Angiotensin II increases vasopressin-stimulated facilitated urea permeability in rat terminal IMCDs

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Kato, Akihiko, Janet D. Klein, Chi Zhang, and Jeff M. Sands. Angiotensin II increases vasopressin-stimulated facilitated urea permeability in rat terminal IMCDs. Am J Physiol Renal Physiol 279: F835–F840, 2000.—Angiotensin II receptors are present along the rat inner medullary collecting duct (IMCD), although their physiological role is unknown. Because urea is one of the major solutes transported across the terminal IMCD, we measured angiotensin II’s effect on urea permeability. In the perfused rat terminal IMCD, angiotensin II had no effect on basal urea permeability but significantly increased vasopressin-stimulated urea permeability by 55%. Angiotensin II, both without and with vasopressin, also increased the amount of 32P incorporated into urea transporter (UT)-A1 in inner medullary tissue exposed to these hormones ex vivo. Because angiotensin II activates protein kinase C, we tested the effect of staurosporine (SSP). In the absence of angiotensin II, SSP had no effect on vasopressin-stimulated urea permeability in the perfused terminal IMCD. However, SSP completely and reversibly blocked the angiotensin II-mediated increase in vasopressin-stimulated urea permeability. SSP and chelerythrine reduced the angiotensin II-stimulated 32P incorporation into UT-A1 in inner medullary tissue exposed ex vivo. We conclude that angiotensin II increases vasopressin-stimulated facilitated urea permeability and 32P incorporation into the 97- and 117-kDa UT-A1 proteins via a protein kinase C-mediated signaling pathway. These data suggest that angiotensin II augments vasopressin-stimulated facilitated urea transport in the rat terminal IMCD and may play a physiological role in the urinary concentrating mechanism by augmenting the maximal response to vasopressin.

inner medullary collecting duct; protein kinase C; urine-concentrating mechanism; phosphorylation; hyperosmolarity; urea transporter

THE RENIN-ANGIOTENSIN SYSTEM may play an important role in the urinary concentrating mechanism because infusing angiotensin II into the renal artery increases urine osmolality in rats (9), whereas subcutaneous injection of an angiotensin-converting enzyme (ACE) inhibitor for 5 days reduces urine osmolality in mice (27). In addition, knockout of the gene for angiotensinogen, ACE, or angiotensin II receptors impairs urine-concentrating ability (7, 8, 15, 19, 26, 27). In some of these knockout mice (angiotensinogen, ACE), the decrease in urine-concentrating ability results from anatomic abnormalities in the renal medulla. However, mice lacking the type 1A angiotensin II receptor or ACE (ACE.2 mice) have a relatively normal medulla and still have a urine-concentrating defect (8, 27).

The preceding findings suggest that angiotensin II may have a functional role in nephron segments involved in generating the hypertonic medullary gradient that is needed to concentrate the urine maximally, i.e., the medullary thick ascending limb (mTAL) and inner medullary collecting duct (IMCD). Angiotensin II receptors are present in the mTAL, and angiotensin II stimulates Na+-K+-2Cl− cotransport (1). Both RT-PCR and in situ hybridization studies show that the mRNA for angiotensin II receptors is present in the IMCD (16, 23, 38), and radioligand binding studies show that angiotensin II receptors are present (24). However, no studies have been performed to determine whether angiotensin II has a functional role in the IMCD.

Urea is one of the major solutes transported across the terminal IMCD and is important for the generation of the hypertonic medulla needed to concentrate the urine maximally [reviewed in (30)]. In rats consuming a standard diet, the terminal IMCD is the only nephron segment in which vasopressin stimulates facilitated urea transport (18, 33). In the present study, we evaluated whether angiotensin II can regulate urea transport in the terminal IMCD by testing its effect on basal and vasopressin-stimulated facilitated urea permeability. We also tested whether any effects of angiotensin II occurred via protein kinase C (PKC) because angiotensin II can activate it (1, 17, 22).

METHODS

Tissue preparation. All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee. Tubules were obtained from pathogen-free male Sprague-Dawley rats (National Cancer Institute, Frederick, MD). Rats were kept in filter-top cages with autoclaved bedding and received free access to water and a standard diet (NIH-31, Ziegler Brothers, Gardner, PA). Kidneys were placed into chilled (17°C), isotonic, dissecting solution to isolate the terminal IMCD (5, 6). The terminal IMCD was
identified by dissecting between 50 and 70% of the distance between the inner-out outer medullary border (0%) and the papillary tip (100%) as measured by using an eyepiece micrometer (32, 33).

The dissecting solution was gassed with 95% O2-5% CO2 and contained (in mM) 118 NaCl, 25 NaHCO3, 2 CaCl2, 2.5 K2HPO4, 1.2 MgSO4, 5.5 glucose, and 4 creatinine. Tubules were transferred into a bath that was continuously exchanged and bubbled with 95% O2-5% CO2 gas and perfused using standard techniques (18, 33). Solution and urine osmolalities were measured by vapor pressure osmometry (model 5500, Wescor, Logan, UT).

Urea measurement. The urea concentration in perfusate, bath, and collected fluid was measured by using a continuous-flow ultramicrofluorimeter as described (18, 32, 33). This assay is capable of resolving differences of 4% or greater in urea concentration (32). Urea flux (Jurea) was calculated as:

\[ J_{\text{urea}} = C_\text{f} V_\text{f} - C_\text{b} V_\text{b} \]

where \( C \) is the urea concentration in perfusate, \( C_\text{f} \) is the urea concentration in collected fluid, \( V \) is the perfusion rate per unit length of tubule, and \( V_\text{b} \) is the collection rate per unit length of tubule.

To study facilitated urea permeability, tubules were perfused with perfusate and bath solutions, the compositions of which were identical to the dissection solution (described above) except that 5 mM urea was added to the bath solution and 5 mM raffinose was added to the perfusate solution to create a 5 mM bath-to-lumen urea gradient without any imposed osmotic gradient (18, 33). Urea permeability was calculated from the urea flux as described (18, 33).

The urea concentration of three to four collections was measured, after which the following compounds (Sigma, St. Louis, MO) were added to the bath: 1) 10 nM arginine vasopressin (AVP; 18, 33); 2) 100 pM or 100 nM angiotensin II (39, 41); or 3) 100 nM staurosporine (SSP) (3, 13, 37); three to four additional collections then were obtained. In some experiments, the osmolality of the perfusate and bath solutions was increased from 290 to 690 mosmol/kgH2O by adding NaCl (10, 34). Twenty minutes were allowed between any solution change and the beginning of measurements.

Phosphorylation of UT-A1. Both inner medullas from a single rat (~50–75 mg) were dissected as previously described (20, 25), cut into small pieces (~5 mg/piece), washed with phosphate (P)-free DMEM, then incubated in P-free DMEM containing 0.1 mM/ml [32P]orthophosphate for 3 h at 37°C and gassed with 5% CO2. Angiotensin II (100 nM), vasopressin (10 nM), SSP (100 nM), chelerythrine (10 μM), or vehicle was added at the end of the 3-h incubation. Unincorporated [32P] was removed from the tissue by six successive washes with P-free DMEM. Tissue was then homogenized in 0.5 ml of isolation buffer (20 mM triethanolamine, 250 mM sucrose, 1 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride, pH 7.5). An equal volume of radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris, pH 7.4, 2.5 mM EDTA; 50 mM NaF; 1 mM Na2P2O7-O,10H2O; 1% Triton X-100; 10% glycerol; 1% deoxycholate, 1 μg/ml aprotinin, 0.18 mg/ml phenylmethylsulfonyl fluoride, and 0.18 mg/ml orthovanadate) was added to the homogenate, and the sample was sheared with a 26-G needle. Samples were centrifuged to remove insoluble particulates, then incubated overnight with our polyclonal anti-urea transporter (UT)-A1 (25) at 4°C with gentle mixing. Immune complexes were precipitated with protein A agarose (Pierce, Rockford, IL) for 2 h at 4°C, then the pelletted beads were washed seven times with RIPA.

Washes were counted to ensure complete removal of unbound radiolabeled material. Laemmli SDS-PAGE sample buffer was added directly to the pellets, samples were boiled for 1 min, and proteins were size-separated on 10% SDS-polyacrylamide gels.

Western blot analysis was performed as previously described (20, 25). Briefly, immunoprecipitated proteins were size-separated by SDS-PAGE on 10% Laemmli gels, electroblotted to polyvinylidene difluoride membranes (Gelman Scientific, Ann Arbor, MI), and incubated for 30 min at room temperature with blocking buffer: 5% nonfat dry milk suspended in Tris-buffered saline (TBS: 20 mM Tris-HCl, 0.5 M NaCl, pH 7.5) (20, 25). The Western blots were incubated with our affinity-purified antibody “C” to rat UT-A1 (20, 25) overnight at 4°C, then washed 3× in TBS-Tween. Blots were then incubated with horseradish peroxidase-linked goat anti-rabbit IgG at a dilution of 1:5,000 (Amersham, Arlington Heights, IL) for 2 h at room temperature, washed 2× with TBS-Tween, and the bound secondary antibody was visualized by using an enhanced chemiluminescence kit (Amersham). Laser densitometry was used to quantify the enhanced chemiluminescence signal. In all cases, parallel gels were stained with Coomassie blue and showed uniformity of loading (data not shown).

Statistics. All data are presented as means ± SE, and n = number of rats. Data from three to four collections were averaged to obtain a single value from each experimental phase in each tubule. To test for statistical significance between two groups, Student’s t-test was used. To test more than two groups, an ANOVA was used, followed by Tukey’s protected t-test (36) to determine which groups were significantly different. The criterion for statistical significance was P < 0.05.

RESULTS

Effect of angiotensin II. Adding either 100 pM or 100 nM angiotensin II to the bath had no effect on basal (no vasopressin) facilitated urea permeability (Fig. 1, solid line). In contrast, either 100 pM or 100 nM angiotensin II significantly increased vasopressin (AVP:10 nM)-stimulated facilitated urea permeability (Fig. 1, dashed line).

Phosphorylation of UT-A1. We divided the inner medullas from two rats (4 inner medullas) into tip and base samples, incubated them with [32P]orthophosphate, and immunoprecipitated the UT-A1 proteins to determine which groups were significantly different. The criterion for statistical significance was P < 0.05.

Fig. 1. Effect of ANG II and vasopressin (AVP; 10 nM) on facilitated urea permeability in the perfused rat terminal inner medullary collecting duct (IMCD). Solid line: without vasopressin. ANG II has no effect on urea permeability (n = 3, P = NS). Dashed line: with 10 nM vasopressin, ANG II increases vasopressin-stimulated urea permeability (n = 5). *P < 0.01 vs. control (vasopressin only).
test whether either the 117- or 97-kDa UT-A1 protein was a phosphoprotein. The Western blot (Fig. 2A) verified that the inner medullary tip contains both the 117- and 97-kDa glycoprotein forms of UT-A1 whereas the inner medullary base contains only the 97-kDa isofrom (29, 31). Both the 117- and 97-kDa UT-A1 proteins incorporated $^{32}$P (Fig. 2B). To verify that UT-A1 was specifically being immunoprecipitated, the precipitating antibody was preadsorbed with the immunizing peptide prior to the onset of immunoprecipitation. Preadsorption of the antibody resulted in a greater than a 90% decrease in immunoprecipitated UT-A1 (Fig. 2C, left and middle lanes). To confirm that the precipitated proteins were not the result of a non-specific association with the protein A bead system, a parallel inner medullary tissue sample was incubated with an unrelated polyclonal antibody, rabbit anti-actin. The Western blot, probed with anti-UT-A1, showed no recognition of 117- or 97-kDa isoforms in this sample (Fig. 2C, right lane).

Rather than pool tissue from two rats, subsequent experiments used inner medullas that were not divided into base and tip. We incubated inner medullary tissue with $^{32}$P orthophosphate and angiotensin II (100 nM). Figure 3A shows that a similar amount of protein was precipitated from each inner medullary sample. Treatment with angiotensin resulted in an increase in $^{32}$P incorporation both the 117- and 97-kDa UT-A1 proteins (Fig. 3B). Next, we incubated the inner medullary tip with $^{32}$P orthophosphate and angiotensin II (100 nM) and vasopressin (10 nM), which resulted in a marked increase in $^{32}$P incorporation both the 117- and 97-kDa UT-A1 proteins (Fig. 3C).

Effect of SSP. We used the PKC inhibitor SSP to test whether angiotensin II increases vasopressin-stimulated urea permeability through a PKC-mediated pathway. SSP (100 nM) had no effect on vasopressin-stimulated facilitated urea permeability but blocked the increase in vasopressin (AVP)-stimulated facilitated urea permeability due to angiotensin II (AVP: 57 ± 14, AVP + SSP: 66 ± 12, AVP + SSP + angiotensin II (100 nM): 82 ± 11 × 10^{-5} cm/s, P = NS, n = 6–10, Fig. 4). Removing SSP restored the angiotensin II-induced increase in facilitated urea permeability (AVP + angiotensin II: 183 ± 26 × 10^{-5} cm/s, P < 0.01 vs. AVP, AVP + SSP, and AVP + SSP + angiotensin II, n = 4, Fig. 4).

Incubating inner medullary tissue with $^{32}$P orthophosphate and 100 nM SSP resulted in a 30% reduc-
tion in the basal phosphorylation of the UT-A1 protein (data not shown). SSP also blocked the angiotensin II-stimulated increase in UT-A1 phosphorylation (Fig. 5A). SSP is known to be somewhat nonspecific, so to confirm the participation of PKC in the phosphorylation of UT-A1, we incubated inner medullary tissue with angiotensin II and 10 μM chelerythrine chloride (Fig. 5B). Chelerythrine is a more specific inhibitor of PKC with an inhibition constant value of 0.66 μM (4, 14); the constant value for inhibition of the nearest alternate substrate [protein kinase A (PKA)] is 170 μM. As with SSP, chelerythrine caused a decrease in basal phosphorylation and a blockade of the angiotensin II-stimulated increase in UT-A1 phosphorylation. In each experiment, parallel Western blots confirmed that the amount of immunoprecipitated UT-A1 was comparable (data not shown).

Hyperosmolality-stimulated facilitated urea transport. Hyperosmolality increases facilitated urea permeability independently of vasopressin in the rat terminal IMCD (10, 34) via an increase in intracellular calcium (11). Because signaling pathways that change intracellular calcium often involve PKC, we used SSP to determine whether PKC may be involved in mediating hyperosmolality’s stimulation of facilitated urea transport.

In the presence of vasopressin and SSP, raising perfusate and bath osmolality from 290 to 690 mosmol/kgH2O by adding NaCl significantly increased facilitated urea permeability (290 mosmol/kgH2O: 70 ± 15, 690 mosmol/kgH2O: 109 ± 31 × 10−5 cm/s, P < 0.01, n = 4, Fig. 6). However, after SSP was washed out, facilitated urea permeability increased further (135 ± 32 × 10−5 cm/s, P < 0.01 vs. AVP + SSP at 290 or 690 mosmol/kgH2O, n = 4, Fig. 6). Thus SSP partially blocked hyperosmolality’s stimulation of facilitated urea permeability.

**DISCUSSION**

The main results in this study are that 1) both the 117- and 97-kDa UT-A1 proteins are phosphoproteins; and 2) angiotensin II increases both vasopressin-stimulated facilitated urea permeability in the rat terminal IMCD.
IMCD and the amount of $[^{32}P]$orthophosphate incorporated into the 117- and 97-kDa UT-A1 proteins. These results show that angiotensin II does affect tubular transport in the terminal IMCD, presumably by activating angiotensin II receptors present in the IMCD (16, 23, 24, 38) and by increasing the phosphorylation of UT-A1. The angiotensin II-mediated increase in facilitated urea permeability was blocked by SSP, suggesting that angiotensin II increases the maximal response to vasopressin by activating PKC.

**Effect of angiotensin II on facilitated urea permeability.** In rats fed a standard diet, vasopressin-stimulated facilitated urea transport is present only in the terminal IMCD (33). It plays an important role in the urinary concentrating mechanism by permitting urea to be rapidly transported down its concentration gradient, via the UT-A1 urea transporter protein, to increase urea delivery to the inner medullary interstitium. In the present study, we measured facilitated urea transport by imposing a bath-to-lumen urea gradient across the terminal IMCD. Previous studies showed that perfusing a rat terminal IMCD with a bath-to-lumen or with a lumen-to-bath urea gradient yielded the same value for urea permeability (32). However, we did not measure active (or net) urea transport, i.e., transport of urea in the absence of an imposed urea concentration gradient, in the present study.

When an animal becomes volume depleted, both the renin-angiotensin system and vasopressin release are stimulated in an effort to conserve salt and water and restore blood pressure. Thus the angiotensin II-induced increase in vasopressin-stimulated facilitated urea permeability (present study) could augment urea reabsorption into the deepest portions of the inner medullary interstitium where it is needed to maintain the osmotic gradient and aid in water reabsorption.

**Role of PKC.** Several studies show that angiotensin II modulates tubular transport processes by activating PKC (1, 22, 40). Even in the absence of angiotensin II, PKC inhibits vasopressin-stimulated osmotic water permeability in rabbit cortical collecting ducts (CCDs) (2, 3), whereas inhibiting PKC with SSP results in an increase in both basal- and vasopressin-stimulated osmotic water permeability in the rat IMCD (28).

Although SSP is not completely specific (like most inhibitors), it did not alter vasopressin-stimulated facilitated urea permeability, suggesting that the concentration of SSP we used in this study had no effect on the PKA pathway. Breyer and colleagues (3, 13, 37) used 100 nM SSP to inhibit PKC effects on vasopressin-stimulated osmotic water permeability in the perfused rabbit CCD. The results of the present study suggest that angiotensin II increases vasopressin-stimulated facilitated urea permeability via a PKC-mediated pathway.

In Chinese hamster ovary cells, angiotensin II can potentiate vasopressin-dependent cAMP production through the activation of PKC (21). Thus it is possible that the additive effect of angiotensin II on vasopressin-stimulated facilitated urea permeability could be due to an effect of angiotensin II on intracellular cAMP production through activation of PKC. The present studies do not determine whether PKC is acting directly or indirectly through changes in intracellular cAMP.

UT-A1 contains consensus phosphorylation sites for both PKC and PKA (35). Thus it is possible that either protein kinase could phosphorylate UT-A1 and increase its activity. The fact that SSP and chelerythrine block the phosphorylation of UT-A1 by angiotensin II suggest that angiotensin II is somehow stimulating the activity of PKC. However, the fact that these PKC inhibitors reduce, but do not eliminate, the basal phosphorylation of UT-A1 suggests that another protein kinase (perhaps PKA) may also be involved in regulating the activity of UT-A1. Future studies will be needed to test this hypothesis.

The rat IMCD also expresses a dual angiotensin II/vasopressin receptor that is coupled to adenylyl cyclase (12). This receptor can be stimulated by either angiotensin II or vasopressin (12). Because we observed no effect of angiotensin II on facilitated urea permeability in the absence of vasopressin, it is unlikely that angiotensin II is working through this dual angiotensin II/vasopressin receptor.

**Role of PKC in hyperosmolality-stimulated facilitated urea permeability.** We previously found that hyperosmolality acutely stimulates facilitated urea permeability in the rat terminal IMCD via increases in intracellular calcium but has no effect on cyclic AMP production (11). Increasing osmolality by adding NaCl (or mannitol) increases facilitated urea permeability, even in the absence of vasopressin (34). In addition, hyperosmolality and vasopressin have additive stimulatory effects on facilitated urea permeability, and this stimulation is stable over the time period needed to perform the present studies (10, 34). Thus hyperosmolality is an independent stimulator of facilitated urea transport.

The results of the present study suggest that the effect of hyperosmolality on facilitated urea transport may be mediated, in part, through activation of PKC. Hyperosmolality also activates the mitogen-activated protein kinase cascade in mouse IMCD cells and rat inner medulla (42). Future studies will be needed to confirm and elucidate the role of PKC and other pathways in hyperosmolality’s stimulation of urea transport.


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