n = 5$), H-QA ($n = 5$), ZnPP-H-QA ($n = 5$), and DFX-H-QA ($n = 3$) groups. Magnification was ×20 (×120 for inserts). Data are means ± SEM. Bars: 20 μm. (a) NeuN (green channel). Neurons staining in contra- and ipsi-lateral striatum (1 and 2) for each group. Data are expressed as relative neuronal loss in the ipsilateral hemisphere versus the contralateral hemisphere (d). $^*P < 0.05$ compared to respective contralateral side. (b) ROS expression analyzed with dihydroethidium (DHE) in the contra- and ipsi-lateral striatum (1 and 2) for each group. ROS measurements were performed at the same location as NeuN counting. Data are expressed as relative augmentation of ROS level in neurons in the ipsilateral hemisphere versus the contralateral hemisphere (e). $^*P < 0.05$. (c) Merge representing neurons and ROS expression in contra- and ipsi-lateral striatum (1 and 2) for each group.

has also been confirmed and quantified in our study where massive brain tissue destruction occurred 3 days after QA injection, amplified by hemin treatment (H-QA group). Since a brief time lapse suggested a fast and highly toxic destruction mechanism, we hypothesized an involvement of ROS in the cerebral tissue loss especially in this group. Because of their brief lifetime, ROS production has been measured 1 h after QA striatal injection by using the dihydroethidium (DHE) method that allows ROS detection only in intact cells. DHE penetrates into the cell where superoxide ion turns it into ethidium bromide which enters the cell nucleus and then inserts into the DNA before emitting a red fluorescence [36]. As the neuronal death was massive at the time of analysis, a double labeling was performed using NeuN coupled to FITC to have fluorescence related to the surviving neuron population. ROS fluorescence rationalized per neuron and compared in ipsi- versus contra-lateral striatum showed no difference between the QA and control groups, demonstrating that QA alone had no significant influence on ROS production. Conversely, chronic hemin treatment significantly promoted ROS production in the injured brain hemisphere in comparison to all other groups. The ROS production increasing in the H-QA group was inhibited in rats receiving the specific HO-1 inhibitor ZnPP, supporting the hypothesis that ROS level augmentation in hemin animals was a direct consequence of HO-1 activation. However, although a reduction of ROS production was observed in DFX-H-QA group, the ROS level remained higher than in the QA or ZnPP groups, suggesting the involvement of another mechanism than iron on ROS production.

Induction of HO-1 results in the catabolism of the prooxidant heme to bile pigments, biliverdin, and bilirubin, which are potent antioxidants [37]. However, HO-1 metabolizes heme into free irons protoporphyrin then releases equimolar amounts of Fe^{2+} [38]. Free iron is well known for its catalyzing reaction that generates ROS and Schipper et al. [39] suggested that the negative effect of HO-1 induction observed in cultures of primary neonatal rat astroglia could be related to iron accumulation. Our results support this hypothesis in an *in vivo* animal model since the DFX iron chelator reduced tissue losses induced by the hemin treatment. Incidentally, it is to be noted that DFX had no effect on tissue destruction in animals receiving QA alone. As no difference was observed between controls and unexposed-to-QA hemin-treated rats (group H), we first hypothesized that QA and hemin developed a synergic destruction pattern. Considering the severity of the striatum lesions and those in the immediate surrounding areas, the microglial activation level measurement was performed in the cortical regions at a distance of 3 mm posterior to the site of injury. The microglia cells at J3 postlesion exhibited thick ramifications matching with activated microglia cells according to Kreutzberg [3]. However, no confusion with infiltrated macrophages could be made in our microscopic analysis as we only identified resting morphology cells in control rats and activated with ramifications microglia cells in H-QA rats. Results showed that microglia was significantly activated in QA animals versus the control in the ipsilateral hemisphere. It is noteworthy that this neuroinflammation was strongly enhanced by hemin in H-QA rats then matching with the tissue loss described. DFX treatment in DFX-H-QA group significantly decreased microglia activation in the ipsilateral hemisphere, strongly suggesting that Fe^{2+} may be implicated in deleterious HO-1 effects. Deleterious effects of microglia activation characterized by proinflammatory cytokines production (e.g., IL-1β, TNFα, iNOS) in striatum area have already been reported at J1 in this QA adult rat model [40]. Unfortunately, the massive tissue destruction observed in H-QA rats in our experimental conditions (J3 after QA injection) did not allow us to quantify the expression level of cytokines. The strong activation of microglia (Ox-42) measured at distance from the lesion (bregma −3 mm) in H-QA animals (Figure 4) was not associated with significant neuronal loss and no difference in neuronal survival in the cortex between the groups was observed. Indeed, the microglia activation in cortical area is not systematically associated with neurons death like in adult rats exposed to temporary brain ischemia [41]. Interestingly, the global level of activated microglia in this area was markedly more important in H versus control animals, suggesting a potential proinflammatory effect of HO-1 induction.

Therefore, we report here that HO-1 induction, ROS production, and cerebral destruction are closely linked in our neuroinflammatory model. ROS production itself could be directly connected to the HO-1 enzyme activity and probably results from a massive release of iron. The striatal injection of QA was responsible for tissue destruction but the induced excitotoxicity did not significantly modify ROS production 1 h after the injection. Nevertheless, hyperactivation of NMDA receptor is associated to massive intracellular Ca^{2+} entrance and accumulation leading to mitochondrial damages and nitric oxide synthase activation which are both responsible for ROS production [20]. So, we thought that the delay between QA injection and ROS production relating to QA effects take longer than one hour before reaching a significant level.

The deleterious effect of HO-1 in this neuroinflammatory model could be explained by a hyperproduction of ROS. The diminution of both tissue loss and ROS production observed in rats receiving both DFX and hemin shows that iron is directly implicated in ROS production caused by HO-1 expression. Given that hemin alone did not induce either microglia activation or detectable neuronal death, it appears that the ROS-HO-1-mediated production created a cerebral destruction pattern in combination with the QA cerebral excitotoxicity. Constitutive heme oxygenase (HO-2) is also expressed in the brain and inhibited by ZnPP [42]. Its involvement in the deleterious effect associated to HO-1 activity cannot be totally rejected. HO-2 is mostly described for its role in the homeostasis of heme in brain [43] and therefore could participate in HO-1 regulation [44]. However, the specific influence of HO-2 in our model was not evaluated.

Recently, it has been proposed that hemin caused microglia to release deleterious inflammatory factors via Toll-like receptor (TLR) 4 in a mouse model of intracerebral

hemorrhage [45]. Indeed, exogenous hemin administration significantly increased microglial activation and exacerbated brain injury in WT mice but not in TLR4$^{-/-}$ mice. Moreover, application of TLR4 antibody suppressed hemin-induced microglial activation in WT mice, suggesting a direct correlation between TLR4 and hemin-induced microglial activation. This was supported by the observation that TLR4 activates NF-κB, which plays a critical role in the inflammatory response by regulating the gene expression of inflammatory mediators such as the cytokines IL-1α and-1β, TNF-α, and IL-6 [46, 47]. Therefore, targeting the TLR4 signaling pathway using anti-TLR4 antibody administration could be a potential therapeutic strategy in our excitotoxic rat model of neuroinflammation.

Complementary experiments could be carried out to determine the optimal hemin doses and administration schedule to achieve a positive benefit-risk balance of the drug. Indeed, most drugs of the Pharmacopoeia can generate deleterious effects if they are not used properly. With the aim of *in vivo* neuroprotection, the drug hemin should be associated to an ROS scavenger or an iron chelator. Thus, HO-1 activity promotes sequestration of redox-active iron in astroglia. This is confirmed by a recent review showing that selective human HO-1 expression in the astrocytes of transgenic mice is associated with iron sequestration in these cells [48]. Glial HO-1 may be a rational therapeutic target in AD, PD, and other human CNS conditions characterized by the unregulated deposition of brain iron.

In conclusion, this study showed first that hemin treatment and HO-1 induction had a deleterious effect in this QA model and enhanced tissue loss and microglia activation. Secondly, we showed that this effect is probably linked to a hyperproduction of ROS and iron accumulation.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Dual Phases of Respiration Chain Defect-Augmented mROS-Mediated mCa^{2+} Stress during Oxidative Insult in Normal and ρ^0 RBA1 Astrocytes

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Mitochondrial respiratory chain (RC) deficits, resulting in augmented mitochondrial ROS (mROS) generation, underlie pathogenesis of astrocytes. However, mtDNA-depleted cells (ρ^0) lacking RC have been reported to be either sensitive or resistant to apoptosis. In this study, we sought to determine the effects of RC-enhanced mitochondrial stress following oxidative insult. Using noninvasive fluorescence probe-coupled laser scanning imaging microscopy, the ability to resist oxidative stress and levels of mROS formation and mitochondrial calcium (mCa^{2+}) were compared between two different astrocyte cell lines, control and ρ^0 astrocytes, over time upon oxidative stress. Our results showed that the cytoplasmic membrane becomes permeated with YO-PRO-1 dye at 150 and 130 minutes in RBA-1 and ρ^0 astrocytes, respectively. In contrast to RBA-1, 30 minutes after 20 mM H_2O_2 exposure, ρ^0 astrocytes formed marked plasma membrane blebs, lost the ability to retain Mito-R, and showed condensation of nuclei. Importantly, H_2O_2-induced ROS and accompanied mCa^{2+} elevation in control showed higher levels than ρ^0 at early time point but vice versa at late time point. Our findings underscore dual phase of RC-defective cells harboring less mitochondrial stress due to low RC activity during short-term oxidative stress but augmented mROS-mediated mCa^{2+} stress during severe oxidative insult.

1. Introduction

Astrocytes are the most abundant type of glial cells that provide support and nutrition for neurons in the central nervous system (CNS). A crosstalk between astrocytes and neurons is crucial to maintain CNS homeostasis. Numerous neurologic disorders are generated due to the disturbance of the interactions between astrocytes and neurons, such as cerebral ischemia, neurodegeneration, cerebral edema, and hepatic encephalopathy [1]. Astrocyte-mediated neuroprotection has been proposed to be due to the maintenance of energy metabolism, adjusting osmolarity for volume regulation [2], the control of synaptic transmission and neurovascular coupling [3–7], and the limiting of neuronal death from excitotoxins and oxidants such as glutamate and reactive oxygen species (ROS) [8, 9]. Crucially, astrocytes enhance neuronal antioxidant defense by releasing high intracellular levels of antioxidants to the extracellular fluid around neurons [10].

The mitochondrial respiratory chain (RC) is crucial for cell survival due to its primary role in ATP generation. In physiological condition, mitochondrial ROS (mROS) are generated by the RC during ATP synthesis due to the leakage of electrons primarily from complex I, complex III and more recently complex II to molecular oxygen (O_2) [11–13]. However, augmented mROS formation is evident in RC defects [14]. Molecular pathogenesis of astrocytes can be initiated and enhanced by mitochondrial oxidative stress,

which is due to augmented mROS generation. Mitochondrial oxidative stress leads not only to the interruption of the energy supply but also to severe damage of mitochondrial components including proteins, lipids, and mitochondrial DNA (mtDNA). In addition, oxidative stress is often accompanied by mitochondrial calcium (mCa^{2+}) overload, which leads to mROS increase [15]. Subsequently, these stresses act synergistically for a vicious amplification of additional oxidative stress which leads to the activation of mitochondrial permeability transition- (MPT-) dependent and/or MPT-independent release of mitochondrial lethal proteins including cytochrome c from the intermitochondrial space; these changes culminate in the "point of no return" apoptosis [16]. Thus, RC injuries could be one of the critical mechanisms impairing astrocyte-mediated neuroprotection.

ρ^0 cells are defined as those lacking mitochondrial genome, encoding for 2 rRNAs, 22 tRNAs, and 13 protein subunits that form RC complexes I, III, IV, and V. Therefore, ρ^0 cell line is an *in vitro* model to investigate the role of RC in CNS injuries and diseases. Controversy exists over the opposite capacity of ρ^0 cells to exert protective effects: they are sensitive [17, 18] or resistant [19, 20] to apoptosis. Since RC is a pivotal component of cell death, its role in apoptotic regulation is warranted to be well understood.

In the current study, we sought to assess the effects of RC-enhanced mitochondrial stress in astrocytes following oxidative insult. For this purpose, we have compared the ability to resist oxidative stress between the two different astrocyte cell lines, normal (control) and ρ^0 astrocytes, over time. Our results showed that ρ^0 astrocytes displayed moderate level of mROS and mCa^{2+} surg under minor oxidative stress but augmented formation of mROS and mCa^{2+} during severe oxidative insult.

2. Materials and Methods

2.1. Cell Preparation. Normal rat brain astrocytes (RBA-1) used for this study were originally established through a continuous passage of primary astrocytes isolated from 3-day-old JAR-2, F51 rat brains by Dr. Jou et al. [21]. All cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum. The cells were plated onto glass coverslips (Model no. 1, VWR Scientific, San Francisco, CA, USA) coated with poly-L-lysine. Experiments were preformed after cells grew to 80–90% (about 2-3 culture days) of confluence. The mtDNA-free cells, ρ^0, were obtained from human 143B osteosarcoma cells treated with ethidium bromide (100 μg/mL). ρ^0 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum supplemented with high glucose (4.5 g/mL), pyruvate (0.11 mg/mL), and uridine (0.1 mg/mL).

2.2. Chemicals and Fluorescent Dyes Loading for Studying Mitochondrial Function. All chemicals were obtained from Sigma (St. Louis, MO, USA) and fluorescent dyes were purchased from Molecular Probes Inc. (Eugene, OR, USA). Mitochondrial function was studied by imaging mitochondrial morphology, mROS formation, mitochondrial membrane potential (DeltaPsi(m)) changes, mCa^{2+} regulation, and the opening of the MPT using specific fluorescent probes. To image mitochondrial morphology, cells were loaded with a mitochondrial-targeted fluorescent probe, Mito-Tracker Green (Mito-G, giving a green fluorescence) or Mito-Tracker Red (Mito-R, giving a red fluorescence), both at a concentration of 100 nM. DeltaPsi(m) was detected using either 200 nM and the ratiometric indicator 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) or 300 nM tetramethyl rhodamine ethyl ester (TMRM). Intracellular ROS formation and mROS were visualized using the acetyl ester form of 2 μMdichlorofluorescein (DCF-DA) and 100 nM dihydrorhodamine 123 (D-123), respectively. mCa^{2+} was detected using 1 μM Rhod-2/AM. Fluorescent probes were all loaded at room temperature for 20–30 min. After loading, cells were rinsed three times with HEPES-buffered saline (in mM : 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 5 HEPES, and pH 7.4). For all ester forms of dyes including DCF-DA and Rhod-2/AM, cells were treated for an extra 40 min after dye loading to allow cleavage of the ester form of dye to its acid form.

2.3. Fluorescence Conventional and Multiphoton Imaging Microscopy. All phase-contrast and conventional fluorescence images were obtained using a Zeiss inverted microscope (Axiovert 200M, Carl Zeiss, Jena, Germany) equipped with a mercury lamp (HBO 103), a cool CCD camera (CoolSNAP fx, Roper Scientific, Tucson, AZ, USA), and Zeiss objectives (Plan NeoFluar 100, NA 1.3 oil). Filters used for detecting DCF were no. 10 (Exi: BP 450–490 nm; Emi: BP 515–565 nm) and for Mito-R and TMRM the filter was no. 15 (Exi: BP 546/12 nm; Emi: LP 590 nm). Confocal images of cells and mitochondria were collected on a Bio-Rad Radiance 2100 using 488 nm Argon laser illumination (Bio-Rad, Hercules, CA, USA). To avoid single-photon-induced autolysis of DCF/DA, multiphoton illumination was applied as previously described [22]. Multiphoton fluorescence images were collected on a Leica SP2 MP (Leica-Microsystems, Mannheim, Germany) fiber-coupling system equipped with a Ti:Sa-Laser system (model: Millennia/Tsunami; Spectra-Physics) providing a pulse repetition rate at 82 MHz, laser pulse width of 1.2 picoseconds, a spectral bandwidth of 1 nm, and object pulse width of 1.3 picoseconds. Wavelength at 800 nm with an average laser power of 600 mW was selected for illumination. During fluorescence imaging, the illumination light was reduced to the minimal level by using appropriate neutral density filters to prevent the photosensitizing effects due to the interaction of light with fluorescent probes including bleaching and autooxidation of the fluorescent probes. All images were processed and analyzed using MetaMorph software (Universal Imaging Corp., West Chester, PA, USA). Intensity levels were analyzed from the original images and graphed using Microsoft EXCEL software and Photoshop. Pseudocolor display with a scale ranged from 0 to 255 units was used to enhance the contrast of the fluorescence changes for each image.

2.4. Quantification of ROS Generation and mCa^{2+} Using a Fluorescent Spectrofluorimeter. The cells cultured in 96-well standard black/clear bottom plate (Greiner Bio-One International AG, Kremsmünster, Austria). The ROS was detected with DCF (excitation and emission wavelengths were 490 and 520 nm, resp.). mCa^{2+} was detected with Rhod-2 (excitation and emission wavelengths were 561 and long pass 575 nm, resp.). The fluorescent intensity of DCF and mCa^{2+} were quantified on a Spectramax Gemini EM spectrofluorimeter (Molecular Devices, Sunnyvale, CA, USA). Results were analyzed by using SoftMax Pro 4.7 software (Molecular Devices) and Microsoft Excel software.

2.5. Measurement of Cell Viability. Cell viability was detected using the colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as previously described [23]. Activity of a mitochondrial reductase to convert a soluble tetrazolium salt into an insoluble formazan precipitate was measured by an ELISA reader (A-5082; TECAN, Grödig/Salzburg, Austria). To investigate the protective effects of RC-defective (ρ^0) astrocytes on hydrogen peroxide- (H_2O_2-) induced cytotoxicity, control and ρ^0 astrocytes were treated with different concentrations of H_2O_2 for different periods of time. MTT assay was performed after 1 hr of H_2O_2 exposure. Activity of mitochondrial reductase was calculated as the amount of MTT dye conversion relative to the changes seen in sham-treated control cells. Data were represented as mean ± SE of at least three independent experiments.

2.6. Measurement of Cellular Oxygen Consumption. Cells ($5 \times 10^5/100\,\mu$L) after trypsinization were immediately transferred to the chamber of Mitocell equipped with a Clark oxygen electrode (782 Oxygen Meter; Strathkelvin Instruments, Glasgow, UK) for the measurement of cellular O_2 consumption as previously described [24]. The rate of O_2 consumption was calculated from the slopes and expressed in % of O_2 consumed per min per 5×10^5 cells.

2.7. Statistical Analysis. The results were expressed as mean ± SEM, and statistical significance was evaluated by either one-way or multifactorial analysis of variance (ANOVA). A value of $P < 0.05$ was considered significant. Each experiment was repeated at least three times.

3. Results

3.1. Slower O_2 Consumption Rate in ρ^0 RBA1 Astrocytes. RC activities are presented with O_2 consumption and ATP production [25, 26]. Therefore, we further examined whether a difference exists in O_2 consumption between control and ρ^0 (RC-lacking) RBA1 astrocytes. The result showed that control cells consumed oxygen faster than ρ^0 astrocytes (Figure 1). However, O_2 consumption compromised significantly during long-term oxidative stress in ρ^0 astrocytes. These findings indicate relatively low level of RC activity, leading to less mitochondrial stress in ρ^0 astrocytes, but the protective effect would be abolished over a long time interval.

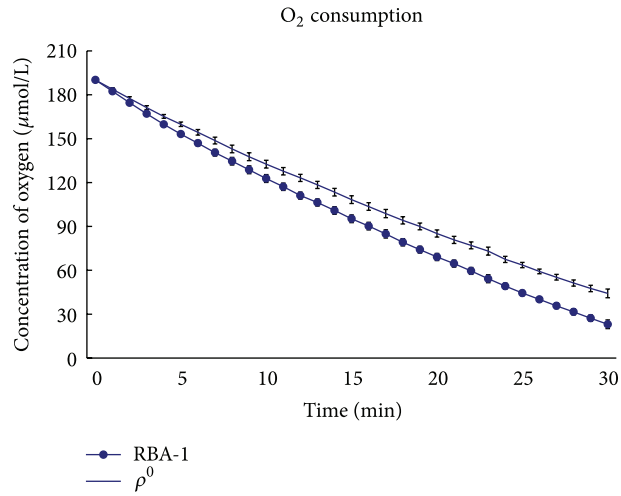

Figure 1: ρ^0 (RC-lacking) RBA1 astrocytes exhibited lower RC activities. RC activities are indicated by O_2 consumption and ATP production. In comparison, ρ^0 astrocytes consumed O_2 slower than control cells. Moreover, O_2 consumption compromised significantly during long-term oxidative stress in ρ^0 astrocytes.

3.2. Dual Phases of ρ^0 RBA1-Astrocytes-Mediated Protection. The extent of oxidative stress depends on toxin concentration and exposure time [27, 28]. To determine the optimal concentration and exposure time, control and ρ^0 RBA1 astrocytes were treated with different concentrations of H_2O_2 (0, 1, 10, 20, and 50 mM) for different periods of time (10, 30 min) to establish an oxidative stress model. Cell viability following H_2O_2 exposure was measured by the MTT assay in the two groups. H_2O_2 (1–50 mM) significantly decreased the cell viability of both cells in a concentration-dependent manner. There were no statistically significant differences between the two groups in the cell viability at different concentration for 10 minutes ($P > 0.05$) (Figure 2(a)). As shown in Figure 2(b), the high concentration of H_2O_2 (50 mM) caused a significant loss in cell viability. We did not use this extremely high dose of H_2O_2 because it was likely that a high concentration would have been excitotoxic to astrocytes. Furthermore, 20 mM H_2O_2 caused a statistically significant loss in cell viability in ρ^0 astrocytes for 30 min when compared to control cells. Thus, optimal H_2O_2 concentration of 20 mM was determined for further experiments.

3.3. RC-Defective Astrocytes Augmented Mitochondrial Damage during Oxidative-Stress-Induced Apoptosis. To compare the capacity to resist to long-term oxidative stress between control and ρ^0 astrocytes, we used time-lapse measurements and recorded the detailed changes in cellular as well as mitochondrial morphology during the entire apoptotic cell death process induced by 20 mM H_2O_2 using both conventional phase-contrast and confocal fluorescence imaging microscopy coupled with a noninvasive mitochondria-targeted fluorescent probe, Mito-R and fluorescent apoptosis marker, YO-PRO-1. Figure 3 shows time-lapse fluorescence images showing dynamic changes in mitochondrial morphology (labeled in red) and apoptotic nuclei (labeled in green)

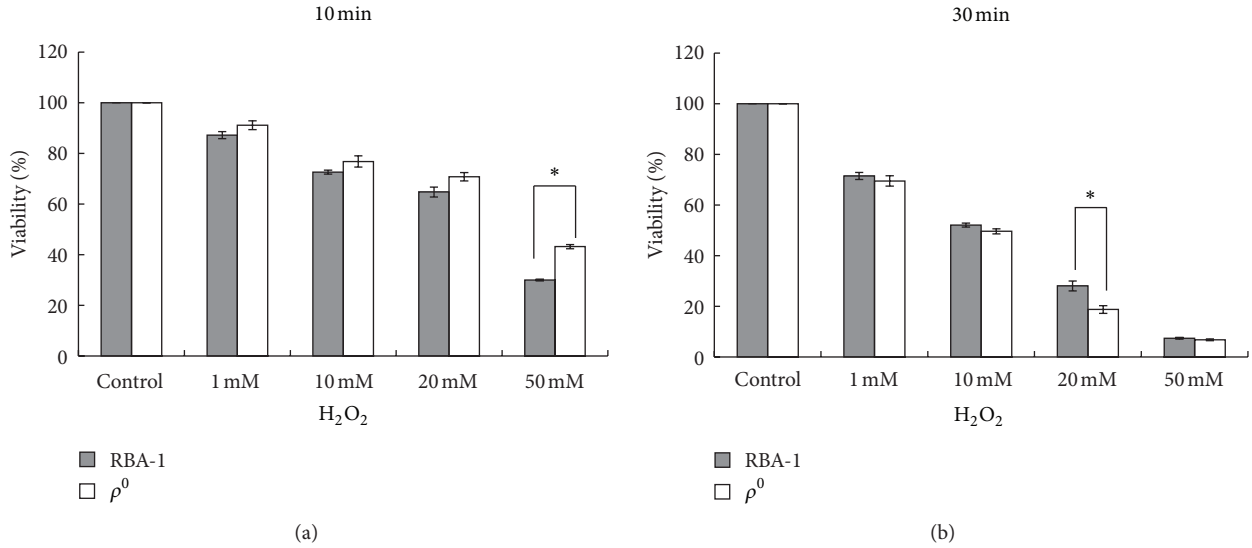

Figure 2: RC defect-augmented loss in cell viability during H_2O_2 stress as measured by the MTT assay. (a, b) Dose-dependent cytotoxic effect induced by H_2O_2 exposure in control (filled columns) and ρ^0 RBA1 astrocytes (empty columns); (a) time point for H_2O_2 exposure: 10 min and (b) 30 min. There were no statistically significant differences between the two groups in the cell viability at different concentration for 10 minutes ($P > 0.05$) (a). 20 mM H_2O_2 caused a statistically significant loss in cell viability in ρ^0 astrocytes for 30 min when compared to control cells ($P < 0.001$) (b). Data were expressed as mean values ± SE of four separate experiments. $^*P < 0.001$.

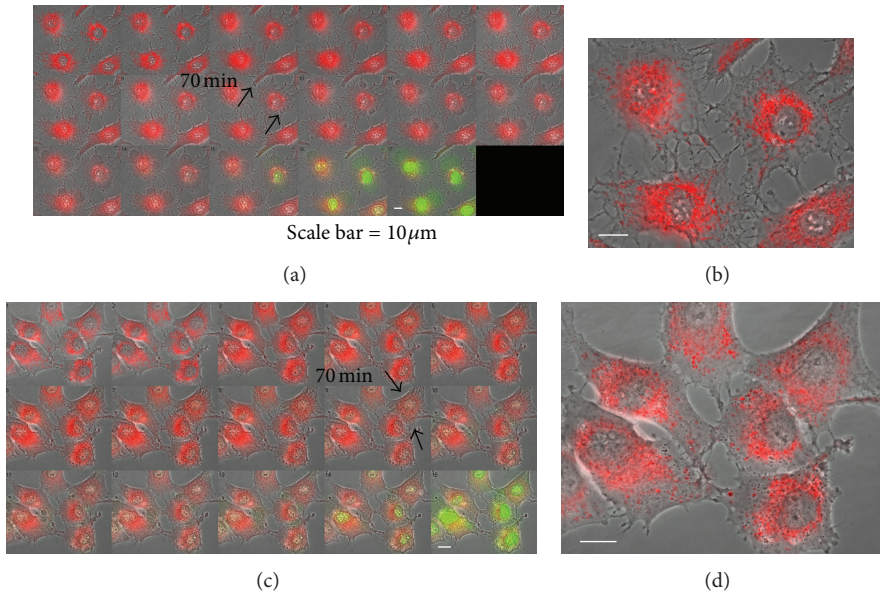

Figure 3: RC-defective astrocytes augmented mitochondrial damage during oxidative stress-induced apoptosis. (a, b) RBA1 astrocytes and (c, d) ρ^0 RBA1 astrocytes. Time-lapse fluorescence images demonstrate dynamic changes in mitochondrial morphology using Mito-R (labeled in red and apoptotic nuclei using YO-PRO-1 (labeled in green color) during H_2O_2 stress. The first image of each image series in (a), (c) is the control. Dual fluorescence time-lapse images of Mito-R and YO-PRO-1 were taken simultaneously at 10 min interval for 150 min. (b), (d) Show magnification 400x of time-lapse fluorescence images at 70 mins in (a), (c), respectively. Scale bar =10 μm.

during H_2O_2 stress in control (a, b) and ρ^0 RBA1 astrocytes (c, d). In Figures 3(a) and 3(c), first image of each image series is the control. Dual fluorescence time-lapse images of Mito-R (for morphology) and YO-PRO-1 (for apoptotic nuclei labeling) were taken simultaneously at 10 min interval for 150 min. For analysis, simultaneous visualization of mitochondrial morphology labeled with Mito-R and apoptotic nuclei labeled with YO-PRO-1 were compared between the two groups over 6 time points: 10, 30, 60, 70, 130, and 150 min in Figure 4. Apoptotic events including swelling of mitochondria, plasma membrane blebbing, YO-PRO-1 stain, and nuclear condensation [29, 30] of the two groups were summarized in Table 1. In

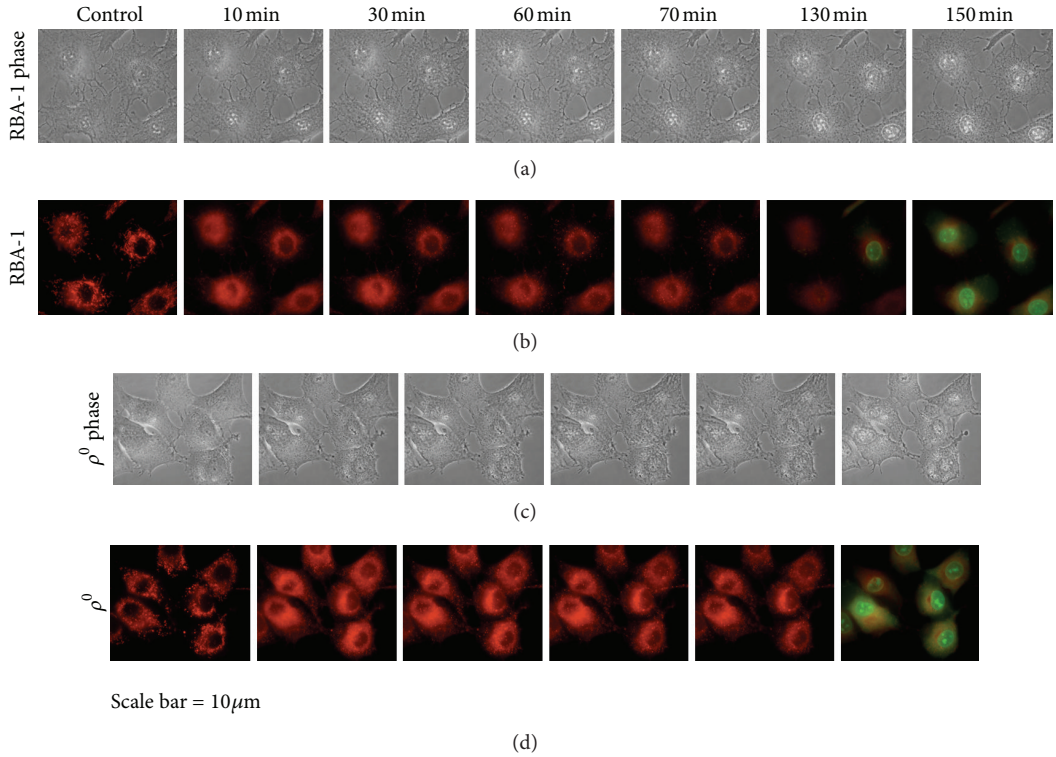

Figure 4: Time-lapse fluorescence images at selected time points. (b) Six time-lapse Mito-R, YO-PRO-1 images of normal RBA1 astrocytes. RBA-1 astrocytes become permanent to YO-PRO-1 after treatment with 20 mM H_2O_2 at 150 min. (d) Five time-lapse Mito-R, YO-PRO-1 images of ρ^0 RBA1 astrocytes. ρ^0 RBA-1 astrocytes become permanent to YO-PRO-1 after treatment with 20 mM H_2O_2 at 130 min. (a) Phase-contrast images of normal RBA1 astrocytes in six time-lapse series. (c) Phase-contrast images of ρ^0 RBA1 astrocytes in five time-lapse series. Images were taken at rest and after exposure of H_2O_2 at 0, 10, 30, 60, 70, 130, and 150 min. Bar =10 μm.

comparison, cellular morphology of both groups after 20 mM H_2O_2 stress which showed phase dense gray mitochondria in the cytosolic area became much lighter due to the swelling of mitochondria over time. Under laser scanning confocal imaging microscopy, mitochondria began to swell earlier on exposure to H_2O_2 in ρ^0 astrocytes than control group (10 min versus 20 min, resp.). There was no significant difference between the two groups for plasma membrane blebbing. Figures 3(b) and 3(d) show magnification 400x of time-lapse fluorescence images at 70 mins in Figures 3(a) and 3(c), respectively. In comparison, ρ^0 astrocytes formed more marked plasma membrane blebs than in control group over time. Moreover, time to become YO-PRO-1-positive was earlier in ρ^0 astrocytes than control group (120 min versus 140 min, resp.). In addition, the percentage for decreased nuclear sizes prior to and after H_2O_2 treatment is much higher in ρ^0 astrocytes than in control group (8.9% versus 7.2%, resp.). These results strongly suggest that ρ^0 astrocytes lacking RC exert protection only in minor oxidative stress while become much vulnerable to secondary oxidative insults in severe oxidative stress.

3.4. Dual-Phase Alteration in mROS and mROS-Dependent mCa^{2+} Formation upon Oxidative Stress Extent in ρ^0 Astrocytes. Next, we investigated whether the resistance to oxidative stress is compromised due to the impact of mitochondrial stress of mROS and mROS-dependent mCa^{2+} stress in ρ^0 astrocytes. mROS formation and mCa^{2+} concentration were concurrently imaged by dual labeling with a mROS probe, DCF, and Ca^{2+} fluorescent probe, Rhod-2 by laser scanning confocal microscopy. Continuous changes in mitochondrial ROS and Ca^{2+} every 6 min for 2 hr after H_2O_2 exposure in both groups were simultaneously analyzed in Figures 5(a) and 5(b), respectively. These results suggest that mROS increased rapidly (within 10 min) after cells were exposed to H_2O_2 and this was later accompanied by the increase of mCa^{2+} level. Importantly, mROS formation and mROS-dependent mCa^{2+} concentration in control cells was higher than in ρ^0 astrocytes during the early stage of 10, 50, and 100 mM H_2O_2 exposure. However, mitochondrial stress of mROS and mROS-dependent mCa^{2+} stress were significantly higher in ρ^0 astrocytes than control during long-term 10, 50, and 100 mM H_2O_2 exposure.

3.5. ρ^0 RBA1 Astrocytes Have Lower DeltaPsi(m) Than Control Cells. We previously demonstrated that resting mROS level in cells harboring large-scale deletion of mtDNA was lower than that found in cells preserving mtDNA. Besides, resting DeltaPsi(m) in the former cells was found to be less hyperpolarized than that detected in the latter [29].

Table 1: Different effects of RC-augmented mitochondrial stress following H_2O_2 treatment on mitochondrial swelling, plasma membrane blebbing, YO-PRO-1 stain, and nuclear condensation in normal and ρ^0 RBA1 astrocytes.

Cell line	Mitochondria swelling	Blebbing	1/2 cells Yo-Pro	Cells Yo-Pro	Condensation of nucleus (%)
RBA-1	20	70	140	150	7.2%
ρ^0 (RBA-1)	10	70	120	130	8.9%

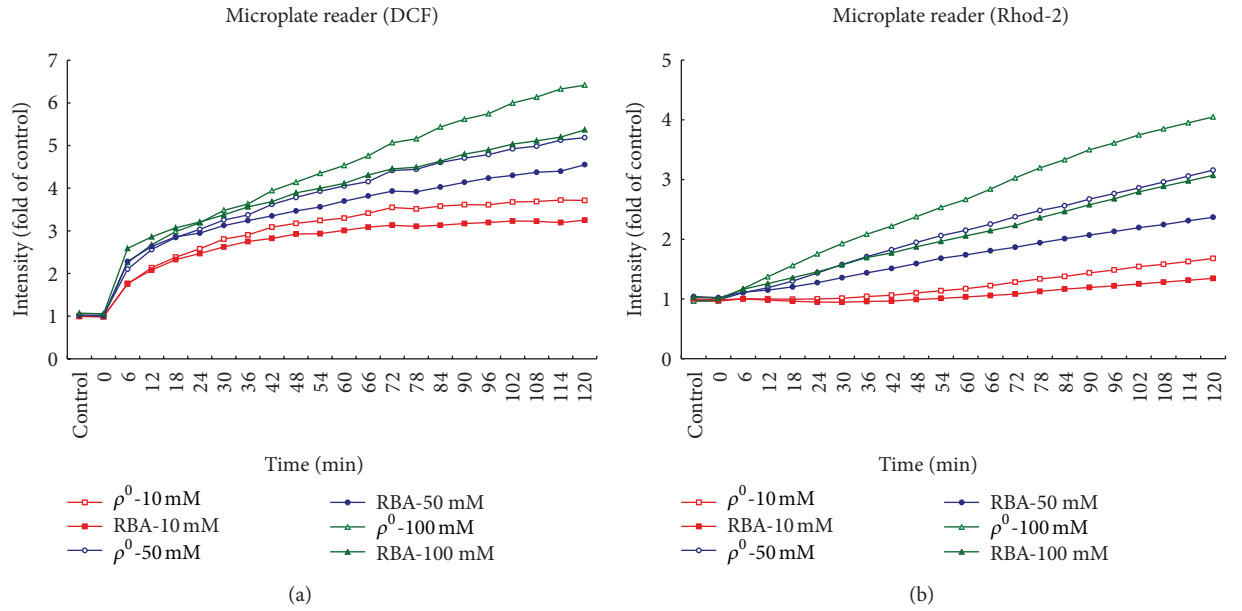

Figure 5: Dual-phase change in mROS and mROS-dependent mCa^{2+} formation during H_2O_2-induced oxidative stress in normal and ρ^0 RBA1 astrocytes. (a) ROS generation detected by DCF. (b) mCa^{2+} detected by Rhod-2. It should be noted that mROS formation and mROS-dependent mCa^{2+}concentration in control cells was higher than ρ^0 astrocytes during the early stage of H_2O_2 exposure. However, mitochondrial stress of mROS and mROS-dependent mCa^{2+} stress were significantly higher in ρ^0 astrocytes than control during long-term H_2O_2 exposure.

Moreover, lower DeltaPsi(m) can produce less driving force for Ca^{2+} to enter the mitochondria and consequently reduces mCa^{2+} stress [31]. To investigate whether ρ^0 astrocytes with lower mROS formation under minor oxidative stress have lower DeltaPsi(m), we detected DeltaPsi(m) changes using a DeltaPsi(m)-sensitive fluorescent probe, JC-1, and evaluated them by laser scanning confocal microscopy. JC-1 measured both high (reported as j-aggregated red fluorescence) and low (reported as monomer green fluorescence) DeltaPsi(m). As indicated in Figure 6, confocal JC-1 imaging demonstrated that DeltaPsi(m) in control cells was heterogeneous (Figures 6(a)–6(c)). Both high (red fluorescence) (Figure 6(b)) and low (green fluorescence) (Figure 6(a)) DeltaPsi(m)s were detected. In comparison, ρ^0 astrocytes showed that JC-1 expression was much reduced in red (Figure 6(e)) and green (Figure 6(d)) fluorescence. Merge images indicated the decrease of both high and low DeltaPsi(m)s in ρ^0 astrocytes (Figure 6(c)) when compared to control cells (Figure 6(f)). Taken together, these data provide illustration for the protective effects of ρ^0 astrocytes via less mROS generation, lower DeltaPsi(m), and therefore lower mCa^{2+} stress. In contrast, ρ^0 astrocytes render their protection due to augmented mROS and mROS-mediated mCa^{2+} stress under long-term mitochondrial oxidative stress.

4. Discussion

In this study, we demonstrated the biphasic effects of RC defect-augmented mROS-mediated mCa^{2+} stress during H_2O_2-induced oxidative damage in ρ^0 RBA1 astrocytes. We compared the impact of oxidative stress on normal and RC-defective astrocytes during short- and long-term exposure to H_2O_2. Our findings indicate that (1) ρ^0 astrocytes exerted slower O_2 consumption than control cells, but O_2 consumption compromised significantly during long-term oxidative stress; (2) cell viability was not different between groups during short-term H_2O_2 exposure; (3) cell survival was decreased in ρ^0 astrocytes incubated with 20 mM H_2O_2 during long-term oxidative stress; (4) ρ^0 astrocytes formed marked mitochondrial swelling, plasma membrane blebs, and earlier positive YO-PRO-1 nuclear staining than control under H_2O_2 treatments; and (5) higher mROS formation, mROS-dependent mCa^{2+} level, and DeltaPsi(m) were detected in control cells in early oxidative stress but vice versa during long-term oxidative insult. These findings underscore dual phases of pathological course in RC-defective cells, which has been an area of significant research interest and has emerged as a potential therapeutic target in the treatment of neurological diseases.

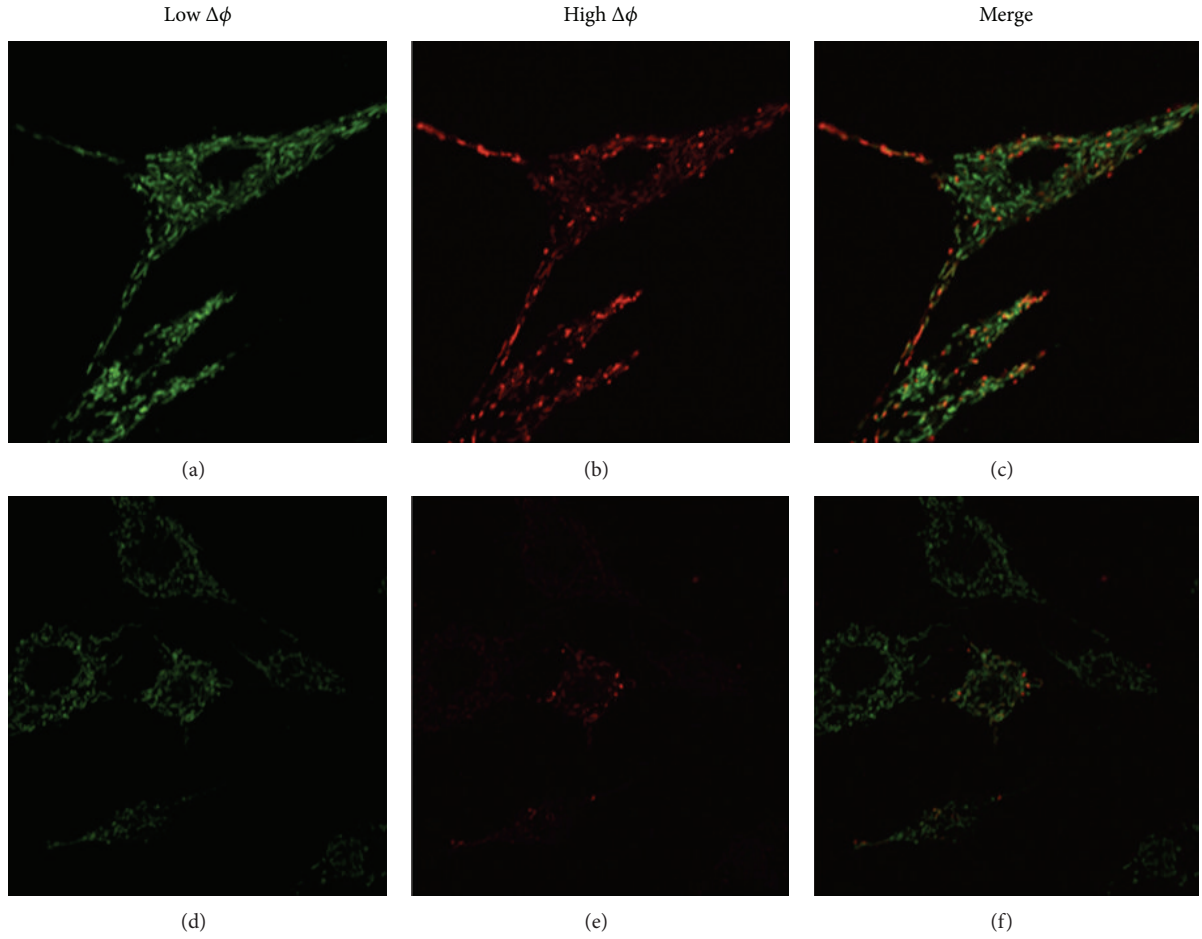

Figure 6: Measurement of DeltaPsi(m) on normal and ρ^0 RBA1 astrocytes cells by the confocal imaging system. (a–c) Normal RBA1 astrocytes. (d–f) ρ^0 RBA1 astrocytes. JC-1 measured both high (J-aggregate red fluorescence) (b and e) and low (monomer green fluorescence) (a and d) DeltaPsi(m).

Using time-lapse dual fluorescence imaging microscopy to simultaneously measure the generation of RC defect-enhanced mROS and mCa^{2+}, changes of them were visualized using DCF and Rhod-2, respectively. In the current study, we found that mROS generation is prior to mCa^{2+} increase in control and ρ^0 astrocytes after H_2O_2 treatment. These findings were compatible with our previous studies on H_2O_2 [32] and photoirradiation-augmented oxidative stress [33–35]. Moreover, our study provided quantitative imaging evidence that dual effect on antioxidation exists in ρ^0 astrocytes during different extent of oxidative stress. In brief, RC defect protects cells due to less mROS, less DeltaPsi(m), and consequently less mROS-mediated mCa^{2+} stress during short-term oxidative insult. However, such protective effect obtained from ρ^0 cells does not last long due to significantly compromised RC activity indicated from O_2 consumption, significantly augmented mROS-enhanced mitochondrial stress, and mROS-dependent mCa^{2+} stress during long-term oxidative stress.

The paradoxical nature of mROS has been described as a double-edged sword: they protect at basal level but may also damage at excessive level [36]. Similarly, moderate increase in mCa^{2+} is physiologically relevant, but Ca^{2+} overload is detrimental to mitochondrial function. The crosstalk between Ca^{2+} and ROS signaling systems is critical to maintain physiological homeostasis. Minor mROS formation can reduce mCa^{2+} level to exert protective preconditioning [37]. In contrast, severe mROS formation can enhance mCa^2increase. The combined effect of mCa^{2+} and mROS may facilitate the opening of the MPT pore [38]. The opening of the MPT pore leads to DeltaPsi(m) depolarization and results in mitochondrial swelling. The outer mitochondrial membrane ruptures due to mitochondrial swelling result in the release of cytochrome c, leading to the inhibition of electron transport and enhancing more ROS production [15]. Thus, oxidative stress resulted from such feed-forward loops could be devastating creating cellular damage far beyond direct Ca^{2+}-induced damage.

Controversy exists as to whether ρ^0 cells are resistant to oxidative stress or not. The effects of these RC-defective cells are diverse, and both detrimental and protective to cell survival have been described. Cells-harboring mtDNA mutations have been shown to induce protective expression of Bcl-2 and Bfl-1, prosurvival proteins [39]. It has been

reported that DeltaPsi(m) in ρ^0 cells might be distinctly lower than that of wild-type cells [40]. In contrast, we previously showed that common deletion- (CD-) induced RC defect results in significantly elevated mROS and depolarized DeltaPsi(m) and can effectively lead to an enhanced apoptotic signaling [29]. As the dual effect of mROS, whether it is protective or detrimental depends on the produced amount. Similarly, whether ρ^0 cells exert protection or damage to cell survival depends on the extent of oxidative stress and consequent mROS production. Future studies are warranted to examine this putative mechanism in ρ^0 cells.

In conclusion, we demonstrated dual phases of RC defect-augmented mROS-mediated mCa^{2+} stress during oxidative insult in a cell culture model of ρ^0 astrocytes. RC defect protects cells due to less mROS and mROS-dependent mCa^{2+} stress in minor oxidative insult. RC-defect-mediated protection is compromised due to significantly augmented mROS and mROS-dependent mCa^{2+} stress during long-term oxidative stress. The key point that ρ^0 astrocytes have lower DeltaPsi(m) than normal RBA1 underscore the dual phases of modulation for cell survival and cell death.

Acknowledgments

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Review Article

Obstructive Sleep Apnea, Oxidative Stress and Cardiovascular Disease: Lessons from Animal Studies

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Obstructive sleep apnea (OSA) is an independent risk factor for cardiovascular (CV) diseases such as arterial hypertension, heart failure, and stroke. Based on human research, sympathetic activation, inflammation, and oxidative stress are thought to play major roles in the pathophysiology of OSA-related CV diseases. Animal models of OSA have shown that endothelial dysfunction, vascular remodelling, and systemic and pulmonary arterial hypertension as well as heart failure can develop in response to chronic intermittent hypoxia (CIH). The available animal data are clearly in favour of oxidative stress playing a key role in the development of all of these CV manifestations of OSA. Presumably, the oxidative stress is due to an activation of NADPH oxidase and other free oxygen radicals producing enzymes within the CV system as evidenced by data from knockout mice and pharmacological interventions. It is hoped that animal models of OSA-related CV disease will continue to contribute to a deeper understanding of their underlying pathophysiology and will foster the way for the development of cardioprotective treatment options other than conventional CPAP therapy.

1. Introduction

Obstructive sleep apnea (OSA) is a highly prevalent sleep-related breathing disorder presenting with the clinical hallmarks of snoring, witnessed nocturnal apneas, and excessive daytime sleepiness. It is caused by repetitive collapse of a narrow upper airway during sleep with the main predisposing factor being obesity [1]. Large-scale epidemiological studies have clearly shown that untreated OSA is an independent cardiovascular (CV) risk factor. Amongst others, severe OSA (i.e., with an apnea-hypopnea-index (AHI) exceeding 30 per hour of sleep) can contribute to the emergence of arterial hypertension, heart failure, stroke, and pulmonary hypertension [2–4]. In addition, otherwise healthy OSA patients can already display more subtle CV changes such as endothelial dysfunction and vascular remodelling [5, 6].

Based on data obtained in patients with OSA, it is currently believed that sympathetic activation, inflammation, and oxidative stress play major roles in the pathophysiology of OSA-related CV diseases [7–10]. However, due to various reasons, the possibilities to conduct further human research into the relationship between OSA and CV diseases are limited. First, OSA patients often present with confounding factors increasing *per se* CV risk such as obesity, concomitant metabolic disease, and smoking. Second, CV diseases in OSA patients often need many years to develop in order to be diagnosed clinically. Third, it is difficult to perform more invasive experimental procedures in these patients.

These drawbacks may be overcome by using animal models of OSA. In most animal studies, only the cyclical pattern of hypoxia characteristic of OSA is simulated [11] (Figure 1). For this purpose, animals are housed in a chamber and cyclically exposed either to normoxia/hypoxia or room air in a computer controlled manner.

Depending on animal species, type of stimuli, and technical approaches, there are many animal models described in the literature. The duration (from 30 seconds up to 30 min) of hypoxic exposure generally varies indirectly to the frequency of events (2 to 120/hour) [12–21], and this issue may contribute to discrepancies in the literature. However, the standard animal model is represented by chronic intermittent exposure of rodents to an FiO2 nadir of 6–10% for 30 sec to 1 min which results in oxyhemoglobin desaturation of about 60% to 80% (Figure 1).

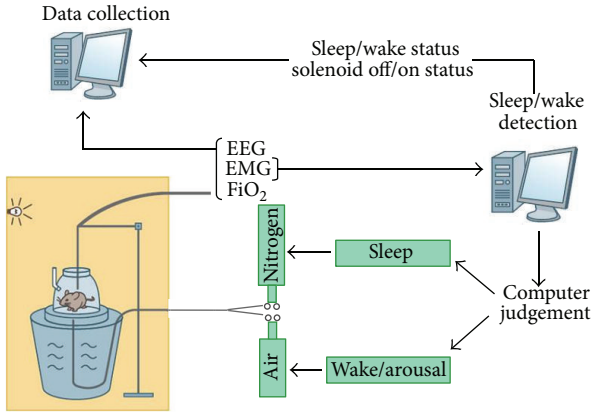

Figure 1: Schematic representation of the mouse/rat model simulating OSA-associated CIH. Animals are housed in plexiglass chambers and are repetitively exposed either to room air (i.e., 21% O_2) or nitrogen (i.e., hypoxia). Exposure to CIH may be synchronized to the sleep-wake cycle by simultaneously measuring EEG and EMG activities of animals.

It should be recognized that the above-mentioned experimental conditions primarily mimic severe forms of human sleep apnea with AHI >30/h. Furthermore, some of the human OSA characteristics are not highlighted in this experimental model. For example, the model fails to reproduce upper airway occlusion, intrathoracic pressure swings, arousals, and hypercapnia. On the other hand, reproducing a single characteristic of clinical OSA by exposure to cyclical hypoxia appears to be relevant in order to induce pathophysiological changes similar to clinical manifestations of patients suffering from OSA. Thus, animals exposed to CIH develop after 3 to 5 weeks pathological changes similar to those found in OSA patients such as endothelial dysfunction, atherosclerosis, arterial hypertension, pulmonary hypertension, and heart failure. Regardless of these considerations, other more complex models have also been developed which additionally reflect the nonhypoxic stimuli linked to OSA and may thus give a more realistic picture of the human disease [22, 23].

In the present paper, we will discuss the animal models of OSA and their respective contributions to a deeper understanding of the CV consequences of OSA. Within this frame, we will focus on oxidative stress as the most relevant intermediary pathogenetic mechanism. The CIH exposure induces tissue deoxygenation followed rapidly by tissue reoxygenation leading to ROS formation.

A few ROS-generating systems are described in the literature including NADPH oxidase (NOX), xanthine oxidase, mitochondrial chain, and uncoupled nitric oxide synthase (NOS). Probably, NOX is the most important enzyme in the setting of CIH as will be discussed in the following sections. The deleterious effects of ROS on the CV system are primarily exerted by unspecific oxidation of biological compounds (DNA, protein, lipid, etc.) and modulation of specific signalling pathways (i.e., redox signalling). In addition, ROS may activate the sympathetic nervous system and enhance inflammatory processes thus acting as master regulators in the pathogenesis of OSA-associated CV diseases.

2. Endothelial Dysfunction

Otherwise healthy, nonsmoking OSA patients have been shown to suffer from endothelial dysfunction, that is, a reduction of endothelial-dependent vasodilation, which may be relieved by effective continuous positive airway pressure (CPAP) therapy [6, 24]. Animal studies clearly suggest that the endothelial dysfunction characteristic of OSA is the result of an increased oxidative stress with reduced bioavailability of nitric oxide (NO).

Rats exposed to chronic intermittent hypoxia (CIH) exhibit a reduced vasodilation in response to infusions of the NO liberator acetylcholine and reduced vasoconstriction following NO synthase inhibition [25]. Furthermore, treatment of CIH-exposed rats with the superoxide dismutase (SOD) mimetic tempol restores vascular reactivity [26]. Investigations of *in vitro* vasoreactivity of isolated coronary and cerebral arteries from mice have found that an activation of NOX is probably responsible for the occurrence of endothelial dysfunction in response to CIH [27].

Similar findings were reported by Liu et al. [28] describing erectile dysfunction in CIH rats due to increased ROS production. In this model, NOX is activated, NOS activity is impaired, and ROS production is increased. Furthermore, treatment of animals with apocynin (a selective NOX inhibitor) normalizes NOX and NOS activities and restores the erectile function. Recent clinical and experimental data suggest that xanthine oxidase-dependent ROS production also induces endothelial dysfunction in muscular skeletal arteries [29, 30] and that xanthine oxidase inhibition by allopurinol reverses this phenomenon.

3. Atherosclerosis

Endothelial dysfunction is a precursor lesion for atherosclerosis. Consequently, OSA patients display increased common carotid artery-intima media thickness when compared with matched controls without sleep-disordered breathing [5]. Furthermore, CPAP therapy leads to a decrease of this noninvasive marker of atherosclerosis [31]. Animal studies support the concept that the enhanced atherosclerosis known to occur in OSA is due to increased vascular inflammation and lipid peroxidation in response to oxidative stress.

In this context, mice exposed to intermittent hypoxia exhibit increased leukocyte adhesion in their cortical venular microcirculation [32]. Another study reported increased flux of leukocytes rolling, a number of rolling leukocytes, and a number of adherent leukocytes in colonic venules following 3 hours of recurrent obstructive apneas in rats [33]. Of note, ROS may act as proinflammatory triggers by inducing NF-κB and subsequently the expression of proinflammatory cytokines such as interleukin-6, tumor necrosis factor alpha and C-reactive protein [34].

In addition, they may exert proatherogenic effects by increasing lipid peroxidation as shown in a mouse model of OSA [35]. In accordance with these assumptions, direct

evidence has been obtained in mice that CIH leads to the formation of atherosclerotic lesions. Exposure to CIH for 12 weeks induced the development of atherosclerotic plaques, but a concomitant high-cholesterol diet was necessary for that effect to occur [36].

4. Arterial Hypertension

Arterial hypertension is the most frequent CV complication of OSA and there is a significant dose-response relationship between the AHI and the odds ratio for developing arterial hypertension [37]. Animal studies have shown that various vasoactive mediator systems may be responsible for the pressor effect of CIH and that this is primarily mediated through an augmentation of carotid chemoreflex function.

In a series of experiments, Flechter et al. were among the first to show the importance of the sympathetic nervous system in this context. They demonstrated that surgical denervation of peripheral chemoreceptors prevented the increase in arterial blood pressure in response to CIH. Adrenal demedullation and chemical denervation of the peripheral sympathetic nervous system by 6-hydroxy dopamine also prevented the increase in blood pressure [38, 39].

Carotid chemoreflex sensitization caused by CIH may also be due to angiotensin-II-induced activation of NOX with subsequent production of ROS. Chronic exposure of rats to CIH results in elevation of plasma renin activity, and pharmacological inhibition of the renin-angiotensin-aldosterone system attenuates CIH-induced arterial hypertension [40]. Similar effects can be observed after ascorbic acid (an antioxidant vitamin [41]), tempol [42], and apocynin [43].

Finally, data from our laboratory show that NOX knockout blocks the development of arterial hypertension in response to CIH [44]. As a more direct evidence, gene transcription of NOX subunits has been found to be upregulated in the carotid body in response to CIH [45].

The current concept is that CIH activates HIF-1α thereby enhancing NOX2 transcription and ROS production [46]. On the other hand, it decreases HIF-2α-dependent SOD activation and thus leads to a reduced clearance of ROS [47]. Importantly, the ROS formed within the carotid body may exert their pressor effects by enhancing central sympathetic activity [48].

Endothelin-1 is another vasoactive mediator which is strongly upregulated in the carotid body in response to CIH and receptor antagonism by bosentan abolished abnormal chemosensitization [49, 50]. In accordance with these findings, other groups have found that systemic administration of endothelin receptor antagonists to rats/mice prevents the increase of blood pressure during CIH [51, 52].

In contrast to angiotensin and endothelin-1, NO inhibits carotid body chemosensitivity [53]. Rats exposed to CIH express less neuronal NO synthase [54], and stimulation of NO production by L-arginine restores the carotid body chemosensitivity [53] thus suggesting that an impaired bioavailability of NO might be responsible for enhanced carotid chemoreflex sensitivity under CIH.

It should be mentioned that the OSA-associated arterial hypertension probably results not only from increased carotid chemoreflex but also from decreased baroreceptor activity. Finally, locally acting processes in peripheral blood vessels as discussed in Section 2 (i.e., reduced NO bioavailability) may play significant roles.

5. Pulmonary Hypertension

20–30% of untreated OSA patients suffer from pulmonary arterial hypertension. It was first thought that this phenomenon is restricted to patients with pulmonary comorbidities such as COPD, but it is now widely accepted that OSA itself can lead to pulmonary hypertension [55]. Up to date, this aspect of OSA-associated CV morbidity has been less extensively investigated in animal models. A histomorphometric study showed that mice exposed to CIH develop characteristic features of pulmonary hypertension such as elevated pulmonary artery pressure, right ventricular hypertrophy, and muscularization of small pulmonary arteries [56]. Quite similar observations were later reported by another group [13]. Furthermore, it was demonstrated that NOX knockout mice are protected against the development of CIH-associated pulmonary hypertension [57]. A more recent study showed that pulmonary vasodilatory capacity is impaired under conditions of CIH and that this is related to increased vascular superoxide anion production [58]. Thus, the same pathogenetic mechanisms which have been proposed for acute hypoxic pulmonary vasoconstriction [59] may be operative in OSA-associated pulmonary hypertension.

6. Heart Failure

OSA patients are also at increased risk for the development of chronic heart failure. This may be due to OSA-related arterial hypertension, coronary artery disease, and the direct negative inotropic effects of breathing against an occluded upper airway [60]. Experimental models of OSA support the notion that CIH negatively affects left ventricular (LV) function and that oxidative stress is an important mediator of myocardial damage.

Exposure of dogs to CIH induces LV hypertrophy and a decrease in ejection fraction [61]. Experimental CIH in rats/mice results in myocardial remodeling with myocyte hypertrophy and interstitial fibrosis finally leading to LV dysfunction [62, 63]. Moreover, these studies suggest that an increased myocardial oxidative stress plays a significant role in this context. Chen et al. observed a significant inverse relationship between LV function and the myocardial content of lipid peroxides [62]. Furthermore, myocardial NOX subunit expression is increased in response to CIH [64, 65], and NOX knock-out mice are obviously protected against the development of LV dysfunction in response to CIH [63]. Finally, treatment of mice with allopurinol significantly attenuates myocardial changes induced by CIH [66]. The exact mechanisms by which ROS induce LV dysfunction are not known, but it is largely accepted that oxidative stress causes a cytotoxic tissue injury by increased lipid peroxidation, protein oxidation, and direct DNA damage leading in

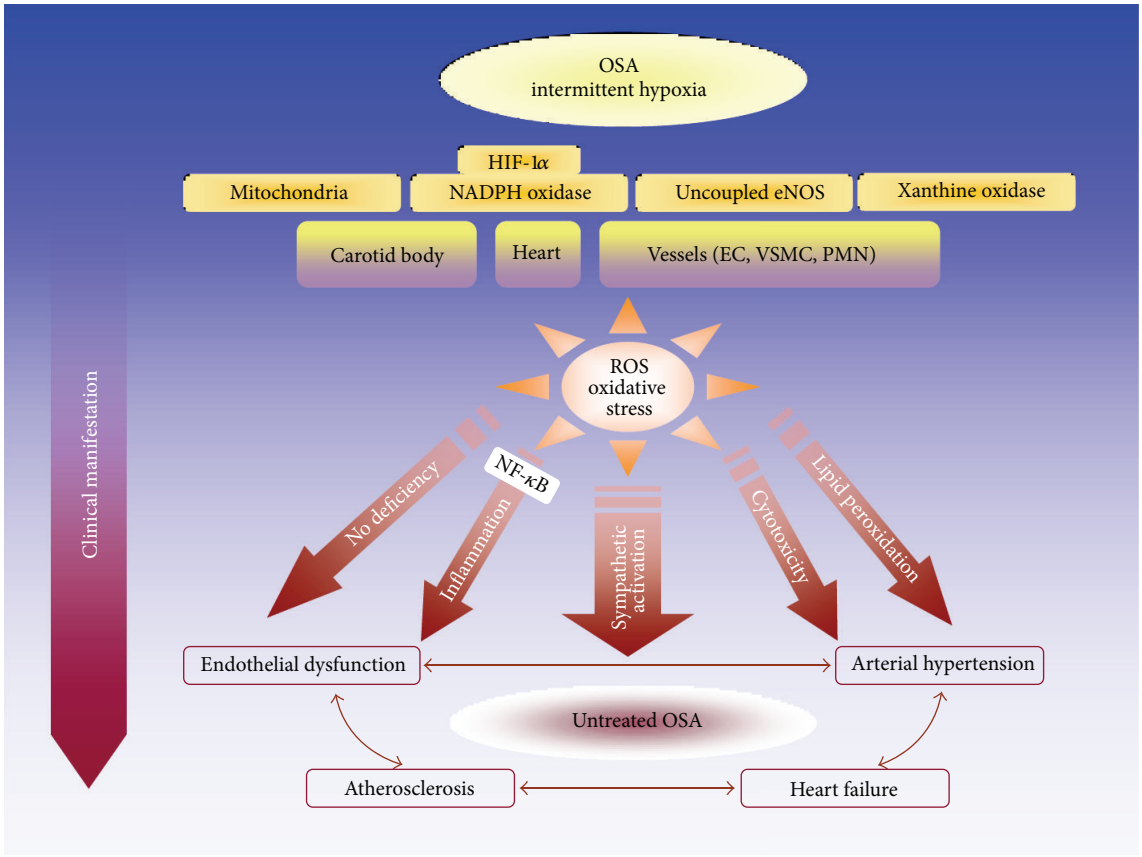

Figure 2: Simplified model of oxidative stress as the central pathogenetic pathway in OSA-associated CV diseases as suggested by animal studies. OSA-associated intermittent hypoxia activates NOX and other ROS-producing enzymes in the carotid body, the heart, and the vessels (PMN: polymorphonuclear neutrophils, EC: endothelial cells, VSMC: vascular smooth muscle cells). The resulting radical flux exerts direct cytotoxic effects, decreases NO bioavailability, enhances lipid peroxidation, increases sympathetic activity, and activates the proinflammatory transcription factor NF-κB. Finally, these changes lead to the well-known clinical manifestations of OSA in the CV system.

this way to apoptosis, necrosis, and abnormal tissue repair processes.

7. Conclusions

In this paper, we have briefly summarized the current state of knowledge about the pathophysiology of OSA-related CV diseases as provided by basic research conducted in animal models of OSA. Almost the complete clinical spectrum of CV diseases known to occur in humans with OSA has been replicated in animals subjected to CIH. The common result of these studies is that an increased oxidative stress, mostly derived from an activation of NOX, seems to play a key role in the development of OSA-associated CV diseases (Figure 2).

It is anticipated that animal studies will continue to enhance our understanding of the pathogenesis of OSA-related CV diseases for instance by investigating knock-out and genetically engineered mice or by performing selective pharmacological interventions. In this way, the fundamental molecular pathways linking OSA to CV diseases may be identified and new cardioprotective treatment options may emerge for the relatively large proportion of OSA patients unable to tolerate CPAP therapy.

Abbreviations

AHI: Apnea-hypopneaindex
CIH: Chronic intermittent hypoxia
CPAP: Continuous positive airway pressure
CV: Cardiovascular
LV: Left ventricular
NO: Nitric oxide
NOS: Nitric oxide synthase
NOX: NADPH oxidase
OSA: Obstructive sleep apnea
ROS: Reactive oxygen species
SOD: Superoxide dismutase.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Clinical Study

Protein Sulfhydryl Group Oxidation and Mixed-Disulfide Modifications in Stable and Unstable Human Carotid Plaques

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Objectives. Oxidative stress has been implicated in the outcome of atherosclerotic plaques. However, at present, no data are available neither on the degree of plaque protein sulfhydryl groups oxidation nor on its relationship with plaque vulnerability. We investigated the entity of protein-SH oxidative modifications, focusing on low molecular weight thiols adduction, in human carotid plaque extracts in relation to plaque stability/instability. *Methods*. Plaque stability/instability was histologically assessed. The extent of protein-SH oxidative modifications was established by a differential proteomic approach on fluorescein-5-maleimide-labeled plaque extracts and corresponding plasma samples from 48 endarterectomized patients. The analysis on protein thiolation was performed by capillary zone electrophoresis. *Results*. We observed a higher protein-SH oxidation of both plasma-derived and topically expressed proteins in unstable plaques, partly due to higher levels of S-thiolation. Conversely, in plasma, none of the investigated parameters discriminated among patients with stable and unstable plaques. *Conclusions*. Our results suggest the presence of a more pronounced oxidative environment in unstable plaques. Identifying specific oxidative modifications and understanding their effects on protein function could provide further insight into the relevance of oxidative stress in atherosclerosis.

1. Introduction

Plaque rupture and thrombosis are the most important clinical complications in the pathogenesis of acute coronary syndromes and peripheral vascular disease [1]. Although the exact mechanisms underlying plaque vulnerability are not completely clear, it is generally held that plaque instability is characterized by a pronounced proteolytic and proinflammatory environment [2]. In a previous study, we provided evidence for a wide fragmentation of some apolipoproteins and arterial proteoglycans and for a pro-inflammatory microenvironment in unstable and much less in stable endarterectomy carotid plaques [3]. Recently, by applying proteomics to the study of carotid plaque vulnerability, we identified a panel of proteins, differently expressed in stable/unstable lesions, with prooxidant and proinflammatory potentials according to our current understanding of the molecular basis of the atherosclerotic process [4]. *In situ* oxidative events may have important functional consequences on protein metabolic fate as well as on their bioactivity and antigenic properties. In this respect, oxidised LDL is readily internalized by macrophages through the so-called "scavenger receptor" pathway [5]. These early modifications could initiate and/or contribute to atherogenesis, mainly when an imbalance between oxidant and antioxidant agents takes place. Many enzymes (such as superoxide dismutases, catalase, peroxidases, glutathione-S transferase and reductase, and peroxiredoxins), as well as nonenzymic proteins (transferrin, ferritin, albumin, and aptoglobin), and nonproteinaceous antioxidants (ascorbic acid, uric acid, and α-tocopherol) are known to participate in maintaining a reductive environment within the arterial wall [6]. Together with these antioxidants, protein sulfhydryl groups (protein-SH groups, PSH) and low molecular weight thiols (LMW-thiols) are involved in the cell regulation of reactive oxygen species levels. Many proteins and enzymes have cysteine residues in their side chain, and their proton

lability makes them chemical hot spots for a wide variety of biochemical interactions such as the reversible reaction of *S*-thiolation (the formation of mixed disulfides among protein thiols and LMW-thiols) [7]. The formation of S-thiolated proteins could be the result of an antioxidant response, and it has been suggested as a possible redox regulation mechanism of protein function [8–10]. So, the reversible covalent modification of some protein cysteine residues may be transitory and have critical modulation effects. It has been suggested that homocysteinylation could activate latent elastolytic metalloproteinase-2 (pro-MMP-2) by disulfide bond formation on the propeptide via the so-called "cysteine switch" mechanism [11]. In this regard, also the S-glutathionylation is thought to play a role [12]. All these evidences have led to the intriguing hypothesis that direct LMW-thiols-mediated matrix metalloproteinases (MMPs) activation could be involved in the extracellular matrix degradation and plaque rupture.

We have previously demonstrated that LDL apolipoprotein B-100 is able to bind all plasma thiols [13, 14] and that human carotid atherosclerotic plaque has different levels and distribution of LMW thiols with respect to plasma [15]. In this respect, the elevated levels of intraplaque glutathione may induce important effects on plaque fate by perturbing the physiological LMW thiol redox state.

The aim of the present work was to investigate the redox status of protein sulfhydryl groups extracted from atherosclerotic plaques in relation to lesion stability. Moreover, we evaluated levels and distributions of both total and protein-bound LMW-thiols to assess the degree of protein mixed-disulfide modification.

2. Methods

2.1. Patient Population. Proteomic and capillary zone electrophoresis (CZE) analyses were conducted on both carotid plaque specimens and plasma samples from forty-eight patients undergoing carotid endarterectomy, enrolled in a previous study and screened for plaque stability by immunohistochemistry [4]. Informed consent was obtained before enrollment. The study was approved by the local Ethical Committee of the University of Milan in accordance with institution guidelines.

2.2. Plaque and Plasma Samples. Tissue extracts from carotid endarterectomy specimens, histologically classified as stable ($n = 19$) or unstable ($n = 29$) plaques, were obtained as previously described [3, 4]. Briefly, the plaque segments were washed in PBS, finely minced, and subjected to protein extraction with a solubilisation buffer (8 M urea, 4% w/v CHAPS, 45 mM Tris), supplemented with 100 μmol/L APMSF, 2 μg/mL KI, and 50 μmol/L leupeptin, under continuous shaking for 1 h at room temperature. Extracts were collected by centrifuging the resulting suspension at 65,000 ×g in a TL-100 Beckman centrifuge for 30 min at 20°C.

Before surgery, blood was collected in Vacutainer tubes containing EDTA. After centrifugation at 1000 ×g at 4°C for 15 minutes, the plasma was separated, supplemented with the aforementioned antiproteolytic agents, and stored at −80°C until analysis.

2.3. Proteomic Analysis. Tissue extracts were delipidated and resolubilized as previously described [4].

PSH of both resolubilized plaque extracts and plasma samples were fluorotagged with fluorescein-5-maleimide (F5M) in the dark following the manufacturer instructions (PIERCE Biotechnology).

8 μg and 50 μg of F5 M-labelled protein were loaded for 1D SDS-PAGE and 2D electrophoresis, respectively. 2D electrophoresis was conducted as already reported [4]. Briefly, IEF was performed using 70 mm, immobilised linear pH 4–8 gradient strips (Nurex srl, Sassari, Italy). IPG strips were rehydrated overnight at 20°C with 50 μg of F5 M-labelled protein diluted in a solubilisation buffer containing 1% w/v DTT and 2% v/v Pharmalyte (pH 3.5–10) and subsequently focused at 50 μA/IPG strip for 22 kVh at 18°C. Once IEF was completed, the strips were equilibrated under continuous shaking for 15 min in 50 mM Tris-HCl buffer containing 6 M urea, 30% v/v glycerol, and 3% w/v SDS with the addition of 1% w/v DTT, followed by an equilibration for 15 min in the same buffer without DTT, but with the addition of 2.5% w/v iodoacetamide. The IPG strips were then sealed with 0.5% low melting point agarose in SDS running buffer at the top of slab gels (8 × 7 × 0.1 cm). SDS-PAGE was performed on 10%T, 3%C polyacrylamide separating gels in a MiniProtean II cell vertical slab gel electrophoresis apparatus (Bio-Rad, Hercules, CA, USA).

Fluorescence images of resolved proteins were acquired by using the Gel Doc XR system (Bio-Rad, Hercules, CA, USA), and, subsequently, gels were stained with Coomassie Brillant Blue G250 (CBB). Images were analysed using Quantity One 4.6.3 software (Bio-Rad, Hercules, CA, USA). Band fluorescence data were normalized for the corresponding intensity after CBB staining. Protein spots identification was performed by peptide mass fingerprinting analysis as reported elsewhere [4].

2.4. Capillary Zone Electrophoresis (CZE) Analysis. Total and protein-bound LMW-thiols in plaque extracts and corresponding plasma samples were assayed as previously described [15].

Briefly, for total LMW-thiols assay, 20 μL of extract were treated with 20 μL of internal standard NAC (6 μM) and 4 μL of 10% TBP in DMF (v/v) for 10 min. Then, proteins were precipitated by adding 1 mL of acetonitrile (ACN), and supernatant containing LMW-thiols was dried under vacuum and resolubilized with 100 μL of 30 mM sodium phosphate buffer (pH 12.5) containing 0.08 mM 5-IAF.

For protein-bound thiols assay, 600 μg of delipidated proteins were resolubilised in 200 μL of 1 mM NaOH and 2 μL of NAC (1.5 μmol/L) at 60°C for 30 min. Disulfide bonds were reduced by incubating with 20 μL of 10% TBP in DMF for 10 min, and proteins were precipitated by adding 900 μL of ACN. Then, supernatant was dried under vacuum, and LMW-thiols were derivatized as previously mentioned.

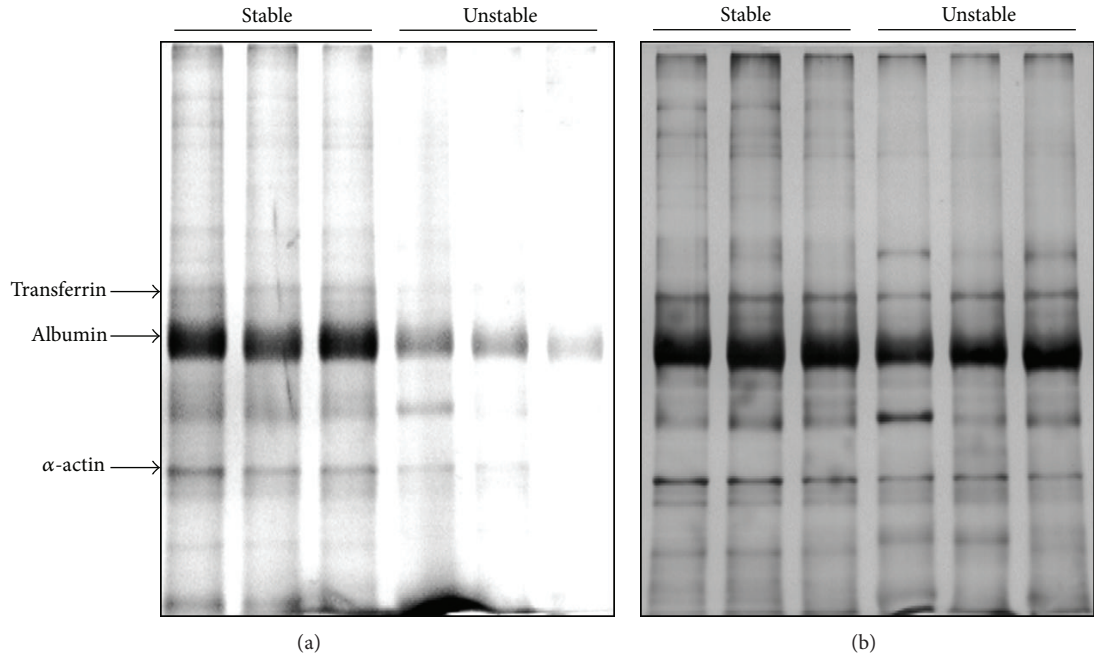

Figure 1: Representative SDS-PAGE patterns of F5M-labeled proteins from stable and unstable plaque extracts (a) and the corresponding CBB staining (b). In (a), inverted fluorescent image is reported.

LMW-thiols measurement was performed by a CE system (P/ACE 5510) equipped with a LIF detector (Beckman, Palo Alto, CA, USA).

Total LMW-thiols were calculated as the sum of free LMW-thiols and protein-bound LMW-thiols.

2.5. Statistical Analysis. Differences between stable and unstable plaques were evaluated by either the Student *t*-test, for normally distributed data, or the Mann-Whitney Rank Sum test, for nonparametric ones. Differences with P values < 0.05 were considered to be significant.

3. Results

3.1. PSH Oxidation (Proteomic Analysis). Differential analysis of F5M-labeled PSH groups by 1D SDS-PAGE indicated deep differences in oxidative state related to plaque stability (Figure 1), whereas no significant difference was observed in plasma samples (data not shown), where only the band corresponding to albumin was detectable. After normalization, the total protein fluorescence signal was significantly different (Figure 2(d)), being approximately twofold lower in unstable plaque extracts than in stable ones ($P = 0.007$). Significant differences were evidenced for transferrin (Figure 2(a), UN/ST = 0.43, $P = 0.006$), albumin (Figure 2(b), UN/ST = 0.48, $P = 0.008$), and α-actin (Figure 2(c), UN/ST = 0.56, $P = 0.02$). Since the fluorescent probe used is known to be effective for labeling reduced protein sulfhydryl groups forming a stable thioether bond [16], the reduced fluorescence intensity observed in unstable plaque extracts, compared to stable ones, reflects a more oxidized status of protein-SH groups in the formers. The lack of differences between the corresponding subsets of plasma samples suggests that PSH oxidative modifications mainly take place within the arterial wall.

2DE analysis allowed a better identification of involved proteins, also confirming the differences in F5M-labeling between the stable and unstable plaque extracts (Figure 3). A total of fourteen F5M-labeled proteins, either filtered or topically expressed, were identified by MALDI-TOF MS analysis (Table 1). On the basis of our previous proteomics data [4], none of them, with the exception of heat shock protein 27, showed significant differential expression between stable and unstable plaque extracts. Overall, the 2DE results corroborated the finding of a higher protein-SH group oxidation in unstable plaques.

3.2. LMW-Thiols Determinations (CZE Analysis). The adopted CZE-LIF method, owing to its elevated sensitivity and selectivity, represents a good tool for an ultrasensitive analysis of LMW-thiols in tissue samples. A representative electropherogram of total and protein-bound LMW-thiols from plaque extracts is shown in Figure 4. Levels of LMW thiols in the atherosclerotic tissue extracts are reported in Table 2. No differences in total LMW thiols were evidenced between stable and unstable plaque extracts. On the contrary, protein-bound LMW thiols levels were significantly higher in unstable than in stable plaque extracts (373 ± 111 versus 283 ± 126 nmol/g prot). The analysis of plasma LMW-thiols did not show differences in both total and protein-bound levels between the two subgroups of patients (data not shown). Moreover, plasma thiols concentration and distribution were similar to those previously obtained in healthy subjects [14]. Interestingly, both levels and

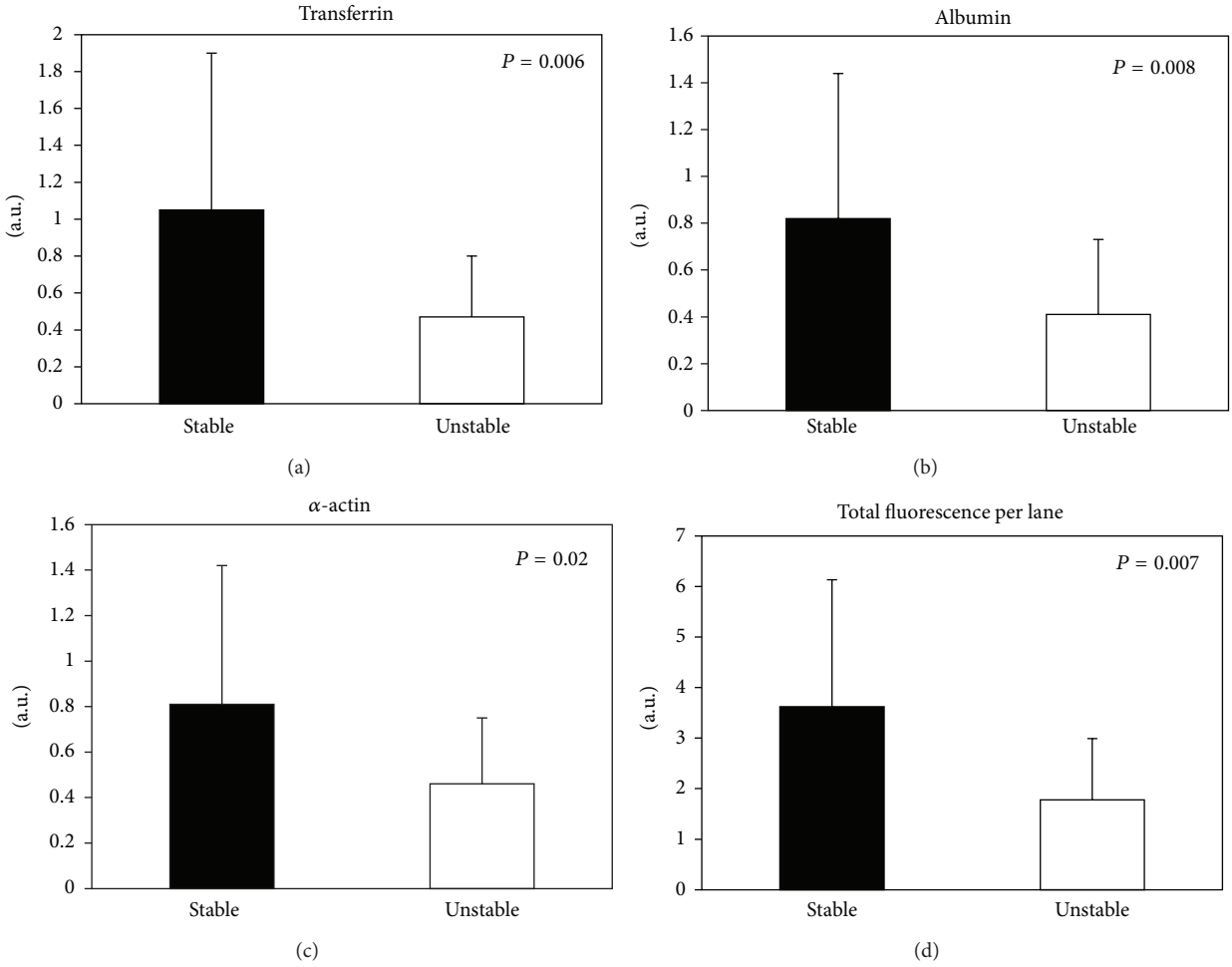

Figure 2: Graphic representation of results obtained by SDS-PAGE of F5M-labelled proteins extracted from stable (black bars) and unstable (empty bars) plaques. The fluorescence intensity signals (arbitrary units) of single bands (a, b, and c) and single lanes (d) were normalized for the corresponding signals obtained after Blue Coomassie G-250 staining.

Table 1: Differentially oxidised proteins identified after 2D electrophoresis by peptide mass fingerprinting analysis.

Spot no.	Identified protein	Accession no. (NCBI)	Theo. Mr (KDa)	Theo. pI	Matched peptides	Coverage (%)
1	Transferrin	gi|4557871|	79.31	6.9	17	25
2	Human serum albumin	gi|31615330|	66.44	5.7	14	21
3, 4	Alpha-2HS-glycoprotein	gi|4502005|	40.11	5.4	3	23
5, 6	Alpha-1-antitrypsin	gi|1703025|	46.89	5.4	11	22
7, 8, 9	Fibrin beta	gi|223002|	51.37	8.3	7	21
10	Vimentin	gi|340219|	53.75	5.0	12	32
11	Vimentin	gi|5030431|	41.66	4.8	12	42
12	Vimentin	gi|340234|	35.09	4.7	10	42
13	Alpha actin	gi|4885049|	42.34	5.2	10	36
14	Tropomyosin 1	gi|88927|	33.03	4.6	8	21
15	SP40 (Apo-J)	gi|338305|	37.00	5.7	7	33
16	Modulator of apoptosis 1 (MAP-1)	gi|19923584|	39.72	5.2	3	14
17, 18	Tropomyosin 4	gi|4507651|	28.62	4.7	7	26
19, 20	Heat shock 27 KDa protein 1	gi|4504517|	22.82	6.0	8	44

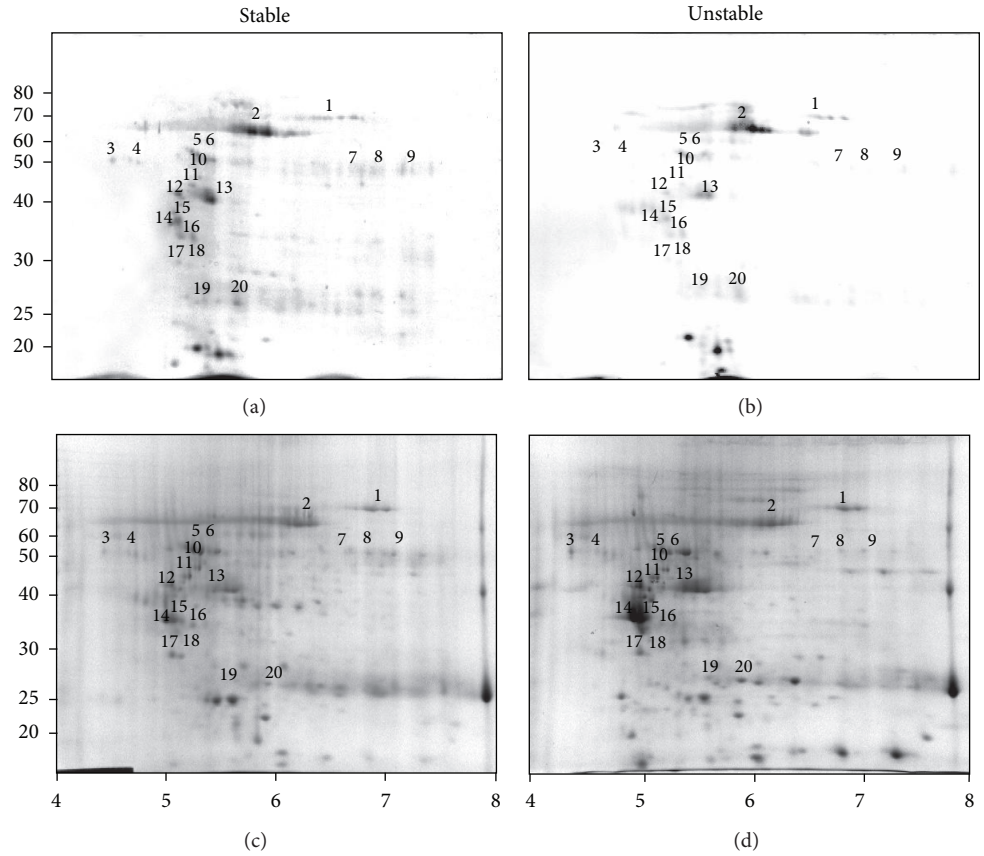

Figure 3: Representative 2D electrophoretic patterns of F5M-labelled proteins and the corresponding CBB staining from stable ((a) and (c)) and unstable ((b) and (d)) plaque extracts. In (a) and (b), inverted fluorescent images are reported. The molecular weight scale was constructed from protein standards (Invitrogen BenchMark Protein Ladder) run alongside the focused strip in the second dimension. The pI scale is based on the linear immobilized pH gradient over 7 cm strips. The numbers indicated on the gels correspond to the spot numbers given in Table 1.

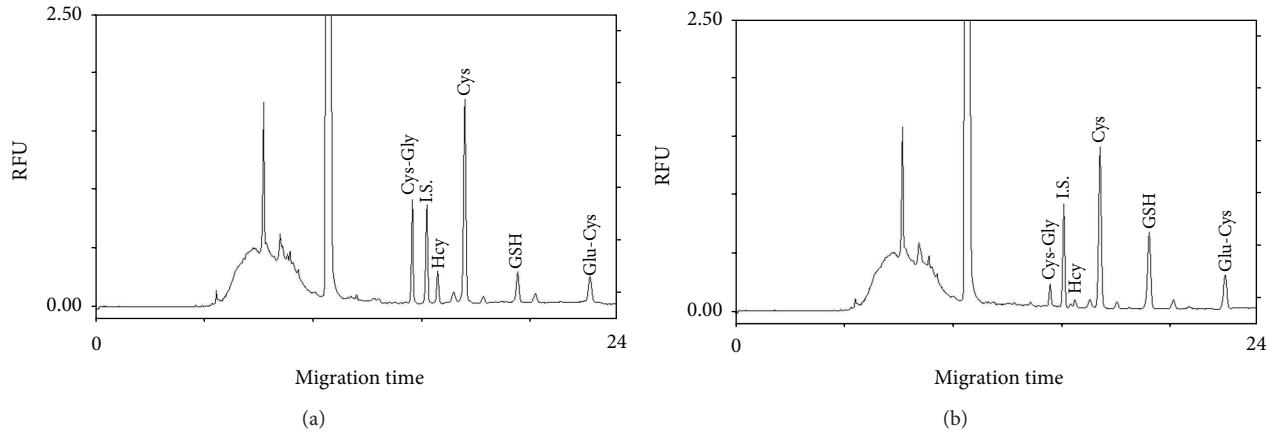

Figure 4: Typical electropherograms of total (a) and protein-bound (b) LMW-thiols from carotid plaque extracts. Cys-Gly: cysteinylglycine; Hcy: homocysteine; Cys: cysteine; GSH: glutathione; Glu-Cys: glutamylcysteine; I.S.: internal standard.

Table 2: Levels of protein-bound and total LMW-thiols from stable and unstable plaque extracts.

	Stable (nmol/g prot)	Unstable (nmol/g prot)	*P* value
Protein-bound LMW-thiols			
Cys-Gly	13.2 (11.3–16.9)	12.1 (8.4–17.1)	0.339
Hcy	5.7 ± 3.2	6.7 ± 4.0	0.468
Cys	146.0 (117.5–211.4)	219.6 (147.6–301.9)	0.089
GSH	41.1 (23.2–77.9)	80.8 (42.0–122.1)	0.168
Glu-Cys	14.1 ± 5.6	15.3 ± 7.0	0.593
Total	283 ± 126	373 ± 111	**0.034**
Total LMW-thiols			
Cys-Gly	53.8 ± 21.1	44.6 ± 19.7	0.205
Hcy	15.0 ± 10.2	20.7 ± 12.3	0.179
Cys	600 ± 276	576 ± 208	0.767
GSH	128.6 (95.1–202.4)	171.2 (97.2–346.4)	0.189
Glu-Cys	69.2 ± 29.4	62.7 ± 29.8	0.534
Total	900 ± 273	960 ± 293	0.555

Values are either mean ± SD or median with ranges (in parenthesis).
LMW-thiols levels were expressed as nmol/g of extracted proteins.
Total LMW-thiols are the sum of free LMW-thiols and protein-bound LMW-thiols.

distribution of LMW-thiols were significantly different between plasma and arterial tissue extracts confirming our previous findings [15].

4. Discussion

A large body of evidences has implicated free radicals and oxidative stress in atherogenesis processes [5, 17]. The endogenous antioxidant capacity of arterial tissues seems to be relevant on this matter since LDL oxidation may occur in sequestered domains of the arterial wall, where a low antioxidant potential and/or a high prooxidant activity could be operative [5, 17–19]. Indeed, it has been shown that human atherosclerotic plaques had low levels of glutathione-related enzyme antioxidant protection [20], so confirming the hypothesis that a specific antioxidant/prooxidant imbalance, operative in the vascular wall, might be involved in atherogenic processes in humans. In this respect, we recently evidenced a reduction in the levels of the enzymes superoxide dismutase 3 and glutathione S-transferase in advanced unstable carotid plaques [4].

Attempting to investigate the relationship between oxidative stress and plaque progression, we studied some post-translational oxidative modifications of extractable proteins from atherosclerotic plaques by means of a differential proteomic approach. To this extent, we preliminarily investigated protein carbonyl groups and HNE adducts as biomarkers of oxidative stress, without detecting substantial differences between stable and unstable plaques (data not shown).

The present study has provided evidence that in the histologically classified unstable carotid plaques, and to a lesser extent in the stable ones, there is a pro-oxidant microenvironment conducive to the formation of ROS- and RNS-mediated protein thiols oxidation products, as well as of mixed disulfides between proteins and LMW thiols.

The significant PSH groups oxidation observed regards both filtered and topically expressed proteins. Although several specific proteins showed a different degree of sulfhydryl group oxidation between stable and unstable plaque extracts, the most pronounced differences regarded albumin, α-actin, and transferrin.

Albumin is a nonglycosylated, single-chain polypeptide tightly folded into three domains that are structurally defined by 17 intra-chain disulfide bonds. Albumin Cys^{34}, the only cysteine residue uninvolved in intrachain disulfide bonds, accounts for the bulk of free-SH groups in plasma. Its pK_a is abnormally low (~5) compared to that of most plasma LMW-thiols [21]. It is present primarily in the reduced form (mercaptalbumin), although about 30%–40% could be variably oxidized, either reversibly (non-mercaptalbumin) as mixed disulfide with LMW-thiols [22], S-nitroso Cys [23], and sulfenic acid or, irreversibly, as sulfinic or sulfonic acid [24]. Furthermore, it has been described that albumin, through nucleophilic residues, and in particular Cys^{34}, is the main plasma target of reactive carbonyl species such as 4-hydroxy-trans-2-nonenal so acting as an endogenous detoxifying agent for these proatherogenic species [25]. Recently, we have developed a new highly sensitive analytical method for the quantification of LMW-thiols bound to both circulating and tissue-retained albumin [26]. Preliminary results suggested that about 35% of filtered albumin carries LMW-thiols showing different thiolation pattern compared with the corresponding circulating form.

Mammalian α-actin contains five cysteine residues in the reduced form [27]. Dalle-Donne et al. [27, 28] have shown how a reversible *S*-glutathionylation of Cys^{374} regulated the actin filament formation by inducing structural changes in the actin molecule.

A human serum purified mature transferrin containing 19 disulfide bonds, and no free sulfhydryl groups have been

described [29]. Indeed, we did not evidence any fluorescent signal from plasma transferrin (data not shown), further confirming these observations. However, the evidences we obtained on tissue samples clearly indicate that arterial transferrin is able to bind the fluorescent probe, suggesting the presence of some reduced –SH groups in the protein structure. The stability of cysteine redox state is dictated by the dihedral strain energy of disulfide bond [30]. It is well known that free homocysteine may not only react with free protein sulfhydryl groups but also disrupt critical Cys-Cys disulfide bonds, so damaging the protein structure and compromising its functionality [31]. These reactions are reversible and can vary depending on the redox potentials of the biological systems [32]. We hypothesize that some disulfide bridges may be destroyed following the transferrin infiltration into the prooxidative microenvironment of the subendothelial space leading to the formation of sites for protein mixed-disulfide modifications and probe binding.

Overall, our proteomic analysis on oxidative modifications of extracted PSH suggested a more pronounced oxidative environment in unstable plaques.

These results were partially confirmed by CZE-LIF analysis of LMW thiols bound to proteins showing higher levels in unstable plaque extracts. Interestingly, such an increase in protein-bound thiols content was not associated with a similar increase in total LMW thiols content. Furthermore, LMW-thiols plasma levels did not discriminate between patients with stable and unstable plaques.

Results obtained by the CZE analysis, compared with those by proteomics, suggest that in unstable plaques, the higher protein-SH thiolation accounts only partially for the total PSH oxidation observed. In this respect, other PSH oxidation processes, either reversible or irreversible, could be implied. Although intraplaque protein S-thiolation is only one of the possible mechanisms of protein-thiols oxidation, we postulate that the more pronounced S-thiolation in the unstable plaques might have important consequences. It is known that the activity of some MMPs is regulated by thiolation of specific cysteine residues according to a cysteine switch mechanism [11, 12, 33, 34]. The degradation of extracellular matrix by these enzymes is a tightly controlled process under normal circumstances. However, within the atherosclerotic plaque, the balance may be shifted towards matrix degradation, particularly at the rupture-prone shoulder regions of the fibrous cap where accumulating macrophages and phenotypically altered smooth muscle cells secrete a plethora of proteinases, including MMPs [1, 2]. It has been proposed that these enzymes contribute to plaque rupture, and, indeed, we previously evidenced a higher proteolytic environment in unstable plaques regarding the fragmentation of apoB, apo(a), apoE, and members of the proteoglycan (PG) families [3]. Due to the higher degree of protein thiolation observed in unstable plaques, we may postulate that the elevated proteolytic activity found in these tissues could be explained at least partly by the activation of MMPs through an increased S-thiolation of cysteine switch.

5. Conclusions

This work describes the extent of oxidative modifications affecting protein-SH groups in atherosclerotic plaques with different vulnerability and the identity of involved proteins. Moreover, the degree of protein mixed-disulfide modifications in relation to atherosclerotic plaque typology is reported.

The elucidation of the mechanisms of protein thiolation in the plaque environment deserves further studies. The identification of specific protein oxidative modifications and the understanding of their effects on protein function could provide further insight into the relevance of oxidative stress in atherosclerosis.

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Review Article

Reactive Oxygen Species in Vascular Formation and Development

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Reactive oxygen species (ROS) are derived from the metabolism of oxygen and are traditionally viewed as toxic byproducts that cause damage to biomolecules. It is now becoming widely acknowledged that ROS are key modulators in a variety of biological processes and pathological states. ROS mediate key signaling transduction pathways by reversible oxidation of certain signaling components and are involved in the signaling of growth factors, G-protein-coupled receptors, Notch, and Wnt and its downstream cascades including MAPK, JAK-STAT, NF-κB, and PI3K/AKT. Vascular formation and development is one of the most important events during embryogenesis and is vital for postnasal tissue repair. In this paper, we will discuss how ROS regulate different steps in vascular development, including smooth muscle cell differentiation, angiogenesis, endothelial progenitor cells recruitment, and vascular cell migration.

1. Introduction

Reactive oxygen species (ROS) are a class of molecules derived from the metabolism of oxygen (O_2), and are characterized by high chemical reactivity. They include free radicals, such as superoxide (O_2^{-}), superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), and peroxynitrate ($ONOO^{-}$), and nonradicals such as hydrogen peroxide (H_2O_2), ozone (O_3), and hypochlorous acid (HOCl). Traditionally viewed as toxic byproducts of metabolism, ROS cause damage to lipids, membranes, proteins, and DNA through free-radical-mediated chain reaction. Over decades, numerous studies showed that increased oxidative stress plays a central role in the pathogenesis of vascular disease, including hypertension, atherosclerosis, and restenosis. Recent evidence, however, clearly demonstrated that that moderate concentration of ROS acts as intracellular signaling molecules and thereby mediates diverse developmental and physiological processes.

ROS are important mediators and signal modifiers during various biological processes. Signal transduction mediated by ROS, known as "Redox signaling," usually involves reversible and oxidation/reduction-based modification of components in signaling pathway. ROS are produced in response to various stimuli, including growth factors, cytokines, chemotactic factors, hypoxia, and shear stress. In turn, many vital biological pathways or cascades are tuned via ROS, such as GPCR, Notch [1] and Wnt-β-catenin [2], MAPK, JAK-STAT, NF-κB, and PI3K/AKT. Transcription factors such as HIF1-α, AP-1, and NF-κB can themselves be directly modified in a redox-sensitive manner, thereby leading an altered transcriptional profile of gene products. Noteworthy, redox singling is spatially regulated and confined in certain subcellular region. The compartmentalization of redox signaling ensures its specificity in gene regulation and cellular functions, and that ROS can participates in more dynamic cell behaviors that needs different parts of the cell to work together, just as in the case of cell migration.

To fulfill the organism's metabolic demand for oxygen and nutrients, blood vessel formation is one of the earliest and most vital events during embryonic development. Vascular formation are coordinated in a number of steps, which include differentiation and proliferation of endothelial cells (vasculogenesis), blood vessel sprouting and branching (angiogenesis), and differentiation and migration of vascular smooth muscle cells (VSMCs) to cover vessel tube (arteriogenesis) [3, 4]. During these processes, ROS can potentiate

angiogenic response by facilitating signaling of multiple angiogenic factors, such as vascular endothelial growth factor (VEGF) and angiopoietin, and enhancing the activity of hypoxia-induced factor (HIF) as well. Vascular repair and regeneration in response to tissue injury or intravascular manipulations also involves angiogenesis. Postnatal neovascularizations are often accompanied by neointimal formation, and these repairing processes involves highly regulated steps, including progenitor cells mobilization and differentiation, vascular cell migration, and VSMCs phenotypic transition, with ROS as an indispensable player. In this paper, we will discuss how ROS regulates various steps of vascular formation and development, including smooth muscle cell differentiation, angiogenesis, endothelial progenitor cells recruitment, and vascular cell migration.

2. ROS Source and Nox Enzyme Family

ROS is generated through a cascade of biological reactions following the formation of superoxide, which can be dismutated to hydrogen peroxide spontaneously or in a reaction catalyzed by superoxide dismutase. Superoxide can also react with nitric oxide to form peroxynitrate. Multiple enzyme systems including nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox) family, xanthine oxidase, mitochondrial respiratory chain, uncoupled eNOS, myeloperoxidase (MPO), lipoxygenase, cyclooxygenase, cytochrome p450, and heme oxygenase have been implicated in the generation of ROS. Among these enzyme systems, the major source of ROS in vascular system, however, is the Noxs.

First characterized in phagocytes like macrophage and neutrophils, the prototypic NADPH oxidase 2 (Nox2, also known as gp91phox) is found to be responsible for antimicrobial defense. With binding to other regulatory subunits, namely, p40phox, p47phox, p67phox, and Rac, the assembled NADPH oxidases complex is able to produce ROS in a "respiratory burst" to kill internalized bacteria. Subsequently, growing biochemical and functional evidence suggests the presence of NADPH oxidase-like activities in nonphagocytic cells, which eventually lead to the discovery of a whole family of NADPH oxidases. The NADPH oxidase family is composed of catalytic subunits termed Nox1-5 and Doux1 and Duox 2, two organizer subunits p47phox and Noxo1, two activator subunits p67phox and Noxa1, and other regulatory subunits like p22phox and p40phox and the binding partner Rac. Different Noxs exhibit tissue-specific distribution and display distinct functions. In vasculature, Nox1, Nox2, Nox4, and Nox5 are mainly expressed. In endothelial cells, Nox2 and Nox4 are responsible for the basal ROS generation [5], but mediate distinctive activation pattern under different stimulation [6].

All Nox family members are transmembrane proteins that contain conserved structures: a C-terminal NADPH binding domain, a flavin adenine dinucleotide (FAD) binding domain, six transmembrane domains, and four highly conserved heme-binding histines in the third and fifth transmembrane domains [7]. Based on predicted domain structures, Nox isoforms can be classified into three groups: (1) Nox1-4 display up to 60% homology in amino acid sequence and are predicted to contain six transmembrane α-helices and an NADPH-binding domain towards the C-terminus; (2) Nox5 has the same basic structure as Nox1-Nox4 but includes an additional four calcium-binding EF-hand motifs within its N-terminus; (3) Duox1 and Duox2 are similar to Nox5 but include an additional N-terminal peroxidase homology domain on the extracellular site of the membrane.

Superoxide is generated by a complex reaction that takes place once NADPH is bounded to the cytosolic COOH terminus. Electron transfer occurs initially from NADPH to reduce FAD to FADH2. Then single electron transfer occurs through the heme groups, which is then accepted by an oxygen which must be bound to the outer heme group on the opposite side of the membrane. For every one NADPH reduced two superoxide molecules are created.

3. ROS and Stem Cell Function Involved in Vascular Formation and Development

3.1. Embryonic Stem Cells. Stem cells possess the ability for indefinite self-renewal and potency of differentiation into specialized cell type. The rapidly advancing research field in stem cells, especially with the advent of induced pluripotent stem cells (iPSCs), holds great promise for tissue engineering and regenerative medicine. The fate of stem cells, that is, whether to self-replicate or to differentiate, is tightly regulated by various extracellular cues and intracellular signaling, in which the role of ROS has recently been discovered.

Embryonic stem cells (ESCs), derived from the inner cell mass of the blastocyst, are pluripotent to generate any cell type from all three primary germ layers. A growing body of evidence suggests that ROS generation and signaling are involved in ESCs differentiation. A single direct current field pulse applied to early embryoid bodies increased intracellular ROS and promotes cardiomyocyte differentiation; this effect can be hampered by free radical scavengers [8]. It was later confirmed NADPH oxidases are vital to drive cardiomyogenesis through MAPK activation and nuclear translocation of cardiac transcription factor myocyte enhancer factor (MEF2C) [9, 10]. Interestingly, mechanical strain-induced cardiovascular differentiation also utilizes Nox-derived ROS family as a signal transducer [11]. Similar evidences of ROS in promoting stem cell differentiation are also demonstrated in many other cell types, including smooth muscle cells [12–14], endothelia cells [15], skeletal muscles [16], neurons [17], adipocyte [18], and chondrocyte [19].

3.2. Stem Cell Niche and Hematopoietic Stem Cells Maintenance. The stem cell niche, defined as local tissue microenvironment that includes cellular and acellular components, integrates systemic and local cues to regulate stem cell biology [20]. Oxygen tension as a component of metabolic milieu, seems to play a role. Early embryogenesis takes place in female reproductive tract with a low oxygen environment of less than 5%, which seems to be the optimum concentration for mammalian embryonic development [21]. In fact, human ESCs (hESCs) are best-maintained pluripotent under 1–4%

oxygen tension with enhanced formation of embryoid bodies and preserved proliferation ability [22]. hESCs began to differentiate spontaneously when culturing under 21% oxygen.

The best-characterized stem cell niche is that of hematopoietic stem cells (HSCs). HSCs with long-term reconstitution activity (LT-HSCs) primarily reside in endosteal zone of bone marrow, where blood perfusion is very limited and oxygen tension can be as low as 1% [23, 24]. Such hypoxic conditions help HSCs maintain slow-cycling proliferation rate and enhanced engraftment ability, while protecting them from potential oxidative stress in more well-oxygenated tissue [20]. HSCs with lower ROS residing in a low-oxygenic osteoblastic niche have a more durable self-renewal activity than those with high ROS [25].

Knockout studies provide more evidence of ROS in regulating HSC fate and function. $Atm^{-/-}$ and $FoxO1/3/4^{-/-}$ mice showed defect in HSCs quiescence maintenance and HSCs exhaustion, due, at least partially, to increased ROS level [26, 27]. Treatment with an antioxidant can reextend the HSCs lifespan and restore cell cycle in these deficient cells. Another knockout study found that $AKT1/2^{-/-}$ HSCs retains in quiescence accompanied by lower ROS content, which can be rescued to differentiate after pharmacologically increasing ROS differentiation [28]. This view is also confirmed in *Drosophila*, as ROS prime hematopoietic progenitor for differentiation through activation of FoxO and JNK and downregulation of polycomb [29]. In addition, in cardiac and embryonic stem cells, physiological levels of intracellular ROS are required for maintaining genomic stability through activating the DNA repair pathway [30]. Thus, fine tuning of ROS levels is essential for stem cell function; with sufficient ROS required for differentiation, and low ROS for stemness maintenance and quiescence.

3.3. Endothelial Progenitor Cells. Postnatal neovascularization in ischemic insults is critical for tissue repair, and involves both angiogenesis from preexisting vessels and *de novo* vasculogenesis to form new vessels. There is firm evidence that various stem/progenitor cells are mobilized from bone marrow to participate in the process, in which endothelia progenitor cells (EPCs) received special attention. However, the nomenclature and characterization of EPCs are rather unambiguous, and many cell lineages claimed to contain EPCs actually do not have direct evidence to differentiate into vascular cells [31, 32]. Here we still use the term EPC for convenience to refer to differently labeled endothelial progenitors in different studies.

As discussed above, an appropriate level of ROS is important for HSCs senescence and differentiation. What is more, hematopoietic progenitors release from bone marrows also depends on ROS signaling, as granulocyte colony-stimulating factor- (G-CSF-) induced mobilization of EPCs ($sca\text{-}1^{+}c\text{-}kit^{+}Lin^{-}$cells) and other progenitors are strongly prevented by antioxidant N-acetyl-L-cysteine (NAC), as well as their chemotactic migration to stromal cell-derived factor-1 (SDF-1) [33]. In a hindlimb ischemia model, Nox2 knockout mice display reduction of ischemia-induced flow recovery and impaired EPCs ($c\text{-}kit^{+}Flk1^{+}$cells) mobilization, both of which can be rescued by transplantation of wild-type bone marrow [34]. Mobilization of $Nox2^{-/-}$ EPCs ($sca\text{-}1^{+}flk\text{-}1^{+}lin^{-}$) is also blocked in hypoxia condition or EPO stimulation, due to defective production of ROS to inactivate SHP-2, which normally dephosphorylates and inactivates STAT5 downstream EPO signaling [35]. Moreover, $Nox2^{-/-}$ $c\text{-}kit^{+}Lin^{-}$ bone marrow stem cells show impaired migration and actin polarization in SDF-1-directed chemotaxis [34].

In bone marrows, matrix degrading and remodeling by protease is important for progenitor cell egress and release of cytokines like VEGF and soluble Kit-ligand (sKitL) [36, 37], which guides activation and chemotactic migration of EPCs. Production of Nox2-derived ROS can be activated by leptin binding to its receptor (ObR) in bone marrow cells [38]. With ROS, matrix metalloproteinase-9 (MMP9) is then upregulated, shedding and releasing sKitL to enhance EPCs ($sca\text{-}1^{+}Flk1^{+}$ cells) mobilization. In addition, the association of EPCs and targeted vessel may also involve ROS, since ROS-dependent expression of vascular cell adhesion molecule-1 (VCAM-1) expression on endothelial cells can promote efficient recruitment and proliferation of LSKCD34- ($Lin^{-}Sca\text{-}1^{+}cKit^{+}CD34^{-}$) hematopoietic cells [39].

4. ROS and Endothelial Cell (EC) Function Involved in Vascular Formation

Vasculogenesis and angiogenesis are core events during embryonic development for supply of metabolic substrate. Postnatal form of angiogenesis, named neovascularization, also has significance implications in various pathophysiological states like ischemia, wound healing and cancer progression. Angiogenesis is a fined regulated process involving multiple steps including EPC mobilization and differentiation, EC proliferation and migration, and matrix remodeling, almost all of which are found to be modulated by redox signaling. In fact, Nox2 knockout mice display impaired neovascularization in hind limb ischemia [40] and their ECs have much reduced VEGF-induced proliferation and migration [41].

4.1. EC Migration. Endothelial cells and progenitor cells migrate following a chemotactic and mechanotactic stimuli to a right place for covering injured portion of a blood vessel or forming new conduits. This highly dynamic process involves complex extracellular matrix-cell and cell-cell interaction and includes chemical sensing of a signal gradient, breaking up intercellular junctions, degradation of extracellular matrix, protrusion of lamellipodia, and cytoskeletal remodeling [42]. There is solid evidence that angiogenic factors like VEGF or angiopoietin-1 utilities ROS for signal transduction and directing cell migration [43–45].

At the very beginning of migration, quiescent endothelial cells lined in parent vessels need to break up their intercellular connections, of which the major adhesion component is vascular-endothelial- (VE-) cadherin [46]. VE-cadherin forms a dimer and bind directly to β-catenin (alternatively to plakoglobin) and to p120, with the latter two also binding to α-catenin to link the actin cytoskeleton.

A scaffold protein called IQGAP1, which binds to actin, β-catenin, CLIP-170, Rac, Cdc42, Calmodulin, and many other cytoskeleton-associated proteins [47], can associate with VE-cadherin and VEGFR in a quiescent endothelial cell [43, 48]. Upon VEGF stimulation, IQGAP1 binds more avidly to activated VEGFR, at same time recruiting Rac1 and Nox subunits to initiate ROS production [43, 49]. Bridging IQGAP1 to VEGFR is further assisted with T-cell-specific adaptor- (TSAd-) dependent activation of c-Src kinase [50, 51], which in turn phosphorylates IQGAP1 [51] and enhances ROS production probably via recruiting more Nox subunits [52, 53] or activating a Rac1-guanine nucleotide exchange factor (GEF) Vav2 [54]. Cysteine sulfenic acid formation in IQGAP by locally produced ROS may also share a role [55]. ROS-dependent phosphorylation of VE-cadherin and catenins leads to disassembly of VE-cadherin-catenin complex and EC junctional breakdown [48, 56–58]. Beta-catenin can be directly phosphorylated by VEGF-induced FAK activation [59], while p120 phosphorylated by thrombin-activated PKC-α [60], all facilitating adherent junctions dissociation and ultimately promoting EC migration. Interestingly, phosphorylation in the cytoplasmic tail of VE-cadherin via VEGF-VEFGR-Src-Vav2-Rac-PAK axis promotes β-arrestin2 dependent of its internalization and disassembly of intercellular junctions [61], which in turn promoted EC migration (see Figure 1).

Migrating cells create focal complexes transiently in leading edges and constantly reorganize cytoskeletons to form filopodia or lamellipoda. Localized production of ROS is essential for their function at precise subcellular compartments. A paradigm used by migrating endothelial cells is to tether Nox subunits by different scaffolds or adaptors to different substructures [62]: IQGAP links Nox2 to actin meshwork at the leading edge [49]; WAVE1 recruits p47phox and binds to Rac1 and Rac1 effector PAK, producing ROS and forming membrane ruffles [63]; TRAF4 associate with focal contact scaffold Hic5 as well as p47phox, promoting p47phox-TRAF-Hic complex formation and PAK1-dependent ROS production at focal complexes [64]. A novel protein Poldip2 in VSMC can associate with p22phox to activate Nox4 and RhoA, thus strengthening focal adhesions and stress fiber to promote cell migration [65]. Even cancers take advantage of this strategy to breed podosomes during invasion. In colon cancer cells, p47phox-related adaptor protein tyrosine kinase substrate (Tks) 4 and Tks5 recruit p22phox and facilitate Rac- and Nox1-dependent ROS generation at invadopodia [66, 67]. Thus, compartmentalization of redox signaling is essential for the highly dynamic feature of a moving cell.

4.2. EC Proliferation and Survival. Proliferating endothelial cells generate higher level of superoxide and hydrogen peroxide than in quiescent cells [68]. ROS produced by Nox2 and Nox4 enhances EC proliferation and survival through activation of receptor tyrosine kinases and phosphorylation of p38, ERK, and Akt [5, 68, 69]. In endothelial cells, Nox2 silencing induces activation of apoptotic marker caspase 3/7 [5], while Nox4 overexpression inhibits their activity during serum deprivation [69], suggesting ROS derived from both Nox isoforms exert antiapoptotic effects.

Under stress condition such as energy deprivation, cells initiate a prosurvival mechanism that degrades damaged cytoplasmic components in lysosomes and recycles new building blocks for renovation, a process known as autophagy [70]. Reactive oxygen species have long been reported to be a signaling mediator of autophagy [71] and to increase endothelial cell survival in response to cell stress [72]. Inhibition of mitochondrial ROS production decreases AMP-activated protein kinase (AMPK) activation, which is involved in chemerin- or 2-Deoxy-D-glucose- (2-DG-) induced endothelial autophagy [72, 73]. Moreover, ROS-mediated autophagy is critical for EC migration and tube formation during angiogenesis [73, 74]. The molecular mechanisms by which ROS regulates autophagy are at least partially due to direct inactivation of a cysteine protease, Atg4, at the site of autophagosome formation, thereby promoting lipidation of Atg8 for autophagosome processing [75]. Excessive oxidative stress, on the other hand, promotes cell apoptosis by activating the death-related pathway, known as type II programmed cell death (PCD). In persistent pulmonary hypertension (PPHN), autophagy of the pulmonary artery endothelial cells (PAECs) is proapoptotic and forms a positive feedback loop with Nox-derived ROS [76].

5. ROS, VEGF Signaling, and HIF Activation in Angiogenesis

5.1. VEGF Signaling. Multiple signaling pathways are activated during angiogenic process by various factors like VEGF, PDGF, angiopoietin, Notch, Wnt, TGF-β, and GPCR agonists, with VEGF as a dominating player. VEGF exerts its action through binding to VEGF Receptor-2 (VEGFR-2, also known as FLK1/KDR) in ECs, causing the latter autophosphorylated in its cytoplasmic tyrosine residues and driving downstream pathway such as PI3K/AKT and MAPK to promote EC proliferation and migration. VEGF stimulates ROS production via Rac-1-mediated NADPH oxidase activation [41, 43] and also increases mitochondria-derived H_2O_2 [77]. ROS, in turn, potentiate VEGFR phosphorylation [41] and is required for downstream cSrc, FAK, PI3K, and ERK signaling [78]. ROS can also upregulate VEGF secretion and VEGFR expression through induction of transcription factors HIF-1 [79–81].

The role of ROS in modulating signaling attributes largely to reversible oxidative inactivation of protein tyrosin phosphatase (PTP), which inhibits signaling by dephosphorylating pathway components [82, 83], including the receptor itself [33]. For VEGFR2, PTP1B and density-enhanced phosphatase-1(DEP-1)/CD148 are the major negative phosphatases, and can be inactivated locally in caveolin-enriched lipid rafts by H_2O_2 generated by extracelluar superoxide dismutase (ecSOD), and thus facilitating VEGFR2 signaling [84]. In addition, growth factor-activated Src kinase can not only stimulated NAPDH for ROS production, but also phosphorylate and inactivate ROS degrading enzyme peroxiredoxin (Prx1), building up a local H_2O_2 gradient

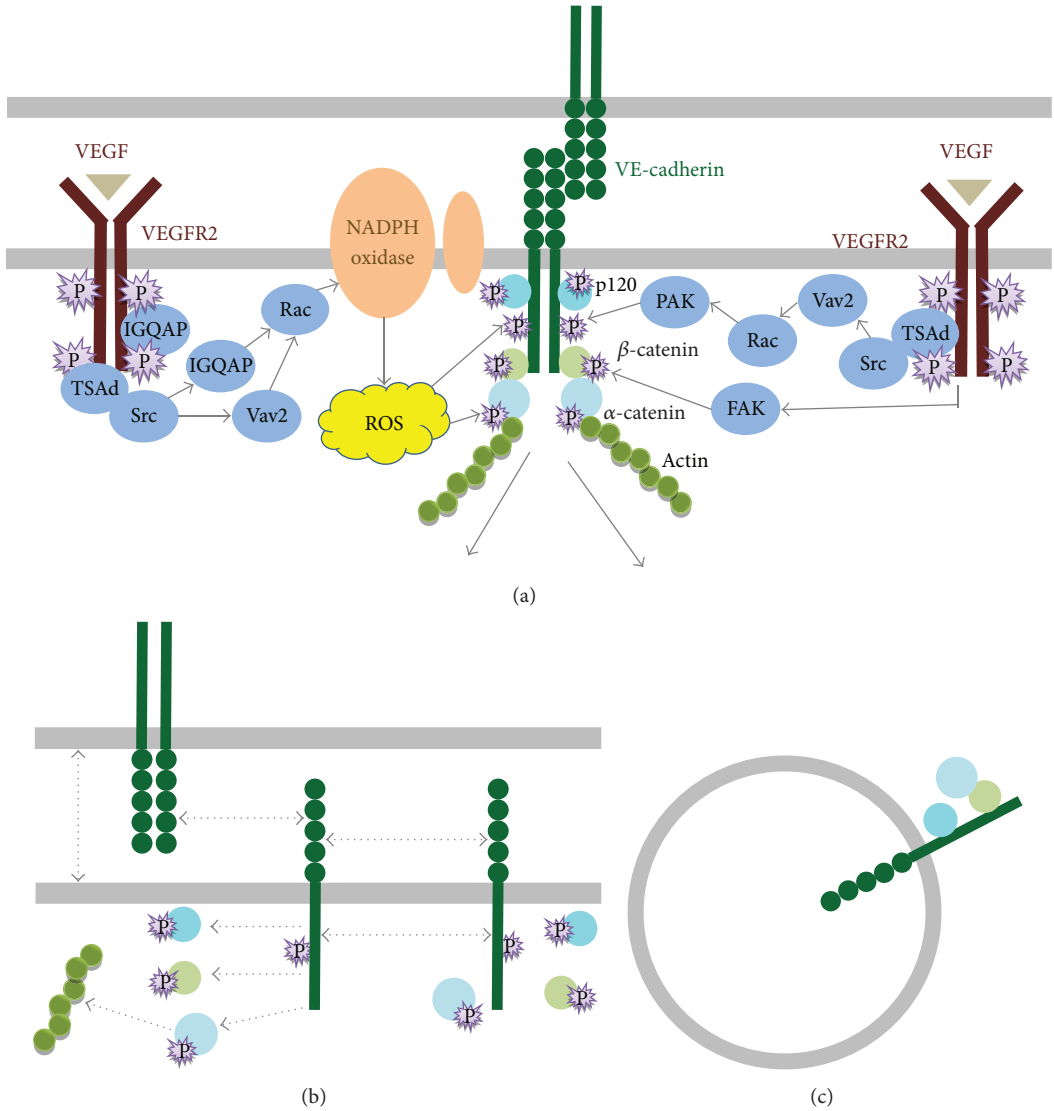

Figure 1: Regulation of intercellular VE-cadherin disruption between endothelial cells by reactive oxygen species and VEGF signaling during EC migration. In basal state, clustering of VE-cadherins between endothelial cells mediate intercellular adhesions. VE-cadherin forms a dimer and bind directly to p120 and β-catenin, with the latter associated with α-catenin to bridge the actin cytoskeleton. Upon VEGF stimulation, TSAd-dependent Src activation recruits IQGAP1, a multifunctional scaffold protein, to assist association of Rac1 with other Nox subunits (a). Subsequent ROS production by NOx phosphorylate VE-cadherin and α-catenins, leading to disassembly of VE-cadherin-catenin complex and EC junctional breakdown, which in turn results in EC migration (b). On the other hand, β-catenin phosphorylation by VEGF-induced FAK activation and p120 phosphorylation by thrombin-activated PKC-α also promotes the breakdown of endothelial cell tight junctions. Moreover, phosphorylation of VE-cadherin in the cytoplasmic tail via VEGF-VEFGR-Src-Vav2-Rac-PAK axis promotes β-arrestin2 dependent of its internalization and disassembly (c).

to inactivate neighboring protein tyrosine phosphatase and sustain tyrosine receptor signaling [85]. Ultimately, such VEGF-ROS signal pathways promote EC migration and proliferation (Figure 2).

5.2. Hypoxia-Induced Factor. Hypoxia, a well-known non-chemical signal for angiogenesis in vascular development and pathological state, also harnesses redox modulating to regulate its responder, hypoxia-induced factor (HIF). HIFs belong to PER-ARNT-SIM (PAS) family of basic helix-loop-helix (b-HLH) transcription factors and have three members: HIF-1, -2, and -3. HIF is a heterodimer composed of an oxygen sensitive HIFα subunits and a constitutively stable HIFβ subunit. Under normal oxygen, HIFα is hydroxylated in its proline residues by prolyl hydroxylate proteins (PHDs), thereby generating a binding site for the von Hippel-Lindau (VHL) tumor suppressor protein, which initiates ubiquitin proteasome pathway for HIFα degradation [86].

Angiogenesis induced by urotensin-II, a potent vasoactive peptide, involves feed-forward enhancement between HIF protein and Nox2 [87]. A rapid increase in nox2-derived ROS in response to urotensin stimulation elevates HIF-1α

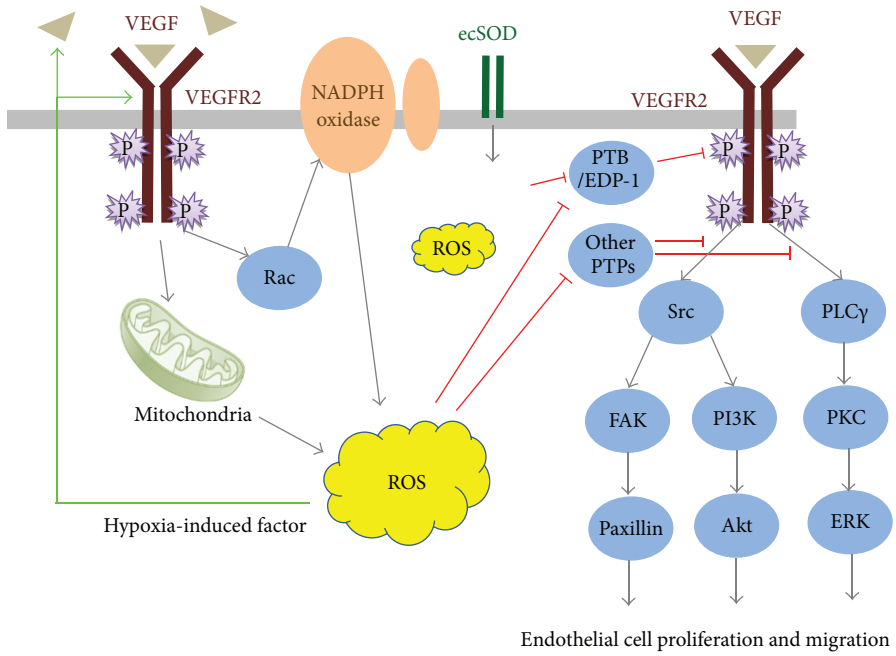

Figure 2: ROS in VEGF signaling in endothelial cell proliferation and migration. Binding of VEGF to VEGFR2 stimulates ROS production via Rac-1 mediated NADPH oxidase activation and through increased mitochondria activity. ROS oxidize and inactivate protein tyrosine phosphatases (PTPs), disinhibiting their negative regulation on downstream signaling pathways, such as Src/PI3K/Akt and PLC/PKC/Raf/ERK. H_2O_2 are also generated extracellularly by ecSOD to locally inactivate DEP1 and PTP1B, two PTPs that dephosphorylate VEGF receptor, thereby promoting VEGF-induced VEGFR2 autophosphorylation. Through induction of transcription factors HIF-1, ROS can also upregulate VEGF secretion and VEGFR expression.

level, leading more binding of HIF-1α to Nox2 promoter. Nox2 transcription is then enhanced and more ROS are generated to activate HIF-1 further, thus maintaining a positive feedback loop for angiogenesis. In another study, ROS produced by Nox4 in cardiomyocyte can stabilize HIF-1α and promote VEGF release to increase myocardial angiogenesis in overload stress [81]. Under hypoxic condition, Nox expression can be readily induced by HIF, participating in cell migration and proliferation. Though this is observed only in pulmonary artery smooth muscle cells, there's reason to expect a similar role in endothelial cells for angiogenesis. How intracellular ROS enhance or stabilize HIF has recently been uncovered. On the one hand, ROS mediate transcriptional activation via NF-κB [88] and translational activation via PI3K/AKt/4E-BP1 pathway [89], increasing HIF production. On the other hand, ROS deplete cellular ascorbate, a cofactor for PHD activity, and inhibit HIFα hydroxylation and VHL binding [81, 90, 91], suppressing HIF degradation (see Figure 3). Increased HIF activity promotes angiogenesis.

6. ROS and SMC Function Involved in Vascular Formation

Vascular smooth muscle cells, as an important component of blood vessels, function to contract or relax vessel, to regulate blood pressure and distribute blood flow. Smooth muscle cells display striking plasticity and can undergo phenotype switch, dedifferentiating from a quiescent contractile state to a highly migratory synthetic state, in response to vascular injury or various disease states [92, 93]. In this section, we discuss how reactive oxygen species regulate SMC differentiation, proliferation, and migration.

6.1. SMC Differentiation. Nascent VSMCs originate from diverge source during mammalian vascular development, including neural crest, proepicardium, mesothelium, secondary heart field, smites, and mesoangioblasts [94]. In injured vasculatures, stem/progenitor cells give rise to smooth muscle cells to form neointima during vascular repair [95]. ES cells can differentiate into SMC in response to growth factors (e.g., PDGF and TGF-β), mechanical forces, and certain extracellular matrix (i.e., collagen IV) [96, 97] by activating various signal pathways or gene programme such as integrins-PDGFR β crosstalk [96], histone deacetylase 7 [98], transcription factor Sp1 [99], nuclear proteins chromobox protein homolog 3 [100], and heterogeneous nuclear ribonucleoprotein A2/B1 [101]. Importantly, during SMC differentiation and phenotypic modulation, ROS mediated by Nox4, Nrf3, Pla2g7, or other regulators also plays a fundamental role [12–14, 102].

TGF-β is a prodifferentiation factor for smooth muscle cells. It activates Nox4 during SMC differentiation from ES cells [13]. Nox4-derived ROS upregulates the expression and phosphorylation of serum response element (SRF) and drives SRF to translocate into nucleus for SMC gene transcription [13]. In addition, Nox4 expression is enhanced by nuclear factor erythroid2-related factor3 (Nrf3) [12], a member of the

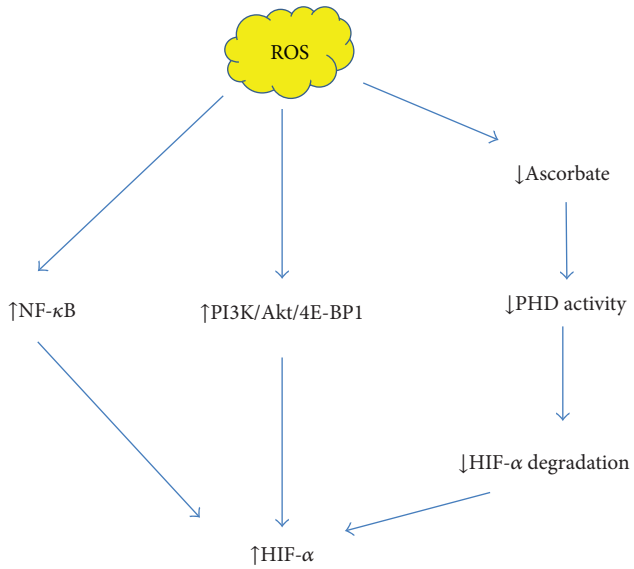

Figure 3: Regulation of hypoxia-induced factor by intracellular reactive oxygen species. Reactive oxygen species positively regulates HIF through enhanced HIF production via activation of NF-κB and PI3K/AKt/4E-BP1 pathway. Meanwhile, ROS inhibits HIF degradation by depleting cellular ascorbate, a cofactor for PHD activity, thus inhibiting HIFα hydroxylation and VHL binding.

cap "N" collar family of transcription factors. Nrf3 can recruit myocardin/SRF complex to CArG box in the promoter region of SMC-specific genes and directly bind to SMαA and SM22α promoter. Our study also demonstrated for the first time that the fine-tuning of Nrf3-Pla2g7- (phospholipase A2-, group VII) Nox4-ROS axis plays a crucial role in SMC differentiation from ES cells *in vitro* and *in vivo* [14], firmly confirming its functional importance of ROS signals in SMC differentiation and development (see Figure 4).

As stated above, VSMCs can exhibit extensive phenotypic diversity and plasticity and are modulated by numerous environmental cues including growth factors and cytokine, inflammatory cell mediators and lipids. Maintenance of differentiated or contractile VSMCs phenotype can be enhanced by PDGF, TGF-β, MMPs, and reactive oxygen species [92]. Nox4 is necessary for smooth muscle markers expression and contractile type stress fibers in VSMCs, through SRF phosphorylation and gene transactivation via p38 MAPK pathway [103, 104]. Notably, the changing of Nox4 localization from stress fibers in differentiated VSMCs to focal adhesions in proliferate cells [103] is reminiscent of the Nox4 translocation into nucleus during SMC differentiation [13]. The subcellular shifting of Nox4 during different cellular state underscores the importance of compartmentalized ROS signaling for specific function [62].

6.2. SMC Proliferation. During normal vascular formation and pathological conditions like hypertension and restenosis, vascular SMCs undergo a phenotypic switch to a migratory or proliferative phenotype in response to a variety of growth factors and inflammatory mediators' stimulations. These factors, including PDGF [105], Ang II [106], urokinase plasminogen activator [107], heme [108], urotensin II [109], TGF-β [110], and thyroid hormone [111], can activate Nox and subsequent ROS production, promoting smooth muscle cell proliferation. The growth-related downstream signaling pathways are varied among different Noxs isoforms and different stimuli. For example, PDGF-induced SMC proliferation mediated by Nox5 involves JAK/STAT pathway [105], while Ang II stimulation leads to p38 and Akt activation through Nox1 in hypertrophic response [112].

6.3. SMC Migration. Migration of smooth cells to cover the preexisting collateral arteriolar network is an essential step in arteriogenesis, and provides mechanical support and contractility for a mature blood vessel. The driving forces for the process include fluid shear stress and growth factors such as PDGF, FGF, and TGF-β. Since cell migration share many similarities and we have already discussed the case of ECs, here we only summarize some common feature and highlight unique aspects in how ROS influence VSMC migration.

First, certain signaling components controlling migration are modulated by ROS, though which the specific pathway can be different. For example, c-Src activation by various agonists such as AngII, PDGF, and thrombin, is ROS-dependent [113–116]. This important signal node has direct impact on downstream cascades like c-Src-PDK1-PAK [114] or c-Src-EGFR-PI3K/ERK [113, 116], all affecting cell motility. Basic fibroblast growth factor (bFGF), however, activates PKC and PI3K/Akt instead of c-Src in smooth muscle cells, but the ultimate JNK activation still requires Nox-derived ROS [117] (see Figure 5).

Second, migration depends on degradation of extracellular matrix and loss of cell-matrix and cell-cell adhesion. This often needs the cleavage activity of metalloproteinase (MMP). Similar to the role of ROS in downregulating VE-cadherin in endothelia cells, N-cadherin shedding in disrupting intercellular junction between VSMCs also involves ROS. By Nox1-dependent transactivation of epidermal growth factor receptor, pro-MMP-9 is activated to cleave N-cadherin to promote SMC migration [116]. Another potentially important MMP subtype produced by SMC is MMP2, which can be induced with transcription factor FoxO3a by urotensin-II. Urotensin drive Nox4-dependent activation of JNK and subsequent phosphorylative inactivation of sequestering protein 14-3-3, thereby allowing FoxO3 into the nucleus to enhance MMP2 transcription [109]. In pathological states like hypertension and acute coronary syndrome, increased MMP release by VSMC may link to abnormal extracellular matrix reorganization, deranged VSMC migration and plaque rupture. This, however, can also be mediated though Nox-derived ROS [118, 119].

Thirdly, in migrating cells, constant reorganization of cell protrusions (filopodia, lamellipoda, stress fiber, and focal complexes) and cytoskeletons are modulated by ROS, indirectly through ROS-dependent activation of downstream effector kinases, small GTPase and cytoskeleton-associated proteins. Moreover, for contractile cells like VSMCs, contraction regime is another significant target for ROS to modulate. $Nox1^{y/-}$ VSMCs present decreased expression of mDia1, a

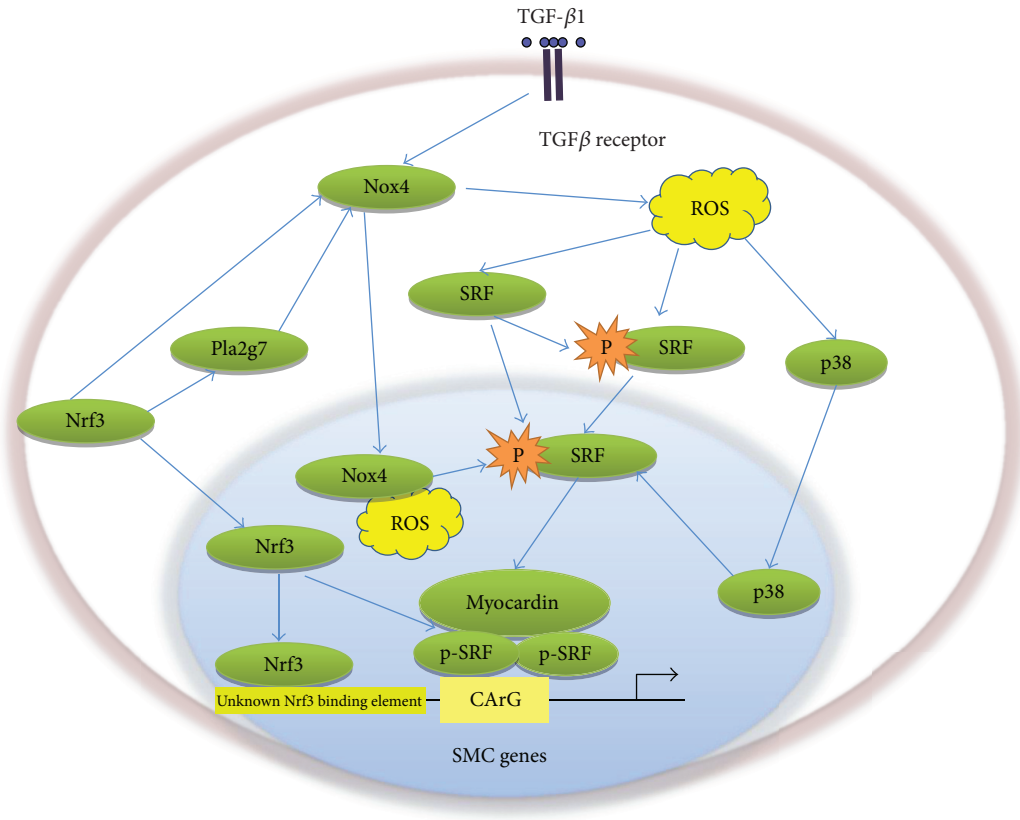

Figure 4: Regulation of SMC differentiation by Nox4-derived ROS. Activated Nox4 by TGF-β via its receptor and/or upregulated Nox4 by Nrf3 and/or Pla2g7 leads to up-regulation and phosphorylation of SRF through ROS. The phosphorylated SRF in the nucleus bind to the CArG recruiting myocardin to the promoter of SMC-specific genes. Meanwhile, Nox4-derived ROS can also activate SMC gene expression via p38MAPK pathway. During differentiation, Nox translocates into nucleus. Moreover, Nrf3 and Pla2g7 promote the recruitment of myocardin/SRF complex to CArG box in the promoter region of SMC genes. Importantly, Nrf3 has been shown to bind directly to the unknown Nrf3 binding element within promoter regions of SMC genes.

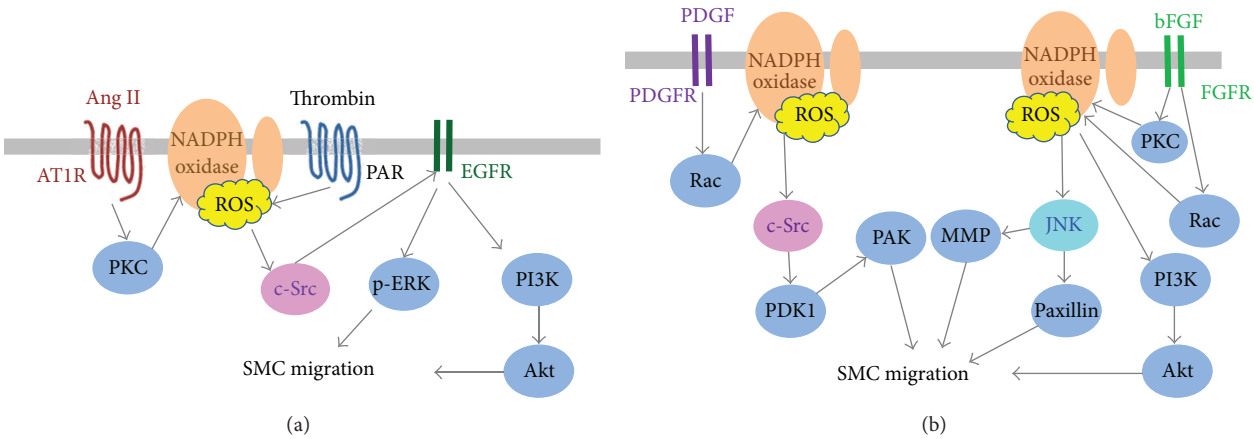

Figure 5: Signaling pathways in ROS-mediated SMC migration activated by agonists. Various agonists such as AngII, PDGF and thrombin activate c-Src in a ROS-dependent way. Signaling cascades downstream of c-Src include EGFR transactivation followed by PI3K/AKT or ERK activation (for AngII) (a) and direct PDK1/PAK phosphorylation (for PDGF) (b). Basic fibroblast growth factor (bFGF) activates PKC and Rac instead of c-Src in smooth muscle cells, but the ultimate JNK or PI3K/Akt activation still requires Nox-derived ROS.

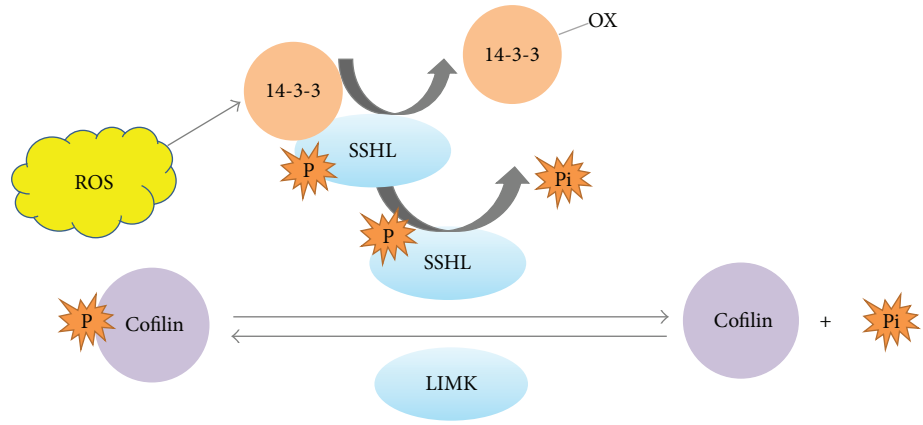

Figure 6: Regulation of slingshot-cofilin pathway by reactive oxygen specious in SMCs. Cofilin is phosphorylated and inactivated by LIM kinase, and p-cofiln is dephosphorylated and activated by phosphatase slingshots-1L. SSHL is sequestered by a regulatory protein 14-3-3. Increasing level of ROS oxidizes 14-3-3, releasing SSH1L phosphatase, which, on the one hand, dephosphorylates other SSH1L molecules to increase SSH1L phosphatase activity. On the other hand, SSH1L dephosphorylates cofilin, which recycles actin monomers for cytoskeletal remodeling during smooth muscle cell migration.

RhoA adaptor protein, and decreased phosphorylation of cofilin, a regulator of actin depolymerization [120]. Cofilin servers to increase the turnover of actin filaments and is essential for maintaining and protruding lamellipodia. Cofilin is phosphorylated and inactivated by LIM kinase (LIMK), and p-cofiln can be dephosphorylated and activated by phosphatase Slingshots-1L (SSH-1L) [121], which is sequestered by a regulatory protein 14-3-3. ROS produced by Nox1 oxidize 14-3-3, thus releasing SSH-1L to activate cofilin and subsequent cytoskeletal remodeling for migration [122, 123] (see Figure 6). Furthermore, ROS increase intracellular Ca^{2+} mobilization partially through Ca^{2+} influx, thereby enhance VSMC contraction [124].

7. Perspective

With years of efforts, ROS is becoming increasingly recognized as key modulator for a variety of biological functions and pathophysiological states. Recent evidence across species suggests an even more general and significant role of ROS, including germ line specification in maize [125], root proliferation/differentiation transition in *Arabidopsis* [126], and wound detection in zebrafish [127]. We have discussed above how ROS regulates vascular development in different aspects, including stem cells and SMC differentiation, angiogenesis, VEGF signaling, endothelia progenitor cells recruitment, and vascular cell migration. Nonetheless, much more details regarding the ROS signaling and pathophysiological functions remain to be learn, for example, how the levels of ROS are balanced not to damage biomolecule but to modify normal signal; how ROS are specified and confined, and how ROS in the nucleus modify epigenetic change. Importantly, different forms of ROS like H_2O_2 and O^{2-} may display opposing effects. Further studies are needed to clarify their respective action, and how transition between different ROS is coordinated by cells to achieve a specific function. More sensitive and specific tools are also needed for detection and visualization of different ROS species.

ROS have long been deemed as noxious molecules in cardiovascular disease, including systemic and pulmonary hypertension, atherosclerosis, cardiac hypertrophy, and heart failure. However, some very recent gene knockout and overexpression studies on Nox4 suggest that Nox4-derived ROS have vascular protective function [81]. Thus, the regulation and function of ROS system seem even more complex and intriguing than we previously thought. A better understanding of how different physiological/pathophysiological state would impact on vascular system may resolve the paradox [128–130]. Lastly, deeper insights into the mechanism of how ROS affect normal vascular development, especially SMC and EC differentiation from stem cells, will promise a more bright future on regeneration medicine for cardiovascular therapy.

Acknowledgments

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Review Article

Oxidative Stress and Immunosenescence: Therapeutic Effects of Melatonin

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Age-associated deterioration in the immune system, which is referred to as immunosenescence, contributes to an increased susceptibility to infectious diseases, autoimmunity, and cancer in the elderly. A summary of major changes associated with aging in immune system is described in this paper. In general, immunosenescence is characterized by reduced levels of peripheral naïve T cells derived from thymus and the loss of immature B lineage cells in the bone marrow. As for macrophages and granulocytes, they show functional decline with advancing age as evidenced by their diminished phagocytic activity and impairment of superoxide generation. The indole melatonin is mainly secreted in the pineal gland although it has been also detected in many other tissues. As circulating melatonin decreases with age coinciding with the age-related decline of the immune system, much interest has been focused on melatonin's immunomodulatory effect in recent years. Here, we underlie the antioxidant and immunoenhancing actions displayed by melatonin, thereby providing evidence for the potential application of this indoleamine as a "replacement therapy" to limit or reverse some of the effects of the changes that occur during immunosenescence.

1. Introduction

All organisms experience the inevitable biological process referred to as aging. In general, aging is characterized by a time-dependent functional decline that leads to increased morbidity and mortality as a consequence of the cell's incapacity to face external and internal challenges. Although aging is an extremely complex, a multifactorial process that has been the subject of considerable speculation, accumulated evidence identifies free radicals as a source of damage to cellular structure and function [1].

Among the countless theories proposed for aging, the free radical theory of aging (also known as oxidative stress theory) put forward by Harman in 1956 [2] has received extensive support. This theory proposes that organismal deterioration that occurs as a result of increasing longevity is specially a consequence of the persistent accumulation of free radical-mediated damage to essential molecules, which gradually compromises the function of cells, of tissues, and eventually of the organism itself [3]. Consequently, aging may be viewed as a process of irreversible injuries associated with accumulated oxidative debris.

Since it was posed, the oxidative stress theory of aging has been continuously studied and modified [4, 5], giving a central involvement of mitochondria in determining the timing of senescence, that is, lifespan, as these organelles generate a disproportionately large amount of oxygen-based free radicals and related nonradical species in cells [6]. Nevertheless, despite the fact that the mitochondrial oxidative stress theory of aging is one of the most plausible theories for explaining aging, it has also received some criticisms in the last few years since some groups have proven that knockout mice for antioxidant enzymes did not show any sign of accelerated aging, thus suggesting that mitochondrial oxidative stress may not be causal for age-related degenerative phenomena [7].

Traditionally, oxygen-based free radicals are designated as reactive oxygen species (ROS), whereas nitrogen-based toxic reactants are generally referred to as reactive nitrogen species (RNS). Both ROS and RNS arbitrarily mutilate

macromolecules in the area of where they are produced, this mutilation leading, in many cases, to death of the cell via programmed cell death or apoptosis [8, 9]. Oxidative stress is a condition in which the redox balance between oxidants and antioxidants is disrupted, thereby tilting the equilibrium towards an oxidized state [10]. To counteract the harmful actions of ROS, aerobic cells are equipped with a series of antioxidant enzymes that metabolize toxic reactants to less reactive or totally innocuous molecules. Superoxide dismutases (SODs), glutathione peroxidase (GPx), and catalase are among these antioxidative enzymes. However, this protective machinery seems to be impaired with aging. In particular, SOD activity has been shown to decrease in aged individuals [11–13] although this finding remains disputed [14]. Conversely, catalase and glutathione peroxidase activities have been reported to be augmented with aging, which might reflect a compensatory response to extremely elevated basal levels of ROS/RNS in cells from aged individuals [13].

Furthermore, melatonin is a powerful antioxidant produced naturally by the pineal gland that exhibits relevant antiaging properties [15–18]. Obviously, the use of therapeutic drugs that are intended to improve the quality of life in the elderly implies the identification of molecules that have both antioxidant and immunoenhancing capabilities. In this sense, some of the evidence suggesting that melatonin is efficient to combat age-related deterioration in immune function will be summarized and discussed in this paper with the objective of fostering melatonin as a potential therapeutic agent for enhancing overall quality of life in the elderly.

2. Aging and the Immune System

During aging, the immune system loses functionality and responsiveness. This deterioration is closely linked to a decreased capacity of the immune system to respond to antigenic stimulation and contributes to the increased susceptibility to infectious diseases and cancer in the elderly [19]. This age-associated decline in immune function, which is known as immunosenescence, results in altered cytokine microenvironment and impairment of both innate and adaptive immunity [20].

In general, all immune cells are affected by aging, thereby contributing to the high vulnerability to infections and increased mortality observed in the elderly [21]. Concerning the macrophage, it has been suggested that the existence of a direct relation between age and macrophage activation seems to be responsible for the presence of a subclinical chronic inflammatory process in the elderly. This increase in proinflammatory status at an organismal level, caused by chronic age-related stimulation of the macrophage, is referred to as "inflamm-aging" [22]. Thus, enhanced macrophage ability to produce proinflammatory mediators such as interleukin (IL)-1, IL-6, and IL-8 occurs in both healthy aged subjects and individuals showing pathological aging [22, 23]. Nonetheless, this phenomenon is only a part of the whole spectrum of change characteristic of immunosenescence, and indeed the macrophage is not the only cell involved in the aging process. The progressive functional T and B lymphocyte deficits have been also suggested as the main responsible factors for age-associated disorders [24]. Certainly, lymphocytes are also largely affected during immunosenescence, and the continuous age-related antigenic stress provokes a variety of changes even in the most evolutionarily recent immune system. These alterations include the expansion of memory B cells, the decrease and even the exhaustion of naïve T cells, and the shrinkage of the T-cell repertoire [25]. Likewise, the reduction both in the number of naïve T cells and in their responsiveness with increased age causes the decline of specific immunization response in aged individuals [26].

As for granulocytes, a functional impairment of these cells has been found in elderly individuals, including diminished intracellular phagocytic capacity, decreased chemotactic activity, degranulation in response to Gram-positive bacteria, and reduced ability to respond to survival factors such as granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and lipopolysaccharides (LPS) [27, 28]. In this vein, the attenuation of Fc-mediated phagocytosis in the elderly has been suggested as the major factor for the age-related decline in neutrophil function [27, 28]. Moreover, a reduction in superoxide production of granulocytes has been reported in centenarians, irrespective of subject's health conditions [29].

Furthermore, the activity of natural killer (NK) cells during aging has been extensively studied, and different results have been reported. Strikingly, the most consistent data indicate an increase in cells with high NK activity with advancing age [29, 30]. In fact, cells from healthy centenarians can efficiently kill target cells [31]. This age-associated increase in NK cell number has been interpreted as a compensatory response to overcome the generally decreased immune function that could otherwise trigger neoplastic growth [32]. However, it has been found that aging may severely affect cytokine production of NK cells. Indeed, NK cells of elderly subjects exhibited a diminished production of cytokines in response to IL-2 [33]. Similarly, it has been shown a substantial impairment in the production of mRNA transcripts encoding several cytokines in NK/LAK (lymphokine-activated killer) cells of aged mouse [34].

3. Oxidative Stress and Immunosenescence

Although aging is not considered as a disease by itself, it makes the organism more vulnerable to many of them, including diabetes, obesity, atherosclerosis, cardiovascular diseases, and neurodegenerative diseases [35]. Two essential biochemical mechanisms link immunosenescence to oxidative stress: a reduction in cellular functions owing to oxidative damage of proteins, lipids, and carbohydrates and apoptotic cell death triggered by the accumulation of oxidative debris. The increased amount of free radicals observed in many aged cells has been reported in cells of the immune system as well [36]. In addition, the levels of MnSOD, which is an antioxidant enzyme located in

the mitochondria and protects macrophages from apoptosis induced by oxidized low-density lipoprotein (LDL), are also decreased in aging macrophages [37] thereby contributing to the increased cellular oxidative stress [36, 38].

The observed oxidative modifications occurring on different macromolecules have been shown to compromise the functionality of subcellular organelles, compartments, and membranes [39]. In this sense, the alterations in membrane lipids composition and function due to an increase in the amount of polysaturated and oxidized fatty acids affect activation of T cells and then contribute to human immunosenescence [40, 41]. Moreover, change and damage to the membrane composition also influence receptor-mediated functions of dendritic cells, including the phagocytic clearance of pathogens [42]. Likewise, a consistent decline in the proteolytic activity of the proteasome has been demonstrated with advancing age, implicating an important role for the proteasome in immunosenescence. Besides the inability to clear-damaged proteins, loss in proteasomal proteolysis has far-reaching implications within the immune system. This includes lowered T-cell functional response, reduced antigenic peptide generation for binding to MHC (major histocompatibility complex) class I molecules, decreased maturation of dendritic cells, and, ultimately, dysregulated proliferation because of altered regulation of the cell cycle [43]. Finally, T and B cell plasma membrane receptors, which are directly involved in immune recognition, have also been shown to be affected by oxidative stress. As a matter of fact, it has been reported that many events of T-cell receptor (TCR) signal transduction, such as protein tyrosine kinase (PTK) and mitogen-activated protein kinase (MAPK) activation, are known to be altered with advancing age due to oxidative modification [44]. Additionally, oxidative inactivation of the CD45 protein tyrosine phosphatase was also described to contribute to T-cell dysfunction in the elderly [45].

A second link between oxidative stress and immunosenescence is the induction of cellular apoptosis following the accumulation of oxidized molecular aggregates. Apoptosis is crucially involved in the age-related remodeling of the immune system, which includes thymic involution and alterations in T cells [46, 47]. In this regard, oxidative stress contributes to damage-induced apoptosis by increasing the number of cells undergoing cell death as a result of the accumulation of oxidatively damaged molecules [48], as shown in aged human leukocytes [49, 50]. In fact, we have observed that unstimulated neutrophils and lymphocytes isolated from elderly patients accumulate higher amounts of ROS, present decreased SOD activity, and are less resistant to cell death compared to those cells obtained from young individuals (Table 1). Furthermore, we have determined that aged neutrophils and lymphocytes are more vulnerable to apoptosis triggered by intracellular calcium overload than those cells obtained from young subjects, as ascertained by the activation of different apoptotic hallmarks (Table 2).

Apart from that, the accumulation of proteins modified with advanced glycation end products (AGEs) has been shown to induce T-cell apoptosis in an oxidative stress-associated and caspase-dependent manner with involvement of the mitochondrial pathway [51]. Similarly, studies in cultured macrophages indicated a positive correlation between exposure to oxidized LDL and cell death [37]. Moreover, the stimulation of macrophages with serum glycated proteins, such as pentosidine, a well-characterized AGE found in plasma and tissue of diabetic and uremic subjects, also leads to a loss of cell viability and presumably to cell death [52].

Table 1: Reactive oxygen species (ROS) levels, superoxide dismutase (SOD) activity and rates of cell death in unstimulated leukocytes from young and elderly individuals.

	Neutrophils		Lymphocytes	
	Young	Aged	Young	Aged
ROS levels	+	+++	+	++
SOD activity	++	+	++	+
Rate of cell death	+	++	+	++

The levels of ROS, SOD activity, and rate of cell death were all classified on a scale of + (indicating lowest level, activity or rate) to +++ (indicating highest intensity level, activity or rate). Data were taken from Espino et al. 2010 [49].

Table 2: Activation of different apoptotic events in thapsigargin-treated leukocytes from young and elderly individuals.

	Neutrophils		Lymphocytes	
	Young	Aged	Young	Aged
MMD	+	+++	+	+++
Caspase-9 activity	+	+++	+	+++
Caspase-3 activity	+	++	+	++
PS exposure	+	++	+	++
DNA fragmentation	+	++	+	++

The degree of apoptosis was classified on a scale of + (indicating lowest apoptosis) to +++ (indicating highest apoptosis). MMD: mitochondrial membrane depolarization; PS: phosphatidylserine. Data were taken from Espino et al. 2011 [50].

4. Synthesis and Function of Melatonin

Melatonin, or *N*-acetyl-5-methoxytryptamine, is a widespread physiological mediator. It has been found in most organisms studied from bacteria to humans. The indole melatonin is mainly secreted in the pineal gland of vertebrates, although it is now known to be produced in many other tissues as well [53]. In the pineal gland, melatonin is converted in two steps from the amino acid tryptophan into serotonin (5-hydroxytryptamine), and then acetylated by arylalkylamine *N*-acetyltransferase (AA-NAT), before finally being converted into melatonin by hydroxyindole-*O*-methyltransferase (HIOMT), which represents the rate-limiting step in magnitude of melatonin biosynthesis [54]. The pineal gland synthesizes and releases melatonin primarily during the dark phase. Thus, melatonin levels in the circulation exhibit a distinctive circadian rhythm in which the highest blood concentration is observed at night, while baseline levels are measured during the day [55].

It is well known that endogenous melatonin production wanes in the elderly [56] and that the total antioxidative

capacity of serum correlates well with its melatonin levels in humans [57]. In this regard, the participation of melatonin in slowing the deterioration of tissues and organs due to aging has been proposed many times. Thus, it has been shown that the removal of the pineal gland early in life exaggerates molecular damage in terms of lipid peroxidation, accumulation of 8-hydroxy deoxyguanosine in the DNA, and levels of protein carbonyls, as well as reducing membrane fluidity in old animals [58], whereas exogenous administration of melatonin reduces lipid peroxidation [59]. These results, considered in light of the free radical theory of aging, suggest that the age-associated melatonin reduction may be linked to the increase in oxidative damage observed with age [60].

From a physiological perspective, melatonin has been classically related to the physiological adjustment in circadian rhythms and mediating seasonal reproductive events in photoperiodically dependent species. It also alters the function of other endocrine organs and may be involved in sleep regulation in at least diurnally active species [61]. Moreover, melatonin interacts with the cardiovascular system [62] and has been implicated in metabolic control [63]. From a pharmacological view, the phase-advancing effects of melatonin have been frequently exploited [64, 65], with the indoleamine proven to be effective in the treatment of insomnia [66, 67] and efficient in limiting jet lag when travelling across time zones [68].

Finally, melatonin has a particular ability to neutralize free radicals [69] and prevent tissue damage associated with oxidative stress. Thus, it exhibits both direct scavenging actions against free radicals and related products [70–72], as well as indirect antioxidative actions via its ability to stimulate the cellular antioxidant defense system by increasing mRNA levels and activities of several important antioxidant enzymes, including SOD [12], to promote the synthesis of another important intracellular antioxidant, that is, gluthatione [73], to reduce the activity of the prooxidative enzyme nitric oxide synthase [74], and to diminish free radical formation at the mitochondrial level by reducing the leakage of electrons from the electron transport chain [75]. Additionally, different studies have demonstrated its protective role against oxidative damage induced by drugs, toxins, and different diseases [49, 76–78]. This combination of actions makes melatonin an important agent in combating some signs of aging and/or the initiation of age-related diseases.

Apart from that, melatonin has been recently proven to exert antisenescence actions through the activation of SIRT1, a sirtuin that promotes cell survival by inhibiting apoptosis or cellular senescence in mammalian cells. Thus, it has been reported that melatonin increases SIRT1 expression, which reduces inflammatory and apoptotic signaling related to p53, and diminishes vasoconstriction via increasing nitric oxide bioavailability [79]. Likewise, in a murine model of senescence (SAMP8), melatonin protects neurons against frailty by enhancing SIRT1 expression [80], and subsequently decreasing the amount of the acetylated (active) form of p53 [81].

5. Therapeutic Effects of Melatonin on Immune Function

As the age-related decline of the immune system first appears around 60 years of age coinciding with the reduction of plasma melatonin concentration, much attention has been devoted to the possible interaction between melatonin and the immune system in the last decade [32, 82]. In 1986, Maestroni and collaborators first showed that blockade of melatonin synthesis causes the inhibition of cellular and humoral responses in mice [83]. From that point on, a variety of investigations has revealed several modulating actions of melatonin on immune system.

Exogenous administration of melatonin has been proven to stimulate the production of cells mediating the nonspecific immunity, that is, NK cells and macrophage/monocyte lineage cells, in both the bone marrow and the spleen [84–86]. As both these populations constitute the first line of defense against neoplastically transformed and virus-transfected cells, these findings account for melatonin's ability to halt neoplastic growth and to destroy virus-infected cells. Additionally, the action of melatonin on NK cells has been proposed to reflect, at least in part, the fact that NK cells are exquisitely sensitive to cytokines produced by melatonin-stimulated T helper cells, including IL-2, IL-6, IL12, and interferon (IFN)-γ [86], since the immunostimulatory role of melatonin is exerted mainly on both T helper cells and T-lymphocyte precursors [32]. Likewise, monocyte production stimulated by melatonin has been suggested to be driven either directly [87], because cells of this lineage do possess melatonin receptors [88], or indirectly, in response to the triggered cascade of monocyte-sensitive stimulants, such as IL-3, IL-4, IL-6, and GM-CSF, set in place by melatonin activation of T helper cells [84–86].

Furthermore, melatonin administration has been revealed to upregulate the level of gene expression of transforming growth factor (TGF)-β, macrophage-colony stimulating factor (M-CSF), tumor necrosis factor (TNF)-α and stem cell factor (SCF) in peritoneal exudates cells, and the level of gene expression of IL-1β, M-CSF, TNFα, IFN-γ, and SCF in splenocytes [89]. In addition, an inhibitory influence of melatonin on parameters of the immune function has also been demonstrated. Thus, melatonin has been shown to inhibit the production of proinflammatory cytokines, such as IL-8 and TNFα, in neutrophils [90], suggesting that the indolamine may help to reduce acute and chronic inflammation. Melatonin has been also reported to counteract the inhibitory effect of prostaglandin E2 on IL-2 production in human lymphocytes via its MT1 membrane receptor [91]. In this sense, it has been suggested that melatonin may be involved in the regulation of cytokine production by modulating the activity of T cells and monocytes via nuclear orphan receptor (RZR/ROR)-mediated transcriptional control [92, 93].

A correct modulation of apoptosis may be useful for prolonging the lifespan or at least reducing age-related degenerative, inflammatory, and neoplastic diseases whose incidence increases with age. In this sense, melatonin's immunoenhancing effect not only depends on its ability to

improve the production of cytokines, but also on its anti-apoptotic and antioxidant actions. The first data supporting a role for melatonin in drug-induced apoptosis appeared in 1994, when Maestroni and coworkers demonstrated that melatonin-rescued bone marrow cells from toxicity caused either *in vivo* or *in vitro* by anticancer compounds, with this mechanism involving the endogenous production of GM-CSF [85]. Additionally, it has been indicated that orally administered melatonin can substantially boost the survival of newly formed B cells in mouse bone marrow, thereby providing evidence for a role of melatonin as a checkpoint regulator in early B-cell development [94]. Interestingly, Tan and colleagues found extremely high levels of melatonin in bone marrow cells of rats, with melatonin concentrations in the bone marrow being two orders of magnitude higher than in the circulation [95]. Given the high sensitivity of bone marrow cells to oxidative agents, for example, anticancer agents, the presence of high melatonin concentrations in bone marrow cells could be important to preserve their integrity.

The severe loss of thymocytes with age is the main cause of structural thymic atrophy and thymic weight loss. In this respect, it has been indicated that melatonin administration rejuvenates degenerated thymus and redress peripheral immune dysfunctions in aged mice [96]. This reversal of age-related thymic involution by melatonin is attributable to increments in thymic cellularity caused by both the antiapoptotic and the proliferative-enhancing effects of melatonin [97] although other mechanisms involving glucocorticoid receptor cannot be ruled out [98].

The antioxidant ability of melatonin and its metabolites may also account for its antiapoptotic actions on immune cells [99]. In fact, we have demonstrated that melatonin is able to inhibit intracellular calcium overload-induced leukocyte apoptosis by blocking caspase-9 and caspase-3 processing, which is mainly due to the modulation of both the opening of the mitochondrial permeability transition pore and the activation of the proapoptotic protein Bax [77]. Moreover, we have also proved that the beneficial effects resulting from melatonin administration on leukocyte apoptosis likely depend on melatonin's antioxidant properties, since this protection is unaffected by the MT1/MT2 antagonist luzindole, that is, independent of plasma membrane MT1/MT2 receptor stimulation [78]. More interestingly, melatonin is able to delay damage-induced apoptosis in aged neutrophils and lymphocytes and therefore may counteract, at the cellular level, age-related degenerative phenomena linked to oxidative stress [50]. This fact is especially remarkable as neutrophils from aged individuals show a diminished rescue capacity when challenged with proinflammatory stimuli, such as GM-CSF, GCSF, LPS, or IL-2 [100, 101].

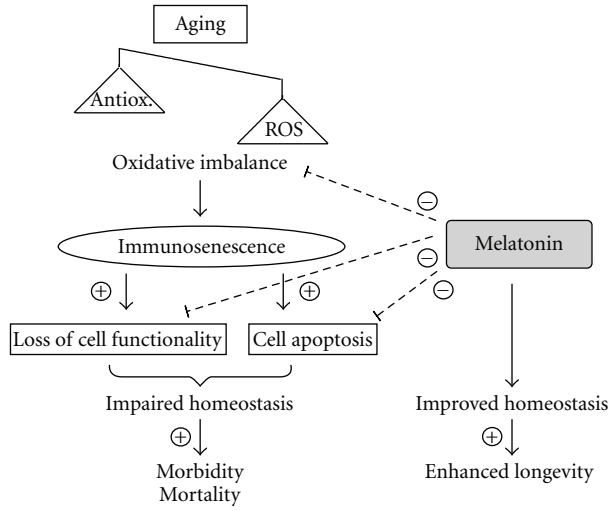

Figure 1: Schematic diagram depicting beneficial effects of melatonin on age-associated deterioration of immune system.

6. Concluding Remarks

The age-associated decline in immune function, known as immunosenescence, is characterized by a decrease in the functional activity of NK cells, granulocytes, and macrophages. In general, these age-associated changes in the immune system render organisms more sensitive to infections, autoimmune diseases, and even to cancer (Figure 1). In the last few years, growing evidence has indicated a tight, cause-effect link between oxidative stress and immunosenescence. Strikingly, several studies have highlighted the reversibility of some of the changes induced by oxidative stress with advancing age, which is particularly important for most cells in the immune system which, having a very short lifespan, are constantly replaced by newly produced elements. Therefore, these cells can potentially benefit from short-term therapies aimed at decreasing oxidative stress. In this sense, it is worth noting the possibility that melatonin supplementation could prevent or delay the functional deterioration of the immune system that accompanies aging and, perhaps, return it to that of the "younger" situation (Figure 1). Indeed, dietary supplementation of melatonin has been shown to ameliorate the attenuated immune responses associated with senescence [102]. Likewise, melatonin-enriched foodstuffs has been proven to modulate serum inflammatory markers in both rats and ringdoves, causing a reduction in proinflammatory markers along with an increase in anti-inflammatory markers which suggests amelioration or reorganization of immunity to a noninflamed state [103, 104]. Based on the experimental data that have accumulated and considering its lack of toxicity, its high lipophilicity, and its large capacity to forestall cell damage, melatonin is one of the most appealing agents to be examined in relation to age-associated deterioration in the immune system and should be considered as a potential agent to improve the quality of life in a rapidly aging population.

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Research Article

Oxidative Stress Contributes to Endothelial Dysfunction in Mouse Models of Hereditary Hemorrhagic Telangiectasia

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Hereditary hemorrhagic telangiectasia (HHT) is a vascular dysplasia caused by mutations in endoglin (*ENG*; HHT1) or activin receptor-like kinase (*ALK1*; HHT2) genes, coding for transforming growth factor-β (TGF-β) superfamily receptors. We demonstrated previously that endoglin and ALK1 interact with endothelial NO synthase (eNOS) and affect its activation. Endothelial cells deficient in endoglin or ALK1 proteins show eNOS uncoupling, reduced NO, and increased reactive oxygen species (ROS) production. In this study, we measured NO and H_2O_2 levels in several organs of adult *Eng* and *Alk1* heterozygous mice, to ascertain whether decreased NO and increased ROS production is a generalized manifestation of HHT. A significant reduction in NO and increase in ROS production were found in several organs, known to be affected in patients. ROS overproduction in mutant mice was attributed to eNOS, as it was L-NAME inhibitable. Mitochondrial ROS contribution, blocked by antimycin, was highest in liver while NADPH oxidase, inhibited by apocynin, was a major source of ROS in the other tissues. However, there was no difference in antimycin- and apocynin-inhibitable ROS production between mutant and control mice. Our results indicate that eNOS-derived ROS contributes to endothelial dysfunction and likely predisposes to disease manifestations in several organs of HHT patients.

1. Introduction

HHT (Rendu-Osler-Weber-syndrome) is inherited in an autosomal dominant fashion and has a prevalence of about 1 in 5–8,000 individuals, worldwide. Mutations in endoglin *(ENG)* and activin receptor-like kinase (*ACVRL1* or *ALK1)* are responsible for HHT1 and HHT2, respectively, and account for approximately 85% of cases [1]. Both endoglin and ALK1 are components of the transforming growth factor-β (TGF-β) superfamily of receptors, predominantly expressed in the vascular endothelium. The receptor complex for TGF-β1 and -β3 isoforms contains the coreceptor endoglin associated with type II (TβRII) and type I receptors ALK1 or ALK5 [2]. Endoglin and ALK1 directly bind to the bone morphogenetic proteins BMP-9 and BMP-10 and mediate their effects in conjunction with BMPRII [3]. ALK1 activation leads to phosphorylation of Smads1/5/8, while ALK5 phosphorylates Smads2/3. Phosphorylated Smads form a heteromeric complex with the common Smad4, accumulate in the nucleus, and regulate gene transcription. MADH4 mutations coding for Smad4 account for about 3%–5% of cases of HHT, those often associated with juvenile polyposis [4]. Two other loci are in linkage disequilibrium with HHT, on chromosomes 5 and 7 and representing HHT3 and HHT4, respectively [5, 6]. The identification of these genes may increase our understanding of the molecular defects responsible for this disease. HHT is characterized by epistaxis, mucocutaneous and gastrointestinal telangiectases, and arteriovenous malformations (AVMs) in visceral organs [1]. Although genetic testing is required to determine the genotype of a patient, the incidence of pulmonary and cerebral AVMs is generally higher in HHT1 while hepatic AVMs and intestinal complications occur more often in HHT2 [7, 8]. The underlying mechanism of disease is haploinsufficiency, indicating that mutations lead to lower

levels of functional endoglin or ALK1 rather than to a dominant negative effect by the mutant protein [9, 10].

Mice totally deficient in endoglin or ALK1 are characterized by an embryonic lethal phenotype due to cardiovascular defects [11, 12]. Endoglin heterozygous ($Eng^{+/-}$) and ALK1 heterozygous ($Alk1^{+/-}$) mice have been used as models for HHT1 and HHT2, respectively, allowing us to dissect the underlying mechanisms of disease [11, 12]. Our earlier studies have revealed that nitric-oxide- (NO-) mediated vasodilation is impaired in $Eng^{+/-}$ mice and that endoglin is important in endothelial NO synthase (eNOS) activation [13, 14]. Moreover, endoglin and ALK1 associate with and stabilize the eNOS activation complex leading to NO production. In endothelial cells deficient in endoglin or ALK1, eNOS becomes uncoupled and produces more superoxide than NO, leading to tissue damage and impaired vascular tone [13–16].

Interestingly, our recent work also revealed that adult $Eng^{+/-}$ and $Alk1^{+/-}$ mice show signs of pulmonary arterial hypertension (PAH) including rarefaction of the distal pulmonary vasculature, muscularization of small arteries, increased right ventricular (RV) systolic pressure, and RV hypertrophy [15, 16]. These disease manifestations were attributable to uncoupled eNOS activity and increased superoxide ($\bullet O_2^-$) production, as antioxidant treatment could prevent the onset of PAH [15, 16].

It is known that increased bioavailability of reactive oxygen species (ROS) and dysregulated redox signalling together with decreased NO production can contribute to endothelial injury in vascular diseases [17]. The major enzymatic sources of ROS production in endothelial cells are mitochondrial respiratory enzymes, NADPH oxidases, and uncoupled eNOS [17]. Excessive ROS production may therefore have an important role in the pathogenesis of endothelial dysfunction leading to diseases such as HHT and PAH. We therefore investigated with the help of inhibitors, the contribution of NO and ROS producing enzymes in various organs of adult $Eng^{+/-}$ and $Alk1^{+/-}$ mice. We report that NO generation is decreased while oxidative stress is increased in several tissues of the mutant mice. Mitochondria were the major source of ROS in liver, while NADPH oxidase produced most of the ROS in other tissues, both in mutant and control mice. We identified eNOS as the main source of ROS overproduction in *Eng* and *Alk1* heterozygous mice. Our findings suggest that oxidative stress may predispose the vascular endothelium of patients with *ENG* and *ALK1* haploinsufficiency to disease manifestations. Antioxidant therapy should be considered for these patients.

2. Materials and Methods

2.1. Chemicals and Reagents. Phosphate buffered saline, (PBS) pH 7.4, was from Life Technologies Inc., Burlington, ON, Canada; S-Nitroso-N-acetylpenicillamine (SNAP50) from World precision Instruments Inc. Sarasota, FL, USA; copper sulfate from Fisher Scientific, Ottawa, ON, Canada; L-Arginine, N_G-nitro-L-arginine methyl ester (L-NAME), Antimycin A, Apocynin, and HEPES from Sigma-Aldrich, Oakville, ON, Canada; EDTA from Ambion, Life Technologies Inc; protease inhibitor cocktail from Fermantas, Burlington, ON, Canada. The Amplex Red assay kit including horseradish peroxidase was from Molecular Probes, Eugene, OR, USA.

2.2. Experimental Animals. All protocols were approved by the Hospital for Sick Children and the Toronto Centre for Phenogenomics Animal Care Committees (AUP#0067) in accordance with the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; A5047-01). N28–N32 $Eng^{+/-}$ and $Eng^{+/+}$ C57BL/6 mice were generated by successive backcrosses and genotyped as previously described [11]. $Alk1^{+/-}$ and $Alk1^{+/+}$ (N13–17) were generated and genotyped as described [18]. Mice ranging from 14 to 18 weeks of age were used for all measurements.

2.3. Tissue Collection. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). For lung tissue collection, mice were perfused with PBS through the right ventricle. For all other organ dissections, PBS perfusion was done through the left ventricle. Organs were kept in cold PBS and used immediately for NO or Amplex red detection.

2.4. NO Measurement. Excised cardiac, pulmonary, and hepatic tissues were incubated in Krebs-Henseleit buffer for 2 hours at 37°C in the presence or absence of L-NAME. NO production was quantified using an NO microsensor ISO-NOP700 attached to an Apollo 4000 Free Radical Analyzer. The microsensor was calibrated using SNAP50 in the presence of copper sulfate.

2.5. H_2O_2 Measurement. H_2O_2 levels were estimated using the Amplex Red assay kit in accordance with manufacturer's instructions. Carefully dissected tissues were flushed with PBS and homogenized in 25 mM HEPES buffer, pH 7.4, plus 1 mM EDTA and protease inhibitors. After centrifugation at 6000 g for 5 minutes at 4°C, the supernatants were incubated in a microtiter plate at 37°C for 1 hour with Amplex red, horseradish peroxidase, and L-Arginine (NOS substrate, 1 mM). The effects of 1 mM L-NAME (NOS inhibitor), 50 μM Antimycin (respiratory chain reaction inhibitor), and 100 μM Apocynin (nicotinamide-adenine dinucleotide phosphate, NADPH oxidase inhibitor) on the reaction were tested. Fluorescence was quantified using excitation at 544 nm and emission at 590 nm; data were normalized for protein content.

2.6. Lipid Peroxidation Measurement in Lung Tissue. The concentration of the lipid peroxidation product 8-iso PGF2α was determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described [19].

2.7. Statistical Analysis. Comparisons were performed by appropriate ANOVA test, and significant differences were

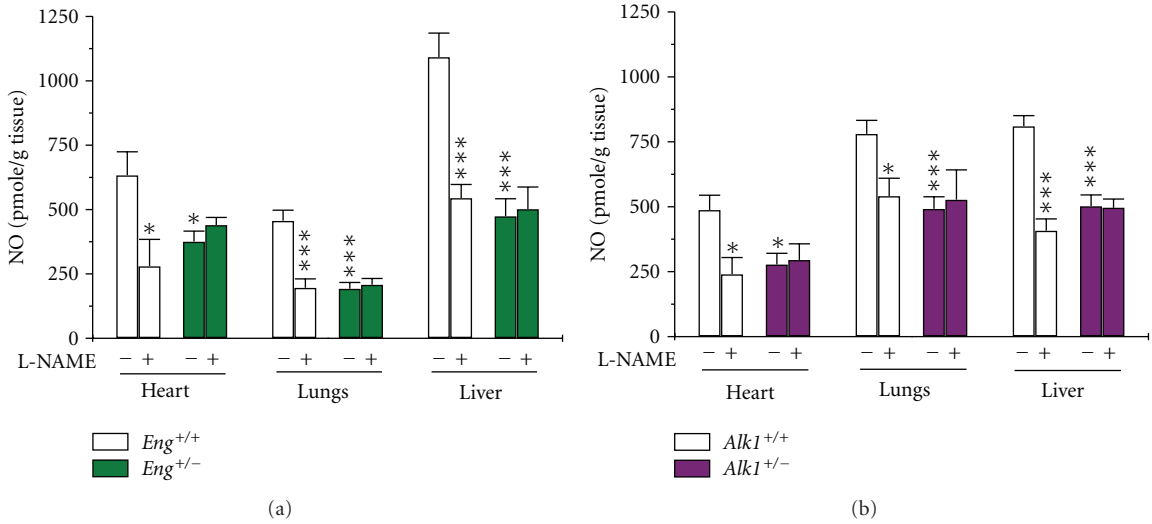

Figure 1: Decreased NO production in tissues of *Eng* and *Alk1* heterozygous mice. NO levels were measured using a microsensor in the presence or absence of the NOS inhibitor L-NAME, 1 mM. NO levels were significantly reduced in heart, lungs, and liver of (a) $Eng^{+/-}$ and (b) $Alk1^{+/-}$ mice compared to their control littermates. Nitric oxide production was inhibited by L-NAME in all tissues of control mice but not in tissues of (a) $Eng^{+/-}$ or (b) $Alk1^{+/-}$ mice. $^{*}P < 0.05$, and $^{***}P < 0.001$ versus +/+ untreated mice; N = 6–8/group for *Eng* mice and 6–10/group for *Alk1* mice.

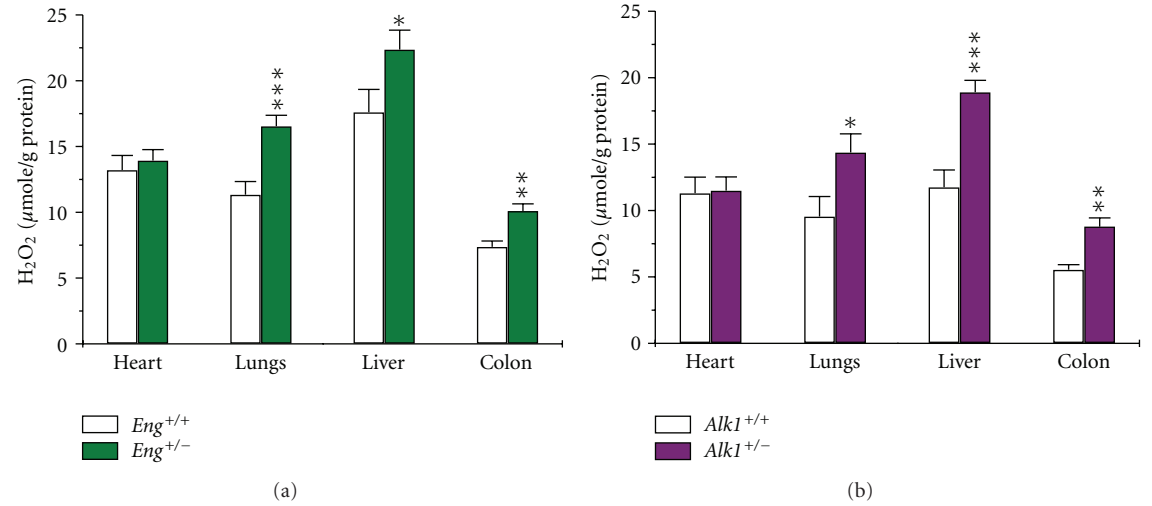

Figure 2: ROS production is increased in tissues of mutant mice. H_2O_2 production was measured by Amplex red, to estimate tissue ROS production. H_2O_2 generation was increased in pulmonary, hepatic, and colonic tissues of (a) $Eng^{+/-}$ and (b) $Alk1^{+/-}$ mice compared to those of corresponding wild-type mice. Cardiac ROS production was similar between mutant and control mice. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus +/+ mice; N = 10–15/group for *Eng* mice and 7–20/group for *Alk1* mice.

evaluated post hoc using the Newman-Keuls test. Results are expressed as the mean ± SEM, with $P < 0.05$ representing significance.

3. Results

3.1. Decreased NO Production in Tissues of Eng and Alk1 Heterozygous Mice. NO levels were decreased in pulmonary, hepatic, and cardiac tissues of $Eng^{+/-}$ and $Alk1^{+/-}$ mice, when compared to respective littermate controls (Figures 1(a) and 1(b)). L-NAME inhibited NO production in heart, lungs, and liver of wild-type mice, indicating NOS involvement. As endoglin and ALK1 are mostly expressed in endothelial cells, eNOS is the enzyme most likely involved. In $Eng^{+/-}$ and $Alk1^{+/-}$ mice, L-NAME did not inhibit NO production, suggesting abnormal eNOS activation and potential uncoupling.

3.2. ROS Production Is Increased in Tissues of Eng and Alk1 Mutant Mice. The Amplex Red assay revealed a substantial increase in H_2O_2 production in lungs, liver, and colon of $Eng^{+/-}$ and $Alk1^{+/-}$ mice, compared to corresponding wild-type mice (Figures 2(a) and 2(b)). This increase in H_2O_2

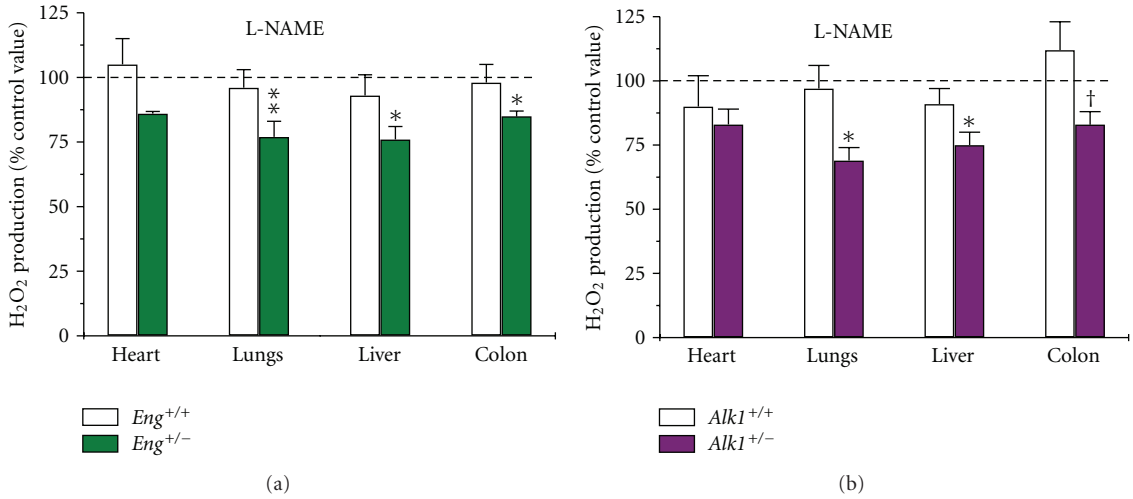

Figure 3: ROS production is inhibited by L-NAME in tissues of mutant mice. H_2O_2 production was measured by Amplex red with and without the NOS inhibitor L-NAME. Results are expressed as a percentage of the respective control values (without inhibitors). L-NAME significantly inhibited H_2O_2 production in pulmonary, hepatic, and colonic tissues of (a) $Eng^{+/-}$ and (b) $Alk1^{+/-}$ mice but had no effect on tissues of control mice. In cardiac tissue, L-NAME effect was not significantly different between mutant and control mice. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{\dagger}P < 0.1$ versus samples from corresponding control values; $N = 6$–13/group for both *Eng* and *Alk1* mice.

likely reflects a rise in ROS production. The highest levels of H_2O_2 were observed in highly vascularized tissues of the heterozygous mice, such as liver, and lungs, suggesting that endothelial cells are the major producers of ROS. This might also explain why despite reduced NO levels, ROS levels were not different between mutant and control mice in cardiac tissue, where cardiomyocytes represent ~75% of total tissue mass.

To confirm that H_2O_2 levels reflect ROS production, we estimated the amount of the lipid peroxidation product 8-iso-PGF2α in lungs of heterozygous mice. We found higher levels of this product in $Eng^{+/-}$ lungs (0.43 ± 0.05 ng/g of tissue) compared to littermate controls (0.28 ± 0.04 ng/g of tissue; $N = 8$, $P < 0.05$). We also observed increased levels of pulmonary 8-iso-PGF2α in $Alk1^{+/-}$ mice as reported previously [16]. These results confirm the higher oxidative stress in lungs of $Eng^{+/-}$ and $Alk1^{+/-}$ mice compared to littermate controls and validate the use of Amplex red assay as a means of assessing ROS.

3.3. L-NAME Decreases ROS Production in Tissues of Eng and Alk1 Heterozygous Mice. To ascertain the source of increased ROS production in $Eng^{+/-}$ and $Alk1^{+/-}$ mice, we compared the ability of various inhibitors to affect H_2O_2 levels. We showed that the NOS inhibitor L-NAME, significantly reduced H_2O_2 generation in lungs, liver, and colon of $Eng^{+/-}$ and $Alk1^{+/-}$ mice, but had no effect on wild-type mice (Figures 3(a) and 3(b)). This result suggests that eNOS is indeed uncoupled in the heterozygous mice leading to ROS production whereas in control mice, eNOS is normal and produces NO and not ROS.

3.4. Mitochondrial and NADPH Oxidase-Mediated ROS Production Is Similar in Tissues of Mutant and Control Mice. Antimycin, a respiratory chain inhibitor, decreased ROS production to the same extent in tissues of $Eng^{+/-}$, $Alk1^{+/-}$ mice and their respective wild-type controls (Figures 4(a) and 4(b)). The strongest effect was seen in liver, where 50%–75% of ROS was antimycin-inhibitable followed by lungs (25%–50%), heart (25%–30%), and colon (5%–25%) (Figures 4(a) and 4(b)). However, the absolute levels of H_2O_2 measured in antimycin-treated samples were still significantly higher in lungs and liver of $Eng^{+/-}$ ($P < 0.01$) and $Alk1^{+/-}$ ($P < 0.05$) mice compared to their respective wild-type controls, indicating higher overall ROS production. Apocynin, an NADPH oxidase inhibitor, blocked half of ROS production in tissues of both mutant and wild-type mice (Figures 4(c) and 4(d)). The biggest inhibitory effect of apocynin was observed in colon, followed by lungs, heart, and liver. There was no significant difference in NADPH-dependent H_2O_2 production between heterozygous and wild-type mice.

4. Discussion

We report that *Eng* and *Alk1* heterozygous mice have decreased NO production and increased ROS levels in several organs, indicating that oxidative stress may lead to endothelial dysfunction. This is the first report comparing the levels of ROS produced in different tissues of these mice and establishing that decreased NO and increased ROS generation can be generalized to several organs. The major source of ROS overproduction in $Eng^{+/-}$ and $Alk1^{+/-}$ mice is NO synthase, suggesting uncoupled eNOS as a major mechanism of increased oxidative stress in lungs, liver, and colon of $Eng^{+/-}$ and $Alk1^{+/-}$ mice. We also showed that in liver, mitochondria contribute most ROS while NADPH oxidase is the major source in other tissues. However, the

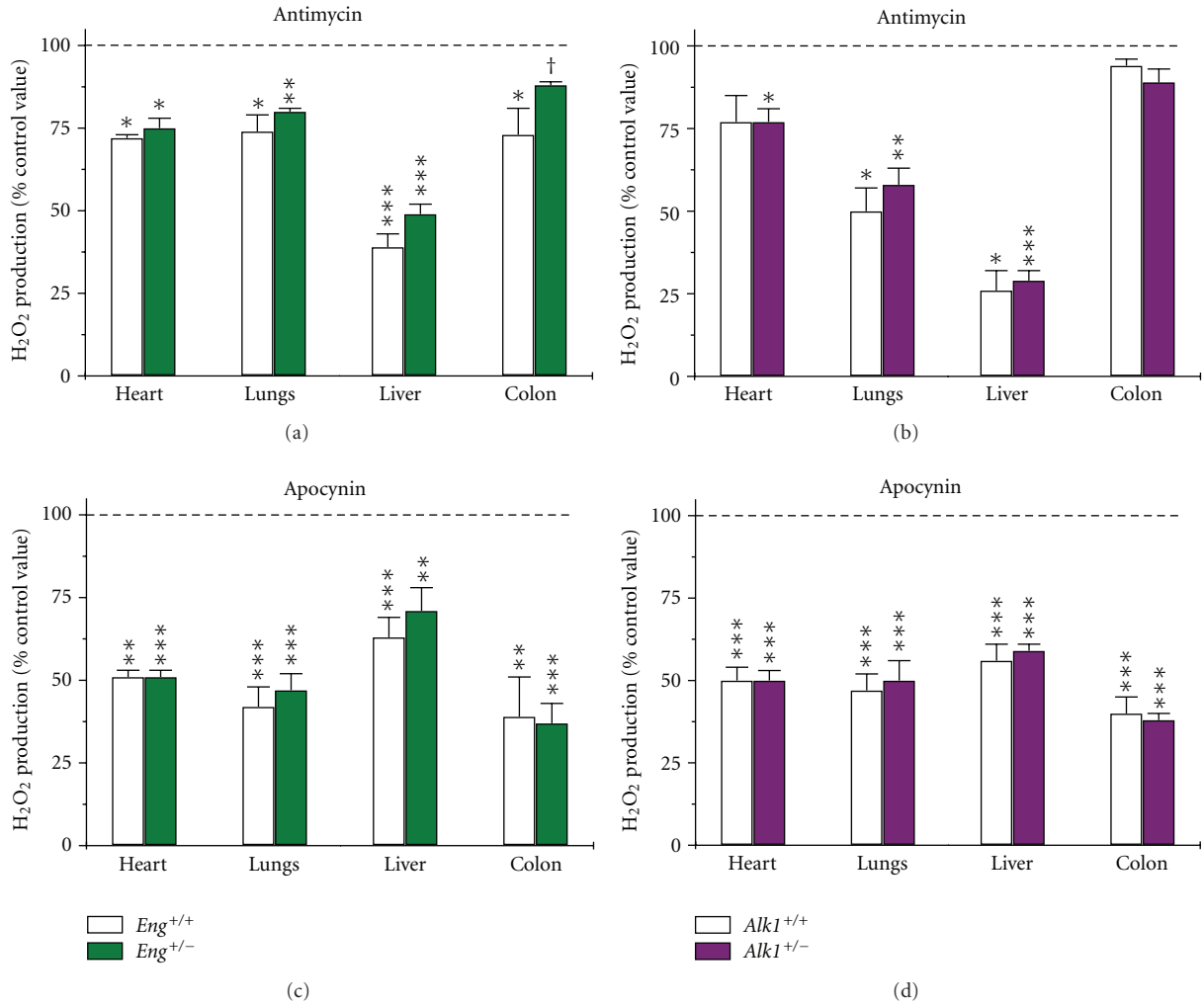

Figure 4: Mitochondrial and NADPH-dependent ROS production does not differ between mutant and wild-type mice. Results are expressed as a percentage of the respective control values in the absence of inhibitor. (a) and (b) Antimycin, 50 μM, inhibited mitochondrial ROS production to the same level in tissues of both $Eng^{+/-}$, $Alk1^{+/-}$ and respective wild-type mice. $N = 5$–11/group for *Eng* mice and 5–15/group for *Alk1* mice, except for liver samples from $Alk1^{+/+}$ mice, where $N = 4$. (c) and (d) Apocynin, 100 μM, inhibited NADPH-oxidase dependent H_2O_2 production in tissues of both mutant and wild-type mice. $N = 5$–8/group for both *Eng* and *Alk1* mice, except for heart samples from both groups of mice, where $N = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{\dagger}P < 0.1$ versus corresponding control values (samples without inhibitor).

percentage of ROS produced by these two sources is similar in HHT and control mice.

Reactive oxygen species have been recognized as important players in both physiological and pathophysiological conditions. A small amount of ROS is necessary and serves as a physiological regulator of intracellular signalling pathways [20]. However, an overproduction of ROS (such as superoxide, hydrogen peroxide, hydroxyl radicals, and peroxynitrite) contributes to the pathogenesis of different diseases [21, 22]. The most prominent role of oxidative stress is in the pathologic mechanisms underlying endothelial dysfunction in cardiovascular disorders such as hypertension, hyperlipidemia, diabetes, arteriosclerosis, vasculitis, and myocarditis, to name a few. Although multiorgan vascular dysplasia and vascular stress are hallmarks of HHT [23], there are few studies investigating ROS involvement in the pathogenesis of this disease. We previously reported that increased H_2O_2 generation in lungs of $Eng^{+/-}$ mice accounts for the heightened pulmonary vasorelaxation [24]. We also showed that the onset of PAH in $Eng^{+/-}$ and $Alk1^{+/-}$ adult mice is attributable to increased endothelial oxidative stress, as it could be prevented by antioxidant treatment [15, 16]. These findings are highly relevant as individuals with *ALK1* and *ENG* mutations can develop PAH, with or without HHT [25–27]. Also, mutations in the gene encoding the BMP receptor type II (*BMPR2*) underlie the majority of cases of familial PAH and up to 40% of idiopathic cases [28]. In addition, increased oxidative stress has been observed in patients with PAH [29, 30]. These findings suggest a functional role for endoglin, ALK1, and BMPR2 in the regulation of vascular function and oxidative stress.

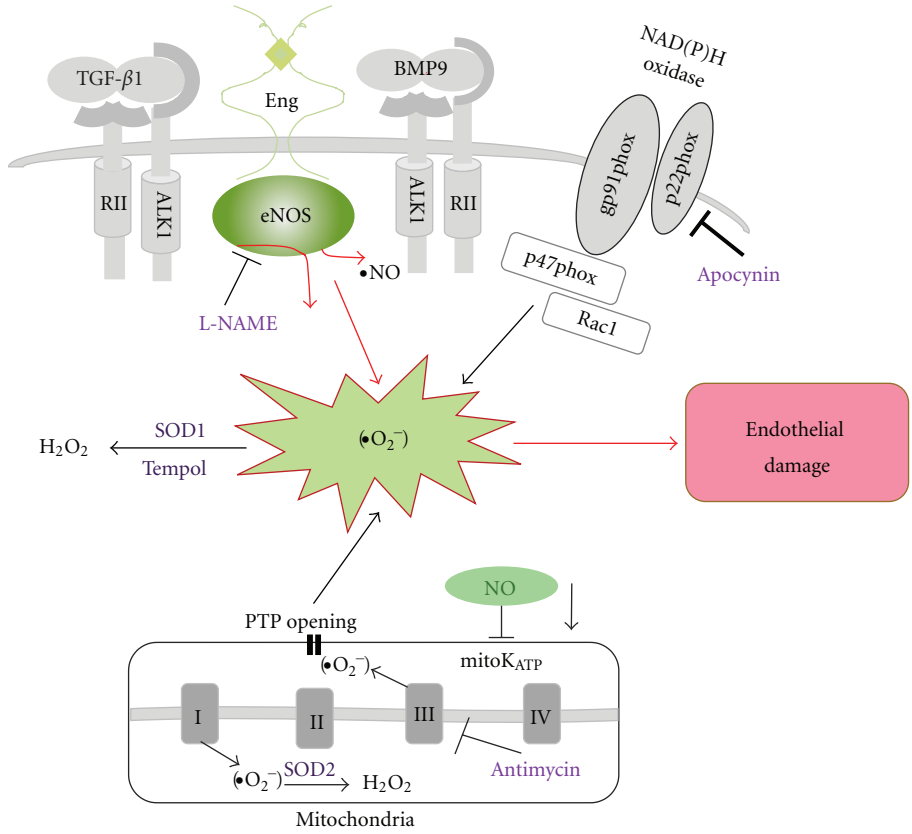

Figure 5: Model of oxidative stress in mutant mice leading to vascular endothelial damage. In *Eng*$^{+/-}$ and *Alk1*$^{+/-}$ mice, altered eNOS activation renders the enzyme refractory to regulation by TGF-β/BMP signaling and represents a critical event leading to excessive oxidative stress. Uncoupled eNOS produces low amounts of NO and high levels of oxygen radicals ($\bullet O_2^-$). The NOS inhibitor, L-NAME, inhibits ROS production in tissues of mutant mice. Superoxide dismutase (SOD) and the SOD mimetic compound Tempol convert $\bullet O_2^-$ into less harmful hydrogen peroxide (H_2O_2). A large portion of ROS is produced by mitochondria (Antimycin-inhibitable) and NADPH oxidases (Apocynin-inhibitable) however that percentage does not differ between mutant and control mice. Low NO cellular level may also inhibit mitochondrial K_{ATP} channel (mitoK_{ATP}) opening, trigger permeability transition pores (PTP) opening, and further increase the oxidative stress caused by mitochondrial ROS release.

To our knowledge, this is the first report showing increased ROS production in several major organs of *Eng*$^{+/-}$ and *Alk1*$^{+/-}$ mice, including lungs, liver, and colon, which are affected in HHT. We estimated ROS levels by measuring H_2O_2 by the Amplex red assay. In a previous paper [16] we observed a good correlation between Amplex red assay results and ROS levels estimated using fluorescent probes (H_2DCFDA and DHE), direct H_2O_2 measurements by ultrasensitive probe, and the lipid peroxidation product 8-iso-PGF2α.

We identified NOS as a major source of ROS overproduction in tissues of *Eng*$^{+/-}$ and *Alk1*$^{+/-}$ mice. Our previous work showed eNOS uncoupling in endothelial cells from HHT patients and from *Eng* and *Alk1* heterozygous mice [14, 16]. eNOS is closely associated with caveolae/lipid rafts in endothelial cells, where endoglin and ALK1 colocalize with eNOS serving as scaffolding molecules for eNOS/Hsp90 association and eNOS activation [14, 15, 31]. In the mutant mice, eNOS uncoupling renders the enzyme refractory to regulation by TGF-β/BMP signaling and represents a critical event leading to excessive oxidative stress. Uncoupled eNOS forms monomers and generates ROS rather than NO, as shown in our proposed model (Figure 5). Neuronal NOS was not observed, and inducible NOS was barely detectable in mouse tissues [13, 16]; therefore, we conclude that the observed increase in L-NAME inhibitable H_2O_2 in tissues of HHT mice (Figure 3) is eNOS-derived. Indeed, eNOS uncoupling has been suggested as one of the main pathomechanisms in a broad range of cardiovascular and pulmonary disorders [32]. Our results strongly suggest that this phenomenon occurs in multiple organs of the mutant mice and can explain the decrease in NO and the increase in ROS production (Figures 1 and 2). We also previously reported a systemic decrease in NO bioavailability as determined by lower plasma nitrate levels in *Eng*$^{+/-}$ mice [13, 14]. NOS-dependent (L-NAME inhibitable) H_2O_2 production was also the only source of ROS that significantly differed between mutant and control mice.

Mitochondria have long been known as an important source of ROS. The role of small quantities of mitochondrial ROS in cellular signaling was recently recognized [33], but it is generally assumed that an increase in mitochondrial

ROS is involved in the pathobiology of many vascular and neurodegenerative disorders [34]. Experiments in isolated mitochondria estimate a 2%-3% leakage of electrons to form $\bullet O_2^-$ [35] that is largely dismutated to H_2O_2 by manganese superoxide dismutase (SOD2) in the mitochondrial matrix (Figure 5). The importance of mitochondrial ROS and their removal is emphasized by the observation that SOD2 null mice exhibit perinatal lethality due to cardiac dysfunction [36]. We found that antimycin, a mitochondrial complex III inhibitor [37], significantly inhibited H_2O_2 production in tissues of mutant and control mice (Figures 4(a) and 4(b)). Despite the fact that the antimycin-inhibitable ROS portion differed between organs, it was similar between heterozygous and control mice. However, despite a similar percentage of inhibition, the absolute amount of H_2O_2 estimated in antimycin-treated samples was higher in lungs and liver of mutant mice. This might be explained by NO reduction indirectly inhibiting the mitochondrial K_{ATP} channel opening (Figure 5), triggering the opening of the permeability transition pores and further increasing oxidative stress caused by mitochondrial ROS release [38, 39].

The NADPH oxidases have emerged as relevant enzymatic sources. In fact, NADPH oxidase is a major ROS generator in endothelial cells [40]. The NADPH oxidase enzyme complex consists of two membrane-bound components, gp91phox (also known as Nox2) and p22phox, and several cytosolic regulatory subunits, including p47phox, p67phox, and the small GTPase Rac (Figure 5). In endothelial cells Nox1, Nox2, Nox4, and Nox5 are expressed [40]. Upon enzyme activation, the cytoplasmic subunits translocate to the cell membrane and the resulting complex transfers electrons from NAD(P)H to molecular oxygen to form $\bullet O_2^-$ [41]. The small amount of ROS produced in endothelial cells by NADPH oxidase may help maintain the critical balance between superoxide and NO levels in vessels. Recently it has been shown that endothelium-dependent coronary vasodilatation requires NADPH oxidase-derived ROS [42]. Excessive amounts of ROS produced by these enzymes contribute to the development of cardiovascular diseases including hypertension and atherosclerosis [41]. However, the use of NADPH oxidase antagonists has been limited to animal studies because the majority of these compounds, including apocynin used in this study and diphenylene iodonium, may possess nonspecific off-target effects. New triazolo pyrimidines, such as VAS3947, are specific for NADPH oxidases but their possible interference with alternative sources of ROS, such as the mitochondrial electron chain, cannot be excluded [43]. On the other hand, it is known that beneficial effects of some antihypertensive drugs (i.e., angiotensin-converting enzyme inhibitors and AT1 receptor antagonists) are partly due to their ability to inhibit NADPH oxidase activation and expression [41]. Results of our study have confirmed that NADPH oxidase is the major source of ROS production in heart, lungs, and colon but not in liver. However, the apocynin-dependent H_2O_2 production was similar in heterozygous and control mice indicating that the portion of NADPH-dependent ROS is not different in tissues of mutant and control mice (Figures 4(c) and 4(d)).

It is well known that intracellular ROS levels are regulated by the balance between ROS generating enzymes and antioxidant enzymes that include SOD (Figure 5) and catalase. In previous studies, we have shown that expression of SOD1, 2, and 3 and catalase is similar in lungs of $Eng^{+/-}$ [24] and $Alk1^{+/-}$ [16] mice when compared to control mice.

In summary, our study demonstrates increased oxidative stress in several organs of $Eng^{+/-}$ and $Alk1^{+/-}$ mice and suggests eNOS uncoupling as the major source of ROS overproduction. This excessive ROS generation may play a pivotal role in the pathobiology of endothelial dysfunction in HHT. Our results also suggest that HHT patients may benefit from antioxidant treatment. We showed previously that the antioxidant Tempol was able to prevent the onset of PAH in $Eng^{+/-}$ and $Alk1^{+/-}$ mice [15, 16]. In HHT patients, the only trial carried out to date was a pilot study in which the antioxidant N-acetylcysteine was given to patients for twelve weeks [44]. The results of this study look promising, with a significant decrease in the frequency and severity of epistaxis in treated HHT patients, improving their quality of life. However, antioxidant treatments used in some clinical studies to ameliorate cardiovascular disease have showed little or no improvement [45]. General strategies with antioxidants used for different diseases may not be very effective because ROS production differs between organs. Effective antioxidant treatment should be tailored differently for various conditions. Our results suggest that antioxidants are promising therapeutic tools in HHT and encourage their use in experimental and clinical studies that will help determine most effective therapeutic strategies.

5. Conclusions

Our study has demonstrated that *Eng* and *Alk1* heterozygous mice show decreased NO and increased eNOS-dependent ROS production in several organs suggesting the role of oxidative stress in endothelial dysfunction in these mice. Results of our studies also suggest that HHT patients may benefit from antioxidant treatment.

Conflict of Interests

The authors declare that they have no conflict of intersts.

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Clinical Study

Decrease of Total Antioxidative Capacity in Developed Low Cardiac Output Syndrome

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Background. It has been known that cardiac surgery induces an oxidative stress. The persistent oxidative stress during reperfusion may lead to depressed myocardial function resulting in low cardiac output syndrome (LCOS) necessitating inotropic or intra-aortic balloon counterpulsation support. Total antioxidant capacity (TAC) is a measurement of oxidative stress in tissues. The purpose of this study was to examine the TAC differences during coronary artery bypass graft (CABG) operation in patients who have developed LCOS and who have not. *Material and Methods*. Seventy-nine patients were enrolled in the study. Central venous blood samples were obtained immediately before surgery, during operation, and at the end of surgery to assess TAC. Clinical data regarding patient demographics and operative outcomes were prospectively collected and entered into our clinical database. *Results*. LCOS developed in 8 patients (10.12%). The TAC has decreased sharply in the LCOS patients compared with those who did not develop LCOS ($P < 0.001$) during operation. In addition, the receiver operating characteristic (ROC) area was 0.879. *Conclusion*. TAC has decreased during operation in a significant proportion of patients undergoing isolated CABG, and this is more prominent and serious and might be an independent variable in patients who have developed LCOS. This may be related to intraoperative misadventure or inadequate myocardial antioxidative protection. Routine measurement of the TAC during operation may provide information for assessment of the LCOS development.

1. Introduction

Ischemia-reperfusion injury may cause a damage to the myocardium following blood flow restoration after a critical period of coronary occlusion [1]. In fact, ischemia-reperfusion is a clinical problem associated with procedures such as thrombolysis, angioplasty, and coronary bypass surgery which are commonly used to establish the blood reflow and minimize the damage of the heart due to severe myocardial ischemia.

Oxidative stress is a one of the hypotheses to explain the pathogenesis of ischemia-reperfusion injury [2, 3]. Oxidative stress, which is usually associated with increased formation of reactive oxygen species (ROS), modifies phospholipids and proteins leading to lipid peroxidation and oxidation of thiol groups [4, 5]. Antioxidant molecules prevent and/or inhibit these harmful reactions [6]. Serum concentrations of different antioxidant molecules can be measured in laboratories separately, but the measurement of these molecules in laboratory is time consuming, labor intensive, and requires complicated techniques. On the contrary, measurement of total antioxidant capacity is easy to use [7]. Advances in myocardial protection have led to lower risk-adjusted rates of morbidity and mortality after coronary artery bypass surgery. Prevalence of postoperative LCOS remains relatively high approximately 9% [8]. In LCOS it is necessary to have intra-aortic balloon counterpulsation or inotropic support for longer than 30 minutes after the patient was returned to the intensive care unit to maintain the systolic blood pressure higher than 90 mm Hg and the cardiac index greater than 2.2 L/min per square meter [8]. The current low rates of perioperative infarction and mortality associated with CABG

Table 1: The preoperative characteristics of the patients.

	n	Ratio (%)
Male	60	75.94
Female	19	24.06
Age (yr)	61.5 ± 9.6	
Timing		
Elective	70	88.6
Emergency	9	11.4
Recent MI	11	13.92
LVEF		
40%–60%	79	100
NYHA class		
I	5	6.3
II	18	22.78
III	24	30.37
IV	32	40.50
CAD		
1 vessel	5	6.3
2 vessel	22	27.84
3 vessel	44	55.69
Left main CAD	8	10.12
Diabetes	14	17.72
Hypertension	35	44.30
PVD	6	7.59

Abbreviations: LVEF: left ventricular ejection fraction; NYHA: New York Heart Association; CAD: coronary artery disease; PVD: peripheral vascular disease.

have required large sample sizes in order to demonstrate clinically significant differences between myocardial protective strategies [3].

In this paper, we have explored if there is a difference between the coronary artery bypass patients who have developed LCOS and who have not.

According to us this is the first report investigated the changes of total antioxidant capacity in patients who have developed LCOS after CABG [4].

2. Material and Methods

Preoperative and operative data were collected prospectively in all patients undergoing CABG between August 2004 and December 2004, at Harran University, Research Hospital. This study was approved by the local ethic committee, and informed consent was obtained from all patients. The preoperative characteristics of the 79 consecutive patients are shown on Table 1.

2.1. Operative Technique. After median sternotomy and full heparinization, cardiopulmonary bypass (CPB) was instituted thorough a single two-stage right atrial cannula and an ascending aortic cannula. During bypass, the hematocrit level, pump flow rates, and mean arterial pressures were maintained between 20% and 25%, 2.0 and 2.5 L/min per square meter, and 50 and 60 mm Hg by use of sodium nitroprusside or phenylephrine hydrochloride as required, respectively. In all patients, the heart was arrested by an aortic root infusion of high potassium (27 mEq/L) blood cardioplegic solution. Distal anastomoses were done under a single cross-clamp. A left internal thoracic artery graft was used in all patients.

2.2. Low Cardiac Output Syndrome. Low cardiac output syndrome was diagnosed in patients who required an intra-aortic balloon pump either in the operating room or in the intensive care unit because of hemodynamic compromise or required inotropic medication to maintain the systolic blood pressure greater than 90 mm Hg and the cardiac output greater than ($\geq 2.5\,\mathrm{L}\cdot\mathrm{min}^{-1}\cdot\mathrm{m}^{-2}$) for at least 30 minutes in the intensive care unit after correction of all electrolytes and blood gas abnormalities while adjusting preload volume to its optimal values [8]. Patients who [5] received dopamine, dobutamine, or epinephrine named inotropic support. Patients who received dopamine lower than 4 mcg/kg/min aimed to increase renal perfusion and received vasodilating agents because of a high cardiac output ($\geq 2.5\,\mathrm{L}\cdot\mathrm{min}^{-1}\cdot\mathrm{m}^{-2}$) and low peripheral resistance were not considered to have LCOS.

2.3. Samples. Blood samples were drawn from central venous catheter immediately before the anesthetic induction, during operation and at the end of operation. Samples were withdrawn from central vein into heparinized tubes and immediately stored on ice at 4°C. The plasma was then separated from the cells by centrifugation at 3000 rpm for 10 min, and the plasma samples were stored at −80°C until analysis.

2.4. Biochemical Parameters' Measurement of TAC. The TAC of the plasma was measured using a novel automated colorimetric measurement method developed by Erel [7]. Accurate measurements of TAC can be obtained in as little as 10 min, making this assay eminently suitable for the clinical biochemistry laboratory [9].

2.5. Statistical Analysis. The data analyzed by repeated measurements' variance analysis, Mann Whitney *U* test, and ROC analysis using SPSS 11.0 and SigmaPlot 8.0 programs [6].

3. Results

The patient demographics are listed in Table 1. Table 2 compares the operative data of patients who developed or not LCOS. Complete revascularization was accomplished in 74 (93.67%) patients. The left anterior descending artery (LAD) territory was revascularized in 98.73% of the patients with LAD disease. Patients who developed LCOS had longer cardiopulmonary bypass time, longer aortic cross-clamp time, longer postoperative intensive care unit stay, increased ventilatory support, and longer hospital stay. TAC was decreased during operation in all patients ($P < 0.001$). It is shown on Table 3 and Figure 1. However, in patients who developed LCOS, TAC presented a sharp initial fall during

Table 2: Operative data of the patients.

	Non-LCOS ($n = 71$)	LCOS ($n = 8$)	P
Age (yr)	61.3 ± 9.6	63.6 ± 9.6	0.51
Grafts	2.03 ± 0.7	1.93 ± 0.7	0.51
Pump time (min)	85 ± 24	110 ± 39	0.27
Crossclamp time (min)	60 ± 18	67 ± 27	0.83
Days in hospital	7 ± 2	13.8 ± 8.4	0.27
Perioperative myocardial infarction	0	2 (25%)	
Mortality	0	1 (12.5%)	

Table 3: TAC differences in non-LCOS and LCOS patients.

TAC	BO	DO	EO	P
Non-LCOS	1.84 ± 0.07	1.66 ± 0.14	1.61 ± 0.08	0.001
LCOS	1.82 ± 0.11	1.52 ± 0.10	1.30 ± 0.16	0.002

Values are mean ± SE.
Abbreviations: BO: before the operation; DO: during the operation; EO: at the end of the operation.

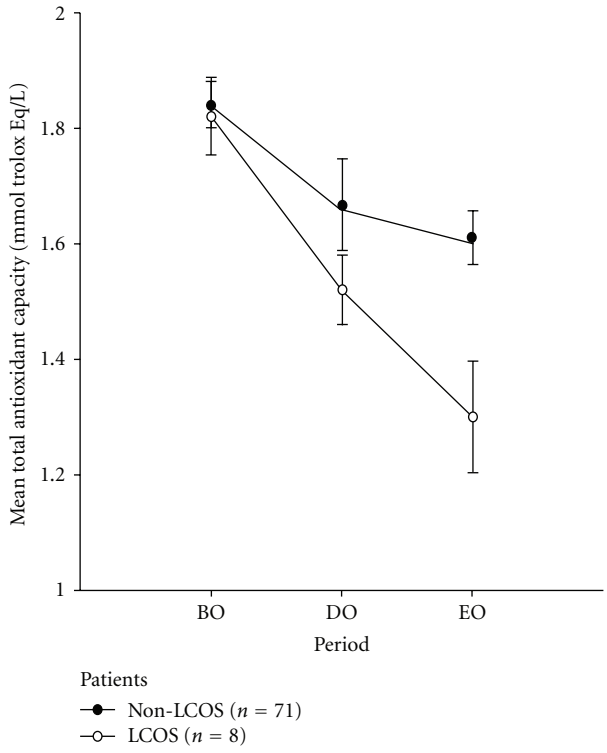

Figure 1: Time changes of TAC during coronary artery bypass surgery. All values showed statistically significant difference from baseline values ($P < 0.05$). The values are mean ± standard error. Abbreviations: BO: before the operation; DO: during the operation; EO: the end of the operation.

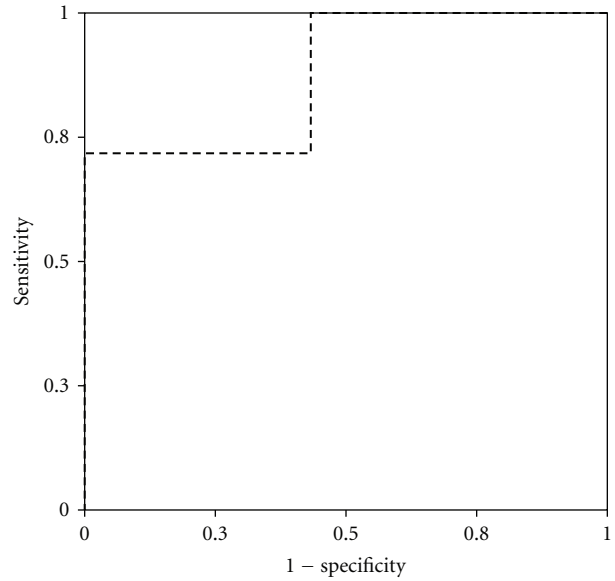

Figure 2: ROC curve of the total antioxidant capacity values obtained from at the end of the surgery.

operation ($P < 0.001$). The area of ROC curve was 0.879 as seen on Figure 2, [7].

4. Discussion

We found that the patients who are undergoing to CABG are exposed to potent oxidative stress. This situation is closely connected with overproduction of reactive oxygen species (ROS). ROS is produced during ischemia/reperfusion process and systemic inflammatory response, associated with cardiac surgery performed with CPB [10, 11]. Preoperative high risk, such as low ejection fraction, recent MI, and renal insufficiency, may lead to inadequate ventricular function in the early postoperative period in patients who developed LCOS undergoing CABG.

In this study LCOS developed in 8 patients (10.12%). The change of TAC was statistically significant in these patients compared with those who did not develop postoperative LCOS. Although the exact mechanisms and interactions among various antioxidants are not fully understood, it is possible that one antioxidant may equilibrate with another to

establish a cellular redox potential, and thus all endogenous antioxidants may act in concert to protect against oxidative insult [1]. Several studies have proposed the essential role of ROS in the pathogenesis of myocardial ischemia-reperfusion injury [10, 11]. In ischemic-reperfused hearts, many alterations such as depression in contractile function, arrhythmias, and loss of adrenergic pathways have been observed [12].

The relationship between contractile function and myocardial metabolism is not clear. Thus, alterations in the myocardium during ischemia-reperfusion were suggested to be in part due to oxidative stress. Ischaemia causes alterations in the defence mechanisms against oxygen free radicals. At the same time, production of oxygen free radicals increases. There is evidence of oxidative stress during surgical reperfusion of the whole heart or after thrombolysis, and it is related to transient left ventricular dysfunction or stunning [13]. Our findings in this study support that in other authors' observations, TAC was decreased during 8 operations in all patients ($P < 0.001$) (Figure 1). However, in patients who developed LCOS, TAC presented a sharp fall during and at the end of the operation ($P < 0.001$) (Figure 1).

Various methods of measurement have been developed for total antioxidant status, but there is no accepted reference method yet [7]. In this study, the total antioxidant status of the plasma was measured using a novel automated colorimetric measurement method for the TAC developed by Erel in patients undergoing CABG [6]. We report here that the novel assay has several major advantages over the other techniques currently available. It is rapid (10 min), simple and inexpensive, and easily fully automated. It is also reliable and sensitive and not subject to interference commonly occurring serum components such as bilirubin, serum lipids, and anticoagulants such as heparin or oxalate [6].

In conclusion, during the coronary bypass surgery, oxidants increase, antioxidants decrease, and oxidative stress develops. This is more prominent and more serious in those who developed LCOS. We think that this difference may be related or may be seconder to the etiopathogenesis of the LCOS. In the coronary bypass surgery, we think that antioxidant vitamin supplementation such as vitamin C and E may be beneficial for these patients. According to our ROC analysis, results of the TAC measurement during the CABG have indicative potential in LCOS development [9].

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Research Article

Simvastatin Attenuates Contrast-Induced Nephropathy through Modulation of Oxidative Stress, Proinflammatory Myeloperoxidase, and Nitric Oxide

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Contrast media- (CM-) induced nephropathy is a serious complication of radiodiagnostic procedures. Available data suggests that the development of prophylaxis strategies is limited by poor understanding of pathophysiology of CM-induced nephropathy. Present study was designed to determine the role of oxidative stress, myeloperoxidase, and nitric oxide in the pathogenesis of iohexol model of nephropathy and its modification with simvastatin (SSTN). Adult Sprague Dawley rats were divided into seven groups. After 24 h of water deprivation, all the rats except in control and SSTN-only groups were injected (10 ml/kg) with 25% glycerol. After 30 min, SSTN (15, 30, and 60 mg/kg) was administered orally, daily for 4 days. Twenty-four hours after the glycerol injection, iohexol was infused (8 ml/kg) through femoral vein over a period of 2 min. All the animals were sacrificed on day 5 and blood and kidneys were collected for biochemical and histological studies. The results showed that SSTN dose dependently attenuated CM-induced rise of creatinine, urea, and structural abnormalities suggesting its nephroprotective effect. A significant increase in oxidative stress (increased lipid hydroperoxides and reduced glutathione levels) and myeloperoxidase (MPO) and decreased nitric oxide in CM group were reversed by SSTN. These findings support the use of SSTN to combat CM-induced nephrotoxicity.

1. Introduction

Contrast media (CM) are frequently used for various diagnostic procedures including X-rays, computed tomography and magnetic resonance imaging. CM-induced nephropathy is a leading cause of acute kidney injury and is associated with significant mortality and morbidity [1, 2]. The mechanism of CM-induced renal impairment at cellular level is not fully understood; recent reports suggested both direct and indirect effects on renal tubules including biochemical and hemodynamic disturbance due to CM-induced prerenal dehydration, hypotension, and medullary ischemia [3, 4]. Jost et al. [5] also reported that contrast media cause impairment of renal perfusion leading to hypoxic conditions resulting in acute tubular necrosis. It has been reported that patients with diabetes and preexisting renal insufficiency may be at a higher risk of CM-induced nephrotoxicity compared to patients with normal renal function [6].

Several experimental and clinical studies suggested beneficial effects of statins against CM-induced nephrotoxicity [7, 8]; however, this claim has been refuted by some of the recent clinical trials [9, 10]. Meta-analysis of available data about the renoprotective effect of statins against CM-induced nephropathy is still conflicting and inconclusive. It has been suggested that the development of highly effective prophylaxis strategies is limited by poor understanding of the pathophysiology of CM-induced renal toxicity. In this paper, attempt has been made to study the role of oxidative

stress, proinflammatory enzyme myeloperoxidase (MPO), and vasoregulatory nitric oxide in the pathogenesis of CM-induced nephropathy and its modification with simvastatin (SSTN). Iohexol (CM) plus glycerol was administered in rats as a model for CM-induced nephrotoxicity according to the method described by Duan et al. [11]. Pretreatment with glycerol was used to simulate preexisting renal insufficiency in clinical setting; this protocol provides for an optimal nephropathy on exposure of rats to contrast media.

2. Material and Methods

2.1. Radio Contrast Medium and Drugs. Contrast medium, iohexol, was obtained from Amersham Health, Cork, Ireland. Glycerol was purchased from Sigma Chemical Co., St. Louis, USA. Simvastatin was purchased from Calbiochem, CA, USA.

2.2. Animals and Treatment Groups. Adult Sprague Dawley male rats (250–300 g) raised in our animal breeding facility were used in this study. The experimental protocol of this study was approved by Hospital Research and Ethical Committee.The animals were randomly divided into 7 groups (G) of 7 animals each; G1 (Control), G2 (Glycerol only), G3 (Glycerol + CM), G4 (Glycerol + CM + SSTN 15 mg/kg), G5 (Glycerol + CM + SSTN 30 mg/kg), G6 (Glycerol + CM + SSTN 60 mg/kg), and G7 (SSTN 60 mg/kg).

2.3. Study Protocol. The protocol suggested by Duan et al. [11] was followed in this study. After 24 h water deprivation, all rats except the control group (G1) and SSTN only group (G7) were injected intramuscularly with 10 mL/kg body weight of 25% glycerol; drinking water and food were then resumed ad libitum. After 30 minutes of glycerol injection, SSTN was administered orally to the animals in groups G4, G5, G6, and G7; the different doses of SSTN were prepared in a volume of 10 mL/kg and administered by gavage using an intubation needle. SSTN treatment was continued daily for 4 days. Twenty-four hours after the glycerol injection, CM were infused through femoral vein in the dosage of 8 mL/kg of body weight over a period of 2 min to the animals in groups G3, G4, G5, and G6. All the animals were weighed and sacrificed on day 5. Animals were killed under deep ethyl ether anaesthesia. Blood was collected through heart puncture and serum separated for renal function test. The kidneys were excised and weighed immediately; one kidney was stored at −70°C for biochemical analysis and the other kidney was preserved in 10% formalin for histopathological studies as shown in Table 1.

2.4. Renal Function Test. Blood urea nitrogen (BUN), serum creatinine (SCr), and serum calcium levels were measured spectrophotometrically (APEL PD-303S Japan) using the commercial kits from United Diagnostics Industry, Riyadh, Saudi Arabia. These values are expressed as mg/dL (Figure 1).

2.5. Determination of Lipid Peroxidation. The level of lipid peroxidation in the kidney tissue was assessed as thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa et al. [12]. The kidney tissue (0.5 g) was homogenized in 1.15% cold KCl using an Ultraturrax homogenizer (Janke and Kunkel, Staufen, Germany). The homogenate was centrifuged at 3000 g for 5 min and an aliquot of supernatant was mixed with 2 mL of reaction mixture (15% trichloroacetic acid and 0.375% thiobarbituric acid in 0.25 N HC1) and heated for 5 minutes in a boiling water bath. The tubes were cooled at room temperature and centrifuged at 1000 g for 10 minutes. The absorbance of supernatant was read at 535 nm on a UV visible spectrophotometer (UV-160A, Shimadzu, Japan), against a blank that contained all reagents except homogenate. Tissue lipid peroxide levels were calculated as nanomoles of MDA using tetramethoxypropane as standard.

2.6. Determination of Glutathione. The reduced glutathione (GSH) in the kidney tissue was measured enzymatically according to the method of Mangino et al. [13]. The kidney tissue was homogenized with 1 mL of 0.1 M perchloric acid plus 0.005% EDTA in a ratio of 1 : 20 (wt/vol). The homogenate was centrifuged at 4000 rpm for 10 min and the supernatant was used for GSH assay. The reaction mixture consisted of the following freshly prepared solutions: solution 1, 0.3 mM, NADPH (Sigma) and solution 2, 6 mM 5.5′-dithio-bis (2-nitrobenzoic acid) (Sigma), and solution 3, 50 U/mL glutathione reductase (Sigma). All three solutions were prepared with a stock buffer consisting of 125 mM NaH_2P0_4 and 6.3 mM EDTA at pH 7.5. For GSH assay, 800 μL of solution 1, 100 μL of solution 2, and 10 μL of solution-3 were mixed in a quartz cuvette and placed in a UV-VIS spectrophotometer at 30°C. The enzymatic reaction was started by the addition of 100 μL of the sample (the supernatant) and the absorbance was monitored for 3 min at 412 nm on a UV-visible Spectrophotometer (UV-160A, Shimadzu, Japan). The slope of the change in absorbance was used to quantitate GSH by comparing the slope of the samples with a standard curve prepared with pure GSH (Sigma).

2.7. Determination of Myeloperoxidase. The activity of inflammatory marker myeloperoxidase (MPO) in the tissue was measured according to the method of Barone et al. [14]. Preweighed tissue was homogenized (1 : 20 wt/vol) in 5 mM potassium phosphate buffer (pH 6.0) using an Ultraturrax homogenizer. The homogenate was centrifuged at 17000 g for 15 min at 4°C. The supernatant was discarded, and the pellet was extracted with 0.5% hexadecyltrimethylammonium bromide (Sigma) in 50 mM potassium phosphate buffer (pH 6.0). Three freeze-thaw cycles were performed, with sonication (10 s, 25°C) between cycles. After the last sonication, the samples were incubated at 4°C for 2 minutes and centrifuged at 12500 g (15 min, 4°C). MPO in the supernatant was assayed by mixing 0.1 mL of supernatant with 2.9 mL of 50 mM potassium phosphate buffer (pH 6.0) containing 0.167 mg/mL o-dianisidine dihydrochloride

Table 1: Drug treatment protocol.

Groups	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Control (G-1)	WD	Saline	Saline	—	—	Sacrificed
Glycerol (G-2)	WD	Gly +	Saline	—	—	Sacrificed
CM + Gly (G-3)	WD	Gly	CM	—	—	Sacrificed
CM + Gly + SSTN 15 (G-4)	WD	Gly + SSTN	CM + SSTN	SSTN	SSTN	Sacrificed
CM + Gly + SSTN 30 (G-5)	WD	Gly + SSTN	CM + SSTN	SSTN	SSTN	Sacrificed
CM + Gly + SSTN 60 (G-6)	WD	Gly + SSTN	CM + SSTN	SSTN	SSTN	Sacrificed
SSTN 60 (G-7)	WD	SSTN	SSTN	SSTN	SSTN	Sacrificed

Abbreviations: WD (Water deprived), Gly (Glycerol), CM (Contrast Media), and SSTN (Simvastatin).

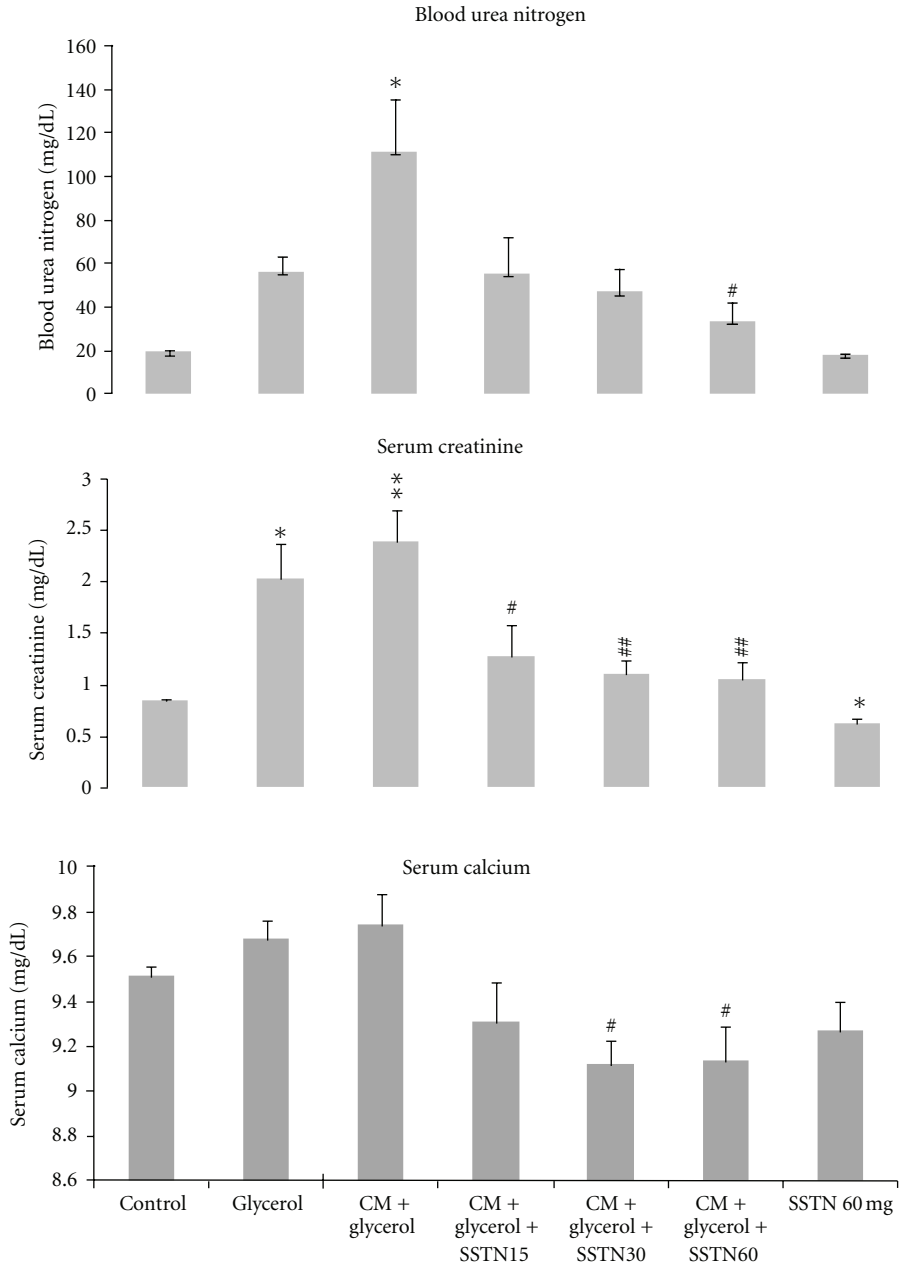


Figure 1: Effect of Simvastatin treatment on blood urea nitrogen, serum creatinine, and calcium in a rat model of contrast media-induced nephropathy. Values are ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with control group, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus glycerol + CM using Tukey's test. CM: contrast media; SSTN: simvastatin, 15, 30, and 60 mg/kg.

(Sigma) and 0.0005% hydrogen peroxide (ICN Pharmaceuticals, Irvine, CA). The change in absorbance at 460 nm was measured for 3 min by using a UV-visible spectrophotometer (UV-160A, Shimadzu, Japan).

2.8. Determination of Nitric Oxide. The levels of nitric oxide (NO) in kidney tissues were determined indirectly using the Griess reaction for simultaneous evaluation of nitrite and nitrate concentrations as described by Moshage et al. [15]. Kidney tissue was homogenized in 0.1 M sodium phosphate buffer pH 7.4 in 1 : 10 (w/v) and centrifuged at 8000 g for 10 minutes. Deproteinization of the sample was carried out by the mixing of 40 μL of 30 g/100 mL zinc sulphate, in 800 μL of sample using vortex and the solution was allowed to stand for 10 min. The tubes were centrifuged at 4000 g for 10 min. For nitrite assay, 500 μL of the clear deproteinized supernatant were transferred into the tubes containing 400 μL distilled water, 500 μL glycine buffer, and 1 mL of Griess reagent (1 g/L sulfanilamide, 25 g/L phosphoric acid, and 0.1 g/L N-1-naphthylethylenediamine). After 15–20 min the absorbance was measured at 545 nm against a blank containing all the reagents except the homogenate sample. For nitrate estimation the same steps were followed but before addition of Griess reagent 2.5–3 g cadmium granules were added in every tube and the samples were kept for 2 hours at room temperature and the absorbance was read at 545 nm on a UV-visible Spectrophotometer (UV-160A, Shimadzu, Japan). Calculation of nitric oxide was based on nitrite and nitrate values.

2.9. Kidney Weight-to-Body Weight Ratio. At the time of sacrifice the body weights and the kidney weights of rats were recorded. The kidney weight body weight ratio was calculated by simple arithmetic calculation of the kidney weight divided by body weight and then converted to percent.

2.10. Histopathological Examination. After recording the kidney weight and morphological examination, the kidney was fixed immediately in 10% formalin, embedded in paraffin, sectioned at 3 μm thickness and the sections were stained with hematoxylin and eosin. The extents of tubular injury, dilatation, vacuolation, and necrosis were evaluated semiquantitatively with a slight modification of the method of Raij et al. [16]. Briefly, the extent of tissue damage was graded from 0–4 according to the severity of tubular necrosis, tubular vacuolation, and tubular dilatation. The scoring system was as follows: 0 = no change in the tubules, 1 $\leq$ 25% of tubular injury (mild), 2 = 25% to 50% of tubular involvement (moderate), 3 = 50% to 75% of tubules showing characteristic change (severe), and 4 $\geq$ 75% of tubular damage (very severe). Fifty fields were counted from each slide. All the assessments were done in a blinded fashion.

2.11. Statistics. The data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test using SPSS (Version 15) statistical package. P values <0.05 were considered as statistically significant.

3. Results

Treatment of rats with SSTN alone did not produce any significant change in serum BUN, SCr, and calcium levels (Figure 1). Administration of Glycerol alone increased the serum BUN levels which were significantly potentiated by CM (ANOVA $F = 3.820$, $P < 0.01$). Treatment with SSTN dose dependently attenuated CM-induced BUN levels (Figure 1). Serum creatinine was significantly increased by glycerol as well as glycerol + CM (ANOVA $F = 7.259$, $P < 0.001$). Treatment with SSTN significantly and dose dependently reduced the SCr levels induced by glycerol + CM and also in SSTN-treated control animals (Figure 1). Treatment of rats with glycerol alone and glycerol plus CM produced a nonsignificant increase in serum calcium. Both the medium (30 mg/kg) and high doses (60 mg/kg) of SSTN were equipotent in reducing serum calcium levels in glycerol + CM treated rats (ANOVA $F = 3.702$, $P < 0.01$) (Figure 1).

Administration of Glycerol with or without CM significantly increased TBARS levels in kidneys of rats which were significantly attenuated by medium and high doses of SSTN (ANOVA $F = 15.239$, $P < 0.001$) (Figure 2). The level of kidney GSH significantly decreased in rats exposed to glycerol or glycerol plus CM. Treatment with medium and high doses of statin significantly and dose dependently reversed the depletion of GSH caused by glycerol and CM (ANOVA $F = 16.223$, $P < 0.001$). Although glycerol alone produced a nonsignificant increase of the activity of MPO in kidneys, combined treatment with glycerol plus CM significantly increased MPO activity (Figure 2) which was significantly reduced by high dose of SSTN (ANOVA $F = 6.380$, $P < 0.001$). A significant reduction in kidney NO level was observed in rats exposed to glycerol alone or in combination with CM. Treatment with medium and high doses of SSTN significantly reversed the effects of Glycerol and CM on kidney NO levels (ANOVA $F = 6.008$, $P < 0.001$).

Administration of glycerol alone or in combination with CM significantly increased kidney weight to body weight ratio (ANOVA $F = 10.228$, $P < 0.001$). Concomitant treatment with SSTN reduced glycerol and CM-induced increase in kidney/body weight ratio; however, the reduction was not significant (Table 2).

3.1. Histological Findings. The light microscopic findings in the kidneys of different treatment groups are given in Table 2. The groups injected with only glycerol produced tubular dilatation and mild tubular vacuolation and necrosis, whereas treatment with glycerol and CM produced significant tubular dilatation, tubular vacuolation, and tubular necrosis. Treatment of rats with SSTN dose dependently reversed CM-induced histological changes (Table 2, Figure 3).

4. Discussions

The findings of this study clearly showed a significant increase in the biomarkers of renal injury including BUN and SCr in the rats exposed to CM plus glycerol (Figure 1). The increase in SCr and BUN following exposure of rats

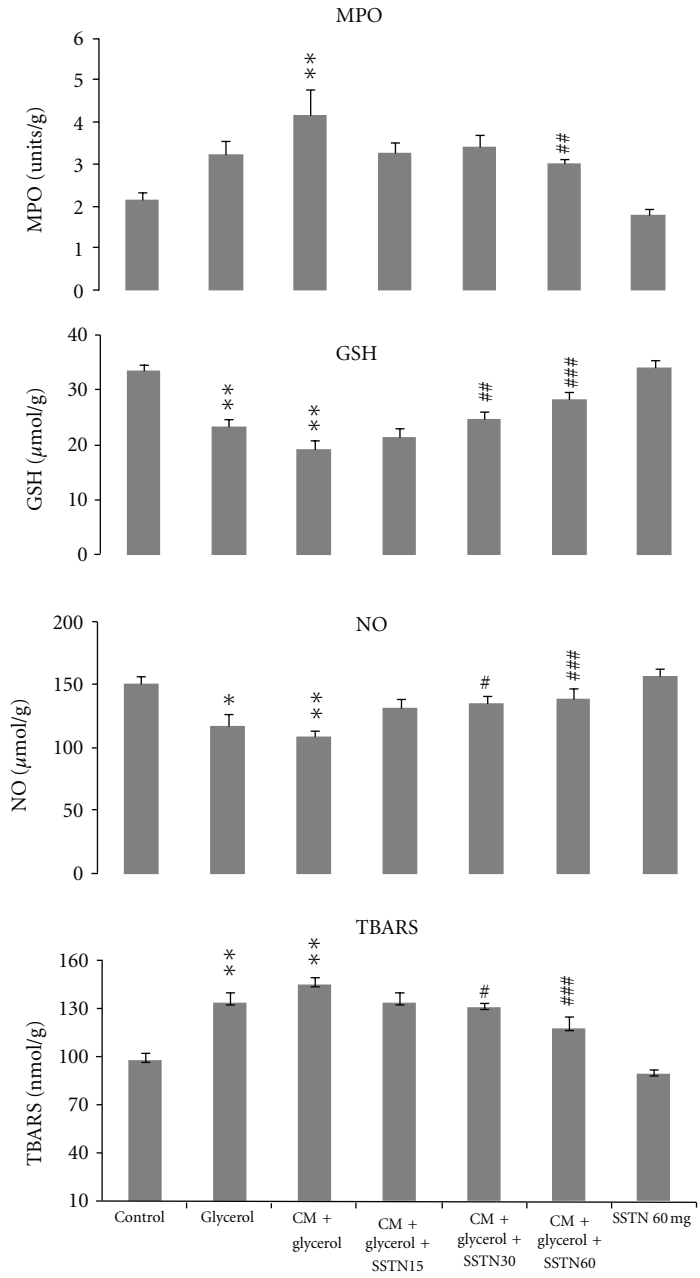
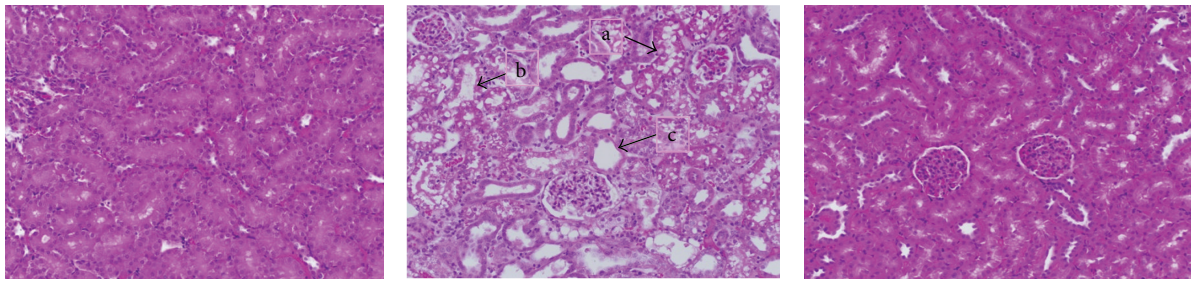


Figure 2: Effect of simvastatin on TBARS, NO, GSH, and MPO in a rat model of contrast media-induced nephropathy. Values are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared to control group and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus glycerol + CM group using Tukey's test. CM: contrast media; SSTN: simvastatin 10, 30, and 60 mg/kg.

to CM and glycerol has been reported earlier [17, 18]. Our histopathological studies also confirm significant tubular vacuolar transformation, interstitial edema, and tubular degeneration in this nephropathy model. Histologic alterations including proximal tubular vacuolar transformation, interstitial edema, and tubular degeneration following contrast administration have also been reported earlier [19]. Development of edema and enlargement of kidney by CM which was evident by significant increase in kidney weight-to-body weight ratio also confirm significant renal toxicity (Figure 3). Pretreatment with SSTN significantly and dose dependently reversed the effects of CM on serum markers of renal injury (Figure 1). Treatment of animals with SSTN has been shown to exert a favorable effect on renal blood flow and glomerular filtration rate by earlier investigators [20], that is evident from a decrease in serum creatinine and blood urea nitrogen levels even in control rats treated with SSTN (Figure 1). There was a nonsignificant increase in the level of serum calcium following exposure to contrast media (Figure 1). On the other hand, treatment with SSTN prevented contrast induced rise in serum calcium. Calcium plays an important role in maintaining cell viability and



(A) (B) (C)

Figure 3: Light microscopy of renal tissue of rats stained with hematoxylin and eosin (×200). (A) Normal histology of kidney tissue. (B) Marked tubular necrosis (a), dilatation (b), and vacuolation (c) in CM model of nephropathy. (C) SSTN-induced reversal of CM-induced nephropathy showing minimal tubular abnormalities.

Table 2: Effect of simvastatin on tubular injury scores in a rat model of CM nephropathy.

Groups	Tubular dilatation	Tubular vacuolation	Tubular necrosis	Kidney wt/Body wt ratio (%)
Control	0	0	0	0.33 ± 0.01
Glycerol	2.7 ± 0.28***	0.6 ± 0.20*	0.70 ± 0.18**	0.58 ± 0.04***
CM + glycerol	3.4 ± 0.32***	2.9 ± 0.29***	3.0 ± 0.26***	0.61 ± 0.04***
CM + glycerol + SSTN 15	2.3 ± 0.28#	1.0 ± 0.01###	0.40 ± 0.20##	0.50 ± 0.03#
CM + glycerol + SSTN 30	1.9 ± 0.22###	0.9 ± 0.01###	0 ± 0###	0.48 ± 0.03#
CM + glycerol + SSTN 60	1.2 ± 0.2###	0.4 ± 0.24###	0	0.47 ± 0.03##
SSTN 60	0	0	0	0.31 ± 0.00

Values are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus glycerol + CM group using Tukey's test.
High scores indicate severe injury.

integrity and alteration of calcium homeostasis may lead to cellular injury through activation of phospholipases, impairment of ion permeability, and their subsequent effect on cytoskeletal structure and function. Calcium channel blocker amlodipine has been shown to attenuate CM-induced nephropathy [18].

The pathogenesis of CM-induced nephropathy is poorly understood; however, numerous pathways have been suggested to participate in the injury process. While some studies support a role for direct action of CM on tubular toxicity, other investigators suggested that the final pathway for contrast-induced renal injury might be due to medullary hypoxia conditioned by medullary ischemia (due to volume depletion and prostaglandin inhibition) affecting metabolically active and hyperfiltering nephrons. CM infusion induces renal vasoconstriction [3], a process that is thought to arise from an imbalance between endothelium-derived vasoconstrictive and vasodilatory factors [21, 22]. Depending on the severity of CM induced vasoconstriction, ischemic tubular injury may result in cell detachment, apoptosis, or necrosis [23, 24]. The ischemic insult is often accompanied by reperfusion injury involving excessive generation of potentially toxic-free radicals and inflammatory mediators [24].

We observed a significant increase in TBARS and decrease in GSH in kidneys of rats exposed to CM, indicating a state of oxidative stress (Figure 2). Treatment of rats with SSTN reversed CM-induced oxidative stress. A significant increase in lipid peroxides has been observed in kidneys of rats treated with CM [11]. Earlier studies also suggested that oxidative stress plays an important role in CM-induced nephrotoxicity [25–27]. The antioxidant, N-acetylcysteine (NAC), has been used clinically for prevention of iothalamate meglumine contrast-induced acute renal failure [28–32]. The protective effect of SSTN against CM can be attributed to its antioxidant property.

There was a significant increase in the proinflammatory enzyme MPO levels in the kidneys of CM-treated rats; this rise in MPO levels was attenuated by SSTN (Figure 2). Increased MPO concentration in the kidney is an indicator of neutrophil infiltration and it is directly proportional to the tissue inflammation caused by CM. MPO activation has been shown to cause glomerular morphologic changes, endothelial and mesangial cell injury, and activation of platelets as well as glomerulonephritis [33]. Higher activity of MPO has been reported in primed macrophages under inflammatory conditions in both patients with chronic kidney disease and experimental studies [34]. The protective effect of SSTN against CM-induced increase in MPO levels can be attributed to its anti-inflammatory property [35]. It has been reported that SSTN may prevent loss of kidney function to a greater extent in individuals with evidence of increased inflammation than in those who show no inflammation [36].

Biochemical analysis of kidney showed a significant decrease in NO levels in our contrast media model of

renal toxicity (Figure 2). A significant decrease in kidney NO following exposure of rats to iodixanol has also been reported by Sendeski et al. [37]. Decreased eNOS expression and increased plasma endothelin-1 have been implicated in nonionic CM-induced endothelial dysfunction and kidney injury [17]. Aggravation of CM-induced nephrotoxicity by hypercholesterolemia has been associated with the reduced production of NO [38]. Administration of L-arginine (NO donor) significantly attenuated hypercholesterolemia-induced aggravation of CM nephrotoxicity in rats [39]. Statins exert a positive influence on NO resulting in improved microcirculation, reduction in platelet aggregation, and enhancing cell proliferation [40–43]. Preservation of renal function by simvastatin in cyclosporine-induced ischemic/reperfusion injury has also been attributed to its vasodilator properties mediated by NO [44]. In conclusion SSTN offers significant nephroprotection against CM-induced nephropathies by inhibiting oxidative stress and inflammation and by improving renal microcirculation. Further clinical trials by using higher doses of statins alone or in combination with other nephroprotective agents should be tested as prophylactic agents to combat contrast-induced nephropathy.

5. Conclusion

In conclusion, the results of this study suggest a significant role of oxidative stress, proinflammatory myeloperoxidase and vasoregulatory nitric oxide in the pathogenesis of contrast-induced nephropathy. Treatment with high dose of SSTN prevents contrast-induced biochemical and structural changes in kidney. Therefore, further studies are warranted to test high doses of SSTN alone or in combination with other antioxidants and/or anti-inflammatory drugs to prevent CM-induced nephropathy.

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