Angiotensin II inhibits HCO$_3^-$ absorption via a cytochrome P-450-dependent pathway in MTAL

DAVID W. GOOD, THAMPI GEORGE, AND DONNA H. WANG
Departments of Medicine and of Physiology and Biophysics,
University of Texas Medical Branch, Galveston, Texas 77555

Angiotensin II inhibits HCO$_3^-$ absorption via a cytochrome P-450-dependent pathway in MTAL. Am. J. Physiol. 276 (Renal Physiol. 45): F726–F736, 1999.—The role of ANG II in the regulation of ion reabsorption by the renal thick ascending limb is poorly understood. Here, we demonstrate that ANG II (10$^{-8}$ M in the bath) inhibits HCO$_3^-$ absorption by 40% in the isolated, perfused medullary thick ascending limb (MTAL) of the rat. The inhibition by ANG II was abolished by pretreatment with eicosatetraenoic acid (10 $\mu$M), a general inhibitor of arachidonic acid metabolism, or 17-octadecynoic acid (10 $\mu$M), a highly selective inhibitor of cytochrome P-450 pathways. Bath addition of 20-hydroxyeicosatetraenoic acid (20-HETE; 10$^{-8}$ M), the major P-450 metabolite in the MTAL, inhibited HCO$_3^-$ absorption, whereas pretreatment with 20-HETE prevented the inhibition by ANG II. The addition of 15-HETE (10$^{-8}$ M) to the bath had no effect on HCO$_3^-$ absorption. The inhibition of HCO$_3^-$ absorption by ANG II was reduced by >50% in the presence of the tyrosine kinase inhibitors genistein (7 $\mu$M) or herbimycin A (1 $\mu$M). We found no role for cAMP, protein kinase C, or NO in the inhibition by ANG II. However, addition of the exogenous NO donor S-nitroso-N-acetylpenicillamine (SNAP; 10 $\mu$M) or the NO synthase (NOS) substrate l-arginine (1 mM) to the bath stimulated HCO$_3^-$ absorption by 35%, suggesting that NO directly regulates MTAL HCO$_3^-$ absorption. Addition of $10^{-11}$ to $10^{-10}$ M ANG II to the bath did not affect HCO$_3^-$ absorption. We conclude that ANG II inhibits HCO$_3^-$ absorption in the MTAL via a cytochrome P-450-dependent signaling pathway, most likely involving the production of 20-HETE. Tyrosine kinase pathways also appear to play a role in the ANG II-induced transport inhibition. The inhibition of HCO$_3^-$ absorption by ANG II in the MTAL may play a key role in the ability of the kidney to regulate sodium balance and extracellular fluid volume independently of acid-base balance.

20-hydroxyeicosatetraenoic acid; tyrosine kinases; nitric oxide; signal transduction; acid-base regulation

Angiotensin II (ANG II) participates in the regulation of renal sodium and water excretion through a variety of physiological mechanisms. These include effects on renal hemodynamics and glomerular filtration rate, regulation of aldosterone secretion, and direct effects on renal tubule transport through interactions with membrane receptors (7, 26, 28). In addition to its effects to promote renal sodium retention, ANG II stimulates H$^+$ secretion and HCO$_3^-$ reabsorption in both proximal and distal tubules (16, 17, 32, 34, 38, 57, 58), regulates H$^+$-ATPase activity in the cortical collecting duct (53), and influences the production and secretion of ammonia by proximal tubule segments (12, 41). These findings suggest that, in addition to its more clearly defined role in the control of sodium excretion and extracellular fluid volume, ANG II also may influence urinary net acid excretion and participate in the regulation of acid-base balance.

Several recent findings suggest that the thick ascending limb of the loop of Henle is a target site for ANG II-dependent regulation in the kidney. First, the thick ascending limb expresses ANG II receptors (5, 40, 44). Second, ANG II has been shown to influence the activity of apical membrane K$^+$ channels (36) and $^{86}$Rb uptake (15) in isolated thick ascending limb segments. Third, ANG II has been reported to regulate a variety of signaling pathways in thick ascending limbs, including intracellular Ca$^{2+}$ activity, NO production, protein kinase C (PKC) activity, and metabolism of arachidonic acid (AA) (3, 5, 15, 36). These studies indicate that ANG II can influence thick ascending limb function. However, the effects of ANG II on transepithelial ion reabsorption by the thick ascending limb have not been examined and the importance of various signal transduction pathways in mediating ANG II-induced effects on transepithelial transport is not understood.

The medullary thick ascending limb (MTAL) of the rat participates in the renal regulation of acid-base balance by reabsorbing much of the filtered HCO$_3^-$ that escapes the proximal tubule (19). Absorption of HCO$_3^-$ in the MTAL is mediated by apical membrane Na$^+$/H$^+$ exchange (23). Furthermore, the regulation of MTAL HCO$_3^-$ absorption is achieved primarily through regulation of this apical exchanger (19, 20, 23). Studies in both proximal and distal tubules have shown that ANG II stimulates HCO$_3^-$ absorption through the stimulation of apical membrane Na$^+$/H$^+$ exchange activity and HCO$_3^-$ absorption in the MTAL. Infusion of ANG II into rats was associated with an increase in HCO$_3^-$ absorption by the loop segment, the portion of the nephron between the late proximal convoluted tubule and early distal tubule (9). However, no studies have examined directly the effects of ANG II on HCO$_3^-$ absorption by the thick ascending limb.

The present study was designed to examine the effects of ANG II on HCO$_3^-$ absorption by the MTAL of the rat and to identify signal transduction pathways involved in ANG II-dependent regulation. The results demonstrate that ANG II inhibits HCO$_3^-$ absorption in the MTAL via a cytochrome P-450-dependent signaling pathway. This inhibition may play an important role in the regulation of renal acid-base balance.
METHODS

Materials. 17-Octadecenoic acid (ODYA) was purchased from Biomol (Plymouth Meeting, PA), 20-hydroxyeicosatetraenoic acid (20-HETE) was from Cayman Chemical (Ann Arbor, MI), and N-acetylpenicillamine (SNAP) was from Calbiochem (La Jolla, CA). ANG II, N-nitro-L-arginine methyl ester (L-NAME), L-arginine, 15(S)-hydroxyeicosatetraenoic acid (15-HETE), 5,8,11,14-eicosatetraynoic acid (ETYA); and palmitic acid were obtained from Sigma Chemical (St. Louis, MO). ANG II was prepared as a 4 × 10⁻⁴ M stock solution in dimethyl sulfoxide. The agents were diluted into bath solutions and SNAP was prepared as a 10 mM stock solution in water up to the time of experiments. MTAL were isolated and perfused in vitro, as previously described (18, 20). In brief, tubules were dissected from the inner stripe of the outer medulla at 10°C in control bath solution (see below), transferred to a bath chamber on the stage of an inverted microscope, and mounted on concentric glass pipettes for microperfusion at 37°C. The length of the perfused segments ranged from 0.49 to 0.64 mm. In all experiments, the lumen and bath solutions contained (in mM) 146 Na⁺, 4 K⁺, 122 Cl⁻, 25 HCO₃⁻, 2.0 Ca²⁺, 1.5 Mg²⁺, 2.0 phosphate, 1.2 SO₄²⁻, 1.0 citrate, 2.0 lactate, and 5.5 glucose. In most experiments, the bath also contained 0.2% fatty acid-free bovine albumin. However, for protocols involving fatty acids (ETYA, ODYA, palmitic acid, 20-HETE, and 15-HETE), albumin was omitted from the bath solutions to prevent binding and inactivation of the experimental lipids. All solutions were equilibrated with 95% O₂:5% CO₂ and ranged between pH 7.45 and 7.47 at 37°C. Bath solutions were delivered to the perfusion chamber via a continuously flowing exchange system (18). Experimental agents were added to the bath and lumen solutions as described in RESULTS.

The protocol for the study of transepithelial HCO₃⁻ absorption was as described previously (18, 20). The tubules were equilibrated for 20–30 min at 37°C in the initial perfusion and bath solutions, and the luminal flow rate (normalized per unit tubule length) was adjusted to 1.5–2.0 nl·min⁻¹·mm⁻¹. Two or three 10-min tubule fluid samples were then collected for each period (initial, experimental, and recovery). The tubules were allowed to reequilibrate for 5–15 min after an experimental agent was added to or removed from the bath solution. The absolute rate of HCO₃⁻ absorption (J [pmol·min⁻¹·mm⁻¹]) was calculated from the luminal flow rate and the difference between total CO₂ concentrations measured in perfused and collected fluids (18). An average HCO₃⁻ absorption rate was calculated for each period studied in a given tubule. When repeat measurements were made at the beginning and end of an experiment (initial and recovery periods), the values were averaged. Single tubule values are presented in Figs. 1–7. Mean values ± SE (n = no. of tubules) are presented in the text. Differences between means were evaluated using the Student’s t-test for paired data, with P < 0.05 considered statistically significant.

RESULTS

Effects of ANG II on HCO₃⁻ Absorption

The effect of ANG II on HCO₃⁻ absorption by the MTAL is shown in Fig. 1A. Addition of 10⁻⁸ M ANG II to the bath decreased HCO₃⁻ absorption by 40%, from 14.6 ± 0.5 to 8.8 ± 1.1 pmol·min⁻¹·mm⁻¹ (n = 5; P < 0.005). The inhibition was observed within 15 min after the addition of ANG II to the bath solution and was reversible. ANG II at 5 × 10⁻⁹ M induced a similar inhibition of HCO₃⁻ absorption (data not shown).

One possible explanation for the inhibition of HCO₃⁻ absorption is that it occurs as the indirect result of an ability of the kidney to regulate sodium and volume balance independently of acid-base balance.
transcellular NaCl absorption. For example, ANG II-induced stimulation of apical membrane Na\(^+-\)K\(^+-\)2Cl\(^-\) cotransport or K\(^+\) channel activity may increase intracellular Na\(^+\) activity (3, 15, 36), an effect that could secondarily reduce the driving force for apical membrane Na\(^+\)/H\(^+\) exchange and decrease HCO\(_3\)\(^-\) absorption. To test this possibility, we examined the effect of ANG II in tubules perfused with furosemide to inhibit net NaCl absorption (18). In MTAL studied with 10\(^{-4}\) M furosemide in the tubule lumen, addition of 10\(^{-8}\) M ANG II to the bath decreased HCO\(_3\)\(^-\) absorption from 12.9 \pm 1.6 to 8.4 \pm 2.1 pmol\(\cdot\)min\(^{-1}\)\(\cdot\)mm\(^{-1}\) (n = 3; P < 0.05; Fig. 1B). Thus the inhibition of HCO\(_3\)\(^-\) absorption occurs independently of effects of ANG II on net NaCl absorption.

In the proximal tubule, ANG II regulates volume and HCO\(_3\)\(^-\) absorption in a concentration-dependent manner: low concentrations (10\(^{-12}\) to 10\(^{-10}\) M) stimulate, whereas high concentrations (10\(^{-8}\) to 10\(^{-6}\) M) inhibit absorption (28, 33, 57). To determine whether a biphasic response was present for the regulation of HCO\(_3\)\(^-\) absorption in the MTAL, we examined the effects of low concentrations of ANG II. Addition of either 10\(^{-11}\) or 10\(^{-10}\) M ANG II to the bath had no effect on HCO\(_3\)\(^-\) absorption [13.5 \pm 1.2 pmol\(\cdot\)min\(^{-1}\)\(\cdot\)mm\(^{-1}\) for control vs. 13.4 \pm 1.2 pmol\(\cdot\)min\(^{-1}\)\(\cdot\)mm\(^{-1}\) for ANG II; n = 4; P = not significant (NS)]. Thus we found no evidence for biphasic regulation of HCO\(_3\)\(^-\) absorption by ANG II in the MTAL.

Signaling Pathways Involved in Inhibition by ANG II

Previously, we demonstrated that cAMP, PKC, and tyrosine kinase pathways play key roles in the regulation of MTAL HCO\(_3\)\(^-\) absorption (18, 20–22). We therefore examined the importance of these and other signaling pathways for regulation by ANG II.

Role of cAMP. cAMP inhibits HCO\(_3\)\(^-\) absorption in the MTAL (18). To determine whether cAMP is involved in the inhibition by ANG II, we examined the interaction between ANG II and arginine vasopressin (AVP). AVP inhibits HCO\(_3\)\(^-\) absorption in the MTAL by increasing cell cAMP, an effect that is maximal with an AVP concentration of 10\(^{-10}\) M (18). The results in Fig. 2A show that, in tubules bathed with 10\(^{-10}\) M AVP, addition of 10\(^{-8}\) M ANG II to the bath decreased HCO\(_3\)\(^-\) absorption from 8.0 \pm 1.5 to 4.4 \pm 0.9 pmol\(\cdot\)min\(^{-1}\)\(\cdot\)mm\(^{-1}\) (n = 3; P < 0.05). Thus the inhibitory effects of ANG II and AVP were additive, suggesting that ANG II inhibits HCO\(_3\)\(^-\) absorption via a signaling pathway independent of cAMP.

To examine further the role of cAMP in the inhibition by ANG II, MTAL were bathed with forskolin or 8-bromo-cAMP (8-BrcAMP), agents that induce maximal cAMP-dependent inhibition of HCO\(_3\)\(^-\) absorption (18). The results in Fig. 2B show that, in the presence of 10\(^{-6}\) M forskolin or 10\(^{-4}\) M 8-BrcAMP, addition of 10\(^{-8}\) M ANG II to the bath decreased HCO\(_3\)\(^-\) absorption from 8.3 \pm 0.9 to 4.2 \pm 1.0 pmol\(\cdot\)min\(^{-1}\)\(\cdot\)mm\(^{-1}\) (n = 4; P < 0.005). Together, these results establish that the inhibition of HCO\(_3\)\(^-\) absorption by ANG II is not mediated by an increase in cAMP.

Role of PKC. PKC has been suggested to play a role in ANG II-dependent regulation of HCO\(_3\)\(^-\) transport in the proximal tubule (28, 35, 57). The role of PKC in the inhibition of HCO\(_3\)\(^-\) absorption by ANG II was examined using staurosporine and chelerythrine chloride, inhibitors of PKC that selectively abolish PKC-dependent regulation of HCO\(_3\)\(^-\) absorption in the MTAL (21, 22). The results in Fig. 3 show that, in tubules bathed with 10\(^{-7}\) M staurosporine or 10\(^{-7}\) M chelerythrine chloride, addition of ANG II to the bath decreased HCO\(_3\)\(^-\) absorption from 10.8 \pm 1.6 to 6.0 \pm 1.3 pmol\(\cdot\)min\(^{-1}\)\(\cdot\)mm\(^{-1}\)
Thus the inhibition by ANG II does not involve PKC.

Role of NO. Recent studies suggest that stimulation of apical membrane K\(^+\) channels by ANG II in the MTAL is mediated by NO (36). To examine the role of NO in the ANG II-induced inhibition of HCO\(_3\)\(^-\) absorption, we performed two series of experiments. In the first series, MTAL were bathed with the NOS inhibitor L-NAME at a concentration that completely eliminated the NO-dependent effect of ANG II on K\(^+\) channel activity (1 mM) (36). The results in Fig. 4A show that, in the presence of L-NAME, addition of 10\(^{-8}\) M ANG II to the bath decreased HCO\(_3\)\(^-\) absorption from 13.7 ± 1.5 to 9.6 ± 1.3 pmol·min\(^{-1}\)·mm\(^{-1}\) (n = 4; P < 0.005). These data suggest that NO is not involved in the ANG II-dependent inhibition of HCO\(_3\)\(^-\) absorption. Addition of 1 mM L-NAME alone to the bath had no effect on HCO\(_3\)\(^-\) absorption (15.1 ± 2.2 pmol·min\(^{-1}\)·mm\(^{-1}\) in control vs. 14.6 ± 1.7 pmol·min\(^{-1}\)·mm\(^{-1}\) in L-NAME; n = 3; P = NS).

In a second series of experiments, we examined the effects on HCO\(_3\)\(^-\) absorption of two compounds that generate NO: the NOS substrate L-arginine and the exogenous NO donor SNAP (51). As shown in Fig. 4B, addition of either 1 mM L-arginine or 10 µM SNAP to the bath reversibly increased HCO\(_3\)\(^-\) absorption from 10.8 ± 1.0 to 14.8 ± 1.0 pmol·min\(^{-1}\)·mm\(^{-1}\) (n = 8; P < 0.001). Addition of 1 mM D-arginine to the bath had no effect on HCO\(_3\)\(^-\) absorption (data not shown). Thus agents that generate NO stimulate HCO\(_3\)\(^-\) absorption in the MTAL, an effect opposite to the inhibition observed with ANG II. Taken together, these results indicate that the inhibition of HCO\(_3\)\(^-\) absorption by ANG II is not mediated by NO.

Role of cytochrome P-450 and 20-HETE. Recent studies indicate that the metabolism of AA via cytochrome P-450 pathways plays a role in the regulation of NaCl absorption in the MTAL (14, 24, 59). To determine whether the metabolism of AA is involved in ANG II inhibition of HCO\(_3\)\(^-\) absorption, we examined the effect of ETYA, a general inhibitor of AA metabolic pathways (10). The results in Fig. 5A show that, in MTAL bathed with 10 µM ETYA, addition of 10\(^{-8}\) M ANG II to the bath had no effect on HCO\(_3\)\(^-\) absorption (10.9 ± 1.5 pmol·min\(^{-1}\)·mm\(^{-1}\) in ETYA vs. 11.1 ± 1.1 pmol·min\(^{-1}\)·mm\(^{-1}\) in ETYA + ANG II; n = 3; P = NS). These data suggest that metabolism of AA plays an important role in the inhibition of HCO\(_3\)\(^-\) absorption by ANG II. Addition of ETYA alone to the bath decreased the basal absorption by 10.2 ± 3.3 pmol·min\(^{-1}\)·mm\(^{-1}\) on August 27, 2017 http://ajprenal.physiology.org/ Downloaded from http://ajprenal.physiology.org/ by 10.220.33.3 on August 27, 2017
HCO₃⁻ absorption rate lines are as in Fig. 1. P values compare compound + ANG II vs. compound alone. Mean values appear in text.

To define further the involvement of the cytochrome P-450 pathway in HCO₃⁻ absorption by ODYA, a highly selective, mechanism-based inhibitor of cytochrome P-450 enzymes (39, 60). As shown in Fig. 5B, ODYA abolished the effect of ANG II to inhibit HCO₃⁻ absorption (11.0 ± 0.3 pmol·min⁻¹·mm⁻¹ in ODYA vs. 10.9 ± 0.5 pmol·min⁻¹·mm⁻¹ in ODYA + ANG II; n = 3; P = NS). To determine whether this may have been the result of a nonspecific effect of ODYA as a fatty acid, additional experiments were performed using palmitic acid, a fatty acid analog of ODYA that is inactive as a P-450 inhibitor (60). The results in Fig. 5C show that palmitic acid (10 µM in the bath) did not prevent the inhibition of HCO₃⁻ absorption by ANG II (11.5 ± 1.2 pmol·min⁻¹·mm⁻¹ in palmitic acid vs. 7.4 ± 0.8 pmol·min⁻¹·mm⁻¹ in palmitic acid + ANG II; n = 3; P < 0.05). Neither ODYA nor palmitic acid alone affected HCO₃⁻ absorption (Fig. 5, B and C). These results indicate that ANG II inhibits HCO₃⁻ absorption via a cytochrome P-450-dependent pathway.

To define further the involvement of the cytochrome P-450 pathway in HCO₃⁻ transport regulation, we examined the effects of 20-HETE, the major P-450 metabolite of AA in the MTAL (11, 14, 36). If ANG II inhibits HCO₃⁻ absorption by increasing the production of 20-HETE, then pretreatment with 20-HETE should prevent ANG II-induced inhibition, and the addition of 20-HETE alone should inhibit HCO₃⁻ absorption. The results in Fig. 6A show that, in tubules bathed with 10⁻⁸ M 20-HETE, addition of 10⁻⁸ M ANG II to the bath had no effect on HCO₃⁻ absorption (11.2 ± 0.5 pmol·min⁻¹·mm⁻¹ in 20-HETE vs. 11.2 ± 0.4 pmol·min⁻¹·mm⁻¹ in 20-HETE + ANG II; n = 4; P = NS). The results in Fig. 6B show that the addition of 10⁻⁸ M 20-HETE to the bath solution decreased HCO₃⁻ absorption from 13.2 ± 1.2 to 9.6 ± 1.1 pmol·min⁻¹·mm⁻¹ (n = 4; P < 0.005). The inhibition by 20-HETE was selective for this metabolite because bath addition of 15-HETE, another endogenously produced HETE (59), had no effect on HCO₃⁻ absorption (11.4 ± 2.0 pmol·min⁻¹·mm⁻¹ in control vs. 11.5 ± 2.2 pmol·min⁻¹·mm⁻¹ in 15-HETE; n = 3; P = NS; Fig. 6C). These results demonstrate that 20-HETE inhibits HCO₃⁻ absorption in the MTAL and that the inhibitory effects of ANG II and 20-HETE are not additive. Taken together, our data support the conclusion that ANG II inhibits HCO₃⁻ absorption via a cytochrome P-450-dependent pathway that involves the production of 20-HETE.

Role of tyrosine kinase pathways. Tyrosine kinase pathways play a key role in the regulation of MTAL HCO₃⁻ absorption (20, 22) and have been implicated in signal transduction by ANG II in cultured proximal tubule and mesangial cells (37, 54). To examine whether tyrosine kinase pathways are involved in the inhibition of HCO₃⁻ absorption, tubules were bathed with genis-
tein or herbimycin A, inhibitors that selectively block tyrosine kinase-dependent regulation of HCO$_3^-$ absorption in the MTAL (20, 22). The results in Fig. 7 show that, in the presence of 7 µM genistein or 1 µM herbimycin A, addition of 10$^{-8}$ M ANG II to the bath decreased HCO$_3^-$ absorption by only 17%, from 17.2 ± 0.6 to 14.4 ± 0.5 pmol·min$^{-1}$·mm$^{-1}$ (n = 5; P < 0.005). This decrease is less than half that observed under identical conditions in the absence of the inhibitors (P < 0.025; Fig. 1A), indicating that the tyrosine kinase inhibitors partially block ANG II action. These results suggest that tyrosine kinase pathways play a role in the inhibition of HCO$_3^-$ absorption by ANG II.

DISCUSSION

The role of ANG II in the regulation of ion reabsorption by the thick ascending limb is poorly understood. The present study demonstrates that ANG II directly inhibits HCO$_3^-$ absorption in the MTAL of the rat. This inhibition is mediated via a cytochrome P-450-dependent signaling pathway that likely involves the production of 20-HETE. Tyrosine kinase pathways also appear to play a role in the ANG II-induced transport inhibition. As discussed below, the effect of ANG II to decrease luminal acidification in the MTAL may play an important role in preserving acid-base balance when sodium intake and extracellular fluid volume are changed.

ANG II Inhibits HCO$_3^-$ Absorption in the MTAL

Our studies identify ANG II as a factor directly involved in the regulation of acid-base transport in the MTAL. We found that ANG II inhibited HCO$_3^-$ absorption to a similar extent in the absence and presence of luminal furosemide, which indicates that the inhibition occurs independent of effects on net NaCl absorption and likely is mediated through regulation of transporters directly involved in transcellular HCO$_3^-$ absorption. In the MTAL, apical membrane Na$^+$/H$^+$ exchange mediates virtually all of the H$^+$ secretion necessary for HCO$_3^-$ absorption (19, 23). Thus it is very likely that ANG II inhibits apical Na$^+$/H$^+$ exchange activity in the MTAL, an effect opposite to its physiological action to stimulate apical Na$^+$/H$^+$ exchange and HCO$_3^-$ absorption in the proximal tubule and early distal tubule (16, 17, 28, 34, 38, 57, 58). At this point, we do not know whether the ANG II signaling pathway is coupled directly to inhibition of the apical Na$^+$/H$^+$ exchanger or if ANG II may act indirectly to reduce the activity of the exchanger through effects on other transporters. In the proximal tubule, ANG II increases HCO$_3^-$ absorption by stimulating directly both apical membrane Na$^+$/H$^+$...
exchange and basolateral membrane Na\(^+\)-HCO\(_3^-\) countertransport (17). The basolateral HCO\(_3^-\) transporters involved in HCO\(_3^-\) absorption in the MTAL are not understood, but may include Na\(^+\)-HCO\(_3^-\) cotransport, K\(^+\)-HCO\(_3^-\) cotransport, and/or Cl\(^-\)/HCO\(_3^-\) exchange (19). Further direct studies of the effects of ANG II on apical membrane Na\(^+\)/H\(^+\) exchange activity and basolateral membrane HCO\(_3^-\) efflux pathways are needed to define the mechanism of HCO\(_3^-\) transport inhibition in the MTAL.

Previous studies in the proximal tubule have demonstrated a dose-dependent, biphasic response to ANG II, with low concentrations (10\(^{-12}\) to 10\(^{-10}\) M) stimulating volume and HCO\(_3^-\) absorption and high concentrations (10\(^{-8}\) to 10\(^{-6}\) M) inhibiting absorption (28, 33, 57). A similar concentration dependence has been reported in the MTAL for ANG II regulation of apical membrane K\(^+\) channels (36), \(^{86}\)Rb uptake (15), and Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport activity (3). These biphasic responses appear to reflect the activation by ANG II of multiple signal transduction pathways (15, 28, 33, 36). In contrast, we found no evidence for biphasic regulation of HCO\(_3^-\) absorption in the MTAL: 5 \times 10\(^{-3}\) to 10\(^{-1}\) M ANG II inhibited HCO\(_3^-\) absorption, whereas 10\(^{-11}\) to 10\(^{-10}\) M ANG II had no effect. Importantly, concentrations of ANG II measured in proximal tubule fluid and star vessel plasma in the rat kidney cortex in vivo ranged from 10 to 40 nM, values several orders of magnitude higher than concentrations in systemic plasma (6, 42, 49). Furthermore, ANG II levels in the renal medulla are even higher than those in the cortex (42). Thus the concentrations of ANG II that inhibit HCO\(_3^-\) absorption in the MTAL are similar to ANG II levels measured in the renal medulla in vivo, suggesting that the transport effects observed represent physiologically relevant regulation.

In recent in vivo microperfusion studies, infusion of ANG II into rats increased HCO\(_3^-\) absorption by the surface loop segment, the portion of the nephron between the late proximal convoluted tubule and early distal tubule (9). Based on this observation, it has been inferred that ANG II stimulates HCO\(_3^-\) absorption in the thick ascending limb (9, 15), a finding in apparent contrast to the results of the present study. It is important to note, however, that the MTAL is short in surface nephrons and does not contribute significantly to net HCO\(_3^-\) reabsorption measured for the surface loop segment as a whole. When this is considered along with our observation that ANG II directly inhibits HCO\(_3^-\) absorption in the MTAL, it appears likely that the stimulation of HCO\(_3^-\) absorption by ANG II in the surface loop may be due to effects on segments other than the thick ascending limb. In particular, the stimulation may take place in the proximal straight tubule and/or early distal tubule, segments in which ANG II has been demonstrated to increase HCO\(_3^-\) absorption (16, 58). Our results establish, however, that the increase in HCO\(_3^-\) absorption observed in the surface loop segment in vivo is unlikely to be the result of a direct stimulation of HCO\(_3^-\) absorption by ANG II in the MTAL.

Two pharmacologically distinct ANG II receptors, AT\(_1\) and AT\(_2\), have been identified and cloned (25, 45). AT\(_1\) is the predominant receptor type in the kidney and is thought to mediate most of the effects of ANG II on transport in the proximal tubule (7, 8, 45). AT\(_1\) receptors also have been localized to the thick ascending limb (5, 44). In recent preliminary studies, we confirmed the expression of AT\(_1\) receptors in the MTAL of the rat and demonstrated that the inhibition of HCO\(_3^-\) absorption by ANG II was blocked by the AT\(_1\) receptor antagonist losartan (55). Thus the regulation of HCO\(_3^-\) absorption by ANG II in the MTAL likely is mediated through intracellular signals generated by the interaction of ANG II with basolateral membrane AT\(_1\) receptors.3

Signal Transduction by ANG II

Our results demonstrate that ANG II inhibits HCO\(_3^-\) absorption in the MTAL via cytochrome P-450- and tyrosine kinase-dependent signaling pathways. In contrast, we found no role for cAMP, PKC, or NO in mediating the ANG II-induced transport inhibition. The lack of involvement of CAMP is noteworthy because the adenyl cyclase-protein kinase A pathway is believed to play an important role in the regulation of apical membrane Na\(^+\)/H\(^+\) exchange and HCO\(_3^-\) absorption by ANG II in the proximal tubule (7, 13, 28, 34). Thus our studies identify a distinct difference in the signaling pathways that couple ANG II to the regulation of HCO\(_3^-\) absorption in the proximal tubule and MTAL. An ancillary finding of our study is that NO appears to be a potent stimulator of MTAL HCO\(_3^-\) absorption. These findings are discussed below in the context of current understanding of signal transduction in the MTAL.

Role of cytochrome P-450 pathways in inhibition by ANG II. The metabolism of AA to biologically active products by cytochrome P-450 enzymes plays a key role in the regulation of a variety of renal processes, including vascular resistance, tubuloglomerular feedback, and sodium reabsorption and excretion (27, 29, 46). The MTAL has a high cytochrome P-450 enzyme activity and has been identified as an important site of endogenous production of P-450 metabolites, predominantly 20-HETE (11, 14, 36, 59). In the present study, we demonstrate that inhibition of HCO\(_3^-\) absorption by ANG II in the MTAL is mediated via a cytochrome P-450-dependent pathway that most likely involves the production of 20-HETE. This conclusion is based on several observations: 1) the ANG II-induced inhibition

---

3In the proximal tubule, ANG II influences ion transport through interactions with either apical or basolateral membrane AT\(_1\) receptors (7). In the MTAL, addition of 10\(^{-8}\) M ANG II to the lumen had no effect on HCO\(_3^-\) absorption (T. George and D. Good, unpublished observations). Thus, if apical receptors for ANG II are present in the MTAL, then they have a different concentration dependence than the basolateral receptors and/or are coupled to signaling pathways that do not influence HCO\(_3^-\) absorption.
of \( \text{HCO}_3^- \) absorption is abolished by ODYA, a highly selective inhibitor of cytochrome P-450 enzymes (39, 60); 2) addition of 20-HETE inhibits \( \text{HCO}_3^- \) absorption, whereas addition of 15-HETE has no effect; 3) the inhibitory effects of 20-HETE and ANG II are not additive, consistent with these factors decreasing \( \text{HCO}_3^- \) absorption via a common mechanism; and 4) ANG II increases the production of 20-HETE in isolated MTAL segments (36). Previous studies in rats have demonstrated that endogenously produced 20-HETE inhibits net chloride absorption by the loop segment in vivo (59) and the MTAL in vitro (24). Our study identifies an additional physiological role for P-450-derived 20-HETE in the MTAL, namely, regulation of acid secretion and transepithelial \( \text{HCO}_3^- \) absorption.

Our experiments demonstrate an important role for the P-450 \( \omega \)-hydroxylase product 20-HETE in mediating ANG II inhibition of \( \text{HCO}_3^- \) absorption; however, other AA products that may affect ion transport are influenced by ANG II in renal tubules. In a recent study in rat MTAL suspensions, inhibition of \( ^{86}\text{Rb} \) uptake by ANG II was mediated through stimulation of PGE\(_2\) production via a cyclooxygenase pathway (15). However, it is unlikely that this pathway is involved in ANG II-induced inhibition of \( \text{HCO}_3^- \) absorption because 1) cyclooxygenase-dependent production of PGE\(_2\) is not inhibited by ODYA (39, 60), and 2) PGE\(_2\) does not inhibit \( \text{HCO}_3^- \) absorption in the MTAL (21). In the proximal tubule, production of epoxyeicosatrienoic acids (EETs) via P-450 epoxygenases is increased by ANG II (13, 43, 46), and EETs have been shown to influence ion transport in proximal tubules and collecting ducts (13, 29, 46). However, production of EETs is low in the MTAL and is not altered by ANG II (11, 36). Also, EETs (5,6- and 11,12-EET) did not affect chloride absorption or \( K^+ \) channel activity in isolated rat MTALs (24, 36). These findings suggest that EETs are unlikely to be involved in the ANG II-dependent regulation of \( \text{HCO}_3^- \) absorption. It should be noted, however, that the MTAL produces other P-450 metabolites, including 19-HETE and 20-COOH-AA, the formation of which is inhibited by ODYA (11, 43, 60). We cannot rule out the possibility that one or more of these products, in addition to 20-HETE, may contribute to the inhibition of \( \text{HCO}_3^- \) absorption.

Although the mechanism by which cytochrome P-450 pathways regulate \( \text{HCO}_3^- \) absorption in the MTAL is not known, some effects of P-450 metabolites on MTAL transport pathways have been described. 20-HETE inhibits apical membrane \( \text{Na}^+\text{-K}^+\text{-2Cl}^- \) cotransport and \( K^+ \) channel activity in the MTAL (2, 14, 36), effects that likely mediate its action to inhibit net chloride absorption (24, 59). As noted above, however, we found that ANG II inhibits \( \text{HCO}_3^- \) absorption to a similar extent in tubules perfused with and without furosemide to block \( \text{Na}^+\text{-K}^+\text{-2Cl}^- \) cotransport, indicating that the ANG II-induced P-450 pathway inhibits \( \text{HCO}_3^- \) absorption independent of effects on net chloride absorption. In segments of the proximal tubule and collecting duct, 20-HETE has been shown to inhibit \( \text{Na}^+\text{-K}^+\text{-ATPase} \) activity (46–48). However, 20-HETE and 20-COOH-AA did not affect \( \text{Na}^+\text{-K}^+\text{-ATPase} \) activity in MTAL suspensions (14), suggesting that inhibition of the \( \text{Na}^+ \) pump does not account for ANG II-induced inhibition of \( \text{HCO}_3^- \) absorption. AA pathways have been suggested to play a role in mediating cAMP-independent regulation of apical membrane \( \text{Na}^+\text{-H}^+ \) exchange activity by ANG II in proximal tubule cells (13, 38). At present, however, the role of cytochrome P-450 pathways in the regulation of \( \text{Na}^+\text{-H}^+ \) exchange in renal tubules is not understood. Thus direct studies of the effects of P-450 metabolites on \( \text{Na}^+\text{-H}^+ \) exchange activity in the MTAL will be important not only to understand the role of P-450 pathways in the regulation of this transport process, but also to identify the mechanism by which ANG II inhibits MTAL \( \text{HCO}_3^- \) absorption.

Role of tyrosine kinase pathways in inhibition by ANG II. We have shown previously that tyrosine kinase pathways play a crucial role in the regulation of \( \text{HCO}_3^- \) absorption by hyperosmolality and growth factors in the MTAL (20, 22). In the present study, the effect of ANG II to inhibit \( \text{HCO}_3^- \) absorption was reduced by >50% in the presence of genistein or herbimycin A, two chemically unrelated tyrosine kinase inhibitors with different mechanisms of action (20). Thus tyrosine kinases appear to be important components of the signaling pathway through which ANG II inhibits \( \text{HCO}_3^- \) absorption. Recent studies indicate that tyrosine kinase pathways are involved in ANG II-induced regulation of ion transport in mesangial cells (37) and OKP cells, a proximal tubule cell line (54). In particular, in OKP cells, the nonreceptor tyrosine kinase c-src appeared to play a role in mediating ANG II regulation of NHE3 (54), the apical \( \text{Na}^+\text{-H}^+ \) exchanger isoform that mediates \( H^+ \) secretion and \( \text{HCO}_3^- \) absorption in the MTAL and proximal tubule (1, 4, 20). Thus c-src may be a component of the tyrosine kinase pathway involved in inhibition of \( \text{HCO}_3^- \) absorption by ANG II in the MTAL.

Our findings indicate that the inhibition of \( \text{HCO}_3^- \) absorption by ANG II likely involves both cytochrome P-450-dependent and tyrosine kinase pathways. The observation that blocking the cytochrome P-450 pathway with ODYA completely eliminated the inhibition of \( \text{HCO}_3^- \) absorption indicates that signaling through the P-450 pathway is necessary for \( \text{HCO}_3^- \) transport regulation, and that the ANG II-induced tyrosine kinase pathway is incapable of inhibiting \( \text{HCO}_3^- \) absorption independent of the cytochrome P-450 pathway. These findings suggest that the cytochrome P-450 and tyrosine kinase effectors may lie within a single regulatory pathway. It is possible that tyrosine kinases may be located upstream or downstream of the cytochrome P-450 enzymes in the ANG II-induced signaling cascade. Alternatively, tyrosine kinases may be activated in a parallel pathway that interacts with and modifies the activity of the cytochrome P-450-dependent AA pathway. Further work is needed to identify the tyrosine kinase(s) regulated by ANG II in the MTAL and to understand the nature of the relationship between the
tyrosine kinase and cytochrome P-450 signaling pathways.

NO stimulates HCO₃⁻ absorption. Based on the recent observation that NO was involved in stimulation of apical membrane K⁺ channels by ANG II in the rat MTAL (36), we investigated its possible role in the regulation of HCO₃⁻ absorption. We found no role for NO in mediating the inhibition of HCO₃⁻ absorption by ANG II. However, we discovered that NO itself appears to be a potent stimulator of MTAL HCO₃⁻ absorption. Specifically, we found that HCO₃⁻ absorption was increased reversibly by the addition of either an exogenous NO donor or the endogenous NO substrate L-arginine. The latter result suggests that the MTAL is capable of producing NO and that the locally produced NO can act directly to regulate HCO₃⁻ absorption. This conclusion is supported by studies demonstrating that NOS isoforms are expressed in MTAL segments (31) and that endogenous NO inhibits chloride absorption in the microperfused rat cortical thick ascending limb (52). In addition, NO has been reported recently to stimulate HCO₃⁻ absorption in the proximal convoluted tubule of the rat in vivo (56). Thus, although more extensive studies clearly are needed, our results identify NO as a factor that may be directly involved in the physiological control of acid secretion and HCO₃⁻ absorption in the MTAL.

If ANG II increases the production of NO in the MTAL, as has been suggested (36), then one might expect that stimulation of HCO₃⁻ absorption via the NO pathway would oppose inhibition via the cytochrome P-450 pathway. We found, however, that the inhibition of HCO₃⁻ absorption by ANG II was not potentiated in tubules pretreated with L-NAME and that ODYA did not unmask stimulation of HCO₃⁻ absorption by ANG II. These findings suggest either that ANG II-induced production of NO is minimal under the conditions of our experiments or that activation of the cytochrome P-450 pathway may suppress the stimulatory effect of NO.

Physiological Significance

Previously, we proposed that the MTAL plays an important role in the ability of the kidneys to maintain acid-base balance when sodium balance and extracellular fluid volume are altered (19). The results of the present study identify ANG II as a factor that may contribute to this process. Activation of the renin-angiotensin system in response to sodium and volume depletion results in several effects on renal acid-base transport: 1) direct stimulation of HCO₃⁻ absorption in proximal and distal tubules by ANG II (16, 32, 34, 57, 58); 2) increased release of aldosterone, which directly stimulates acid secretion by the collecting ducts (19); and 3) stimulation of ammonium production by ANG II in the proximal tubule (12, 41). Other effects of volume depletion, such as changes in hemodynamics and catecholamine levels, also act to enhance proximal tubule HCO₃⁻ absorption (50). Together, these effects would act synergistically to increase urinary net acid excretion and promote the development of metabolic alkalosis.

We suggest, however, that changes in acid excretion are minimized because the above effects, which tend to increase urinary acidification, are opposed by a decrease in luminal acidification along the MTAL, mediated in part by the direct inhibitory action of ANG II. That is, when sodium intake and extracellular fluid volume are decreased, the effect of ANG II to inhibit HCO₃⁻ absorption in the MTAL would offset ANG II stimulation of HCO₃⁻ absorption in the proximal and distal tubules and aldosterone stimulation of H⁺ secretion in the collecting ducts to stabilize net acid excretion. When sodium intake and volume are increased, net acid excretion would be maintained through changes opposite to those described above. In this way, the direct action of ANG II to inhibit HCO₃⁻ absorption in the MTAL would play a key role in enabling the kidney to regulate extracellular fluid volume independently of acid-base balance. Finally, in addition to this physiological role, the inhibition of HCO₃⁻ absorption by ANG II may contribute to changes in net acid excretion under pathophysiological conditions. For example, an increase in luminal acidification along the MTAL mediated by a decrease in ANG II levels may contribute to the metabolic alkalosis observed in primary hyperaldosteronism.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-38217 (to D. W. Good).

Address for reprint requests and other correspondence: D. W. Good, Division of Nephrology, 4.200 John Sealy Annex, Univ. of Texas Medical Branch, Galveston, TX 77555.

Received 10 December 1998; accepted in final form 12 February 1999.

REFERENCES


