NO Inhibits NaCl Absorption by Rat Thick Ascending Limb Through Activation of cGMP-Stimulated Phosphodiesterase

Pablo A. Ortiz, Jeffrey L. Garvin

Abstract—In the isolated, perfused rat thick ascending limb (THAL), L-arginine (L-Arg) stimulates endogenous nitric oxide (NO) production, which inhibits NaCl absorption. However, the intracellular cascade responsible for the effects of NO has not been studied. We hypothesized that endogenous NO inhibits THAL NaCl transport by increasing cGMP, which activates protein kinase G (PKG) and cGMP-stimulated phosphodiesterase (PDE II), which, in turn, decreases cAMP levels. THALs from rats were isolated and perfused, and net chloride flux (JCl) was measured. L-Arg was used to stimulate NO production. Adding L-Arg (0.5 mmol/L) to the bath decreased JCl from 154.4±9.9 to 101.9±14.1 pmol·mm⁻¹·min⁻¹, a 35.2% decrease (n=6; P<0.05). In the presence of the soluble guanylate cyclase inhibitor LY-83583 (10 μmol/L), adding L-Arg to the bath did not affect THAL JCl (143.7±28.1 versus 136.7±22.2 pmol·mm⁻¹·min⁻¹; n=6). LY-83583 alone had no effect on JCl. In the presence of the PDE II inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) 50 μmol/L, L-Arg reduced JCl by only 13% (142.1±8.9 versus 122.7±11.5 pmol·mm⁻¹·min⁻¹; P<0.05; n=6). EHNA alone had no effect on THAL JCl. In the presence of 10⁻⁵ mol/L dibutyryl (dB)-cAMP, L-Arg did not significantly reduce JCl (116.3±18.2 versus 102.6±15.6 pmol·mm⁻¹·min⁻¹; n=6). dB-cAMP (10⁻³ mol/L) had no effect on THAL JCl. In the presence of the PKG inhibitor KT-5823 (2 μmol/L), L-Arg lowered JCl from 142.6±14.1 to 85.9±8.3 pmol·mm⁻¹·min⁻¹, a decrease of 35.6% (n=8; P<0.05). We conclude that (1) endogenous NO inhibits THAL JCl by stimulating soluble guanylate cyclase and increasing cGMP; (2) NO inhibits THAL JCl by stimulation of PDE II, which, in turn, decreases cAMP levels; and (3) PKG does not mediate NO-induced inhibition of THAL JCl. (Hypertension. 2001;37[part 2]:467-471.)

Key Words: limb, thick, ascending ▪ nitric oxide ▪ cGMP ▪ phosphodiesterases ▪ transport, chloride

Several studies have demonstrated that nitric oxide (NO) exerts natriuretic effects in vivo. In vitro, NO inhibits transport in the proximal tubule, cortical collecting duct, and inner medullary collecting duct. We previously reported that endogenously produced NO inhibits chloride absorption in isolated, perfused thick ascending limbs (THAL).

Both cGMP-dependent and cGMP-independent mechanisms mediate NO effects in different tissues. However, in vivo studies suggest that the natriuretic and diuretic effects of NO are mediated by cGMP. Using isolated THALs, we and other investigators have shown that NO donors stimulate production of cGMP. However, the cascade beyond cGMP has not been studied in the THAL.

cGMP can alter the activity of several enzymes, including protein kinase G (PKG) and cGMP-stimulated and cGMP-inhibited phosphodiesterases (PDE II and PDE III, respectively). In cardiac myocytes and platelets, nitric oxide (NO) prevents the effects of cAMP through activation of PDE II. In the isolated perfused cortical collecting duct, NO inhibits arginine vasopressin–stimulated osmotic water permeability by decreasing cAMP production through activation of PKG. Because hormones that increase cAMP production stimulate chloride absorption in the THAL, we hypothesize that endogenously produced NO inhibits THAL chloride absorption by stimulating soluble guanylate cyclase, which increases cGMP, thus activating PKG and PDE II and thereby decreasing cAMP.

Methods

THAL Isolation and Perfusion

THALs were obtained from male Sprague-Dawley rats weighing 120 to 150 g (Charles River Breeding Laboratories, Wilmington, Mass) maintained on a diet that contained 0.22% sodium and 1.1% potassium (Ralston Purina) for ≥5 days. Each rat was anesthetized with ketamine 100 mg/kg body wt and xylazine 20 mg/kg body wt. The abdominal cavity was opened and flushed with ice-cold 150 mmol/L NaCl. The left kidney was removed and bathed in ice-cold perfusion solution. Coronal slices were cut and THALs dissected from medullary rays and perfused at 37°C as described previously. All protocols were performed in accordance with the guidelines of the Henry Ford Hospital Animal Care and Use Committee.

In all experiments, both lumen and bath contained (in mmol/L) 114 NaCl, 25 NaHCO₃, 2.5 NaH₂PO₄, 4 KCl, 1.2 MgSO₄, 6 alanine, 1.8 glutamine, 0.4 glucose.
1 trisodium citrate, 5.5 glucose, and 2 calcium dilactate. All solutions were gassed with 95% O₂/5% CO₂ before the experiment. Osmolality of the solution was 290±3 mOsmol/kg H₂O as measured by freezing-point depression, pH 7.4. The basolateral bath was exchanged at a rate of 0.5 mL/min by means of a continuously flowing exchange system, and tubules were perfused at 5 to 10 mL·mm⁻¹·min⁻¹.

L-Arginine (L-Arg) was added to the bath to stimulate endogenous NO production. We tested the effects of L-Arg (Sigma Chemical Co) on chloride absorption by isolated perfused THALs alone and in the presence of the soluble guanylate cyclase inhibitor LY-83583 (Bi-omol), PKG inhibitor KT-5823 (Biomol), phosphodiesterase II inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) (Biomol) and membrane-permeable cyclic nucleotide analogues dibutyryl-cGMP (db-cGMP) and dibutyryl-cAMP (db-cAMP) (Sigma). Typically, tubules were equilibrated for 20 to 25 minutes in the presence of each inhibitor at 37°C, and ≥4 measurements corresponding to basal reabsorption rates were taken. L-Arg was then added to the bath, and after a 20-minute reequilibration period, 4 additional collections were made. In control experiments, we determined the effect of each inhibitor alone on THAL chloride absorption. Additionally, time-control experiments were performed in the presence of each inhibitor.

Chloride concentration in the perfusate and collected fluid was measured by microfluorometry. All data were recorded and stored on data acquisition software (DATAQ Instruments). Data analysis was performed with newly developed software specifically designed for voltage-spike analysis.

Statistics
Results are expressed as mean±SE. Differences between means were evaluated with Student’s paired t test. *P<0.05 was considered significant.

Results

Because we were interested in studying the second-messenger cascade activated by endogenously produced NO, we first tested the effects of L-Arg (0.5 mmol/L) on THAL chloride absorption (JCl⁻). In isolated perfused THALs, addition of L-Arg (0.5 mmol/L) to the basolateral bath significantly decreased JCl⁻ from 154.4±9.9 to 101.9±6 pmol·mm⁻¹·min⁻¹. Time controls showed no reduction in chloride absorption over a 2-hour period. Thus, the presence of 0.5 mmol/L L-Arg in the bath significantly decreased THAL chloride absorption, by 36.9±11.6% (*P<0.025; n=6; Figure 1).

In many cell types, including tubular epithelial cells, NO stimulates soluble guanylate cyclase. To determine whether endogenous NO inhibits THAL chloride absorption by stimulating soluble guanylate cyclase, we tested whether LY-83583, a soluble guanylate cyclase inhibitor, could block the effects of L-Arg on THAL JCl⁻. In the presence of LY-83583 (10 μmol/L), chloride absorption averaged 143.7±28.1 pmol·mm⁻¹·min⁻¹. After 0.5 mmol/L L-Arg was added to the bath, chloride absorption was 136.7±22.2 pmol·mm⁻¹·min⁻¹ (Figure 2). Control experiments showed that blocking soluble guanylate cyclase with LY-83583 did not modify basal THAL JCl⁻ (n=7). These results indicate that the effect of L-Arg on THAL JCl⁻ is mediated by stimulation of soluble guanylate cyclase.

LY-83583 has been reported to have effects other than inhibition of soluble guanylate cyclase. Therefore, we studied whether the effects of L-Arg and cGMP are additive. In the presence of 50 μmol/L db-cGMP, chloride absorption averaged 132.7±17.1 pmol·mm⁻¹·min⁻¹. After L-Arg 0.5 mmol/L was added to the bath, THAL JCl⁻ did not change significantly (n=6). Control experiments in the presence of db-cGMP showed no significant change in THAL JCl⁻ over time (n=4). Taken together, these data indicate that cGMP mediates all of the effects of NO on THAL JCl⁻.

cGMP may activate either PKG or the cGMP-dependent phosphodiesterases, PDE II and PDE III. Stimulation of PDE II decreases intracellular levels of cAMP, which is known to stimulate THAL chloride transport. To test whether inhibition of PDE II activity could block the effects of L-Arg, we used the PDE II inhibitor EHNA. During incubation with EHNA 50 μmol/L, chloride absorption averaged 142.1±8.9 pmol·mm⁻¹·min⁻¹. After L-Arg 0.5 mmol/L was added to the bath, THAL JCl⁻ was 122.7±11.5 pmol·mm⁻¹·min⁻¹, a 13% decrease (Figure 3). Adding EHNA (50 μmol/L) alone to the bath did not significantly affect JCl⁻.

Figure 1. Effect of L-Arg on THAL JCl⁻. Addition of 0.5 mmol/L L-Arg to the bath decreased THAL JCl⁻ by 36.9±11.6% (*P<0.05; n=6).

Figure 2. Effect of L-Arg on THAL JCl⁻ during soluble guanylate cyclase (sGC) blockade. In the presence of the sGC inhibitor LY-83583 (10 μmol/L), addition of 0.5 mmol/L L-Arg to the bath did not change THAL JCl⁻.
change THAL $J_{Cl^{-}}$ (n=4). Time-control experiments in the presence of EHNA (50 μmol/L) showed no significant change in THAL $J_{Cl^{-}}$ (n=4). These data show that PDE II inhibition blunts l-Arg-induced inhibition of THAL transport.

Because activation of PDE II mediates the effects of NO, we next tested whether we could block the effects of l-Arg by treating tubules with a cAMP analogue not hydrolyzed by PDE II. For this purpose, we performed dose-response experiments to determine the maximum concentration of db-cAMP that does not stimulate transport by itself and used it in the following experiments. In the presence of db-cAMP $10^{-5}$ mol/L, chloride absorption averaged 116.3 ± 18.2 pmol · mm$^{-1}$ · min$^{-1}$. After l-Arg 0.5 mmol/L was added to the bath, THAL $J_{Cl^{-}}$ did not change significantly (n=6; Figure 4). Time-control experiments in the presence of db-cAMP $10^{-5}$ mol/L showed no significant change in THAL $J_{Cl^{-}}$ during the experimental period (n=4). These results indicate that preventing the fall in intracellular cAMP blocks NO-induced inhibition of chloride absorption.

Because activation of PKG decreases cAMP in the cortical collecting duct, we determined whether PKG activation is a necessary step in the NO second messenger cascade. For this purpose we tested the effect of l-Arg 0.5 mmol/L on THAL $J_{Cl^{-}}$ in the presence of KT-5823, a PKG inhibitor. During incubation with KT-5823 2 μmol/L, THAL $J_{Cl^{-}}$ averaged 142.6 ± 14.1 pmol · mm$^{-1}$ · min$^{-1}$. As shown in Figure 5, addition of l-Arg 0.5 mmol/L to the bath significantly decreased chloride absorption to 85.9 ± 8.3 pmol · mm$^{-1}$ · min$^{-1}$, a 35.6% decrease (n=8; $P<0.05$). These results indicate that PKG does not play a major role in the NO-induced inhibition of $J_{Cl^{-}}$.

**Discussion**

In many cell types, NO activates soluble guanylate cyclase and increases cGMP production. Our data show that inhibition of THAL soluble guanylate cyclase completely blocks NO-induced inhibition of THAL chloride absorption and that the effects of NO and cGMP on chloride absorption are not additive. Taken together, these data indicate that NO stimulates soluble guanylate cyclase and increases cGMP. This, in turn, mediates all of the effects of NO on THAL chloride transport. Our results are consistent with others showing that cGMP inhibits transport in the THAL and other nephron segments. The NO donor spermine NONOate increases cGMP production in the cortical collecting duct while sodium nitroprusside (SNP) increases cGMP production in the proximal tubule, and this effect is blocked by inhibiting soluble guanylate cyclase with LY-83583. Although we know of no reports regarding the amount of...
soluble guanylate cyclase enzyme present in the THAL, Terada et al. found reverse transcription PCR products for soluble guanylate cyclase in microdissected THALs. In agreement with the presence of soluble guanylate cyclase in the THAL, we and others have reported an increase in cGMP production after incubation of THALs with NO donors.

It has been reported that NO can exert its effects by cGMP-dependent and cGMP-independent mechanisms. cGMP-dependent mechanisms are mediated by phosphodiesterases, PKG, and cGMP-gated ion channels. cGMP-independent mechanisms include K⁺ channel activation, modification of sulphydryl groups, and changes in intracellular calcium. We found that NO-induced inhibition of chloride absorption is completely mediated by cGMP. However, our data do not rule out the possibility that NO can affect other physiological functions directly.

Hormones that increase cAMP production stimulate THAL chloride absorption. CAMP can interact with the cAMP pathway by modulating the activity of the two different phosphodiesterases. PDE II is stimulated by cGMP to break down both cAMP and cGMP. PDE III is inhibited by cGMP, and it mainly degrades cAMP. Because NO inhibits THAL J_cl, and cAMP stimulates THAL J_cl, activation of PDE III is not likely to mediate the effects of NO in the THAL. Therefore, we tested whether the effects of NO are mediated by activation of PDE II. When PDE II was inhibited by EHNA, l-Arg only produced a small reduction in J_cl, which indicated that the effect of NO on chloride absorption is mediated mainly by activation of this enzyme. Researchers have reported that part of the effect of NO on platelet aggregation is mediated by stimulation of PDE II and can be prevented by treatment with EHNA. In isolated cardiac myocytes, activation of PDE II mediates the effect of NO on L-type Ca²⁺ channels. Velardez et al. found that the NO-induced inhibition of prolactin release from the pituitary gland is caused by a reduction of cAMP that can be prevented by blocking PDE II.

The fact that EHNA does not completely block the effect of l-Arg could be due to failure of EHNA to inhibit the enzyme completely. The inhibitory effect of EHNA has been reported to be achieved by competition of EHNA with cAMP for the catalytic site of PDE II. Therefore, it is possible that at 50 μmol/L, enzyme catalysis was not completely inhibited. Concentrations of EHNA > 80 μmol/L have been reported to inhibit other families of PDE enzymes. Thus, to avoid misinterpretation of the results, we did not test concentrations >50 μmol/L. EHNA is also known to inhibit adenosine deaminase. However, we found no effect of EHNA on chloride transport in control experiments. In addition, inhibition of adenosine deaminase increases adenosine content and inhibits THAL J_cl. Because EHNA blunts NO-induced inhibition of J_cl, inhibition of adenosine deaminase by EHNA is unlikely to have affected our results.

Activation of PDE II by cGMP may cause a decrease in cAMP levels in the THAL as it does in other cells. To test whether a decrease in cAMP is necessary for NO-induced inhibition of THAL J_cl, we treated tubules with a cAMP analogue that is not hydrolyzed by PDE II. Our results show that a nonstimulatory concentration of db-cAMP prevented the effects of l-Arg. Our results are supported by De Jesus Ferreira and Bailly, who showed that under basal conditions, with no stimulation of cAMP, inhibition of protein kinase A decreases basal chloride absorption in the THAL.

cGMP can exert intracellular effects by interacting with PKG. In cortical collecting ducts, the inhibitory effect of NO on arginine vasopressin–stimulated osmotic water permeability is mediated by stimulation of PKG, which also decreases cAMP. In the proximal tubule, ANP inhibits fluid reabsorption by stimulation of PKG. Our results show that blockade of PKG had no effect on NO-induced inhibition of chloride absorption, which indicates that PKG does not mediate the effects of NO on THAL chloride transport. It is unlikely that the concentration of KT-5823 we used is not sufficient to inhibit PKG. First, we used a KT-5823 concentration that is 10 times the inhibition constant for PKG inhibition. Second, we recently reported that NO-induced inhibition of THAL bicarbonate transport is mediated by PKG activation, because 2 μmol/L KT-5823 blocked the effect.

In summary, we conclude that (1) endogenous NO inhibits THAL chloride absorption by stimulating soluble guanylate cyclase and increasing cGMP; (2) NO inhibits THAL J_cl by stimulation of PDE II, which, in turn, decreases cAMP levels; and (3) PKG does not mediate NO-induced inhibition of THAL chloride transport.

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References


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