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## The vasoprotective axes of the renin-angiotensin system: physiological relevance and therapeutic implications in cardiovascular, hypertensive and kidney diseases

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### Abstract

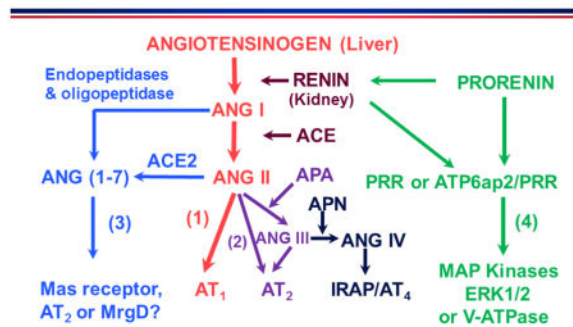
The renin-angiotensin system (RAS) is undisputedly one of the most prominent endocrine (tissue-to-tissue), paracrine (cell-to-cell) and intracrine (intracellular/nuclear) vasoactive systems in the physiological regulation of neural, cardiovascular, blood pressure, and kidney function. The importance of the RAS in the development and pathogenesis of cardiovascular, hypertensive and kidney diseases has now been firmly established in clinical trials and practice using renin inhibitors, angiotensin-converting enzyme (ACE) inhibitors, type 1 (AT<sub>1</sub>) angiotensin II (ANG II) receptor blockers (ARBs), or aldosterone receptor antagonists as major therapeutic drugs. The major mechanisms of actions for these RAS inhibitors or receptor blockers are mediated primarily by blocking the detrimental effects of the classic angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub>/aldosterone axis. However, the RAS has expanded from this classic axis to include several other complex biochemical and physiological axes, which are derived from the metabolism of this classic axis. Currently, at least five axes of the RAS have been described, with each having its key substrate, enzyme, effector peptide, receptor, and/or downstream signaling pathways. These include the classic angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor, the ANG II/APA/ANG III/AT<sub>2</sub>/NO/cGMP, the ANG I/ANG II/ACE2/ANG (1–7)/Mas receptor, the prorenin/renin/prorenin receptor (PRR or Atp6ap2)/MAP kinases ERK1/2/V-ATPase, and the ANG III/APN/ANG IV/IRAP/AT<sub>4</sub> receptor axes. Since the roles and therapeutic implications of the classic angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor axis have been extensively reviewed, this article will focus primarily on reviewing the roles and therapeutic implications of the vasoprotective axes of the RAS in cardiovascular, hypertensive and kidney diseases.

### Graphical abstract

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**The vasopressor and vasoprotective axes of the RAS:  
physiological relevance and therapeutic implications**



## Keywords

Angiotensin 1-converting enzyme; ACE2; alamandine; angiotensin (1–7); angiotensin II; cardiovascular disease; dipeptidyl peptidase III; hypertension

## 1. Introduction

The renin-angiotensin system (RAS) is undisputedly one of the most important endocrine (tissue-to-tissue), paracrine (cell-to-cell) and intracrine (intracellular/nuclear) vasoactive systems in the physiological regulation of cardiovascular, blood pressure, and kidney function, and the development of cardiovascular, hypertensive, and renal diseases [1–5]. The discovery of each of key members of the RAS, uncovering their cardiovascular, blood pressure, and renal actions, and developing pharmacological drugs to target the key enzyme(s) and receptor(s) of the RAS to treat cardiovascular, hypertensive and renal diseases have become a successful story in the cardiovascular, hypertensive, and renal research [1–5]. The earliest and most significant member of the RAS, the rate-limiting enzyme renin, was first discovered by Robert Tigerstedt and P. G. Bergman at Karolinska Institute in 1898 [6]. The foremost contribution of Tigerstedt and Bergman to the RAS research was their discovery of renin as a pressor substance released from the kidney, which increased blood pressure systemically [6]. However, it took almost 40 years until 1934, when Harry Goldblatt demonstrated in a landmark study that a vasopressor substance from the kidney induced hypertension in a dog model of two-kidney, one-clip (2K1C) renovascular hypertension [7]. Subsequent studies suggested that renal ischemia not only caused the release of renin from the kidney, but also a heat-stable, short-lived plasma pressor substance, which was initially named angiotonin or hypertensin, but was later widely recognized as angiotensinogen [8–10]. The liver was then considered to be the primary source of plasma angiotensinogen, with the first twelve amino acids, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-..., being the most important for its biological activity. It was in early 1950s that Skeggs and colleagues purified this peptide and found that there were actually two peptides, termed angiotensin I (ANG I) with ten amino acids, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu, and ANG II with eight amino acids, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, and that ANG I had to be cleaved to form ANG II to be active by angiotensin-converting enzyme (ACE) [11,12]. Interestingly, ACE is also known as Kininase II [13,14],

which metabolizes the vasodepressor peptide bradykinin; thus ACE is not only responsible for ANG II formation, but also for degradation of bradykinin [14]. Therefore the RAS and the kinin–kallikrein–bradykinin system interact to regulate cardiovascular, blood pressure, and renal function in health and disease [15,16].

In the tissues which express an enzyme named aminopeptidase A (APA), especially in the kidney, ANG II is metabolized to form des-aspartyl<sup>1</sup>-ANG II, also termed ANG III with seven amino acids, Arg-Val-Tyr-Ile-His-Pro-Phe [17–19]. ANG III is further metabolized by the action of Aminopeptidase N (APN) to form ANG IV with six amino acids, Val-Tyr-Ile-His-Pro-Phe, ANG (3–8) [17,19,20]. Unlike ANG II, both ANG III and ANG IV were previously considered partial agonists for ANG II receptors with lesser vasopressor activity [21–23]. More recently, ANG III has been recognized as a major ligand for the AT<sub>2</sub> receptor/NO/cGMP signaling cascade [18,24,25], whereas ANG IV is thought to activate the AT<sub>4</sub> receptor [23,26–28]. In late 1980s and early 1990s, through the study of ANG II receptor pharmacology it was discovered that ANG II acted on at least two different classes of G protein-coupled receptors, which led to the development of two classes of nonpeptide ANG II receptor antagonists, losartan as a representative blocker for type 1 (AT<sub>1</sub>) and PD123319 as a representative type 2 (AT<sub>2</sub>) receptor blockers [1,29–31]. The AT<sub>1</sub> receptor was successfully cloned in 1991 by Murphy et al. [32] and Sasaki et al [33], respectively, which shares the seven-transmembrane-region motif with the G protein-coupled receptor superfamily. The AT<sub>1</sub> receptor mediates the well-recognized effects of ANG II on cardiovascular, blood pressure, and renal systems, such as vasopressor, cardiac hypertrophic, hypertensive, renal vasoactive and salt-retaining actions, as well as aldosterone biosynthesis and release [1,5,29,31]. The AT<sub>2</sub> receptor was successfully cloned by Mukoyama M et al. [34], Nakajima M [35], and Kambayashi Y [36], respectively. The cloned AT<sub>2</sub> receptor has 34% identical sequence to the cloned AT<sub>1</sub> receptor, shares a seven-transmembrane domain topology [34], and appears to mediate ANG II-induced inhibition of protein tyrosine phosphatase in COS-7 cells [36]. The 3<sup>rd</sup> ANG receptor termed “AT<sub>3</sub>” was reportedly cloned to encode a Mr 40,959 protein with 95% amino acid identity to the rat smooth muscle AT<sub>1</sub> receptor, which also mediates ANG II-induced Ca<sup>2+</sup> mobilization [37]. However, the so-called “AT<sub>3</sub>” receptor has not been recognized in the field. The 4<sup>th</sup> ANG receptor was identified in 2001 as insulin-regulated aminopeptidase (IRAP) by Albiston et al. using protein purification and peptide sequencing [26]. ANG (3–8) was found to bind and activate this receptor in the brain and plays an important role in learning and memory [26,38]. Most recently, the *Mas* oncogene was identified by Santos et al. as the specific receptor for ANG (1–7) [39], playing a key role in mediating ANG (1–7)-induced cardiovascular, vasodepressor, and renal responses [39–42].

Collectively, it is now well understood that the classic RAS axis includes the substrate angiotensinogen primarily from the liver, which is cleaved by the rate-limiting enzyme renin released primarily from the juxtaglomerulus apparatus (JGA) of the kidney to form the biologically inactive ANG I. ACE, which is primarily localized in the vascular endothelium of major target organs including lung, heart, kidney, adrenal glands, brain etc., converts ANG I to the most potent vasopressor of the RAS, ANG II, by removing two amino acids, His-Leu, from ANG I. ANG II acts on two classes of G protein-coupled receptors (GPCR), AT<sub>1</sub> and AT<sub>2</sub> and possibly the Mas-related G-protein-coupled receptor (MrgD) as well, to

play important “YIN” and “YANG” counter-regulatory roles in maintaining normal cardiovascular, blood pressure and renal function, as well as in the development of cardiovascular, hypertensive and renal diseases [1,5,29,43]. In addition to the classic axis, the RAS has evolved well beyond its classic paradigms primarily as a powerful vasopressor, growth/fibrosis promoter, a potent aldosterone stimulator, or a sodium-retaining hormone [25,39,41,42]. New members of the RAS are continuously discovered with different roles during last few decades, so that ANG II is no longer the only active peptide of the RAS. Figure 1 summarizes the current understanding on the entire RAS superfamily, including the classic angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor axis, the prorenin/renin/prorenin receptor (PRR or Atp6ap2)/MAP kinases ERK1/2/V-ATPase axis, the ANG II/APA/ANG III/AT<sub>2</sub>/NO/cGMP axis, the ANG I/ANG II/ACE2/ANG (1–7)/*Mas* receptor axis, and the ANG III/APN/ANG IV/IRAP/AT<sub>4</sub> receptor axis. The first two axes represent the powerful vasopressor systems, which are physiologically required to maintain normal cardiovascular, blood pressure, and renal homeostasis [1,5,29,44]. However, overactivation of these two axes of the RAS plays a critical role in the development of cardiovascular and kidney diseases and hypertension. By contrast, the other two axes of the RAS, including the ANG II/APA/ANG III/AT<sub>2</sub>/NO/cGMP axis [24,25,45] and the ANG I/ANG II/ACE2/ANG (1–7)/*Mas* receptor axis [39–42] may serve as the vasodepressor and cardiorenal protective arms of the RAS acting to counteract the detrimental effects of the renin/ACE/ANG II/AT<sub>1</sub> receptor axis and the prorenin/renin/PRR/MAP kinases ERK1/2/V-ATPase axis. The ANG III/APN/ANG IV/IRAP/AT<sub>4</sub> receptor axis is unlikely classified as an independent member of the vasopressor or vasodepressor system, since it appears to play a primary role in learning and memory [26,31,38]. Recently, a number of new enzyme(s) and peptides have been reported to possess the vasodepressor properties, which may have novel therapeutic implications in cardiovascular, hypertensive, and renal diseases. Among these newly described enzyme(s) or peptides, dipeptidyl peptidase III, or DPP III [46], alamandine [47–49], and angiotensin [50,51], have been described as “new members” of the RAS or associated with the vasoprotective arms of the RAS. These enzyme(s) or peptides act to counter or opposing the actions of ANG II and may have a potential therapeutic role in treating hypertension associated with the activation of RAS [46–48].

The roles and the therapeutic implications of the classic angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor axis in the cardiovascular, blood pressure, and renal physiology and diseases have been extensively reviewed elsewhere, and therefore are not the subject of this review article. Instead, we focus our reviews and discussions primarily on recent advances in the research of the roles, insights, and potential therapeutic implications of the vasodepressor axes of the RAS.

## 2. New insights into the roles and therapeutic implications of the ANG II/APA/ANG III/AT<sub>2</sub>/NO/cGMP axis in cardiovascular, hypertensive and renal diseases

### 2.1. Role of aminopeptidase A

To maintain physiological cardiovascular, blood pressure and renal homeostasis by the RAS, a balance between the formation and degradation of the effector peptide ANG II is critical. ANG II is increased in the plasma and tissues either by increased ACE expression and actions or by decreasing the expression and activity of key enzymes that metabolize ANG II. Increased ANG II formation over its degradation leads to elevation of circulating and tissue ANG II levels, which play a key role in animal models of hypertension with 2-kidney, 1-clip [52–54] or ANG II infusion [55–57]. ACE is the primary enzyme responsible for ANG II formation, and its biochemistry, localization and its implications in cardiovascular, hypertensive and kidney diseases have been studied and reviewed extensively during last several decades in the context of the classic angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor axis [58–60]. Thus the role of ACE in the classic angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor axis is not the focus of discussion in this article. By comparison, the roles of ANG II degradation in cardiovascular, blood pressure and renal regulation have not been well studied. ANG II is metabolized to form des-aspartyl<sup>1</sup>-ANG II, ANG III, primarily by aminopeptidase A (APA) [17–19,61]. Aminopeptidase A, also named glutamyl aminopeptidase (EC 3.4.11.7), aspartate aminopeptidase, or angiotensinase A, is a zinc-dependent membrane-bound enzyme [17–19,61]. Its primary action is to cleave glutamatic and aspartatic amino acid residues from the N-terminus of polypeptides, such as ANG II. Aminopeptidase A circulates in the plasma and hydrolyzes ANG II reportedly at the rate of  $4.1 \pm 0.5$  nmol/min/ml and a  $K_m$  of  $90.3 \pm 14.3$   $\mu$ M in the rat plasma [62]. Aminopeptidase A is expressed widely in tissues including the kidney, vessels, and brain [17,63,64]. Within the kidney, the glomeruli and the proximal tubules in Spontaneously hypertensive rats (SHR) and ANG II-infused rats expresses high levels of APA and high APA activity [17,20,61]. In the extrarenal tissues, the pituitary and the circumventricular organs express high levels of APA or APA activity, followed by the median eminence, the area postrema, and paraventricular nuclei, which are associated with the central regulation of cardiovascular function and blood pressure [64,65]. Indeed, it is interesting that APA activity was higher in SHR in these brain nuclei, where AT<sub>1</sub> receptors have been localized [65].

Since APA is the key enzyme for the degradation of ANG II, its implications in cardiovascular diseases have been studied in APA-KO mice [66,67]. Lin et al. first generated mice with deficiency of APA by gene targeting in embryonal stem cells to study its role in normal B and T cell development [66]. The absence of APA in these mice was confirmed in the lack of APA proteins and activity in bone marrow-derived stromal cells, thymic cortical epithelial cells, and renal proximal tubular cells [66]. This study showed that APA is not essential for normal B and T cell development, but it did not study any cardiovascular, blood pressure and kidney phenotypes [66]. Mitsui et al. showed that basal systolic blood pressure was significantly elevated in APA-KO mice, compared with heterozygous mutant and wild-type littermate mice [67]. Furthermore, infusion of ANG II further increased blood pressure

in APA-KO mice, an enhanced response due to the lack of APA to metabolize ANG II [67]. Interestingly, deletion of APA did not increase the heart or kidney wt. to body wt. ratio, nor altered urinary sodium and water excretion and osmolality, in the presence of significant elevation of blood pressure [67]. But a later study reported that water consumption in APA-KO mice was significantly elevated, suggesting that ANG II rather than ANG III regulates water drinking behavior or thirst [68]. Kubota et al. demonstrated that ischemia-induced angiogenesis is impaired in APA-KO mice via down-regulation of HIF-1 $\alpha$ , suggesting a potential role of ANG III in ischemia-induced angiogenesis [69]. While these findings from APA-KO mice provide the proof of the concept for a physiological role of APA in the blood pressure regulation, thirst response, or ischemia-induced angiogenesis, the potential therapeutic implications of APA in cardiovascular, hypertensive and kidney diseases remain to be further determined. However, the clinical benefits of APA as a therapeutic target may most likely be limited, since it acts to decrease circulating and tissue ANG II levels primarily by degrading ANG II, rather than by inhibiting ANG II formation. Although overexpression of APA in tissues may increase ANG II degradation to attenuate the effects of ANG II, such as in cultured mouse mesangial cells [70], its feasibility in humans remains uncertain. Indeed, if the goal is to decrease circulating and tissue ANG II levels either by inhibition of its formation or by increasing its degradation, the renin inhibitors and ACE inhibitors, especially the latter, have been widely used to block the RAS in treating cardiovascular, hypertensive and kidney diseases with proven clinical beneficial outcomes [71–74]. A potential pharmacological strategy to upregulate APA expression or enhance its activity to further decrease circulating and tissue ANG II levels may be warranted, if ACE inhibitors and/or renin inhibitors are inadequate to treat hypertension or cardiovascular and kidney diseases associated with the activation of the RAS.

## 2.2. Role of angiotensin III

Angiotensin III (ANG III) is the key metabolite of the effector peptide ANG II derived from the enzymatic action of APA to remove its 1<sup>st</sup> amino acid to des-aspartyl<sup>1</sup>-ANG II [20,64,75]. The metabolism of ANG II and generation of ANG III and their structure and activity relationship have been extensively investigated from 1970s to present [18,20,21,76,77]). Although ANG III is thought to circulate in the blood as well as is generated in tissues, few studies have specifically measured and compared the levels of ANG III in the plasma and tissues relative to ANG II. The reported levels of ANG III in the plasma and kidney varied widely from fmol/ml or fmol/g to nmol/ml or nmol/g due to different assays or approaches being used [78–80]. Using Liquid chromatography–tandem mass spectrometry (LC-MS/MS), Wysocki et al. reported that the level of plasma ANG III was remarkably higher than that of ANG II in wild-type mice on the C57BL6 genetic background ( $1,047 \pm 178$  vs.  $7.8 \pm 2.0$  pg/ml), whereas the kidney ANG III level was much lower than that of plasma ( $172 \pm 12$  pg/g) [81]. Also using LC-MS/MS, Olkowicz et al. reported the plasma ANG III level nearly 35-fold lower at  $30.3 \pm 3.8$  pg/ml in wild-type mice [82]. More reliable ANG II levels in the plasma and kidney may be obtained by the gold-standard high-performance liquid chromatography (HPLC)-based RIA or ELISA approach [78–80]. Campbell and colleagues simultaneously measured and compared angiotensin peptides and their carboxy-truncated metabolites in human and rat plasma using N-terminal-directed antisera and HPLC [78,79]. In the plasma of normal male human

subjects, the level of ANG II is reportedly five-fold higher than that of ANG III ( $13.9 \pm 2.0$  vs.  $2.9 \pm 1.0$  fmol/ml) [78]. In normal rat plasma, the ANG II level is about 50% of that for ANG I, whereas the ANG III is about 50% of ANG II ( $47 \pm 17$  vs.  $29 \pm 8$  fmol/ml), respectively [79]. In the normal rat kidney, ANG II is the major ANG peptide and the ANG II level is much higher than in the plasma, whereas the level of ANG III is much lower than ANG II ( $338 \pm 33$  vs.  $<15$  fmol/g) [79]. Kemp et al. appeared to confirm the finding of lower kidney ANG III, but the levels of renal cortical interstitial ANG II and ANG III were comparable ( $76 \pm 12.0$  and  $77.4 \pm 12.1$  fmol/ml) [18]. In the rat proximal tubule fluid collected from free-flow micropuncture, ANG II and III concentrations were estimated at 6–8 nM and 14–25 nM, respectively [83]. Thus based on the available data, ANG III levels are most likely lower in the plasma and kidney than ANG II.

The physiological roles of ANG III as a vasopressor or a vasodepressor remain incompletely understood. It also remains unknown whether there is a specific receptor for ANG III [1,4,31]. One exception is that an “AT<sub>3</sub>” receptor was reportedly cloned with 95% amino acid identity to the AT<sub>1</sub> receptor, but it remains to be recognized [1,37]. It is likely that ANG III may bind and activate AT<sub>1</sub> and AT<sub>2</sub> receptors with lower affinity than ANG II [1,84], and its vasopressor or vasodepressor effects may be dose-dependent on whether the AT<sub>1</sub> or AT<sub>2</sub> receptor is activated [4,18,76,85,86]. Previous studies in 1970s and 1980s have shown that although not as potent as ANG II, up to 40% to 50% of the pressor activity of ANG III was retained with removal of the aspartic acid from ANG II [21,87]. In a human study, Carey et al. carefully compared the effect of [des-Asp]-ANG II (ANG III) and ANG II on blood pressure and aldosterone production, and demonstrated that ANG III is less efficacious than ANG II in increasing blood pressure and stimulating aldosterone production [87]. In rats, Gardiner et al. showed that although less potent than ANG II, ANG III induced dose-dependent pressor and renal and mesenteric vasoconstrictor effects [88]. In the rat kidney, we previously found that unlike ANG II, equimolar ANG III failed to completely displace <sup>125</sup>I-[Sar<sup>1</sup>,Ile<sup>8</sup>]-ANG II receptor binding in the glomeruli, proximal tubules, and the inner stripe of the outer medulla [84] or raise blood pressure induced by ACE inhibition with enalaprilat, suggesting that ANG III acts as a weaker agonist of ANG II [76].

More recently, Carey’s group has been instrumental to uncovering a novel role of ANG III in the kidney in inducing natriuresis via activation of AT<sub>2</sub> receptors [18,89,90]. To demonstrate the roles of ANG III and AT<sub>2</sub> receptors, Padia et al. first treated rats with candesartan to block AT<sub>1</sub> receptors before ANG III was infused directly into the renal cortical interstitium to stimulate AT<sub>2</sub> receptors [89]. Interestingly, ANG III significantly increased urinary sodium excretion in the presence of candesartan, suggesting a possible role of AT<sub>2</sub> receptors in mediating ANG III-induced natriuretic response unmasked by AT<sub>1</sub> receptor blockade [89]. Subsequent studies by Carey’s group further confirmed these ANG III-induced natriuretic responses in Wistar-Kyoto, but not in spontaneously hypertensive rats (SHR), implying an important role for a defect in ANG III/AT<sub>2</sub> receptor signaling in the development of hypertension in SHR [90]. A further support for the interaction between AT<sub>2</sub> receptor and ANG III came from a study that administration of a highly selective nonpeptide AT<sub>2</sub> receptor agonist, C21, was able to recapitulate the ANG III-induced natriuretic effect [24]. These studies lead to the hypothesis that in the kidney, ANG III is the predominant ligand for proximal tubule AT<sub>2</sub> receptors, and activation of which induces natriuresis

responses and contributes to blood pressure regulation [18]. However, how ANG III preferably binds and stimulates AT<sub>2</sub> receptors in the kidney remains to be elucidated.

### 2.3. Role of the AT<sub>2</sub> receptor

The AT<sub>2</sub> receptor plays a key role in mediating the vasodepressor responses, and cardiorenal protective effects to the activation of the APA/ANG III/AT<sub>2</sub>/cGMP axis [1,91–94]. While the angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor axis acts as a “YANG” factor to induce vasoconstriction, increase blood pressure, and promote growth and fibrotic and proinflammatory responses, the APA/ANG III/AT<sub>2</sub>/cGMP axis acts as a “YIN” factor to counteract the vasopressor effect of ANG II/AT<sub>1</sub> activation to induce vasodilation and decrease blood pressure to maintain normal blood pressure homeostasis [25,95–97]. Before the AT<sub>2</sub> receptor was molecularly cloned, this class of ANG II receptors was primarily discriminated by using nonpeptide antagonists PD123177 and PD123319 or a peptide agonist CGP42112A in ANG II receptor binding assays [1,29,84,98]. Using radioreceptor binding assays and autoradiography with <sup>125</sup>I-[Sar<sup>1</sup>,Ile<sup>8</sup>]-ANG II as the radioligand and losartan, PD123317 or PD123319 as ANG II receptor blockers, we have localized the distribution of AT<sub>2</sub> receptors in the kidneys, heart, blood vessels, adrenal glands, and brain (Fig. 2) [98–102]. Notably, AT<sub>2</sub> receptor binding is widely localized in fetal and neonatal tissues days after birth, but it was primarily localized in cell- or tissue-specific manners in certain tissues of adult humans and rodents. Inagami’s group first cloned the rat AT<sub>2</sub> receptor cDNA from a rat pheochromocytoma cell line [36] and isolated the human AT<sub>2</sub> receptor from a genomic DNA library of human placenta [103]. Both rat and human AT<sub>2</sub> cDNAs encode a 363-amino acid protein with 7-transmembrane domains and a 32% identity of amino acid sequence of the rat and mouse AT<sub>1</sub> receptor [36,103]. In COS-7 cells stably expressing the rat AT<sub>2</sub> receptor, the AT<sub>2</sub> receptor blocker PD123319, but not the AT<sub>1</sub> blocker losartan blocked its binding to ANG II [36]. Although the signaling transduction mechanisms, upon which ANG II binds and activates AT<sub>2</sub> receptors, have been extensively studied in cultured cells in vitro and in tissues in vivo, they remain incompletely understood. Unlike the well-established signaling pathways for the AT<sub>1</sub> receptor, the most commonly described signaling pathways for the AT<sub>2</sub> receptor include ANG II-induced inhibition of the protein phosphotyrosine phosphatase (PTP) activity, phospholipase A(2), nitric oxide, and cyclic guanosine monophosphate (cGMP) [1,36,95,96].

The physiological or vasodepressor role of the ANG II/ANG III/AT<sub>2</sub>/cGMP axis has been extensively investigated during last two decades. Although it was widely believed that the primary action of the AT<sub>2</sub> receptor activation is to oppose the vasopressor action of the angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor axis on the cardiovascular, blood pressure, and kidney functions, the results of early animal studies were not very conclusive [1,29,30]. Indeed, it is very difficult to elicit the vasodepressor effect of the ANG II/ANG III/AT<sub>2</sub>/cGMP axis due to the dominant role of the angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor axis in cardiovascular, blood pressure and kidney regulation [92,104]. For example, activation of the AT<sub>2</sub> receptor with the AT<sub>2</sub> receptor-selective agonist CGP42112A or pharmacological blockade of the AT<sub>2</sub> receptor with the AT<sub>2</sub> receptor-selective antagonists, PD123177 or PD123319, alone doesn’t alter blood pressure in animal studies [1,29,92]. Barber et al. demonstrated that AT<sub>2</sub> receptor-mediated depressor responses to CGP42112A



were demonstrated only in the presence of AT<sub>1</sub> receptor blockade in both SHR and WKY rats [104]. Li and Widdop further showed that in the presence of AT<sub>1</sub> receptor blockade with candesartan, CGP42112 caused a marked depressor effect, which was abolished by the coadministration of the AT<sub>2</sub> receptor antagonist, PD123319 (Fig. 3) [92]. The unmasking of the vasodepressor role of the AT<sub>2</sub> receptor during AT<sub>1</sub> receptor blockade has important clinical therapeutic implications, because AT<sub>1</sub> receptor blockers (ARBs) are currently widely prescribed to treat cardiovascular, hypertensive and kidney diseases [92,94,104,105]. ARBs selectively blocks the actions of the angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor axis by binding and occupying AT<sub>1</sub> receptors in target tissues, which leads to several-fold increases in the circulating ANG II levels [106,107]. ANG II then may bind and activate the unopposed AT<sub>2</sub> receptors in cardiovascular and kidney tissues to induce vasodepressor effects.

The evidence supporting the vasodepressor role of AT<sub>2</sub> receptors is best appreciated in genetically modified mice lacking [91,108] or overexpressing AT<sub>2</sub> receptors [109]. Although AT<sub>2</sub> receptors are abundantly expressed in most of fetal tissues [1,36,99], one would expect that genetic deletion of AT<sub>2</sub> receptors may lead to the impairment of the development and growth of organs or tissues. However, AT<sub>2</sub> receptor-null mice develop normally and show no structural defects in the brain, heart, vessels, and kidneys [91,108]. The lack of developmental defects in AT<sub>2</sub> receptor-null mice is surprising, given that the structural or developmental abnormalities in the kidney have been reported in mice lacking angiotensinogen [110,111], ACE [58,112], and both AT<sub>1a</sub>/AT<sub>1b</sub> receptor double-null mice [113,114]. One study shows that basal blood pressure remains unaltered [108], while the other shows elevated basal blood pressure in AT<sub>2</sub> receptor-null mice [91]. Although these results on basal blood pressure seem conflicting, both studies demonstrated increased vasopressor response to ANG II stimulation in AT<sub>2</sub> receptor-null mice [91,108]. Siragy et al. subsequently confirmed that basal systolic blood pressure in AT<sub>2</sub>-null mice was slightly elevated, compared with wild-type mice, but showed sustained hypertensive and antinatriuretic responses to ANG II [45]. In transgenic mice overexpressing AT<sub>2</sub> receptors in vascular smooth muscle cells, the AT<sub>1</sub>-mediated pressor effect of ANG II was completely abolished in AT<sub>2</sub> transgenic mice via the bradykinin/NO/cGMP signaling [109]. While the latter study may seem too perfect, all available data appear to be consistent with the concept that AT<sub>2</sub> receptors may be physiologically important and beneficial in counteracting the vasopressor effect of ANG II [45,91,108].

Whether the ANG II/APA/ANG III/AT<sub>2</sub>/cGMP axis may be targeted in preventing and treating cardiovascular, hypertensive, and kidney diseases has been a subject of continuous debates for two decades [93,96,105]. The fundamental premise of therapeutically targeting the ANG II/APA/ANG III/AT<sub>2</sub>/cGMP axis is primarily based on the “YIN” and “YANG” balance hypothesis, in that as a “YANG” factor, the angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor axis plays a key role in the pathogenesis of cardiovascular, hypertensive, diabetic and kidney diseases, whereas as an “YIN” factor, the ANG II/APA/ANG III/AT<sub>2</sub>/cGMP axis acts to counteract the detrimental effects of ANG II/AT<sub>1</sub> activation [25,95–97]. Indeed, one of the most successful stories in the drug development for cardiovascular, hypertensive, diabetic and kidney diseases during last three decades is the successful development of ACE inhibitors, ARBs, renin inhibitors, and aldosterone receptor antagonists to treat

hypertension, ischemic heart disease or heart failure, and diabetic nephropathy in humans [72,74,115–117]. However, it has been questioned whether AT<sub>2</sub> receptor activation may have deleterious effects in cardiovascular diseases [105]. AT<sub>2</sub> receptors have been implicated in programmed cell death [118], cardiomyocyte enlargement during pressure overload [119], left ventricular hypertrophy and cardiac fibrosis during ANG II-induced hypertension [120], and cellular proliferation in the kidney [121]. Nevertheless, most of these reported “deleterious effects” of AT<sub>2</sub> receptors were mainly deduced from animal studies using the AT<sub>1</sub> and AT<sub>2</sub> receptor-selective blockers or AT<sub>2</sub> receptor-null mice as the experimental model.

#### 2.4. Therapeutic role of CGP42112A as a peptide AT<sub>2</sub> receptor agonist

Perhaps a more direct and clinically relevant approach will be to investigate whether either peptide or non-peptide AT<sub>2</sub> receptor agonists are antihypertensive, cardiovascular and renal protective in animal models and human trials. Two different AT<sub>2</sub> receptor-selective agonists, one peptide and the other nonpeptide form, have been developed for targeting AT<sub>2</sub> receptors. CGP42112A, an AT<sub>2</sub> receptor-selective peptide agonist, has been widely used along with the nonpeptide AT<sub>2</sub> receptor antagonist PD123319 to localize and characterize AT<sub>2</sub> receptors in cells and tissues [84,122,123]. In vivo administration of CGP42112A has been shown to induce renal natriuretic responses via AT<sub>2</sub> receptor-mediated NO/cGMP signaling and inhibition of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity and oxidative stress in obese Zucker rats [124–126], lower blood pressure in SHR [92;104], and inhibit vascular endothelial growth factor-induced migration in human endothelial cells [127]. However, while it is useful as an agonist for AT<sub>2</sub> receptor binding assay and for studying the roles of AT<sub>2</sub> receptors in vitro and in vivo animal models, CGP42112A, as a peptide, may not be practical in human studies to activate the ANG II/APA/ANG III/AT<sub>2</sub>/cGMP axis.

#### 2.5. Therapeutic role of Compound 21 as a novel nonpeptide AT<sub>2</sub> receptor agonist

Recently, Vicore Pharma in Sweden has designed and patented a unique nonpeptide AT<sub>2</sub> receptor agonist, named Compound 21, to target AT<sub>2</sub> receptors for therapeutic application to treat pulmonary fibrosis or hypertension, renal failure, spinal cord injury and acute ischemic stroke [128]. Wan et al. first reported that Compound 21 is an ANG II receptor agonist with a  $K_i$  value of 0.4 nM for the AT<sub>2</sub> receptor and a  $K_i > 10$  μM for the AT<sub>1</sub> receptor [128,129]. They found that Compound 21 has a bioavailability of 20–30% after oral administration and a half-life of 4 h in rat, induces neurite outgrowth, and lowers blood pressure in anesthetized SHR, some well-described effects of AT<sub>2</sub> receptor activation [128]. Further studies in animal models and humans reveal some very encouraging cardiovascular, blood pressure and renal effects of Compound 21, showing some potential therapeutic implications. Compound 21 dose-dependently inhibits tumor necrosis factor- $\alpha$  (TNF $\alpha$ )-induced interleukin 6 production and nuclear factor  $\kappa$ B in primary human and murine dermal fibroblasts [130], decreases early renal inflammatory responses in renovascular hypertension via production of NO and cGMP [131,132], inhibits endothelial inflammation and leukocyte adhesion in human umbilical vein endothelial cells (HUVECs) and in ApoE<sup>-/-</sup> mice [133], and prevents vascular dementia induced by bilateral common carotid artery stenosis in mice [134]. Additionally, Compound 21 is protective against high salt diet-induced kidney injury in obesity Zucker rats [135]. Whether Compound 21 is antihypertensive in animal models and humans in vivo remains uncertain despite the well-recognized vasodepressor and natriuretic

effects of AT<sub>2</sub> receptor activation [94,136]. Compound 21 has no significant effect on blood pressure in stroke-prone spontaneously hypertensive rats (SHR) [137] or on renal vasodilator responses in SHR [138]. High blood pressure in 2K1C rats is not altered by Compound 21 either [131]. By contrast, Kemp et al. demonstrated that C21 lowered blood pressure in ANG II-dependent hypertensive rats and mice through AT<sub>2</sub> receptor-mediated natriuresis [24,94]. Taken together, the available evidence suggests that it is unlikely that Compound 21 may be useful as an antihypertensive agent, but it may be used as a potentially therapeutic agent to help prevent and treat hypertension-induced cardiovascular and kidney organ damages through its anti-fibrotic and anti-inflammatory actions [131,133,135,136], or to counteract AT<sub>1</sub> receptor-mediated ANG II-dependent hypertension [24,94].

### **3. New insights into the roles and therapeutic implications of the ACE2/ANG (1–7)/Mas receptor axis in cardiovascular and renal diseases**

#### **3.1. ACE2**

In addition to the well-recognized angiotensin-converting enzyme 1 (ACE), which converts the biologically inactive ANG I into the potent vasopressor ANG II, a new ACE isoform, named angiotensin-converting enzyme 2 (ACE2) was discovered in 2000 by two different groups of scientists [139,140]. The discovery of ACE2, along with the *Mas* receptor [39], as a key enzyme in generating the vasodepressor peptide ANG (1–7) was viewed as a scientific breakthrough in the RAS research [60,139,141]. Indeed, ACE2 is the key enzyme playing a crucial physiological role of the ACE2/ANG (1–7)/*Mas* receptor axis, the vasodepressor or cardiovascular, blood pressure and renal protective arm of the RAS [39,40,42]. Donoghue et al. were the first to clone ACE2, a human homologue of ACE, from 5' sequencing of a human heart failure ventricle cDNA library [139], found that ACE2 contains a signal peptide, a single metalloprotease active site, and a transmembrane domain [139]. Although ACE2 and ACE share only 42% of amino acid identity, they both act as carboxypeptidases to cleave amino acids from the peptides' carboxyl terminal [139]. However, ACE and ACE2 differ in their enzymatic selectivity in that ACE cleaves two amino acids from ANG I to form ANG II at a time, whereas ACE2 cleaves only one amino acid from ANG I to form ANG (1–9), which is then converted to ANG (1–7) by ACE [60,139]. A further important enzymatic action of ACE2 is to metabolize ANG II to form the vasodepressor peptide ANG (1–7) to decrease the vasopressor peptide ANG II levels in the circulation and tissues [139,142]. Additionally, the activity and action of ACE2 are not affected by ACE inhibitors, further distinguishing ACE2 from the classic ACE [139,140].

#### **3.2. Localization of ACE2 in cardiovascular and kidney tissues**

Donoghue et al. first reported that ACE2 is expressed mainly in the heart, kidney, and testis of 23 human tissues in their landmark study [139]. In the heart, Northern blot analysis revealed that ACE2 mRNA is expressed as a 3.4-kb transcript, and ACE2 immunohistochemical analysis showed its primary localization in the ventricular myocardium, the endothelium of capillaries, and the coronary arteries [139]. Lower levels of ACE2 expression was found in vascular smooth muscle cells and in the adventitia of larger blood vessels. One interesting finding is that the distribution or level of ACE2 protein

expression is not different in failing and nonfailing human hearts [139]. In the kidney, ACE2 is primarily localized in the endothelium of intrarenal blood vessels and the proximal tubule epithelial cells [139,143,144], with very low levels of ACE2 proteins in the glomeruli [139]. Similar findings were observed in biopsied samples obtained from normal and diseased human kidneys [145], though no differences in ACE2 expression was found in human kidneys of normal and various renal diseases [145]. In the mouse kidney, ACE2 was localized in glomerular podocytes, whereas ACE was localized in glomerular endothelial cells [143], consistent with the finding that ANG I or ANG II was metabolized to form ANG (1–7) in the glomerular podocytes [146]. In the rat kidney, ACE2 and ACE proteins were colocalized predominantly in the proximal tubule, in which ACE2 expression was downregulated in diabetes [144]. These animal studies were supported by findings that glomerular and tubular ACE2 expression was decreased in patients with type 2 diabetes and kidney disease [147,148]. In the mouse brain, ACE2 was localized in the cytoplasm of neuronal cells, especially in the subfornical organ (SFO), a neural structure outside the blood-brain barrier, the nucleus of tractus solitarius (NTS), dorsal motor nucleus of the vagus, and the ventrolateral medulla [149]. The localization of ACE2 in these brain structures is physiologically relevant, since these brain structures are closely involved in the neural control of cardiovascular function by the RAS.

### 3.3. Therapeutic implications of ACE2 in cardiovascular, hypertensive and kidney diseases

**3.3.1. New insights from ACE2-KO mice**—In contrast to the classic ACE, which converts inactive ANG I to the active ANG II, ACE2 cleaves ANG I to ANG (1–9) and ANG II to ANG (1–7) [139,140]. Thus, whilst ANG II is the effector peptide for the angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor axis, ANG (1–7) is the effector peptide for the ANG I/ACE2/ANG (1–7)/Mas receptor axis [39,40,42]. The roles and therapeutic implications of ACE2 in cardiovascular, hypertensive and renal diseases are best appreciated from studying the phenotypes of ACE2 knockout mouse models [150–152]. ACE2 knockout mice were first generated by Crackower et al. in 2002 to determine its roles in the regulation of cardiac function [150]. ACE2 knockout in mice showed severe cardiac contractility defect with significantly increased ANG II levels in the heart and kidney [150]. The left ventricular wall of ACE2-null mice was slightly thinner, whereas the chamber dimension was increased, but no cardiac hypertrophy or dilated cardiomyopathy was observed [150]. Blood pressure was normal in 3 month-old male, but significantly decreased in 6 month-old male ACE2-null mice, though the reason is unknown. Since genetic ablation of ACE on an ACE2 mutant background completely rescues the cardiac phenotype of ACE2-null mice, increased cardiac ANG II levels may contribute to the cardiac phenotypes of ACE2-null mice [150]. However two subsequently studies showed completely different cardiac phenotypes in their independent ACE2-null mice [151,152]. Yamamoto et al. reported that their ACE2-null mice showed completely normal cardiac histology and function, including heart wt. and cardiac morphology, systolic left ventricular pressure, though the hypertrophic response to pressure overload induced by transverse aortic constriction was accelerated in these mice [152]. Gurley et al. generated ACE2-null mice on two different genetic background, one on C57BL/6 and the other on 129/SvEv [151]. No abnormal cardiac morphological and functional phenotypes were observed. Blood pressure was moderately increased in ACE2-null mice on the C57BL/6 genetic background, but not in ACE2-null mice on the 129/SvEv

genetic background, although hypertension induced by ANG II infusion was more severe in ACE2-null than in wild-type mice [151]. The reasons underlying these significant differences in cardiac morphological and functional phenotypes between these studies remain unresolved, but likely due to the differences in target-deletion approaches, genetic backgrounds, age of animals, genders used, and experimental designs [150–152].

Although ACE2 is widely localized in the renal glomeruli, intrarenal blood vessels, and tubular epithelial cells, there are few studies have determined the roles of renal ACE2 on renal blood flow, glomerular filtration rate, or tubular transporter functions using ACE2-null mice [139,143,144]. The renal histological and functional phenotypes have not been studied in those three different lines of ACE2-null mice [150–152]. This is very surprising, given the importance of ACE2 in the metabolism of ANG I and ANG II to produce the vasodepressor and natriuretic ANG (1–7) in the kidney [39,42,153].

**3.3.2. ACE2 as a therapeutic target**—The therapeutic potentials of targeting ACE2 in treating cardiovascular, hypertensive and kidney diseases remain to be determined. It is attractive to speculate that any pharmacological approaches to upregulate ACE2 expression via overexpression or to stimulate ACE2 activity with chemical compounds to markedly increase the vasodepressor peptide ANG (1–7) would be clinically beneficial. Some proof of concept studies recently published have provided some support of the concept. In the mouse brain, Feng et al. used the adenovirus-mediated approach to overexpress the human ACE2 cDNA upstream of an enhanced green fluorescent protein (eGFP) reporter gene, Ad-hACE2-eGFP, in the subformal organ, and reported attenuated pressor and drinking responses to ANG II [154]. In a further study, Feng et al. used a synapsin promoter to drive neuron-specific overexpression of hACE2 throughout the brain in mice, which also attenuated ANG II-induced hypertension [155]. In the kidney, Wsocki et al. tested whether a soluble human recombinant ACE2 (rACE2) may be used to decrease ANG II and increase ANG (1–7) levels in plasma and tissues, and whether rACE2 may be used to prevent ANG II-induced hypertension in mice [156]. Interestingly, this study found that rACE2 infusion induced a dose-dependent increase in serum ACE2 activity, but had no effect on kidney or cardiac ACE2 activity [156]. rACE2 infusion alone had no effect on blood pressure, but it normalized the hypertensive response to ANG II infusion [156]. Nadarajah et al. used a nephrin promoter to generate a transgenic mouse model with overexpression of human ACE2 specifically in podocytes of the glomerulus, and treated these transgenic mice with STZ to induce type 1 insulin-dependent diabetes [157]. Although urinary albumin excretion was significantly attenuated in hACE2 transgenic diabetic mice, compared with wild-type nondiabetic mice, at 4 weeks, this anti-albumin effect was not long-lasting [157]. Indeed, the differences in proteinuria were no longer observed between hACE2 transgenic and nontransgenic diabetic mice by 16 weeks [157]. The clinical relevance or practicality of using hACE2 to treat cardiovascular, hypertensive and kidney diseases has been evaluated in a clinical trial [158]. Haschke et al. designed a randomized, double-blind, placebo-controlled, single-dose, dose-escalation study in five cohorts of four subjects each, who were intravenously treated with single doses of rhACE2 at a starting dose of 100 µg/kg body weight in the first cohort over 30 min, whereas cohorts 2–4 were treated with 200, 400, and

800 µg /kg body weight, respectively [158]. Surprisingly, rhACE2 had no effect on Blood pressure and heart rate [158].

**3.3.4. Therapeutic potentials of ACE2 activators**—Recently, efforts have been made to identify so-called ACE2 activators for therapeutic application [159–161]. Hernandez et al. first used a novel conformation-based drug discovery strategy to identify compounds that increase ACE2 activity and to determine whether they may reverse hypertension-induced pathophysiologies [159]. Two compounds, xanthenone and resorcinolnaphthalein, were identified and found to increase ACE2 activity in a dose-dependent manner. Xanthenone showed a remarkable antihypertensive effect, as it acutely decreased blood pressure in SHR by a massive 71 mmHg, but its long-term antihypertensive effect was only moderate [159]. Xanthenone was also found to improve cardiac function and reverse myocardial, perivascular, and renal fibrosis in SHR [159]. However, despite of its impressive antihypertensive and cardiovascular protective effects, there are very few follow-up studies to confirm these findings by this and other groups. Additionally, a chemical compound, diminazene aceturate (DIZE), has been promoted as a potential ACE2 activator to treat ischemia-induced cardiac pathophysiology, pulmonary hypertension, and ischemic stroke [160–163]. DIZE is a widely used antiprotozoal agent to treat trypanosomiasis in domestic livestock, but rarely used in humans [164]. Mecca et al. first reported that intracerebroventricular infusion of either ANG (1–7) or DIZE significantly attenuated the cerebral infarct size and neurological deficits, which were reversed by co-intracerebroventricular administration of the Mas receptor inhibitor, A-779 [162]. This was interpreted as that DIZE may have cerebroprotective properties against endothelin 1-induced ischemic stroke [162]. Subsequent studies by the same group of investigators have demonstrated that DIZE significantly protected rats from monocrotaline (MCT)-induced pulmonary hypertension (PH) [163,165], and decreased myocardial infarction (MI)-induced infarct area, LV remodeling post-MI, and restored normal balance of the cardiac renin-angiotensin system [160]. Recently, the cardiovascular, hypertensive, and renal effects of targeting ACE2 with DIZE in various animal models have been comprehensively reviewed elsewhere often with mixed or conflicting results [161]. In twenty in vivo studies reviewed, only six studies reported a significant decrease in blood pressure in response to DIZE administration, whilst conflicting beneficial effects of DIZE on other cardiovascular, renal, or inflammatory responses were demonstrated [161]. Overall, the potential therapeutic use of DIZE as an ACE2 activator to treat cardiovascular, hypertensive, and diabetic, and kidney diseases remains to be determined for a number of reasons. One of the significant weaknesses of the DIZE story is that the off-target effects of DIZE on ACE2 activity have not been excluded in cultured cell models with absence of ACE2 expression in vitro or in ACE2-null mouse model, nor has it been compared with well-recognized renin and ACE inhibitors or ARBs. Another significant weakness is that most of these studies simply focused on the effect of DIZE on ACE2 activity, but paid no attention to the potential effects of DIZE on the expression of production of ANG I, Mas receptor, ACE, ANG II, AT<sub>1</sub> or AT<sub>2</sub> receptors. Increases in ANG (1–7) or Mas receptor expression, or downregulation of the angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor axis would help explain the in vivo effects of DIZE. An easy argument against the use of DIZE as a therapeutic agent is that if the therapeutic goal of using DIZE is to activate ACE2 to increase the vasodepressor peptide

ANG (1–7) level and lower the vasopressor peptide ANG II level, ACE inhibitors have already been widely used clinically, not mentioning that DIZE may not be safe for use in humans.

### 3.4. Localization, roles, and therapeutic implications of the Mas receptor in cardiovascular, hypertensive and kidney diseases

**3.4.1. New insights from Mas-KO mice**—*Mas*, or the *Mas* receptor, is the first discovered member of a subfamily of *Mas*-related G protein-coupled receptors (Mrgprs or *Mas*-related genes) [40,43], and a protooncogene encoding a protein with seven transmembrane domains [166]. In late 1980s, the *Mas* receptor was thought to mediate the effects of ANG II and ANG III on intracellular accumulation of inositol phosphates (IP<sub>3</sub>) and calcium (Ca<sup>2+</sup>) after in *Mas*-transfected cells, suggesting that the *Mas* oncogene may encode a different ANG receptor [167]. However, two subsequent studies in the early 1990s found no evidence of *Mas*-induced ANG II receptor and/or Ca<sup>2+</sup> responses in *Xenopus* oocytes or in most of transfected cells [168,169]. Thus the *Mas* receptor and ANG II receptor are not identical in their molecular structure, function and signaling [168,169]. To determine whether the *Mas* receptor plays a central role in anxiety, spatial learning, and memory responses, Walther et al. performed targeted deletion of the *Mas* receptor in mice and demonstrated sustained long term potentiation and anxiety in mice lacking the *Mas* protooncogene [170]. No direct interactions or associations in the localization and function were found between the *Mas* receptor and the ANG II/AT<sub>1</sub> receptor axis in the brain [170]. It was Santos et al. who first reported that ANG (1–7) is an endogenous ligand for the *Mas* receptor, thus uncovering for the first time that *Mas* is the specific receptor for the vasopressor peptide ANG (1–7) [39]. However, there is evidence that some neuropeptides (beta-alanine, alamandine, GABA, cortistatin-14, metabolites of proenkephalin, pro-opiomelanocortin, prodynorphin), may also be potential ligands for the Mrgprs family including the *Mas* receptor [40,43]. These ligands activate the Mrgprs to initiate the signaling cascades coupled to G<sub>q/11</sub> and *pertussis* toxin (PTX)-sensitive G<sub>i/o</sub> proteins, activation of which mobilizes PTX-insensitive intracellular Ca<sup>2+</sup> and PTX-sensitive inhibition of forskolin-induced activation of adenylyl cyclase [43]. There is also evidence that additional receptors, such as AT<sub>2</sub> receptors and MrgD receptors, may be involved in mediating the effects of ANG (1–7) [47,48,171–173].

Previous studies have shown that most of the Mrgprs family are expressed in primary sensory neurons in the brain [40,43,174]. *Mas* is also expressed in mast cells in other tissues, such as the testis, heart and kidney [39,40,43]. In the kidney, Santos et al. demonstrated that the *Mas* receptor was localized in all regions of the mouse kidney, though some of <sup>125</sup>I-labelled ANG (1–7) receptor binding persisted in the kidney of *Mas* receptor-deficient mice [39]. Using the immunofluorescent approach with a *Mas* receptor antibody, Gwathmey et al. localized *Mas* antibody binding in the sheep kidney primarily in the proximal tubules, thick limb of Henle, and distal tubules [175], consistent with a natriuretic action of ANG (1–7) especially in the proximal tubules [176]. By contrast, we used [<sup>125</sup>I]-ANG (1–7) as a radioligand to map the distribution of ANG (1–7) receptor binding in the rat kidney, and demonstrated its predominant localization in the proximal tubules in the inner cortex (Fig. 4) [5]. Thus the results strongly suggest that ANG (1–7) may only be one of endogenous

ligands for the *Mas* receptor, activation of which may lead to some off-target responses [39,40,43].

**3.4.2. The *Mas* receptor as a therapeutic target**—The roles and therapeutic implications of the *Mas* receptor in the cardiovascular, blood pressure and renal regulation may be best appreciated from our understanding the physiological roles of the ANG (1–7) (see discussion below) and the cardiovascular, blood pressure, and renal phenotypes demonstrated in *Mas* receptor-deficient mice [39,41,42,153]. The *Mas* receptor was first knocked out by Walther et al., but unfortunately cardiovascular, blood pressure and renal phenotypes were not studied [170]. In a subsequent study, Walther et al. reported that neither heart rate nor blood pressure were significantly different between adult *Mas* receptor-deficient mice and wild-type controls [177]. However, further analysis by these authors found that female *Mas* receptor-deficient mice showed reduced heart rate variability, and increased sympathetic tone in male and female *Mas* receptor-deficient mice [177]. Heringer-Walther et al. also failed to uncover any basal blood pressure phenotype in *Mas* receptor-deficient mice [178]. In the kidney, Santos reported that ANG (1–7) induced significant antidiuretic effect due to increased tubular water reabsorption, whereas the *Mas* receptor knockout in mice significantly attenuated this antidiuretic effect of ANG (1–7) [39]. The *Mas* receptor knockout abolished ANG (1–7)-induced, but not acetylcholine-induced endothelium-dependent relaxation in *Mas* receptor-deficient mice [39,179]. The lack of a blood pressure phenotype in *Mas* receptor-deficient mice is unexpected, because if the vasodepressor peptide ANG (1–7) is the endogenous ligand for the *Mas* receptor, targeted deletion of the *Mas* receptor is expected to elevate basal blood pressure. Likewise, the reported abolishment of ANG (1–7)-induced endothelium-dependent relaxation and ANG (1–7)-induced antinatriuretic effect is also expected to lead to increases in basal arterial pressure in *Mas* receptor-deficient mice [39]. The mechanisms underlying the absence of a clear blood pressure phenotype in *Mas* receptor-deficient mice remain to be determined. One reason may be due to the activation of other compensatory mechanisms to offset the effects of globally knocking out the *Mas* receptor. The other reason may be due to the different genetic backgrounds, mixed 129/C57Bl/6 or FVB/N, on which *Mas* receptor-deficient mice were generated [180]. To further uncover the cardiovascular and blood pressure phenotypes in *Mas* receptor-deficient mice, Xu et al. generated a new strain of *Mas*-deficient mice by backcrossing *Mas*-deficient mice for 7 generations onto the FVB/N background [180]. With the change in the genetic background, these authors were able to demonstrate that *Mas* deficient mice had elevated basal blood pressure, endothelial dysfunction, and impaired NO production [180]. Furthermore, intracellular Ca<sup>2+</sup> responses, eNOS expression and NO production in cardiomyocytes were significantly impaired in *Mas*-deficient mice on the FVB/N background [181], whereas PPAR $\kappa$  expression was decreased in adipocytes of *Mas*-deficient mice [182]. Taken together, these results are largely consistent with the well-recognized roles of ANG (1–7) in the cardiovascular, blood pressure and renal regulation, but need to be confirmed by others.



### 3.5. Roles and therapeutic implications of ANG (1–7) in cardiovascular, hypertensive and kidney diseases

**3.5.1. ANG (1–7) as the effector peptide of the ACE2/ANG (1–7)/Mas receptor axis**—In the ACE2/ANG (1–7)/Mas receptor axis, ANG (1–7) is considered to be the most important effector peptide. The importance of ANG (1–7) in the neural, cardiovascular, blood pressure, and renal regulation and its therapeutic implications has been extensively investigated since 1970s [41,42,85,183]. Indeed, a careful search of PubMed easily yielded over 3300 published studies directly and indirectly involving ANG (1–7). ANG (1–7) was previously considered to be an inactive component of the RAS in the 1970s, because structure and activity studies showed that removal of phenylalanine (position 8) or the dipeptide, Pro-Phe (positions 7 and 8) from the potent vasopressor ANG II completely abolished the vasoconstrictor, pressor or thirst-promoting effects of ANG II [184]. However, Ferrario's group at Cleveland Clinic and then at Wake Forest University has been instrumental in uncovering the vasodepressor roles and mechanisms of ANG (1–7) in the neural, cardiovascular, blood pressure, and renal regulation due to their persistent effort from 1980s to present [41,42,85,183]. Recent identification of the Mas receptor for ANG (1–7) [39], and ACE2 as the key enzyme for the formation of ANG (1–7) [139] adds further enthusiasm to the ongoing research on the roles and therapeutic implications of the ACE2/ANG (1–7)/Mas receptor axis. Now it appears to be well accepted that the ACE2/ANG (1–7)/Mas receptor axis may act as the protective arm of the RAS, counteracting the detrimental effects of the activation of the angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor axis [41,185]. Whilst ANG II induces potent vasoconstriction and cardiac hypertrophy [186–189], renal vasoconstriction and antinatriuresis [3,190,191], promotes central thirst and sympathetic nerve activity [192,193], and increases aldosterone biosynthesis and release [194,195], ANG (1–7) appears to oppose and attenuate these harmful effects of ANG II [41,185,196]. Over the last two decades, the biochemistry, physiological roles and signaling mechanisms underlying the ANG (1–7) have been comprehensively reviewed elsewhere, and therefore not a focus of discussion in this article [41,42,183].

**3.5.2. ANG (1–7) as a therapeutic agent**—The potential implications in using exogenous ANG (1–7) as a therapeutic agent or increasing endogenous ANG (1–7) formation alone in treating hypertension, cardiovascular and kidney diseases remain a challenging issue. In theory, in vivo administration of ANG (1–7) would be beneficial to oppose or attenuate the harmful effects of ANG II, but in practice it may be difficult to administer a peptide in a clinical setting. The questions remain with respect to how much ANG (1–7) should be given and via what route the vasodepressor peptide ANG (1–7) should be administered to induce beneficial cardiovascular or renal protective effects. Campbell et al. have previously shown that under basal conditions, ANG (1–7) levels were about one-tenth of ANG II in both the rat plasma and the kidney, as measured by HPLC-based radioimmunoassays [78,79]. Chappell showed that ANG (1–7) and ANG II levels were largely similar in the plasma and kidney, but ANG II levels are higher than ANG (1–7) in the heart, adrenal glands, lung and liver [80]. However, plasma and tissue ANG (1–7) levels are significantly increased during ACE inhibition, which leads to the notion that the beneficial effects of ACE inhibitors may be mediated in part by ANG (1–7) [80,197].

Although global or cell-specific ACE2 overexpression may be beneficial in SHR [198,199] or diabetic nephropathy [200], not all animal studies in vivo support the therapeutic premise of the ACE2/ANG (1–7)/Mas receptor axis in protecting cardiovascular, pulmonary, and renal systems, or in treating hypertension. Indeed, inconsistent findings have been reported in mutant animals overexpressing ANG (1–7) [201–203] or ACE2 [157,200,204] with markedly increased circulating and tissue ANG (1–7) levels. Indeed, overexpression of ANG (1–7) had no effect on blood pressure in TGR(A1–7)3292 rats, despite of significant increased stroke volume and cardiac index and decrease in total peripheral resistance [201,202]. Rentzsch et al. overexpressed the human ACE2 gene selectively in rat vascular smooth muscle cells using of the SM22 promoter on a SHRSP genetic background [204]. Circulating ANG (1–7) was significantly elevated; whereas basal blood pressure was only slightly lower [204]. Liu et al. overexpressed ACE2 globally via the adenoviral gene delivery, and found no differences in blood pressure between control and ACE2-overexpressing rats [200]. Breitling et al. found insignificant therapeutic usefulness of ANG (1–7) in an animal model of experimental pulmonary hypertension [205], whereas Zhang et al. reported that activation of *Mas* during myocardial infarction contributed to, rather than attenuated, ischemia-reperfusion injury in rats [206]. In the latter study, inhibition of *Mas* G-protein signaling surprisingly improved coronary blood flow, reduced myocardial infarct size, and provided long-term cardioprotection [206].

**3.5.3. (ANG (1–7) mimetics as therapeutic drugs—**A novel nonpeptide compound, AVE0991, has been developed as a mimetic of ANG (1–7) for therapeutic application [207]. AVE0991 was found to be 10-fold more potent than ANG (1–7) in competing for [<sup>125</sup>I]-ANG (1–7) binding to, and 5-fold more potent than ANG (1–7) to induce NO release from bovine aortic endothelial cells [207], suggesting that AVE0991 may act as a nonpeptide agonist for the *Mas* receptor [208,209]. Subsequent in vivo studies have reported that AVE0991 significantly protected postischemic heart failure in rats [210], prevented diabetes-induced cardiovascular dysfunction [211], inhibited atherogenesis in apoE-knockout mice [212,213], ameliorated inflammation in experimental arthritis [214], and attenuated cardiac hypertrophy [215]. Despite these reported protective effects in different animal models or diseases, no clinical trial for AVE0991 is currently registered since it was developed more than 10 years ago [207]. Although these results from animal studies are very promising, they are basically very similar to those well-described responses to ACE inhibitors or ARBs. Whether these findings will be confirmed in humans in clinical trials remains unknown.

#### **4. New insights into the roles and therapeutic implications of dipeptidyl peptidase III in cardiovascular, hypertensive and renal diseases**

Recently, Dipeptidyl peptidase III (DPP III, EC 3.4.14.4) has been reported to have anti-hypertensive, anti-cardiac hypertrophic and anti-fibrotic effects in mice [46]. DPP III is zinc-dependent aminopeptidase and acts to preferentially cleave two amino acids (dipeptide residues) from the N-terminus of the oligopeptides in the serum, placenta, liver and brain [46,216–218]. The oligopeptides subjected to metabolism by DPP III include ANG II and its metabolites ANG III and ANG IV, and enkephalins and endorphins [46]. Degradation of the vasopressor peptide ANG II is expected to lower circulating and tissue ANG II levels,

leading to the same cardiovascular, blood pressure and renal protective effects induced by ACE inhibitors and ARBs. Although DPP III was discovered and characterized several decades ago [216–218], there is only one study investigating its roles and therapeutic implications in hypertension [46]. Pang et al. first studied the enzymatic properties of DPP III for degrading the vasopressor peptide ANG II, and found that DPP III potently metabolized ANG II with a  $K_m$  of  $3.7 \times 10^{-6}$  mol/L and more effectively ANG IV with a  $K_m$  of  $1.7 \times 10^{-6}$  mol/L in vitro [46]. In ANG II-induced hypertensive mice, intravenous bolus injection of DPP III at 8  $\mu$ g/g body wt (or  $\sim 200$   $\mu$ g for a 25 g mouse) markedly decreased systolic blood pressure by almost 60 mmHg one hr after injection, and the response returned to pre-injection level one day later [46]. More interestingly, repeated DPP III injection every other day for 4 weeks significantly attenuated ANG II-induced cardiac fibrosis and hypertrophy, albuminuria and kidney injury were significantly attenuated [46]. These results strongly suggest that DPP III may be a therapeutic target for ANG II-dependent hypertension and target organ damage [46,219].

However, there are a number of caveats with the potential roles or therapeutic implications of DPP III that need to be further discussed. First, only high pressor doses of ANG II or high doses of DPP III were used in the above-mentioned study [46]. Second, DPP III not only metabolizes the vasopressor arm of the key RAS peptide ANG II, but likely also degrades the vasodepressor and protective arm of ANG fragments, ANG (1–7), ANG III, which offsets its potential therapeutic effects [220]. Third, as an enzyme DPP III has to be given intravenously and daily, it may not be practical in clinical settings, and perhaps a nonpeptide mimetic must be developed for long-term oral use. Fourth, the cardiovascular, antihypertensive and renal protective effects of DPP III were reportedly similar to those induced by the  $AT_1$  receptor blocker candesartan [46]. If DPP III mediates these effects by increasing ANG II degradation and lowering systemic and tissue ANG II levels, then ACE inhibitors and ARBs are readily available for use and should be more superior to DPP III as antihypertensive drugs. Nevertheless, more studies are required to determine whether DPP III may be suitable for treating other cardiovascular and renal diseases not associated with ANG II.

## 5. New insights into the roles and therapeutic implications of a novel ANG peptide alamandine in cardiovascular, hypertensive and renal diseases

Alamandine is another recently discovered ANG peptide reportedly having vasodepressor properties [47,48]. Alamandine is a heptapeptide derived from the catalytic hydrolysis of the octapeptide Ala<sup>1</sup>-ANG II (ANG A) by human ACE2 and structurally similar to ANG (1–7), except that aspartic acid in position 1 of ANG (1–7) is substituted with alanine in position 1 of alamandine [48,49,221]. Lautner et al. demonstrated that alamandine can be generated from ANG (1–7) in the rat heart and is circulating in human blood with increased levels in nephropathic patients [48]. Unlike ANG (1–7), which binds and activates the *Mas* receptor, alamandine reportedly binds and activates the *Mas*-related G-protein-coupled receptor, member D (MrgD), which may be blocked by D-Pro<sup>7</sup>-ANG (1–7),  $\beta$ -alanine, and PD123319 [48]. Since the *Mas* antagonist A-779 had no effect in blocking alamandine binding, this suggests that alamandine and ANG (1–7) act on two different receptors, yet they induce

similar vasodepressor and cardiovascular protective effects [47,48]. In the in vivo setting, oral administration of alamandine/ $\beta$ -hydroxypropyl cyclodextrin decreased blood pressure by <20 mmHg in SHR, and showed antifibrotic effects in isoproterenol-treated rats, but not in human tumoral cell lines [48]. How this structural substitution of aspartic acid in position 1 of ANG (1–7) with alanine leads to the vasodepressor effect in alamandine remains poorly understood. Another newly identified ANG peptide, ANG A (Ala-Arg-Val-Tyr-Ile-His-Pro-Phe), with the substitution of alanine for aspartate acid in position 1 of ANG II, shows similar vasoconstrictive response to ANG II, rather than a vasodepressor effect [222]. This is also inconsistent with previous studies in 1970s, which consistently showed that substitution of the amino acid in position 1 had no effect on the vasopressor activity of ANG II [21]. An interesting study even suggests that like ANG II, alamandine acts on both AT<sub>1</sub> and AT<sub>2</sub> receptors to cause both vasopressor and vasodepressor effects in 2K1C hypertensive rats [223]. Taken together, the findings that the AT<sub>2</sub> receptor antagonist PD123319 completely displaced alamandine receptor binding and blocked alamandine-induced aortic vasorelaxation, and that the *Mas* antagonist A-779 had no effect in blocking alamandine binding, raise more questions than answers on whether alamandine is a truly endogenous or physiological agonist for the alamandine/MrgD axis or the ANG II/ANG III/AT<sub>2</sub> receptor/cGMP axis [48,49,224]. More studies are required to confirm the structure, biochemistry, the physiological role and therapeutic implications of alamandine in cardiovascular, hypertensive and kidney diseases.

## 6. New insights into the roles and therapeutic implications of angiotensin in cardiovascular and renal diseases

In 2011, a novel endogenous ANG II-like octapeptide, angiotensin (MW 1001.5 Da), was identified by Jankowski et al. in blood of healthy humans and patients with end-stage renal failure using chromatographic purification and structural analysis by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) [50]. Compared with the sequence of ANG II, angiotensin has the sequence of Pro-Glu-Val-Tyr-Ile-His-Pro-Phe, with its Pro<sup>1</sup>-Glu<sup>2</sup> substituting Asp<sup>1</sup> and Arg<sup>2</sup> in ANG II, raising the possibility that angiotensin may be derived from ANG II [50]. In fluorescent receptor binding assays in mouse endothelial cells, unlabeled ANG II did not displace Cy3-angiotensin binding, but unlabeled angiotensin blocked FAM-ANG (1–7) binding to the *Mas* receptor [50]. These results implicate that like ANG (1–7), angiotensin may represent an additional agonist for the *Mas* receptor. Indeed, this study showed that angiotensin induced a significant vasodilatory effect in wild-type mice at  $\mu\text{mol/L}$  concentrations, but it had no similar vasodilatory effect in *Mas* receptor-deficient mice [50]. This study also showed dose-dependent antihypertensive effects in SHR with a peak vasodepressor response to angiotensin at 30–300 pmol/kg/min, i.v. [50]. In healthy humans, the plasma angiotensin and ANG II levels were reportedly similar at 3–7 pmol/L, whereas they were about 10–12 pmol/L in patients with end-stage renal diseases [50]. These levels of angiotensin and ANG II levels, as determined by MALDI-TOF/TOF, are largely similar to those measured using HPLC-based RIAs [78,80,225]. Finally, a cDNA encoding angiotensin or RNA-splicing variants for angiotensin has not been identified in the human genome, raising a doubt whether it is an endogenous vasodepressor peptide [50].

Since its discovery in 2011, there have been very few studies reporting its true levels in rodent and human plasma and tissues. Accordingly, the molecular, biochemical and physiological or pathological roles and therapeutic implications of angiotensin remain to be further studied and independently confirmed by others [51].

## 7. Conclusions

In summary, the extensive research during last three decades has firmly established at least five axes of the RAS, with each having its primary substrate, key enzyme, the major effector peptide, specific receptor, and downstream signaling pathways. These RAS axes may be divided into two different classes of vasoactive systems, one “YIN” and one “YANG”. The “YANG” vasopressor system includes the classic angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor axis and the newly discovered prorenin/renin/prorenin receptor (PRR)/MAPK/V-ATPase axis, and possibly includes the newly described ANG fragment, ANG A, if the latter’s structure and role are confirmed by others. The ANG IV/AT<sub>4</sub> receptor axis may also belong to the vasopressor system, since at the pharmacological concentrations, ANG IV activates not only the AT<sub>4</sub> receptor, but also the AT<sub>1</sub> receptor, in blood vessels and the kidney to increase blood pressure and induce renal vasoconstriction (Fig. 4 & Fig. 5) [21–23]. The “YIN” vasodepressor system includes the ANG II/APA/ANG III/AT<sub>2</sub> receptor/NO/cGMP axis and the ANG I/ANG II/ACE2/ANG (1–7)/*Mas* receptor axis. The newly described DPP III, alamandine and angiotensin, together may be “assigned” to the “YIN” vasodepressor system. It is now beyond any doubt that the “YANG” vasoactive system plays the most critical role, contributing to the overall physiological regulation of central, cardiovascular and kidney function, and blood pressure homeostasis. However, the upregulation or overactivation of the “YANG” vasoactive system contributes to the pathogenesis and underlying mechanisms of most well-recognized cardiovascular, hypertensive and kidney diseases. The development of orally active therapeutic drugs to target the “YANG” vasoactive system by inhibiting the rate-limiting enzyme, renin, and the key ANG II-generating enzyme, ACE, and blocking the receptors for ANG II and aldosterone, has been one of the most successful stories in pharmacological and pharmaceutical research during last three decades. By contrast, although tremendous efforts and progress have been made in identifying key members of the “YIN” vasoactive system, and uncovering its respective roles and signaling mechanisms of actions, the therapeutic implications of targeting the ANG II/APA/ANG III/AT<sub>2</sub> receptor/NO/cGMP axis, the ANG I/ANG II/ACE2/ANG (1–7)/*Mas* receptor axis, DPP III, alamandine and angiotensin in treating cardiovascular, hypertensive, and kidney diseases still remain to be determined. Nevertheless, recent research on the nonpeptide AT<sub>2</sub> receptor agonists, ACE2 activators, ANG (1–7) mimetic, dipeptidyl peptidase III, alamandine and angiotensin has provided some proof of concept support of using the “YIN” vasoactive system to counteract the detrimental effects of the vasopressor peptide ANG II and activation of AT<sub>1</sub> receptors. However, to be as successful as the well-established renin and ACE inhibitors, ARBs and aldosterone receptor antagonists in treating cardiovascular, hypertensive and kidney diseases, the therapeutic usefulness and effectiveness of the nonpeptide AT<sub>2</sub> receptor agonists, ACE2 activators, ANG (1–7) mimetic, DPP III, alamandine or angiotensin will

have to be confirmed in multi-center, randomized and double-blinded clinical trials in humans.

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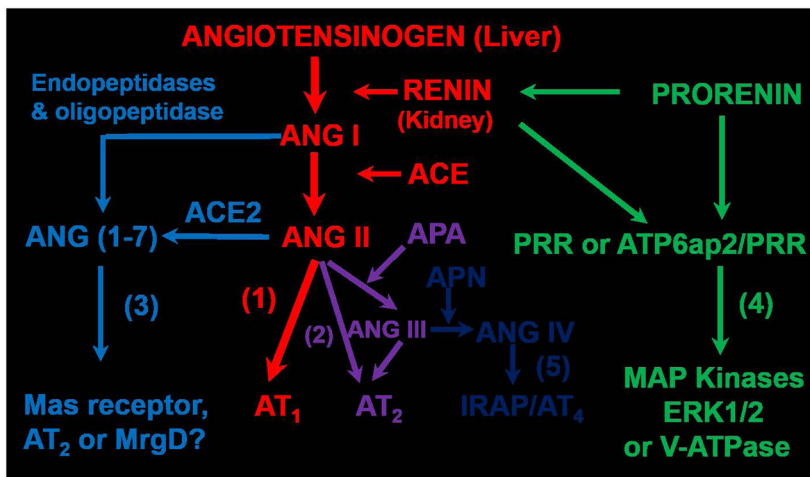
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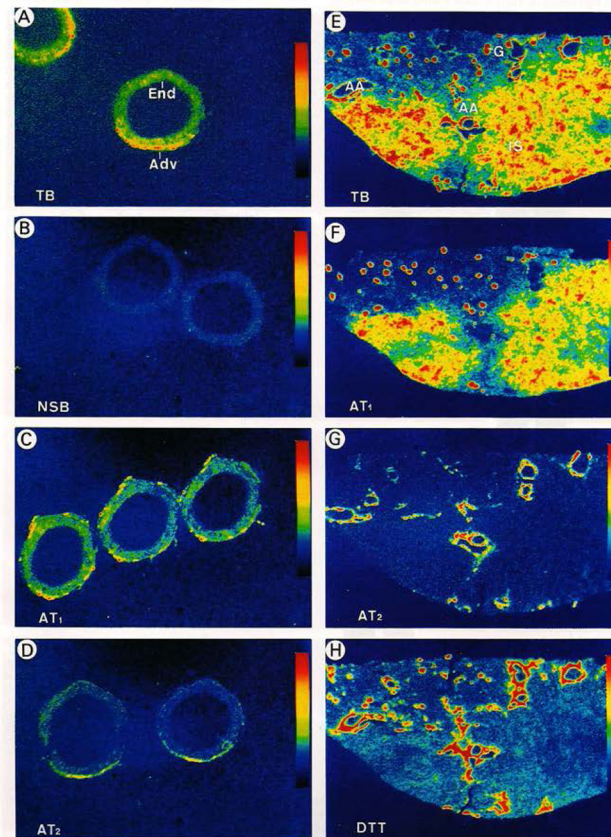


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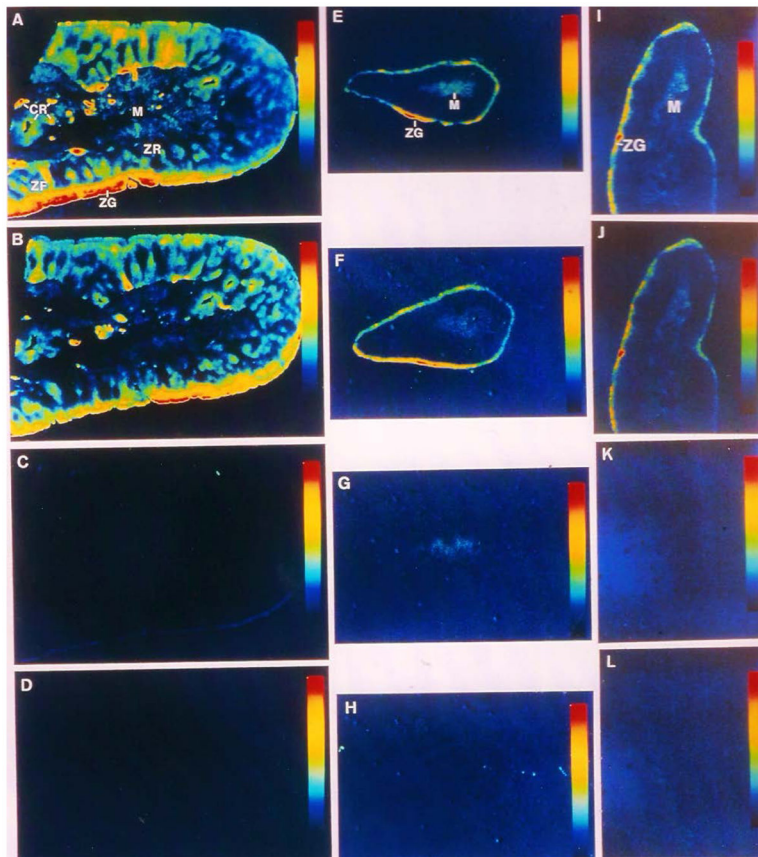


**Figure 1.** Classical and new paradigms of the evolving renin-angiotensin system. (1): receptor axis. (2): the ANG the classical angiotensinogen/renin/ACE/ANG II/AT<sub>1</sub> receptor/NO/cGMP axis. (3): the ANG I/ANG II/ACE2/ANG (1–7)/Mas II/APA/ANG III/AT<sub>2</sub> receptor axis. (4): the prorenin/renin/prorenin receptor (PRR or ATP6ap2)/MAP kinases receptor/IRAP axis. Modified ERK1/2/V-ATPase axis. (5): the ANG III/APN/ANG IV/AT<sub>4</sub> from reference [5] with permission.



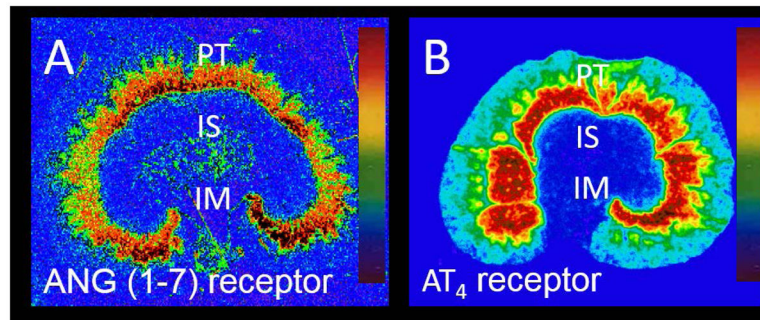
**Figure 2.**

In vitro autoradiographic localization of AT<sub>1</sub> and AT<sub>2</sub> receptors in the human kidney and renal artery using [<sup>125</sup>I]-[Sar<sup>1</sup>,Ile<sup>8</sup>]-ANG II as the radioligand. A & E: total ANG II (AT<sub>1</sub> and AT<sub>2</sub>) receptors. C & F: AT<sub>1</sub> receptor binding in the presence of 10 μM of the AT<sub>2</sub> receptor blocker PD123319 (10 μM). D & G: AT<sub>2</sub> receptor binding in the presence of 10 μM of the AT<sub>1</sub> receptor blocker losartan. Modified from reference [100] with permission.



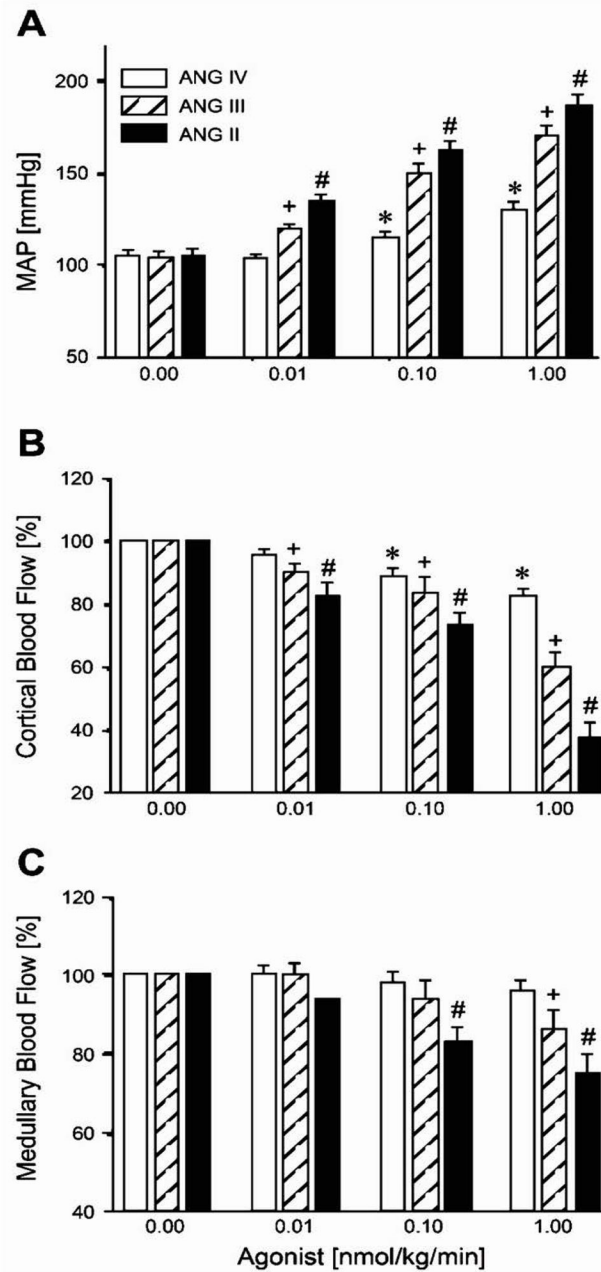
**Figure 3.**

In vitro autoradiographic localization of AT<sub>1</sub> and AT<sub>2</sub> receptors in bovine (A–D), monkey (E–H) and human adrenal glands (I–L) using [<sup>125</sup>I]-[Sar<sup>1</sup>,Ile<sup>8</sup>]-ANG II as the radioligand. A, E & I: total ANG II (AT<sub>1</sub> and AT<sub>2</sub>) receptors. B, F & J: AT<sub>1</sub> receptor binding in the presence of 10 μM of the AT<sub>2</sub> receptor blocker PD123319 (10 μM). C, G & K: AT<sub>2</sub> receptor binding in the presence of 10 μM of the AT<sub>1</sub> receptor blocker losartan. D, H & L: nonspecific binding in the presence of 10 μM of unlabeled ANG II. Modified from [99] with permission.



**Figure 4.**

In vitro autoradiographic localization of ANG (1–7) (A) and AT<sub>4</sub> receptors (B) in the rat kidney using [<sup>125</sup>I]-ANG (1–7) and [<sup>125</sup>I]-ANG (3–8) as the radioligands, respectively. Modified from [5] and [23] with permission.



**Figure 5.**

Dose-dependent pressor and renal cortical and medullary vasoconstrictor responses to intravenous infusion of increasing concentrations of ANG II, ANG III, and ANG IV. ANG II, ANG III, and ANG IV increased mean arterial pressure (MAP) and decreased renal cortical blood flow (CBF) in a dose-dependent manner, exhibiting potency in the order: ANG II > ANG III > ANG IV. ANG II and ANG III also decreased renal medullary blood flow (MBF) at higher doses, whereas ANG IV had no effect.  $P < 0.05$  vs. baseline ANG IV

response (\*), vs. baseline ANG III response (+), and vs. baseline ANG II response (#).  
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