

# The renin–angiotensin system and its involvement in vascular disease

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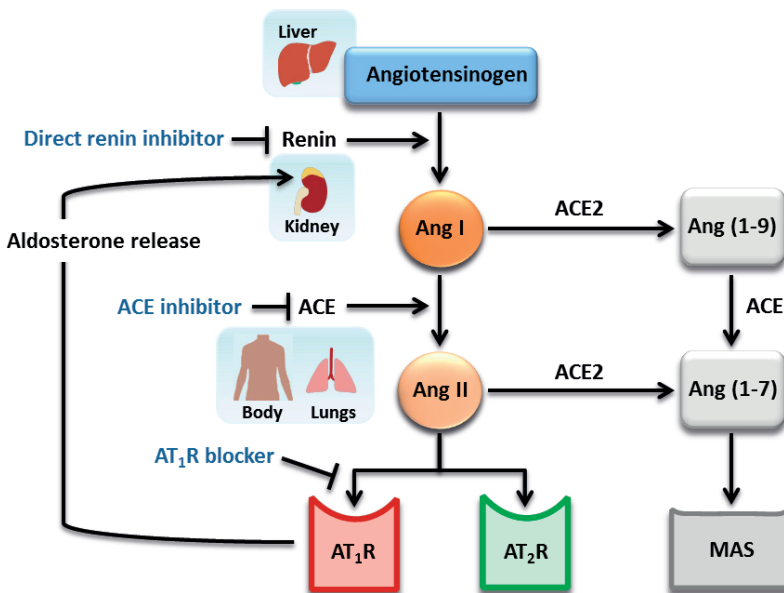
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## ABSTRACT

The renin-angiotensin system (RAS) plays a critical role in the pathogenesis of many types of cardiovascular diseases including cardiomyopathy, valvular heart disease, aneurysms, stroke, coronary artery disease and vascular injury. Besides the classical regulatory effects on blood pressure and sodium homeostasis, the RAS is involved in the regulation of contractility and remodeling of the vessel wall. Numerous studies have shown beneficial effect of inhibition of this system in the pathogenesis of cardiovascular diseases. However, dysregulation and overexpression of the RAS, through different molecular mechanisms, also induces, the initiation of vascular damage. The key effector peptide of the RAS, angiotensin II (Ang II) promotes cell proliferation, apoptosis, fibrosis, oxidative stress and inflammation, processes known to contribute to remodeling of the vasculature. In this review, we focus on the components that are under the influence of the RAS and contribute to the development and progression of vascular disease; extracellular matrix defects, atherosclerosis and aging. Furthermore, the beneficial therapeutic effects of inhibition of the RAS on the vasculature are discussed, as well as the need for additive effects on top of RAS inhibition.

## 1. GENERAL FUNCTION OF THE RENIN-ANGIOTENSIN SYSTEM

The renin-angiotensin system (RAS) is a peptide cascade well known for its critical role in the regulation of arterial blood pressure and sodium homeostasis, as well as cardiovascular regulation and remodeling. It regulates fluid and electrolyte balance through coordinated effects on the heart, blood vessels and kidneys. Angiotensin II (Ang II) is the primary effector hormone of this system that can act either as a systemic hormone or as a locally produced factor. Ang II is generated in two sequential steps: renin, secreted from the juxtaglomerular apparatus of the kidney, cleaves angiotensin I (Ang I) from liver-derived angiotensinogen, and Ang I is subsequently hydrolyzed by endothelial angiotensin-converting enzyme (ACE) to form Ang II. Ang II stimulates the adrenals to produce aldosterone and acts on cardiovascular and other tissues to regulate blood pressure and remodeling (Fig. 1). The recent identification of angiotensin-converting enzyme 2 (ACE2), which is responsible for the conversion of Ang II to angiotensin (1-7), suggest that this enzyme is a negative regulator of Ang II production, and thus the balance between ACE and ACE2 is important in the regulation of Ang II levels, as depicted in Figure 1. In addition to



**Figure 1.** Schematic overview of the renin-angiotensin system (RAS) and its intervening compounds. Liver-derived angiotensinogen is cleaved into angiotensin I (Ang I) by renin that is secreted from the kidney. Ang I is subsequently hydrolyzed by endothelial angiotensin-converting enzyme (ACE) to form angiotensin II (Ang II). Ang II stimulates the adrenals, via the angiotensin type 1 receptor (AT<sub>1</sub>R), to produce aldosterone and acts on cardiovascular and other tissues to regulate blood pressure and remodeling via the AT<sub>1</sub>R and angiotensin type 2 receptor (AT<sub>2</sub>R). Parts indicated in grey are only briefly discussed in the review.

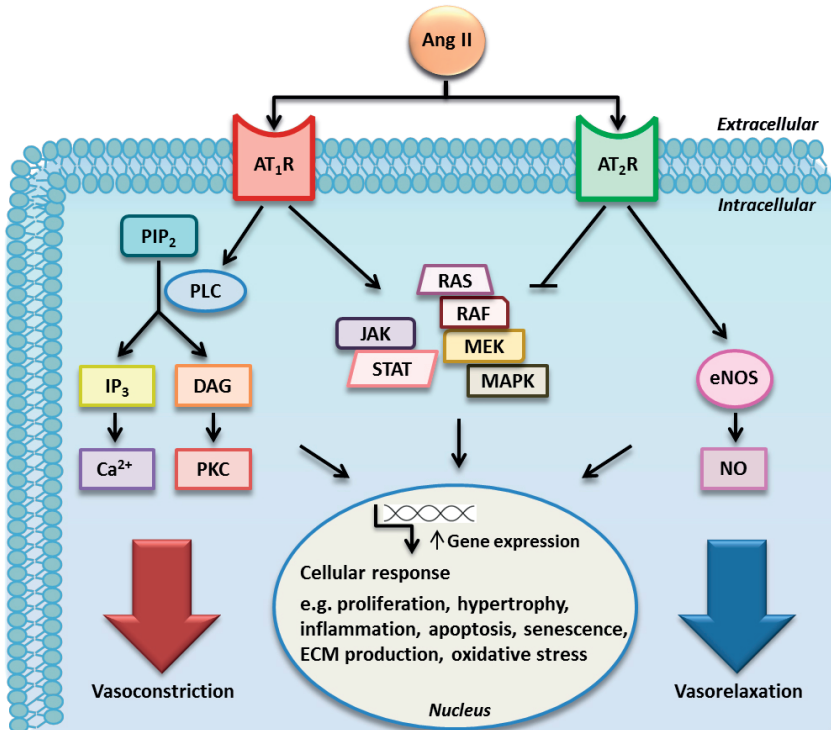
the circulating RAS, there is increasing evidence for the existence of local or tissue RAS, which generate the Ang II that is involved in paracrine and/or autocrine signaling within organs and tissues. Tissue RAS is thought to be present in all major organs, including brain, heart, blood vessels, adrenals and the kidney.<sup>1</sup> The exact function of vascular tissue RAS remains elusive, but it most likely contributes to the fine-tuning of Ang II actions on vascular tone and remodeling. A schematic representation of the RAS, based on what is currently known, is depicted in Figure 1.

Ang II mediates its physiological actions mainly via two distinct receptors: angiotensin II type 1 (AT<sub>1</sub>) and type 2 (AT<sub>2</sub>) receptors. The majority of the functions of Ang II are mediated through AT<sub>1</sub> receptor binding, while the role and biological function of the AT<sub>2</sub> receptor is less well-defined. Binding of Ang II to the AT<sub>1</sub> receptor activates a series of signaling cascades leading to tissue remodeling and acute vasoconstriction, whereas binding to the AT<sub>2</sub> receptor is believed to have counteractive effects, as it has been reported to inhibit and antagonize the AT<sub>1</sub> receptor-mediated functions.<sup>2, 3</sup>

### 1.1 Role of the RAS in the pathogenesis of vascular disease

Cardiovascular diseases are a major cause of mortality worldwide. It is well established that RAS dysregulation and/or overexpression leads to a variety of harmful vascular effects, thereby contributing to the pathophysiology of cardiovascular diseases including hypertension, aneurysms, congestive heart failure, stroke, coronary artery disease and vascular injury.<sup>4</sup> Besides the classical regulatory effects on blood pressure and sodium homeostasis, the RAS is involved in the regulation of vascular tone and remodeling of the vessel wall. Activation of the AT<sub>1</sub> receptor by Ang II induces its well-known actions such as vasoconstriction, aldosterone and vasopressin release, renal tubular sodium reabsorption, renal blood flow reduction, and production of reactive oxygen species. The signal transduction pathway for vasoconstriction is established by stimulation of the AT<sub>1</sub> receptor which in turn activates phospholipase C, which cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate into inositol-1,4,5-trisphosphate and diacylglycerol. Inositol-1,4,5-trisphosphate induces Ca<sup>2+</sup> release into the cytosol, thereby activating myosin light chain kinase which phosphorylates myosin. Diacylglycerol activates protein kinase C, which phosphorylates C-kinase potentiated protein phosphatase-1 which directly inhibits the activity of myosin light chain phosphatase.<sup>5</sup> Both processes result in phosphorylation of myosin and thereby induce smooth muscle contraction. Moreover, AT<sub>1</sub> receptor stimulation has been implicated to mediate tissue remodeling as it promotes vascular smooth muscle cell migration and senescence, vascular hypertrophy, endothelial dysfunction, oxidative stress and the synthesis and release of extracellular matrix protein.<sup>6</sup> Multiple signal transduction cascades with complex interactions are activated by the AT<sub>1</sub> receptor (Fig. 2), including the mitogen activated protein kinase (MAPK) and the janus kinase/signal transducers and activators of transcription pathway<sup>7</sup>, which together induce

cell growth, migration, proliferation and other processes linked to vascular remodeling. In addition, it is reported that Ang II acts pro-inflammatory and pro-atherogenic, both contributing to vascular remodeling and damage. Accordingly, overstimulation of the AT<sub>1</sub> receptor has been linked to various cardiovascular and renal pathologies such as left ventricular hypertrophy, vascular media hypertrophy, cardiac arrhythmias, atherosclerosis and glomerulosclerosis.<sup>6</sup>



**Figure 2.** Effect of angiotensin II (Ang II) signaling on vascular tone and remodeling of the vessel wall, via the angiotensin type 1 and 2 receptors (AT<sub>1</sub>R and AT<sub>2</sub>R). AT<sub>1</sub> receptor activation induces vasoconstriction, mediated by the inositol trisphosphate (IP<sub>3</sub>)-Ca<sup>2+</sup> and diacylglycerol (DAG)-protein kinase C (PKC) pathways. This effect is counteracted by AT<sub>1</sub> receptor-induced activation of nitric oxide synthase (NOS) leading to vasorelaxation. AT<sub>1</sub> receptor stimulation also activates several signal transduction pathways which regulate the expression of target genes promoting cell proliferation, migration and senescence, vascular hypertrophy, inflammation, apoptosis, oxidative stress and the synthesis and release of extracellular matrix (ECM) proteins. Stimulation of the AT<sub>2</sub> receptor inhibits these processes by blocking the mitogen-activated protein kinase (MAPK) signaling pathway. Abbreviations: janus kinase (JAK), nitric oxide (NO), phospholipid phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>), phospholipase C (PLC), signal transducers and activators of transcription (STAT)

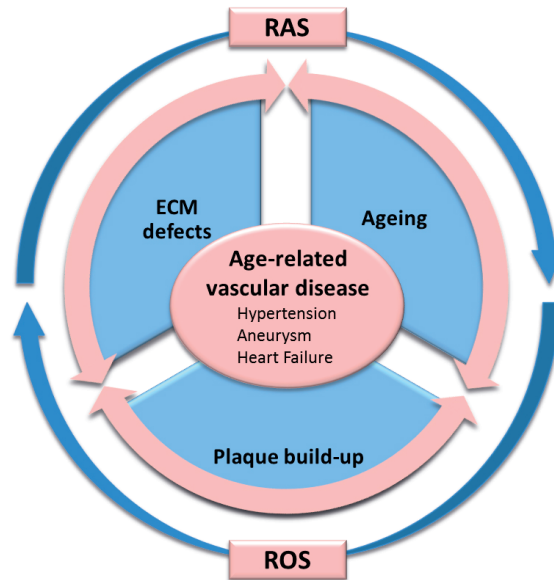
As mentioned above, besides the AT<sub>1</sub> receptor, Ang II can also mediate its effect via the AT<sub>2</sub> receptor. Generally, the counteractive effects of the AT<sub>2</sub> receptors, opposing those of AT<sub>1</sub> receptors, lead to vasodilatation<sup>8</sup>, and suppression of growth, fibrosis and inflammation (Fig. 2). However, the latter is not a uniform finding, because it has been shown that under certain conditions, e.g. in the spontaneously hypertensive rat, AT<sub>2</sub> receptors may become AT<sub>1</sub> receptor-like.<sup>9, 10</sup> The mechanism behind this phenotypic change is unclear, but most likely involves a difference in location of endothelial cell versus vascular smooth muscle cell and/or heterodimerization with AT<sub>1</sub> receptor.<sup>3</sup> Therefore, whether upregulation of AT<sub>2</sub> receptors under pathological conditions is always beneficial, should be questioned.<sup>11</sup> Similar opposing findings are found in the heart; upregulation of AT<sub>2</sub> receptors in the post-myocardial infarction area has beneficial effects, but a massive increase of 9-fold overexpression of AT<sub>2</sub> receptors did not yield a positive effect anymore.<sup>12</sup> Thus, the balance between the AT<sub>1</sub>/AT<sub>2</sub> receptors in traumatized tissue will probably determine whether the net effect is adaptive or maladaptive.

## 2. COMPONENTS CONTRIBUTING TO THE DEVELOPMENT AND PROGRESSION OF VASCULAR DISEASE

Several components which are under the influence of the RAS contribute to the development and progression of vascular disease (Fig. 3). The key components oxidative stress, extracellular matrix defects, atherosclerosis and aging and their effect on the vasculature will be discussed in this review.

### 2.1 Oxidative stress leading to vascular damage

It is well established that reactive oxygen species play a fundamental role in vascular damage and the development of cardiovascular disease.<sup>13, 14</sup> Reactive oxygen species include free radicals, mainly superoxide anions and hydroxyl radicals, and other molecules such as hydrogen peroxide and ozone. They are generated during cellular metabolism, in the vessel wall by all vascular cells, including endothelial cells, smooth muscle cells and adventitial fibroblasts. Several cellular sources are known to produce reactive oxygen species, with mitochondria as a major site of production. In general, reactive oxygen species are essential in the functioning of cells as they modulate many downstream signaling molecules regulating cell growth and vascular contraction and relaxation. However, an imbalance between reactive oxygen species generation and antioxidant protection, resulting in increased bioavailability of reactive oxygen species, leads to a state of oxidative stress. Oxidative stress contributes to vascular remodeling and dysfunction as it activates a series of signaling pathways involving MAPK, tyrosine kinases, protein tyrosine phosphatases, calcium channels and redox-sensitive transcription factors. Activation of all these factors



**Figure 3.** Interplay between the renin angiotensin system (RAS) and reactive oxygen species on three important components; extracellular matrix (ECM) defects, plaque build-up and aging, together contributing to the development and progression of age-related vascular disease. Abbreviations: reactive oxygen species (ROS).

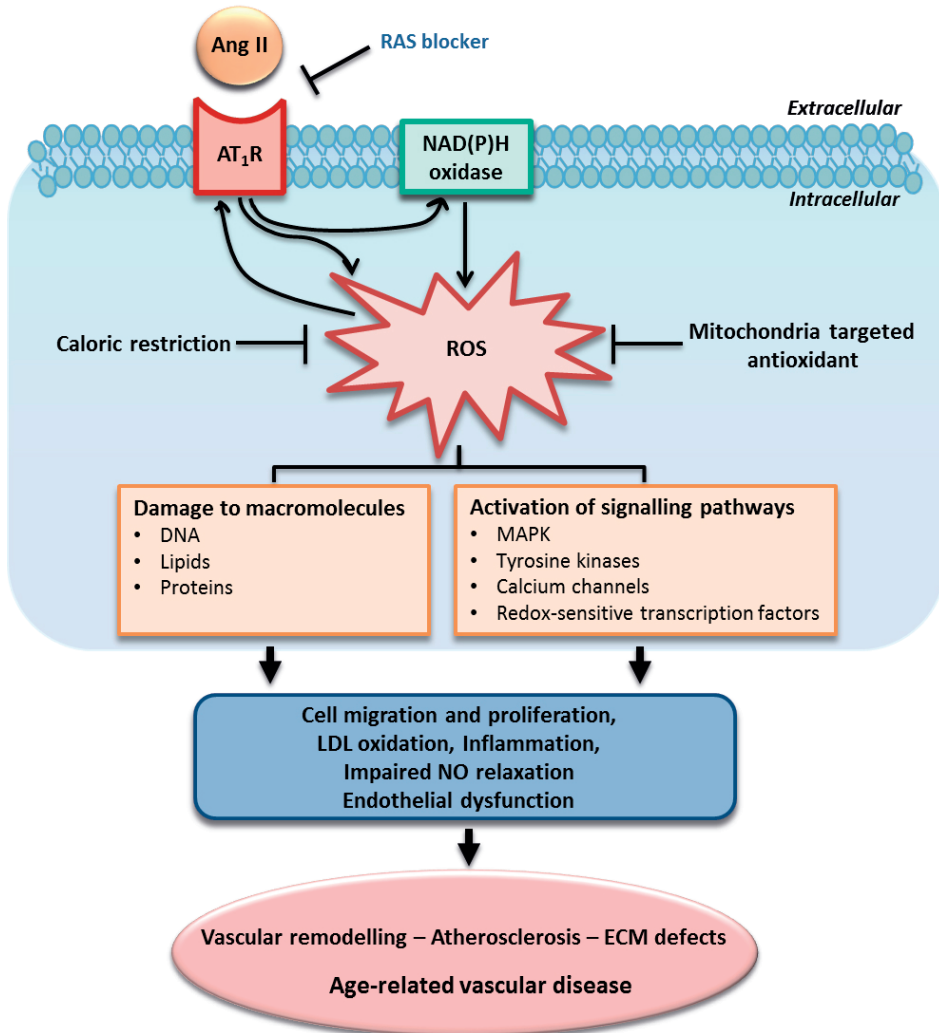
results in increased cell migration and proliferation, expression of pro-inflammatory genes, extracellular matrix production and apoptosis in the vessel wall, which all play an important role in vascular injury.<sup>15,16</sup> In addition, oxidative stress induces enhanced oxidation of low-density lipoproteins and inactivation of endothelial derived nitric oxide, processes known to be involved in atherosclerotic disease. Moreover, oxidative stress is known to induce DNA damage. As such, reactive oxygen species are the most likely trigger of DNA damage in atherosclerosis<sup>17</sup>, where DNA damage is present in both plaques of patients with atherosclerosis as well as in the circulating blood cells of these patients.<sup>18</sup> Multiple lines of evidence indicate that the RAS contributes to reactive oxygen species generation and its deleterious effects. One of the effects of AT<sub>1</sub> receptor activation in cardiovascular tissue is the generation of reactive oxygen species<sup>19</sup>, as Ang II stimulates the expression and activation of nicotinamide adenine dinucleotide phosphate-oxidase (NAD(P)H) in various cells (Fig. 4).<sup>20,21</sup> In several Ang II-associated vascular diseases the occurrence of oxidative stress has been shown to be the result of activation of NAD(P)H oxidase, mitochondrial dysfunction, inflammation and the reduction of endogenous antioxidant enzymes.<sup>22</sup> In various pathological conditions it was found that elevated Ang II levels lead to upregulation of the NAD(P)H oxidase subunits in endothelial, adventitial and vascular smooth muscle cells, resulting in increased levels of reactive oxygen species in the vessel wall.<sup>15</sup>

Of note, reactive oxygen species in turn have been shown to cause increased expression of the AT<sub>1</sub> receptor, thereby modulating the generation of reactive oxygen species, creating a vicious circle.<sup>23, 24</sup> Moreover, in recent years it has become evident that Ang II not only activates NAD(P)H but also stimulates mitochondrial reactive oxygen species production and induces mitochondrial dysfunction, presented as increased mitochondrial hydrogen peroxide production, and decreased mitochondrial glutathione, state 3 respiration and membrane potential.<sup>25, 26</sup> Molecular mechanisms that are involved in Ang II-induced mitochondrial dysfunction include protein kinase C activation, which in turn activates NAD(P)H and stimulates peroxynitrite formation.<sup>25</sup> Moreover, Ang II-derived reactive oxygen species production triggers mitochondrial ATP-dependent potassium (mitoK<sub>ATP</sub>) channel opening, which further promotes mitochondrial reactive oxygen species generation.<sup>27</sup> Research has shown that mitochondria-derived reactive oxygen species play an important role in the development of cardiovascular disease, and therapeutic targeting of mitochondrial reactive oxygen species by using a mitochondria-targeted antioxidant, significantly decreased blood pressure in Ang II-induced hypertension, and improved endothelial dysfunction.<sup>28, 29</sup> Moreover, it is suggested that mitochondrial dysfunction initiated by reactive oxygen species is one of the causes of aging, implying an important role of Ang II in aging and age-related diseases (Fig. 4).

## 2.2 Extracellular matrix defects and vascular disease

The extracellular matrix is composed of numerous macromolecules, including collagens, elastin and proteoglycans. These extracellular matrix molecules not only provide structural support to cells and tissues, but also exhibit important functional roles that control the behavior of cells such as adhesion, migration, proliferation and differentiation. Moreover, the extracellular matrix provides mechanical properties required for the functioning of the vasculature.<sup>30</sup> Minor alterations in extracellular matrix composition of the vasculature can lead to changes in cellular phenotype and function, which can ultimately lead to development of vascular disease. Diseases that are associated with an extracellular matrix defect include cutis laxa, osteogenesis imperfecta, Ehlers-Danlos and Marfan syndrome.<sup>31</sup> Moreover, it is suggested that alterations towards the breakdown of the extracellular matrix contributes to the progression of atherosclerosis and plaque instability<sup>32</sup>, and to the formation of aortic aneurysms.<sup>33, 34</sup> It is suggested that the RAS plays a role in the alteration of extracellular matrix components as numerous studies have shown that blockade of the RAS reduces the incidence and progression of aortic aneurysms (which is discussed in section 3 of this review), although the precise role of the RAS in the onset of extracellular matrix defects is not well-understood yet.





**Figure 4.** Effects of angiotensin type 1 receptor (AT<sub>1</sub>R) activation -via angiotensin II (Ang II) receptor binding- on reactive oxygen species generation, leading to age-related vascular diseases. AT<sub>1</sub>R stimulation upregulates nicotinamide adenine dinucleotide phosphate-oxidase (NAD(P)H), thereby increasing the formation of reactive oxygen species. Reactive oxygen species in turn activates several signalling pathways and induces damage to macromolecules, eventually contributing to the pathogenesis of age-related vascular disease. Abbreviations: extracellular matrix (ECM), low density lipoprotein (LDL), mitogen-activated protein kinase (MAPK), nitric oxide (NO), renin angiotensin system (RAS), reactive oxygen species (ROS).

### 2.2.1 The involvement of RAS in alterations of the extracellular matrix

Accumulating evidence indicates that increased Ang II signaling in the vessel wall results in the release of inflammatory and pro-fibrotic factors, and regulate the genetic expres-

sion of extracellular matrix proteins, which might lead to defects in the build-up of the extracellular matrix. As such, Ang II has been shown to promote vascular smooth muscle cells to synthesize extracellular matrix components.<sup>35</sup> For instance, Ang II-induced cultured rat vascular smooth muscle cells display increased levels of collagen, fibronectin, laminin and tenascin mRNA and protein.<sup>36-38</sup> Additionally, *in vivo* Ang II infusion results in increased fibronectin mRNA and protein.<sup>39</sup> Furthermore, Ang II stimulates the induction of various growth factors, inflammatory and pro-fibrotic factors including transforming growth factor beta (TGF- $\beta$ )<sup>40</sup>, platelet-derived growth factor<sup>41</sup>, basic fibroblast growth factor<sup>40</sup>, vascular endothelial growth factor<sup>42</sup> and insulin-like growth factor.<sup>43</sup> Ang II-induced TGF- $\beta$  mRNA expression in vascular smooth muscle cells is mediated by activation of extracellular-signal-regulated kinase (ERK) and activator protein-1.<sup>44</sup> Ang II stimulates mRNA expression and activity of plasminogen activator inhibitor-1 and -2 in rat aortic smooth muscle cell.<sup>45</sup> It is intriguing that all these factors could play an important role in Ang II-mediated vascular disease.

### 2.2.2 Effect of Ang II induced TGF- $\beta$ activation in extracellular matrix disruption and vascular disease

TGF- $\beta$  production by Ang II has a pivotal role in vascular disease and fibrosis. TGF- $\beta$  is a pleiotropic cytokine that regulates diverse functions such as proliferation, differentiation and apoptosis. Increased TGF- $\beta$ -signaling results in increased pSmad2/3 signaling, the canonical pathway, and recent work has also shown an increase in pERK1/2 signaling, the so-called non-canonical pathway.<sup>46-48</sup> Activation of these pathways leads to changes in adhesion, proliferation, migration and differentiation. Moreover, TGF- $\beta$  signaling plays a crucial role in the regulation of the extracellular matrix, mainly by stimulating the expression of collagens, fibronectin and proteoglycans. Furthermore, it induces the production of metalloproteinases that affect extracellular matrix breakdown. Thus, persistent activation of TGF- $\beta$  receptors leads to an abnormal deposition of connective tissue, which is associated with fibrotic disease and vascular disease.<sup>49, 50</sup>

### 2.2.3 Involvement of Ang II-mediated extracellular matrix defects in aortic aneurysms

The strength and elasticity of our blood vessels is mainly established by the extracellular matrix components elastin and collagen, which originate in the medial layer of the vessel wall. Degeneration of the medial layer of the aorta allows the development of an aneurysm, which is characterized by elastic fiber fragmentation, loss of smooth muscle cells, and accumulation of amorphous extracellular matrix.<sup>51</sup> Two main types of aortic aneurysms can be distinguished; abdominal aortic aneurysm and thoracic aortic aneurysm. Abdominal aortic aneurysms are usually caused by multiple environmental factors, such as smoking, high blood pressure and inflammation, while the development of thoracic aortic aneurysms often has a genetic origin. Different experimental mouse models for abdominal

aortic aneurysms exist, for instance the well-recognized mouse models with infusion of Ang II in atherosclerotic apolipoprotein E - or low density lipoproteins (LDL) receptor knock out mice resulting in abdominal aneurysm formation. In contrast, most mouse models for thoracic aortic aneurysms are genetically engineered, containing mutations in for example extracellular matrix proteins and TGF- $\beta$  receptors. A well-known genetic disease characterized by thoracic aortic aneurysms is Marfan syndrome, which is caused by a mutation in the extracellular matrix protein fibrillin-1.<sup>52</sup> Accumulating evidence in different thoracic aortic aneurysms mouse models has shown that increased TGF- $\beta$ -signaling in the vasculature is responsible for the development of aneurysms, which will be discussed in more detail below. It is suggested that this increase in TGF- $\beta$ -signaling leading to aortic aneurysm development is initiated by Ang II.<sup>48, 53, 54</sup> Moreover, recently it has been shown that blockade of the RAS downregulates pSmad2/3 and pERK1/2 signaling in aortic aneurysm, which gave rise to a new proposed signaling mechanism in which the AT<sub>1</sub>/AT<sub>2</sub> receptors are directly involved in Smad2/3 and ERK1/2 activation.<sup>46, 47</sup>

### 2.3 Involvement of the RAS in atherosclerosis

Atherosclerosis refers to the build-up of fat, cholesterol and other substances in and around the vasculature. Over time this build-up, so-called plaques, causes thickening and stiffening of the vessel wall. Moreover, as these plaques grow larger and larger, they eventually partially or totally block the blood flow through an artery. Numerous cardiovascular diseases are a direct consequence of the atherosclerotic process. Diseases that could develop as a result of this plaque build-up include coronary heart disease, carotid artery disease, peripheral artery disease and chronic kidney disease. Two types of plaques are described in literature; stable and unstable/vulnerable plaques, the latter having a high risk of rupture.<sup>55</sup> Plaque rupture and subsequent thrombus formation are among the main causes of acute cardiovascular events like unstable angina, acute myocardial infarction and sudden cardiac death.<sup>56</sup> It is suggested that loss of vascular function together with oxidation and accumulation of low-density lipoprotein and endothelial damage promotes an inflammatory vascular response, which plays an essential role in the development of atherosclerotic plaques. Several risk factors are strongly associated with the onset of plaque build-up such as aging, smoking, lack of physical activity, unhealthy diet, hypercholesterolemia, hypertension and genetic background. In addition, it is proposed that the RAS, and particularly Ang II, is involved in the initiation and progression of atherosclerotic plaques, since various atherogenic stimuli are mediated by RAS activity.<sup>57</sup> Ang II stimulates the atherogenic process not only through its hemodynamic effects but also through various effects on the vessel wall itself.<sup>58</sup> In particular, Ang II promotes the generation of oxidative stress in the vasculature, which plays a pivotal role in endothelial dysfunction and lipoprotein oxidation. Furthermore, Ang II induces the expression of cellular adhesion molecules and pro-inflammatory cytokines, which contribute to the induc-

tion of the inflammatory process in the vessel wall. Ang II also triggers vascular smooth muscle cells to proliferate and migrate, subsequently leading them to produce growth factors and extracellular matrix components. It was also reported that overexpression of ACE2, which converts Ang II to Ang-(1-7), improves endothelial function and decreases plaque formation in atherosclerotic mice.<sup>59,60</sup> Moreover, several studies suggest that Ang II may be involved in the acute complications of atherosclerosis by promoting plaque vulnerability, eventually resulting in plaque rupture.<sup>61-64</sup>

### *2.3.1 Evidence for the contribution of the RAS in atherosclerotic plaque build-up*

The initial steps of the atherogenic process include endothelial damage and dysfunction, which allows the migration of inflammatory cells and lipid particles into the damaged part of the vessel wall, where they accumulate and form a 'fatty streak'. These lipid particles are taken up by macrophages and smooth muscle cells, which become fat-laden foam cells and release substances which trigger a greater inflammatory response. Next, smooth muscle cells migrate to the inner layer of the vessel wall, where they proliferate, produce extracellular matrix components and contribute to the formation of the fibrous cap covering the plaque. These processes together result in growth of the plaque.<sup>65</sup> Over time the plaque may become destabilized, resulting in plaque rupture which manifests as acute cardiovascular events.

It has become apparent that one of the most important mechanisms whereby Ang II exerts vascular damage, is the production and release of reactive oxygen species via stimulation of NAD(P)H.<sup>19</sup> Oxidative stress, which is caused by an imbalance between reactive oxygen species and antioxidants, induces nitric oxide inactivation, lipid oxidation, modifications in DNA and proteins, and activation of adhesion molecules, pro-inflammatory cytokines and matrix metalloproteinases. As discussed, Ang II induces reactive oxygen species generation in various vascular cells including smooth muscle cells, endothelial cells and adventitial fibroblasts by binding to the AT<sub>1</sub> receptor, expressed by these cells.<sup>66</sup> Enhanced levels of reactive oxygen species are an important feature of atherosclerosis. Reactive oxygen species are detected in all layers of the atherosclerotic vessel wall, particularly at pathophysiological relevant locations of the atherogenic process, such as the shoulder region of coronary atherosclerotic plaques where they co-localize with Ang II.<sup>67-69</sup>

An additional mechanism by which Ang II promotes atherosclerosis is endothelial dysfunction, which is considered one of the earliest steps in the atherosclerotic process. Several animal studies show that Ang II causes endothelial dysfunction, measured by impaired vasorelaxation in response to acetylcholine, which is an endothelium-dependent vasodilator.<sup>20, 70, 71</sup> Endothelial cells are the major regulators of vascular homeostasis and anticoagulant properties of the vessel. Endothelial dysfunction and/or apoptosis is considered to be an initial step in the development and progression of atherosclerotic plaques as it promotes abnormal vasomotion, a procoagulant state, and infiltration of inflammatory

cells into the vessel wall.<sup>72</sup> Oxidative stress is recognized as one of the main factors that promotes vascular endothelial dysfunction, as elevated levels of reactive oxygen species caused by Ang II induce impaired endothelial relaxation and vascular function. Nitric oxide, which is a potent vasodilator produced by endothelial cells, is inactivated in response to reactive oxygen species. Additionally, it is suggested that a more direct effect of Ang II on endothelial cells exist, as Ang II stimulates them to express various adhesion molecules and atherogenic genes. For instance, Ang II stimulates the mRNA and protein expression of vascular cell adhesion molecule-1, which leads to the recruitment of inflammatory cells to the site of plaque formation.<sup>73, 74</sup> Furthermore, Ang II was shown to regulate the expression of plasminogen activator inhibitor-1<sup>75, 76</sup> and stimulate endothelial cell apoptosis<sup>77</sup>, resulting in an alteration in fibrinolytic balance which could lead to a highly thrombogenic state.

Another phase in the atherosclerotic process is fatty streak formation, characterized by oxidation of LDL, which Ang II facilitates by promoting reactive oxygen species formation. Oxidized LDL particles have important atherogenic properties as they can penetrate the endothelial layer after which they are taken up by macrophages and vascular smooth muscle cells, contributing to the creation of so-called foam cells. Moreover, Ang II increases the uptake of oxidized LDL by endothelial cells and macrophages, as it upregulates the expression of receptors that take up oxidized LDL, in the end leading to endothelial dysfunction.<sup>78</sup> Furthermore, oxidized LDL also triggers an inflammatory process that accelerates the formation of atherosclerotic plaques.

In atherosclerosis, vascular smooth muscle cells are involved in the stability of the plaque as they contribute to the formation of the fibrous cap. It is reported that Ang II triggers vascular smooth muscle cells to proliferate and migrate to the outer layer of the atherosclerotic plaques, where they produce growth factors and extracellular matrix proteins.<sup>21, 79</sup> With the secretion of extracellular matrix components by smooth muscle cells, the plaques increase in size and eventually become occlusive resulting in the occurrence of acute complications. Thus, inhibition of smooth muscle cell migration and proliferation may be beneficial to prevent early lesion formation, though, it might influence the stability of the plaque at the same time.

The progression of atherosclerosis is considered to be inflammation-driven, as advanced lesions of atherosclerosis are predominantly constituted of macrophages and lymphocytes. As mentioned, activation of the RAS increases the expression of adhesion molecules and pro-inflammatory chemokines and cytokines, leading to the recruitment and activation of various inflammatory cells into the vessel wall.<sup>80</sup> For example, Ang II activates nuclear factor  $\kappa$ B (NF $\kappa$ B) which promotes the expression of adhesion molecules such as vascular cell adhesion molecule-1, intercellular adhesion molecule-1, E-selectin, and chemoattractant proteins such as monocyte chemoattractant protein-1.<sup>81</sup> Moreover, a study by Daugherty et al. shows that Ang II infusion in mice promoted rapid formation

of atherosclerotic plaques, and that these lesions were mainly dominated by lipid-laden macrophages and lymphocytes.<sup>82</sup> In addition, transiently heightened levels of Ang II in apolipoprotein E-deficient mice already caused a profound increase in atherosclerosis, attributable to stimulated expression of various immunological markers, including monocyte chemoattractant protein-1.<sup>83</sup> Ang II was also found to induce interleukin-6 expression in cultured vascular smooth muscle cells<sup>84</sup> and in advanced atherosclerotic lesions Ang II stimulates the expression of matrix metalloproteinase and plasminogen activator inhibitor-1, which leads to the destabilization of these plaques.<sup>76, 85</sup> Thus, Ang II stimulates the interaction between vascular cells and leukocytes contributing to the pathophysiology of atherosclerosis.

#### 2.4 Aging: a key player in vascular damage

Aging is a natural biological process that is associated with diverse detrimental changes in cells and tissues resulting in an increased risk of health complications and disease with increasing age. As such, with age, various cardiovascular diseases including heart failure, myocardial infarction, atherosclerosis and hypertension increase tremendously.<sup>86</sup> During aging, a general decline in organ function occurs, and alterations in structure and function of the heart and the vasculature will eventually affect cardiovascular performance. For instance, elderly people are more affected by cardiac rhythm disturbances such as atrial fibrillation and most often have a reduced cardiac output. Additionally, age-related changes in the vasculature often relate to arterial stiffening, atherosclerosis, as well as an increased blood pressure. Whether the prevalence of cardiovascular disorders in the elderly is due to the aging process or whether these disorders occur more frequently because of longer exposure to risk factors is not well-defined yet. Research has shown that age-associated changes in structure and function of the vasculature share similarities with vascular changes seen in early stages of cardiovascular disease.<sup>87, 88</sup> With aging, the vessel wall gradually thickens and becomes stiffer, accompanied by impairment of vascular tone due to endothelial dysfunction. Moreover, age-related vascular damage is associated with increased extracellular matrix deposition, apoptosis, cell senescence and fibrosis.<sup>89-91</sup> The mechanisms underlying age-related vascular disease involve multiple factors and pathways, including oxidative stress, mechanical fatigue and environmental factors (e.g. food intake and diet). Further, it is reported that the systemic RAS is suppressed during normal aging. However, the activity of tissue RAS is not well defined yet. Research has shown that during aging, ACE is increased in vascular smooth muscle cells as well as vascular endothelial cells.<sup>92-94</sup> Additionally, upregulation of chymase is observed in the vessel wall during aging.<sup>93</sup> Although chymase is capable of converting Ang I to Ang II *in vitro*, its role *in vivo* is still controversial.<sup>95, 96</sup> Microarray analysis confirmed the upregulation of several genes within the RAS pathway in the vessel wall of aged mice.<sup>97</sup> Consequently, Ang II

is increased in the vasculature during advanced aging and was found to induce arterial remodeling in young animals, which mimicked features of vascular aging.<sup>98</sup>

#### 2.4.1 *The involvement of RAS components in aging and the effect on vascular damage*

During aging, dysfunction of both endothelial cells as well as vascular smooth muscle cells occurs. Advanced aging leads to impaired endothelial nitric oxide synthesis resulting in a decline in endothelium-dependent vascular dilatation and eventually vascular stiffness.<sup>99, 100</sup> Additionally, the endothelial barrier becomes porous allowing the migration of vascular smooth muscle cells into the intima layer of the vessel wall where they proliferate and deposit extracellular matrix proteins resulting in vessel wall thickening. It is demonstrated that Ang II induces expression and activation of matrix metalloproteinase 2 and calpain-1 in the arterial wall, which has been linked to an age-related increase in migration capacity of vascular smooth muscle cells.<sup>98, 101</sup> The migration of these vascular smooth muscle cells is accompanied by Ang II-mediated increase in TGF- $\beta$ -1 activity and collagen deposition, leading to thickening of the vessel wall mimicking features of the aging vasculature.<sup>98</sup> Furthermore, Kunieda and co-workers showed that Ang II signaling promotes a vascular aging phenotype by inducing vascular cell senescence.<sup>102</sup>

It has been proposed that a prominent cause of aging is the accumulation of unrepaired DNA damage<sup>103-105</sup>, and considerable evidence supports a crucial role of DNA damage in the development of vascular disease, especially atherosclerosis.<sup>106</sup> DNA damage can be induced by exogenous and endogenous sources, including reactive oxygen species. It has been widely postulated that oxidative stress is a major determinant of lifespan, as it triggers mitochondrial dysfunction and cellular injury by targeting DNA, protein, lipids and other components of the cell, and thus is causatively involved in the aging process.<sup>25, 107, 108</sup> Oxidative stress not only leads to accumulation of reactive oxygen species but can also modify or damage DNA, processes known to be involved in aging. Research has linked enhanced reactive oxygen species to many age-related degenerative diseases including atherosclerosis, stroke and heart disease. Locally, an unbalance in reactive oxygen species can have deleterious effects as it can disturb cell signaling and can trigger apoptosis, cellular senescence and inflammation. It has been demonstrated that the RAS plays a role in age-related upregulation of reactive oxygen species, as increased Ang II/AT<sub>1</sub> receptor signaling activates NAD(P)H, leading to increased reactive oxygen species production in both vascular endothelial cells and vascular smooth muscle cells.<sup>109</sup> Benigni et al. reported that disruption of the AT<sub>1</sub> receptor in mice promotes longevity, probably through reduced oxidative stress and overexpression of pro-survival genes.<sup>110</sup> During aging, these mice also develop less atherosclerotic plaques and cardiac injury. As mentioned, oxidative stress also modifies and/or damages DNA, leading to altered gene expression which contributes to the aging process. As such, it was shown that Ang II induces DNA damage in epithelial human and porcine kidney cell lines, as well as in isolated mouse kidneys.<sup>111, 112</sup> In addition,

Ang II-induced reactive oxygen species results in DNA damage, leading to senescence and an accelerated aging phenotype of vascular smooth muscle cells.<sup>102, 113</sup> Thus, Ang II increases oxidative stress in the vasculature which leads to cell and organ deterioration, thereby accelerating the aging process resulting in an increased risk for vascular disease.

Moreover, mitochondrial dysfunction represents a common feature of the aging process. Mitochondria itself are a major source of reactive oxygen species during aging, and age-related mitochondrial dysfunction closely correlates with enhanced mitochondrial reactive oxygen species production reviewed by.<sup>114, 115</sup> reactive oxygen species production by mitochondria contributes to Ang II-induced vascular alterations and dysfunction<sup>116</sup> and Ang II blockade can protect against age-related mitochondrial dysfunction.<sup>117</sup> A schematic representation of this interaction is given in Figure 4. Furthermore, transgenic mice over-expressing mitochondrial superoxide dismutase 2, the critical scavenger of mitochondrial reactive oxygen species, demonstrated attenuated Ang II-induced hypertension and vascular oxidative stress.<sup>28</sup>

#### 2.4.2 *The role of RAS and aging in atherosclerosis*

Especially atherosclerosis is an age-related disease as the prevalence and severity of atherosclerosis strikingly increase with age. There is evidence of accelerated cellular aging in atherosclerosis which includes impaired proliferation, cell senescence and DNA damage.<sup>118</sup> It was also found by Wang et al. that age-associated arterial remodeling and the development and progression of experimental atherosclerosis in young animals share common mechanisms, such as increased matrix metalloproteinase activity and Ang II signaling.<sup>93</sup> Moreover, it is suggested that aging prolongs the exposure to risk factors, which can cause increased production of reactive oxygen species within the vessel wall and the atherosclerotic plaque.

#### 2.5 **The role of RAS in age-associated gender differences in vascular disease**

Growing evidence suggests that the development of vascular disease in women is different from that in men. Sex hormones play a key role in the gender-associated difference in pathophysiology of cardiovascular diseases.<sup>119</sup> Young, premenopausal women have a lower cardiovascular risk compared to men, which points to estrogen as a protective factor. For instance, blood pressure is found to be higher in men compared to age-matched women, while after menopause blood pressure rises sharply in women, again suggestive for a role of estrogen.<sup>120</sup> Furthermore, in menopausal and aged female rats, age-related vascular dysfunction is increased due to lack of estrogen.<sup>121, 122</sup> Moreover, studies have shown that estrogen is involved in regulation of the RAS: estrogens increase angiotensinogen and AT<sub>2</sub> receptor density, while they decrease renin, ACE and AT<sub>1</sub> receptor density.<sup>123</sup> In animal models of menopause, chronic replacement of estrogen reduces ACE activity in the aorta.<sup>124, 125</sup> Hence, as estrogen inhibits ACE expression in the vasculature<sup>124</sup>, it subsequently reduces



the production of Ang II. Additionally, estrogen weakens the response and expression of the AT<sub>1</sub> receptor in the heart and the aorta.<sup>126, 127</sup> Thus, loss of estrogen production with menopause is associated with increased RAS signaling, leading to an increased risk for developing vascular disease.

Research has shown that the systemic RAS is suppressed during normal aging, which could be related to an increase in systolic blood pressure as described in the elderly.<sup>128, 129</sup> An increase in blood pressure during aging may suppress renin release from the kidney due to a higher perfusion pressure at the juxtaglomerular cells, contributing to the decline in circulating RAS. However, the activity of tissue RAS during aging is not yet well-defined. In humans, increased levels of Ang II, AT<sub>1</sub> receptor, ACE and adventitial chymase are found in the aortic wall of older donors.<sup>94</sup> Activation of these components not only leads to elevated blood pressure, but also results in harmful effect on the vasculature. These results are consistent with findings in postmenopausal women, and in men mentioned above, when compared to premenopausal women. Though it is known that aging exerts different effects on males compared to females, the precise actions of aging on the RAS and gender in different tissues are not well-established yet, and should be further elucidated.

### 3. THERAPEUTIC APPROACHES TO TARGET RAS IN VASCULAR DISEASE

Current medical therapies for managing Ang II-associated vascular diseases include  $\beta$ -blockers, statins, diuretics, calcium channel blockers and RAS inhibitors. In recent years, components of the RAS have become important targets in cardiovascular disease. Inhibition of the RAS is recommended for managing most of the cardiovascular diseases, such as hypertension, heart failure, acute myocardial infarction and stroke. Therapeutic interventions that control the effects of the RAS are often used for treatment of high blood pressure, particularly in young patients. In addition to their blood pressure-lowering effects, RAS inhibitors are also suggested to have additional cardioprotective effects. Both animal and human studies show that RAS blockade not only lowers blood pressure but may also prevent age-related structural and functional alterations in several organs.<sup>130</sup>

#### 3.1 Current RAS-related therapeutic interventions for age-related vascular disease

There are several classes of RAS inhibitors available, including ACE inhibitors, angiotensin receptor blockers, direct renin inhibitors and mineralocorticoid receptor antagonists. All four of these inhibitors interrupt the formation or block the effect of Ang II and/or aldosterone. Monotherapy using ACE inhibitors cannot completely block the persistent activation of the RAS, due to either renin upregulation and/or the existence of ACE-independent pathways that convert Ang I to Ang II. Therefore, dual RAS blockade, in particular by combining ACE inhibitors and angiotensin receptor blockers, has been proposed in patients

with cardiovascular and renal disease, to obtain a more complete blockade of the RAS. Because of the reported deleterious effects of Ang II on cardiovascular tissue, it seems logical to target the RAS and thereby reduce the development of cardiovascular damage.

Several studies already demonstrated that ACE inhibitors might inhibit atherosclerosis in animal models independent of blood pressure lowering.<sup>131-133</sup> Additionally, renin inhibition and angiotensin receptor blockers reduced atherosclerotic lesion size in cholesterol fed mice susceptible for atherosclerosis.<sup>134-137</sup> Moreover, research in aneurysmal mouse models has shown that inhibition of the RAS reduces the formation and progression of aortic aneurysms. Inhibition and/or blockade of the RAS reduces the incidence, progression and mortality in mouse models of abdominal aortic aneurysms.<sup>138, 139</sup> Especially in mouse models of thoracic aortic aneurysms, inhibition of the RAS by AT<sub>1</sub> receptor blockade (by losartan) or TGF- $\beta$ -signaling (by TGF- $\beta$ -neutralizing antibodies) effectively blocks the production of downstream TGF- $\beta$  and thereby inhibits aortic root dilatation and aneurysm formation.<sup>46-48</sup> This new mechanism of decreased TGF- $\beta$ -signaling by blocking the RAS has initiated numerous clinical trials and different aneurysmal mouse model studies to investigate the potency of RAS blockade.<sup>140, 141</sup> Yet, the efficacy of several Ang II receptor blockers varies between different animal models and patients with aneurysm disease<sup>142, 143</sup>, indicating that further investigation into the role of RAS targeting in cardiovascular disease is warranted.

Controversy remains as research has shown that dual RAS blockade gave conflicting results in different patient populations and was associated with adverse side effects. Combination of ACE inhibitors with angiotensin receptor blockers in the elderly with cardiovascular complications, is linked to an increased risk of adverse renal outcomes with higher rate of hyperkalemia, renal dysfunction and no observed benefit with respect to overall mortality.<sup>144-147</sup> Additionally, several clinical studies on dual RAS blockade were terminated early due to adverse side effects, implying that dual RAS blockade is not recommended. This might relate to the fact that a certain level of RAS activity is still required for proper physiological functioning of tissues, e.g., in the kidney<sup>148</sup>, and that blockade of the RAS should be optimal rather than maximal. Moreover, some patients fail to respond positively to these treatments. As described above, variation exists in RAS between men and women but also at different ages, which complicates the prediction of how elderly will react to therapeutic intervention strategies for vascular disease. Thus, blocking and/or inhibiting the RAS should take disease, age and gender into account.<sup>149</sup>

Although the standard therapeutic interventions to control the effects of RAS activation, i.e. ACE inhibitors, angiotensin receptor blockers, direct renin inhibitors and mineralocorticoid receptor antagonists, are effective in controlling the progression of vascular disease in some patients, they are not effective in preventing the onset of new cardiovascular diseases, and vascular disease still persists as a leading cause of illness and death. Moreover, many RAS inhibiting therapies are not 100 percent effective in all patients and additionally give adverse side effects. Thus, it is necessary to identify bet-

ter therapeutic targets and strategies, which more successfully prevent and/or slow the progression of age-related vascular disease.

### 3.2 Indirect modulation of RAS in vascular disease: caloric restriction and anti-oxidants

One of the best anti-aging strategies so far has been shown to be associated with diet intake. Research has discovered that our diet has a great impact on life span and age-related diseases. Caloric restriction, defined as a reduction of 70% in calorie intake without malnutrition, has been shown to be the most potent strategy to slow the aging process and it extends life span in different short lived species, such as mice and rats. Moreover, caloric restriction has a number of beneficial effects on the aging cardiovascular system (reviewed by Weiss and Fontana).<sup>150</sup> In rhesus monkeys it has been shown that caloric restriction delays the onset of age-associated pathologies, which not only prolongs lifespan but also protects against the onset of cardiovascular disease.<sup>151</sup> The biological mechanisms that induce the beneficial effects include modifications in energy metabolism and insulin sensitivity, increased oxidative stress resistance, reduced production of mitochondria-derived reactive oxygen species and reduced inflammation.<sup>152-154</sup> In the vasculature, caloric restriction also enhances endothelial function and reduces the size and progression of atherosclerotic plaque formation in apolipoprotein E-deficient mice.<sup>155</sup> Furthermore, in the aorta it was shown that caloric restriction not only attenuates the production of reactive oxygen species and oxidative damage, but it also increases levels of the endogenous antioxidant glutathione and ascorbate.<sup>156</sup> Additionally, Finckenberg et al. showed that caloric restriction effectively ameliorates Ang II-induced mitochondrial remodeling and cardiac hypertrophy in transgenic rats expressing human renin and angiotensin genes, leading to a reduction in overall mortality.<sup>157</sup> This study also showed that caloric restriction attenuates fibrosis and cardiomyocyte apoptosis. Even though not much is known about the effect of caloric restriction on Ang II-induced vascular damage, it is suggested that caloric restriction may have beneficial effects as it attenuates oxidative damage, enhances endothelial function and preserves mitochondrial function, processes known to be negatively influenced by Ang II signaling.

Still, a caloric restriction diet does not have the same impact on life span in humans, possibly due to the fact that most people would not submit to such a rigorous dietary program. Therefore, research is aimed at determining the feasibility and efficacy of caloric restriction mimetics, both drugs and natural compounds, without lowering caloric intake. Resveratrol is one of the compounds that mimics the cardiovascular protective effects of calorie restriction, including the attenuation of mitochondrial oxidative stress in coronary arterial endothelial cells.<sup>158, 159</sup> Currently, several studies are in progress investigating the effect of caloric restriction and different caloric restriction mimetics in humans.<sup>160, 161</sup> Regardless of the results of these studies, a healthy, balanced and sensible diet combined with enough physical activity, is still very important to maintain overall health.<sup>162</sup>

Additionally, to improve current medical therapies for managing Ang II-associated cardiovascular disease, attention has been placed on antioxidant-based therapies and reactive oxygen species scavenging molecules to decrease oxidative stress associated with vascular damage. Overproduction of reactive oxygen species has been shown to be an important factor in the development of age-related cardiovascular diseases. As mentioned, reactive oxygen species plays a central role in cellular signaling when maintained at normal tissue levels, while during times of cell stress, excessive amounts of reactive oxygen species cause harmful effects on the vasculature. Increased levels of reactive oxygen species have been implicated in the pathogenesis of diverse diseases including cancer, diabetes mellitus, atherosclerosis and aging. During aging, the production of reactive oxygen species is increased, while some of the endogenous defense mechanisms decrease, leading to progressive damage of cellular structures and eventually an aging phenotype. As mentioned, especially mitochondrial-derived reactive oxygen species play an important role in aging and age-related cardiovascular disease.<sup>163, 164</sup> Therefore, it is logical to suggest that application of antioxidants or reactive oxygen species scavengers could be useful in the treatment of age-related vascular disease. Research already demonstrated that several RAS inhibitors have an antioxidant effect. In clinical studies, it is shown that administration of AT<sub>1</sub> receptor blocker candesartan mediates an antioxidant effect, resulting in less oxidative stress and inflammation, independent from its effect on blood pressure.<sup>165</sup>

Antioxidant therapies or reactive oxygen species scavengers may have an additional advantage over the current RAS therapies, because they can prevent vascular damage by direct interaction with reactive oxygen species, not only those produced by Ang II, but also by inflammatory cells or through the regulation of reactive oxygen species-dependent molecular signaling cascades. Several clinical trials and animal models of cardiovascular diseases have focused on antioxidant-based therapies to decrease oxidative stress. Strategies to deliver antioxidants include gene therapy, dietary sources, low-molecular-weight free radical scavengers, polyethylene glycol conjugation, and nanomedicine-based technologies, as reviewed recently by.<sup>22</sup> Although many studies have shown therapeutic benefits for monotherapy with antioxidant use on vascular disease, others have failed to show any beneficial effects. This might be because of incorrect dosing, the lack of interaction between the antioxidant and reactive oxygen species or due to the fact that a state of 'antioxidative' stress can occur, in which the antioxidants attenuate or block adaptive stress responses.<sup>166</sup> Thus, specific targeting of cells and locations where oxidative stress occurs might improve the efficacy of antioxidant therapies.

### 3.3 Combining RAS blockade with reactive oxygen species inhibition

Considering the above, combined inhibition of reactive oxygen species and RAS would be expected to have further beneficial effects on vascular damage, to a greater degree than RAS inhibition alone. Not only reactive oxygen species produced by Ang II signaling

via the AT<sub>1</sub> receptor will be reduced, but also reactive oxygen species produced by other sources which are independent of RAS signaling, including inflammatory cells and high pressure-derived reactive oxygen species. Indeed, recently it was shown that combining a mitochondria-targeted antioxidant, MitoQ<sub>10</sub>, with an angiotensin receptor blocker, losartan, has an additive therapeutic benefit on attenuating development of hypertension and reducing left ventricular hypertrophy in stroke-prone spontaneously hypertensive rats.<sup>167</sup> Thus, combined RAS/reactive oxygen species blocking therapy might be a new strategy to prevent cardiovascular events.

#### 4. CONCLUSION

Clearly, the RAS plays a critical role in the pathogenesis of many types of age-related vascular diseases. This review discussed that the RAS is involved in components that contribute to the development and progression of vascular disease; i.e. extracellular matrix defects, atherosclerosis and aging. Oxidative stress seems to be related to all of these components, subsequently contributing to the onset of vascular disease. Though, the precise mechanisms by which these components induce vascular damage still need further study.

Yet, it is not entirely clear which pathogenic mechanism should be targeted and which treatments should be used in prevention of cardiovascular events. Although numerous RAS inhibiting therapies have been developed and used in clinical settings for treatment of cardiovascular disease, they are not 100 percent effective in all patients and, particularly when given in combination, give rise to adverse side effects, including hyperkalemia and renal dysfunction. Moreover, the regulation of the RAS in the elderly is not fully understood yet and should be further explored, as many vascular diseases are age-related. Thus, it is necessary to further explore optimal strategies of (combined) RAS blockade to prevent or stop the progression of vascular disease. It would be particularly interesting to test the efficacy of combined RAS/reactive oxygen species suppressing therapy on cardiovascular disease in animal models of aging, as it might give further beneficial effects on the vasculature.

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