

Renal nitric oxide (NO) is an important controller of urinary sodium excretion. NO may enhance natriuresis by inhibiting transport along the nephron as well as altering renal hemodynamics and glomerular filtration rate. Several in vivo studies support the hypothesis that NO directly inhibits tubular sodium reabsorption. Urinary sodium excretion increases when NO production is stimulated and decreases when NO synthase (NOS) is inhibited in the absence of altered renal hemodynamics.

In vitro studies indicate that NO directly affects tubular absorption. We recently reported that L-Arginine (L-Arg) inhibits chloride flux through the thick ascending limb of the loop of Henle (THAL) of rats via an L-NAME–sensitive mechanism. These data indicate that endogenously produced NO inhibits THAL transport. However, these studies did not address the NOS isoform(s) mediating tubular NO synthesis.

mRNA for each of the NOS isoforms has been detected in THALs. With the use of reverse transcription polymerase chain reaction (RT-PCR) of microdissected nephrons, Ujiie et al detected endothelial (eNOS) mRNA in rat THALs, whereas Mohaupt et al used competitive RT-PCR to demonstrate high expression of inducible (iNOS) mRNA in medullary THALs of rats. Finally, NOS transcript was detected by in situ hybridization in THALs and macula densa. NOS protein expression has also been demonstrated in the THAL. Tojo et al described positive immunolabeling of constitutive NOS in THALs. More recently, Mattson and Higgins, with the use of Western blots, showed that the rat outer medulla expresses all 3 NOS isoforms.

Currently, the isoform(s) of NOS mediating the effects of L-Arg on THAL transport have not been extensively studied. We hypothesized that L-Arg inhibits mouse THAL chloride absorption through stimulation of eNOS rather than either iNOS or the neuronal (nNOS) isoform. We studied genetically mutated mice to directly evaluate the effects of L-Arg on THAL transport in the absence of specific isoforms of NOS. Our findings indicate that L-Arg inhibits THAL chloride absorption through activation of eNOS.

### Methods

**Preparation of Isolated Nephron Segments**

eNOS- and iNOS-homozygous knockout mice on a C57BL/6J background and their wild-type controls were bred in the Henry Ford Hospital animal facility. Homozygous nNOS knockout mice on a
hybrid B6129S background and B6129S hybrid wild-type controls were obtained commercially (Jackson Laboratory). Thick ascending limbs were obtained from 6-week-old male mice (19 to 23.5 g) maintained on a diet containing 0.22% sodium and 1.1% potassium (Purina) with water ad libitum for at least 5 days. On the day of the experiment, mice were anesthetized with ketamine (150 mg/kg body wt IP) and xylazine (30 mg/kg body wt IP), and the abdominal cavity was opened. The left kidney was bathed in ice-cold saline and removed. Coronal slices were placed in oxygenated physiological saline at 12°C. Thick ascending limbs were dissected in the same solution under a stereomicroscope.

**Thick Ascending Limb Perfusion**

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. The composition of the basolateral bath and perfusate (in mmol/L) was NaCl, 114; NaHCO₃, 25; NaH₂PO₄, 2.5; KCl, 4; MgSO₄, 1.2; alanine, 6; Na₃ citrate, 1; glucose, 5.5; Ca-lactate, 2; raffinose, 5. The solution was bubbled with 5% CO₂-95%O₂ before and during the experiment, and the pH of the bath was 7.4. The osmolality of the bath solution was 290±3 mosmol/kg H₂O 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 to 10 nL/mm per minute. Time-control experiments were conducted for each protocol to determine the stability of tubular transport.

An NO donor, 1,3-propanediamine, N-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosodihydrino]-butyl][C₆H₄]NO (spermine NONOate) was purchased from Cayman Chemical. The substrate for NOS, L-Arg, and its stereoisomer D-arginine (D-Arg) were purchased from Sigma Chemical Co.

**Net Chloride Flux**

Chloride concentrations were determined in samples of perfusate and collected fluid with the use of a previously described fluorometric technique. Because chloride reabsorption was not accompanied by significant fluid reabsorption, net chloride flux (JCl) was calculated according to the formula

\[ J_{Cl} = PR ([Cl_i] - [Cl_o]) \]

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

The typical experimental protocol was as follows. After a 20-minute equilibration period, 3 basal measurements were performed (control period). One of the compounds was then added to the bath, and 20 minutes later 3 additional collections were made (experimental period). Spermine NONOate (SPM), L-Arg, and D-Arg were added to the bath as indicated in the text.

**Blood Pressure Measurement**

Arterial blood pressure was measured in separate groups of knockout and corresponding control mice under inactin (25 mg/kg SC) anesthesia. Briefly, after a stable plane of anesthesia was obtained, a cervical midline incision was made and a catheter (PE-10) was inserted into the carotid artery. The catheter was advanced to the aortic arch. Mean arterial pressure values for the eNOS knockout mice (n=12) was 102±2 mm Hg, whereas the C57Bl6j controls (n=6) averaged 86±2 mm Hg. The iNOS knockout mice (n=3) averaged 99±5 mm Hg, whereas their C57Bl6j controls (n=4) averaged 101±3 mm Hg. Last, the nNOS knockout mice (n=5) averaged 83±5 mm Hg, whereas their B6129S controls (n=5) averaged 93±4 mm Hg.

**Statistics**

Experimental results are expressed as mean±SEM. 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 rat thick ascending limb contains active NOS and that locally produced NO inhibits thick ascending limb transport. In addition, others have reported detection of both transcript and protein for all 3 isoforms of NOS in the thick ascending limb. Accordingly, we evaluated the response to L-Arg in isolated perfused thick ascending limbs from control and selective NOS isoform knockout mice to L-Arg.

We first determined whether the mouse thick ascending limb contains active NOS and whether endogenously produced NO inhibits transport. Figure 1 illustrates the effect of the substrate for NOS, L-Arg (0.5 mmol/L), on chloride flux in 6 isolated thick ascending limbs from C57BL/6j controls. During the control period, tubules absorbed chloride at a rate of 105.8±17.5 pmol/mm per minute. After 0.5 mmol/L L-Arg was added to the bath, tubules absorbed chloride at a rate of 79.2±15.8 pmol/mm per minute. Perfusion rates did not differ between the 2 periods. To determine whether the inhibitory effects were specific to the L-isomer, we next evaluated the effects of D-Arg on thick ascending limb JCl. During the control period, tubules absorbed chloride at a rate of 152.5±21.5 pmol/mm per minute. After the tubules were treated with 0.5 mmol/L D-Arg, they absorbed chloride at a rate of 166.8±30.3 pmol/mm per minute (n=4). Thus 0.5 mmol/L L-Arg inhibited chloride flux by 26.9±5.5% (P<0.01), indicating that the transport-inhibiting effects of arginine are specific for the L-isomer and that the mouse thick ascending limb possesses a constitutively active isoform of NOS.

We next evaluated the role of endothelial NOS in the inhibitory effects of L-Arg on THAL chloride flux. Figure 2 illustrates the effect of the substrate for NOS, L-Arg (0.5 mmol/L), on chloride flux in 6 isolated thick ascending limbs from eNOS knockout mice on a C57BL/6j background. During the control period, tubules absorbed chloride at a rate of 102.0±26.8 pmol/mm per minute. After 0.5 mmol/L L-Arg was added to the bath, tubules absorbed chloride at a rate of 111.1±19.9 pmol/mm per minute. Perfusion rates did not differ between the 2 periods. These data indicate that selective genetic ablation of the endothelial isoform of NOS prevents the inhibitory effects of L-Arg on THAL JCl.
To determine whether the absence of a reaction to L-Arg was due to a defect in responsiveness of eNOS knockout mice to NO, we next evaluated the effect of an NO donor on eNOS thick ascending limb chloride flux. Figure 3 illustrates the effect of the NO donor SPM (10 μmol/L) on chloride flux in 6 isolated thick ascending limbs from eNOS knockout mice. During the control period, tubules absorbed chloride at a rate of 111.5 ± 14.7 pmol/mm per minute. After the tubules were treated with 10 μmol/L SPM, they absorbed chloride at a rate of 74.2 ± 4.7 pmol/mm per minute. Perfusion rates did not differ between the 2 periods. Thus 10 μmol/L SPM inhibited chloride flux by 29.0 ± 8.1% (*P < 0.05). The inhibition of eNOS knockout THAL chloride flux by exogenous NO indicates that the lack of responsiveness of eNOS knockouts to L-Arg was not due to an inability to respond to NO.

THALs also have been reported to express iNOS. Therefore we next evaluated the effects of L-Arg on THAL chloride flux in iNOS knockout mice on a C57BL/6J background. Figure 4 illustrates the effect of 0.5 mmol/L L-Arg on chloride flux in 5 isolated thick ascending limbs from iNOS knockout mice. During the control period, tubules absorbed chloride at a rate of 126.9 ± 28.2 pmol/mm per minute. After 0.5 mmol/L L-Arg was added to the bath, tubules absorbed chloride at a rate of 72.4 ± 9.7 pmol/mm per minute. Perfusion rates did not differ between the 2 periods. Thus 0.5 mmol/L L-Arg inhibited chloride flux in iNOS knockout THALs by 37.7 ± 6.4% (*P < 0.05). These data indicate that selective genetic ablation of the inducible isoform of NOS does not alter the inhibitory effects of L-Arg on THAL JCl.

Expression of nNOS has also been reported in the thick ascending limb. Therefore we next evaluated the effects of L-Arg on chloride flux by THALs from hybrid nNOS knockout mice on a B6129S background. Figure 5 illustrates the effect of 0.5 mmol/L L-Arg on chloride flux in 6 isolated thick ascending limbs from nNOS knockout mice. During the control period, tubules absorbed chloride at a rate of 162.7 ± 27.3 pmol/mm per minute. After 0.5 mmol/L L-Arg was added to the bath, tubules absorbed chloride at a rate of 114.9 ± 23.2 pmol/mm per minute. Perfusion rates did not differ between the 2 periods. Thus 0.5 mmol/L L-Arg inhibited chloride flux in nNOS knockout THALs by 31.8 ± 8.3% (*P < 0.05). These data indicate that selective genetic ablation of the neuronal isoform of NOS does not alter the inhibitory effects of L-Arg on THAL JCl.

Discussion
Our data show that (1) L-Arg inhibits chloride flux by isolated mouse thick ascending limbs, whereas D-Arg does not. (2) L-Arg inhibits chloride flux in THALs from eNOS knockout mice. (3) L-Arg inhibits chloride flux in THALs from iNOS knockout mice. (4) L-Arg inhibits chloride flux in THALs from nNOS knockout mice.
Our findings suggest that L-Arg inhibits thick ascending limb transport, supporting in vivo data\(^1\) that suggest that renal L-Arg exerts a direct effect on urinary sodium excretion. When NOS inhibitors are administered intrarenally, they lower urinary sodium excretion,\(^2\) whereas intrarenal infusion of L-Arg induces natriuresis.\(^3\) These data suggest that L-Arg affects urinary sodium excretion by a direct tubular effect. Our own data indicate that at least part of this effect may reside in the thick ascending limb, where L-Arg inhibits NaCl absorption.

Regulation of thick ascending limb function is critical in the control of urinary sodium excretion because this nephron segment absorbs \(\approx 25\%\) of the filtered sodium load. Because it is impermeable to water, its absorption of salt both establishes and maintains a hypertonic medullary solute gradient as well as generating dilute tubular fluid.\(^4\) Therefore the ability of L-Arg to directly alter thick ascending limb absorption could have potent effects on urinary NaCl excretion and concentrating ability.

Numerous studies have demonstrated the expression of endothelial, inducible, and neuronal NOS in the thick ascending limb.\(^5\) Our current data demonstrating inhibition of chloride absorption with L-Arg and the absence of this response in eNOS knockout mice suggest that the endothelial isoform of NOS is responsible for L-Arg–induced inhibition in chloride flux. These data appear to conflict with other reports that eNOS requires increased intracellular calcium to become activated.\(^6\) In contrast, the inducible isoform does not have that requirement\(^7\) but is dependent on substrate availability in vivo\(^8\) and in vitro.\(^9\) However, normal thick ascending limb intracellular calcium concentrations are \(\approx 100\) nmol/L,\(^10\) whereas the \(K_{1/2}\) of NOS for calcium is 200 nmol/L.\(^11\) Thus, based on Michalis-L-Menten kinetics, addition of excess substrate (0.5 mmol/L) in the presence of basal intracellular calcium concentrations should be sufficient for 33% of maximal NOS activity. Therefore elevation of intracellular calcium may be unnecessary for activation of thick ascending limb NOS, provided that adequate substrate is available. Further study into the mechanism of L-Arg–induced activation of tubular NOS is needed.

The mechanism by which L-Arg ultimately inhibits chloride absorption in the thick ascending limb is unknown, although the abolition of this response in eNOS knockout mice suggests it is secondary to NO production. NO has been shown to act through a variety of second-messenger cascades, although most of its effects are mediated by cGMP.\(^12\) In particular, NO-induced natriuresis is linked to increased cGMP production in the kidney.\(^13\) Our laboratory has previously shown that NO increases cGMP in collecting duct cells by activating soluble guanylate cyclase\(^14\) and that NO increases cGMP in the thick ascending limb.\(^15\) Thus it is possible that L-Arg–mediated NO inhibits transport in the thick ascending limb through stimulation of soluble guanylate cyclase, resulting in an increase in cGMP. We have previously demonstrated that NO stimulates activation of cGMP-dependent protein kinase in cortical collecting ducts.\(^16\) Because thick ascending limb sodium chloride absorption depends on the Na-K-2Cl cotransporter, Na-K-ATPase, apical K channel, and basolateral Cl channels, it could be affected by a change in cGMP concentration and in turn decrease chloride transport.

In conclusion, we found that L-Arg–induced inhibition of chloride absorption by isolated mouse thick ascending limbs is stereospecific and dependent on the presence of eNOS. Such inhibition of chloride transport is not altered by the absence of either the inducible or neuronal isoform of NOS and is not secondary to an inability to respond to NO. These findings indicate that eNOS is involved in L-Arg–induced inhibition of thick ascending limb transport under basal conditions. Thus L-Arg may be a physiological regulator of thick ascending limb NO production through activation of eNOS, and the inhibitory effects of L-Arg on thick ascending limb chloride absorption may partially explain the ability of L-Arg to increase urinary sodium excretion in vivo.

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