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The Intratubular and Intracrine Renin-Angiotensin System in the Proximal Tubules of the Kidney and Its Roles in Angiotensin II-Induced Hypertension

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Abstract

The kidney plays a fundamental role in the physiological regulation of basal blood pressure and the development of hypertension. Although the mechanisms underlying hypertension are very complex, the renin-angiotensin system (RAS) in the kidney, especially intratubular and intracellular RAS, undoubtedly plays a critical role in maintaining basal blood pressure homeostasis and the development of angiotensin II (ANG II)-dependent hypertension. In the proximal tubules, ANG II activates two G protein-coupled receptors, AT₁ and AT₂, to exert powerful effects to regulate proximal tubular sodium and fluid reabsorption by activating cell surface as well as intracellular AT₁ receptors. Increased production and actions of ANG II in the proximal tubules may cause salt and fluid retention, impair the pressure-natriuresis response, and consequently increase blood pressure in hypertension. The objectives of this chapter are to critically review and discuss our current understanding of intratubular and intracellular RAS in the kidney, and their contributions to basal blood pressure homeostasis and the development of ANG II-dependent hypertension. The new knowledge will likely help uncover novel renal mechanisms of hypertension, and develop kidney- or proximal tubule-specific strategies or drugs to prevent and treat hypertension in humans.

Keywords: angiotensin II, blood pressure, hypertension, kidney, proximal tubule

1. Introduction

According to the most recent American College of Cardiology (ACC)/American Heart Association (AHA) reports, 46% of U.S. adults now develop

hypertension and take antihypertensive drugs in their lifetime [1, 2]. Prevention and treatment of hypertension and its target organ complications cost several hundreds of billion dollars a year to the U.S. economy [3–6]. Although the causes of hypertension are multifactorial, the activation of circulating (endocrine), tissue (paracrine) and intracellular (intracrine) RAS via angiotensin II (ANG II) remains one of most important contributing mechanisms [1–7]. Indeed, angiotensin-converting enzyme (ACE) inhibitors, ANG II receptor blockers (ARBs), and renin inhibitors, which block the RAS at the enzymatic or receptor levels, are widely used to treat hypertension, reduce cardiovascular and renal disease risks, and prevent target organ damage [1–7]. However, clinical trials have shown that not all RAS-targeting drugs have the same efficacy of blocking the actions of ANG II and afford the same degree of cardiovascular, blood pressure and renal protection [1–6]. Some patients continue to develop cardiovascular and renal complications despite being treated with one or more than two of these blockers [7, 8]. The underlying mechanisms responsible for these clinical observations are not well understood. One of the possibilities may be that not all ARBs have the same ability to enter the cells to block intracellular ANG II. Some, but not all, ARB(s) such as telmisartan and losartan may exert therapeutic effects beyond the classic ARBs' properties.

There is accumulating evidence that ANG II acts not only as an endocrine or paracrine hormone activating cell surface ANG II receptors, but also as an intracellular or intracrine peptide activating intracellular ANG II receptors, though the precise roles of the latter remain largely unknown [9–11]. Indeed, in addition to activating cell surface ANG II receptors, circulating and paracrine ANG II can readily enter the cells via AT₁ receptor-mediated endocytosis. The ANG II/AT₁ receptor complex internalized into endosomes may continue to transmit signals from endosomes or be translocated to the nucleus to induce long-lasting genomic effects [12, 13]. Recently, we and others have used innovative in vitro cell expression system [14–16], in vivo adenoviral gene transfer of an intracellular ANG II protein selectively in proximal tubule cells of the rat and mouse kidneys [17, 18], or genetically modified mouse models to investigate the physiological roles and mechanisms of actions of intratubular and intracellular ANG II in the proximal tubules of the kidney, with a focus on basal blood pressure homeostasis and ANG II-induced hypertension [19, 20]. Specifically, we have determined whether intracellular ANG II is derived from AT₁ (AT_{1a}) receptor-mediated uptake by the proximal tubule cells, and whether proximal tubule-selective expression of an intracellular ANG II fusion protein in the rat and mouse kidney increases the expression and activity of NHE3, promotes proximal tubular sodium and fluid reabsorption, and therefore elevates arterial blood pressure [17–23]. These new studies have generated new knowledge to improve, and provided new insights into our understanding of renal mechanisms of hypertension involving both endocrine, paracrine and intracellular ANG II, and perhaps aid the development of new classes of multifunctional drugs to treat ANG II-induced hypertension and its target organ damage by blocking not only extracellular but also intracellular and nuclear actions of ANG II. Accordingly, the objectives of this chapter are to critically review, analyze, and discuss the recent developments and progresses in the studies of novel renal mechanisms of hypertension with a focus on the roles of intratubular and intracellular ANG II in the proximal tubules of the kidney.

2. Localization of intratubular and intracellular RAS and its receptors in the proximal tubules of the kidney

2.1 Angiotensinogen

Angiotensinogen, a ~60 kDa α_2 globulin in the serpin family, is the primary, if not the only, substrate for the RAS super family. It is well-recognized that angiotensinogen is primarily expressed or produced in the liver under physiological conditions. Human angiotensinogen consists of 452 amino acids, whereas rodent's angiotensinogen may vary in its molecular size slightly from human form [24–27]. Angiotensinogen, not active in itself, is released from the liver and cleaved in the circulation by the rate-limiting enzyme renin to form the still inactive decapeptide ANG I. This is followed by the conversion of inactive ANG I to the active and potent peptide ANG II, initiating important biological and physiological actions. A second enzyme called angiotensin I-converting enzyme (ACE) acts to convert ANG I to form the biologically active ANG II, initiating an important biochemical and physiological angiotensinogen/renin/ANG I/ACE/ANG II cascade (see below section on ACE). Accordingly, the recognized and primary role of angiotensinogen is to serve as a key substrate to the production of ANG II in the circulation and tissues.

In the kidney, angiotensinogen mRNAs and proteins have been localized in the kidney, primarily in the proximal tubules [28–30]. Immunohistochemistry, immunoelectron microscopy and non-isotopic hybridization histochemistry have demonstrated the localization of angiotensinogen mRNAs and proteins in the proximal convoluted and straight tubules of the cortex, with glomerular mesangial cells and medullary vascular bundles also being immunopositive in neonatal rat kidney [29, 30]. In the adult rat kidney, however, angiotensinogen mRNA expression was localized primarily in the proximal convoluted tubules, whereas electron-microscopic immunohistochemistry localized angiotensinogen immunostaining in the apical membrane of proximal convoluted tubules [29, 30]. By contrast, few if any angiotensinogen mRNAs and proteins are localized in the glomeruli, mesangial cells, or distal nephrons under physiological conditions [29, 30].

Although most of angiotensinogen in the circulation is derived from the liver, there is evidence showing that angiotensinogen is also expressed and produced in the kidney [28, 31–33]. Kobori et al. have consistently shown that angiotensinogen mRNA expression and proteins are increased in the proximal tubules of the kidney in ANG II-infused rats [28, 31–33]. However, Matsusaka et al. have demonstrated that there were no significant differences in the levels of angiotensinogen and ANG II proteins in the kidney between wildtype mice and mice with kidney-specific angiotensinogen knockout [34]. It was further found that angiotensinogen protein and ANG II levels in the kidney were nearly abolished in mice with liver-specific knockout of angiotensinogen [34]. The studies of Kobori et al. and Matsusaka et al. suggests that liver-derived angiotensinogen is the primary source of renal angiotensinogen protein and ANG II under physiological conditions, but during the ANG II-induced hypertension, angiotensinogen mRNAs and proteins are also expressed in the kidney proximal tubules.

2.2 Renin

Renin, the rate-limiting enzyme first discovered to increase blood pressure in rabbits by Tigerstedt and Bergman in 1898 [35], is an aspartyl proteinase or angiotensinogenase. Renin plays the most critical role in the initiation of the

angiotensinogen/renin/ACE/ANG II/AT₁ receptor activation in the cardiovascular, kidney, and other major target tissues. Human renin precursor consists of 406 amino acids with a pre- and a pro-segment of 20 and 46 amino acids, respectively [36]. Mature human renin contains 340 amino acids and a molecular wt. of 37 kDa [36]. Renin, renin activity, and its mRNA have been localized in the kidney, sub-maxillary glands, blood vessels, heart, adrenal glands, and brain tissues by enzymatic assays, immunohistochemistry, in situ hybridization histochemistry etc. [37–39]. In the kidney, active renin is primarily localized in the juxtaglomerular apparatus (JGAs) in the afferent arterioles of the kidney under both physiological and diseased conditions [40–42]. For example, light and electron microscopic immunocytochemistry with an antibody to purified human renal renin localized renin in the secretion granules of the epithelioid cells of the afferent arteriole of the JGAs, in renal artery stenosis, or in Bartter's syndrome [36, 37]. In the dog kidney, we have used an in vitro autoradiographic approach to localize active renin using radiolabeled renin inhibitors [40–42]. High resolution light microscopic autoradiography specifically localized active renin to the vascular pole of the glomeruli, or the JGAs (**Figure 1**) [40–42].

In the proximal tubule of the kidney, renin mRNAs have been reported [43, 44]. Renin activity and mRNAs were detectable in cultured rabbit proximal tubule cells [45], in isolated proximal convoluted and straight tubules, but not in outer medullary collecting ducts [44]. Tang et al. reported that all major components of the RAS, including angiotensinogen, angiotensin converting enzyme, and renin, were expressed in an immortalized rat proximal tubule cell line [45]. However, there is also evidence that renin localized in the proximal tubules may be due to the uptake of circulating renin after filtration [46, 47]. Taugner et al. demonstrated that the reabsorptive pinocytosis of the filtered renin was the primary source of tubular renin in the kidney [46], whereas Iwao et al. used light and electron microscopic autoradiography to localize ¹²⁵I-labeled renin accumulated in the apical membranes of the proximal convoluted tubules [47]. Taken together, these studies strongly support the concept that in addition to local biosynthesis and expression, circulating or interstitial renin may be taken up by the proximal convoluted tubules in the kidney.

2.3 Angiotensin I-converting enzyme (ACE)

The 2nd key enzyme for the activation of the RAS is ACE, a dipeptidyl carboxypeptidase I, kininase II and EC 3.4.15.1 [48]. Corvol's group first molecularly cloned ACE from human vascular endothelial cells [48], whereas Bernstein's

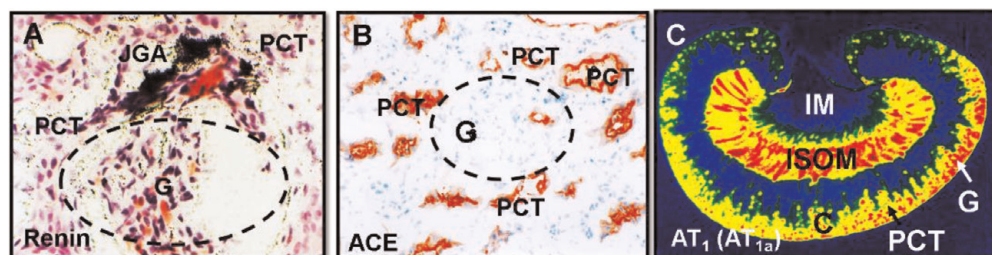


Figure 1. Intrarenal localization of renin in the juxtaglomerular apparatus (A: JGA), angiotensin-converting enzyme (B: ACE), and angiotensin II AT₁ receptors in the kidney (C: AT₁ or AT_{1a}) using quantitative in vitro autoradiography. C, renal cortex; G, glomerulus; IM, inner medulla; ISOM, inner stripe of the outer medulla; PCT, proximal convoluted tubule.

group cloned ACE from the mouse kidney in 1988, respectively [49]. ACE in humans consists of 1306 residues with a signal peptide of 29 amino acids [48], whereas ACE in mice contains 1278 amino acids [49]. Approximately 80% of the amino acid sequences are similar between human and mouse ACE. There are two ACE isozymes, one somatic isozyme in the lung, vascular endothelial cells, renal epithelial cells, and testicular Leydig cells, and the other germinal isoenzyme solely in sperm [50–52]. The key actions of ACE are to convert the biologically inactive ANG I to the active peptide ANG II, and to degrade the vasoactive peptide bradykinin. Thus, ACE is most critical for the generation of ANG II in the circulation and tissues.

Abundant ACE is expressed and localized in the kidney, especially in the proximal tubules and glomerular and vascular endothelial cells of intrarenal blood vessels [53–57]. We and others have localized ACE proteins and its mRNA expression in the kidney using quantitative in vitro autoradiography, immunohistochemistry, and in situ hybridization histochemistry (**Figure 1**). For example, the Mendelsohn's group first localized ACE in the rat kidney using quantitative in vitro autoradiography with the radiolabeled ACE inhibitor lisinopril, ¹²⁵I-351A [53]. ACE was localized primarily to the inner cortex, corresponding to the proximal tubules and blood vessels [53]. We found that infusion of ANG II for 2 weeks significantly increased, rather than downregulated, ACE in the proximal tubules of the rat kidney [54]. At higher resolutions, Brunevaly et al. and others showed ACE primarily in the microvilli and brush borders of the proximal tubules in the human kidney [55–57]. In the vasculature, ACE was localized to the vascular endothelial cells especially in the peritubular capillaries, but not glomerular capillaries of the kidney [53–57]. ACE was also localized inside the renal vascular endothelial and proximal tubular cell in endoplasmic reticulum, endosomes, and nuclear envelope, suggesting the presence of intracellular and/or nuclear ACE [53–57]. However, only very low levels of ACE were detected in the inner medulla.

2.4 Angiotensin II (ANG II)

Angiotensin II (ANG II) is undoubtedly the most powerful peptide in the RAS super family, playing a key role in regulating renal blood flow, glomerular filtration, and proximal tubular reabsorption of sodium and fluid, contributing to normal blood pressure and body salt and fluid homeostasis [58–64]. It is well-recognized that the levels of ANG II in the kidney, especially in the proximal tubules, are higher than in the plasma or other tissues. Indeed, local expression and biosynthesis of angiotensinogen, renin, and ACE in the proximal tubules of the kidney significantly contribute to high levels of ANG II levels in the kidneys under physiological conditions [64–68]. Furthermore, ANG II levels are further increased in the kidney of animal models of ANG II-dependent hypertension, even though the circulating and JGA renin and ACE are suppressed [67–73]. This is likely due to the fact that the proximal tubules express all major components of the RAS necessary for the formation of ANG II [38, 47, 54, 59, 67, 74, 75], the proximal tubules have a greater capacity to take up circulating ANG II via AT₁ (AT_{1a}) receptor-mediated mechanisms [14, 19, 20, 67], and to augmentation of the expression or generation of angiotensinogen, ACE and ANG II in ANG II-induced hypertension [54, 67, 70, 73]. Finally, ANG II is not only generated in the intratubular fluid compartment, but also localized in intracellular organelles, such as endosomes, mitochondria, and nuclei [15, 67, 71, 74, 75], where it serves as an important intracellular or intracrine peptide.

2.5 AT₁ and AT₂ receptors

It is now well-accepted that ANG II binds to and activates two different classes of G protein-coupled receptors (GPCRs) to induce well-recognized cardiovascular, renal and blood pressure responses, following the successful development of nonpeptide ANG II type 1 and type 2 receptor antagonists [76–78]. Molecular cloning of AT₁ and AT₂ receptors and studies of animal models with genetically knockout of these receptors further confirms their pharmacological characterization. Murphy et al. [79] and Sasaki et al. [80] successfully cloned the AT₁ receptor in 1991, showing that the AT₁ receptor shares the seven-transmembrane-region motif of the GPCR superfamily. AT₁ receptors mediate the well-known actions of ANG II on vasoconstriction, cardiac hypertrophy, hypertensive, renal salt retention, as well as aldosterone biosynthesis [76–78, 81]. The AT₂ receptor was cloned by Mukoyama et al. [82], Nakajima et al. [83], and Kambayashi et al. [84], respectively. The AT₂ receptor was found to have 34% of the identical sequence to the AT₁ receptor, sharing a seven-transmembrane domain topology of GPCRs [82–84]. However, the roles and signal transduction pathways for the AT₂ receptor remain incompletely understood.

In the kidney, the AT₁ receptor is widely expressed and localized in different structures or cell types, most prominent in three anatomical regions, that is, the glomerulus, proximal tubules, and the inner stripe of the outer medulla, corresponding the vasa recta blood vessels and renomedullary interstitial cells (**Figure 1**) [85–87]. We and others have consistently localized the AT₁ receptor in the rodent and human kidneys using quantitative in vitro and in vivo autoradiography, with high levels of these receptors in the glomerulus, proximal tubules, and renomedullary interstitial cells (**Figure 1**) [85–87]. Other anatomical regions or renal structures may express low levels of AT₁ receptor expression, detectable with RT-PCR or immunohistochemistry. AT₁ receptors have also been localized in intracellular organelles, for example, endosomes, mitochondria, and nuclei in the proximal tubule cells, suggesting an important intracellular roles [67, 74, 88–90]. By contrast, the levels of AT₂ receptor expression in the kidney are species-related or closely associated with the kidney development. Indeed, high levels of AT₂ receptors are expressed extensively in the developing fetal and neonatal tissues, but most of them disappear before reaching the adulthood [87]. Nevertheless, the expression of AT₂ receptors appears to persist in the adrenal medulla, proximal tubules, and the adventitia of human kidney blood vessels, suggesting potential roles for these receptors in these target tissues [85–87, 91–93].

3. Intratubular and intracellular ANG II: the long-term genomic effects induced by endocrine, paracrine and intracellular ANG II

In contrast to the classic dogma that ANG II only binds to and activates cell surface GPCRs to initiate downstream signaling responses, ANG II can also bind and activate intracellular GPCRs to induce long-term genomic effects. The RAS includes an extracellular system and an intracellular system. ANG II acts as the principle effector of both extracellular and intracellular RAS. Extracellular ANG II includes circulating (endocrine) and paracrine ANG II, which plays the classical roles of the RAS through activation of cell surface GPCRs [76–78, 81, 94, 95]. Intracellular ANG II includes intracellularly formed ANG II (intracrine) and ANG II internalized through AT₁ (AT_{1a}) receptor-mediated endocytosis [96–101]. The roles of circulating and paracrine ANG II and its GPCR-mediated signaling mechanisms via cell surface receptors have been extensively investigated. By contrast, the roles of intracellular ANG II and its mechanisms of actions remain poorly understood.

This disparity in our understanding extracellular versus intracellular ANG II has led many to assume that ANG II only activates cell surface receptors to induce all of its biological and physiological responses, and that all ARBs would only block cell surface receptors to produce the same beneficial effects. Thus, an intracellular ANG II system is thought to be unnecessary in the regulation of cardiovascular, blood pressure, and renal physiology and diseases.

However, recent studies strongly suggest that these views may be revised for a number of reasons [96–101]. First, it is well-recognized that extracellular ANG II is continuously internalized with its receptors after it activates cell surface receptors. This has long been interpreted only as required for the desensitization of cell surface receptors to repetitive stimulation by extracellular ANG II by moving the ANG II/AT₁ complex into the lysosomal pathway for degradation. There is evidence, however, that the activated agonist/receptor complex internalized into the endosomes may continue to transmit ras/mitogen-activated protein kinase (MAPK) signaling [12, 13]. Ras and MAPK signaling for AT_{1a}, vasopressin V₂, and β 2 adrenergic receptors (β 2AR) have been reported in endosomal membranes [12, 13, 15, 16], the endoplasmic reticulum, the Golgi or the nucleus independent of cell surface receptor-initiated signaling [81, 88, 89, 102]. Second, ANG II exerts long-lasting genomic or transcriptional effects, which may be independent from the well-recognized effects induced by activation of cell surface receptors [97–99, 102, 103]. ANG II induces the expression or transcription of many growth factors and proliferative cytokines including nuclear factor- κ B (NF- κ B) [104–107], monocyte chemoattractant protein-1 (MCP-1) [106, 108], TNF- α [107], and TGF- β 1 [102, 109, 110]. While hemodynamic responses to ANG II often occur in seconds or minutes, cellular growth, mitogenic, proliferative and fibrotic responses to ANG II may last from hours to weeks and months. Since the cell surface AT₁ (AT_{1a}) receptors may be desensitized in response to sustained exposure to endocrine and paracrine ANG II, the long-term genomic effects of ANG II, as observed in cardiovascular, hypertensive, and renal diseases, are at least in part mediated by intracellular ANG II system. Third, not all ARBs, ACE or renin inhibitors are created equal to block both extracellular and intracellular ANG II systems. ARBs may differ in their lipophilic ability to enter the cells to block intracellular AT₁ receptors [111–113]. Indeed, ARBs show different effects on uric acid metabolism, cell proliferation, oxidative stress, nitric oxide production and PPAR- γ activity [111–113]. We and others have shown that losartan internalized with AT_{1a} and AT_{1b} receptors, albeit at a slower rate than ANG II [19, 20, 67, 103, 114], and to attenuate ANG II-induced intracellular and nuclear effects [15, 88, 89, 102, 103]. Moreover, telmisartan not only blocks AT₁ receptors, but also acts as a partial activator of liver-specific peroxisome proliferator-activated receptor γ (PPAR- γ) [111, 115–157]. Finally, some clinical studies have shown that even treated with renin inhibitors, ACE inhibitors or ARBs, there are some patients who still progress to hypertension and suffer from cardiovascular and renal complications [111, 115–117]. These data suggest that additional mechanisms should be involved and studied accordingly. Thus, the new challenges to the field are to study whether and how intracellular ANG II may contribute to these mechanisms and design multifunctional drugs to block both extracellular and intracellular ANG II-induced effects.

4. Intratubular and intracellular ANG II: AT_{1a} receptor-mediated uptake of circulating and paracrine ANG II in the proximal tubules

We and others have investigated whether circulating and local paracrine ANG II is taken up by the proximal tubules of the kidney via AT₁ (AT_{1a}) receptor-mediated endocytosis [19, 20, 118–121], and whether internalized ANG II and AT_{1a} receptors

are co-localized in the endosomal compartment and nucleus (**Figure 2**) [67, 74, 88, 89]. Our studies demonstrated that global deletion of AT_{1a} receptors blocked the uptake of unlabeled Val⁵-ANG II [19] or [¹²⁵I]Val⁵-ANG II in the kidney of AT_{1a}-KO mice [20]. However, these studies focused only on the entire kidney, and what nephron segments involved in taking up unlabeled Val⁵-ANG II or [¹²⁵I]Val⁵-ANG II could not be determined using these approaches [19, 20]. We further used cultured proximal tubules cells to test whether proximal tubule cells take up extra-cellular ANG II and the mechanisms involved (**Figure 2**) [14, 100, 122–126]. The advantages of using these cells for the proposed studies are that ANG II receptors are abundantly expressed and localized in both apical (AP) and basolateral (BL) membranes [127–131]. However, it has not been determined whether ANG II receptors in AP or BL membranes mediate ANG II uptake in the proximal tubules. In a previous study using a porcine proximal tubule cell line expressing a rabbit AT₁ receptor, AT₁-mediated uptake of [¹²⁵I]-ANG II was found to be significantly different between AP and BL membranes [130]. AT₁-mediated uptake of [¹²⁵I]-ANG II was more robust and efficient in AP membranes than in BL membranes [130]. Conversely, ANG II-induced AT₁ receptor internalization was reportedly much faster in BL membranes than in AP membranes of OK cells [131]. Thus these differences in AT₁-mediated uptake of [¹²⁵I]-ANG II or ANG II-induced AT₁ receptor endocytosis or internalization may underscore the differences in the cell types used or experimental conditions.

In addition to AT₁ (AT_{1a}) receptors, other factors may also regulate the uptake of extracellular ANG II by proximal tubule cells. AP membranes of proximal tubule cells express abundant endocytic receptor megalin, which plays a crucial role in mediating the uptake of low molecular weight (LMW) proteins in proximal tubule cells [132–136]. Deletion of megalin in mice led to the development of LMW proteinuria [135]. Interestingly, megalin also binds and internalizes ANG II in immortalized yolk sac cells (BN-16 cells) [136]. We have demonstrated that siRNA knockdown of megalin expression or caveolin 1 in proximal tubule cells significantly attenuated ANG II uptake by proximal tubule cells [122, 123]. However, the

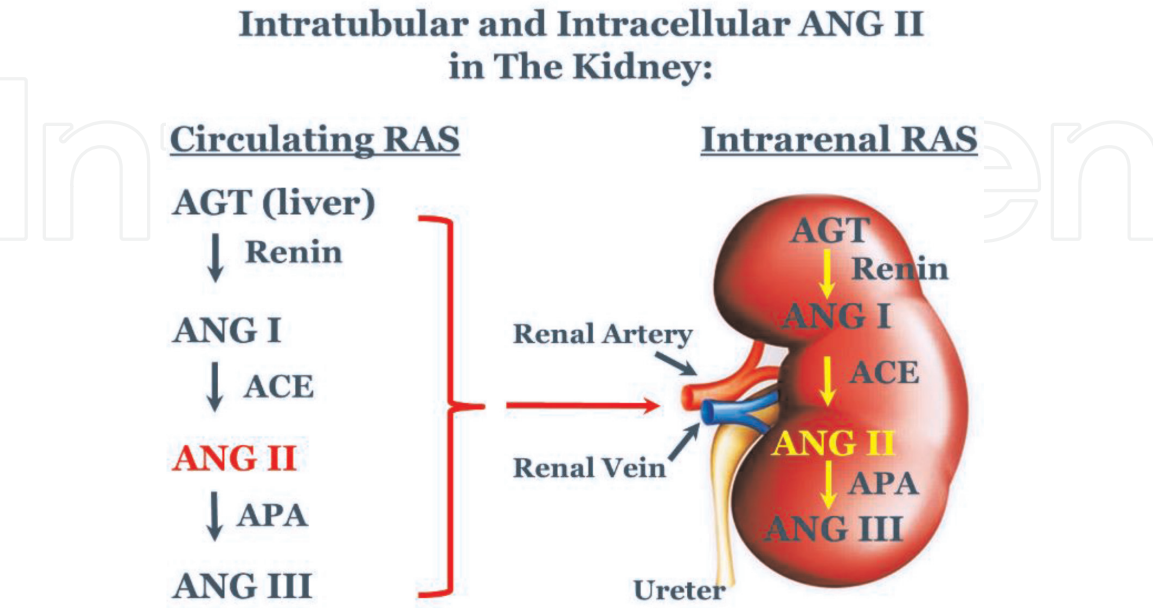


Figure 2. All major components of the circulating RAS, including angiotensinogen (AGT), renin, angiotensin I (ANG I), and ANG II, may be filtered by the kidney glomerulus and taken up by the proximal tubules. Alternatively, all major components of the RAS may be expressed and localized in the proximal tubules of the kidney. ACE, angiotensin-converting enzyme and APA, aminopeptidase A.

extent to which megalin- and caveolin 1-mediate ANG II uptake in proximal tubule cells is significantly smaller than that mediated by AT₁ (AT_{1a}) receptor-dependent mechanism [19, 20, 122, 123].

5. Intratubular and intracellular ANG II: canonical versus noncanonical endocytic pathways in mediating ANG II uptake in the proximal tubules

We have mechanistically investigated that AT₁ (AT_{1a}) receptor-mediate the uptake of extracellular ANG II by proximal tubule cells in vitro and circulating ANG II in vivo [19, 20, 122–126]. It has been previously shown that in vascular smooth muscle cells (VSMCs), cardiomyocytes, and COS-7 cells, β 2 adrenergic receptors, AT_{1a}, epidermal growth factor receptors, and insulin receptors are internalized via the canonical clathrin-dependent pathway [137–144]. Clathrin-coated pits play an important role in invaginating and pinching off the plasma membranes to form coated vesicles and targeted to endosomes [138, 140, 142]. GPCR kinases (GRKs), small GTP-binding proteins, such as Rab5, and β -arrestins are reportedly involved in clathrin-dependent AT_{1a} endocytosis [145, 146]. However, dominant-negatives, siRNAs or knockout targeting dynamin, GRKs or β -arrestins have little effects on AT_{1a} receptor endocytosis in some studies, suggesting that alternative (non-canonical) pathways may also be involved in AT_{1a} receptor endocytosis [137–146].

There is evidence to suggest that tyrosine phosphatases may be involved in ANG II-induced AT₁ receptor endocytosis in AP and BL membranes, since the endocytic response was inhibited by the tyrosine phosphatases inhibitor, phenylarsine oxide (PAO), rather than by pertussis toxin [147–151]. Colchicine, an inhibitor of cytoskeleton microtubules [148], also appeared to inhibit AT₁ receptor-mediated ANG II uptake and its effects in rat proximal tubule cells [150, 151]. The role of clathrin-coated pits in mediating AT₁ receptor-mediated ANG II uptake was also investigated, but we found that deletion of clathrin-coated pits with sucrose or specific siRNAs to knock down clathrin light (LC) or high chain subunits (HC) failed to alter AT₁-mediated uptake of Val⁵-ANG II [151]. However, AT₁-mediated uptake of Val⁵-ANG II was significantly inhibited by colchicine or siRNA knocking down of microtubule-associated proteins, MAP-1A or MAP-1B, in proximal tubule cells [151]. Our studies therefore support the scientific premise that the noncanonical microtubule-dependent endocytic pathway may be involved in mediating the AT₁-mediated uptake of ANG II in proximal tubule cells.

How ANG II and AT₁ receptors are internalized into the endosomal compartments and transported to other organelles or the nucleus in proximal tubule cells remains incompletely understood. Intravenous infusion of ¹²⁵I-labeled ANG II was previously detected in the nuclei of rat vascular smooth muscle cells (VSMCs) and cardiac myocytes [152] or the Golgi of adrenal cells [153]. Cook et al. showed that ANG II and its AT_{1a} receptor were translocated to the nuclei of hepatocytes and VSMCs [154]. In AT_{1a} receptor-expressing HEK 293 cells, internalized AT_{1a} receptors were detected in perinuclear areas as well as in the nuclei [155, 156]. In supporting the above-mentioned studies, we also reported high levels of internalized FITC-labeled ANG II in perinuclear areas and the nucleus, which was inhibited by colchicine and siRNA knockdown of MAP-1A [14, 122, 123, 151]. Taken together, our results strongly suggest that the microtubule-dependent pathway may play an important role in mediating the nuclear translocation of internalized ANG II/AT₁ receptor complex in proximal tubule cells. Indeed, a nuclear localization sequence (NLS, KKFKKY, aa307-312) has been identified

within the AT_{1a} receptor, which may mediate nuclear trafficking and activation of AT_{1a} receptors by ANG II [155, 156].

6. Intratubular and intracellular ANG II: intracellular versus extracellular effects and signaling mechanisms in the proximal tubules

In the proximal tubules of the kidney, extracellular ANG II has been reported to stimulate the expression of Na⁺/H⁺ exchanger 3 (NHE3) [14, 16, 102, 125], AP insertion of NHE3 [157], Na⁺/H⁺ exchanger activity [158–161], or NHE3-induced ²²Na⁺ uptake in cultured or isolated proximal tubule cells [162, 163]. The signaling mechanisms by which extracellular ANG II increases the expression and activity of NHE3 in proximal tubule cells have been well studied and documented [164–169]. The most well-described signal mechanism is that ANG II activates cell surface receptor-coupled G proteins, with subsequent increases in IP₃ and [Ca²⁺]_i, generation of DG, and activation of PKC [164–169]. The other well-recognized downstream signaling pathways for extracellular ANG II to induce biological or physiological responses also include activation or inhibition of calcium-dependent calcineurin [170], cAMP-dependent protein kinase A (PKA) [169, 171], Ca²⁺-independent PLA₂ [172], PI 3-kinase [157], c-Src/MAP kinases ERK 1/2 [165], or nuclear factor-κB [173].

According to the principles of the G protein-coupled receptor pharmacology, ANG II must bind to its cell surface receptors to activate intracellular signaling mechanisms in order to induce responses [76–78, 138]. Upon internalization, however, ANG II may act as an intracellular peptide to induce biological or physiological responses. Indeed, blockade of the endocytosis of AT₁ receptors is associated with inhibition of PKC, IP₃ formation, and Na⁺ flux in proximal tubule cells [14, 16, 122–126, 149, 150]. Furthermore, ANG II-induced AT₁ receptor endocytosis is also associated with activation of PLA₂ [147, 172], inhibition of adenylyl cyclase [151, 169, 171], and increases in Na⁺ uptake from AP membranes [149–151]. We have recently shown that AT₁-mediated uptake of extracellular Val⁵-ANG II was indeed associated with inhibition of basal and forskolin-stimulated cAMP accumulation [125, 151], ANG II-stimulated NHE3 expression [14, 16, 122, 123], and ANG II-induced activation of MAP Kinases ERK1/2 and nuclear factor-κB in proximal tubule cells [14, 16, 124, 126, 151].

Nevertheless, these approaches are unlikely able to distinguish the effects of ANG II mediated by cell surface or intracellular receptors. Previous studies have shown that single cell microinjection or microdialysis of ANG II directly into the cells may distinguish between the effects induced by extracellular ANG II from those induced by intracellular ANG II [15, 102, 174–177]. Indeed, we have demonstrated that intracellular microinjection of ANG II directly into single rabbit proximal tubule cells induced intracellular [Ca²⁺]_i responses (**Figure 3**) [10, 15, 16, 81, 177]. We further reported that microinjection of the AT₁ blocker losartan abolished the [Ca²⁺]_i response induced by microinjected ANG II, but it only partially blocked the effects of extracellular ANG II [15]. In further proof-of-the concept studies, we showed that ANG II stimulated nuclear AT_{1a} receptors to increase in vitro transcription of mRNAs for TGF1, MCP-1 and NHE3 in isolated rat renal cortical nuclei [102]. These studies provide evidence that intracellular ANG II may activate cytoplasmic and nuclear AT₁ receptor to induce important genomic effects in proximal tubule cells [15, 102, 174–177].

Whether intracellular ANG II may alter biological responses in a cell culture model has been determined by directly expressing an intracellular ANG II fusion

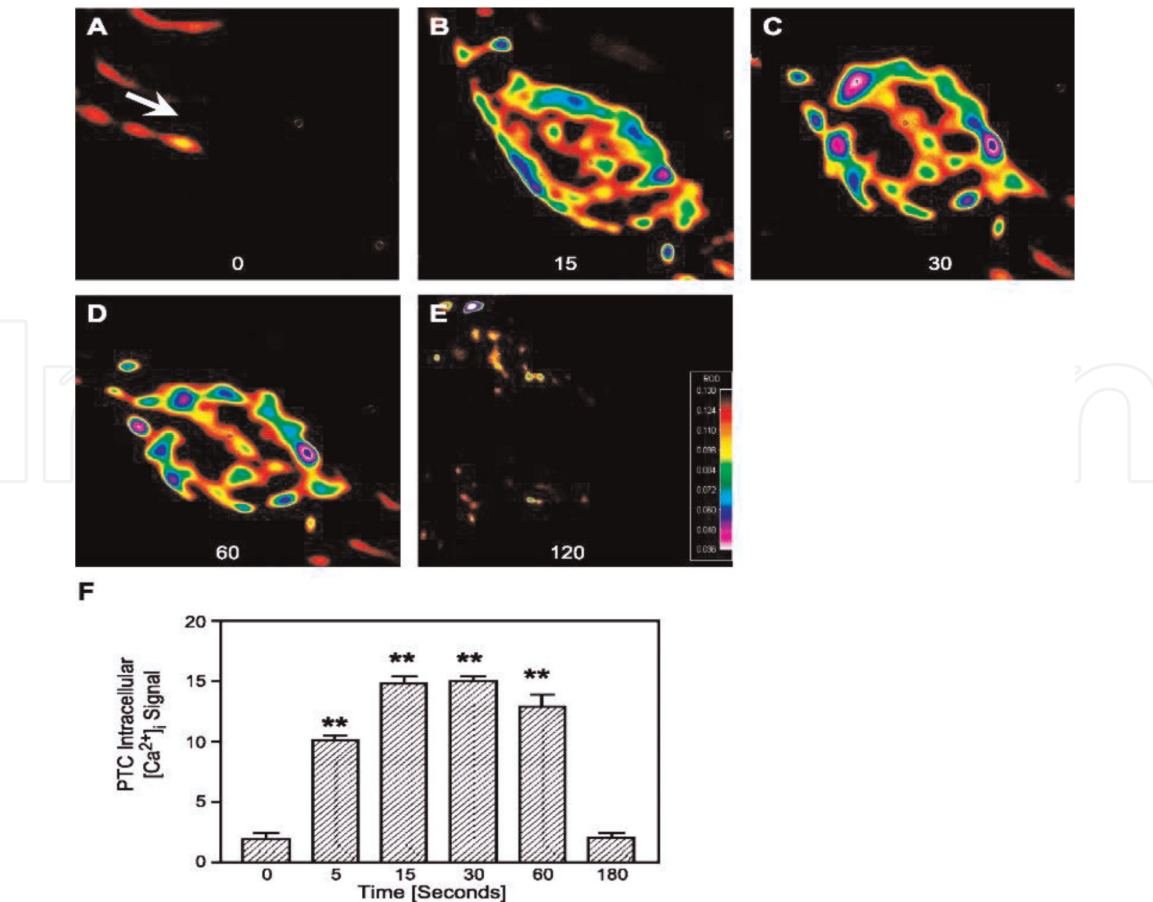


Figure 3.
Intracellular microinjection of angiotensin II induces intracellular calcium mobilization in cultured rabbit proximal tubule cells. Adapted from Zhuo et al. with permission [15].

protein [9, 11, 15, 88–90, 102]. Cook et al. overexpressed a cyan fluorescent, intracellular ANG II construct (ECFP/ANG II) with or without a rat yellow fluorescent AT_{1a} receptor ($AT_{1a}R$ /EYFP) in rat VSMCs or hepatocytes [9, 97, 98]. They demonstrated that intracellular ANG II induced the proliferation of VSMC via activation of cAMP response element-binding protein (CREB), p38 MAP kinase, and MAP kinases ERK 1/2 [9, 97, 98]. In another study, an intracellular ANG II (pcDNA/TO-iAng II) was expressed in CHO cells to induce cell proliferation, but none of ARBs was found to attenuate the effect of intracellular ANG II on cell proliferation [178, 179]. Nevertheless, these early proof of concept studies suggest that in vitro or in vivo expression of a cyan fluorescent intracellular ANG II fusion protein (ECFP/ANG II) in the proximal tubule cells of wild-type and AT_{1a} -KO mice may be an innovative approach to distinguish the effects of intracellular versus extracellular ANG II.

7. Intratubular and intracellular ANG II: physiological effects of intracellular versus extracellular ANG II on proximal tubule Na^+ reabsorption and blood pressure

The physiological roles of intracellular ANG II in the regulation of proximal tubule Na^+ reabsorption and normal blood pressure homeostasis remain to be determined. Whether intracellular and/or internalized ANG II may physiologically regulate proximal tubule Na^+ transport and blood pressure has not been studied until recently. Indeed, this line of research has been long stymied due to the lack of suitable animal models that express an intracellular ANG II protein, which is not

secreted outside the cells and only acts intracellularly. Dr. Reudelhuber's group was the first to generate genetically modified mouse model that expresses an ANG II-producing fusion protein in the cardiomyocytes of the rat heart [180, 181]. They used the α myosin heavy chain promoter to control the expression of ANG II-releasing fusion protein in the cardiomyocytes. Cardiac specific expression of this ANG II fusion protein led to 10-fold increases in ANG II levels in the heart of these transgenic mice, but it did not elevate ANG II levels in the plasma [180, 181]. This approach is very unique to construct this cardiac-specific ANG II fusion protein with a signal peptide sequence derived from human prorenin and a furin cleavage site. Thus, the expressed ANG II fusion protein will be cleaved by furin, and released into the secretory pathway and the cardiac interstitium [180, 181]. It is expected that this cardiac-specific ANG II fusion protein activates cell surface, but not intracellular receptors. In a different study, Baker et al. expressed an intracellular ANG II peptide in the mouse cardiomyocytes using an adenoviral vector [178]. Cardiac-specific expression of this intracellular ANG II peptide in mice induced cardiac hypertrophy, but not altered blood pressure and plasma ANG II [99, 178]. Furthermore, the AT₁ receptor blocker failed to block the cardiac hypertrophic effect of this peptide, suggesting that AT₁ receptor may not be involved [99, 178].

In the kidney, a proximal tubule cell-specific promoter may be an ideal approach to express an intracellular ANG II protein selectively in the proximal tubules. For example, the kidney androgen-regulated protein gene (KAP) has been used to drive "proximal tubule-specific" expression of human angiotensinogen and renin in the kidney [182, 183]. It has been shown that the KAP gene is widely expressed in the kidney, with its expression reportedly confined to the proximal tubules and regulated by androgen and estrogen [184, 185]. The advantages of this approach are its usefulness for studying the sexual dimorphic regulation of angiotensinogen expression in the proximal tubules of the kidney [182, 183].

We have collaborated with Dr. Julie Cook of Ochsner Clinic and Dr. Isabelle Rubera of University of Nice-Sophia, France to develop an adenoviral construct

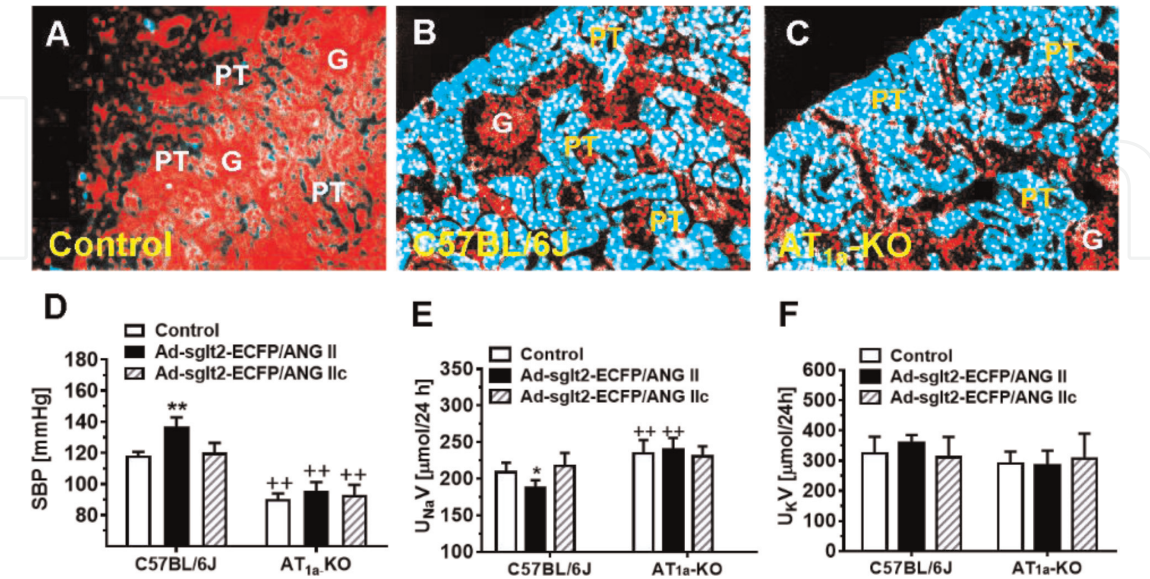


Figure 4. Overexpression of an intracellular ECFP/ANG II fusion protein selectively in the proximal tubule of the kidney in C57BL/6J or AT_{1a}-KO mice. ECFP/ANG II increased systolic blood pressure and had a significant antinatriuretic response in C57BL/6J but not in AT_{1a}-KO mice. Green blue represents ECFP/ANG II expression in the proximal tubules, whereas Red represents DAPI-stained nuclei in the cortex after conversion from blue color. G, glomerulus. PT, proximal tubule. ***p* < 0.01 versus control, whereas ++*p* < 0.01 versus C57BL/6J mice. Reproduced from Zhuo et al. with permission [15].

(Ad-sglt2-ECFP/ANG II), which encodes a cyan fluorescent intracellular ANG II fusion protein (ECFP/ANG II) [17, 18]. The sodium and glucose cotransporter 2 promoter, sglt2, was used to drive the expression of ECFP/ANG II selectively in the proximal tubule cells of the rat and mouse kidneys. Sglt2 is expressed almost exclusively in S1 and S2 segments of the kidney proximal tubules [186]. Using this approach, we have determined whether intrarenal adenovirus-mediated expression of intracellular ECFP/ANG II selectively in the proximal tubules of the rat and mouse kidneys increases the expression and activity of NHE3, stimulate proximal tubule sodium reabsorption, and increase blood pressure in rats and mice. We demonstrated that expression of intracellular ECFP/ANG II selectively in the proximal tubules of rats and mice significantly increased NHE3 expression, proximal tubule sodium reabsorption, and blood pressure (**Figure 4**) [17, 18]. We further showed that AT₁ receptor blocker losartan and deletion of AT_{1a} receptors in mice significantly attenuated intracellular ANG II-induced NHE3 expression, proximal tubule sodium reabsorption, and blood pressure responses, suggesting an AT₁ (AT_{1a}) receptor-mediated mechanisms.

8. Intratubular and intracellular ANG II: role of NHE3 in maintaining normal blood pressure homeostasis and ANG II-induced hypertension

The Na⁺/H⁺ exchanger 3 (NHE3) is the most important Na⁺ transporter in AP membranes of the proximal tubules of the kidney [187–190]. NHE3 is directly and indirectly responsible for reabsorbing approximately 50–60% of filtered load of NaCl and 70–80% of filtered load of bicarbonate (HCO₃[−]) [187–190]. Indeed, nearly all of the measured Na⁺/H⁺ exchanger activity in AP membrane vesicles of proximal tubules are mediated by NHE3 [187–190]. The importance of proximal tubule NHE3 in maintaining body salt and fluid balance and blood pressure homeostasis has not been well studied until recently. Overall, global deletion of the NHE3 gene in all tissues of mice (*Nhe3*^{−/−}) leads to ~50% decreases in fluid, Na⁺ and HCO₃[−] absorption in proximal convoluted tubules, causes salt wasting from the digestive system, and significantly decreases basal blood pressure [191–194]. One of striking phenotypes is absorptive defects in the small intestines due to intestinal NHE3 deletion [191–194]. Moreover, the transgenic rescue of the NHE3 transgene in small intestines in *Nhe3*^{−/−} mice, tg*Nhe3*^{−/−}, failed to rescue the structural and absorptive defects of global NHE3 deletion, with basal blood pressure being similar to those of *Nhe3*^{−/−} mice [195, 196]. These abnormal phenotypes have been confirmed by us recently [21–23].

However, these studies using either *Nhe3*^{−/−} or tg*Nhe3*^{−/−} mice are unable to determine the roles of NHE3 in the proximal tubules of the kidney, since NHE3 is abundantly expressed not only in the proximal tubules of the kidney, but also in small intestines of the gut. To overcome this limitation, we have generated mutant mice with deletion of NHE3 selectively in the proximal tubules of the kidney, PT-*Nhe3*^{−/−}, using the state of the art Sglt2-Cre/LoxP approach [23]. We directly tested the hypothesis that deletion of NHE3 selectively in the proximal tubules of the kidney would lower basal blood pressure by inhibiting proximal tubule Na⁺ reabsorption and increasing the pressure natriuresis response in mice [23]. We demonstrated that under basal conditions, PT-*Nhe3*^{−/−} mice had significantly lower systolic, diastolic, and mean arterial blood pressure than WT mice, accompanied by significantly greater diuretic and natriuretic responses than WT mice, without altering 24 h fecal Na⁺ excretion, plasma pH, Na⁺, and bicarbonate levels. Furthermore, we demonstrated that the pressure-natriuresis response, as well natriuretic

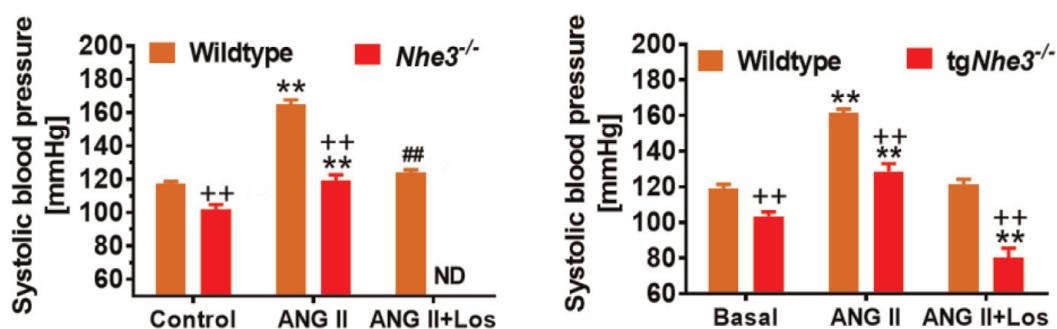


Figure 5. Global (*Nhe3*^{-/-}) or “kidney-selective” deletion of the Na⁺/H⁺ exchanger 3 (NHE3) (*tgNhe3*^{-/-}) in mice significantly attenuates systolic blood pressure response to angiotensin II infusion for 2 weeks (ANG II), 1.5 mg/kg/day, i.p. ^{**}*p* < 0.01 versus their control or basal; ⁺⁺*p* < 0.01 versus wildtype; ^{##}*p* < 0.01 versus ANG II.

responses to acute volume expansion and a high salt diet, were significantly augmented in PT-*Nhe3*^{-/-} mice [23]. Thus, our data support the scientific premise and physiological relevance that NHE3 in the proximal tubules plays an important role in maintaining basal blood pressure homeostasis, and genetic deletion of NHE3 selectively in the proximal tubules of the kidney lowers blood pressure by increasing the pressure-natriuretic response.

Recently, we further investigated whether NHE3 in small intestines and proximal tubules of the kidney plays a key role in ANG II-induced hypertension using *Nhe3*^{-/-}, *tgNhe3*^{-/-}, and PT-*Nhe3*^{-/-} mice [21, 22]. As expected, infusion of a pressor dose of ANG II, 1.5 mg/kg/day, i.p., via an osmotic minipump for 2 weeks markedly increased blood pressure and caused hypertension in C57BL/6J mice (Figure 5) [21, 22]. These hypertensive responses were significantly attenuated in conscious and anesthetized *Nhe3*^{-/-}, *tgNhe3*^{-/-}, and PT-*Nhe3*^{-/-} mice [21, 22, 197]. These results strongly support an important role of NHE3 not only in small intestines, but also in the proximal tubules of the kidney in maintaining basal blood pressure homeostasis and in the development of ANG II-induced hypertension.

9. Future perspectives and conclusions

Taken together, there is accumulating evidence to support the existence of the circulating (endocrine), local intratubular (paracrine), and intracellular RAS system in the kidney, especially in the proximal tubules. All major components of the RAS, including the substrate angiotensinogen, renin, ACE, ANG II, AT₁ and AT₂ receptors, have been localized in the circulation, the kidney, and in the proximal tubule. The roles of the circulating and intratubular RAS in the cardiovascular and kidney, and blood pressure regulation have been extensively studied using molecular, cellular, genetic and pharmacological approaches. It is now well-understood that AGT, prorenin, renin, ACE, ANG II and AT₁ and AT₂ receptors are not only expressed and localized in the proximal tubules under physiological conditions, but the levels of intratubular angiotensinogen, renin, ACE, and ANG II proteins are also significantly increased in the kidney in response to ANG II infusion in spite of suppression of the circulating RAS. Furthermore, there is also increasing evidence supporting the genomic roles of intracellular and nuclear ANG II in the regulation of proximal tubule reabsorption, blood pressure and the development of hypertension. Future studies should focus more on the long-term genomic and hypertensive roles of intracellular, mitochondrial and nuclear ANG II and the underlying signaling mechanisms in ANG II-dependent hypertension and target organ injury.

Acknowledgements

This work was supported in part by NIH grants, 2R01DK102429-03A1, 2R01DK067299-10A1, and 1R56HL130988-01 to Dr. Zhuo. Ana Paula de Oliveira Leite was supported by scholarships from the Ministry of Education, Brazilian Federal Agency for Support and Evaluation of Graduate Education—CAPES, and Hospital do Rim, Sao Paulo, Brazil, respectively. Drs. Chunling Zhao, Xiaowen Zheng, Jianfeng Zhang were visiting scholars from the Department of Emergency Medicine, The 2nd Affiliated Hospital, Guangxi Medical University, Nanning, Guangxi, China.

Conflict of interest

The authors declare no conflict of interest.

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