REVIEW SERIES

ACE2–angiotensin-(1–7)–Mas axis and oxidative stress in cardiovascular disease

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The renin–angiotensin–aldosterone system (RAAS) is a pivotal regulator of physiological homeostasis and diseases of the cardiovascular system. Recently, new factors have been discovered, such as angiotensin-converting enzyme 2 (ACE2), angiotensin-(1–7) and Mas. This newly defined ACE2–angiotensin-(1–7)–Mas axis was shown to have a critical role in the vasculature and in the heart, exerting mainly protective effects. One important mechanism of the classic and the new RAAS regulate vascular function is through the regulation of redox signaling. Angiotensin II is a classic prooxidant peptide that increases superoxide production through the activation of NAD(P)H oxidases. This review summarizes the current knowledge about the ACE2–angiotensin-(1–7)–Mas axis and redox signaling in the context of cardiovascular regulation and disease. By interacting with its receptor Mas, angiotensin-(1–7) induces the release of nitric oxide from endothelial cells and thereby counteracts the effects of angiotensin II. ACE2 converts angiotensin II to angiotensin-(1–7) and, thus, is a pivotal regulator of the local effects of the RAAS on the vessel wall. Taken together, the ACE2–angiotensin-(1–7)–Mas axis emerges as a novel therapeutic target in the context of cardiovascular and metabolic diseases.

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THE CLASSIC PATHWAY AND THE NOVEL COMPONENTS OF THE RENIN–ANGIOTENSIN–ALDOSTERONE SYSTEM (RAAS)

In recent decades, cardiovascular disease has been considered the main cause of morbidity and mortality worldwide. Hypertension is a critical risk factor for these diseases, which include coronary and peripheral arterial disease, stroke and heart failure.¹ One of the major regulatory mechanisms of cardiovascular homeostasis is the RAAS.^{2,3} The classic pathway involves a two-step enzymatic pathway (Figure 1). First, the aspartyl protease renin, which is primarily released by the kidneys, cleaves a hepatic protein, angiotensinogen, to angiotensin I (Ang I).^{2,3} The second step involves hydrolysis of Ang I by angiotensin-converting enzyme (ACE), resulting in the production of the bioactive octapeptide angiotensin II (Ang II), which is a potent vasoconstrictor and stimulates the release of aldosterone from the adrenal cortex.^{2–5} Moreover, ACE inactivates the vasodilator bradykinin by degradation of the peptide.²

The discovery of angiotensin-(1–7) (Ang-(1–7)) by Santos *et al.*⁶ and the subsequent cloning of angiotensin-converting enzyme 2 (ACE2)^{7,8} shed new light on angiotensin metabolism and the regulation of the RAAS. ACE2, a zinc metalloprotease with carboxypeptidase activity, catalyzes the conversion of Ang I to the non-peptide Ang-(1–9) or the conversion of Ang II to Ang-(1–7) by the removal of a single carboxy-terminal amino acid (phenylalanine; Figure 1).⁸ This

heptapeptide can also be formed from Ang I by the action of neprilysin (also known as neutral endopeptidase 24.11).⁹ It has been suggested that Ang-(1–7) mediates its effects by interacting with the G-protein-coupled receptor Mas,¹⁰ a prototypic seven-transmembrane domain receptor (Figure 1), which is predominantly expressed in the brain and testis¹¹ but is also found in the kidney, heart and vessels.^{11–13} Moreover, several studies have shown that the interaction of Ang-(1–7) with Mas evokes numerous protective cardiovascular actions, such as nitric oxide (•NO)^{14,15} release, Akt phosphorylation¹⁶ and vasodilation (Figure 2).¹⁷ Nevertheless, other studies indicate that Ang-(1–7) may function through angiotensin type 2 receptor¹⁸ and that Mas can antagonize the actions of the angiotensin type 1 receptor.^{19,20}

The local activity of ACE2 determines the relative levels of the vasoconstrictor and pro-oxidative peptide Ang II and its vasodilatory and antioxidative metabolite Ang-(1–7) at the corresponding receptors (Figure 2).^{21,22} There is now a very large body of evidence showing that the newly discovered angiotensin system, ACE2–Ang-(1–7)–Mas, is pivotal for physiological homeostasis.²³

ENDOTHELIAL DYSFUNCTION AND OXIDATIVE STRESS IN THE ETIOLOGY OF CARDIOVASCULAR DISEASE

Strategically located between the circulating blood and the other vascular layers, the endothelium is a sensor of hemodynamic changes

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Figure 1 Classic pathway and new components of the RAAS. Three main enzymes are involved in the generation of active angiotensin peptides: First, renin cleaves angiotensinogen into angiotensin I (Ang I). The second step involves hydrolysis of Ang I by angiotensin-converting enzyme (ACE), resulting in the production of the bioactive octapeptide angiotensin II (Ang II), which interacts with angiotensin type I (AT₁-R) and angiotensin type II (AT₂-R) receptors. Third, ACE2 catalyzes the conversion of Ang II to Ang-(1-7), which mediates its effects by interaction with the G-protein-coupled receptor Mas.

and is known to have a central role in vascular homeostasis. However, for a long time, this layer was seen 'only' as a cluster of cells that separates the circulating blood from the other layers. Exactly 30 years ago, a seminal paper revised this foundational idea. The seminal experiments of Furchgott and Zawadzki²⁴ first demonstrated the existence of endothelium-derived relaxing factor, which was subsequently identified as nitric oxide (•NO; nitrogen monoxide).²⁵ In the endothelium, this free radical is produced from L-arginine by endothelial nitric oxide synthase (eNOS) in the presence of cofactors, mainly tetrahydrobiopterin (BH₄) (Figure 2).^{26–29} Interestingly, Landmesser *et al.*,³⁰ in an elegant study, showed that oxidation of this biopterin induces eNOS uncoupling. In this structural state, the enzyme is an important source of reactive oxygen species (ROS).^{31,32} Thus, in the absence of sufficient levels of cofactors, such as BH₄ for enzymatic catalysis, eNOS may reduce molecular oxygen rather than transfer electrons to the substrate L-arginine, which results in the generation of superoxide anion (•O₂⁻).^{30–32} Interestingly, BH₄ is believed to be deficient in conditions associated with altered endothelial function,³⁰ such as hypercholesterolemia,³³ diabetes,³⁴ high blood pressure³⁵ and cigarette smoking.^{36,37}

Both •NO and •O₂⁻ are radicals. These molecules react rapidly with each other to generate peroxynitrite, •ONOO⁻³⁸ (Figure 2), which is an important lipid peroxidation mediator. Peroxynitrite oxidizes low-density lipoprotein, a pivotal event for atherogenesis.³⁹ However, it is well established that •NO exerts pleiotropic effects in the regulation of vascular function. Under normal physiological conditions, this molecule serves as a vasodilator and platelet inhibitor, exhibiting antiproliferative,⁴⁰ antithrombotic,⁴¹ antiatherogenic⁴² and antioxidant capacities.⁴³ Thus, the balance between levels of •O₂⁻ and released •NO has a critical role in the maintenance of normal endothelial function.^{39,43,44}

In physiological conditions, a certain amount of intracellular •O₂⁻ is required for normal redox homeostasis in the vessel wall.^{45–47} Therefore, this reactive species is an important scavenger of the free radical signaling performed by •NO.⁴⁸ However, in pathological conditions, the extracellular increase in •O₂⁻ decreases the bioavailability of •NO, reducing its diffusion into the vascular smooth muscle.⁴⁹ Indeed, in the endothelium, excessive •O₂⁻ stimulates vasoconstriction and inflammation,⁴⁶ which mutually reinforce each other, resulting in endothelial dysfunction, mainly through the reduction of •NO bioavailability and the imbalance between ROS and antioxidant capacity.⁴⁸ Therefore, a vicious cycle pivotal in numerous disease processes is established.

According to Dröge,⁴⁵ the term redox signaling is used to describe a regulatory process in which the signal is delivered through reduction–oxidation (redox) chemistry. The cellular concentration of ROS is determined by the balance between producing sources and the rate of clearance by antioxidant compounds and enzymes. Direct ROS scavenging antioxidant enzymes include superoxide dismutase, glutathione peroxidase and catalase.^{44–47} Superoxide dismutase represents the first defense against •O₂⁻ and converts superoxide radicals to hydrogen peroxide (H₂O₂); catalase and glutathione peroxidase are two different but kinetically complementary enzymes that can eliminate H₂O₂ by breaking it down directly to H₂O and O₂ (Figure 2).^{45,46} On the other hand, oxidative stress emerges when the production of ROS, most notably •O₂⁻, exceeds the quenching antioxidant capacity of the protective systems of the cell.^{44–47}

Recent evidence suggests that oxidative stress, which is elevated in cardiovascular disease, contributes to endothelial dysfunction. This disorder is a common feature of hypertension and results from the imbalance in the release of endothelium-derived relaxing factors, mainly •NO^{32,44–46} and endothelium-derived contracting factors,

such as cyclooxygenase-derived constrictors, endothelins and $\bullet\text{O}_2^{-48-50}$ (Figure 2). Indeed, clinical studies show that ROS has a significant role in several cardiovascular and metabolic diseases.^{27,51-53} On the other hand, impaired endothelium-mediated vasodilation in hypertension has been linked to decreased $\bullet\text{NO}$ bioavailability. This may be secondary to decreased synthesis or to increased $\bullet\text{NO}$ degradation because of its interaction of $\bullet\text{NO}$ with $\bullet\text{O}_2^{-}$ to form $\bullet\text{ONOO}^{-}$.³⁸ Thus, the status of the redox system and $\bullet\text{NO}$ bioavailability are key factors controlling the influence of oxidative stress on cardiovascular function.

Accumulating evidence suggests that NAD(P)H oxidase is a major source of ROS, most notably $\bullet\text{O}_2^{-}$, in both endothelial and vascular smooth muscle cells.⁵⁴⁻⁶⁰ Ang II promotes ROS production by the activation of membrane-bound NAD(P)H oxidase.^{54,57,59} This activation was first demonstrated by Griendling *et al.*⁵⁹ in vascular smooth muscle cells. The H_2O_2 generated from the $\bullet\text{O}_2^{-}$ produced by NAD(P)H oxidase is involved in vasoconstriction and vascular hypertrophy⁵⁷ (Figure 2). Thus, the classic RAAS is a potent prooxidant system in vessels causing endothelial dysfunction and consequently cardiovascular disease.

REDOX-SENSITIVE SIGNALING BY THE ACE2-ANG-(1-7)-MAS AXIS

In 1992, Santos *et al.*⁶¹ demonstrated for the first time that Ang-(1-7) is present in vessels. After this first report, vasodilatory actions of the heptapeptide have been demonstrated in animals in several vascular beds.^{21,22,62} In addition, numerous studies showed that Ang-(1-7) functions mostly as an antithrombotic,^{63,64} antiproliferative^{65,66} and antioxidative agent.⁶⁷ The mechanisms of action in the vasculature has not yet been well established. However, several studies have focused on control of the $\bullet\text{NO}$ and $\bullet\text{O}_2^{-}$ balance, which regulates cardiovascular function.

We have recently demonstrated a reduction in superoxide dismutase and catalase activity in *Mas*-deficient mice from two different genetic backgrounds, demonstrating impaired antioxidant properties in these animals.⁶⁷ In addition, TBARS levels, which are most widely used as lipid peroxidation markers, were increased in aorta of *Mas*-null mice on the FVB/N genetic background. Accordingly, isoprostanes, other sensitive and stable oxidative stress biomarkers, are significantly upregulated in the urine of this strain. Moreover, ROS levels are increased in both FVB/N- and C57Bl/6 *Mas*-deficient animals,^{67,68} but

Figure 2 Role of ACE2-Ang-(1-7)-Mas axis in vascular function and redox signaling in vessels. Under normal physiological conditions (a), $\bullet\text{NO}$ exerts pleiotropic effects in the regulation of vascular function. The balance between levels of $\bullet\text{O}_2^{-}$ and released $\bullet\text{NO}$ has a critical role in the maintenance of normal endothelial function. However, in pathological conditions (b), excessive $\bullet\text{O}_2^{-}$, mainly produced by NAD(P)H oxidase, stimulates vasoconstriction and inflammation, resulting in endothelial dysfunction, mainly through the reduction of $\bullet\text{NO}$ bioavailability and the imbalance between ROS and antioxidant capacity. Thus, the classic RAAS is a potent prooxidant system in vessels, causing endothelial dysfunction. The ACE2-Ang-(1-7)-Mas axis counteracts these effects. For more details, see text. AT₁-R, angiotensin type I receptor; Cat, catalase; COX, cyclooxygenase; GPX, glutathione peroxidase; Ec-SOD, extracellular-superoxide dismutase; LPO, lipid peroxidation; MPO, myeloperoxidase; XO, xanthine oxidase.

Figure 2 Continued.

this difference is more pronounced in mice on the FVB/N background. Consequently, these *Mas*-knockout animals exhibit impaired *in vivo* endothelial function and increased blood pressure. One potential mechanism accounting for these observations is a decrease in •NO bioavailability. To find out if more •O₂⁻ is produced by NAD(P)H oxidases, the expression level of the main catalytic subunit of this enzyme, gp91phox (NOX2), was quantified and found to be increased in *Mas*-null mice. Furthermore, tempol, a stable superoxide dismutase mimetic, reduced blood pressure in *Mas*^{-/-} mice to levels comparable with those observed in wild-type animals.⁶⁷ Taken together, these data suggested a causative link between ROS/•NO imbalance and endothelial dysfunction in *Mas*-deficient mice.

To confirm these findings, our group developed a transgenic model overexpressing human ACE2 in vascular smooth muscle cells, using spontaneously hypertensive stroke-prone rats as a background strain.⁶⁹ These transgenic rats showed improved endothelial function and decreased vasoconstriction response to Ang II. One potential mechanism underlying these actions involves a reduction in oxidative stress by a decrease in Ang II and/or an increase in Ang-(1-7). Nevertheless, additional studies are needed to clarify this issue.

Benter *et al.*⁷⁰ showed that streptozotocin-treated spontaneously hypertensive rats (diabetic spontaneously hypertensive rats) chronically treated with Ang-(1-7) showed improved endothelial dysfunction of the renal artery, mediated primarily by the reduction of NAD(P)H-mediated oxidative stress, which resulted in improved

renal function. In contrast, another study suggests that Ang-(1-7) stimulates oxidative stress in rat kidney.⁷¹ Thus, the role of the ACE2-Ang-(1-7)-Mas axis and oxidative stress in kidney pathophysiology is still controversial.

It has been demonstrated that the vascular actions of the ACE2-Ang-(1-7)-Mas axis could be mediated by different pathways, such as antagonism of angiotensin type 1 receptor,^{19,20} by vasoactive peptides and increases in the release and/or bioavailability of •NO. Indeed, a bradykinin-potentiating effect of Ang-(1-7) was reported in certain rodent models.^{72,73} This peptide evokes endothelium-dependent relaxation in several vascular beds.⁷⁴⁻⁷⁶ In addition, Ang-(1-7) improves endothelial function *in vivo*,⁷⁷ induces coronary vasodilation by •NO release^{72,78} and activates eNOS in Chinese hamster ovary cells.¹⁶ Carvalho *et al.*⁷⁹ showed that short-term infusions of AVE 0991, a Mas agonist, increase the hypotensive effect of bradykinin in normotensive rats; one plausible mechanism for this effect involves an increase in •NO levels.

In atherosclerosis, ACE2 protects endothelial cells from Ang II-mediated macrophage infiltration and oxidative stress in an Ang-(1-7)-dependent manner.^{80,81} Corroborating these findings, Tesanovic *et al.*⁸² recently reported that long-term Ang-(1-7) treatment induces atheroprotective effects in ApoE^{-/-} mice, the most widely used animal model for atherosclerosis. According to the authors, the improvement in endothelial function resulted from an increase in •NO bioavailability, as the heptapeptide increased NOS expression and decreased

•O₂⁻ production. Concurrently, it was recently shown that genetic deletion of ACE2 significantly increases⁸³ plaque formation in atherosclerotic animals, which is decreased by targeted vascular ACE2 overexpression.^{80,84} Consequently, these actions of the ACE2-Ang-(1-7)-Mas axis may be exploited in the treatment of atherosclerosis and other vascular diseases.

As mentioned above, the activation of NAD(P)H oxidase has a key role in the intracellular signaling that leads to hypertension-induced oxidative stress and endothelial dysfunction.⁸⁵⁻⁸⁸ Sampaio *et al.*⁸⁹ showed that the activation of this enzymatic complex by Ang II is attenuated by Ang-(1-7) in human endothelial cells. The authors suggest that the principal mechanism involves the interaction of this peptide with Mas, leading to the activation of AKT. This kinase mediates the dual phosphorylation of the Ser1177/Thr495 sites in eNOS and thereby stimulates eNOS activity and increases •NO release.

Current findings also suggest that the ACE2-Ang-(1-7)-Mas axis may participate in pathomechanisms of cerebrovascular disorders. Increased levels of ACE2 and Ang-(1-7) were found in the cerebrospinal fluid of multiple sclerosis patients.⁹⁰ Moreover, Lu *et al.*⁹¹ showed that chronic central administration of Ang-(1-7) reduces the infarct area and provides cerebroprotection following focal cerebral ischemia/reperfusion in rats. The authors suggest that the heptapeptide modulates *in vivo* •NO release and eNOS expression in this stroke model. Corroborating these findings, another group showed in transgenic mice that the overexpression of ACE2 was associated with significant upregulation of neuronal NOS and eNOS in the brainstem.⁹² In addition, ACE2 overexpression in the brain prevented the Ang II-mediated decrease in NOS expression in regions involved in blood pressure regulation.⁹³ Zhang *et al.*⁹⁴ reported that the central administration of Ang-(1-7) stimulates •NO release and upregulates eNOS in a rat model of cerebral ischemia/reperfusion, further corroborating the antioxidant actions of ACE2 in the brain.

On the basis of above findings, there are at least three potential mechanisms by which ACE2 may exert protective cardiovascular effects via the reduction and/or prevention of oxidative stress: first, Ang II is cleaved to Ang-(1-7) by ACE2, thereby attenuating Ang II-induced actions; second, Ang-(1-7) may reduce the effects of Ang II by downregulating the signaling of angiotensin type 1 receptor; and third, the production of •NO is potently induced by Ang-(1-7), which might protect against endothelial dysfunction and oxidative stress (Figure 2).^{3,84-89}

CONCLUSIONS

In summary, Ang-(1-7) is generated in the vascular wall and other tissues from Ang II by ACE2, resulting in a decrease in Ang II levels and an increase in Ang-(1-7) concentrations in the vessels. Thus, the ACE2-Ang-(1-7)-Mas axis counterregulates the classic RAAS in tissues responsible for blood pressure regulation and cardiovascular homeostasis. It decreases the production of ROS and increases endothelial •NO synthesis, improving the antioxidant capacity of cardiovascular tissues. This leads to an improvement of endothelial function and, consequently, to a decrease in arterial stiffness and, therefore, beneficially alters overall arterial function.

The net cardiovascular effect of angiotensin peptides in the vascular wall depends on the relative expression of classic and novel components of the RAAS in vascular layers, as well as their effects on the redox balance. As the ACE2-Ang-(1-7)-Mas axis exerts primarily beneficial effects in the vasculature, it may become an important therapeutic target for cardiovascular protection.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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