Nitric Oxide Decreases the Permselectivity of the Paracellular Pathway in Thick Ascending Limbs

Casandra M. Monzon, Jeffrey L. Garvin

Abstract—Thick ascending limbs reabsorb 25% to 30% of the filtered NaCl. About 50% to 70% is reabsorbed via the transcellular pathway and 30% to 50% is reabsorbed through the Na-selective paracellular pathway. Nitric oxide (NO) inhibits transepithelial Na reabsorption, but its effects on the paracellular pathway are unknown. We hypothesized that NO decreases the selectivity of the paracellular pathway in thick ascending limbs via cGMP-dependent protein kinase. To assess relative Na/Cl permeability ratios (P_{Na}/P_{Cl}), we perfused rat thick ascending limbs and measured the effect of reducing bath NaCl on transepithelial voltage, creating dilution potentials, with vehicle, NO donors, and endogenous NO. P_{Na}/P_{Cl} was calculated using the Goldman–Hodgkin–Katz equation. Reducing bath Na/Cl to 16/8, 32/24, and 64/56 mmol/L created dilution potentials of −13.6±2.2, −10.8±3.0, and −6.1±0.9 mV, respectively. Calculated P_{Na}/P_{Cl} were 2.0±0.2, 2.2±0.5, and 1.9±0.2. The NO donor spermine NONOate (200 μmol/L) blunted the dilution potential caused by 32/24 mmol/L Na/Cl from −11.1±2.1 to −6.5±1.6 mV (P<0.004) and P_{Na}/P_{Cl} from 2.2±0.4 to 1.5±0.2. Nitroglycerin (200 μmol/L), another NO donor, also reduced P_{Na}/P_{Cl}. Controls showed no significant changes. Dibutyryl-cGMP decreased dilution potentials from −13.4±2.9 to −7.5±1.8 mV (n=6; P<0.01). cGMP-dependent protein kinase inhibition with KT5823 (4 μmol/L) blocked the effect of spermine NONOate, whereas phosphodiesterase 2 inhibition did not. Endogenously produced NO mimicked the effect of the NO donors. In conclusion, NO reduces the selectivity of the paracellular pathway in thick ascending limbs via cGMP and cGMP-dependent protein kinase. (Hypertension. 2015;65:00-00. DOI: 10.1161/HYPERTENSIONAHA.115.05356.)

Key Words: hypertension ■ kidney ■ nitric oxide ■ permeability

Thick ascending limbs reabsorb ≈30% of the filtered load of NaCl. About 50% to 70% of the Na traverses the transcellular pathway, entering the cell via Na/K/2Cl cotransporter and Na/H exchange, and exiting via Na/K ATPase. Transcellular NaCl reabsorption generates a lumen-positive potential that drives Na reabsorption via the paracellular pathway. Up to 50% of the total Na reabsorbed by thick ascending limbs traverses the paracellular pathway. The paracellular pathway in thick ascending limbs is selective for Na over Cl with a Na/Cl permeability ratio (P_{Na}/P_{Cl}) of ≈2.3,5,6

Renal NO causes natriuresis and diuresis.7–9 A major part of this is because of inhibition of NaCl reabsorption by the thick ascending limb.10 We have shown that NO inhibits net NaCl11 and Na bicarbonate12 reabsorption in this segment. We have also shown that NO inhibits Na/K/2Cl cotransporter13 and Na/H exchange activity.14 The former was because of an increase in cGMP, activation of phosphodiesterase 2, and consequent decreases in cAMP.15 The latter was also because of an increase in cGMP with subsequent activation of cGMP-dependent protein kinase (PKG) rather than phosphodiesterase 2.12 Although as much as 50% of reabsorbed Na traverses the paracellular pathway, the effects of NO on transport via this route are unknown.

Although there have been no studies of the effect of NO on the properties of the paracellular pathway in native renal epithelia, several reports have shown that it regulates the permeability and selectivity of the paracellular pathway in other cells. NO increases the permeability of the paracellular pathway in endothelial cells16 as the initiating step in angiogenesis.17,18 NO has also been shown to reduce the assembly of the proteins that form the paracellular pathway of Sertoli cells via a cGMP and PKG-dependent process.19 Finally, in intestinal epithelial cells, NO has been reported to both increase and decrease total permeability and permselectivity of the paracellular pathway in both physiological and pathological states.20–24

We hypothesized that NO reduces the permselectivity of the paracellular pathway in thick ascending limbs via a process involving cGMP and PKG.

Methods

An expanded Methods section is available in the online-only Data Supplement.
Animals

We used male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 110 to 150 g for this study. Animals were maintained on a diet containing 0.22% sodium and 1.1% potassium (Purina, Richmond, IN) for at least 4 days. All protocols involving animals were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Results

Effect of Different NaCl Concentrations on Dilution Potential and PNa/PCl

To set the most adequate experimental conditions for subsequent protocols, we first measured the dilution potentials generated by replacing the basolateral solution for 1 containing either 16/8, 32/24, or 64/56 mmol/L Na/Cl. The dilution potentials were −13.6±2.2 mV (n=5), −10.8±3.0 mV (n=6), and −6.1±0.9 mV (n=4), respectively. The calculated PNa/PCls were 2.0±0.2, 2.2±0.5, and 1.9±0.2. We decided to use the solution containing 32/24 mmol/L Na/Cl in all subsequent experiments.

Effect of NO Donors on the Permselectivity of the Paracellular Pathway

We first evaluated the effect of 200 µmol/L spermine NONOate (SPM), an NO donor, on the dilution potential caused by reducing the bath to 32/24 mmol/L Na/Cl and the calculated PNa/PCl. During the control period, the dilution potential was −11.6±1.7 mV. After the addition of nitroglycerin, the dilution potential was −9.5±1.5 mV, a reduction of 18.1% (Figure 2; P<0.02; n=5). The calculated PNa/PCl in the control period was 2.1±0.2, whereas that after nitroglycerin was 1.8±0.2 (P<0.03).

Effect of Endogenously Produced NO on the Permselectivity of the Paracellular Pathway

Thick ascending limbs produce NO from l-arginine. Thus, we next tested whether endogenously produced NO reduced the permselectivity of the paracellular pathway. During the control period, the dilution potential was −11.0±1.7 mV. When 0.5 mmol/L l-arginine was added to stimulate NO production, the dilution potential was −8.0±1.0 mV (Figure 3; n=6; P<0.03), a 27.3% reduction. The calculated PNa/PCl was 2.0±0.2 in the control period, whereas that after l-arginine was 1.6±0.1 (P<0.05).

Signaling Cascade Mediating the Effect of NO on the Paracellular Pathway

NO exerts most of its effects via cGMP. Thus, we tested whether cGMP mediates its effects on PNa/PCl. We found that during the control period, the dilution potential was −13.4±2.9 mV. In the presence of the membrane-permeant cGMP analog, dibutyryl-cGMP (500 µmol/L), the dilution potential was −7.5±1.8 mV, a reduction of 44% (Figure 4; P<0.02; n=6). Controls showed no significant changes with time.

cGMP can activate 2 downstream mediators, PKG12 and phosphodiesterase 2.15 Thus, we next tested which of these mediates the actions of NO. First, we studied the effect of the PKG inhibitor KT5823 (4 µmol/L). During the control period, in the presence of KT5823, the dilution potential was −8.6±0.1 mV. When SPM was added, the dilution potential was −7.1±0.9 mV, not significantly different (Figure 5; n=6). KT5823 alone did not affect the dilution potential.

Next, we tested the effect of phosphodiesterase 2–specific inhibitor BAY60-7550 (10 µmol/L). The dilution potential in
the control period with BAY60-7550 present was $-7.0 \pm 0.5$ mV. After the addition of SPM (200 µmol/L), the dilution potential was $-4.1 \pm 0.5$ mV (Figure 6; n=6; $P<0.005$), a reduction of 41.4%. Time controls showed no significant differences.

**Discussion**

Our hypothesis was that NO decreases the permselectivity of the paracellular pathway in thick ascending limbs. We found that (1) the PNa/PCl (a measure of permselectivity of the paracellular pathway) of perfused thick ascending limbs measured under different conditions was $\approx 2$; (2) NO donors reduce PNa/PCl; (3) endogenously produced NO reduces the PNa/PCl of the paracellular pathway; (4) cGMP mimicks the effects of the NO donors; (5) inhibiting PKG blocks the effect of NO; and (6) inhibiting phosphodiesterase 2 does not block the effect of NO. These are the first results showing that both exogenously added and endogenously produced NO reduce the permselectivity of the paracellular pathway in the thick ascending limb or any nephron segment. They are also the first data showing that cGMP and PKG mediate these effects.

We studied the effects of NO on the paracellular pathway by measuring Na/Cl dilution potentials and calculating PNa/PCl. The ionic permeability of the paracellular pathway is a result of charge selectivity and steric hindrance. Dilution potentials result from the differential permeabilities of Na and Cl of the paracellular pathway. Thus, they are a direct measure of a physiologically relevant parameter which takes into account both charge selectivity and steric hindrance. We found that the PNa/PCl was $\approx 2$ under a variety of conditions and did not change significantly with time. Our results are comparable with those previously reported$^{3,6}$ and indicate that the paracellular pathway of thick ascending limbs is selective for Na over Cl by a factor of 2. The fact that PNa/PCl was stable over time indicates that cell viability was maintained throughout the protocol. This is consistent with our previous data, which show that isolated, perfused thick ascending limbs remain viable for 85 to 90 minutes.$^{12,15,25,26}$

NO has been shown to reduce net transepithelial NaCl reabsorption$^{10,27}$ and Na/K/2 Cl cotransport activity$^{13}$ in thick ascending limbs. However, its effect on the paracellular pathway, which is responsible for as much as 50% of Na reabsorption, is unknown. Our data show that the NO donor SPM decreased both the magnitude of the Na/Cl dilution potential and the calculated PNa/PCl. Nitroglycerin had a similar effect.

The fact that 2 chemically distinct NO donors similarly reduced PNa/PCl is strong evidence that the effect was because of NO and not a byproduct of either SPM’s or nitroglycerin’s metabolism/degradation. The larger inhibitory effect observed with SPM despite using equimolar concentrations of both compounds can most likely be explained by the fact that although SPM spontaneously degrades releasing NO with...
a half-time of 40 minutes in aqueous solutions, nitroglycerin needs to be metabolized to produce NO. The concentration of SPM we used produces an NO concentration of ≈1.6 µmol/L. This is within the physiological range of 0.6 to 9 µmol/L previously reported for the renal medulla.34,29

Because thick ascending limbs produce NO and physiologically relevant concentrations of NO from donors reduced PNA/PCI, we next tested whether the results observed with exogenously added NO were mimicked by endogenously produced NO. We found that endogenously produced NO significantly decreased both dilution potentials and PNA/PCI.

We tested the effects of endogenously produced NO on PNA/PCI by first depleting tubules of l-arginine by perfusing and bathing them in l-arginine-free solution, and then adding it back to the bath. This protocol works because the Y+ transporter that moves l-arginine into the cell will also remove it from the cell if the chemical gradient favors exit. Because the bath is constantly exchanged, the concentration of l-arginine in the bath is effectively zero. Thus, the chemical gradient always favors l-arginine exit from the cell. When l-arginine is added back to the basolateral bath NO production begins because NOS is already activated by luminal flow and only lacks substrate to produce NO.30

Our finding that addition of l-arginine to the bath reduces PNA/PCI and the conclusion that this is because of NO is based on our previously published work showing that (1) l-arginine stimulates NO production31; (2) l-arginine inhibits net NaCl reabsorption and this is blocked by L-NAME, an inhibitor of NOS; (3) d-arginine does not mimic the effects of l-arginine on net NaCl reabsorption.32 If l-arginine per se rather than NO was affecting the paracellular pathway in the experiments reported here, we would have been able to observe this effect in the previously reported experiments in which net NaCl reabsorption was measured. Such an effect would have reduced net reabsorption, not been inhibitable by L-NAME, and may have been mimicked by d-arginine.

The concentration of l-arginine in the outer medulla of the kidney is not clear. Recently, it has been reported that the concentration in the entire kidney may be as high as 0.5 mmol/L.33 If this value is accepted for whole kidney, one would expect that in the chemical gradient favors exit. Because the bath is constantly exchanged, the concentration of l-arginine in the bath is effectively zero. Thus, the chemical gradient always favors l-arginine exit from the cell. When l-arginine is added back to the basolateral bath NO production begins because NOS is already activated by luminal flow and only lacks substrate to produce NO.30

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In our experimental design, luminal flow serves as the stimulus for NO production.30,34 Flow in the nephron is not static but oscillates,35–37 and in some nephron segments it stops periodically.35,37 Thus, it is likely that the NO signaling also oscillates and therefore the effect of NO on the paracellular pathway is presumably always present but variable.

There are at least 3 possible explanations for the fact that NO reduces PNA/PCI. First, PNA may be reduced. Second, PCI may be increased. Third, both PNA and PCI may be affected in opposite ways. Given that NO reduces net transepithelial NaCl reabsorption, it is likely that NO decreases PNA rather than increasing PCI. Such an effect would be expected to contribute to the reduction in net NaCl reabsorption caused by NO.

The permselectivity of the paracellular pathway is because of the claudin family of proteins. Thick ascending limbs express several claudins including claudin-3, claudin-10, claudin-16, claudin-18, and claudin-19.38 Recently it was reported that claudin-10b, an isoform of claudin-10, is the primary regulator of Na selectivity of the paracellular pathway.38 Thus, NO may be affecting this protein.

Although we have previously shown that NO inhibits active, transcellular NaCl reabsorption,3,13,14,32 this is likely to have a negligible effect on the dilution potentials and calculated PNA/PCI in our experiments because of the nature of our experimental design. The dilution potentials that we used to calculate PNA/PCI in the presence of NO take into account any effect NO may have on the spontaneously developed transepithelial voltage because of active transport. Furthermore, because we diluted the concentration of NaCl in the bath rather than in the luminal perfusion solution, Na/K/2Cl cotransport activity is not diminished by the reduction in NaCl concentration, and, in fact, if anything it would be enhanced. This would lead to an underestimation of the true dilution potential, and thus our experimental design is conservative by nature. We do not think this is a major issue, however, because our calculated control PNA/PCI ratios are consistent with those previously reported in the literature.3,5,6,39

Our study is the first to investigate whether NO regulates the permselectivity of the paracellular pathway in isolated, perfused tubules. Besides our study, there is little evidence about the effects of NO on paracellular permeability in the kidney. As far as we know, there is only a single report by Liang et al.40 These authors showed a dose-dependent effect of NO on paracellular permeability of immortalized opossum kidney cells in culture. In this study, high concentrations of the NO donor Na nitroprusside increased permeability, increased lipid peroxidation, and reduced ATP levels.40 Given the long incubation times used in this study and the fact that the effects were reversed by superoxide dismutase, it is unclear whether the results were because of NO per se or ONOO−, produced when NO and O2− react.

Outside the kidney NO has been shown to regulate the permselectivity of the paracellular pathway in numerous systems. In epithelial cells of the gastrointestinal tract, NO has been shown to both increase and decrease permeability.20–23,41–45 The precise explanation for these data is unclear; however, it may be related to the source of the NO, and therefore its concentration. Most studies in which constitutive NO synthases, including neuronal NO synthase and endothelial NO synthase, report that NO decrease permeability.46 In contrast, when inducible NO synthase is activated, NO seems to increase permeability.20–22,45,46 It is known that constitutive NO synthases produce low, levels of NO, whereas inducible NO synthase is a high output enzyme involved in pathophysiological disorders.47,48 Thus, low levels of NO produced by constitutive NO synthase play a crucial role in the maintenance and regulation of normal gut function, whereas lack of constitutive NO synthase activity or excess of inducible NO synthase activity leads to gut inflammation.48 cGMP mediates the effects of NO on Na transport in both proximal tubules49–51 and thick ascending limbs.1,52 We found that NO reduces the permselectivity of the paracellular pathway via cGMP. Our data show that the membrane-permeant cGMP analog dibutyryl-cGMP reproduced the effects of NO indicating that cGMP mediates the first step of the signaling pathway. These data are consistent with our previous data showing that NO inhibits net bicarbonate and NaCl reabsorption via cGMP in this nephron segment. They are also consistent with our data showing that NO reduces NHE3 (Na+/H+ exchange),...
exchanger type 3) and NKCC2 (Na+/K+/2Cl– cotransporter) activity via cGMP. In fact, the magnitude of the decrease in the dilution potentials was larger, probably because of the fact that a relatively high concentration of the second messenger was used to assure a visible result in the case of an effect. Both cGMP-dependent and independent pathways have been linked to changes in permeability in a variety of cell types. Increased cAMP levels have been consistently shown to preserve paracellular barrier integrity.53–55 Specifically in the kidney, cAMP has been shown to mediate changes in paracellular permeability in the proximal tubule, leading to changes in transport.36 The relationship between cGMP levels and changes in the paracellular pathway is not as clear. Increases in cGMP have shown to both increase and decrease permeability and permselectivity in different cell types.57–60 Our results showing that cGMP regulates the paracellular pathway in the thick ascending limb are in agreement with other reports showing a NO/cGMP cascade involved in the decrease of permeability in endothelial cells,57,58 as well as permeability and permselectivity23,24 of the intestinal barrier.

The effects of NO on the paracellular pathway in other cells are mediated by PKG; however, NO inhibits NaCl reabsorption by thick ascending limbs via phosphodiesterase 2. As a result, we next evaluated which mediates the actions of NO on the permeability of the paracellular pathway in isolated thick ascending limbs. We found that PKG mediates the effects of NO on the paracellular pathway, based on the fact that the effect of NO was prevented in the presence of a PKG inhibitor. In contrast, when we used a phosphodiesterase 2 inhibitor, NO still reduced PNa/PCI. Together, these data indicate that PKG rather than phosphodiesterase 2 mediates the effect of NO on the permeability of the paracellular pathway in thick ascending limbs. These data are consistent with our previous data showing that NO reduces bicarbonate reabsorption in this segment via phosphodiesterase 2 mediates the inhibitory action of NO on Cl reabsorption. The results of NO were in agreement with those of others who have implicated PKG as the downstream mediator of the effects of NO on the permeability of the paracellular pathway.18,19

Perspectives

In summary, our results show that NO reduces the permselectivity of the paracellular pathway in thick ascending limbs via cGMP and PKG. The data presented in this report may provide evidence of an additional antihypertensive action exerted by NO in the thick ascending limb. To our knowledge, it is the first work showing a regulatory effect of NO on the permselectivity of the paracellular pathway in native renal tubules.

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Disclosures

None.

References


Novelty and Significance

**What is New?**

- NO decreases the permeability of the paracellular pathway in thick ascending limbs.
- This mechanism is mediated by cGMP and cGMP-dependent protein kinase.
- Phosphodiesterase 2 does not mediate this process

**What is Relevant?**

- We have previously shown that NO decreases net NaCl and Na bicarbonate reabsorption in thick ascending limb by inhibiting transcellular Na/K/2Cl cotransport and Na/H exchange activity. Up to 50% of reabsorbed Na traverses the paracellular pathway. In this article, we show that NO inhibits the permeability of the paracellular pathway and its mechanism of action, which may provide evidence of an additional anti-hypertensive action exerted by NO in this segment.

**Summary**

Our results show that NO reduces the permeselectivity of the paracellular pathway in thick ascending limbs via cGMP and cGMP-dependent protein kinase. This data presented in this report may provide evidence of an additional antihypertensive action exerted by NO in the thick ascending limb. To our knowledge, it is the first work showing a regulatory effect of NO on the permeability of the paracellular pathway in native renal tubules.
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Nitric oxide decreases the permselectivity of the paracellular pathway in thick ascending limbs

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Short title: NO reduces $P_{Na}/P_{Cl}$ in thick ascending limbs
**Methods**

**Chemicals and Solutions.** The physiological saline solution used to perfuse and bathe thick ascending limbs contained (in mmol/l): 130 NaCl, 4 KCl, 2.5 NaH₂PO₄, 1.2 MgSO₄, 6 L-alanine, 1 Na₃citrate, 5.5 glucose, 2 Ca(lactate)₂, and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4 at 37°C. The final concentration of Na and Cl in this solution was 142 and 134 mmol/l respectively. To generate dilution potentials, the NaCl concentration of the basolateral bath was reduced to either 16/8; 32/24; or 64/56 mmol/l Na/Cl, with all other compounds remaining the same. All solutions were adjusted to 290 ± 3 mOsm/kg H₂O as measured by vapor pressure osmometry using mannitol. The NO donor spermine NONOate and the phosphodiesterase 2 inhibitor BAY-60-7550 were obtained from Cayman Chemical (Ann Arbor, MI). Spermine NONOate was prepared immediately before use. Dibutyryl-cGMP and the PKG inhibitor KT5823 were from Enzo Life Sciences (Farmingdale, NY). L-arginine and nitroglycerin from Sigma-Aldrich (Milwaukee, WI).

**Isolation and Perfusion of Thick Ascending Limbs.** Rats were anesthetized with ketamine and xylazine (100 and 20 mg/kg i.p. body weight, respectively). The abdominal cavity was opened and the left kidney was placed in ice-cold 150 mmol/l NaCl. The capsule was removed and coronal slices cut. Cortical tissue was discarded and thick ascending limbs were dissected from the outer medulla under a stereomicroscope at 4–10°C. Tubules were transferred to a temperature-regulated chamber (37 ± 1°C) with a bath flowing at 1 ml/min. Tubules were held and perfused between glass pipettes as previously described. Measurements of Dilution Potentials. Tubules were initially bathed and perfused in symmetric physiological saline for a 15-min equilibration period during which transepithelial voltage was measured with a set of two calomel electrodes and 150 mmol/l NaCl, 4% agar bridges connected to an electrometer (Axon Instruments, Burlingame, CA). The bath was grounded. Voltages were recorded with a PowerLab acquisition system and PowerChart 8 software (AD Instruments, Colorado Springs, CO). Thick ascending limbs were then bathed for an additional 12 min, and basal voltages were recorded during the last min of this period. Afterwards, the bath was changed to a solution with reduced NaCl, as indicated in the results section, for 12 min. The resulting difference in transepithelial voltage measured 3 min after the exchange was considered as the dilution potential of the control period. The bath was then restored to physiological saline for 12 min to allow tubules to recover. When L-arginine was used to stimulate endogenous NO production physiological saline was restored for 20 min to allow time for the L-arginine to enter the cells and be metabolized to NO. The process was then repeated in the presence of test compounds as indicated in the text. All dilution potentials were corrected for liquid junction potentials.

**PNa/PCI Calculation.** PNa/PCls were calculated from the dilution potentials using the Goldman-Hodgkin-Katz equation:

\[ \Delta V = (RT/zF \log \frac{P_{Na}*[Nain]}{P_{Na}*[Naout]} - P_{Cl}*[Clout]/P_{Cl}*[Clin]) \]

Where: \( \Delta V \) is change in voltage caused by diluting the bath; \( R \) is the ideal gas constant; \( T \) is the temperature in Kelvin; \( F \) is Faraday's constant; \( z \) is the valence of the ions; \( P_{Na} \) and \( P_{Cl} \) are the Na and Cl permeabilities respectively; \( [Naout] \) is the Na concentration in the basolateral bath; \( [Nain] \) is the Na concentration in the lumen; \( [Clout] \) is the Cl concentration in the basolateral bath; \( [Clin] \) is the Cl concentration in the lumen.
Statistical Analysis. All data were processed using GraphPad Prism® 4.0 (GraphPad Software Inc., La Jolla, CA). Two-tailed Student’s t-test for paired experiments was used to analyze the data. Results are presented as means ± SEM. A p value of <0.05 was considered significant.


