SPECIAL TOPIC | Intracrine Renin-Angiotensin System: A New Paradigm in Cardiovascular and Renal Control

Novel roles of nuclear angiotensin receptors and signaling mechanisms

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Gwathmey TM, Alzayadneh EM, Pendergrass KD, Chappell MC. Novel roles of nuclear angiotensin receptors and signaling mechanisms. Am J Physiol Regul Integr Comp Physiol 302: R518–R530, 2012. First published December 14, 2011; doi:10.1152/ajpregu.00525.2011.—The renin-angiotensin system (RAS) constitutes an important hormonal system in the physiological regulation of blood pressure. The dysregulation of the RAS is considered a major influence in the development and progression of cardiovascular disease and other pathologies. Indeed, experimental and clinical evidence indicates that blockade of this system with angiotensin-converting enzyme (ACE) inhibitors or angiotensin type 1 receptor (AT1R) antagonists is an effective therapy to attenuate hypertension and diabetic renal injury, and to improve heart failure. Originally defined as a circulating system, multiple tissues express a complete RAS, and compelling evidence now favors an intracellular system involved in cell signaling and function. Within the kidney, intracellular expression of the three predominant ANG receptor subtypes is evident in the nuclear compartment. The ANG type 1 receptor (AT1R) is coupled to the generation of reactive oxygen species (ROS) through the activation of phosphoinositol-3 kinase (PI3K) and PKC. In contrast, both ANG type 2 (AT2R) and ANG-(1–7) (AT7R) receptors stimulate nitric oxide (NO) formation, which may involve nuclear endothelial NO synthase (eNOS). Moreover, blockade of either ACE2—the enzyme that converts ANG II to ANG-(1–7)—or the AT7 receptor exacerbates the ANG II-ROS response on renal nuclei. Finally, in a model of fetal programmed hypertension, the nuclear ROS response to ANG II is enhanced, while both AT2 and AT7 stimulation of NO is attenuated, suggesting that an imbalance in the intracellular RAS may contribute to the development of programming events. We conclude that a functional intracellular or nuclear RAS may have important implications in the therapeutic approaches to cardiovascular disease.

renin-angiotensin system; angiotensin converting enzyme; ACE2; neprilysin; kidney; nuclei; AT1; AT2; AT7.

FROM THE EARLY STUDIES OF Tigerstedt and Bergman (101) that described renin activity in the kidney to the characterization of a pressor substance subsequently identified as ANG II by the laboratories of Braun-Menendez (6) (hypertensin) and Page (angiotonin) 50 years later (8), the characterization of the renin-angiotensin system (RAS) within the kidney and other tissues continues unabated to broaden our understanding of the functional aspects of this important hormonal system in blood pressure regulation and cardiovascular pathologies. Coupled with the discoveries of ANG I-converting enzyme (ACE) as the primary enzyme that forms ANG II in the circulation and tissue and the molecular identification of the ANG II type 1 receptor (AT1) that confers the predominant actions of ANG II, the “classical” RAS was regarded as a sequential endocrine system to form ANG II for the maintenance of blood pressure through renal, vascular, and central mechanisms. There is, however, overwhelming evidence for a “nonclassical” RAS that results in the formation of novel peptide products with functional properties distinct from that of the ANG II-AT1 receptor pathway (5, 12, 14, 67, 80, 94). This nonclassical RAS includes the endogenous peptide angiotensin-(1–7) [ANG-(1–7)] (15), angiotensin-converting enzyme 2 (ACE2) (27, 102), the ANG-(1–7) [AT7] or Mas receptor (81), the AT2 receptor subtype (69), and the prorenin receptor (68), which are all expressed within the kidney. Emerging studies also reveal the RAS to exhibit intracellular actions within multiple cell types and the localization of various components, including the three principal AT receptor subtypes in the nucleus (24, 29, 50, 77). Indeed, the density of AT1 receptors within the nuclear fraction

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of the rat renal cortex is comparable, if not greater, than that of the plasma membrane fraction (60, 61, 71). In addition, nuclear angiotensin receptors are evident in extra-renal cell types, including cardiomyocytes, cardiomyocytes, lymphocytes, smooth muscle cells, hepatocytes, and neurons (4, 17, 45, 62, 65, 96, 98). Thus, our view of the RAS should encompass a biological set of subsystems or functional paths that elicit actions through both extracellular and intracellular events. Moreover, the various components of the RAS that comprise the classical and nonclassical pathways may exert competing or buffering capacities to modulate the functional output of this system. The current review focuses on the evidence to date for the functional roles of the intracellular RAS within the kidney, a key target organ of the RAS and critical in the maintenance of fluid and electrolyte balance, as well as the development and progression of renal injury.

**Nuclear Receptors**

**AT$_1$ receptors.** Although the RAS is traditionally viewed as an endocrine system, whereby circulating renin and angiotensinogen initiate an enzymatic cascade to form active products, it is apparent that multiple tissues contain the necessary components for the local generation of ANG peptides. These tissue systems may release the precursor angiotensinogen or the active peptides to bind to cell surface receptors in an autocrine (same cell) or paracrine (adjacent cell) manner (Fig. 1). Alternatively, the intracellular processing to form ANG II or ANG-(1–7) would imply the localization of ANG receptor subtypes on distinct intracellular organelles. Within the kidney, we and others document a surprisingly high density of ANG II binding sites in the nuclear fraction from both the cortical and medullary areas of the rat kidney (Fig. 2) (60, 61, 71). The density of ANG II receptors in the nuclear fraction of the renal cortex is at least twofold higher than in the plasma membrane fraction and 20-fold greater than the nuclear fraction of rat liver (4, 71, 98). The nuclear ANG II binding sites exhibit conventional receptor kinetics with a high affinity ($K_D \sim 10^{-9} \text{M}$) and moderate density ($B_{max} \sim 250 \text{ fmol/mg protein}$) (Fig. 2A). Our studies further demonstrate that the majority of the nonselective ANG receptor antagonist $^{125}$I-labeled [Sarcosine$^1$-Thr$^8$]-ANG II (Sarthran) bound to rat cortical nuclei was inhibited by the AT$_1$ receptor antagonists losartan and candesartan, but not the AT$_2$ antagonist PD123319 or the AT$_7$ blocker [D-Ala$^7$]-ANG-(1–7) (DALA) (Fig. 2B). Isolated nuclei from the renal medulla of rat also exhibit AT$_1$ binding sites, although the overall receptor density was considerably less than that in the renal cortex (71). As shown in Fig. 3A, flow cytometry with differentially labeled antibodies to the AT$_1$ receptor and the nuclear pore complex Nup62 reveal that essentially all of the nuclei (>95%) in the rat renal cortex express the AT$_1$ receptor subtype. Interestingly, two populations of AT$_1$ sites that differed by a density of threefold were evident in the nuclear compartment of the renal cortex, as revealed by flow cytometry of isolated nuclei (Fig. 3A). The AT$_1$ antibody used for flow cytometry yielded a single band (52 kDa) by immunoblots, suggesting a mature form of the receptor on cortical nuclei (Fig. 3B).

Validation of functional AT$_1$ binding sites (receptors) on renal nuclei was initially established by Li and Zhuo (60) and Zhuo et al. (107). These investigators demonstrated that incubation of ANG II with isolated nuclei stimulated an immediate increase in calcium as detected by Ca$^{2+}$ indicator fluo 3 (107). The ANG II-dependent increase in nuclear calcium was abolished by the coadministration of the AT$_1$ antagonist losartan (107). Studies by Li and Zhuo (60) also demonstrate that the ANG II-AT$_1$ receptor axis mediates the expression of various mRNA transcripts that are linked to the pathological actions of ANG II, including monocyte chemotactic protein, trans-
forming growth factor and the sodium hydrogen exchange transporter (NHE3). Moreover, intracellular expression of ANG II within proximal tubules by an adenoviral vector for a nonsecreted form of ANG II (developed by Dr. Julia Cook) induces a sustained increase in blood pressure that was associated with higher expression of NHE3 and enhanced sodium retention (56). Because many of the actions of the ANG II-AT1 receptor pathway are associated with an increase in reactive oxygen species (ROS), we assessed whether the nuclear AT1 receptor mediates ANG II-dependent stimulation of ROS. In isolated nuclei of the rat renal cortex loaded with the ROS fluorophor dichlorofluorescein (DCF) and analyzed by flow cytometry, we show that a low dose of ANG II (10^{-9} M) resulted in the immediate increase in DCF fluorescence (72). ANG II-dependent stimulation of ROS was blocked by losartan and the NAD(P)H oxidase inhibitor diphenyleneiodonium (DPI) (Fig. 3C).

The nuclear source of ROS in rat cortical nuclei is not currently known; however, the NAD(P)H oxidase isoform NOX4 is expressed in the nuclear compartment of human endothelial (51), human aortic smooth muscle cells (44), and mouse hepatocytes (92). The latter study localized NOX4 to

Fig. 2. AT1 receptors are expressed on the nuclear fraction of the rat kidney. A: saturation binding and Scatchard analysis (inset) of [125I]-labeled Sarthran reveal high affinity (K_D of 0.7 nM) and density (B_max of 270 fmol/mg protein) of binding sites on renal cortical nuclei. B: competition of Sarthran binding in renal cortical nuclei by PD123319 (PD), D-Ala^7-ANG-(1–7) (DALA); ANG II; losartan (LOS) and candesartan (CV). *P < 0.01 vs. control. C: comparison of AT1 receptor densities in nuclear (NUC) and plasma membrane (PM) fractions prepared from the rat renal cortex and medulla. [Adapted from Pendergrass et al. (71).]
both the outer and inner nuclear envelope, consistent with the perinuclear generation of superoxide (92). Moreover, NOX4 is considered the predominant NOX isoform in the kidney, specifically localized to the proximal tubule epithelium and mesangial cells of the glomerulus and forming primarily hydrogen peroxide (39). We assessed NOX4 expression in the nuclear fraction of rat kidney by flow cytometry and immunoblot methods. NOX4 overlapped 75% with the nuclear marker Nup62, indicating extensive nuclear expression of the NAD(P)H oxidase within the renal cortex (Fig. 3D). Immuno- blots revealed a single immunoreactive band at 65 kDa for NOX4 in isolated nuclei of rat renal cortex (Fig. 3E) (72). Although our studies do not establish a de facto role for NOX4 in the stimulation of ROS by ANG II in the nucleus, they portend the potential contribution of NAD(P)H oxidase to the generation of ROS within this compartment. In this regard, Abboud and colleagues (38) demonstrated that anti-sense inhibition of NOX4 conveys protective actions within the diabetic kidney, a model of increased oxidative stress. Indeed, pharmacological blockade of PKC by GF109203X or phosphoinositide 3-kinase (PI3K) by LY294002 abrogated the ANG II-dependent stimulation of ROS in isolated nuclei, while the PKC agent phorbol myristate acid (PMA) enhanced ROS (Figs. 1 and 3F). Our preliminary studies also show PMA-dependent stimulation of ROS in isolated nuclei of NRK52E renal epithelial cells that predominantly express NOX4 and PKC-δ in the nuclear fraction (104). These data support earlier studies for the upstream participation of PI3K and PKC for ROS stimulation by ANG II in smooth muscle cells, as well as a more recent report on differential activation of PKC isoforms within the kidney by pressor and nonpressor doses of ANG II (85). The latter study suggests the contribution of different PKCs to the actions of ANG II. Moreover, PKC inhibitors,
and other forms of organ damage (32).

**AT2 receptors.** The second major receptor subtype for ANG II is the AT2 receptor, a G protein-coupled receptor (GPCR) that is expressed in the kidney, as well the adrenal gland, heart, pancreas, and brain (70). In general, activation of the AT2 receptor may serve to counterbalance the actions of the AT1 receptor pathway (12, 47). Although the AT2 receptor does not contain a canonical nuclear localization sequence (NLS), as does the AT1 or AT7 subtypes, we demonstrate AT2 receptor expression on renal cortical nuclei of both fetal and adult sheep (41). Compared with AT1 (losartan-sensitive) sites, Sarthran binding that was sensitive to the AT2 antagonist PD123319 accounted for 60% of the sites on cortical nuclei of young adult sheep (41). In contrast, the sheep medullary nuclei contained essentially all losartan-sensitive binding sites (41). One of the signaling pathways associated with activation of the ANG II-AT2 receptor axis is the stimulation of nitric oxide (NO); therefore, we assessed NO release in the nuclear compartment. In cortical nuclei loaded with the NO fluorophor diaminofluorescein (DAF), ANG II (10⁻⁹ M) stimulated NO release that was subsequently blocked by the AT2 antagonist PD123319 (but not losartan) and the general NOS inhibitor nitro-l-arginine methyl ester (l-NAME) (41). In addition to the AT2 receptor protein, immunoblots revealed expression of the type III endothelial NO synthase (eNOS) and soluble guanylate cyclase on isolated nuclei from sheep kidney (41). Our data support an earlier study demonstrating bradykinin-dependent stimulation of NO and cGMP in isolated liver nuclei, as well as the nuclear expression of eNOS (35, 82). The AT2-dependent generation of NO and potentially cGMP may have direct signaling effects within the nucleus to influence transcription, as previously demonstrated for bradykinin (35). Alternatively, the formation of NO may interact with locally produced superoxide (SO⁻²) to quench both species. It is quite feasible that the AT2 receptor itself, by competing for ANG II binding, may directly limit the actions of the ANG II-AT1 receptor axis. In isolated nuclei, blockade of the AT2 site with PD123319 exacerbated the ROS response to ANG II (42). These studies suggest the potential impact of the AT2 receptor to buffer oxidative stress in renal nuclei. Abadir et al. (1) also confirm the intracellular expression of functional AT2 receptors in various cell types, including mouse cardiomyocytes and proximal tubule cells, as well as human fibroblasts (1). Similar to our findings in cortical nuclei, stimulation of the AT2 receptor by the agonist CPG4211A increased NO formation in isolated mitochondria that was blocked by the PD123319 antagonist (1). Moreover, AT2 receptor activation significantly attenuated mitochondrial respiration rate, which was reversed by both the PD123319 compound and the NOS inhibitor l-NAME. Finally, these authors show that mitochondrial AT2 receptor expression in mouse proximal tubules was lower in older 70-wk-old mice (compared with 20 wk) and that chronic losartan treatment prevented the age-related decline in receptor density (1).

**AT7 receptors.** ANG-(1–7) is the other major peptide product of the RAS, and the GPCR Mas conveys the biological actions of this peptide hormone (14, 81). Expression of the AT7/Mas receptor is significant within the kidney, particularly the proximal tubule elements of the renal cortex (21, 43, 106). The Mas receptor contains a canonical NLS, and ANG-(1–7)-mediated internalization of the receptor was recently demonstrated in HEK 293 cells transfected with a Mas chimera protein; however, nuclear translocation of the internalized receptor was not evident (34, 55). The initial evidence for intracellular ANG-(1–7) was demonstrated in studies by Casa- rini and colleagues (9) that revealed perinuclear immunostaining of the peptide in mesangial cells. We assessed binding sites for ANG-(1–7) on isolated nuclei of the sheep kidney and revealed significant attenuation of Sarthran binding by the selective ANG-(1–7) receptor antagonist D-Ala³-ANG-(1–7) (DALA or A779) (43). Functional receptor studies on isolated nuclei revealed that very low concentrations of ANG-(1–7) in the femtomolar to picomolar (10⁻¹⁵ – 10⁻¹² M) range stimulated NO release (increased DAF fluorescence) (Fig. 4A).

Fig. 4. ANG-(1–7) stimulates nitric oxide in isolated nuclei of sheep renal cortex. A: ANG-(1–7) exhibits greater potency than ANG II to stimulate nitric oxide (NO) as detected by diaminofluorescein (DAF) [*P < 0.05 vs. ANG-(1–7)]. B: DALA and NOS inhibitor nitro-l-arginine methyl ester (l-NAME) block NO response to ANG-(1–7) [*P < 0.05 vs. 10⁻⁹ M ANG-(1–7)]. The AT2 antagonist LOS and AT2 antagonist PD had no effect. C: immunoblots of purified nuclei from sheep renal cortex for the Mas receptor (33 kDa) and the nuclear protein lamin (72 kDa). [Adapted from Gwathmey et al. (43).].

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NO response to ANG-(1–7) exhibited greater sensitivity than that for the ANG II-AT2 receptor pathway, which may, in part, reflect active enzymatic processing on the nuclear membrane (see proceeding section). The ANG-(1–7)-NO response was abolished by the DALA antagonist and the NOS inhibitor l-NAME but was not influenced by either losartan or PD123319 (Fig. 4B) (43). Immunoblot analysis revealed the expression of the AT7/Mas receptor of the appropriate molecular size (33 kDa) in sheep cortical nuclei that stain positive for the nuclear protein laminin (Fig. 4C). These findings suggest that ANG-(1–7) maintains its unique selectivity for ANG receptor sites on the nucleus of the sheep kidney. We speculate that the ACE2-ANG-(1–7)-AT7 receptor axis functionally balances the output of the ANG II-AT1 receptor pathway (14). Recent studies in isolated nuclei of older sheep cortex support this tenet. As shown in Fig. 5A, the ANG II-AT1 receptor-dependent ROS stimulation was markedly increased in cortical nuclei form older sheep, as well as the response to PMA. The pharmacological blockade of ACE2 with the MLN inhibitor, which prevents the processing of ANG II to ANG-(1–7), significantly augmented the ROS response to ANG II in cortical nuclei (Fig. 5B). Moreover, the administration of the ANG-(1–7) antagonist DALA enhanced the ANG II-ROS increase to a similar extent as the ACE2 inhibitor (Fig. 5B). These findings suggest an active ACE2-ANG-(1–7) pathway on nuclei that attenuates ANG II-dependent generation of oxidative stress. Indeed, we also demonstrate protein expression for the Mas receptor and ACE2, as well as ACE2 activity in the isolated nuclei from proximal tubules (Fig. 5C). In support of a pathological role for oxidative stress within the nucleus, Schupp and colleagues find that ANG II-induced DNA damage in the perfused kidney, and LLC-PK proximal tubule cells was attenuated by either AT1 receptor blockade or antioxidant treatment (83, 84). Associated with the increase in DNA damage, ANG II also stimulated intracellular ROS (increased DCF fluorescence), although the ANG II concentration in the intact LLC-PK cells was 170-fold greater than that used in the isolated nuclei (83). In cardiomyocytes, ANG II also was more potent to stimulate the transcription factor NF-kB when applied to isolated nuclei than to the intact cells (96). We have as yet to define the mechanisms of the nuclear ANG-(1–7) pathway to attenuate ROS. ANG-(1–7) may stimulate NO to quench the generation of SO2 by ANG II. Alternatively, the downstream actions of ANG-(1–7) may result in activation of nuclear phosphatase activity that abrogates kinase signaling and the stimulation of NAD(P)H oxidase. Several studies reveal that ANG-(1–7) induces phosphatase activity in human endothelial cells, rat cardiomyocytes, and porcine proximal tubules (30, 79, 95). Sampaio et al. (79) report that knockdown of the phosphatase SHP-2 abolished the inhibitory effects of ANG-(1–7) on ANG II-dependent oxidative stress in intact endothelial cells. In lieu of the localization of various phosphatases in the nuclear compartment, the intracellular signaling for ANG-(1–7) may involve an influence on phosphatase tone in the nucleus (Fig. 1) (74).

**Distribution and trafficking.** The nucleus is a complex structure that is composed of two bilayer membranes (outer and inner membrane) completely traversed by nuclear pores that facilitate access between the cytosol and the nuclear matrix. Portions of the outer nuclear membrane are continuous with the endoplasmic reticulum (ER), such that peri-nuclear space is shared with the ER. The inner nuclear membrane overlies a mesh of proteins termed lamins that comprise the nuclear lamina, and integral proteins penetrate the lamina to associate with chromatin (23, 105). The region surrounded by the nuclear lamina is termed the nuclear matrix and contains the chromatin-DNA complex. Definive localization of angiotensin receptors on domains of the

**Fig. 5.** ANG-(1–7) attenuates the ANG II-stimulated increase in ROS in isolated nuclei from renal cortex of older sheep. A: ANG II and PMA increase ROS to a greater extent in cortical nuclei of older sheep (*P < 0.01 vs. 10–8 M ANG II). B: The AT1 antagonist LOS blocks the ANG II stimulation of ROS; the AT7 antagonist D-Ala2-ANG-(1–7) (DALA) and ACE2 inhibitor MLN exacerbate the response (#P < 0.05 vs. ANG II), while the AT2 antagonist PD had no effect. C: HPLC chromatograph of conversion of ANG II to ANG-(1–7) in isolated nuclei from sheep proximal tubules and inhibition by the ACE2 inhibitor MLN. Inset: immunoblot of ACE2 in isolated nuclei at the molecular weight of 120 kDa (lanes 1–3 are 3 distinct preparations). (Reprinted and adapted from Gwathmey TM, Pendergrass KD, Reid SD, Rose JC, Diz DI, Chappell MC. Angiotensin-(1–7)-angiotensin-converting enzyme 2 augments reactive oxygen species formation to angiotensin II within the cell nucleus. *Hypertension* 55: 166–171 (40)).
renal nuclei is not currently known; however, the seven-transmembrane motif of the intact receptor would predict their localization to the outer or inner nuclear membrane domains for activity. Functional studies on isolated renal nuclei suggest that at least a portion of the receptors may reside on the outer nuclear membrane with the ligand binding site oriented to the cytoplasm. Indeed, we find an immediate response in the fluorescent signal for ROS or NO upon addition of ANG II or ANG-(1–7) to rat and sheep renal nuclei (41–43). Moreover, the functional data are consistent with the demonstration of high-affinity binding sites on intact renal nuclei obtained from different laboratories using sucrose or iodixanol density gradient methods (43, 61, 71, 107).

Apart from their nuclear distribution, the cellular mechanisms for expression of angiotensin receptors within the nucleus are not resolved. It is well established that rapid internalization of the ANG II-AT1 receptor complex occurs in a variety of cells and that the AT1 receptor may associate with the perinuclear or nuclear area (46, 99). Morinelli et al. (65) demonstrated that a single point mutation in the NLS (Lys305 to Gln) of the AT1 receptor did not influence ANG II-dependent internalization of the receptor but abrogated the subsequent association with the perinuclear membrane. Interestingly, ANG II stimulation of the COX2-synthesizing enzyme PTGS-2 was attenuated but not the expression of the early growth response gene (EGR-1) or the calcium response in cells transfected with the mutant NLS AT1 receptor (65). Cook et al. (18) find agonist-dependent proteolytic cleavage of the AT1 receptor and translocation of the resultant 7-kDa COOH-terminal fragment to the nucleus. Moreover, overexpression of the AT1 receptor COOH-terminal fragment stimulated the apoptotic pathway in several cell lines, suggesting a novel action of the internalized receptor (19). It is not clear, however, whether internalized AT1 receptors or the COOH-terminal region exclusively traffic to the nucleus in all cells or that trafficking results in functional expression of receptors on the nuclear membrane. Lee et al. (55) show expression of the GFP-tagged AT1 receptors on the nuclear following transfection in various cell lines that was not dependent on prior ANG II stimulation. The distribution of nuclear and cell surface receptors varied in the different cells, suggesting cell-specific regulation of nuclear expression. Natel and colleagues (96) observed that internalized ANG II-AT1 receptor complex did not traffic to the nucleus of cardiomyocytes, suggesting that the presence of AT1 receptors on the nuclear membrane may arise from intracellular synthesis and transport (96). Furthermore, Re et al. (76) recently described the localization of functional GNRH receptors on the nuclear membrane of HEK293 cells; however, deletion of the NLS did not influence the distribution of the receptor to the nucleus. As previously mentioned, AT2 receptors lack a canonical NLS and do not internalize following ligand activation, yet functional receptors are present on both renal and cardiac nuclei (42, 96). The inability to demonstrate that internalized endothelin receptors traffic from the cell surface to the nucleus further supports an intracellular mechanism for nuclear expression of both ET-A and ET-B receptors on cardiomyocytes (3). In lieu of these studies, we favor a cellular mechanism for intracellular synthesis and trafficking of the mature receptor from the post-Golgi complex that ultimately inserts into the nuclear membrane and associates with the appropriate signaling components to influence ROS or NO formation within renal nuclei. We acknowledge that further studies are certainly warranted to establish the discrete distribution of angiotensin receptors within the nucleus and the associated signaling components of the RAS, as well as to define the cellular mechanisms that account for their nuclear expression in different cell types within the kidney.

**Intracellular Formation**

The presence of intracellular receptors for ANG II and ANG-(1–7) within the kidney necessitates the delivery and/or intracellular generation of the requisite ligands. Binding of ANG II to the AT1 receptor on the cell surface induces rapid internalization of the ligand-receptor complex (46, 59, 99). Chronic AT1 receptor blockade or AT1 receptor deletion significantly reduces renal content of ANG II, suggesting that receptor-mediated uptake of ANG II contributes directly to intracellular peptide levels and/or stimulates intracellular synthesis (10, 13, 57, 58, 63, 78). Our preliminary studies find that ANG II administration restores the depleted levels of ANG II in the kidney of tissue ACE knockout mice to that of the wild-type mice (64). Similar to other peptidergic GPCR systems, the internalized AT1 receptor complex is sorted into either endosomal or lysosomal compartments (7, 46, 100). In the kidney, endosomal sorting of ANG II may protect the peptide from immediate metabolism and provide a mechanism for the subsequent release into the intracellular compartment (48). Gonzalez-Villalobos et al. (36, 37) demonstrate that transport protein megalin can promote uptake of ANG-(1–7), as well as ANG II in the rat proximal tubules. However, it is not clear whether either peptide escapes from lysosomal sorting and subsequent metabolism to bind to intracellular receptors. Moreover, megalin transports a number of tubular fluid proteins, including angiotensinogen, and the extent that these proteins compete for the uptake of ANG II or ANG-(1–7) is not known (75).

In addition to the internalization of the ANG II-AT1 receptor complex, the functional effects of ACE2 blockade on isolated nuclei imply that some degree of intracellular or nuclear processing of angiotensins may occur (40). Therefore, we assessed the intracellular components of the RAS in isolated proximal tubules from the sheep kidney. The proximal tubules are considered one of the primary cell types within the kidney to express all components of the RAS (66). In isolated nuclei from proximal tubules, we utilized the endogenous peptide substrates to demonstrate processing of ANG I to ANG II by ACE, direct formation of ANG-(1–7) from ANG I by the endopeptidase nephrilysin, and the conversion of ANG II to ANG-(1–7) by ACE2 (42, 43). Importantly, the activities of all three enzymes were comparable to that in whole proximal tubules using identical enzyme assays (88). As shown in Fig. 6, A and B, immunoblot studies revealed protein expression for both renin and the precursor protein angiotensinogen in the nuclear compartment of isolated proximal tubules. The antibody directed to the immunogenic site of angiotensinogen distal to the ANG I sequence revealed a 60-kDa band, consistent with the mature or glycosylated form of the protein (Fig. 6B). The antibody directed against the ANG I sequence failed to detect a protein band in the nuclear fraction (Fig. 6C), yet the antibody revealed intact angiotensinogen in sheep and...
Fig. 6. Immunoblots reveal the presence of renin and angiotensinogen in the nuclear fraction from proximal tubules. The nuclear fractions from three distinct proximal tubule preparations (lanes 1–3) and a rat kidney homogenate (lane 5) were separated on two SDS gels and probed with antibodies against renin (A) and angiotensinogen (Aogen, B and C). A: renin antibody revealed a single band of ~55 kDa in the nuclei of the tubules (lanes 1–3) and rat kidney homogenate (lane 5). B: Aogen antibody against the NH2 terminal distal to ANG I (Aogen-NT) revealed a ~60-kDa band in the nuclear fraction of the tubules (lanes 1–3) and the kidney homogenate (lane 5). C: no bands were detected using the angiotensinogen antibody that recognizes the ANG I-containing sequence in the nuclear or cortical extracts (Aogen-Al). D: Aogen-Al antibody recognized nephrectomized sheep plasma (lanes 1–3), intact sheep plasma (lane 4), and rat plasma (lane 5). [Adapted from Gwathmey et al. (43)].

rat serum collected with a renin inhibitor to prevent ANG I release (Fig. 6D). The time period required to isolate intact proximal tubules and obtain the nuclear fraction, particularly in the absence of any peptidase inhibitors, may have allowed for the renin-dependent processing of angiotensinogen to the des-ANG I form of the protein. Moreover, this indirect evidence may portend a source of intracellular angiotensin peptides to target nuclear receptors. Cook et al. (20) utilized a novel approach to express a nonsecreted form of angiotensinogen that increased cell growth of transfected cells, which was abrogated by either the AT1 receptor antagonist losartan or knockdown of renin (20). The latter studies clearly support a functional intracellular processing pathway for the generation of ANG II within these cells, as well intracellular receptors that mediate the growth response to ANG II. Preliminary studies in the NRK52E cell line, a well-characterized model of rat epithelial cells, support findings for a nuclear RAS in proximal tubules (11). As shown in Fig. 7, A and B, intracellular staining for both renin and angiotensinogen is evident in the perinuclear compartment of the NRK52E cells. Immunoblots of purified nuclei from the NRK cells reveal a single band for both proteins (Fig. 7C). Immunostaining of nuclei in the NRK cells is positive for ANG-(1–7) and the AT2/Mas receptor protein (Fig. 7, D and E, respectively). Although the direct demonstration of ANG II or ANG-(1–7) within the nuclear compartment of the kidney awaits further studies, identification of a renal cell line that expresses these RAS components may facilitate the elucidation of the processing pathways that contribute to the intracellular/nuclear generation of angiotensins. In this regard, Sigmund and colleagues (89) reported the presence of angiotensinogen in the nuclear compartment of glial cells and identified a putative NLS for the protein; however, the extent of processing of the precursor in the glial nuclei is not known. Both immunoreactive ANG-(1–7) and ANG II were detected in the perinuclear region of rat mesangial cells, in addition to the presence of intracellular ACE and ACE2 (9). Immunoreactive ANG II was localized to the inner membrane of the mitochondria in close association with the AT2 receptor of mouse proximal tubules and myocytes (1). Nattel and colleagues (96) demonstrated significant ANG II content in the nuclear fraction of isolated rat cardiomyocytes, as well as the functional expression of AT1 and AT2 receptors. Furthermore, Singh et al. (90, 91) find that isolated cardiomyocytes from the diabetic rat exhibit increased intracellular content for ANG II that was associated with a greater extent of oxidative stress. In the diabetic cardiac cells, the increase in intracellular ANG II was mediated through a chymostatin-dependent enzyme distinct from ACE. Lavrentyev and colleagues (53, 54) also report similar findings for intracellular ANG II in rat vascular smooth muscle cells chronically exposed to high glucose conditions, although lysosomal enzyme cathepsin D and not renin catalyzed the formation of ANG I from angiotensinogen. These latter studies emphasize the diversity of enzymes, such as chymase and cathepsin D that may participate in intracellular processing of angiotensins, particularly in extrarenal tissues.

Angiotensin Receptor Regulation in Fetal Programming

The exact influence of the intracellular RAS on cardiovascular disease or other pathologies is not clear at this point. There is strong evidence that ACE inhibitors or AT1 receptor antagonists have a profound impact on the treatment of cardiovascular disease. Emerging data suggest that the RAS may influence the course of other pathologies, including cancer, diabetes, pulmonary injury and aging (25, 26). Indeed, AT1 receptor knockout mice exhibit a significant increase in life span when maintained on a normal chow/caloric diet, which supports earlier studies on the protective effects of ACE inhibitors in aging rats (2, 26, 28, 33). Our studies in older adult sheep also revealed evidence for an altered balance in the nuclear expression of ANG receptors, as well as the functional responses of the ANG II-AT1 receptor axis (40). The ratio of AT1 to AT2 sites was greater in the renal nuclei of older sheep, suggesting that alterations in receptor expression may underlie the greater ROS response in the older animals (40).

An imbalance in angiotensin receptors within the nuclear compartment was also evident in the kidney of sheep exposed to glucocorticoids in utero (42). Betamethasone exposure in pregnant sheep that parallels the gestational age for premature infant administration of glucocorticoids results in a “fetal programming phenotype,” in which the offspring exhibit reduced nephron number, glucose insensitivity, attenuated baroreflex sensitivity, and elevated blood pressure (16, 42, 86, 87). Altered renal mechanisms that favor increased sodium retention with an enhanced response
to ANG II and a reduced natriuretic effect of ANG-(1–7) are evident in this model (16, 97). Moreover, renal expression of ACE2 is significantly reduced in the renal cortex and urine of adult sheep exposed to betamethasone in utero, which may influence the renal expression of ANG II and ANG-(1–7) (86). Premature infants are now routinely administered glucocorticoids to accelerate pulmonary development and increase survival of the neonate. Despite the immediate beneficial effects of glucocorticoid exposure, the long-term consequences of this therapy may have adverse cardiometabolic consequences in adulthood (22). We recently showed an enhanced ratio of AT1 to AT2 and AT7 receptors in isolated renal nuclei of betamethasone-exposed adult sheep (42). Functional AT1-dependent responses in ROS to ANG II were augmented in the nuclear compartment of exposed sheep. In contrast, both the AT2 and AT7-dependent responses for NO were significantly reduced following betamethasone exposure (42). The AT1-ROS response was positively correlated to mean arterial blood pressure in the experimental animals; conversely, blood pressure was negatively associated to the nuclear NO response mediated by the AT2 and AT7 receptors (42). Although it is not currently known whether alterations in angiotensin receptor signaling within the nuclear compart-

Fig. 7. Evidence for intracellular RAS components in the rat proximal tubule NRK52E cell line. NRK52E cells were maintained in serum-free conditions prior to immunocytochemical and immunoblot characterization. A, B, D, and E: immunofluorescent staining for renin, angiotensinogen (Aogen, NT-Aogen antibody), ANG-(1–7), and the AT7/Mas receptor protein. Arrows indicate perinuclear or nuclear staining for each component. C: immunoblots for renin and Aogen in nuclear (lanes 1–3) and cytosolic (lanes 4–6) fractions of NRK52E cells from three different passages. Antibodies for RAS components are previously described (43).
ment contribute to the development of fetal programmed hypertension and cardiometabolic disorders, this study illustrates the dynamics of the intracellular RAS to influence local NO or ROS production.

Perspectives and Significance

The current therapies to attenuate an activated RAS include ACE inhibitors, AT1 receptor antagonists (ARBs) and more recently, agents to inhibit renin, such as aliskiren. In general, this approach targets the ACE-ANG II-AT1 receptor axis of the RAS; however, ACE inhibitors dramatically increase circulating levels of ANG-(1–7) by blocking the metabolism of ANG-(1–7) from ACE and shunting ANG I through a nephrilysin or endopeptidase path independent from ACE2 (14). ARBs, however, stimulate marked increases in circulating ANG II and to a lesser extent, ANG-(1–7) through the disinhibition of renin release. Thus, the effectiveness of these therapies may reflect, in part, the participation of either the AT7 receptor or AT2 receptor pathways. Renin inhibitors are obviously designed to block the initial enzymatic cascade in RAS activation, and subsequently, the downstream pathways stimulated by ANG II and ANG-(1–7) would be attenuated by this approach. There are now agonists to both the AT7 and AT2 receptors that may potentially be combined with a renin inhibitor to preserve these receptor pathways while blocking the ANG II-AT1 axis of the RAS. Moreover, both the AT7 agonist AVE0992 and the AT2 agonist C21 are nonpeptides that should access the cell membrane and activate intracellular or nuclear receptors (49, 73, 93, 103). Aliskiren and ARBs are also nonpeptides that may target intracellular as well as extracellular sites, although ARBs exhibit different degrees of lipophilicity, which may influence the extent of their tissue permeability (52). Agents with low lipophilicity may require higher doses to inhibit the intracellular RAS and provide maximal benefit against renal damage. Provided that the intracellular RAS significantly contributes to cardiovascular pathologies, the effective targeting of these pathways may convey additional benefit above that obtained for extracellular inhibition. Our concept of the RAS not only encompasses multiple subsystems, such as the ANG II-AT1 receptor and ANG-(1–7)-AT7 receptor pathways that may oppose one another to influence blood pressure and organ injury, but that these systems may be functionally relevant within the cell and exhibit actions on the nuclear compartment or other intracellular organelle through the regulation of calcium, reactive oxygen species, and nitric oxide that may directly influence gene transcription.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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