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REVIEW

Renin-angiotensin system in the kidney: What is new?

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Abstract

The renin-angiotensin system (RAS) has been known for more than a century as a cascade that regulates body fluid balance and blood pressure. Angiotensin Ⅱ (Ang Ⅱ) has many functions in different tissues; however it is on the kidney that this peptide exerts its main functions. New enzymes, alternative routes for Ang Ⅱ formation or even active Ang Ⅱ-derived peptides have now been described acting on Ang Ⅱ AT_1 or AT_2 receptors, or in receptors which have recently been cloned, such as Mas and AT_4. Another interesting observation was that old members of the RAS, such as angiotensin converting enzyme (ACE), renin and prorenin, well known by its enzymatic activity, can also activate intracellular signaling pathways, acting as an outside-in signal transduction molecule or on the renin/(Pro)renin receptor. Moreover, the endocrine RAS, now is also known to have paracrine, autocrine and intracrine action on different tissues, expressing necessary components for local Ang Ⅱ formation. This *in situ* formation, especially in the kidney, increases Ang Ⅱ levels to regulate blood pressure and renal functions. These discoveries, such as the ACE2/Ang-(1-7)/Mas axis and its antangonistic effect rather than classical deleterious Ang Ⅱ effects, improves the development of new drugs for treating hypertension and cardiovascular diseases.

Key words: Renin-angiotensin system; Angiotensin Ⅱ; Kidney; Hypertension treatment; Receptor

Core tip: Activation of the angiotensin converting enzyme (ACE)/ Angiotensin Ⅱ (Ang Ⅱ)/AT_1 axis leads to vasoconstriction, anti-diuresis, anti-natriuresis, release of aldosterone and anti-diuretic hormone, which can result in hypertension, renal and cardiovascular diseases. Inhibition of renin and ACE or blocking AT_1 receptor is the most used therapies for heart failure and hypertension. Nevertheless, the discovery of local Ang Ⅱ synthesis, new Ang Ⅱ metabolites, receptors and axis of this system, makes possible the development of new drugs and strategies for renal and cardiovascular diseases treatment, such as activation of ACE2/Ang-(1-7)/Mas axis, which presents opposite effects of AT_1 activation by Ang Ⅱ.

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RAS IS NOT ONLY AN ENDOCRINE SYSTEM

The first observation that the arterial blood pressure could be regulated was in 1898 after the discovery of a soluble protein extracted from the kidney that increased

Table 1 Comparison of the components from classic and recent renin-angiotensin system

	Classic RAS	Recent RAS
Hormone process	Endocrine	Paracrine Autocrine Intracrine
Bioactive peptide	Ang Ⅱ	Ang Ⅱ Ang III Ang IV Ang-(1-7) Ang-(3-4) Ang A Alamandine
Receptor	AT_1 AT_2	AT_{1a} AT_{1b} AT_2 Mas Mrg AT4 PRR ACE

Ang: Angiotensin; ACE: Angiotensin converting enzyme; AT_1: Angiotensin type 1 receptor; AT_2: Angiotensin type 2 receptor; AT_4: Angiotensin type 4 receptor; Mas: Ang-(1-7) Mas receptor; Mrg: Mas-related G-protein-coupled receptor; PRR: Renin/(Pro)renin receptor; RAS: Renin-angiotensin system.

blood pressure in rabbits, called "renin"[1]. Over 30 years later, Goldblatt *et al*[2] associated the decrease of blood pressure in kidneys with hypertension by using a silver clamp to partially constrict dogs renal arteries, resulting in reno-vascular hypertension. Using the same methodology as Goldblatt, Braun-Menendez *et al*[3] in 1940 isolated a vasoconstrictor substance responsible for the reno-vascular hypertension from renal venous blood of the hypertensive kidney dog, calling it "hypertensin". Page *et al*[4] independently described a vasoconstrictor substance by injecting renin into cats, called "angiotonin". The same group also described angiotensinogen, first referred to as a "renin activator"[4]. The name "angiotensin" for the vasoconstrictor substance "hypertensin" by Braun-Menendez and "angiotonin" by Page emerged in 1958 after they both agreed on this hybrid name, since these 2 substances proved to be the same potent vasoactive octapeptide. The World Health Organization and the American Heart Association in 1987 suggested the abbreviation Ang for angiotensin, numbering the amino acids residues of angiotensin I (Ang I) as a reference for all angiotensin-derived peptides[5]. The decapeptide Ang I has no physiological effect, but is hydrolyzed by angiotensin converting enzyme (ACE) generating angiotensin Ⅱ (Ang Ⅱ), which was considered the only peptide in renin-angiotensin system (RAS) with biological actions[6].

More than a century since the discovery of renin by Robert Tigerstedt and Bergman[1], the RAS, remains a fascinating subject for research. Although it is well known the distinct roles of RAS in different tissues, such as brain, adipose tissue, gastrointestinal tract and cardiovascular system[7-10], it is on the kidney that Ang Ⅱ has its main function on regulating body fluid content and blood pressure by alterating Na^+ and water homeostasis, intrarenal hemodynamics and glomerular filtration[11,12]. Ang Ⅱ stimulates anti-diuretic hormone secretion in the pituitary gland with increased water reabsorption in the collecting duct, and also increases aldosterone secretion, a steroid hormone synthesized mainly by the adrenal cortex, and a downstream effector of Ang Ⅱ that induces sodium reabsorption and concomitant potassium and hydrogen ion excretion by the kidney[13].

Many new findings suggest new properties of this system, with new enzymes, different routes for Ang Ⅱ formation, new receptors and active Ang Ⅱ-derived peptides (Table 1). The classical axis ACE/Ang Ⅱ/AT_1 is not the only signaling pathway within RAS, since others such as angiotensin converting enzyme 2 ACE2/Ang-(1-7)/Mas receptor and Angiotensin IV/AT_4 indicate new activities for this cascade[14,15]. Besides the inhibition of renin and ACE, and also angiotensin type 1 receptor (AT_1) blockade, activation of the ACE2/Ang-(1-7)/Mas axis is a possible alternative target for new drugs, since some protective effects on renal and cardiovascular function have been reported[14,16-18]. Ang Ⅱ is not the only active peptide of the RAS, there now being physiological properties associated with many Ang Ⅱ-derived peptides[14,15,19]. Ang Ⅱ can be hydrolyzed by > 13 "angiotensinases", proteolytic enzymes such as aminopeptidases, carboxipeptidases, endopeptidases, ACE2 and neprilysin, generating angiotensin Ⅲ (Ang Ⅲ), angiotensin Ⅳ (Ang Ⅳ), angiotensin-(1-7) [Ang-(1-7)], angiotensin-(3-4) [Ang-(3-4)], angiotensin A (Ang A), and alamandine, which can bind to specific receptors or act on the same receptors as Ang Ⅱ[14,15,19-22]. Although AT_1 and AT_2 receptors are the most studied receptors for Ang Ⅱ, two other receptors - Mas receptor for Ang-(1-7), and AT_4 receptor for Ang Ⅳ - have been cloned[14,15]. Ang Ⅱ-derived peptides could have similar effects to Ang Ⅱ, or counteract its effects on renal function. For instance, like Ang Ⅱ, Ang-(1-7) can increase intracellular Ca^{2+} *via* AT_1 receptor, but has the opposite effect to Ang Ⅱ, since it can induce antiproliferative and protective effect through the Mas receptor[23,24]. New functions for well known members of the RAS have been found. For example, ACE, known for its catalytic action on Ang I, also functions as a signal transduction molecule, initiating a series of intracellular events when stimulated[25,26]. Besides increasing catalytic activity of renin and prorenin, the renin/(Pro)renin receptor (PRR), cloned in 2002[27], can also induce an intracellular signaling pathway generating effects in an angiotensin-independent manner[6,27].

It is now considered that RAS assumes paracrine, autocrine and intracrine mechanisms of action in hormone signaling[6,28]. Many tissues and cells, including kidneys, have all the necessary RAS components to form Ang Ⅱ *in situ*[29-31]. Renal levels of Ang Ⅱ are much higher than in the plasma[32], indicating that the source of Ang Ⅱ within the kidney is not only provided by filtered plasma Ang Ⅱ. The kidney expresses all the major components of the RAS, such as angiotensinogen, renin and

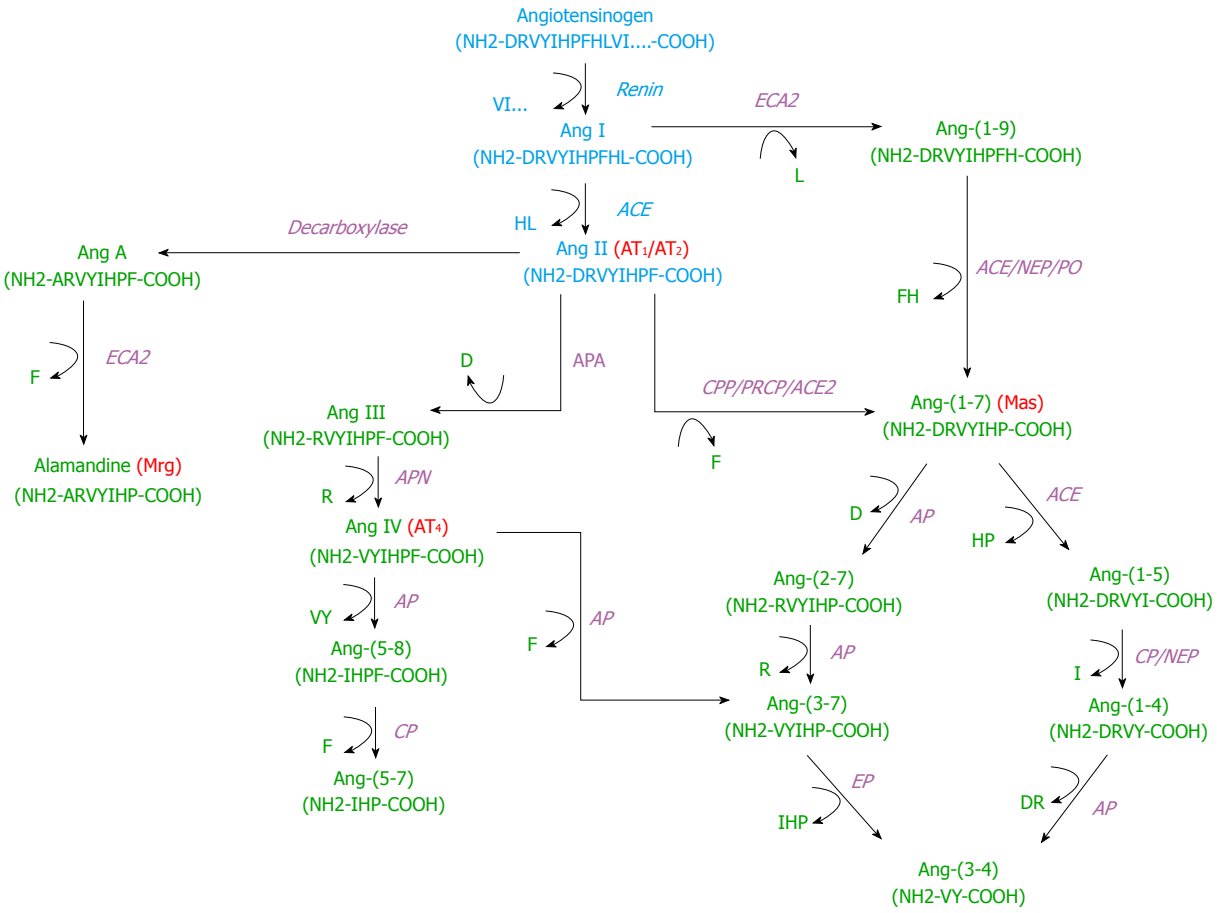

Figure 1 Classic view of renin-angiotensin system cascade (blue) and recent view of renin-angiotensin system cascade (green). AP: Aminopeptidase; APA: Aminopeptidase A; APN: Aminopeptidase N; CP: Carboxypeptidase; EP: Endopeptidase; ACE: Angiotensin converting enzyme; ACE2: Angiotensin converting enzyme 2; CPP: Carboxypeptidase P; PRCP: Prolyl carboxypeptidase; NEP: Neprilysin; PO: Prolyl oligopeptidase; Mas: Ang-(1-7) Mas receptor. Adaptated from Axelband *et al*[20] with permission.

ACE[29-31]. Locally synthesized Ang Ⅱ can act on cell surface, nuclear and cytoplasmic AT_1 and AT_2 receptors[33-35].

We will describe a novel view of the classic RAS that includes new members, routes, receptors, and new drugs and targets for the treatment of heart failure and hypertension. Due to the high Ang Ⅱ concentration in different compartments of the kidney, and the importance of Ang Ⅱ effects on renal function in physiological and physiopathological conditions, the focus will be on the intrarenal RAS, especially its paracrine and intracrine functions. This new aspect of RAS will improve our present understanding of RAS and the role of its new members, which should benefit the development of new treatments for hypertension and kidney diseases.

NEW MEMBERS OF RAS: ANG Ⅱ-DERIVED PEPTIDES

Classically, renin is secreted by juxtaglomerular cells in response to 3 stimuli: (1) decreased arterial blood pressure, detected by baroreceptors; (2) decreased sodium levels in the macula densa ultrafiltrate; and (3) increased sympathetic nervous system activity. Renin is an enzyme with only one known substrate, angiotensinogen. The reaction catalyzed by renin, generating the decapeptide Ang Ⅰ, is the rate-limiting step in Ang Ⅱ formation. Ang Ⅰ is then converted to Ang Ⅱ by ACE, a monomeric glycoprotein that acts as an exopeptidase to cleave dipeptides from the C-terminus of Ang Ⅰ -(1–10) (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) into the octapeptide Ang Ⅱ-(1-8)[36] (Figure 1). The main Ang Ⅱ effects are mediated by the AT_1 receptor, such as vasoconstriction, anti-diuresis, anti-natriuresis, release of aldosterone and anti-diuretic hormone, whereas AT_2 activation counterbalances these effects[19,36,37].

It is widely accepted that small peptides derived from Ang Ⅱ have local physiological effects, especially in the kidney (Figure 1). ACE2 is a transmembrane glycoprotein that shares a 42% of homology with ACE and contains a single active site domain more closely to the N domain of ACE[16,38]. Unlike ACE, ACE2 is a monocarboxypeptidase, generating Ang-(1-7) by the cleavage of a single Phe residue from Ang Ⅱ, and Ang-(1-9), removing the C-terminal Leu residue from Ang Ⅰ [16,38]. Within the renal brush-border vesicles of the rat, Ang-(1-7) is preferentially hydrolyzed by aminopeptidases and neprilysin (NEP) after aminopeptidase blockade, generating Ang-(1-4)[39]. In the basolateral membrane, brush-border vesicles of the pig and purified preparations of

renal NEP Ang I is hydrolyzed primarily to Ang-(1-7) and Ang-(1-4)[40,41]. In sheep proximal tubules, urine and serum, Ang Ⅱ is converted to Ang-(1-7) by both membrane-bound and soluble forms of ACE2[38].

The physiological importance of Ang-(1-7) has become increasingly evident, especially after Santos *et al*[14] found a G protein-coupled receptor for Ang-(1-7), the Mas receptor, using a selective Ang-(1-7) antagonist. The Mas protooncogene was cloned and sequenced in 1986, after being detected by its tumorigenicity in mice[42]. This gene encodes a protein with 7 hydrophobic transmembrane domains, first considered as an "orphan" G protein-coupled receptor[43]. Ang-(1-7) exerts many effects on renal function, such as diuresis and natriureses, and it can be detected in human urine[44]. This peptide is of importance during late gestation in rats, where RAS overactivity is associated with increased kidney and urine levels of Ang-(1-7) and enhanced kidney immunostaining of Ang-(1-7) and ACE2[45].

Diuretic/natriuretic effects of Ang-(1-7) may also be due to the regulation of Na^+ reabsorption within the proximal tubule. *In vivo* and *in vitro* studies showed that Ang-(1-7) is a potent inhibitor of Na^+ reabsorption in this nephron segment, acting on different receptors[46-49]. Ang-(1-7) can bind to distinct receptors and induces different cellular responses depending on the cell type. For instance, in distal tubule cell (MDCK), Ang-(1-7) inhibits ($Na^+ + K^+$)-ATPase activity through the AT_1 receptor to stimulate the PI-PLC/PKC signaling pathway[47], whereas in the proximal tubule, it inhibits Na^+-ATPase *via* the AT_2/G(i/o) protein/cGMP/PKG pathway[48]. Moreover, at different concentrations of Ang-(1-7) (10^{-12}, 10^{-9}, or 10^{-6} mol/L) used in intratubular perfusion in the absence or presence of the Mas receptor antagonist (A779) of rat isolated proximal tubules, it was shown that Ang-(1-7) has a biphasic dose-dependent effect on the Na^+/H^+ exchanger mediated by Mas receptor and gave a moderate increase in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$)[49]. Increased $[Ca^{2+}]_i$ stimulated by Ang-(1-7) also occurred in MDCK cells, but through the AT_1 receptor, which in turn stimulated Ca^{2+} release from endoplasmic reticulum *via* the PLC pathway and Ca^{2+} influx through PLA2-dependent store-operated Ca^{2+} entry[24]. In this way, ACE2/Ang-(1-7)/Mas axis can counteract most of the deleterious effects of ACE/Ang Ⅱ/AT_1. It has been corroborated that acute intravenous infusion of Ang-(1-7) induces diuresis, natriuresis and renal vasodilatation[50].

Like to Ang-(1-7), there is another heptapeptide derived from Ang Ⅱ having the opposite effect to Ang Ⅱ, namely Ang-(2-8), also known as Ang Ⅲ. Ang Ⅱ can be hydrolyzed by aminopeptidase A, generating Ang Ⅲ[51] (Figure 1). Heretofore there has been no evidence of a specific receptor for Ang Ⅲ, and Ang Ⅲ normally binds to AT_1 with greater affinity than to the AT_2 receptor inducing natriuresis on rats[52,53]. Intrarenal Ang Ⅲ induces natriuresis *via* the AT_2 receptor in the proximal tubule by a cGMP-dependent mechanism[51].

Ang Ⅲ can be hydrolyzed by aminopeptidase N generating Ang-(3-8), also called Ang IV, which can be also generated directly from Ang Ⅱ by D-aminopeptidase[20,54]. The receptor for Ang IV, AT_4, was initially detected in the guinea pig hippocampus[15]. Protein purification and peptide sequencing showed that the AT_4 receptor is an insulin-regulated aminopeptidase[54]. AT_4 receptor is also found in the kidney, where this angiotensin-derived fragment can elicit many responses[55]. Aminopeptidases A and N are abundant in the kidney, especially in proximal nephron, and Ang Ⅳ is formed in the glomerulus[56,57]. Ang Ⅳ increases blood flow in the kidney and decreases in Na^+ transport in proximal tubules[55]. Ang Ⅳ induces Ca^{2+} mobilization in human proximal tubule cells[58] through the AT_1 receptor. In AT_4 knockout (-/-) mice, Ang Ⅳ mediated its renal vasoconstrictor effects through AT_{1a} receptors[59].

Ang Ⅱ can also be hydrolyzed to dipeptides that are biological active, and we have found an alternative pathway for Ang-(1-7) formation from Ang Ⅱ by carboxypeptidase N, and posterior generation of Ang-(3-4) with Ang-(1-5) and Ang-(1-4) as intermediate peptides[20] (Figure 1), using isolated basolateral membranes from sheep proximal tubules and different peptidase inhibitors. Ang-(3-4) could counteract inhibition of plasma membrane Ca^{2+}-ATPase promoted by nanomolar concentrations of Ang Ⅱ through conformational changes in the AT_2 receptor and the cAMP/PKA pathway[19,20,37].

Ang (3-4) is remarkably stable in human blood serum and has antihypertensive effects in spontaneously hypertensive rats (SHR)[60,61]. Dias *et al*[62] showed that oral administration of Ang-(3-4) inhibited Na^+-ATPase activity in membranes of SHR and blocked the stimulation of Na^+-ATPase induced by Ang Ⅱ in normotensive rats *via* the AT_2 receptor and the PKA signaling pathway. This effect leads to increased urinary Na^+ concentration, and simultaneous decrease in systolic arterial blood pressure in SHR, but not in normotensive rats[62].

The presence of another angiotensin derived fragment, known as Ang A (Ala-Arg-Val-Tyr-Ile-His-Pro-Phe), occurs in the plasma of healthy humans and in high levels in end-stage patients with renal failure[21,63]. Decarboxylation of Asp^1 of Ang Ⅱ, in the presence of mononuclear leukocytes leads to Ang A generation, which has higher affinity for AT_2 than Ang Ⅱ and the same affinity for the AT_1 receptor[21,63]. As the other Ang Ⅱ-derived peptides, Ang A exerts its effects on the kidney, inducing renal vasoconstrictor responses in normotensive and hypertensive rats, and also in genetically modified mice[64]. Ang A can also be hydrolyzed by ECA2 in rats, mice and humans generating the heptapeptide alamandine (Ala-Arg-Val-Tyr-Ile-His-Pro), a novel peptide of the RAS[22]. Alamandine has long-term antihypertensive effect in SHRs and antifibrotic effects in isoproterenol-treated rats *via* the Mas-related G-protein-coupled receptor, member D (MrgD), and independent of Mas and AT_2 receptor, the known vasodilator receptors of the RAS, since it is blocked by $D\text{-}Pro^7$-angiotensin-(1-7) and PD123319, but not by the Mas antagonist A-779[22]. Most members of Mas-related

G-protein-coupled receptor (Mrg), a novel class of RAS-related receptor, are orphan, with no identified endogenous ligand, but MrgD has been identified as a binding site for alamandine[22].

NEW MEMBERS OF RAS: RECEPTORS

Classically, there are 2 well described Ang Ⅱ receptors, AT_1 and AT_2 receptors. However, newer work on RAS and its effects shows that there are novel members of this system.

Besides the newly described Ang Ⅱ-derived peptides and their corresponding receptors, there are enzyme members of RAS whose actions depend on interaction with receptors. Nguyen *et al*[27] in 2002 cloned the PRR, which contains a specific binding site for renin and its inactive precursor, prorenin; this interaction stimulates their catalytic activity, increasing RAS activation. Prorenin has a "handle" region that binds to the receptor with a 3-4 fold higher affinity than renin and is important in enzymatic activation of prorenin[65].

After binding, renin and prorenin can also act as agonists to its receptor, generating effects in an Ang Ⅱ-independent manner. In the human kidney, PRR is expressed in glomerular mesangial cells, the subendothelium of renal arteries[27], in the distal nephron[66], collecting ducts, and mostly at the apical surface of intercalated cells, where, due to its high expression it stimulates cyclooxygenase-2 (COX-2)-derived prostaglandins to attenuate the anti-natriuretic and vasopressor effects of Ang Ⅱ[67].

However, activation of PRR in kidney is also associated with many pathological conditions. Activation of human PRR and MAPK through an Ang Ⅱ-independent mechanism contributes to the development of nephropathy in prorenin/renin transgenic rats overexpressing the human receptor[68]. PRR is important through the same signaling pathway in diabetic nephropathy by its activation of glomerular ERK. These studies used an AT_{1a} receptor-deficient mice[69] and db/db mice to show that the receptor-bound prorenin leads to the development of nephropathy in type 2 diabetes[70]. In HEK cells, renin and prorenin activate its receptor to promote fibrosis in an Ang II-independent manner[71].

Kohlstedt *et al*[25] in 2004 revealed another unexpected function of the RAS enzymes. Human ACE, usually known by its catalytic action on Ang I in generating Ang Ⅱ, could also function as an outside-in signal transduction molecule. Binding of ACE substrates or inhibitors to this enzyme can stimulate intracellular signaling pathways: ACE inhibitors (perindoprilat and ramiprilat), like the ACE substrate (bradykinin), could also increase COX-2 expression, ACE phosphorylation at Ser1270 and activation of JNK in endothelial cells[25]. The modulation of gene expression in endothelial cell by ACE inhibitors and JNK/c-Jun pathway requires ACE dimerization through the C domain of the enzyme[26]. This indicates that, although ACE is not a cell surface receptor, it is involved in cell functions. Nevertheless, whether ACE works only as a catalytic enzyme or as a signaling molecule in the kidney remains to be elucidated.

BREAKING PARADIGMS

A newly recognized view of RAS assumes that Ang Ⅱ acts beyond cell surface receptors, with endocrine and paracrine action of RAS. Ang Ⅱ also acts through intracellular receptors. Local RAS was first described within the kidney over 20 years ago[29-32], where the levels of Ang Ⅱ are much higher than in plasma[32,72]. Intrarenal Ang Ⅱ levels and local formation in the kidney have been reported by Navar and colleagues[11,32,73-76].

In addition to Ang Ⅱ synthesis in the kidney, there are other well-described mechanisms that play a critical role in high renal Ang Ⅱ levels, and these occur after Ang II endocytosis with the AT_1 receptor[77,78]. Since AT_1 receptors are expressed in different parts of the kidney, such as in the mesangial cells, afferent and efferent arterioles, glomerular podocytes, macula densa and both basolateral and luminal membranes of different nephron segments[79,80], intracellular Ang Ⅱ accumulation by coupled-receptor internalization is one of main sources of renal Ang Ⅱ accumulation.

In Ang Ⅱ-dependent hypertension several groups have shown that Ang Ⅱ can positively amplify it, leading to its high intrarenal levels. Zhuo *et al*[77] showed increased intracellular Ang Ⅱ levels in cortical endosomes, and Ang Ⅱ-infused hypertensive rats mediated by AT_1 receptors. Ang Ⅱ-infused rats through an osmotic minipump also had increased Ang Ⅱ levels in renal interstitial fluid, which is mediated by the AT_1 receptor[81]. Ang Ⅱ endocytosis with AT_1 receptor has been confirmed by the absence of renal Ang Ⅱ accumulation in AT_{1a} receptor-deficient mice ($Agtr1a^{-/-}$)[82,83]. Another possible pathway for increasing the intrarenal Ang Ⅱ level is due to endogenous Ang Ⅱ production, *via* markedly augmentation on angiotensinogen[11,84] and renin expression in collecting ducts[85,86], the secretion of renin and prorenin by these cells into the luminal fluid, leading to its increased urinary levels in Ang Ⅱ-infused hypertensive rats[87]. These results indicating a positive feedback by Ang Ⅱ in the kidney contradict the well-established view that Ang Ⅱ has a negative feedback mechanism in the expression and activity in the RAS, where high Ang Ⅱ levels suppress the release of renin in juxtaglomerular cells and Ang Ⅱ production in the kidney[88], demonstrating the complexity of the system.

Both Ang Ⅱ receptors (AT_1 and AT_2) are expressed in adult kidneys, although AT_2 receptor is less expressed than AT_1 receptor[79]. This intensely local synthesis of high renal levels of Ang Ⅱ, and the wide expression of Ang Ⅱ receptors within the kidney, provides evidence of the pivotal role of Ang Ⅱ in renal physiology, regulating water and solute reabsorption and renal hemodynamic processes that contribute to Na^+ balance and blood pressure regulation. AT_1 receptors in the kidney are responsible for the development of hypertension[89-91]. And AT_1 receptors within the kidney are necessary for cardiac

hypertrophy and hypertension[90,92].

Ang Ⅱ has many effects on different parts of the kidney. As in the systemic circulation, intrarenal Ang Ⅱ also is important in renal hemodynamics. Thereby, long-term treatment with Ang Ⅱ receptor blockers induced unusual proliferative changes in afferent arteriolar smooth muscle cells, narrowing arteriolar lumens and reducing glomerular pressure[93]. Administration of Ang Ⅱ through an osmotic minipump in hypertensive rats leads to marked suppression of Na^+ excretion as well as renal and medullary blood flow[94]. Peritubular capillary Ang Ⅱ infusion enhanced proximal tubular reabsorption and reduced single nephron glomerular filtration rate in rats[95].

Different targets and signaling pathways regulate Na^+ balance within the kidney; rats infused with Ang Ⅱ showed enhanced ENaC expression[96] and activation of the renal Na^+:Cl^- cotransporter[97,98]. *In vitro* studies using isolated basolateral membrane fractions from pig kidney have demonstrated that Ang II stimulates the renal proximal tubule Na^+-ATPase activity *via* PI-PLCβ/PKC pathway[99,100].

It is widely known that intracellular Ca^{2+} mobilization in proximal tubule cells leads to the activation of many Ca^{2+}-dependent intracellular signaling pathways, including those associated with Na^+ reabsorption[101]. Ang Ⅱ microperfusion techniques in rabbit superficial segment of proximal tubules *in vitro* regulated Na^+ reabsorption *via* PKC and intracellular Ca^{2+}[102]; low concentrations of Ang Ⅱ inhibited membrane Ca^{2+}-ATPase *via* AT_1/AT_2 receptors heterodimers and PKC in isolated fractions of basolateral membranes of proximal tubule, increasing cytosolic Ca^{2+} concentration in proximal tubule cells[37,103]. Luminal Ang Ⅱ stimulates AT_1/AT_2 receptors heterodimerization that increases sarco/endoplasmic reticulum Ca^{2+}-ATPase activity and promotes Ca^{2+} mobilization in proximal tubule cells[101].

The intracrine/intracellular system is new paradigm. Cells that express all the necessary components for synthesis can generate Ang Ⅱ internally[28,29]. Ang Ⅱ can be secreted and exert autocrine effects, or remain inside the cell and have its effects[6,35]. An alternative way for the intracellular source of Ang Ⅱ is the internalization of extracellular Ang Ⅱ after binding to the AT_1 surface receptor[82,83]. Not all internalized Ang Ⅱ-AT_1 complex is degraded in lysosomes, thereby increasing its concentration within the cell, and the AT_1 receptor may be relocated to other organelles, including the nucleus[101,104-108]. Indeed, subcellular localization of ^{125}I-labeled Ang Ⅱ in the pig kidney indicates that Ang Ⅱ generation is predominantly extracellular, followed by AT_1 receptor-mediated endocytosis leading to higher intracellular Ang Ⅱ levels[109]. In accord with this, internalization is seen to be important for AT_{1a} receptor function in polarized proximal tubule epithelial cells, where apical AT_{1a} receptor internalize before interaction with G proteins, which stimulates phospholipase C and cAMP to increase proximal tubule Na^+ reabsorption[110,111].

Within the kidney, cells from different segments can generate Ang Ⅱ or internalize Ang Ⅱ through the AT_1 receptor[109-111]. *In vitro* and *in vivo* studies showed that extracellular Ang Ⅱ accumulates within the kidney *via* AT_{1a} receptor-mediated endocytosis[82,83,107]. Although many have demonstrated different Ang Ⅱ intracellular effects, the precise role of intracellular Ang Ⅱ in nephron segments remains poorly understood. Renal intracellular Ang Ⅱ increases blood pressure and decreases 24 h urinary Na^+ excretion in rats and mices[89,105], suggesting that, like intrarenal Ang Ⅱ, intracellular Ang Ⅱ within the kidney also increases Na^+ reabsorption and blood pressure.

Endocytosis of Ang Ⅱ through the AT_1 receptor within proximal tubule cells occurs through 2 main pathways: the clathrin-dependent and the microtubule-associated pathway[106]. The canonical clathrin-dependent endocytosis pathway for Ang Ⅱ occurs in different cell types, such as vascular smooth muscle and human embryonic kidney (HEK-293) cells through the AT_1 receptor, c-Src and clathrin Adapter Protein 2[112]. In rabbit proximal tubule cells, the alternative microtubule-associated endocytic pathway rather than the clathrin-dependent pathway participates in the AT_1 receptor-mediated uptake of Ang Ⅱ[113].

Another alternative endocytic pathway for Ang Ⅱ internalization in proximal tubule cells has been described by Gonzalez-Villalobos *et al*[114], where anti-megalin antisera interferes with Ang Ⅱ binding in cell brush-border membrane vesicles extracted from mice, indicating that Ang Ⅱ internalization is a megalin-dependent process.

Angiotensin receptors are present in the intracellular organelles, including the sarco/endoplasmic reticulum, Golgi apparatus and the nucleus, indicating that Ang Ⅱ can have many intracellular effects, including modulation of gene expression[33-35]. Proximal tubule cells express angiotensinogen, renin, and ACE mRNAs, suggesting high levels of intracellular Ang Ⅱ[28,32,73]. Thus, microinjection of Ang Ⅱ directly in single rabbit proximal tubule cells increased intracellular Ca^{2+} mobilization through its intracellular AT_1 receptors and Ca^{2+} release from intracellular stores[115]. Ang Ⅱ induced transcriptional responses of mRNAs for MCP-1, NHE-3 and TGF-β1 stimulating the AT_{1a} receptor in freshly isolated intact rat renal cortical nuclei, indicating that internalized and/or intracellular Ang Ⅱ acts on nuclear receptors to mediate growth, proinflammatory responses and Na^+-retaining effects[108]. Furthermore, in isolated nuclei from kidney cortex from sheep in the absence of cytoplasm, all RAS components (angiotensinogen, ACE and renin) have been identified[116], showing that Ang Ⅱ can indeed be synthesized within the nucleus.

Another interesting role for intracellular Ang Ⅱ is encountered in pathological situations. It is thought that intracellular Ang Ⅱ levels could be altered in different diseases, such as diabetic nephropathy and cardiomyopathy, where hyperglycemia might induce intracellular Ang Ⅱ production. Indeed, a high glucose concentration induced an increase of ACE mRNA, synthesis and secretion of renin and Ang Ⅱ in an immortalized murine mesangial cell line[117-119]. Interestingly, an alternative pathway

Table 2 Most common drugs already established for clinical use and emerging drugs and new targets for the treatment of hypertension, cardiovascular and renal diseases

Target	Drug	Therapy	Clinical use
Renin	Aliskiren	HTN, RF	+
	Remikiren, enalkiren	HTN	+
ACE	Captopril, lisinopril, trandolapril	HTN, HF, LVD, DN	+
	Enalapril, enalaprilat, fosinopril, ramipril	HTN, HF	+
	Moexipril, quinapril, perindopril, benazepril	HTN	+
AT_1	Losartan, azilsartan, valsartan, ibesartan, candesartan, telmisartan, eprosartan, omesartan	HTN, HF	+
Mas	AVE 0991	HTN	-
	Ang-(1-7)-CyD	HF	-
ACE2	Xanthenone	HTN, RF, HF	-

+: Already used in clinic; –: Not used in clinic yet. ACE: Angiotensin converting enzyme; ACE2:Angiotensin converting enzyme 2; AT1: Angiotensintype 1 receptor; Mas: Ang-(1-7) Mas receptor; RF: Renal failure; HF: Heart failure; HTN: Hypertension; LVD: Left ventricular dysfunction; DN: Diabetic nephropathy.

was found for the synthesis of intracellular Ang Ⅱ in the presence of high glucose in vascular smooth muscle cells. Under normal glucose levels, Ang Ⅱ is generated by cathepsin D and ACE; however, Ang Ⅱ is obtained by cathepsin D and chymase action in the presence of high glucose[120,121].

NEW TARGETS FOR HYPERTENSION TREATMENT

RAS is important in the development of hypertension and cardiovascular diseases[1-4,90]; one of the most common treatments for these diseases is pharmacological inhibition of enzymes and blockade receptors of RAS[122]. Inhibition of renin, the enzyme that initiates RAS, presents a strategy for hypertension therapy (Table 2). Aliskiren is a more selective and potent inhibitor of human renin than other orally active renin inhibitors, remikiren and enalkiren[123]; it can block the generation of active renin in both normotensive and hypertensive human subjects[124]. Aliskiren is as effective as losartan, valsartan and ibesartan (AT_1 receptor blockers), atenolol (β blocker) and amlodipine (Ca^{2+} channel blocker), and has an antihypertensive effect comparable to other major classes of antihypertensive drugs[124,125]. Besides decreasing blood pressure, aliskiren is also renoprotective in diabetic and nondiabetic models of chronic kidney disease, preventing albuminuria in rats[126]. In humans, aliskiren significantly decreases blood pressure, and also the urinary albumin and creatinine ratio in 15 patients with type 2 diabetes mellitus[127].

ACE is another enzyme of the RAS that can be pharmacology inhibited so as to decrease hypertension (Table 2). A total of 17 small orally active ACE inhibitors have recently been synthesized for clinical use, all binding to the active site of the enzyme and interfering with ACE's ability to bind and cleave its substrates (Ang I and bradykinin, among others)[128,129]. Many ACE inhibitors were approved for hypertension treatment, heart failure and left ventricular dysfunction (*e.g.*, captopril, lisinopril, trandolapril), as also captopril for diabetic nephropathy[129].

Ang Ⅱ promotes cardiovascular disorders and hypertension *via* the AT_1 receptor, which can be blocked to treat these pathological conditions (Table 2). A total of 8 non-peptide angiotensin-receptor blockers (ARBs) orally active are used clinically for hypertension and cardiovascular diseases (namely losartan, azilsartan, valsartan, ibesartan, candesartan, telmisartan, eprosartan, omesartan), which are all well-tolerated[129,130]. Telmisartan seems more efficacious in decreasing blood pressure than the other ARBs[131,132].

Many patients with hypertension require combination regimens to achieve a significant decrease in blood pressure. In this case, the most commonly used drugs are ARBs and ACE inhibitors, Ca^{2+} channel blockers (CCB) and diuretics[130]. Long-term treatment triple therapy with olmesartan medoxomil (ARB), amlodipine besylate (CCB) and hydrochlorothiazide (diuretic) in 2112 hypertensive patients with moderate to severe hypertension resulted in 44.5%-79.8% of participants having a decreased the mean blood pressure from 168.6/100.7 mm Hg to 125.0-136.8/77.8-82.5 mmHg, reaching the blood pressure goal[133]. The same triple therapy also proved to be efficient in hypertensive Hispanic/Latin patients[134].

However, even with the successful results obtained by inhibiting the enzymes and receptors of the RAS, many patients do not respond as expected, and cardiovascular disease risks have not decreased to those in normotensive people. Due to the high death rates by heart diseases in the world, which are higher than from many cancers[135], it is important to devise new strategies for the treatment of cardiovascular diseases and hypertension.

Because of the discovery of new components in the RAS that have herein been described, novel Ang Ⅱ-derived peptides have emerged as excellent target for heart diseases. Since the ACE2/Ang-(1-7)/Mas axis has an opposite and protective effect from the deleterious ACE/Ang Ⅱ/AT_1 axis, it is now the main target for these drugs[14]. Besides inhibiting ACE activity and blocking AT_1 receptors responsible for the inhibition of ACE/Ang Ⅱ/AT_1 axis, activation of the ACE2/Ang-(1-7)/Mas axis is a promising alternative means for the treatment of the heart diseases. Nevertheless, this new strategy presents certain problems. First, as a peptide, Ang-(1-7) is proteolytically degraded in the gastrointestinal tract[18]; and second, Ang-(1-7) has a short half-life, complicating its use as an oral pharmacotherapy for hypertension and cardiovascular disease.

The difficulty was overcome after the synthesis of the first nonpeptide compound able to mimic Ang-(1-7) and bind selectively to the Mas receptor[136], the AVE 0991 5-formyl-4-methoxy-2-phenyl-1-{[4-(2-ethyl-ami-

nocarbonylsulfonamido-5-isobutyl-3-thienyl)-phenyl]-methyl}-imidazole (Table 2)[137]. Although this molecule is an antihypertensive candidate because it stimulates NO release in endothelial cells[137], promotes vasorelaxation in mouse and rat aortic rings[138], and attenuates hypertension in SHR[139], clinical trials are needed to see its effects in humans.

Another important achievement has been the inclusion of the heptapeptide in hydroxypropyl-β-cyclodextrin [Ang-(1-7)-CyD], avoiding its proteolytic degradation in the gastrointestinal tract and permitting its oral administration (Table 2)[18]. Cyclodextrins are amphiphilic oligosaccharides that increase drug stability and absorption[140]; after oral administration, they are split up into small saccharides in the colon, leaving Ang-(1-7) to be absorbed[18]. Chronic oral administration of Ang-(1-7)-CyD in isoproterenol-treated rats increases plasma Ang-(1-7) levels, with attenuation of myocardial infarction associated with cardioprotective effects[141].

Another option for the treatment of the deleterious effects of Ang Ⅱ is activation of ACE2, which, besides increasing Ang Ⅱ degradation, enhances Ang-(1-7) production; ACE2 activators are an alternative source for controlling hypertension (Table 2). Acute intravenous administration of xanthenone (XNT), which interact with ACE2 in specific sites, promotes conformational changes and increases ACE2 activity. Consequently, it decreases blood pressure, improves cardiac function and decreases renal fibrosis in SHR[142]. It also has antihypertensive effects in rats with pulmonary hypertension[143].

These results together suggest that, besides inhibition of renin and ACE, associated or not with the blocking of AT_1 receptor, activation of the ACE2/Ang-(1-7)/Mas axis and its protective effects is emerging as an excellent alternative therapy for the treatment of hypertension and cardiovascular diseases.

CONCLUSION

The data presented herein show that RAS has passed from being simply an endocrine system to one with paracrine, autocrine and intracrine functions, increasing Ang Ⅱ concentration in different tissues including the kidney. After years of research, the RAS - previously seen as a simple system with only 2 receptors (AT_1 and AT_2), and one active peptide (Ang Ⅱ), turns out to be a complex system, with many new members continuing to be described. In addition to (ACE)/Ang Ⅱ/AT_1 and AT_2 axis, other signaling pathways in the RAS, such as ACE2/angiotensin-(1-7)/Mas and Ang IV/AT_4, and other active peptide of the RAS, with physiological relevance as Ang Ⅲ, Ang-(3-4), Ang A and alamandine, are now widely recognized. These newly discovered fragments derived from Ang Ⅱ can act on the same classic Ang Ⅱ receptors, AT_1 and AT_2, or on specific receptors (Mas and AT_4) having the same or the opposite effects of Ang Ⅱ depending on the triggered signaling pathway, in the kidney and other tissues, with many roles seen in physiological and physiopathological conditions. The discovery of renin and prorenin as agonists of PRR receptor, stimulating intracellular pathways and having effects on different cells types in an Ang Ⅱ-independent manner, raised another axis for this system, namely the prorenin/PRR/MAPK ERK1/2 axis.

Finally, activation of the new ACE2/Ang-(1-7)/Mas axis with opposite and protective effects, compared with ACE/Ang Ⅱ/AT_1 axis, with different drugs such as AVE 0991, the nonpeptide compound mimicking Ang-(1-7) effects, the Ang-(1-7)-CyD, and the XNT, the activator of ACE2 activity, now leading to improved and greater fall in blood pressure creates new possibilities for patients who do not respond as expected to conventional antihypertensive drugs.

A thorough understanding of RAS and all the new possibilities described on this review will certainly contribute to the development of pharmacological approaches, discovery of new drugs and alternative treatments for hypertension, cardiovascular and kidney diseases.

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