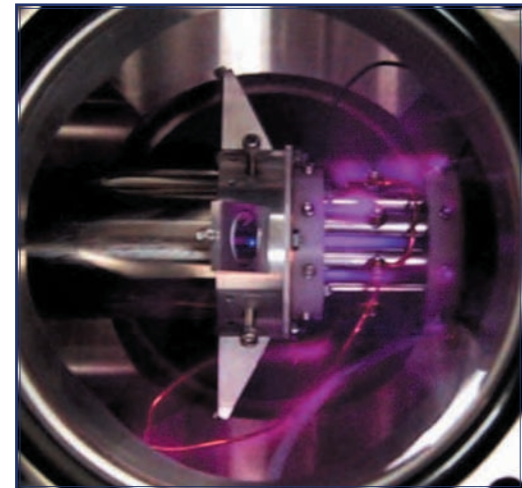
CARDIOVASCULAR PROTEOMICS

Oxidative Post-Translational Modifications of Proteins in Cardiovascular Disease



October 3 - 6, 2006

Boston University School of Medicine

Co-Directors

Catherine E. Costello, PhD
Professor of Biochemistry
Director, Center for Biological Mass Spectrometry

Richard A. Cohen, MD
Professor of Medicine
Director, Vascular Biology Unit

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Welcome Message

Welcome to Boston University and to the second meeting on "Oxidative Post-Translational Modifications of Proteins in Cardiovascular Disease." Organization of this meeting has been undertaken as an activity of our four-year-old BUSM-NHLBI Cardiovascular Proteomics Center whose focus is the same as this meeting. We and our colleagues here at Boston University School of Medicine have long and special interests in the physiological and pathological roles of reactive oxygen and nitrogen species in the cardiovascular system, and in the development and application of sophisticated analytical methods for structural studies of biopolymers. Many of us are convinced that oxidative modifications of proteins represent important molecular mechanisms for physiological regulation and pathological dysfunction of cells and tissues in the cardiovascular system. Furthermore, detecting oxidant-mediated modifications of key proteins in the cardiovascular system may enable mechanism-based diagnosis of disease.

The subject of this meeting represents an exciting, cutting edge field that demands understanding and integration of concepts of physiology, pathology, biochemistry, and cell biology, as well as mastery of technologies of protein separation and mass spectrometry. A major goal of this meeting is to foster the integration of these disciplines. A second goal is to broaden the understanding of this field amongst its participants. A third is to entice young scientists into the field. To these ends we have added a pre-conference methodologies workshop to the program. We are pleased that so many of you chose to attend the first meeting and have come back for the second; we also extend a warm welcome to new faces. We hope that the stimulation for research and enthusiasm for interaction will continue to grow between now and our next meeting in 2008.

Please let us know if we can make your visit more pleasant or more fruitful — and do suggest what we can all do together to make the next meeting even more stimulating and productive!

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Professor of Biochemistry
Director, Center for Biological Mass Spectrometry

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Professor of Medicine
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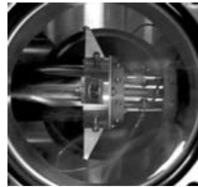
Acknowledgements

The "Oxidative Post-Translational Modifications of Proteins in Cardiovascular Disease" symposium is sponsored by the:

NHLBI Proteomics Initiative



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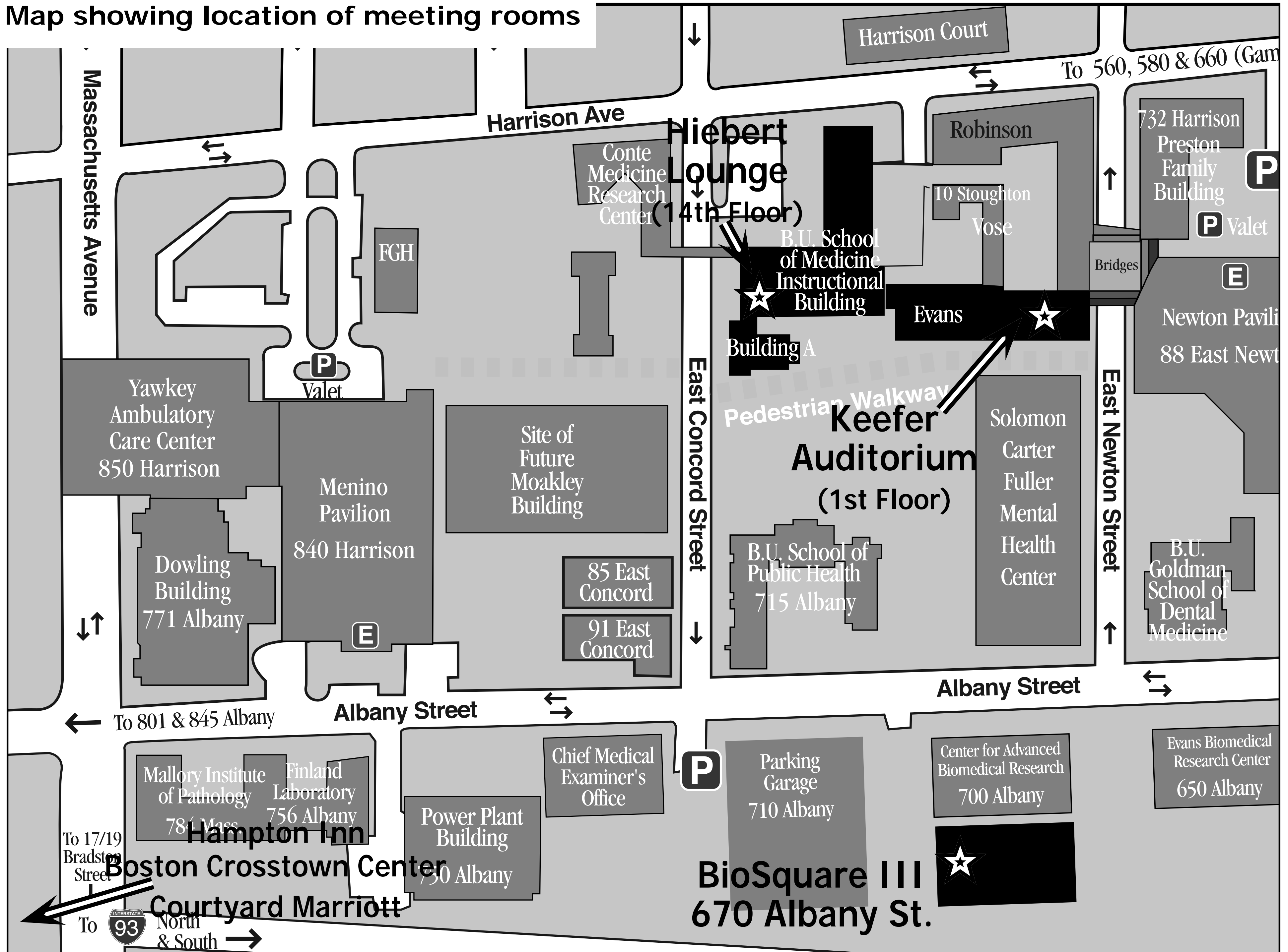
Scientific Advisory Committee

"Oxidative Post-Translational Modifications of Proteins in Cardiovascular Disease"

Symposium October 3-6, 2006

1. Steven Barnes, Ph.D., Professor, Pharmacology/Toxicology, University of Alabama at Birmingham
2. Joseph S. Beckman, M.D., Professor, Linus Pauling Institute, Department of Biochemistry and Biophysics, Oregon State University
3. Bradford C. Berk, M.D., Ph.D., Chairman and Director, Center for Cardiovascular Research, University of Rochester School of Medicine
4. Aruni Bhatnagar, Ph.D., Professor, Medicine-Cardiology, University of Louisville
5. Richard A. Cohen, M.D., Professor of Medicine and Director, Vascular Biology Unit, Boston University School of Medicine
6. Catherine E. Costello, Ph.D., Professor of Biochemistry and Director, Center for Biological Mass Spectrometry, Boston University School of Medicine
7. Victor Darley-Usmar, Ph.D., Professor, Molecular and Cellular Pathology, University of Alabama at Birmingham
8. Philip Eaton, Ph.D., Department of Cardiology, King's College, London
9. Martin Feelisch, Ph.D., Professor, Whitaker Cardiovascular Institute, Boston University School of Medicine
10. Gerald W. Hart, Ph.D., Professor and Chairman, Department of Biological Chemistry, Johns Hopkins University
11. Stanley L. Hazen, M.D., Ph.D., Director, Center for Cardiovascular Diagnostics, Cleveland Clinic Foundation, Case Western Reserve University
12. Jay W. Heinecke, M.D., Professor, Metabolism, University of Washington
13. Harry Ischiropoulos, Ph.D., Assistant Professor, Abramson Research Center, Pediatrics/Neonatology, Children's Hospital of Philadelphia
14. Joseph Loscalzo, M.D., Ph.D., Hersey Professor of the Theory and Practice of Medicine and Chairman, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School
15. Mark E. McComb, Ph.D., Research Assistant Professor of Medicine, Cardiovascular Proteomics Center, Boston University School of Medicine
16. Peter B. O'Connor, Ph.D., Research Associate Professor of Biochemistry, Center for Biological Mass Spectrometry, Boston University School of Medicine
17. Maria J.T. Saraiva, Ph.D., Professor, Amyloid Unit, Institute for Molecular and Cell Biology, University of Porto
18. Christian Schoneich, Ph.D., Professor, Pharmaceutical Chemistry, University of Kansas
19. Roland Stocker, Ph.D., Senior Principal Research Fellow, Centre for Vascular Research, School of Medical Sciences, University of New South Wales
20. Jennifer E. Van Eyk, Ph.D., Associate Professor, Department of Medicine/Cardiovascular and Director, JHU NHLBI Proteomic Group, Johns Hopkins University

Map showing location of meeting rooms



Meeting Information

Meeting Venues and Schedule

The conference plenary sessions take place in the auditorium of Boston University's new BioSquare III building, 670 Albany Street. The auditorium is on the ground floor just past the security desk, on the left. The conference poster session takes place in the Hiebert Lounge, located within the School of Medicine main complex, 715 Albany Street (14th floor, Instructional Building), across the street from the plenary sessions. Conference registration is located in the inner lobby of the auditorium, where attendees can pick up the Abstract Book and conference information. These areas are starred on the map on the previous pages.

The plenary session oral presentations are scheduled as 30-minute keynote talks followed by 10 minutes of discussion, 20-minute talks followed by 10-minute discussions, or 15-minute poster-related presentations followed by 5 minutes of discussion. Additional general discussion periods are scheduled at the end of each oral session.

Speakers and session chairs should come to the auditorium at least thirty minutes before the start of their session to meet with the audiovisual technician staffing the session. Speakers are requested to send their presentations in advance (to klevy@bu.edu) or bring them (on a flash drive or CD) to the technician early enough to allow time for verifying that the files will project properly. Files should be saved in PC-compatible format.

Lunch and social hour refreshments will be provided on Wednesday and Thursday in Hiebert Lounge.

The poster session begins at noon on Wednesday in the Hiebert Lounge. Posters are open for viewing throughout the meeting. Posters are arranged in numerical order, according to the poster numbers on page 19. During the luncheons on Wednesday and Thursday, discussion time with poster authors is included in the program. Corporate display booths are set up in Hiebert Lounge, near the posters. Tours of the Cardiovascular Proteomics Center and the Mass Spectrometry Resource are available during the lunch hour.

The conference adjourns Friday after Grand Rounds (in Keefer Auditorium in the Evans building, signs will be posted).

Abstracts

Abstracts of the oral and poster presentations are grouped separately in the pages following the Scientific Program, one abstract to a page. Abstracts of oral presentations are organized according to the order of their presentation in the Scientific Program; following these, the abstracts of poster presentations are placed in alphabetical order according to the first author's last name. Entries in the Author Index list the authors' names and the abstract page numbers.

Poster Presentations

Posters will be on display in the Hiebert Lounge, located on the 14th floor of the School of Medicine's main building, on Wednesday and Thursday. To avoid conflicts with student examinations, poster authors are requested to mount their posters on Tuesday afternoon or at the start of the poster session. Posters may be taken down Thursday evening or Friday between 12:00 and 2:30 p.m. Authors of the short orals are considered part of the poster session and are expected to present their data as a poster, in addition to delivering their talk.

Conference Dinner

The Conference Dinner will be held on the evening of Wednesday, October 4th, at 6:30 pm at a local favorite spot, Amrhein's, 80 W. Broadway, South Boston. Tickets can be purchased on the morning of October 4th at the registration desk in the 670 Albany Street auditorium inner lobby. The dinner ticket, depending upon menu choice of three items, costs $30 or $36.50. We hope all meeting attendees will participate in the dinner as it promises to be a very good time to get acquainted during a lovely time of year in Boston! Maps and directions to the restaurant may be picked up at the OPTM registration desk. Ample parking is available. The room reserved for the dinner will be open from 6:00 pm to accommodate guests who wish to arrive early and socialize prior to the meal.

Hotel Shuttle

The two hotels, Hampton Inn Boston Crosstown Center, and Courtyard Marriott, have hourly shuttle service to and from Logan International Airport. Shuttle information and schedules are available at the hotel reception desks and the OPTM conference registration desk.

Departing from Hampton Inn, there will be two shuttles, at 7:30 am and 7:45 am. Return trips on Wednesday and Thursday will be from the same drop-off point at 5:30 p.m., as needed.

Departing from Courtyard Marriott, OPTM guests can use the hotel shuttle which departs for the airport on the hour and comes by the medical center about 10 minutes later. Let the hotel know that you need transportation to 670 Albany St. You probably will want to take the 8:00 am shuttle which will pass by at 8:10 am approximately. To take the shuttle back to the hotel from the conference, first speak to the hotel to schedule a stop since the shuttle does not stop at the medical center automatically on the return. When ready to return to the hotel, call the hotel telephone number and let them know where you are. En route to the hotel, the shuttle reaches the medical center approx 15 minutes before the hour.

Support

OPTM is sponsored by the NHLBI Proteomics Initiative, Cardiovascular Proteomics Center, Boston University School of Medicine, and two NIH institutes, the National Heart Lung and Blood Institute, and the National Institute of Diabetes and Digestive and Kidney Diseases. Corporate supporters include Wyeth Research, Applied Biosystems/MDS SCIEX, PerkinElmer Life and Analytical Sciences, the Nest Group, and Waters Corp.

Scientific Program

Tuesday afternoon
METHODOLOGIES WORKSHOP
Session 1, Wednesday morning
LIPIDS
Session 2, Wednesday afternoon
OXIDATION AND SIGNALING
Session 3, Thursday morning
MASS SPECTROMETRY AND PROTEOMICS
Session 4, Thursday afternoon
TYROSINE NITRATION
Session 5, Friday morning
THIOLS
Session 6, Friday afternoon
GRAND ROUNDS

METHODOLOGIES WORKSHOP
Tuesday, October 3, 2006, 1-5 pm

Pre-conference proteomics methodologies workshop (held at 670 Albany St. in conference rooms on the first floor adjacent to the auditorium): The curriculum covers the complex of mass spectral technologies that the Cardiovascular Proteomics Center staff are utilizing to create an integrated overall proteomics approach. The workshop is two-thirds lecture and one-third wet lab. Attendance is limited in order to ensure the appropriate environment. Some of the technologies that will be covered include:

- New Proteome Lab Protein Fraction 2-Dimension (PF2D) protein fractionation methods to analyze protein abundance and detect protein modifications;
- Capillary Liquid Chromatography MS and MS/MS to automate sequencing, variant identification, and identification of post-translational modifications;
- Matrix-Assisted Laser Desorption/Ionization Time-of-Flight MS, for peptide mass mapping;
- MALDI FT MS, for more accurate peptide mass measurement;
- Electrospray Ionization Fourier Transform (FT) Mass Spectrometry (MS), for mass measurement of intact protein samples , top-down sequencing and peptide mapping;
- Thiol modifications and thiol labeling;
- Use of MALDI and ESI hybrid FTMS instruments constructed in-house within Boston University School of Medicine’s Mass Spectrometry Resource, an NIH-NCRR Research Resource. The present BUSM instrumentation and the new cryoFTMS now under construction at the CPC will be described in the tutorial, as well as the use of commercial instruments.

Lunch will be served for workshop participants prior to the session, starting at 12:15 pm.

Wednesday, October 4, 2006

7:30 am **Registration (inner lobby of Auditorium)**

Session 1: LIPIDS, Bruce A. Freeman and Jennifer E. van Eyk, chairs (Auditorium)

8:30 Keynote presentation, "Nitric Oxide Regulation of Lipid Signaling," Bruce A. Freeman, Ph.D., Professor and Chair of Pharmacology, University of Pittsburgh School of Medicine

9:10 Batthyány Carlos, Schopfer Francisco, Baker Paul R, and Freeman Bruce A. Protein-Nitroalkylation Mediates the Anti-Inflammatory Properties of Nitrated Fatty Acids.

9:40 Gao Xuan, Jayaraman Shobini, and Gursky Olga. Effect of Oxidation on the Structure and Stability of High-Density Lipoprotein.

10:00 Trostchansky Andrés, Souza José M, Ferreira Ana, Ferrari Mariana, Blanco Fabiana, Baker Paul R, O´Donnell Valerie B, and Rubbo Homero. Chemical and Biological Characterization of Nitroarachidonate: Isomer Distribution and Modulation of Inflammation.

10:20 Hill Bradford G, Srivastava Sanjay, Feldman Andrew, and Bhatnagar Aruni. Oxidative Protein Modification by Lipid-Derived Aldehydes, such as 4-Hydroxy-Trans-2-Nonenal (HNE), Promote ER Stress Responses.

10:40 Eliuk Shannon M, Renfrow Matthew B, Kirk Marion C, Barnes Stephen, Kim Helen. Inactivation of Creatine Kinase by 4-Hydroxy-2-Nonenal and Identification of 4HNE-Modified Residues by FT-ICR MS.

11:00 Shonsey Erin M, Kirk Marion, Renfrow Matthew B, and Barnes Stephen. Detection of Modifications of Human Bile Acid COA: Amino Acid N-Acyltransferase By 4HNE Using LTQ-FTMS Analysis.

11:20 Discussion

12 - 1:45 pm Luncheon and Poster Session with Authors (Hiebert Lounge)

Tours of Cardiovascular Proteomics Center and Mass Spectrometry Resource

Session 2: OXIDATION AND SIGNALING, Richard A. Cohen and Stephen Barnes, chairs (Auditorium)

2:00 Handy Diane E, Yang Yi, Lubos Edith, Galbraith John D, Leopold Jane A, Loscalzo Joseph. Glutathione Peroxidase-1 Over-Expression Alters Receptor-Mediated Signaling by Regulating Intracellular Levels of Reactive Oxygen Species.

2:30 El-Remessy Azza B and Caldwell Ruth B. Dual Role of Peroxynitrite in Mediating VEGF Signaling: Oxidation Versus Nitration.

3:00 Deng Haiteng. Nitrite Anions Induce Protein and Peptide Modifications.

3:30 Snook Jeremy H, Evangelista Alicia, Guilford William H. Effects of Peroxynitrite on Cardiac Myofibrillar Protein Mechanics.

4:00 Ying Jia, Tong Xiaoyong, Pimental David R, Weisbrod Robert M, Adachi Takeshi, and Cohen Richard A. SERCA Cys-674 Is Required for the Inhibition by Nitric Oxide of Cell Migration.

4:20 Pinzar Elena, Communi David, Csibi Alfredo, Urrecheaga Daniela, Garrido Maria, and Bottari Serge P. Tyrosine Nitration of MEK and ERK Induces Their Autophosphorylation and Activation *In Vivo* and *In Vitro*.

4:50 Close

5:20 Social Hour with poster viewing, refreshments (Hiebert Lounge)

6:30 Conference Dinner at Amrhein's Restaurant, South Boston

Thursday, October 5, 2006

8:00 am **Registration (inner lobby of Auditorium)**

Session 3: MASS SPECTROMETRY AND PROTEOMICS, Bradford W. Gibson and Catherine E. Costello, chairs (Auditorium)

8:30 Keynote presentation, "Aging, Oxidative Stress and Your Brain," Bradford W. Gibson, Ph.D., Professor and Director of Chemistry, Buck Institute for Age Research

9:10 Bigelow Diana J, Knyushko Tatyana V, Kowalska Malgorzata, Sacksteder Colette. Endogenous Nitroproteomes of Mouse Heart and Skeletal Muscle: Distinct Signatures of Their Nitrative Environment.

9:40 McComb Mark E, Dauly Claire, Odhiambo Adam, Perlman David H, Huang Hua, Steinberg Martin H, Farber Harrison W, Klings Elizabeth S, and Costello Catherine E. Comparative Proteomics: Post-Translational Modifications in Sickle Cell Disease.

10:10 Cournoyer Jason J, Lin Cheng, Zhao Cheng, O'Connor Peter B. Deamidation and Isoaspartic Acid Formation in Proteins.

10:30 Hesketh Geoffrey G, Shah Manish, Guo Yurong, Tomaselli Gordon F, Van Eyk Jennifer E. Identification of Novel Phosphorylation Sites Within the Carboxyl-Terminus of Connexin 43 Immunoprecipitated from Canine Heart.

10:50 Perlman David H, Bauer Selena, Bryan Nathan S, Garcia-Saura Maria F, Fernandez Bernadette O, McComb Mark E, Costello Catherine E, Feelisch Martin. Proteomic and Metabonomic Analyses of the Cardioprotective Effects of Nitrite Administration in the Heart.

11:10 Greco Todd M, Hodara Roberto, Parastatidis Ioannis, Heijnen Harry FG, Dennehy Michelle K, Liebler Daniel C, and Ischiropoulos Harry. Site-Specific Mapping of the *S*-Nitrosocysteine Proteome in Human Vascular Smooth Muscle Cells.

11:30 Discussion

12 - 2:00 pm Luncheon and Poster Session with Authors (Hiebert Lounge)

Tours of Cardiovascular Proteomics Center and Mass Spectrometry Resource

Session 4: TYROSINE NITRATION, Joseph S. Beckman and Harry Ischiropoulos, chairs (Auditorium)

2:15 Przybylski Michael, Petre Alina, Dragusanu Mihaela, Weber Reinhold, Bachschmid Markus, Ullrich Volker, Ulrich Martina, and Doering Gerd. Mass Spectrometric Identification and Structural Characterization of Tyrosine Nitration in Cellular Proteins.

2:45 Knyushko Tatyana V, Londono Monica P, Xiong Yijia, Stenoien David L, Sacksteder Colette, and Bigelow Diana J. Robust Mechanisms in the Heart for Repair and Degradation of Nitrated Proteins.

3:15 Schöneich Christian, Gokulrangan Giridhan, Kanski Jarowslaw, Li Xiaobao, Pennington Justin, Killmer Jaque, and Stobaugh John. Proteomic Analysis of Age-Dependent Protein Tyrosine Nitration: A Novel ICAT Method Selective for 3-Nitrotyrosine.

3:45 Lourette Natacha M, Smallwood Heather S, Boschek Curt B, Smith Richard D, Squier Thomas C, and Paša-Tolic Ljiljana. Dynamics of Site-Specific Calmodulin Nitration and Oxidation Determined Using LC-FTICR MS.

4:05 Koeck Thomas, Stuehr Dennis, and Aulak Kulwant S. Protein Tyrosine Nitration/Denitration--An Oxygen Regulated Process.

4:25 Petre Alina, Weber Reinhold, Ulrich Martina, Döring Gerd, and Przybylski Michael. Identification and Characterization of Tyrosine Nitration in Human Eosinophils Using FTICR Mass Spectrometry in Combination with Immunoanalytical Procedure.

4:45 Parastatidis Ioannis, Thomson Leonor, Fries Diana, Moore Ryan, Tohyama Junichiro, Hazen Stanley L, Heijnen Harry FG, Dennehy Michelle K, Liebler Daniel C, Rader Daniel, and Ischiropoulos Harry. Proteomic Profiling of Nitrated Proteins in Atherosclerotic Lesions and Plasma Reveals An Anti-Oxidative Role of Apolipoprotein A-I.

5:05 Hong Sung Jung, Gokulrangan Giri, and Schöneich Christian. A Proteomic Approach for the Study of Age Dependent Protein Nitration in Heart.

5:25 Close

5:45 Social Hour with poster viewing, refreshments (Hiebert Lounge)

Friday, October 6, 2006

8:00 am **Registration desk (inner lobby of Auditorium)**

Session 5: THIOLS, Aruni Bhatnagar and Victor Darley-Usmar, chairs (Auditorium)

8:30 Chen C-L, Zweier JL, Chen Y-R. Site-Specific *S*-Glutathiolation of Mitochondrial Complex I.

9:00 Clavreul Nicolas, Adachi Takeshi, Sethuraman Mahadevan, Heibeck Tyler, Ido Yasuo, Pimental David, Kuster Gabriela, Colucci Wilson, Zhao Cheng, O'Connor Peter B, McComb Mark E, Costello Catherine E, Schöneich Christian, and Cohen Richard A. *S*-Glutathiolation of P21ras Initiates Oxidant-Mediated Signaling and Pathology.

9:30 Ullrich Volker and Frein Daniel. *S*-Nitrosation and Redox Regulation.

10:00 Andringa Kelly K, Diers Anne R, Upton Ashley N, Bailey Shannon M. Analysis of Liver Mitochondria Protein Thiol Modifications in Response to Chronic Alcohol and *S*-Adenosylmethionine (SAM) Administration.

10:20 Burgoyne Joseph, Madhani Melanie, Brennan Jonathan P, Cuello Friederike, Eaton Philip. Interprotein Disulfide Bond Formation Activates PKG1α Independently of cGMP.

10:40 Charles Rebecca, May Georgina L, Free Paul, Gaffney Piers RJ, Eaton Philip. Biotinyl Dimedone (BD) -- A Novel Reagent for Studying Protein Sulfenic Acids.

11:00 Zhao Cheng, Sethuraman Mahadevan, Clavreul Nicolas, Kaur Parminder, Cohen Richard A, O'Connor Peter B. A Detailed Map of Oxidative Post-Translational Modifications of Human P21ras Using Fourier Transform Mass Spectrometry.

11:20 General discussion, conference summary and conclusion

12:00 **Session 6: GRAND ROUNDS (Keefer Auditorium)**
OPTM symposium participants join Grand Rounds in the School of Medicine, "Systems Biology and Personalized Medicine," presented by Joseph Loscalzo, M.D., Ph.D., Hersey Professor of the Theory and Practice of Medicine and Chairman, Department of Medicine, Brigham and Women's Hospital, Harvard Medical SchoolPoster Session

Poster Session

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Corporate Booths

1. **PerkinElmer Life and Analytical Sciences, Wellesley, Mass.**
2. **The Nest Group, Southborough, Mass.**
3. **Waters Corp., Milford, Mass.**

OPTM Oral Session Abstracts

Session 1:

LIPIDS

NITRIC OXIDE REGULATION OF LIPID SIGNALING

Freeman Bruce. Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA.

Nitric oxide (NO) and NO-derived reactive species react with fatty acid oxidation intermediates, yielding nitrated products from all major unsaturated fatty acids that exert potent cell signaling actions. Multiple free and esterified nitrated fatty acid species (generically termed LNO_2) are detectable by HPLC-MS in red cell membranes, plasma lipids, urine and other tissue compartments during basal and clinical inflammatory conditions. LNO_2 derivatives modulate cell function via both cGMP-dependent and -independent mechanisms. In the latter regard, LNO_2 derivatives are potent endogenous ligands for peroxisome proliferator activated receptor (PPAR) γ and α, and to a lesser extent, δ. Gene expression array, Northern and Western blot analyses reveal that LNO_2 either enhances or suppresses the expression of metabolic, transcriptional regulatory and inflammatory-related proteins by up to 80-fold. Control studies revealed that neither fatty acid oxygenation products nor NO accounted for this activity of LNO_2. Additional properties of nitrated fatty acids responsible for their potent cell signaling actions include an ability to decay via the Nef reaction to release NO, and a reversible electrophilic reactivity with key target proteins including transcriptional regulatory factors and enzymes. In aggregate, current data indicate that an oxidative inflammatory milieu generates unsaturated fatty acid-nitrating products from oxides of nitrogen yielding a novel class of NO_2-containing unsaturated fatty acid signaling mediators that link fatty acid and NO signaling pathways.

PROTEIN-NITROALKYLATION MEDIATES THE ANTI-INFLAMMATORY PROPERTIES OF NITRATED FATTY ACIDS. Batthyány Carlos; Schopfer Francisco; Baker Paul R. and Freeman Bruce A.

Nitrated fatty acids are potent anti-inflammatory molecules that signal *via* receptor-dependent and independent mechanisms. The olefinic nitro group renders these derivatives electrophilic at the carbon β to the nitro group, thus competent for Michael addition reactions with nucleophilic amino acids residues. **Objective:** Analyze the electrophilic properties of nitrated fatty acids, potential cell targets and their impact in cell signaling events. **Methods and Results:** Nitro-linoleic acid (LNO_2; *m/z* 324.2) and nitro-oleic acid ($OA\text{-}NO_2$; *m/z* 326.2) reacted with GSH (*m/z* 306.1), yielding adducts with *m/z* of 631.3 and 633.3, respectively, as studied by LC-MS. At physiological concentrations, nitrated fatty acids also inhibited glyceraldehyde-3phosphate dehydrogenase (GAPDH, IC_{50} of ~ 3 μM), which contains a critical catalytic Cys (Cys-149). By nanospray LC-MS analysis, 3 Cys- and 3 His-residues were found to be modified, including the catalytic Cys149. GAPDH inhibition and concomitant mass changes were found to be reversible upon treatment with low concentrations of thiol-containing reducing agents. Adduction of GAPDH and GSH by nitroalkenes significantly increased the hydrophobicity inducing translocation to membranes. Electrophilic nitrated fatty acids also induced the nitroalkylation of NF-κB p65 *in vitro* and in macrophages, inhibiting DNA binding activity and repressing NF-κB–dependent target gene expression. **Conclusion:** Nitroalkylation of proteins affect protein subcelluar distribution and functions, revealing a new mechanism by which nitrated fatty acids exerts anti-inflammatory cellular response.

EFFECT OF OXIDATION ON THE STRUCTURE AND STABILITY OF HIGH-DENSITY LIPOPROTEIN

Gao Xuan, Jayaraman Shobini and Gursky Olga

Oxidation of LDL is widely recognized as a key event in atherogenesis, yet the role of HDL oxidation is less well-understood. In contrast to ox-LDL that readily fuse in the arterial wall, ox-HDL remain in circulation and hence may provide useful markers of atherosclerosis. Our goal is to analyze the structure and stability of HDL at various stages of oxidation. Cu^{2+}-mediated oxidation was monitored by UV absorbance for conjugated diene formation. At ambient temperatures, HDL oxidation induced 20% loss of α-helical content monitored by far-UV CD. Trp fluorescence spectra show 9 nm red shift and a large reduction in the emission intensity. SDS gel shows formation of high-molecular-weight aggregates. Importantly, HDL oxidation leads to large changes in the particle stability revealed in thermal denaturation studies. Our recent analysis of intact HDL at near-physiological conditions revealed two kinetic phases in HDL protein unfolding: a fast phase (τ_1 ~ 5 min) involves partial protein unfolding, and a slow phase (τ_2 ~ hours) involves complete protein unfolding, dissociation, and HDL fusion. Interestingly, ox-HDL show only the fast phase. Thus, in contrast to intact HDL, in ox-HDL the proteins do not dissociate even upon prolonged incubation at high temperatures. As a result, ox-HDL undergo no morphologic transitions such as fusion below 100°C, which was indicated by light scattering and negative staining electron microscopy. Consequently, oxidation hampers HDL fusion and apolipoprotein exchange; this may be due to surface protein polymerization or to reduced lipid fluidity. This enhanced stability reinforces the utility of ox-HDL as diagnostic markers of atherosclerosis.

CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF NITROARACHIDONATE: ISOMER DISTRIBUTION AND MODULATION OF INFLAMMATION

Trostchansky Andrés, Souza José M, Ferreira Ana, Ferrari Mariana, Blanco Fabiana, Baker Paul R, O´Donnell Valerie B, and Rubbo Homero

Nitrated lipids have been recently detected in cell membranes and human plasma. In particular, nitration of arachidonic acid (AA) under oxidative/nitrative conditions could redirect AA-dependent cell signaling pathways. Herein, we synthesized and characterized the major isomers of nitroarachidonate ($AANO_2$) and demonstrated its ability to modulate inflammation. Synthesis of mononitrated nitroalkenes was achieved by AA incubation with sodium nitrite at acidic pH following TLC and/or HPLC separation. Mass spectrometry analysis showed the characteristic MS/MS transition of $AANO_2$ (m/z 348/301). Moreover, the IR spectra at 1378.3 cm^{-1} and NMR confirmed the presence of a nitroalkene. The position of the NO_2 group was determined by MS fragmentation with lithium. In fact, nitroalkenes yield aldehydes or oxymes when the nitro group is bounded to the closer or farer carbon with respect of the carboxyl group of the fatty acid. In this way, four different isomers (9-, 12-, 14- and 15-$AANO_2$) having biological activity were identified. $AANO_2$ isomers released nitric oxide

OXIDATIVE PROTEIN MODIFICATION BY LIPID-DERIVED ALDEHYDES, SUCH AS 4-HYDROXY-*TRANS*-2-NONENAL (HNE), PROMOTE ER STRESS RESPONSES

Hill Bradford G, Srivastava Sanjay, Feldman Andrew, and Bhatnagar Aruni.

Objective: The formation of lipid peroxidation products such as HNE is associated with cardiovascular disease, but how such aldehydes contribute to tissue injury or response remains unclear. In this study, we tested the hypothesis that the downstream effects of HNE are educed via covalent protein modification. Methods: The formation and metabolism of HNE-protein adducts in rat aortic smooth muscle cells was assessed by immunoblotting and by measuring protein-bound radioactivity. The proteins modified by HNE were identified by MALDI-TOF/MS analysis. Results: HNE-adducts generated in the cells were removed with half-life of 4-6 h. Treatment of the cells with proteosome/lysosome inhibitors MG132 (10 μM) and clasto-lactacystin (10 μM) delayed adduct removal. Adduct removal was significantly accelerated in the presence of 0.2 μM rapamycin, and partially prevented by insulin. Several peptides corresponding to Grp78, Grp58, and PDI, were identified from 2-D gel analysis of HNE-modified proteins in lysates of HNE-exposed cells. Exposure to HNE activated the PERK/eIF2α pathway; however, CHOP and Grp78 expression were not increased. Conclusions: These studies suggest that aldehyde adducts are metabolized by several protein degradation pathways and that these pathways may be regulated by signaling kinases. Reactive aldehydes such as HNE stimulate the ER stress response, possibly by covalent modification of Grp78.

INACTIVATION OF CREATINE KINASE BY 4-HYDROXY-2-NONENAL AND IDENTIFICATION OF 4HNE-MODIFIED RESIDUES BY FT-ICR MS

Eliuk Shannon M, Renfrow Matthew B, Kirk Marion C, Barnes Stephen, Kim Helen.

Objective: The aims of this study are to determine the functional consequences of 4-hydroxy-2-nonenal (4HNE) adduct formation on creatine kinase (CK) and subsequently identify the 4HNE-modified residues.

Methods: 10 μM CK was incubated with increasing concentrations (10-1000 μM) of 4HNE and assayed for changes in enzymatic activity by use of a coupled spectrophotometric assay. Identification of modified amino acids was performed by use of 7 T LTQ-FT MS. Tryptic and chymotryptic peptides were directly infused by use of a chip-based electrospray ionization source (ESI) (Triversa NanoMate) with gas phase separation. For LTQ-MS and MS/MS analysis, samples were analyzed by a modified data-dependent triple play method.

Results: Incubation of CK with 4HNE resulted in significant reduction of CK activity. The number of 4HNE-modified residues identified by MS analysis decreased with decreasing 4HNE concentration from 15 sites at 300 μM to 5 sites at 100 μM. Even at the lowest concentration analyzed, however, active site modifications were identified. Further MS studies are ongoing with CK modified with lower levels of 4HNE.

Conclusions: Organs with a high energy demand, including heart and brain, depend on CK for rapid ATP generation. Several conditions, including atrial fibrillation, heart failure and Alzheimer's disease, that are correlated with increased oxidative stress have decreased CK function. The data shown here support the hypothesis that oxidative modification could play a role in reduced function of CK.

DETECTION OF MODIFICATIONS OF HUMAN BILE ACID COA:AMINO ACID N-ACYLTRANSFERASE BY 4HNE USING LTQ-FTMS ANALYSIS

Shonsey Erin M, Kirk Marion, Renfrow Matthew, Barnes Stephen

Objective: To determine the effects of modification of hBAT on the activity of the enzyme and to correlate these effects with the sites of modification that can be identified using LTQ-FT fragmentation techniques.

Methods: Recombinant human bile acid CoA:amino acid N-acyltransferase (hBAT) was modified *in vitro* with 4HNE. Following modification, the protein underwent activity analysis, and was then digested with trypsin and/or chymotrypsin and analyzed by a combination of LTQ-FT LC MS methods. First used was the standard data-dependent triple play LC method with a broad FT-ICR scan (200-2000 m/z). They were then analyzed with an ECD LC MS method which included a broad FT-ICR MS scan followed by data-dependent ECD of the top two ions. Results from both methods were analyzed with modified TurboSequest searches within the BioWorks 3.2 browser.

Results: Inactivation was determined to occur in a dose dependent manner with an IC_{50} of 8 microM. Modifications were found at all levels of HNE treatment, ranging from 10 to 3 adducts on different amino acids, including the active site histidine at the highest level of treatment, with an overall sequence coverage of 64.4%.

Conclusions: LTQ-FTMS analysis of 4HNE modified hBAT has led to the discovery of modification within the active site of the protein correlating to a complete lack of activity in the enzyme. At lower levels of 4HNE treatment, adducts occur around the active site, which may be responsible for the lower activity levels seen.

OPTM Oral Session Abstracts

Session 2:

OXIDATION AND SIGNALING

GLUTATHIONE PEROXIDASE-1 OVER-EXPRESSION ALTERS RECEPTOR-MEDIATED SIGNALING BY REGULATING INTRACELLULAR LEVELS OF REACTIVE OXYGEN SPECIES

Handy Diane E, Yang Yi, Lubos Edith, Galbraith John D, Leopold Jane A, Loscalzo Joseph.

Glutathione peroxidase (GPx-1) is a selenocysteine containing enzyme that plays a major role in the detoxification of peroxides in vascular cells. Intracellular reactive oxygen species (ROS), especially hydrogen peroxide, are important mediators of normal cellular events, such as growth factor stimulation and disulfide bond formation. To study the role of ROS in cellular function, we overexpressed GPx-1 in permanently transfected cells and compared the response to hydrogen peroxide of the overexpressing cells (GPx-1 OE) to that of cells similarly transfected with empty vector. GPx-1 OE significantly reduced the accumulation of intracellular ROS (as measured by DCF fluorescence) in response to exogenous hydrogen peroxide: basal accumulation of DCF fluorescence was unchanged after a 45 minute incubation. In GPx-1 OE, hydrogen peroxide activation of Akt , as measured by phosphorylation at serine 473, was significantly reduced. Overexpression of GPx-1 did not decrease the formation of oxidized PTEN, an oxidant-sensitive phosphatase that antagonizes PI3K-Akt activation; however, overexpression did reduce EGF receptor activation following hydrogen peroxide treatment or EGF stimulation. These data show that ROS signaling is downregulated in GPx-1 overexpressing cells, in part, via altering EGF receptor mediated signaling events, and suggest that modulating ROS flux can affect functional cellular responses.

DUAL ROLE OF PEROXYNITRITE IN MEDIATING VEGF SIGNALING: OXIDATION VERSUS NITRATION

El-Remessy Azza B. and Caldwell Ruth B.

Objective: VEGF is one of the most potent survival and angiogenesis growth factors. Peroxynitrite causes oxidation of protein-associated thiol groups and nitration of tyrosine residues. We have shown that peroxynitrite (0.5 mM) inactivate VEGF survival signaling and accelerate endothelial cell death via tyrosine nitration of p85 subunit of PI3-kinase. We have shown also that peroxynitrite (1μM) mimics the effects of VEGF in causing immediate tyrosine phosphorylation of VEGFR2, c-Src and focal adhesion kinase (FAK). The purpose of this study is to elucidate the molecular mechanisms by which peroxynitrite mediates VEGF/FAK activation. **Methods:** The effects of the specific peroxynitrite decomposition catalyst (FeTTPs, 2.5 μM), the thiol donor N-acetyl cysteine (NAC, 1mM) and the specific nitration inhibitor (epicatechin, 100 μM) were studied on VEGF-mediated phosphorylation of VEGFR2, Src, and FAK in microvascular endothelial cells. 5-IAF was used to label free thiols of the low molecular weight PTP (LWT-PTP), the specific phosphatase of FAK using fluorescein antibody. **Results:** While activation of VEGFR2, cSrc and FAK was blocked by FeTTPs, it was enhanced by epicatechin. FAK activation was blocked by NAC suggesting an oxidation mediated action of peroxynitrite. Treatment with VEGF or 1 μM peroxynitrite resulted in oxidation of the free thiols of LMW-PTP at 1 and 15 minutes which co-insides with peak of VEGFR2 and FAK phosphorylation. Cell migration assay revealed that FeTTPs and NAC blocked VEGF-stimulated cell migration while epicatechin did not alter VEGF's effects. **Conclusion:** Taken together, these results indicate a dual role of peroxynitrite as signaling molecule interacting with VEGF: an inhibitory role of nitration and a novel role mediating VEGF angiogenic signal via thiol oxidation.

NITRITE ANIONS INDUCE PROTEIN AND PEPTIDE MODIFICATIONS

Deng Haiteng

Objective: Understanding of Chemical Basis of Nitrite-Induced Peptide/Protein Modifications

Methods: MALDI-TOF and LC-MS/MS

Results: In the present study, nucleophilic reactions of nitrite anions with proteins/peptides were characterized with mass spectrometry. The reaction generates two major products: replacement of the amino group by hydroxyl group and formation of an alkene derivative by loss of a NH_3 group at the N-terminus and the side chain of lysine residues of proteins/peptides. The reaction proceeds rapidly in weak acidic solution and at 37 ºC in the presence of millimolar concentration of nitrite. The reactivities of nitrite anions in nitrosive deamination, nitrosylation, and nitration are discussed.

Conclusion: The results show that nitrite induced modification of amino groups of protein/peptides that changes chemical nature of proteins and have various applications in peptide synthesis, analytical chemistry, and protein engineering. It also provides information to enhance our understanding of functions of nitrite anions in biology and food preservation.

EFFECTS OF PEROXYNITRITE ON CARDIAC MYOFIBRILLAR PROTEIN MECHANICS

Snook Jeremy, Evangelista Alicia, Guilford William

Objectives: Determine the effects of peroxynitrite ($ONOO^-$) on cardiac myosin, actin and thin filaments in order to more clearly delineate its role in left ventricular dysfunction after myocardial infarction. Methods: Cardiac myosin, actin and thin filaments were purified from rat myocardium and subsequently treated with varying concentrations of $ONOO^-$ in the presence of 2 mM bicarbonate. Protein function (filament velocity) and calcium sensitivity were assessed by in vitro motility assay. Results: The velocity with which a-cardiac myosin propelled actin filaments was significantly reduced when myosin was exposed to [$ONOO^-$] of 10 μM and above – a molar ratio of >10:1 $ONOO^-$:heavy chain. Both the number of filaments moving and the velocity of moving filaments were affected, and the deficit was not reversible by exposure to DTT. Actin filaments exhibited similar $ONOO^-$ sensitivity. The velocities of regulated thin filaments at saturating [Ca^{2+}] were slightly reduced at [$ONOO^-$] between 1 and 10 μM. However, at 10 μM [$ONOO^-$] the calcium sensitivity remained unchanged (6.60 vs 6.57 pCa_{50}). Exposing both thin filaments and myosin to ONOO did not lower the threshold [$ONOO^-$] necessary to elicit a deficit, but made the deficit more severe. Conclusions: $ONOO^-$ significantly inhibits the ability of myofibrillar proteins to generate motion. Because some modifications are irreversible by reducing agents, when generated continuously even low concentrations of $ONOO^-$ may affect function. However, these data call into question a familiar hypothesis that reactive species lower the calcium sensitivity of thin filaments after myocardial infarction, when ONOO may be abundant.

SERCA CYS-674 IS REQUIRED FOR THE INHIBITION BY NITRIC OXIDE OF CELL MIGRATION

Ying Jia, Tong Xiao Yong, Pimental David, Weisbrod Robert, Adachi Takeshi, Cohen Richard

Nitric oxide inhibits smooth muscle cell migration after arterial injury, but the mechanism is not understood. Our previous studies showed that NO lowers intracellular Ca^{2+} by increasing sarcoplasmic reticulum calcium ATPase (SERCA) activity by inducing reversible S-glutathiolation of Cys-674. Because Ca^{2+} is an important second messenger for cell migration, we hypothesized that NO also inhibits cell migration through redox regulation of SERCA activity via Cys-674. To test our hypothesis, wildtype (WT) or mutant SERCA in which cysteine-674 was mutated to serine was stably transfected in HEK 293 cells or expressed by adenovirus in rat aortic smooth muscle cells (VSMC). Biotinylated-iodoacetamide and glutathione labeling of SERCA was decreased in cells expressing mutant SERCA, and NO failed to increase $^{45}Ca^{2+}$ uptake activity. In the absence of NO, serum increased migration of both cell types expressing WT or C674S SERCA at similar rates over 6 h. The NO donor, S-nitrosopenicillamine inhibited migration of cells with WT SERCA, but had no effect on the migration of either HEK cells or VSMC with mutant SERCA C674S. The same result was achieved not only with the NO donor, but also in VSMC in which endogenous NO was produced by iNOS induced by IL-1β. Blocking cyclic GMP did not prevent the inhibition of migration by NO. Therefore, we conclude that SERCA mediates inhibition of cell migration by both exogenous and endogenously generated NO, and that redox regulation of SERCA by *S*-glutathiolation of cysteine-674 is required.

TYROSINE NITRATION OF MEK AND ERK INDUCES THEIR AUTOPHOSPHORYLATION AND ACTIVATION IN VIVO AND IN VITRO.

Pinzar Elena, Communi David, Csibi Alfredo, Urrecheaga Daniela, Garrido Maria, Bottari Serge P.

Angiotensin II (Ang II) has been shown to activate MAPK pathways and to induce production of reactive oxygen and nitrogen species. NO and O_2^-, as well as $ONOO^-$, have been previously reported to stimulate MAPKs. Stimulation of VSMC with 10 nM Ang II induced a rapid and sustained nitration of ERK1/2 and MEK1/2 as revealed by immunoprecipitation and immunoblotting. ERK nitration was accompanied by its activation. Both Ang II-induced ERK nitration and phosphorylation were mediated by the AT_1 receptor. Both nitration and phosphorylation of MEK and ERK1/2 by Ang II were significantly inhibited by the NAD(P)Hoxidase inhibitors AEBSF and DPI as well as by the ROS scavengers ebselen and myricetin. Moreover, the selective iNOS inhibitor 1400W also completely inhibited their nitration and activation. Conversely, the MEK inhibitor U0126, did not affect ERK nitration, but completely blocked ERK Thr/Tyr phosphorylation. Therefore ERK nitration appears to be a prerequisite for its activating phosphorylation by MEK in response to Ang II in vivo since nitration inhibitors prevent its activating phosphorylation. The $ONOO^-$ donor SIN-1 also stimulated nitration and phosphorylation of recombinant ERK and MEK in vitro, indicating that nitration of these kinases leads to their autophosphorylation on $Ser^{217+221}$ for MEK and Thr^{202} + Tyr^{204} for ERK. MS analysis of tryptic peptides allowed the identification of two nitrated tyrosines: Tyr^{156} located in the kinase domain and another Tyr at position 119 or 130 present only in ERK 1. Similar results were obtained with insulin stimulation.

OPTM Oral Session Abstracts

Session 3:

MASS SPECTROMETRY AND PROTEOMICS

AGING, OXIDATIVE STRESS AND YOUR BRAIN

Gibson Bradford W.
Buck Institute for Age Research, Novato, CA.

Theories on aging are complex and often contradictory, ranging from evolutionary arguments to biochemical process to entropy. Many of these theories center around oxidative stress as an underlying or contributing mechanism in the aging process, as best embodied in the 'free radical theory of aging', as well as other arguments such as those that focus on role of mitochondria or overall system failure. Another important consideration in studying aging mechanisms is to explain the exponential risk in the incidence of various age-related diseases such as cancer or neurodegenerative diseases with age. In this talk, I will discuss some of our recent work at the Buck Institute for Age Research in studying mitochondrial dysfunction, Parkinson's and Huntington's disease, as well as other topics that relate to aging or age-related diseases. My primary focus will be on the development and application of proteomic technologies to study protein post-translational modifications, such as phosphorylation and oxidative damage, as well as the dynamics of protein turnover.

ENDOGENOUS NITROPROTEOMES OF MOUSE HEART AND SKELETAL MUSCLE: DISTINCT SIGNATURES OF THEIR NITRATIVE ENVIRONMENT

Bigelow Diana J, Knyushko Tatyana V., Kowalska Malgorzata, and Sacksteder Colette

OBJECT: In order to characterize the heart's nitrative environment in comparison with that of the less aerobic skeletal muscle, endogenous levels of nitrotyrosine modified proteins were identified in vivo in these two muscle types. METHODS: Both soluble and membrane fractions of balb/c mouse hearts and skeletal muscle were analyzed by gel-free 2-D LC MS/MS. RESULTS: From stringent criteria for SEQUEST search results, we identified 17,226 peptides corresponding to 3,492 unique proteins in the heart; from skeletal muscle, 15, 423 peptides and 2,992 proteins were identified including 206 and 221 nitrotyrosine-containing peptides, respectively. The more modest overall nitrotyrosine load in the heart as compared with skeletal muscle is consistent with its more robust activities for clearance of protein nitration. Moreover, the heart exhibits a striking preference for nitration of mitochondrial proteins, whereas cytoskeletal, nuclear, and SR proteins are preferentially nitrated in skeletal muscle. Methionine sulfoxide, a marker of the nitrating species, peroxynitrite, as well as other ROS, exhibit similar patterns of occurrence in proteins and cellular locations as does nitrotyrosine. CONCLUSION: These results suggest differential points of vulnerability in heart and skeletal muscle under conditions of chronic inflammation.

COMPARATIVE PROTEOMICS: POST-TRANSLATIONAL MODIFICATIONS IN SICKLE CELL DISEASE

McComb Mark E; Dauly Claire; Ohiambo Adam; Perlman David H; Huang Hua; Steinberg Martin H; Farber Harrison W; Klings Elizabeth S; Costello Catherine E

Objective: Apply differential plasma proteomics to characterize PTMs associated with sickle cell disease (SCD).
Methods: Plasma from Sickle Cell Disease (SCD) patients with pulmonary hypertension (PH) and healthy controls, were albumin-depleted and separated by two-dimensional HPLC. Differential 2D expression map targeted fractions were analyzed by mass spectromery.
Results: We identified several abundant and medium-abundance proteins that appear to vary with SCD and PH, and we have identified several protein post-translational modifications which also correlated to the disease phenotype. Modifications of transferrin, an important molecule in iron metabolism and transport, and apolipoprotein A1, a molecule required for HDL-mediated activation of endothelial nitric oxide synthase, were observed and could play a role in the pathogenesis of PH in SCD, or could serve as important biomarkers of disease.
Conclusions: Differential plasma proteomics such as this study may have a significant impact on the detection and treatment of SCD, PH and other human diseases. This project was funded by NIH grants P41-RR10888 (to C.E.C.), S10-RR15942 (to C.E.C.), U54 HL070819 (to M.H.S.), and NHLBI contract N01-HV-28178 (to C.E.C.).

DEAMIDATION AND ISOASPARTIC ACID FORMATION IN PROTEINS.

Cournoyer Jason J, Lin Cheng, Zhao Cheng
O'Connor Peter B.

Deamidation of proteins is THE most common post-translational modification, proceeding via nucleophilic attack of the backbone amide on the sidechain carbonyl of asparagine or glutamine residues. This reaction results in loss of ammonium and formation of a cyclic succinimide intermediate, which rapidly hydrolyzes to form isomeric product mixtures. Asparagine forms aspartic and isoaspartic acid; glutamine forms glutamic and γ-glutamic acid. Detection of deamidation requires some effort, but distinguishing the products is extremely difficult. The new method of Electron Capture Dissociation, however, has been shown to readily differentiate the isomeric products.

The methodology for differentiating aspartic and isoaspartic acid has been worked out, and recently it has been improved to allow relative quantitation of the products. The method has been applied to a number of different proteins and we are now in the position to be able to study the correlation between oxidative stress and formation of these products. Our most current results on these methods will be shown.

IDENTIFICATION OF NOVEL PHOSPHORYLATION SITES WITHIN THE CARBOXYL-TERMINUS OF CONNEXIN 43 IMMUNOPRECIPITATED FROM CANINE HEART

Hesketh Geoffrey G, Shah Manish, Guo Yurong, Tomaselli Gordon F, Van Eyk Jennifer E

Changes in the phosphorylation state of Connexin 43 (Cx43) have been associated with conduction slowing and arrhythmogenesis in heart failure (HF). To identify specific phosphorylated amino acids within Cx43 that may contribute to these phenomena Cx43 was immunoprecipitated from canine ventricular tissue, separated by SDS-PAGE, and Cx43 specific bands were excised and subjected to in-gel tryptic digestion. Phosphorylated peptides were enriched by immobilized metal affinity chromatography (IMAC) and analyzed by liquid chromatography coupled tandem mass spectrometry (LC-MS/MS). Eight phosphorylated amino acid residues were identified within the carboxyl-terminus of Cx43 from canine heart. Two sites were consensus PKA phosphorylation sites (Ser365 and Ser373) and two were casein kinase 1 sites (Ser 328 and Ser 330), all previously described only in non-cardiac cell lines. Four phosphorylation sites not previously described in the literature were also identified (Ser296, Ser297, Ser306 and Thr327). None of the identified sites have been previously described in heart muscle. Further understanding of the phosphorylation state of Cx43 may provide insight into the underlying mechanisms of conduction slowing and arrhythmogenesis in HF.

PROTEOMIC AND METABONOMIC ANALYSES OF THE CARDIOPROTECTIVE EFFECTS OF NITRITE ADMINISTRATION IN THE HEART

Perlman David H, Bauer Selena, Bryan Nathan S, Garcia-Saura Maria F, Fernandez Bernadette O, McComb Mark E, Costello Catherine E, Feelisch Martin

Objectives: Nitrite has recently been shown to offer protection from ischemia-reperfusion injury in the heart, liver and brain, and to have signaling molecule-like properties, affecting sGC, CYP450, HSP-70 and HO-1 proteins. Intrigued by these findings, we have used an integrated proteomics and metabonomics approach to further characterize the impact of changes in systemic nitrite availability on the heart.

Methods: Cellular redox status and concentrations of NO-related metabolites were assessed in cardiac tissue from rats after systemic administration of sodium nitrite. Crude cardiac mitochondria and post-mitochondrial supernatants were subjected to proteomic analysis using 2D-PAGE, in-gel trypsin digestion, MALDI-TOF MS, and PMF analyses.

Results: Systemic administration of nitrite induced short-term spikes in the levels of cardiac *S*-nitroso, *N*-nitroso, and heme-nitrosyl species, followed by a large and persistent increase in the ratio of oxidized to reduced ascorbate. These changes were accompanied by significant alterations to protein expression and post-translational modifications within the cardiac proteome, involving proteins in energy metabolism, redox balance, chaperone activity, cell structure, contractility, and nitric oxide metabolism.

Conclusions: This integrated proteomic and metabonomic approach is a step toward elucidating the scope and mechanism of cardioprotection and the potential physiological activity of nitrite.

SITE-SPECIFIC MAPPING OF THE S-NITROSOCYSTEINE PROTEOME IN HUMAN VASCULAR SMOOTH MUSCLE CELLS

Greco Todd M., Hodara Roberto, Parastatidis Ioannis, Heijnen Harry F.G., Dennehy Michelle K., Liebler Daniel C., Ischiropoulos Harry

OBJECTIVE: S-nitrosylation, the selective modification of cysteine residues in proteins to form S-nitrosocysteine, is a major emerging mechanism by which nitric oxide acts as a signaling molecule, yet its role in the regulation of vascular smooth muscle cell biology is not clear. Therefore, the potential protein targets and the corresponding modified cysteine residues were explored. METHODS: We employed a proteomic approach using chemical derivitization, selective peptide enrichment, and LC-MS-MS to identify the targets of S-nitrosylation in human aortic smooth muscle cells upon exposure to S-nitrosocysteine and propylamine propylamine NONOate. RESULTS: Twenty unique S-nitrosocysteine-containing peptides belonging to 18 proteins were identified, including cytoskeletal proteins, chaperones, proteins of the translational machinery, vesicular transport, and signaling. High-resolution immunogold electron microscopy supported the cellular localization of several of these proteins. In addition, sequence analysis of the S-nitrosocysteine-containing peptides revealed the presence of acid-base motifs, as well as hydrophobic motifs surrounding the identified cysteine residues. CONCLUSION : Seven of the 18 proteins identified are localized within the ER/Golgi complex, suggesting a role for S-nitrosylation in membrane trafficking and ER stress response in vascular smooth muscle.

OPTM Oral Session Abstracts

Session 4:

TYROSINE NITRATION

MASS SPECTROMETRIC IDENTIFICATION AND STRUCTURAL CHARACTERISATION OF TYROSINE NITRATION IN CELLULAR PROTEINS.

Przybylski Michael, Petre Alina, Dragusanu Mihaela, Weber Reinhold, Bachschmid Markus, Ullrich Volker, Ulrich Martina and Doering Gerd.

Oxidative modification of proteins may cause substantial biochemical and pathophysiological changes, however, structural identification is often tedious and requires methods of high sensitivity and specificity. Tyrosine nitration has been associated to pathophysiological effects, e.g. in neurodegeneration, atherosclerosis, and broncho-alveolar diseases. While immuno-analytical methods suffer from low detection specificity of antibodies, mass spectrometric methods are hampered by low stabilities and levels of modification. High resolution FTICR-MS, using ESI and IR-MALDI ionisation, has been found a powerful tool for unequivocal identification of Tyr-nitrations. Recent applications include the identification of nitration (i), upon peroxynitrite treatment at the active site Tyr-430 of prostacyclin synthase and (ii), specific endogenous nitration in human eosinophil-peroxidase (EPO). Structural studies of Tyr-nitrated peptides, prepared by chemical synthesis and *in vitro oxidation,* were performed by 2D-NMR and FTICR-MS, in combination structure modelling and H/D exchange-MS. 3-Nitrotyrosyl-peptides undergo photochemical fragmentation by UV-MALDI-MS, making standard UV-MALDI critical for identification of nitrations; however, stable molecular ions were obtained by IR-MALDI-MS. New cellular Tyr-nitration substrates were recently identified in blood eosinophils such as eosinophil-cationic protein, suggesting a possible function of nitration in host defense in providing enhanced surface binding in eosinophil granules.

ROBUST MECHANISMS IN THE HEART FOR REPAIR AND DEGRADATION OF NITRATED PROTEINS

Knyushko Tatyana V, Londono Monica P, Xiong Yijia, Stenoien David L, Sacksteder Colette, and Bigelow Diana J.

OBJECT: Cellular mechanisms responsible for the clearance of nitrated proteins from muscle were examined using both C2C12 myocytes in culture and heart and skeletal muscle tissues. METHODS & RESULTS: Acute responses to nitrative stress induced with peroxynitrite (PN) reveal rapid and efficient clearance of the resulting protein nitration. Efficient penetration of PN into the cell's interior was indicated by FRAP measurements showing the loss of lateral mobility of expressed GFP-tagged SERCA; in addition, a proteomic screen of PN treated cells shows nitration of multiple intracellular proteins. The rapid removal of protein nitration was largely dependent upon the 20S proteasome. However, an enzymatic denitrating activity was not detected in lysates from PN-treated myocytes in contrast to muscle where tissue homogenates exhibit measurable denitrating activity with heart homogenates exhibiting five-fold higher rates as compared with those of skeletal muscle homogenates. In parallel, more rapid protein turnover is observed in the heart for nitration sensitive proteins, the SR Ca-ATPase and the ryanodine receptor calcium release channel as compared with skeletal muscle. CONCLUSIONS: These robust cellular mechanisms for removal of nitrated proteins in heart explain their lower overall load of nitrated proteins as compared with skeletal muscle.

PROTEOMIC ANALYSIS OF AGE-DEPENDENT PROTEIN TYROSINE NITRATION: A NOVEL ICAT-METHOD SELECTIVE FOR 3-NITROTYROSINE

Schöneich Christian, Gokulrangan Giridhan, Kanski Jarowslaw, Li Xiaobao, Pennington Justin, Killmer Jaque, Stobaugh John.

The accumulation of oxidative protein modifications is a hallmark of many pathologic conditions and aging. Any mechanistic correlation of biologic dysfunction with the formation of such modifications requires the identification of target proteins and the localization and quantification of the respective modification(s). 3-Nitrotyrosine represents a tyrosine modification resulting from the increased generation/availability of nitric oxide and its oxidation products, commonly referred to as reactive nitrogen species (RNS). Through several complementary multidimensional proteomic methods we show that biologic aging leads to increased accumulation of 3-NY on *selected* proteins in skeletal muscle, heart and specific brain regions (such as cerebellum). The specific sites of 3-NY formation on some of these proteins could be located through detailed MS/MS analysis. However, the acquisition of good quality MS/MS spectra proved difficult specifically for lower abundance proteins with lower levels of 3-NY. Therefore, we developed a novel tagging procedure for 3-NY, which allows us to *selectively* convert peptide and protein 3-NY into a mass spectrometry-friendly, fluorescent benzoxazole derivative, which can be obtained in its protonated (H-10) and deuterated (D-10) form for relative quantification. Synthetic procedures were developed to append the derivatization reagents with various functional groups for (a) improving chromatographic behaviour and (b) affinity enrichment. [Support: NIH AG23551, AG25350].

DYNAMICS OF SITE-SPECIFIC CALMODULIN NITRATION AND OXIDATION DETERMINED USING LC-FTICR MS

Lourette NM, Smallwood HS, Boschek CB, Smith RD, Squier TC, and Paša-Tolić L

Reactive oxygen species can induce post-translational modifications (PTMs) to calmodulin (CaM), a ubiquitous calcium regulator in eukaryotes. These PTMs interfere with cellular signaling, may contribute to pathologies, and are associated with cardiovascular diseases.

We characterized the dynamics of PTMs in CaM to gain insight into oxi- and nitro-CaM forms in response to oxidative stress; specifically, whether PTMs are reversible and/or whether they induce turnover of modified CaM by degradation processes.

CaM was treated with peroxynitrite to model common PTMs and subsequent modifications were determined using immunoblotting and capillary reversed phase liquid chromatography coupled with (12T) FTICR MS.

FTICR MS of intact CaM allowed the extent of PTM heterogeneity to be resolved prior to and post introduction of modified CaM to the intracellular milieu of macrophages under conditions of oxidative stress. Upon introduction of modified CaM to the lysate, we observed a reduction in modifications, with minimal degradation of unmodified CaM. We also eliminated chemical reversion to amino tyrosine as a pathway of nitrotyrosine loss. Additionally, we identified a modification dependent C-terminal lysine cleavage that is likely to have functional implications in cells, as well as make a useful biomarker for oxidative stress conditions in a wide variety of cells. We are currently in the process of quantifying the PTMs in modified CaM following exposure to macrophages.

PROTEIN TYROSINE NITRATION / DENITRATION – AN OXYGEN REGULATED PROCESS

Koeck Thomas, Stuehr, Dennis and Aulak Kulwant S.

Objectives: Hypoxia is a frequently encountered stress in intense exercise, stroke and cardiovascular disease. NO is known to play a critical role in adaptive responses to this stress. NO and reactive oxygen species, generated during oxygen depletion and reoxygenation, result in protein tyrosine nitration. Up until recently, protein tyrosine nitration was viewed as an indiscriminate process leading to a cumulative dead end product that was destined for degradation. This study focuses on tyrosine denitration as an alternative response that establishes tyrosine nitration as a regulatory post-translational modification during adaptation to alterations in the oxygen partial pressure. **Methods:** Cells and Mitochondria were exposed to hypoxia under various conditions and alterations in tyrosine nitration analyzed by 2D SDS-PAGE, Western blotting, LC-MS/MS and MALDI-TOF/TOF. **Results:** We show that tyrosine nitration during hypoxia is a rapid and highly protein target-selective process. We further show that this process is partially reversible, especially in mitochondria. While some targeted proteins in cells undergo proteolysis through the proteosome most are denitrated by an uncharacterized mechanism. **Conclusions:** Our data reveal that protein tyrosine nitration can be controlled, target-selective, rapid and dynamic enough to meet the criteria for a “nitrative” regulatory signaling process. It likely impacts cellular energy and redox homeostasis during hypoxia. Excessive or inappropriate nitration exceeding the denitration capacity therefore can lead to disease or acute pathological conditions.

IDENTIFICATION AND CHARACTERISATION OF TYROSINE NITRATION IN HUMAN EOSINOPHILS USING FTICR MASS SPECTROMETRY IN COMBINATION WITH IMMUNOANALYTICAL PROCEDURE

Petre Alina, Weber Reinhold, Ulrich Martina, Döring Gerd, Przybylski Michael

High resolution FTICR mass spectrometry in combination with immuno-analytical procedures have been developed as powerful tools for unequivocal and sensitive identification of Tyr-nitration, (i) upon peroxynitrite treatment at the active site Tyr-430 residue of bovine prostacyclin synthase, and (ii) specific endogenous nitration at Tyr-349 of human eosinophil-peroxidase (EPO). A series of peptides containing 3-nitro-tyrosine were synthesized by solid phase peptide synthesis using Fmoc strategy and standard side chain protection chemistry. Detailed structural studies were performed with Tyr-nitrated model peptides, using FTICR-MS in combination with 2D-NMR, with the aim to reveal possible structural differences introduced by the 3-nitro-Tyr group. In addition to ESI-MS unequivocal identifications of 3-nitro-tyrosyl-peptides were obtained by (IR)-MALDI-FTICR-MS with a 2.97 µm NdYAG-laser. Specificity of anti-3-NitroTyrosine antibodies to nitrated/non-nitrated peptides was verified by affinity – mass spectrometry and ELISA. Recent applications will be presented showing that, in resting human blood eosinophils; eosinophil cationic protein and eosinophil peroxidase are 3-nitro-tyrosine modified. Furthermore, studies in mice strains lacking EPO or functional NADPH oxidase revealed that tyrosine nitration of eosinophil granule toxins is mediated by EPO which was identified to contain a single, surface-exposed nitrotyrosine modification at Tyr349.

PROTEOMIC PROFILING OF NITRATED PROTEINS IN ATHEROSCLEROTIC LESIONS AND PLASMA REVEALS AN ANTI-OXIDATIVE ROLE OF APOLIPOPROTEIN A-I.

Parastatidis Ioannis, Thomson Leonor, Fries Diana, Moore Ryan, Tohyama Junichiro, Hazen Stanley L., Heijnen Harry F.G., Dennehy Michelle K., Liebler Daniel C., Rader Daniel, Ischiropoulos Harry

OBJECTIVE: To evaluate the proposed antioxidant function of apoA-I in atherosclerosis, proteins modified by nitrating oxidants were evaluated in the aortic tissue and plasma of mice lacking the low-density lipoprotein receptor and apobec (LA) and LA mice with genetic deletion of apoA-I (LA-apo A-I-/-). METHODS: Affinity enrichment of nitrated proteins in plasma and aortic roots followed by site specific adduct mapping were employed to identify the modified proteins. LC/ESI/MS/MS using stable isotope dilution methodology was used to quantify nitrotyrosine burden in aortic roots. RESULTS: The levels of nitrated proteins in aortic tissue were six-fold higher in the LA-apoA-I-/- as compared to the LA mice. The quantitative analyses were corroborated by immunohistochemical and high-resolution immuno-electron microscopic evaluation of the lesions. Site-specific adduct mapping identified 13 specific proteins targets for nitration in the aortic root lesions of the LA-apo A-I-/-. Four of the nitrated proteins identified were unique targets, not found in the lesions of LA. The same approach also identified three plasma and three tissue-derived proteins modified by nitrating oxidants in the LA-apoA-I-/- plasma. CONCLUSIONS: The results suggest a protective function of apo-A-I against nitrative oxidants in these models of atherosclerosis.

A PROTEOMIC APPROACH FOR THE STUDY OF AGE DEPENDENT PROTEIN NITRATION IN HEART

Hong Sung Jung, Gokulrangan Giri, Schöneich Christian

Objectives. To identify and characterize nitrotyrosine (3-NY) containing proteins in cardiac tissue *in vivo*, and study the differences in nitration as a function of aging.

Methods. Cardiac tissues were obtained from young and old Fisher 344/BN F1 rats. Tissues were homogenized in a urea lysis buffer. Soluble proteins were collected by ultracentrifugation and were then separated by solution isoelectric focusing. Following separation, proteins were collected into 20 different fractions of discrete pH ranges. Proteins were submitted to reduction and alkylation and were then precipitated out in EtOH at -20 ^{o}C overnight. Finally, proteins were resolubilized in NH_4HCO_3 buffer and were submitted to tryptic digest and analysis by a nano-LC-ESI-MS/MS. Results were submitted to a database search through a Finnigan protein database search tool, SEQUEST/BIOWORKS.

Results. The solution phase separation method allowed us to work with sufficient protein amounts to obtain reliable MS/MS data. The nitrated cardiac proteins that were identified in both aged cardiac tissue and young cardiac tissue included myosin heavy chain and neurofibromin. Additional nitrated proteins found in aged cardiac tissue included N-RAP, tropomyosin, and dynein, among others.

Conclusion. With the in-solution approach we were able to load sufficient protein levels to allow MS/MS analysis of 3-NY containing proteins *in vivo.* Site-specific identification of 3-NY is essential in order to understand the effects of protein nitration on protein structure and function.

OPTM Oral Session Abstracts

Session 5:

THIOLS

SITE-SPECIFIC S-GLUTATHIOLATION OF MITOCHONDRIAL COMPLEX I

Chen Chwen-Lih, Zweier Jay L, Chen Yeong-Renn

Reactive oxygen species in mitochondria acts as a redox signal in triggering apoptosis and senescence. Complex I is the major host of reactive/regulatory protein thiols. An important response of protein thiols to oxidative stress is to reversibly form protein mixed disulfide via S-glutathiolation. Exposure of Complex I to GSSG resulted in specific S-glutathiolation at the 51 kDa and 75 kDa subunits. To investigate the molecular mechanism of S-glutathiolation of Complex I, we employed the technique of mass spectrometry. LC/MS/MS analysis of tryptic digests of the 51 kDa and 75 kDa polypeptides from glutathiolated Complex I (GS-NQR) revealed that two specific cysteines (C_{206} and C_{187}) of the 51 kDa subunit and one specific cysteine (C_{367}) of the 75 kDa subunit were involved in redox modifications with GS binding. The electron transfer activity (ETA) of GS-NQR was significantly enhanced. However, superoxide generation (SGA) mediated by GS-NQR suffered a mild loss. Exposure of NADH dehydrogenase (NDH), the flavin subcomplex of Complex I, to GSSG resulted in specific S-glutathiolation on the 51 kDa subunit. Both ETA and SGA of GS-NDH decreased in parallel as the dosage of GSSG increased. LC/MS/MS analysis of a tryptic digest of the 51 kDa subunit from GS-NDH revealed that C_{206}, C_{187}, and C_{425} were glutathiolated. C_{425} of the 51 kDa subunit is one of the 4Fe-4S ligands, suggesting that destruction of 4Fe-4S is the major mechanism of oxidative damage. In conclusion, S-glutathiolation of the 75 kDa subunit may play a role in protecting the 4Fe-4S cluster of the 51 kDa subunit from redox modification when Complex I is exposed to redox change in the GSH pool.

S-GLUTATHIOLATION OF P21RAS INITIATES OXIDANT-MEDIATED SIGNALING AND PATHOLOGY

Cohen Richard, Clavreul Nicolas, Adachi Takeshi, Sethuraman Mahadevan, Heibeck Tyler, Ido Yasuo, Pimental David, Kuster Gabriela, Colucci Wilson, Zhao Cheng, O'Connor Peter, McComb Mark, Costello Catherine, and Schöneich Christian

p21Ras is a small GTPase whose activity leads to signaling which regulates growth and hypertrophy. Reactive oxygen and nitrogen species (RO/NS) generated endogenously, including H_2O_2 and $ONOO^-$ form glutathione adducts (GSS-) with exposed reactive cysteine thiols; Cys-118, in the GTP binding site, and the C-terminal Cys-181,184,186. The GSS-adduct, demonstrated by labeling and MS methods, is stable in cellular concentrations of GSH, but can be reduced chemically or enzymatically with glutaredoxin. Increases in RO/NS caused by angiotensin II, β-adrenoceptors, and mechanical strain initiate p21Ras/Mek/Erk and PI3 kinase/Akt signaling that mediates hypertrophy of vascular smooth muscle and cardiomyocytes. The oxidant-activated Erk activity increases phosphorylation of IRS-1 and thereby contributes to impaired endothelial cell insulin signaling. The response to oxidants is reproduced on recombinant p21Ras or in cells by exposure to GSSG or GSNO, and is suggested to occur due to slower secondary reactions of these species with the reactive Cys-118, rather than it reacting directly with oxidants. The RO/NS mediated signaling events and pathological endpoints are prevented by overexpression of antioxidant enzymes, glutaredoxin-1, or a p21ras C118S mutant, supporting a major role of oxidant-mediated GSS-p21Ras Cys-118 as the underlying mechanism.

S-NITROSATION AND REDOX REGULATION

Ullrich Volker and Frein Daniel
Department of Biology, University of Konstanz

Equal fluxes of superoxide and nitric oxide in cells lead to peroxynitrite formation, causing the sulfoxidation of methionines, the nitration of tyrosines, and the oxidation of thiols to disulfides. How the necessary concentrations of peroxynitrite can be built up under the reducing conditions of the cellular milieu remains obscure. We and others have postulated that nitrosations occur at fluxes of about 3:1 nitric oxide and superoxide, in agreement with the formation of dinitrogen trioxide as the nitrosating species. However some findings are not in accord with this assumption and ask for NO+ as the nitrosating intermediate. Following the concept of redox regulation, the process of *S*-nitrosation should precede peroxynitrite-dependent oxidations if increasing superoxide levels are causing oxidative cell activation. This hypothesis is supported by the described enzymatic effects of *S*-nitrosation on the redox state of the cell.

ANALYSIS OF LIVER MITOCHONDRIA PROTEIN THIOL MODIFICATIONS IN RESPONSE TO CHRONIC ALCOHOL AND S-ADENOSYLMETHIONINE (SAM)

Andringa Kelly K, Diers Anne R, Upton Ashley N, Bailey Shannon M.

Studies support the efficacy of SAM as a therapeutic agent for treating alcoholic liver disease. The molecular mechanisms that govern SAM-associated hepatoprotection are unknown, but may involve prevention of alcohol-associated alterations to the redox status of mitochondrial protein thiols. Thiol status of liver mitochondria proteins was determined using, 4-iodobutyl triphenylphosphonium (IBTP), which labels reduced protein thiols. Thus, oxidized/modified thiols are identified by decreased IBTP labeling via immunoblotting with anti-IBTP. Liver mitochondria from male Sprague-Dawley rats fed control and alcohol liquid diets with/without SAM for 5 weeks were IBTP-labeled and used to generate high-resolution 2-D protein gels. Comparable spot patterns were observed for all four groups. Immunoblot analyses revealed decreased IBTP labeling in proteins from alcohol-fed rats compared to controls, indicating oxidation/modification of protein thiols due to chronic alcohol consumption. Modifications were prevented by co-administration of SAM. We have shown that this technique can be used to determine the thiol redox status of individual mitochondrial proteins as a consequence of chronic alcohol induced oxidant stress. These data demonstrate the hepatoprotective mechanisms of SAM may work via effects on the mitochondrial proteome. Protein identification will provide new insight regarding the role of oxidative stress and thiol modifications in the mechanism of alcohol-induced mitochondrial dysfunction and liver disease.

INTERPROTEIN DISULFIDE BOND FORMATION ACTIVATES PKG1α INDEPENDENTLY OF cGMP

Burgoyne Joseph, Madhani Melanie, Brennan Jonathan P, Cuello Friederike, Eaton Philip.

Isolated rat hearts were treated with H_2O_2 at constant flow (5min, 100μM), and non-reducing immunoblotting showed this caused 86% ($P<0.01$) of the PKG to form a disulfide dimer (36% in controls). H_2O_2 decreased coronary perfusion pressure (vasodilation) by 32%, implicating PKG activation via disulfide oxidation. Using purified PKG1α and $AT^{32}P$ assays with Glasstide substrate we found that cGMP increased the Vmax of the kinase by 45±1%, with little affect on its Km for substrate. In contrast disulfide oxidation had little influence on Vmax, but decreased its Km for substrate from 247 to 37μM. This decrease in Km would account for a H_2O_2-induced activation of PKG. It also explains the translocation of disulfide PKG from the cytosol to membrane and myofilament fractions (sites where many PKG substrates are located) we observed. Vasorelaxation, PKG oxidation and PKG substrate (VASP) phosphorylation also occurred in aortic rings treated with H_2O_2. NO (0.1μM-0.1mM spermine NONOate) mediated relaxation was attenuated ($P<0.01$) by inhibition of soluble guanylate cyclase (ODQ, 5μM) or PKG (Rp-8-Bromo-cGMPS, 100μM). In contrast H_2O_2 relaxation was only inhibited by Rp-8-Bromo-cGMPS, but not ODQ, indicating H_2O_2 relaxation is independent of cGMP, consistent with direct activation by oxidation. Cys-42 to Ser PKG mutants expressed in HEK cells confirmed this was the redox active residue, as it did not disulfide bond basally or with H_2O_2. H_2O_2 causes vasodilation and operates as an endothelium-derived hyperpolarizing factor distinct from NO, by activating PKG by a novel cGMP-independent mechanism involving oxidation.

BIOTINYL DIMEDONE (BD) - A NOVEL REAGENT FOR STUDYING PROTEIN SULFENIC ACIDS

Charles Rebecca, May Georgina L, Free Paul, Gaffney Piers RJ, Eaton Philip.

Protein sulfenic acids are reactive intermediates in the catalytic cycles of many enzymes, as well as the formation of other redox states. Sulfenates also have potential in post-translational regulation. Dimedone (5,5-dimethyl-1,3-cyclohexanedione) is used in vitro to study sulfenation of purified proteins, selectively 'tagging' them with monitoring by MS. However this reagent is of little use in complex protein mixtures, as selective monitoring of labeling is not possible. To address this issue, we synthesized BD, keeping the sulfenate reactivity but adding a biotin tag. Biotin-amido(5-methyl-5-carboxamido cyclohexane 1,3-dione) tetragol (BD) was prepared in six steps, combining 3,5-dimethoxybenzoic acid (Birch reduction, ultimately leading to the dimedone unit with a carboxylate functionality), 1-amino-11-azido-3,6,9-trioxaundecane (a differentially substituted tetragol spacer) and biotin. We loaded BD (0.1mM, 30min) into rat ventricular myocytes, treated them with H_2O_2 (0.1-10,000μM, 5min) and monitored derivatization on Westerns using streptavidin-HRP. There was a dose-dependent increase in labeling of multiple proteins, which was maximal at 0.1 or 1mM H_2O_2, declining sharply below basal with 10mM treatment. Cell wide labeling was observed in fixed cells, probed with avidin-FITC using a fluorescence microscope. Similar H_2O_2-induced labeling was observed in isolated rat hearts. Hearts loaded and subjected to hypoxia showed a striking (almost absent) loss of labeling, highlighting the protein sulfenates as oxygen sensors. These proteins have been avidin-agarose purified for identification using mass spectrometry.

A DETAILED MAP OF OXIDATIVE POST-TRANSLATIONAL MODIFICATIONS OF HUMAN P21RAS USING FOURIER TRANSFORM MASS SPECTROMETRY

Zhao C, Sethuraman M, Clavreul N, Kaur Parminder, Cohen RA, and O'Connor PB.

Objective: Develop a method to create a detailed map of oxidative post-translational modifications of human P21ras with high resolution and high accuracy Fourier Transform Mass Spectrometry (FTMS).
Methods: Recombinant p21ras was treated with peroxynitrite or GSSG and analyzed by "bottom-up" and/or "top-down" MS strategies.
Results: 5 oxidized Met, 5 nitrated Tyr and at least 2 oxidized Cys (including Cys118) were identified in peroxynitrite-treated p21ras using bottom up analysis. Cys118 was revealed as the major glutathiolated Cys in glutathiolated p21ras using top down analysis.
Conclusion: This study combined bottom-up and top-down MS methods on a recently developed FTMS instrument to study the post-translational modifications of p21ras. Peroxynitrite-treated p21ras included multiple complex modifications that, despite their complexity, were analyzed using a bottom up MS/MS approach. Also, from top-down analysis, Cys-118 is identified as the major glutathiolated cysteine on p21ras. This study provides a paradigm for an effective and efficient method for mapping the post-translational modifications of proteins. With appropriate software and search engines, this method also can be used to identify unknown proteins and peptides.

OPTM Oral Session Abstracts

Session 6:

GRAND ROUNDS

SYSTEMS BIOLOGY AND PERSONALIZED MEDICINE

Loscalzo Joseph, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

The conventional scientific approach involves experimental reductionism followed by inductive generalization. While this Cartesian paradigm has been effective in understanding and advancing the field of biomedicine, it is inconsistent with biological reality. Most biological systems respond to multiple inputs that vary simultaneously and can interact, i.e., they are complex systems and, thus, require different experimental and analytical approaches to predict their behavior optimally. Systems biology is defined most broadly as the science of integrating genomic, proteomic, metabolomic, biochemical, cellular, physiological, and clinical data to create a network that predicts biological phenomena. In this presentation, I will discuss the application of systems biology and network modeling to human pathobiology, and will consider the potential utility of these principles in predicting and treating human disease with special emphasis on their application to the individual patient.

OPTM Poster Session Abstracts

Note:

Grouped here are abstracts of poster-only presentations. Abstracts of poster-related oral presentations, from the Oral Session, are grouped in preceding pages according to order of presentation.

DETECTION AND IDENTIFICATION OF *S*-GLUTATHIOLATED PROTEINS IN ENDOTHELIAL CELLS EXPOSED TO OXIDANTS

Clavreul Nicolas, Dauly Claire, McComb Mark E, Costello Catherine E, and Cohen Richard A.

Bovine Aortic Endothelial Cells were loaded with biotin labeled glutathione ethyl ester and exposed to oxidant conditions (Lysophosphatidylcholine). The cells were harvested in a buffer containing iodoacetamide to block excess of glutathionyl and proteinyl free cysteines, and proteins purified on PD 10 columns. The *S*-glutathiolated proteins were pulled-down using streptavidin beads and released in 95% formamide and 10 mM EDTA. Samples were run consecutively on a non porous C18 column and the fractions were collected every 15 sec in a Beckman PF-2D chromatographic system. Because the procedure leaves the biotin labeled glutathione linked to the reactive cysteine, the labeled cysteine could be identified by peptide mass. The theoretical mass of the biotin-*S*-glutathione adduct as well as the ability for the modified peptides to be detected in the TOF spectrometer was verified using recombinant p21ras chemically *S*-glutathiolated in presence of oxidized biotin labeled glutathione. The adduct (+531 or +559) was easily detectable with MALDI-TOF and Q-TOF MS/MS. *S*-glutathiolated proteins contained in 10 mg of protein lysate purified using this method revealed that only a small fraction of the proteome was significantly *S*-glutathiolated in these conditions confirming the specificity of this modification. Mass spectrometric analysis identified proteins already known to be *S*-glutathiolated as well as numerous new candidates.

PROTEIN CHARACTERIZATION IN 3-DIMENSIONS VIA 2-D HPLC AND MASS SPECTROMETRY

Dauly Claire; Perlman David H; Costello Catherine E; McComb Mark E

Objective: Develop methodology for 2D-protein HPLC coupled with intact protein MALDI and PMF for differential proteomics.
Methods: Endothelial cells were subjected to ultra-centrifugation, washed at high pH to remove membrane associated proteins, de-lipidated and separated by SEC and RP chromatography. Fraction were characterized by MALDI-TOF MS for protein mass and PMF.
Results: Our 2-D HPLC methodology yielded orthogonal separation and gave high peak capacity. Post separation, proteins were directly characterized as intact entities by MS, enabling further resolution of several proteins: hence the 3rd dimension of separation was provided by MS. Differences between theoretical and experimental masses were attributed to the presence of isoforms, truncation and post-processing/post-translational modifications. The proteins were then digested by trypsin and peptide mass fingerprint (PMF) analysis was performed using MALDI-TOF MS, allowing correlation of the intact protein masses with the PMF results.
Conclusions: Overall we demonstrated that the 2D-HPLC MALDI-TOF MS system allows complex protein samples to be rapidly fractionated and identified by integration of the information on intact molecular weights and peptide mass database searches. This project was funded by NIH grants P41-RR10888, S10-RR15942, and NHLBI contract N01-HV-28178 (to C.E.C.).

EPITOPE IDENTIFICATION OF 3-NITRO-TYROSINE PEPTIDES BY MONOCLONAL 3-NITRO-TYROSINE ANTIBODIES USING PROTEOLYTIC EPITOPE-EXCISION MASS SPECTROMETRY METHOD

Dragusanu M, Petre A, Przybylski M

Tyrosine nitrations in proteins play an important role in diseases such as Alzheimer's disease, Parkinson, atherosclerotic and the bronchio-alveolar diseases. Prostacyciln synthase (PGI_2), together with nitric oxide (NO), is an essential factor for a functional endothelium by providing anti-aggregatory, antiadhesive, anti-proliferative and vasorelaxing properties to the vessel wall. Specific nitration of Tyrosine residues have been detected by bovine prostacyclin synthase (PCS) at the active site Tyr-430 upon peroxynitrite treatment using proteolytic digestion and ESI-FTICR-MS at isotopic resolution. After identification of specific nitration site Tyr-430 several nitrated peptide were synthesized by SPPS according to Fmoc strategy and characterized by high resolution mass spectrometry in combination with immunological methods. The specificity of 3-nitro tyrosine antibody to the nitrated and non-nitrated peptide was characterized by Dot blot and affinity-mass spectrometry. The major goal of this study was to identify the epitope motif of the nitrated PCS peptides bound to the 3-nitrotyrosine antibody. We performed epitope-excision mass spectrometry using a micro affinity column of a monoclonal anti-3-nitroTyr antibody. The protease digests the PCS peptide, the epitope-antibody complex was dissociated and the fragments were analyzed by mass spectrometry. The mass spectra of the elution fraction show the peptide fragment containing 3-nitro-tyrosine which was bound to the 3-nitrotyrosine antibody column.

PROTEOME CHANGES OF RAT AORTA ASSOCIATED WITH AGING

Fu Zongming, Wang Mingyi, O'Meally Robert, Zhang Jing, Lakatta Edward and Van Eyk Jennifer

Objective: To understand aging effect on the vascular proteome.
Methods: A comprehensive quantitative two-dimensional gel electrophoresis (2DE) proteomic analysis (pH4-7 and 6-11; 10%bis-TRIS) was carried out on aortas obtained from young (8 months) and old (30 months) rats. Abdominal aortas were isolated from healthy young (n=7) and old (n=7) rats. Gels were visualized with silver stain and using 2-D DIGE and protein identifications were carried on the tryptic fragments using MALDI-TOF and/or electrospray ion trap mass spectrometry.
Results: Greater than 300 proteins have been identified from 2-D gels and approximately 20 proteins were found to be present at different quantities between the two age groups. The proteins having different abundance play important roles in metabolism, anti-oxidant defense and apoptosis. For example, milk fat globule-EGF factor 8 Protein (MFG-E8), which is a factor that links apoptotic cells to phagocytes, is found to be more abundant in old rat aorta. Glutathione S-transferase Mu 2, aldehyde dehydrogenase, and transketolase are found to be less abundant in the aortas with age.
Conclusion: Selective changes to antioxidant defense, metabolism and apoptosis pathways occur in old aorta.

A PROTEOMIC ANALYSIS OF HUMAN LIVER MITOCHONDRIA DURING NASH

Johnson Michelle S, Zaragoza Corinne, Darley-Usmar Victor, Abrams Gary, and Landar, Aimee

Objectives: Obesity and type-2 diabetes are associated with a spectrum of nonalcoholic fatty liver (NAFLD) pathologies encompassing fatty liver (FL) and nonalcoholic steatohepatitis (NASH). Mitochondrial perturbations in NASH have been reported based upon the decrease in activity of the proteins of oxidative phosphorylation. Methods: Proteomics approaches were used to examine mitochondria prepared from human liver biopsies to test the hypothesis that changes in mitochondria associated with NASH impact the mitochondrial proteome.The first step was to determine the effects of freezing the biopsy on the proteomic pattern. Mitochondria prepared from fresh or frozen biopsies were analyzed by Blue Native PAGE, where intact mitochondrial protein complexes are separated in the first dimension, followed by SDS-PAGE which resolves individual subunits in the second dimension. Results: Interestingly, the quality and pattern of the protein complex bands obtained from both samples were remarkably similar. In the next series of experiments, a comprehensive proteomic analysis of liver mitochondria isolated from patients with FL or NASH was performed using both 2D Blue Native PAGE and 2D-isoelectric focusing PAGE. Conclusions: These data will provide insight into the mechanisms through which mitochondria contribute to the progression from fatty liver to NASH. In addition, they may provide the basis for the development of potential therapeutic markers and approaches to treatment to prevent these changes.

OxICAT: A GLOBAL TOOL TO MONITOR OXIDATIVE THIOL MODIFICATIONS IN PROTEINS

Leichert Lars I, Blackwell Tom, Gehrke Florian, Strahler John, Walker Angela, Xi Guohua, Jakob Ursula

Objectives: Oxidative stress is considered to be one of the major factors that contribute to the damaging effects of many diseases, among them the most common causes of death in the United States, heart disease, cancer and stroke. Very few techniques exist, however, that allow determining the proteins affected by oxidative stress and quantifying reliably the extent of oxidative damage.

Methods: We have now combined a differential thiol trapping technique with ICAT chemistry, a novel approach we termed OxICAT. With OxICAT we can specifically label all *in vivo* reduced cysteines in a sample with light ^{12}C-ICAT reagent and all *in vivo* oxidized cysteines in the very same sample with heavy ^{13}C-ICAT reagent. This allows us to use mass spectrometry to quantify the *in vivo* ratio of reversible thiol modifications in hundreds of individual proteins in a single experiment.

Results: In an animal model, we now used OxICAT to define the set of redox-sensitive proteins in brain tissue and to monitor the global changes in the thiol redox state of proteins that occur under hemorrhagic stroke.

Conclusions: The thiol oxidation state of protein cysteines as measured with OxICAT serves as a highly sensitive, quantifiable and specific read-out for the presence of reactive oxygen and nitrogen species in tissues and organs. OxICAT enables us to draw conclusions about redox potentials of proteins and allows us to pinpoint redox sensitive features in proteins down to the cysteine sidechain.

DECREASED OXIDATIVE STRESS IN ANGIOTENSIN II-INFUSED GLUTAREDOXIN-1 KNOCKOUT MOUSE

Matsui Reiko, Bachschmid Marcus, Clavreul Nicholas, Xu Shanqin, Maitland-Toolan Karline, Pimental David, Ho Yo-Shi, Cohen Richard A

Glutaredoxin-1 may regulate oxidative signaling by catalyzing de-glutathiolation of some proteins. To examine roles of glutaredoxin in oxidative stress, we infused angiotensin II (Ang II, 0.7 mg/kg/min) for 6 days in glutaredoxin-1 knockout (GRx KO) and wild type (WT) control mice. Blood pressure and body weight were not different between two groups, but heart weight was significantly smaller in the GRx KO mice. Immunohistochemistry for nitrotyrosine showed less staining of GRx KO aorta, and oxidation of dihydroethidium was less fluorescent in GRx KO aortas. These results unexpectedly indicate less oxidants in GRx KO mouse after Ang II infusion. In preliminary experiments, biotinylated iodoacetamide labeling in aortic homogenates indicate lower free thiols of β-actin in GRx KO. In addition, embryonic fibroblasts derived from GRx KO and WT mice were exposed to H2O2 and *S*-glutathiolation of cellular proteins was studied by Western blot using anti-GSH-protein adduct antibody. Staining with the antibody was prevented by DTT consistent with its specificity. The blots indicate that the cells from GRx KO show higher *S*-glutathiolation of proteins including actin. Polymerization of actin is known to be prevented by *S*-glutathiolation. Because NADPH oxidase is the major source of oxidants induced by Ang II in the aorta, we speculate that higher *S*-glutathiolation of actin results in impaired polymerization and membrane assembly of NADPH oxidase, resulting in less oxidant production by Ang II in GRx KO mice.

PATTERN-BASED IDENTIFICATION OF PROTEIN STRUCTURAL VARIATIONS BY MASS SPECTROMETRY

McComb Mark E, Hayete Boris, Huang Hua, Perlman David H, Luo Hongyuan, Skelton Timothy P, Steinberg Martin H, Chui David HK, Collins, James J, Costello Catherine E

Objective: Develop pattern-based identification for important protein structural changes in biological samples by MALDI-TOF-MS
Methods: We targeted hemoglobin for our initial studies. Whole blood was cleaned, digested with trypsin and subjected to MALDI-TOF MS.
Results: MALDI-TOF MS yielded high sequence coverage for both alpha and beta globin chains based on PMF analysis. Use of PCA and factor analysis yielded an additional level of detail within the analyses. We identified variants and modifications by a pattern-based identification methodology either in intensity or in isotopic peak distribution, or both. Structural variations identified include the beta globin chain mutation Asp52 to Asn with a 1 mass unit decrease and the beta globin chain Lys95 mutated to Glu with a 1 mass unit increase. Additionally, post-translational modifications have also been observed in these biological samples.
Conclusions: This complementary methodology combining pattern-based identification for MALDI-TOF-MS data PMF information has demonstrated its power for characterizing subtle primary structural changes in proteins and peptides. This project was funded by NIH grant P41 RR10888 and NHLBI contract N01 HV28178.

ON THE ROLE OF FREE RADICAL RE-ARRANGEMENTS IN PEPTIDE DISSOCIATION.

O'Connor Peter B, Lin Cheng, Cournoyer Jason J, Zhao Cheng

Electron capture dissociation (ECD) is a new method for sequencing peptides and characterizing their post-translational modifications, such as those generated by oxidative stress. The ECD method involves generation of a radical cation as one of the main dissociation products. Our recent results have shown that this radical is involved in multiple rearrangements during the ECD process, and we have managed to determine some of the features of this process through the use of some advanced mass spectrometry techniques. These results and their implications for sequencing peptides involved in oxidative stress will be discussed.

IDENTIFICATION OF OXIDATIVE POST-TRANSLATIONAL MODIFICATIONS OF PLASMA ALBUMIN IN PULMONARY HYPERTENSION OF SICKLE CELL ANEMIA

Odhiambo Adam, Perlman David, McComb Mark, Huang Hua, Costello Catherine E, Farber Harrison W, Steinberg Martin H, Klings Elizabeth S

Objective: Pulmonary hypertension (PH) in sickle cell anemia (SCA) is characterized by decreased nitric oxide bioavailability and increased oxidative stress. We hypothesized that, in patients with PH of HbSS, oxidative PTMs occur and are important in disease pathogenesis. To examine this hypothesis, we chose to use differential proteomics to study a large-abundance protein, albumin, for PTMs that might reflect the presence of more widespread protein oxidative damage. Methods: Plasma was obtained from subjects with: 1) SCA and PH; 2) SCA without PH; 3) Pulmonary Arterial Hypertension (PAH); 4) no cardiopulmonary disease (n=4) and separated into albumin enriched and albumin-depleted fractions. The albumin fraction was studied by MALDI-TOF MS and LC-MS/MS. MS database searches were performed and PTMs identified. Results: In the albumin fraction, we identified differentially expressed peaks on peptide 146-159 only in PAH and SCA+PH samples consistent with a malondialdehyde (MDA) adduct; results were confirmed by Western analysis. Conclusions: We have utilized MS to identify a MDA adduct in plasma albumin which appears to be a common link between PH of SCA and PAH. Further use of this technology will likely lead to the identification of other oxidative targets in PH of SCA and a greater understanding of disease pathogenesis.

THE IDENTIFICATION OF CARBOXYLASES AS POTENTIAL TARGETS FOR POST-TRANSLATIONAL MODIFICATION BY CYCLOPENTENONE ELECTROPHILIC LIPIDS IN ENDOTHELIAL CELLS

Oh JooYeun, Johnson Michelle, Darley-Usmar Victor and Landar Aimee
Department of Pathology, Center for Free Radical Biology,
University of Alabama at Birmingham, U.S.A.

Objectives: Dysfunction of redox signaling has been shown to be involved in pathologies such as atherosclerosis that are associated with increased formation of reactive lipid oxidation products, particularly electrophiles. These lipids contain functional groups that can react with the nucleophilic residues of proteins, particularly thiols, and induce post-translational modifications leading to change in function. We have shown in previous studies that electrophilic lipids colocalize to mitochondria, and in isolated mitochondria have identified some proteins which may be targets. However, proteomic approaches have not been successfully applied to identify lipid-protein adducts in endothelial cells. Methods: The focus of this study was to combine proteomic techniques with tagging strategies using biotin or BODIPY to follow lipid-protein adduct formation. Results: Our results show that one of the protein targets in endothelial cells is a member of the carboxylase protein family. Conclusions: Interestingly, carboxylases contain active site thiol residues, which we postulate may be reactive with electrophilic lipids. Carboxylases are known to play an important role in a number of pathways including glycolysis, TCA cycle and fatty acid synthesis. The potential implications of these findings will be discussed.

SIMPLE, RAPID COUPLING OF PROTEIN HPLC TO MALDI-TOF MS USING AN ON-TARGET NINETY-SIX-WELL ELASTOMERIC DEVICE

Perlman David H, Huang Hua, Dauly Claire, Costello Catherine E, McComb Mark E

Objectives: Preparation of complex mixtures for MS-based proteomics commonly involves sample fractionation, followed by multiple steps of sample manipulation including fraction collection, concentration, buffer exchange, digestion with a protease, desalting, and, in the case of MALDI-MS, matrix/analyte co-crystallization on target. Here, we demonstrate the application of a novel, simple, and inexpensive (non-robotic), on-target ninety-six-well elastomeric technology, the BD™ MALDI Sample Concentrator, for use in conducting one-pot sample preparation from the collection of HPLC fractions to MALDI-MS analyses. **Methods:** Protein solutions were added directly or protein mixtures were fractionated by millibore HPLC and collected directly into the device. Samples were subjected to a one-pot protocol of concentration, trypsin digestion, cleanup, and matrix co-crystallization, which was optimized for proteolysis and sample recovery. Resulting spots were subjected to MALDI-TOF MS and PMF analyses. **Results:** We have demonstrated that this methodology enables the rapid digestion of proteins and the analysis of peptides from low starting amounts of protein. Furthermore, we have demonstrated its efficacy through the rigorous characterization of an HPLC-fractionated protein mixture by MALDI-TOF MS and PMF analyses.

Conclusions: This methodology allows the inexpensive and facile coupling of HPLC to MALDI-MS with minimal sample preparation time, little sample handling, and without costly robotics.

IMMUNE VS. THROMBOTIC STIMULATION OF PLATELETS DIFFERENTIALLY REGULATES INTRACELLULAR PROTEIN ASSOCIATIONS AND PROTEIN RELEASE: A PROTEOMICS ANALYSIS

Rex S, Perlman DH, Vitseva O, Blair PS, McComb ME, Costello CE, Freedman JE
Whitaker Cardiovascular Institute and Cardiovascular Proteomics Center, BUSM, Boston MA, USA

Objectives: In addition to hemostasis, platelets mediate inflammation and clearance of bacteria from the bloodstream. These processes involve platelet granular release and cytoskeletal rearrangements. It is not known if the activation of platelets by thrombotic or immune pathways leads to differential regulation of protein interactions and/or granule release. Thus, these pathways were compared using a proteomics approach with focus on FXIIIA as a candidate protein.
Methods: We immunoprecipitated FXIIIA from resting and activated platelets to study FXIIIA-associated proteins by gel electrophoresis, followed by mass spectrometric identification of protein spots. Results were confirmed by Western blotting and confocal microscopy. Proteins released after stimulation with Pam_3CSK_4 or thrombin were analyzed by 2D gels.
Results: We identified 6 proteins that associate with cytosolic or granular FXIIIA in resting platelets. Several novel protein interactions were found such as FAK, GAPDH, and gelsolin. Specific protein associations were differentially altered by immune or thrombotic stimulation including gelsolin (20% decrease vs. no change) and GAPDH (80% vs. 30% decrease). In addition, about 80 released proteins from Pam_3CSK_4- vs. thrombin-stimulated platelets were differentially displayed, including FXIIIA.
Conclusions: Our findings highlight the differences in the platelet's inflammatory vs. thrombotic responses.

IDENTIFICATION AND QUANTIFICATION OF OXIDATIVE POST-TRANSLATIONAL MODIFICATIONS OF CYSTEINE THIOLS OF P21RAS THAT ARE RESPONSIBLE FOR REDOX MODULATION OF ITS ACTIVITY

Sethuraman Mahadevan, Clavreul Nicolas, Huang Hua, McComb Mark E , Costello Catherine E and Cohen Richard A. Boston University Medical School

Objectives: To identify and quantitate the reversible and irreversible oxidative post-translational thiol modifications of p21ras caused by peroxynitrite ($ONOO^-$) and oxidized glutathione (GSSG) that are responsible for redox modulation of its activity. Methods: H-p21ras (10-20 µg) was treated with peroxynitrite ($ONOO^-$, 100-250 µM) for 5 min at 37 ^oC or with oxidized glutathione. p21ras activity was assayed by association with Raf-1 and by GTP/GDP exchange. H-p21ras under control and oxidant stress conditions was thiol labeled with isotope-coded affinity tag (ICAT) and analyzed by ESI QoTOF LC-MS/MS. Results: The activity of p21ras was significantly increased following exposure to GSSG but not with $ONOO^-$ alone. In $ONOO^-$-treated samples, ICAT labeling of Cys^{118} was decreased to 47 % whereas labeling of the non-reactive Cys^{80} was not affected significantly. Also Cys^{118} has been identified as a site for *S*-glutathiolation and extent of the glutathiolation of Cys^{118} by GSSG was estimated by our ICAT method to be 53 % that accompanies activation of p21ras. The terminal cysteines were also shown to be *S*-glutathiolated by 85 %, suggesting that their normal prenylation in vivo might be influenced by oxidative modification. Conclusions: By applying our ICAT procedure we even identified and quantified glutathiolated Cys^{118} and other terminal cysteines of p21ras.

PROTEOMIC ANALYSIS OF SMOOTH MUSCLE CELL DIFFERENTIATION IN A P19 MODEL SYSTEM: PRELIMINARY RESULTS

Strong Bethany L, Wang Guoshen, Pattabiraman Vaishnavi, Anderson Leonard

In the United States, African Americans suffer more than any other minority group from cardiovascular diseases. The P19 embryonal carcinoma stem cell line is obtained from teratocarcinomas in mice. Studying the differentiation of P19 cells is similar to observing the process of vascular regeneration and acts as a model system for experimentation. The long term application for cells of this nature is transplantation and restoration of blood vessel function as an alternative to vascular grafting. In this study, the proteomic changes that occur within the P19 cell line during the process of differentiation were determined. It was hypothesized that differences would exist and be more indicative of vascular smooth muscle for the treated cells. Using the Sigma Panorama Antibody Array protocol, the total protein content from adherent P19 cells was extracted on Day0 - Day3, treatment with retinoic acid (RA) or vehicle. The samples were then labeled with Cy3 (vehicle) or Cy5 (RA) fluorescent dye and hybridized on the antibody array slide. The four slides were then scanned with an Agilent scanner and analyzed. Differences in protein abundance during smooth muscle cell differentiation were successfully detected. The total number of exclusive proteins was 27, 0, 3, and 3, for Days 0, 1, 2, and 3, respectively. Protein interaction networks revealed potential complex protein signaling events which may be important for vascular smooth muscle cell lineage commitment. Bioinformatics analysis revealed significant cell growth and development function during vascular smooth muscle lineage commitment.

BUDSS: A SOFTWARE SHELL FOR MS DATA PROCESSING AND MANAGEMENT

Su Yang, Huang Sequin, Huang Hua,
Perlman David H, West James, Costello Catherine E,
McComb Mark E

Objective: Here we present a stand-alone software application named BUDSS (Boston University Database Search Shell) which is designed to aid the processing and management of various MS data files.
Methods: BUDSS was developed using Microsoft Visual Studio 6.0. MSXML 4.0 was used as an XML parser to decode Base64 encoded peak list data in the mzXML/mzData file. A C++ library was also built to improve computational efficiency of decoding.
Results: We tested BUDSS using large volumes of MALDI-TOF MS, MALDI-FT MS and LC MS/MS data sets obtained in house. After initial processing using vendor-specific programs BUDSS converted processed files to the formats of several commercially and publicly available search engines. Files were then submitted for protein identification to the search engines with the search settings specified by the user. Results files are automatically saved in HTML format and can then be viewed directly inside the program. Both data conversion and database searching were run in the batch mode to handle large amount of data with little manual intervention (BUPID).
Conclusions: BUDSS provides an easy-to-use graphical interface for performing MS data processing and management in unattended batch mode. It can also be easily be expanded for more MS data types and linked to more database search engines.

NOVEL FLUORESCENT TAG FOR LABELING OXIDATIVE POST-TRANSLATIONAL MODIFICATIONS OF TYROSINE RESIDUES IN AGING TISSUE.

Thorson Maria, Hong Sung Jung, Killmer Jacque, Pennington Justin, Li Xiaobao, Stobaugh John, Schöneich Christian.

Objectives: Nitrotyrosine and dihydroxytyrosine are hallmarks for protein oxidation, which leads to changes in protein structure and function. An enrichment method using benzylamine-dependent chemistry to label nitrotyrosine and dihydroxytyrosine for both fluorescence detection and affinity-based separation methods has been developed. Successful labeling of model proteins has been observed, and the present study applies the method to *in vivo* samples from both old and young rats in order to study age-dependent differences in protein oxidation.
Methods: Soluble protein fractions from rat cardiac and skeletal muscle tissue are reacted with potassium ferricyanide and excess benzylamine to fluorescently label dihydroxytyrosine. In order to label nitrotyrosine, samples are first reduced with dithionite. These samples are desalted with zeba cartridges prior to reaction with potassium ferricyanide and excess benzylamine. After tagging, all samples are desalted with zeba cartridges just prior to chromatography with fluorescence detection. Mass spectrometry is used to detect and identify specific sites of modification.
Results: Tagged proteins have been separated using chromatography with fluorescence detection and identified by mass spectrometry.
Conclusions: This method allows for the enrichment of nitrotyrosine and dihydroxytyrosine, which are in low abundance *in vivo*. This enrichment aids in detection and identification by mass spectrometry.

HIGH GLUCOSE PREVENTS INHIBITION OF SMOOTH MUSCLE CELL MIGRATION BY NITRIC OXIDE: ROLE OF SERCA CYSTEINE-674

Tong Xiaoyong, Ying Jia, Xu Shanqin, Pimental David, Adachi Takeshi, Cohen Richard

Vascular smooth muscle cell (VSMC) migration is an important pathological process in neointimal hyperplasia and atherosclerosis, and acceleration of these processes underlies the higher rates of cardiovascular disease in diabetes. This study tested the role of SERCA Cys-674 in the inhibition of VSMC migration by nitric oxide and determined the effect of high glucose (HG). Rat aortic VSMC were cultured in normal glucose and SERCA WT, C674S mutant, or GFP were overexpressed with adenovirus. Two days later, cells were switched to medium containing normal 5.5, or 25 mmol/L glucose (HG) for an additional 3 days. To study cell migration a scratch wound was made 5 min after adding the NO donor, SNAP or 24 h after inducing iNOS with IL-1β. In normal glucose, SNAP or IL-1β significantly inhibited migration in cells transfected with GFP or SERCA WT, but not with SERCA C674S mutant. The iNOS inhibitor, L-NIL, blocked the inhibition of migration by IL-1β confirming the role of endogenous NO. In HG, NO failed to inhibit migration of cells expressing either GFP or SERCA C674S mutant. In contrast, overexpression of SERCA WT maintained the inhibition of migration by NO despite exposing the cells to HG. Thus, HG prevents the inhibition of migration by NO that depends on SERCA Cys-674. The impaired response to NO caused by HG can be rescued by overexpression of SERCA, but this also depends on Cys-674. These results suggest that failure of NO to inhibit migration in diabetes might be due to abnormal redox regulation of SERCA Cys-674.

CHRONIC HYPOXIA INCREASES CARBONYLATED PROTEINS IN RAT PULMONARY ARTERY

Wong Chi Ming and Suzuki Yuichiro Justin

OBJECTIVES: Chronic hypoxic pulmonary hypertension is associated with profound vascular remodeling and vasoconstriction. It has been shown that the increased generation of reactive oxygen species (ROS) induced by hypoxia in smooth muscle cells is linked to hypoxic pulmonary vasoconstriction and vascular remodeling. The objective of this study is to examine the downstream target proteins modified by hypoxia-induced ROS. METHODS: Male SD rats were placed in a hypoxic chamber and exposed to either normoxia or hypoxia 10% O2 for 0, 2, 7 and 15 days to induce hypoxic pulmonary hypertension. Then, rats were killed for dissection of the pulmonary arteries followed by protein extraction. The whole-cell lysates were derivatized by reaction with dinitrophenylhydrazine (DNPH), and separated by polyacrylamide gel electrophoresis followed by Western blotting. RESULTS: The total protein carbonyl contents in pulmonary arteries of the hypoxia-treated rats did not have significant change. However, analyses of individual bands revealed that the levels of carbonylation of the band 15 (14 kDa), band 16 (11 kDa) and band 17 (9 kDa) were increased at 2 days and then sustained at 7 days and 15 days. The levels of carbonylation of the band 7 (52 kDa), band 8 (47 kDa) and band 14 (21 kDa) transiently increased at 7 days while band 11 (30 kDa) increased at 7 days and sustained at 15 days. The remaining bands did not have any significant change. CONCLUSIONS: Hypoxia treatment to the rats probably increased ROS generation to enhance the levels of the carbonylated proteins in pulmonary arteries.

DETECTION OF SULFONIC OXIDATION OF SERCA CYSTEINE-674 BY IMMUNOHISTOCHEMISTRY

Xu Shanqin, Jiang Bingbing, Ying Jia, Tong Xiaoyong, Adachi Takeshi, McIntosh Eric, Cohen Richard

Objectives: The SERCA Cys-674 thiol is reversibly *S*-glutathiolated under physiological conditions, but subject to irreversible oxidation in pathological conditions. This study aimed to develop an immunohistochemical method to identify the irreversible sulfonic oxidation of SERCA Cys-674 in tissues exposed to oxidative stress. **Methods:** A polyclonal anti-peptide SERCA Cys-674-SO_3H antibody was developed against an immunogenic peptide synthesized with sulfonated Cys-674 and affinity-purified after eliminating antibodies against the same peptide with reduced Cys-674. Immunohistochemical staining was done on atherosclerotic diabetic pig and rabbit aorta, and young and aging rat skeletal muscle. Among these, Cys-674 sulfonic acid has been verified by mass spectrometry. **Results:** Treatment of normal tissue sections with H_2O_2 resulted in intense positive staining. Staining for SERCA Cys-674-SO_3H was either negative or rare in aortic smooth muscle from normal rabbits or pigs. However, there was intense staining in the aorta of diseased animals, particularly in the atherosclerotic plaque and underlying smooth muscle. Incubating normal rabbit aorta for 6 h in high, but not normal glucose resulted in intense staining of endothelium and smooth muscle. In rat skeletal muscle, staining increased with age (6< 22 < 34 mos). Staining with non-immune rabbit IgG was negative in simultaneous control sections in all of the above tissue sections. **Conclusions:** Sulfonic thiol oxidation of SERCA Cys-674 in pathological conditions can be detected by immunohistochemistry.

OPTM Participant Directory

Aesif, Scott W. M.S.
MD/PhD Student
Pathology
University of Vermont
HSRF 214
89 Beaumont Avenue
Burlington, VT 05405 USA
802-656-8976
scott.aesif@uvm.edu

Anathy, Vikas Ph.D.
Pathology
University of Vermont College of Medicine
89 Beaumont Avenue 203 E Given Bldg.
Burlington, VT 05405 USA
802-656-8976
vikas.anathy@med.uvm.edu

Andringa, Kelly K. Ph.D.
Postdoctoral Trainee
Environmental Health Sciences
University of Alabama at Birmingham
1530 3rd Ave. South
Room 629 RPHB
Birmingham, AL 35294 USA
(205) 913-5237
andringa@uab.edu

Aulak, Kulwant Ph.D.
Project Staff
Pathobiology
Cleveland Clinic Res. Foundation
9500 Euclid Ave.
Cleveland, Oh 44195 USA
216-444-5602
aulakk@ccf.org

Bachschmid, Markus M.
Vascular Biology
Boston University School of Medicine
650 Albany Street
Boston, MA 02118 USA
617-638-2797
bach@bu.edu

Barnes, Stephen Ph.D.
Professor
Pharmacology/Toxicology
University of Alabama at Birmingham
1918 University Blvd.
MCLM 452
Birmingham, AL 35294 USA
205-934-7117
sbarnes.uab@gmail.com

Bartoli, Manuela Ph.D.
Associate Professor
Ophthalmology
University of South Carolina
4 Medical Park, Suite 301
Columbia, SC 29203 USA
706-267-3107
mbartoli@gw.med.sc.edu

Batthyany, Carlos M.D., Ph.D.
Research Instructor
Pharmacology
University of Pittsburgh
200 Lothrop St. BST E1314
University of Pittsburgh
Pittsburgh, PA 15213 USA
412-648-9671
batti@pitt.edu

Beckman, Joseph S. M.D.
Professor
Linus Pauling Institute
Dept. of Biochemistry and Biophysics
Oregon State University
2011 Agricultural and Life Sciences
2141 ALS
Corvallis, OR 97331 USA
541-737-8867
Joe.Beckman@oregonstate.edu

Benjamin, Emelia J. M.D.
Professor
Framingham Heart Study
Boston University School of Medicine
73 Mt Wayte Ave.
Framingham, MA 01702 USA
617-638-8968
emelia@bu.edu

Benjwal, Sangeeta
Department of Biophysics
Boston University School of Medicine
710 Albany Street
Boston, MA 02118 USA
617-638-4247
sbenjwal@bu.edu

Bhatnagar, Aruni Ph.D.
Professor
Medicine-Cardiology
University of Louisville
421F Delia Baxter Bldg, MS06-09
580 So. Preston St.
Louisville, KY 40202 USA
502-852-5966
aruni@louisville.edu

Bigelow, Diana Ph.D.
Staff Scientist
Cell Biology & Biochemistry
Pacific Northwest National Laboratory
PO Box 999, MSP7-56
Richland, WA 99352 USA
509-376-2378
diana.bigelow@pnl.gov

Biolo, Andreia M.D.
Cardiology
Boston University School of Medicine
88 East Newton Street
Cardiology C818
Boston, MA 02118 USA
617-638-8738
abiolo@bu.edu

Blakely, Kent
Student
Biology
UCLA
250 De Neve Dr.
Los Angeles, CA 90024 USA
310-975-9702
kentmoney@gmail.com

Bonacci, Gustavo Ph.D.
Posdoctoral Associate
Pharmacology
University of Pittsburgh
200 Lothrop St. BST E1314
University of Pittsburgh
Pittsburgh, PA 15213 USA
412-648-9671
gub4@pitt.edu

Bottari, Serge P. M.D., Ph.D.
Associate Professor
Cell Biology
Medical School, Grenoble
Faculté de Médecine
Domaine de la Merci
La Tronche, 38700 France
+33 476 63 75 17
Serge.Bottari@ujf-grenoble.fr

Bourque, Karen M.
Pharmacology
Boston University School of Medicine
205 Great Rd. C8
Acton, MA 01720 USA
617-665-5417
kbourque@wyeth.com

Branchaud, Bruce Ph.D.
Director
Global Chemistry
Molecular Probes/Invitrogen
29851 Willow Creek Rd.
Eugene, OR 97402 USA
541-335-0309
bruce.branchaud@invitrogen.com

Burgoyne Jr, Joseph
Cardiology, Biochemistry
Kings College London
The Rayne Institute
St Thomas' Hospital
London, SE1 7EH UK
+44 (0) 207-188-0969
joseph.burgoyne@kcl.ac.uk

Castillo, Leticia M.D.
Pediatrics
Baylor College of Medicine
Texas Children's Hospital
6621 Fannin St. WT6
Houston, Texas 77030 USA
832-826-6230
lcastill@bcm.tmc.edu

Charles, Rebecca
Cardiology, Biochemistry
King's College London
The Rayne Institute
St Thomas' Hospital
London, SE1 7EH UK
+44 (0) 207-188-0969
rebecca.charles@kcl.ac.uk

Chen, Chun-An
DHLRI
Ohio State University
473 W. 12th Ave.
Columbus, OH 43210 USA
614-292-7375
chun-an.chen@osumc.edu

Chen, Yeong-Renn Ph.D.
Assistant Professor
Internal Medicine
Davis Heart and Lung Research Institute
The Ohio State University
607 DHLRI
473 W. 12th Ave.
Columbus, OH 43210 USA
614-688-4054
yeong-renn.chen@osumc.edu

Ckless, Karina Ph.D.
Pathology
University of Vermont
89 Beaumont Ave.
HRSF Room 214
Burlington, VT 05405 USA
802-656-8976
kckless@zoo.uvm.edu

Clavreul, Nicolas
Vascular Biology Unit
Boston University School of Medicine
650 Albany street
Boston, MA 02118 USA
617-638-7116
clavreul@bu.edu

Cohen, Richard A. M.D.
Professor
Medicine
Boston University School of Medicine
Vascular Biology Unit
650 Albany St. X720
Boston, MA 02118 USA
617-638-7115
racohen@bu.edu

Collinge, Mark Ph.D.
Research Scientist
Cardiology/Internal Medicine
Yale University
TAC S463, 333 Cedar Street
PO Box 208017
New Haven, CT 06520 USA
203-785-7544
mark.collinge@yale.edu

Collins, James J. Ph.D.
Professor
Biomedical Engineering
Boston University
44 Cummington St.
Boston, MA 02215 USA
617-353-0390
jcollins@bu.edu

Colucci, Wilson M.D.
Professor and Chief, Cardiovascular Medicine
Clinical Cardiology
Boston University School of Medicine
715 Albany St. C-8
Boston, MA 02118 USA
617-638-8706
wilson.colucci@bmc.org

Costello, Catherine E. Ph.D.
Professor
Biochemistry
Boston University School of Medicine
670 Albany Street, Rm 511
Boston, MA 02118 USA
617-638-6490
cecmsms@bu.edu

Cournoyer, Jason J.
Graduate student
Chemistry
Boston University School of Medicine
670 Albany St.
Boston, MA 02118 USA
617-638-6760
jcournoy@bu.edu

Cunningham, Jacobi I. B.S.
Ph.D. Candidate
Pharmacology and Experimental Therapeutics
Boston University School of Medicine
715 Albany Street L-613
Boston, MA 02118 USA
617-438-4956
cunningj@bu.edu

Darley-Usmar, Victor Ph.D.
Professor
Pathology
University of Alabama at Birmingham
901 19th Street Southq
BMR2 room 312
Birmingham, AL 35294 USA
205-975-9686
darley@path.uab.edu

Dauly, Claire M.S.
Research Assistant
Cardiovascular Proteomics Center
Boston University School of Medicine
670 Albany Street
Boston, MA 02118 USA
617-614-8220
cdauly@bu.edu

Deng, Haiteng Ph.D.
Director
Proteomics Resource Center
Rockefeller University
1230 York Ave
New York,, NY 10021 USA
212-327-7525
dengh@rockefeller.edu

Dragusanu, Mihaela B.Sc.
Student
Analytical Chemistry
University of Konstanz
Univeritaetsstrasse,10,
Konstanz, Germany 78467 Germany
0049-7531-883391
mihaela.dragusanu@uni-konstanz.de

Eaton, Philip Ph.D.
Department of Cardiology
King's College London
The Rayne Institute
St Thomas' Hospital
London, SE1 8RH UK
44-20-7928-9292
philip.eaton@kcl.ac.uk

Eliuk, Shannon B.Sc.
Student
Pharmacology & Toxicology
University of Alabama at Birmingham
MCLM 453
1918 University Blvd.
Birmingham, AL 35294 USA
205-975-3244
sme@uab.edu

El-Remessy, Azza Ph.D., R.Ph.
Assistant Professor
Clinical Pharmacy
University of Georgia
CJ-1033
Medical College of Georgia
Augusta, GA 30912 USA
706-721-6760
aelremessy@mcg.edu

Evangelista, Alicia BS
Molecular Medicine
Boston University
43 Glenville Ave. #6
Allston, MA 02134 USA
203-215-0159
a.m.evangelista@gmail.com

Farber, Harrison W. M.D.
Professor
Pulmonary Center
Boston University School of Medicine
715 Albany St. R-2
Boston, MA 02118 USA
617-638-4860
hfarber@bu.edu

Feelisch, Martin Ph.D.
Professor
Whitaker Cardiovascular Institute
Boston University School of Medicine
650 Albany St. X-305
Boston, MA 02118 USA
617-414 8150
feelisch@bu.edu

Freedman, Jane M.D.
Medicine
Boston University School of Medicine
700 Albany Street, W507
Boston, MA 02118 USA
617-638-4022
freedmaj@bu.edu

Freeman, Bruce A. Ph.D.
Professor and Chair
Pharmacology
University of Pittsburgh
E1340 Biomedical Science Tower
200 Lothrop St
Pittsburgh, PA 15213 USA
412-648-9319
freerad@pitt.edu

Friedman, Alan PhD
Assistant Professor
Environmental Medicine
University of Rochester
601 Elmwood Ave Box 611
Rochester, NY 14642 USA
585-273-4066
alan_friedman@urmc.rochester.edu

Fu, Zongming Ph.D.
Postdoctoral fellow
Medicine
Johns Hopkins University
601 Mason Lord Center Tower
5200 Eastern Avenue
Baltimore, MD 21224 USA
410-550-8507
zfu1@jhmi.edu

Gao, Xuan
Biophysics
Boston University School of Medicine
700 Albany St. W321
Boston, MA 02118 USA
617-638-4247
gaoxuan@bu.edu

Gibson, Bradford W. Ph.D.
Professor and Director of Chemistry
Chemistry
Buck Institute for Age Research
8001 Redwood Blvd.
Novato, CA 94945 USA
415-209-2032
bgibson@buckinstitute.org

Giroux, Kevin B.S.
Account Manager
Sales
PerkinElmer
8 Cutters Sound Drive
Bourne, Ma 02532 USA
508-887-0619
kevin.giroux@perkinelmer.com

Greco, Todd M. B.S.
Pediatrics
Children's Hospital of Philadelphia
3516 Civic Center Blvd.
ARC 416A
Philadelphia, PA 19104 USA
215-590-3593
tgreco@mail.med.upenn.edu

Guan, Zhanjun M.D., Ph.D.
Trainee
Internal Medicine/Cardiology
University of Texas Medical Branch
301 University Blvd
Galveston, TX 77555 USA
409-747-0683
zhguan@utmb.edu

Guilford, William H. Ph.D.
Associate Professor
Biomedical Engineering
University of Virginia
Box 800759
Charlottesville, VA 22908 USA
434-243-2740
guilford@virginia.edu

Guo, Jinjin M.D.
Postdoctoral Fellow
Pathology
Boston University School of Medicine
715 Albany St. L804
Boston, MA 02118 USA
617-638-5603
jinjin@bu.edu

Handy, Diane E. Ph.D.
Assistant Professor
Medicine
Brigham and Women's Hospital
Harvard Medical School
77 Ave Louis Pasteur
NRB0630
Boston, MA 02115 USA
617-525-4845
dhandy@rics.bwh.harvard.edu

Heckendorf, Amos Ph.D.
President
Technical Support
The Nest Group, Inc.
45 Valley Road
Southborough, MA 01772 USA
508-481-6223
amos@nestgrp.com

Heibeck, Tyler M.A.
PhD candidate
Chemistry
Boston University
650 Albany St. X-704
Boston, MA 02118 USA
617-638-2796
heibeck@bu.edu

Hesketh, Geoffrey G. B.S.
Cardiology
Johns Hopkins University
5200 Eastern Ave.
MFLB Center Tower 601
Baltimore, MD 21224 USA
410-550-8507
ghesketh@jhmi.edu

Hill, Bradford G. M.A.
Medicine-Cardiology
University of Louisville
580 S. Preston St.
Baxter II Bldg. Rm. 421
Louisville, KY 40202 USA
502-852-5724
bghill01@louisville.edu

Hong, Sung Jung M.A.
Pharmaceutical Chemistry
University of Kansas
3801 Clinton Parkway
Apt. J2
Lawrence, KS 66047 USA
785-979-0314
sungjung@ku.edu

Ischiropoulos, Harry Ph.D.
Assistant Professor
Abramson Research Center
Children's Hospital of Philadelphia
3517 Civic Ctr Blvd.
Philadelphia, MA 19104 USA
215-590-5320
ischirop@mail.med.upenn.edu

Jayaraman, Shobini Ph.D.
Physiology and Biophysics
Boston University School of Medicine
715 Albany Street W-302
Boston, MA 02118 USA
617-638-4247
shobini@bu.edu

Jiang, Zhenghui Ph.D.
Postdoctoral Fellow
Physiology and Biophysics
Boston University School of Medicine
715 Albany St.
Boston, MA 02118 USA
617-638-4093
jiangzh@bu.edu

Jiang, Yan
Chemistry
Boston University School of Medicine
670 Albany St, Suite 504
Boston, MA 02118 USA
617-414-8230
yanjiang@bu.edu

Johnson, Michelle S. B.S.
Research Assistant
Pathology
University of Alabama at Birmingham
901 19th Street South
BMR II, Room 342
Birmingham, AL 35294 USA
205-975-9576
mnsmith@uab.edu

Kang, Hee Chol Ph.D.
Technical Area Manager
Dye Chemistry
Molecular Probes/Invitrogen
29851 Willow Creek Rd
Eugene, OR 97402 USA
541-335-0342
heechol.kang@invitrogen.com

Keaney, Jr., John F. M.D.
Professor and Vice-Chair of Research
Whitaker Cardiovascular Institute
Boston University School of Medicine
700 Albany St. W-5
Boston, MA 02118 USA
617-638-4885
jkeaney@bu.edu

Kim, Julia B.S.
Graduate Student
Pharmacology and Experimental
Therapeutics
Boston University School of Medicine
715 Albany Street
Boston, MA 02118 USA
310-710-4790
juliahk@bu.edu

Kim, Helen Ph.D.
Associate Professor
Pharmacology and Toxicology
University of Alabama at Birmingham
1918 University Blvd
McCallum Bldg, room 460
Birmingham, AL 35294 USA
205-934-3880
helenkim@uab.edu

Kirber, Michael T. Ph.D.
Associate Professor
Molecular Medicine
Boston University School of Medicine
650 Albany St. X-7
Boston, MA 02118 USA
617-638-7153
mkirber@bu.edu

Klings, Elizabeth S. M.D.
Assistant Professor
Medicine/Pulmonary
Boston University School of Medicine
The Pulmonary Center, R-304
715 Albany Street
Boston, MA 02118 USA
617-638-4860
klingon@bu.edu

Knyushko, Tatyana V. Ph.D.
Senior Scientist
Cell Biology and Biochemistry
Pacific Northwest National Laboratory
902 Battelle Blvd
Richland, WA 99352 USA
509-376-5635
Tatyana.Knyushko@pnl.gov

Koeck, Thomas Ph.D.
Research Associate
Pathobiology
Cleveland Clinic Foundation
9500 Euclid Ave, NC22
Cleveland, OH 44195 USA
216-445-9761
koeckt@ccf.org

Kolmakova, Julia M.D.
Molecular Medicine
Boston University School of Medicine
88 Gordon Street Apt 202
Brighton, MA 02135 USA
617-854-7161
mazjul@yahoo.com

Landar, Aimee Ph.D.
Research Instructor
Pathology
University of Alabama at Birmingham
901 19th Street South
BMR2 rm 329
Birmingham, AL 35294 USA
205-975-9507
landar@uab.edu

Larson, Martin G. Ph.D.
Research Professor
Framingham Heart Study
Boston University School of Medicine
73 Mt. Wayte Ave.
Framingham, MA 01702 USA
508-935-3456
mlarson@bu.edu

Leichert, Lars I. Ph.D.
Molecular, Cellular and Developmental Biology
University of Michigan
830 N. University
Ann Arbor, MI 48109 USA
734 615-1957
leichert@umich.edu

Levy, Kirsten
Administrator
Cardiovascular Proteomics Center
Boston University School of Medicine
670 Albany Street Suite 504
Boston, MA 02118 USA
617-638-4019
klevy@bu.edu

Loscalzo, Joseph M.D., Ph.D.
Chairman
Department of Medicine
Brigham and Women's Hospital
75 Francis Street
Boston, MA 02115 USA
617-732-6340
jloscalzo@partners.org

Lourette, Natacha Ph.D.
Fundamental Science Directorate
Pacific Northwest National Laboratory
K8-98, P.O.Box 999
Richland, WA 99352 USA
509-376-0580
natacha.lourette@pnl.gov

Lui, Charles Y. M.D., M.Sc.
Professor of Medicine
Internal Medicine
U. Texas Medical Branch
301 University Blvd
Division of Cardiology
Galveston, TX 77555 USA
409-772-2410
cylui@utmb.edu

Mainolfi, Elizabeth M.S.
Senior Scientist
Cardiovascular Disease
Boehringer Ingelheim Pharmaceutical Co.
175 BriarRidge Road
Ridgefield, CT 06877 USA
203-798-4261
emainolf@rdg.boehringer-ingelheim.com

Martin, LeRoy Ph.D.
Sr. Marketing Manager
Waters Corp.
100 Cummings Ctr Ste 407N
Beverly, MA 01915 USA
508-482-4609
roy_martin@waters.com

Matsui, Reiko M.D.
Vascular Biology
Boston University School of Medicine
650 Albany St, X-729
Boston, MA 02118 USA
617-638-7114
rmatsui@bu.edu

McComb, Mark Ph.D.
Research Assistant Professor
Medicine
Boston University School of Medicine
670 Albany Street, suite 504
Boston, MA 02118 USA
617-638-4280
mccomb@bu.edu

Meng, Jianmin
Physiology and Biophysics
Boston University School of Medicine
715 Albany St, W302
Boston, MA 02118 USA
617-638-4093
jmmeng@bu.edu

Myers, Richard Ph.D.
Professor
Neurology
Boston University School of Medicine
715 Albany Street E-304
Boston, MA 02118 USA
617-638-5376
rmyers@bu.edu

Namakkal Soorappan, Rajasekaran Ph.D.
Postdoctoral Fellow
Cardiology, Internal Medicine
University of Utah
4A 100 SOM
Univeristy of Utah
Salt Lake City, UT 84132 USA
801-585-1724
Raj.Soorappan@hsc.utah.edu

O'Connor, Peter B. Ph.D.
Research Associate Professor
Biochemistry
Boston University School of Medicine
670 Albany St. Rm 507
Boston, MA 02118 USA
617-638-6705
poconnor@bu.edu

Odhiambo, Adam B.Sc.
Sr. Research Technician
Pulmonary, Biotechnology
Boston University School of Medicine
715 Albany St
Boston, MA 02118 USA
617-638-5229
aodhiambo1@hotmail.com

Oh, Joo Yeun M.S.
Graduate student
Pathology
University of Alabama at Birmingham
901 19th Street South
BMR2 Rm 342
Birmingham, AL 35294 USA
205-975-9576
jyoh@path.uab.edu

Ospina, Maria Ph.D.
Research Chemist
Clinical Chemistry Branch
CDC
4770 Buford Highway
MS-F25
Atlanta, GA 30341 USA
770-488-7407
mospina@cdc.gov

Ottaviano, Filomena Ph.D.
Graduate Student
Medicine
Boston University School of Medicine
715 Albany Street W-507
Boston, MA 02118 USA
617-525-4834
ottavpg@bu.edu

Parastatidis, Ioannis
Neonatology
Children's Hospital of Philadelphia
34th & Civic Center Blvd.
Philadelphia, PA 19104 USA
215-590-3046
parastatidis@email.chop.edu

Perlman, David H. Ph.D.
Research Associate
Cardiovascular Proteomics Center
Boston University School of Medicine
670 Albany Street, Rm 504
Boston, MA 02118 USA
617-414-8220
dperlman@bu.edu

Petre, Alina C. Brindusa M.Sc.
Ph.D. student
Analytical Chemistry
University of Konstanz
Universitaetsstrasse, 10
Konstanz, Germany 78457
0049-7531-882690
Brindusa-Alina.Balan@uni-konstanz.de

Przybylski, Michael Ph.D.
Professor
Department of Chemistry
University of Konstanz
Laboratory of Analytical Chemistry
and Biopolymer Structure Analysis
Konstanz, 78457 Germany
+49-7531-882249
michael.przybylski@uni-konstanz.de

Ramachandran, Vasan M.D.
Professor
Framingham Heart Study
Boston University School of Medicine
73 Mt. Wayte Ave.
Framingham, MA 01702 USA
508-935-3450
vasan@bu.edu

Rex, Sybille Ph.D.
Research Associate
Department of Medicine
Boston University School of Medicine
Whitaker Cardiovascular Institute
700 Albany Street, W533
Boston, MA 02118 USA
617-638-4106
sybrex@hotmail.com

Safaya, Surinder Ph.D.
Research Associate
Medicine/Hematology Oncology
Boston University School of Medicine
715 Albany St. E-2
Boston, MA 02118 USA
617-414-1020
ssafaya@bu.edu

Sam, Flora M.D.
Assistant Professor
Clinical Cardiology
Boston University School of Medicine
715 Albany St. X-704
Boston, MA 02118 USA
617-638-8700
florasam@bu.edu

Schildknecht, Stefan
Vascular Biology
Boston University School of Medicine
1175 Boylston St. Apt. 5
Boston, MA 02215 USA
617-638-4038
ssbu@bu.edu

Schoneich, Christian Ph.D.
Professor
Pharmaceutical Chemistry
University of Kansas
2095 Constant Avenue
Lawrence, KS 66047 USA
785-864-4880
schoneic@ku.edu

Schopfer, Francisco Ph.D.
Research Instructor
Pharmacology
University of Pittsburgh
E1314 Biomedical Sciences Tower
200 Lothrop St
Pittsburgh, PA 15213 USA
412-648-9671
fjs2@pitt.edu

Seal, Jennifer Ph.D.
Postdoctoral Fellow
Biological Engineering
MIT
77 Massachusetts Ave
Cambridge, MA 02139 USA
617-253-3106
jseal@mit.edu

Sethuraman, Mahadevan Ph.D.
Research Assistant Professor of Medicine
Vascular Biology Unit
Boston University School of Medicine

650 Albany Street EBRC X726
Boston, MA 02118 USA
617-638-2798
smdeva@bu.edu

Shimasaki, Yukio M.D.
Research Associate
Boston University School of Medicine
700 Albany St., W-632
Boston, MA 02118 USA
617-638-4836
yuki7nao@bu.edu

Shonsey, Erin
Pharmacology and Toxicology
University of Alabama at Birmingham
1918 University Blvd MCLM 435
Birmingham, AL 35205 USA
205-934-2338
eshonsey@uab.edu

Steinberg, Martin H. M.D.
Professor and Sickle Cell Center Director
Medicine/Hematology Oncology
Boston University School of Medicine
715 Albany St. E-2
Boston, MA 02118 USA
617-414-1020
mhsteinb@bu.edu

Strong, Bethany L.
Biology
Spelman College
836 Maner Terrace SE
Smyrna, GA 30080 USA
405-627-1365
bethany_strong@yahoo.com

Su, Yang
Cardiovascular Proteomics Center
Boston University School of Medicine
670 Albany Street
Boston, MA 02118 USA
617-638-6704
yangsu@bu.edu

Theberge, Roger Ph.D.
Sr. Research Associate
Biochemistry
Boston University School of Medicine
670 Albany Street
Boston, MA 02118 USA
617-803-1394
byron197@bu.edu

Thomas, Shane R. PhD
NHMRC Research Fellow
Centre for Vascular Research
University of New South Wales
Centre for Vascular Research
Sydney, NSW 2052 Australia
+61-2-9385 2582
shane.thomas@unsw.edu.au

Thorson, Maria B. B.S.
Graduate Student
Pharmaceutical Chemistry
University of Kansas
2095 Constant Ave.
Lawrence, KS 66047 USA
785-864-4826
thorsonm@ku.edu

Tong, Xiaoyong M.D., Ph.D.
Research Associate
Medicine
Boston University School of Medicine
650 Albany St., X704
Boston, MA 02118 USA
617-638-7116
xytong@bu.edu

Trostchansky, Andrés
Center for Free Radical and Biomedical Research
Facultad de Medicina, Universidad de la República
Avda. General Flores 2125
Montevideo, 11800 Uruguay
598-2-9249562
trocha@fmed.edu.uy

Ullrich, Volker Ph.D.
Professor
Chemistry
University of Konstanz
Universitätsstraße 10
Konstanz, D-78464 Germany
+49 (0)7531 / 88 - 0
Volker.Ullrich@uni-konstanz.de

Van Eyk, Jennifer E. Ph.D.
Associate Professor
Department of Medicine/Cardiovascular
Johns Hopkins University
School of Medicine
602 Mason F. Lord Building, Center Tower
Baltimore, MD 21224 USA
410-550-8510
jvaneyk1@jhmi.edu

Vita, Joseph A. M.D.
Professor
Medicine
Boston University School of Medicine
88 East Newton Street
Boston, MA 02118 USA
617-638-8742
jvita@bu.edu

Walsh, Kenneth Ph.D.
Professor and Director of Molecular Cardiology
Whitaker Cardiovascular Institute
Boston University School of Medicine
700 Albany St. W-6
Boston, MA 02118 USA
617-414-2390
kxwalsh@bu.edu

Wang, Zhongyan M.D., Ph.D.
Surgery
Boston University Shool of Medicine
700 Albany St, W402
Boston, MA 02118 USA
617-638-5046
wangzhy@bu.edu

Weng, Zhiping Ph.D.
Associate Professor
Biomedical Engineering
Boston University
44 Cummington St. Room 223
Boston, MA 02215 USA
617-353-3509
zhiping@bu.edu

West, James
Cardiovascular Proteomics Center
Boston University School of Medicine
670 Albany Street
Boston, MA 02118 USA
617-414-8228
west@bu.edu

Williams, Michelle
Biological Engineering
MIT
77 Massachusetts Ave
Rm 56-722
Cambridge, MA 02139 USA
617-253-3106
miwillia@mit.edu

Wong, Chi Ming Ph.D.
Postdoctoral Fellow
Pharmacology
Georgetown University
NW404 Medical Dental Building
3900 Reservoir Road NW
Washington, DC 20057 USA
202-687-9709
cmw45@georgetown.edu

Xu, Shanqin M.D.
Vascular Biology Unit, Dept of Med
Boston University School of Medicine
650 Albany Street, X-704
Boston, MA 02118 USA
617-638-2796
shanqin@bu.edu

Ying, Jia
Vascular Biology Unit
Boston University School of Medicine
650 Albany Street 704
Boston, MA 02215 USA
617-638-7116
ardelley@bu.edu

Yuan, Shujun
Biophysics
Boston University School of Medicine
700 Albany St. W-302
Boston, MA 02118 USA
617-638-5210
shujuny@bu.edu

Zhao, Cheng Ph. D.
Biochemistry
Boston University School of Medicine
670 Albany St.
Boston, MA 02118 USA
617-414-8230
czhao@bu.edu

Zou, Ming-Hui M.D., Ph.D.
Professor
Medicine
University of Oklahoma Health Science Center
941 Stanton L Young Blvd
Oklahoma City, OK 73104 USA
405-271-3974
ming-hui-zou@ouhsc.edu

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