Angiotensin I Conversion to Angiotensin II Stimulates Cortical Collecting Duct Sodium Transport

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Abstract—Angiotensin (Ang) II directly stimulates epithelial sodium channel activity in the rabbit cortical collecting duct. Because Ang I and converting enzyme analogues might be present in the distal nephron, this raises the possibility of intraluminal generation of Ang II. Conversion of Ang I to Ang II was monitored by Ang II–dependent changes in intracellular sodium concentration as a reflection of sodium transport across the apical membrane. This involved imaging-based fluorescence microscopy with sodium-binding benzofuran isophthalate in isolated, perfused, cortical collecting-duct segments from rabbit kidney. Principal and intercalated cells were differentiated by rhodamine-conjugated peanut lectin. Control principal cell intracellular sodium concentration, during perfusion with 25 mmol/L NaCl and zero sodium in the bath plus monensin (10⁻³ mol/L) averaged 5.8±0.14 mmol/L (n=156). The increase in intracellular sodium concentration, when luminal NaCl was increased from 25 to 150 mmol/L, was elevated by 3.5-fold in the presence of intraluminal Ang I (10⁻⁶ mol/L). Also, the effects of Ang I on sodium transport were not significantly different from the effects of Ang II (10⁻⁹ mol/L). Ang I was used in micromolar concentrations to ensure that there was sufficient substrate available for conversion to Ang II. Inhibition of the angiotensin-converting enzyme with captopril reduced the stimulatory effect of Ang I. These results suggest that intraluminal conversion of Ang I to Ang II can occur in the cortical collecting duct, resulting in enhanced apical sodium entry. (Hypertension. 2003;42:195-199.)

Key Words: angiotensin-converting enzyme □ imaging □ kallikrein □ kidney □ rabbits □ renin-angiotensin system

The renin-angiotensin system (RAS) functions as both a circulating endocrine and a tissue paracrine system. As an endocrine peptide, angiotensin (Ang) II exerts a prominent influence on blood pressure control and body fluid and electrolyte balance, whereas as a locally formed paracrine peptide, it is involved in the regulation of regional hemodynamics, tissue remodeling, neurotransmitter release, cell growth, and ion transport.¹ In the kidney, besides its role in the juxtaglomerular apparatus, accumulating evidence indicates the importance of intratubular RAS in the control of salt and water transport. In addition to the presence of a fully functional RAS in the proximal tubule,² there is evidence that Ang II can also be formed in the distal nephron.³ Angiotensinogen, which is produced and secreted by the proximal tubule, traverses the nephron to the connecting tubule. At this site, renin, which is secreted into the distal tubular fluid, cleaves angiotensinogen, forming Ang I. Because of technical difficulties, it has not been possible to obtain precise measurements of Ang II and its precursors in the distal nephron. However, indirect data suggest that tubular Ang I concentration is much higher than in plasma.⁴

Recent studies have demonstrated the existence of alternative Ang II–forming pathways, in addition to angiotensin-converting enzyme (ACE). Serine proteases, such as chymase⁵ and glandular kallikrein,⁶ are candidates responsible for ACE-independent Ang II formation in the heart. The existence and level of expression of Ang II–forming enzymes differ markedly among species and organs. In rabbit kidney, aprotinin-inhibitable kallikrein has been localized to the distal nephron, including the first part of the cortical collecting duct (CCD).⁷,⁸ Kallikrein appears to be located at the apical membrane, with its catalytic active site directed into the lumen.⁹ ACE was also demonstrated to be secreted into the tubular lumen of the collecting duct,¹⁰ where its suggested role was in kinin hydrolysis.¹¹ Along with its effects on proximal tubular transport, Ang II has been shown to act on various ion channels and transporters in the CCD.¹²–¹⁴ Ang II was suggested to stimulate amiloride-sensitive Na⁺ channels in the initial part of the collecting tubule.¹⁵ Previous work from our laboratory has shown that Ang II, added to luminal fluid, exerts a direct stimulatory effect on the epithelial sodium channel (ENaC) in the rabbit CCD.¹⁶ In the current studies, we hypothesized that the various proteinases present in the tubular cells and/or tubular fluid might convert Ang I to Ang II in the lumen of the CCD, thereby resulting in an Ang II–mediated increase in ENaC activity.

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Methods

Materials
All materials were purchased from Sigma unless otherwise stated.

Tubule Perfusion
We isolated and perfused individual CCD segments dissected from rabbit (New Zealand, Myrtle’s Rabbitry, Thompson Station, Tenn; 800 to 1000 g body weight, n=37 animals) kidneys as described previously.16 This preparation allowed us to manipulate the composition of the tubular fluid at the apical side (perfusate) independently from the basolateral interstitium (bath). The dissection solution was composed of (in mmol/L) 25 NaCl, 125 N-methyl-D-glucamine cyclamate, 5 KCl, 1 MgSO₄, 1.6 Na₂HPO₄, 0.4 NaH₂PO₄, 1.5 CaCl₂, 5 D-glucose, and 10 HEPES and was adjusted to a pH of 7.4. Tubes (0.8 to 1.2 mm long) were bathed and perfused with this same solution. For the experiments, CCDs were bathed in a modified solution in which NaCl was isosmotically replaced with N-methyl-D-glucamine cyclamate to achieve an NaCl concentration of 0 mmol/L and containing the Na⁺ ionophore monensin (10⁻⁵ mol/L). This maneuver effectively eliminated the basolateral membrane as a barrier for Na⁺ movement, and the absence of bath Na⁺ resulted in a lowering of principal cell intracellular sodium concentration ([Na⁺]). Under these conditions, Na⁺ entry across the apical membrane is the only means of altering [Na⁺], and because changes in [Na⁺], are blocked with luminal benzamyl, this strongly suggests that what we are measuring is a reflection of apical entry via the ENaC. As shown in Figure 1A, principal and intercalated cells were differentiated by selective, apical, PNA binding. After removal of basolateral NaCl and permeabilization of the basolateral membrane with the Na⁺ ionophore monensin (10⁻⁵ mol/L), steady-state [Na⁺], did not differ between principal and intercalated cells (5.8±0.14 vs 5.7±0.16 mmol/L; n=156 and 154, respectively) during perfusion with 25 mmol/L luminal NaCl concentration ([NaCl]ₗ). Representative recordings in Figures 1B and 1C illustrate that increasing [NaCl]ₗ from 25 to 150 mmol/L caused rapid and sustained elevations in both principal and intercalated cell

Fluorescence Microscopy
[Na⁺], in CCD cells was measured with imaging-based, dual-excitation-wavelength fluorescence microscopy with use of the fluorescent probe sodium-binding benzofuran isophthalate (SBFI, Teflabs). After cannulation, tubes were loaded with 5×10⁻⁸ mol/L of the acetoxymethyl ester of SBFI added to the luminal perfusate. Studies were performed in a chamber mounted on an inverted epifluorescence microscope (Eclipse TE2000, Nikon), which was linked to a cooled charge-coupled-device camera (SenSys, Photometrics) interfaced with a digital imaging system (Photon Technologies, Inc.). To identify principal and intercalated cells, we labeled intercalated cells by adding rhodamine-labeled peanut lectin (PNA, Vector Labs) to the luminal perfusate for 5 minutes and observed which cells were fluorescent with excitation and emission wavelength of 545 and 620 nm, respectively.17 SBFI fluorescence ratios (340/380 nm) were converted to [Na⁺] values, as described previously.18 Two to 4 cells were analyzed in each CCD preparation, and 2 or 3 preparations were used from each animal.

Immunofluorescence
Rabbit kidneys were perfusion-fixed with paraformaldehyde, and tissue sections were processed as described earlier.19 After subsequent washings in phosphate-buffered saline, tissues were treated with a goat polyclonal ACE-1 antibody (C-20, 1:100, Santa Cruz Biotechnology) for 1 hour. This was followed by incubation with Alexa594-conjugated donkey anti-goat immunoglobulin G (1:500, Molecular Probes) for 40 minutes. Sections were mounted with an appropriate medium (Vectashield) containing 4,6-diamino-2-phenylindole for nuclear staining (Vector Labs). Tissue sections were examined with an inverted epifluorescence microscope (IX70, Olympus) equipped with a UAp0/340 40× objective. Images were captured with the digital camera described previously (SenSys) and IPLab Spectrum software (Signal Analytics Corp).

Statistical Analyses
Data are expressed as mean±SE. Statistical significance was tested by ANOVA. Significance was defined as P<0.05.

Results
Control and Ang II–Facilitated [Na⁺] Transport
As shown in Figure 1A, principal and intercalated cells were differentiated by selective, apical, PNA binding. After removal of basolateral NaCl and permeabilization of the basolateral membrane with the Na⁺ ionophore monensin (10⁻⁵ mol/L), steady-state [Na⁺], did not differ between principal and intercalated cells (5.8±0.14 vs 5.7±0.16 mmol/L; n=156 and 154, respectively) during perfusion with 25 mmol/L luminal NaCl concentration ([NaCl]ₗ). Representative recordings in Figures 1B and 1C illustrate that increasing [NaCl]ₗ from 25 to 150 mmol/L caused rapid and sustained elevations in both principal and intercalated cell

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This increase in principal cell [Na\(^+\)]\(_i\) was elevated by ~3.5-fold in the presence of luminal Ang II (10\(^{-9}\) mol/L); however, there was no effect of Ang II on sodium transport in intercalated cells.

**Effect of Ang I**

Ang I (10\(^{-6}\) mol/L) added to the luminal perfusate increased [Na\(^+\)]\(_i\) in principal cells by ~3.5-fold when [NaCl] \(_L\) was increased from 25 to 150 mmol/L (Figures 1B and 2). Thus, the effects of micromolar Ang I on Na\(^+\)/H\(^+\) transport were nearly identical to those found with nanomolar Ang II. This increase in [Na\(^+\)]\(_i\), responses with Ang I was almost completely abolished by luminal addition of 10\(^{-7}\) mol/L benzamyl, a specific inhibitor of ENaC. Also, coadministration of luminal Ang I with the Ang II type 1 (AT\(_1\)) receptor blocker losartan (10\(^{-6}\) mol/L) reduced the stimulatory effects of Ang I on [Na\(^+\)]\(_i\), in principal cells to a level not significantly different from control (Figure 2).

**ACE Blockade**

In principal cells, addition of the ACE inhibitor captopril (10\(^{-4}\) mol/L) or the protease inhibitor aprotinin (10\(^{-5}\) mol/L) to the luminal perfusate blocked the Ang I–induced [Na\(^+\)]\(_i\) responses by ~50% (Figure 3A). Coadministration of both drugs plus Ang I attenuated the increases in [Na\(^+\)]\(_i\), by 85% in response to an elevation in [NaCl]\(_L\), compared with Ang I alone, although this inhibition was not statistically different from the inhibition obtained with either captopril or aprotinin alone. Various controls were performed to determine the specificity of our observations. First, in PNA-positive intercalated cells, with or without inhibitors, Ang I did not alter the magnitude of increase in [Na\(^+\)]\(_i\), in response to an elevation in [NaCl]\(_L\), (Figure 3B). Second, captopril plus aprotinin had no significant effect on Ang II stimulation of [Na\(^+\)]\(_i\), responses (Figure 4) in principal cells. This eliminates the possibility that the effects of these agents to inhibit Ang I–mediated increases in [Na\(^+\)]\(_i\), responses were due to some nonspecific action of these drugs. Third, in the presence of captopril and aprotinin and either Ang I or Ang II, [Na\(^+\)]\(_i\), responses with elevations in [NaCl]\(_L\), were the same as in controls in the presence of losartan, the AT\(_1\) receptor blocker (data not shown). Fourth, captopril and aprotinin in the absence of Ang I did not significantly alter [Na\(^+\)]\(_i\), responses (data not shown).

**Immunofluorescence Labeling of ACE-1**

Figure 5 shows ACE-1 labeling within the collecting duct at the corticomedullary boundary in rabbit kidney, with strong labeling at the apical membrane and cytoplasm. At more cortical segments, the apical staining was less prominent.
Discussion

The present studies were designed to determine whether intratubular Ang II generation from Ang I occurs in the distal nephron. We measured changes in [Na\(^+\)]\(_i\), by using SBFI and image analysis fluorescence microscopy as a reflection of Na\(^+\) transport across the apical membrane of the CCD during luminal addition of Ang II or its precursor. With imaging and rhodamine-conjugated PNA fluorescence characteristics, we could easily differentiate principal and intercalated cell populations in the rabbit CCD.\(^{20}\) In a previous study with photometry, both the change in [Na\(^+\)]\(_i\), and the rate of increase (\(\Delta[Na^+]_i/\Delta t\)) in CCD cell [Na\(^+\)], with Ang II were measured.\(^{16}\) Nevertheless, previous work as well as the current studies both show that Ang II, when given into the tubular fluid, directly increases Na\(^+\) transport in the CCD. The current studies extend this finding by localizing the effects of Ang II on Na\(^+\) transport to principal cells and not to intercalated cells.

In the current studies, luminal Ang I (10\(^{-6}\) mol/L) increased [Na\(^+\)]\(_i\), in principal cells similar to what was obtained with Ang II (10\(^{-9}\) mol/L; Figures 1B and 2). The 3 orders of magnitude higher concentration of Ang I, compared with that of Ang II, was chosen to provide enough substrate to the convertases to produce Ang II in effective amounts. The similarity of responses evoked by Ang II at nanomolar and Ang I at micromolar concentrations might suggest that only a small fraction of Ang I was converted to Ang II in our system. However, precise estimates of conversion efficacy should not be drawn from the aforementioned data. Consistent with what has been reported for Ang II, the ENaC blocker benzamyl (10\(^{-7}\) mol/L) nearly abolished the effect of Ang I on principal cell [Na\(^+\)], but had no effect on [Na\(^+\)]\(_i\), in intercalated cells (data not shown). These results further support the conclusion that Ang II increases Na\(^+\) transport in the CCD via stimulation of ENaC. In other studies, the effect of luminal Ang I on principal cell [Na\(^+\)], was inhibited by the AT\(_1\) receptor blocker losartan. Again these results are consistent with our previous study that suggested a role for AT\(_1\) receptors in mediating the effects of Ang II on ENaC.

Therefore, these data strongly support the view that Ang I exerts its effect on ENaC through apical AT\(_1\) receptors by conversion of Ang I to Ang II. To further test this hypothesis, we sought to characterize the enzymatic activities responsible for Ang I conversion. With angiotensinogen from the proximal tubule and renin from the connecting tubule, the CCD could then be a site for the production of Ang I and the possible conversion to Ang II. ACE is reported to be secreted in the collecting duct and was identified immunologically along the whole length of swine nephron.\(^{21}\) Besides observing the highest concentration of ACE in the brush border of proximal tubules (data not shown), we confirmed its presence in the cytoplasm of tubular cells in rabbit CCD and found increasingly strong staining at the apical membrane toward the corticomedullary boundary (Figure 4). ACE does convert Ang I to Ang II, but it might influence other processes as well. For instance, ACE (kininase II) might also degrade bradykinin in the distal nephron. However, bradykinin does not appear to affect Na\(^+\) transport in the rat CCD.\(^{22}\) In our studies, captopril, which inhibits ACE, was added to the tubular lumen in the presence of Ang I. We found an \(\approx\)50% reduction in Ang I–enhanced Na\(^+\) influx in principal cells (Figure 3A), suggesting that intraluminal or apical membrane ACE is responsible for the conversion of Ang I to Ang II in the CCD.

Glandular kallikrein is also capable of converting Ang I to Ang II, and this enzyme is insensitive to captopril.\(^{23}\) Similar to ACE, kallikrein is reported to be expressed and secreted in the CCD, and this occurs mainly in cortical segments. Aprotinin is a widely used though not entirely specific blocker of glandular kallikrein. Therefore, whereas captopril is a specific blocker of ACE, aprotinin has additional effects besides blockade of glandular kallikrein. For instance, aprotinin might interfere with the actions and secretion of ACE.\(^{24}\) However, in swine kidney, ACE secretion is not influenced by aprotinin.\(^{25}\) In our studies, there was also a 50% inhibition of [Na\(^+\)]\(_i\), elevation in principal cells on addition of aprotinin and Ang I to the tubular lumen. Thus both inhibitors, captopril and aprotinin, appear to be active in the lumen and/or at the apical membrane of CCD cells, and both appear to block, at least to some extent, conversion of Ang I to Ang II. Simultaneous addition of both captopril and aprotinin appeared to further reduce the stimulatory effects of Ang I on principal cell [Na\(^+\)], although the effects of both agents together did not achieve statistical significance compared with either agent alone. It should be noted that aprotinin was used to test the effects of another proteinase on Ang I to Ang II conversion. It is possible that the effects of aprotinin occurs through kallikrein; however, it is equally possible that it also blocks ACE or other proteinases. In addition to ACE and possibly kallikrein, other enzymes are also capable of converting Ang I to Ang II, including chymase.\(^{26}\) It has been reported that certain proteinases (including trypsin and tonin\(^{23}\)) might convert angiotensinogen directly to Ang II, thus bypassing the step of conversion to Ang I. The existence and distribution of the various enzymes that form Ang II, in terms of specific tubular segments, location within defined nephron segments (cortical versus medullary and proximal versus distal, for example), and cellular distribution (apical versus cytoplasmic), might vary among species and with physiologic conditions. Thus, it might not be possible to generalize regarding the exact enzyme or enzymatic process that results in the local generation of Ang II from Ang I in the CCD.
Nevertheless, these studies do suggest that conversion of Ang I to Ang II can occur in the lumen of the CCD and that the Ang II that is formed stimulates ENaC activity via activation of AT1 receptors.

Perspectives

Ang II is not only a vasoactive hormone but also is important in controlling renal tubular salt reabsorption. Recent evidence suggests that the enzymatic components exist in renal tubules that might lead to formation of Ang II. The collecting duct is responsible for determining the final composition of urine, controlling extracellular volume and electrolyte composition, and blood pressure control. This work identified that Ang II can be formed intraluminally in the collecting duct and that this hormone can directly increase sodium reabsorption in this segment. Thus, these studies have identified the existence of a collecting duct–Ang II system that might play an important role in the regulation of collecting duct reabsorptive function, homeostasis, and blood pressure regulation.

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