Nitric oxide inhibits sodium/hydrogen exchange activity in the thick ascending limb

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Garvin, Jeffrey L., and Nancy J. Hong. Nitric oxide inhibits sodium/hydrogen exchange activity in the thick ascending limb. Am. J. Physiol. 277 (Renal Physiol. 46): F377–F382, 1999.—Nitric oxide (NO) inhibits transport in various nephron segments, and the thick ascending limb (TAL) expresses nitric oxide synthase (NOS). However, the effects of NO on TAL transport have not been extensively studied. We tested the hypothesis that NO inhibits apical and basolateral Na+/H+ exchange by the TAL by measuring intracellular pH (pHi) of isolated, perfused rat TALs using the fluorescent dye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF). The NO donor spermine NONOate (SPM, 10 µM) decreased steady-state pHi in medullary TALs from 7.18 ± 0.13 to 7.13 ± 0.14 (P < 0.02), whereas controls did not decrease significantly. We next measured the buffering capacity of medullary TALs and the rate at which they recovered from acid loads to investigate the mechanism whereby NO reduces steady-state pHi. SPM decreased H+ flux (JH+) from 2.41 ± 0.66 to 0.97 ± 0.19 pmol·min⁻¹·mm⁻¹, 55%. To assure that the decrease in JH+ was due to NO, another donor, nitroglycerin (NTG; 10 µM), was used. NTG decreased JH+ from 1.65 ± 0.11 to 1.07 ± 0.24 pmol·min⁻¹·mm⁻¹, 37%. To determine the relative contributions of the apical and basolateral Na+/H+ exchangers, 5-(N,N-dimethyl)-amiloride (DMA; 100 µM) was added to either bath or lumen. With DMA added to the bath, SPM decreased JH+ from 4.78 ± 1.08 to 2.74 ± 0.54 pmol·min⁻¹·mm⁻¹, an inhibition of 41% and with DMA added to the lumen, SPM decreased JH+ from 2.31 ± 0.29 to 1.74 ± 0.27 pmol·min⁻¹·mm⁻¹, a reduction of 26%. Addition of DMA alone to both bath and lumen resulted in an 87% inhibition of JH+. We conclude that NO inhibits both apical and basolateral Na+/H+ exchangers and consequently may play an important role in regulating pHi, and may alter acid/base balance by directly affecting bicarbonate absorption in the TAL.

Several recent studies indicate that NO directly affects transport in various nephron segments. NO has been shown to have an inhibitory effect on Na+/K+-ATPase in cultured mouse proximal tubule cells (15) and in the renal medulla (24) and on sodium and water reabsorption in the rat proximal convoluted tubule (9). Our laboratory has found that NO inhibits sodium reabsorption in cultured mouse cortical collecting duct cells (29) and in the rat CCD (30). In addition, NO inhibits chloride transport in the rat cortical thick ascending limb (TAL) (27).

NO has also been shown to play an important role in regulating Na+ absorption by inhibiting Na+/H+ exchange in the rabbit proximal tubule, where the Na+/H+ exchanger (NHE) mediates most proximal tubule Na+ reabsorption (28). Since the Na+/H+ exchanger is also involved in bicarbonate absorption, NO may also play a role in regulation of acid/base balance in the kidney. This is supported by the finding that NO inhibits H+/ATPase activity in the rat CCD (31).

The TAL exhibits both apical and basolateral Na+/H+ exchange activity. Two of the four NHE isoforms known to be expressed in the kidney have been found in the rat medullary TAL (6). NHE3 is most likely the apical isoform, and NHE1 is likely the basolateral exchanger. Currently, the effects of NO on TAL Na+/H+ exchange have not been extensively studied. We hypothesized that NO inhibits both apical and basolateral Na+/H+ exchange in the TAL. Our findings indicate that exogenous NO inhibits Na+/H+ exchange activity in isolated perfused TALs.

METHODS

Preparation of isolated nephron segments. Medullary TALs were obtained from male Sprague-Dawley rats weighing 120–150 g (Charles River Breeding Laboratories, Wilmington, MA), which had been fed a diet containing 0.22% sodium and 1.1% potassium (Purina, Richmond, IN) for at least 4 days. On the day of the experiment, rats were injected intraperitoneally with 2 mg furosemide, and 15 min later they were anesthetized with ketamine (100 mg/kg body wt ip) and xylazine (20 mg/kg body wt ip). The abdominal cavity was opened, and the left kidney was bathed in ice-cold saline and removed. Coronial slices were placed in oxygenated HEPES-buffered physiological saline at 12°C. TALs were dissected from the inner stripe of the outer medulla at 12°C in the same solution under a stereomicroscope.

Solutions. The composition of the basolateral bath and perfusate (perfusion solution) was (in mM) 130 NaCl, 2.5 NaH₂PO₄, 4 KCl, 1.2 MgSO₄, 6 alanine, 1 trisodium citrate, 5.5 glucose, 2 calcium dilactate, and 10 HEPES (pH 7.4). The composition of the basolateral bath used to acid-load cells (acetate solution) was the same, except that 20 mM sodium acetate was added and NaCl was decreased from 130 to 110 mM. NH₃/NH₄⁺ was not used to acid-load cells, to decrease the
roles of the Na⁺-K⁺-2Cl⁻ cotransporter and Na⁺-K⁺-ATPase pump in pH recovery. 5-(N,N-dimethylamino)lauryl (DMA; Sigma Chemical, St. Louis, MO) was prepared daily as a 10 mM stock in warm perfusion solution and diluted to 100 µM just before the experiment. The pH-sensitive fluorescent dye 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR) was prepared daily as a 5 mM stock in DMSO and diluted in perfusion solution to a final concentration of 1 µM. Perfusion solution containing an NO donor, either spermine NONOate (SPM; 1.3-propanediamine, N-[4-(1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino)-butyl], C₁₀H₂₆N₆O₂; Cayman Chemical, Ann Arbor, MI) or nitroglycerin (NTG), was prepared daily as a 5 mM stock in warm perfusion solution and diluted to 100 µM just prior to use.

The pH calibration solutions contained (in mM) 95 KCl, 5 NaCl, 30 N-methyl-D-glucamine, 2.5 NaH₂PO₄, 1.5 MgSO₄, 5 glucose, 2 CaCl₂, 25 HEPES, and 0.01 nigericin and were titrated to selected pH values between 6.3 and 7.5. Nigericin (Molecular Probes) was prepared as a 10 mM stock in methanol and diluted in calibration solution just prior to use. All solutions had an osmolality of 290 ± 3 mosmol/kg H₂O and were equilibrated with 100% O₂ until the experiment.

TAL perfusion. Medullary TALs (ranging from 0.5 to 1.0 mm in length) were transferred to a temperature-regulated chamber and perfused between concentric glass pipettes at 37 ± 1°C as described previously (12). The normal flow rate of the basolateral bath was ~1.0 ml/min and was increased to ~4.0 ml/min for acid-loading.

Measurement of intracellular pH. Once the TALs were perfused, cells were loaded by bathing the tubules in 1 µM BCECF-AM at 37 ± 1°C for 15 min and then washed for 20 min. Excitation and emission were controlled via a system composed of a xenon arc lamp, excitation filters connected to a Uniblitz shutter driver system (Vincent Associates, Rochester, NY), and a 510-nm dichroic mirror. Intracellular dye was excited alternately at 500 and 450 nm, and fluorescence was imaged digitally with an image intensifier (Video Scope International, Herndon, VA) and a charge-coupled device camera (Hamamatsu, Hamamatsu City, Japan). Imagermetric measurements of the images (500/450 nm) were recorded utilizing an Image One Metalfluor system (Universal Imaging, West Chester, PA).

Typically, after the wash period, measurements were taken once every 15 s for 1 min, then every 3 s as the bath was switched to acetate solution to initiate acid-loading of the cells. This maneuver caused rapid acidification of intracellular pH (pHi), as indicated by a drop in the 500/450 nm fluorescence ratio. Measurements were taken for an additional 60 s. After 2 min, the bath was switched back to normal perfusion solution for a wash period of 5 min. Subsequently an NO donor was added to the bath. After 5 min, the acid-loading and recovery measurements were repeated. At the end of the experiment, a three-point in situ calibration was performed using pH calibration solutions and the K⁺/H⁺ ionophore nigericin.

Determination of intrinsic buffering capacity. The intrinsic buffering capacity (βi) of medullary TALs was determined using a method similar to that used by Weintraub and Machen (34), except that a weak acid was used to change pHi instead of a weak base. To exclude HCO₃⁻/CO₂ as a buffering component and block Na⁺-dependent pH regulatory mechanisms, HEPES-buffered solutions containing 100 µM DMA were used in the luminal perfusate and basolateral bath. Addition of a range of acetate concentrations to the bath caused pHi to decrease. The pKₐ of acetic acid (4.74) was used to calculate the intracellular acetate concentration at each extracellular acetate concentration. For the midpoint of each range of pHi decrease, βi was calculated as the change in intracellular acetate concentration divided by the change in pHi; βi ranged from ~33 to 43 mM/pH unit over the range of pHi observed in the experiments.

Calculation of proton flux rates. Proton flux rates (JH⁺, expressed in pmol·min⁻¹·mm⁻¹) were calculated using the equation

\[ J_H^+ = \frac{d \text{pHi}}{dt} \times \beta_i \times V \]

where dpHi/dt is the initial recovery rate (in pH units/min), βi is the intrinsic buffering capacity (in mM/pH unit), and V is the cell volume per 1 mm of tubule length (in nl/mm). V was calculated by determining the volume of the tube [π × (radius of tube)² × length] and subtracting the volume of the lumen [π × (radius of lumen)² × length]. Initial recovery rates were calculated from the regression line fit to the first 6–10 measurements (corresponding to the first 18–30 s) following the minimum pHi reached after an acid load.

Statistics. Results are expressed as means ± SE. Data were evaluated with Students' paired t-test. P < 0.05 was considered significant.

RESULTS

First, we examined whether the NO donor SPM alters steady-state pHi. SPM was found to decrease steady-state pHi of medullary TALs from 7.18 ± 0.13 to 7.13 ± 0.14 (n = 6; P < 0.02). To investigate the mechanism by which NO decreases steady-state pHi, we studied the effect of SPM on pHi recovery from an acid load. Figure 1 shows the effect of SPM (10 µM) on pHi recovery rate in isolated perfused medullary TALs. Adding acetate caused an immediate intracellular acidification of 0.19 pH units (from 7.19 to 7.00), followed by pHi recovery at an initial rate of 0.067 pH units/min. Addition of 10 µM SPM followed by acetate caused pHi to drop from 7.12 to 6.98 and decreased the pHi recovery rate to 0.043 pH units/min. H⁺ flux rates (JH⁺) were calculated using the equation:

\[ J_H^+ = \frac{d \text{pHi}}{dt} \times \beta_i \times V \]

where dpHi/dt is the initial recovery rate (in pH units/min), βi is the intrinsic buffering capacity (in mM/pH unit), and V is the cell volume per 1 mm of tubule length (in nl/mm). V was calculated by determining the volume of the tube [π × (radius of tube)² × length] and subtracting the volume of the lumen [π × (radius of lumen)² × length]. Initial recovery rates were calculated from the regression line fit to the first 6–10 measurements (corresponding to the first 18–30 s) following the minimum pHi reached after an acid load.

Statistics. Results are expressed as means ± SE. Data were evaluated with Students' paired t-test. P < 0.05 was considered significant.

RESULTS

Fig. 1. Effect of spermine NONOate (SPM) on intracellular pH (pHi) recovery. Addition of 20 mM sodium acetate (NaAc) caused immediate acidification, followed by recovery of pHi. Treatment with 10 µM SPM resulted in a decrease in recovery rate, from 0.067 to 0.043 pH units/min. This trace represents 1 of 9 similar experiments.
determined from calculated β values for the medullary TAL (see METHODS) are shown in Fig. 2. SPM caused a 55% decrease in $J_H$, from $2.41 \pm 0.66$ to $0.97 \pm 0.19$ pmol·min$^{-1}$·mm$^{-1}$ ($n = 9; P < 0.05$).

To investigate whether the effect of SPM was due to NO, we used another NO donor, NTG. Figure 3 shows the effect of NTG (10 µM), which caused a 37% decrease in $J_H$ (from $1.65 \pm 0.11$ to $1.07 \pm 0.24$ pmol·min$^{-1}$·mm$^{-1}$; $n = 6; P < 0.05$). Taken together, these data indicate that the effects of SPM are due to NO and that NO inhibits $H^+$ efflux from TALs.

Because the TAL exhibits both apical and basolateral Na$^+$/H$^+$ exchange activity, we added DMA to either bath or lumen to characterize the individual effect of NO on each of these exchangers. First, we examined the effect of inhibiting both apical and basolateral exchangers on recovery of pH$_i$ after an acid load. Adding DMA alone to both bath and lumen resulted in a 87% inhibition of $J_H$. These data indicate that essentially all $H^+$ efflux is due to inhibition of DMA-sensitive Na$^+$/H$^+$ exchangers rather than other transporters that may contribute to the regulation of pH$_i$. We next investigated whether NO affected the apical Na$^+$/H$^+$ exchanger. Figure 4 illustrates the effect of NO when DMA was added to the bath to block the basolateral Na$^+$/H$^+$ exchanger. In the presence of DMA, acetate decreased pH$_i$ from 7.51 to 7.06, and then pH$_i$ recovered at a rate of 0.370 pH units/min. After treatment with SPM, acetate reduced pH$_i$ from 7.41 to 6.97; the recovery rate was 0.239 pH units/min. In five experiments, SPM decreased $J_H$ from $4.78 \pm 1.08$ to $2.74 \pm 0.54$ pmol·min$^{-1}$·mm$^{-1}$ ($P < 0.05$), a reduction of 41% (Fig. 5). These data indicate that NO inhibits the apical Na$^+$/H$^+$ exchanger.

Next, we studied the effect of NO on the basolateral Na$^+$/H$^+$ exchanger by adding DMA to the lumen to block the apical Na$^+$/H$^+$ exchanger. Figure 6 depicts a representative experiment. Acid-loading decreased pH$_i$ from 7.40 to 7.19. pH$_i$ then recovered at an initial rate of 0.090 pH units/min. After SPM treatment, addition of acetate resulted in a pH$_i$ decrease from 7.34 to 7.09 and a subsequent recovery rate of 0.039 pH units/min. The average decrease in $J_H$ induced by SPM was from $2.31 \pm 0.29$ to $1.74 \pm 0.27$ pmol·min$^{-1}$·mm$^{-1}$ ($n = 8; P < 0.01$), an inhibition of 26% (Fig. 7). Thus NO also

Fig. 2. Effect of SPM on $H^+$ flux. Addition of 10 µM SPM caused a 55% reduction in $H^+$ flux, from $2.41 \pm 0.66$ to $0.97 \pm 0.19$ pmol·min$^{-1}$·mm$^{-1}$ ($n = 9; P < 0.05$).

Fig. 3. Effect of nitroglycerin (NTG) on $H^+$ flux rate. Addition of 10 µM NTG resulted in a 37% decrease in $H^+$ flux, from $1.65 \pm 0.11$ to $1.07 \pm 0.24$ pmol·min$^{-1}$·mm$^{-1}$ ($n = 6; P < 0.05$).

Fig. 4. Effect of SPM on apical pH$_i$ recovery. With 100 µM 5-(N,N-dimethyl)amiloride (DMA) in the bath, addition of 20 mM sodium acetate (NaAc) caused immediate acidification, followed by recovery of pH$_i$. Treatment with 10 µM SPM resulted in a decrease in recovery rate, from 0.370 to 0.239 pH units/min. This trace represents 1 of 5 similar experiments.
inhibited basolateral Na⁺/H⁺ exchange as well as apical exchange.

**DISCUSSION**

We found that NO reduced steady-state pHᵢ of isolated, perfused TALs. Additionally, we found that inhibition of apical and basolateral Na⁺/H⁺ exchangers prevented recovery of pHᵢ from an acid load and that NO reduces proton efflux across the apical and basolateral membranes. These data indicate that NO inhibits both apical and basolateral Na⁺/H⁺ exchange activity in the medullary TAL.

To assure that the effects of SPM on Na⁺/H⁺ exchange activity were due to the generation of NO and not some other degradation product of SPM, we also examined the ability of NTG to inhibit the recovery from an acid load. Both SPM and NTG decreased J_H⁺; however, NTG did not cause as great a reduction as 10 µM SPM. We previously have reported differences in equimolar concentrations of these donors to inhibit transport (10). Given that they release NO via different mechanisms, it is also likely that the magnitude of the effects is related to the amount of NO released.

Our data indicate that apical Na⁺/H⁺ exchange activity is greater than basolateral Na⁺/H⁺ exchange activity and that both apical and basolateral exchangers are inhibited by NO. The former data are consistent with studies that suggest a more active role for the apical exchanger in regulation of transport and acid/base balance (1, 3, 16). The apical Na⁺/H⁺ exchanger appeared to be inhibited to a greater extent than the basolateral exchanger. The varying effect of NO on the exchanger activity of the apical and basolateral membranes may be due to a difference in the type of Na⁺/H⁺ exchanger present on each membrane. Reverse-transcriptase polymerase chain reaction analysis of Na⁺/H⁺ exchanger mRNAs indicates that NHE3 is the dominant isoform in the rat medullary TAL and that the NHE1 isoform is also expressed in this nephron segment (6). Several studies suggest that NHE3 is the apical isoform and NHE1 is the basolateral exchanger (2–4, 7). Differences in the proton affinity of these NHE isoforms may indicate that they are also differentially affected by NO and other regulators.

The effect of NO on Na⁺/H⁺ exchange that we observed could be a direct inhibition of the Na⁺/H⁺ exchanger or an indirect effect caused by inhibition of...
the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, thereby causing a decrease in the Na\textsuperscript{+} concentration gradient. NO most likely directly inhibits the Na\textsuperscript{+}/H\textsuperscript{+} exchanger. The inhibition of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger by NO in cultured proximal tubule cells has been observed to be amiloride sensitive, indicating a direct effect on the exchanger rather than the pump (28). Furthermore, other studies indicate that there is no effect of NO on the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (30). Finally, if NO directly inhibited the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase rather than the Na\textsuperscript{+}/H\textsuperscript{+} exchanger, then apical and basolateral Na\textsuperscript{+}/H\textsuperscript{+} exchange should have been inhibited by the same amount, which was not indicated by our data. Although NO has been shown to inhibit the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (15, 24), this may be due to the high concentration of NO donors used.

With relation to in vivo studies, interpretations of our data obtained using isolated perfused TALs should be made with caution, because numerous hormones and other regulators affect the TAL. The absolute \(J_{\text{H}}\) values we observed are lower than in vivo values due to a lack of bicarbonate in the perfusion and bath solutions (8).

Several studies indicate that NO inhibits solute and water reabsorption (9, 27–30). Eitel et al. (9) used split-drop micropuncture to determine the effect of NO in the rat proximal tubule and found that the NO donor sodium nitroprusside reduced the proximal tubular fluid absorption rate whether administered at the luminal or peritubular side. Furthermore, NO inhibited angiotensin II-stimulated fluid absorption. Since the Na\textsuperscript{+}/H\textsuperscript{+} exchanger mediates most Na\textsuperscript{+} and bicarbonate transport in this nephron segment, these data indicate that NO may play a key role in regulation of acid/base balance and/or natriuresis. In support of this, Roczniak and Burns (28) demonstrated that NO donors inhibit apical Na\textsuperscript{+}/H\textsuperscript{+} exchange in the rabbit proximal tubule. Even though the Na\textsuperscript{+}/H\textsuperscript{+} exchanger accounts for only ~20% of the sodium absorption observed in the TAL, the associated bicarbonate flux is extremely important in acid/base balance. An important role for NO in regulation of acid/base excretion is also supported by the finding that NO inhibits H\textsuperscript{+} transport in the collecting duct (31).

Although most studies indicate that NO has an inhibitory effect, there are data which suggest that NO stimulates transport in the kidney. A stimulatory effect of NO has been found for HCO\textsubscript{3}\textsuperscript{-} absorption in rat medullary TALs (13), and a biphasic effect has been found for HCO\textsubscript{3}\textsuperscript{-} absorption in rat proximal tubule (33). Lu et al. (18, 19), using patch clamp, found that NO stimulates K\textsuperscript{+} channels in both apical and basolateral membranes of the rat CCD as well as in the apical membrane of the TAL. The explanation for the discrepancy regarding the inhibitory effect of NO on transport and the stimulatory effect observed by others is not known. However, in our experiments, we observed a stimulatory effect of NO in 2 of 29 tubules. It is possible that under some conditions NO can exert a stimulatory rather than inhibitory effect. Because evidence suggests that NO has a dose-dependent biphasic effect (20, 33), a difference in the actual amount and/or stability of NO delivered to the cells by the various NO donors used in these studies might account for these discrepancies.

Additionally, effects of NO not directly due to actions in the proximal tubule cannot be ruled out in micropuncture experiments (33). Finally, NO has been reported to inhibit K\textsuperscript{+} channels when peroxynitrite forms from NO and superoxide (20). Thus variability in superoxide generation by the different preparations may also play a role in the discrepancy.

Although the effect of NO on the kidney has been well established through the use of NO donors and NO synthase (NOS) inhibitors, it is not clear where NO would be produced that would be physiologically important in the regulation of TAL Na\textsuperscript{+}/H\textsuperscript{+} exchange activity. NO has been reported to be synthesized in many nephron segments as well as the vasculature (23, 25, 32). Additionally, our laboratory has found that cortical TALs produce NO in the presence of the NOS substrate \(l\)-arginine, which decreases chloride absorption (27).

Therefore, we propose that endogenous NO is also produced by the medullary TAL and that it, acting as an autacoid, is the primary regulator of transport.

The mechanism by which NO inhibits transport in the TAL is currently unknown. Most evidence suggests that NO increases cGMP production in the kidney (5, 14, 18). cGMP has been found to inhibit TAL chloride absorption (26) and to inhibit the Na\textsuperscript{+}/H\textsuperscript{+} exchanger in the proximal tubule (28). Our laboratory has shown that NO activates soluble guanylate cyclase and thereby increases cGMP in cultured collecting duct cells (29), that NO increases cGMP in the TAL (11), and that NO stimulates cGMP-dependent protein kinase (10). Thus, taken together, these findings support the hypothesis that NO inhibits TAL transport by activating soluble guanylate cyclase and increasing cGMP.

In summary, NO decreases \(pH_{\text{i}}\) due to inhibition of both apical and basolateral Na\textsuperscript{+}/H\textsuperscript{+} exchanger activity in medullary TAL. Thus NO may play a role in not only natriuresis but also acid/base regulation.

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