ACE2 and vasoactive peptides: novel players in cardiovascular/renal remodeling and hypertension

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***Abstract*:** The renin–angiotensin system (RAS) is a key component of cardiovascular physiology and homeostasis due to its influence on the regulation of electrolyte balance, blood pressure, vascular tone and cardiovascular remodeling. Deregulation of this system contributes significantly to the pathophysiology of cardiovascular and renal diseases. Numerous studies have generated new perspectives about a noncanonical and protective RAS pathway that counteracts the proliferative and hypertensive effects of the classical angiotensin-converting enzyme (ACE)/angiotensin (Ang) II/angiotensin type 1 receptor (AT1R) axis. The key components of this pathway are ACE2 and its products, Ang-(1-7) and Ang-(1-9). These two vasoactive peptides act through the Mas receptor (MasR) and AT2R, respectively. The ACE2/Ang-(1-7)/MasR and ACE2/Ang-(1-9)/AT2R axes have opposite effects to those of the ACE/Ang II/AT1R axis, such as decreased proliferation and cardiovascular remodeling, increased production of nitric oxide and vasodilation. A novel peptide from the noncanonical pathway, alamandine, was recently identified in rats, mice and humans. This heptapeptide is generated by catalytic action of ACE2 on Ang A or through a decarboxylation reaction on Ang-(1-7). Alamandine produces the same effects as Ang-(1-7), such as vasodilation and prevention of fibrosis, by interacting with Mas-related GPCR, member D (MrgD). In this article, we review the key roles of ACE2 and the vasoactive peptides Ang-(1-7), Ang-(1-9) and alamandine as counter-regulators of the ACE–Ang II axis as well as the biological properties that allow them to regulate blood pressure and cardiovascular and renal remodeling.

Keywords: ACE2, angiotensin-(1-7), angiotensin-(1-9), alamandine, blood vessels, heart, hypertension, kidney, renin–angiotensin system, tissue remodeling

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Introduction

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, representing 30% of all deaths [WHO, 2011]. Atherosclerosis and hypertension (HT) are the most common causes of CVD [Poulter, 2003; Coffman, 2011]. These pathologies predominantly affect the heart and blood vessels, through cardiovascular remodeling [Kearney *et al.* 2005], a process characterized by structural and functional changes in the arteries and heart [Creemers and Pinto, 2011; Fan *et al.* 2012; Heusch *et al.* 2014]. Cardiac remodeling involves the development of cardiac hypertrophy, which is initially is a compensatory process to reduce ventricular wall stress, improve cardiac output and maintain energy requirements [Berenji *et al.* 2005]. Vascular remodeling affects artery function and involves changes in vascular smooth muscle cell (VSMC) migration, proliferation, cell death and extracellular matrix protein secretion [Gibbons and Dzau, 1994]. Vascular remodeling can trigger kidney remodeling, characterized by parenchymal destruction and fibrosis. This complex interaction has been described as cardiorenal syndrome [Ronco *et al.* 2008; Campbell *et al.* 2009].

In this article, we review the key role of the angiotensin-converting enzyme 2 (ACE2) and the vasoactive peptides angiotensin-(1-7) [Ang-(1-7)], angiotensin-(1-9) [Ang-(1-9)] and alamandine as counter-regulators of the ACE–angiotensin II

(Ang II) axis as well as the biological properties that allow them to regulate blood pressure (BP) and cardiovascular and renal remodeling.

Canonical renin–angiotensin system (RAS)

HT is one of the most common factors that triggers cardiovascular remodeling. This disease is characterized by chronically elevated BP and mainly affects the cardiovascular system. HT occurs due to increased mechanical and hemodynamic stress, pressure overload and activation of vasoactive substances. Ang II, a component of the RAS, is one of most important vasoactive substances and influences the development of HT and cardiovascular remodeling [Touyz, 2003; Montezano *et al.* 2014].

The RAS is a fundamental component of cardiovascular physiology and homeostasis due to its influence on the regulation of electrolyte balance, BP, vascular tone and cardiovascular remodeling. Deregulation of this system contributes significantly to the pathophysiology of CVD [McKinney *et al.* 2014]. The canonical RAS pathway begins with the formation of the decapeptide angiotensin I (Ang I) in the circulation from angiotensinogen of hepatic origin, *via* the action of renin. ACE then catalyzes the cleavage of two amino acids from the *C*-terminal of Ang I to form Ang II, which is involved in sodium homeostasis, aldosterone secretion and regulation of BP by action in VSMC [Varagic *et al.* 2014].

Ang II binds with high affinity to its two different cell–surface receptors, subtypes AT1R and AT2R, which are members of the seven transmembrane domain G-protein-coupled receptors (GPCRs) superfamily, through Gq and Gi, respectively [Kaschina and Unger, 2003]. These receptors differ with respect to molecular weight, signaling mechanisms and tissue-specific expression [Li *et al.* 2012]. AT1R mediates most of the physiological and pathological effects of Ang II and is widely expressed in the cardiovascular cells [Mehta and Griendling, 2007]. The binding of Ang II to the AT1R results in coupling of G proteins (Gq/G11and/or Gi/Go) to the *C*-terminal of the receptor, inducing many intracellular effects through phospholipase C (PLC)/inositol trisphosphate (IP3)/diacylglycerol (DAG)/Ca^{2+} pathway, adenylyl cyclase, MAP kinases, tyrosine kinases, tyrosine phosphatases, PLC/phospholipase A2 (PLA2), Janus kinase (JAK) signal transducer and activator of transcription (STAT) pathway, NADPH oxidase, RhoA/Rho kinase among others [Higuchi *et al.* 2007], causing cardiovascular remodeling [Suzuki *et al.* 2005]. Some tyrosine kinase receptors in VSMCs, such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) are transactivated by AT1R to induce growth and migration [Higuchi *et al.* 2007].

AT2R shares only 34% sequence identity with AT1R and forms heterodimers with AT1R [Funke-Kaiser *et al.* 2010]. The human *AGTR2* gene that encodes for AT2R is located on the X chromosome and consists of three exons with an uninterrupted coding region that encodes a 363 amino acid receptor [Funke-Kaiser *et al.* 2010]. Whereas AT1R mediates most of the recognized detrimental effects of Ang II, it seems that the AT2R opposes, in part, the AT1R mediated effects [Li *et al.* 2012]. AT2R is expressed in adult tissues in smaller amounts than AT1R [Horiuchi *et al.* 1999]. The expression of AT2R undergoes a complex tissue specific regulation that appears to influence vascular development and repair. AT2R is expressed by cardiomyocyte, cardiac fibroblast, aorta, coronary and resistant arteries. AT2R is regulated during HT, inflammation, myocardial infarction and vascular injury [Faria-Costa *et al.* 2014]. AT2R effects and cell signaling have been less well characterized than those of AT1R [Savoia *et al.* 2005; Hu *et al.* 2008]. Current knowledge suggests that AT2R stimulation mediates vasodilation, antigrowth, proapoptotic and anti-inflammatory effects [Touyz and Schiffrin, 2000; Henrion *et al.* 2001]. Hence, AT2R can modulate cardiovascular remodeling as well as progression of atherosclerosis [Faria-Costa *et al.* 2014]. Recent studies suggest that AT2R regulates BP, cardiac hypertrophy and fibrosis, and cell death after myocardial infarction [Li *et al.* 2012;Tavares *et al.* 2013].

AT2R stimulation activates the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) pathway [Abadir *et al.* 2003]. This occurs either directly or indirectly through bradykinin (BK) or by increased endothelial nitric oxide synthetase (NOS) activity or expression. AT2R activation is associated with phosphorylation of protein phosphatases, IκBα (inhibitor of NF-κB), the transcription factor ATF2, and dephosphorylation of JNK, p38MAPK, ERK1/2 and STAT3, which are linked to antiproliferative and anti-inflammatory effects and apoptosis [Brassard *et al.* 2005; Savoia *et al.* 2006; Dandapat *et al.* 2008; Hu *et al.* 2008].

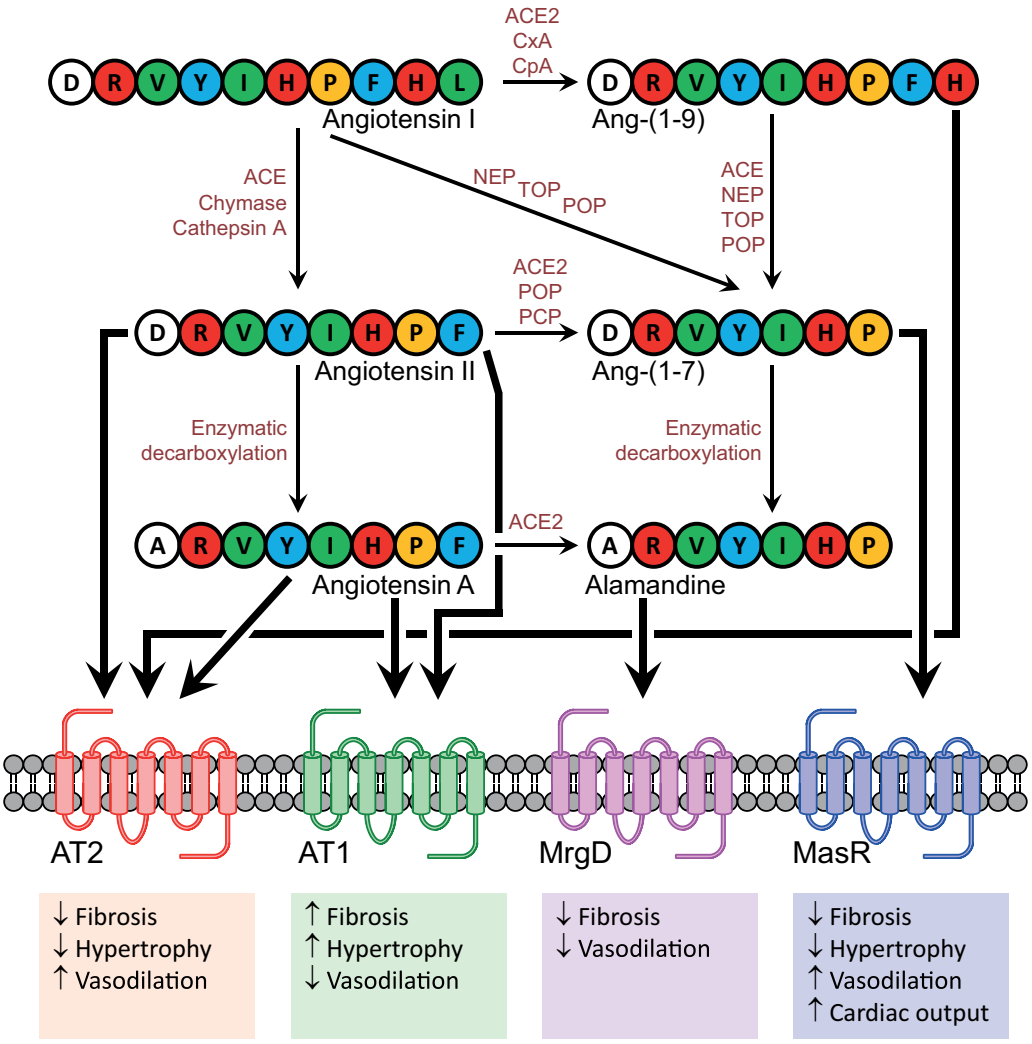

Figure 1. Protective RAS pathway: ACE2 and vasoactive peptides.
ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; AT2R, angiotensin type 2 receptor; CxA, carboxypeptidase A; CpA, cathepsin A; NEP, neutral endopeptidase; MasR, Mas receptor; MrgD, Mas-related G protein-coupled receptor member D; PCP, prolyl carboxypeptidase; POP, prolyl endopeptidase; TOP, thimet oligopeptidase.

AT2R stimulation induces relaxation by large-conductance Ca^{2+}-activated K^+ channels (BKCa) opening [Dimitropoulou *et al.* 2001] and by negative regulation of the vascular RhoA/Rho kinase pathway [Savoia *et al.* 2006]. AT2R also enhances tyrosine phosphatases and vanadate-sensitive phosphatases MKP1 (DUSP1), SHP1 (PTPN6) and PP2A activities and inhibits cell growth [Bedecs *et al.* 1997; Horiuchi *et al.* 1997].

Noncanonical RAS

The key components of this pathway are ACE2, Ang-(1-7) and Ang-(1-9). Ang-(1-7) can be produced from Ang I by different endopeptidases and from Ang II by ACE2 (Figure 1) [Santos *et al.* 1988; Ferrario *et al.* 1991]. This heptapeptide has positive cardiovascular and renal effects such as vasodilation and antifibrosis [Santos *et al.* 1992; Ferreira *et al.* 2010]. Ang-(1-9) is generated from Ang I by ACE2, regulates cardiac hypertrophy and has antihypertensive effects [Varagic *et al.* 2014]. Ang-(1-7) and Ang-(1-9) produce biological effects through the Mas receptor (MasR) and AT2R, respectively [Santos *et al.* 2003; Flores-Muñoz *et al.* 2011].

The GPCR MasR is encoded by the Mas proto-oncogene [Young *et al.* 1986]. Among its physiological ligands are Ang-(1-7) and the neuropeptide FF (NPFF) [Santos *et al.* 2003]. Most effects of MasR are mediated by Ang-(1-7) [Santos *et al.*

2006]. However, Ang III and Ang IV activate MasR generating similar effects to those found for Ang-(1-7) [Gembardt *et al.* 2008]. The nonpeptide compound AVE 0991, an agonist of MasR, mimics Ang-(1-7) effects in blood vessels [Lemos *et al.* 2005], heart [Benter *et al.* 2006] and kidney [Pinheiro *et al.* 2004]. Kostenis and colleagues showed that MasR hetero-oligomerizes with AT1R and inhibits the actions of Ang II [Kostenis *et al.* 2005].

A novel peptide from the noncanonical pathway, alamandine was recently identified in rats, mice and humans [Lautner *et al.* 2013]. This heptapeptide is generated by the catalytic action of ACE2 on Ang A or through a decarboxylation reaction on Ang-(1-7) in the *N*-terminal aspartate amino acid residue. Alamandine produces the same effects as Ang-(1-7), such as vasodilation and antifibrosis [Villela *et al.* 2014], through interaction with the Mas-related GPCR member D (MrgD), also known as hGPCR45 [Takeda *et al.* 2002] and TGR7 [Shinohara *et al.* 2004]. This receptor was recently identified as a novel GPCR in murine and human genomes [Dong *et al.* 2001].

Noncanonical RAS as counter-regulator of the canonical RAS

Recent studies have generated new perspectives about a noncanonical and protective RAS pathway (Figure 1) that counteracts the proliferative and hypertensive effects of the classical ACE/Ang II/AT1R axis [Donoghue *et al.* 2000; McKinney *et al.* 2014]. The ACE2/Ang-(1-7)/MasR and ACE2/Ang-(1-9)/AT2R axes have effects opposite to those of the ACE/Ang II/AT1R axis, such as decreased proliferation and cardiovascular remodeling, increased production of NO and vasodilation [Ocaranza *et al.* 2014b, 2014c; Varagic *et al.* 2014].

There are various conjectures about the selective activity of Ang-(1-9) on AT2R, as compared with Ang II, which shows a higher affinity for AT2R and produces different effects. AT1R and AT2R share structural hallmarks of GPCRs; AT2R contains equivalent residues in the critical positions Asn^{111}, Lys^{199} and Asp^{281}, but it lacks an equivalent residue for His^{256}, conferring it with binding properties similar to those of a constitutively-activated AT1R. This phenotype, described as relaxed conformation, might be susceptible to stabilization by the extra histidine residue present in Ang-(1-9), leading to the activation of pathways that counter-regulate the effects of Ang II and the AT1R [Miura and Karnik, 1999]. Furthermore, there could be a ligand-specific conformation triggered by Ang-(1-9) that results in differential signaling pathway activation [Clarke and Bond, 1998]. Another possibility is that Ang-(1-9) exerts its effects by being metabolized to Ang-(1-7) or another unknown peptide with great affinity for the AT2R, or an alternative receptor that is also affected by the AT2R inhibitor PD123319. This alternative receptor could be the MrgD [Lautner *et al.* 2013].

ACE2

Like ACE, ACE2 belongs to the family of zinc metalloproteases. ACE2 is a membrane-bound ecto-enzyme that can be found in soluble form in the plasma and tissues such as heart, liver, kidney, brain and blood vessels [Ocaranza *et al.* 2014a]. ACE2 gene expression is high in the kidney and low in the heart, aorta, lung and retina. ACE2 is not expressed in the brain, adrenals, skeletal muscle or fatty tissue [Yagil *et al.* 2003]. ACE and ACE2 have 40% amino acid sequence homology but have different substrate specificities [Chamsi-Pasha *et al.* 2014]. ACE2 is a monocarboxypeptidase with a single active site and greater (~400-fold) affinity for Ang II than Ang I [Tipnis *et al.* 2000; Rice *et al.* 2004] and is insensitive to ACE inhibitors (ACEIs) [Donoghue *et al.* 2000].

ACE2 regulation

The metalloproteinase ADAM17 cleaves the membrane-bound ectodomain of ACE2, which releases soluble ACE2 into the plasma [Iwata *et al.* 2009]. Levels of this form of the enzyme are elevated after myocardial infarction (MI) and Ang II promotes ACE2 shedding by increasing ADAM17 activity [Patel *et al.* 2014]. This could be one of the mechanisms by which Ang II induces heart injury.

ACEIs and AT1R antagonists (ARAs) increase ACE2 activity in the heart and kidneys in normotensive and hypertensive rats and in rats with MI [Ishiyama *et al.* 2004; Ferrario *et al.* 2005a, 2005b; Ocaranza *et al.* 2006]. ACEIs and ARAs can also modulate both the ACE2/Ang-(1-7)/MasR and ACE2/Ang-(1-9)/AT2R axes. ACEIs increase levels of Ang I, which can then be converted to Ang-(1-9) by ACE2 or to Ang-(1-7) by other endopeptidases [Moon, 2013]. The effect of

the ACEI captopril may be accompanied by increased excretion of Ang-(1-7) [Castro-Moreno *et al.* 2012]. ARAs increase the availability of Ang II so that it can be metabolized to Ang-(1-7). Ang-(1-7) can also be an Ang II antagonist, because it binds with low affinity to the AT1R [Moon, 2013].

Several ACE2 activators have been tested, such as XNT and NCP-2454. XNT decreases BP and improves cardiac function in hypertensive rats [Hernandez Prada *et al.* 2008]. NCP-2454 prevents pulmonary HT by inhibiting inflammatory cascades and restoring caveolin 1 expression [Haga *et al.* 2014]. However, recombinant ACE2 attenuates diabetic nephropathy by decreasing Ang II and increasing Ang-(1-7) levels [Oudit *et al.* 2010].

ACE2 in HT

The potential role of ACE2 in essential HT is based on the findings that this enzyme is present in the heart and kidney [Donoghue *et al.* 2000; Tipnis *et al.* 2000] and that the ACE2 gene is associated with the BP locus on the X chromosome [Crackower *et al.* 2002]. Both spontaneously hypertensive rats (SHR) and spontaneously hypertensive stroke-prone rats (SHRSP) show reduced renal ACE2 protein levels compared with normotensive Sabra and Wistar Kyoto (WKY) strains [Crackower *et al.* 2002; Zhong *et al.* 2004; Tikellis *et al.* 2006]. Renal expression of ACE2 is higher in newborn and prehypertensive SHR rats and diminishes as the rat reaches adulthood and becomes hypertensive [Tikellis *et al.* 2006]. However, some investigators have been unable to detect any difference in renal ACE2 mRNA, protein or activity between adult hypertensive rats and their normotensive controls [Hamming *et al.* 2005]. Transgenic SHRSP-ACE2 rats on a SHRSP genetic background expressing the human ACE2 in VSMCs have significantly elevated circulating Ang-(1-7) levels. Mean arterial BP and vasoconstrictive response to Ang II are reduced, while endothelial function is improved in SHRSP-ACE2 compared with SHRSP rats [Rentzsch *et al.* 2008]. Gain-of-function experiments in SHRs indicated that ACE2 expression in the central nervous system is associated with substantially decreased BP [Yamazato *et al.* 2007] and improves of endothelial function in hypertensive rats [Gallagher *et al.* 2008]. These effects were accompanied with increased levels of Ang-(1-7) in both plasma and kidney [Moon, 2013]. Indeed, ACE2-deficient C57BL/6 mice have raised basal BP with notable amplifications of angiotensin-II-induced HT and have increased accumulation of Ang II in plasma and the kidney after Ang II infusion [Gurley *et al.* 2006].

Based on these observations, low ACE2 expression and activity have been associated with HT, suggesting that ACE2 could prevent the development of HT. Therefore, ACE2 has been proposed as a novel therapeutic target for HT treatment [Diez-Freire *et al.* 2006]. In fact, ACE2 overexpression in SHR and WKY rats, produced using a lentiviral vector, results in significantly decreased systolic BP. However, genetic studies in humans have not shown an association between ACE2 and HT. Indeed, four single nucleotide polymorphisms (SNPs) in the ACE2 gene were evaluated and none showed any association with HT [Benjafield *et al.* 2004]. The disparity between animal and human results regarding ACE2's relationship with HT could be due to the complex compensatory mechanisms present in humans. In genetic hypertensive animal models, these compensatory mechanisms are less prevalent, as animals display spontaneous HT, while human essential HT is multifactorial and is the result of both environmental and genetic factors [Jacob, 1999; Stoll *et al.* 2000, 2001]. Decreased ACE2 expression could be a predisposing rather than a causal factor in essential HT.

The manipulating of ACE2 gene expression in adult animal is a useful alternative approach in studying this enzyme's role in controling BP and cardiovascular functions. In ACE2 knockout mice, BP was significantly increased following Ang II infusions [Gurley *et al.* 2006]. In contrast, a spontaneous hypotension was observed in ACE knockout mice [Krege *et al.* 1995]. These data suggest that, in addition to the Ang II system, ACE2 might regulate BP.

Recently, Sriramula and colleagues showed that ACE2 overexpression in the brain attenuated the development of deoxycorticosterone acetate (DOCA) salt HT, a neurogenic HT model with enhanced brain RAS and sympathetic activity [Sriramula *et al.* 2014]. DOCA salt induces a reduction of neuronal NOS expression and phosphorylated endothelial NOS/total endothelial NOS, and enhanced phosphorylation of Akt and ERK 1/2 in the paraventricular nucleus; these effects are reversed by ACE2 overexpression. ACE2 overexpression blunts the HT-mediated

increase in the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) gene and protein expression in the paraventricular nucleus. Furthermore, gene silencing of either COX-1 or COX-2 in the brain reduces microglial activation and accompanied neuroinflammation, ultimately attenuating DOCA salt HT. These data provide evidence that brain ACE2 overexpression reduces oxidative stress and COX-mediated neuroinflammation, improves antioxidant and NO signaling, and thereby attenuates the development of neurogenic HT.

ACE2 in cardiac remodeling

Because cardiomyocytes express a complete RAS, the heart was the first organ in which ACE2 physiology was studied [Averill *et al.* 2003]. In the heart, ACE2 participates in the structural and functional regulation. ACE2 knockout mice present a deficiency in cardiac contractility accompanied by high levels of Ang II in the plasma and heart [Crackower *et al.* 2002]. ACE2 gene expression is abundant in the heart [Ferrario, 2006]. The severity of cardiac dysfunction depends on the age and sex of the animals, with abnormalities most pronounced in adult male mice. However, the hearts of these animals do not show dilatation, hypertrophy, fibrosis or excessive collagen deposits, although Ang II levels are elevated in the heart and plasma [Crackower *et al.* 2002]. The cardiac phenotype found in ACE2$^{-/-}$ mice is similar to that observed in cardioplegia, representing human adaptive responses to coronary disease and bypass surgery [Hsueh *et al.* 1998; Kloner *et al.* 1998]. These mice also show a greater expression of genes induced by hypoxia, a condition that causes endothelial dysfunction, vasoconstriction and cardiac hypoperfusion [Crackower *et al.* 2002].

The ventricular dysfunction observed in ACE2$^{-/-}$ mice can be completely reversed by a second mutation that causes ACE gene deficiency [Crackower *et al.* 2002]. These results imply an important regulatory mechanism involving an interaction between ACE and ACE2 in normal control of cardiac function [Crackower *et al.* 2002]. Similarly in MI, loss of ACE2 accelerates maladaptive left ventricular remodeling [Kassiri *et al.* 2009]. ACE2's role in cardiac physiopathology has also been supported by observations of increased cardiac ACE2 activity in patients with terminal idiopathic dilated cardiomyopathy [Zisman *et al.* 2003] and with ischemic heart disease [Goulter *et al.* 2004]. These observations suggest that a greater expression of ACE2 in damaged tissue can result from the participation of ACE2 as compensatory mechanism.

Although most studies suggest that ACE2 may have cardioprotective effects after myocardial injury, studies in transgenic mice with increased cardiac ACE2 expression have shown an increased incidence of sudden death that correlates with ACE2 expression levels [Donoghue *et al.* 2003]. Electrophysiological studies have revealed alterations in heart rhythm, including ventricular tachycardia that can progress to fibrillation and death. Interestingly, Ang-(1-7) has been shown to reverse cardiac arrhythmia [Santos *et al.* 2004]. Additionally, the administration of Ang-(1-7) preserves cardiac contractility after experimental MI in rats [Loot *et al.* 2002].

To understand the importance of ACE2 in cardiac remodeling, the effect of cardiac ACE2 overexpression using a lentivirus [Huentelman *et al.* 2005], as well as evaluating the effect of ACE inhibitors [Ocaranza *et al.* 2006]. ACE2 overexpression significantly reduces the development of left ventricular hypertrophy (LVH) and myocardial fibrosis triggered by Ang II [Huentelman *et al.* 2005]. Further, in MI rats randomized to receive either vehicle or ACEI for 8 weeks, enalapril, prevented LVH and increased ACE2 expression several fold [Ocaranza *et al.* 2006]. These results suggest a cardioprotective role of ACE2 in those models. There is evidence that ACE2 is upregulated post-MI in both a rat model and in human patients and in the failing heart [Karram *et al.* 2005; Ocaranza *et al.* 2006; Epelman *et al.* 2009; Kim *et al.* 2010; Burchill *et al.* 2012; Ortiz-Perez *et al.* 2013]. During the early post-MI period (1 week) in rats, ventricular dysfunction is associated with significantly increased plasma and LV ACE and ACE2 activity, as well as elevated Ang II/Ang I and Ang-(1-9)/Ang I ratios, but reduced Ang-(1-7)/Ang I ratios [Ocaranza *et al.* 2006]. However, during the long-term post-MI stage (8 weeks), the development of LVH is associated with marked myocardial dysfunction [Ocaranza *et al.* 2006]. Plasma and LV ACE and Ang II levels remain high during this stage, but plasma and LV ACE2 activity and plasma Ang-(1-9) levels are lower than those of controls, with no change in plasma Ang-(1-7) levels observed at any time [Ocaranza *et al.* 2006]. Similar results have also been found in a HF model induced by volume overload [Karram *et al.* 2005]. The increase in cardiac and plasma ACE2 activity

noted after experimental MI is consistent with studies in patients [Ortiz-Perez *et al.* 2013]. In this study, the novel findings were that ACE2 is upregulated in the acute phase of ST segment elevation MI (STEMI) in humans. Serum ACE2 activity was found to correlate with the infarct size and ACE2 activity measured acutely is associated with late LV systolic function and occurrence of adverse LV remodeling. However, varied cardiac effects of ACE2 on post-MI remodeling have been shown. The inhibition of ACE2 by the compound C16 increased the infarct size and reduced the LV contractile function, while resulted in less myocardial hypertrophy and fibrosis suggesting that ACE2 activity has diverse effects on post-MI remodeling [Kim *et al.* 2010]. Also Burchill and colleagues found that the beneficial effects of ACEI and ARA or in combination on cardiac remodeling were similar and occurred with no further increase in cardiac ACE2 expression [Burchill *et al.* 2012] .

ACE2 genetic deletion or pharmacological inhibition has been associated with LVH, dysfunction in response to MI and diabetic cardiomyopathy [Kim *et al.* 2010; Patel *et al.* 2012; Moritani *et al.* 2013]. ACE2 overexpression prevented collagen accumulation, cardiomyocyte hypertrophy and myocardial fibrosis. Moreover, it also improved left ventricular remodeling and function in a rat model with diabetic cardiomyopathy [Dong *et al.* 2012]. Furthermore, SNPs in the ACE2 gene are also associated with LVH, impaired cardiac function and HT [Patel *et al.* 2012; Wang *et al.* 2013; Yang *et al.* 2014]. Because of these and other findings, ACE2 gene is considered a functional candidate for CVD [Burrell *et al.* 2013].

ACE2 in vascular remodeling

ACE2 is present in human coronary arteries and arterioles, the *vasa vasorum* of most organs and large conduit arteries of SHRs [Donoghue *et al.* 2000; Hamming *et al.* 2004; Igase *et al.* 2005]. ACE2 localizes preferentially to endothelial cells and arterial VSMCs [Donoghue *et al.* 2000; Hamming *et al.* 2004]. Vascular endothelial damage induces vascular ACE [Shi *et al.* 1994]. Reducing BP with drugs that block the RAS such as ACEI [Thybo *et al.* 1995; Rizzoni *et al.* 1998a, 1998b] or ARAII [Rizzoni *et al.* 1998a, 1998b; Schiffrin *et al.* 2000] and calcium channel antagonists [Porteri *et al.* 1998] reverses the eutrophic inward vascular remodeling [Hamming *et al.* 2004].

As a specific Ang II-degrading enzyme, ACE2 suppresses VSMC proliferation and vascular hypertrophy. Loss of ACE2 led to vascular proliferation and elevated migration of VSMC, while ACE2 overexpression inhibited vascular proliferation and hypertrophy by preventing aortic wall thickening [Jin *et al.* 2012; Patel *et al.* 2012]. In hypertensive animal models, ACE2 mRNA and protein have been associated with immunoreactive Ang-(1-7) in the large conduit arteries of SHRs. Treatment with an AT1R blocker induces a five-fold increase in ACE2 mRNA and is associated with a significant increase in aortic Ang-(1-7) protein expression. This effect is associated with decreased aortic medial thickness, suggesting that this may be a protective mechanism to prevent cardiovascular events during HT [Igase *et al.* 2005]. 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 [Igase *et al.* 2008]. ARA-induced increase in neointimal ACE2 was associated with a reduction in neointima thickness [Igase *et al.* 2008]. This association between an increase in ACE2 protein in the injured carotid artery of SHR and vascular remodeling during blockade of Ang II receptors could indicate the possibility that ACE2 plays a role in mediating the local effects of reversal of vascular hypertrophy in the carotid artery.

Aortic wall from hypertensive rats shows increased ACE levels, decreased ACE2 mRNA and protein levels, with no differences in the other angiotensins. These findings are associated with increased mRNA and protein levels of transforming growth factor (TGF) β1, plasminogen activator inhibitor-1 (PAI-1) and monocyte chemoattractant protein-1 (MCP-1) [Ocaranza *et al.* 2011]. These results strongly suggest that vascular remodeling could be dependent on the tissular ACE2 axis rather than the plasmatic RAS [Ocaranza *et al.* 2011].

Ang-(1-7)

Ang-(1-7) is produced from Ang I by different endopeptidases which cleave the Pro^{7}-Phe^{8} bond to remove the last three amino acids. The role of the prolyl endopeptidase (PEP) [Welches *et al.* 1991], thimet oligopeptidase (TOP) and neutral endopeptidase (NEP) [Chappell *et al.* 1998] in the conversion of Ang I into Ang-(1-7) depends on tissue enzyme distribution and substrate availability. Neprilysin is highly active in the vascular endothelium whereas the hydrolytic activity of

TOP is important in the formation of Ang-(1-7) in VSMC [Welches *et al.* 1993; Chappell *et al.* 1994]. Ang-(1-7) is also produced from Ang II by ACE2 [Tipnis *et al.* 2000]. The level of Ang-(1-7) is regulated by the action of ACE which hydrolyzes Ang-(1-7) to Ang-(1-5) [Chappell *et al.* 1998].

The functional role of Ang-(1-7) in the antagonism of the actions of Ang II was known before the discovery of ACE2 and MasR [Sampaio *et al.* 2003]. Ang-(1-7) induces regional and systemic vasodilation, diuresis and natriuresis, and also has antiproliferative and antimigratory effects on smooth muscle cells, cardiomyocytes, fibroblasts and glomerular cells. The cardioprotective effects of Ang-(1-7) are mediated by MasR to different signaling pathways involving MAPK, phosphoinositide 3-kinase (PI3K)/Akt and NADPH oxidase [Nemoto *et al.* 2014; Zheng *et al.* 2014]. The interaction Ang-(1-7)/MasR leads to activation of different effectors such as forkhead box protein O1 (FOXO1), COX-2 and vasodilator mediators such as prostanoids and NO. Furthermore, this heptapeptide has positive effects on metabolism, increasing the glucose uptake and lipolysis [Passos-Silva *et al.* 2013].

Ang-(1-7) in HT

Ang-(1-7) may act as an antihypertensive hormone [Moriguchi *et al.* 1995], based on its potent natriuretic and diuretic actions [Dellipizzi *et al.* 1994; Hilchey and Bell-Quilley, 1995; Handa *et al.* 1996] and vasodepressor effects in the coronary and mesenteric vascular beds [Osei *et al.* 1993; Porsti *et al.* 1994; Brosnihan *et al.* 1996]. Moreover, low concentrations of Ang-(1-7) enhance the vasodepressor actions of BK, potentiating BK vasodilatory effects [Paula *et al.* 1995; Abbas *et al.* 1997; Li *et al.* 1997]. This interaction is exaggerated after ACE inhibition. Although the precise mechanisms of this potentiation remain controversial [Greco *et al.* 2006; Carvalho *et al.* 2007], data suggest that the release of prostaglandins, NO and endothelium-derived hyperpolarizing factor [Oliveira *et al.* 1999], as well as the ability of Ang-(1-7) to inhibit ACE activity [Tom *et al.* 2001], may be involved in these effects.

Rakusan and colleagues evaluated the effects of target disruption of the MasR for Ang-(1-7) in knockout mice on the course of two-kidney, one-clip Goldblatt HT model. Their results showed that MasR knockout mice responded to clipping by accelerated increases in BP and the final BP was significantly higher than that in wild-type mice [Rakusan *et al.* 2010].

Although most evidence indicates that the effects of Ang-(1-7) are due to its interaction with the MasR, there is evidence to suggest that Ang-(1-7) also signals through the AT2R pathway in spite of having a relatively low affinity for this receptor [Bosnyak *et al.* 2011]. Ang-(1-7) reduces BP in HT rats during blockage of the AT1R in a similar fashion to that seen with AT2R agonist CGP42114. Furthermore, the vasodepressor effect of Ang-(1-7) is blocked by AT2R antagonist PD123319, but not by the MasR antagonist A779 [Li and Widdop, 2004; Walters *et al.* 2005]. These effects appear to be preserved in aged rats both by the MasR and AT2R [Bosnyak *et al.* 2012]. However, the importance of the AT2R to the signaling of and the effects triggered by Ang-(1-7) remains controversial, because studies have shown that the effects of Ang-(1-7) on BP are independent of the AT2R [Gembardt *et al.* 2012]. The fact that Ang-(1-7) generates effects *via* the MasR and AT2R could be explained by the similarity in the signaling pathways of these two receptors, with the result that antagonists for one receptor inhibit the action of agonists for the other receptor. According to Villela and colleagues, there may be a functional interaction between the two receptors, but this hypothesis should be tested in future studies [Villela *et al.* 2015].

Ang-(1-7) in cardiac remodeling

Chronic infusion of Ang-(1-7) prevents the development of heart failure (HF) after MI [Averill *et al.* 2003]. In ACE2$^{-/-}$ mice, AT1R blockade and Ang-(1-7) administration in a pressure overload-induced HF model suppresses NADPH oxidase, reduces cardiac hypertrophy and recovers systolic dysfunction [Patel *et al.* 2012]. Treating MI rats with Ang-(1-7) in hydroxypropyl β-cyclodextrin (HPβCD) improves cardiac function [Marques *et al.* 2012]. Ang-(1-7) reduces pathological cardiac remodeling by attenuating interstitial and perivascular fibrosis. In cardiac fibroblasts, Ang-(1-7) reduces DNA, protein and collagen synthesis upon stimulation with serum and endothelin-1 (ET-1) [McCollum *et al.* 2012]. In type 2 diabetes patients, plasma Ang-(1-7) level is negatively and significantly associated with ratio of early diastolic mitral inflow to annular velocity (E/Ea) and Log *N*-terminal pro-B-type natriuretic

peptide (NT-proBNP), but positively associated with left ventricular ejection fraction (LHEF) and ratio of early to late left ventricular filling velocity (E/A). These data suggest a protective action of Ang-(1-7) in cardiac remodeling and function in diabetic patients [Hao *et al.* 2013].

Ang-(1-7) agonists have been tested, including peptides CGEN-856 and CGEN-857 [Shemesh *et al.* 2008] and nonpeptide molecule AV0991. Oral treatment with AV0991 reduces cardiac remodeling in hypertensive rats [Cunha *et al.* 2013]. Moreover, Ang-(1-7) prevents organ damage in hypertensive rats by activating nitric oxide synthase. These effects are inhibited in MasR knockout mice [Lemos *et al.* 2005; Carvalho *et al.* 2007].

Most of the data related to MasR and its ligand Ang-(1-7) in the heart have shown cardioprotective effects of Ang-(1-7). Accordingly, cardiomyocytes from MasR-KO mice presented a smaller peak Ca^{2+} transient and slower Ca^{2+} uptakes due to a decreased expression of SERCA2 [Gomes *et al.* 2012]. These changes were turned into a decreased heart function in MasR-KO mice [Santos *et al.* 2006; Botelho-Santos *et al.* 2012; Gava *et al.* 2012]. The changes in the Ca^{2+} handling proteins were paralleled by changes in NO production [Dias-Peixoto *et al.* 2008]. Cardiomyocytes from MasR-KO mice presented normal endothelial NOS (eNOS) protein levels. However, MasR deficiency resulted in a 70% increase in caveolin 3 expression and a decrease in heat shock protein 90 [Dias-Peixoto *et al.* 2008]. These two alterations may lead to a decrease in eNOS activity, because caveolin 3 prevents calmodulin interaction with NOS and heat shock protein 90 act as a scaffold protein for the recruitment of Akt to the eNOS complex [Wu, 2002; Takahashi and Mendelsohn, 2003]. Likewise, the antihypertrophic effect of Ang-(1-7) on cardiomyocytes was blunted by downregulation of Mas by antisense RNA [Tallant *et al.* 2005].

Ang-(1-7) in vascular remodeling

Ang-(1-7) is produced and metabolized in vascular endothelial cells [Santos *et al.* 1992]. This heptapeptide produces relaxation in canine and porcine coronary arteries, rat aortic rings and mesenteric microvessels of normotensive and hypertensive rats [Brosnihan *et al.* 1996; Gorelik *et al.* 1998; Fernandes *et al.* 2001; Haulica *et al.* 2003]. Ang-(1-7) mediates the chronic hypotensive effects of losartan in normal rats [Collister and Hendel, 2003] and vasodilation in forearm circulation of normotensive subjects and patients with essential HT through a pathway that does not involve NO synthesis [Sasaki *et al.* 2001].

Ang-(1-7) potentiates the vasodilator and hypotensive effects of BK in normotensive and hypertensive rats [Paula *et al.* 1995], in porcine [Tom *et al.* 2001] and rat coronary vessels [Almeida *et al.* 2000]. Ueda and colleagues demonstrated that Ang-(1-7) potentiates the vasodilating effect of BK in human forearm resistance vessels through a mechanism that appears to involve NO release [Ueda *et al.* 2001]. BK-potentiating activity of Ang-(1-7) also involves inhibition of ACE [Tom *et al.* 2001] and a crosstalk between MasR and kinin B_2 receptor [Brosnihan *et al.* 1996; Soares De Moura *et al.* 2004]. Recent observations indicate that Ang-(1-7)/MasR pathway is mainly involved in the effects of ACEI because of Ang-(1-7) antagonist (A779) reverses the potentiation of BK in mesenteric microvessels by enalapril or enalaprilat [Fernandes *et al.* 2001] or attenuates the potentiated hypotensive response to BK in captopril-treated rats [Maia *et al.* 2004]. But Ang-(1-7) infusion after balloon catheter injury of rat carotid artery reduces neointima formation [Strawn *et al.* 1999]. This effect is likely mediated by inhibition of VSMC proliferation [Freeman *et al.* 1996].

The protective role of Ang-(1-7) on vascular beds by controling vascular tone and its relationship with the genesis of vascular dysfunction were first shown in MasR KO mice by Xu and colleagues and by Rabelo and colleages [Xu *et al.* 2008; Rabelo *et al.* 2008]. The results showed that MasR KO mice impair the *in vivo* endothelium-dependent vasolkrelaxant response to acetylcholine, reduce the urinary excretion rates of nitrate and nitrite and the plasma levels of nitrite [Rabelo *et al.* 2008; Xu *et al.* 2008], increase the aortic thiobarbituric reactive substances levels [Xu *et al.* 2008], upregulate the protein and gene expression of the NADPH oxidase catalytic subunit gp91phox (Nox2) [Rabelo *et al.* 2008; Xu *et al.* 2008] and reduce the aortic SOD activity [Xu *et al.* 2008]. Taken together, these findings point to the protective role played by MasR on endothelial function by inhibiting the vascular oxidative stress mediated by NADPH oxidase-derived O_2^-.

Ang-(1-9)

At early 1970s, Oparil and colleagues reported the rapid appearance of Leu after injection of radiolabeled Ang I in dog renal and pulmonary arteries [Oparil *et al.* 1970, 1971]. They suggested a carbopeptidase was breaking down Ang I forming des-Leu^{10} Ang I, now known as Ang-(1-9). Shortly afterwards Ang-(1-9) was detected in human and rat plasma [Johnson *et al.* 1989]. Initially Ang-(1-9) was thought to be biologically inactive, operating indirectly by competing with Ang I for the ACE active site and therefore reducing Ang II levels while increasing those of Ang-(1-7) [Drummer *et al.* 1990; Donoghue *et al.* 2000; Rice *et al.* 2004]. However, increasing evidence has shown that Ang-(1-9) has cardiovascular effects *in vivo* and *in vitro*, acting through AT2R [Ocaranza *et al.* 2010; Flores-Muñoz *et al.* 2012b; Cha *et al.* 2013].

Ang-(1-9) in HT

Studies in MI rats have shown that ACE2 activity positively correlates with Ang-(1-9) levels [Ocaranza *et al.* 2006]. Circulating Ang-(1-9) levels and both plasma and aortic wall ACE2 activities are significantly decreased in DOCA salt hypertensive rats. However, no change in Ang-(1-7) plasma level has been observed in this experimental model. Administration of fasudil, a Rho kinase inhibitor, reduces BP by 13% and ACE activity by 15% but increases ACE2 activity in the plasma and aortic wall [Ocaranza *et al.* 2011]. Chronic administration of Ang-(1-9) significantly reduces HT in experimental models of HT induced by Ang II infusion and the Goldblatt model (2K-1C) [Ocaranza *et al.* 2014c]. This antihypertensive effect of Ang-(1-9) involves the AT2R and is independent of Ang-(1-9) conversion to Ang-(1-7) [Ocaranza *et al.* 2014c]. These findings suggest an excellent opportunity to simultaneously measure ACE2 and Ang-(1-9) in hypertensive patients [Ocaranza *et al.* 2014b]. Therefore, this second axis involving ACE2 and Ang-(1-9) could be also an important target for HT therapy.

Ang-(1-9) in cardiac remodeling

The first observations on the role of the Ang-(1-9) in counter-regulating of the ACE–Ang II axis were made by Ocaranza and colleagues [Ocaranza *et al.* 2006] . In MI rats, circulating and LV ACE2 activities are downregulated in the long-term phase of LV dysfunction. These effects are prevented by the conventional ACEI, enalapril [Ocaranza *et al.* 2006]. Plasma Ang-(1-9) levels increase significantly when MI or sham-operated rats are treated with enalapril, but circulating Ang-(1-7) levels remain constant. Based on these findings, Ocaranza and colleagues proposed that Ang-(1-9) rather than Ang-(1-7) counter-regulates Ang II in this experimental model of HF [Ocaranza *et al.* 2006]. Ocaranza and colleagues also showed that Ang-(1-9) regulates cardiac hypertrophy *in vivo* and *in vitro* [Ocaranza *et al.* 2010]. In rats with MI randomized to receive vehicle, enalapril or the ARA candesartan, both drugs prevented LVH and increased plasma Ang-(1-9) levels several-fold [Ocaranza *et al.* 2010]. Ang-(1-9) levels correlate inversely with different LVH markers, even after adjusting for BP reduction. This effect of Ang-(1-9) is specific, as there was no correlation with LVH for Ang-(1-7), Ang II or BKs. In other experiments, chronic administration of Ang-(1-9) to MI rats has been shown to decrease plasma Ang II levels, inhibit plasma and LV ACE activity, and prevent cardiomyocyte hypertrophy [Ocaranza *et al.* 2010]. Interestingly, in two experimental HT models (Ang II infusion and Goldblatt model 2K-1C), chronic administration of Ang-(1-9) significantly reduced hypertensive cardiac damage [Ocaranza *et al.* 2014c]. These effects of Ang-(1-9) are mediated by AT2R, but not by Ang-(1-7) [Flores-Muñoz *et al.* 2011, 2012a, 2012b].

Ang-(1-9) in vascular remodeling

It has recently been shown that inhibition of the RhoA/Rho-associated, coiled-coil containing protein kinase (ROCK) signaling pathway increases ACE2 activity and Ang-(1-9) plasma levels, reducing BP and vascular remodeling in DOCA hypertensive rats [Ocaranza *et al.* 2011]. Furthermore, overexpression of vascular remodeling promoting genes was normalized and mRNA eNOS levels increased, revealing a novel role of Ang-(1-9) in vascular protection further than regulating HT. In the same vein, Ang-(1-9) infusion improved vasorelaxation and NO levels on SHRSP [Flores-Muñoz *et al.* 2012b]. Ang-(1-9) may increase NO bioavailability by stimulating BK release [Erdös *et al.* 2002; Jackman *et al.* 2002]. Also, Flores-Muñoz and colleagues reported an Ang-(1-9)-induced increase in NADPH oxidase 4 expresion [Flores-Muñoz *et al.* 2012b], which has been previously associated with endothelium promoted vasodilation by the release of NO [Ray *et al.* 2011]. However, the molecular mechanisms by

which Ang-(1-9) causes those effects remain to be elucidated.

ACE2 and vasoactive peptides in renal remodeling

In male ACE2$^{-/-}$ mice, structural changes in the glomerulus included increased collagen type I and III and fibronectin content. These animals show signs renal dysfunction, such as increased albumin excretion in the urine compared with control groups. Treatment with the AT1R antagonist irbesartan prevents these structural and functional changes [Oudit *et al.* 2006]. The role of renal ACE2 in humans is unclear. The only available study used immunostaining of ACE2 in renal biopsies of 58 patients with primary renal disease or renal disease secondary to glomerulopathy and diabetic kidney disease [Lely *et al.* 2004]. ACE2 was found in tubular cells, glomerular epithelial cells, VSMCs and the endothelial cells of interlobular arteries. Immunohistochemical studies in the kidney show that ACE and ACE2 are located predominantly in the epithelial cells of the proximal tubule [Donoghue *et al.* 2000; Tikellis *et al.* 2003]. Both primary and secondary renal disease is associated with the expression of the *de novo* ACE2 in glomerular endothelial cells and peritubular capillaries.

The kidney is an important organ in HT. It is also a well-known target for end organ damage in HT, but several studies suggest that the kidney also plays an active role in the pathogenesis of HT. For example, in salt-sensitive HT, a high-salt diet results in decreased renal blood flow [Campese, 1994] and altered-pressure natriuresis curves are observed in essential HT [Guyton, 1992]. Ali and colleagues recently demonstrated that Ang-(1-7) concentration and ACE2 activity in the renal cortex were reduced in high-sodium diet (HSD) induced HT and that this was reversed by C21 (AT2R selective agonist) treatment [Ali *et al.* 2015]. Reduced nephron numbers are also associated with essential HT and this finding may be partly attributable to enhanced Ang II generation [Brenner and Chertow, 1994; Keller *et al.* 2003].

Emerging evidence shows that ACE2 plays an important role in negatively regulating HT. In rat HT models, renal ACE2 mRNA and protein levels are decreased, although this finding could not be confirmed in human hypertensive nephropathy [Lely *et al.* 2004]. Furthermore, in rats treated with ACEI and ARA, local renal ACE2 activity is elevated [Ferrario *et al.* 2005a, 2005b]. ACE is upregulated in human diabetic nephropathy accompanied by HT, a condition associated with high Ang II levels [Huang *et al.* 2003]. Taken together, these findings suggest that HT is associated with an alteration in renal ACE/ACE2 balance towards increased Ang II generation (i.e. ACE upregulation) and decreased Ang II degradation (i.e. ACE2 downregulation).

In order to understand factors that may downregulate renal ACE2 during HT, Koka and colleagues explored *in vivo* and *in vitro* regulation of ACE2 under hypertensive conditions [Koka *et al.* 2008]. In patients with hypertensive cardiomyopathy and nephropathy and *in vitro* in a human tubular cell line (HK-2), Ang II upregulates ACE and downregulates ACE2 expression levels under hypertensive conditions [Koka *et al.* 2008]. The AT1R-mediated ERK/p38MAPK kinase signaling pathway is a key mechanism by which Ang II downregulates ACE2 expression, implicating low ACE2 in hypertensive CV and renal disease [Huang *et al.* 2003]. The RAS is also involved in the pathogenesis of diabetic nephropathy [Brenner *et al.* 2001] and ACE inhibition has a significant effect on kidney protection. In rodent experimental models of diabetes mellitus type 1, ACE and ACE2 mRNA and protein levels are decreased. This effect is prevented by ACEI, suggesting that ACE2 could have a renoprotective effect in diabetes [Ferrario, 2003]. Opposite results have been presented by Ye and colleagues, who showed that ACE expression is increased in the renal tubules of diabetic mice (*db*/*db*) at 8 weeks of age compared with the control group (*db*/*m*), likely as an early renoprotective effect [Ye *et al.* 2004].

Alamandine and cardiovascular remodeling

A new member of the RAS has been discovered recently, the heptapeptide Ala-Arg-Val-Tyr-Ile-His-Pro, known as alamandine. Lautner and colleagues characterized alamandine as an endogenous peptide in rat and mouse cardiac tissue and human plasma [Lautner *et al.* 2013]. Using mass spectrometry, they identified alamandine as a product of catalytic hydrolysis of the octapeptide angiotensin A by ACE2. Its sequence is very similar to Ang-(1-7), differing only by the presence of an alanine residue in place of an aspartate residue in the amino terminus. Alamandine may be synthesized by decarboxylation of the Ang-(1-7) *N*-terminal aspartate amino acid residue. The enzyme responsible for the

latter reaction remains unknown [Lautner *et al.* 2013]. Alamandine degradation has not yet been elucidated. However, aminopeptidases may play an important role, since the removal of Ala1 could lead to the formation of Ang-(2-7), which considered an inactive peptide, although it shows ACE inhibitory activity [Paula *et al.* 1999]. Other Ang-(1-7) degrading enzymes, such as NEP or neprilysin, may also participate, given alamandine's similarity to Ang-(1-7) [Etelvino *et al.* 2014].

Alamandine produces vasorelaxation in phenylephrine-contracted aortic rings and when microinjected into central areas critically involved in BP control, such as caudal ventrolateral and rostral ventrolateral medulla. Alamandine produces a decrease *versus* increase in BP in the former *versus* the latter, respectively, revealing that it acts locally and centrally in a manner similar to Ang-(1-7). Additionally, oral alamandine administration produces a long-term antihypertensive effect in SHR rats [Lautner *et al.* 2013], suggesting a therapeutic potential for the conditions underlying cardiovascular remodeling. Furthermore, alamandine has a direct effect on remodeling by diminishing collagen I, III and fibronectin accumulation in rats with isoproterenol-induced heart fibrosis [Lautner *et al.* 2013]. Since alamandine is a very new member of the RAS, there is scarce information about its involvement in disease, although it has been reported that its plasma concentration is elevated in nephropathy patients, suggesting that alamandine may participate in pathological conditions [Lautner *et al.* 2013].

In 2001, researchers identified a large family of genes that code for GPCRs and have a strong sequence homology with the MasR, called Mas-related genes (Mrg) [Dong *et al.* 2001]. Among the Mrg-receptor family, the Mrg type D (MrgD) was identified as the binding site for alamandine [Lautner *et al.* 2013]. Interestingly, although alamandine residue's sequence differs only slightly from Ang-(1-7), its effects are mediated through the MrgD and its binding to the Mas is weak or nonexistent [Villela *et al.* 2015]. In aortic ring vasorelaxation assays, the Mas antagonist A-779 does not block alamandine-induced vasorelaxation. Furthermore, alamandine-induced vasorelaxation is preserved in aortic rings of Mas-deficient mice, showing that alamandine does not produce these effects through the Mas receptor. However, D-Pro7-Ang-(1-7) is capable of blocking alamandine's effects, implying that Ang-(1-7) is also a ligand for MrgD [Lautner *et al.* 2013]. Ang-(1-7) has been reported to be a weak agonist of the MrgD [Gembardt *et al.* 2008]. Moreover, the amino acid β-alanine is an MrgD agonist, although with a higher effective concentration [Milasta *et al.* 2006]. However, β-alanine does not produce relaxation itself, but rather blocks the effect of alamandine [Lautner *et al.* 2013]. Finally, the AT2R antagonist PD123319 also acts as antagonist/ligand for MrgD; alamandine causes effects in AT2R-deficient mice, but PD123319 can block these effects [Lautner *et al.* 2013]. This result suggests that alamandine rather than Ang-(1-7) could be involved in these actions. The conditions that stimulation alamandine's formation and the players involved in this process remain unknown.

Future directions for ACE2 and its vasoactive peptides in cardiovascular and renal remodeling

RAS activation has an important role in the development of HT and secondary target organ damage (TOD), characterized by cardiovascular and renal remodeling [Touyz, 2003; Montezano *et al.* 2014]. Currently, pharmacologic blockade of the RAS is one of the most useful tools for treating HT and TOD. However, current HT treatment has significant limitations that jeopardize clinical success and compliance. Antihypertensive drugs are not always effective in normalizing BP, and two or three drugs are often required [Mancia, 2009]. Moreover, these drugs are not particularly effective in reducing TOD and adverse effects are common [Jamerson *et al.* 2008]. Thus, there is a need to develop more effective antihypertensive drugs to normalize BP, reduce cardiovascular and renal remodeling, and increase compliance.

In light of the limited efficacy of currently available treatment options for cardiovascular and renal damage, it is fundamental to identify new molecules to prevent and reduce HT and the resulting pathologic cardiovascular and renal remodeling. Therefore, the discovery of ACE2, Ang-(1-7), Ang-(1-9) and alamandine is significant. Moreover, in patients with HT, HF or kidney damage currently on RAS blocker therapy, ACE2 overexpression and treatment with Ang-(1-7), Ang-(1-9) and alamandine might have additive effects to ACEI and AT1R blockers. We can speculate that the antiremodeling mechanisms triggered Ang-(1-7), Ang-(1-9) and alamandine act by AT1R antagonism lowering Ang II levels and ACE inhibition. The effects of even higher

Ang-(1-7), Ang-(1-9) and alamandine levels on these clinical situations and the appropriate strategies to increase these levels should be further investigated.

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Conflict of interest statement

The authors declare no conflicts of interest in preparing this article.

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