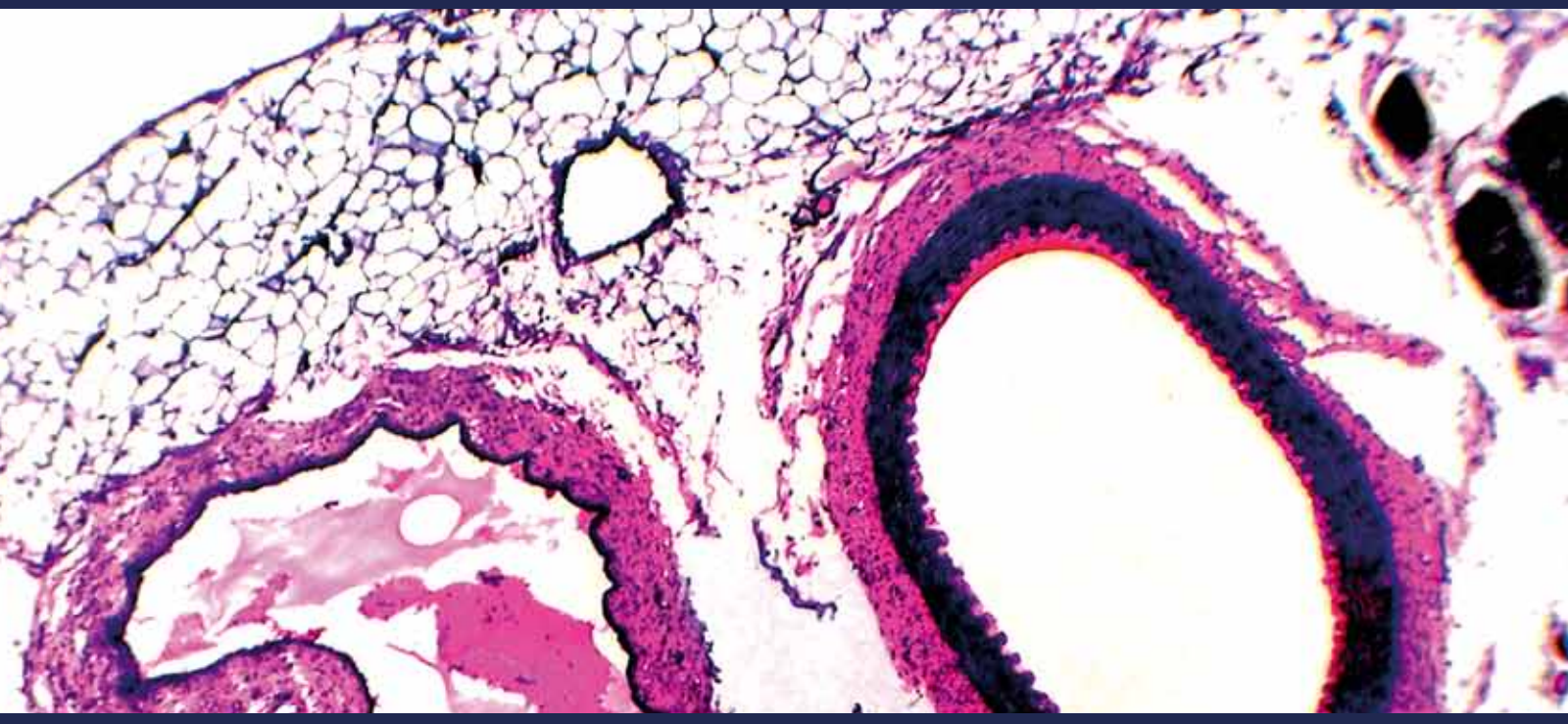
International Journal of Hypertension

Angiotensin-(1-7)/ Angiotensin-Converting Enzyme 2/Mas Receptor Axis and Related Mechanisms

Guest Editors: Anderson J. Ferreira, Robson A. S. Santos, and Mohan K. Raizada



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Editorial Board

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Editorial

Angiotensin-(1-7)/Angiotensin-Converting Enzyme 2/Mas Receptor Axis and Related Mechanisms

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Cardiovascular disease is America's leading health problem and the leading cause of death. One person in three suffers from some form of cardiovascular disease. There is an urgent need to develop new therapeutic strategies to improve the cardiovascular diseases outcome [1]. Very recent studies have demonstrated that the heptapeptide angiotensin (Ang)-(1-7) holds important cardiovascular beneficial effects [2]. These actions are generally mediated by activation of the G protein-coupled receptor Mas [3]. As a result of these novel findings, Ang-(1-7), in combination with Mas and angiotensin-converting enzyme (ACE) 2 [4, 5], the main enzyme involved in its formation, is thought to compose a cardioprotective branch within the renin-angiotensin system (RAS), which balances the ACE/Ang II/AT_1 receptor axis effects [6]. Consequently, new therapeutic approaches targeting the ACE2/Ang-(1-7)/Mas axis have been proposed.

In view of these new and exciting findings, we proposed a special issue in the International Journal of Hypertension. Our main objective was to compile original research articles as well as review articles from outstanding investigators around the World contributing to the continued efforts to understand the role of this axis in the cardiovascular system. Particularly, our intention was to give some insights into the advances in therapeutic approaches based on the ACE2/Ang-(1-7)/Mas axis to treat cardiovascular diseases.

This special issue is composed of 15 papers (original articles and reviews). Importantly, part of them was presented in the VIII Vasoactive Peptides Symposium which was held in Brazil in April, 2011. This meeting was a unique opportunity for clinicians and researchers to exchange their experience within the cardiovascular field focusing on the RAS.

The paper provided by N. E. Clarke and J. Turner is an overview of the biological roles of ACE2. They focused on the entrance of recombinant human ACE2 (rhACE2) into clinical trials discussing the potential use of this enzyme as a therapeutic strategy. A. J. Ferreira et al. expand this view to the whole ACE2/Ang-(1-7)/Mas axis highlighting the initiatives to develop potential therapeutic approaches based on this axis. Other three papers are original articles evaluating examples of these strategies. M. Durik et al. investigated the effects of a stabilized, thioether-bridged analogue of Ang-(1-7) called cyclic Ang-(1-7) [cAng-(1-7)] in rat model of myocardial infarction (MI). They found that MI increased the heart weight and myocyte size which was restored by cAng-(1-7) to sham levels. In addition, cAng-(1-7) lowered left ventricular end-diastolic pressure and improved endothelial function. The second strategy discussed in this special issue was the Ang-(1-7) included in hydroxypropyl β-cyclodextrin (HPβCD). F. D. Marques et al. evaluated the chronic cardiac effects of this compound in infarcted rats. Once-a-day oral HPβCD/Ang-(1-7) administration improved the cardiac function and reduced the deleterious effects induced by MI on TGF-β and collagen type I expression. Finally, the antiproliferative effects of the nonpeptide AVE 0991, an agonist of Mas, were investigated by C. Sheng-Long et al. It was observed that

AVE 0991 attenuates the Ang II-induced vascular smooth muscle cells proliferation in a dose-dependent fashion and that this effect is associated with the Mas/heme oxygenase-1/p38 MAPK signaling pathway.

In this special issue, the role of the ACE2/Ang-(1-7)/Mas axis in the cardiovascular function was extensively discussed in review articles and new findings regarding this axis were presented. E. R. M. Gomes et al. revised the actual knowledge about Ang-(1-7)-mediated signaling in cardiac cells, as well as the discoveries made in cardiomyocyte physiology through the use of genetic approaches. Interestingly, M. Poglitsch et al. reported significant differences in the conversion rates of recombinant human and recombinant murine ACE2 (rhACE2 and rmACE2, resp.) for diverse natural peptide substrates in plasma samples. They found species-specific differences in substrate specificities, probably leading to functional differences in the alternative axis of the RAS. Specially, in contrast to rhACE2, rmACE2 is substantially less potent in transforming Ang-(1-10) to Ang-(1-9). Of note, this enzymatic pathway was reviewed by M. P. Ocaranza and J. E. Jalil. They presented current experimental evidence suggesting that activation of the ACE2/Ang-(1-9) pathway might protect the heart and vessels from adverse cardiovascular remodeling in hypertension and in heart failure.

In addition to the cardiovascular system, some insights were given in the renal system in this special issue. S. V. B. Pinheiro and A. C. Simões e Silva highlighted the current understanding of the ACE2/Ang-(1-7)/Mas axis in renal physiology and in the pathogenesis of primary hypertension and chronic kidney disease. Also, L. C. Barroso et al. presented some data demonstrating the renal effects of acute administration of AVE 0991 in a murine model of renal ischemia/reperfusion. Administration of AVE 0991 promoted renoprotective effects evidenced by improvement of renal function, decreased tissue injury, prevention of local and remote leukocyte infiltration, and release of the chemokine CXCL1.

In the past few years, the understanding of the RAS has improved significantly. This is not only a consequence of the identification of novel members, but also due to advances in the knowledge concerning the role of these components in many tissues and cells [2, 6]. Here, M. A. F. Godoy et al. provided an overview of the functional role and the molecular pathways involved in the biosynthesis and activity of Ang-(1-7) in diverse systems, including its actions in gastrointestinal smooth muscles. Also, S. E. Thatcher et al. evaluated the effects of ACE2 deficiency in bone marrow-derived stem cells on adipose inflammation and glucose tolerance in mice fed a high-fat diet. They concluded that ACE2 deficiency in these cells promotes inflammation in adipose tissue and augments obesity-induced glucose intolerance. Interestingly, J. W. Prokop et al. demonstrated that many different genes participating in the RAS can be affected by testis determining protein (SRY), apparently in coordinated fashion, to produce more Ang II and less Ang-(1-7).

In certain circumstances and in some tissues, AT_2 receptors appear to be involved in the Ang-(1-7) effects [7]. Furthermore, physical interaction between Mas and AT_2 in selected tissues such as heart has been suggested as a putative mechanism for Ang-(1-7) actions [8]. In this special issue, R. E. Widdop's group presents an original article demonstrating the relationship between Ang-(1-7) and AT_2 receptors. S. Bosnyak et al. reported that the hypotensive effect of Ang-(1-7) was dependent on the background dose of candesartan and that this effect was reversed by AT_2 receptor blockade. In aged rats, the depressor effect of Ang-(1-7) was evident but was inhibited by either AT_2 or Mas receptors blockade. Furthermore, E. S. Jones et al. reported that AT_2 receptor stimulation does not significantly influence the antihypertensive effect of chronic AT_1 receptor blockade but plays a role in the regulation of vascular structure, as well as in cardiac perivascular fibrosis.

Thus, we hope that this special issue is able to make a picture of the role of the ACE2/Ang-(1-7)/Mas axis and of the potential therapeutic of this concept for treating cardiovascular and related diseases. Our expectation is that this special issue might serve as an important reference to scientists and physicians to update their knowledge about this contemporary theme.

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Review Article

Angiotensin-(1-7)-Mediated Signaling in Cardiomyocytes

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The Renin-Angiotensin System (RAS) acts at multiple targets and has its synthesis machinery present in different tissues, including the heart. Actually, it is well known that besides Ang II, the RAS has other active peptides. Of particular interest is the heptapeptide Ang-(1-7) that has been shown to exert cardioprotective effects. In this way, great compilations about Ang-(1-7) actions in the heart have been presented in the literature. However, much less information is available concerning the Ang-(1-7) actions directly in cardiomyocytes. In this paper, we show the actual knowledge about Ang-(1-7)-mediated signaling in cardiac cells more specifically we provide a brief overview of ACE2/Ang-(1-7)/Mas axis; and highlight the discoveries made in cardiomyocyte physiology through the use of genetic approaches. Finally, we discuss the protective signaling induced by Ang-(1-7) in cardiomyocytes and point molecular determinants of these effects.

1. Introduction

Arterial hypertension is an important cardiovascular risk factor and contributes to the development of cardiovascular event. Despite the substantial advances in antihypertensive drug therapy, the number of patients with uncontrolled hypertension remains high around the world [1].

The renin-angiotensin system (RAS) is an important classical player that directly contributes to the development and maintenance of essential hypertension [2]. This system is classically known as a hormonal system, involved in salt and water regulation and blood pressure control. Angiotensin (Ang) II, one of the main components of the RAS, exerts its biological effects by binding with high affinity to two distinct subtypes of receptor, the angiotensin II type 1 receptor (AT_1R) and the angiotensin II type 2 receptor (AT_2R) [3, 4]. Under physiological and pathological states, it is recognized that AT_1R plays a critical role in Ang II-mediated actions in the cardiovascular system [3, 4]. On the other hand, a large body of evidence suggests that AT_2R antagonizes the effects of AT_1R preventing, between other effects, its hypertrophic and angiogenic effects [5, 6].

Although Ang II is the major effector of this system, several other peptides are now recognized as being biologically important. Of particular importance is the heptapeptide Ang-(1-7) that decades ago emerged as a new metabolite of the RAS. Ang-(1-7) was initially detected as an Ang I metabolite in canine brain homogenates [7]. This discovery led to the later demonstration of its action in releasing vasopressin from hypophyseal-hypothalamic explants [8] and in counteracting the pressor and baroreflex effects of Ang II [9–11]. Later on, Ang-(1-7) was finally recognized as a putative biologically active component of the RAS [12–14]. Since then, the physiological actions of Ang-(1-7) have been extensively investigated. The heart is an important target for Ang-(1-7), which exerts direct effects on cardiomyocytes. The following sections focus on the cellular mechanism and signaling pathways involved in Ang-(1-7) actions in the cardiac cell, with particular emphasis on recent discoveries made through the use of genetic approaches.

2. The Angiotensin-Converting Enzyme2/Angiotensin-(1-7)/Mas Axis

Ang-(1-7) can be formed directly from Ang I or Ang II and indirectly from Ang I having as an intermediate step the formation of Ang-(1-9) [14, 15]. Angiotensin-converting enzyme (ACE) 2, as well as prolylcarboxypeptidase (PCP) and prolylendopeptidase (PEP), can generate Ang-(1-7) directly from Ang II. Apparently, the principal enzyme and pathway involved in the Ang-(1-7) generation is ACE2 through the hydrolysis of Ang II [16, 17]. However, Campbell et al. [18] have suggested that PEP is the main enzyme responsible for generating Ang-(1-7) from Ang II in human coronary vessels. In addition, it appears that the generation of Ang-(1-7) involving an intermediate step, including the hydrolysis of Ang I to Ang-(1-9), is less important [19]. Ang-(1-7) is also produced directly from Ang I by neutral endopeptidase (NEP) and PEP through hydrolysis of the Pro7-Phe8 bond [19, 20]. A schematic representation of the enzymatic pathways involved in the generation of Ang-(1-7) is presented in Figure 1.

In cardiomyocytes the expression of ACE2, the main enzyme involved in Ang-(1-7) formation, was already demonstrated [21, 22], as well as NEP [23]. In addition, ACE mRNA has been consistently demonstrated in cardiac cells [24, 25]. However, the ACE mRNA levels were not supported by protein measurements in human hearts. No immunoreactivity for ACE was found in ventricular myocytes from human control hearts, with ACE detected only in cardiomyocytes from hearts after myocardial infarction [26]. Nevertheless, ACE has been demonstrated in cardiomyocytes from rats and mice by enzyme-kinetic and immunohistochemical methods [27]. The presence in the cardiomyocyte of these specific enzymes indicates that Ang-(1-7) and some of RAS components can be locally synthesized in the heart. It remains to be determined which components are produced locally and in which conditions this production is activated.

The identification of ACE2 as an important Ang-(1-7)-forming enzyme [28, 29], and of Mas as a G protein-coupled receptor for Ang-(1-7) [30], contributed to establish Ang-(1-7) as a biologically active component of the RAS. In 2005, Ferreira and Santos advanced the hypothesis that ACE2, Ang-(1-7), and Mas could be considered as components of a novel axis of the RAS, the so-called ACE2/Ang-(1-7)/Mas axis [31]. For the heart, this concept is now quite well accepted in the literature [13, 32–35]. But, at present, only limited information is available regarding the direct effects of ACE2/Ang-(1-7)/Mas axis activation for cardiomyocyte function during physiological as well as in pathological conditions. In addition to ACE2, the presence of Mas has been demonstrated in cardiomyocytes from different species [21, 22, 36, 37], including humans [38].

A variety of vasoactive peptides and hormones can regulate ACE2 mRNA levels in cardiomyocytes. Modulation of ACE2 mRNA levels by aldosterone has been demonstrated in neonatal cardiomyocytes treated with this mineralocorticoid [22]. Accordingly, aldosterone decreased ACE2 mRNA levels in these cells, an effect apparently mediated by the mineralocorticoid receptor. In the same study, ACE2 mRNA modulation was not affected by Ang II treatment, suggesting that ACE2 mRNA expression is under differential modulation by endocrine molecules in cardiomyocytes. Considering the Ang II actions on ACE2 mRNA levels, opposing results were obtained by Gallagher et al. [21], who found a decrease in ACE2 activity and downregulation of its mRNA by Ang II. Importantly, this effect was mediated by AT_1R and blocked by inhibitors of mitogen-activated protein kinase kinase 1 (MAPKK1). Considering that differences in experimental conditions can explain the contrasting results regarding Ang II modulation of ACE2, further investigation will be necessary to elucidate the specific mechanism involved in ACE2 downregulation in cardiomyocytes. Endothelin-1 (ET-1) also significantly reduced myocyte ACE2 mRNA via MAPKK1 activation [21]. Apparently Ang-(1-7) has no direct effect on ACE2 mRNA regulation, although this peptide, through Mas receptor, blocked the Ang II and ET-1 mediated downregulation of ACE2 expression [21]. Collectively, these results indicate that ACE2 expression in cardiomyocytes is tightly regulated by important modulators of cardiovascular system, highlighting its importance in cardiac disease establishment and progression. Since ACE2 converts Ang II to Ang-(1-7), it is plausible that ACE2 downregulation by Ang II serves as a mechanism to favor Ang II-mediated responses, by preventing its degradation to Ang-(1-7). Thus, conditions favoring excess Ang II generation and reduced Ang II breakdown would likely lead to more deleterious effects on the heart. Figure 2 summarizes the information regarding the modulation of ACE2 expression in cardiomyocytes.

3. Protective Signaling Induced by ACE2/Ang-(1-7)/Mas Axis in Cardiomyocytes

In the past two decades, since the detection of the Ang-(1-7) as a product of the metabolism of Ang I, the physiological actions of Ang-(1-7) have been extensively investigated, and Ang-(1-7) was finally recognized as a putative biologically active component of the RAS [12–14]. However, besides this great advance in the understanding of Ang-(1-7) actions, especially in the heart, only a few reports have explored the Ang-(1-7) actions directly in cardiomyocytes. This section will focus on signaling pathways and molecular determinants of Ang-(1-7) signaling in cardiomyocytes. This will be accomplished by highlighting the following effects: (1) anti-hypertrophic, (2) anti-inflammatory, and (3) antioxidative. In addition, we summarize current knowledge regarding Ang-(1-7) modulation of Ca^{2+} handling in cardiomyocytes. Initial studies were performed by Tallant et al. [39], who confirmed the presence of the Mas receptor in neonatal cardiomyocytes and showed a direct effect of Ang-(1-7) in these cells, by preventing cell growth, through inhibition of the MAPK ERK1/2 activity. Later on, it was demonstrated the presence of Mas receptor in adult ventricular myocytes [40]. Continuing the exploration of Ang-(1-7) actions and pathways in cardiomyocytes, some information coming from

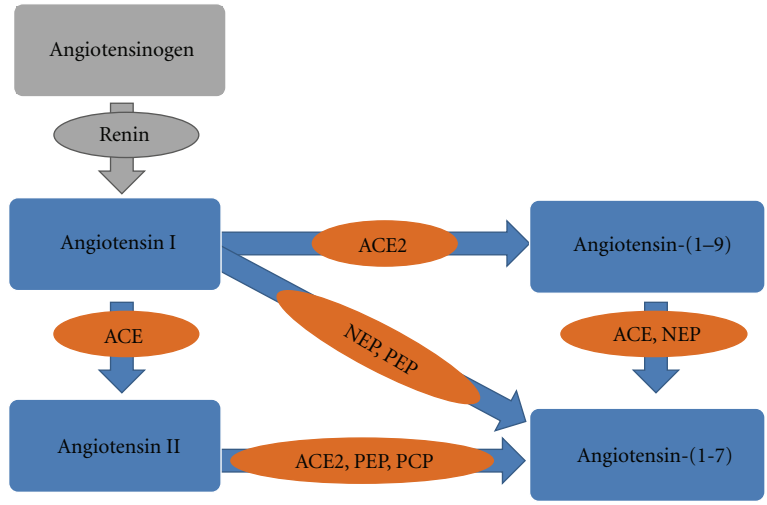

Figure 1: Schematic representation of the enzymatic pathways involved in the generation of Ang-(1-7). Ang-(1-7) can be formed by at least three different pathways: directly from Ang I by NEP and PEP, by hydrolysis of Ang II by ACE2, PEP, and PCP, and finally by hydrolysis of Ang-(1-9) by ACE and NEP. ACE, ACE2, and NEP are found in cardiomyocytes.

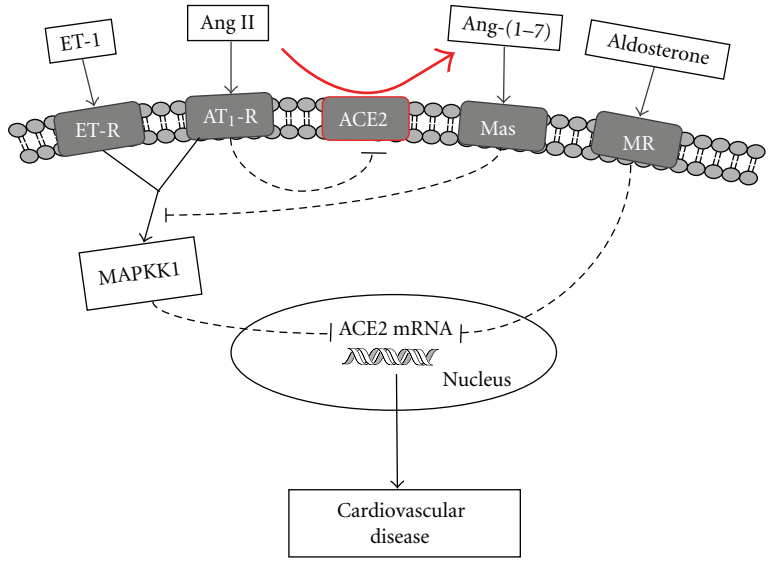

Figure 2: Schematic illustration of ACE2 modulation in cardiomyocytes by different molecules. Ang II, and ET-1 through MAPKK1 activation, and aldosterone lead to ACE2 mRNA downregulation. To date no reported effects of Ang-(1-7) on ACE2 levels have been demonstrated, although this peptide is capable of antagonizing Ang II and ET-1 effects on ACE2 mRNA. Conditions that favor ACE2 downregulation would likely lead to more deleterious effects during cardiovascular disease development. → = activation; - -⊣= inhibition; ET-1 = endothelin; Ang II = angiotensin II; Ang-(1-7) = angiotensin-(1-7); ET-R = endothelin receptor; AT_1-R = AT_1 receptor; Mas = Mas receptor; MR = mineralocorticoid receptor; MAPKK1 = mitogen-activated protein kinase kinase 1.

different types of cell supported the next steps in the understanding of the signaling molecules involved in the Ang-(1-7) effects. Sampaio et al. [41] showed that in endothelial cells Ang-(1-7) was able to generate nitric oxide (NO). In the same way, Dias-Peixoto et al. [36] demonstrated that Ang-(1-7) was able to activate the phosphatidylinositol 3-kinase (PI3-K)-protein kinase B (Akt)-pathway, resulting in nitric oxide synthase (NOS) 3 activation and NO generation in adult ventricular cardiomyocytes. It should be noted that cardiomyocytes express distinct subtypes of PI3-K, and some of them are activated by Ang II [42, 43]. Therefore, is of particular importance to investigate which specific pools of PI3-K are regulated by Ang II and Ang-(1-7). In addition, Dias-Peixoto et al. [36] have shown that expression levels

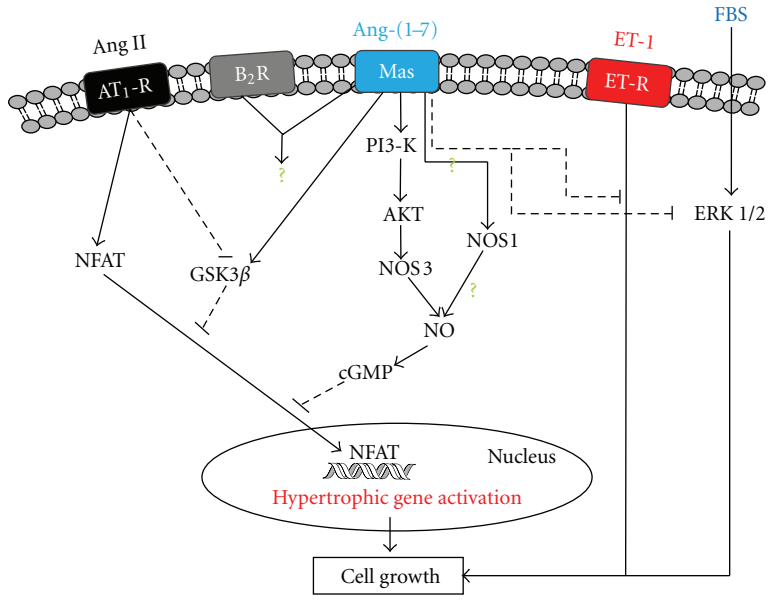

Figure 3: Ang-(1-7) signaling in cardiomyocytes. Ang-(1-7) activates the PI3-K/AKT/NOS3 pathway leading to NO generation and cGMP production. Activation of this pathway culminates with inhibition of Ang II-induced NFAT translocation. Preliminary evidence suggests that NOS1 expression may be modulated by Ang-(1-7). Ang-(1-7) also inhibits ET-1 and FBS activation of cell growth. The consequences of Ang-(1-7)/Mas and B_2R cross-talk for cardiomyocyte function are still unknown (?). → = activation; - -⊣= inhibition; ET-1 = endothelin; Ang II = angiotensin II; Ang-(1-7) = angiotensin-(1-7); ET-R = endothelin receptor; AT_1-R = AT_1 receptor; Mas = Mas receptor; B_2R = bradykinin receptor type 2; FBS = fetal bovine serum; NFAT = nuclear factor of activated T cells; GSK3β = glycogen synthase kinase 3β; PI3-K = phosphatidylinositol 3-kinase; AKT = protein kinase B; NOS3 = nitric oxide synthase 3; NOS1 = nitric oxide synthase 1; NO = nitric oxide; cGMP = cyclic guanosine monophosphate; ERK 1/2 = extracellular signal regulated kinase 1/2. The question marks denote areas in which the current state of knowledge is still preliminary.

of proteins involved in the NOS3 macromolecular complex, such as caveolin-3, heat shock protein (HSP)-90, and protein kinase B (AKT), were altered in ventricular myocytes from *Mas*$^{-/-}$ (Mas knockout) mice, indicating an important relationship between NOS3 activity and Ang-(1-7)/Mas axis. Initial investigation into the cellular mechanisms underlying protective effects of Ang-(1-7) against Ang II signaling was recently performed by our laboratory. Gomes et al. [37] have demonstrated, in cardiomyocytes, that Ang-(1-7) prevention of Ang II-induced pathological remodeling is mediated by NO/cGMP (cyclic guanosine monophosphate) pathway. This result identifies a role of NO as mediator of Ang-(1-7) beneficial effects and extends the concept that cGMP is another key molecule in this signaling pathway. In addition, this study showed that transgenic rats presenting increased Ang-(1-7) plasmatic levels have higher levels of NOS1 in ventricular cardiomyocytes, showing that besides NOS3, NOS1 shall be involved in NO generation elicited by Ang-(1-7). Ang-(1-7) also modulated the activity of the transcription factor NFAT (nuclear factor of activated T cells), preventing its translocation to the nucleus, and the activation of hypertrophic gene program by Ang II [37]. Stimulated by calcium signals, NFAT is translocated to the nucleus where it can regulate hypertrophic genes. In cardiomyocytes, NFAT nuclear localization is tightly controlled at multiple levels [44–46]. Glycogen synthase kinase 3β (GSK3β), in particular, is considered a potent inhibitor of this pathway downstream of calcineurin. In the nucleus, GSK3β phosphorylates NFAT, thereby promoting its nuclear export [44]. Moreover, GSK3β has been shown to regulate hypertrophy development by restraining gene expression [47]. Gomes et al. [37] have shown that Ang-(1-7) modulates the activity of GSK3-β, by preventing its inactivation by Ang II. The modulation of these two proteins, NFAT and GSK3- β, supports the anti-hypertrophic effect of Ang-(1-7) observed in the heart [37] and in cardiomyocytes [37, 39]. Corroborating these findings Flores-Muñoz et al. [48] reported that Ang-(1-7) was able to block the increase in cell size induced by Ang II in H9c2 cardiomyocytes. These effects were mediated by Mas receptor, since Mas antagonist A779 efficiently blocked the antihypertrophic effects of Ang-(1-7). Importantly, these authors have also shown that Ang-(1-7) anti-hypertrophic activity was inhibited in the presence of the bradykinin type 2 receptor (B_2R) antagonist, HOE140, suggesting a cross-talk between Mas and B_2R in response to Ang-(1-7). Figure 3 shows recent data about Ang-(1-7) signaling and cross-talk in cardiomyocytes.

Recently, Qi et al. have provided evidence for an anti-inflammatory role of angiotensin-(1-7) at the cardiomyocyte level [49]. By using neonatal cardiomyocyte culture, the authors demonstrated that protective effects of Ang-(1-7) against hypoxia-induced cell death were mediated, at least in part, through modulation of cytokine production. This beneficial effect was associated with decreased expression

of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) and increased gene expression of ACE2, bradykinin type 2 receptor, and interleukin-10 (IL-10). Taken together, these data show that Ang-(1-7) regulates cytokine responses, which could contribute to its cardioprotective effects.

Considering the critical role of Ca^{2+} ions for cardiomyocyte contraction [50], some studies have addressed whether Ang-(1-7) modulates Ca^{2+} handling in ventricular cardiomyocytes. Recent work by our group has shown that acute Ang-(1-7) treatment does not significantly alter Ca^{2+} transient amplitude or kinetics of decay [36]. We extended these findings to *in vivo* conditions and showed that cardiomyocytes from transgenic (TG) rats with chronic elevated plasmatic Ang-(1-7) do not show alteration in cytosolic Ca^{2+} transient parameters [37]. Interestingly, cardiomyocytes from mice with genetic ablation of Ang-(1-7) Mas receptor ($Mas^{-/-}$) presented a Ca^{2+} signaling dysfunction represented by a smaller peak Ca^{2+} transient and slower Ca^{2+} uptake. This Ca^{2+} signaling dysfunction was accompanied by decreased protein levels of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 2 (SERCA2) [36]. SERCA2 is responsible for Ca^{2+} reuptake by the sarcoplasmic reticulum (SR), thereby setting SR Ca^{2+} load, which is an important determinant of Ca^{2+} release in cardiomyocytes [50]. The reduction in the Ca^{2+} transient is consistent with the depression of contractility that was previously observed in $Mas^{-/-}$ hearts [40]. This finding was particularly important since it suggested that the Ang-(1-7)/Mas axis is critical for long-term maintenance of normal Ca^{2+} handling in the cardiac cell. However, there was still the possibility that the alterations in Ca^{2+} handling found in $Mas^{-/-}$ cardiomyocytes were secondary to the cardiac dysfunction observed in these hearts.

Adding further complexity to the understanding of Ang-(1-7)/Mas modulation of cardiomyocyte Ca^{2+} signaling, it was also shown that cardiomyocytes from TG rats with cardiac specific overexpression of Ang-(1-7) presented higher Ca^{2+} transient amplitude, faster Ca^{2+} uptake, and increased levels of SERCA2 [51], suggesting that chronic local increase of Ang-(1-7) in the heart was associated to enhanced Ca^{2+} handling. Are these changes in Ca^{2+} handling a direct consequence of local Ang-(1-7) increase in the heart? These findings contrasted with the lack of effect on Ca^{2+} signaling found in cardiomyocytes from TG rats with chronic elevated plasmatic Ang-(1-7) levels. As it stands, the relationship between Ang-(1-7) and Ca^{2+} signaling is more complex than one may have anticipated. It is also plausible, that Ang-(1-7) effects on Ca^{2+} handling observed in an *in vivo* model of chronic Ang-(1-7) overexpression in the heart are consequences of long-term changes in expression levels of Ca^{2+} handling proteins. Future studies are needed to demonstrate whether Ang-(1-7) prevents Ca^{2+} signaling dysfunction in ventricular myocytes from animal models of heart failure.

NO has been attributed as a key mediator of Ang-(1-7) effects on different cell types, including cardiomyocytes, and it is known to interact with proteins involved in Ca^{2+} handling and regulate cardiac contractility. The question remains whether long-term Ang-(1-7) effects on Ca^{2+} handling are mediated by NO or other signaling molecules in cardiomyocytes. In this way, the regulation of some key proteins involved in cardiomyocyte Ca^{2+} handling, such as ryanodine receptor (RyR), phospholamban (PLN), Na^{+}/Ca^{2+} exchanger (NCX), and troponins by Ang-(1-7) must be investigated in order to provide a deeper understanding of Ang-(1-7) actions on Ca^{2+} signaling. A summary of current knowledge regarding Ang-(1-7)/Mas modulation of cardiomyocyte Ca^{2+} signaling is shown in Figure 4.

Evidence for a direct role of ACE2/Ang-(1-7)/Mas axis against oxidative stress in cardiomyocytes was also obtained [52]. Experiments on adult ventricular myocytes demonstrated that Ang II-mediated superoxide generation and extracellular signal-regulated kinase 1/2 (ERK 1/2) activation were inhibited by recombinant ACE2 (rhACE2). Importantly, these effects were mediated by Ang-(1-7), since preincubation with the Mas receptor peptide antagonist, D-Ala7-Ang-(1-7), largely prevented rhACE2 suppression of Ang II-induced responses in cardiomyocytes. These *in vitro* findings correlated with *in vivo* data showing that treatment with rhACE2 prevented Ang II–induced hypertrophy and myocardial fibrosis. Thus, these findings give further support to the fact that enhanced Ang-(1-7) signaling at the cardiomyocyte level prevents Ang II pathological effects and highlight Ang-(1-7) anti-oxidative actions on cardiomyocytes.

Contrasting to the view of a protective role of ACE2/Ang-(1-7)/Mas axis some reports have shown hypertrophic effects of ACE2 and Mas overexpression in cardiomyocytes. Masson et al. [53] reported that adenoviral-mediated gene transfer of ACE2 in rabbit cardiomyocytes leads to cellular hypertrophy. In the same study, *in vivo* ACE2 overexpression in the myocardium of stroke-prone spontaneously hypertensive rats resulted in profound cardiac dysfunction. The authors have argued that the detrimental effects of ACE2 overexpression were possibly due to higher amounts of protein expressed using this approach. Using similar overexpression strategy, neonatal rat cardiomyocytes were infected with adenovirus encoding the human Mas receptor. Intriguingly, overexpression of Mas induced a significant increase in IP3 accumulation and cellular hypertrophy. These responses were due to enhanced Gq-mediated signaling via Mas receptor [38]. Whether this response is a consequence of exacerbated Mas signaling or is a result of promiscuous signaling activation caused, for example, by heterodimerization, it is a topic that needs further clarification. Therefore, understanding the role of "physiological" versus "supraphysiological" levels of ACE2 and Mas, its downstream signaling pathways and their functional outcomes are crucial for clarifying the role of ACE2/Ang-(1-7)/Mas axis for cardiomyocyte function.

4. Conclusions and Perspectives

In cardiomyocytes, the actual knowledge of Ang-(1-7) protective effects was mainly focused on the modulation of Ang II signaling with emphasis on anti-hypertrophic actions. This effect was dependent on Mas, indicating that important

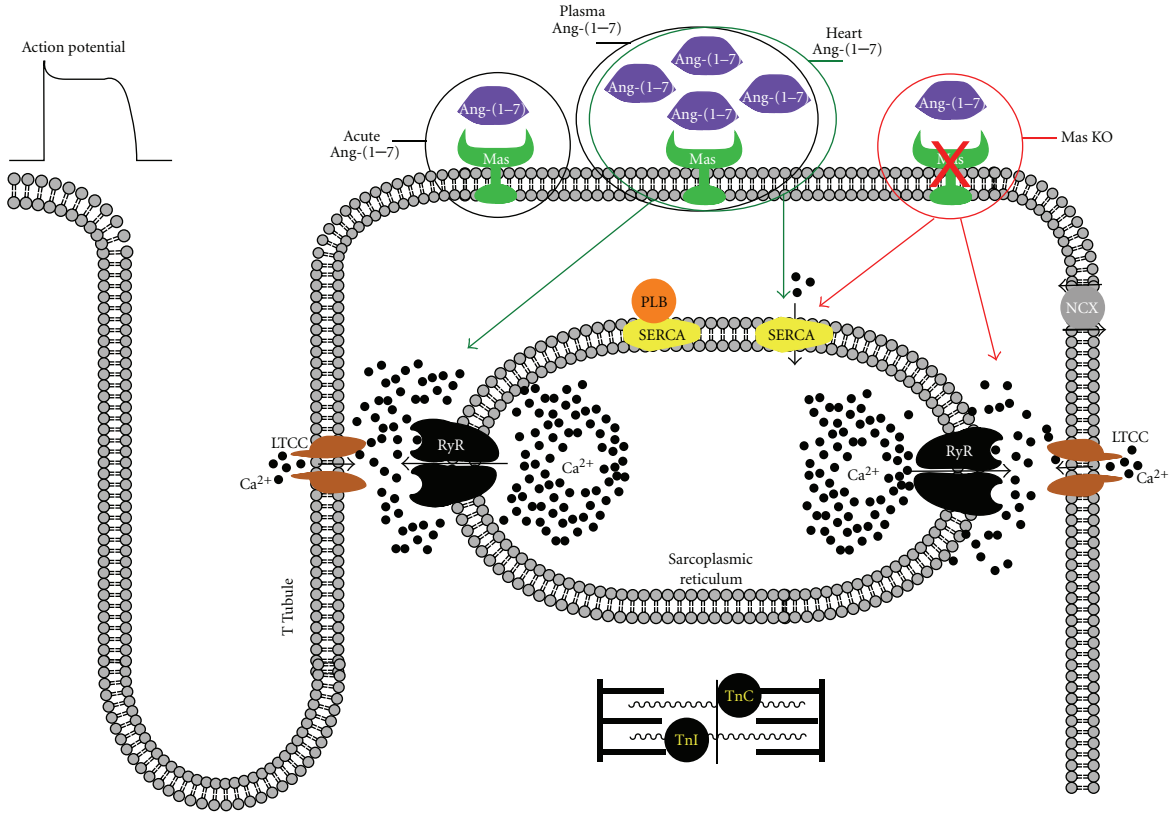

Figure 4: Summary of current knowledge regarding Ang-(1-7) modulation of Ca^{2+} handling in ventricular myocytes and the underlying mechanisms. Acute treatment of cardiomyocytes with Ang-(1-7) apparently has no direct effect on Ca^{2+} handling (black circle, left). Lack of effect on Ca^{2+} levels was also observed in cardiomyocytes from TG rats with increased circulating levels of Ang-(1-7) (black circle, middle). In contrast, Ang-(1-7) signaling disruption through Mas genetic ablation (Mas KO) leads to Ca^{2+} dysfunction (red circle, right). Cardiomyocytes from Mas KO mice present reduced SERCA expression levels and Ca^{2+} transients (red arrows). Cardiac specific overexpression of Ang-(1-7) enhances Ca^{2+} release and SERCA levels in ventricular myocytes (green circle and arrows). Data regarding Ang-(1-7) modulation of some other key proteins involved in Ca^{2+} handling in ventricular myocytes, such as PLN, NCX, TnI, and TnC, are still missing. Black filled circles = calcium ions; LTCC = L-type Ca^{2+} channels; Mas = Mas receptor; NCX = Na^{+}/Ca^{2+} exchanger; PLB = phospholamban; RyR = ryanodine receptor; TnC = troponin C; TnI = troponin I.

cardioprotective aspects of Ang-(1-7) signaling are mediated through Mas receptor, and involved NO and cGMP generation. It remains to be elucidated whether antioxidative and anti-inflammatory responses of Ang-(1-7) also depend on NO/cGMP production. A direct acute effect of Ang-(1-7) on Ca^{2+} signaling in cardiomyocytes seems unlikely. However, there still are many ways by which Ang-(1-7) may regulate Ca^{2+} signaling in ventricular myocytes. Chronic increase in local Ang-(1-7) levels could be a mechanism by which Ang-(1-7) enhances Ca^{2+} handling, as observed in cardiomyocytes from transgenic rats with cardiac specific overexpression of Ang-(1-7). To understand how Ang-(1-7) regulates Ca^{2+} handling in ventricular myocytes is of fundamental importance in light of Ang-(1-7) therapeutic potential in several disease conditions. The literature shows a tight control of ACE2 synthesis and activity in cardiomyocytes. How this enzyme expression is modulated, the signaling pathways involved in this regulation, and whether this occurs *in vivo* are questions that remain to be answered, considering the pivotal role of ACE2 as modulator of Ang II/Ang-(1-7) levels. The actions of Ang-(1-7) on cardiomyocytes are just beginning to unravel, dissecting the signaling pathways, and the conditions under which Ang-(1-7) signaling is turned on will be a major issue to be addressed in the future.

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Research Article

Influence of Angiotensin II Subtype 2 Receptor (AT_2R) Antagonist, PD123319, on Cardiovascular Remodelling of Aged Spontaneously Hypertensive Rats during Chronic Angiotensin II Subtype 1 Receptor (AT_1R) Blockade

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Cardiac AT_2R expression is upregulated in the normal process of aging. In this study we determined the contribution of AT_2R to chronic antihypertensive and remodelling effects of AT_1R blockade in aged hypertensive rats. Adult (20 weeks) and senescent (20 months) spontaneously hypertensive rats (SHRs) were treated with either the AT_1R antagonist, candesartan cilexetil (2 mg/kg/day), the AT_2R antagonist, PD123319 (10 mg/kg/day), or a combination of the 2 compounds. Mean arterial pressure (MAP) and left ventricular volume were markedly decreased by candesartan cilexetil, however, simultaneous treatment with PD123319 had no additional effect on either parameter. Perivascular fibrosis was significantly reduced by candesartan cilexetil in aged animals only, and this effect was reversed by concomitant PD123319 administration. Vascular hypertrophy was reduced by candesartan cilexetil, and these effects were reversed by simultaneous PD123319. These results suggest that AT_2R stimulation does not significantly influence the antihypertensive effect of chronic AT_1R blockade, but plays a role in the regulation of vascular structure. The severe degree of cardiac perivascular fibrosis in senescent animals was regressed by AT_1R blockade and this effect was reversed by simultaneous AT_2R inhibition, demonstrating an antifibrotic role of AT_2R stimulation in the aging hypertensive heart.

1. Introduction

The incidence of hypertension, cardiac hypertrophy, and heart failure increases significantly with aging [1], and age-related structural adaptations may contribute to deteriorating function of the cardiovascular system. The aging heart is characterised by myocyte loss, hypertrophy of remaining cells, and exaggerated accumulation of extracellular (ECM) proteins [1, 2], which is associated with increased incidence of both contractile and conductile dysfunction of senescent hearts [2]. In addition, structural modifications of the aorta and coronary vasculature, particularly involving hypertrophy/hyperplasia of smooth muscle cells and increased collagen deposition within and surrounding the media of vessels [3], result in arterial stiffening, alterations in vascular permeability, and deterioration of coronary haemodynamics [4].

Ang II is known to promote cardiovascular hypertrophy and fibrosis via AT_1R stimulation [5, 6], whereas the role of AT_2R has been less conclusively defined [7]. AT_2R activation is thought to oppose AT_1R-mediated hypertrophic and fibrotic effects; however, studies in transgenic mouse models of targeted deletion [8, 9] or overexpression [10] of AT_2R have reported contrasting effects on cardiovascular structure, emphasising the need for further pharmacological investigation and elucidation of AT_2R function.

AT_1R antagonists increase circulating levels of Ang II, which may stimulate unopposed AT_2R and potentially contribute to the effects of AT_1R blockade [11]. We have previously shown that impaired *in vitro* AT_2R-mediated relaxation in SHRs was restored by antihypertensive treatment [12]. Furthermore, AT_2R stimulation may influence cardiovascular function and structure during chronic AT_1R

blockade [13–15]. These studies have been performed in animal models of genetic hypertension or following cardiovascular infarct and have deduced various degrees of AT_2R-mediated antihypertrophic and antifibrotic effects, depending on the study.

Importantly, although cardiac AT_2R expression is relatively low in the adult rat heart [16], expression may be upregulated in certain disease states and has been particularly associated with conditions of increased fibrosis [17], cardiac hypertrophy [18], heart failure [19], and also with increasing age [20, 21]. Moreover, increased myocardial angiotensinogen and ACE indicate that intracardiac production of Ang II may also be potentiated with senescence [22].

Given the possibility of augmented cardiac RAS activity with increased age, and also the fact that chronic AT_1R blockade increased longevity in rodent models of aging and was associated with cardiovascular protective effects [23, 24], we reasoned that a greater AT_2R contribution to AT_1R inhibition may be manifest in the aged hypertensive state. Therefore, the aims of this study were to determine the contribution of the AT_2R to the antihypertensive and cardiovascular remodelling effects of chronic AT_1R blockade in aged SHRs.

2. Materials and Methods

2.1. Animals and Treatment. Male SHRs (12 weeks) were obtained from the Animal Resource Centre, Western Australia and were maintained on a 12-hour day/night cycle with free access to food and water until animals were either 20 weeks or 20 months of age. Senescent animals were used at 20 months, as at this age, SHRs display many of the features of hypertensive and age-related cardiac remodelling (including cardiovascular hypertrophy and fibrosis) but are yet to complete the transition to heart failure [25].

Radiotelemetry transmitters (TA11PA-C40, Data Sciences) were inserted into the abdominal aorta of SHRs under isoflurane anaesthesia (2–4%, O_2), as previously described [26]. Animals were allowed to recover for 1 week, after which time a continuous baseline recording of MAP and HR was made for a further week. Animals were then given the AT_1R antagonist, candesartan cilexetil (2 mg/kg/day), its vehicle, or the nonangiotensin antihypertensive, hydralazine (30 mg/day), in drinking water. At the same time, senescent SHRs were also briefly anaesthetised with isoflurane, and osmotic mini pumps containing either PD123319 (10 mg/kg/day) or saline vehicle were inserted into a subcutaneous pocket formed between the scapulae. Doses of candesartan cilexetil and PD123319 were based on previous studies performed in senescent Wistar Kyoto rats [26]. Adult SHRs were treated for 2 weeks with candesartan cilexetil (2 mg/kg/day), before implantation of osmotic mini pumps, such that animals received the combination of 6-week candesartan cilexetil and 4-week PD123319 treatment. In senescent SHRs, all drug treatments were initiated simultaneously and continued for 4 weeks duration. MAP and HR were recorded continuously during the entire 4- or 6-week treatment period. Treatment groups were as follows:

Adult (20 weeks) SHRs

(i) control ($n = 6$),
(ii) candesartan cilexetil alone ($n = 7$),
(iii) candesartan cilexetil + PD123319 ($n = 7$),
(iv) PD123319 alone ($n = 7$).

Senescent (20 months) SHRs

(i) control ($n = 10$),
(ii) candesartan cilexetil alone ($n = 9$),
(iii) candesartan cilexetil + PD123319 ($n = 9$),
(iv) PD123319 alone ($n = 4$),
(v) hydralazine ($n = 7$).

2.2. Determination of Plasma Ang II Levels. At the end of the treatment period, a sample of blood was collected directly from the catheterised aorta of each animal into chilled, heparinised tubes, and then centrifuged at 4000 rpm at 4°C for 10 minutes to isolate plasma. The resultant plasma sample was stored at −80°C for later analysis. Ang II concentrations were analysed in duplicate by RIA as described previously [27]. Briefly, plasma (100 μL) was equilibrated with antibody raised in rabbit against Ang II, which was N-terminally conjugated to bovine thyroglobulin. Monoiodinated ^{125}I-Ang II tracer (10 000 cpm in 100 μL) was added and allowed to equilibrate for 16 hrs at 4°C, whereupon bound and free phase was separated using Dextran 10-coated charcoal and centrifugation. Sensitivity was 3.5 pg/mL. Intra- and interassay variabilities were 6.4 and 12.0%. Cross reactivity to other angiotensins were Ang I = 0.52%, Ang (1–7) = 0.01%, and to all other pertinent hormones less than 0.10%.

2.3. Perfusion Fixation. After 4 or 6 weeks treatment, animals were anaesthetised (ketamine/xylazine; 100 mg/10 mg per kg), and the abdominal aorta briefly ligated to enable removal of the radiotelemetry probe. A catheter was inserted into the abdominal aorta, and a sample of blood was collected into a heparinised tube. Heparin sodium (1 IU/g body weight), papaverine hydrochloride (1.2 mg/rat), and potassium chloride (60 mM in 0.1 mL) were administered via the catheterized aorta to prevent blood from clotting, maximally dilate blood vessels, and arrest the heart in diastole, respectively. Organs were cleared of blood with physiological saline, and then perfusion fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. Perfusion pressure was maintained at a pressure corresponding to the *in vivo* systolic pressure of adult and aged SHRs by use of a perfusion apparatus attached to a sphygmomanometer. Hearts and blood vessels were then excised and stored immersed in paraformaldehyde at 4°C for later processing.

2.4. Cardiac Remodelling. Both left and right atria were removed from fixed hearts, and the remaining left ventricle (LV), right ventricle (RV), and septum were weighed. Hearts were then cut into approximately twelve 1.5 mm thick slices using a razor blade slicing device. Each slice was then

Table 1: Effect of drug treatments on body weight, ventricular weight, and plasma Ang II of adult and senescent SHRs.

	Control	Candesartan cilexetil	Candesartan cilexetil + PD123319	PD123319	Hydralazine
Body weight (g)					
Adult SHRs	411 ± 11	415 ± 5	410 ± 5	414 ± 3	—
Senescent SHRs	417 ± 9	427 ± 7	428 ± 10	399 ± 9	419 ± 11
Ventricular weight (mg)					
Adult SHRs	1423 ± 23	1229 ± 39*	1207 ± 30*	1474 ± 48	—
Senescent SHRs	2114 ± 74	1796 ± 85*	1962 ± 86	2136 ± 163	1952 ± 49
Plasma Ang II (pg/mL)					
Adult SHRs	220 ± 100	1597 ± 234*	2785 ± 817*	68 ± 18	—
Senescent SHRs	80 ± 10	383 ± 89*	271 ± 51*	64 ± 23	91 ± 16

Values are mean ± SEM. *$P < 0.05$ versus age-matched control (1-way ANOVA).

placed on a light table, images were captured using a video camera module (Sony, XC-77CE CCD, Japan) displayed on a monitor, and analysed using imaging computer software (Microscope Computed Imaging Device M4 (MCID), Imaging Research, Canada). Sampled cross-sectional areas of the LV, RV, and both LV and RV chambers were then multiplied by slice thickness to calculate the volume of each sampled area. Total volumes of LV, RV, LV chamber and RV chamber of each heart were determined by adding measurements taken from heart slices throughout the entire heart. Ventricular weight and volume measurements were normalized to body weight for each animal.

2.5. Interstitial and Perivascular Fibrosis in the Heart. After heart volumes had been determined, five 1.5 mm heart slices from each animal were embedded in paraffin, sectioned at 5 μm and stained for collagen with 0.001% Picrosirius Red. Each section was viewed under a light microscope (Olympus, BH-2, Japan) with a video camera module interfaced to a computer. Images were displayed onto a monitor and analysed using imaging computer software (MCID). All sections were examined under ×200 magnification.

The area of interstitial fibrosis in 6 fields of view of the LV per section and 2 fields of view of the RV per section were sampled, and the percentage of fibrosis within each sampled area was averaged for each animal. Collagen volume fraction (%) was calculated by determining the area stained for collagen as a percentage of the total area of sampled tissue, per field of view. Perivascular fibrosis was investigated in the LV only. Two intramyocardial arterioles (measuring 100–200 μm in diameter) were randomly selected per section. The cross-sectional area (CSA) of adventitia (representing perivascular fibrosis), media, and lumen were determined and averaged for each animal. Perivascular fibrosis was normalised to lumen area. As an index of microvascular remodelling, media-to-lumen ratio of intramyocardial vessels was determined from CSA measurements and averaged for each animal.

2.6. Aortic Hypertrophy. Segments of fixed vessel were dissected from the thoracic portion of the aorta of each animal, embedded in epon-araldite, cut at 1 μm, and stained with toluidine blue. Each section was viewed under a light microscope and analysed using imaging computer software (MCID). All sections were examined under ×100 magnification. CSA of the media was determined for each vessel and normalised to lumen CSA.

2.7. Statistics. The effect of drug treatments on MAP and HR over time was assessed by one- or two-way analysis of variance (ANOVA) with repeated measures, as appropriate. Differences in morphometric data between treatments were determined using one-way ANOVA, followed by a Bonferroni post hoc test. Results are expressed as mean ± standard error of the mean (SEM). Statistical significance was accepted as a probability of $P < 0.05$.

3. Results

3.1. Body Weight and Plasma Ang II. Body weight was not affected by any drug treatments (Table 1). Compared to age-matched untreated animals, plasma Ang II levels were increased more than 7- and 3-fold by candesartan cilexetil treatment, either alone or in combination with PD123319, in adult and senescent SHRs, respectively (Table 1). Both PD1231319 alone and hydralazine had no effect on plasma Ang II levels (Table 1).

3.2. Blood Pressure and Heart Rate in Senescent SHRs. Candesartan cilexetil caused a marked reduction in MAP compared to control animals, and administration of PD123319 did not reverse this antihypertensive effect, in either adult or senescent animals (Figures 1(a) and 1(c)). MAP was unaffected by PD123319 alone. In senescent SHRs, hydralazine caused a reduction in MAP that was similar in magnitude to that caused by AT_1R blockade (Figure 1(c)). Candesartan cilexetil and hydralazine increased HR at the initiation of antihypertensive treatment in both age groups, which most likely represents a reflex tachycardia which persisted for 2-3 days until resetting of the baroreflex occurred. HR after this initial period was unaffected by treatments (Figures 1(b) and 1(d)).

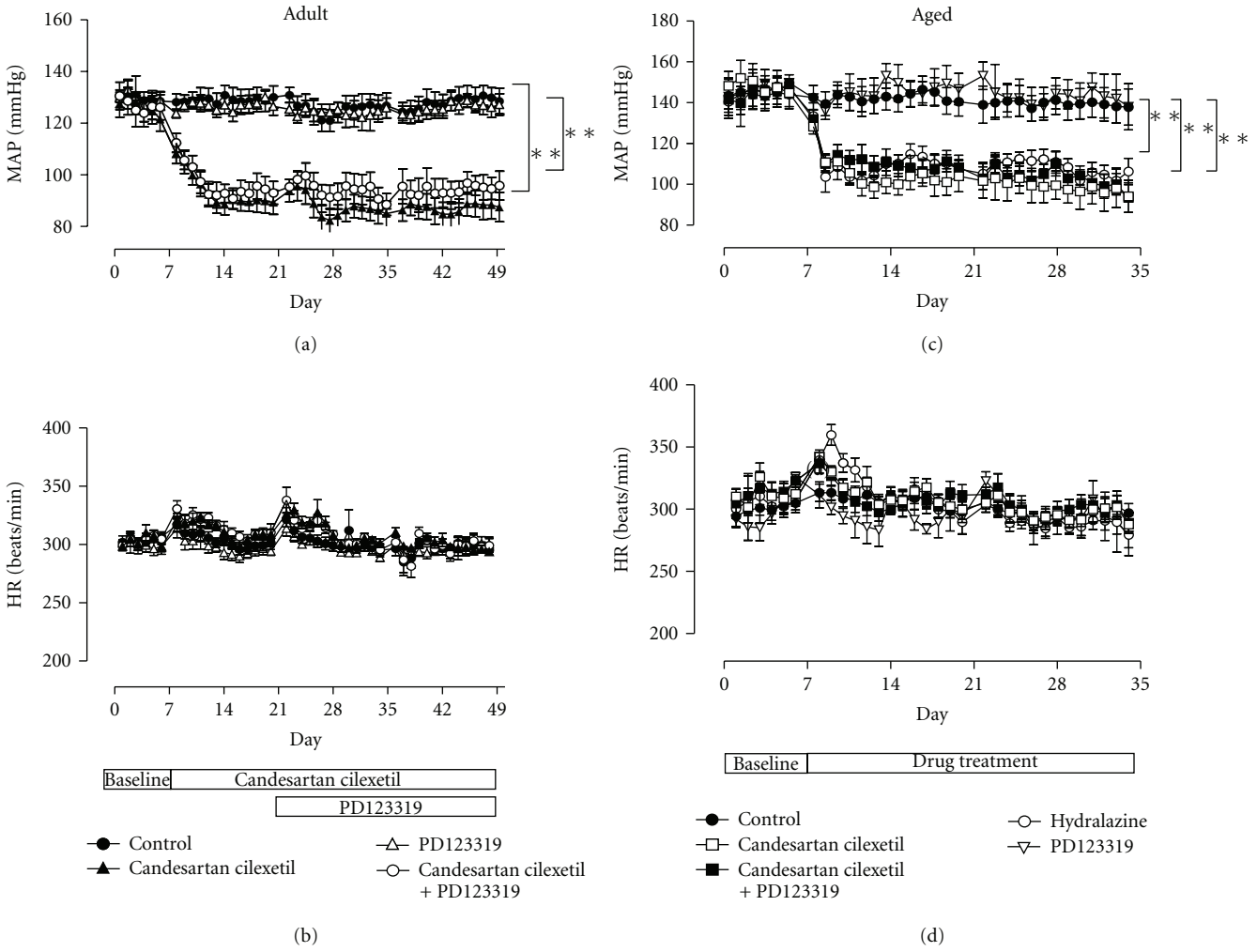

Figure 1: Radiotelemetry recordings of (a) MAP and (b) HR of adult SHRs, at baseline and during treatment with vehicle (control, $n = 6$), candesartan cilexetil (2 mg/kg/day) alone ($n = 7$) or in combination with PD123319 (10 mg/kg/day, $n = 7$), or PD123319 alone. Analogous radiotelemetry recordings of (c) MAP and (d) HR of senescent SHRs, at baseline and during treatment with vehicle (control, $n = 10$), candesartan cilexetil alone ($n = 9$) or in combination with PD123319 ($n = 9$), PD123319 alone ($n = 4$), or hydralazine (30 mg/kg/day, $n = 7$). $^{**}P < 0.01$ versus control (2-way ANOVA).

3.3. Cardiac Remodelling. Ventricular weight (Table 1), ventricular weight to body weight ratio (Figure 2(a)) and LV volume to body weight ratio (Figure 2(b)) of adult SHRs were reduced by candesartan cilexetil; however, this antihypertrophic action was not further influenced by simultaneous AT_2R inhibition. Similarly, ventricular weight (Table 1), ventricular weight to body weight ratio (Figure 2(c)), and LV volume to body weight ratio (Figure 2(d)) of senescent SHRs were also decreased by AT_1R blockade. Furthermore, the regression of both ventricular weight and ventricular weight to body weight ratio were partially reversed by concurrent PD123319 treatment, such that these indices were not significantly different from control values. There were no effects of drug treatments on RV, LV chamber or RV chamber, volume to body weight ratios (data not shown).

3.4. Interstitial and Perivascular Fibrosis. Representative light micrographs of perivascular and interstitial fibrosis of senescent SHRs are shown in Figure 3. Group data shows that neither left (Figures 4(a) and 4(c)) nor right (Figures 4(b) and 4(d)) ventricular interstitial fibrosis of adult and senescent SHRs were altered by any drug treatments. Likewise, perivascular fibrosis of adult SHRs was not influenced by AT_1 or AT_2R inhibition (Figure 5(a)). In contrast, perivascular fibrosis was significantly decreased by ~28% in senescent SHRs receiving candesartan cilexetil, and this effect was completely reversed by simultaneous AT_2R blockade (Figure 5(b)).

3.5. Vascular Hypertrophy. Media to lumen ratios of aortic vessels in both adult and senescent SHRs (Figures 6(a) and 6(c)) and intramyocardial vessels of senescent SHRs (Figure 6(d)) were decreased by candesartan cilexetil, and this antihypertrophic effect of AT_1R blockade was reversed by concomitant PD123319 administration. Hydralazine also caused a significant reduction in media-to-lumen ratios

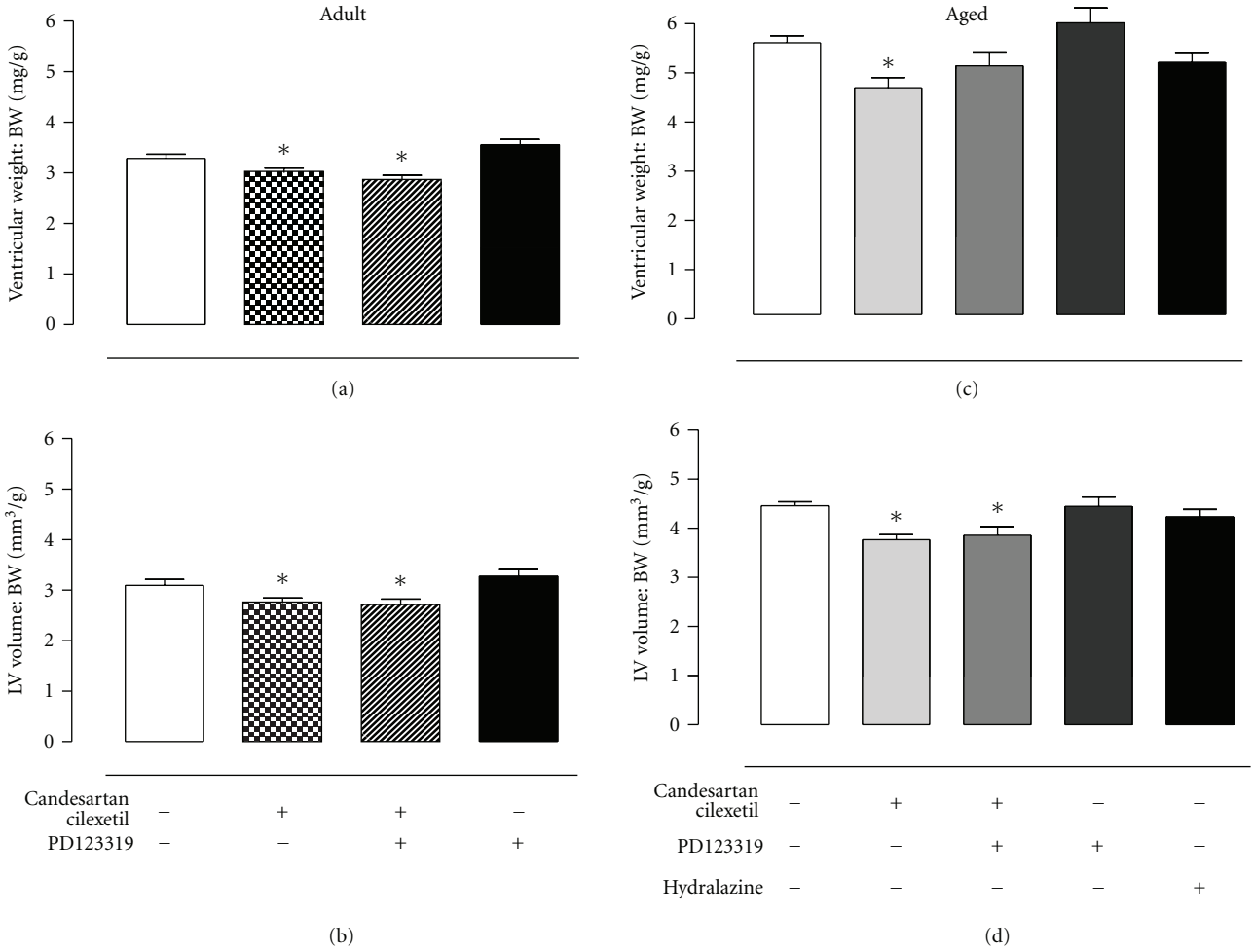

Figure 2: (a) Ventricular weight to body weight (BW) ratio and (b) left ventricular (LV) volume to body weight ratio of adult SHRs, at baseline and during treatment with vehicle (control, $n = 4$), candesartan cilexetil (2 mg/kg/day) alone ($n = 4$), or in combination with PD123319 (10 mg/kg/day, $n = 4$), and PD123319 alone ($n = 7$). (c) Ventricular weight to BW ratio and (d) LV volume to BW ratio of senescent SHRs, at baseline and during treatment with vehicle (control, $n = 10$), candesartan cilexetil (2 mg/kg/day) alone ($n = 9$), or in combination with PD123319 (10 mg/kg/day, $n = 9$), PD123319 alone ($n = 4$), or hydralazine (30 mg/kg/day, $n = 7$). $^*P < 0.05$ versus control (1-way ANOVA).

of aortic (Figure 6(b)) and intramyocardial (Figure 6(d)) vessels of senescent SHRs.

4. Discussion

We have shown for the first time, a role for AT_2R in cardiac and vascular remodelling in a clinically relevant animal model of aging and hypertension. Notably, AT_2R stimulation by endogenously raised Ang II levels contributed to the cardiac antifibrotic and vascular antihypertrophic effects of chronic AT_1R blockade. Thus, this study highlights the importance of AT_2R in the chronic regulation of cardiovascular structure in the aging hypertensive heart and vasculature.

Candesartan cilexetil caused a marked reduction in MAP in both adult and senescent SHRs, which was not further affected by AT_2R blockade. These results imply that stimulation of the AT_2R does not significantly influence chronic blood pressure regulation and is consistent with other long-term studies that showed either no [13, 14, 28] or minimal [15] reversal of AT_1R-blocker- (ARB-) mediated blood pressure-lowering by simultaneous AT_2R blockade in SHRs. These findings are in direct contrast to the acute setting, in which the antihypertensive effect of ARB compounds was reversed by simultaneous AT_2R blockade with PD123319 [29–31]. In addition, acute stimulation of AT_2R has also been shown to lower blood pressure in rats, supporting a role for AT_2R in acute blood pressure regulation [32–35].

Since both human and animal studies have shown circulating Ang II and renin levels to be reduced with increasing age [22, 36, 37], it is possible that the absence of AT_2R-mediated actions on blood pressure in aged SHRs is due to depressed systemic RAS activity in senescence. However, in this study we have shown a similar inability of PD123319 to reverse the ARB-induced reduction in

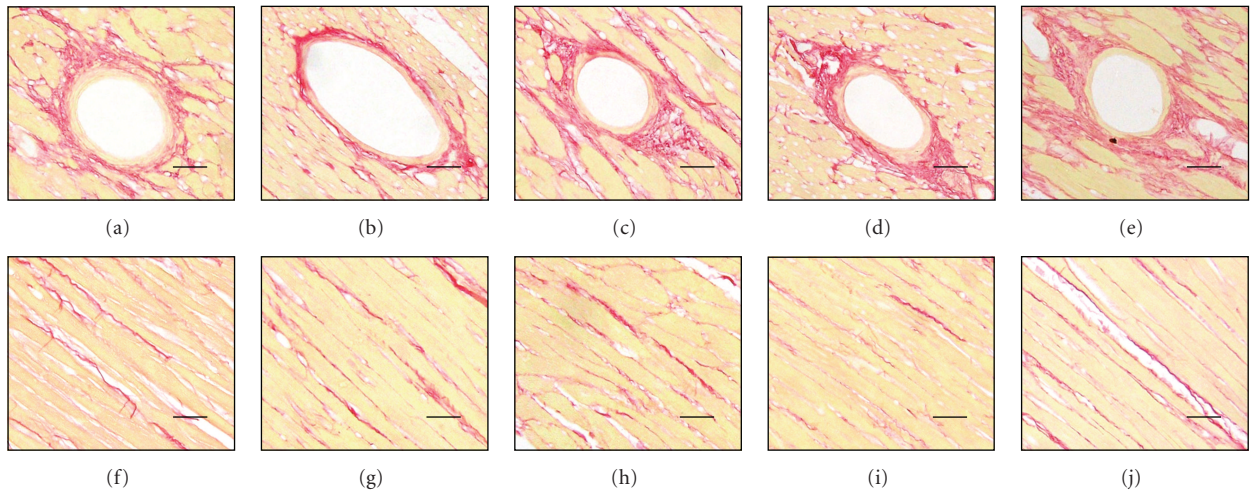

Figure 3: Representative light micrographs of cardiac (a–e) perivascular and (f–j) interstitial fibrosis in senescent SHRs treated with (a, f) vehicle (control), (b, g) candesartan cilexetil (2 mg/kg/day), (c, h) candesartan cilexetil in combination with PD123319 (10 mg/kg/day), (d, i) PD123319 alone, or (e, j) hydralazine (30 mg/kg/day). Scale bar = 50 μm.

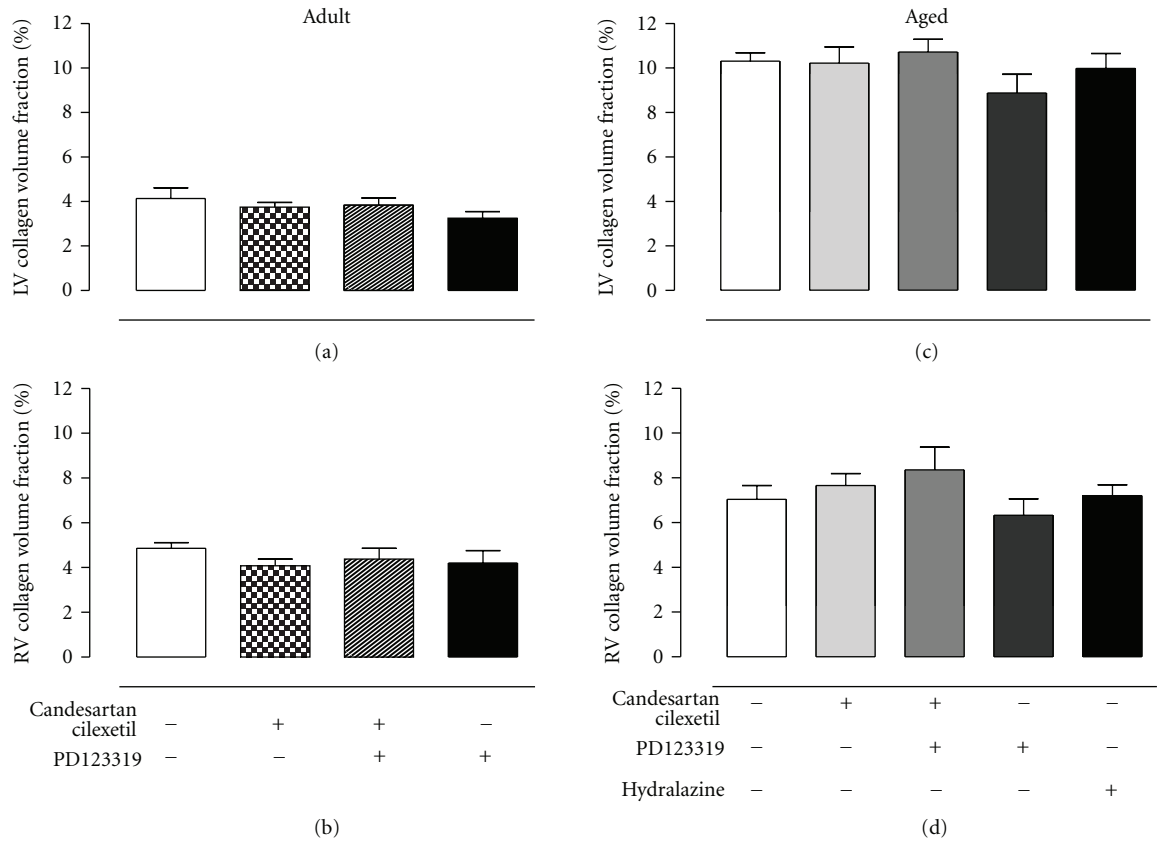

Figure 4: Mean data of interstitial collagen volume fraction of (a) left and (b) right ventricles of adult SHRs treated with vehicle (control, $n = 6$), candesartan cilexetil (2 mg/kg/day) alone ($n = 7$) or in combination with PD123319 (10 mg/kg/day, $n = 7$) or PD123319 alone. Interstitial collagen volume fraction of (c) left and (d) right ventricles of senescent SHRs treated with vehicle (control, $n = 10$), candesartan cilexetil alone ($n = 9$) or in combination with PD123319 ($n = 9$), PD123319 alone ($n = 4$), or hydralazine (30 mg/kg/day, $n = 7$).

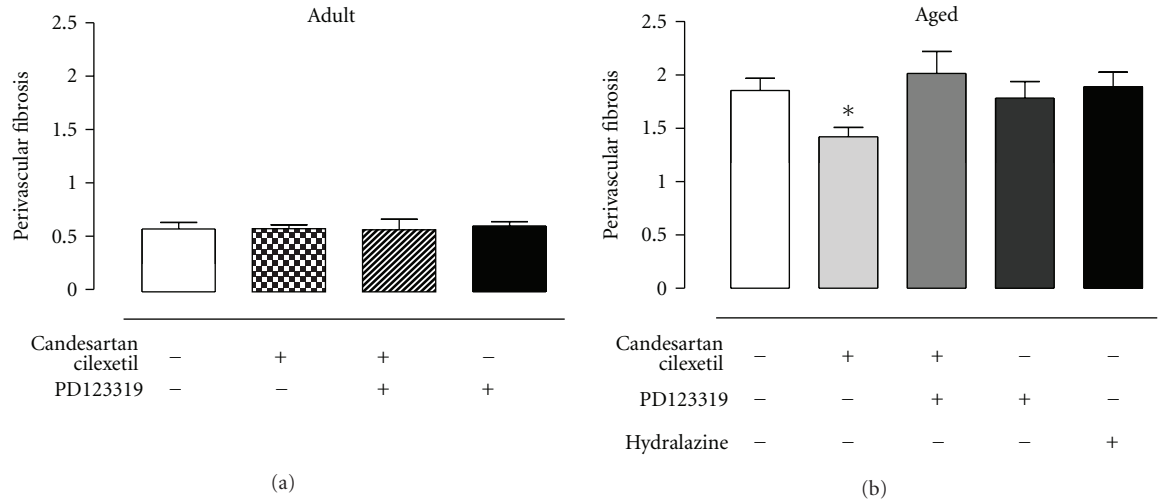

FIGURE 5: (a) Perivascular fibrosis of adult SHRs treated with vehicle (control, $n = 6$), candesartan cilexetil (2 mg/kg/day) alone ($n = 7$) or in combination with PD123319 (10 mg/kg/day, $n = 7$), or PD123319 alone ($n = 7$). (b) Perivascular fibrosis of senescent SHRs treated with vehicle (control, $n = 10$), candesartan cilexetil alone ($n = 9$) or in combination with PD123319 ($n = 9$), PD123319 alone ($n = 4$), or hydralazine (30 mg/kg/day, $n = 7$). Perivascular fibrosis of intramyocardial arterioles, calculated as cross-sectional area of adventitia to lumen ratio. $^*P < 0.05$ versus control (1-way ANOVA).

blood pressure in both adult and senescent rats. Moreover, even though baseline levels of Ang II are relatively low in aged SHRs compared to adult SHRs (~3-fold lower than adult SHRs, Table 1), AT_1R inhibition caused an increase in plasma Ang II of 3-4-fold, suggesting that the RAS is still sensitive to perturbation in aged SHRs. Moreover, local tissue production of Ang II has been shown to be elevated in aged humans [6] and rodents [38]. Thus it is more likely that the inability of PD123319 administration to reverse ARB-induced antihypertensive effects in the current context reflects a subtle influence of AT_2R stimulation on blood pressure regulation being masked by the dominant impact of AT_1R blockade.

Candesartan cilexetil decreased indices of cardiac growth of adult rats (ventricular weight, and LV volume to body weight ratios), and PD123319 administration had no further influence on these parameters, suggesting no major role for AT_2R in cardiac hypertrophy. Other studies in hypertensive models have also reported that PD123319 administration did not significantly reverse cardiac hypertrophy [15, 19, 28], and additionally, AT_2R were deduced to have no major function in the regulation of cardiac mass from studies in transgenic mice models of targeted deletion or cardiac-specific over expression of AT_2R [10, 39, 40]. In contrast, a dependence on AT_2R for ARB-mediated cardiac remodelling following MI has been demonstrated in rats [13] and AT_2R knock out mice [40]. These mismatches in reported AT_2R influence on cardiac hypertrophy most likely reflect the gross measures of cardiac hypertrophy made in the majority of studies, as heart mass is commonly employed as a surrogate marker for cardiac hypertrophy (i.e., increased cardiomyocyte size) but is unable to distinguish between changes in proportion of specific components within the heart.

Indeed, ventricular weight and LV volume to body weight ratios were also reduced by AT_1R blockade in senescent SHRs; however, in these aged animals, simultaneous AT_2R inhibition caused a partial reversal of ventricular weight to body weight ratio. Given that LV volume is heavily influenced by changes in cardiomyocyte area [2], and that LV volume was not influenced by AT_2R blockade, PD123319-mediated reversal of heart weight to body weight ratio most likely reflects changes in the nonmyocyte components of the heart, rather than a true effect on cardiac hypertrophy. Indeed, we have shown that perivascular fibrosis of coronary microvessels is decreased by AT_1R blockade and that this effect is reversed by concomitant AT_2R blockade, but only in senescent hearts. We have previously shown a similar mismatch between LV volume and ventricular weight following AT_1R blockade in senescent normotensive WKY rats [26], which also coincided with a cardiac AT_2R-mediated antifibrotic action. Thus the PD122319-mediated increase in ventricular weight to body weight ratio during AT_1R blockade may in fact be due to inhibition of an AT_2R-mediated antifibrotic action in senescent SHRs.

Surprisingly, candesartan cilexetil did not reduce interstitial fibrosis in either adult or senescent SHRs. In the present study, particularly high levels of LV and RV interstitial fibrosis (collagen volume fraction ~7–10%) were seen in control senescent animals. These relatively high levels of interstitial fibrosis in aged hearts are entirely consistent with previous studies [15, 20], and contrast with the degree of fibrosis in adult hypertensive SHRs (interstitial collagen volume fraction ~4-5%). We have previously shown that an identical treatment regime markedly reduced interstitial fibrosis from similar levels in aged normotensive WKY (interstitial collagen volume fraction ~4-5%), and this effect was also reversed by PD123319 [26]. Thus in the current

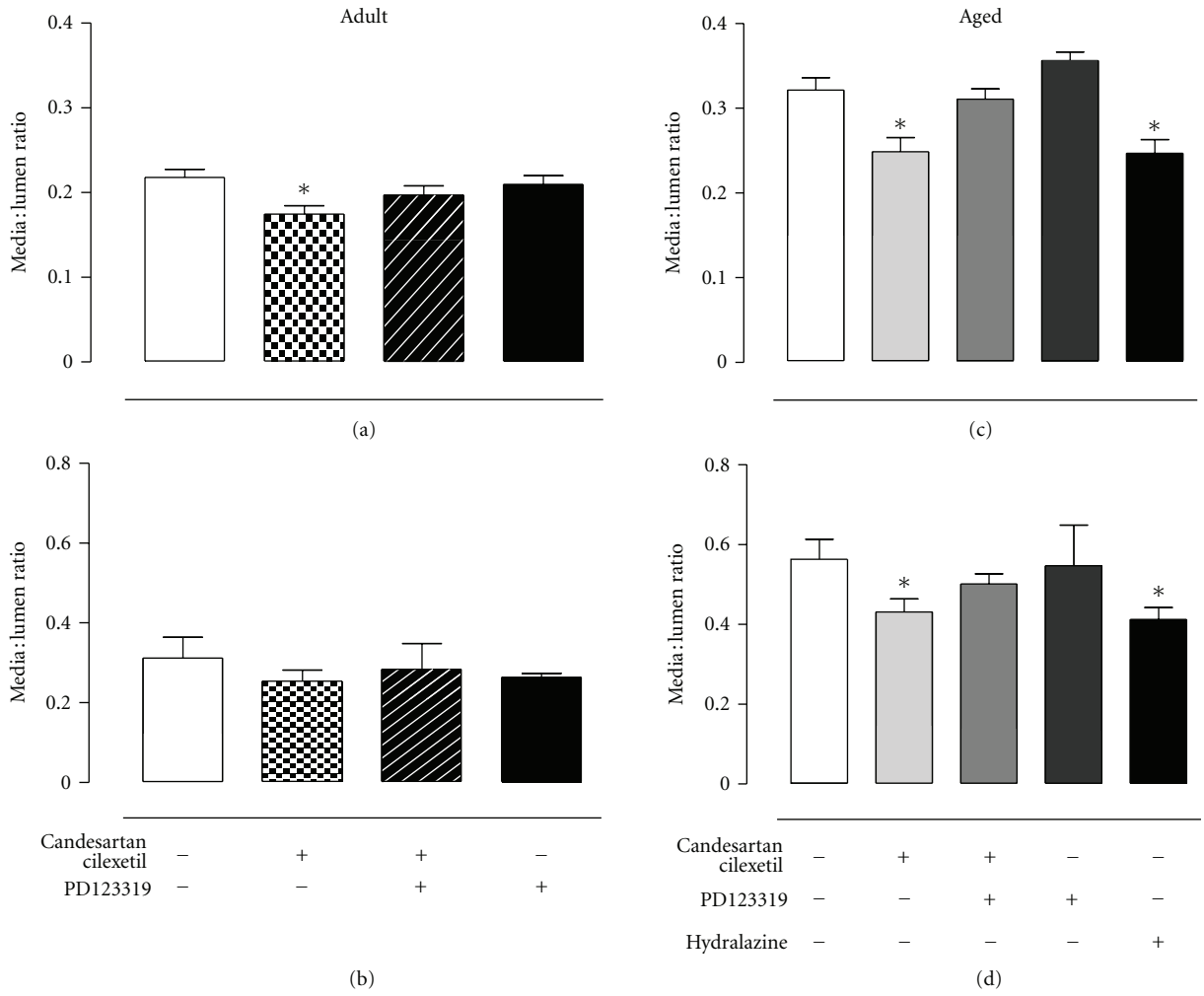

Figure 6: Media to lumen ratio of (a) aortic and (b) intramyocardial vessels of adult SHRs treated with vehicle (control, $n = 6$), candesartan cilexetil (2 mg/kg/day) alone ($n = 7$) or in combination with PD123319 (10 mg/kg/day, $n = 7$) or PD123319 alone ($n = 7$). Media to lumen ratio of (c) aortic and (d) intramyocardial vessels of senescent SHRs treated with vehicle (control, $n = 10$), candesartan cilexetil alone ($n = 9$) or in combination with PD123319 ($n = 9$), PD123319 alone ($n = 4$), or hydralazine (30 mg/kg/day, $n = 7$). $^*P < 0.05$ versus control (1-way ANOVA).

study, it appears that the inability of candesartan cilexetil to reduce interstitial fibrosis in both adult and senescent SHRs, results from modifications of the ECM specifically related to hypertension, rather than particularly high pretreatment basal levels of fibrosis. Indeed, collagen cross-linking has been shown to be augmented by hypertension [41, 42], and increased cross-linking is associated with diminished susceptibility of the ECM to proteolytic degradation [43]. Alteration in ECM degradation due to increased glycation cross-linking has been associated with decreased activity of proteolytic enzymes such as matrix metalloproteinase 1 and 2 (MMP-1 and MMP-2) [44], and findings of decreased activity of MMP-1 and MMP-2 by 40–45% in aged, hypertensive rats [45] further support the notion of impaired collagen degradative mechanisms in senescent hypertensive hearts.

On the other hand, the antifibrotic action of AT_2R stimulation on perivascular fibrosis in senescent rats, as demonstrated in the current investigation, is in accordance with other chronic *in vivo* studies, which have also shown increased cardiac fibrosis during AT_2R blockade [15, 19, 26]. Similarly, investigators who have used either targeted deletion [8, 40, 46] or cardiac overexpression [10] of AT_2R in mice have also deduced an antifibrotic role of the AT_2R. Importantly, cardiac fibrosis induced by circulating humoral factors such as Ang II, typically initiates around blood vessels and then progresses to infiltrate interstitial areas, resulting in a temporal divergence in onset (and thus conceivably also of regression) of the two types of fibrosis related to location [47]. In this context, it is possible that interstitial fibrosis may have been reduced by a longer duration treatment with an AT_1R antagonist, as has been reported by other investigators following AT_1R blockade for 12 weeks [15].

In the current study, media-to-lumen ratio of both aortae and coronary vessels was decreased by candesartan cilexetil treatment and also by hydralazine in aged SHRs.

This vascular antihypertrophic effect is consistent with previous reports that increased medial thickness due to hypertrophy/hyperplasia of smooth muscle cells is closely related to pressure [48, 49]. However, the other major modification of vascular structure that occurs in hypertension and senescence is an increase in vascular collagen content, the levels of which have been shown to be poorly associated with MAP, but sensitive to AT_1R inhibition [48]. As the vascular antihypertrophic effect of candesartan cilexetil was reversed, but MAP was unchanged by PD123319, it is reasonable to suggest that the effect of AT_2R inhibition on vascular remodelling was pressure-independent and thus may be via a reduction in vascular collagen. Furthermore, such a pressure-independent influence of AT_2R on collagen accumulation in aged SHRs is consistent with effects on perivascular fibrosis in this study, which were decreased by AT_1R blockade but unaffected by hydralazine, despite both treatments resulting in similar reductions in MAP.

A limitation of this study was that we did not confirm that the reversal of ARB-mediated antifibrotic effects by PD123319 is solely via AT_2R mechanisms. Indeed, we [50] and others have shown that in certain situations, PD123319 may inhibit the effects of Ang 1–7, which is considered the endogenous ligand for the *Mas* receptor (*Mas*R). However, we have also recently reported that Ang 1–7 shows significant AT_2R binding [51], which is consistent with PD123319-mediated reversal of Ang 1–7 effects being due to inhibition of AT_2R rather than a nonselective action at MasR. Nevertheless, definitive elucidation of this issue regarding selectivity of PD123319 requires future determination of *Mas*R binding.

The present study demonstrates an important role for AT_2R in cardiovascular remodelling in senescent SHRs, as evidenced by the fact that AT_2R inhibition with PD123319 reversed ARB-mediated regression of perivascular fibrosis in aged SHRs only. Furthermore, we have shown an inhibitory influence of AT_2R in vascular remodelling, which was apparent in both adult and senescent SHRs, and occurred despite a lack of AT_2R-mediated effects on blood pressure. Given that our population is aging and that AT_1R antagonists are commonly used antihypertensives in this demographic, this study provides information regarding the functional relevance of AT_2R in the physiologically relevant setting of hypertension and senescence, which may have important implications for optimising cardiovascular therapeutics in the elderly.

Acknowledgments

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Research Article

Recombinant Expression and Characterization of Human and Murine ACE2: Species-Specific Activation of the Alternative Renin-Angiotensin-System

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Angiotensin-converting enzyme 2 (ACE2) is a monocarboxypeptidase of the renin-angiotensin-system (RAS) which is known to cleave several substrates among vasoactive peptides. Its preferred substrate is Angiotensin II, which is tightly involved in the regulation of important physiological functions including fluid homeostasis and blood pressure. Ang 1–7, the main enzymatic product of ACE2, became increasingly important in the literature in recent years, as it was reported to counteract hypertensive and fibrotic actions of Angiotensin II via the MAS receptor. The functional connection of ACE2, Ang 1–7, and the MAS receptor is also referred to as the alternative axis of the RAS. In the present paper, we describe the recombinant expression and purification of human and murine ACE2 (rhACE2 and rmACE2). Furthermore, we determined the conversion rates of rhACE2 and rmACE2 for different natural peptide substrates in plasma samples and discovered species-specific differences in substrate specificities, probably leading to functional differences in the alternative axis of the RAS. In particular, conversion rates of Ang 1–10 to Ang 1–9 were found to be substantially different when applying rhACE2 or rmACE2 *in vitro*. In contrast to rhACE2, rm ACE2 is substantially less potent in transformation of Ang 1–10 to Ang 1–9.

1. Introduction

The classical renin-angiotensin-system (RAS) is a proteolytic cascade which is constituted by multiple enzymes and effector peptides. The cascade starts when Angiotensin I (Ang 1–10) is released from the propeptide angiotensinogen by kidney-secreted renin. The peptide metabolites produced from Ang 1–10 by a variety of proteases act as ligands for angiotensin receptors in different tissues leading to a diversified panel of physiological functions mediated by angiotensin peptides [1].

Angiotensin II (Ang 1–8) is one of the most extensively studied angiotensin peptides. It is mainly produced by the proteolytic action of angiotensin-converting enzyme (ACE) by removal of the two C-terminal amino acids from Ang 1–10. Ang 1–8 is able to bind to several cellular receptors leading to a variety of physiologic effects among different tissues and cell types [2]. Importantly, increased levels of Ang 1–8 are reported to be associated with life-threatening pathologic conditions including hypertension, congestive heart failure, chronic kidney disease, and also tumor progression [3]. Ang 1–8 was described to directly increase blood pressure and vessel permeability, to induce Na reabsorption and ROS production and excert proinflammatory and proliferative effects on various cell types [4, 5].

The disease-promoting functions of Ang 1–8 convert it to a favorable therapeutic target in the treatment of many diseases mainly by preventing its formation by low-molecular-weight compounds inhibiting appropriate enzymes of the RAS cascade. An alternative way of decreasing Ang 1–8 levels

became available over the recent years and uses recombinant angiotensin-converting enzyme 2 (ACE2) to lower Ang 1–8 levels. ACE2 inactivates Ang 1–8 by clipping off one C-terminal phenylalanine [6], while Ang 1–7 is generated. Ang 1–7 is known to take over Ang 1–8 antagonistic functions by activating the MAS receptor [7–9] and therefore is thought to be the key effector peptide of the so-called alternative RAS.

Therefore, the monocarboxypeptidase ACE2 is a key activator of the alternative RAS and is critically involved in the regulation of the classical RAS, which is known to be functionally important in the vascular system and in a variety of organs [6, 10, 11]. The biological function of the RAS has been investigated in cardiovascular [12, 13], pulmonary [14], fibrotic [15], nephrologic [13], and artheriosclerotic [16] models.

Throughout all these studies the loss of ACE2 activity in knock-out variants induced pathologies which could be restored by systemic administration of the recombinant enzyme. ACE2 therefore can be regarded as one of the key players of the renin-angiotensin-system (RAS) being responsible for fluid homeostasis, blood pressure regulation, inflammatory processes, and cell proliferation. ACE2 is a membrane anchored glycoprotein which is expressed in most organs and blood vessels and recognizes multiple peptide substrates within the RAS and other peptide hormone systems. Among its substrates beside Ang 1–8, Ang 1–10, and des-Arg-bradykinin, Apelins and Dynorphins have been reported to be cleaved by ACE2 *in vitro* [17] with Ang 1–8 being the preferred substrate regarding conversion rates [18].

We recombinantly expressed both human ACE2 (rhACE2) and murine ACE2 (rmACE2) and compared their substrate conversion rates *in vitro* and in blood plasma which represents the natural compartment of enzyme action. In previously mentioned murine knock-out models, rhACE2 was frequently used to restore ACE2 activity. Despite the fact that sequence coverage between murine and human ACE2 is only 83% [19], it has been assumed that the enzyme has the same catalytic activity and function. In this work we will highlight species-specific differences between human and murine ACE2 regarding their function of keeping the balance between the classical and the alternative RAS.

2. Material and Methods

2.1. Recombinant Expression of Murine and Human ACE2. The extracellular domains of human or murine ACE2 [6] were recombinantly expressed in CHO cells under serum-free conditions. The sequence identity between rhACE2 and rmACE2 accounts to 84% which leads to minor alterations in physicochemical properties and altered patterns in post-translational modifications, especially N-glycosylation. Both expression products were purified by sequentially performing a capture step on a DEAE-Sepharose, ammonium sulfate precipitation, followed by a purification step on a HIC-Phenyl Sepharose column and a final polishing step on a Superdex 200 gel filtration column. The purity of rhACE2 and rmACE2 was determined by high-performance liquid chromatography (HPLC) and was found to exceed 98%. The concentrations of final ACE2 preparations were determined by size-exclusion chromatography (SEC) and in line with photometric measurement at 280 nm and peak integration (OD_{280}: rhACE2: $\varepsilon = 1621\ L*mol^{-1}*cm^{-1}$, rmACE2: $\varepsilon = 1750\ L*mol^{-1}*cm^{-1}$).

2.2. Native PAGE. 2 μg of rmACE2 and rhACE2 were applied on a precast native 3–12% gradient gel (Invitrogen). Anode buffer (50 mM Bis/Tris, 50 mM Tricine) and cathode buffer (Invitrogen NativePAGE Cathode Buffer Additive, 50 mM Bis/Tris, 50 mM Tricine) were used to run the gel. 40% glycerol, 200 mM Bis/Tris, and 200 mM Tricine were used as a loading buffer. NativeMark Unstained Protein Standard (Thermo Scientific) was used for estimation of molecular weights in Coomassie Blue-stained gels. The gel was run at 150 V for 80 min. Proteins were stained in gel using NOVEX Colloidal Blue Staining Kit according to manufacturers' recommendations.

2.3. SDS-PAGE. Samples were analyzed by SDS-PAGE using a 4–12% precast gradient gel (NuPage) following reductive denaturation for 5 min at 95°C. BenchMark Protein Ladder was run on the same gel to allow molecular weight estimations. The gel was run in NuPage MES SDS Running Buffer (Invitrogen) at 150 V for 80 min. In gel protein staining was performed using a NOVEX Colloidal Blue Staining Kit according to manufacturer's protocol.

2.4. Kinetic Assays for rmACE2 and rhACE2. Substrate specific turnover rates for rhACE2 and rmACE2 were determined by *in vitro* kinetic analysis of Ang 1–8 and Ang 1–10 cleavage followed by HPLC-based quantification of substrate and product concentrations. Enzyme reactions were started by adding a defined amount of enzyme to substrate dilutions in MES-buffer (50 mM MES, 300 mM NaCl, 10 μM $ZnCl_2$, 0.01% Brij-35, pH 6.5) which were previously equilibrated at 37°C. Aliquots of the reaction mixes were taken every 10 minutes and stopped by addition of 0.5 M EDTA to a final concentration of 100 mM before HPLC-based quantification of peptides.

2.5. HPLC-Based Quantification of Angiotensin Peptides. The concentration of peptides in enzymatic reactions was quantified by detection of peaks eluted from the HPLC column using an in-line diode array detector. Chromatography was performed by running a gradient on a reversed-phase matrix (Source 5RPC, 4.6×150 mm, 5 μm) with 0.08% H_3PO_4 in water as mobile phase A and 40% acetonitrile in water and 0.08% H_3PO_4 as mobile phase B. The optical density at 280 nM was recorded inline for all eluting peaks, and peptide concentrations were calculated via calibration curves for each individual peptide.

2.6. Ex Vivo Incubation of Plasma Samples. Anticoagulated blood was collected from healthy volunteers, and plasma was separated by 10 minutes centrifugation at 3000 RCF. Following addition of 100 pg/mL recombinant human renin (Sigma) to isolated blood plasma, rmACE2 or rhACE2 was added to the samples. After 10 minutes of incubation at 37°C,

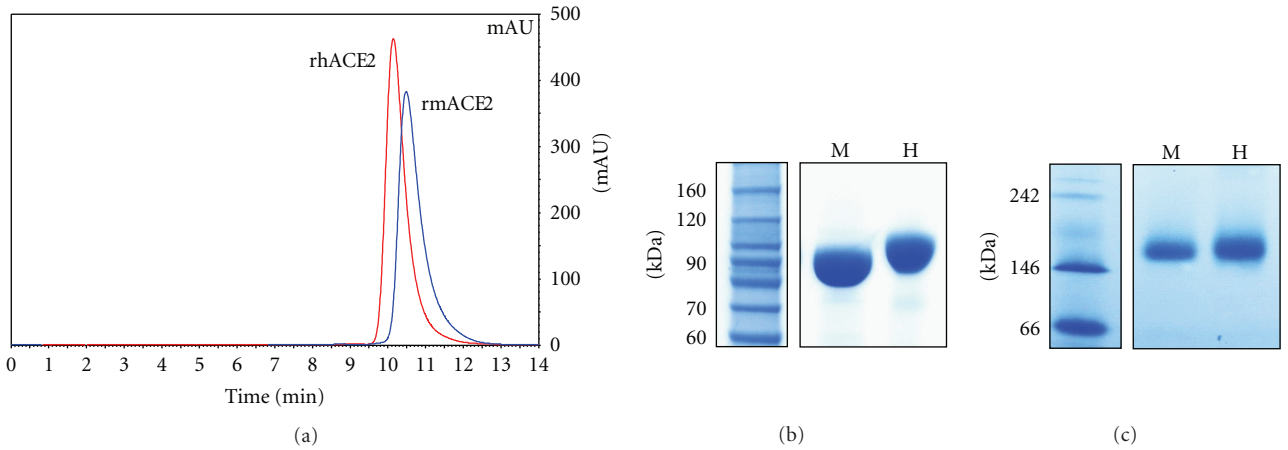

Figure 1: Batch quality of recombinant human and murine ACE2. (a) Equal amounts (5 μg) rmACE2 and rhACE2 were analyzed by size-exclusion chromatography. The elution was continuously monitored by in-line measurement of the absorbance at 280 nm. The eluted peaks for purified rmACE2 (blue) and rhACE2 (red) are shown in the chromatogram and given in absorption units (mAU). (b) Equal amounts (7 μg) of rmACE2 (m) and rhACE2 (h) were subjected to SDS-PAGE analysis followed by in-gel protein staining with Coomassie Brilliant Blue. Selected marker bands are depicted in the graph to allow the estimation of molecular weights. (c) Equal amounts (2 μg) of rmACE2 (m) and rhACE2 (h) were analyzed in native PAGE followed by in-gel protein staining with Coomassie Brillant Blue as described in Section 2.

in the presence or absence of Lisinopril (Sigma), samples were chilled on ice and immediately subjected to LC-MS/MS analysis.

2.7. RAS-Fingerprinting: LC-MS/MS Quantification of Angiotensin Peptides. Plasma samples were spiked with 100 pg/mL stable-isotope-labeled internal standards and subjected to solid-phase extraction using Sep-Pak cartridges (Waters) according to manufacturers protocol. Following elution and solvent evaporation, samples were reconstituted in 50 μL 50% acetonitrile/0.1% formic acid and subjected to LC-MS/MS analysis using a reversed-phase analytical column (Luna C18, Phenomenex) using a gradient ranging from 10% acetonitrile/0.1% formic acid to 70% acetonitrile/0.1% formic acid in 9 minutes. The eluate was analyzed in line with a QTRAP-4000 mass spectrometer (AB Sciex) operated in the MRM mode using dwell times of 25 msec at a cone voltage of 4000 volts and a source temperature of 300°C. For each peptide and corresponding internal standards, two different mass transitions were measured. Angiotensin peptide concentrations were calculated by relating endogenous peptide signals to internal standard signals provided that integrated signals achieved a signal-to-noise ratio above 10. The quantification limits for individual peptides were found to range between 1 pg/mL and 5 pg/mL undiluted plasma.

3. Results

3.1. Quality Control of rmACE2 and rhACE2 Reveals a High Degree of Purity and Functional Protein Structure. The quality of the frozen enzyme batches used for later functional analysis was analyzed regarding enzyme purity and characteristics. The investigation of rmACE2 and rhACE2 by size-exclusion chromatography revealed that no detectable contaminations were present in the enzyme preparations. The protein concentration in the enzyme batches was determined by measuring the peak absorbance inline at 280 nm, peak integration, and subsequent calculation based on the corresponding extinction coefficients. Both ACE2 variants appeared as baseline separated sharp peaks. rhACE2 and rmACE2 were found to slightly differ in retention times, pointing to a difference in their hydrodynamic molecular diameter which was found to be lower for rmACE2 (Figure 1(a)). In order to further investigate this observation, we employed SDS-PAGE analysis, revealing a mass difference under denatured conditions (Figure 1(b)), indicating the presence of additional covalent mass-increasing modifications in rhACE2. The mass shift was found to be caused by two additional glycosylation sites in the human enzyme (data not shown). According to our results, both recombinant ACE2 versions apparently occur as noncovalent homodimers in physiological solution. These findings and their possible implications will be discussed in detail elsewhere. The calculated molecular weights of monomeric rmACE2 and rhACE2 are 85.2 kDa and 85.3 kDa, respectively. RmACE2 and rhACE2 were both found to give a single band at approximately 170 kDa in native PAGE, giving evidence for a homodimer occurrence of both recombinant ACE2 versions (Figure 1(c)). These findings indicate that the recombinant enzymes, produced and purified according to our protocol, are free of contaminants and possess their natural folding and tertiary structure.

3.2. Recombinant ACE2 of Mouse and Human Origin Possess Diverging Turnover Rates for Natural Peptide Substrates In Vitro. Based on the concentrations and purity of enzyme batches previously determined, we investigated the biological activity of rhACE2 and rmACE2 in an *in vitro* system. Therefore, we coincubated defined amounts of purified enzymes with an excess of Ang 1–8 and Ang 1–10, respectively, which

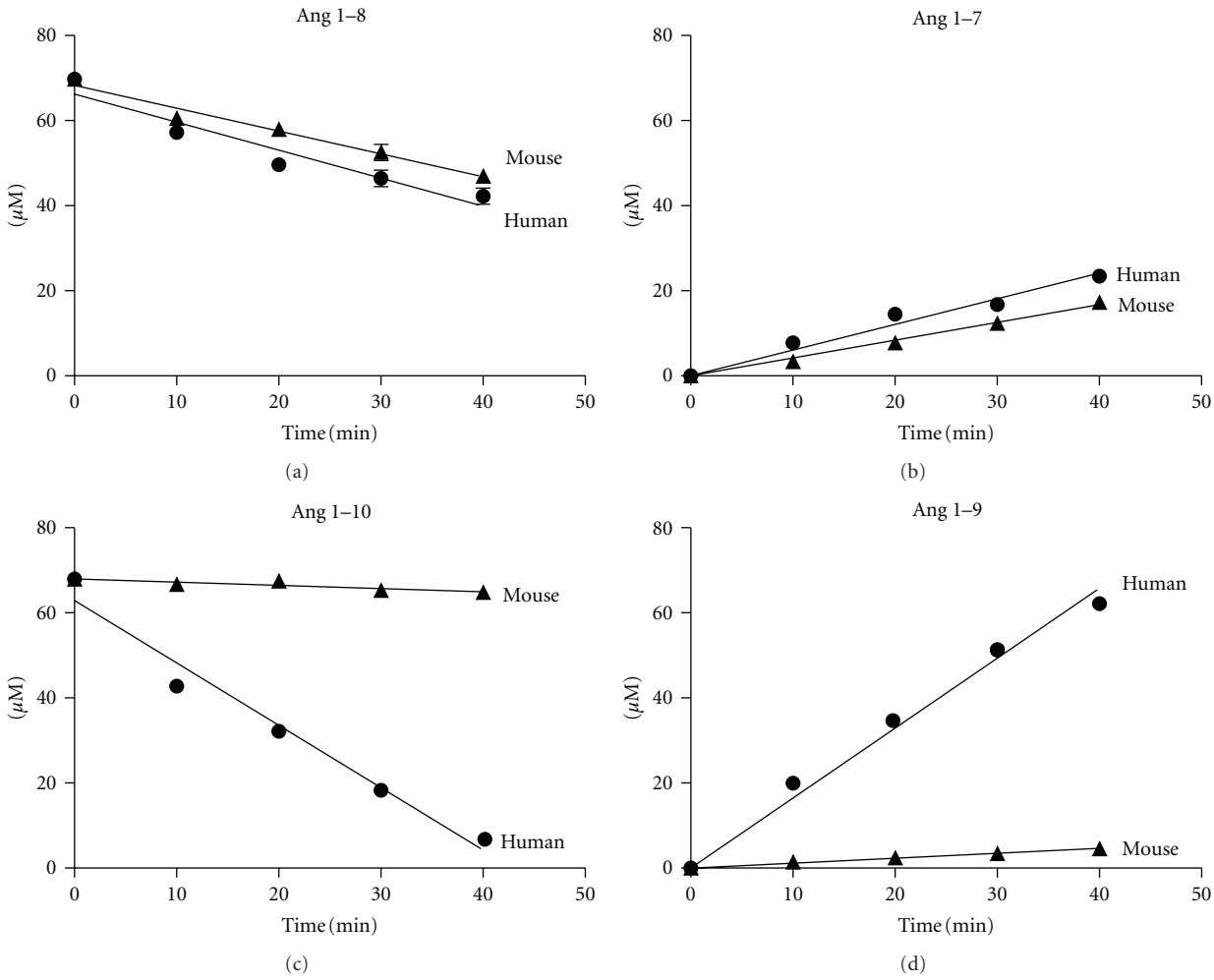

Figure 2: *In vitro* turnover rates of rhACE2 and rmACE2. The turnover rates for natural ACE2 substrates were determined by *in vitro* incubation with excess amounts of substrate followed by reversed-phase HPLC analysis. Therefore, 14 nM rmACE2 or rhACE2 were coincubated with 65 μM Ang 1–8 (a) in MES buffer and aliquots were taken at indicated time points. The resulting time courses are shown in the graphs. The turnover rates for Ang 1–10 were determined in MES buffer containing 1.5 μM rmACE2 or rhACE2 and 65 μM Ang 1–10 (b) as described in Section 2. Each time point was analyzed in true triplicates and standard deviations are given in the graphs together with linear regressions of the measured values.

represent natural substrates for ACE2. We found that rhACE2 as well as rmACE2 converted Ang 1–8 to Ang 1–7 at comparable rates (Figure 2(a)). The calculation of k_{cat} via the graphically determined product formation rate and substrate degradation rate revealed that the turnover number of rhACE2 for Ang 1–8 was 1.2-fold higher than that for rmACE2 (Table 1). As an alternative natural angiotensin substrate for ACE2, we also employed Ang 1–10 cleavage *in vitro*. Surprisingly, rhACE2 turned out to be much more effective in performing the cleavage of Ang 1–10 to Ang 1–9 compared to rmACE2 (Figure 2(b)). The calculation of Ang 1–10 related turnover rates for rhACE2, and rmACE2 revealed that the Ang 1–10 related k_{cat} for rhACE2 was 15-fold higher than that for rmACE2 (1.8×10^{-2} versus 1.2×10^{-3} s^{-1}). Furthermore, the comparison of turnover numbers for different substrates revealed that Ang 1–8 is the preferred substrate for both enzymes *in vitro* with a 42-fold higher turnover number for rhACE2 (0.77 s^{-1}) and a 492-fold higher turnover number for rmACE2 (0.62 s^{-1}) compared to Ang 1–10 (Table 1). These results demonstrate that human and murine ACE2 possess substantially different turnover numbers for Ang 1–10, pointing to a species-specific functional diversity of the enzyme.

3.3. Species-Specific Substrate Specificity of ACE2 Affects Endogenous Plasma Angiotensin Levels Ex Vivo. In order to investigate the substrate specificity of rhACE2 and rmACE2 under physiologic conditions, we assessed the impact of the two enzymes on the human RAS in blood plasma. Therefore, we simulated a pathological hyperactivated RAS by addition of recombinant human renin to anticoagulated human blood plasma. Going in line with our previous *in vitro* findings, the addition of rhACE2 or rmACE2 to *ex vivo* incubated plasma samples revealed that both enzymes effectively degraded Ang 1–8 to yield Ang 1–7 and Ang 1–5 when compared to the enzyme-free control sample (Figure 3(a)). Although the plasma

Table 1: Enzymatic properties of rhACE2 and rmACE2. The substrate turnover rates and product formation rates were determined for the kinetic experiments in Figure 2. Therefore, the slope of the linear regressions across all data points was determined for both substrate-product pairs and both enzymes. The mean of the absolute values for the substrate degradation rates and product formation rates for each enzyme were related to the molar concentration of rhACE2 or rmACE2. The resulting values for k_{cat} are given in the table.

	k_{cat} [s^{-1}]	
	rhACE2	rmACE2
Ang 1–8 ≥ Ang 1–7	7.7×10^{-1}	6.2×10^{-1}
Ang 1–10 ≥ Ang 1–9	1.8×10^{-2}	1.3×10^{-3}

concentration of Ang 1–10 was only 161 pg/mL (124 pM), a concentration of 5 μg/mL (58,8 nM) rhACE2 was found to efficiently convert Ang 1–10 to Ang 1–9, as indicated by the peptide levels depicted in the RAS-Fingerprints (Figure 3(a), right). In contrast to rhACE2, rmACE2 was unable to decrease Ang 1–10 concentrations in plasma and failed to induce detectable Ang 1–9 levels (Figure 3(a), middle). Of note, the increase of Ang 1–7 and Ang 1–5 in the presence of rhACE2 was even more prominent, due to this second pathway of Ang 1–7 production via Ang 1–9, which was selectively supported only by rhACE2.

As *in vitro* experiments revealed that rmACE2 was capable of converting Ang 1–10 to Ang 1–9, although to a much lower extent compared to rhACE2, we further investigated the capability of rmACE2 for Ang 1–9 formation in human plasma at increased Ang 1–10 concentrations. We added the ACE-inhibitor Lisinopril to our *ex vivo* setting, in order to prevent ACE-mediated degradation of ACE2-produced Ang 1–9 and to increase Ang 1–10 levels by preventing its degradation by endogenous ACE. The presence of Lisinopril led to significantly increased Ang 1–10 peptide levels compared to untreated control samples (710 pg/mL versus 161 pg/mL) (Figures 3(a) and 3(b) left). Comparison of rhACE2 and rmACE2 activities in Lisinopril-treated complete human plasma revealed that rhACE2 effectively converts large amounts of Ang 1–10 to Ang 1–9 in the physiological matrix while rmACE2 was found to be much less effective in catalysing this reaction (Figure 3(b)).

Interestingly, Lisinopril was not able to increase Ang 1–7 concentrations in our experimental settings, which was in contrast to several published reports. No Ang 1–7 was detectable in plasma samples incubated with Lisinopril in the absence or presence of rhACE2 or rmACE2 (Figure 3(b)) meaning that the concentration was below the quantification limit of 2 pg/mL plasma. For further investigation of these surprising results, the experiment was repeated for rhACE2 in whole blood instead of plasma. Whole blood incubations gave similar results as previously reported by other groups, showing an increase of Ang 1–7 concentrations in control and rhACE2 samples in response to Lisinopril (see Supplementary Figure 1 available online at doi:10.1155/2012/428950).

For further investigation of rmACE2 and rhACE2 substrate specificities, different states of RAS activity were simulated by addition of lower amounts of recombinant human renin in the presence of Lisinopril, confirming our findings about strongly diverging conversion rates for Ang 1–10 between rhACE2 and rmACE2 in a substrate concentration-dependent manner (Figure 3(c)). These results demonstrate that Ang 1–10 serves as a natural substrate for rhACE2 which is efficiently processed under physiological conditions. In contrast to that, rmACE2 is much less effective regarding this catalytic conversion, strongly supporting a species-specific role of ACE2 in the activation of the alternative RAS pathway.

4. Discussion

We expressed and purified both rhACE2 and rmACE2 in CHO cells under serum-free conditions. Both cell lines were stably secreting high levels of recombinant proteins for at least two months of roller bottle cultivation. The quality of the expression products did not change from early to the latest passages. Both rhACE2 and rmACE2 appeared as stable homo-dimers, while we did not identify monomeric or other multimeric forms. We evaluated the quality of the enzyme preparations by multiple methods including HPLC, SEC, SDS-PAGE, and native PAGE which all confirmed the purity of the final products and their homo-dimeric tertiary structure (Figure 1). Despite the similarity of the calculated molecular weights for human and murine monomers, surprisingly high mass differences between rhACE2 and rmACE2 were observed in SEC and could be finally identified to be caused by species-specific sequence variations which lead to a different number in N-glycosylation sites in human and murine ACE2.

ACE2 is known to cleave a variety of peptide substrates *in vitro* [18], which are involved in a broad panel of physiological functions [17]. Based on our findings about the differences in tertiary structure between rhACE2 and rmACE2, we hypothesized that the well-known sequence diversity between the two species might have an impact on the functional characteristics of the enzymes. Therefore, we assessed the turnover rates for rhACE2 and rmACE2 for two natural and physiologically important substrates (Ang 1–10 and Ang 1–8) in a well-defined *in vitro* model system (Figure 2). We selected these substrates for ACE2 characterization because of their functional importance in maintaining RAS peptide levels. It has been described previously that the angiotensin peptides Ang 1–8 and Ang 1–10 are cleaved by ACE2 *in vitro* [6]. As the conversion rates of ACE2 for Ang 1–10 were reported to be substantially slower than those for Ang 1–8, this enzyme reaction was supposed to take over a minor role in the formation of Ang 1–7 than the direct production by Ang 1–8 cleavage. We could confirm previous findings regarding substrate preferences and found a 42-fold higher turnover number for Ang 1–8 compared to Ang 1–10 when cleaved by rhACE2 (Table 1). Interestingly, our values for k_{cat} were lower compared to previous publications which might have been caused by differences in the employed experimental settings, in particular because of different buffer

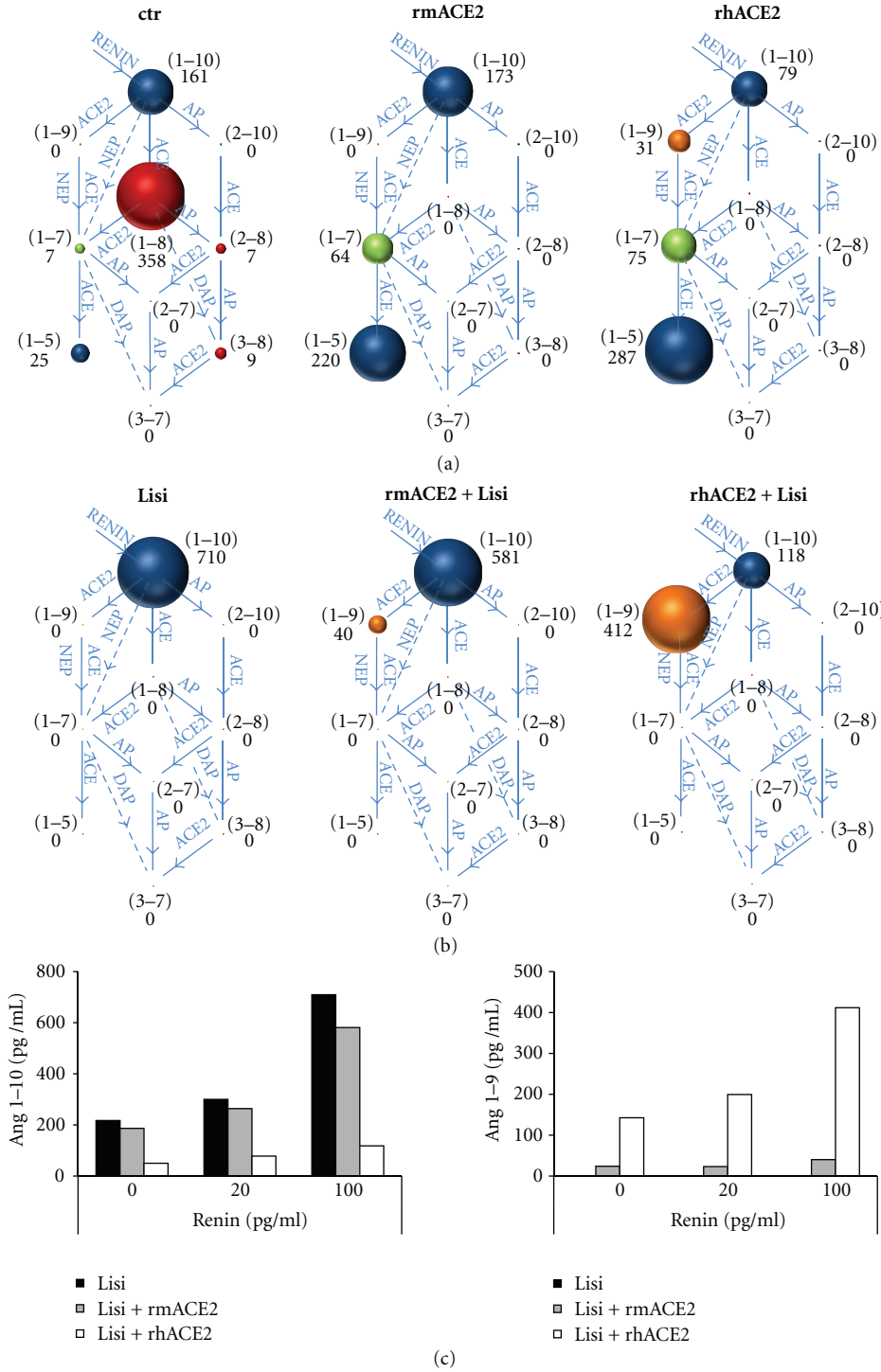

Figure 3: Interference of human and murine ACE2 with the endogenous RAS in human plasma. Anticoagulated plasma samples were incubated at 37°C in the presence of 100 pg/mL recombinant human renin (a, b). rhACE2 or rmACE2 were added as indicated at a final plasma concentration of 5 μg/mL. RAS-Fingerprints were measured by LC-MS/MS as indicated in Section 2. Samples were incubated in the absence (a) or presence (b) of 10 μM of the ACE inhibitor Lisinopril. In this figure, the diameter of the spheres reflects the concentration of the respective peptide metabolite, which is also given in pg/mL next to each individual sphere. 0 pg/mL indicates concentrations below quantification limits, which are defined by a signal-to-noise ratio below 10. Furthermore, the amino acid sequence of each angiotensin metabolite is schematically given in brackets beside the corresponding sphere. The sequence annotation is based on the decapeptide Angiotensin I (1–10) which is N- or C-terminally cleaved by the indicated proteases. Proteases are illustrated by arrows connecting their substrate and product. AP: aminopeptidases; NEP: neutral endopeptidase; DAP: di-aminopeptidase; ACE: angiotensin-converting enzyme. (c) Indicated concentrations of recombinant human renin were added to Lisinopril-treated plasma samples. Control samples (black bars), rmACE2- (grey bars), and rhACE2- (white bars) treated samples were subjected to RAS-Fingerprinting, and resulting concentrations for Ang 1–10 (left) and Ang 1–9 (right) are given in pg/mL.

systems. Nevertheless, the qualitative conclusions were comparable and internally controlled. While showing comparable turnover rates for Ang 1–8, the Ang 1–10-related turnover rate for rmACE2 was found to be only 7% of the respective rate for rhACE2. This fundamental difference in the substrate conversion rates of the two enzymes might also have substantial impact on the regulation of the RAS under physiologic conditions in the two different species.

Unfortunately, only limited conclusions about physiological consequences can be drawn out of *in vitro* experiments. An important feature of the physiologic conditions in blood plasma is that all RAS enzymes except renin are present in excess compared to their substrates, which is the exact opposite of the *in vitro* situation. Although *in vitro* investigations are very useful for the comparison of enzyme characteristics in one and the same model system, they tell us very little about the *in vivo* situation.

Therefore, we developed an *ex vivo* experimental setup which allowed us to investigate the enzymatic function of the two recombinant enzymes in their physiological environment, with their natural substrates being present at picomolar concentrations. We would like to point out that these *ex vivo* conditions reflect the human *in vivo* plasma conditions regarding circulating enzyme concentrations after systemic administration of rhACE2 (data not shown). Although *ex vivo* incubations are very reproducible and reflect an integrated picture of soluble enzyme activities throughout the RAS in undiluted plasma, the angiotensin peptide concentrations are clearly higher in *ex vivo* incubated plasma samples, which might be caused by a lack of the peptide flow towards organs or endothelial surfaces *ex vivo*.

We investigated the RAS in these samples by means of a newly developed LC-MS/MS method, which allows the quantification of multiple angiotensin metabolites simultaneously in one single sample of blood plasma. The obtained RAS-Fingerprints revealed that, in contrast to rmACE2, rhACE2 is capable to generate Ang 1–9 from Ang 1–10 at physiologic peptide concentrations (Figure 3). This activity even more gains importance in the presence of the ACE-inhibitor Lisinopril, which blocks the formation of Ang 1–8. Under latter conditions, large amounts of Ang 1–9 are generated in the plasma samples by rhACE2, while rmACE2 is much less effective in its formation of Ang 1–9 from Ang 1–10.

Although significant amounts of Ang 1–10 and Ang 1–9 were present in samples treated with Lisinopril alone or in combination with rhACE2, no Ang 1–7 could be detected in these samples. These findings were in contrast to previously published reports on Ang 1–7 accumulating effects of ACE inhibitors *in vivo* [20, 21]. In our experimental setting, we employed heparinized blood plasma as a sample matrix for ACE2 characterization which is reflecting *in vivo* conditions very well. However, plasma lacks all blood cells which might carry receptors and angiotensin peptide converting enzymes being able to affect angiotensin peptide concentrations *in vivo*. Comparison of plasma and whole blood samples revealed that Lisinopril-induced Ang 1–7 accumulation is strictly dependent on blood cell-associated angiotensin peptide converting enzymes, as it was exclusively observed in whole blood *ex vivo* incubations (Figure 3(b), Supplementary Figure 1). As neutral endopeptidase (NEP, CD10) is known to be expressed on the cell surface of leukocytes [22, 23] and that it is able to convert Ang 1–10 and Ang 1–9 to Ang 1–7 *in vitro* [18], NEP is very likely to be responsible for Ang 1–7 accumulation also *in vivo,* especially in the presence of ACE inhibitors which block the formation of Ang 1–8 which is an important precursor for Ang 1–7.

In human plasma, the ACE2-mediated formation of Ang 1–9 from Ang 1–10 represents a significant route of establishment of the alternative RAS. This may be, for example, of particular importance *in vivo*, when ACE inhibitors are used for antihypertensive treatment. As ACE2 is primarily expressed as a membrane-attached enzyme in several organs [24], the local production of Ang 1–9 from Ang 1–10 which is increased when ACE inhibitors are present, might become an important mechanism of action for ACE inhibitors action *in vivo in humans*. In addition to Ang 1–7, also Ang 1–9 has been reported to possess protective effects in cardiovascular disease models [25]. These mechanistic considerations seem to be of particular importance in humans, while murine model systems for the investigation of ACE inhibitor efficacy might be reconsidered in respect to the species-specific lack of Ang 1–10 cleavage by murine ACE2.

Altogether, our findings describe important species-specific differences in the fine specificity of ACE2. Thus, the murine RAS is likely to function differently when compared to its human counterpart. Furthermore, our data point to the importance of further investigations and improved understanding of the human RAS, while data generated in murine model systems might be partially reconsidered in respect to different enzyme properties. Deciphering the functional characteristics of the human RAS using new analytical possibilities reveals previously invisible features of the system. The future generation of human-derived data describing RAS function in health and disease will pave the way for new concepts of therapeutic manipulations of the system which are more specifically designed for application in humans.

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Research Article

AVE0991, a Nonpeptide Compound, Attenuates Angiotensin II-Induced Vascular Smooth Muscle Cell Proliferation via Induction of Heme Oxygenase-1 and Downregulation of p-38 MAPK Phosphorylation

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The nonpeptide AVE0991 is an agonist of the angiotensin-(1–7) (Ang-(1–7)) Mas receptor and is expected to be a putative new drug for treatment of cardiovascular disease. However, the mechanisms involved in the antiproliferative effects of AVE0991 are not fully understood. We saw that the compound attenuated proliferation in an angiotensin II-induced rat vascular smooth muscle cells (VSMC) proliferation model. Moreover, treatment with AVE0991 (10^{-5} mol/L or 10^{-7} mol/L) significantly attenuated reactive oxygen species (ROS) production, phosphorylation of p38 MAPK, and dose-dependently (10^{-8} to 10^{-5} mol/L) inhibited Ang II-induced VSMC proliferation. Meanwhile, heme oxygenase-1 (HO-1) expression increased in the AVE0991 + Ang II group (10^{-5} mol/L or 10^{-6} mol/L). However, the beneficial effects of AVE0991 were completely abolished when the VSMC were pretreated with A-779 (10^{-6} mol/L). Furthermore, treatment with the HO-1 inhibitor ZnPPIX attenuated the inhibitory effect of AVE0991 on Ang II-induced p38MAPK phosphorylation. These results suggest that AVE0991 attenuates Ang II-induced VSMC proliferation in a dose-dependent fashion and that this effect is associated with the Mas/HO-1/p38 MAPK signaling pathway.

1. Introduction

Angiotensin-(1–7) (Ang-(1–7)) is a potent, endogenous effector hormone of the renin angiotensin system (RAS) pathway. It can be formed directly from Ang I or Ang II, or indirectly from Ang I, where Ang-(1–9) is produced in an intermediate step [1]. Santos et al. demonstrated that a G-protein-coupled receptor is the specific Ang-(1–7) receptor in *Mas*-deficient mice [2]. Previous studies have shown that Ang-(1–7) has opposite effects to Ang II, which induces myocardial hypertrophy and ultimately cellular proliferation [3, 4]. A-779 is a selective antagonist to the Ang-(1–7) Mas receptor and can therefore prevent effects of Ang-(1–7) by preventing ligand/receptor interactions [5].

Because the Ang-(1–7) peptide is not resistant to proteolytic enzymes, its clinical application is limited. Wiemer et al. have shown that a nonpeptide compound (AVE0991) produces similar effects as Ang-(1–7) in biological systems [6]. Previous studies have shown that AVE0991 ameliorated hepatic fibrosis in the bile-duct-ligated rat model and improved myocardial fibrosis induced by isoproterenol in male Wistar rats [7, 8]. AVE0991 is an orally available compound that has a wider clinical application and thus could be a putative new drug for the treatment of cardiovascular disease. However, the antiproliferative mechanisms of AVE0991 are still not fully understood.

Numerous studies have shown that Ang II plays a critical role during proliferation in vascular smooth muscle cells

(VSMCs) [9–12]. The molecular and cellular mechanisms underlying the Ang II-dependent processes in vascular remodeling have not been fully elucidated. However, the mitogen-activated protein kinase (MAPK) cascade, particularly the p38 MAP kinase, may play a role in mediating responses that are related to cell growth and differentiation [13]. Ang II is a well-known activator of this signaling pathway.

Heme oxygenases (HOs), which catalyze the breakdown of heme to equimolar quantities of carbon monoxide (CO), biliverdin, and ferrous iron, are the rate-limiting enzymes in heme degradation. These enzymes have antioxidative and anti-inflammatory effects, and several studies have suggested that HO-1 has a cytoprotective role [14, 15]. Additionally, many studies have indicated that HO-1 has a beneficial antiproliferative effect in VSMCs and that this effect can be abolished by the HO-1 inhibitor ZnPPIX [16].

Moreover, Ang II-stimulated growth of VSMCs has an essential redox-sensitive component that is mediated by activation of MAPK-dependent signaling pathways, while HO-1 attenuates the Ang II-induced damage in VSMCs [17]. In addition, Sun et al. have shown that HO-1 attenuated Ang II-induced VSMCs proliferation via inhibiting the expression of MAPK [18]. Hence, we hypothesize that the HO-1/p38 MAPK signaling pathway may be involved in the inhibition of VSMCs proliferation.

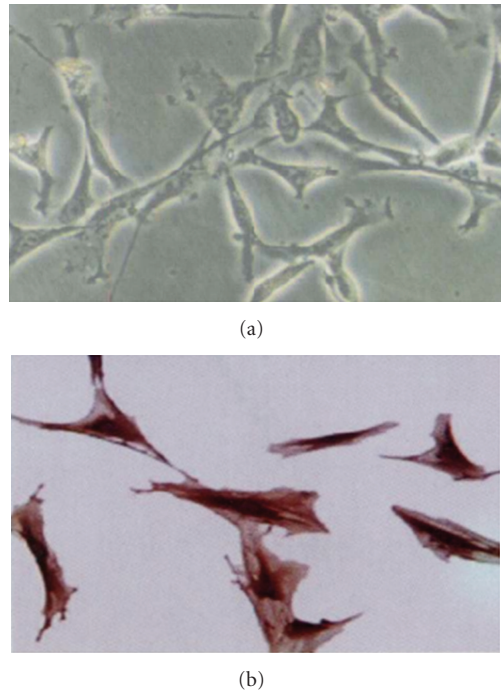
The objectives of this study were to determine whether the nonpeptide compound AVE0991 could inhibit Ang II-induced VSMCs proliferation and if the HO-1/p38 MAPK signal pathways are involved in the AVE0991-mediated effects.

2. Materials and Methods

The investigation was carried out according to the Guide for Care and Use of Laboratory Animals that was published by the US National Institutes of Health (NIH Publication no. 85-23, revised in 1996).

2.1. Materials. The peptides Ang II and the Ang-(1–7) Mas receptor antagonist A779 were obtained from Bachem (King of Prussia, PA, USA). The β-actin monoclonal antibody and HO-1 inhibitor ZnPPIX were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) and trypsin were obtained from Invitrogen (Carlsbad, CA, USA). [^{3}H]Thymidine (20 Ci/mmol) was obtained from the Atomic Energy Institute of China (Wuhan, China). Fetal bovine serum (FBS) was purchased from the Sijiqing Company (Hangzhou, China). AVE0991 was kindly provided by Dr. Juergen Puenter of Aventis Pharma (Frankfurt, Germany) and male Sprague-Dawley rats (10 to 12 weeks old) were obtained from the Experimental Animal Facility of Sun Yat-sen University, China.

2.2. Cell Culture and Experimental Designs. VSMCs were isolated from the thoracic aorta of 10- to 12-week-old male Sprague-Dawley rats by an explant culture method. The cells were seeded in DMEM/Ham's F-12 (1 : 1) that was supplemented with 10% FBS, 100 μg/mL penicillin, and 100 U/mL streptomycin (Invitrogen). The cell preparations were cultured at 37°C in a humidified atmosphere with 5% CO_2. The culture purity was assessed by immunostaining with a monoclonal antibody against smooth muscle α-actin, followed by an anti-mouse fluorescein-conjugated goat IgG antibody. Using these methods, the purity of VSMCs reached more than 98%. Cells between passages 4 and 7 were used for all of the experiments [19] (Figure 1).



(a)

(b)

Figure 1: The analysis of cultured VSMCs in morphology and identification. (a) The morphology of cultured VSMCs detected by phase-contrast microscope. Magnification of light microscopy images is ×100. (b) SM-α actin immunocytochemical staining of cultured VSMCs. Magnification of light microscopy images is ×400.

The cells were rendered quiescent by serum starvation for 36 hours before ROS, HO-1, and p38 phosphorylation detection, and for 48 hours before thymidine incorporation experimentation. The VSMCs were treated according to the following experimental protocols.

Protocol 1. Effect of various concentrations of AVE0991 on the VSMCs [^{3}H]thymidine incorporation efficiency.

The cells were pretreated with Ang II for 24 hours and then followed by AVE0991 (10^{-5} mol/L, 10^{-6} mol/L, 10^{-7} mol/L, or 10^{-8} mol/L) for 24 hours.

Protocol 2. Effects of AVE0991 on Ang II-induced VSMC proliferation, ROS production, HO-1 protein, and p38 phosphorylation expression.

For Ang II group, the cells were treated with Ang II for 48 hours. For Ang II + AVE0991-H (10^{-5} mol/L) group and Ang II + AVE0991-L (10^{-7} mol/L) group, the cells were pretreated

with Ang II for 24 hours and followed by AVE0991 for 24 hours. And for Ang II + AVE0991-H (10^{-5} mol/L) + A-779 group, the cells were pretreated with A-779 for 30 min, followed by Ang II for 24 hours, and then AVE0991 for 24 hours.

Protocol 3. Effects of AVE0991 on Ang II-induced VSMCs proliferation, ROS production, and p38 phosphorylation expression with pretreatment of HO-1 inhibitor ZnPPIX.

For Ang II group, the cells were treated with Ang II for 48 hours. For Ang II + AVE0991 (10^{-5} mol/L) group, the cells were pretreated with Ang II for 32 hours and followed by treatment with AVE0991 for 16 hours. For Ang II + AVE0991 (10^{-5} mol/L) + A-779 group, the cells were pretreated with A-779 for 30 min, then treated with Ang II for 32 hours, and then followed by treatment with AVE0991 for 16 hours. And for Ang II + AVE0991 (10^{-5} mol/L) + ZnPPIX group, the cells were treated in the following order: Ang II for 24 hours, HO-1 inhibitor ZnPPIX for 8 hours, and AVE0991 for 16 hours.

2.3. [^{3}H]Thymidine Incorporation. De novo DNA synthesis was measured via incorporation of tritiated thymidine by VSMCs that were grown in 24-well culture plates. The cells were plated at a density of 2000 cells/well and subconfluent monolayers were made quiescent by serum starvation for 48 h. The monolayers were then treated with or without Ang II for 48 h and were subsequently treated with various concentrations of AVE0991, A-779, and/or ZnPPIX. During the last 24 h, 0.25 μCi of [^{3}H]thymidine/mL culture medium was added to the growth medium. The incorporation of [^{3}H]thymidine was determined after precipitation of acid-insoluble material with ice-cold 5% trichloroacetic acid. The acid-insoluble material was dissolved in 0.25% NaOH and counted in a liquid scintillation spectrometer in the presence of 5 mL Ecolite.

2.4. Detection of ROS Production. VSMCs were preloaded with 30 μmol/L 2′-7′-dichlorofluorescein diacetate at 37°C for 75 min and were treated with the indicated agents for an additional 2 h in serum-free medium. After washing with Hanks, the cells were lysed with Tris-HCl (10 mmol/L, pH 7.4, containing 0.5% Tween-20) and were centrifuged at 10,000 g for 10 min. The fluorescence intensity of the supernatants was determined with a spectrofluorometer [20].

2.5. Western Blotting. The cytosolic proteins were extracted, and the total protein concentration was determined with a BCA Protein Assay kit (Pierce, Rockford, IL, USA). Thirty micrograms of crude protein extract was loaded on to a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. The membrane was blocked for 2 h at room temperature and incubated with an anti-HO-1 (1 : 1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phosphorylated p38 or p38 (1 : 800 dilution; Cell Signal, Beverly, MA, USA), or anti-GAPDH (1 : 10 000 dilution; Boshide, Wuhan, China) antibody diluted in Tris-buffered saline/Tween-20 (TBS-T). After washing, the membranes were subsequently incubated for 1 h with rabbit anti-mouse or goat anti-rabbit secondary antibodies diluted 1 : 2000 or 1 : 3000, respectively, in TBS-T. The bands were visualized with an ECL kit (GE Healthcare, Chalfont St Giles, Bucks, UK) according to the manufacturer's instructions, and GAPDH was used as a loading control. The results were analyzed with a gel image analysis system (Bio-Rad, Richmond, CA, USA).

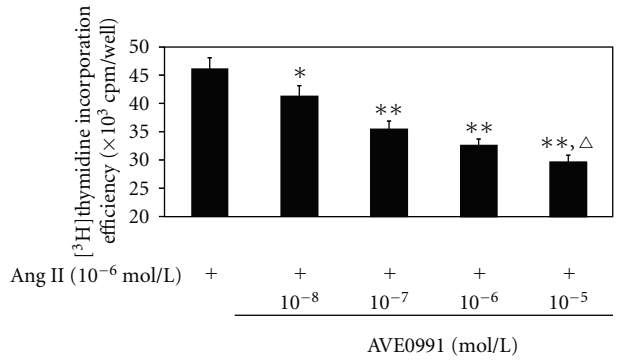


Figure 2: The effect of various concentrations of AVE0991 on the VSMCs [^{3}H]thymidine incorporation efficiency when stimulated with Ang II. $^*P < 0.05$; $^{**}P < 0.01$ versus control group; $^{\triangle}P < 0.05$ versus Ang II + AVE0991 (10^{-6} mol/L) group; $n = 8$.

2.6. Statistical Analysis. The data are expressed as the mean ± standard deviation (SD). Differences between groups were evaluated by two-tailed unpaired Student's t-test, and a value of $P < 0.05$ was interpreted as being statistically significant. Statistical analyses were performed using SPSS 13.0 statistics software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effects of AVE0991 on Ang II-Induced VSMC Proliferation and ROS Production. Firstly, we evaluated the inhibition effect of AVE0991 on Ang II-induced VSMCs proliferation. Ang II (10^{-6} mol/L) and AVE0991 (10^{-5} mol/L, 10^{-6} mol/L, 10^{-7} mol/L, or 10^{-8} mol/L) were added to culture fluid. The [^{3}H]thymidine incorporation efficiency of VSMCs was inhibited by AVE0991 in a dose-dependent manner (Figure 2).

Secondly, treatment with 10^{-6} mol/L of Ang II significantly increased the [^{3}H]thymidine incorporation efficiency of VSMCs compared to the control group ($n = 8$, $P < 0.01$) (Figure 3). When the VSMCs were treated with the same dose of Ang II combined with AVE0991 (10^{-5} mol/L and 10^{-7} mol/L), the [^{3}H]thymidine incorporation efficiency decreased significantly when compared to the Ang II group ($n = 8$, $P < 0.01$), although the efficiency was still higher than the control group. Nevertheless, when the cells were treated with Ang II + AVE0991 + A-779 (10^{-6} mol/L), this AVE0991-mediated effect which inhibited [^{3}H]thymidine incorporation efficiency of VSMCs was abolished. However,

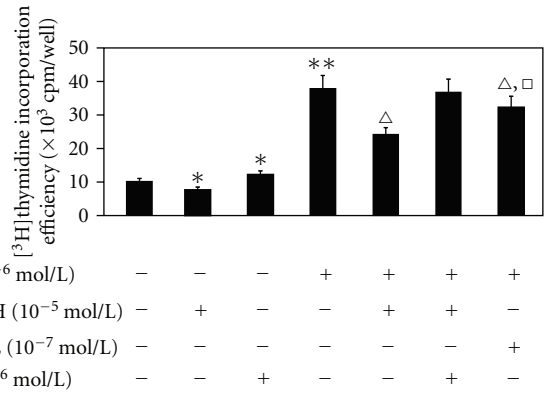


Figure 3: The effect of AVE0991 on the [^{3}H]thymidine incorporation efficiency of VSMCs that were stimulated by Ang II. $^*P < 0.05$; $^{**}P < 0.01$ versus control group; $^{\triangle\triangle}P < 0.01$ versus Ang II group; $^{\square}P < 0.05$ versus Ang II + AVE0991-H group; $n = 8$.

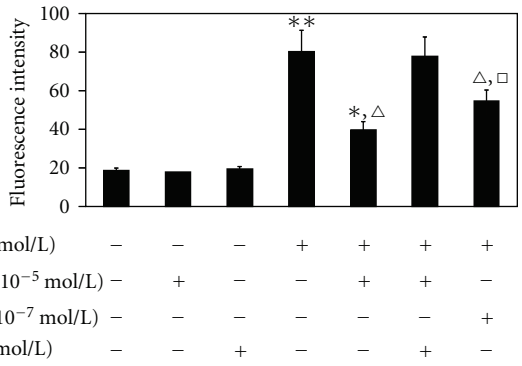


Figure 4: The effect of AVE0991 on Ang II-stimulated ROS production in VSMCs. $^*P < 0.05$; $^{**}P < 0.01$ versus control group; $^{\triangle}P < 0.01$ versus Ang II group; $^{\square}P < 0.05$ versus Ang II + AVE0991-H group; $n = 8$.

when the VSMCs were treated with 10^{-5} mol/L AVE0991 or 10^{-6} mol/L A-779, both reagents affected the [^{3}H]thymidine incorporation efficiency of the VSMCs. Compared to the control group, the [^{3}H]thymidine incorporation efficiency was decreased when the cells were treated with AVE0991 alone ($n = 8$, $P < 0.05$). Conversely, the efficiency increased when the cells were treated with A-779 alone ($n = 8$, $P < 0.05$).

We sought to determine whether AVE0991 inhibition of Ang II-stimulation has any effect on ROS production afterwards. The ROS level was significantly increased in the Ang II group (10^{-6} mol/L) compared to the control group (Figure 4). AVE0991 significantly inhibited Ang II-stimulated ROS expression, especially in the Ang II (10^{-6} mol/L) + AVE0991-H (10^{-5} mol/L) group ($n = 8$, $P < 0.01$). However, neither AVE0991 nor A-779 alone had a significant effect on ROS expression.

3.2. Effects of AVE0991 on Ang II-Induced VSMC Proliferation and ROS Production with Pretreatment of HO-1 Inhibitor ZnPPIX. In this experiment, in the Ang II + AVE0991 + ZnPPIX group, the [^{3}H]thymidine incorporation efficiency

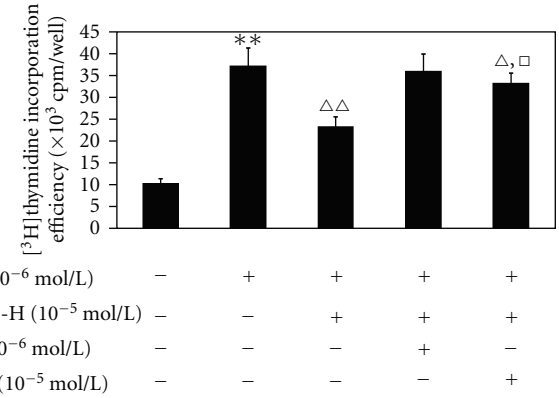


Figure 5: The effect of AVE0991 on the Ang II-induced VSMCs [^{3}H]thymidine incorporation efficiency with pretreatment with the HO-1 inhibitor ZnPPIX. $^{**}P < 0.01$ versus control group; $^{\triangle}P < 0.05$; $^{\triangle\triangle}P < 0.05$ versus Ang II group; $^{\square}P < 0.01$ versus Ang II + AVE0991 group; $n = 8$.

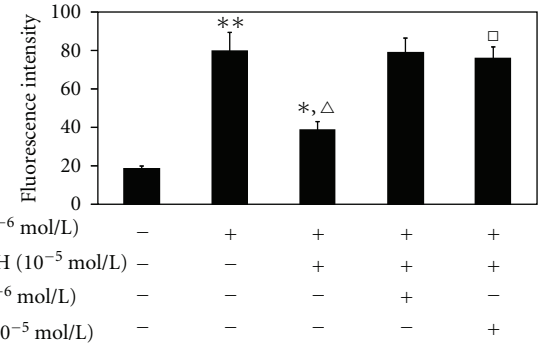


Figure 6: The effect of AVE0991 on Ang II-stimulated ROS production in VSMCs that were pretreated with the HO-1 inhibitor ZnPPIX. $^*P < 0.05$; $^{**}P < 0.01$ versus control group; $^{\triangle}P < 0.01$ versus Ang II group; $^{\square}P < 0.01$ versus Ang II + AVE0991 group; $n = 8$.

of the VSMCs that were pretreated with ZnPPIX increased significantly when compared to the Ang II + AVE0991 (10^{-5} mol/L) group ($n = 8$, $P < 0.01$), although the efficiency was lower than the Ang II group ($n = 8$, $P < 0.05$) (Figure 5).

To determine whether AVE0991 inhibits Ang II-stimulated ROS production via modulating HO-1 expression, we pretreated VSMCs with the HO-1 inhibitor ZnPPIX (10^{-5} mol/L). The ROS level was significantly higher in the Ang II + AVE0991 (10^{-5} mol/L) + ZnPPIX group compared to the Ang II + AVE0991 (10^{-5} mol/L) group ($n = 8$, $P < 0.01$). However, this AVE0991-mediated effect was also abolished by the Ang-(1–7) Mas receptor antagonist A-779 (Figure 6).

3.3. Effect of AVE0991 on p38 Phosphorylation. The phosphorylation of p38 was significantly higher in the Ang II (10^{-6} mol/L) group compared to the control group ($n = 8$, $P < 0.01$) (Figure 7). AVE0991 treatment attenuated this

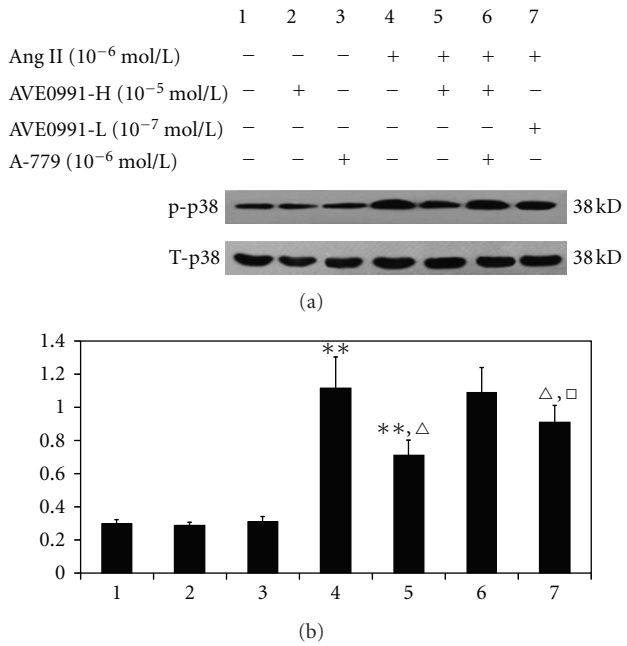


Figure 7: The effect of AVE0991 on the p38 phosphorylation level in VSMCs that were induced by Ang II. Lanes 1–7 represent the control, AVE0991, A-779, Ang II, Ang II + AVE0991-H, Ang II + AVE0991-H + A-779, and the Ang II + AVE0991-L group. $^{**}P < 0.01$ versus control group; $^{\triangle}P < 0.01$ versus Ang II group; $^{\square}P < 0.01$ versus Ang II + AVE0991-H group; $n = 8$.

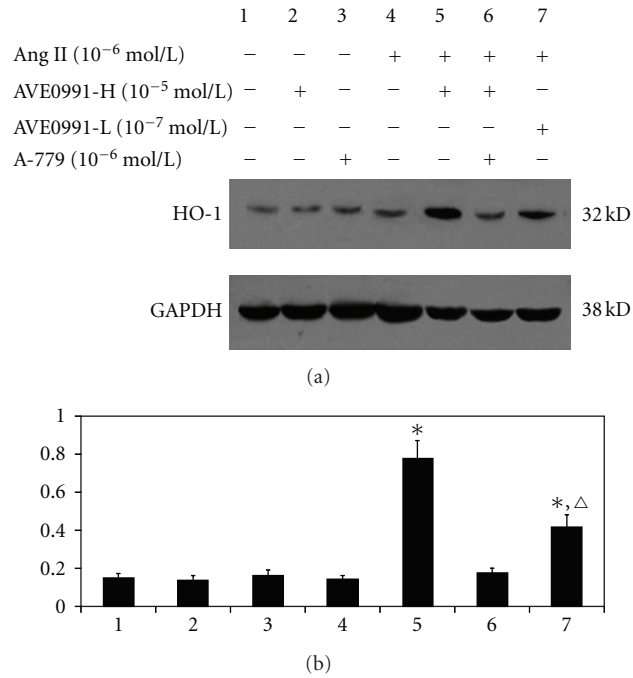


Figure 8: The effect of AVE0991 on HO-1 protein expression in VSMCs that were induced by Ang II. $^{*}P < 0.01$ versus control group; $^{\triangle}P < 0.01$ versus Ang II + AVE0991-H group; $n = 8$.

increase in p38 phosphorylation, and attenuation of p38 phosphorylation was more pronounced in the AVE0991 higher-concentration treatment group (AVE0991-H, 10^{-5} mol/L) compared to the AVE0991 low-concentration treatment group (AVE0991-L, 10^{-7} mol/L). However, when the cells were treated with Ang II + AVE0991 and 10^{-6} mol/L of A-779, there was no change in the level of p38 phosphorylation compared to the Ang II + AVE0991 group.

3.4. Effect of AVE0991 on HO-1 Protein Expression. HO-1 protein expression was not significantly different between the Ang II (10^{-6} mol/L) and control groups (Figure 8). However, AVE0991 treatment significantly increased VSMCs HO-1 protein expression in the Ang II + AVE0991 group when compared to the control group ($n = 8$, $P < 0.01$). This increase in HO-1 protein expression was more pronounced in the AVE0991-H treatment group (10^{-5} mol/L) compared to the AVE0991-L treatment group (10^{-7} mol/L) ($n = 8$, $P < 0.05$). However, when the cells were treated with Ang II + AVE0991 (10^{-5} mol/L) + A-779 (10^{-6} mol/L), there was no change in HO-1 expression compared to the Ang II + AVE0991 (10^{-5} mol/L) group. Neither AVE0991 nor A-779 treatment alone has a significant effect on HO-1 protein expression.

3.5. Effect of AVE0991 on p38 Protein Phosphorylation When the VSMC Were Pretreated with the HO-1 Inhibitor ZnPPIX. We next sought to determine whether AVE0991 modulates p38 phosphorylation via HO-1 expression. As Figure 9 indicates, the level of p38 phosphorylation in the Ang II (10^{-6} mol/L) group was significantly higher than that in the control group. AVE0991 treatment (10^{-5} mol/L) significantly inhibited Ang II-mediated p38 phosphorylation ($n = 8$, $P < 0.01$), although p38 phosphorylation was higher than that in the control group ($n = 8$, $P < 0.01$). However, when the cells were pretreated with the HO-1 inhibitor ZnPPIX (10^{-5} mol/L), the p38 phosphorylation level in the Ang II + AVE0991 + ZnPPIX group was significantly higher than that in the Ang II + AVE0991 group ($n = 6$, $P < 0.01$), although the phosphorylation level was lower than that in the Ang II group.

4. Discussion

The major finding of this study is that AVE0991, a nonpeptide analog of Ang (1–7), attenuates Ang II-induced VSMCs proliferation by inducing heme oxygenase-1 expression and by downregulating p-38 MAPK phosphorylation. In addition, the present study shows that AVE0991 suppresses the Ang II-stimulated ROS production in VSMCs. Furthermore, experiments with the HO-1 inhibitor ZnPPIX indicate that AVE0991 decreases p-38 MAPK phosphorylation via induction of HO-1 protein expression in Ang II-induced VSMCs proliferation. In addition, treatment with AVE0991 attenuated proliferation of the VSMCs in a dose-dependent manner. However, all of the beneficial effects of AVE0991 were completely blocked by pretreatment with the Ang-(1–7) receptor antagonist A-779.

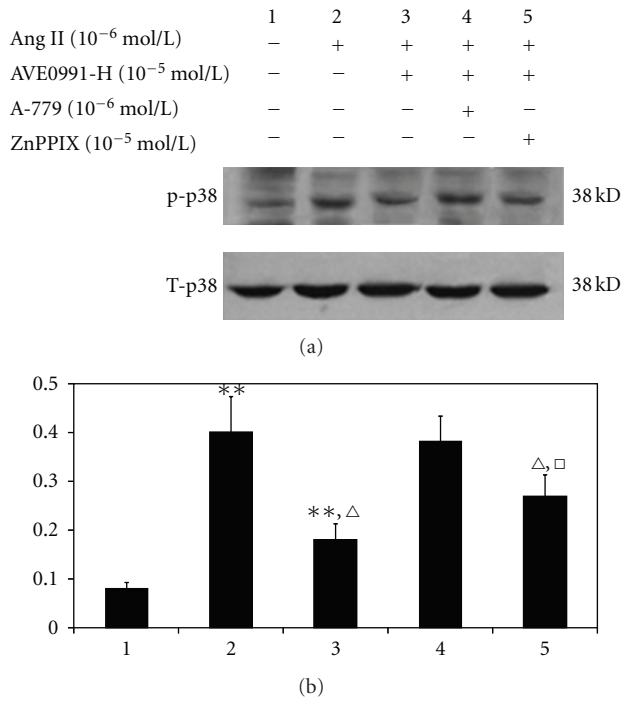


Figure 9: The effect of AVE0991 on the p38 phosphorylation level in VSMCs when combined with the HO-1 inhibitor ZnPPIX. $^{**}P < 0.01$ versus control group; $^{\triangle}P < 0.01$ versus Ang II group; $^{\square}P < 0.01$ versus Ang II + AVE0991 group; $n = 8$.

AVE0991 mimics many of the biological actions of Ang-(1–7) and acted like an Ang-(1–7) Mas receptor agonist in Mas-knockout mice and Mas-transfected cells [1, 2, 6, 21]. Ang-(1–7) has been shown to improve vascular endothelial dysfunction, delay the development of cardiac hypertrophy, and attenuate the development of heart failure [3, 22]. EJ Freeman reported that Ang-(1–7) pretreatment also inhibited the proliferative effects of Ang II-treated VSMCs as measured by [^{3}H]leucine incorporation and [^{3}H]-Thymidine incorporation [19]. Thus, Ang-(1–7) interacts with specific receptors on VSMCs to exert antiproliferative effects that can reverse the Ang II-mediated effects [19]. However, because it is a peptide that is rapidly degraded when orally administered, Ang-(1–7) has limited clinical use. In contrast, AVE0991, the nonpeptide analog of Ang-(1–7), is resistant to proteolytic enzymes and can thus be clinically oral applied to treat cardiovascular and related diseases.

Previous studies have shown that Ang II plays an important role in VSMCs proliferation [10, 12]. In vitro, Ang II is one of the most important factors that contribute to VSMCs proliferation by increasing protein and DNA synthesis through the type 1 Ang II receptor [23]. Because Ang II induces a significant increase in VSMCs protein synthesis in conditioned medium [24], the VSMCs were starved in DMEM medium without FBS for 36 h in our experiments to minimize the medium-induced myocyte proliferative effects. According to our previous studies and other published results, the cells were starved for 48 hours prior [^{3}H]-Thymidine incorporation experiments [19]. In addition, a VSMCs purity of greater than 98% was controlled in every experiment group to decrease false results that could be caused by contamination.

The present study indicates that Ang II promotes VSMCs DNA synthesis (measured via [^{3}H]-Thymidine incorporation) in vitro compared to the control group, which agrees with the previously published findings. Treatment with AVE0991 + Ang II significantly suppressed DNA synthesis. Moreover, when the concentration of AVE0991 was increased from 10^{-8} mol/L to 10^{-5} mol/L, AVE0991 inhibited the Ang II-mediated increase in DNA synthesis of the VSMCs in a dose-dependent manner. When the cells were pretreated with the Ang-(1–7) Mas receptor antagonist A-779, the beneficial effect of AVE0991 was completely abolished, which also suggested that the AVE0991 is a nonpeptide Mas receptor agonist. In addition, treatment with AVE0991 alone significantly suppressed VSMCs DNA synthesis. Conversely, A-779 alone also elicited a significant increase in DNA synthesis. However, differently to the results that were seen with DNA synthesis, neither treatment with AVE0991 alone nor treatment with A779 alone can alter ROS production and p38 phosphorylation. Future studies are needed to confirm these results.

Although AVE0991 treatment is antiproliferative in VSMCs, the mechanisms of this effect remain unclear. Previous studies have shown that Ang II activates the NAD(P)H oxidase enzyme system and promotes the generation of ROS, such as the superoxide anion and hydrogen peroxide which stimulate smooth muscle cell proliferation [10, 11, 25]. In addition, the mitogen-activated protein kinase cascade, particularly p38 MAPK, may be an important intracellular mediator of responses that are related to cell growth and differentiation [18]; Ang II is a well-known activator of this signaling pathway. According to this evidence, we question whether AVE0991 inhibits Ang II-stimulated VSMCs proliferation by modulating ROS production or by modulating the p38 pathway. In our study, Ang II promoted ROS production compared to the control group. However, treatment with AVE0991 significantly suppressed Ang II-mediated ROS production in a dose-dependent manner. Similarly, the nonpeptide AVE0991 also inhibited Ang II-mediated phosphorylation of p38MAPK. Nevertheless, pretreatment with the Ang-(1–7) Mas receptor antagonist A-779 also abolished the inhibitory effects of AVE0991. Hence, the present study shows that AVE0991 may inhibit Ang II-mediated VSMCs proliferation by decreasing ROS production and altering the p38 pathway. However, as an important intracellular second messenger, ROS can activate many downstream signaling molecules, including MAPK; thus, there may be a relationship between the ROS and p38 mechanisms of AVE0991. Nevertheless, more direct evidence should be established to fully understand these mechanisms.

Recently, induction of heme oxygenase- (HO-) 1 expression in vivo has been reported to suppress NADPH oxidase-derived oxidative stress. Additionally, HO-1 overexpression suppressed the Ang II-induced hypertrophic response in cardiomyocytes via decreasing the ROS production stress [20]. Ang II-stimulated growth of VSMCs has an essential redox-sensitive component that is mediated by activation of

the MAPK-dependent signaling pathways [26]. In addition, HO-1 attenuates Ang II-induced VSMCs proliferation that involves MAPK inhibition [18]. In neutrophils, HO-1 attenuates infiltration during sepsis via inactivation of p38 MAPK [27]. Hence, we wonder whether there is any relationship between HO-1 and p38 MAPK in Ang II-induced VSMCs proliferation with AVE0991 treatment. In the present study, the nonpeptide AVE0991 dose-dependently increased HO-1 protein expression in the Ang II + AVE0991 group. However, Ang II alone has no significant effect on HO-1 protein expression, which agrees with the previous data in cardiomyocytes [20]. In addition, pretreatment with the HO-1 inhibitor ZnPPIX significantly attenuated the inhibitory action of AVE0991 on Ang II-induced VSMCs DNA synthesis, which indicates that AVE0991 inhibits Ang II-induced VSMCs proliferation partly via induction of HO-1. On the other hand, ZnPPIX pretreatment significantly increased the Ang II-induced p38 phosphorylation level. This indicates that AVE0991 attenuates phosphorylation of p38 partly via induction of HO-1 expression in Ang II-induced VSMCs proliferation.

In summary, our results suggest that the ACE2-Ang-(1–7)-Mas pathway may play a more important antiproliferative role than our current understanding of endogenous Ang-(1–7) in RAS. Moreover, Ang II treatment upregulates p38 phosphorylation and ROS production, which contribute to VSMCs proliferation. Treatment with AVE0991 attenuates Ang II-induced VSMCs proliferation in a dose-dependent manner, which may be associated with regulation of Mas/HO-1/p38 signaling pathway.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

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Research Article

Beneficial Effects of Long-Term Administration of an Oral Formulation of Angiotensin-(1–7) in Infarcted Rats

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In this study was evaluated the chronic cardiac effects of a formulation developed by including angiotensin(Ang)-(1–7) in hydroxypropyl β-cyclodextrin (HPβCD), in infarcted rats. Myocardial infarction (MI) was induced by left coronary artery occlusion. HPβCD/Ang-(1–7) was administered for 60 days (76 μg/Kg/once a day/gavage) starting immediately before infarction. Echocardiography was utilized to evaluate usual cardiac parameters, and radial strain method was used to analyze the velocity and displacement of myocardial fibers at initial time and 15, 30, and 50 days after surgery. Real-time PCR was utilized to evaluate the fibrotic signaling involved in the remodeling process. Once-a-day oral HPβCD/Ang-(1–7) administration improved the cardiac function and reduced the deleterious effects induced by MI on TGF-β and collagen type I expression, as well as on the velocity and displacement of myocardial fibers. These findings confirm cardioprotective effects of Ang-(1–7) and indicate HPβCD/Ang-(1–7) as a feasible formulation for long-term oral administration of this heptapeptide.

1. Introduction

Cardiovascular diseases remain the leading cause of morbidity and mortality worldwide mainly due to their ischemic conditions [1]. Moreover, coronary artery disease is the most common reason of heart failure in Westernized nations [2]. Thus, the continuous search for therapies that are effective in reducing the incidence of these pathologies is still imperative.

Since its discovery in 1988 [3], the biologically active heptapeptide angiotensin(Ang)-(1–7) has been widely studied. This is mainly due to the observation that its effects are often opposite to those attributed to Ang II, whose actions favor the development of pathologic conditions in the heart [2, 4] and in other organs [5, 6] by binding to the AT_1 receptor. In fact, several studies have demonstrated that Ang-(1–7) exerts beneficial effects in various organs [7, 8], including the heart. In this organ, it promotes antiarrhythmogenic effects [9], potentiation of the bradykinin vasodilatory effect [10], improvement of the cardiac function [11–15], reduction of the release of norepinephrine [16], and regulation of the cell growth and cardiac remodeling [4, 17–20]. Many of these effects are mediated by the activation of the Mas receptor [4, 9, 10, 14, 18] which was identified as an endogenous binding site for Ang-(1–7) [21].

In the heart, an increased activity of the classical axis of the renin-angiotensin system (RAS) composed by angiotensin-converting enzyme (ACE), Ang II, and AT_1 receptor leads to ventricular hypertrophy, heart failure

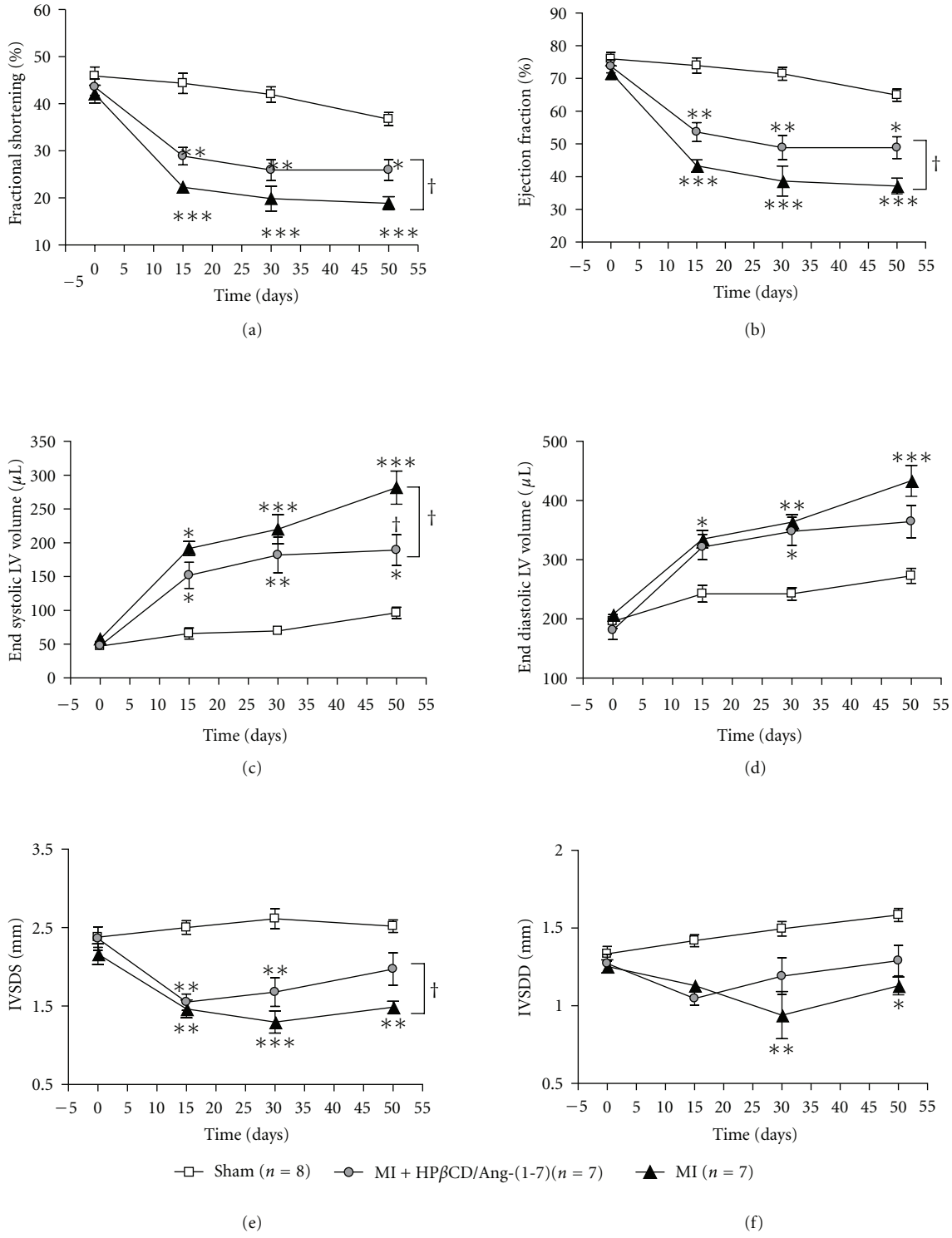

Figure 1: Effects of HPβCD/Ang-(1–7) on echocardiographic parameters after left coronary artery ligation in rats followed for up to 50 days. (a) Fractional shortening, (b) ejection fraction, (c) end systolic left ventricular volume, (d) end diastolic left ventricular volume, (e) interventricular septal dimension in systole, and (f) interventricular septal dimension in diastole. $^{*}P < 0.05$ versus sham; $^{**}P < 0.01$ versus sham; $^{***}P < 0.001$ versus sham; $^{\dagger}P < 0.05$ versus MI (two-way ANOVA followed by the Bonferroni posttest).

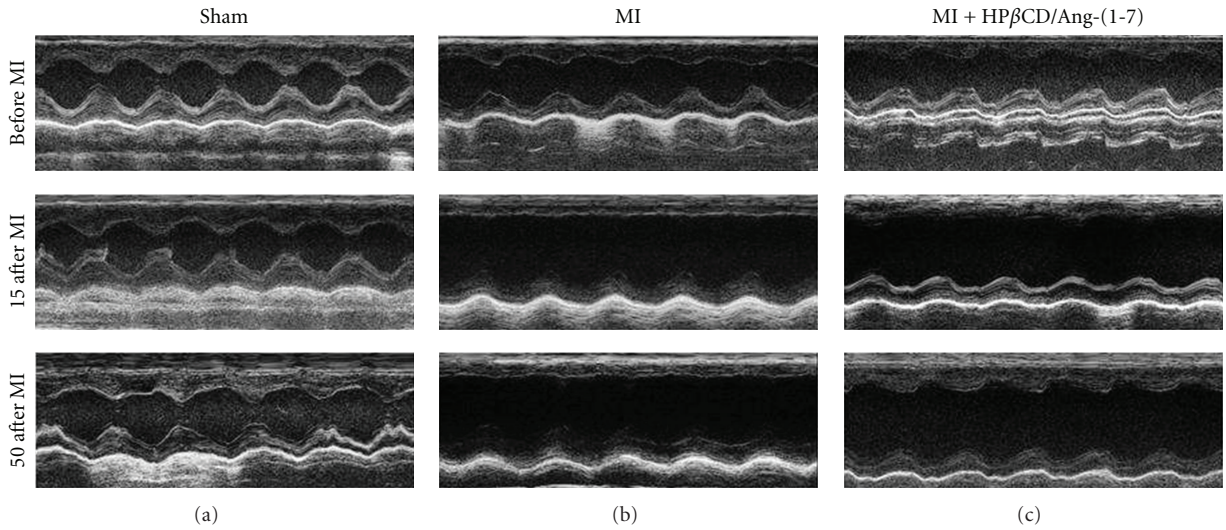

Figure 2: Representative M-mode images showing cardiac function and left ventricle chamber dimensions in sham, MI, and MI + HPβCD/Ang-(1–7)-treated rats. Note the marked increase in end-systolic dimension (ESD) and in end-diastolic dimension (EDD) after infarction and the improvement in the cardiac function after 50 days in rats treated with HPβCD/Ang-(1–7).

[22, 23], and fibrosis [4, 19]. The excessive fibrosis caused by maladaptive remodeling processes contributes to the diastolic and systolic dysfunction by increasing the myocardial stiffness and by reducing the pumping capacity [24]. The locally produced cytokine transforming growth factor β (TGF-β) is a major mediator of this process [25]. Its expression is increased in many cardiac pathologies such as hypertrophic and dilated cardiomyopathy [26–28] and myocardial infarction [29, 30]. In this latter condition, evidences suggest that TGF-β has a central role in the inflammatory and fibrotic phase of the healing process and may critically modulate many cellular steps of the postinfarction repair process by mediating cardiomyocyte growth, fibroblast activation, and extracellular matrix deposition [30]. Furthermore, TGF-β is considered an important marker for the transition course of stable hypertrophy to heart failure [31].

Recently, we have demonstrated that the inclusion of Ang-(1–7) into the oligosaccharide hydroxypropyl β-cyclodextrin (HPβCD) [32] is an effective formulation for oral administration of this heptapeptide [12]. Here, we aimed to evaluate the effects of long-term administration of the HPβCD/Ang-(1–7) inclusion compound on cardiac dysfunction and fibrosis caused by myocardial infarction (MI) in rats. Additionally, the expression levels of collagen type I and TGF-β were also analyzed in the hearts.

2. Methods

2.1. Animals. Male Wistar rats weighing 180 to 210 g (approximately 3 months of age) were used in this study. The animals were provided by the animal facilities of the Biological Sciences Institute (CEBIO, Federal University of Minas Gerais) and housed in a temperature- and humidity-controlled room maintained on a 12:12-h light-dark schedule with free access to food and water. All animal procedures were performed in accordance with institutional guidelines approved by local authorities.

2.2. Experimental Groups. The animals were divided into three groups: sham surgery treated with HPβCD ($n = 8$), vehicle-treated MI (infarction plus HPβCD, $n = 7$), and MI + HPβCD/Ang-(1–7) [infarction plus HPβCD/Ang-(1–7), $n = 7$]. The treatment with vehicle (HPβCD; 46 μg/kg/day in distilled water by gavage) or HPβCD/Ang-(1–7) (76 μg/kg/day in distilled water by gavage) started in the first day of MI, and the rats were killed, and the hearts were harvested for real-time PCR analysis 60 days after the beginning of the treatment. The final volume of gavage [HPβCD and HPβCD/Ang-(1–7)] was approximately 0.5 mL. Thirty-one animals initiated the experimental protocols. Three rats died within 48 hours after the MI surgery and one sham-operated animal died after one week. Additionally, five animals were excluded due to abnormal increases in the right atria detected by the echocardiographic exam at the initial examination or due to marked weight loss during the period of treatment.

2.3. Myocardial Infarction. MI was induced by proximal left anterior descending (LAD) coronary artery occlusion and performed under anesthesia with 10% ketamine/2% xylazine (4 : 3, 0.1 mL/100 g, i.p.). The animals were placed in supine position on a surgical table, tracheotomized, intubated, and ventilated with room air using a respirator for small rodents. The chest was opened by a left thoracotomy at the third or fourth intercostal space. After the incision of the pericardium, the heart was quickly removed from the thoracic cavity and moved to the left to allow access to the proximal LAD coronary artery. A 4-0 silk suture was snared around the LAD and carefully ligated to occlude the vessel. The heart was then placed back, and the chest was closed with

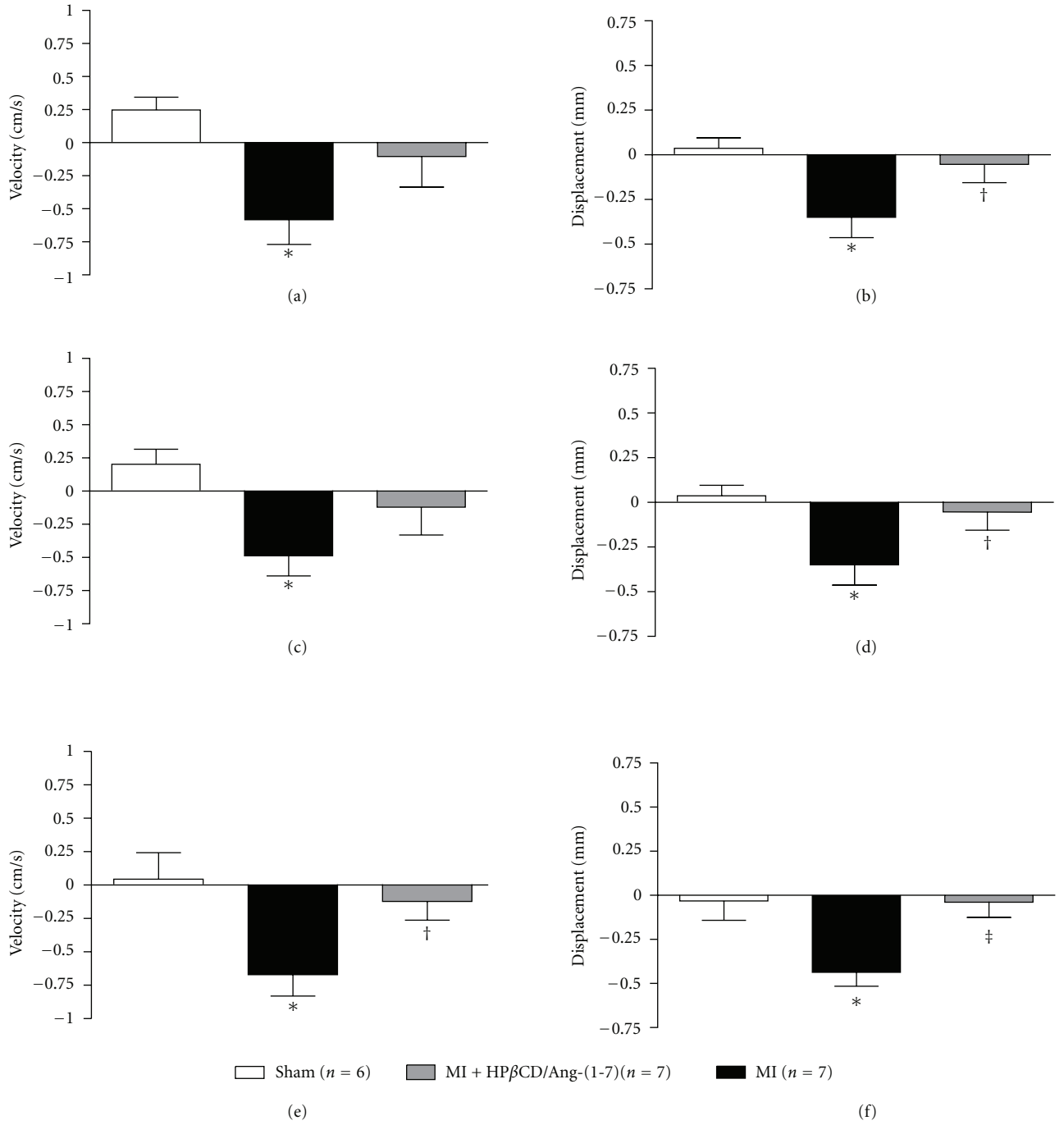

Figure 3: Effects of HPβCD/Ang-(1–7) on the variation of the velocity and displacement in rats after (a and b) 15 days; (c and d) 30 days, and (e and f) 50 days of treatment. $^{*}P < 0.05$ versus sham; $^{\dagger}P < 0.05$ versus MI; $^{\ddagger}P < 0.01$ versus MI (one-way ANOVA followed by the Newman-Keuls posttest).

4-0 silk sutures. Sham-operated rats were treated in the same manner, but the coronary artery was not ligated.

2.4. Echocardiography Analysis. Animals underwent transthoracic echocardiographic examination before the surgery and after 15, 30, and 50 days of LAD coronary artery ligation. *In vivo* cardiac morphology and function were assessed non-invasively using a high-frequency, high-resolution echocardiographic system consisting of a VEVO 2100 ultrasound machine equipped with a 16–21 MHz bifrequencial transducer (Visual Sonics, Toronto, Canada). The rats were anaesthetized with 3.5% isoflurane for induction, the anterior chest was shaved, and the rats were placed in supine position on an imaging stage equipped with built-in electrocardiographic electrodes for continuous heart rate monitoring and

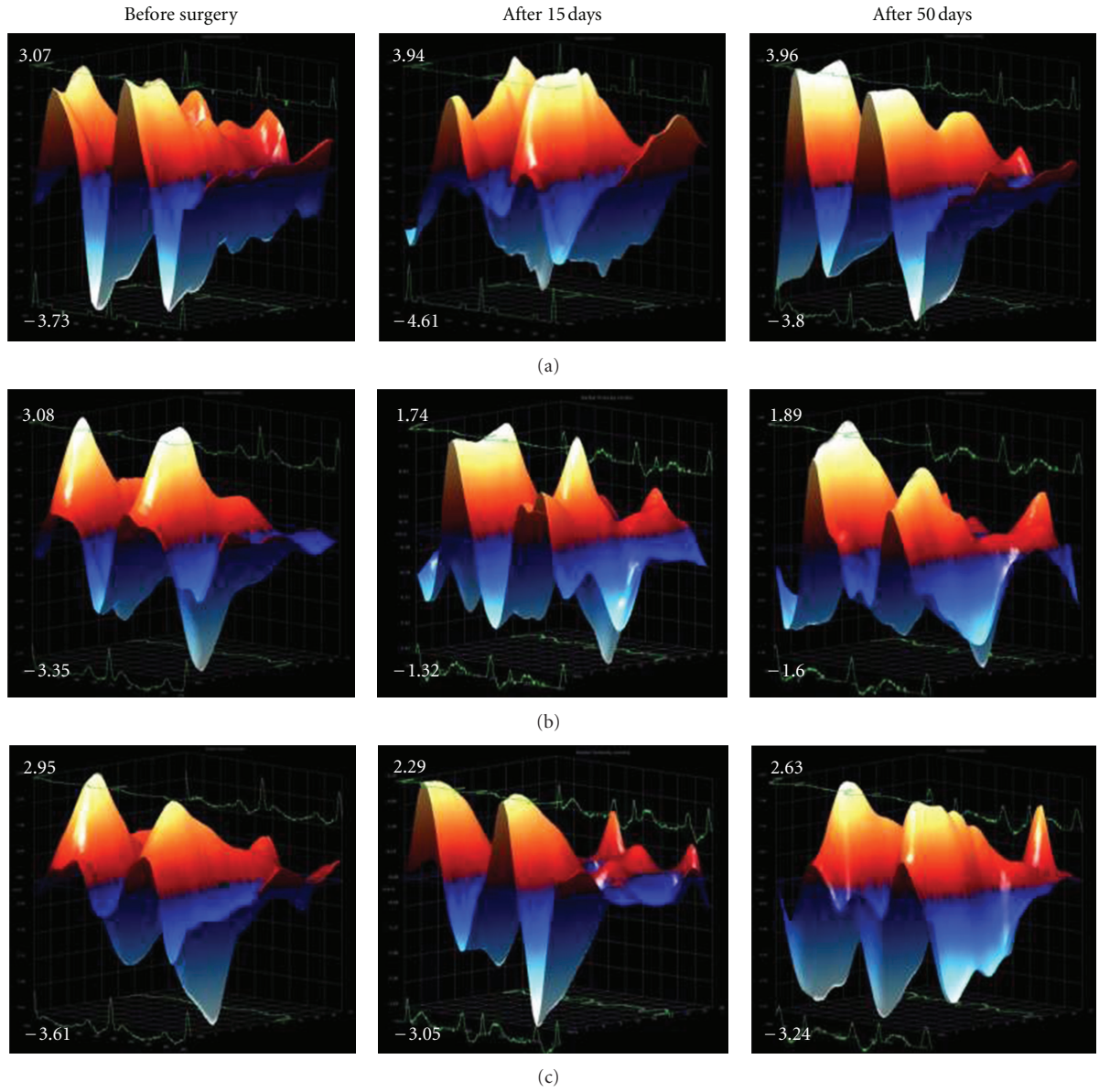

Figure 4: Representative images of the radial strain analysis of the velocity (cm/s) at basal conditions and after 15 and 50 days of MI. (a) sham group, (b) MI group, and (c) MI + HPβCD/Ang-(1–7).

a heater to maintain the body temperature at 37°C. Anesthesia was sustained via a nose cone with 2.5% isoflurane. High-resolution images were obtained in the right and left parasternal long and short axes and apical orientations. Standard B-mode images of the heart and pulsed Doppler images of the mitral and tricuspid inflow were acquired. Left ventricular (LV) dimensions and wall thickness were measured at the level of the papillary muscles in left and right parasternal short axis during the end systole and end diastole. LV ejection fraction (EF), fractional shortening (FS), and mass were measured. All the measurements and calculations were done in accordance with the American Society of Echocardiography. The following M-mode measurements were performed: LV internal dimensions at diastole and systole (LVIDD and LVIDS, resp.), LV posterior wall dimensions at diastole and systole (LVPWD and LVPWS, resp.), and interventricular septal dimensions at diastole and systole (IVSDD and IVSDS, resp.). Based on these parameters, end diastolic and end systolic LV volumes (EDLVV and ESLVV, resp.), FS, EF, stroke volume (SV), and cardiac output (CO) were calculated. Also, the radial strain from the bidimensional long axis view of the left ventricle was performed using the Vevostrain software. The following parameters were evaluated: velocity, displacement, strain, and strain rate.

2.5. Plasma Ang-(1–7) Levels Measurement. Blood samples were collected in tubes through a polypropylene funnel after the decapitation of the animals. These tubes

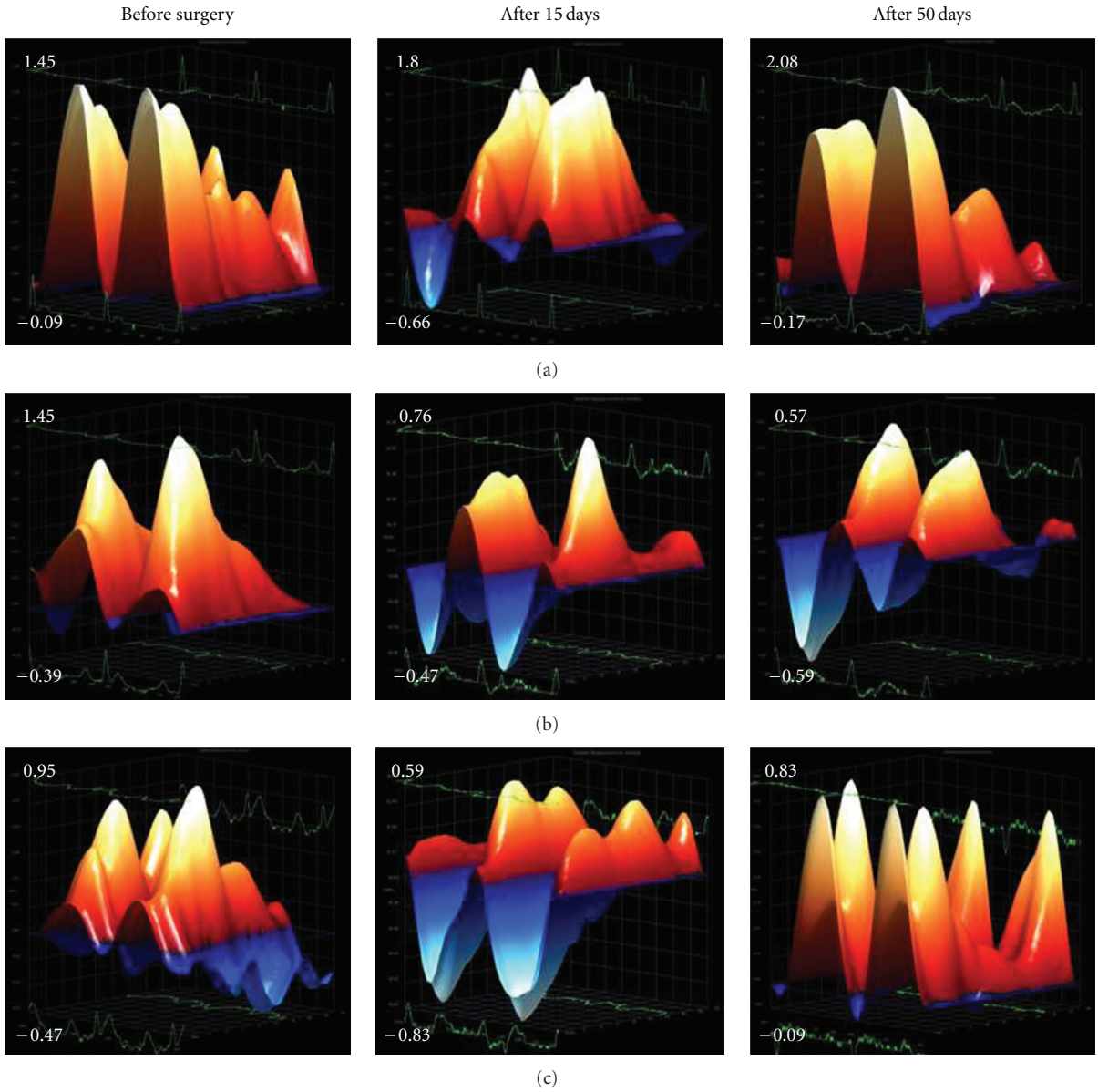

Figure 5: Representative images of radial strain analysis of the displacement (mm) at basal conditions and after 15 and 50 days of MI. (a) sham group, (b) MI group, and (c) MI + HPβCD/Ang-(1–7).

contained 1 mmol/L *p*-hydroxymercuribenzoate, 30 mmol/L 1,10-phenanthroline, 1 mmol/L PMSF, 1 mmol/L pepstatin A, and 7.5% EDTA (50 μL/mL of blood). After centrifugation, plasma samples were frozen in dry ice and stored at −80°C. Peptides were extracted onto a BondElut phenylsilane cartridge (Varian). The columns were preactivated by sequential washes with 10 mL of 99.9% acetonitrile/0.1% heptafluorobutyric acid (HFBA), and 10 mL of 0.1% HFBA. After sample application, the columns were washed with 20 mL of 0.1% HFBA and 3 mL of 20% acetonitrile/0.1% HFBA. The adsorbed peptides were eluted with 3 mL of 99.9% acetonitrile/0.1% HFBA into polypropylene tubes rinsed with 0.1% fat-free BSA. After evaporation, the Ang-(1–7) levels were measured by radioimmunoassay (RIA), as previously described [33].

2.6. Reverse Transcription and Real-Time PCR. To perform the real-time PCR analysis, the hearts were cut transversally approximately 1 mm below the suture point and 3 mm above the apex; thereby, they were divided in 3 parts: basal, middle, and apical. Only the middle portion, where the majority of the infarcted tissue was localized, was used for real-time PCR analysis. Total mRNA isolation was performed following the TRIzol reagent protocol (Invitrogen Life Technologies). Seven hundred nanograms of mRNA treated with DNAse were

used as template for M-MLV reverse transcriptase (ArrayScript, Ambion) using the following antisense primers: Mas (3′-GGTGGAGAAAAGCAAGGAGA-5′), TGF-β (3′-GGTTCATGTCATGGATGGTGC-5′), collagen I (3′-CCTTAGGCCATTGTGTATGC-5′), and S26 (3′-CGTGCTTCCCAAGCTCTATGT-5′). Real-time PCR was carried out immediately after the synthesis of the first strand cDNA. The sense primers used were Mas (5′-ACTGTCGGGCGGTCATCATC-3′), TGF-β (5′-TGACGTCACTGGAGTTGTACGG-3′), collagen I (5′-TGTTCAGCTTTGTGGACCTC-3′), and S26 (5′-CGATTCCTGACAACCTTGCTATG-3′) and their respective antisense primers as mentioned above. The PCR reactions containing 300 μM of each primer (sense and antisense), 50–100 ng of cDNA, and SYBR Green PCR Master Mix (Applied Biosystems) were run under standard conditions in an ABI Prism 7000 Sequence Detector. The threshold cycle (CT) was determined for each sample, and the CT values of the S26 were subtracted from the CT values of the experimental samples to obtain ΔCT values. Transcript levels in left ventricles were expressed as fold relative to the S26 values (2-$\Delta\Delta$CT).

2.7. Statistical Analysis. All data are expressed as means ± SEM. Echocardiographic data were estimated using two-way ANOVA followed by the Bonferroni posttest. The real-time PCR data, variation of velocity, and displacement of each time in relation to the initial time were analyzed using one-way ANOVA followed by the Newman-Keuls posttest. The level of significance was set at $P < 0.05$ (GraphPad Prism 4.0).

3. Results

3.1. Plasma Ang-(1–7) Levels. Plasma levels of Ang-(1–7) in blood samples collected 24 hours after the last dose of HPβCD/Ang-(1–7) in MI rats were higher than those observed in vehicle-treated rats; however, the difference did not reach significant statistical difference (80.16 ± 18.4 pg/mL versus 49.7 ± 13.6 pg/mL). Plasma levels of Ang-(1–7) in vehicle-treated sham rats averaged 63 ± 11.7 pg/mL.

3.2. Effects of HPβCD/Ang-(1–7) Long-Term Administration on Echocardiographic Parameters. The echocardiographic analysis at the initial time (before the surgery) showed that SV, HR, CO, EF, FS, EDLVV and ESLVV, LV mass, IVSDD, and IVSDS were similar in all three groups evaluated (data not shown). The success of the MI procedure was confirmed by the presence of one of the following changes in the myocardial kinetics observed during the echocardiographic analysis: (i) hypokinesia caused by reduction in the thickness or wall motion, (ii) akinesia represented by absence of thickening and/or movement, and (iii) dyskinesia characterized by changes in the movement in one or more segments or regions of the heart. Although the body weight gain was less pronounced in MI vehicle-treated animals, no significant differenceswere detected among the groups during the treatment period (Table 1). Also, no significant changes were observed in the LV mass, SV, HR, and CO during the treatment period. However, the administration of the inclusion compound to infarcted animals allowed them to better recover the SV and CO over time (Table 1). As expected, MI caused a progressive impairment of the cardiac function evidenced by decreases in the FS (Figure 1(a)), EF (Figure 1(b)), IVSDS (Figure 1(e)), and IVSDD (Figure 1(f)) and increases in the ESLVV (Figure 1(c)) and EDLVV (Figure 1(d)). The administration of HPβCD/Ang-(1–7) significantly improved the FS, EF, IVSDS, and ESLVV of MI animals (Figure 1). Specifically, after 15 days of surgical procedure, MI induced a significant reduction in the FS (50%), EF (42%), IVSDS (44%), and a significant increase in the EDLVV (40%) and ESLVV (189%) (Figure 1). The HPβCD/Ang-(1–7) treatment ameliorated the decrease of FS, EF, and ESLVV; that is, FS increased 32% (29 ± 2% versus 22 ± 1% in vehicle-treated MI rats), EF increased 26% (54 ± 3% versus 43 ± 2% in vehicle-treated MI rats), and ESLVV decreased 20% (152 ± 19 μL versus 191 ± 10 μL in vehicle-treated MI rats). Thirty days after MI induction, control infarcted animals showed a similar profile as observed at the 15 days of MI. An increase of 52% and 214% in the EDLVV and ESLVV was observed, respectively. Furthermore, an additional reduction in the IVSDS of untreated MI rats was observed (36%). Again, the treatment with HPβCD/Ang-(1–7) induced an improvement in all parameters analyzed, including a significant attenuation of the reduction in the IVSDD (1.2 ± 0.11 mm versus 0.9 ± 0.13 mm in vehicle-treated MI rats). At the end of the 50 days postinfarction period, no further alterations in the cardiac function of the vehicle-treated infarcted rats were observed; in contrast, the HPβCD/Ang-(1–7)-treated rats showed an improvement of all cardiac parameters evaluated, that is, FS (26 ± 2% versus 19 ± 1% in vehicle-treated MI rats), EF (49 ± 3% versus 37 ± 2% in vehicle-treated MI rats), IVSDS (2.0 ± 0.21 mm versus 1.5 ± 0.08 mm in vehicle-treated MI rats), IVSDD (1.3 ± 0.1 mm versus 1.1 ± 0.06 mm in vehicle-treated MI rats), EDLVV (364 ± 27 μL versus 445 ± 27 μL in vehicle-treated MI rats), and ESLVV (189 ± 23 μL versus 282 ± 25 μL in vehicle-treated MI rats) (Figures 1 and 2).

3.3. Effects of Long-Term Administration of HPβCD/Ang-(1–7) on Radial Strain Parameters. Radial strain analysis of the bidimensional long axis view of the LV revealed that MI induced a significant decrease in the velocity and displacement of myocardial fibers at 15, 30, and 50 days after surgery. HPβCD/Ang-(1–7) treatment completely reversed the reduction observed in the displacement of the myocardial fibers at all periods of evaluation after infarction (15, 30, and 50 days after surgery—Figures 3(b), 3(d), and 3(f), resp.) as well as in the velocity of myocardial fibers at 50 days after MI induction (Figure 3(e)). In addition, the velocity of myocardial fibers was improved in infarcted rats treated with HPβCD/Ang-(1–7) after 15 and 30 days of MI induction (Figures 3(a) and 3(b), resp.). Three-dimensional representative diagrams of velocity and displacement obtained by radial strain are shown in Figures 4 and 5, respectively.

Table 1: Functional parameters during long term of continuous treatment with HPβCD/Ang-(1–7) in infarcted rats.

Parameters	Sham				MI				MI + HPβCD/Ang-(1–7)			
	Before surgery	Days after surgery			Before surgery	Days after surgery			Before surgery	Days after surgery		
		15	30	50		15	30	50		15	30	50
Body weight (g)	188 ± 6	264 ± 6	342 ± 12	404 ± 11	191 ± 12	243 ± 11	307 ± 12	351 ± 14	213 ± 14	272 ± 10	334 ± 9	380 ± 11
LV mass (mg)	531 ± 39	695 ± 29	740 ± 34	793 ± 44	503 ± 31	740 ± 44	715 ± 26	901 ± 62	479 ± 54	640 ± 43	747 ± 46	780 ± 60
SV (μL)	147 ± 9.6	179 ± 10	173 ± 7.8	181 ± 7.8	148 ± 10	147 ± 10.3	141 ± 13.8	163 ± 9.5	133 ± 12	170 ± 8.5	166 ± 8.9	175 ± 11
HR (bpm)	399 ± 17	395 ± 11	403 ± 16	380 ± 12	377 ± 10	367 ± 23	359 ± 10	351 ± 11	410 ± 6	366 ± 10	378 ± 11	368 ± 11
CO (mL/min)	58 ± 4	70 ± 4	70 ± 4	69 ± 4	56 ± 4	55 ± 7	51 ± 6	57 ± 3	54 ± 4	61 ± 3	63 ± 4	65 ± 5

Data are expressed as mean ± SEM. No significant differences were observed among any of the groups (two-way ANOVA followed by Bonferroni posttest). MI: myocardial infarction; LV: left ventricle; SV: stroke volume; HR: heart rate; CO: cardiac output.

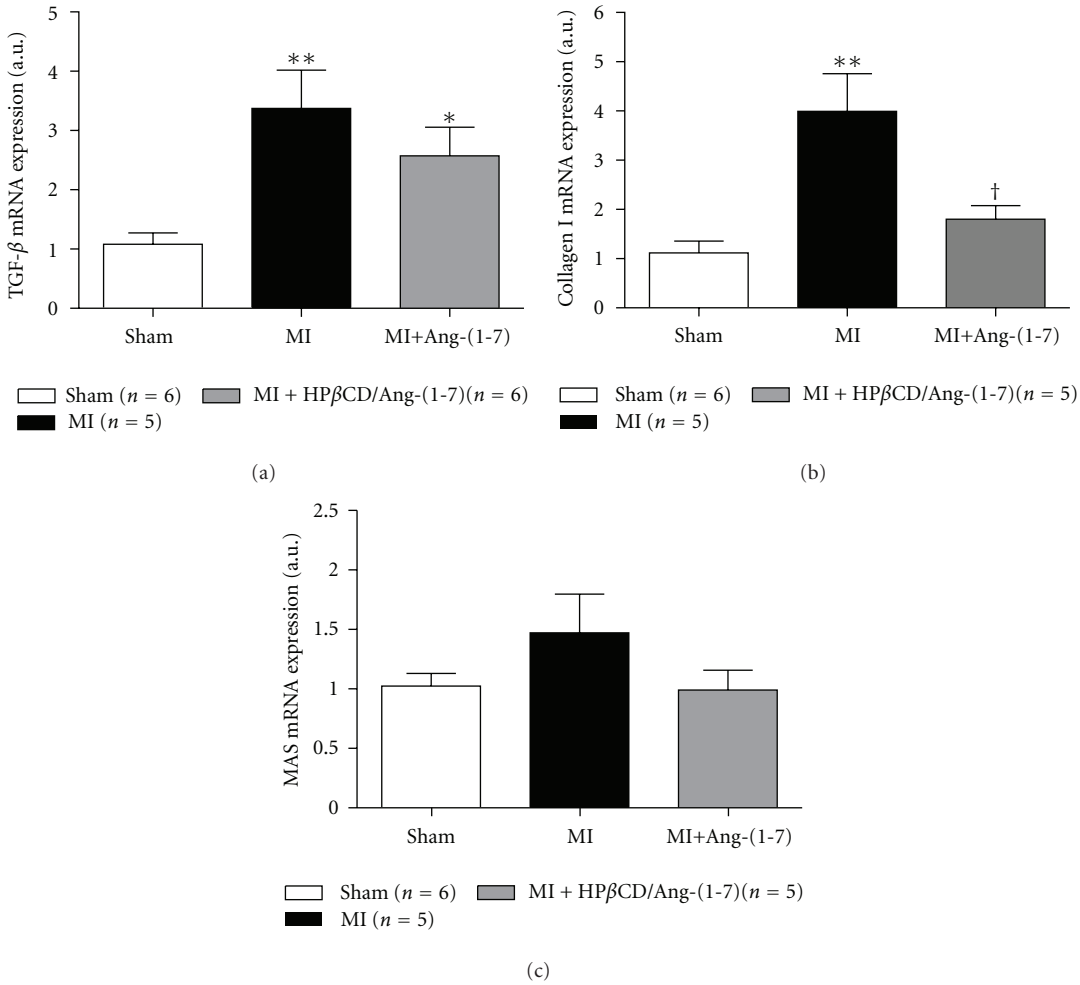

Figure 6: Effects of long-term administration of HPβCD/Ang-(1–7) on mRNA expression of (a) TGF-β, (b) collagen type I, and (c) Mas in infarcted animals. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus sham; $^{\dagger}P < 0.01$ versus MI (one-way ANOVA followed by the Newman-Keuls posttest) (a.u) = Arbitrary Units.

3.4. Effects of HPβCD/Ang-(1–7) Long-Term Administration on Mas, TGF-β, and Collagen Type I mRNA Expression. At the end of the treatment, real-time PCR assays were performed in order to evaluate the Mas, TGF-β, and collagen type I gene expression. We found that vehicle-treated infarcted animals presented an increased expression of TGF-β (Figure 6(a)) and collagen type I (Figure 6(b)) in the heart as compared with sham-operated rats. The administration of HPβCD/Ang-(1–7) in MI animals abolished the increase of collagen type I mRNA expression and reduced the increase in the expression of TGF-β mRNA. Although the expression of Mas tended to increase in MI rats, it did not reach statistical significance when compared with sham-operated animals (Figure 6(c)).

4. Discussion

In the present study, we demonstrated that once-a-day chronic oral administration of the inclusion compound HPβCD/Ang-(1–7) produced progressive time-dependent cardioprotective effects in MI animals. Specifically, we found that chronic oral administration of HPβCD/Ang-(1–7) improves the diastolic and systolic function and reduces the expression of fibrosis scar markers (TGF-β and collagen type I).

MI is a common cause of heart failure in humans [2, 34, 35], and the rat model of MI produced by coronary artery ligation has been used extensively to study the pathophysiology of this condition as well as new approaches to its treatment [12, 35, 36]. In keeping with a previous study [37], our results indicate that the progressive increase in diastolic LV volume was the main mechanism underlying the maintenance of the stroke volume in the presence of a prominent decrease in the FS. Importantly, the treatment with HPβCD/Ang-(1–7) caused a significant time-dependent improvement in LV dilation demonstrated by the attenuation of the changes in ESLVV and EDLVV.

It has been recently proposed that measurement of the myocardial deformation (velocity, displacement, strain, and

strain rate) is a powerful technique to measure heart function disturbances [38, 39]. In this study, we used this approach to evaluate functional changes induced by MI and the effects of HPβCD/Ang-(1–7) on these alterations. A significant improvement in the velocity and displacement of the cardiac fibers in animals treated with this heptapeptide was observed.

The increased deposition of collagen in the heart and abnormal extracellular matrix structure result in myocardial stiffness, leading to ventricular systolic and diastolic dysfunction [24, 35, 37]. Our data are in keeping with these concepts since we observed that chronic treatment with HPβCD/Ang-(1–7) attenuated these alterations, evidenced by a smaller fall in EF and FS and by a smaller reduction in the thickness of the cardiac fibers in both systole (IVSDS) and diastole (IVSDD), which was accompanied by a decrease expression of TGF-β and collagen type I.

Cardiac fibroblasts are the primary source of TGF-β in the heart [30], and it was demonstrated that this cytokine is absolutely required for the Ang II-induced cardiac hypertrophy *in vivo* [30], and regulates the collagen synthesis in cardiac fibroblasts [40, 41]. Thus, there are evidences supporting a direct functional association between the RAS and TGF-β pathways [42]. It has been suggested that Smad proteins are the main downstream mediators of the cardiac Ang II/TGF-β1 pathway in the chronic phase of MI [28]. Furthermore, it was observed that TGF-β1/TAK1/p38MAPK-signaling pathway is activated in spared cardiomyocytes following MI and can play an important role in the development of hypertrophy in the remodeling myocardium [43]. On the other hand, it is well documented that Ang-(1–7) acting through the Mas receptor counter regulates the Ang II effects [4, 9, 10, 14, 18, 19, 44, 45]. Although our results did not show statistical differences in the expression of Mas among any of the groups, they clearly demonstrated that the administration of HPβCD/Ang-(1–7) in infarcted animals induced a reduction in the increase of TGF-β mRNA expression. These findings can explain, at least partially, the beneficial effects of the inclusion compound in MI. Moreover, the improvement in the heart function of MI rats by HPβCD/Ang-(1–7) treatment could be related to the reduction of the infarcted area as demonstrated in our previous study [12] which is in keeping with the strain analysis showing an improvement of the ventricular wall displacement in the HPβCD/Ang-(1–7)-treated MI rats.

One special feature of the Ang-(1–7) is its long-term effectiveness, as demonstrated in this study. Indeed, this observation is in keeping with previous studies showing beneficial effects of chronic Ang-(1–7) administration in different models of cardiovascular diseases [4, 11, 13, 14, 20, 44, 46]. Chronic Ang-(1–7) administration improved LV function of Wistar rats [47] and of diabetic spontaneously hypertensive rats (SHRs) [14] after global ischemia, attenuated the heart failure induced by MI [11], prevented the development of severe hypertension and end-organ damage in SHR treated with L-NAME [13], and reduced the cardiac remodeling in DOCA-salt and in Ang II-infused rats [4, 20]. In addition, an antifibrotic effect was observed in transgenic animals, which chronically present an increased plasma Ang-(1–7) levels [46].

In summary, this study showed that long-term treatment with HPβCD/Ang-(1–7) was able to attenuate the maladaptive remodeling events caused by MI, thereby indicating that Ang-(1–7) holds beneficial effects in hearts and that this inclusion compound constitutes an effective strategy to orally administer this heptapeptide.

Acknowledgments

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Review Article

Angiotensin Converting Enzyme 2, Angiotensin-(1-7), and Receptor Mas Axis in the Kidney

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In the past few years the understanding of the renin-angiotensin system (RAS) has improved, helping to better define the role of this system in physiological conditions and in human diseases. Besides Angiotensin (Ang) II, the biological importance of other Ang fragments was progressively evidenced. In this regard, Angiotensin- (Ang-) (1-7) was recognized as a biologically active product of the RAS cascade with a specific receptor, the G-protein-coupled receptor Mas, and that is mainly formed by the action of the angiotensin-converting enzyme (ACE) homolog enzyme, ACE2, which converts Ang II into Ang-(1-7). Taking into account the biological effects of these two mediators, Ang II and Ang-(1-7), the RAS can be envisioned as a dual function system in which the vasoconstrictor/proliferative or vasodilator/antiproliferative actions are primarily driven by the balance between Ang II and Ang-(1-7), respectively. In this paper, we will discuss our current understanding of the ACE2/Ang-(1-7)/Mas axis of the RAS in renal physiology and in the pathogenesis of primary hypertension and chronic kidney disease.

1. Introduction

1.1. Historical Background of the ACE2/Ang-(1-7)/Mas Axis of the RAS. In the past few years the understanding of the renin-angiotensin system (RAS) has improved, helping to better define the role of this system in physiological conditions and in human diseases. Following the seminal study of Schiavone and coworkers [1] demonstrating that Angiotensin- (Ang-) (1-7) is a biologically active peptide of the RAS, several reports have clearly shown that this heptapeptide plays important functions in cardiovascular and renal system [2, 3].

The identification of the angiotensin-converting enzyme (ACE) homologue, ACE2, as the main Ang-(1-7)-forming enzyme was essential to establish a preferential enzymatic pathway for the production of this angiotensin peptide [4, 5]. ACE2 can cleave Ang I to form Ang-(1-9) [4], which is subsequently converted to Ang-(1-7) through ACE and neutral-endopeptidase 24.11 (NEP) activity [6]. However, the main substrate for ACE2 is Ang II, which is converted into Ang-(1-7) [7]. Consequently, ACE2 plays a pivotal role in the balance between both RAS mediators, Ang II and Ang-(1-7), once this enzyme can convert Ang II, a vasoconstrictor peptide, into Ang-(1-7), a vasodilator peptide. However, it should be mentioned that, besides ACE2, other enzymes might contribute to Ang-(1-7) formation such as prolylendopeptidase (PEP), prolylcarboxypeptidase (PCP), and NEP [8–10].

Further support for the relevance of Ang-(1-7) was achieved with the description of the orphan receptor Mas as a functional ligand site for this angiotensin [11]. This discovery was a confirmation of results previously obtained with the Ang-(1-7) antagonists, suggesting that Ang-(1-7) exerted its actions through a specific receptor, distinct from Ang II receptors type 1 (AT_1) and type 2 (AT_2) [12, 13].

It is now conceived that the RAS axis formed by ACE2, Ang-(1-7), and Mas is able to counter balance many of the well-established actions of the ACE-Ang II-AT_1 receptor axis [2, 3, 14, 15]. Accordingly, the activation of the vasodilator/antiproliferative axis might represent an endogenous protective mechanism against the deleterious effects elicited by the ACE-Ang II-AT_1 receptor axis, especially in pathological

conditions [2, 3, 14]. However, the role of ACE2-Ang-(1-7)-Mas axis appears to go far beyond a counterregulatory action.

This paper will briefly highlight recent findings concerning the renal effects of the ACE2-Ang-(1-7)-Mas axis in renal physiology and discuss its potential role in disease states.

1.2. The Role of ACE2/Ang-(1-7)/Mas Axis in Renal Physiology. A growing body of evidence supports the relevance of Ang-(1-7) for the regulation of renal function. Ang-(1-7) is present in the kidney at concentrations that are comparable to Ang II [8, 15]. The processing pathways for Ang-(1-7) in the circulation and kidney appear to be distinct. In the circulation, NEP is one of the major enzymes that produce Ang-(1-7) from Ang I or Ang-(1-9) [8]. In the kidney, NEP may contribute to both the synthesis as well as the degradation of Ang-(1-7). This enzyme cleaves Ang I to Ang-(1-7) and also metabolizes the peptide at Tyr^4-Ile^5 bond to form Ang-(1-4) and Ang-(5-7) [16, 17]. ACE2 seems to be the primarily responsible for Ang-(1-7) synthesis in the renal tissue [15].

It should be pointed that there are gender differences in renal activity of ACE2 and in the mRNA expression for this enzyme at renal tissue. In this regard, Ji and coworkers showed that ovariectomy decreased ACE2 protein (30%) and mRNA expression (36%) in renal wrap hypertension in rats, while 17-beta-estradiol replacement prevented these effects [18]. In addition, the infusion of Ang-(1-7) attenuated renal injury which was exacerbated by ovariectomy in this experimental model [18]. The authors concluded that 17-beta-estradiol-mediated upregulation of renal ACE2 and the consequent increased Ang-(1-7) production might protect against hypertensive renal disease. More recently, Liu and coworkers found that ACE2 activity was higher in the kidney of male mice compared to the kidney of females [19]. These authors believe that sex differences in renal ACE2 activity in intact mice are due, at least in part, to the presence of 17-beta-estradiol in the ovarian hormone milieu and not to the testicular milieu or to differences in sex chromosome dosage (2X versus 1X; 0Y versus 1Y) [19]. Therefore, the regulation of renal ACE2 by 17-beta-estradiol has particular implications for women across their life span since this hormone changes radically during puberty, pregnancy, and menopause.

Ang-(1-7) is the main product obtained in preparations of isolated proximal tubules and exists in urine at higher concentrations than Ang II [17]. The heptapeptide is also present in the distal convoluted tubules and collecting ducts [20]. Chappell et al. [15] demonstrated that the distribution of ACE2 within renal tubules is similar to that of Ang-(1-7). This finding was a preliminary evidence for the direct conversion of Ang II to Ang-(1-7) in the kidney. In keeping with these observations, Ferrario et al. [21] supported a role for ACE2 in Ang-(1-7) formation from Ang II in the kidney of normotensive rats. This study showed an increased ACE2 activity measured in renal tissue of rats given either lisinopril or losartan [21]. These data further suggest that increased levels of Ang-(1-7) in the urine of animals under ACE inhibition or AT_1 receptor blockade might reflect an intrarenal formation of this heptapeptide [21].

Many studies have addressed the complexity of renal actions of Ang-(1-7) [8, 15, 22–26]. Differences between species, local and systemic concentrations of Ang-(1-7), nephron segment, level of RAS activation, and sodium and water status can be responsible for discrepant results concerning renal effects of Ang-(1-7). A diuretic/natriuretic action of Ang-(1-7) has been described in several *in vitro* [27–30] and *in vivo* experimental models, mostly by inhibition of sodium reabsorption at proximal tubule [28, 31, 32]. Ang-(1-7) seems to be a potent inhibitor of Na-K-ATPase activity in the renal cortex [33] and in isolated convoluted proximal tubules [34]. In renal tubular epithelial cells, Ang-(1-7) inhibited transcellular flux of sodium, which was associated with activation of phospholipase A_2 [27]. *In vitro* studies also indicated that Ang-(1-7) modulates the stimulatory effect of Ang II on the Na-ATPase activity in proximal tubule through an A779-sensitive receptor. In this regard, Bürgelová et al. [34] showed that intrarenal administration of Ang-(1-7) produced natriuresis and blocked the antinatriuretic actions of Ang II.

On the other hand, our group and other investigators have observed an antidiuretic/antinatriuretic effect induced by Ang-(1-7), especially in water-loaded animals [11, 22, 23, 32, 35–41]. Ang-(1-7) has a potent antidiuretic activity in water-loaded rats [38, 39] and mice [35] probably mediated by the receptor Mas [11]. *In vitro*, Ang-(1-7) increased the water transport in the inner medullary collecting duct through an interaction between receptor Mas and the vasopressin type 2 receptor with subsequent adenylate cyclase activation [41]. These data were in accordance with the renal effects produced by the selective Ang-(1-7) receptor Mas antagonists, the compounds A-779 [12, 38–41] and D Pro^7-Ang-(1-7) [42]. The administration of these antagonists exerts a diuretic effect associated with an increase in glomerular filtration rate and in water excretion [38–42]. These findings suggest that endogenous Ang-(1-7) takes part in the regulation of glomerular filtration and of water handling at renal level.

The physiological relevance of Ang-(1-7) was further corroborated by the demonstration that Ang-(1-7) is an endogenous ligand for the G-protein-coupled receptor Mas in the kidney [11]. Immunocytochemical data reveal a similar distribution for Ang-(1-7), ACE2, and the Mas receptor within the tubular epithelium of the kidney [15]. Experimental data obtained with receptor Mas agonists and antagonists help understanding the role of this receptor in renal physiology. In water-loaded C57BL/6 mice, the administration of the oral agonist of receptor Mas, the compound AVE 0991, produced a significant reduction in urinary volume, associated with an increase in urinary osmolality [35]. The receptor Mas antagonist, A-779, completely blocked the antidiuretic effect of AVE 0991 [35]. As observed previously for Ang-(1-7) [11], the antidiuretic effect of AVE 0991 after water load was blunted in mice with genetic deletion of receptor Mas [35]. *In vitro* receptor autoradiography in C57BL/6 mice showed that the specific binding of ^{125}I-Ang-(1-7) to mouse kidney slices was displaced by AVE 0991, whereas no effects were observed in the binding of ^{125}I-Ang II or ^{125}I-Ang IV [35]. More recently, these findings were

further corroborated taking advantage of a novel transgenic rat model, TGR(A1-7)3292, that expresses an Ang-(1-7)-producing fusion protein which produces chronic elevation in Ang-(1-7) plasma concentration [43]. In this study, transgenic rats presented a significant reduction of basal urinary volume and of free water clearance, without changing plasma levels of vasopressin and the mRNA expression of Mas and vasopressin type 2 receptors in renal tissue [43].

Beside important tubular actions, Ang-(1-7) also contributes to renal hemodynamic regulation. The ability of the kidney to generate high intratubular and interstitial concentrations of Ang II and Ang-(1-7) allows the kidney to regulate intrarenal levels of these angiotensins in accord with the homeostatic needs for the regulation of renal hemodynamics, tubular reabsorption, and sodium balance. When the RAS is inappropriately stimulated, high intrarenal Ang II levels, acting on AT_1 receptors, can lead to both systemic and glomerular capillary hypertension, which can induce hemodynamic injury to the vascular endothelium and glomerulus [44, 45]. In addition, direct profibrotic and proinflammatory actions of Ang II may also promote kidney damage [44–46]. On the other hand, Ren et al. [47] reported that Ang-(1-7)-induced dilatation of pre-constricted renal afferent arterioles in rabbits and Sampaio et al. [48] showed that an infusion of low concentrations of Ang-(1-7) increased renal blood flow in rats. Ang-(1-7) also attenuated the effect of Ang-II-induced pressor responses and Ang-II-enhanced noradrenaline release to renal nerve stimulation in rat isolated kidney [49]. These results opened the possibility that Ang-(1-7) can also act as a physiological regulator of intraglomerular pressure, probably opposing the hypertensive and fibrogenic effects of Ang II.

2. The Role of ACE2/Ang-(1-7)/Mas Axis in Renal Diseases

2.1. Current Experimental Evidence. Experimental studies have also indicated a role for the Ang-(1-7)-Mas interaction in the regulation of matrix proteins deposition in the heart and liver [50, 51]. Our group has shown fibronectin and collagen III deposition in the kidney of mice with genetic deletion of receptor Mas, suggesting that these genetic modified animals exhibit a phenotype predisposition to renal fibrosis [52]. Accordingly, at initial stages of collagen deposition and renal fibrosis, type III collagen appears in greater amounts than do type I. As renal fibrosis progresses, there is a proportional decrease in type III collagen, and tubulointerstitial fibroblasts secrete collagen types I, III, IV, and V in response to TGF-beta, epidermal growth factor, and interleukin-2 [53, 54]. More recently, Zhang et al. demonstrated that infusion of angiotensin-(1-7) reduces glomerulosclerosis through counteracting angiotensin II in experimental glomerulonephritis [55], suggesting that Ang-(1-7) is also relevant for modulating renal fibrosis in disease states.

Although a protective role for Ang-(1-7) in renal fibrosis remains speculative, our findings in animals with genetic deletion of receptor Mas support this hypothesis [52]. In addition, many studies have shown that Ang-(1-7) exerts inhibitory effects on vascular and cellular growth mechanisms. The molecular mechanisms for the antiproliferative action of Ang-(1-7) include the stimulation of prostaglandin and cAMP production as well the inhibition of mitogen-activated protein (MAP) kinases [56]. The antiproliferative effects of Ang-(1-7) in vascular smooth muscle cells [57], liver tissue [51], and cardiomyocytes [58] seem to be mediated by receptor Mas. Moreover, Mas-deficient mice exhibited an impairment of heart function associated with changes in collagen expression toward a profibrotic profile [50]. Gallagher and Tallant [59] also reported the inhibition of human lung cell growth by Ang-(1-7) through a reduction in the serum-stimulated phosphorylation of extracellular signal-regulated kinase (ERK) 1 and ERK2. As the ERK cascade is activated in response to different stimuli, such as growth factors, cytokines, or DNA-damaging agents, the stimulation of the ACE2-Ang-(1-7)-Mas axis could be effective in halting glomerulosclerosis. Su et al. [60] have reported that Ang-(1-7) inhibits Ang II-stimulated MAP kinases phosphorylation in proximal tubular cells. Thus, the generation of Ang-(1-7) by proximal tubular ACE2 could counteract the proliferative effects of locally produced Ang II [60].

In keeping with this possibility, recent studies suggested a protective role for ACE2 in the kidney. Kidney diseases have been associated with a reduction in renal ACE2 expression, possibly facilitating the damaging effects of Ang II. Acquired or genetic ACE2 deficiency also appears to exacerbate renal damage and albuminuria in experimental models, supporting this hypothesis [61–67]. In addition, chronic blockade of ACE2 with the enzyme inhibitor MLN-4760 in control or diabetic mice produced albuminuria and matrix protein deposition [65]. More recently, Dilauro et al. [66] showed that ACE2 is downregulated in the renal cortex of mice that underwent subtotal nephrectomy. The reduction of renal ACE2 in nephrectomized animals led to proteinuria via an AT_1 receptor dependent mechanism [66]. Accordingly, the renal expression of ACE2 was also reduced in an experimental model of renal ischemia/ reperfusion [67]. Taken together, these findings suggest that decreased ACE2 activity may be involved in the pathogenesis of kidney disease, possibly by disrupting the metabolism of angiotensin peptides [68]. Taking into account the enzymatic properties of the two ACEs and of the two main mediators Ang II and Ang-(1-7), the RAS can be envisioned as a dual function system in which the vasoconstrictor/proliferative or vasodilator/antiproliferative actions are primarily driven by the ACE–ACE2 balance. Accordingly, an increased ACE/ACE2 activity ratio will lead to increased Ang II generation and increased catabolism of Ang-(1-7), favoring vasoconstriction, while a decreased ratio will decrease Ang II and increase Ang-(1-7) levels, facilitating vasodilatation [68–70].

On the other hand, some studies pointed to a deleterious role for Ang-(1-7) at renal system. For instance, the study of Esteban and coworkers using mice with genetic deletion of receptor Mas showed very discrepant results in relation to renal function when compared to our findings [71]. While our research group [52] showed that the genetic deletion of receptor Mas in C57Bl/6 mice led to glomerular hyperfiltration, proteinuria and renal fibrosis, Esteban et al. [28] reported that renal deficiency of Mas diminished renal

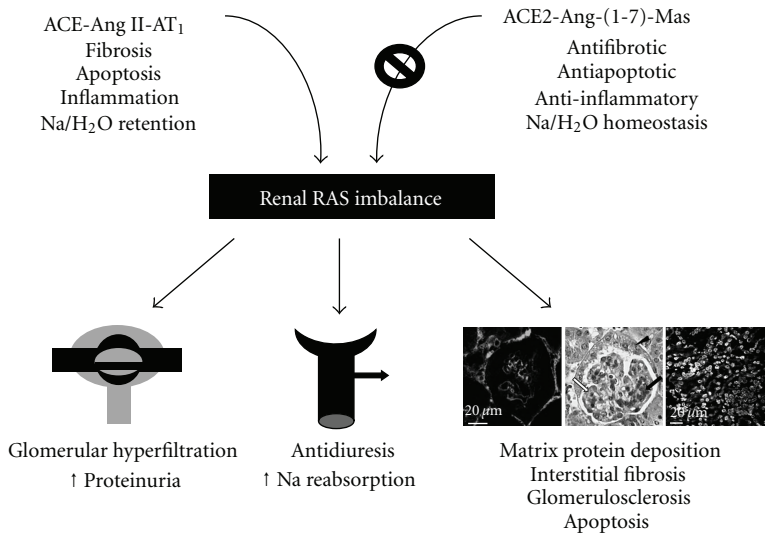

Figure 1: Proposed mechanisms for the role of ACE-Ang II-AT_1 receptor axis in excess of ACE2-Ang-(1-7)-Mas receptor axis at renal level.

damage in unilateral ureteral obstruction and in ischemia/reperfusion injury, and that the infusion of Ang-(1-7) to wild-type mice elicited an inflammatory response. Furthermore, animal models of renal diseases have also showed discrepant findings. Zhang et al. [55] showed that a 5-day infusion of Ang-(1-7) improved glomerulosclerosis in a rat model of thy-1-induced glomerulonephritis, whereas Velkoska et al. [72] verified that a 10-day infusion of the same concentration of Ang-(1-7) in rats with subtotal nephrectomy was associated with deleterious effects on blood pressure and the heart, with increase in cardiac ACE, and decrease in cardiac ACE2 activity. Although these findings are conflicting, cell-specific signaling pathways associated with Ang-(1-7) in the kidney could play a role in the variable response. In this regard, in the proximal tubule Ang-(1-7) displays growth inhibitory properties and antagonizes the effects of Ang II [60], whereas in mesangial cells, it appears to stimulate cell growth pathways [73]. In addition, the vascular and tubular effects of Ang-(1-7) in the kidney appear to be importantly influenced by experimental conditions and the level of RAS activation [74].

2.2. Current Clinical Evidence. Agonists and antagonists of the Ang-(1-7)-Mas axis probably possess a therapeutic potential for the modulation of sodium and water excretion in many physiologic and pathologic renal conditions, such as arterial hypertension, nephrogenic diabetes insipidus, glomerular diseases, chronic kidney disease (CKD), and diabetic nephropathy (see [68–70], for review). Ang-(1-7) can be measured in plasma and urine samples collected in healthy subjects and in patients with diverse clinical conditions (see [68–70], for review). Changes in blood pressure, in blood volume, in sodium intake and in renal function were able to modify the levels of Ang-(1-7) measured in plasma, renal tissue, and urine [75–81]. In addition, the concentration of the heptapeptide may differ in plasma and urine samples of the same subject. Accordingly, Ferrario and coworkers have reported that Ang-(1-7) is excreted in the urine of normal healthy adult volunteers in amounts 2.5-fold higher than those measured in plasma [82]. In the same study, it was also observed that untreated adults with primary hypertension exhibited a lower urinary excretion of Ang-(1-7) than normotensive controls and urinary concentrations of Ang-(1-7) were inversely correlated with blood pressure [82].

In pediatric patients, Simões e Silva and coworkers have reported significant differences among circulating Ang II and Ang-(1-7) levels in renovascular disease and in primary hypertension [83]. Children with renovascular hypertension had plasma levels of Ang II higher than of Ang-(1-7) and the successful correction of unilateral renal artery stenosis produced a return of circulating angiotensins to levels similar to those in healthy subjects. In contrast, patients with primary hypertension had significant elevation of circulating Ang-(1-7), while the levels of Ang I and Ang II were within the same range as in healthy subjects. In addition, the achievement of blood pressure control with calcium channel blockers did not change plasma concentration of Ang-(1-7) and Ang II. The physiopathological meaning of increased levels of only Ang-(1-7) in pediatric primary hypertension is still unknown and raises the question whether this elevation is a compensatory mechanism that opposes deleterious renal and cardiovascular effects of Ang II or whether, at supra physiological concentrations, Ang-(1-7) could act as another RAS mediator of renal dysfunction.

In addition, Simões e Silva et al. have demonstrated a significant increase in plasma Ang-(1-7) and Ang II levels among hypertensive children with CKD stage III when compared to normotensive CKD patients with the same stage of renal dysfunction [76]. While the presence of hypertension affected plasma concentration of both peptides, the progression to end stage renal disease was accompanied by more pronounced elevation only in Ang-(1-7) levels. Ang II levels were similarly elevated despite the level of renal dysfunction. Taken together, these data support a preferential production of Ang-(1-7) in end stage renal disease [76]. Future studies

are needed to elucidate the physiopathological role of this heptapeptide in human CKD.

Another important aspect to be considered is the elevation of plasma Ang-(1-7) during chronic RAS inhibition [76–79]. The renoprotective actions of ACE inhibitors and AT_1 receptor blockers clearly involve multiple pathways including antiproliferative and antifibrogenic effects [78, 80, 81]. In particular, an altered balance between Ang II and Ang-(1-7) might be related to the mechanism of action of ACE inhibition and AT_1 receptor blockade. Studying healthy subjects, Kocks et al. [79] showed that, during ACE inhibition, the administration of a low sodium diet did not affect plasma levels of Ang II but induced a significant elevation in Ang-(1-7) concentration. Consequently, the combination of ACE inhibition and a low sodium diet appeared to shift the balance between Ang-(1-7) and Ang II towards Ang-(1-7), which in turn might contribute to the therapeutic benefits of ACE inhibition [79].

3. Concluding Remarks

The current evidence supports the existence of a counterregulatory axis within the RAS formed mainly by the ACE2-Ang-(1-7)-receptor Mas axis. The primary function of this axis is to oppose the effects of the major component of the RAS, Ang II. Experimental and clinical studies have demonstrated a role for the ACE2/Ang-(1-7)/Mas axis in the regulation of renal function, in arterial hypertension, and in the progression of CKD. Figure 1 shows the proposed mechanisms for the role of ACE-Ang II-AT_1 receptor axis in excess of ACE2-Ang-(1-7)-Mas receptor axis at renal level. Therefore, the disproportion between both RAS axes might represent an important pathway for CKD progression (Figure 1). Further research on the contribution of the ACE2/Ang-(1-7)/Mas axis to renal pathophysiology should lead to the development of new pharmacologic approaches resulting in the design of molecular or genetic means to increase the expression of ACE2, allow for increased tissue levels of Ang-(1-7), or both.

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Research Article

Deficiency of ACE2 in Bone-Marrow-Derived Cells Increases Expression of TNF-α in Adipose Stromal Cells and Augments Glucose Intolerance in Obese C57BL/6 Mice

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Deficiency of ACE2 in macrophages has been suggested to promote the development of an inflammatory M1 macrophage phenotype. We evaluated effects of ACE2 deficiency in bone-marrow-derived stem cells on adipose inflammation and glucose tolerance in C57BL/6 mice fed a high fat (HF) diet. ACE2 activity was increased in the stromal vascular fraction (SVF) isolated from visceral, but not subcutaneous adipose tissue of HF-fed mice. Deficiency of ACE2 in bone marrow cells significantly increased mRNA abundance of F4/80 and TNF-α in the SVF isolated from visceral adipose tissue of HF-fed chimeric mice, supporting increased presence of inflammatory macrophages in adipose tissue. Moreover, deficiency of ACE2 in bone marrow cells modestly augmented glucose intolerance in HF-fed chimeric mice and increased blood levels of glycosylated hemoglobin. In summary, ACE2 deficiency in bone marrow cells promotes inflammation in adipose tissue and augments obesity-induced glucose intolerance.

1. Introduction

Angiotensin-converting enzyme-2 (ACE2) is a monocarboxypeptidase which is responsible for converting angiotensin II (AngII) to angiotensin 1–7 (Ang-(1–7)). Previous studies demonstrated expression of a complete renin-angiotensin system (RAS) in bone marrow cells, including renin, angiotensin converting enzyme (ACE), ACE2, AngII, and angiotensin receptors (AT1 and AT2) [1, 2]. Recent studies in our laboratory demonstrated ACE2 enzymatic activity in macrophages and localization of ACE2 immunoreactivity to macrophage-containing atherosclerotic lesions [2]. Moreover, deficiency of ACE2 in bone-marrow-derived stem cells promoted the development of diet-induced atherosclerosis in low-density-lipoprotein-receptor (LDLR-)deficient mice [2]. Peritoneal macrophages from ACE2-deficient LDLR−/− mice exhibited increased release of AngII, IL-6, and plasminogen activator inhibitor-1 (PAI-1), and conditioned media from ACE2-deficient macrophages promoted monocyte adhesion to endothelial cells [2]. These results suggest that elevated levels of AngII in ACE2-deficient leukocytes may promote adhesion of monocytes to vascular endothelial cells. Using bone-marrow-derived macrophages from mice with combined deficiency of apolipoprotein E and ACE2, Thomas et al. demonstrated enhanced lipopolysaccharide-(LPS-) induced mRNA abundance of tumor necrosis factor-alpha (TNFα), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), and matrix metalloproteinase-9 (MMP-9) [3]. In addition, the mas receptor was localized to peritoneal macrophages and Ang-(1–7) decreased LPS-induced inflammatory responses [4]. Collectively, these results suggest that macrophage ACE2 influences levels of AngII/Ang-(1–7), potentially contributing to macrophage-mediated inflammation.

Obesity is known to increase macrophage infiltration into adipose tissue, and adipose tissue macrophages (ATMs) are mainly derived from the bone marrow [5, 6]. Infiltration of macrophages with obesity promotes inflammation in adipose tissue and has been linked to the development of insulin resistance and type 2 diabetes [7]. Adipocytes secrete a number of different cytokines that can influence the polarization state of macrophages in adipose tissue.

Macrophages that are recruited to adipose tissue display an activated state, termed M1 polarization [8–10]. Activated M1 macrophages have increased expression of IL-6, inducible nitric oxide (iNOS), and C-C chemokine receptor 2 (CCR2) [8]. Alternatively activated macrophages (M2 polarization) counterbalance the proinflammatory status in adipose tissue [9]. Since peritoneal macrophages from ACE2-deficient mice displayed increased expression and/or release of several M1 macrophage-related cytokines, macrophages from ACE2-deficient mice have been suggested to exhibit M1 polarization [2].

Previous studies demonstrated that diet-induced obesity is associated with an activated systemic and adipose renin-angiotensin system (RAS) [11, 12]. Increased plasma concentrations of AngII in male C57BL/6 mice with diet-induced obesity were associated with dysregulated ACE2 in adipose tissue and the development of obesity hypertension [12]. These results suggest that obesity is associated with changes in the adipose RAS, including ACE2. Since obesity is associated with increased macrophage infiltration into adipose tissue, the specific cell type(s) experiencing previously observed alterations in ACE2 function in adipose tissue of obese mice is unclear [12]. Moreover, the role of macrophage-derived ACE2 in obesity-induced inflammation of adipose tissue has not been defined. Bone marrow transplantation in irradiated mice has been extensively employed to define the role of leukocytes in various disease pathologies. The purpose of this study was to define the effect of leukocyte deficiency of ACE2, using bone marrow transplantation from ACE2-deficient mice, on the development of obesity, adipose inflammation, and glucose intolerance in high-fat-(HF-) fed C57BL/6 mice.

2. Methods

2.1. Mice and Bone Marrow Transplantation. All experiments involving mice conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kentucky Institutional Animal Care and Use Committee. Male, 8-week-old C57BL/6 mice were purchased from Jackson Labs (Bar Harbor, MA) and housed in a temperature-controlled room with a 12 : 12-h light-dark cycle. *Ace2*$^{+/y}$ or *Ace2*$^{-/y}$ C57BL/6 mice were 10-times backcrossed onto a C57BL/6 background [13]. Initial studies examined ACE2 activity in the stromal vascular fraction (SVF) isolated from adipose tissues of male C57BL/6 mice (2 months of age; $N = 10$ /group) fed a low fat (LF; 10% kcal as fat, D12450, Research Diets Inc, New Brunswick, NJ) or high fat (HF, 60% kcal as fat, D12492, Research Diets Inc, New Brunswick, NJ) diet for 16 weeks. For bone marrow transplantation, C57BL/6 male mice (2 months of age) were pretreated with antibiotic water (sulfatrim, 4 μg/mL) for 1 week prior to irradiation [2, 14]. Bone marrow was extracted from the tibias and femurs of *Ace2*$^{+/y}$ or *Ace2*$^{-/y}$ male mice (2 months of age) and injected into gamma-irradiated recipient C57BL/6 males ($N = 15$ mice/donor genotype) at a dose of 10^7 cells per mouse. Recipient mice were given antibiotic water for 8 weeks to allow for efficient repopulation [14]. Mice in each donor genotype were fed the HF diet for 4 months. Body weight was recorded weekly. To define fat/lean mass, Dual Energy X-ray Absorptiometry (DEXA) was performed on anesthetized mice prior to initiation of the HF diet and at study endpoint. At study endpoint, mice were anesthetized (ketamine/xylazine 100/10 mg/kg, ip) for exsanguination to obtain blood for white cell counts (WBCs, K indicates 1000 per microliter), hemoglobin concentration (grams per deciliter), and bone marrow (femur) was harvested to confirm effective bone marrow repopulation (data not shown).

Table 1: Characteristics of chimeric HF-fed mice transplanted with *Ace2*$^{+/y}$ or $^{-/y}$ bone marrow.

	Ace2$^{+/y}$	*Ace2*$^{-/y}$
Body weight (g)	45 ± 1	46 ± 1
Body fat by DEXA (%)	40 ± 1	39 ± 1
Serum cholesterol (mg/dL)	227 ± 9	240 ± 12
Serum triglycerides (mg/dL)	91 ± 9	77 ± 8
Serum NEFAs (mEq/L)	4.3 ± 0.3	4.2 ± 0.4
Plasma renin (ng/mL)	8 ± 1	5 ± 1
Plasma AngII (pg/mL)	243 ± 50	154 ± 35
Serum ACE activity (nmol/mL/min)	742 ± 77	686 ± 96

Data are mean ± SEM from $N = 3$–11 mice/donor genotype.

2.2. Measurement of Plasma and Serum Parameters. Fasting (6 hr) blood glucose concentrations (mg/dL) were measured with a glucometer (FreeStyle Strips, Abbott Labs, Alameda, CA) at 1, 2, and 3 months of HF-feeding. During month 4 of HF feeding, a glucose tolerance test (GTT) was performed on fasted (6 hr) mice. Blood glucose concentrations were quantified at 0, 15, 30, 60, 90, 125, 160, and 220 minutes after glucose administration (2 mg/g glucose, ip). Percent glycosylated hemoglobin (%GHb) was quantified in whole blood according to the manufacturer's instructions (Glycohemoglobin Reagent Set-Unitized, cat no. G7540-100, Pointe Scientific, Inc., Canton MI). Plasma concentrations of insulin were quantified in nonfasted mice by ELISA according to the manufacturer's instructions (Millipore Inc., Billerica, MA). Serum concentrations of cholesterol, triglyceride, and free fatty acids were quantified using colorimetric kits from Wako Pure Chemical Industries (Osaka, Japan). Serum ACE activity (Table 1) was quantified using 5 mM N-Hippuryl-His-Leu as a substrate in 0.4 M sodium borate, pH 8.3 (30 min incubation at 37°C), with 2% o-phthaldialdehyde added to measure the fluorescence of the reaction for 10 minutes at room temperature. Reactions were preincubated with and without captopril (1 μM) for 30 minutes at 37°C to assess specificity. Absorbance was measured at an excitation of 365 nm and an emission of 495 nm. Specific activity was normalized to total volume of serum added to the reaction (2 μLs). Plasma renin concentrations were quantified by incubating mouse plasma (8 μLs) with an excess of partially purified rat angiotensinogen (from nephrectomized rats) in the presence of ACE inhibition (EDTA, captopril, 1 μM), followed by quantification of angiotensin

I concentrations by radioimmunoassay (DiaSorin CA-1553, Stillwater, MN) [15]. To quantify plasma concentrations of AngII, plasma was first processed over mini-C18 columns to concentrate peptides, followed by quantification of AngII by radioimmunoassay using an anti-rabbit AngII antibody (1 : 40,000 dilution; Bachem, Torrance, CA) that exhibits cross reactivity to AngIII (100%), AngIV (75%), but minimal reactivity to other angiotensins [14, 16].

2.3. Isolation of Stromal Vascular Fraction (SVF) from Adipose Tissue and Quantification of ACE2 Enzymatic Activity. Adipose tissue (epididymal fat, EF; retroperitoneal fat, RPF; subcutaneous fat, SubQ) was minced and digested with Type I collagenase (1 mg/mL; 60 min at 37°C) in buffer containing fatty acid-free bovine serum albumin (1%) [17]. Digested material was filtered (100 μm nylon mesh) and centrifuged (500 g) for 10 minutes to pellet SVF (frozen at −70°C unless used for ACE2 activity). ACE2 enzymatic activity was quantified in SVF by examining the conversion of [^{125}I]-AngII to [^{125}I]-Ang-(1–7) [2, 12]. Briefly, SVF was homogenized in Tris buffer (100 mM) containing NaCl (0.3 M), $ZnCl_2$ (10 μM), and Z-proprolinal (10 μM). Following centrifugation (30,000 g for 20 minutes, 4°C), pellets were reconstituted in the above buffer containing 0.5% Triton-X and incubated overnight at 4°C. Samples were again centrifuged (5,000 g for 10 minutes, 4°C) and the supernatant containing solubilized membrane was used for measurement of protein (BCA assay, ThermoFischer) and ACE2 enzymatic activity. SVF protein (0.05 mg/mL) was added to tubes with Tris buffer (total volume was 250 μLs) containing the following inhibitors: thiorphan (0.1 mM), phosphoramidon (0.1 mM), bestatin (100 μM), pepstatin A (100 μM), and captopril (10 μM) (pH = 7.0). [^{125}I]-AngII (2×10^6 cpms) was incubated with samples for 30 minutes at 37°C and the reactions were stopped by adding 50 μLs of 1% phosphoric acid. Samples were centrifuged, filtered, and injected onto a Beckman reverse-phase HPLC to resolve angiotensins [12]. Retention times for [^{125}I]-Ang-(1–7) (6.6 minutes) and [^{125}I]-AngII (13.6 minutes) were used to define HPLC fractions containing angiotensins, and radioactivity was quantified by gamma counting. ACE2 activity is expressed as femtomoles per milligram protein per minute, based on the specific activity of [^{125}I]-AngII [12].

2.4. Quantification of mRNA Abundance by RT-PCR. SVF pellets were placed in RNA lysis buffer (Promega, Madison, WI), and RNA was extracted using an SV Total RNA isolation kit (Promega, Madison, WI). RNA absorbance was measured at 260/280 nm and reverse transcription reactions were conducted on 0.5 μg of total RNA. Reverse transcription reactions were performed using a RETROscript kit (Ambion Inc, Austin, TX) through use of random decamers and heat denaturation of the RNA. Subsequent PCR analysis was performed using SYBR Green PCR core reagents (Applied Biosystems, Foster City, CA), and real-time conditions were as follows: 2.5 minutes at 95°C, 40 cycles of 1 minute at 94°C, 1 minute at reannealing temperature, 1 minute at 72°C, and a final elongation step at 72°C for 10 minutes.

Table 2: Primers used in this study.

Gene	Primers
F4/80	Forward 5′-CTTTGGCTATGGGCTTCCAGTC-3′ Reverse 5′-GCAAGGAGGACAGAGTTTATCGTG-3′
TNF-α	Forward 5′-CCCACTCTGACCCCTTTACTC-3′ Reverse 5′-TCACTGTCCCAGCATCTTGT-3′
TNF-α receptor	Forward 5′-CAGTCTGCAGGGAGTGTGAA-3′ Reverse 5′-CACGCACTGGAAGTGTGTCT-3′
CCR2	Forward 5′-AGAGAGCTGCAGCAAAAAGG-3′ Reverse 5′-GGAAAGAGGCAGTTGCAAAG-3′
CCL2	Forward 5′-CCTGCTGCTACTCATTCACC-3′ Reverse 5′-TGTCTGGACCCATTCCTTCT-3′
IL-1beta	Forward 5′-ATCTGGGATCCTCTCCAGCCAAGC-3′ Reverse 5′-AAAGGTTTGGAAGCAGCCCTTCAT-3′
PAI-1	Forward 5′-ACTGCAAAAGGTCAGGATCG-3′ Reverse 5′-ACAAAGGCTGTGGAGGAAGA-3′
Mgl-1	Forward 5′-ATGATGTCTGCCAGAGAACC-3′ Reverse 5′-ATCACAGATTTCAGCAACCTTA-3′
IL-4	Forward 5′-AGAAGGGACGCCATGCACGG-3′ Reverse 5′-ATGCGAAGCACCTTGGAAGCCC-3′
YM-1	Forward 5′-GACCTGCCCCGTTCAGTGCC-3′ Reverse 5′-TGCCAGTCCAGGTTGAGGCCA-3′
Mrc-1	Forward 5′-AGGGCAAGCTGCAAGCAGCA-3′ Reverse 5′-CCCACTGCCAACCACTGCGT-3′

Cyclophilin A was used to control for loading material and mRNA abundance was quantified using the $2^{-\Delta\Delta Ct}$ method. Primers for specific genes are listed in Table 2.

2.5. Statistical Analysis. Data are expressed as mean ± SEM. All data were analyzed using Sigma Stat. For one factor analysis (diet or genotype), a *t*-test was used to analyze endpoint measures. For GTTs, a repeated measures two-way ANOVA was performed with a Holm-Sidak test for multiple comparisons. Significance was accepted at $P > 0.05$.

3. Results

3.1. ACE2 Activity is Increased in SVF from Adipose Tissue of HF-fed Mice. We quantified the effect of HF feeding on ACE2 activity in peritoneal macrophages (MPM), bone marrow (BM), and the SVF isolated from different adipose depots of LF and HF-fed mice. In MPMs and BM, ACE2 activity was not influenced by HF feeding. In contrast, the SVF isolated from visceral (RPF) adipose tissue, but not gonadal (EF) or subcutaneous (SubQ) adipose tissue of HF-fed mice, exhibited significantly increased ACE2 activity compared to LF-fed controls (Figure 1; $P < 0.05$ for RPF; $P = 0.06$ for EF; $P > 0.05$ for SubQ).

3.2. Bone Marrow Deficiency of ACE2 Promotes Increases in Macrophage and Inflammatory Markers and Glucose Intolerance in HF-fed Mice. Deficiency of ACE2 in bone-marrow-derived cells had no significant effect on body weight or fat mass in HF-fed mice (Table 1). In addition, ACE2 deficiency in bone marrow cells had no significant

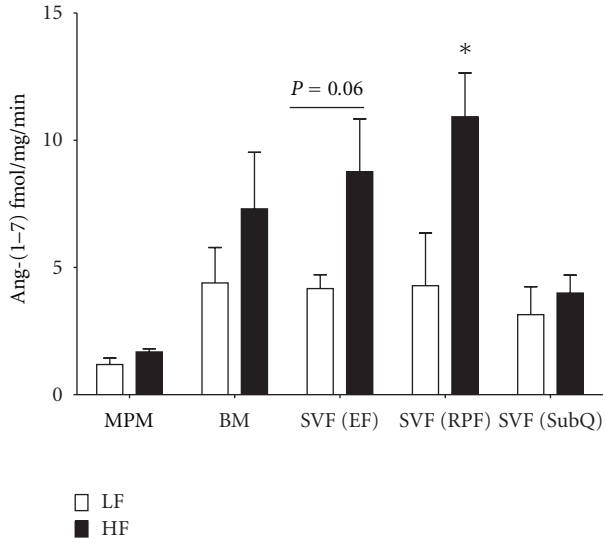

Figure 1: ACE2 activity is increased in the stromal vascular fraction (SVF) isolated from visceral adipose tissue of HF-fed C57BL/6 mice. ACE2 activity was quantified in mouse peritoneal macrophages (MPMs), bone marrow (BM), and SVF isolated from epididymal (EF), retroperitoneal (RPF) or subcutaneous (SubQ) adipose tissue from LF or HF-fed mice. Data are mean ± SEM from N = 3-4 mice/diet group. *: $P < 0.05$ compared to LF.

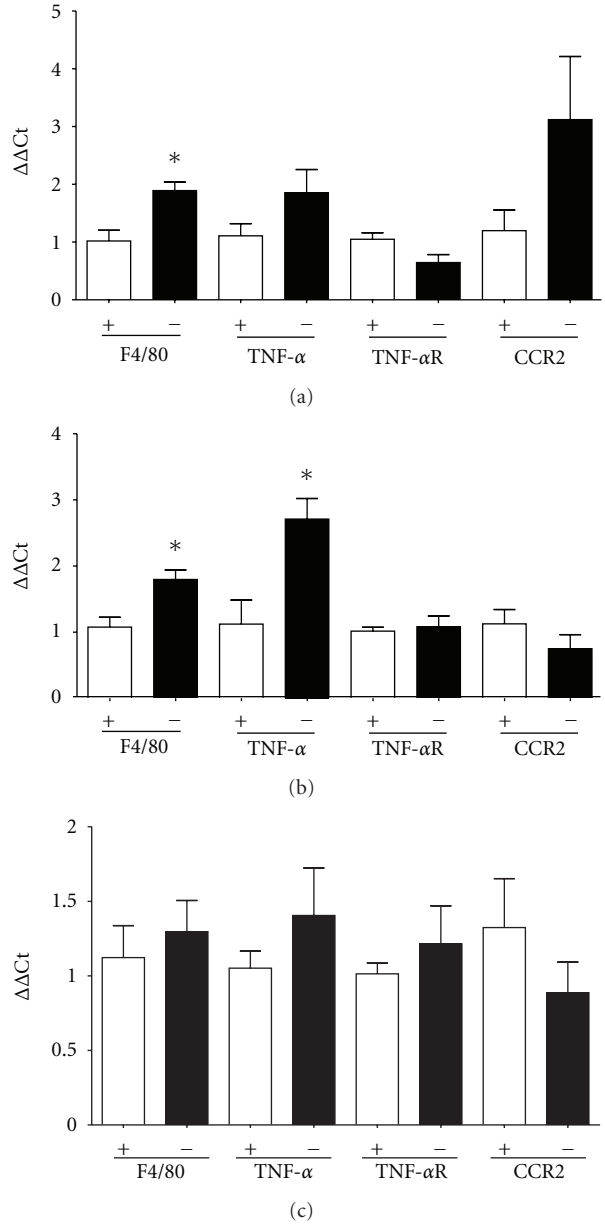

Figure 2: Bone marrow deficiency of ACE2 increases F4/80 and TNF-α mRNA abundance in the stromal vascular fraction (SVF) isolated from visceral adipose tissue of HF-fed C57BL/6 mice. Quantification of mRNA abundance in the SVF isolated from epididymal adipose (EF, a), retroperitoneal adipose (RPF, b), or subcutaneous fat (SubQ, c) of chimeric mice transplanted with $Ace2^{+/y}$ (depicted on the x-axis as +) or $^{-/y}$ (depicted on the x-axis as−) bone marrow. Data are mean ± SEM from N =3–11 mice/donor genotype. *: $P < 0.05$ compared to +.

effect on several plasma parameters measured in HF-fed mice (Table 1). Plasma renin ($P = 0.06$) or AngII concentrations ($P = 0.148$) were not statistically different between groups indicating that the systemic RAS was not influenced by the bone marrow repopulation. Bone marrow transplantation had no effect on WBC cell counts (WBC: $Ace2^{+/y}$, 3.9 ± 1; $Ace2^{-/y}$, 3.7 ± 0.2 K/μL). However, irradiated mice receiving donor marrow from each genotype exhibited a significant reduction in blood hemoglobin (normal range 11–15 g/dL), but there were no differences between genotypes ($Ace2^{+/y}$, 7.6 ± 0.6; $Ace2^{-/y}$, 8.7 ± 0.6 g/dL, $P = 0.22$).

To define effects of ACE2 deficiency in leukocytes on expression of M1 polarization markers in adipose tissue, we quantified mRNA abundance in the SVF isolated from EF (Figure 2(a)), RPF (Figure 2(b)), and SubQ (Figure 2(c)) of HF-fed mice transplanted with $Ace2^{+/y}$ or $^{-/y}$ bone marrow. Expression of the macrophage marker, F4/80, was significantly increased in the SVF isolated from EF and RPF from HF-fed mice transplanted with bone marrow from $Ace2^{-/y}$ compared to $^{+/y}$ mice (Figures 2(a) and 2(b); $P < 0.05$). In contrast, deficiency of ACE2 in leukocytes had no significant effect on F4/80 mRNA abundance in the SVF isolated from SubQ tissue of HF-fed chimeric mice (Figure 2(c)). In the SVF isolated from RPF of chimeric $Ace2^{-/y}$ mice, mRNA abundance of TNF-α was significantly increased compared to controls. However, other M1 markers (CCR2, EF, $P = 0.282$, TNFα receptor, Figures 2(a)–2(c); IL-6, IL-1β, CCL2, PAI-1, Mgl-1(data not shown)) did not exhibit significant differences in the SVF isolated from chimeric $Ace2^{-/y}$ compared to $^{+/y}$ mice. We also quantified M2 markers (IL-4, Ym-1, and Mrc-1) with no significant differences between genotypes (data not shown).

Chimeric mice transplanted with $Ace2^{-/y}$ bone marrow exhibited significantly increased blood glucose concentrations at 15, 160, and 220 minutes after a glucose challenge compared to mice transplanted with $Ace2^{+/y}$ bone marrow

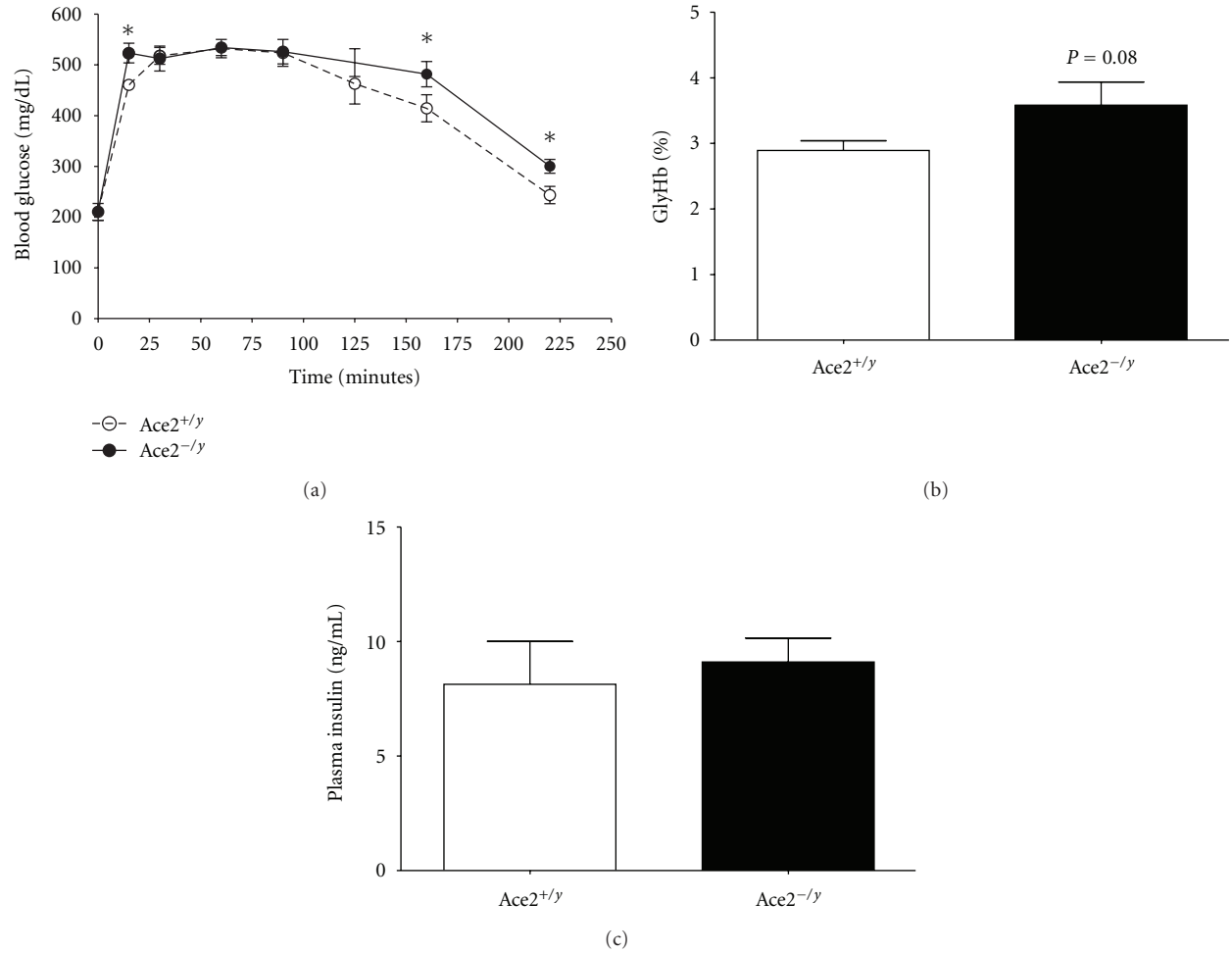

Figure 3: Bone marrow deficiency of ACE2 augments glucose intolerance at specific time points following glucose injection in HF-fed C57BL/6 mice. (a) Glucose tolerance tests (GTT) in HF-fed chimeric mice transplanted with bone marrow from *Ace2*$^{+/y}$ or $^{-/y}$ mice. (b) Percent glycosylated hemoglobin levels and plasma insulin concentrations (c) in *Ace2*$^{+/y}$ and $^{-/y}$ chimeric mice. Data are mean ± SEM from N =8–15 mice/donor genotype. *: $P < 0.05$ compared to *Ace2*$^{+/y}$.

(Figure 3(a); $P < 0.05$). In addition, blood levels of glycosylated hemoglobin were modestly, but not significantly, increased in plasma from *Ace2*$^{-/y}$ chimeric mice (*Ace2*$^{+/y}$, 2.9 ± 0.1; *Ace2*$^{-/y}$, 3.6 ± 0.4%, Figure 3(b); $P = 0.08$). In contrast, plasma concentrations of insulin were not significantly different between groups (*Ace2*$^{+/y}$, 8±2; *Ace2*$^{-/y}$, 9 ± 1 ng/mL, $P = 0.66$).

4. Discussion

Results from this study demonstrate that deficiency of ACE2 in leukocytes promotes inflammation in adipose tissue and results in a modest impairment of glucose tolerance in obese mice. Initial studies identified an increase in ACE2 activity in heterogeneous stromal cells isolated from visceral adipose tissue, but not in more purified populations of peritoneal or bone marrow macrophages from HF compared to LF-fed mice. Notably, deficiency of ACE2 in bone-marrow-derived cells increased F4/80 expression in the SVF of RPF tissue, suggesting increased infiltration of macrophages into adipose tissue of chimeric mice lacking ACE2 in leukocytes. Moreover, expression of TNF-α was increased in the SVF of RPF tissue from chimeric mice lacking ACE2 in bone marrow, supporting increased adipose inflammation from deficiency of ACE2. Finally, obesity-induced impairment of glucose homeostasis was modestly augmented in chimeric ACE2-deficient mice. These results suggest that deficiency of ACE2 in leukocytes promotes adipose tissue inflammation and augments the development of obesity-induced diabetes.

Initial studies examined effects of diet-induced obesity on ACE2 activity in macrophages isolated from the peritoneal cavity, bone marrow, or the stromal vascular fraction of adipose tissue from different regions. Of these different cell isolations, the SVF included several other cell types in addition to macrophages. Notably, ACE2 activity was increased by HF-feeding in the SVF isolated from visceral, but not subcutaneous adipose tissue. Deposition of excess adipose tissue in the visceral cavity has been linked to several obesity-associated diseases, including hypertension and diabetes [18, 19]. Mechanisms for increased risk from

visceral adipose accumulation are not clear, but may relate to increased metabolic activity of visceral adipose tissue [20]. Region-specific effects of obesity to increase visceral, but not subcutaneous ACE2 activity, are consistent with increased metabolic activity and a more pronounced impact of visceral adipose tissue on obesity-associated diabetes. However, since increased ACE2 activity would predictably lower local levels of AngII, these results suggest that activated ACE2 may serve as a compensatory protective mechanism. Alternatively, since F4/80 mRNA abundance was increased in visceral adipose tissue from HF-fed mice, increased ACE2 activity in the SVF may have resulted from increased macrophage infiltration to visceral adipose tissue of HF-fed mice. This finding is consistent with results from studies demonstrating that obesity is associated with more pronounced infiltration of macrophages in visceral compared to subcutaneous adipose tissue [21]. Moreover, this conclusion is supported by data demonstrating that ACE2 activity in more purified populations of macrophages, such as peritoneal macrophages or the bone marrow, was not increased by HF feeding. Thus, increased ACE2 activity in the stromal vascular fraction of HF-fed mice most likely arose from increased macrophage infiltration to adipose tissue.

Given findings of increased ACE2 activity in the SVF from visceral adipose tissue of HF-fed mice, we quantified effects of ACE2 deficiency in infiltrating leukocytes on adipose inflammation and glucose homeostasis using bone marrow transplantation. Bone marrow transplantation has been previously employed to define the source of macrophages infiltrating into adipose tissue with obesity [5] and by our laboratory to determine the role of leukocyte components of the RAS on developing atherosclerotic lesions [2, 14]. Our results do not support a role for leukocyte ACE2 in the development of obesity in HF-fed mice. In addition, consistent with previous studies, [2] systemic concentrations of cholesterol and/or fatty acids were not influenced by ACE2 deficiency in leukocytes. Moreover, deficiency of ACE2 in bone-marrow-derived stem cells had no effect on systemic concentrations of renin or AngII, similar to previous studies examining effects of renin deficiency in bone-marrow-derived cells on atherosclerosis in western diet-fed LDLR−/− mice [22].

A novel finding in this study was that ACE2 deficiency in bone-marrow-derived stem cells increased mRNA abundance of a macrophage marker (F4/80) and TNF-α in the SVF from visceral adipose tissue. As described above, increased mRNA abundance of F4/80 in the SVF from ACE2 chimeric mice supports increased infiltration of macrophages into adipose tissue. Increased infiltration of macrophages into adipose tissue was associated with elevated expression of TNF-α, a marker of M1 polarized macrophages. TNF-α has a well-defined role in the development of insulin resistance in both mice and humans [23–26]. Moreover, previous studies demonstrated that AngII increases secretion of TNF-α from RAW 264.7 macrophages [27] and that blockade of AT1 receptors in bone marrow stromal cells decreased TNF-α mRNA abundance [28]. Thus, it is conceivable that increased levels of AngII released from macrophages of ACE2-deficient mice [2] contributed to elevated mRNA abundance of TNF-α in the SVF. Alternatively, increased TNF-α mRNA abundance may have resulted from increased macrophage infiltration into adipose tissue of ACE2 chimeric mice. This possibility is unlikely since other markers of M1 macrophage polarization, such as CCR2, were not increased in the SVF from ACE2 chimeric mice.

It should be noted that effects of leukocyte ACE2 deficiency to promote F4/80 mRNA abundance in SVF of high fat-fed mice, while significant, were relatively modest (2-fold increase in F4/80 mRNA abundance in ACE2-deficient chimeras). Previous investigators have used bone marrow transplantation with creation of chimeric mice to examine effects of CCR2 deficiency in leukocytes on F4/80 expression in adipose tissue of high-fat-fed mice. Leukocyte deficiency of CCR2, the major receptor for the chemokine MCP-1, resulted in a 50% reduction in F4/80 expression in adipose tissue [29]. Thus, the magnitude of the effect of ACE2 deficiency to promote F4/80 mRNA abundance is consistent with other leukocyte chimeric manipulations.

To our knowledge, this is the first report that deficiency of leukocyte ACE2 can modulate the development of glucose intolerance in obese mice. Moreover, the magnitude of effect of ACE2 deficiency in bone-marrow-derived cells to promote glucose intolerance in HF mice was most likely underestimated in the present study due to the pronounced glucose intolerance present in irradiated 4 month HF-fed mice. Indeed, impaired glucose tolerance in chimeric mice lacking ACE2 in leukocytes was supported by increased blood concentrations of glycosylated hemoglobin, a more stable blood predictor of type 2 diabetes [30]. Since plasma insulin concentrations in the present study were not influenced by leukocyte ACE2 deficiency, then these results suggest that the degree of glucose intolerance in chimeric ACE2-deficient mice was not sufficient to further augment hyperinsulinemia in chronic 4 month HF-fed mice. Indeed, plasma insulin concentrations in the present study (>8 ng/mL) in 4 month HF-fed mice were greater than those observed in 2 month HF-fed mice (2 months, 5 ng/mL [31]). Importantly, glucose intolerance was slightly augmented in chimeric mice lacking ACE2 in leukocytes even though these mice exhibited a similar level of obesity compared to wild type controls. In comparison to other studies examining leukocyte deficiency of proteins known to regulate glucose homeostasis, previous studies demonstrated that deficiency of IL-10 [32], leptin [33], toll 4 receptors [34], or PAI-1 [35] had no effect on glucose tolerance in HF-fed mice. Thus, given negative results from leukocyte deficiency of these well-known regulators of glucose homeostasis, it is interesting that deficiency of ACE2 in leukocytes modestly impaired glucose homeostasis in obese mice.

A limitation of this study is that the relative role of AngII versus Ang-(1–7) in the effects of bone marrow ACE2 deficiency on adipose tissue inflammation and glucose homeostasis was not defined. Previous studies demonstrated that mas receptor deficiency in FVB/N mice resulted in marked changes in lipid profiles and glucose homeostasis [36]. In addition, chronic infusion of Ang-(1–7) to fructose-fed rats reduced fasting insulin levels and enhanced insulin signaling pathways (IRS-1/PI3K/Akt) in liver, skeletal

muscle, and adipose tissue [37]. An elegant study by Santos et al. utilized a transgenic Sprague-Dawley rat to overexpress Ang-(1–7) systemically and found that plasma triglyceride and cholesterol levels were decreased and whole body insulin sensitivity was enhanced [38]. This group also noted that Ang-(1–7) significantly increased adiponectin levels in rat adipocytes [38]. These results suggest that reductions in Ang-(1–7) may have contributed to the observed effects of leukocyte ACE2 deficiency. Alternatively, since AngII, but not Ang-(1–7), increased adhesion of monocytes to endothelial cells, [2] then AngII may have mediated effects of chimeric ACE2 deficiency. Further studies are needed to clarify the role of the AngII/Ang-(1–7) balance in effects of ACE2 deficiency on adipose inflammation and glucose homeostasis in obese mice.

5. Conclusions

In conclusion, deficiency of ACE2 in leukocytes modestly promoted inflammation in the stromal vascular fraction from visceral adipose tissue and augmented glucose intolerance in mice with diet-induced obesity. Future studies should address the role of ACE2 in inflammation of human adipose tissue and type 2 diabetes.

Acknowledgments

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Research Article

Renoprotective Effects of AVE0991, a Nonpeptide Mas Receptor Agonist, in Experimental Acute Renal Injury

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Renal ischemia and reperfusion (I/R) is the major cause of acute kidney injury in hospitalized patients. Mechanisms underlying reperfusion-associated injury include recruitment and activation of leukocytes and release of inflammatory mediators. In this study, we investigated the renal effects of acute administration of AVE0991, an agonist of Mas, the angiotensin-(1–7) receptor, the angiotensin-(1–7) receptor, in a murine model of renal I/R. Male C57BL/6 wild-type or $Mas^{-/-}$ mice were subjected to 30 min of bilateral ischemia and 24 h of reperfusion. Administration of AVE0991 promoted renoprotective effects, as seen by improvement of function, decreased tissue injury, prevention of local and remote leucocyte infiltration, and release of the chemokine, CXCL1. I/R injury was similar in WT and $Mas^{-/-}$ mice, suggesting that endogenous activation of this receptor does not control renal damage under baseline conditions. In conclusion, pharmacological interventions using Mas receptor agonists may represent a therapeutic opportunity for the treatment of renal I/R injury.

1. Introduction

Acute kidney injury (AKI) is defined as a rapid decrease of the glomerular filtration rate that occurs in minutes or days. Renal ischemia and reperfusion (I/R) is a major cause of AKI in hospitalized patients [1–3]. Renal I/R triggers an inflammatory cascade clearly involved in the pathogenesis of AKI [4–6]. The control of inflammatory responses emerges as a putative therapeutic target to halt AKI [7]. Many regulatory systems modulate inflammation in renal tissue [4]. Among these systems, the Renin Angiotensin System (RAS) seems to exert a pivotal action in this scenario [8]. RAS is now regarded as a dual system with two opposite arms: the classical one formed by angiotensin converting enzyme (ACE), Angiotensin II (Ang II), and receptor type 1 (AT_1) and the counter-regulatory arm comprising the enzyme ACE2, Angiotensin-(1–7) [Ang-(1–7)], and Mas receptor [9–13].

Besides opposing AT_1 receptor [14], the activation of Mas receptor by Ang-(1–7) or Ang-(1–7) agonists elicits renal effects [15–18]. Concerning experimental AKI, our team has recently shown that renal levels of Ang-(1–7) and ACE2 were significantly reduced whereas Mas receptor expression was increased [19]. However, our study was not able to clarify the precise function of the RAS at the early stages of renal I/R. In addition, data on the endogenous relevance of Mas receptor activation in renal tissue are still

controversial [20, 21]. While Pinheiro et al. [20] showed that the genetic deletion of Mas receptor in C57Bl/6 mice led to glomerular hyperfiltration, proteinuria, and renal fibrosis, Esteban et al. [21] reported, otherwise, that renal deficiency of Mas diminished renal damage in unilateral ureteral obstruction and in I/R. Infusion of Ang-(1–7) to wild-type mice appeared to worsen the inflammatory response [21].

More recently, we have shown that administration of Ang-(1–7) or the orally active agonist, the non-peptide compound AVE0991, exerted anti-inflammatory effects in experimental models of arthritis [19] and of nephritic syndrome [22]. Therefore, the purpose of the present study was to evaluate the renal effects of AVE0991 administration in a mice model of AKI, induced by renal ischemia following reperfusion.

2. Materials and Methods

2.1. Animals. Eight- to 10-week-old C57BL/6 wild-type male mice ($Mas^{+/+}$) weighing 20–25 g or mice with genetic deletion of Mas receptor ($Mas^{-/-}$) were obtained from the animal facility of the Universidade Federal de Minas Gerais. Animals were maintained under temperature-controlled conditions with an artificial 12-h light/dark cycle and were allowed standard chow and water ad libitum. The study was approved by the Ethics Committee of our Institution.

2.2. Renal Ischemia/Reperfusion Injury (I/R). Mice were anesthetized with ketamine and xylazine (150 mg/kg and 10 mg/kg, resp.). Abdominal incision was performed to expose both renal pedicles. Ischemia was induced by totally occluding the renal pedicle for 30 minutes, using microsurgical clamps. After inspection for signs of ischemia, the wound was covered with cotton soaked with sterile PBS. After 30 min, clamps were released and blood-flow was reestablished, as confirmed by visual inspection of the kidneys. The wound was sutured in two layers using 5.0 sutures (Procare, Brazil). Throughout the experiments, body temperature was kept at 36–38°C by placing mice on a heating pad. Sham-operated animals were used as controls in all experimental protocols. These animals were also anesthetized and subjected to abdominal incision, exposing, and superficial manipulation of the renal pedicles. After recovery from anesthesia, mice were accommodated in individual metabolic cages (Tecniplast, Italy) for evaluation of renal function parameters. Urine was collected for the first 24 h after which mice were killed. Samples of plasma, renal, and lung tissues were collected and stored at −20°C for posterior analysis.

2.3. Study Protocol. The first set of experiments was to evaluate the effect of AVE0991 treatment in renal I/R injury. Therefore, wild type ($Mas^{+/+}$) mice submitted to I/R were treated with subcutaneous (sc) injection (200 μL) of AVE0991 (9.0 mg/kg) (Aventis Pharma Deutschland, Frankfurt, Germany), AVE group; or vehicle (10 mM KOH in 0.9% NaCl), VE group, immediately following 30 minutes of the renal ischemia and 12 h after renal reperfusion (AVE group) or vehicle (VE group). As control group, sham-operated $Mas^{+/+}$ mice were also treated with vehicle (CT group).

The second set of the experiments had the aim to verify the endogenous role of Mas receptor in modulating experimental AKI. Thus, mice with genetic deletion of Mas receptor were submitted to the same protocol of renal I/R.

2.4. Determination of the MPO Activity. The extent of neutrophil accumulation in the kidney and lung tissue was measured by assaying myeloperoxidase (MPO) activity, as described previously [22]. Briefly, a portion of the kidney or lungs was removed and frozen in liquid nitrogen, homogenates were prepared in 1 mL of PBS containing 0.5% hexadecyltrimethyl ammonium bromide (HTAB) and 5 mM EDTA using a Dispomix tissue homogenizer (Medic Tools), and the protocol was followed as already described. Neutrophil number in each sample was calculated from a standard curve obtained from the peritoneal cavity after stimulation with 5% casein. The results were expressed as relative unit.

2.5. Differential Blood Cell Count. The total number of leukocytes was counted in a Neubauer chamber after staining with Turk's solution, and differential leukocyte counts were obtained after staining with May-Grunwald-Giemsa using standard morphologic criteria.

2.6. Assessment of CXCL1 in Serum. Levels of the chemokine CXCL1/KC were measured in serum using a commercial available enzyme-linked immunosorbent assay (ELISA) in accordance with the procedures supplied by the manufacturer (R&D Systems, Minneapolis, MN), as previously described by Souza et al. [14]. Results were expressed as picograms of cytokine per mL of serum.

2.7. Renal Function. To evaluate the effects of renal ischemia and reperfusion, as well as of the treatment with AVE0991 on renal physiology, several parameters were evaluated. First, mice were housed individually in metabolic cages (Tecniplast, Italy) three days before I/R procedure to adapt to the cages. I/R was then performed and animals were placed in the metabolic cages soon after recovering from anesthesia. Groups of animals were killed 24 h after reperfusion.

At the end, 24 h urine samples were collected and centrifuged at 3,000 g for 5 min. Urine was used to determine osmolality and creatinine concentrations. Blood samples were collected from the lower abdominal cava vein, under ketamine and xylazine anesthesia (150 mg/kg and 10 mg/kg, resp.), and centrifuged at 2,000 ×g for 15 min at 4°C. The resulting plasma was used to measure osmolality and creatinine concentrations. Samples of urine and plasma were stored at −20°C until assessments.

Creatinine concentrations were determined using an enzymatic kit (Bioclin/Quibasa, Belo Horizonte, MG, Brazil), and osmolality was determined by a freezing-point osmometer (microOsmetter, Calumet City, IL, USA). Osmolar clearance and free-water clearance were calculated. Finally, body weights were recorded daily.

Table 1: Effect of renal ischemia and reperfusion on renal function parameters, local CXCL1 production, and local and remote neutrophil accumulation. Urinary creatinine (mg/dL), serum creatinine (mg/dL), serum osmolality (mOsm/Kg H_2O), urinary osmolality (mOsm/Kg H_2O), serum CXCL1 (pl/mL serum), and renal and pulmonary neutrophils (relative units).

	Control	I/R	n	P
Urinary creatinine (mg/dL)	700 ± 5.1	170 ± 1.2*	6	0.001
Serum creatinine (mg/dL)	0.29 ± 0.1	5.34 ± 1.2*	4	0.016
Serum osmolality (mOsm/Kg H_2O)	317 ± 10.4	360 ± 17.8*	6	0.006
Urinary osmolality (mOsm/Kg H_2O)	3996 ± 340	905 ± 69*	6	0.06
Serum CXCL1/KC (pg/mL)	94 ± 70	940 ± 164*	6	0.008
Renal neutrophils (relative units)	0.26 ± 0.13	5.3 ± 1.2*	4	0.016
Lung neutrophils (relative Units)	2.2 ± 0.5	5.6 ± 0.5*	5	0.0015

2.8. Renal Histopathology. Paraffin-embedded sections (4 mm thick) were deparaffinized with xylene and rehydrated through a descending ethanol gradient. Histological sections were examined following H&E staining, and classified according to published standards [15, 16]. The degree of segmental glomerulosclerosis (glomerulosclerosis and nephron damage) was assessed by computer-aided image analysis of H&E-stained kidney sections. A semiquantitative score (glomerular and tubular injury index) was used to evaluate the degree of scarring as previously described [15, 16]. All scoring was performed in a blinded manner. The damage was scored semiquantitatively on a scale of 1 to 5.

2.9. Statistical Analysis. All results are presented as the mean ± SEM. Normalized data were analyzed by one-way ANOVA, and differences between groups were assessed using Student-Newman-Keuls post-test. The level of significance was set at $P < 0.05$.

3. Results and Discussion

It is well known that the classical RAS axis, composed of ACE/Ang II/AT_1 receptor, plays a role in mediating AKI [17, 18, 23]. Recently, studies have also demonstrated an important role of the counterregulatory RAS axis, ACE2/Ang-(1–7)/Mas receptor, in several renal disorders, including AKI [19, 21, 24–28]. However, there are inconsistencies on the role of Mas receptor activation in renal disorders [20, 21]. As example, Pinheiro et al. [20] showed that genetic deletion of Mas receptor in C57BL/6 mice led to glomerular hyperfiltration, proteinuria, and renal fibrosis. In contrast, Esteban et al. [21] reported that renal deficiency for Mas diminished renal damage in unilateral ureteral obstruction and ischemia/reperfusion injury and the infusion of Ang-(1–7) to wild-type mice elicited inflammatory response. Therefore, whether the actions of Ang-(1–7) on renal function do indeed counter act those of Ang II in the context of disease states remains to be shown [20, 21]. In the present study, we investigated the renal effects of acute administration of the Mas receptor agonist, AVE0991, in a murine model of AKI caused by reperfusion of ischemic kidneys. Our major findings can be summarized as follows: (1) treatment with AVE0991 improves renal function and attenuates renal tissue damage; (2) the compound reduces infiltration of leukocytes in the kidney and overall tissue inflammation. On the other hand, despite the effectiveness the agonist of Mas receptor, AVE0991, in protecting the kidney against I/R injury, the same degree of renal damage was obtained in $Mas^{-/-}$ mice submitted to experimental AKI. This finding suggests that there is no protective role for endogenous Mas activation.

3.1. Renal Function and Inflammatory Parameters. As displayed in Table 1, bilateral ischemia (30 minutes) followed by reperfusion (24 h) causes an acute decrease of glomerular filtration characterized by the accumulation of serum creatinine (Table 1). Serum and urinary osmolality were also altered by I/R injury. The osmolality was elevated in the serum and reduced in the urine of mice submitted to I/R in comparison to sham-operated animals. The increase in serum osmolality can be attributable to the failure of the injured kidney to excrete nitrogen waste products and other osmotic active molecules, while the reduction in urinary osmolality probably reflects the loss of urinary concentration ability by ischemic renal tubules. Taken together, these functional data confirm the acute and significant impairment in renal function as a result of I/R injury [1, 2].

I/R of the kidney is followed by a robust inflammatory reaction in the renal tubulointerstitium [29]. Studies in rodents have suggested that many components of the innate immune system contribute to renal injury after I/R, including neutrophils [30] and monocytes [31, 32]. Inflammatory cytokines and chemokines are generated in the ischemic kidney [30, 33] and likely orchestrate this broad inflammatory response. CXCL1/KC is a chemokine that promotes the recruitment and activation of inflammatory cells into renal tissue during I/R process [7]. In addition to renal damage, it was previously shown that extensive I/R, such as that of the renal or intestinal vascular beds, may be accompanied by remote organ (lung) and systemic inflammation [34, 35]. In the present study, we demonstrated an important increase in renal CXCL1/KC levels (Table 1). There was an increase in systemic and local (in renal tissue) accumulation of neutrophils, as detected by myeloperoxidade assay, which is in agreement with other studies [31, 35]. Pulskens et al. [36] and Roelofs et al. [37] found that neutrophils are the first inflammatory cells infiltrating the damaged kidney after reperfusion injury and there is much evidence suggesting that neutrophils are crucial for the development of reperfusion injury [37–39].

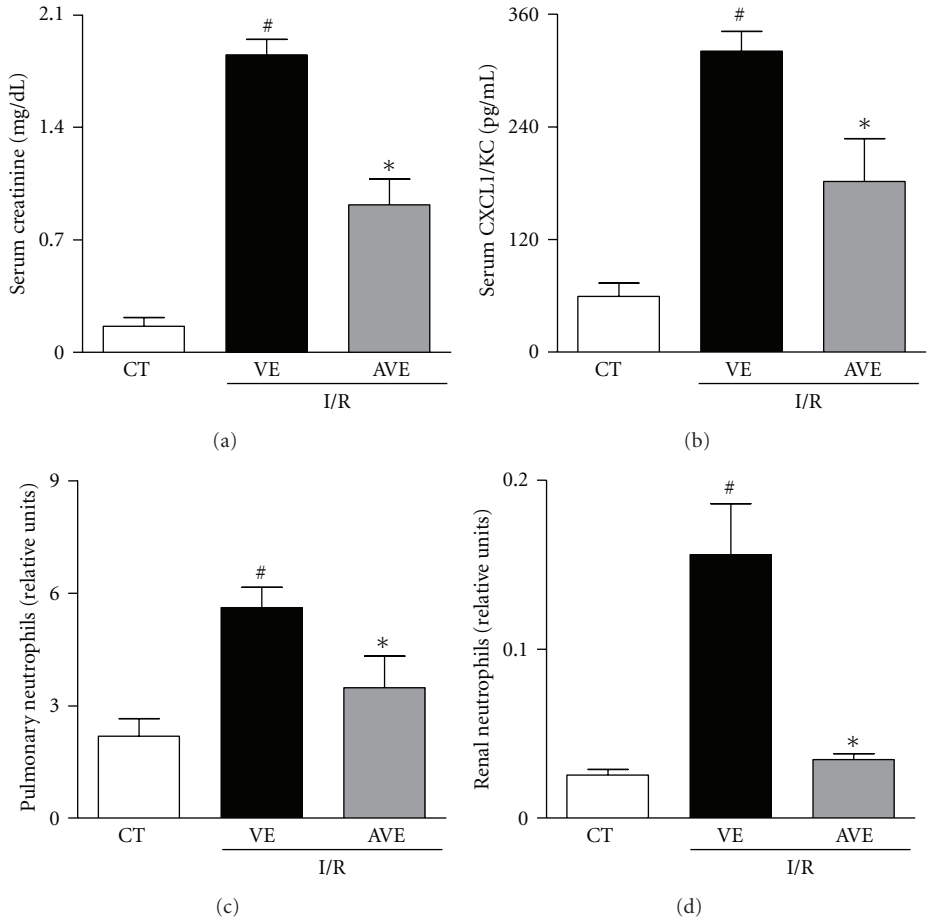

Figure 1: Effects of the treatment with AVE0991 in a model of renal ischemia and reperfusion in mice. AVE0991 (AVE, 9.0 mg/kg) or vehicle (VE; 10 mM KOH in 0.9% NaCl) was given immediately after ischemia and 12 h after reperfusion. All analyses were performed at 24 h after reperfusion. Serum creatinine (a), number of neutrophils in blood (b), levels of CXCL1/KC (c), and relative number of neutrophils in renal tissue (d) are shown as the mean ± SEM from six mice per group. $^{*}P < 0.05$ when compared with VE-treated animals; $^{\#}P < 0.05$ when compared with sham-operated animals (CT, control).

3.2. Effect of the Mas Receptor Agonist, AVE0991, in Renal I/R. Recent studies have evidenced a renoprotective and anti-inflammatory effect of ACE2/Ang-(1–7)/Mas receptor axis activation in the context of numerous disease models [26, 40]. These findings encouraged us to investigate the effect of an agonist of Mas receptor, AVE0991, in experimental AKI. The elevation of serum creatinine is one of the most important biomarkers of impaired glomerular filtration present in AKI [41]. We demonstrated that AVE0991 was able to attenuate the increase of serum creatinine, when compared to the vehicle-treated group (Figure 1(a)).

There was a marked infiltration of neutrophils in renal and pulmonary tissues after I/R injury (Figures 1(b)-1(c)). Treatment with AVE0991 significantly decreased neutrophil influx in both the kidney and lungs (Figures 1(b) and 1(c)). Decrease in neutrophil accumulation in both organs was associated with reduced production of CXCL1/KC, as seen by lower circulating levels of this chemokine in AVE-treated animals than in vehicle group (Figure 1(d)). Systemic and remote organ inflammation frequently accompanies severe I/R injury [34, 42] and may contribute to the fatal outcome observed in reperfused mice. The lung is the most commonly affected remote organ during systemic inflammation [34, 35, 43, 44]. Therefore, protection against systemic inflammation may have accounted for the overall beneficial effects of AVE0991 treatment. We have recently shown that Mas receptor activation decreased neutrophil migration and accumulation in models of arthritis [45], suggesting the overall capacity of Mas receptor activation in controlling the influx of these cells during acute inflammatory response.

As an attempt to quantify the protective action of AVE0991 at the level of the renal tissue, the degree of tubular and glomerular injuries was evaluated in mice subjected to I/R. As expected, sham-operated animals (control group) had well-preserved glomerular and tubular architecture (Figures 2(a)-2(b)). Thirty minutes of ischemia followed by 24 h of reperfusion caused focal acute tubular necrosis, intense tubular vacuolization in proximal tubules (asterisks), cast formation (arrows), and tubular dilatation as observed in vehicle-treated animals submitted to I/R (Figures 2(c)-2(d)). On the other hand, the administration of AVE0991 improved renal damage in animals submitted to I/R (Figures 2(e)-2(f)).

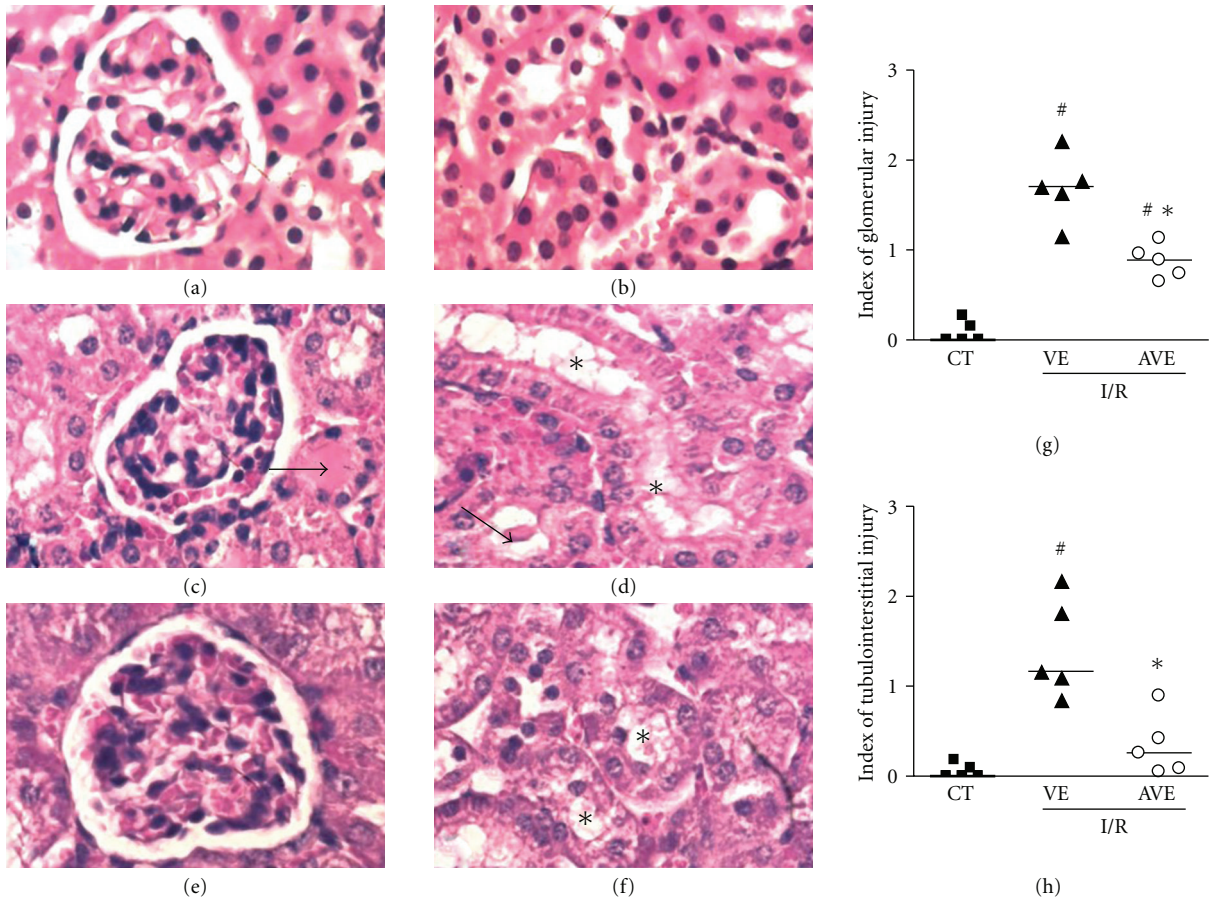

Figure 2: Effects of the treatment with AVE0991 in histological injury in a model of renal ischemia and reperfusion in mice. AVE0991 (AVE, 9.0 mg/kg) or vehicle (VE; 10 mM KOH in 0.9% NaCl) was given immediately after ischemia and 12 h after reperfusion. All analyses were performed at 24 h after reperfusion. Representative photomicrographs show H&E-stained sections from sham-operated animals (CT, a, b) and animals subjected to I/R, which were treated with vehicle (VE, c, d) and AVE0991 (AVE, 9 mg/kg, e, f). There was severe renal damage with vacuolization of tubular epithelium (c) (insert) and tubular dilation and protein casts (arrow) and extensive tubular necrosis (d) in mice subjected to I/R. Original magnification: ×600. Index of glomerular injury (g) and index of tubulointerstitial injury (h) were graded in a blind manner, as described in Material and Methods. Symbols represent results in single animals, and the trace is median value for all animals. $^{*}P < 0.05$ when compared with VE-treated group; $^{\#}P < 0.05$ when compared with CT.

The renal tissue of AVE-treated animals had a significant decrease in the indexes of renal injury at both glomerular and tubular sites when compared to vehicle group (Figure 2(h)).

Studies evaluating the effect of Ang-(1–7) or AVE0991 in models of chronic kidney disease have shown that these molecules might be renoprotective [25, 26]. In this regard, Zhang et al. showed that Ang-(1–7) infusion ameliorates glomerular sclerosis in experimental glomerulonephritis [24]. In addition, the beneficial effect of ACE2 overexpression in experimental diabetic nephropathy may also be due to the increase of Ang-(1–7) levels [24]. These studies concur with our findings that the agonist of Mas receptor, AVE0991, attenuates renal damage in this model of I/R injury. Based on our previous results with experimental arthritis [45], we believe that AVE0991 exerted renoprotective actions mainly through Mas receptor activation. To corroborate this idea, our group has previous shown that the mRNA expression for Mas receptor is increased in I/R model [19]. However, we cannot rule out the possibility that other mechanisms could contribute to the beneficial effects of AVE0991. In this regard, AVE0991 could interact with ACE by inhibiting the renal activity of this enzyme.

To verify the endogenous role of Mas receptor activation in the pathogenesis of renal I/R, we induced AKI in mice with genetic deletion of this receptor ($Mas^{-/-}$). As demonstrated in Figure 3, the absence of Mas receptor did not change the degree of renal function impairment, as assessed by serum creatinine concentration (Figure 3(a)). Moreover, there was no change in inflammatory response, as measured by the number of systemic and renal neutrophils (Figures 3(b)-3(c)). The increase of Mas receptor could represent more a compensatory response to renal damage rather than an endogenous regulatory mechanism. In this regard, we have previously obtained the same finding in a model of arthritis induced by adjuvant [45]; that is, the phenotype of Mas-deficient mice was not major when compared to pharmacological administration of AVE0991, which were able to efficiently control articular inflammation through Mas

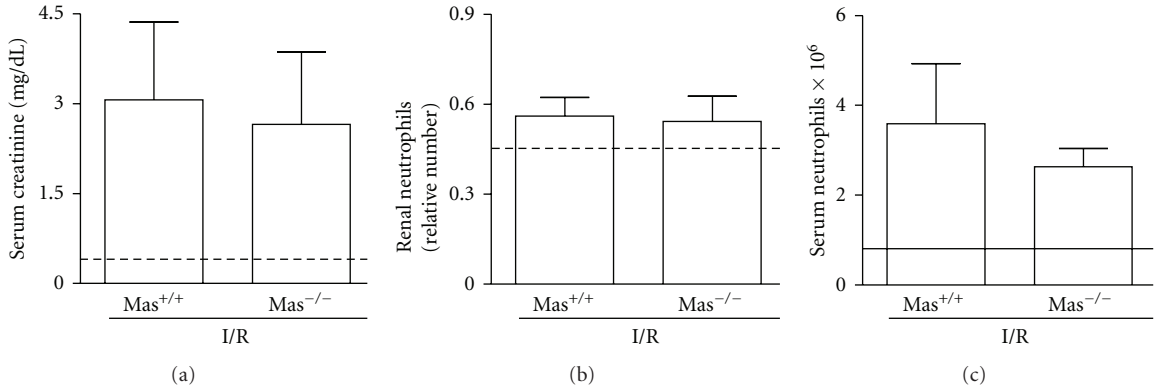

Figure 3: Ischemia and reperfusion in mice with genetic deletion for the Ang-(1–7) Mas receptor ($Mas^{-/-}$) or WT mice ($Mas^{+/+}$). Animals were subjected to 30 min of ischemia followed by 24 h of reperfusion. Serum creatinine (a), relative number of neutrophils in renal tissue (b), and number of neutrophils in blood (c) are shown as the mean ± SEM of six mice per group.

receptor activation. Similarly, endogenous activation of Mas receptor does not appear to exert a major role in I/R model.

The present results are in contrast to those of Esteban et al. [21], which showed less intense reperfusion injury in $Mas^{-/-}$ mice. These data are difficult to reconcile. However, there are substantial differences between the experimental protocols for producing I/R model and for the Mas receptor agonist administration such as which agonist was administered, AVE0991 or Ang-(1–7), the via of administration, and the dose used. For example, in the present study, we have used a model of bilateral I/R (30 min), while Esteban et al. performed unilateral I/R (25 min) with contralateral nephrectomy [21] that could at least in part account for the differences observed. Renal effects of Mas receptor agonists, Ang-(1–7) and AVE0991, appear to be importantly influenced by experimental conditions and the level of RAS activation. Despite these inconsistencies, our results corroborate emerging data showing that the major overall effects of ACE2/Ang-(1–7)/Mas receptor axis stimulation are anti-inflammatory [19, 21, 24–28]. As such, one would expect that absence of this pathway would be associated with no phenotype, as observed here, or even worsening of the inflammatory response [37].

In conclusion, treatment with AVE0991, a nonpeptide Mas agonist, attenuated renal functional impairment, decreased the local and systemic inflammatory responses, and reduced glomerular and tubulointerstitial damage in a murine model of AKI induced by bilateral I/R injury. These results show that activation of Mas receptor is renoprotective, at least in part by anti-inflammatory actions, in mice subjected to AKI, a tenet that clearly deserves further investigation in the context of human disease.

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Review Article

New Cardiovascular and Pulmonary Therapeutic Strategies Based on the Angiotensin-Converting Enzyme 2/Angiotensin-(1–7)/Mas Receptor Axis

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Angiotensin (Ang)-(1–7) is now recognized as a biologically active component of the renin-angiotensin system (RAS). The discovery of the angiotensin-converting enzyme homologue ACE2 revealed important metabolic pathways involved in the Ang-(1–7) synthesis. This enzyme can form Ang-(1–7) from Ang II or less efficiently through hydrolysis of Ang I to Ang-(1–9) with subsequent Ang-(1–7) formation. Additionally, it is well established that the G protein-coupled receptor Mas is a functional ligand site for Ang-(1–7). The axis formed by ACE2/Ang-(1–7)/Mas represents an endogenous counter regulatory pathway within the RAS whose actions are opposite to the vasoconstrictor/proliferative arm of the RAS constituted by ACE/Ang II/AT_1 receptor. In this review we will discuss recent findings concerning the biological role of the ACE2/Ang-(1–7)/Mas arm in the cardiovascular and pulmonary system. Also, we will highlight the initiatives to develop potential therapeutic strategies based on this axis.

1. Introduction

The renin-angiotensin system (RAS) plays a key role in several target organs, such as heart, blood vessels, and lungs, exerting a powerful control in the maintenance of the homeostasis [1–4]. This system is activated by the conversion of the angiotensinogen to the inactive peptide angiotensin (Ang) I through the renin action [5]. Subsequently, Ang I is cleaved by the angiotensin-converting enzyme (ACE) generating Ang II [6], the main angiotensin peptide, whose actions are mediated by two G protein-coupled receptors (GPCR), AT_1 and AT_2 [7, 8] (Figure 1). The major physiological functions of Ang II are mediated by AT_1 receptor [9, 10]. In pathological conditions, activation of this receptor induces deleterious effects, such as vasoconstriction, fibrosis, cellular growth and migration, and fluid retention [11, 12]. On the other hand, Ang II binding to the AT_2 receptor generally causes opposite effects when compared with those actions mediated by the AT_1 receptor [13, 14].

Recently, it has been proposed that, in addition to the ACE/Ang II/AT_1 receptor axis, the RAS possesses a counter regulatory axis composed by ACE2, Ang-(1–7), and Mas receptor (Figure 1). Ang-(1–7) is a biologically active component of the RAS which binds to Mas inducing many beneficial actions, such as vasodilatation, antifibrosis, and antihypertrophic and antiproliferative effects [15–23]. This peptide is produced mainly through the action of ACE2, which has approximately 400-fold less affinity to Ang I than to Ang II [24–26]; thereby, Ang II is the major substrate for Ang-(1–7) synthesis. In fact, the conversion of Ang II to

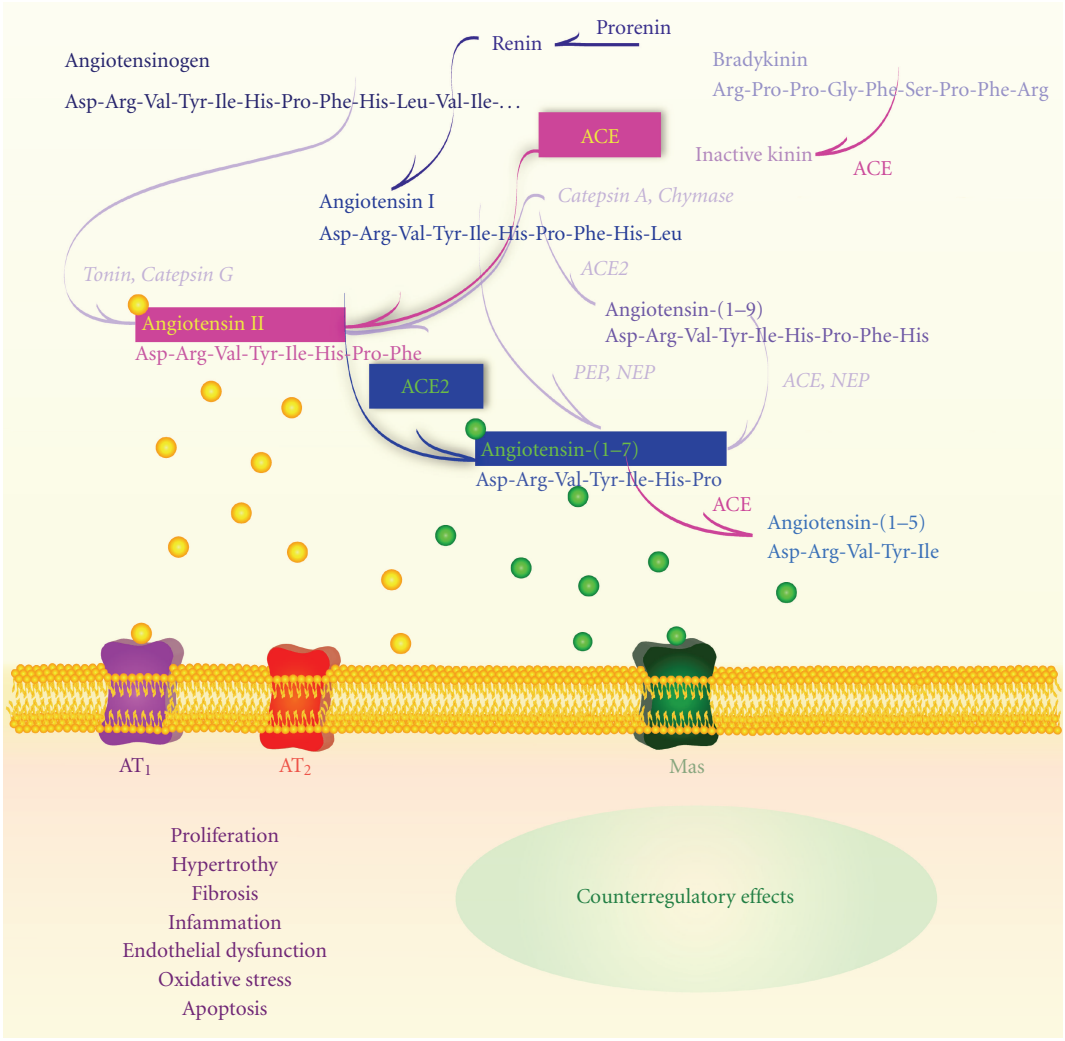

Figure 1: Schematic representation of the renin-angiotensin system (RAS) cascade. The counterregulatory axes of the RAS are composed by ACE/Ang II/AT_1 and ACE2/Ang-(1–7)/Mas. ACE: angiotensin-converting enzyme; Ang: angiotensin; AT_1: Ang II type 1 receptor; AT_2: Ang II type 2 receptor; Mas: Ang-(1–7) receptor; PCP: prolylcarboxypeptidase; PEP: prolyl-endopeptidase; NEP: neutral-endopeptidase 24.11.

Ang-(1–7) by ACE2 is important to regulate the RAS activity since Ang-(1–7) induces opposite effects to those elicited by Ang II [16–24]. Additionally, ACE2 can form Ang-(1–7) less efficiently through hydrolysis of Ang I to Ang-(1–9) with subsequent Ang-(1–7) formation [24].

The relevance of the RAS is highlighted by the success obtained in therapeutic strategies based on the pharmacological inhibition of this system in cardiovascular and respiratory diseases [27–32]. Blockade of the RAS with ACE inhibitors (ACEi) or AT_1 receptor antagonists (ARBs) improves the outcomes of patients with hypertension, acute myocardial infarction, and chronic systolic heart failure [33–35]. Furthermore, based on the involvement of the ACE/Ang II/AT_1 axis in respiratory diseases and the crucial role of the lungs in the RAS metabolism, several studies have reported the contribution of the RAS in lung pathophysiology [28, 30, 31, 36–40]. Importantly, it has been shown that administration of ACEi and ARBs causes substantial increases in plasma Ang-(1–7) levels, leading to the assumption that part of their clinical effects might be mediated by this heptapeptide [41–43]. Indeed, some effects of ACEi and ARBs can be blocked or attenuated by A-779, a Mas antagonist, confirming the role of Ang-(1–7) in the actions of these compounds [44]. The beneficial effects of Ang-(1–7), as well as its likely participation in the effects of the ACEi and ARBs, represent evidences for the potential of the ACE2/Ang-(1-7)/Mas axis as a therapeutic target.

In this review, we will focus on the recent findings related to the pathophysiology actions of the ACE2/Ang-(1–7)/Mas axis in the cardiovascular and respiratory system. Also, we will discuss the promising initiatives to develop new therapeutic strategies based on this axis to treat pathological conditions.

2. Cardiac ACE2/Ang-(1–7)/Mas Axis

The heart is one of the most important targets for the actions of the ACE2/Ang-(1–7)/Mas axis. In the heart, ACE2

is expressed in the endothelium [45], myofibroblasts [46], cardiomyocytes, and fibroblasts [47, 48]. Classical pharmacotherapeutic agents used to treat heart failure, including ACEi, ARBs, and aldosterone receptor blockers, increase ACE2 activity and/or expression, indicating its importance in the cardiac diseases establishment and progression [49–51].

Additionally, pharmacological and genetic (transgenic animals and gene transfer) approaches have evidenced the significance of ACE2 in cardiac pathologies. Despite some controversies concerning the consequences of the ACE2 deficiency, in general, evidences indicate a protective role of ACE2 in the heart [48, 52–57]. Crackower and colleagues [52] were the first to demonstrate that genetic ablation of ACE2 results in severe blood-pressure-independent systolic impairment. Also, disruption of ACE2 was able to accelerate cardiac hypertrophy and shortened the transition period to heart failure in response to pressure overload by increasing local Ang II [54]. Recently, it has been demonstrated that loss of ACE2 enhances the susceptibility to myocardial infarction, with increased mortality, infarct expansion and adverse ventricular remodeling [56]. In keeping with these genetic findings, pharmacological inhibition of ACE2 exacerbated cardiac hypertrophy and fibrosis in Ren-2 hypertensive rats [58]. On the other hand, cardiac overexpression of ACE2 prevented hypertension-induced cardiac hypertrophy and fibrosis in spontaneously hypertensive rats (SHR) and in Ang-II-infused rats [59, 60]. Indeed, transfection of Lenti-ACE2 (lentivirus containing ACE2 cDNA) or Ad-ACE2 (recombinant adenovirus carrying the murine ACE2) into the surrounding area of the infarcted myocardium was protective against pathological remodeling and cardiac systolic dysfunction in a rat model of myocardial infarction [61, 62]. This effect was associated with decreased expression of ACE and Ang II and increased expression of Ang-(1–7) [62]. Collectively, these observations reveal that ACE2 effectively plays a protective role in the cardiac structure and function.

Since the discovery of Ang-(1–7) in the late 1980s [63, 64], several studies have demonstrated important effects of this peptide in hearts. The presence of Ang-(1–7) and its receptor Mas in the heart [65, 66] and the ability of this organ to produce Ang-(1–7) [55, 67] are evidences of the role of this peptide in cardiac tissues. Functionally, Ang-(1–7) induces an antiarrhythmogenic effect against ischemia/reperfusion injuries in rats [17, 68] as well as prevents atrial tachycardia and fibrillation in rats and dogs [69, 70]. Treatment with Ang-(1–7) improved the coronary perfusion and cardiac function in rats after myocardial infarction [71] and after ischemia/reperfusion injury [72]. Increases in circulating Ang-(1–7) levels in transgenic rats reduced the cardiac hypertrophy [17] and fibrosis [20, 22] induced by isoproterenol administration. These effects are apparently independent of changes in blood pressure since Grobe and colleagues [18] have demonstrated that the antifibrotic and antihypertrophic actions of Ang-(1–7) are still observed in Ang-II-infused hypertensive rats. Local overexpression of Ang-(1–7) in hearts of mice and rats improved the myocardial contractility and prevented the isoproterenol- and hypertension-induced cardiac remodeling [19, 21]. Altogether, these findings support a direct effect of Ang-(1-7) in the heart.

Further evidence for the role of Ang-(1–7)/Mas in the pathophysiology of the heart came from experimental protocols utilizing mice with genetic deficiency of Mas. They revealed that the cardiac function is impaired in Mas knockout mice likely due to the increased extracellular matrix proteins deposition in the heart [66, 73]. This profibrotic phenotype may be related to changes in matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) levels and/or activities [74, 75].

Although further elucidations regarding the signaling pathways involved in Mas activation are necessary, some mechanisms have been proposed. Overexpression of Ang-(1–7) in hearts of rats causes an improvement in the $[Ca^{2+}]$ handling in cardiomyocytes and increases the expression of SERCA2a [21]. In keeping with these results, cardiomyocytes from Mas-deficient mice present slower $[Ca^{2+}]_i$ transients accompanied by a lower Ca^{2+} ATPase expression in the sarcoplasmic reticulum [66, 76]. Although acute Ang-(1–7) treatment failed to alter Ca^{2+} handling in ventricular myocytes of rats [76], these findings suggest an important role of the Ang-(1–7)/Mas in the long-term maintenance of the Ca^{2+} homeostasis in the heart.

One of the mechanisms by which Ang-(1–7) plays its effects in the heart is stimulating the nitric oxide (NO) production. Indeed, it has been demonstrated that Ang-(1–7) via Mas increases the synthesis of NO through a mechanism involving the activation of the endothelial NO synthase (eNOS). These effects were abolished by A-779 and are absent in cardiomyocytes from Mas-deficient mice [76]. Recently, Gomes et al. [77] found that the treatment of isolated cardiomyocytes of rats with Ang-(1–7) efficiently prevents the Ang-II-induced hypertrophy by modulating the calcineurin/NFAT signaling cascade. These effects were blocked by NO synthase inhibition and by guanylyl cyclase inhibitors, indicating that these effects are mediated by the NO/cGMP pathway.

Also, Ang-(1–7) inhibits serum-stimulated mitogen-activated protein kinase (MAPK) activation in cardiac myocytes [78] and prevents the Ang-II-mediated phosphorylation of ERK1/2 and Rho kinase in hearts in a dose-dependent manner [79]. In line with these data, activation of endogenous ACE2 significantly reduced the phosphorylation of ERK1/2 in hearts of hypertensive rats (SHRs) [48]. However, Mercure et al. [19] reported that overexpression of Ang-(1–7) in hearts of rats decreases the Ang-II-induced phosphorylation of c-Src and p38 kinase, whereas the increase in ERK1/2 phosphorylation was unaffected by the expression of the transgene, thereby suggesting a selective effect of Ang-(1–7) on intracellular signaling pathways related to cardiac remodeling.

Overall, these data reveal a key role of the ACE2/Ang-(1–7)/Mas axis in the pathophysiology of the cardiac structure and function. Activation of this axis might be an important strategy to develop a new generation of cardiovascular therapeutic agents against cardiac dysfunction and pathological remodeling of the heart.

3. Vascular ACE2/Ang-(1–7)/Mas Axis

Early studies have reported the endothelium as the major site for generation [67] and metabolism [41] of Ang-(1–7). In addition to Ang-(1–7), endothelial cells also express ACE2 and Mas [80, 81]. Thus, now it is recognized that the ACE2/Ang-(1–7)/Mas axis is present in vascular endothelial cells and modulates its function promoting vasorelaxation [82], reduction of the oxidative stress [83, 84], and antiproliferative effects [85, 86].

The vasodilatory actions of Ang-(1–7) have been reported in many studies in several vascular beds and preparations, including mouse [16, 23] and rat [15] aortic rings, canine [87] and porcine [88] coronary arteries, canine middle cerebral artery [89], porcine piglet pial arterioles [90], feline mesenteric vascular bed [91], rabbit renal afferent arterioles [92], and mesenteric microvessels of normotensive [93] and hypertensive [94] rats. Vascular Ang-(1–7) actions are still controversial in human. For example, it has been shown that Ang-(1–7) causes vasodilation in forearm circulation of normotensive subjects and patients with essential hypertension [95] while other studies were unable to report any significant effect of Ang-(1–7) in the same vascular territory in ACEi-treated patients [43].

The Mas receptor is critically involved in the vascular effects of Ang-(1–7). In fact, many of these actions are completely abolished by A-779 or partially blocked by this antagonist [3, 86, 96]. Importantly, the endothelium-dependent relaxation induced by Ang-(1–7) in mouse aortic rings is absent in vessels derived from Mas-knockout mice [16]. However, other studies have shown that Ang-(1–7) also interacts with ACE, AT_1, and AT_2-like receptors, suggesting the existence of additional sites of interaction for Ang-(1–7) [3, 97, 98]. Indeed, Silva et al. [99] reported evidence for the presence of a distinct subtype of Ang-(1–7) receptor sensible to D-pro^7-Ang-(1–7), a second Mas antagonist, but not to A-779 in aortas of Sprague-Dawley rats.

The vascular effects of Ang-(1–7) are endothelium dependent and involve the production of vasodilator products, such as prostanoids, NO, and endothelium-derived hyperpolarizing factor (EDHF) [16, 81, 100]. Pinheiro and coworkers [101] found that Ang-(1–7) promotes an increase in NO release in Mas-transfected chinese hamster ovary (CHO) cells [101]. Furthermore, short-term infusion of Ang-(1–7) improved the endothelial function by a mechanism involving NO release in rats [102]. Mas deletion resulted in endothelial dysfunction associated with an unbalance between NO and oxidative stress [83]. Also, Mas activation by Ang-(1–7) in human endothelial cells stimulated eNOS phosphorylation/activation via the Akt-dependent pathway [81]. Other mechanisms appear to be involved in the Ang-(1–7) vascular actions. Roks et al. [103] have shown that Ang-(1–7) inhibits the vasoconstriction induced by Ang II in human internal mammary arteries, thereby suggesting that Ang-(1–7) can regulate the Ang II effects [103]. In fact, Ang-(1–7) negatively modulates the Ang II type 1 receptor-mediated activation of c-Src, and its downstream targets ERK1/2 and NAD(P)H oxidase [104]. The counterregulatory action of Ang-(1–7) on Ang II signaling has been also observed in cardiomyocytes [77], vascular smooth muscle cells [105], and fibroblasts [106]. Additionally, an interaction between Mas and bradykinin (Bk) type 2 (B_2) receptors may modulate some of the Ang-(1–7) effects in blood vessels [107]. Indeed, it has been demonstrated that Ang-(1–7) potentiates the vasodilator and hypotensive effects of Bk in several vascular beds [93, 108–110].

As the major enzyme involved in Ang-(1–7) formation, ACE2 has also a crucial role in vessels. Lovren et al. [111] have demonstrated that ACE2 ameliorates the endothelial homeostasis via a mechanism involving reduction of the reactive oxygen species production [111]. Of note, this effect was attenuated by A-779 [111]. Moreover, overexpression of ACE2 in vessels of hypertensive rats resulted in reduction in the arterial blood pressure and improvement of the endothelial function associated with increased circulating Ang-(1–7) levels [112]. Overall, these data indicate that the beneficial effects of ACE2 are, at least in part, mediated by Ang-(1–7). Recently, we have demonstrated that activation of endogenous ACE2 causes a dose-dependent hypotensive effect in normotensive and hypertensive rats [113]. Also, the response to Bk administration was augmented in rats chronically treated with XNT, an ACE2 activator [113]. However, we were unable to demonstrate any significant effect of XNT on blood pressure in response to the administration of Ang II or Losartan in normotensive and hypertensive rats (Figure 2).

4. Pulmonary ACE2/Ang-(1–7)/Mas Axis

In the past few years, the participation of the ACE2/Ang-(1–7)/Mas axis in the establishment and progression of pulmonary diseases has become evident. Indeed, the important role of the RAS in the lung pathophysiology and the side effects and pulmonary toxicity induced by the ACEi raised the interest to evaluate the activation of the ACE2/Ang-(1–7)/Mas axis as an alternative target to treat pulmonary pathologies. Thus, it has been reported beneficial outcomes induced by the activation of this axis in animal models of acute respiratory distress syndrome (ARDS), pulmonary hypertension (PH), fibrosis, and lung cancer [31, 37, 114–117]. These studies pointed out that the imbalance between the ACE/Ang II/AT_1 and the ACE2/Ang-(1–7)/Mas axes of the RAS might be relevant in lung diseases. Taking into account that systemic hypotension is an important limitation to the use of ACEi and ARBs in pulmonary patients, therapies based on the ACE2/Ang-(1–7)/Mas axis emerge as a safe and efficient approach since studies using the ACE2 activator XNT or ACE2 gene transfer have shown that these strategies induce beneficial pulmonary outcome without changes in systemic blood pressure in rats and mice [39, 117, 118].

Imai and colleagues [37] demonstrated the role of ACE2 in ARDS pathogenesis. They found that a more severe ARDS was reached in ACE2 knockout mice, and this phenotype was reversed by double genetic deletion of the ACE2 and ACE genes or by the treatment with recombinant human ACE2 (rhACE2). Furthermore, Ang II levels were related

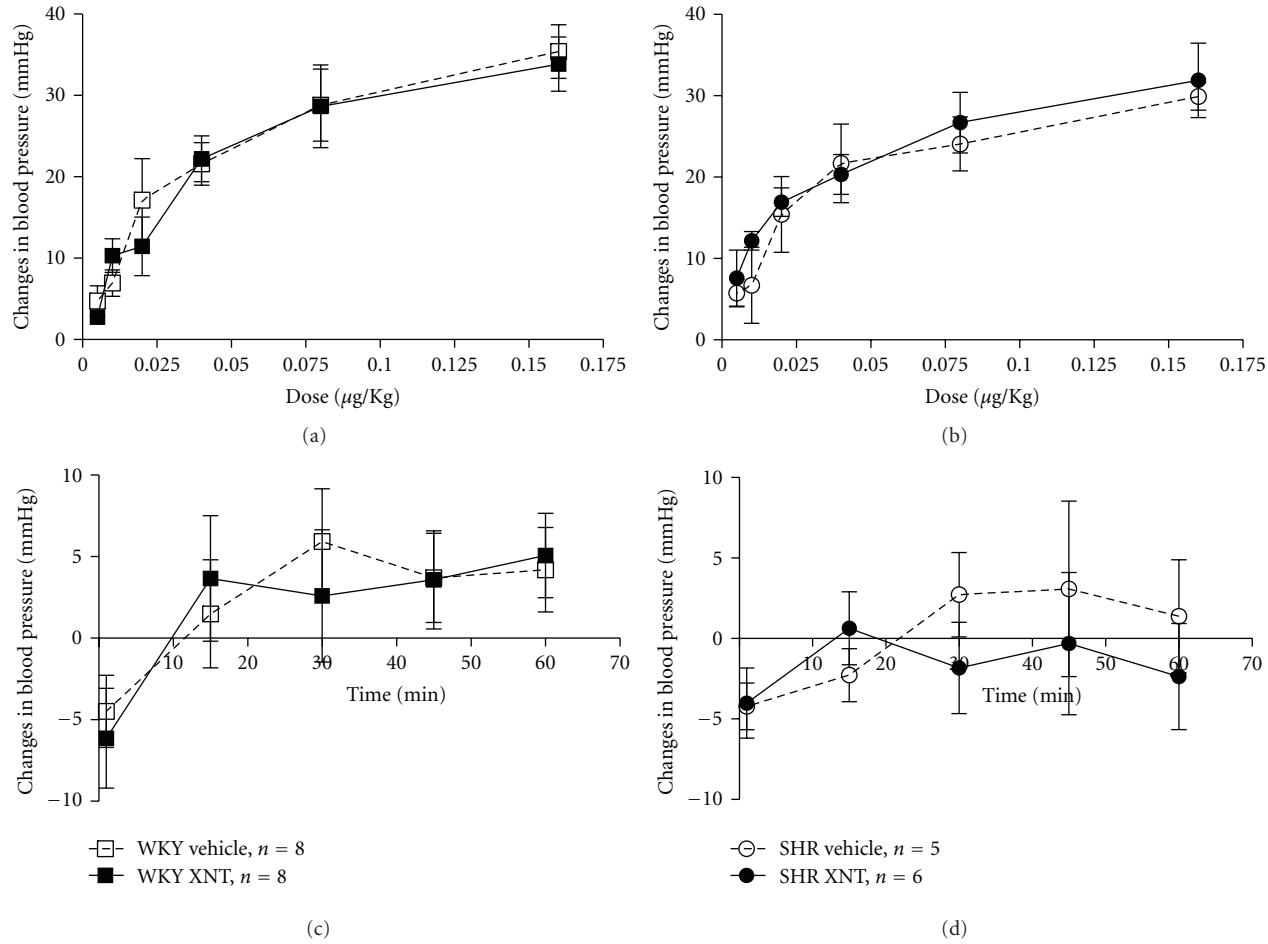

Figure 2: Effects of Ang II and Losartan on arterial blood pressure of rats chronically treated with XNT. The responses to increasing doses of Ang II were similar in vehicle- and XNT-treated (a) normotensive (Wistar-Kyoto rats—WKY) and (b) hypertensive (spontaneously hypertensive rats—SHR) rats. Likewise, the response to Losartan (0.25 mg/kg) was similar in vehicle- and XNT-treated (c) normotensive (WKY) and (d) hypertensive (SHRs) rats. The blood pressure was measured through a catheter inserted into the carotid artery and Ang II and Losartan were administrated *in bolus* using the jugular vein.

to the severity of the lung injury. Of note, ACE2 is widely expressed in the pulmonary endothelium, vasculature, and pneumocytes [119, 120]. Also, rhACE2 inhibited the increase of Ang II and TNF-α levels, attenuated the arterial hypoxemia and PH, and ameliorated the distribution of the pulmonary blood flow in lipopolysaccharide-induced lung injury in piglets [121]. Therefore, these studies suggest that ACE2 is a suitable target to arrest the development of ARDS in patients at risk.

The stimulation of the ACE2/Ang-(1–7)/Mas axis has been successful used to prevent and reverse PH and fibrosis in animals. ACE2 activation using the compound XNT or induction of ACE2 overexpression by gene transfer efficiently prevented and, more importantly, reversed the increase of the right systolic ventricular pressure (RSVP), pulmonary fibrosis, imbalance of the RAS, and inflammation in animals (rats and mice) with PH induced by monocrotaline (MCT) or in rats with pulmonary fibrosis caused by bleomycin treatment [39, 117, 118]. In keeping with these findings, Ang-(1–7) gene transfer into the lungs triggered similar protective actions in MCT-treated rats [39]. In addition, Ang-(1–7) via Mas prevented the apoptosis of alveolar epithelial cells and the Jun N-terminal kinase (JNK) activation induced by bleomycin [122]. The involvement of the Ang-(1–7)/Mas in PH was further evidenced by the observation that the XNT effects are blocked by A-779 [117]. Furthermore, in both lung specimens from patients with idiopathic pulmonary fibrosis and from animals with bleomycin-induced pulmonary fibrosis were reported a reduction in mRNA, protein, and activity of ACE2 with a reciprocal increase in Ang II level [116].

A growing body of studies has focused on the relevance of the ACE2/Ang-(1–7)/Mas axis in the pulmonary cancer pathophysiology. The protein expression of ACE2 is reduced in non-small-cell lung carcinoma (NSCLC) along with an increase in Ang II levels. Moreover, overexpression of ACE2 in cultured A549 lung cancer cells and in human lung cancer xenografs inhibited the cell growth and the vascular endothelial growth factor-a (VEGFa) expression induced by Ang II [123, 124]. Gallagher and Tallant [125] evaluated the

effects of several angiotensin peptides [Ang I, Ang II, Ang-(2–8), Ang-(3–8), and Ang-(3–7)] in SK-LU-1 cancer cells growth, and only Ang-(1–7) showed significant attenuation of the DNA synthesis and proliferation. The antiproliferative effect of Ang-(1–7) was mediated by its receptor Mas and inhibition of the ERK1/2 pathway. Neither the blockage of AT_1 nor AT_2 succeeded in inhibiting the action of Ang-(1–7). In keeping with these data, the antiproliferative effect of Ang-(1–7) was observed in human A549 lung tumor xenograft growth along with a marked decrease in the vessel density in mice through a mechanism involving cyclooxygenase-2 (COX-2) [126, 127]. Of note, in a nonrandomized phase I clinical trial conducted by Petty and colleagues [38], subcutaneous injections of Ang-(1–7) were administered in 18 patients with advanced solid tumors refractory to standard therapy. Despite the mild adverse effects observed with the Ang-(1–7) treatment, generally it was well tolerated. There were no treatment-related deaths. Clinical benefits were observed in 27% of the patients. Altogether, these studies provide insights into the involvement of the ACE2/Ang-(1–7)/Mas axis in lung cancer.

5. Pharmacological Therapeutic Strategies Based on the ACE2/Ang-(1–7)/Mas Axis

Many advances have been achieved regarding the therapeutic regulation of the RAS. Current therapies based on the modulation of the RAS include the ACEi, ARBs, and renin inhibitors. In general, these drugs prevent or reverse endothelial dysfunction and atherosclerosis, reduce cardiovascular mortality and morbidity of patients with coronary artery disease, and hold antihypertensive effects [128].

Classically, the mechanisms of action of the ACEi and ARBs involve the blockade of the synthesis and actions of Ang II, respectively. However, the RAS is a complex hormonal system and, consequently, other mechanisms are likely implicated in the actions of these drugs [42, 86, 129]. They cause substantial increase in plasma levels of Ang-(1–7), leading to the assumption that their clinical effects might be partly mediated by this heptapeptide [42, 130]. Indeed, a variety of effects of the ACEi and ARBs can be abolished or attenuated by Mas antagonism, confirming the role of Ang-(1–7) in the actions of these compounds [129, 131]. The beneficial effects of Ang-(1–7) as well as its likely involvement in the effects of the ACEi and ARBs represent a strong evidence for the therapeutic potential of the activation of the ACE2/Ang-(1–7)/Mas axis (Figure 3).

5.1. Ang-(1–7) Formulations. The beneficial effects of Ang-(1–7) are well known; however, the therapeutic utilization of this peptide is limited due to its unfavorable pharmacokinetic properties. Ang-(1–7) has a short half-life (approximately 10 seconds) since it is rapidly cleaved by peptidases [132]. Furthermore, Ang-(1–7) is degraded during its passage through the gastrointestinal tract when orally administrated. Thus, new strategies are crucial to make feasible the clinical application of Ang-(1–7).

Recently, a formulation based on the Ang-(1–7) included into hydroxypropyl β-cyclodextrin [HPβCD/Ang-(1–7)] was developed by Lula and colleagues [133]. Cyclodextrins are pharmaceutical tools used for design and evaluation of drug formulations, and they enhance the drug stability and absorption across biological barriers and offer gastric protection [134]. The amphiphilic character of cyclodextrins allows the possibility of formation of supramolecular inclusion complexes stabilized by noncovalent interactions with a variety of guest molecules [133, 134]. In this regard, the formulation HPβCD/Ang-(1–7) allowed the oral administration of Ang-(1–7). Pharmacokinetic and functional studies showed that oral HPβCD/Ang-(1–7) administration significantly increases plasma Ang-(1–7) levels and promotes an antithrombotic effect that was blunted in Mas deficient mice [135]. Marques and colleagues [136] have found that chronic oral administration of HPβCD/Ang-(1–7) significantly attenuates the heart function impairment and cardiac remodeling induced by isoproterenol treatment and myocardial infarction in rats [136].

In addition, liposomal delivery systems represent an alternative method to administer Ang-(1–7) [137]. Administration of liposomes containing Ang-(1–7) in rats led to prolonged hypotensive effect for several days in contrast to the response observed when the free peptide was used [137, 138].

A strategy used to protect the Ang-(1–7) against proteolytic degradation was proposed by Kluskens and coworkers [139]. Using the ability of prokaryotes to cyclize peptides, they synthesized a cyclic Ang-(1–7) derivative [thioether-bridged Ang-(1–7)] which presented an increased stability in homogenates of different organs and plasma and enhanced the Ang-(1–7) bioavailability in rats [139]. Furthermore, cyclized Ang-(1–7) induced a relaxation in precontracted aorta rings of rats which was blocked by the Ang-(1–7) receptor antagonist D-Pro^7-Ang-(1–7), providing evidence that cyclized Ang-(1–7) also interacts with Mas [139].

5.2. Synthetic Mas Receptor Agonists. AVE 0991 was the first nonpeptide synthetic compound developed with the intention of stimulating the Mas receptor. This compound mimics the Ang-(1–7) effects in several organs such as vessels [140, 141], kidney [101], and heart [142, 143]. Similar to Ang-(1–7), AVE 0991 induced a vasodilation effect which was absent in aortic rings of Mas-deficient mice [140]. Moreover, its effects in aortic rings were blocked by the two Ang-(1–7) receptor antagonists, A-779 and D-Pro^7-Ang-(1–7) [140]. AVE 0991 potentiated the acetylcholine-induced vasodilation in conscious normotensive rats, and this effect was abolished by A-779 and L-NAME [102]. Similarly, it was able to increase the hypotensive effect of Bk in normotensive rats, and A-779 also blocked this effect [107]. Ferreira et al. [142, 143] reported that AVE 0991 protects the heart against cardiac dysfunction and remodeling caused by isoproterenol treatment or by myocardial infarction in rats [142, 143]. In *Mas*-transfected cells, AVE 0991 induced NO release which was blunted by A-779 and not by AT_2 or AT_1 antagonists [101]. All these data support the concept that AVE 0991 is

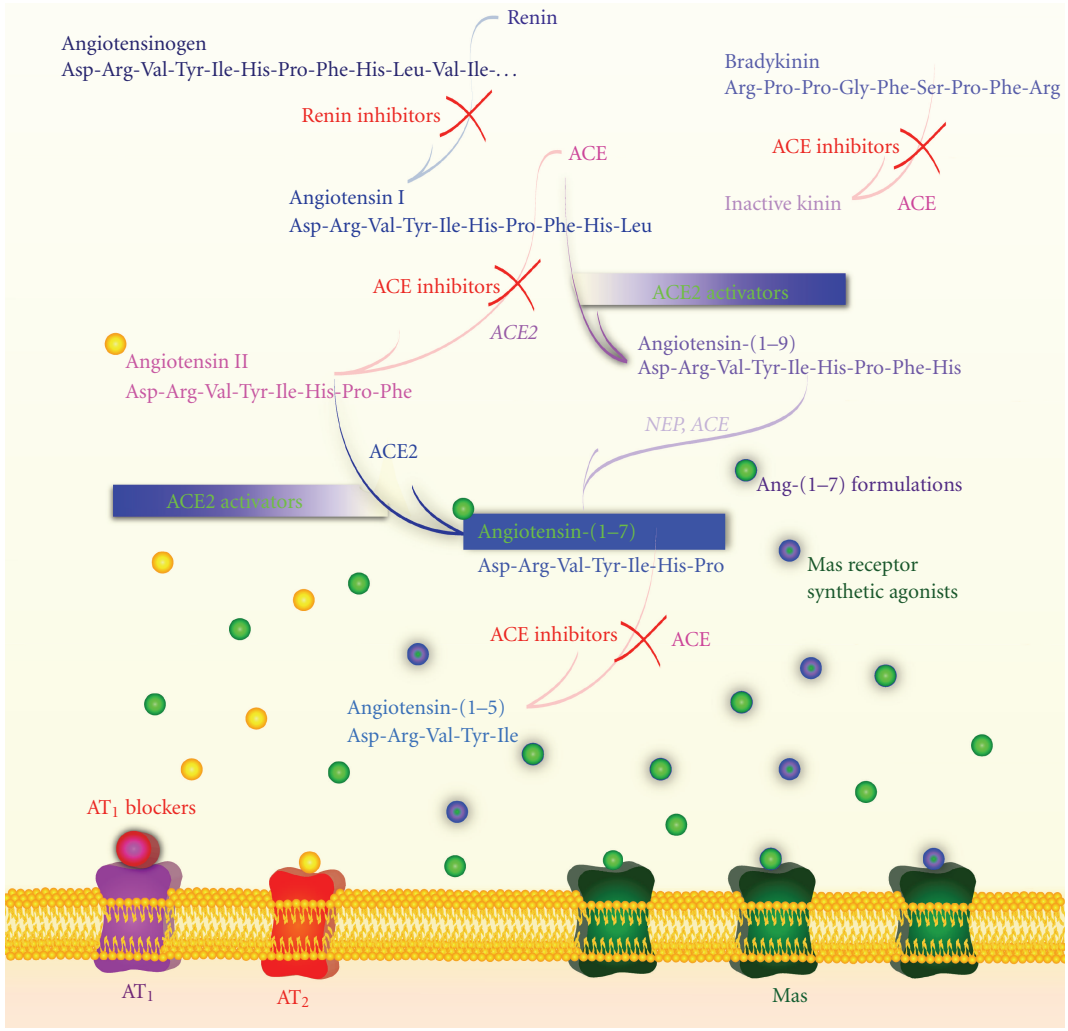

Figure 3: Schematic diagram showing the therapeutic strategies to modulate the activity of the renin-angiotensin system (RAS). In addition to the classical RAS blockers, that is, ACE inhibitors and AT_1 receptor blockers, the figure highlights the renin inhibitors, the Ang-(1–7) formulations [HPβCD/Ang-(1-7) and cyclic Ang-(1-7)], the synthetic Mas receptor agonists (AVE 0991 and CGEN-856S), and the ACE2 activator (XNT). ACE: angiotensin-converting enzyme; AT_1: Ang II type 1 receptor; AT_2: Ang II type 2 receptor; Mas: Ang-(1–7) receptor; NEP: neutral-endopeptidase 24.11.

an Ang-(1–7) mimetic and that its actions are mediated by the interaction with Mas.

Using a computational discovery platform for predicting novel naturally occurring peptides that may activate GPCR, two novel peptides, designated as CGEN-856 and CGEN-857, with amino acid sequence unrelated to angiotensin peptides, were found to display high specificity for Mas [23]. These peptides elicited Ca^{+2} influx in CHO cells overexpressing Mas without any activity in AT_1 or AT_2 receptors [144]. CGEN-856S, a derivative of the CGEN-856 peptide, induced beneficial cardiovascular effects similar to those caused by Ang-(1–7) [23]. This compound competes with Ang-(1–7) for the same bind site in *Mas*-transfected cells. Furthermore, similar to Ang-(1–7), CGEN-856S produced a vasodilation effect which was absence in Mas-deficient mice, indicating that this compound also acts via Mas [23]. This was confirmed by the inhibition of the CGEN-856S effects by the Mas antagonist A-779. Importantly, Savergnini et al. [23] showed that CGEN-856S promotes antiarrhythmogenic effects and produces a small dose-dependent decrease in arterial pressure of conscious SHR [23].

5.3. ACE2 Activators. A new approach addressing the therapeutic potential of the activation of the ACE2/Ang-(1–7)/Mas axis was proposed by Hernández Prada et al. [113]. Based on the crystal structure of ACE2 and using a virtual screening strategy, it was identified small molecules that may interact with this enzyme leading to changes in its conformation and, consequently, enhancing its activity [113]. Thus, the ACE2 activator, namely XNT, was identified and its administration in SHR decreased blood pressure, induced an improvement in cardiac function, and reversed the myocardial and perivascular fibrosis observed in these animals [48, 113]. The beneficial effects of XNT were also

observed in rats with PH induced by MCT [117]. Furthermore, this compound attenuated the thrombus formation and reduced the platelet attachment to vessels in hypertensive rats [145].

It appears that the pharmacological activation of ACE2 promotes its beneficial effects due to an increased Ang-(1–7) production with concomitant degradation of Ang II. In fact, coadministration of A-779 abolished the protective effects of XNT on PH [117]. In addition, the antifibrotic effect of XNT observed in hearts of SHR was associated with increases in cardiac Ang-(1–7) expression [48]. However, it is also pertinent to point out that off-target effects of XNT on these beneficial outcomes cannot be ruled out at the present time.

6. Conclusions

The complexity of the RAS is far beyond we could suspect few years ago. There is growing evidence that changes in the novel components of the RAS [Ang-(1–7), ACE2, and Mas] may take part of the establishment and progression of cardiovascular and respiratory diseases. Importantly, these new components of the RAS, due to their counter regulatory actions, are candidates to serve as a concept to develop new cardiovascular and respiratory drugs.

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Research Article

From Rat to Human: Regulation of Renin-Angiotensin System Genes by Sry

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The testis determining protein, Sry, has functions outside of testis determination. Multiple Sry loci are found on the Y-chromosome. Proteins from these loci have differential activity on promoters of renin-angiotensin system genes, possibly contributing to elevation of blood pressure. Variation at amino acid 76 accounts for the majority of differential effects by rat proteins Sry1 and Sry3. Human SRY regulated rat promoters in the same manner as rat Sry, elevating *Agt, Ren*, and *Ace* promoter activity while downregulating *Ace 2*. Human SRY significantly regulated human promoters of *AGT, REN, ACE2, AT2,* and *MAS* compared to control levels, elevating *AGT* and *REN* promoter activity while decreasing *ACE2, AT2,* and *MAS*. While the effect of human SRY on individual genes is often modest, we show that many different genes participating in the renin-angiotensin system can be affected by SRY, apparently in coordinated fashion, to produce more Ang II and less Ang-(1–7).

1. Introduction

Many genes on the Y chromosome that are expressed in tissues not involved in testis formation could contribute to sex differences in blood pressure and other disease phenotypes. *Sry* is believed to have evolved from the X chromosome gene *Sox3* during the process of Y-chromosome formation in therian mammals [1]. Since Sox3 has functions other than testis determination [2], *Sry* may also have additional functions outside testis determination. Sry and other Sox proteins are architectural transcription factors that bind to the minor groove of DNA, changing gene regulation through inducing a bend in the DNA [3].

The spontaneously hypertensive rat (SHR) has at least seven expressed *Sry* loci whereas the normotensive Wistar Kyoto (WKY) rat has at least six [4], lacking *Sry3*. Sry transcripts have been observed in adult rat tissues consistent with blood pressure regulation [5], and one or more of the *Sry* loci play a role in the development of hypertension in SHR [6]. Many rodent species have multiple *Sry* loci, while human and mouse have only one known locus. Sry proteins in human, rat, and other placental mammals have a homologous HMG box, hinge region, and bridge domain, with little homology in the N and C terminal ends (Figure 1(a)). The role of Sry regulation of blood pressure in humans has not been studied directly. However, with the high degree of conservation in the DNA-binding domain between human and rat, functions of Sry seen in rat are likely to translate into clinical relevance for human male blood pressure regulation.

Of the rat Sry proteins we have examined, Sry3 proteins have the largest effect on promoters of the renin-angiotensin system (RAS) genes [7]. Additionally, the Sry3 locus is found only in SHR, making it a prime candidate for elevating

blood pressure. Sry1 [8] and Sry3 [9] proteins of rat have been shown to elevate blood pressure in normotensive rats. Delivery of Sry1 is known to regulate tyrosine hydroxylase promoter activity in cultured cells [10] and in rats [9]. Two amino acid differences are found between Sry1 and Sry3, a histidine (Sry1) to glutamine (Sry3) at amino acid 38 and a proline (Sry1) to threonine (Sry3) at amino acid 76 [4]. At these two amino acids, humans share one with each rat Sry protein: proline at amino acid 131 is the same as amino acid 76 of Sry1 and glutamine at amino acid 93 is the same as amino acid 38 of Sry3. The aim of this study was to identify the role of these two amino acid differences in rat, and to address the role of human SRY on regulation of rat and human RAS promoter constructs identifying possible conserved functions of Sry in blood pressure regulation across species.

2. Methods

2.1. Sry Modeling. Models for Sry1, Sry3 and hSRY proteins were created with iTASSER [11]. Models that contained a DNA-binding cleft with the best confidence score were selected. Manipulations and highlighting of structures were performed with YASARA (http://www.yasara.org/). Models were superposed to DNA using PDB structure 1j46 [12]. 1j46 is a structure determined through NMR of the human SRY HMG box bound to a fragment of DNA containing the SRY binding element. To produce structure 1j46 P76T, proline 76 was swapped with a threonine using YASARA Structure. Both 1j46 and 1j46 (P76T) were energy minimized with AMBER03 force field [13].

2.2. Cloning. Mutant Sry constructs were designed using pEF-1 expression vectors containing rat *Sry1* (EU984075), *Sry3* (EU984077), or human *SRY* (NM_003140) through primer directed mutagenesis. Constructs are shown in Table 1, identifying the amino acid found at 76 (131 of human). Primers were phosphorylated with T4 polynucleotide kinase (Fermentas) and used in PCR with Phusion Hot Start Taq (Finnzymes). T4 DNA ligase (Fermentas) was used for ligations. Following transformation into TAM-1 competent *E. coli* (Active Motif), clones were sequenced with BigDye Terminator chemistry on ABI 3130xl genetic analyzer (Applied Biosystems). Luciferase reporter vectors (pGL3) for rat *angiotensinogen* (*Agt), renin (Ren), angiotensin-I converting enzyme (Ace)*, and *angiotensin-2 converting enzyme* (*Ace2)* were previously described [6]. Human promoters of *AGT, REN, ACE, ACE 2, MAS1 oncogene* (*MAS*), and *angiotensin II subtype 2 receptor* (*AT2*) were cloned into pGL3 using primers and restriction enzymes listed in Table 2.

Because of the way the constructs were made and the way the luciferase assays were carried out, the levels of induction on these promoters can only be compared to the control for each promoter. Levels of activity on one promoter cannot be compared to those of another promoter construct.

2.3. Cotransfections. CHO-K1 cells (ATCC) were plated at 5×10^4 cells per well into 24 well plates (COSTAR) with HAMs F12K media (Sigma) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 10 mmol/L HEPES, and 30 mmol/L sodium bicarbonate and cultured in 5% CO_2 and 95% humidity. Twenty four hours after plating, cotransfections were performed using 50 ng pEF-1 effector vector, 500 ng pGL3 reporter vector, 500 pg phRL-null Renilla control vector, 2 μL TurboFect (Fermentas), and serum-free HAMs-F12K media to 100 μL. After twenty four hours, cells were lysed and light intensity measured with Dual-Luciferase Reporter Assay System (Promega).

Table 1: Sry expression constructs of rat and human showing the amino acid at 76 in rat or 131 in human.

Construct	76 (131)
Sry1	P
Sry1 P76T	T
Sry3	T
Sry3 T76P	P
hSRY	P
hSRY P131T	T

2.4. Statistics. Results for each promoter were analyzed with JMP by comparing relative intensity to an empty pEF-1 control vector or respective nonmutated vector. All ANOVAs that showed significance ($P \leq 0.05$) were followed by Students t-tests. For all luciferase assays $n = 3$–5, each n represents an individual experiment of dual measurements from two duplicate wells, and error bars are standard error of the mean (SEM) and $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

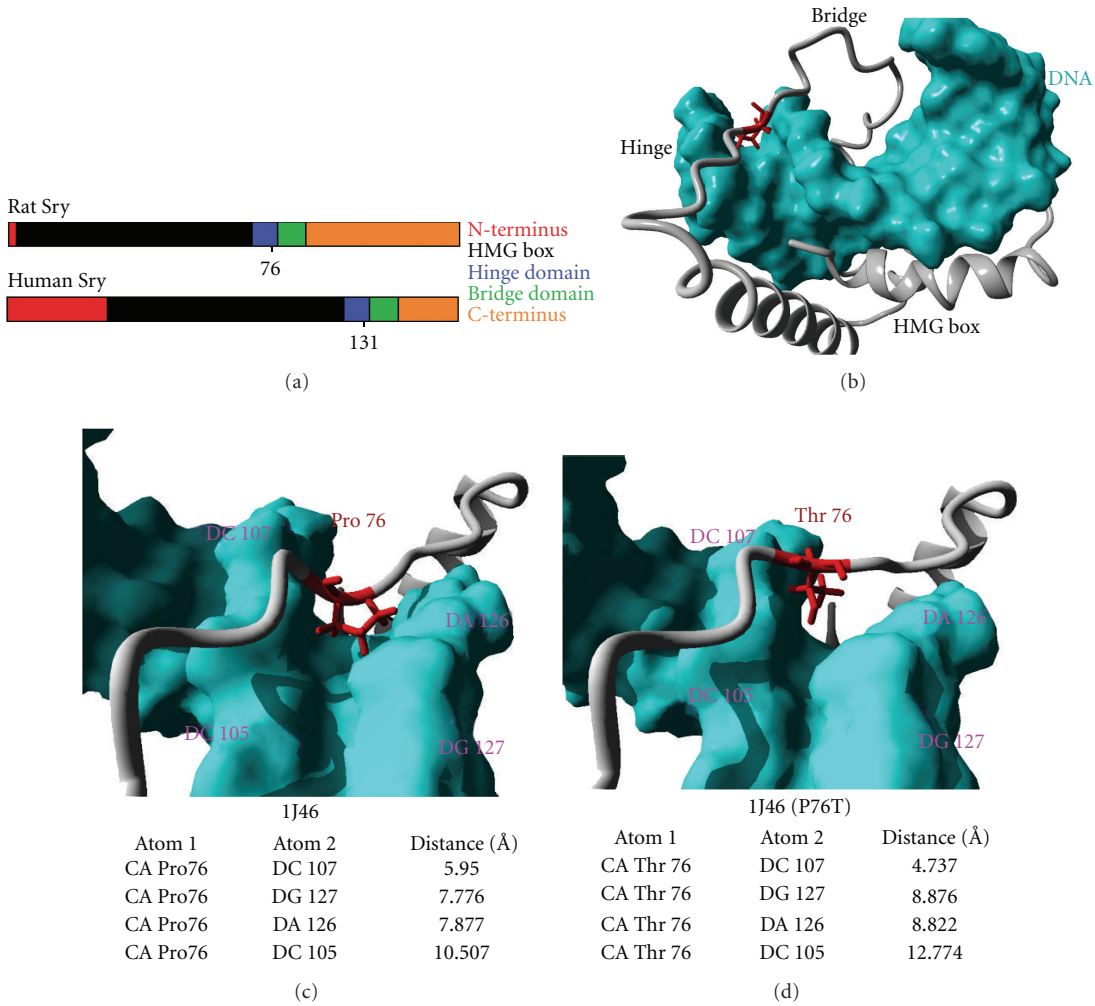
3. Results

Models of variations at amino acids 38 and 76 previously described were created to verify whether the variation could potentially alter DNA interaction. This was determined by either modeling the protein backbone independent of DNA interaction with iTASSER or swapping amino acids in the known hSRY structure (1j46) of Sry-DNA interaction. When Sry is interacting with DNA, amino acid 38 (found in the HMG box) is found on the opposite side of the DNA interaction of the HMG box. Therefore, it does not appear to contribute to DNA interaction. Amino acid 76 of rat Sry (131 of human) is in the hinge domain (Figure 1(b) red) and interacts with DNA. A proline at amino acid 76 interacts with the DNA minor groove base pairs in a non-DNA sequence-specific manner [12]. Models of Sry3, containing a threonine at amino acid 76, show an altered protein backbone, which would change the interaction of amino acid 76 from the minor groove to the phosphate backbone of DNA (data not shown).

Exchanging the proline of hSRY 1j46 (Figure 1(c)) for a threonine (Figure 1(d)) and energy minimizing the protein bound to DNA led to the same shift in DNA interaction as seen with modeling approaches. Bond distances between the alpha carbon (CA, atom one) of either the proline

Table 2: Cloning of the human renin-angiotensin system gene promoters. The start and stop are the base pairs from the transcriptional start site of each gene.

Gene	Restriction enzymes	Right primer	Left primer	Size (bp)	Stop	Start
AGT	NcoI/SacI	ATACCATGGGGCCACTTCTGACCCTGCTG	GCCGAGCTCTAGAAGATCCCCCAGCTGATAG	1610	34	−1576
REN	NcoI/MluI	GTTCCATGGGAGGTTCTGTGGCTCCCTTAG	CGCACGCGTCTTCTTATGGGAAGCCCATTTA	1452	8	−1444
ACE	BglII/KpnI	CAGAGATCTGTGCTCGGCTCTGCCCCTTCTC	CGAGGTACCCCAAGCTGTTAGGACCCCTGAG	1700	21	−1691
ACE 2	HindIII/NheI	CCGAAGCTTTCCTGATCCTCTGTAGCCATGGGA	CGAGCTAGCAGGGCAGGCAGCATCTGACT	1932	34	−1899
AT2r	NcoI/MluI	CCGCCATGGGTCCACTGGGAGCCTTCAACCT	CGAACGCGTGGTGGAGGTGAGGCGGCAAA	2906	3	−2903
Mas	HindIII/NheI	CCGAAGCTTCCATGAGGAGGCCTCAGGTTGGA	CGAGCTAGCGCCCGTTTGGACCTGGTCGC	2204	18	−2186



Atom 1	Atom 2	Distance (Å)
CA Pro76	DC 107	5.95
CA Pro76	DG 127	7.776
CA Pro76	DA 126	7.877
CA Pro76	DC 105	10.507

(c)

Atom 1	Atom 2	Distance (Å)
CA Thr 76	DC 107	4.737
CA Thr 76	DG 127	8.876
CA Thr 76	DA 126	8.822
CA Thr 76	DC 105	12.774

(d)

Figure 1: Modeling Sry variations of Sry1, Sry3, and hSRY. (a) Schematic of the rat Sry and human SRY proteins with amino acid 76 of rat (131 of human) shown. In red is the N-terminus, black the HMG box, blue the hinge domain, green the bridge domain, and orange the C-terminus. (b) Amino acid 76 (red) can be seen to interact with DNA (cyan) on modeled rat Sry1. (c) 1j46 (human SRY bound to DNA) was energy-minimized and the distances of the carbon alpha of the proline, amino acid at 76 (131), were measured from four different phosphates of DNA (DC 105, DC 107, DG 127, and DA 126). (d) 1j46 (P76T), in which the proline was substituted with a threonine, was energy-minimized and distance-measured.

(Figure 1(c)) or threonine (Figure 1(d)) with four different phosphates of DNA bases (atom two) confirm the shift relative to DNA. The CA of threonine (Figure 1(d)) is shifted closer (by 1.213 Å) to the phosphate of DC 107 (4.737 Å compared to 5.950 Å), while farther from DG127 (by 1.100 Å), DA126 (by 0.945 Å), and DC105 (by 2.267 Å) compared to proline (Figure 1(c)). This suggests the CA is shifting out of the minor groove and closer to the phosphate backbone when changed to threonine.

Mutation of amino acid 76 from proline to threonine in Sry1 (Sry1 P76T) significantly increased promoter activity from *Ren, Ace* and *Ace2* relative to nonmutated Sry1 ($P \leq 0.05$, Figure 2(a)). Mutation of Sry3 at amino 76 from threonine to proline (Sry3 T76P) decreased *Agt*, *Ace*, and *Ace2* promoter activity ($P \leq 0.05$, Figure 2(b)). Mutagenesis of rat amino acid 38 (93 of human) showed no significant effect on activity of any promoters constructs tested (data not shown). Cotransfecting hSRY expression constructs with rat RAS promoters significantly regulated each construct in the same manner as rat Sry [7] but with different relative intensities (Figure 3). Mutation of hSRY at amino acid 131 from proline to threonine (hSRY P131T) caused a significant increase in both *Ren* and *Ace2* promoter activities.

Human SRY significantly regulated the human promoters of *AGT* ($P \leq 0.001$), *REN* ($P \leq 0.001$), *ACE2* ($P \leq 0.001$), *AT2* ($P \leq 0.001$), and *MAS* ($P \leq 0.001$) compared to control levels (Figure 4), elevating AGT and REN promoter activity while decreasing *ACE2*, *AT2*, and *MAS*. Mutation P131T caused an alteration in hSRY regulation on all these promoters. Sry3 significantly regulated *AGT, ACE2, AT2*, and *MAS*. The human ACE promoter construct used in these studies was not regulated by hSRY or Sry3.

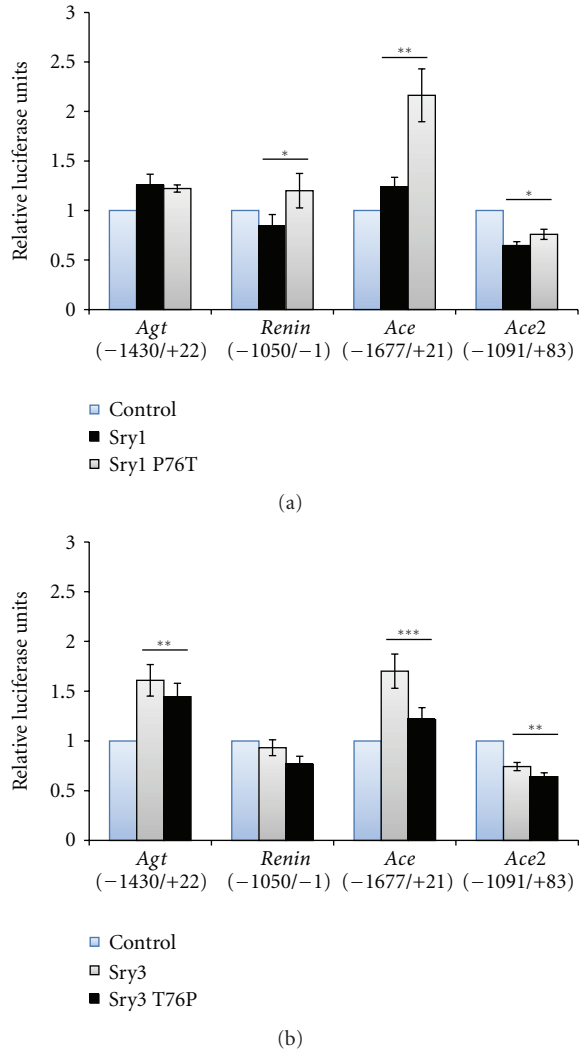


Figure 2: Variation at amino acid 76 of rat Sry proteins effects on rat RAS gene promoters. Amino acid variation between Sry1 and Sry3 results in differential activity on promoters of the RAS with Sry1 P76T (a) and Sry3 T76P (b). (a) Sry1 P76T significantly increased activity on *Ren, Ace*, and *Ace2*. (b) Sry3 T76P significantly decreased activity on *Agt*, *Ace*, and *Ace2*. Asterisks indicate significance from respective nonmutant Sry. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. Blue bars are the control pEF-1 vector and black bars represent proteins with a proline at amino acid 76.

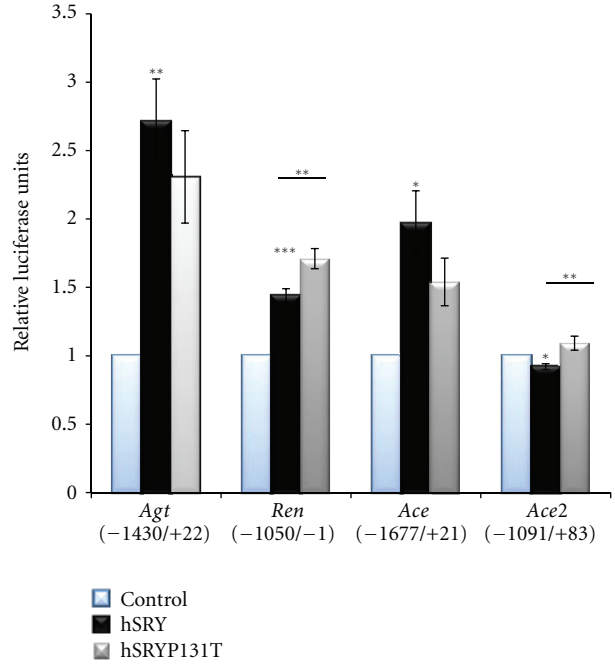


Figure 3: Human SRY regulation of rat RAS gene promoters. Human SRY (black) increases promoter activity of rat *Agt, Ren*, and *Ace* while decreasing *Ace2* as previously seen with Sry1 and Sry3. Mutations to the proline 131 of hSRY to threonine (gray) significantly increased activity in *Ren* and *Ace2* while they had a trend in decreasing activity on *Agt* and *Ace*. Significance of hSRY is based on comparison to the control vector (blue) or for hSRY P131T to the hSRY values. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

4. Discussion

Hypertension is a complex disease, and distinguishing among a myriad of genetic and environmental factors is difficult. In humans and other animal models, blood pressure is higher in males than premenopausal females [14, 15]. This may be due to a genetic component that is male specific such as the Y-chromosome gene Sry. Accumulating evidence suggests that Sry has functions not directly related to testis determination [16]. This study, as well as others from our lab, suggests that Sry is a contributor to increasing blood pressure in SHR. Here we show that this Sry-mediated increase in blood pressure may translate into humans since both rat and human proteins seem to function similarly. Sry activation of the RAS gene promoters [7], combined with the effects of Sry on the sympathetic nervous system [8, 10], could contribute to sex differences in hypertension.

In SHR males, androgens have been shown to increase plasma Ren, renal Agt, and hepatic Agt [17]. Sry is known to interact with the androgen receptor (AR) and affect AR gene regulation [18]. It is thus probable that Sry and androgens coregulate the RAS and other genes.

In this study we have identified and characterized the amino acid in the rat Sry1 and Sry3 proteins that cause their differential effects on regulation of RAS gene promoters. Rat amino acid 38 (93 of human) has no published function or natural variation resulting in phenotypic change. Known structures of Sry and the models created here show that this amino acid is not likely to contribute to DNA interactions, and promoter activity assays support this.

The majority of variation of rat Sry protein differences on the RAS could be explained from results of mutations at amino acid 76 of rat (131 of human). In humans, a mutation of the proline to an arginine at amino acid 131 was present in an individual with sex reversal [19], showing significance of this amino acid. Modeling Sry1, Sry3, and hSRY suggests that the proline and threonine differences could account for altered interactions with DNA. NMR experiments showed that the proline of Sry interacts with

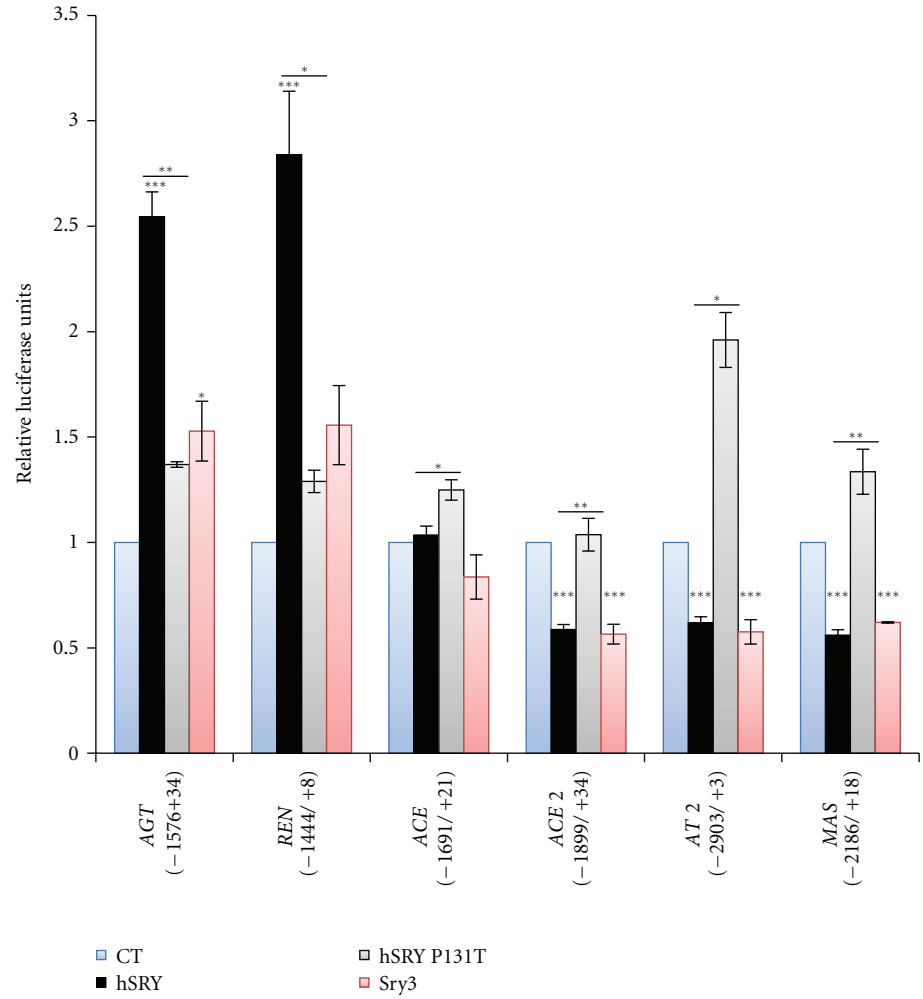


Figure 4: Human SRY regulation of the human RAS gene promoters. Human SRY (hSRY, black) significantly regulated human *AGT*, *REN*, *ACE 2*, *AT2*, and *MAS* promoters. Significance of hSRY is based on comparison to the control vector (blue). The mutation at amino acid 131 (hSRY P131T, gray) significantly diminished function in *AGT*, *REN*, *ACE 2*, *AT2*, and *MAS* constructs relative to hSRY. Sry3 (red) significantly regulated *AGT*, *ACE2*, *AT2*, and *MAS* compared to control (blue). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

the base pairs of the minor groove and the sugars of the DNA backbone [12]. Modeling variation through prediction of structure with iTASSER or changing structure 1j46 both showed a shift in the threonine of Sry3 to be more likely to interact with the phosphate backbone of DNA. The hinge region serves as a kinetic clamp for Sry-DNA interaction [20]. The hydrophobicity of the proline, at position 76/131, allows this residue to fit into the DNA minor groove, reducing the solvent accessible surface compared to the threonine (data not shown) and improving the stability of this Sry-DNA interaction. Alternatively, the presence of threonine may alter the solvent accessible surface and change the thermodynamics and kinetics of the Sry-DNA complex by potentially reducing kinetic clamp ability in Sry. In general, hSRY behaves similar to the rat Sry proteins with the exception that a change to threonine at amino acid 131 (correlating to rat amino acid 76) alters gene regulation from nonmutated much greater than in the rat. We believe that variations in the divergent bridge domain between rat and human may stabilize rat proteins containing a threonine, for example Sry3, more strongly than human SRY.

Although CHO cells are not normally used in analysis of the RAS, they provide us with several benefits. Co-transfection efficiency is very high in CHO cells. Also they are efficient for recombinant protein expression. Since CHO cells are from the ovary and lack the Y chromosome, there is no endogenous Sry expression. There is an ovarian tissue RAS, with ovary tissue or CHO cells shown to express prorenin, Ren, Ang, Ace, Ace 2, Ang II, and Ang II receptors [21–24]. Our promoter constructs contain only the proximal promoter region and a few hundred bases upstream, thus they cannot tell us if Sry activates RAS promoters differently *in vitro* or *in vivo*. Electroporation of Sry3 into the kidney increased Ang II and plasma Ren activity [9], which we believe is likely through the gene regulation we see in this study and thus provides *in vivo* gene support of the validity of our promoter activity assays.

We have long been interested in determining how human and mouse, with only a single *Sry* gene, can potentially perform all the functions of *Sry* encompassed by the multiple copies in rat. Human SRY behaves in a manner similar to both Sry1 and Sry3 in regulation of rat *Agt, Ren, Ace*, and *Ace2* promoters. Promoters of the human RAS showed similar responses to hSRY and Sry3, with hSRY P131T losing regulation. The human *ACE* promoter we used showed no significant change in activity with hSRY, and after analysis of Sry-binding sites using Genomatix MatInspector we could not identify any Sry-binding elements in the *ACE* promoter used in our experiments. Several potential Sry-binding sites are present in longer promoter sequences of human *ACE*. Additional experiments using longer *ACE* promoter sequences are necessary to address this issue, which will likely yield results similar to the rat *Ace* promoter. All other promoters used contained Sry and AP1-binding sites required for cis-Sry activation. In a previous study we showed that Sry acts primarily through the AP1 binding site for activation of the tyrosine hydroxylase promoter [10].

The data presented here suggest that human and rat Sry proteins and their genome targets (either cis or trans) regulating the RAS have remained functionally conserved. Regulation of the RAS by Sry appears to favor the production of Ang II, possibly reducing Ang-(1–7) and MAS levels [7]. MAS is known to be the receptor of Ang-(1–7) which can counter the effects of Ang II [25], thus the gene regulation by Sry suggests not only increasing the Ang II signaling, which has been shown *in vivo*, but also decreasing the counter pathway of Ang-(1–7). In addition, AT2 receptor promoter activity was decreased by human SRY, supporting its repressing effects on RAS components that oppose Ang II actions. Despite past uncertainty about the role of AT2 receptor, recent studies have confirmed AT2 receptor as a vasodilator mediator [26]. Human SRY likely plays a role similar to that of rat Sry in the regulation of blood pressure. It will be interesting to explore further the role this may serve in human hypertensive disease phenotypes.

5. Conclusions

Variations of amino acid 76 in rat (131 in human) led to differential regulation of rat RAS gene promoters. This study is the first to show that hSRY can regulate RAS promoters in the same pattern as rat Sry. In addition, by analyzing the human promoters of RAS, we show that potential cis- or trans-binding sites for Sry are conserved, indicating a conserved role of Sry in human blood pressure.

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Review Article

Protective Role of the ACE2/Ang-(1–9) Axis in Cardiovascular Remodeling

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Despite reduction in cardiovascular (CV) events and end-organ damage with the current pharmacologic strategies, CV disease remains the primary cause of death in the world. Pharmacological therapies based on the renin angiotensin system (RAS) blockade are used extensively for the treatment of hypertension, heart failure, and CV remodeling but in spite of their success the prevalence of end-organ damage and residual risk remain still high. Novel approaches must be discovered for a more effective treatment of residual CV remodeling and risk. The ACE2/Ang-(1–9) axis is a new and important target to counterbalance the vasoconstrictive/proliferative RAS axis. Ang-(1–9) is hydrolyzed slower than Ang-(1–7) and is able to bind the Ang II type 2 receptor. We review here the current experimental evidence suggesting that activation of the ACE2/Ang-(1–9) axis protects the heart and vessels (and possibly the kidney) from adverse cardiovascular remodeling in hypertension as well as in heart failure.

1. Introduction

All epidemiological studies show that the risk of adverse cardiovascular (CV) outcomes, such as stroke, myocardial infarction (MI), heart failure (HF), and kidney disease [1], increase progressively with increasing blood pressure (BP). On the other hand, clinical trials demonstrate that lowering BP reduces such risks [1]. All antihypertensive medications lower BP, but specific drug classes display effects beyond BP reduction (pleiotropic effects) that might contribute to cardiovascular risk reduction.

Remodeling of the cardiovascular structure occurs in response, not only to changes in BP and flow, but also to modifications in the neurohormonal environment, in which the rennin-angiotensin-aldosterone system (RAAS) exerts a most predominant influence [2].

The RAAS is a major regulator of BP [3, 4]. In addition, the RAAS has a role in the vascular response to injury and inflammation [4]. Chronic RAAS activation, through both angiotensin (Ang) II and aldosterone, leads to hypertension and perpetuates a cascade of proinflammatory, prothrombotic, and atherogenic effects associated with end-organ damage [3, 4]. Based on these facts, several drugs have been developed that work by (a) reduction of Ang II levels, (b) inhibition of the Ang II type 1 receptor (AT1R), (c) blockade of the aldosterone receptor, and (d) renin receptor blockade [5, 6]. During the last 25 years several clinical trials have shown the benefits with these drugs that inhibit the RAAS with regard to BP reduction, regression of cardiac hypertrophy, prevention of kidney damage and reduction of cardiovascular morbidity reduction in hypertensive patients. Besides, with most of these RAAS blockers, quality of life as well as survival has been significantly improved in patients with heart failure. Consequently, the RAAS is currently a main therapeutic target in hypertension treatment [3, 4]. Aggressive BP control improves outcomes in patients with CV disease, stroke, and nephropathy and might have beneficial effects beyond BP lowering [7].

Despite the reduction of CV events and end-organ damage with the current pharmacologic strategies, CV disease remains the primary cause of death in the world, and more than 94,000 Americans annually experience progression to end-stage renal disease (ESRD). As population ages, the proportion affected by end-organ damage is expected to grow [8]. Thus, it is most relevant to find new molecules

in order to prevent and reduce hypertension as well as pathologic CV and kidney remodeling and dysfunction. In this regard, activation of the new ACE2/Ang-(1–9) pathway seems to counterbalance the damage due to the RAAS system activation.

We review here the current experimental evidence suggesting that activation of the ACE2/Ang-(1–9) pathway protects the heart and vessels (and possibly the kidney) from adverse cardiovascular remodeling in hypertension as well as in heart failure.

2. Angiotensin-Converting Enzyme 2

The discovery of angiotensin-converting enzyme homologue, ACE2, added further complexity to the main axis of the RAAS, in which Ang II and its forming enzyme ACE play major roles [9, 10]. A growing body of evidence points to a possible promising role for this new member of the RAAS by opposing to the effects of the main axis [11, 12]. ACE2 has dramatically changed the direction of cardiovascular and renal research in view of the pivotal role of this enzyme in the regulation of the RAAS [12, 13].

ACE2 is the newest member of the RAAS and shares approximately 40% similarity with the somatic form of ACE [9, 10]. ACE2 is a membrane-bound carboxypeptidase and its cellular and tissue distribution is different from that of ACE. While ACE is expressed in the endothelium throughout the vasculature, ACE2 is distributed in tissues with the most abundant expression in heart, kidney, lung, small intestine, and testis [14]. ACE2 can be released into the circulation and urine by shedding [15]. Tumor necrosis factor-alpha-converting enzyme (TACE/ADAM17) is the sheddase responsible for the ectodomain cleavage and shedding of ACE2 [16].

However, normal ACE2 enzymatic activity in plasma is very low, probably due to the presence of an endogenous inhibitor [17–19]. ACE2 is different from ACE in both substrate specificity and functions [9, 20, 21]. ACE2 can form (a) Ang-(1–7) through hydrolysis of Ang II and (b) Ang-(1–9) through hydrolysis of Ang I. This last reaction is negligibly slow and is several hundred times slower than Ang II hydrolysis by ACE2 to form Ang(1–7)—a vasodepressor peptide counterbalancing the vasopressor effect of Ang II [20, 21]. Ang-(1–7) can be subsequently converted to Ang-(1–5) by ACE [9, 20] or by neutral endopeptidases [9], while Ang-(1–9) may be converted to Ang-(1–7) by ACE [9]. There is little evidence proving the existence of alternative hydrolysis of Ang-(1–9) to Ang II in some tissues. Drummer et al. [22] proved that homogenates of rat kidney, and in a lesser extent of lung, convert Ang-(1–9) to Ang II due to an ACE-independent aminopeptidase and N-like carboxypeptidase. Singh et al. [23] confirmed that the pathway Ang I-Ang-(1–9)-Ang II really exists in glomeruli of streptozotocin-induced diabetes mellitus rats. Moreover, in human heart tissue the main products of Ang I degradation are both Ang-(1–9) and Ang II generated by heart chymase, ACE and a poorly identified carboxypeptidase A [24]. Although the data proving the existence of alternative pathways of Ang II production, in clinical practice we can still block only ACE or AT1R.

ACE2 does not act on bradykinin metabolism and its activity is not inhibited by classic ACE inhibitors (ACEIs) [9]. Thus it has been proposed that ACE2 activity may counterbalance the effects of ACE by preventing the accumulation of Ang II in tissues where both ACE2 and ACE are expressed [25, 26]. ACE2 has several biological substrates and it is considered a multifunctional enzyme. Acting as a monocarboxypeptidase, it cleaves several other non-RAAS peptides which have roles in maintaining cardiovascular homeostasis such as (des-Arg9)-bradykinin, a member of the kininogen-kinin system [13]. (des-Arg9)-Bradykinin is formed from bradkinin by the action of carboxypeptidases and is an agonist of the B1 receptor, which is induced after tissue injury [27]. Bradykinin, a vasodilator which acts through the B2 receptor, is produced from its precursor kininogen by kallikrein and is degraded by ACE [13]. While, degradation of bradykinin by ACE is known to be an important aspect of BP regulation, the significance of the degradation of (des-Arg9)-bradykinin by ACE2 remains to be established. In addition to (des-Arg9)-bradykinin, ACE2 is also able to degrade apelin-13, a peptide proposed to cause vasoconstriction and known to regulate fluid homeostasis, and other non-RAAS peptides such as kinetensin, dynorphin A and neurotensin [20].

For a long time, Ang-(1–7) was thought to be devoid of biological activity, in spite of early reports on biological effects [28]. The importance of Ang-(1–7) was emphasized by the discovery of ACE2. Ang-(1–7) has been shown to release vasopressin as effectively as Ang II from neurohypophyseal explants [28] and to have actions opposing those of Ang II, namely vasodilation, antitrophic effects and implications of vasodilation caused by bradykinin [29, 30].

Several experiments suggest an important interaction between Ang-(1–7) and prostaglandin-bradykinin-nitric oxide (NO) systems. Ang-(1–7) binds to the Mas receptor (G protein-coupled receptor) which mediates vasodilating and antiproliferative actions of this peptide [31]. The Mas receptor can hetero-oligomerize with the AT1 receptor and acts as a physiological antagonist of Ang II [32]. Studies revealed that Ang-(1–7) activated endothelial nitric oxide synthase and NO production via Akt-dependent pathways [33]. Furthermore, Tallant et al. [34] showed that the presence of an antisense probe directed against Mas abolished the Ang-(1–7)-induced inhibition of protein synthesis in cardiomyocytes. This study also revealed that Ang-(1–7) decreased serum-stimulated ERK1/ERK2 mitogen-activated protein kinase activity, a response that was blocked by D-Ala 7-Ang-(1–7), an antagonist of Mas receptor.

Ang II binds with high affinity to two different receptor subtypes—AT1R and AT2R—which are members of the seven-transmembrane-domain G-protein-coupled receptors (GPCR) superfamily, through Gq and Gi, respectively [35]. Whereas the AT1R mediates most of the recognized actions of Ang II, it appears that the AT2R opposes, in part, to the effects mediated by the AT1R. As the AT2R is expressed in adult tissues in smaller amounts than the AT1R, the actions and cell signaling of AT2R have been less well characterized

than those of AT1R [36–38]. Current knowledge suggests that AT2R stimulation mediates vasodilation, antigrowth, proapoptotic and antiinflammatory effects [39, 40]. Hence, the AT2R can modulate cardiovascular remodeling as well as progression of atherosclerosis.

AT2R stimulation activates the NO-cGMP-dependent pathway [41]. This occurs either directly or indirectly through bradykinin or by increased endothelial NOS activity or expression. AT2R activation is associated with phosphorylation of JNK, PTPs, IκBα (inhibitor of NF-κB), and the transcription factor ATF2, and dephosphorylation of p38MAPK, ERK1/2, and STAT3, which are linked to antiproliferative and antiinflammatory effects and apoptosis [38, 42–44]. AT2R may induce relaxation by opening large-conductance Ca^{2+}-activated K^+ channels (BKCa) [45] and by negative regulation of the vascular Rho A/Rho kinase pathway. The AT2R also enhances the activity of tyrosine phosphatases and vanadate-sensitive phosphatases MKP1 (DUSP1), SHP1 (PTPN6) and PP2A [46, 47].

3. Ang-(1–9)

There is little information in the literature with respect to Ang-(1–9) probably because this peptide was initially thought to be active only after conversion to Ang-(1–7). Ang-(1–9) can be generated by several carboxypeptidase-type enzymes including ACE2 or cathepsin A [48, 49]. Ang-(1–9) is present in healthy volunteers, in patients or in animals treated with ACE inhibitors (ACEIs) or AT1 receptor blockers (ARBs) [50–52], and its circulating levels are increased by pathological conditions (i.e., early after MI) [51]. However, very little is currently known about Ang-(1–9) biological effects [50, 53].

Initial studies showed that incubation of Chinese hamster ovary cells (CHO) with Ang-(1–9) potentiated the release of arachidonic acid by [$Hyp^3Tyr(Me)^8$]BK, elevated [Ca^2]i and also resensitized the B2 receptor desensitized by BK [48]. At the same time, Jackman et al. [54] showed in CHO cells and in human pulmonary endothelial cells that Ang-(1–9) was significantly more active than Ang-(1–7) enhancing the effect of an ACE-resistant bradykinin analogue on the B2 receptor and that Ang-(1–9) also augmented arachidonic acid and NO release by kinin [54].

Some studies have suggested that Ang-(1–9) may be an endogenous inhibitor of ACE. Donoghue et al. [9] proposed that Ang-(1–9) is a competitive inhibitor of ACE because it is by itselfan ACE substrate. Under conditions of ACE inhibition, such as after long-term administration of an ACEI in rats, Ang-(1–9) levels increased in plasma and kidney [50, 53]. This increase in Ang-(1–9) steady-state levels could be due to decreased catabolism of Ang-(1–9) by ACE. Conversely, the increased levels of Ang-(1–9) could be due to increased production by ACE2 as a result of increased availability of Ang I substrate. These results indicate that an alternate pathway of Ang I metabolism by ACE2 exists and that this pathway may be amplified in the presence of ACE inhibitors.

To determine whether Ang-(1–9) is active per se or it becomes active only after conversion to Ang-(1–7), Chen et al. [55] examined the metabolism of Ang I, Ang-(1–9) and Ang-(1–7) in stably transfected CHO cells that express human ACE and human bradykinin B2 receptors coupled to green fluorescent protein (B2GFP). They found that Ang-(1–9) was hydrolyzed 18 times slower than Ang I and 30% slower than Ang-(1–7). Ang-(1–9) inhibited ACE and it resensitized the desensitized B2GFP receptors, independently of ACE inhibition [55]. This is reflected by release of arachidonic acid through a mechanism involving cross-talk between ACE and B2 receptors. They concluded that Ang-(1–9) enhanced bradykinin activity, probably by acting as an endogenous allosteric modifier of the ACE and B2 receptor complex. Therefore, when ACE inhibitors block conversion of Ang I, other enzymes like ACE2 can still release Ang I metabolites like Ang-(1–9) and enhance the efficacy of ACEIs.

Recently, Flores-Muñoz et al. [56] using radioligand binding assays observed that Ang-(1–9) is able to bind the Ang II type 2 receptor (AT2R) ($pKi = 6.28 \pm 0.1$). They demonstrated that Ang-(1–9) and not Ang II, affected hypertrophy through the AT2R, as PD123319 (an AT2 receptor blocker) did not alter Ang II-mediated growth but did block the effects of Ang-(1–9). Despite having ~100-fold lower affinity than Ang II for the AT2R [57], the selective AT2R activity of Ang-(1–9) is not inconsistent with current pharmacological models of G protein-coupled receptor signalling and activation. Indeed, the concept of functional selectivity, where individual receptor ligands have the capacity to selectively stabilize conformations which lead to distinct signalling outcomes [57–59], is supported by a previous study in which the critical amino acids and the mode of binding of ligands at the AT1R and AT2R were investigated [60]. While agonist activation of the AT1R was particularly sensitive to peptide modifications that disrupted contact points between Ang II and its receptor, substitutions within Ang II were far better tolerated by the AT2R [60]. The AT2R exists in a relaxed conformation and Ang II therefore binds to multiple indistinct contact points [60]. Since Ang-(1–9) contains the entire Ang II sequence plus a C-terminal histidine, these observations indicate that this difference may stabilize the AT2R in a conformation able to counteract hypertrophic signalling in cardiomyocytes. Flores-Muñoz et al. [56] did not observe functional competition between Ang II and Ang-(1–9) at the AT2R and they concluded that that Ang-(1–9) is able to antagonize Ang II signalling in cardiomyocytes selectively via the AT2R, highlighting that Ang-(1–9), along with Ang-(1–7), makes up part of the counter-regulatory arm of the RAS. What remains to be determined is the downstream signalling effects from Ang-(1–9). Preliminary studies indicate that the classical pathways via PKC translocation and ERK1/2 activation [61–63] are not different between Ang II-, Ang-(1–7)- and Ang-(1–9) stimulated cells. Since the downstream signalling from the AT2R is unclear at present, future studies will be required to establish these mechanisms.

4. Role of the ACE2/Ang-(1–9) Axis in Hypertension

Crackower et al. [64] were the first to test ACE2 as the gene underlying the blood pressure locus on the X chromosome. They showed reduced expression of renal ACE2 in the salt-sensitive Sabra hypertensive rat compared with the normotensive rat. Both spontaneously hypertensive rats (SHR) and spontaneously hypertensive stroke-prone rats (SHRSP) rats showed reduced renal ACE2 protein levels compared with the normotensive Sabra and Wistar Kyoto (WKY) strains. Two other groups confirmed some of these findings showing lower renal ACE2 mRNA, protein, and activity in the SHR compared to WKY rats [65, 66]. However, other investigators were unable to detect any difference in renal ACE2 mRNA, protein, and activity between adult hypertensive rats and their normotensive controls [67].

Rentzsch et al. [68], assessed in SHRSP (that display reduced ACE2 mRNA and protein expression compared with control animals in the kidney) the role of ACE2 in the pathogenesis of hypertension. They generated transgenic rats on a SHRSP genetic background expressing the human ACE2 in vascular smooth muscle cells by the use of the SM22 promoter, called SHRSP-ACE2. In these transgenic rats, vascular smooth muscle cells (VSMC) expression of human ACE2 was confirmed by RNase protection, real-time RT-PCR, and ACE2 activity assays. Transgene ACE2 expression leads to significantly increased circulating levels of Ang-(1–7), a prominent product of ACE2. Mean arterial blood pressure was reduced in SHRSP-ACE2 compared to SHRSP rats, and the vasoconstrictive response to intraarterial administration of Ang II was attenuated. The latter effect was abolished by previous administration of an ACE2 inhibitor. To evaluate the endothelial function in vivo, endothelium-dependent and endothelium-independent agents such as acetylcholine and sodium nitroprusside, respectively, were applied to the descending thoracic aorta and blood pressure was monitored. Endothelial function turned out to be significantly improved in SHRSP-ACE2 rats compared to SHRSP. These data indicate that vascular ACE2 overexpression in SHRSP reduces hypertension probably by local Ang II degradation and by improving endothelial function [68].

A target gene therapy strategy holds significant potential to translate the available fundamental research of ACE2 into therapeutics. In fact, initial animal experiments have been extremely encouraging. For example, in SHR, viral-mediated ACE2 overexpression in the heart decreased high BP [69]. This strategy also preserved cardiac function, as well as left ventricular wall motion and contractility, and attenuated left ventricular wall thinning induced by myocardial infarction [70]. ACE2 overexpression in the rostral ventrolateral medulla causes significant decreases in BP and heart rate (HR) [71].

Compared with ACEIs and ARBs, the targeting of ACE2 has the following potential therapeutic advantages, first, it degradates both Ang I to generate Ang-(1–9) and Ang II to generate Ang-(1–7). Thus, targeting ACE2 would not only produce the antihypertrophic peptide Ang-(1–9) [52] and the vasoprotective/antiproliferative peptide Ang-(1–7) [72–74], but would also influence the vasoconstrictive/proliferative effects of the ACE/Ang II/AT1R axis [75]. Second, it is a multifunctional enzyme with many biologically active substrates [9, 20]. Third, unlike ARB/ACEI therapy, ACE2 is an endogenous regulator of the RAS [75]. Fourth, it is a part of the vasodilatory/antiproliferative axis of the RAS [20] and fifth, although treatment with ACEIs or ARBs indirectly increases ACE2 expression, direct activation of this enzyme could result in a better outcome in cardiovascular diseases [68, 75]. Thus, the activation of the ACE2 axis may be a novel therapeutic strategy in hypertension.

So far, all attention has been focused on Ang-(1–7), that opposes the pressor, proliferative, profibrotic, and prothrombotic actions mediated by Ang II [76]. Experimental and clinical studies have demonstrated a role for the Ang-(1–7)/ACE2/Mas axis in the evolution of hypertension, the regulation of cardiovascular and renal function, and the progression of cardiovascular and renal disease including diabetic nephropathy [77]. Additional evidence suggests that a reduction in the expression and activity of this vasodepressor component may be a critical factor in mediating the progression of cardiovascular and renal disease. These findings support a role for the Ang-(1–7)/ACE2/Mas axis and, in particular, on its putative role as an ACE-Ang II-AT1 receptor counter-regulatory axis within the RAS [76, 77].

Recently, the alternative angiotensin peptide, Ang-(1–9) has shown relevant biological functions. Ocaranza et al. [51] have observed increased ACE2 activity and Ang-(1–9) plasma levels in MI and sham rats treated with enalapril for 8 weeks while circulating Ang-(1–7) levels did not change in any phase after MI [51] (Figure 1). These findings support the hypothesis that, in this second arm of the RAS, ACE2 through Ang-(1–9) instead of Ang-(1–7), could act as a counterregulator of the first arm, where ACE catalyzes the formation of Ang II.

Besides, in experimental hypertension (DOCA salt model) and in normotensive sham animals, RhoA/Rho-kinase inhibition (a signaling pathway that participates in pathological cardiovascular and renal remodeling and also in blood pressure regulation) by fasudil reduced BP and increased vascular and plasma ACE2 enzymatic activity. At the same time, fasudil reduced Ang II and increased Ang-(1–9) plasma levels (Figure 2) [78]. No modifications were observed here in Ang-(1–7) levels despite increased ACE2 levels with RhoA/Rho-kinase inhibition [78]. Thus, RhoA/Rho-kinase inhibition, by increasing eNOS and/or by reducing both ACE and Ang II, does not activate the Ang-(1–7) pathway. This novel effect of RhoA/Rho-kinase inhibition on both ACE2 expression and Ang-(1–9) levels might additionally contribute to the antihypertensive effects of RhoA/Rho-kinase inhibitors. Besides, these results strongly suggest that in this experimental model, hypertension is more dependent on ACE2 and Ang-(1–9) levels than on ACE and Ang II levels. Therefore, this second RAAS axis through ACE2 and Ang-(1–9) could be an important target for the treatment of hypertension.

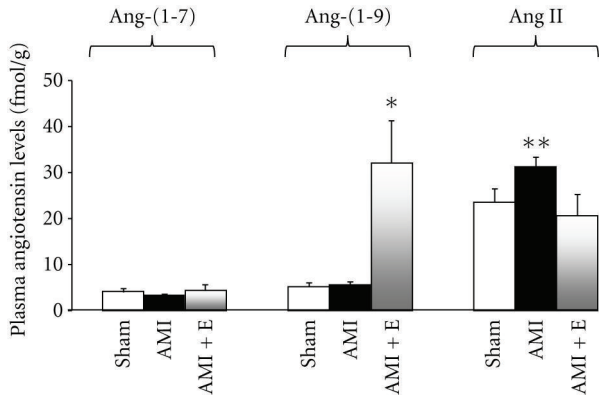

Figure 1: Plasma levels of Ang-(1–7), Ang-(1–9) and Ang II in rats with myocardial infarction treated with the ACE inhibitor enalapril (8 weeks). Increased plasma levels of Ang-(1–9) were observed in rats with myocardial infarction treated with the ACE inhibitor enalapril. Myocardial infarction was induced by coronary artery ligation. Data are presented as mean ± SEM ($n = 12$/group). AMI: acute myocardial infarction, E: enalapril. $^*P < 0.05$ compared to both Sham and untreated myocardial infarction groups; $^{**}P < 0.05$ compared to both Sham and enalapril-treated myocardial infarction groups. (adapted with permission from [51]).

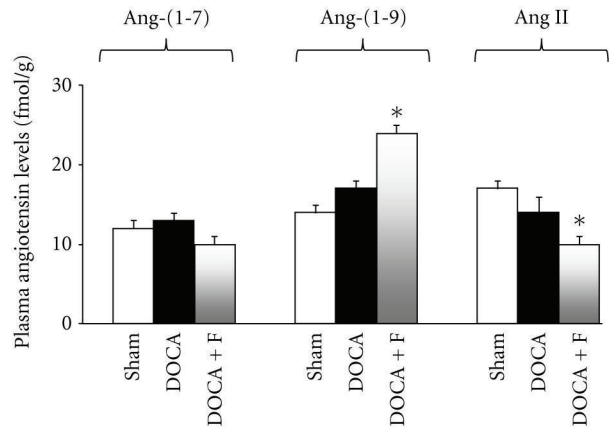

Figure 2: Plasma levels of Ang-(1–7), Ang-(1–9) and Ang II in DOCA salt hypertensive rats treated with the Rho kinase inhibitor fasudil. Increased plasma levels of Ang-(1–9) were observed in DOCA salt hypertensive rats treated with the Rho kinase inhibitor fasudil. Fasudil (100 mh/kg/day) by gavage was administered during 3 weeks, starting on the third week after DOCA administration. Data are presented as mean ± SEM ($n = 8$–11/group). DOCA: deoxycorticosterone, F: fasudil. $^*P < 0.05$ compared to both Sham and untreated DOCA groups (adapted with permission from [78]).

5. Role of ACE2/Ang-(1–9) Axis in Vascular Remodeling

The vascular wall is continuously exposed to hemodynamic forces such as the luminal pressure and shear stress. Changes in these forces, either physiological or pathological, lead to functional and/or structural alterations of the vascular wall [79]. Acute changes in hemodynamic forces can modify vessel diameter. Chronic changes in hemodynamic forces result in structural alterations of the vessel wall, indicated by changes in wall diameter and thickness. In addition, changes in vascular structure are not solely determined by hemodynamic forces [80], but also by inflammatory responses and changes in extracellular matrix components [81]. Structural changes of the medial layer of the vascular wall during hypertension are termed "eutrophic remodeling" [82] and subsequently translate to other vascular pathologies. This involves an inward encroachment of the arterial wall thereby, reducing the diameter of the lumen [83].

Several RAAS components are involved in neointimal formation after vascular endothelial damage [84]. In particular, Rakugi et al. [85] observed that vascular endothelial damage results in the induction of vascular ACE. Their results suggested that inhibition of vascular ACE might be critical in the prevention of restenosis after balloon injury. Patients with previously untreated essential hypertension and eutrophic inward remodeling appears to respond to antihypertensive medication. Reduction in BP with drugs that block the RAAS such as ACEIs [86–88] or ARBs [86, 87, 89] and calcium channel antagonists [90] are able to reverse the eutrophic inward remodeling [88].

The protein and mRNA of ACE2 are expressed in human coronary arteries and arterioles and the vasa vasorum of most organs [9, 91]. Recently, ACE2 expression has also been observed in the large conduit arteries (aorta and carotid) in the HR [92]. ACE2 localizes preferentially in endothelial cells and arterial smooth muscle cells (SMCs) [9, 91]. As for the role of ACE2 in vascular remodeling, the effect of ACE2 on neointima formation has not yet been studied, but Ang-(1–7) infusion after balloon-catheter injury of the rat carotid artery reduced neointima formation [93]. This effect was probably mediated by its inhibition of vascular SMC proliferation [94]. In hypertensive animal models, ACE2 mRNA and protein were associated with immunoreactive Ang-(1–7) in the large conduit arteries of SHRs. Treatment with an ARB induced a fivefold increase in ACE2 mRNA and was associated with a significant increase in aortic Ang-(1–7) protein expression. This effect was associated with a decrease in aortic medial thickness, suggesting that this may be a protective mechanism in the prevention of cardiovascular events during hypertension [94]. Igase et al. [95] showed that ACE2 protein is expressed not only in the media of the carotid artery but also in the neointima of the balloon-injured carotid artery in SHR. The increase in ACE2 protein expression in the neointima following exposure of the rats to an ARB compared to vehicle was associated with a reduction in neointima thickness. These results lead to the hypothesis that there is a strong correlation between the increase in ACE2 protein in the injured carotid artery of SHR and vascular remodeling during blockade of Ang II receptors [95].

There is known the prothrombotic effect of Ang II [96, 97] and the antithrombotic action of Ang-(1–7) [98] in renovascular hypertensive rats. Thus, in this context, the question arises whether Ang-(1–9) effects are similar to Ang II or to Ang-(1–7) in in vivo conditions. Kramkowski et al. [99] described that Ang-(1–9) enhances electrically

stimulated thrombosis in rats and that this effect was abolished by losartan—an antagonist of the AT1 receptor. The prothrombotic activity of Ang-(1–9) was accompanied by the enhancement of ex vivo platelet aggregation and in vitro Ang-(1–9) increased platelet aggregation. However, there are some points in this paper that should be clarified. First, thrombus formation was initiated by electrical stimulation producing arterial injury that is unrelated to a clinical situation. Second, the prothrombotic effect of Ang-(1–9) was much weaker, to the prothrombotic action of Ang II [96, 97]. Third, Ang-(1–9) slightly increased platelet aggregation in in vitro conditions.

On the contrary Ocaranza et al. [78] showed that by inhibiting the RhoA/Rho-kinase pathway with fasudil, gene expression and enzymatic ACE activity and plasma levels of Ang II were reduced (Figure 2) and whereas aortic gene expression and ACE2 activity were importantly increased. Simultaneously, plasma levels of Ang-(1–9) (Figure 2), mRNA eNOS levels increased and the aortic overexpression of the remodeling promotion proteins TGF-β1, PAI-1, and MCP-1 as well as the increased aortic NADPH oxidase activity and O^{2-} production were reduced, as a consequence of direct RhoA/Rho-kinase inhibition [100]. This novel effect of RhoA/Rho-kinase inhibition on ACE2 gene expression, enzymatic activity, and Ang-(1–9) levels might additionally contribute to its benefits in hypertension, atherosclerosis, and in cardiovascular and renal pathologic remodeling. This is the first observation concerning a pharmacologic ACE2 and Ang-(1–9) levels activator, both in normotensive and in hypertensive animals, one of the most interesting findings of that study (Figure 2). Additionally, in experimental hypertension, direct RhoA/Rho-kinase inhibition also normalizes overexpression of genes that promote vascular remodeling. Interestingly, the observed changes in ACE/ACE2 and in Ang-(1–9) levels were present only during fasudil treatment both in sham and in the DOCA hypertensive rats [78]. Thus, vascular remodeling could be more dependent on the tissue ACE2/Ang-(1–9) axis than on Ang-(1–7) levels in normotensive as well as in hypertensive rats.

In vessels, new members of the RAS have been detected, including ACE2, Ang-(1–7) and Mas. Vascular ACE2 is functionally active and generates Ang-(1–7) from Ang II. Ang-(1–7) is found in the endothelium and vascular wall [101–103] and immunohistochemical staining shows abundant presence in aortic perivascular adventitial tissue [104, 105]. Ang-(1–7), by binding to receptor Mas on endothelial cells, opposes Ang II actions by mediating vasodilation, growth-inhibition, antiinflammatory responses, antiarrhythmogenic and antithrombotic effects [33, 68] through NOS-derived NO production, activation of protein tyrosine phosphatases, reduced MAPK activation and inhibition of NADPH oxidase-derived generation of reactive oxygen species (ROS) [106, 107]. Overexpression of ACE2 in the vascular wall of SHR is associated with improved endothelial function and attenuated development of hypertension [68]. Ang-(1–7)-Mas can hetero-oligomerize with AT1R, thereby inhibiting Ang II actions. The ACE2/Ang-(1–7)-Mas axis is now considered as a counter-regulatory system to the ACE-Ang II-AT1R axis in the vasculature [107], although some evidence indicates that Ang-(1–7) may also promote fibrosis and inflammation in certain conditions [108, 109].

6. Role of the ACE2/Ang-(1–9) Axis in Cardiac Remodeling

After myocardial injury or in response to chronically increased hemodynamic load, cardiac mass increases as a result of cardiomyocyte hypertrophy and ventricular wall thickening. Initially these changes are compensatory mechanisms which help to maintain ejection performance and heart function. With continued hemodynamic overload the heart becomes dilated and its walls thinner, resulting in a geometry that contributes to systolic dysfunction by increasing wall stress [110]. At the cellular level, cardiac myocytes increase in size (hypertrophy), rearrange within the myocardial matrix (cell slippage), and die, to be replaced by fibrous tissue, which include fibroblasts and collagen. These changes are collectively referred to as "remodeling" [111]. Cardiac remodeling has been consistently associated with an impaired prognosis in patients with hypertension, MI and chronic heart failure (CHF) [112].

Despite recent advances in our understanding of the ACE2/Ang-(1–7)/axis, the functional role of ACE2 in the heart is somewhat controversial. Crackower et al. [64] originally reported a progressive reduction in LV contractile function in ACE2-null mice without significant changes in fibrosis, left ventricular and cardiac myocyte hypertrophy, or in mean arterial pressure [64]. Interestingly, whereas plasma and tissue levels of Ang II were increased, a decrease in blood pressure was only observed in 6-month-old male ACE−/− homozygote mice but not in age-matched females or 3-month-old males. Conversely, Gurley et al. [113] reported that ACE2 deletion enhanced the susceptibility to Ang II-induced hypertension but had no effect on cardiac structure or function [113]. Huentelman et al. [114] showed that the ACE2 overexpression protects the heart from Ang II-induced hypertrophy and fibrosis. More recently, in SHR hypertensive rats Díez-Freire et al. by using lentiviral-based ACE2 gene transfer, attenuated cardiac fibrosis and hypertrophy [70] and also improved LV and remodeling after experimental MI [115]. Finally, Yamamoto et al. [116] reported that ACE2 deletion exacerbated pressure overload-induced cardiac dysfunction and remodeling that was associated with increased intracardiac Ang II levels and AT1R activation. The reasons for these discrepancies seem to be: (a) the genetic background of the mice used for ACE2 gene deletion [113], (b) global versus tissue-specific ACE2 manipulation, or (c) the cardiac responses were monitored under basal or pathophysiological conditions.

In MI Ocaranza et al. [51] observed that (a) circulating and LV enzymatic activities of ACE2 were downregulated in the long-term phase of LV dysfunction in rats, (b) these effects were prevented by the conventional ACE inhibitor enalapril, (c) plasma Ang-(1–9) levels were significantly increased when MI rats or sham-operated rats were treated with enalapril for 8 weeks but circulating Ang-(1–7) levels did not change at that time (Figure 1) [51]. Based on these

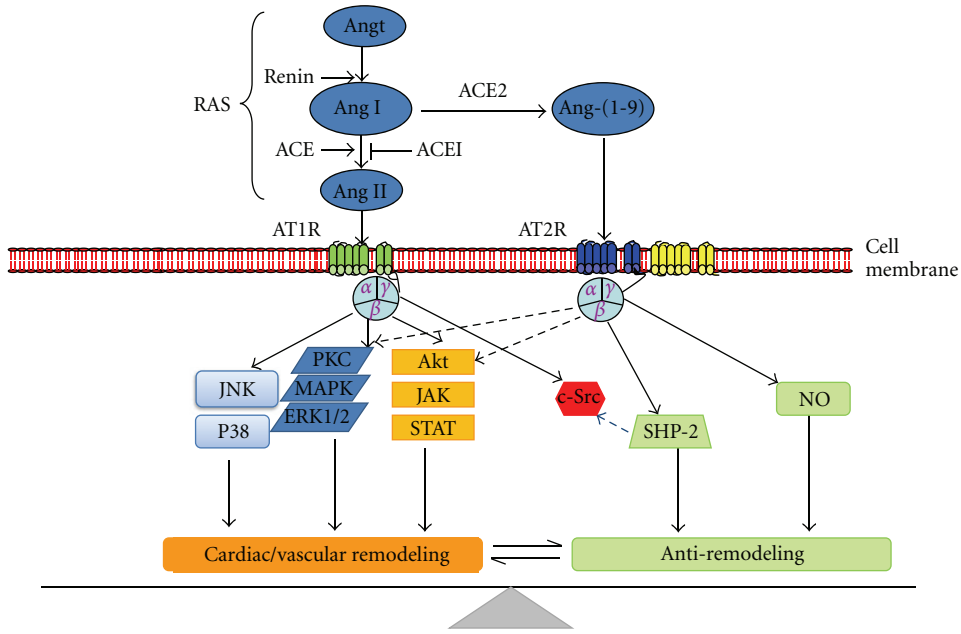

Figure 3: Signaling events and cellular effects induced by Ang II via AT1R and opposing effects of Ang-(1–9) acting through AT2R. Proposed Ang-(1–9)-dependent mechanisms that antagonize the cardiovascular remodeling effects of Ang II. ACE2 can directly cleave Ang I to form Ang-(1–9). This peptide activates the AT2R to initiate signaling pathways that antagonize AT1R-mediated tyrosine kinase cascades. In this simplified scenario, Ang-(1–9) increases SHP-1 tyrosine phosphatase activity to inactivate src-dependent signaling. AT2R activation also acts other pathways such as NO-AKT. AT1R: Ang II type 1 receptor; AT2R: Ang II type 2 receptor; ERK1/2: extracellular signal-regulated kinase 1/2; JAK: Janus-activated kinase; MAPK: mitogen-activated protein kinase; p38: p38 MAPK; PKC: protein kinase C; STAT: signal transducer and activator of transcription; NO: nitric oxide; SHP-1: protein tyrosine phosphatase SH2 domain-containing phosphatase 1; MEK: mitogen/ERK kinase. Solid arrows indicates activation broken arrows indicates inactivation.

findings, it was proposed in this model of HF, that Ang-(1–9) rather than Ang-(1–7) acts as a counterregulator of Ang II [51].

Recently, in MI rats randomized to receive either vehicle, the ACEI enalapril, or the ARB candesartan for 8 weeks, Ocaranza et al. [52] observed that both drugs prevented LVH and increased plasma Ang-(1–9) levels by several folds. Ang-(1–9) levels correlated negatively with different LVH markers with or without adjustment for BP reduction. This effect was specific as neither Ang-(1–7), Ang II nor bradykinins were correlated with LVH. Chronic administration of Ang-(1–9) to MI rats by osmotic minipumps versus vehicle for two weeks decreased plasma Ang II levels, inhibited ACE activity and also prevented cardiac myocyte hypertrophy. Because there are in vitro evidences that the incubation of Ang-(1–9) with ACE generates Ang-(1–7) [9], and Ang-(1–7) negatively regulates hypertrophy [34, 117], the authors used the Ang-(1–7) receptor blocker A779 to investigate whether Ang-(1–7) could mediate the effects of Ang-(1–9). Even though A779 was bioactive, with significant increase in circulating Ang-(1–7) levels by 2.7 fold, this compound did not modify the Ang-(1–9)-dependent suppression of cardiac myocytes hypertrophy induced by MI [52]. In in vitro experiments with cardiac myocytes incubated with norepinephrine (10 μM) or with IGF-1 (10 nM), Ang-(1–9) also prevented hypertrophy and this effect was not modified by the coincubation with Ang-(1–9) and A779 [52].

To further understand the role of Ang-(1–9) compared to Ang-(1–7) in cardiomyocyte hypertrophy, Flores-Muñoz et al. [56] studied Ang-(1–9) effects in rat neonatal H9c2 and in rabbit left ventricular cardiomyocytes. Cardiomyocyte hypertrophy was stimulated with Ang II or vasopressin, significantly increasing cell size by approximately 1.2-fold as well as stimulating expression of the hypertrophy gene markers atrial natriuretic peptide, brain natriuretic peptide, β-myosin heavy chain and myosin light chain (2- to 5-fold). Both Ang-(1–9) and Ang-(1–7) were able to block hypertrophy induced by either agonist. The effects of Ang-1–9) were not inhibited by captopril, supporting previous evidence that Ang-(1–9) acts independently of Ang-(1–7). The authors investigated receptor signalling via angiotensin type 1 and type 2 receptors (AT1R, AT2R) and Mas. The AT1R antagonist losartan blocked Ang II-induced, but not vasopressin-induced, hypertrophy. Losartan did not block the antihypertrophic effects of Ang-(1–9), or Ang-(1–7) on vasopressin-stimulated cardiomyocytes. The Mas antagonist A779 efficiently blocked the antihypertrophic effects of Ang-(1–7), without affecting Ang-(1–9). Furthermore, Ang-(1–7) activity was also inhibited in the presence of the bradykinin type 2 receptor antagonist HOE140, without affecting Ang-(1–9). Moreover, Flores-Muñoz et al. [56] observed that the AT2R antagonist PD123,319 abolished the antihypertrophic effects of Ang-(1–9), without affecting Ang-(1–7), suggesting Ang-(1–9) signals via the AT2R. Radioligand binding assays

demonstrated that Ang-(1–9) was able to bind the AT2R (pKi = 6.28 ± 0.1). The data indicate that ACE2/Ang-(1–9) axis, acting as a counterregulator of Ang II, is an effective, and possibly direct novel anticardiac hypertrophy axis.

7. Conclusions

Pharmacological treatments based on the RAS blockade are used extensively for the treatment of hypertension and CV remodeling. However, in spite of their success in pharmacological blockade of the RAS, the prevalence of end-organ damage has risen steadily in the last several decades. These observations indicate that novel and innovative approaches must be used in an attempt to promote a more effective treatment for the residual CV remodeling. In this environment, the ACE2/Ang-(1–9) axis is an important target, that is critical in tipping the balance of vasoconstrictive/proliferative to vasodilatory/antiproliferative axis of the RAS. Conceptually, the ACE2/Ang-(1–9)/AT2 axis balances the adverse effects of the ACE-Ang II-AT1 receptor axis (Figure 3). Accumulating evidence suggests that ACE2 expression and Ang-(1–9) levels are altered in diastolic and systolic dysfunction and remodeling and the activation of the ACE2/Ang-(1–9) axis protects the heart and vessels from cardiovascular remodeling. In conclusion, the noncanonical RAS arm has new biological effector Ang-(1–9) to counterregulate the classical RAS.

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Research Article

Differential Mechanisms of Ang (1-7)-Mediated Vasodepressor Effect in Adult and Aged Candesartan-Treated Rats

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Angiotensin (1-7) (Ang (1-7)) causes vasodilator effects in Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs) via angiotensin type 2 receptors (AT_2R). However, the role of vascular AT_2R in aging is not known. Therefore, we examined the effect of aging on Ang (1-7)-mediated vasodepressor effects and vascular angiotensin receptor localization in aging. Blood pressure was measured in conscious adult (~17 weeks) and aged (~19 months) normotensive rats that received drug combinations in a randomised fashion over a 4-day protocol: (i) Ang (1-7) alone, (ii) AT_1R antagonist, candesartan, alone, (iii) Ang (1-7) and candesartan, or (iv) Ang-(1-7), candesartan, and the AT_2R antagonist, PD123319. In a separate group of animals, the specific *Mas*R antagonist, A779, was administered in place of PD123319. Receptor localisation was also assessed in aortic sections from adult and aged WKY rats by immunofluorescence. Ang (1-7) reduced blood pressure (~15 mmHg) in adult normotensive rats although this effect was dependant on the background dose of candesartan. This depressor effect was reversed by AT_2R blockade. In aged rats, the depressor effect of Ang (1-7) was evident but was now inhibited by either AT_2R blockade or *Mas*R blockade. At the same time, AT_2R, *Mas*R, and ACE2 immunoreactivity was markedly elevated in aortic sections from aged animals. These results indicate that the Ang (1-7)-mediated depressor effect was preserved in aged animals. Whereas Ang (1-7) effects were mediated exclusively via stimulation of AT_2R in adult WKY, with aging the vasodepressor effect of Ang (1-7) involved both AT_2R and *Mas*R.

1. Introduction

It is well known that Angiotensin II (Ang II) mediates its physiological functions via two main receptor subtypes, the type 1 (AT_1R) and type 2 (AT_2R) angiotensin receptors where it has similar affinity for both the AT_1R and AT_2R. However, there is now increasing evidence suggesting that angiotensin peptides other than Ang II can evoke cardiovascular effects that oppose the effects mediated by the AT_1R via a number of non-AT_1R mechanisms. In fact, heptapeptide Angiotensin (1-7), (Ang (1-7)), a biologically active metabolite of angiotensin I (Ang I) and Ang II [1, 2] has been shown to possess biological activity in its own right [3]. Interest in Ang (1-7) has surged since the discovery of angiotensin converting enzyme type 2 (ACE2) and recognition that Ang (1-7) can be produced directly from Ang II via ACE2 [1, 2]. Although, Ang (1-7) differs to Ang II by only one amino acid, Ang (1-7)-mediated effects are markedly different to those of Ang II, and it has been suggested that Ang (1-7) may in fact play a counterregulatory role to Ang II [4], mediating a range of effects such as vasodilatation, inhibition of vascular smooth muscle proliferation, and fluid and electrolyte homeostasis [5]. The cardiovascular effects of Ang (1-7) are often reported to be inhibited by the D-Ala^7 Ang (1-7) analogue, known as A779 [6]. Recently, Ang (1-7) was identified as an endogenous ligand for the Ang (1-7)/*Mas*R (*Mas*R), since Ang (1-7)-mediated vasorelaxation was impaired in $MasR^{-/-}$ mice [7]. However, under some circumstances, Ang (1-7) can mediate its effects via AT_2R [8–10]. In fact, we have shown that Ang (1-7)-mediated vasodepressor effect was via an AT_2R sensitive pathway [11]. In that study, Ang (1-7) acutely lowered blood pressure in spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) adult rats during concomitant AT_1R blockade, [11] in a similar manner to that seen with AT_2R agonist, CGP42114 [12, 13], and more recently with selective nonpeptide AT_2R agonist,

Table 1: Resting MAP recorded on separate days before drug treatments, as indicated.

Treatment	MAP (mmHg)
Group 1 ($n = 4$)	
Saline	132 ± 14
Ang-(1-7) (15 pmol/kg/min)	124 ± 4
Candesartan (0.01 mg/kg)	131 ± 6
Ang-(1-7) and candesartan	134 ± 4
Group 2 ($n = 7$)	
Saline	131 ± 13
Ang-(1-7) (15 pmol/kg/min)	136 ± 13
Candesartan (0.01 mg/kg)	126 ± 15
Ang-(1-7) and candesartan	136 ± 16
Group 3 ($n = 6$)	
Ang-(1-7) (15 pmol/kg/min)	138 ± 8
Candesartan (0.1 mg/kg)	135 ± 8
Ang-(1-7) and candesartan	139 ± 11
Ang-(1-7), candesartan, and PD123319 (50 μg/kg/min)	139 ± 5
Group 4 ($n = 7$)	
Ang-(1-7) (15 pmol/kg/min)	143 ± 10
Candesartan (0.1 mg/kg)	142 ± 10
Ang-(1-7) and candesartan	142 ± 9
Ang-(1-7), candesartan, and PD123319 (50 μg/kg/min)	137 ± 9
Group 5 ($n = 8$)	
Ang-(1-7) (15 pmol/kg/min)	128 ± 11
Candesartan (0.1 mg/kg)	122 ± 10
Ang-(1-7) and candesartan	133 ± 5
Ang-(1-7), candesartan, and A779 (15 pmol/kg/min)	132 ± 2
Group 6 ($n = 8$)	
Ang-(1-7) (15 pmol/kg/min)	129 ± 5
Candesartan (0.01 mg/kg)	126 ± 10
Ang-(1-7) and candesartan	132 ± 6
Ang-(1-7), candesartan, and A779 (15 pmol/kg/min)	125 ± 8

Values are ± SEM.

Compound 21 [14]. Furthermore, the AT_2R antagonist, PD123319, but not the *Mas*R antagonist, A779, blocked this vasodepressor effect of Ang (1-7) [11].

While it is well recognized that the renin-angiotensin system (RAS) has a critical role in the cardiovascular system; its role in the aging process is still under investigation**.** During aging, circulating levels of Ang II are downregulated while local production of Ang II is increased in the aorta and other vessels [15] suggesting an essential role of local RAS in the vasculature during aging. However, there is little functional evidence about angiotensin receptors and their role during aging. In this context, we have shown that AT_2R expression was increased in both endothelial and vascular smooth muscle of aortae obtained from aged WKY rats [16].

Given that there was an increased vascular AT_2R expression in aging [16], the current study was designed to test our hypothesis that AT_2R-mediated depressor function was preserved with aging. In the present study, we have used Ang (1-7) as an endogenous ligand for the AT_2R, as we have previously reported in adult rats [11]. In preliminary experiments, we have determined that vascular expression of both AT_2R and *Mas*R/ACE2 axis was upregulated with aging. Therefore, this strategy of using Ang (1-7) will also determine whether or not there was a role for *Mas*R to evoke vasodepressor effects with aging.

2. Methods

2.1. Animals. All animal care and experimental procedures were approved by the Monash University Animal Ethics Committee and performed according to the guidelines of the National Health and Medical Research Council of Australia for animal experimentation.

16- to 18-week-old WKY male rats (300 to 350 g) and 20-month-old WKY male rats (450–500 g) were obtained from the Animal Resource Centre (Perth, Wash, USA) and were used to represent adult and aged normotensive rats, respectively. Animals were maintained on a 12-hour day/night cycle with standard laboratory rat chow and water available *ad libitum*.

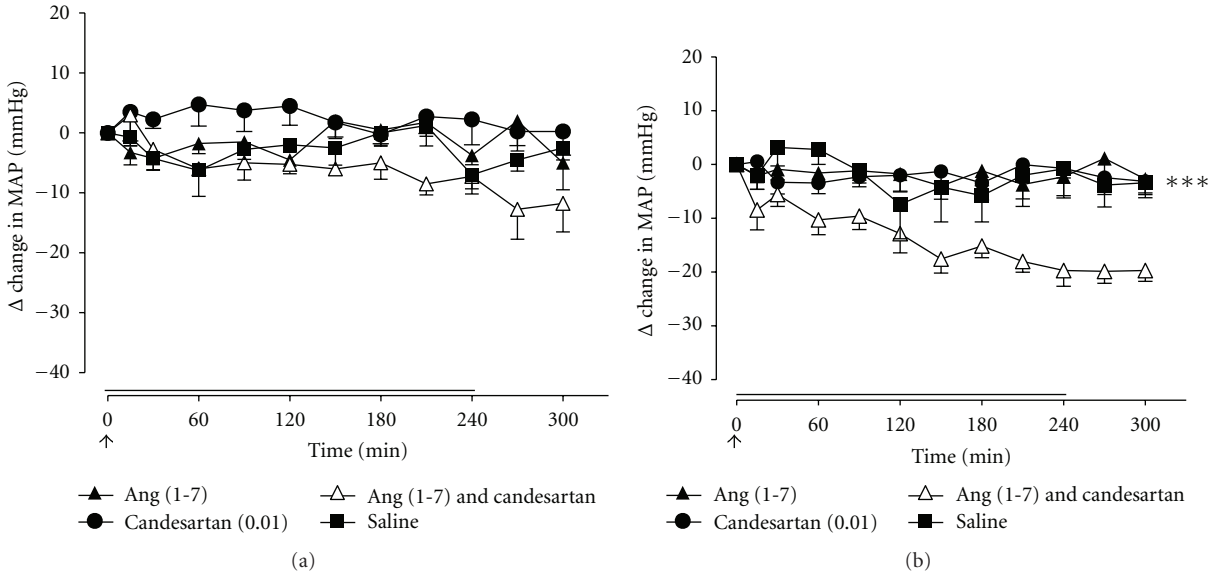

Figure 1: Effect of the AT_1R Ang (1-7) (15 pmol/kg/min; 4-hour infusion depicted by full line), AT_1R antagonist, candesartan (0.01 mg/kg bolus IV; depicted by an arrow), saline (0.1 mL/kg 0.9% NaCl IV for 4 hours), and Ang (1-7) + candesartan on MAP in (a) adult WKY rats ($n = 4$) and (b) aged WKY rats ($n = 7$). Values represent mean ± SEM. $^{***}P < 0.001$, for treatment effect of Ang (1-7) + candesartan versus all other treatments (2-way RM ANOVA).

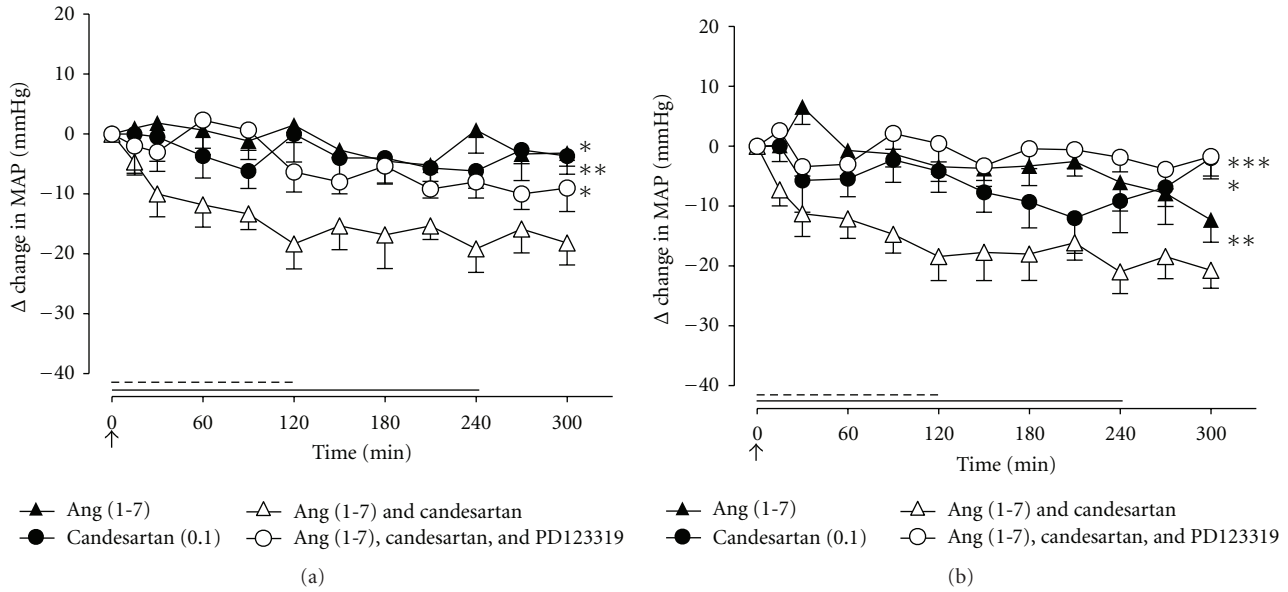

Figure 2: Effect of Ang (1-7) (15 pmol/kg/min; 4-hour infusion depicted by full line), AT_1R antagonist, candesartan (0.1 mg/kg bolus IV; depicted by an arrow), Ang (1-7) + candesartan, and Ang (1-7) + candesartan + AT_2R antagonist, PD123319 (50 μg/kg/min for 2 hours; depicted by dashed line), on MAP in (a) adult WKY rats ($n = 6$) and (b) aged WKY rats ($n = 7$). Values represent mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, for treatment effect of Ang (1-7) + candesartan versus all other treatments as indicated (2-way RM ANOVA).

2.2. In Vivo Procedures. Rats were anesthetised (ketamine and xylazine; 75 mg/kg and 10 mg/kg, i.p, resp.; supplemented as required). Two catheters were inserted into the right jugular for intravenous drug administration. A catheter was implemented into the right carotid artery for direct blood pressure measurement as described previously [11–14]. Rats were housed in individual cages and allowed free access to food and water while maintained on 12-hour day/night cycle. The arterial catheter was infused overnight with heparinised saline using an infusion pump.

24 hours after the surgery, the arterial catheter was attached to a pressure transducer (Gould Inc), connected to a MacLab-8 data acquisition system (ADInstruments, Sydney), interfaced to a Macintosh computer. Mean arterial pressure

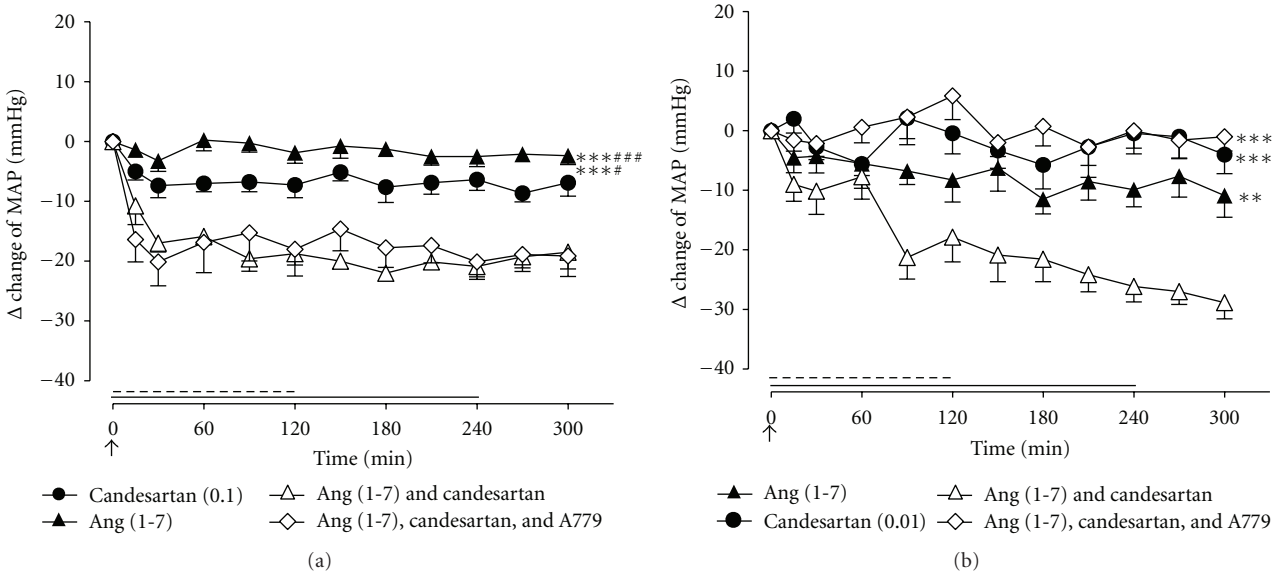

Figure 3: Effect of Ang (1-7) (15 pmol/kg/min; 4-hour infusion depicted by full line), AT_1R antagonist, and candesartan in (a) adult WKY rats (0.1 mg/kg bolus IV; depicted by an arrow) and (b) aged WKY rats (0.01 mg/kg bolus IV; depicted by an arrow), together with Ang (1-7) + candesartan and Ang (1-7) + candesartan + *Mas*R antagonist A779 (15 pmol/kg/min for 2 hours; depicted by dashed line), on MAP ($n = 8$ for both groups). Values represent mean ± SEM. (a) $^{***}P < 0.001$, for treatment effect of Ang (1-7) + candesartan versus Ang (1-7) or candesartan alone (2-way RM ANOVA), † <0.05; ††† <0.001, for treatment effect of Ang (1-7) + candesartan +A779 versus candesartan or Ang (1-7) alone (2-way RM ANOVA). (b) $^{**}P < 0.01$; *** <0.001 for treatment effect of Ang (1-7) + candesartan versus Ang (1-7), candesartan, or Ang (1-7) + candesartan + A779 (2-way RM ANOVA).

(MAP) and heart rate (HR) were computed from the phasic blood pressure signal.

2.3. Experimental Protocol. Rats received drug combinations in a randomised fashion over a 4-day protocol, as we have performed previously [11, 12, 14]. Doses for candesartan and PD123319 were chosen on the basis of previous studies [11, 12, 14]. Six groups of rats underwent experimental protocols during which basal MAP and HR were recorded. Adult and aged WKY rats (Groups 1 and 2, resp.) were randomized to receive the following treatments on different days: (1) candesartan (0.01 mg/kg), (2) Ang (1-7) infusion (15 pmol/kg per minute for 4 hours), (3) Ang (1-7) infusion together with candesartan, and (4) a 4-hour infusion (0.1 mL/kg per hour IV) of saline (0.9% NaCl) to confirm a lack of effect on MAP. Animals in Group 3 (adult WKY rats) and Group 4 (aged WKY rats) were randomized to receive the following treatments: (1) candesartan at a 10-fold higher dose (0.1 mg/kg), (2) Ang (1-7) infusion (15 pmol/kg per minute for 4 hours), (3) Ang (1-7) infusion together with candesartan, and (4) Ang (1-7) infusion in the presence of candesartan and PD123319 infusion (50 μg/kg per minute for 2 hours). In analogous experiments in additional adult and aged WKY rats (Groups 5 and 6), the putative Ang (1-7) antagonist, A779 (15 pmol/kg per minute), was used instead of PD123319. Doses of Ang (1-7) and A779 are based on our previous study [11].

2.4. Localization of ACE2, AT_1, AT_2, and Mas Receptors. Localization of ACE2, AT_1, AT_2, and *Mas* receptors using immunofluorescence was performed using thoracic aortic sections taken from naïve aged and adult rats to determine changes in expression levels between the two age groups. Male adult and aged WKY rats were killed by isoflurane inhalation followed by decapitation, and the thoracic aorta was removed in order to dissect 3–5 mm long sections. Immunofluorescence was performed using 10 μm thick section of thoracic aorta cut on Cryostat. Aortic sections were incubated overnight at 4°C with 1/500 dilution of polyclonal rabbit antibodies raised against AT_1R, AT_2R, *Mas*R, and ACE2. Following overnight incubation, sections were incubated for 2.5 hour with a goat anti-rabbit secondary antibody conjugated with Alexa 568 flurophore. Rabbit IgG antibody was used as negative control. Sections were mounted with antifade medium (VectorShield) and cover slipped. Sections were imaged using Olympus Fluoview 500 confocal microscope equipped with a krypton/argon laser. Fluorescence intensity was quantified using analysis professional software (Soft Imaging System, Singapore) with identical measurement settings.

2.5. Statistical Analysis. All data are presented as mean responses ± standard error of the mean (SEM). Differences in MAP between treatments were analysed using a 2-way ANOVA repeated measure analysis of variance. Differences in fluorescence intensity were analysed using 1-way ANOVA with Bonferroni corrections where appropriate. Statistical analysis was performed using GraphPad Prism (Version 5.0c). P values <0.05 were deemed statistically significant.

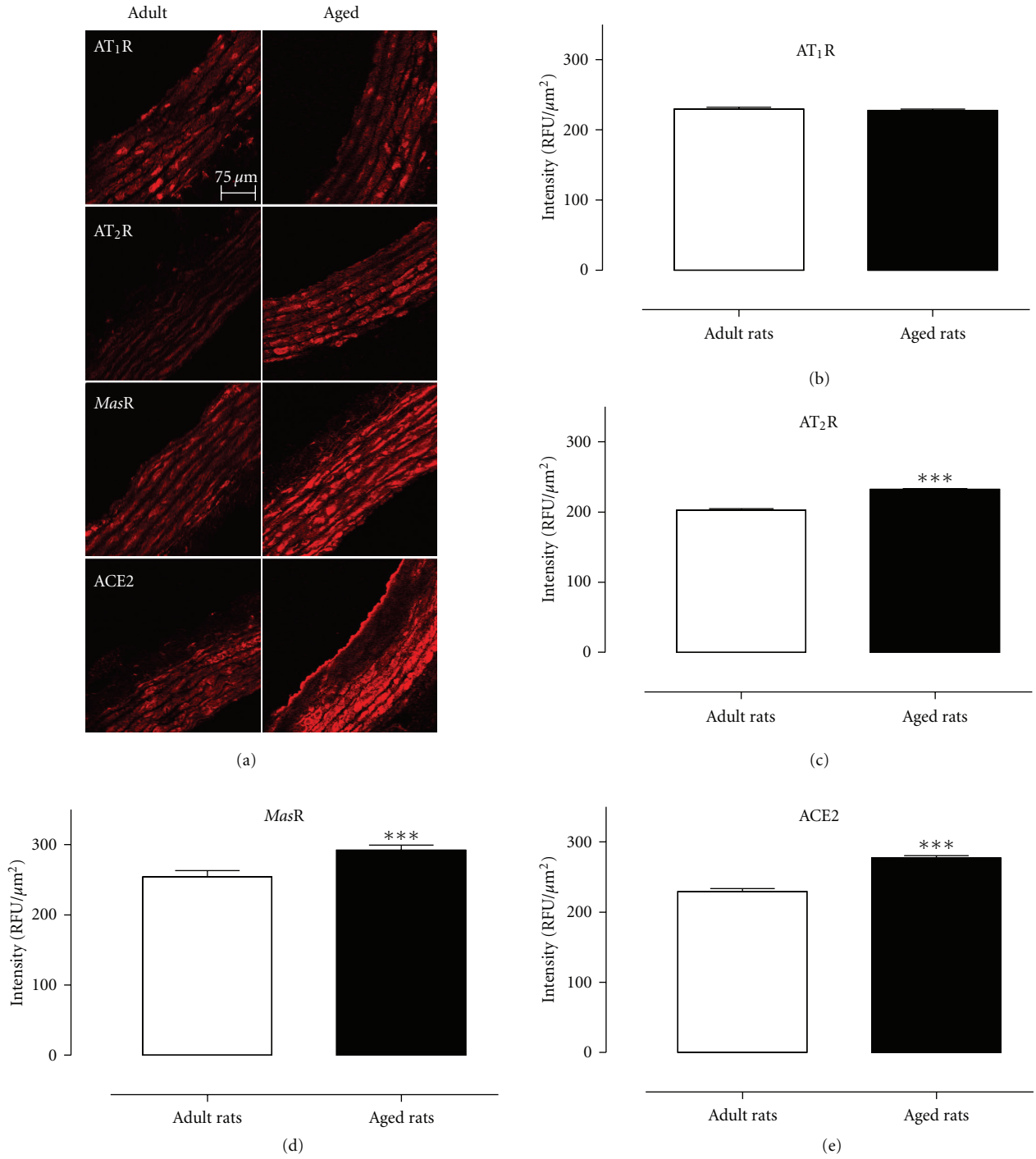

FIGURE 4: (a) Representative immunolocalisation images of AT_1R, AT_2R, *Mas*R, and ACE2 in adult WKY rats and aged WKY rats. Mean data for aortic expression of the (b) AT_1R, (c) AT_2R, (d) *Mas*R, and (e) ACE2 expressed as relative fluorescent units in adult ($n = 5$) and aged ($n = 4$) WKY rats. $^{***}P < 0.001$ versus adult WKY rats.

2.6. Materials. PD123319 and candesartan were kind gifts from Pfizer and AstraZeneca, respectively. All other chemicals were purchased from commercial sources: ketamine (Troy Laboratories, Australia), xylazine (Troy Laboratories), isoflurane (Baxter, USA), Ang (1-7) (Ausep, Australia), A779 (Auspep, Australia), rabbit polyclonal antibodies raised against AT_1R, AT_2R, and ACE2 (Santa Cruz Biotechnology Inc., Catalogue no. SC1173, SC9040, and SC2099), rabbit polyclonal antibody raised against *Mas*R (Novus Biologicals, USA, Catalogue no. NLS1531), secondary goat anti-rabbit Alexa 568 antibody (Invitrogen, USA, Catalogue no. A-21069).

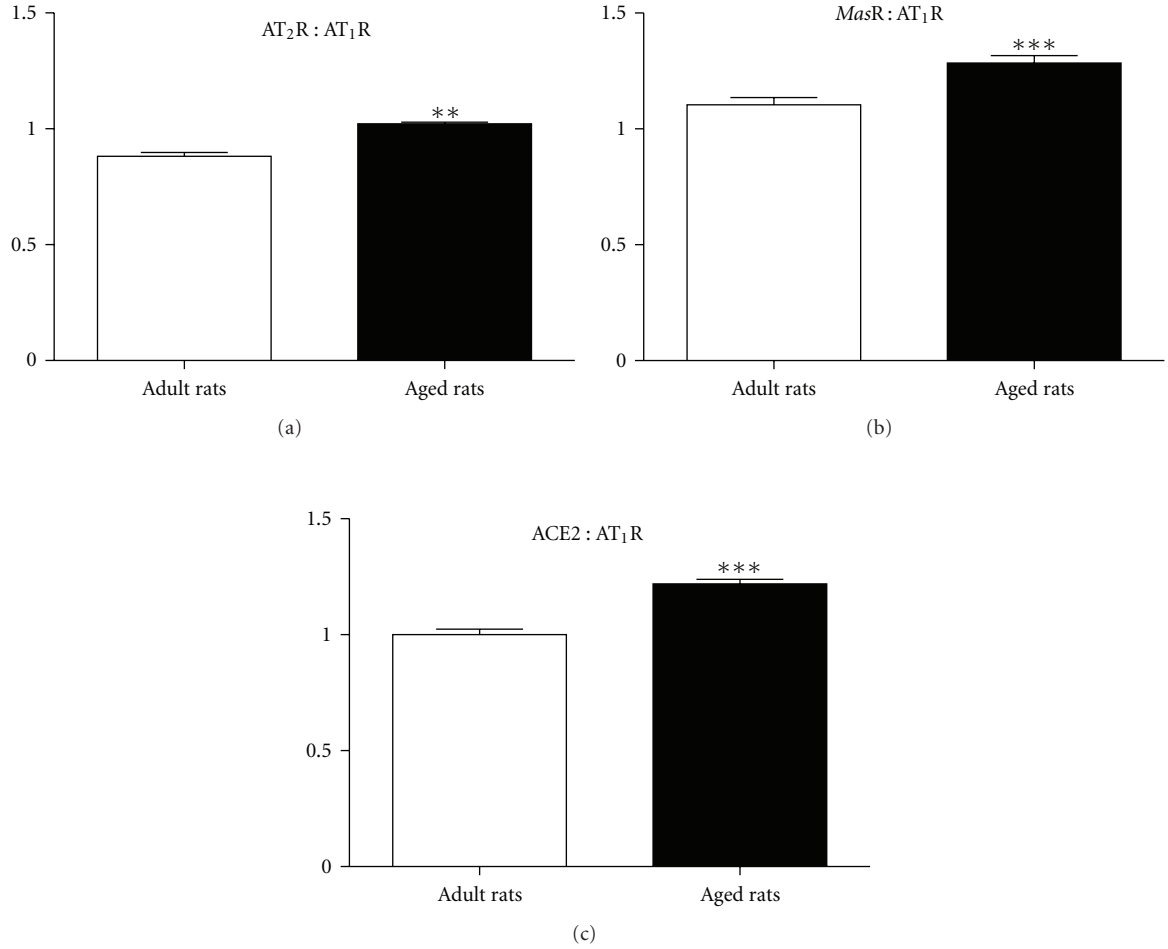

Figure 5: Ratios of (a) $AT_2R : AT_1R$, (b) *Mas*R : AT_1R, and (c) ACE2 : AT_1R in adult and aged WKY rats. $^{**}P < 0.01$; $^{***}P < 0.001$ versus adult WKY rats.

3. Results

3.1. In Vivo Effect of Ang (1-7) in Conscious Normotensive Rats. Basal MAPs of WKY rats over the 4 experimental days for each group are listed in Table 1. There was no significant difference between resting MAPs over the experimental period for any of the treatment groups, suggesting that none of the acute treatments had long-lasting effects and, therefore, did not influence baseline MAP on subsequent days.

In groups 1 and 2, infusion of saline had no significant effect on MAP (Figure 1). Therefore, this treatment was not performed in subsequent groups in order to include additional treatment arms. In all groups, infusion of Ang (1-7) (15 pmol/kg/min) or candesartan (0.01 or 0.1 mg/kg IV) had no significant effect on MAP. Coinfusion of Ang (1-7) and candesartan (0.01 mg/kg IV) had no effect on MAP in adult WKY rats (Figure 1(a)) whereas, in aged WKY rats, combined administration of Ang (1-7) and candesartan (0.01 mg/kg IV) significantly decreased MAP ($P < 0.001$) (Figure 1(b)). When Ang (1-7) was combined with a 10-fold higher dose of candesartan (0.1 mg/kg IV), there were significant reductions in MAP in both adult and aged WKY male rats compared with Ang (1-7) alone or candesartan alone ($P < 0.01$). Moreover, this depressor effect of Ang (1-7) was abolished by the addition of the AT_2R antagonist, PD123319 (50 μg/kg/min), (Figures 2(a) and 2(b)).

In separate groups of animals, we examined the ability of the *Mas*R antagonist A779 to modify the Ang (1-7)-mediated depressor effect. Coinfusion of the Ang (1-7) antagonist A779 with the Ang (1-7)/candesartan combination in adult WKY male rats did not affect Ang (1-7)-mediated depressor response (Figure 3(a)). By contrast, the Ang (1-7)-evoked depressor response, during AT_1R blockade, in aged WKY rats was in fact abolished by the addition of A779 (Figure 3(b)).

3.2. Localization of ACE2, AT_1, AT_2, and Mas Receptors. Expression levels of ACE as well as angiotensin levels were determined using thoracic sections taken from naïve adult WKY rats ($n = 5$) and aged WKY rats ($n = 4$). ACE2, AT_1R, AT_2R, and *Mas*R were all localised throughout the entire aortic sections (Figure 4(a)). Expression levels of the AT_1R were not changed between adult and aged WKY male rats, whereas ACE2, AT_2R, and *Mas*R expression levels were

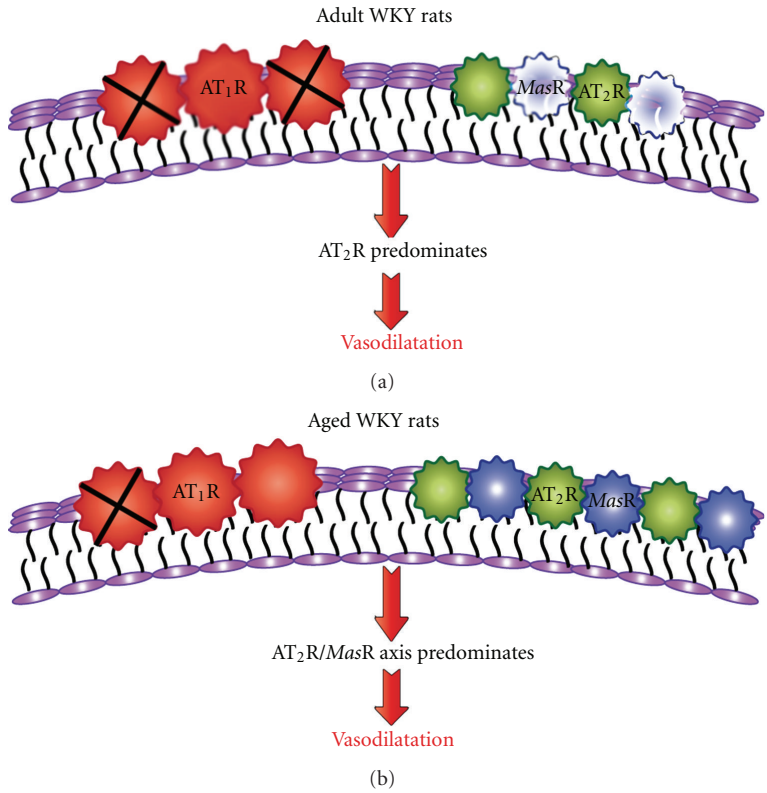

Figure 6: Schema depicting differential mechanisms of Ang (1-7)-mediated vasodepressor effect in adult and aged candesartan-treated rats. AT_1R expression was similar in aortae from adult and aged rats whereas there was upregulation of AT_2R, MasR, and ACE2. Therefore, a lower level of AT_1R blockade with candesartan (**X**) was required in aged animals (b) compared with adult animals (a) in order to unmask the vasodilator axis. MasR was not functionally active in adult rats.

all significantly upregulated in aged WKY rats compared to adult WKY rats (Figures 4(b)–4(e)). Therefore, when expressed relative to AT_1R levels, each of the vasodilator non-AT_1R components of the RAS was significantly increased in aged WKY rats compared to adult WKY rats (Figure 5).

4. Discussion

The main findings of the current study demonstrate for the first time that the depressor effect evoked by Ang (1-7) is preserved in aged normotensive candesartan-treated animals and was sensitive to both AT_2R and *Mas*R blockade which contrasts with the involvement of only AT_2R in the effects of Ang (1-7) in adult candesartan-treated rats. Moreover, these findings were consistent with increased AT_2R, *Mas*R, and ACE2 expression in the thoracic aorta of aged WKY rats.

AT_2R-mediated relaxation is a well-established effect in isolated resistance vessels [17–21]. Previous studies have shown AT_2R-mediated vasodilatation in adult conscious rats [11–14, 22]. The AT_2R-mediated reduction in blood pressure was likely to be a result of direct vasodilatation, rather than a result of decrease in cardiac output, as CGP42112 increased mesenteric and renal conductance in SHR, which was indicative of regional vasodilatation [13]. Furthermore, it is well documented that, in order to unmask any AT_2R-mediated vasodilatation, there needs to be a removal of a tonic AT_1R-mediated vasoconstriction induced by endogenous Ang II [23].

In the current study, acute Ang (1-7) infusion against a background of AT_1R blockade resulted in a decrease in MAP in adult WKY male rats, and this Ang (1-7) response was mediated exclusively via AT_2R in adult WKY male rats since the AT_2R antagonist, PD123319, abrogated this Ang (1-7)-depressor response, which is consistent with previous findings obtained in both SHR and WKY rats [11]. Of note, the Ang (1-7) antagonist, A779, failed to inhibit vasodepressor responses induced by Ang (1-7) during AT_1R blockade in adult rats, which confirmed our previous study that also found a 10-fold higher dose of A779 failed to block Ang (1-7) [11]. Thus, at least in this adult model, an exclusive role for Ang (1-7) as an endogenous ligand for the AT_2R was demonstrated.

In contrast, in the aged setting, the vasodepressor effect of Ang (1-7) was mediated by both AT_2R and MasR stimulation. Moreover, both candesartan doses (0.01 and 0.1 mg/kg) were effective in unmasking Ang (1-7)-mediated vasodepressor responses in aged rats. These results are consistent a 10-fold lower dose of candesartan being used to reveal Ang (1-7)-mediated vasodepressor effects via AT_2R in SHR compared with WKY rats [11] and point towards an increased sensitivity to AT_1R blockade in aged rats, as

we have noted previously [24]. Increased vascular expression of AT_2R in aging was seen in mesenteric resistance arteries [25] and in thoracic aorta [16]. Thus, Ang (1-7) infusion reduced MAP via AT_2R in aged WKY rats irrespective of the background dose of candesartan. However, there are numerous reports suggesting the *Mas*R as the functional binding site for Ang (1-7) [7]. For example, Peiro et al. (2007), observed comparable impairment in Ang (1-7)-mediated vasorelaxation as a result of pharmacologic or genetic inhibition of MasR using A779 and MasR-deficient mice, respectively [26]. However, Ang (1-7) evoked vasorelaxation in pig coronary arteries that was attenuated by the AT_2R antagonist, PD123319, suggesting an AT_2R involvement [27]. Subsequent studies confirmed that Ang (1-7) can mediate its effects via AT_2R [8–10]. Ang (1-7)-stimulated NO release in bovine aortic endothelial cells was markedly attenuated by AT_2R inhibition (~90%) [28, 29] and to a lesser extent by *Mas*R inhibition (~50%) [28], suggesting activation of multiple receptors by Ang (1-7) which is also consistent with Ang (1-7)-stimulated arachidonic acid release in rabbit vascular smooth muscle cells [30].

More recently, we have demonstrated that chronic treatment with Ang(1-7) was both vaso- and atheroprotective in Apolipoprotein E-deficient mice via both *Mas*R and AT_2R [31]. Similarly, in the current study, we found that Ang (1-7) evoked a vasodepressor responses in aged candesartan-treated rats that was sensitive to both the AT_2R antagonist PD123319 and the *Mas*R antagonist A779. This finding suggests that, unlike that in adult normotensive rats, Ang (1-7) can act via AT_2R and/or MasR during aging. Therefore, we also examined relative expression levels of the AT_1R, AT_2R, and MasR as well as ACE2 to determine if this could account for the age-related differences in the cardiovascular effects of Ang (1-7). We have now confirmed an increased AT_2R expression in aortae from aged WKY rats [16], and in addition we have shown, for the first time, a marked increase in expression levels of both *Mas*R and ACE2 in aortic sections from aged WKY rats. Future studies will need to confirm these findings using RT-PCR. These changes in ATR subtype expression fit with our *in vivo* results and also with other evidence for increased AT_2R function in aging. For example, PD123319 can potentiate AT_1R-mediated contractions, which is an indirect measure of AT_2R relaxation [32, 33], and this "PD123319 potentiation" was enhanced in human coronary microvessels and was positively correlated with age [34]. To our knowledge, there are no reported functional correlates for enhanced Ang (1-7) in aging. At the same time, there was no difference in the expression levels of AT_1R between adult and aged WKY rats, although a lower level of the AT_1R block was required to unmask the depressor effect of Ang (1-7) in aged rats. One possible explanation for this difference between aged and adult WKY rats is due to the presence of several potential vasodilator pathways (AT_2R, *Mas*R) resulting in preserved vasodilatation in aged WKY rats. This hypothesis is strengthened by the increased ratio of non-AT_1R components to AT_1R in aged WKY rats (Figure 6).

In conclusion we have found that Ang (1-7)-mediated vasodepressor activity is preserved with aging. Thus, we can postulate that an increased AT_2R/MasR/ACE2 vasodilator axis relative to AT_1R in aged rats is in part responsible for the ability of Ang (1-7) to operate via multiple mechanisms in aging, as opposed to only AT_2R in adult normotensive candesartan-treated rats.

Acknowledgments

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Review Article

Biosynthetic Pathways and the Role of the Mas Receptor in the Effects of Angiotensin-(1–7) in Smooth Muscles

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Ang-(1–7) is produced via degradation of Ang II by the human angiotensin converting enzyme, also known as ACE2. In the cardiovascular system, Ang-(1–7) has been shown to produce effects that are opposite to those of Ang II. These include smooth muscle relaxation and cardioprotection. While the roles of Ang-(1–7) in other systems are currently topic of intense research, functional data suggest a relaxation action in gastrointestinal smooth muscles in a way that corroborates the results obtained from vascular tissues. However, more studies are necessary to determine a relevant role for Ang-(1–7) in the gastrointestinal system. The Ang-(1–7) actions are mediated by a distinct, functional, Ang-(1–7) receptor: the *Mas receptor* as shown by diverse studies involving site-specific binding techniques, selective antagonists, and targeted gene deletion. This paper provides an overview of the functional role and the molecular pathways involved in the biosynthesis and activity of Ang-(1–7) in diverse systems.

1. A Brief Historical Overview

Until the late 80s, it was thought that most of the biological activity of angiotensin peptides was based on their interaction with the AT_1 receptor at the C-terminal side chain of a phenylalanine residue in the position 8 (Phe^8) [1, 2]. Consequently, it was assumed that fragments of Ang II lacking the C-terminal Phe^8 were biologically inactive [3]. A number of studies have shown that the N-terminal heptapeptide angiotensin-(1–7) [Ang-(1–7)], also named as des-[Phe^8]-angiotensin II [4], lacked vasopressor effect [5, 6], aldosterone release activity [5], and central dipsogenic action [7].

However, in 1988, Santos and coworkers showed that Ang-(1–7) was produced as the main metabolite of angiotensin I (Ang I) in dog brainstem and spinal cord, which is produced even in the presence of angiotensin-converting enzyme (ACE) inhibitors, suggesting an ACE-independent route [8]. Further studies have shown that Ang-(1–7) stimulates arginine vasopressine (AVP) release from the rat hypothalamo-neurohypophysial system (HNS) with potency comparable to angiotensin II (Ang II) [9]. These findings triggered general scientific interest in the area with a series of studies involving site-specific, functional antagonism, and targeted-gene deletion that, among other techniques, have resulted in the identification of ACE2 and of the *Mas* receptor as the main agents responsible for the biosynthesis and actions of Ang-(1–7) at the molecular level.

This paper provides an overview of the molecular pathways involved in the biosynthesis and activity of Ang-(1–7) in the vascular and gastrointestinal systems with emphasis on their smooth muscle structures, and the limited availability of such information in the gastrointestinal tract.

2. Biosynthesis and Degradation of Ang-(1–7)

ACE2 is the enzyme responsible for the biosynthesis of Ang-(1–7). ACE2 is a membrane-associated zinc metalloprotease and a homologue of the human ACE isoforms, which is highly expressed in several tissues such as human heart, kidney, lungs, and testis [10, 11]. There is also evidence for the presence of ACE2 in smooth muscles: in the cardiovascular system, studies have shown the expression of ACE2 in the media of thoracic aorta and common carotid arteries from spontaneously hypertensive rats (SHRs) [12]. In line with these studies, ACE2 has also been shown to be expressed in vascular smooth muscle cells (VSMCs) isolated from rat aorta [13, 14], and in renal and mesenteric arteries from spontaneously hypertensive stroke-prone rats (SHRSP) [15]. In the gastrointestinal system, studies suggest a role for local biosynthesis of Ang-(1–7). These include an inhibitory effect in the basal tone of the internal anal sphincter [16] and the identification of ACE2 mRNA in the stomach wall of rats [17]. In addition, studies using quantitative real-time polymerase chain reaction (QRT-PCR) have made the novel observation that ACE 2 shows levels of expression in the gastrointestinal system that are comparable to those in the cardiovascular system. Particularly high levels of ACE2 have been found in duodenum, jejunum, ileum, caecum, and colon. Therefore, consideration should also be given to a potential role for ACE2 in gastrointestinal physiology and pathophysiology [18]. However, other than this, there is relatively scarce information about formation and actions of Ang-(1–7) in the gastrointestinal system.

Despite its homology with ACE, ACE2 is functionally different as it acts as a C-terminal carboxypeptidase rather than a C-terminal dipeptidase by cleaving the C-terminal of a residue of Leu in the structure of Ang I or a residue of Phe in Ang II. This activity, respectively, generates angiotensin-(1–9) [Ang-(1–9)] [10, 11] or Ang-(1–7) [11, 19].

There are also pharmacological differences between ACE and ACE2. ACE2 is insensitive to classic ACE inhibitors such as lisinopril [10, 11], enalaprilat, or captopril [11] as well as other ACE inhibitors [20]. The differential sensitivity to ACE inhibitors results from amino acid substitutions in the substrate binding site from ACE2. Studies show that ACE2 has an Arginine (Arg) residue in the position 273 instead of the Glutamine (Gln) residue normally found in ACE [21]. This substitution allosterically impairs the interaction between ACE2 and classic ACE inhibitors because of the larger size of Arg^{273}, which limits the size of the S_2' substrate subsite in ACE2 [21].

As mentioned above, ACE2 catalyzes the conversion of both Ang I and Ang II into smaller fragments. Donoghue et al. [10] first showed that ACE2 is able to hydrolyze Ang I. Following this, Vickers et al. [19] showed that ACE2 more efficiently hydrolyzes Ang II than Ang I since the latter is only partially hydrolyzed. ACE2 and the resulting formation of Ang-(1–7) play an important role in the cardiovascular system. Studies by Igase et al. [12] have shown that blockade of AT_1 receptors in the media layer of thoracic aortas isolated from SHR resulted in significant increases in the expression of ACE2 at the mRNA and protein levels. This effect was consistently associated with increased levels of Ang-(1–7), suggesting that ACE2 generates Ang-(1–7) locally. Corroborating these results, studies by Lavrentyev and Malik [14] have found that reduction in ACE2 expression is followed by a reduction in the cellular levels of Ang-(1–7) in rat aorta VSMC. Together, these studies suggest the involvement of ACE2 in the generation of Ang-(1–7) within the VSMC. Similar results have been found in human coronary vessels [22] and rat stomach wall [17].

In addition to ACE2, there are other enzymes that have been reported to release Ang-(1–7). Endopeptidases are the first example and have been implicated in the biosynthesis of Ang-(1–7) mainly from Ang I. As an example, prolyl endopeptidase converts Ang I into Ang-(1–7) as shown in NG108-15 neuroblastoma versus glioma hybrid cells [23] and in endothelial cells from human and bovine aorta as well as in umbilical veins [24]. As another example, neutral endopeptidase (NEP or neprilysin) accounts for the generation of most of the circulating Ang-(1–7) derived from Ang I as suggested by studies in SHR and Wistar-Kyoto (WK) rats [25]. In addition, thimet oligopeptidase (EC 3.4.24.15) forms Ang-(1–7) from Ang I in VSMC [26]. The catalytic action of these peptidases is responsible for the efficiency of the Ang I-dependent pathways in the biosynthesis of Ang-(1–7) because ACE2 hydrolyzes Ang II rather than Ang I [19, 20].

ACE has also been reported to be able to release Ang-(1–7). Even though it does not catalyze the generation of Ang-(1–7) from Ang I or Ang II, ACE is able to cleave Ang-(1–9) leading to the generation of Ang-(1–7) [20]. Furthermore, ACE is an important component in the catabolism of Ang-(1–7).

The first evidence that pointed a potential role for ACE in the degradation of Ang-(1–7) came from the findings obtained by Kohara et al. [27]. In this study, the authors observed that the chronic treatment of SHR or WK rats with the ACE inhibitors ceranopril or lisinopril augmented the circulating levels of Ang-(1–7). Corroborating with Kohara et al. [27], Luque et al. [28] showed that the chronic therapy of essential hypertensive subjects with captopril reduced the diastolic blood pressure without changing the plasma levels of Ang II while increasing the levels of Ang-(1–7). Taken together, these findings suggest a potential role for ACE in the degradation of Ang-(1–7), which may be involved in the antihypertensive effects from ACE inhibitors.

Indeed, *in vitro* experiments obtained by Chappell et al. [29] showed that ACE cleaves the Isoleucine $(Ile)^5$-His^6 bond of Ang-(1–7) with a high specificity constant. The hydrolysis of Ang-(1–7) catalyzed by ACE involves the N-domain of the enzyme [30] and leads to the formation of angiotensin-(1–5) [Ang-(1–5)] [29].

3. Effects Elicited by Ang-(1–7)

Studies show that Ang-(1–7) has a cardioprotective role mainly attributable to counteraction of the Ang II effects, which contribute to maintaining the vascular homeostasis and attenuating the progression of atherogenesis [31, 32] among other effects as further explained below.

Campagnole-Santos et al. [33] were the first to demonstrate the vascular effects induced by Ang-(1–7). The authors observed that the injection of Ang-(1–7) in the rat dorsal motor nucleus of the vagus elicited a centrally mediated hypotension similar to that induced by Ang II. Later, the authors showed that intracerebroventricular injections of Ang-(1–7) increased the baroreflex sensitivity for the control of the heart rate in conscious rats, suggesting that Ang-(1–7) facilitates the baroreflex by inducing depressor effects into the dorsal medulla [34]. In this context, Benter et al. [35] found that systemic injections of Ang-(1–7) transiently reduced the systolic pressure in SHR, suggesting a potential counterregulatory effect of Ang-(1–7) on the pressor effects elicited by the main agents that increase the total peripheral resistance during hypertension, like Ang II and α_1-adrenergic agonists. Later, the authors confirmed their hypothesis [36] by showing that the intravenous administration of Ang-(1–7) reduced the pressor responsiveness to the α_1-adrenergic agonist phenylephrine, and to Ang II, and improved the sensitivity of the reflex control of the heart rate in SHR. These results were suggestive that Ang-(1–7) activated antihypertensive mechanisms.

Additional studies showed that Ang-(1–7) was responsible for the hypotensive effects of ACE inhibitors like lisinopril and losartan. Experiments using monoclonal antibodies selective for Ang-(1–7) showed that the antihypertensive effects of lisinopril and losartan were reversed by scavenging the circulating Ang-(1–7) in SHR [29, 30, 37, 38]. Following diverse studies showed that Ang-(1–7) has a relaxation effect of its own. Meng et al. showed that Ang-(1–7) caused a mild dilatation of cerebral arterioles via a mechanism that involves the release of cyclooxygenases (COX) metabolites [39]. Pörsti et al. [40] demonstrated that Ang-(1–7) induced an endothelium- and nitric oxide synthase (NOS) metabolites-mediated relaxation in porcine coronary arteries. Similar findings were obtained by Brosnihan et al. [41], who showed an endothelium- and NOS metabolites-mediated relaxation evoked by Ang-(1–7) in canine coronary artery. Comparable results were found in other animal species using a number of tissues suggesting that Ang-(1–7) has a widespread relaxation effect in the cardiovascular system via NO release [42–44]. In the vascular system, the counterregulatory actions of Ang-(1–7) over the effects produced by Ang II occur at the molecular level. Sampaio et al. [45] have shown that Ang-(1–7) inhibits the assembly and activation of NAD(P)H oxidase induced by Ang II by inhibiting Ang II-induced phosphorylation of p47phox, which is crucial to the NAD(P)H oxidase activation. This Ang II-mediated mechanism results in O_2^- generation [46], NO inactivation [47], and has been correlated with diabetes. During diabetes mellitus, the vascular endothelium is an important source of NAD(P)H oxidase-derived O_2^-, which is involved in the reduction of endothelial NO availability and in the consequent endothelial dysfunction [47, 48]. Therefore, the counteractive effects by Ang-(1–7) over Ang II make it a potentially important therapeutic target to attenuate the endothelial dysfunction and in treating diabetes mellitus.

Ang-(1–7) also has antiproliferative effects in the cardiovascular system. Freeman et al. [49] have shown that Ang-(1–7) inhibits incorporation of mitogen-stimulated thymidine in rat aortic VSMC thus inhibiting cellular growth. The mechanism was characterized by Tallant and Clark [50], who showed that Ang-(1–7) attenuates the mitogenic activity of MAPK by a cAMP-dependent protein kinase (PKA), which is activated after a PGI_2-mediated increase in cAMP production. In addition, Strawn et al. [51] have shown that Ang-(1–7) treatment reduced the DNA synthesis and the cross-sectional area of neointima in rat carotid artery injured by balloon catheter. In line with these results, Langeveld et al. [52] observed that Ang-(1–7) treatment reduces the neointimal thickness in rat abdominal aorta after stent implantation and restores the impaired endothelial function. Many other studies also support the concept that Ang-(1–7) plays an important role in neointimal re-endothelization and inhibition of neointimal formation and restenosis [52–54].

Studies also provide evidence for a role for Ang-(1–7) in reducing thrombus formations. Experiments in rat vena cava have shown that Ang-(1–7) reduces the thrombus weight and platelet adhesion to fibrilar collagen by a mechanism involving NOS and COX metabolites [55]. In addition, Tesanovic et al. [56] found that the chronic treatment with Ang-(1–7) reduced the development of atherosclerotic lesion in ApoE (−/−) and high-fat diet-fed mice, followed by an increase in the local expression of *e*NOS. These findings have opened new perspectives on the promising vasculo- and atheroprotective effects of Ang-(1–7) suggesting therapeutic potentials for the atherosclerosis, thrombosis, and atherothrombosis.

4. *Mas* Receptor

The *Mas* receptor was first cloned and sequenced by Young et al. [57] in cotransfected NIH 3T3 cells from nude mice and revealed a very hydrophobic protein, containing seven potential transmembrane domains.

A connection between the *Mas* receptor and Ang-(1–7) was first established by Santos et al. [58]. Using binding studies performed in kidney sections from wild-type and *Mas*-deficient mice, the authors demonstrated that Ang-(1–7) binding was absent in kidneys from *Mas*-deficient mice, but preserved in wild-type membranes. In addition, the authors also showed that Ang-(1–7) binding was preserved in membranes isolated from AT_1- or AT_2-deficient animals, suggesting a twofold conclusion: (1) that Ang-(1–7) has limited interactions with AT_1 and AT_2 receptors and (2) that it mainly binds to *Mas* receptors [58]. These results were confirmed by further studies showing high affinity sites for Ang-(1–7) in *Mas* receptors. The results also showed that the *Mas* receptor has very low affinity for AT_1 or AT_2 ligands, excluding the hypothesis that Ang-(1–7) would directly interact with AT_1 or AT_2 receptors [58].

The first evidence for a functional role for the *Mas* receptor as the mediator of the Ang-(1–7) effects in the vascular system was also provided by Santos et al. [58]. The authors showed that the relaxation induced by Ang-(1–7) in mouse aorta was absent in *Mas*-deficient mice.

The authors were also the first group to develop a selective antagonist for the *Mas* receptor called D-Ala^7-Ang-(1–7) also known as A-779 [58, 59]. These findings fostered publication of many functional studies supporting a role for *Mas* receptors in the effects induced by Ang-(1–7). These include indirect (centrally mediated) antihypertensive effects [60] and local effects at the cellular [61] and molecular [44] levels. Tirapelli et al. [61] have shown that A-779 inhibited vasorelaxant effects caused by Ang-(1–7) in rat carotid artery in a concentration-dependent fashion. Sampaio et al. [44] demonstrated that A-779 blocks the phosphorylation of *e*NOS induced by Ang-(1–7) in human aortic endothelial cells, suggesting the involvement of *Mas* receptors in this effect. A-779 also blocked the inhibitory effect of Ang-(1–7) on VSMC growth, suggesting the participation of *Mas* receptors in the antiproliferative action of Ang-(1–7) [62].

There is also functional evidence directly supporting a role for *Mas* receptors in the antithrombotic effect of Ang-(1–7). A-779 dose-dependently inhibits the Ang-(1–7)-induced reduction in venous thrombus weight [55]. In addition, A-779 reduces the development of atherosclerotic lesion by Ang-(1–7) in mice. Interestingly, this effect was more evident when A-779 was combined with the AT_2-antagonists, suggesting an important interaction between *Mas* and AT_2 receptors in mediating the atheroprotective effect of Ang-(1–7) [56].

Multipronged studies also show a potential role for *Mas* receptors and Ang-(1–7) in the regulation of gastrointestinal smooth muscle motility. Studies by De Godoy et al. in Dr. Rattan's laboratory [16] have shown that Ang-(1–7) dose-dependently reduces the basal tone of spontaneously contracted internal anal sphincter of rats. The studies also show that this relaxation effect is abolished by A-779. In addition, the studies have shown that A-779 had no significant effect on the contractile response of Ang II [16]. Whether Ang-(1–7) has an important role in the gastrointestinal system remains to be determined.

In closing, Ang-(1–7) relaxes smooth muscles via interactions with the *Mas* receptor that elicit well-known molecular mechanisms of relaxation such as the release of NO. Limited studies suggest a potential role for Ang-(1–7) in regulating motility of gastrointestinal smooth muscles, but additional studies are necessary to further determine Ang-(1–7) actions and therapeutic potentials in the gastrointestinal system. On the other hand, the effects of Ang-(1–7) in the cardiovascular system are better understood and provide strong evidence for the cardiovascular protective action that can be selectively modulated via *Mas* ligands and have important therapeutic implications for human therapy.

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Review Article

Angiotensin-Converting Enzyme 2: The First Decade

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The renin-angiotensin system (RAS) is a critical regulator of hypertension, primarily through the actions of the vasoactive peptide Ang II, which is generated by the action of angiotensin-converting enzyme (ACE) mediating an increase in blood pressure. The discovery of ACE2, which primarily metabolises Ang II into the vasodilatory Ang-(1-7), has added a new dimension to the traditional RAS. As a result there has been huge interest in ACE2 over the past decade as a potential therapeutic for lowering blood pressure, especially elevation resulting from excess Ang II. Studies focusing on ACE2 have helped to reveal other actions of Ang-(1-7), outside vasodilation, such as antifibrotic and antiproliferative effects. Moreover, investigations focusing on ACE2 have revealed a variety of roles not just catalytic but also as a viral receptor and amino acid transporter. This paper focuses on what is known about ACE2 and its biological roles, paying particular attention to the regulation of ACE2 expression. In light of the entrance of human recombinant ACE2 into clinical trials, we discuss the potential use of ACE2 as a therapeutic and highlight some pertinent questions that still remain unanswered about ACE2.

1. Introduction

When angiotensin-converting enzyme-2 (ACE2) was serendipitously discovered ten years ago, neither of the two groups at the centre of its discovery [1, 2] could have guessed at the disproportionate number of distinct roles it plays in biology, from cardiovascular regulation to viral infection. As so often happens in modern biological research two independent approaches converged on the same discovery, to give us ACE2 or angiotensin-converting enzyme homologue (ACEH), back in 2000. Over the past ten years our knowledge of this protein's role in the body has increased exponentially, resulting in recombinant ACE2 protein entering clinical trials back in 2009. This paper will focus on what we currently know about ACE2 and its regulation, highlighting some of the gaps and discrepancies that still remain in our knowledge.

2. Biochemistry and Cell Biology of ACE2: Comparisons and Distinctions from ACE

ACE inhibitors have been the first line of treatment against hypertension for decades, and their success has served to place ACE and its biologically active product, angiotensin II (Ang II), as central regulators of the renin-angiotensin system (RAS). Ang II is produced by ACE through hydrolysis of its precursor Ang I. Ang II is the major vasoactive peptide in the RAS, acting as a potent vasoconstrictor through its receptor AT1R (Figure 1). Hence, inhibition of the production of Ang II and more recently its receptor-induced signalling, through the use of AT1R blockers, have been highly successful treatments in hypertension. Consequently there was immediate commercial interest in ACE2, as another likely therapeutic target, when it was discovered as an active homologue of ACE. However, as the initial publications observed to their surprise, despite high similarity to ACE (Figure 2), ACE2 did not convert Ang I to Ang II nor was it inhibited by ACE inhibitors [1, 2]. A major difference in substrate specificity was immediately noticed, namely, that ACE2 acted as a carboxypeptidase removing a single amino acid from the C-terminus of susceptible substrates whereas ACE acts as a carboxy-dipeptidase (more correctly, peptidyl-dipeptidase), removing a C-terminal dipeptide. ACE2 does hydrolyse the decapeptide Ang I, albeit relatively poorly, but converts it to Ang-(1-9) rather than Ang II (Ang-(1-8)).

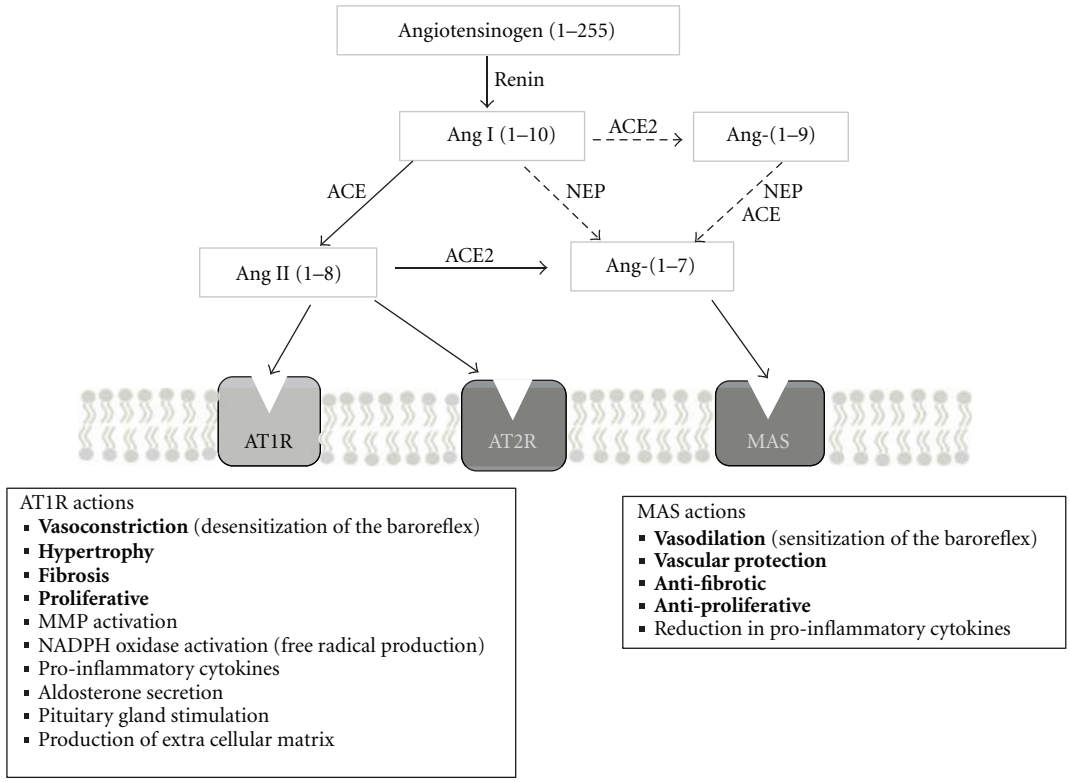

Figure 1: Schematic representation of the renin-angiotensin system (RAS). ACE: angiotensin-converting enzyme; ACE2: angiotensin-converting enzyme 2; NEP: neprilysin; AT1R: Ang II type 1 receptor. Angiotensinogen is cleaved by renin in the circulation to generate Ang I. Ang I is cleaved to yield Ang II by ACE, Ang-(1-7) by NEP, or Ang (1-9) by ACE2; this reaction is much less favourable than the production of Ang-(1-7) from Ang II. Ang-(1-9) is then cleaved by either NEP or ACE to yield Ang-(1-7) in a minor pathway. Ang II exerts its main actions by binding to the AT1R. Ang II can also be further cleaved by ACE2, into Ang-(1-7), which exerts its effects through its receptor (Mas). The opposing actions of the two receptors are listed above.

It was initially hypothesised that ACE2 counterbalanced the actions of ACE as Ang-(1-9) is also metabolised by ACE and therefore competes with Ang I for its active site, thus providing a novel regulatory arm to the RAS (Figure 1). Studies revealed that ACE2 hydrolyses a number of substrates [3] and preferentially cleaves terminal amino acids from peptides ending in Pro-X, where X is a hydrophobic amino acid [4]. The hydrolysis of some ACE2 substrates is chloride-dependent, as is the case for ACE, and the structural basis for this selectivity has been proposed [5]. Of the biologically active peptides that ACE2 cleaves, the most relevant are apelin-13 [6] and Ang II [3]. In order to further understand the biological relevance of ACE2 an inhibitor was developed based on the C-terminal dipeptide (His-Leu) of Ang I. This allowed development of the potent and specific inhibitor, MLN-4760 [4], which has been used in numerous studies of ACE2 action *in vivo* and *in vitro*, although the compound is not currently commercially available.

The elucidation of the structure of ACE2, and subsequent comparative modelling studies, explained its distinct specificity by revealing subtle differences in the active sites of ACE and ACE2 [7–10]. A single amino acid substitution in ACE2 sterically hinders the entrance of the penultimate substrate amino acid into the active site, thereby eliminating the ACE-like peptidyl-dipeptidase activity [7]. The substrate specificity of ACE2 was clarified when it was shown that ACE2 had a much higher catalytic efficiency for hydrolysis of Ang II (400-fold) compared with Ang I [3]. Only under conditions of elevated Ang I concentrations (such as in patients on ACE inhibitor therapy) is the conversion of Ang I to Ang-(1-9) by ACE2 (Figure 1.) likely to be of any physiological significance [11]. The revelation that the main product of the catalytic activity of ACE2 was Ang-(1-7) (Figure 1), a vasodilatory peptide, led to a complete reevaluation of its therapeutic potential. Currently strategies are aimed at upregulation of ACE2 expression and activity, technically more complex than enzyme inhibition. This does not rule out any potential application of ACE2 inhibitors which have recently been proposed as possible anti-inflammatories [12], having initially but unsuccessfully been tested as potential antiobesity drugs.

The main tissue sites of expression of ACE2 were originally identified as testis, heart, and kidney [1], where it was shown to be localised on the apical membrane of polarised cells whereas ACE is equally distributed between apical and basolateral membranes [13]. The molecular basis for this differential localization has not been addressed but presumably relates to determinants in the C-terminal

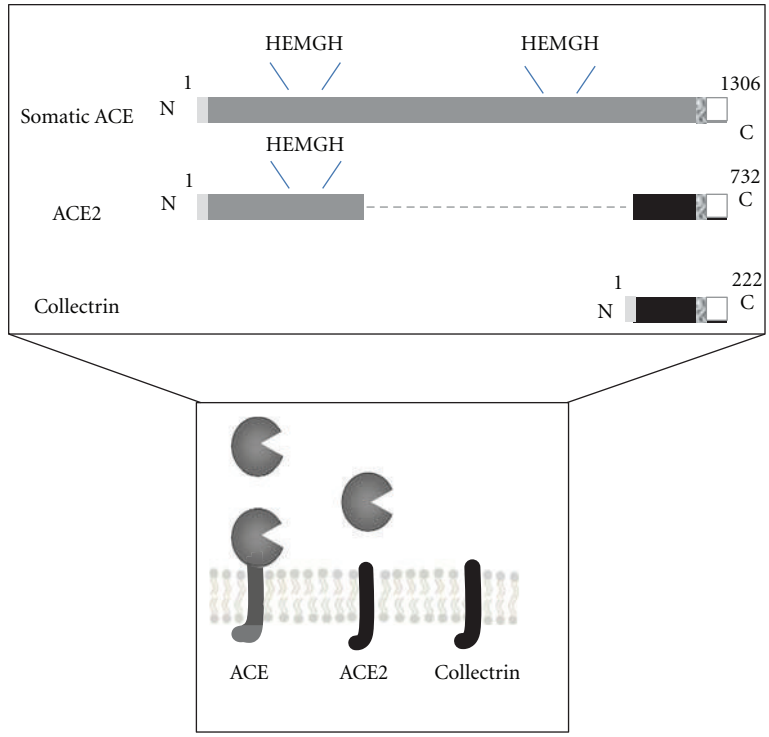

Figure 2: The domain structure and membrane topology of somatic ACE, ACE2, and collectrin. Each protein is a type I integral-membrane protein with an N-terminal ectodomain, a transmembrane region, and a short C-terminal cytoplasmic tail. Residue numbers are indicated. Both ACE and ACE2 contain zinc-binding motifs (HEMGH), which form the active sites of the enzyme: somatic ACE has two active sites whereas ACE2 only has one. Collectrin contains no catalytic residues. ACE2 is homologous to the N-terminal ectodomain of ACE but has no homology with its C-terminal cytoplasmic domain. Instead, it shares a number of residues with the intracellular domain of collectrin. Signal peptide in light grey; transmembrane domain in textured grey.

cytoplasmic tails of the two enzymes which are quite distinct in sequence. The tissue distribution of ACE2 has now been catalogued more widely, for example, in liver, intestine, and lung [14, 15]. More recently ACE2 has been localized in the brain [16], where it appears to act as a central regulator of cardiovascular function [17–20]. ACE2 is a type 1 transmembrane protein (N-terminus outside, C-terminus intracellular), predominantly localised on endothelial cells where its catalytic site, like that of ACE, is exposed (so-called "ectoenzyme") to circulating vasoactive peptides [13]. The activity of ACE2 can therefore be modulated via its expression on the cell surface, through its expression levels and also through its cleavage from the cell membrane. This cleavage or shedding releases the catalytically active ectodomain and when stimulated, for example, by phorbol esters, is mediated by a disintegrin and metalloprotease (ADAM 17) [21]. ACE also undergoes constitutive and regulated shedding from the cell surface into plasma although the enzymes responsible in this case have not been identified.

3. ACE2 and Cardiovascular Function

The success of ACE inhibitors has shown that Ang II is a key mediator of hypertension, and, hence, by metabolising Ang II into Ang-(1-7), ACE2 is crucial in the modulation of blood pressure. The role of ACE2 in hypertension has been clarified by its overexpression *in vivo*, reducing blood pressure in hypertensive models [22–24] but not in normotensive animals [25]. This reduction in blood pressure may be the result of increased sensitivity of the baroreflex, which has been seen upon ACE2 delivery in hypertensive models [26], and a reduction in neuronally induced hypertension has been observed in transgenic mice [19]. Central blood pressure regulation is controlled in part by the actions of Ang II on the AT1R. Ang II acts through the AT1R to desensitise the baroreflex, stimulate water uptake, and increase vasopressin release and sympathetic activation, ultimately leading to increased blood pressure [27]. The actions of Ang II are in part modulated by the increase in baroreflex sensitivity mediated by Ang-(1-7) [28, 29]. Comparison of hypertensive models to normotensive rodents has revealed decreased ACE2 protein expression by up to 40%, in the brain, of the hypertensive models [20, 22]. Moreover, overexpression of ACE2 in the brain attenuates hypertension, via an increase in nitric oxide production [19] and improved baroreflex [20]. Accordingly, injection of the ACE2 inhibitor MLN-4760 into the brain of rodents attenuates the baroreflex [18]. Site-specific overexpression of ACE2 at a locus controlling

sympathetic nerve activity reduces the overall hypertensive state of rats [22]. For a review of the roles of ACE2 in central blood pressure regulation, see [30].

Soon after its discovery, gene deletion studies established ACE2 not only as a modulator of blood pressure but also as an essential regulator of cardiovascular function [31]. The progressive cardiac dysfunction observed in the first ACE2 mouse knockout model resembled that of tissue subjected to long-term hypoxia of the type that occurs after coronary artery disease or bypass surgery in humans [32]. As a result of these observations ACE2 was immediately proposed as a cardioprotective protein. This hypothesis was strengthened by the observation that ACE2-null phenotypes were reversed by concurrent knockout of the ACE gene. This evidence appeared to demonstrate unequivocally that the primary role of ACE2 was to counterbalance that of ACE [31]. The initial hypothesis that ACE2 plays a critical role in cardiac function primarily by counterbalancing the effects of ACE was not, however, entirely supported by subsequent gene deletion models. The discrepancies between the initial study and the phenotypes are described elsewhere, which saw no obvious functional or morphological changes [33, 34], were initially proposed to be due to differing genetic backgrounds in their models. This potential mechanism was investigated by backcrossing the hybrid model used in both studies with an initial parental line; however, both backcrossed models showed no cardiac changes [34]. Interestingly subsequent studies using the original ACE2-deficient mice described by Crackower et al. also showed no overt cardiac changes, suggesting that the phenotype is lost over time [35, 36]. Despite seeing no overt phenotypic change in deletion models, subsequent groups have shown a reduced ability to respond to injury in ACE2-null mice. Together these studies suggest that, rather than being a key mediator of cardiac phenotype, ACE2 is essential in modulating responses to injury [33, 37].

In fact ACE2 deletion models have a significantly higher mortality rate after myocardial infarction (MI) than wild-type mice, associated with adverse ventricular remodelling and worsening ventricular function following MI [38]. An increase in matrix metalloproteinase2 (MMP2) and MMP9 activation, free radical production, and upregulation of proinflammatory cytokines, in the hearts of ACE2-knockout mice, were postulated to mediate the adverse remodelling after MI. These events, and the adverse remodelling they cause, were reversed upon administration of an AT1R blocker, and therefore the pathology of ACE2 deletion in states of injury can be attributed, for the most part, to increases in the local levels of Ang II [38].

The ability of ACE2 to improve responses to injury is not only the result of clearing Ang II, thereby limiting its pathological potential, but also by producing Ang-(1-7). The conversion of Ang II to Ang-(1-7) by ACE2 is not the only physiological route to Ang-(1-7) production. For example, the zinc metallopeptidase neprilysin (NEP) can convert Ang I to Ang-(1-7) efficiently [39], and both ACE and NEP can convert Ang-(1-9) to Ang-(1-7). The relative importance of these various enzymes to Ang-(1-7) production will vary dependent on their relative expression levels in different tissues (e.g., kidney versus heart versus brain) and on physiological status. Like Ang II the actions of Ang-(1-7) extend beyond vasopressor control. Infusion of Ang-(1-7) reduces interstitial fibrosis in Ang II-independent [40] and Ang II-dependent hypertension [41]. Interestingly in both studies there was no effect on the blood pressure of hypertensive animals when infused with chronic levels of Ang-(1-7). There was, however, a discrepancy in the effects of Ang-(1-7) on cardiac hypertrophy between the two studies. Ang-(1-7) had no effect on the salt-induced hypertrophy in Ang II-independent hypertension but it significantly reduced myocyte hypertrophy in Ang II-induced hypertension. Cardiac-specific overexpression of Ang-(1-7) was observed to reduce the hypertrophic response to Ang II concurrently with a reduction in hypertrophic markers, atrial natriuretic peptide and brain natriuretic peptide, transcript levels and activation of hypertrophic signalling pathways, c-src and p38 MAPK [42]. Ang-(1-7) inhibits myocyte cell growth *in vitro* through the actions of the MAS receptor [43] and accordingly prevents ventricular hypertrophy *in vivo*, when stimulated by myocardial infarction (MI) [44]. The reduction in myocyte diameter and ventricular weight of mice virally expressing Ang-(1-7) was associated with a decrease in proinflammatory cytokines (TNFα and IL-6) compared to control. It is worth noting that Ang-(1-7) overexpression slightly reduced exogenous ACE mRNA levels and ablated the approximate twofold increase in expression resulting from MI, whilst increasing ACE2 expression levels in response to MI [44].

ACE2 levels have consistently been shown to alter in cardiovascular disease states. In light of the counterbalancing hypothesis it could be presumed that, since ACE is consistently reported to increase in damaged cardiac tissue [45–47], ACE2 levels would also increase as a homeostatic response to offset the rise in Ang II concentration. This hypothetical upregulation is supported by evidence from human nonischaemic cardiomyopathy, which has consistently shown increased ACE2 levels in the failing human heart compared to control patients [48–50]. However, in contrast, in ischaemic cardiomyopathy, there is currently conflicting evidence for the changes in expression levels of ACE2 [48, 49]. Where ACE2 upregulation has been seen in these studies, the mechanism of this damage-induced increase has been investigated using *in vivo* models of MI. ACE2 upregulation has been repeatedly shown in rat models of MI [38, 51, 52]. Discrepancies between the mRNA and protein levels seen in the infarct zone have suggested that the increase in ACE2 protein is mediated by a posttranscriptional mechanism [38]. However, time-course investigations reveal that the increases seen at eight weeks after MI were followed by a decrease in ACE2 expression in MI models compared to control after 28 weeks [52]. Although not entirely consistent these results on balance seem to indicate a compensatory role for ACE2 in conditions of myocardial injury.

Given its role in removing Ang II, ACE2 was identified as a candidate gene underlying the loci linked to hypertension [31], following its initial mapping to the X chromosome [1]. Comparison of ACE2 expression levels in the kidneys of three rat strains showed that ACE2 expression was

lower in the hypertensive-prone strains and moreover that ACE2 expression decreased significantly when hypertension was initiated in salt-sensitive hypertensive rats. Decreased endogenous ACE2 expression has been noted in spontaneously hypertensive rats compared to Wistar-Kyoto [53]. The initial study did not see any genetic changes associated with the ACE2 gene in these hypertensive strains, supporting subsequent data, which have, up until now, failed to show any link between ACE2 polymorphisms and hypertension [54].

4. ACE2, the Kidney, and Diabetes

ACE2 is abundantly expressed in the kidneys, where its expression is inversely correlated with hypertension [55, 56]. The local RAS within the kidneys is activated by hyperglycemic conditions, which model the environment in type 2 diabetes [57]. Studies using models of type 2 diabetes have shown at early stages, prior to diabetic nephropathy developing, that ACE2 expression is reduced in the kidney, while ACE expression is elevated [58]. Similarly in models of type 1 diabetes ACE2 expression is elevated in early [59] and decreased in late stage of diabetic nephropathy [60]. Additionally, studies on human samples have shown *de novo* expression of ACE2 in the glomerular endothelium and mesangial cells of diabetic patients [61]; however, this expression was not seen in type 2 diabetic renal biopsies [57]. One study carried out by taking biopsies of twenty type 2 diabetic patients and twenty healthy donors showed decreased ACE2 and increased ACE in tubulointerstitium and glomeruli in the diabetic patients with nephropathy indicating a pathologically important balance between the two enzymes [62]. The hypothesis that kidney disease and the pathogenesis of diabetes are mediated by an upregulation of ACE and a downregulation of ACE2 was originally suggested by Mizuiri et al. [62].

As in the heart, loss of ACE2 in the kidneys is again associated with increased susceptibility to injury. ACE2-knockout mice have been shown to have enhanced susceptibility to glomerulosclerosis, coupled with increased collagen and fibronectin deposition [63]. Filtration dysfunction, evidenced by urinary albumin, was pronounced in the male mice whereas the female mice appeared to be protected. Pharmacological inhibition of ACE2, by MLN-4760, has been shown to have similar effects, increasing urinary albumin and mesangial cell expansion and vascular thickness, in both type 1 and type 2 diabetic models [58, 64]. All these studies attributed the pathology seen when ACE2 is lost to increases in levels of Ang II [58, 63]. In order to further confirm the renoprotective role of ACE2, Akita mice (a type 1 model of diabetes) were crossed with ACE2-knockout mice and kidney function observed. This model showed an increase in urinary albumin, glomerular basement membrane thickness, fibronectin, and smooth muscle α-actin compared to diabetic mice expressing ACE2 [35]. Surprisingly they did not see any change in Ang II in ACE2-knockouts, or in the diabetic model; despite this, they did show that use of an Ang II receptor blocker was able to attenuate some of the markers of glomerular injury and urinary albumin seen in the ACE2 knockout diabetic mice. Conversely, ACE2 deletion disrupted the benefits of ACE inhibition on diabetic nephropathy in streptozotocin-induced diabetes [65] suggesting that ACE inhibition may enhance ACE2 activity. Interestingly, in the same diabetic model, Ang-(1-7) infusion resulted in pronounced renal injury [66]. This may not be as contradictory as it first appears as they also saw a downregulation in the MAS receptor, the proposed receptor for Ang-(1-7) [67]. These current findings suggest that ACE2 may participate in a compensatory mechanism in the diabetic kidney prior to the onset of diabetic nephropathy.

More direct involvement of ACE2 in diabetes, through its pancreatic expression, has been investigated [68]. ACE2 expression is elevated in the islets of type 2 diabetic rats, which correlates with an increase in ACE, collagen IV, and TGF-β1 levels [68]. ACE2-null mice have significantly increased fasting blood glucose compared to their wild-type littermates [69]. No direct role for ACE2 in the pancreas has yet been identified; in contrast its homologue collectrin is heavily implicated in insulin exocytosis. When discovered, collectrin excited interest due to its high homology to the cytoplasmic tail of ACE2 [70]. SiRNA knockdown of collectrin results in a reduction of insulin exocytosis in insulin-secreting INS-1 cells [71]. *In vivo*, overexpression of collectrin led to significant increases in insulin secretion [71]. Collectrin was implicated in the insulin secretory pathway through an association between collectrin and snapin, part of the SNARE complex [70–72]. However, collectrin-knockout mice revealed no difference in insulin secretion from wild-type, only a decrease in insulin sensitivity [73].

ACE2 is not only homologous to ACE but is a chimaera of ACE, with which it has close homology in the catalytic domains of the N-terminus, and of collectrin, which closely resembles the transmembrane and intracellular C-terminal domains of ACE2 (Figure 2). Collectrin was first identified as an unknown protein upregulated in a model of partial nephrectomy, its function remaining elusive for four years until crystals of tyrosine and phenylalanine were detected in the urine of collectrin-null mice [74]. Further investigation revealed that the levels of the neutral amino acid transporter, B^0AT1, which reached the plasma membrane were significantly decreased in collectrin-null mice [75]. This suggested that collectrin may act as a molecular chaperone for B^0AT1 in the kidney, implicating ACE2 in a similar role, because of their close homology. An elegant set of studies subsequently revealed that ACE2 did in fact act as the molecular chaperone for B^0AT1 in the small intestine, where collectrin is not expressed. This interaction was shown to underlie the pathology of the aminoaciduria seen in Hartnup disorder. Hartnup disorder is caused by a mutation on the outer edge of B^0AT1 resulting in its failure to reach the plasma membrane [76]. It was revealed that this mutation disrupts the ACE2/B^0AT1 complex and therefore prevents ACE2 from acting as a molecular chaperone delivering the transporter to the intestinal brush border membrane.

Outside the cardiovascular system another noncatalytic function of ACE2 had previously been shown. In 2003 a new disease termed "severe acute respiratory syndrome (SARS)" caused by a novel coronavirus (SARS-CoV) spread quickly

around the world, causing more than 800 deaths. ACE2 was identified as the receptor for SARS virus *in vitro* [77, 78] and also acts as receptor for the NL63 virus. Soon after, studies confirmed that ACE2 was essential for SARS infection *in vivo* using *ACE2*-knockout mice [79]. Concurrently it was discovered that ACE2 protects murine lungs from severe acute injury [80] and subsequently that SARS-CoV infections and the SARS spike protein itself downregulate ACE2 expression (Figure 3) [81].

5. ACE2 Regulation

5.1. Transcriptional Regulation of ACE2. As mentioned above there is circumstantial but not entirely consistent evidence in the literature that ACE and ACE2 are coregulated. In human hypertensive patients, ACE2 levels are lower in both kidney and heart compared to normotensive volunteers [82]. A growing body of *in vitro* evidence suggests that this decrease is mediated at least in part by Ang II [82–84]. The proposed mechanism for this involves AT1R signalling via ERK/p38 MAP [82] and/or by elevated ERK1/2 and JNK phosphorylation [85]. Furthermore, administration of an AT1R blocker has been shown to result in an increase in ACE2 levels [84, 86]. As such there is linked regulation of both ACE2 and ACE, as the catalytic product of ACE, Ang II, regulates ACE2. However, the role Ang II plays in regulating ACE2 is not yet fully elucidated; despite decreasing ACE2 expression in response to Ang II in most models there is evidence of Ang II-mediated increases in ACE2 in hepatic stellate cells [87].

Relatively little is known about the detailed transcriptional regulation of ACE2. Although angiotensin peptides, as well as other peptide and steroid hormones, appear to modulate its expression, few studies have been done on other factors that may control its regulation, such as hormones and oxygen levels. For example, although hypoxia decreases the transcription of ACE2, further investigation has revealed that hypoxia-induced HIF-1α increases ACE expression which, in turn, leads to an increased concentration of Ang II. It is this Ang II that then mediates a decrease in ACE2 [88]. Ang-(1-7) has also been shown to affect ACE2 expression: cardiac and renal ACE2 were decreased in both hypertensive and normotensive rat models in response to Ang-(1-7) infusion although no effects on blood pressure were demonstrated and no mechanism of action was proposed [89].

Administration of aldosterone or endothelin-1 to rat myocytes has also been shown to downregulate ACE2 mRNA levels [90]. Micromolar concentrations of aldosterone were shown to decrease ACE2 mRNA expression significantly in the myocytes of hormone-infused rats although, in contrast to other models, no change in ACE2 mRNA levels were seen when these rats were infused with Ang II [83]. When treated with endothelin-1, myocytes isolated from neonatal rats decrease ACE2 expression via ERK1/ERK2 signalling, a decrease that was blocked by cotreatment with Ang-(1-7) [90]. The effect of oestrogen on ACE2 expression has recently also been explored in light of clinical evidence which has established that hormone replacement therapy is protective against cardiovascular disease. Treating rats with oestrogen was shown to reduce cardiac remodelling and interstitial fibrosis [91]. Previous *in vitro* studies had shown that oestradiol (E2) treatment was protective against Ang II-induced fibroblast proliferation [92]. The beneficial effects of oestrogen were coupled with a dose-dependent increase in ACE2 but no significant change in blood pressure was seen and no protective mechanism proposed [91].

Components of the RAS are also expressed in adipose tissue [93]. A high-fat diet has been shown to increase ACE2 mRNA levels in mouse adipocytes both *in vivo* and *in vitro* [94] although these changes were not evident in the adipose tissues of mice with heart failure. Tissue culture models of adipose differentiation have shown that the increase in ACE2 mRNA over time was accompanied by an increase in ADAM 17 and no increase in ACE2 activity [94]. Thus the activity levels of ACE2 remain constant via coregulation with ADAM 17, which cleaves ACE2 at the cell membrane. An increase in ACE2 expression has been reported in adipose tissue when rats were fed a high-sucrose diet, although in preliminary form only [95].

The effects of all-trans-retinoic acid have been investigated on ACE2 expression revealing an increase in ACE2 mRNA levels and reportedly in protein [53]. A decrease in blood pressure in the treated rats was also seen, which was attributed to the increased ACE2 levels.

5.2. Posttranslational Regulation of ACE2. ACE2 expression is not only subject to posttranslational modifications, such as glycosylation and phosphorylation, but also subject to posttranslational regulation, when released from the cell membrane by shedding through the action of ADAM 17 as described above [21, 96, 97]. Cleavage of ACE2 occurs at the juxtamembrane region. Short peptide mimics around the likely cleavage site region are hydrolysed by recombinant ADAM 17 at an Arg-Ser bond (corresponding to Arg^{708} and Ser^{709} in ACE2), in a sequence-dependent manner [93, 94]. However, mutation of these critical cleavage residues in a cell-based system failed to inhibit shedding suggesting that the specificity of ADAM 17 is topographically determined, rather than sequence dependent [97, 98]. The function (if any) of the catalytically active soluble, shed form is unknown, although for some other proteins, for example, the amyloid precursor protein and acetylcholinesterase, the released protein acts as a ligand for stimulating cell-cell interactions. The retention of ACE2 on the cell membrane is regulated by calmodulin binding [99]. Inhibition of calmodulin binding increases the cellular release of ACE2. Elevated levels of shed ACE2 have been associated with increased myocardial dysfunction [100]. The catalytic activity of any shed ACE2 may be masked by the presence of an endogenous inhibitor of ACE2 in the plasma, which currently remains uncharacterised [101].

As previously mentioned SARS virus downregulates cellular expression of ACE2 [102]. Binding of the SARS spike protein induced ADAM-17-dependent shedding of ACE2 N-terminal domain [103] (Figure 3). This shedding has been reported by different groups to be both essential for viral replication [104] and unnecessary [97].

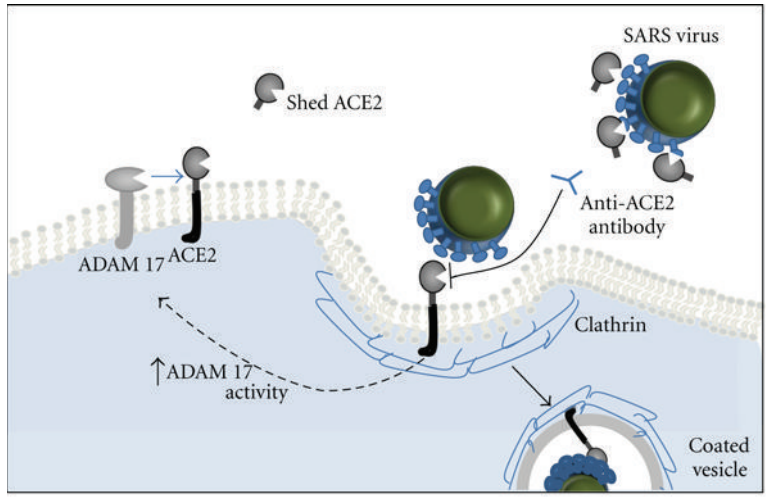

Figure 3: ACE2 acts as the host cell receptor for SARS-CoV, by binding to the spike protein on the viral capsid. Binding to ACE2 stimulates clathrin-dependent endocytosis of both ACE2 and the SARS-CoV, which is essential for viral infection. Binding of the spike protein to ACE2 induces ADAM 17 activity, thereby reducing the amount of ACE2 expressed on the cell surface. Treatment with soluble ACE-2 or anti-ACE-2 antibodies disrupts the interaction between virus and receptor.

The SARS virus undergoes clathrin-dependent endocytosis upon receptor binding; this process internalises both the SARS virus and its receptor further clearing ACE2 from the cell membrane (Figure 3) and hence allowing enhanced (and damaging) reactivity towards circulating Ang II. In contrast to ectodomain shedding, the cytoplasmic domain of ACE2 appears to play no role in the regulation of internalisation [105].

6. Unanswered Questions

Currently the most pertinent of all questions about ACE2 is whether it is going to be a useful therapeutic target, and so far all data suggest that increased expression would be beneficial in a number of diseases. Until now an increase in the level of ACE2 has been achieved by viral delivery [106], application of allosteric activators of ACE2 catalysis [107], or administration of human recombinant ACE2 [108]. Aside from its effects on hypertension [22–25], viral overexpression of ACE2 has shown reduced collagen production in cultured fibroblasts [106] as well as inhibition of Ang-II-induced fibrosis and hypertrophy *in vivo* [109], stabilisation of atherosclerotic plaques [110], and renoprotection [24]. Viral delivery of ACE2 after induction of myocardial infarction is protective, reducing the adverse cardiac remodelling and fibrosis [111, 112]. Similar antifibrotic effects have been seen with an ACE2 activator [113, 114] along with attenuation of Ang-II-induced thrombus in hypertensive rats [115] and a modest reduction in the blood pressure of spontaneously hypertensive rats [107]. Over the past 18 months a number of studies have been carried out examining the effect of recombinant human ACE2 on a range of disease conditions. To date, administration of ACE2 to mouse models of Ang-II-induced diseases has been shown to reverse the pathological effects of Ang II in diabetic nephropathy [116], heart disease [117], renal oxidative stress [118] as well as reversal of Ang-II-induced hypertension [108]. Interestingly, infusion of ACE2 does not appear to have any effect on nondisease states or on the basal level of Ang II in wild-type mice or ACE2-knockout mice, making it a potentially valuable therapeutic.

More fundamental questions about the cellular biology of ACE2 remain. As is evident from this paper, questions need answering about underlying mechanisms of ACE2 regulation, which could help clarify our understanding of the RAS as a whole, for example, are there antagonistic transcription factors regulating ACE and ACE2? Are components of the RAS co-ordinately regulated and by which signalling pathways? Do microRNAs regulate the RAS coordinately? Do cytokines modify the expression of ACE2? What posttranslational changes apart from shedding regulate ACE2 including phosphorylation and ubiquitination?

Some analogies are provided by the regulation of ACE. ACE is known to signal through phosphorylation of its cytoplasmic tail modulating its own retention on the cell membrane [119, 120] and also to mediate transmembrane signalling, increasing its own transcription in response to ACE inhibitors [121, 122]. Exogenous ACE has been shown to have transcriptional effects independent of its catalytic activity when VSMC and endothelial cells are treated with ACE [123, 124]. Does the cytoplasmic tail of ACE2, despite sharing no homology with ACE, have similar signalling properties? Both ACE and ACE2 are shed from the membrane to release their ectodomains. The fate of these ectodomains is unknown and it may be a mechanism of rapid clearance, or perhaps these ectodomains are endocytosed and trafficked to the nucleus, where they elicit transcriptional changes, as has been shown with exogenous ACE [124]. For that matter the destiny of the retained cytoplasmic sections of these

proteins is not known. The intracellular domains of other shed proteins, such as APP and Notch, are released by γ-secretase cleavage, after initial ADAM ectodomain cleavage, a process referred to as intramembrane proteolysis [125]. The intracellular domains of both APP and Notch travel to the nucleus, stabilised by chaperones, where they elicit transcriptional changes [126]. Perhaps a similar fate awaits the ACE intracellular domain, if generated.

Novel roles for ACE2 may yet remain to be discovered and a new twist to the ACE2 story has emerged with the discovery of autoantibodies targeting ACE2 in the sera of patients with connective tissue diseases [127]. This, coupled with results showing that administration of an ACE2 inhibitor improved the pathology of inflammatory bowel disease [12], suggests that inhibition of ACE2 may be beneficial in inflammatory diseases such as arthritis.

7. Summary

The diversity of the roles of ACE2 continues to surprise those in the field. Given the apparent success of recombinant human ACE2 in animal models, ACE2 could be a valuable therapeutic. However, here it is worth noting that so far the only disease model recombinant ACE2 has shown success in is type 1 diabetes. All other studies have, as yet, only shown that ACE2 reverses the effects of exogenous Ang II infusion. Until work is done on hypertensive-prone models, as with viral delivery and ACE2 activator-mediated induction, it is hard to judge the clinical relevance of this treatment. Although no immune response to recombinant ACE2 or the viral delivery systems has been reported upon infusion into rodents, this is always a concern with such strategies. Given these caveats perhaps a priority focus for future research should be upregulation of endogenous ACE2 gene expression or catalytic activity. As highlighted in this paper, more work is required to determine how the diverse roles of ACE2 interlink in order to allow chronic modulation of ACE2 levels to proceed with confidence.

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Research Article

The Effect of the Thioether-Bridged, Stabilized Angiotensin-(1–7) Analogue Cyclic Ang-(1–7) on Cardiac Remodeling and Endothelial Function in Rats with Myocardial Infarction

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Modulation of renin-angiotensin system (RAS) by angiotensin-(1–7) (Ang-(1–7)) is an attractive approach to combat the detrimental consequences of myocardial infarction (MI). However Ang-(1–7) has limited clinical potential due to its unfavorable pharmacokinetic profile. We investigated effects of a stabilized, thioether-bridged analogue of Ang-(1–7) called cyclic Ang-(1–7) in rat model of myocardial infarction. Rats underwent coronary ligation or sham surgery. Two weeks thereafter infusion with 0.24 or 2.4 μg/kg/h cAng-(1–7) or saline was started for 8 weeks. Thereafter, cardiac morphometric and hemodynamic variables as wells as aortic endothelial function were measured. The average infarct size was 13.8% and was not changed by cAng-(1–7) treatment. MI increased heart weight and myocyte size, which was restored by cAng-(1–7) to sham levels. In addition, cAng-(1–7) lowered left ventricular end-diastolic pressure and improved endothelial function. The results suggest that cAng-(1–7) is a promising new agent in treatment of myocardial infarction and warrant further research.

1. Introduction

Myocardial infarction is a leading cause of mortality and morbidity in western society. Current intervention relies on prevention of myocardial hypertrophy and fibrosis and of thrombosis. Since these processes are partially mediated by an increase of the renin-angiotensin system (RAS) hormone; angiotensin (Ang) II, inhibition of this hormone through drugs; that decrease its production or its signaling via the Ang II type 1 (AT1) receptor; forms an important part of the applied pharmacotherapy. The ever culminating knowledge of RAS; brought about by relentless research of a vast group of scientists; has raised the awareness that there is more to achieve than with classical RAS intervention only. Possible novel intervention strategies have emerged, of which those based on stimulation of angiotensin-(1–7) (Ang-(1–7)) function as one of the most appealing [1, 2].

Angiotensin-(1–7) (Ang-(1–7)) is a hormone that in general counteracts Ang II through its own signaling pathways, which involves the Mas receptor [3]. Studies in animal models show that it has ample therapeutic potential in cardiovascular disease, in particular diseases that are featured by malignant remodeling of the heart. We showed that chronic infusion of Ang-(1–7) in rats or mice with myocardial infarction improves cardiac and endothelial function [4, 5]. The beneficial effect of Ang-(1–7) infusion after myocardial infarction relies on the versatile bioactivity of the hormone, which comprises antihypertrophic, -fibrotic,

and -thrombotic function, improvement of eNOS function, blockade of Ang-II-induced ROS production, and stimulation of endothelial-progenitor-cell-mediated angiogenesis [1, 2, 5–9]. In spite of being a therapeutic prodigy, Ang-(1–7) does not offer ideal prospects for clinical use because of its pharmacokinetical and pharmacodynamical properties (as is also elaborated in [1]). Firstly, the peptide is rapidly metabolised in plasma and tissue. Second, beneficial effects of Ang-(1–7) take place at low concentrations at which Mas receptors are stimulated. At higher concentrations Ang-(1–7) becomes aspecific for receptor subtype binding, being a partial Ang II type 1 receptor agonist and an Ang II type 2 receptor agonist. Thus, overdosing might interfere with its Mas receptor-associated functions.

To improve the pharmacological profile we have developed cyclic Ang-(1–7) (cAng-(1–7)), an Ang-(1–7) analogue in which amino acid residues 4 and 7 have been linked with a thioether bridge, thus forming a lanthionine [10]. The strategy of thioether bridging is used by bacteria to stabilize peptides, and we previously showed that enzymatically synthesized cAng-(1–7) was fully resistant against degradation by angiotensin-converting enzyme and had enhanced resistance against breakdown by other proteases. It displayed 34-fold enhanced presence in the blood circulation in Sprague-Dawley (SD) rats during continuous intravenous infusion. The thioether ring did not prevent cAng-(1–7) from agonistically interacting with the Mas receptor, the receptor of native angiotensin-(1–7). cAng-(1–7) even induced a twofold larger relaxation of precontracted SD rat aorta rings than native Ang-(1–7). Moreover, it is a specific agonist for Ang-(1–7) receptors. Therefore, cAng-(1–7) holds promise for use in cardiovascular therapy. In this study we have tested the effect of chronic cAng-(1–7) infusion on hemodynamic function after myocardial infarction in the rat.

2. Methods

2.1. Animals. Male Sprague-Dawley rats weighing 280–300 grams were obtained from Harlan (Horst, the Netherlands). Animals were put on standard rat chow and water, available ad. libitum. Housing was at room temperature with a 12 h light–12 h dark cycle. After at least one week of acclimatization in the caretaking facility, the rats were operated to induce left ventricular myocardial infarction (MI) or underwent a sham procedure.

2.2. Surgery to Induce MI and Surgical Procedures. Prior to surgery 0.01 mg/kg buprenorphine was given subcutaneously for postoperative analgesia, which was repeated after surgery for 2 days, 2 times daily. Operations were performed under 2.5% isoflurane in air ventilation anesthesia for which the rats were intubated. Through an opening in the left 4th intercostal space of the chest, MI was induced by ligation of the left coronary artery with a 6/0 silk suture. After induction of MI, as witnessed by bleaching of the myocardium, the chest was closed and animals were withdrawn from anesthesia. Sham-operated animals (SHAM) underwent an identical procedure, however, without tying the silk suture to close the coronary artery. MI surgery was performed in 106 animals, 8 animals were sham-operated. Perioperative mortality was 45% in the MI group.

2.3. Treatment with cAng-(1–7). Two weeks after induction of MI, rats were randomly allocated to intravenous infusion of either 0.24 (low dose or low cAng-(1–7)) or 2.4 μg/kg/h (high dose or high cAng-(1–7)) of cAng-(1–7) $n = 12$ for each dose), or of saline ($n = 25$) by 4-week osmotic minipumps (Alzet model 2004). Sham-operated controls ($n = 8$) received saline or high dose of cAng-(1–7) (2.4 μg/kg/h). Animals were infused for 8 weeks, changing pumps at week 4. To accomplish intravenous infusion a polyethylene tube was implanted in the left jugular vein. cAng-(1–7) was made by BiOMade/LanthioPep, Groningen.

2.4. Measurements of Hemodynamic and Vascular Function. After 8 weeks of treatment animals were weighed (body weight: BW) and hemodynamic studies were performed under isoflurane anesthesia (2.5% in air) with a 2F catheter-based, microtip pressure transducer (Millar, Houston, Tex, USA) that was introduced into the left ventricle via intraluminal passing through the right carotid artery. Rats were anesthetized for 20 minutes before the start of the measurement.

After measurement of hemodynamic function the heart was excised for histological studies. After removal of ventricular blood; the heart was weighed to obtain total heart weight (HW). The thoracic aorta was isolated to perform functional studies. To this end the aorta was kept in Krebs solution in mmol/L: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4. Surrounding periaortic adipose tissue was carefully removed with small scissors. Rings of 2 mm length were cut and mounted in small wire organ baths containing Krebs at 37°C. To investigate the contribution of dilator signaling factors nitric oxide (NO) production was blocked using L-NAME (100 μmol/L), and endothelium-derived hyperpolarizing factor (EDHF) was blocked with apamin (0.5 μmol/L) and charybdotoxin (0.1 μmol/L). Subsequently, in the absence or presence of these inhibitors, concentration-response curves were constructed to methacholine and SNP after preconstruction with phenylephrine. All chemicals were from Sigma-Aldrich, the Netherlands.

2.5. Histology. Midventricular slices of the heart were fixed with 4% formaldehyde, embedded in paraffin and processed for histochemical analysis. Infarct size was determined on picrosirius red/fast green-stained sections and was expressed as the percentage of scar length of the average of left ventricular internal and external circumference. Rats with all infarct sizes were included in the analysis. The cross-sectional area of the individual cells was measured on gomori-stained sections. Myocyte density was determined by assessment of the number of cells per tissue area for each slide and subsequent conversion to mm^2. Fibrosis was measured on picrosirius red/fast green-stained sections from three randomly selected regions of the surviving myocardium.

2.6. Statistical Analysis. Data are presented as mean ± SEM. Statistical differences between the groups were evaluated by

Table 1: Weight, basic histological and cardiac parameters.

	Sham saline	MI saline	Sham high cAng-(1–7)	MI low cAng-(1–7)	MI high cAng-(1–7)
BW, g	477.1 ± 6.6	475.3 ± 6.4	484.6 ± 7.4	458.6 ± 10	492.3 ± 9.5
Infarct size, %	0 ± 0	15.853 ± 3.17	0 ± 0	9.721 ± 2.89	14.535 ± 4.10
Fibrosis, %	3.896 ± 0.57	3.992 ± 0.39	4.289 ± 0.54	4.695 ± 0.65	4.479 ± 0.34
HR beats/min	255.12 ± 16.0	260.3 ± 5.5	240.4 ± 9.8	274.7 ± 6.2	266.7 ± 6.2
MaxP, mmHg	107.8 ± 3.8	103.5 ± 2.9	98.6 ± 8.3	93.0 ± 4.7	96.4 ± 4.7
ESPress, mmHg	103.6 ± 4.2	100.1 ± 3.0	93.5 ± 9.3	89.4 ± 5.3	92.4 ± 5.1
dpdtMax, mmHg/sec	5572.0 ± 188.2	5456.8 ± 198.7	5533.0 ± 530.7	5026 ± 269.3	5111.3 ± 345.1
dpdtMin, mmHg/sec	−5189.1 ± 178.8	−4919.1 ± 265.2	−6123.5 ± 1268.9	−4615.4 ± 361.8	−4742.6 ± 404.9
N	8	25	5	12	12

t-test or by 1-way ANOVA for hemodynamic and histological variables, using Dunnett's *t*-test or Bonferroni correction where appropriate. One-sided testing was applied in all bar graphs as the effects were in the expected direction. For testing of trend; linear regression analysis was applied. Differences in concentration-response curves to methacholine were tested by general linear model ANOVA for repeated measures. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Weight and Histological Characteristics. General parameters at the end of treatment are shown in Table 1. No differences were observed in body weight between the 4 groups.

Infarct sizes were in general small and did not differ significantly between the cAng-(1–7) and saline-treated group. Similarly, fibrosis did not differ between the groups (Table 1).

Despite the small infarct sizes total heart weight to body weight ratio has modestly but significantly increased in saline-treated MI group compared with SHAM (Figure 1(a)). Both doses of cAng (1–7) abolished the significant difference between MI and SHAM. However, only the higher dose of 2.4 μg/kg/h cAng-(1–7) resulted in a lower heart weight compared to saline, though not significantly different.

To further determine the cause of the weight differences the effect of cAng-(1–7) on myocyte size measured. Myocardial infarction increased myocyte cross-sectional area and decreased myocyte cell density (Figures 1(b) and 1(c)). Treatment with both doses of cAng-(1–7) restored myocyte cross-sectional area to the level of saline-treated sham (Figure 1(b)). Myocyte density was only restored by the higher dose of cAng-(1–7) (Figure 1(c)). In sham-operated animals, cAng-(1–7) treatment showed a trend towards a decrease in myocyte size, but this effect did not reach a statistical significance (Figures 1(b) and 1(c)).

3.2. Hemodynamics. After 8 weeks of treatment, cardiac function was measured in vivo in anesthetized rats. In accordance with the small infarct size, cardiac function was not significantly impaired in untreated MI rats as compared with SHAM (Table 1). In agreement with the absence of systolic or diastolic heart failure MI did not significantly change left ventricular end diastolic pressure (LVEDP) or left ventricular minimal pressure (Pmin) (*t*-test, $P = 0.199$ for LVEDP; $P = 0.090$ for Pmin), and therefore the effect of cAng-(1–7) was tested within the MI and sham group, respectively (Figure 2). In the MI group, cAng(1–7) treatment lowered LVEDP which was significant at the highest doses (Figure 2(a)). Since there seemed to be a dose-dependent effect we tested for a trend line, which resulted in a significance for trend. Pmin seemed also to be lowered in MI animals, but this effect did not reach statistical significance (Figure 2(b)). In sham animals, cAng-(1–7) given at a doses of 2.4 μg/kg/h lowered both LVEDP and Pmin (Figures 2(a) and 2(b)). All other measured pressure variables were not changed by cAng-(1–7) treatment as compared to MI saline (Table 1).

3.3. Endothelial Function. Endothelial dysfunction is a key feature in the development of heart failure after MI since it contributes to the increase of peripheral vascular resistance that leads to increased cardiac workload resulting in hypertrophy and contractile dysfunction of the myocardium. Therefore, we investigated endothelium-dependent relaxation in isolated aortic rings.

Phenylephrine (1 μmol/L) caused similar contractile responses in all groups (data not shown). The responses of aortic rings to endothelium independent vasodilator SNP were not changed between groups (data not shown). Responses to the endothelium-dependent vasodilator methacholine were unchanged in saline-treated MI animals when compared with SHAM (data not shown). However, both doses of cAng (1–7) showed increased responsiveness to methacholine when compared to saline-treated MI group, which was most pronounced and only significant in the higher dose (Figure 3(a)). After blocking the NO production of endothelium with L-NAME, the response to methacholine was greatly suppressed in all the groups, however the increased responsivity of high-dose cAng (1–7) treated animals remained present (Figure 3(b)). After blocking both NO and EDHF, leaving prostaglandins as the remaining dilator factor, the difference between saline and cAng-(1–7)-treated animals disappeared (Figure 3(c)) indicating that cAng works via EDHF.

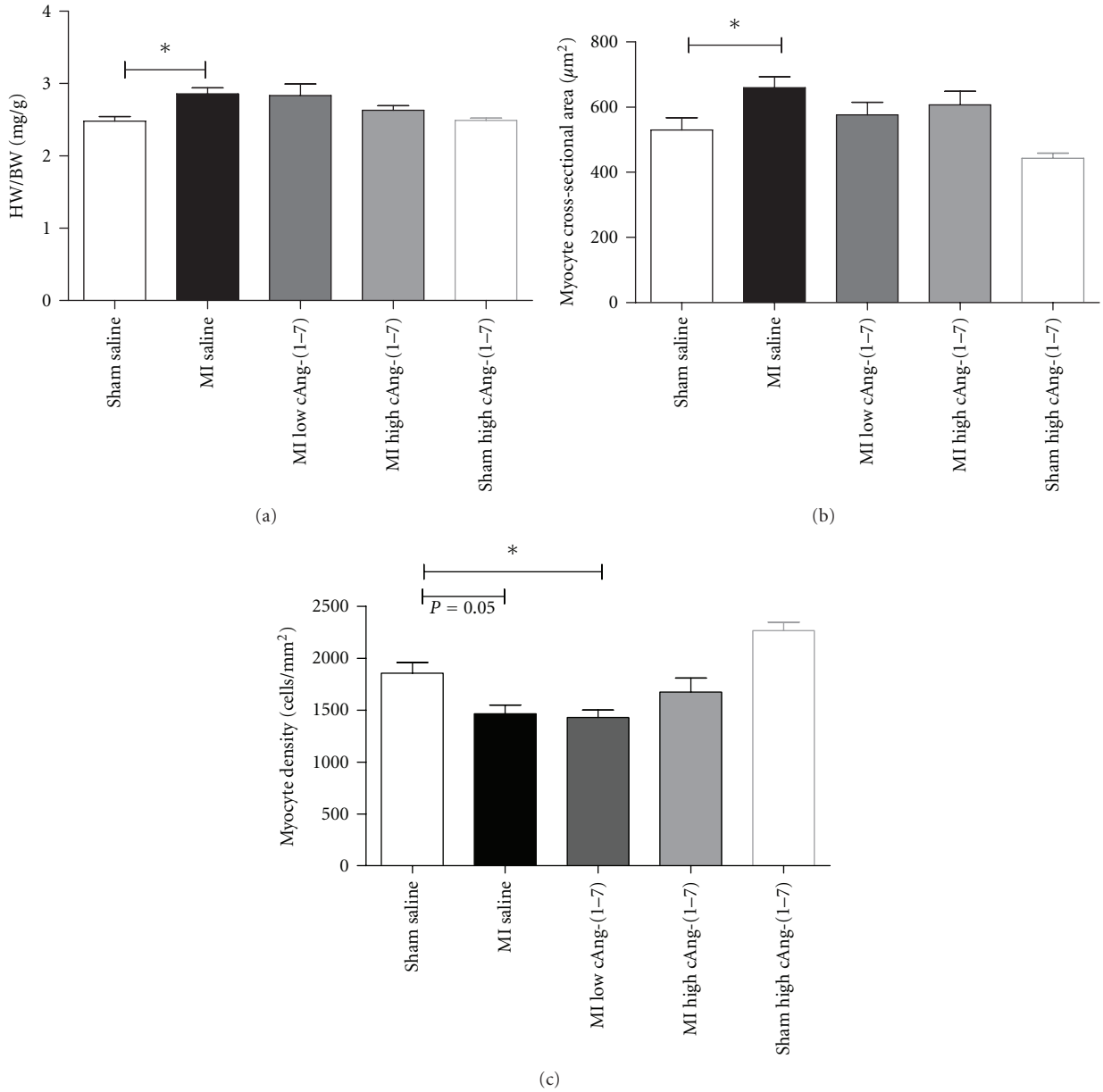

Figure 1: Comparison of heart weight/body weight ratios between the different treatments (a), variables of cardiac hypertrophy: myocyte, cross-sectional area (b), and myocyte density (c). (*$P < 0.05$, One way ANOVA, Dunnett's post hoc testing).

4. Discussion

Stimulation of the Ang-(1–7)/Mas receptor axis is a promising therapeutic strategy for treatment of MI and prevention of heart failure. For this purpose we tested the effect of the metabolically protected and Mas receptor-specific compound cAng-(1–7). Given at doses that were, respectively, 10 and 100 times lower than the minimally effective doses of native Ang-(1–7) [11], cAng-(1–7) dose-dependently lowered left ventricular weight and diastolic pressure in an MI model in which no contractile failure had yet occurred. The effect on cardiac weight seemed to depend at least partially on reduction of cardiomyocyte hypertrophy, as evidenced by the decrease in myocyte dimensions. The effects on the heart morphology and function were independent from the presence of an infarction since they also occurred in sham animals. In addition to effects on the heart, cAng-(1–7) improved peripheral endothelium-dependent vasodilation, as measured in isolated aortic rings; an effect that predominantly involved EDHF. cAng-(1–7) therefore shows favorable characteristic with regard to improvement of cardiovascular function after MI.

The present results with respect to cardiac improvement are in accordance with previous results in the MI model obtained after infusion of native Ang-(1–7) [4, 5]. A limitation of the present study, however, is the fact that infarct sizes

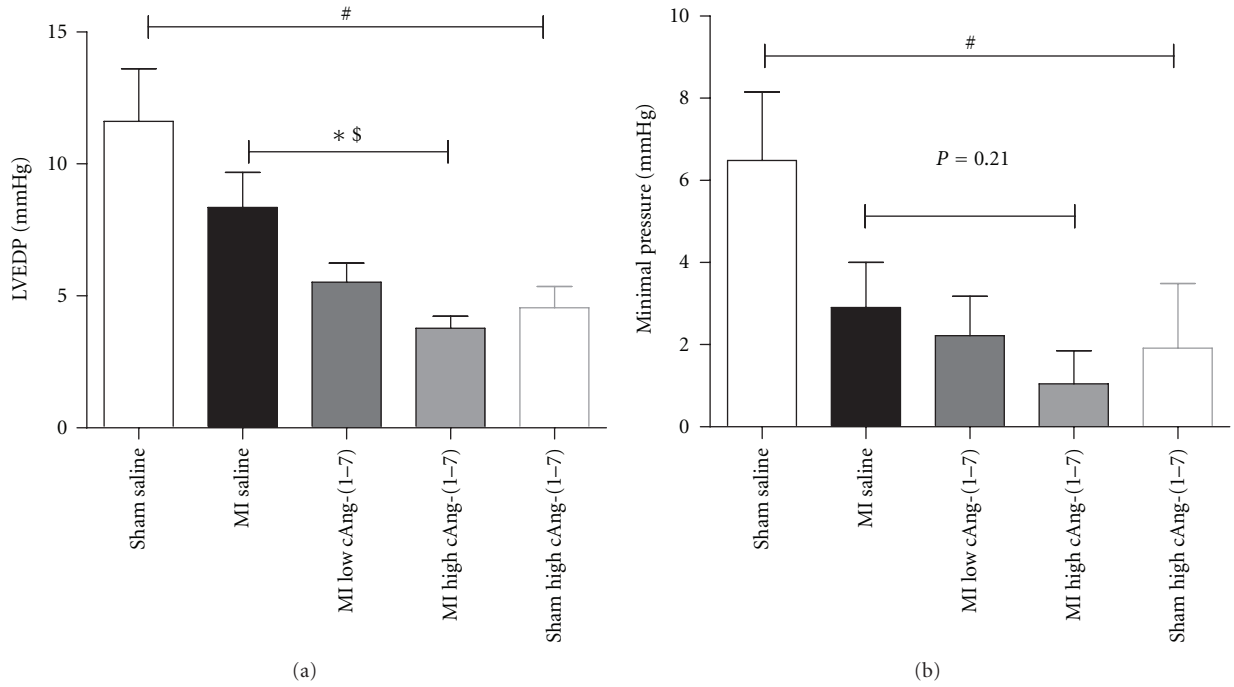

Figure 2: Effects of cAng 1–7 on left ventricular end-diastolic pressure and minimal pressure in both sham-operated rats and rats with myocardial infarction. ($^{\#}P < 0.05$ t-test sham saline versus sham cAng-(1–7); $^{*}P < 0.05$, One way ANOVA for MI groups, Dunnett's post hoc testing; $^{\$}P < 0.05$ for linear trend for MI groups).

were relatively small as compared to the previous studies, thus not allowing us to study possible beneficial effects of the compound on systolic function and cardiac fibrosis [4, 12, 13]. Nevertheless, the implications of the present study are relevant since patient populations also comprise subjects with relatively small infarct sizes but who will eventually develop heart failure, albeit after a relatively longer period. The full potential of cAng-(1–7) as an experimental drug can be appreciated from evaluation in a model of heart failure or cardiac fibrosis. The present data warrant such studies.

Endothelial dysfunction is an important hallmark in heart failure caused by MI and is believed to be pivotal in malignant cardiac remodeling due to increased afterload. Ang-(1–7) was shown to restore endothelium-dependent vasodilator function in heart failure, after stent placement, after a high salt diet and in the atherosclerosis-prone ApoE knockout mouse when infused chronically [4, 11, 14, 15]. Vascular upregulation of ACE2, which increases Ang-(1–7) levels, improves endothelial function in hypertensive rats [16]. Conversely, Mas receptor knockout or chronic treatment with A779, an antagonist of Mas receptor-associated effect diminishes endothelial function [17–19]. In accordance with the suggested role of Mas receptor signaling in improvement of endothelial function cAng-(1–7) infusion led to improved endothelial function in our rats with small MI. The improvement that was observed by us appears to be mainly caused by an increase of endothelium-derived hyperpolarizing factor (EDHF), and not through prostaglandin release. In a previous study, which involved relatively older rats that developed endothelial dysfunction after stent placement, chronic infusion of native Ang-(1–7) mainly increased prostaglandin [20]. Furthermore, short-term infusion of the native peptide improves the hypotensive response to acetylcholine through NO signaling, whilst Mas receptor knockout results in impaired NO bioavailability [19, 21]. Thus, the model that is used for studying the effect of Ang-(1–7) mediated seems to determine the signaling pathway that is improved. Our present results are to our knowledge the first to show an increased contribution of EDHF and emphasize the versatility of the therapeutic potential of the Ang-(1–7)/Mas receptor axis towards endothelial function.

As noted above, cAng-(1–7) was intravenously administered by osmotic minipump in a dose that was 10 to 100 times lower than in previous studies the lowest efficacious dose for native Ang-(1–7). This approach allowed us to make comparisons with these previous studies and indicate that the pharmacological properties of cAng-(1–7) seem to be superior to those of native Ang-(1–7). To provide conclusive evidence it will be necessary to test cAng-(1–7) in a model of heart failure. Furthermore, a clinically relevant method of drug delivery will have to be developed. Most commonly, clinically applicable peptides are administered subcutaneously where the peptide is not degraded and which allows manipulation of the rate of peptide release, such as in the case of insulin formulations. In a recent study it was shown that subcutaneous cAng-(1–7) resulted in a 98% bioavailability. Although less efficient, oral and especially pulmonary delivery (28% bioavailability) of cAng-(1–7) appeared possible too. Therefore translation to the clinic is

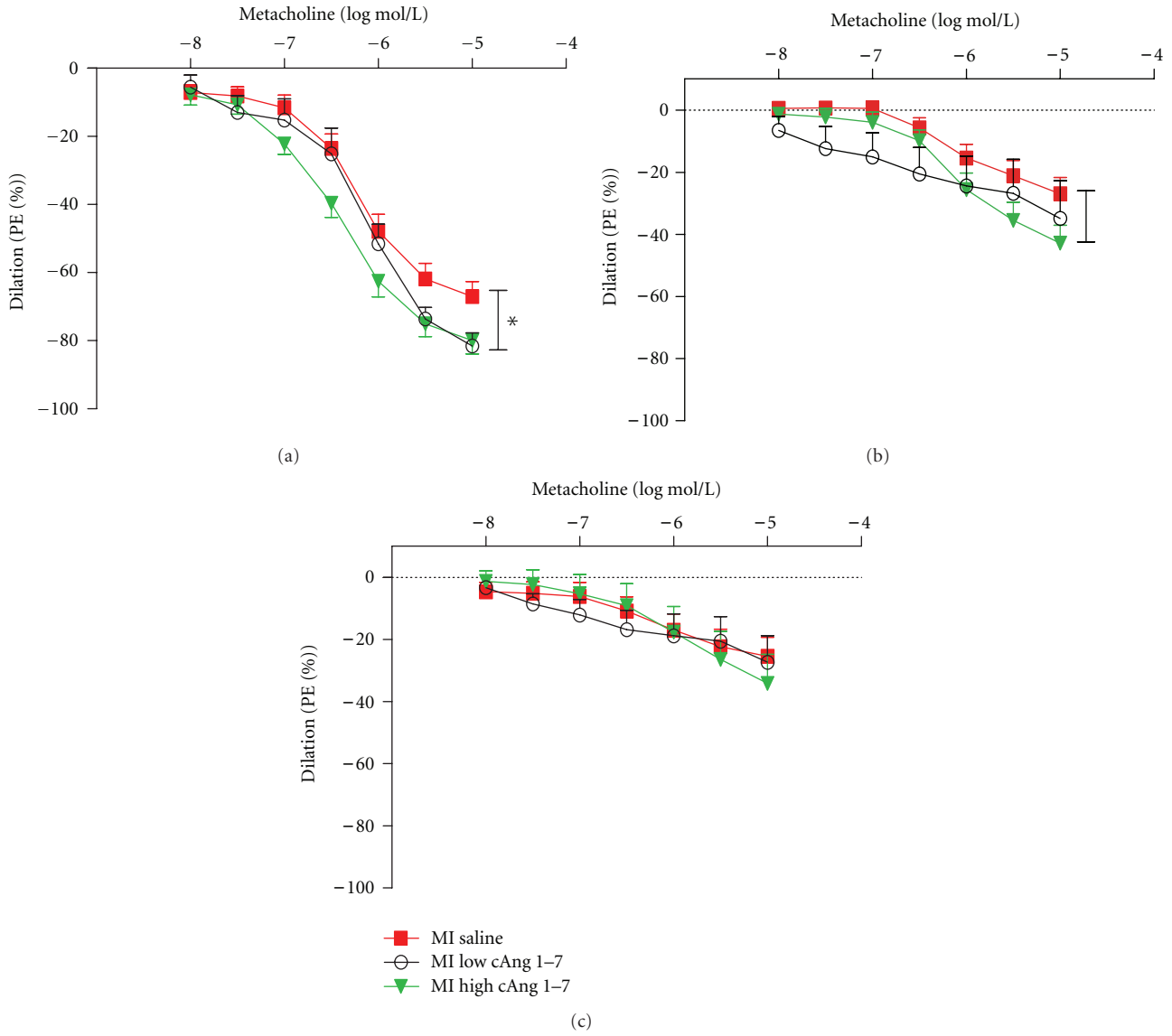

FIGURE 3: Endothelial-dependent dilator function of rat aorta to metacholine (a), after blockade of eNOS/NO signaling (b), and after combined blockade of eNOS/NO and EDHF vasodilator mechanisms (c). (*$P < 0.05$, GLM-RM).

feasible [22]. There are other approaches to design a clinically relevant delivery method to exploit the Ang-(1–7)/Mas axis. These designs fall into four main categories: local delivery of the native peptide, nonpeptide analogues, protective incapsulation of the native peptide, and upregulation of the Ang-(1–7)-synthesizing enzyme ACE2. Local delivery is an elegant way to circumvent loss of bioavailability of Ang-(1–7). This approach has been explored to counteract problems that are associated with stent placement and has led to prevention of endothelial dysfunction [23]. Theoretically, this strategy should also be applicable for solid tumors. Peptide incapsulation includes PEG-liposome complexes that can be delivered intravenously [23], but most promising appears to be the use of hydroxypropyl β-cyclodextrin, which has led to successful cardioprotection after infarction or chronic isoproterenol infusion in rats when delivered orally [24]. Nonpeptide analogues include AVE 0991 and CGEN-856S, which show vasodilatory and cardioprotective properties (less arrythmias during recovery from I/R) in vitro, and antihypertensive effects in vivo [23, 25]. However, oral delivery has not been attempted with these compounds. Last, upregulation of ACE2 has been successfully attempted as intervention in cardiac and pulmonary fibrosis models, and in Ang-II-dependent renal fibrosis. Of particular interest is the use of 1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one (XNT), an ACE2 ligand and activator of the enzyme. Until present, XNT was shown effective against cardiac and pulmonary fibrosis and against pulmonary hypertension when administered subcutaneously with minipumps [26, 27].

In summary, we here present the first data showing that lanthionine-bridged Ang-(1–7), shortly cAng-(1–7), holds

promise as a therapeutic agent after MI, as it improves cardiac remodeling and endothelial function and since it has previously [22] been demonstrated that it can be delivered orally and pulmonarily. Our present results warrant further testing of this compound in various models of heart failure and possible other diseases that can be a target of beneficial Ang-(1–7)/Mas receptor axis signaling.

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