Endogenous flow-induced nitric oxide reduces superoxide-stimulated Na/H exchange activity via PKG in thick ascending limbs

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Hong NJ, Garvin JL. Endogenous flow-induced nitric oxide reduces superoxide-stimulated Na/H exchange activity via PKG in thick ascending limbs. Am J Physiol Renal Physiol 308: F444–F449, 2015. First published December 10, 2014; doi:10.1152/ajprenal.00583.2014.—Luminal flow stimulates endogenous nitric oxide (NO) and superoxide (O2-) production by renal thick ascending limbs (TALs). The delicate balance between these two factors regulates Na transport in TALs; NO enhances natriuresis, whereas O2- augments Na absorption. Endogenous, flow-stimulated O2- enhances Na/H exchange (NHE). Flow-stimulated NO reduces flow-induced O2-, a process mediated by cGMP-dependent protein kinase (PKG). However, whether flow-stimulated, endogenously-produced NO diminishes O2- stimulated NHE activity and the signaling pathway involved are unknown. We hypothesized that flow-induced NO reduces the stimulation of NHE activity caused by flow-induced O2- via PKG in TALs. Intracellular pH recovery after an acid load was measured as an indicator of NHE activity in isolated, perfused rat TALs. L-Arginine, the NO synthase substrate, decreased NHE activity by 34% (n = 5; P < 0.04). The O2- scavenger tempol decreased NHE activity by 46% ± 8% (n = 6; P < 0.004) in the absence of NO. In the presence of L-arginine, the inhibitory effect of tempol on NHE activity was reduced to −19 ± 6% (n = 6; P < 0.03). The soluble guanylate cyclase inhibitor LY-83583 blocked the effect of L-arginine thus restoring tempol’s effect on NHE activity to −42 ± 4% (n = 6; P < 0.0005). The PKG inhibitor KT-5823 also inhibited L-arginine’s effect on tempol-reduced NHE activity (−43 ± 5%; n = 5; P < 0.03). We conclude that flow-induced NO reduces the stimulatory effect of endogenous, flow-induced O2- on NHE activity in TALs via an increase in cGMP and PKG activation.

The thick ascending limb of the loop of Henle is a nephron segment that is important in salt, water, and acid/base homeostasis (22, 23, 43). Na is reabsorbed by thick ascending limbs as NaCl and Na bicarbonate via Na-K-2Cl cotransport (43) and Na/H exchange (NHE) (22, 23), respectively. We showed that NHE activity is inhibited by NO (20, 46) and enhanced by O2- (26, 32) in this nephron segment. Increases in luminal flow stimulate NO (7–9, 49, 50) and O2- (1, 19, 27, 28) production. The O2- scavenger tempol reduces net NaCl reabsorption (48) and NHE activity (26) by isolated, perfused thick ascending limbs. We showed that flow-induced NO prevents flow from stimulating O2- production via a cGMP-dependent protein kinase (PKG)-dependent process (29). However, the effects of luminal flow-enhanced NO and O2- production on thick ascending limb transport and the mechanisms involved have not been thoroughly studied. Moreover, whether flow-induced NO reduces flow-stimulated O2- to affect NHE activity and the mechanisms involved are unclear.

We hypothesized that endogenous, flow-stimulated NO reduces the O2- sensitive stimulation of NHE activity and that this process is mediated by a cGMP/PKG signaling pathway. To test this hypothesis, we examined the effect of tempol on the recovery rate of intracellular pH (pHi) after an acid load in isolated, perfused rat thick ascending limbs. We then repeated the experiments in the presence of L-arginine. Finally, we used L-arginine in conjunction with a soluble guanylate cyclase or PKG inhibitor to clarify the signaling pathway involved. Our findings indicate that NO reduces the stimulation of NHE activity caused by endogenous, flow-induced O2- production in thick ascending limbs via a PKG-dependent mechanism.

MATERIALS AND METHODS

Chemicals and solutions. L-Arginine and 4-hydroxy-TEMPO (tempol) were purchased from Sigma (St. Louis, MO). Dibutylryl-cGMP (db-cGMP) was from Enzo Life Sciences (Farm ingdale, NY). The soluble guanylate cyclase inhibitor LY-83583 and the PKG inhibitor KT-5823 were obtained from Cayman Chemical (Ann Arbor, MI). The pH-sensitive fluorescent dye 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was purchased from Life Technologies (Eugene, OR). Thick ascending limbs were perfused and bathed in physiological saline composed of (in mM) 130 NaCl, 2.5 NaH2PO4, 4 KCl, 1.2 MgSO4, 6 L-alanine, 1 N-acetate, 5.5 glucose, 2 (lactate)2, and 10 HEPES, pH 7.4 at 37°C. The composition of the basolateral bath used to acid-load cells was the same, except that 10 mM NH4Cl was added and NaCl was decreased from 130 to 120 mM. 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 (lactate)2, and 25 HEPES and were titrated to selected pH values between 6.5 and 7.5. Nigericin (Life Technologies) was prepared as a 10-mM stock in methanol and diluted in calibration solution to a final concentration of 10 μM just before use. All solutions used to perfuse and bathe thick ascending

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limbs were adjusted to 290 ± 3 mosmol/kg H2O as measured by vapor pressure osmometry.

**Animals.** Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were maintained on a diet containing 0.22% sodium and 1.1% potassium (Purina, Richmond, IN) for at least 5 days. All protocols involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Case Western Reserve University in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Isolation and perfusion of thick ascending limbs. Rats weighing 120–150 g were anesthetized with ketamine and xylazine (100 and 20 mg/kg body wt, respectively) via intraperitoneal injection. After the abdominal cavity was opened, the left kidney was bathed in ice-cold saline and removed. Coronal slices were placed in physiological saline, and medullary thick ascending limbs were dissected from the outer medulla under a stereomicroscope at 4–10°C. Tubules ranging from 0.5 to 1.0 mm were transferred to a temperature-regulated chamber (37°C) or H11006 from 0.5 to 1.0 mm were transferred to a temperature-regulated chamber (37°C) and perfused using concentric glass pipettes as described previously (18). Luminal flow rate was 20 nl/min.

**Measurement of pH.** Thick ascending limbs were loaded for 5 min with 0.5 μM BCECF-AM in physiological saline and then washed for 20 min in dye-free solution. A Xenon arc lamp and a Lambda 10–2 filter wheel (Sutter Instrument, Novato, CA) were used to excite the dye alternately at 488 and 440 nm. Emitted fluorescence was collected with a ×40 oil immersion objective mounted on an inverted microscope (Nikon Eclipse TE300) equipped with a 505-nm dichroic mirror and a 520–560-nm barrier filter and digitally imaged with a CoolSnap HQ digital camera (Photometrics, Tucson, AZ). Ratiometric data (488 nm/440 nm fluorescence) were recorded with MetaFluor version 6.2r6 imaging software (Molecular Devices, Downingtown, PA) and were converted to pH values using a three-point nigericin/high-K+ technique for in situ calibration (20).

**Measurement of NHE activity.** After the dye-loading/washing period, measurements were taken once every 3 s to establish baseline pH. Then, cells were acid-loaded using the NH4Cl prepulse method (10) in which the basolateral bath was switched to NH4Cl solution for 28 s and then switched back to physiological saline while continuing to measure every 3 s. The initial rate of pH recovery that follows the intracellular acidification was taken as a measurement of NHE activity. After 10 data points beyond the nadir of pH were recorded, measurements were stopped. Thick ascending limbs were treated with tempol (100 μM) via the basolateral bath and the NH4Cl prepulse/measurement procedure was repeated after 5 min. Recovery rates for the two periods were calculated and compared. The substrate for NO synthase, L-arginine (0.5 mM), was present in the basolateral bath throughout the experiments in which the effect of NO on O2-stimulated NHE activity was studied. For experiments using db-cGMP (0.5 mM), the chemical was present in the basolateral bath throughout the experiment. For experiments in which LY-83583 (10 μM) or KT-5823 (5 μM) were used, the inhibitors were present in the basolateral bath throughout the experiment.

**Statistical analysis.** Results are expressed as means ± SE. Data were evaluated using two-tailed Student’s t-test for paired experiments with a P < 0.05 criterion for significance.

**RESULTS**

To begin to test our hypothesis, we studied the effect of endogenous, flow-stimulated NO on NHE activity by using the NO synthase substrate L-arginine (Fig. 1). The basal pH recovery rate was 0.411 ± 0.114 pH U/min. Treatment with L-arginine (0.5 mM) in the bath caused a 34 ± 5% decrease to 0.276 ± 0.085 (Δ = −0.135 ± 0.043; P < 0.04; n = 5). These data suggest that endogenous, flow-induced NO inhibits NHE activity.

We next studied the effect of endogenous, flow-induced O2 production in the absence of L-arginine, and therefore NO. Figure 2 shows that 0.1 mM tempol, a O2- scavenger, caused a 46 ± 8% decrease in pH recovery rate (from 0.672 ± 0.147 to 0.393 ± 0.128 pH U/min; Δ = −0.278 ± 0.054 pH U/min; P < 0.004; n = 6). These data indicate that endogenous O2 stimulates NHE activity.

When the tempol experiments were repeated in the presence of L-arginine (Fig. 3), addition of the O2- scavenger decreased pH recovery rate by only 19 ± 6% (0.576 ± 0.119 pH U/min for arginine alone vs. 0.463 ± 0.103 pH U/min; Δ = −0.113 ± 0.037 pH U/min for arginine and tempol; P < 0.03; n = 6). These data indicate that endogenous, flow-induced NO diminishes the stimulatory effect of O2- on NHE activity in thick ascending limbs.

NO activates soluble guanylate cyclase causing an increase in cGMP. One of the ways in which NO can reduce O2- in the thick ascending limb is via a cGMP/PKG signaling mechanism (29). We began to examine the first step in this pathway by testing the effect of the soluble guanylate cyclase inhibitor LY-83583 on the ability of endogenous, flow-induced NO to reduce the stimulatory effect of O2- on NHE activity (Fig. 4). In Fig. 4A, tempol reduced pH recovery rate by 45 ± 6% (from 0.347 ± 0.110 to 0.185 ± 0.055 pH U/min; Δ = −0.162 ± 0.057 pH U/min; P < 0.04; n = 6). When tubules were treated with LY-83583 (10 μM), L-arginine’s ability to reduce tempol’s effect was blocked (Fig. 4B). In the presence
of LY-83583 and l-arginine, pH\textsubscript{i} recovery rate was 0.396 ± 0.042 pH\textsubscript{i} U/min. The addition of tempol in the presence of these drugs caused a 42 ± 4% reduction to 0.233 ± 0.034 pH\textsubscript{i} U/min (Δ = −0.164 ± 0.020 pH\textsubscript{i} U/min; \(P < 0.0005; n = 6\)), similar to the effect of tempol in the absence of l-arginine.

Next, we tested whether the cGMP analog db-cGMP could mimic the effect of l-arginine on the stimulatory effect of O\textsubscript{2}\textsuperscript{-} on NHE activity. As shown in Fig. 5, db-cGMP (0.5 mM) blocked tempol’s ability to reduce pH\textsubscript{i} recovery rate (0.150 ± 0.027 pH\textsubscript{i} U/min for db-cGMP alone vs. 0.145 ± 0.026 pH\textsubscript{i} U/min; Δ = −0.004 ± 0.002 pH\textsubscript{i} U/min for db-cGMP and tempol; \(n = 6\)). Taken together, these data suggest that NO reduces O\textsubscript{2}\textsuperscript{-}’s effect on NHE activity in a cGMP-dependent manner rather than acting as a scavenger of O\textsubscript{2}\textsuperscript{-}.

The increase in cGMP stimulated by NO production can activate a PKG signaling cascade. Therefore, we next studied whether NO’s ability to diminish O\textsubscript{2}\textsuperscript{-} -stimulated NHE activity was dependent on PKG. With the PKG inhibitor KT-5823 in the basolateral bath, we tested the ability of l-arginine to reduce the effect of tempol on NHE activity (Fig. 6). KT-5823 (5 μM) blocked l-arginine’s effect, thereby restoring tempol’s effect on pH\textsubscript{i} recovery rate to −43 ± 5% (0.299 ± 0.076 pH\textsubscript{i} U/min for KT-5823 and l-arginine vs. 0.161 ± 0.039 pH\textsubscript{i} U/min for KT-5823 and l-arginine plus tempol; Δ = −0.138 ± 0.041 pH\textsubscript{i} U/min; \(n = 5; P < 0.03\)). These data suggest that the inhibitory effect of flow-induced endogenous NO on enhancement of NHE activity caused by flow-induced endogenous O\textsubscript{2}\textsuperscript{-} is PKG-dependent and that scavenging of O\textsubscript{2}\textsuperscript{-} by NO plays a minor role.
DISCUSSION

The effect of the interaction between endogenous, flow-stimulated NO and O$_2^-$ on thick ascending limb NHE activity and the signaling pathway involved are unclear. Our hypothesis was that endogenous, flow-induced NO reduces the stimulation of NHE activity caused by endogenous, flow-induced O$_2^-$ production, and this effect is due to a cGMP/PKG-dependent mechanism rather than scavenging.

To begin to test this hypothesis, we first measured the effect of L-arginine on NHE activity. We found that it caused $\sim$35% reduction in NHE activity. These results provide an explanation for our previous data showing that L-arginine inhibits thick ascending limb HCO$_3^-$ transport (46). Our findings are also similar to our previous results with NO donors on apical NHE activity (20).

Next, we studied the effect of O$_2^-$ on NHE activity. We found that tempol treatment caused $\sim$45% decrease in NHE activity, suggesting that O$_2^-$ stimulates NHE activity. These data are in agreement with our previous results showing that exogenously added O$_2^-$ stimulates apical NHE activity (32). In addition, these data are consistent with our recent report that endogenous, flow-induced O$_2^-$ stimulates NHE activity (26).

To study the interaction between NO and O$_2^-$, we tested the effect of tempol on NHE activity in the presence of L-arginine, and found that tempol’s effect was reduced by more than half to 19%. These data indicate that NO attenuates the stimulatory effect of O$_2^-$ on NHE activity.

While our data show that endogenous NO inhibits the effect of endogenous O$_2^-$ on NHE activity, it must be noted that the simple calculation performed above actually underestimates the real effect. To calculate the real effect of tempol in the presence of L-arginine, one must take into account that L-arginine alone reduces NHE activity to 65%. As a result, the basal rate of NHE activity in the absence of both L-arginine and tempol for the experiments depicted in Fig. 3 would be 0.886 pHi/min, which translates to $0.886/0.65 = 0.886$. Thus, tempol only really reduces NHE activity by 12% rather than 19% in the presence of L-arginine ($\Delta0.113/0.886 \times 100 = 12\%$). This calculation indicates that endogenous, flow-induced NO actually prevents nearly 90% of the stimulatory effect of O$_2^-$ on NHE activity.

To study whether NO’s ability to reduce O$_2^-$’s effects on NHE activity was due to scavenging or true inhibition, we used pharmacological inhibitors of components of the NO/cGMP/PGK pathway. The soluble guanylate cyclase inhibitor LY-83523 and the PKG inhibitor KT-5823 each blocked virtually all of NO’s effect on O$_2^-$-stimulated NHE activity. Thus, endogenous, flow-induced NO reduces O$_2^-$-sensitive stimulation of NHE activity in thick ascending limbs and this process is cGMP- and PKG-dependent. This is the first study to show that endogenous, flow-induced NO and O$_2^-$ interact to affect luminal NHE activity primarily by a nonscavenging process.

From the current data, we cannot conclude whether NO inhibits the ability of flow-induced O$_2^-$ to stimulate NHE activity by: 1) a direct action on NHE; 2) an indirect effect on some other regulatory protein; or 3) PKG inhibiting O$_2^-$ production by NADPH oxidase directly. However, the latter is most likely because of several previous studies. We showed that flow-stimulated O$_2^-$ production by thick ascending limbs is due to activation of NADPH oxidase (27, 28) and that endogenous NO reduces flow-stimulated O$_2^-$ production primarily via a PKG-mediated process (29). Additionally, Fujii et al. (17) found that in the renal cortex of Dahl salt-sensitive rats, arginine inactivates the NADPH oxidase subunit p47phox, which would reduce O$_2^-$ production by preventing NADPH oxidase assembly. Muzaffar et al. (45) demonstrated in human vascular smooth muscle cells that NO acutely inhibits O$_2^-$ production by blocking the translocation of p47phox via a PKG-dependent mechanism. Thus, it is possible that in thick ascending limbs PKG inhibits the activation of NADPH oxidase in a similar manner.

Protein kinase C (PKC) may also play a role in the effect of NO on O$_2^-$’s actions in the thick ascending limb. PKG phosphorylates (21) PKC in PC12 cells and it inhibits PKC (34) in vascular smooth muscle cells. PKC is required for assembly of p47phox with other subunits to form active NADPH oxidase (6, 59). Additionally, we found that PKC mediates flow-induced increases in O$_2^-$ production in thick ascending limbs (30). Therefore, it is possible that NO increases cGMP and thereby PKG, which in turn phosphorylates and inhibits PKC. As a result p47phox activity and NADPH oxidase assembly are reduced to diminish flow-stimulated increases in O$_2^-$ production.

We observed that endogenous, flow-induced NO diminishes but does not completely block O$_2^-$-stimulated NHE activity (Fig. 3). This finding is similar to our previous findings that showed flow-stimulated NO reduces flow-stimulated O$_2^-$ production by thick ascending limbs primarily via PKG-mediated process rather than scavenging (29).

While this is the first study to demonstrate the effect of the interaction of flow-induced NO and O$_2^-$ on thick ascending limb transport, it must be noted that the data gathered from in vitro experiments may not reflect what occurs in vivo. The in vivo system is more complex and several other variables may be involved. However, this study strengthens what has been ascertained previously with regard to NO and O$_2^-$ interaction. Most importantly, this study shows the effect of flow-induced NO and O$_2^-$ production on renal tubule transport, which had not previously been demonstrated. As is the case with any in vitro study, the limitation in terms of impact and interpretation in an in vivo system must be considered.

The effect of luminal flow on ion transport has been shown in several nephron segments. Increases in luminal flow enhance Na reabsorption in the proximal tubule (13, 62), thick ascending limb of the loop of Henle (52), and cortical collecting duct (16, 57). In proximal tubules, luminal flow stimulates bicarbonate reabsorption (2, 38) via changes in both NHE and H-ATPase activity (13). Flow also affects chloride reabsorption in proximal tubules (24, 65). In rabbit cortical collecting ducts, luminal flow regulates apical epithelial Na channels (57), enhances net Na absorption (16, 57) and BK channel-mediated K secretion (64). Increases in luminal flow also stimulate Na and Cl transport in avian thick ascending limbs (52).

Although several studies have implicated the interaction effects of NO and O$_2^-$ on renal function (41, 42, 63, 68), most of the effects of NO and O$_2^-$ interaction on ion transport within the kidney have been studied in the thick ascending limb. We found that tempol enhanced the NO-induced inhibition of Cl reabsorption by thick ascending limbs (47). Mori and Cowley (44) found that angiotensin II stimulates O$_2^-$ production in thick ascending limbs via the NADPH oxidase pathway and...
that interactions of O$_2^-$ and NO determine the effectiveness of cross-talk between the thick ascending limb and vasa recta. Recently, De Miguel et al. (11) showed that the interaction of NO and O$_2^-$ during diabetes affects Na transport in thick ascending limbs.

Little is known about the effect of interactions of NO and O$_2^-$ on transport in other nephron segments. Lu and Wang (39) found that the effect of NO on basolateral K channels in the collecting duct is modulated by O$_2^-$. NO stimulation of COX-2 in collecting duct cells appears to be mediated through mechanisms involving MAP kinase and O$_2^-$, rather than a GMP (66).

In summary, we found that endogenous, flow-induced NO reduces the enhancement of NHE activity stimulated by endogenous, flow-induced O$_2^-$ and this process is dependent on PKG. The interaction between these two reactive oxygen species is important in regulating renal water and salt reabsorption. Understanding the processes involved in maintaining the balance between NO and O$_2^-$ may give insight into the pathogenesis and treatment of diseases associated with oxidative stress and Na retention.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: N.J.H. performed experiments; N.J.H. analyzed data; N.J.H. and J.L.G. interpreted results of experiments; N.J.H. prepared figures; N.J.H. drafted manuscript; N.J.H. and J.L.G. edited and revised data; N.J.H. and J.L.G. interpreted results of experiments; N.J.H. prepared figures; N.J.H. and J.L.G. edited and revised manuscript; N.J.H. and J.L.G. approved final version of manuscript; J.L.G. conceived and designed the research.

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