NO/cGMP signaling modulates regulation of Na\(^+\)-K\(^+\)-ATPase activity by angiotensin II in rat proximal tubules

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Zhang, Chaojie, and Philip R. Mayeux. NO/cGMP signaling modulates regulation of Na\(^+\)-K\(^+\)-ATPase activity by angiotensin II in rat proximal tubules. Am J Physiol Renal Physiol 280: F474–F479, 2001.—ANG II exerts a biphasic effect on Na\(^+\) transport in the kidney through its effects on Na\(^+\)-K\(^+\)-ATPase activity. Beginning at 10\(^{-11}\) M, ANG II increased Na\(^+\)-K\(^+\)-ATPase activity in freshly isolated rat proximal tubules to a maximum stimulation at 10\(^{-10}\) M of 1.43 ± 0.08-fold above control stimulation. Stimulation decreased progressively at concentrations >10\(^{-10}\) M to a value of 0.96 ± 0.1-fold at 10\(^{-7}\) M. In the presence of additional L-arginine, the substrate for NO synthesis, the stimulatory effect of ANG II (10\(^{-10}\) M) was lost. Conversely, N-monomethyl-L-arginine (L-NMMA), the nitric oxide (NO) synthase inhibitor, unmasked the stimulatory effect of ANG II at 10\(^{-7}\) M (1.40 ± 0.1-fold). 1H-[1,2,4]oxadiazole-[4,3-a]quinoxalin-1-one, the soluble guanylyl cyclase inhibitor, like L-NMMA, unmasked the stimulatory effect of ANG II at 10\(^{-7}\) M (1.30 ± 0.1-fold). The intracellular cGMP concentration was increased 1.58 ± 0.28-fold at 10\(^{-7}\) M ANG II. The ANG II AT\(_1\) receptor antagonist SK&F 108566 blocked the stimulatory effect of ANG II at 10\(^{-10}\) M. These data suggest that the NO/cGMP signaling pathway serves as a negative component in the regulation of Na\(^+\)-K\(^+\)-ATPase activity by ANG II.

N-monomethyl-L-arginine: 1H-[1,2,4]oxadiazole-[4,3-a]quinoxalin-1-one; SK&F 108566; angiotensin II

The effects of ANG II are, at least in part, a direct result of altered Na\(^+\)-K\(^+\)-ATPase activity (4). The mechanism behind this biphasic effect of ANG II on Na\(^+\)-K\(^+\)-ATPase activity is unclear, but the stimulatory effect on Na\(^+\)-K\(^+\)-ATPase activity is thought to be mediated by a pertussis toxin-sensitive G protein pathway (4) coupled to a decrease in intracellular cAMP formation (1, 4). Nitric oxide (NO) and cGMP antagonize many physiological effects of ANG II such as regulation of blood pressure and Na\(^+\) transport. In previous studies, we showed that ANG II, acting through the AT\(_1\) receptor, activates the NO/cGMP signaling pathway in the isolated rat proximal tubule (31). Studies by McKee et al. (19) showed that NO and cGMP reduce Na\(^+\) transport in the renal cortex by decreasing Na\(^+\)-K\(^+\)-ATPase activity. Studies by Liang and Knox (17) showed that NO and cGMP reduce the molecular activity of Na\(^+\)-K\(^+\)-ATPase in the cultured opossum kidney cells, thus reducing Na\(^+\) transport. Taken together, these studies suggest that the NO/cGMP signaling pathway may be an important physiological regulator of Na\(^+\) transport. The purpose of the present study was to examine the role of the NO/cGMP signaling pathway on the ANG II biphasic regulation of Na\(^+\)-K\(^+\)-ATPase activity in the rat proximal tubule.

METHODS

Materials and reagents. Collagenase A was purchased from Boehringer Mannheim (Indianapolis, IN). ANG II was purchased from Novabiochem (La Jolla, CA). SK&F 108566 was a generous gift from SmithKline Beecham Pharmaceuticals (King of Prussia, PA). 1H-[1,2,4]oxadiazole-[4,3-a]quinoxalin-1-one (ODQ) was purchased from Alexis (San Diego, CA). IBMX was purchased from Research Biochemicals International (Natick, MA). cGMP immunoassay kit was purchased from Amersham Life Science (Arlington Heights, IL). N-monomethyl-L-arginine (L-NMMA), L-arginine, and other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Isolation of proximal tubules. All animal experiments were approved by the University of Arkansas for Medical Sciences' Animal Care and Use Committee. Animals were housed and killed in accord with the National Institutes of Health (NIH)
Guide for the Care and Use of Laboratory Animals (NIH publication 86–23 revised 1985). Rat proximal tubules were isolated from the kidneys of male Sprague-Dawley rats (200–250 g) by collagenase digestion and Percoll density gradient centrifugation using our published methods (21, 31). The buffer used was a modified Krebs buffer (MKB) containing (in mM) 103 NaCl, 5.0 KCl, 2.0 NaH₂PO₄, 1.0 MgSO₄, 1.0 CaCl₂, 5.0 glucose, 5.0 malate, 4.0 lactate, 1.0 alanine, 20 NaHCO₃, and 10 HEPES (pH 7.4).

Measurement of cGMP. Isolated tubules were resuspended to a concentration of 1–1.5 mg/ml in MKB and preincubated with the phosphodiesterase inhibitor IBMX (1 mM) for 15 min at 37°C. Aliquots were washed one time and resuspended in the same buffer warmed to 37°C. After a 30-min incubation with ANG II, the reaction was stopped by placing each sample on ice. The aliquots were then centrifuged at 12,000 g for 2 min at 4°C. cGMP content in the cell pellet was assayed by immunoassay as described by the manufacturer. Protein concentration was measured for each sample using a 10% TCA precipitate.

Measurement of Na⁺⁻K⁺-ATPase activity. Na⁺⁻K⁺-ATPase activity was measured in a crude membrane preparation using published methods (2, 14, 17). After the incubation period in phosphate-free MBK, the tubules were allocated into two tubes and frozen in liquid nitrogen. After being thawed, the tubes were centrifuged for 2 min at 8,000 g, and the supernatant was preincubated for 5 min before the addition of ANG II. L-Arginine (1 mM, −10-fold higher than physiological concentration; see Refs. 6 and 23) was preincubated with the tubules for 5 min before the addition of ANG II. L-Arginine blocked the stimulatory effect of ANG II at 10⁻⁷ M (Fig. 2) without altering basal Na⁺⁻K⁺-ATPase activity in the proximal tubules (180 ± 7 nmol·min⁻¹·mg⁻¹, n = 7, with L-arginine compared with 199 ± 16 nmol·min⁻¹·mg⁻¹, for control). D-Arginine was used as a control for L-arginine because D-arginine cannot be metabolized by NO synthase (NOS) to yield NO. A 5-min preincubation with D-arginine (1 mM) did not alter the stimulatory effect of ANG II (10⁻¹¹ M). Values were 1.43 ± 0.05-fold above control for ANG II

RESULTS

Biphasic effect of ANG II on Na⁺⁻K⁺-ATPase activity. Na⁺⁻K⁺-ATPase activity (ouabain-inhibitable ATPase activity) was determined after incubation with various concentrations of ANG II for 30 min in freshly isolated rat proximal tubules. At the end of the 30-min incubation period, tubule viability, as measured by LDH release, was 90% or greater. Viability was unaffected by incubation with ANG II. For example, at concentrations of 10⁻⁷ and 10⁻¹¹ M, LDH release was 10.5 ± 3.0 and 9.1 ± 3.8%, respectively, compared with a control value of 9.6 ± 3.3% (n = 4). ANG II caused maximal stimulation of Na⁺⁻K⁺-ATPase activity at 10⁻¹¹ M (1.43 ± 0.08-fold increase above control, Fig. 1). The stimulatory effect began to decrease with ANG II concentrations >10⁻⁹ M. At a concentration of 10⁻⁷ M ANG II, Na⁺⁻K⁺-ATPase activity was 0.95 ± 0.08% of control. The control (basal) activity of Na⁺⁻K⁺-ATPase was 199 ± 16 nmol·min⁻¹·mg⁻¹ protein⁻¹ (n = 8) and represented ~45–50% of the total ATPase activity.

Effect of L-arginine on ANG II-stimulated Na⁺⁻K⁺-ATPase activity. The role of NO on ANG II regulation of Na⁺⁻K⁺-ATPase activity was examined using L-arginine, the substrate of NO synthesis. L-Arginine (1 mM, −10-fold higher than physiological concentration; see Refs. 6 and 23) was preincubated with the tubules for 5 min before the addition of ANG II. L-Arginine blocked the stimulatory effect of ANG II at 10⁻¹¹ M (Fig. 2) without altering basal Na⁺⁻K⁺-ATPase activity in the proximal tubules (180 ± 7 nmol·min⁻¹·mg⁻¹, n = 7, with L-arginine compared with 199 ± 16 nmol·min⁻¹·mg⁻¹, for control). D-Arginine was used as a control for L-arginine because D-arginine cannot be metabolized by NO synthase (NOS) to yield NO. A 5-min preincubation with D-arginine (1 mM) did not alter the stimulatory effect of ANG II (10⁻¹¹ M). Values were 1.43 ± 0.05-fold above control for ANG II

Data analysis. Data are reported as means ± SE. Each n represents an individual tubule preparation. Data were analyzed using the t-statistic for comparison between two groups or by a one-way ANOVA followed by the Student-Newman-Keuls test for comparison of more than two groups. P < 0.05 was considered statistically significant.
not affect the stimulatory effect of 10^{-11} M (1.32 ± 0.12 in the presence of L-NMMA). In addition, L-NMMA alone did not alter basal Na^+ -K^+ -ATPase activity in the proximal tubule (1.07 ± 0.1-fold above control, n = 7, P > 0.05).

**ANG II-stimulated formation of cGMP.** Formation of cGMP was measured 30 min after addition of various concentrations of ANG II. These data are shown in Fig. 4. ANG II at 10^{-7} M increased cGMP formation to 1.58 ± 0.22-fold above control (n = 5, P = 0.05 compared with control). ANG II at 10^{-11} and 10^{-10} M did not show any significant increase in cGMP formation (1.09 ± 0.13- and 1.04 ± 0.10-fold above control, n = 5, P > 0.05). The basal levels of cGMP after a 30-min incubation in the absence of ANG II were 0.68 ± 0.09 pmol/mg protein (n = 5).

**Effect of soluble guanylyl cyclase inhibition.** The role of cGMP on the regulation of Na^+ -K^+ -ATPase activity by ANG II was examined using ODQ, the soluble guanylyl cyclase inhibitor (15). ODQ (50 μM) was preincubated with the tubules for 5 min before the addition of ANG II (Fig. 5). ODQ restored the stimulatory effect of ANG II at 10^{-7} M (1.31 ± 0.07-fold above control, n = 5, P < 0.05 compared with control) to a value not different from that produced by 10^{-11} M of ANG II. Furthermore, ODQ did not affect the stimulatory effect of 10^{-11} M (1.25 ± 0.12 in the presence of ODQ). Also, ODQ alone did not alter basal Na^+ -K^+ -ATPase activity in the proximal tubule (1.11 ± 0.09-fold above control, n = 5, P > 0.05).

**Effects of AT 1 receptor antagonism on ANG II-stimulated Na^+ -K^+ -ATPase activity.** Our previous studies showed that the stimulation of NO and cGMP formation by ANG II (10^{-7} M) was mediated by the AT1 receptor (31). SK&F 108566, the AT1 receptor-selective antagonist (10), was used to examine the role of AT1 receptors on the regulation of Na^+ -K^+ -ATPase by ANG II. SK&F 108566 was preincubated for 5 min before the

![Fig. 2. Effect of L-arginine on ANG II-stimulated Na^+ -K^+ -ATPase activity. Tubules were preincubated with L-arginine (1 mM) for 5 min before addition of ANG II. Data (means ± SE) are expressed as the degree of increase above control and were analyzed by 1-way ANOVA followed by the Student-Newman-Keuls test. *P < 0.05 compared with all other conditions (n = 5–7).](http://ajprenal.physiology.org/)

![Fig. 3. Effect of N-monomethyl-L-arginine (L-NMMA) on ANG II-stimulated Na^+ -K^+ -ATPase activity. Tubules were preincubated with L-NMMA (2 mM) for 5 min before the addition of ANG II. Data (means ± SE) are expressed as the degree of increase above control and were analyzed by 1-way ANOVA followed by the Student-Newman-Keuls test. *P < 0.05 compared with all other conditions (n = 5–7).](http://ajprenal.physiology.org/)

![Fig. 4. ANG II-induced cGMP formation in rat proximal tubules. Tubule suspensions were incubated with various concentrations of ANG II in the presence of IBMX (1 mM) for 30 min. Data (means ± SE) are expressed as the degree of increase in cGMP levels above basal (0.68 ± 0.09 pmol·mg^{-1} min^{-1}, n = 5). Data were analyzed by 1-way ANOVA followed by the Student-Newman-Keuls test. *P = 0.05 compared with all other conditions (n = 5).](http://ajprenal.physiology.org/)
addition of ANG II. These data are presented in Fig. 6. SK&F 108566 blocked the stimulatory effect of ANG II at $10^{-11}$ M ($1.06 \pm 0.04$-fold above control, $n = 4$, $P > 0.05$ compared with control). SK&F 108566 alone did not alter the basal Na$^+/K^+$-ATPase activity in the proximal tubules ($1.14 \pm 0.06$-fold above control, $n = 4$, $P > 0.05$).

Effects of l-arginine, l-NMMA, and ODQ on intracellular Ca$^{2+}$. Data from our previous studies in the proximal tubule showed that an increase in intracellular Ca$^{2+}$ mediated by AT$_1$ receptor activation is associated with NO and cGMP generation (31). To address the possibility that the site of action of l-arginine, l-NMMA, or ODQ was at the level of Ca$^{2+}$ signaling, their effects on ANG II-induced changes in intracellular Ca$^{2+}$ concentration were determined. l-Arginine, l-NMMA, or ODQ was preincubated for 5 min with fura 2-loaded proximal tubules before the addition of ANG II. Neither of these agents affected basal intracellular Ca$^{2+}$ levels during the 5-min incubation period. The rise in intracellular Ca$^{2+}$ concentration elicited by ANG II ($10^{-7}$ M) was $244 \pm 58$ nM ($n = 3$) above basal levels. Neither agent had any effect on the rise in intracellular Ca$^{2+}$ concentration elicited by ANG II (data not shown).

**DISCUSSION**

ANG II is an important regulator of Na$^+$ transport in the kidney. Interestingly, ANG II exerts a biphasic regulation on proximal tubule Na$^+/K^+$-ATPase activity (4). At picomolar concentrations, ANG II stimulates Na$^+/K^+$-ATPase activity, whereas at nanomolar concentrations stimulation is lost. Bharatula and co-workers (4) showed that stimulation of Na$^+/K^+$-ATPase activity by ANG II was mediated by AT$_1$ receptors coupled to the inhibition of adenyl cyclase via a pertussis toxin-sensitive G protein. It is proposed that the decrease in cAMP formation can result in decreased phosphorylation of Na$^+/K^+$-ATPase through protein kinase A (1, 3, 11). Additional studies have also shown that phosphorylation of the pump by protein kinase A or protein kinase C can decrease activity (7, 12). Thus inhibition of cAMP may explain the stimulatory effects of ANG II at picomolar concentrations. The present data suggest that activation of NO/cGMP signaling is responsible, at least in part, for the loss of the stimulatory effects of ANG II at nanomolar concentrations.

In a previous study, we showed that activation of AT$_1$ receptors led to a rise in intracellular Ca$^{2+}$ concentration and the activation of cGMP formation in the rat proximal tubule (31). Studies by McKee et al. (19) showed that NO and cGMP reduced Na$^+$ transport in the rat renal cortex by decreasing Na$^+/K^+$-ATPase activity. They further showed the regulation of Na$^+/K^+$-ATPase activity involved cGMP- and cGMP-dependent protein kinase. Studies by Liang and Knox (17) reported that NO and cGMP reduced the molecular activity of Na$^+/K^+$-ATPase activity in the cultured opossum kidney cells. They also showed that NO donors acting through the cGMP pathway can activate protein kinase C-α, resulting in the inhibition of Na$^+/K^+$-ATPase activity (16). Studies with the NO donor sodium nitroprusside have suggested that NO inhibits both basal and ANG II-stimulated fluid absorption by the proximal tubule (13). However, in other studies, sodium nitroprusside was shown to produce a biphasic effect on fluid absorption in the absence of ANG II (27). Our studies reveal that endogenous synthesis of NO and the activation of the NO/cGMP signaling pathway by ANG II represents an important physiological modulator of proximal tubule Na$^+/K^+$-ATPase activity.

The following three approaches were used to evaluate the role of NO signaling: 1) increasing the substrate for NOS, 2) inhibiting NOS activity, and 3) inhibiting cGMP synthesis. Rat proximal tubules express a con-
stimulative Ca2+-regulated NOS (9, 18). In a previous study, we showed that ANG II at concentrations >10^-10 M increase intracellular Ca2+ concentration and the generation of cGMP (31). However, we and others have shown that basal levels of intracellular Ca2+ are enough to support low levels of spontaneous NO generation and cGMP formation (26, 29–31). Freshly isolated rat proximal tubules are capable of generating NO even in the absence of added l-arginine (26, 29–31) because, as the primary site of l-arginine biosynthesis (8), they can carry enough intracellular l-arginine for NO synthesis (23). Although intracellular l-arginine concentration in freshly isolated rat proximal tubules is reported to be ~2 µmol/mg protein (23), the intracellular concentration of l-arginine available for NO synthesis is not known. Nevertheless, the addition of l-arginine can increase NO synthesis (25, 30). Because l-arginine did not affect intracellular Ca2+ levels, a likely explanation for the inhibitory effects of added l-arginine is increased NO synthesis. This is supported by the lack of inhibitory effect of d-arginine on the ANG II response.

In the present study, we show that activation of NOS opposes the stimulatory effects of ANG II on Na+-K+-ATPase activity. This is supported by the observation that increasing the substrate for NOS prevents the stimulatory effects of ANG II at a concentration (10^-11 M) that under control conditions activates Na+-K+-ATPase activity. In contrast, the NOS inhibitor L-NMMA unmasksthe stimulatory effect of ANG II (10^-7 M). Thus modulation of NO synthesis modulates the effects of ANG II on Na+-K+-ATPase activity. The ability of the soluble guanylyl cyclase inhibitor ODQ to also unmask the stimulatory effect of ANG II suggests that the effects of NO are mediated by cGMP. As expected, neither L-NMMA nor ODQ affected the stimulatory effects of ANG II at 10^-11 M, a concentration that did not cause an increase in cGMP. To confirm that L-NMMA, ODQ, and l-arginine were acting at the level of NO and not AT1 receptors, we showed that these agents did not affect the ANG II-stimulated rise in intracellular Ca2+ concentration.

Interactions between the NO/cGMP signaling and the ANG II signaling pathways in the proximal tubule appear to be an example of a homeostatic mechanism to regulate Na+-K+-ATPase activity. This occurs through activation of AT1 receptors coupled to a rise in intracellular Ca2+ concentration as the concentrations of ANG II rise above 10^-10 M (EC50 for ANG II is 1.7 nM; see Ref. 31). The nonpeptide AT1 receptor antagonist SK&F 108566 has been well characterized in other tissues as a selective AT1 antagonist (10). SK&F 108566 blocks the stimulatory effects of high concentrations of ANG II on intracellular Ca2+ concentration and the generation of cGMP in the proximal tubule (31). We now show that SK&F 108566 also blocks low concentrations of ANG II that stimulate Na+-K+-ATPase activity. Thus the studies with SK&F 108566 indicate that AT1 receptors mediate both the stimulation of Na+-K+-ATPase activity and the activation of NO synthesis. NO/cGMP signaling serves to dampen the effects of increasing concentrations of ANG II. As the concentration of ANG II rises to critical levels above 10^-10 M, NO synthesis is triggered. This results in cGMP generation and inhibition of Na+-K+-ATPase activity. Because peritubular concentrations of ANG II are in the nanomolar concentration range (5, 20, 24), our data suggest that the effects of fluctuations in ANG II concentration on Na+-K+-ATPase activity are buffered by the activation of NO/cGMP signaling. The interaction between these two signaling pathways may represent an important physiological mechanism to regulate Na+-K+-ATPase activity in the proximal tubule.

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