Endothelin inhibits thick ascending limb chloride flux via ETₐ receptor-mediated NO release

CRAIG F. PLATO,¹ DAVID M. POLLOCK,² AND JEFFREY L. GARVIN¹
¹Division of Hypertension and Vascular Research, Henry Ford Hospital, Detroit, Michigan 48202; and ²Vascular Biology Center, Medical College of Georgia, Augusta, Georgia 30912

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Plato, Craig F., David M. Pollock, and Jeffrey L. Garvin. Endothelin inhibits thick ascending limb chloride flux via ETₐ receptor-mediated NO release. Am J Physiol Renal Physiol 279: F326–F333, 2000.—Endothelin-1 (ET-1) inhibits transport in various nephron segments, and the thick ascending limb of the loop of Henle (TALH) expresses ET-1 receptors. In many tissues, activation of ETₐ receptors stimulates release of NO, and we recently reported that endogenous NO inhibits TALH chloride flux (J_Cl). However, the relationship between ET-1 and NO in the control of naphron transport has not been extensively studied. We hypothesized that ET-1 decreases NaCl transport by cortical TALHs through activation of ETₐ receptors and release of NO. Exogenous ET-1 (1 nM) decreased J_Cl from 118.3 ± 15.0 to 62.7 ± 13.6 pmol · mm⁻² · min⁻¹ (48.3 ± 8.2% reduction), whereas removal of ET-1 increased J_Cl in a separate group of tubules from 87.6 ± 10.7 to 115.2 ± 10.3 pmol · mm⁻² · min⁻¹ (34.5 ± 6.2% increase). To determine whether NO mediates the inhibitory effects of ET-1 on J_Cl, we examined the effect of inhibiting of NO synthase (NOS) with N⁵-nitro-L-arginine methyl ester (L-NAME) on ET-1-induced changes in J_Cl. L-NAME (5 mM) completely prevented the ET-1-induced reduction in J_Cl, whereas D-NAME did not. L-NAME alone had no effect on J_Cl. These data suggest that the effects of ET-1 are mediated by NO. Blockade of ETₐ receptors with BQ-788 prevented the inhibitory effects of 1 nM ET-1. Activation of ETₐ receptors with sarafotoxin S6c mimicked the inhibitory effect of ET-1 on J_Cl (from 120.7 ± 12.6 to 75.4 ± 13.3 pmol · mm⁻² · min⁻¹). In contrast, ETₐ receptor antagonism with BQ-610 did not prevent ET-1-mediated inhibition of TALH J_Cl (from 96.5 ± 10.4 to 69.5 ± 8.6 pmol · mm⁻² · min⁻¹). Endothelin increased intracellular calcium from 96.9 ± 14.0 to 191.4 ± 11.9 nM, an increase of 110.8 ± 26.1%. We conclude that exogenous endothelin indirectly decreases TALH J_Cl by activating ETₐ receptors, increasing intracellular calcium concentration, and stimulating NO release. These data suggest that endothelin acts as a physiological regulator of TALH NO synthesis, thus inhibiting chloride transport and contributing to the natriuretic effects of ET-1 observed in vivo.

kidney; nitric oxide; tubular transport

THE ENDOTHELINS (ET) are a family of three 21-amino-acid peptides (19) that have potent effects on the cardiovascular system. Receptors for ET-1 have been identified in the kidney (11, 25), where both ETₐ and ETₐ receptors are expressed (3, 18). ET-1 promotes natriuresis and diuresis (21, 42) at concentrations that do not alter systemic or renal hemodynamics, suggesting that ET-1 directly inhibits tubular sodium and water reabsorption. Tomita et al. (47) and Zeidel et al. (50) reported that ET-1 decreased transport in cortical and inner medullary collecting ducts, respectively. More recently, de Jesus Ferreira and Bailly (4) demonstrated that ET-1 inhibited transport in mouse cortical and medullary thick ascending limbs.

Nitric oxide (NO) is synthesized from L-arginine (L-Arg) by nitric oxide synthases (NOS) (32) and exerts significant effects on the cardiovascular system. NOS mRNA has been detected in various nephron segments, including the thick ascending limb (30). Protein expression of all three NOS isoforms has been detected in the outer medulla (which contains thick ascending limbs) using Western blots (29). Tojo et al. (46) described positive immunolabeling of constitutively expressed NOS in the thick ascending limb, and more recently endothelial NOS has been specifically localized to the thick ascending limb using immunocytochemical techniques (1).

Previous studies have demonstrated that NO plays an important role in the control of renal sodium excretion both in vivo (27) and in vitro (40, 44, 51). We recently reported that thick ascending limb transport is directly inhibited by endogenously produced NO (37). Although the physiological regulation of tubular NOS is poorly understood, ET-1 acts via stimulation of NO release in several tissues. For example, ET-1 administration in vivo causes transient vasodilation followed by sustained vasoconstriction (49). The initial vasodilation is attenuated by blocking NO synthesis (5) but is dependent on activation of ETₐ receptors (17). These findings suggest that endothelial ETₐ receptor activation stimulates NO release.

The thick ascending limb expresses ETₐ receptors (45) and produces NO (37); however, it is not clear whether ET-1 can inhibit transport via an NO-dependent mechanism. We hypothesized that ET-1 decreases sodium chloride absorption in the thick ascending limb...
by activating ETB receptors, stimulating NOS, and increasing the production of endogenous NO. Our findings indicate that exogenous ET-1 inhibits chloride absorption in isolated perfused thick ascending limbs via activation of ETB receptors, acting through an NOS-dependent mechanism. Thus ET-1 may function as a physiological regulator of thick ascending limb NOS activity.

METHODS

Preparation of isolated nephron segments. Cortical thick ascending limbs were obtained from male Sprague-Dawley rats weighing 120–150 g (Charles River, Wilmington, MA) maintained on a diet containing 0.22% sodium and 1.1% potassium (Purina, Richmond, IN) with water ad libitum for at least 5 days. On the day of the experiment, rats were anesthetized with ketamine (100 mg/kg body wt ip) and xylazine (20 mg/kg body wt ip) and the abdominal cavity was opened. The kidney was bathed in ice-cold saline and removed. Coronal slices were placed in oxygenated physiological saline at 12°C. Cortical thick ascending limbs were dissected from medullary rays in the same solution under a stereomicroscope.

Thick ascending limb perfusion. Cortical thick ascending limbs (0.5 to 0.9 mm in length) were transferred to a temperature-regulated chamber and perfused between concentric glass pipettes at 37°C as described previously (37). The composition of the basolateral bath and perfusate (in mM) was 114 NaCl, 25 NaHCO3, 2.5 NaH2PO4, 4 KCl, 1.2 MgSO4, 6 alanine, 1 trisodium citrate, 5.5 glucose, 2 calcium dilactate, 5 raffinose. Endothelin (ET-1), the NOS inhibitor L-NAME (N-nitro-l-arginine methyl ester) and its enantiomer D-NAME (N-nitro-d-arginine methyl ester), the substrate for NOS, i.e., l-arginine, and the ETB-specific agonist sarafotoxin S6c (S6c) were all purchased from Sigma Chemical (St. Louis, MO). The ETA- and ETB-selective antagonists (BQ-610 and BQ-788, respectively) were purchased from Peninsula Labs (Belmont, CA).

We recently demonstrated that the ability of renal tubules to synthesize NO in vitro is substrate limited (37). Thus, in the current studies, we included 4 μM L-arginine in the basolateral bath and bath to approximate the K_m of NOS for l-arginine (41). To determine the dependence of the ET-1 response on NO production, a separate series of experiments was conducted, measuring thick ascending limb J_cl in the absence of exogenous l-arginine. Solutions were bubbled with 5% CO₂ and 95% O₂ before and during the experiment. The pH of the bath was 7.4 and the osmolality was 290 ± 3 mosmol/kgH₂O as measured by freezing-point depression. The basolateral bath was exchanged at a rate of 0.5 ml/min, and tubules were perfused at 5–10 nl · mm⁻¹ · min⁻¹. Time-control experiments were conducted for each protocol to determine the stability of tubular transport.

Net chloride flux. Chloride concentrations were determined in samples of perfusate and collected fluid using a fluorometric technique described elsewhere (8). Because chloride reabsorption was not accompanied by significant fluid reabsorption, net chloride flux (J_cl) was calculated according to the formula

\[ J_{cl} = PR([Cl_i] - [Cl_l]) \]

where PR is the perfusion rate normalized for tubule length, Cl_i is the chloride concentration in the perfusion fluid, and Cl_l is the chloride concentration in the collected tubular fluid.

Experimental protocols. The transepithelial chloride flux studies consisted of two types of protocols. The first protocol was conducted to determine the effects of 1) endothelin receptor agonists and antagonists and 2) NOS inhibition on thick ascending limb chloride flux. After a 20-min equilibration period, three basal measurements were performed (control period). ET-1, an ETB receptor agonist (S6c) or antagonist (BQ-788), an ETA receptor antagonist (BQ-610), or an NOS inhibitor (l-NAME) was then added to the bath. Following a second 20-min equilibration period, three additional collections were made (experimental period). To evaluate the reversibility of the response to ET-1, a separate protocol was conducted with ET-1 in the bath during the initial period (basal), and then ET-1 was removed from the bath (recovery).

The second protocol was conducted to determine the effect of the selective ETA or ETB receptor antagonists or NOS inhibition on the response of thick ascending limb chloride flux to stimulation with ET-1. After a 20-min equilibration period with l-NAME, BQ-688, or BQ-610 in the bath, there was a control period consisting of three basal measurements. ET-1 (1 nM) was then added to the bath in the presence of the antagonist or inhibitor. Following a 20-min equilibration period, three additional collections were performed.

Intracellular calcium concentration. The effects of ET-1 on intracellular calcium concentration were determined in individual isolated, perfused thick ascending limbs using a ratiometric fluorescent indicator technique (16). Briefly, tubules were isolated, perfused, and superfused as described above and incubated with 5 μM fura-2 AM (Molecular Probes, Eugene, OR) for 1 h. After washing for 30 min, basal intracellular calcium concentration was determined. Following 5 min of stable basal recording, tubules were exposed to 1 nM ET-1, and the response was recorded. Calcium concentrations were calibrated for each tubule by using 10 μM 4-Br-A23187 and 5 mM EGTA. Wavelength intensities and ratios were sampled every 20 s by use of the MetaFluo (Universal Imaging, West Chester, PA) imaging software. Intracellular calcium concentration was calculated according to the formula

\[ [Ca^{2+}]_i = K_m(F_{\text{min}}/F_{\text{max}})(R - R_{\text{min}}/R_{\text{max}} - R) \]

where K_m is the affinity of Fura-2 for calcium (224 nM), F_{\text{min}} is the intensity of the 340 nm signal during EGTA, F_{\text{max}} is the intensity of the 380-nm signal during 4-Br-A23187, R is the 340/380 ratio during sampling, R_{\text{min}} is the minimum 340/380 ratio during EGTA, and R_{\text{max}} is the maximum 340/380 ratio during 4-Br-A23187.

Statistics. Experimental results are expressed as means ± SE. Data were evaluated with Student’s paired t-test. The criterion for statistical significance was P < 0.05 in all experiments.

RESULTS

We have previously reported that the thick ascending limb contains active NOS and that locally produced NO inhibits thick ascending limb transport (37). In addition, others have reported that ET-1 stimulates NO release (5). Thus we hypothesized that ET-1 stimulates thick ascending limb NOS activity via activation of ETB receptors and that the resulting NO inhibits chloride transport. Accordingly, we evaluated the response of isolated perfused thick ascending limbs to ET-1 both alone and in the presence of NOS inhibitors or ET-1 receptor antagonists.

Figure 1 illustrates the effect of 1 nM ET-1 on chloride flux in isolated thick ascending limbs. During the control period, tubules absorbed chloride at a rate of
ET-1 inhibits TALH $J_{\text{Cl}}$ via NO

Fig. 1. Exogenous endothelin-1 (ET-1) inhibits net chloride flux ($J_{\text{Cl}}$) in isolated perfused cortical thick ascending limbs of the loop of Henle (TALHs). Addition of 1 nM ET-1 to the basolateral bath resulted in a 48.3% reduction in $J_{\text{Cl}}$, from 118.3 ± 15.0 to 62.7 ± 13.6 pmol mm$^{-1}$ min$^{-1}$ ($n = 8$; *$P < 0.01$).

118.3 ± 15.0 pmol mm$^{-1}$ min$^{-1}$. After 1 nM ET-1 was added to the bath, tubules absorbed chloride at a rate of 62.7 ± 13.6 pmol mm$^{-1}$ min$^{-1}$. Perfusion rates did not differ during the two periods, and time controls showed no reduction in chloride absorption over a 2-h period. Thus 1 nM ET-1 inhibited chloride flux by 48.3 ± 8.2% ($P < 0.01$; $n = 8$).

To examine the possibility that the reduction in transport observed was not secondary to any cytotoxic effects of ET-1, we evaluated the ability of cortical thick ascending limbs to increase chloride flux following recovery from ET-1 exposure. Figure 2 illustrates the effects of removing 1 nM ET-1 from the bath. During the initial period in the presence of 1 nM ET-1, tubules absorbed chloride at a rate of 87.6 ± 10.7 pmol mm$^{-1}$ min$^{-1}$. Forty-five minutes following removal of 1 nM ET-1 from the bath, the tubules significantly increased chloride flux to a rate of 115.2 ± 10.3 pmol mm$^{-1}$ min$^{-1}$, an increase of 34.5 ± 6.2% ($P < 0.05$; $n = 5$). These findings indicate that the reductions in chloride flux observed in response to 1 nM ET-1 administration were not secondary to cytotoxic effects.

Our laboratory has previously reported a biphasic effect of ET-1 on tubular transport, with low concentrations stimulating and higher concentrations inhibiting proximal tubular reabsorption (7). Thus we next determined whether a lower concentration of ET-1 might exert a different effect on thick ascending limb transport. We evaluated the response of isolated thick ascending limbs to 1 pM ET-1, which had been demonstrated to stimulate tubular sodium chloride reabsorption. During the control period, tubules reabsorbed chloride at a rate of 57.5 ± 7.1 pmol mm$^{-1}$ min$^{-1}$. Following the addition of 1 nM ET-1, the thick ascending limb chloride reabsorption rate was unchanged from control (55.0 ± 6.1 pmol mm$^{-1}$ min$^{-1}$; $n = 4$). These data indicate that, unlike the proximal tubule, the thick ascending limb does not exhibit a biphasic response to ET-1 in vitro.

Because we hypothesized that ET-1 inhibited thick ascending limb $J_{\text{Cl}}$ by activation of NOS combined with increased NO production, we next examined the effect of L-NAME on ET-1-mediated inhibition of thick ascending limb chloride absorption (Fig. 3). In the presence of 5 mM L-NAME, tubules absorbed chloride at a rate of 89.2 ± 10.7 pmol mm$^{-1}$ min$^{-1}$ ($n = 5$). After adding 1 nM ET-1 to the bath, thick ascending limb chloride absorption did not change significantly from the basal rate (96.0 ± 16.8 pmol mm$^{-1}$ min$^{-1}$). Addition of 5 mM L-NAME alone did not significantly alter chloride flux from control (114.3 ± 15.5 vs. 119.9 ± 15.8 pmol mm$^{-1}$ min$^{-1}$; $n = 8$). Taken together, these findings suggest that ET-1 inhibits thick ascending limb transport through an NOS-dependent mechanism, whereas L-NAME itself does not effect thick ascending limb transport under basal conditions.

The possibility exists that L-NAME nonspecifically inhibited the effects ET-1 on chloride flux. Thus a...
separate series of experiments evaluated the effects of the enantiomer of the NOS inhibitor, D-NAME, on ET-1-mediated inhibition of thick ascending limb chloride flux. During the control period with 5 mM D-NAME in the bath, tubules reabsorbed chloride at a rate of 110.2 ± 16.3 pmol·mm⁻¹·min⁻¹. After the addition of 1 nM ET-1, tubules significantly reduced their rate of chloride absorption to 69.6 ± 13.9 pmol·mm⁻¹·min⁻¹ (P < 0.05; n = 4). Thus D-NAME had no effect on ET-1-mediated inhibition of thick ascending limb chloride flux, whereas inhibition of thick ascending limb NOS by equimolar L-NAME abolished the reduction in chloride absorption induced by 1 nM ET-1.

Because NOS inhibition abolished the ability of ET-1 to inhibit thick ascending limb transport, we evaluated the role of exogenous L-arginine in the ET-1 response. As stated in the METHODS, the previous experiments were conducted with 4 μM L-arginine in the bath. Therefore, we measured the effect of ET-1 on thick ascending limb J_{Cl} in the absence of exogenous L-arginine, the substrate for NOS (Fig. 4). During the control period, tubules absorbed chloride at a rate of 124.2 ± 12.8 pmol·mm⁻¹·min⁻¹. After adding 1 nM ET-1 to the bath, chloride absorption was not significantly different from the basal rate (130.8 ± 20.8 pmol·mm⁻¹·min⁻¹; n = 7). Thus removal of the substrate for NOS prevented the reduction in thick ascending limb chloride absorption induced by 1 nM ET-1.

To determine which endothelin receptor subtype mediates ET-1 activity in the rat thick ascending limb, we measured its effect on chloride absorption in the presence of different endothelin receptor antagonists. Others have reported that activating the ET_{B} receptor stimulates NO production (18), and the thick ascending limb contains ET_{B} receptors (45). Therefore, we hypothesized that the ET_{B} receptor mediates the ability of ET-1 to inhibit thick ascending limb chloride absorption. To test this, we examined the effect of the selective ET_{B} receptor antagonist BQ-788 on ET-1-mediated inhibition of chloride absorption (Fig. 5). Initially, tubules pretreated with 100 nM BQ-788 absorbed chloride at a rate of 96.3 ± 21.2 pmol·mm⁻¹·min⁻¹. After adding 1 nM ET-1 to the bath, chloride absorption was not significantly different from the basal rate (93.2 ± 18.1 pmol·mm⁻¹·min⁻¹; n = 6). In a separate series of experiments, addition of 100 nM BQ-788 alone did not alter chloride absorption (61.9 ± 9.7 vs. 66.9 ± 7.7 pmol·mm⁻¹·min⁻¹; n = 6), indicating that BQ-788 exerted no indirect effects on chloride flux. Thus selective antagonism of ET_{B} receptors abolished the inhibitory effect of ET-1 on thick ascending limb chloride absorption.

We reasoned that if ET-1 inhibits thick ascending limb chloride absorption via activation of ET_{B} receptors, a selective ET_{B} agonist should mimic the effect of ET-1 and inhibit thick ascending limb chloride absorption. Therefore, we measured the chloride absorption response to selective activation of ET_{B} receptors with sarafotoxin S6c. However, sarafotoxin S6c had no significant effect on chloride absorption at the concentration tested (Fig. 6). This suggests that ET-1 inhibits thick ascending limb chloride absorption via NO production, and not through direct activation of ET_{B} receptors.

![Fig. 4](image-url) Removal of the substrate for NOS, L-arginine (L-Arg), prevents the inhibitory effect of ET-1 on TALH J_{Cl}. Addition of 1 nM ET-1 to the basolateral bath in the absence of exogenous L-Arg did not alter the J_{Cl} of isolated perfused cortical TALHs (n = 7).

![Fig. 5](image-url) An ET_{B} receptor antagonist abolishes the ET-1-induced decrease in J_{Cl} in isolated perfused TALHs. Addition of 1 nM ET-1 to the basolateral bath of cortical TALHs pretreated with 100 nM BQ-788, a selective ET_{B} receptor antagonist, did not alter J_{Cl} (n = 6).

![Fig. 6](image-url) Activation of ET_{B} receptors inhibits J_{Cl} in isolated perfused cortical TALHs. Addition of 1 nM of the selective ET_{B} receptor agonist sarafotoxin S6c to the basolateral bath resulted in a 40% reduction in J_{Cl}, from 120.7 ± 12.6 to 75.4 ± 13.3 pmol·mm⁻¹·min⁻¹ (n = 6; *P < 0.002).
ETB receptors mediate the inhibitory effects of ET-1 on intracellular calcium concentration. Figure 7 illustrates a typical intracellular calcium response to ET-1. Other investigators have demonstrated that activation of endothelial NOS mediates the inhibitory effects of L-arginine on thick ascending limb chloride absorption.

We have recently reported that the endothelial isoform of NOS mediates the inhibitory effects of L-arginine in the thick ascending limb. Our current findings indicate that ET-1 inhibits thick ascending limb transport. These data support in vivo data which suggest that renal ET-1 exerts a direct effect on urinary sodium excretion. Previous investigators have demonstrated that when endothelin receptor antagonists are administered intrarenally, they lower urinary sodium excretion (12), whereas intrarenal infusion of ET-1 induces natriuresis without decreasing glomerular filtration rate (21, 42). These data suggest that ET-1 regulates urinary sodium excretion by a direct tubular effect. Our own data indicate that at least part of this effect may originate in the thick ascending limb, where ET-1 inhibits NaCl absorption. Since sodium is required for chloride transport across the apical membrane and the Na-K-2Cl cotransporter, sodium reabsorption must accompany thick ascending limb chloride reabsorption.

The thick ascending limb is critical in the control of sodium excretion, absorbing ~25% of the filtered sodium load (22). The thick ascending limb is impermeable to water, and thus absorption of salt by this nephron segment both establishes and maintains the hypertonic medullary solute gradient as well as generating dilute tubular fluid (14, 31). Therefore, factors such as ET-1 that directly alter thick ascending limb calcium responses of isolated thick ascending limbs to 1 nM ET-1. During the control period, thick ascending limb intracellular calcium concentration was 96.9 ± 14.0 nM. After 1 nM ET-1 was added to the bath, calcium concentration peaked at 191.4 ± 11.9 nM, an increase of 110.8 ± 26.1% (P < 0.05; n = 5). These findings indicate that thick ascending limbs increase intracellular calcium concentration in response to exogenous ET-1.

**DISCUSSION**

Our data show that: 1) ET-1 inhibits chloride flux by isolated thick ascending limbs; 2) this inhibition could be blocked by a competitive inhibitor of NOS or removal of substrate for this enzyme; 3) an ET_A receptor antagonist was unable to block ET-1-induced decreases in chloride flux, whereas an ET_B receptor antagonist did block the decrease, and an ET_A agonist mimicked the effect of ET-1; and 4) ET-1 increased thick ascending limb calcium concentrations. Taken together, these findings suggest that ET-1 inhibits transport by enhancing endogenous NOS activity and releasing NO via activation of the ET_B receptor, an effect associated with increases in intracellular calcium. Thus the current data indicate that ET-1 may be one of the physiological regulators of thick ascending limb NOS. We believe these are the first data showing that ET-1 acts via NO to inhibit transport in any nephron segment.

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sodium chloride absorption may have potent effects on urinary sodium chloride excretion and concentrating ability. Because tubular ET-1 production may be influenced by interstitial osmolality (23), in vivo ET-1 may alter tubular NO production through a paracrine or autocrine feedback mechanism, responding to changes in sodium or water intake in a way that affects thick ascending limb transport and thereby maintaining sodium and water balance.

Endothelin stimulates NO production in a variety of renal cells (35, 39). This effect has been assayed by measuring NO metabolites (17) as well as the second messenger of NO, cGMP. Edwards et al. (6) described a dose-dependent increase in cGMP in intact glomeruli in response to ET-1 and the selective ETB receptor agonist sarafotoxin S6c. This increase was abolished by the NOS inhibitor nitro-L-arginine (L-NNA) and was equipotent to ET-1, ET-3, and S6c, indicating that ETB receptors bind and inhibit eNOS activity (34). Furthermore, L-NNA-mediated inhibition of the ET-1-induced response was reversed by high concentrations of L-Arg.

The specific isoform(s) and mechanism(s) by which thick ascending limb NOS is stimulated by ET-1 are currently unknown. However, our data showing 1) attenuation of ET-1-mediated inhibition of thick ascending limb chloride flux by L-NAME and substrate deprivation; and 2) no effect of d-NAME on ET-1-mediated inhibition of thick ascending limb chloride flux indicates that this response is dependent upon activation of NOS and increased NO production. White et al. (48) have demonstrated that the so-called constitutively expressed NOS isoforms [i.e., endothelial (eNOS) and neuronal (nNOS)] require increased intracellular calcium to become activated; in contrast, inducible NOS (iNOS) does not, but is dependent upon substrate availability (48). We have recently reported that eNOS mediates the inhibitory effects of exogenous L-arginine on thick ascending limb chloride flux (36).

We found that ET-1 increases thick ascending limb intracellular calcium concentration, and others have reported that ET-1 increases calcium concentrations in endothelial and epithelial cells (38). Because the $K_{m}$ of NOS for calcium is 200 nM (15), increasing intracellular calcium from 100 to 200 nM would be sufficient to increase NOS activity from 33% to 50% of its maximal rate. The current findings therefore support the possibility of calcium-activated NOS activity. In addition, recent evidence obtained from endothelial cells indicates that ETB receptors bind and inhibit eNOS activity in the absence of ET-1, an effect that is relieved following receptor occupancy by ligand (28). The current studies indicate that the thick limb appears to possess a calcium-activated isoform of NOS that, when activated, inhibits chloride reabsorption. Therefore, in the thick ascending limb, NOS activity may be suppressed under basal conditions, activated following agonist binding, and stimulated by increased intracellular calcium concentrations.

In agreement with the current findings, various investigators have shown that ET-1 increases calcium in collecting duct cells (26, 33). In contrast, not all investigators have documented an increase in intracellular calcium concentrations in the thick ascending limb (4, 33). The reason for this discrepancy is currently unknown. However, the moderate increases and transient nature of the calcium responses observed in the current studies may have contributed to previous negative findings. Alternatively, there may be strain-dependent responses to ET-1. Significant strain differences have been observed in mice. For example, C57B1/6 mice possess a single renin locus, whereas DBA/2J mice possess two renin loci (43). Thus comparisons of strains and species may be required to reconcile these apparent differences in calcium responsiveness to ET-1.

The mechanism by which ET-1-stimulated NO ultimately inhibits chloride absorption in the thick ascending limb is unknown. NO has been shown to act via a variety of second messenger cascades, although most of its effects are mediated by cGMP (2). In particular, NO-induced natriuresis is linked to increased cGMP production in the kidney (13). We have shown that NO increases cGMP in collecting duct cells by activating soluble guanylate cyclase (44) and also in the thick ascending limb (9), whereas others have reported that cGMP inhibits thick ascending limb chloride absorption (34). Thus it is possible that ET-1-mediated NO inhibits transport in the thick ascending limb via stimulation of soluble guanylate cyclase, resulting in increased cGMP. We reported that NO stimulates activation of cGMP-dependent protein kinase in cortical collecting ducts (10). The Na-K-2Cl cotransporter, Na-K-ATPase, apical K channel, or basolateral Cl channels could be directly phosphorylated by this enzyme and in turn decrease chloride transport.

Endothelin may activate two different receptors, ETA or ETB. Our data indicate that an ETB receptor antagonist can block the ability of ET-1 to inhibit thick ascending limb chloride transport. Additionally, an ETB receptor agonist, S6c, also inhibited chloride absorption, mimicking the effects of ET-1. Taken together, these findings suggest that ET-1 inhibits chloride transport by activating the ETB receptor.

Our findings agree with other reports showing that ETB receptor activation inhibits transport. de Jesus Ferreira and Bailly (4) found that selective ETB receptor stimulation inhibited chloride absorption in the mouse thick ascending limb. Similarly, Kohan et al. (24) found that selective activation of ETB receptors with S6c inhibited arginine vasopressin-stimulated...
cAMP accumulation in rat inner medullary collecting duct cells, whereas the ET$_{A}$ antagonist BQ-123 had no effect. When all reports are taken into account, they indicate that ET$_{B}$ receptor activation is generally responsible for inhibition of transport. This leads to the possibility that ET-1 may act via NO in other nephron segments, especially the inner medullary collecting duct which has been shown to produce NO (39).

In conclusion, we found that exogenous ET-1 inhibits chloride absorption by the isolated perfused thick ascending limb via activation of ET$_{B}$ receptors. Such inhibition is significantly attenuated by L-NAME and requires the substrate for NOS activity, L-arginine. These findings indicate that the thick ascending limb responds to ET-1 by increasing NO production, and the inhibitory effects of ET-1 on thick ascending limb chloride absorption may partially explain the ability of ET-1 to increase urinary sodium excretion in vivo.

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