Regulation of thick ascending limb transport: role of nitric oxide

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Herrera, Marcela, Pablo A. Ortiz, and Jeffrey L. Garvin. Regulation of thick ascending limb transport: role of nitric oxide. Am J Physiol Renal Physiol 290: F1279–F1284, 2006; doi:10.1152/ajprenal.00465.2005.—Nitric oxide (NO) plays a role in many physiological and pathophysiological processes. In the kidney, NO reduces renal vascular resistance, increases glomerular filtration rate, alters renin release, and inhibits transport along the nephron. The thick ascending limb is responsible for absorbing 20–30% of the filtered load of NaCl, much of the bicarbonate that escapes the proximal nephron, and a significant fraction of the divalent cations reclaimed from the forming urine. Additionally, this nephron segment plays a role in K+ homeostasis. This article will review recent advances in our understanding of the role NO plays in regulating the transport processes of the thick ascending limb. NO has been shown to inhibit NaCl absorption primarily by reducing Na+-K+-2Cl⁻ cotransport activity. NO also inhibits bicarbonate absorption by reducing Na+/H⁺ exchange activity. It has also been reported to enhance luminal K⁺ channel activity and thus is likely to alter K⁺ secretion. The source of NO may be vascular structures such as the afferent arteriole or vasa recta, or the thick ascending limb itself. NO is produced by NO synthase 3 in this segment, and several factors that regulate its activity both acutely and chronically have recently been identified. Although the effects of NO on thick ascending limb transport have received a great deal of attention recently, its effects on divalent ion absorption and many other issues remain unexplored.

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NITRIC OXIDE (NO) IS A KEY regulator of physiological processes, ranging from long-term potentiation of synaptic transmission in the brain (7, 9, 86) to dilatation of resistance vessels in the circulatory system (14, 43, 44). In the kidney, NO reduces renal vascular resistance (64, 87), increases glomerular filtration rate (5, 45, 46, 60, 80), and appears to have a biphasic effect on renin release (48, 82, 91). NO also regulates solute and solvent transport along the nephron (71). The importance of NO as a regulator of ion and water transport was first demonstrated by in vivo experiments showing that stimulating NO production increased urinary Na⁺ and water excretion (6, 50, 62, 96), whereas inhibiting NO generation decreased excretion (49–51). In many situations, the natriuretic and diuretic effects of NO were not accompanied by changes in glomerular filtration rate or renal blood flow (58, 59), suggesting that NO directly inhibits Na⁺ and water transport along the nephron. The source of NO in these early studies was not investigated and could have involved one or more of the three NO synthase (NOS) isoforms expressed in the kidney: NOS1, NOS2, and NOS3. Originally, these enzymes were referred to as inducible (iNOS), neuronal (nNOS), and endothelial (eNOS) after the tissues where they were identified; but this nomenclature now creates confusion, as each NOS isoform is expressed in many cell types (37). The thick ascending limb of the loop of Henle absorbs 20–30% of the filtered load of NaCl (27), and because it is water impermeable it generates most of the osmotic gradient that drives fluid absorption in the collecting duct system. Thus it is logical to assume that some of the effects of NO on urinary Na⁺ excretion and urinary volume are due to its actions in the thick ascending limb.

TRANSPORTERS INVOLVED IN NaCl ABSORPTION

NaCl absorption in the thick ascending limb occurs via both transcellular and paracellular pathways. Transcellular absorption is an active process in which Na⁺ and Cl⁻ enter thick ascending limb cells via luminal Na⁺-K⁺-2Cl⁻ cotransporters. Cl⁻ ions exit across the basolateral plasma membrane via Cl⁻ channels and/or K⁺-Cl⁻ cotransport (26). The basolateral Na⁺-K⁺-ATPase generates the driving force for NaCl entry by extruding Na⁺ across the basolateral membrane (63). Na⁺ is also absorbed via the paracellular pathway due to the lumen-positive voltage caused by recycling of K⁺ across the luminal membrane and back into the forming urine (29–34).

The effects of NO on NaCl absorption were first demonstrated in isolated, perfused tubules. NO from a donor was found to inhibit Cl⁻ flux (JCl) (78). These data indicated that NO from other sources, such as the afferent arterioles, descending vasa recta, or other nephron segments, may regulate NaCl transport in the thick ascending limb via generation and diffusion of NO. Addition of L-arginine, the substrate of NOS, also decreased JCl, and this effect was blocked by the NOS inhibitor N⁶-nitro-L-arginine methyl ester (L-NAME). These data suggest that NO is produced by thick ascending limbs and acts as an autacoid to inhibit NaCl absorption (78). The conclusion...
that NO is produced by the thick ascending limb was confirmed by direct measurements of NO in isolated, perfused thick ascending limbs as well as in thick ascending limb suspensions using two different techniques: a NO-specific fluorescent dye (67, 85) and a NO-selective electrode (38, 73).

The inhibitory effects of NO on thick ascending limb J_{CI} could be due to inhibition of one of the luminal membrane transporters (Na^+-K^+-2Cl^- cotransporter or K^+ channels) or one of the basolateral transporters (Na^+-K^+^-ATPase; Cl^- channels or K^+^-Cl^- cotransporter) (Fig. 1). Data showing that NO decreased both intracellular Na^+ and Cl^- indicate that NO decreases NaCl entry rather than exit. Furthermore, because the Na^+-K^+-2Cl^- cotransporter links Na^+ and Cl^- entry, the effect of NO on J_{CI} was most likely due to inhibition of the luminal Na^+-K^+-2Cl^- cotransporter. Studies directly investigating Na^+-K^+-2Cl^- cotransport activity confirmed this. Measurements of initial Na^+ influx when luminal NaCl was increased from 0 to 130 mM in isolated, perfused thick ascending limbs revealed that the rate was lower after NO treatment (72).

The direct measurements of inhibition of Na^+-K^+-2Cl^- cotransport by NO conflict with the predicted effect based on patch-clamp studies of luminal K^+ channels. In patch-clamp studies, NO donors have been reported to stimulate the inwardly rectified K^+ channel (ROMK) (57). This led to the prediction that NO would stimulate Na^+-K^+-2Cl^- cotransport in the thick ascending limb and thus NaCl absorption, because inhibiting ROMK reduces Na^+-K^+-2Cl^- cotransport (40). However, this reasoning was proven incorrect, because increasing luminal K^+ depolarized the luminal membrane potential to the same extent before and after treatment of isolated, perfused tubules with NO, suggesting that NO did not have a significant effect on luminal K^+ permeability. Additionally, increasing luminal membrane K^+ permeability with valinomycin did not block the inhibitory action of NO (72). Why patch-clamp studies show that NO stimulates ROMK, whereas other studies do not remains an unanswered question.

Although NO reduces intracellular Na^+ and Cl^- and inhibits Na^+-K^+-2Cl^- cotransport, this does not necessarily indicate that NO has no effect on Na^+-K^+^-ATPase, only that the magnitude of the effect is relatively less than its effect on the cotransporter. However, at least acutely, NO does not appear to have an effect on Na^+-K^+^-ATPase activity. In suspensions of thick ascending limbs, in the presence of 10.5 mM extracellular Na^+, NO inhibited oxygen consumption to the same extent as furosemide. Addition of nystatin to equilibrate extra- and intracellular Na^+ returned oxygen consumption to control levels in both NO- and furosemide-treated tubules. These data indicate that NO does not affect the affinity constant of Na^+-K^+^-ATPase for intracellular Na^+. Additionally, nystatin stimulated oxygen consumption in NO- and furosemide-treated suspensions to the same extent in the presence of 145.5 mM Na^+. These data indicate that NO does not affect the maximum rate of Na^+-K^+^-ATPase (72).

Although NO does not appear to inhibit Na^+-K^+^-ATPase acutely, this may not be the case chronically. After exposure of thick ascending limbs to a NO donor for 2 h, pump activity was reduced by 32% (90), similar to cultured mouse proximal tubule cells (28). Inhibition in both studies depended on superoxide, because superoxide scavengers abolished the inhibitory effects of NO on Na^+-K^+^-ATPase activity. These results indicate that OONO^- rather than NO per se was responsible. This raises the possibility that inhibition of Na^+-K^+^-ATPase by OONO^- is an artifact of the experimental design due to nonphysiological Po_2 rather than a physiologically significant regulatory process. Po_2 values of the outer medulla are 10–20 mmHg (94) and even those of the cortex are only 40 mmHg (93); whereas Po_2 in both studies was ~160 mmHg, the same as room air.

Besides the potential chronic effects of NO on Na^+-K^+^-ATPase, Turban et al. (88) reported that systemic blockade of NOS by infusion of L-NAME decreased the renal abundance of Na^+-K^+-2Cl^- cotransporters during aldosterone escape, suggesting that NO stimulates thick ascending limb cotransporter expression. However, this observation could have been the result of the increased blood pressure rather than a direct effect of NO on cotransporter expression.

The signaling cascade whereby NO inhibits the Na^+-K^+-2Cl^- cotransporter involves a decrease in cAMP (70), as does inhibition of vasopressin-stimulated osmotic water permeability in the collecting duct (18). NO increases cGMP by stimulating soluble guanylyl cyclase. In turn, cGMP activates cGMP-stimulated phosphodiesterase (phosphodiesterase II). This enhances the degradation of cAMP and thus reduces Na^+-K^+-2Cl^- activity (70). Because cAMP stimulates the
Na⁺-K⁺-2Cl⁻ cotransporter and has recently been shown to increase membrane expression of Na⁺-K⁺-2Cl⁻ cotransporters (68), the mechanism whereby NO decreases Na⁺-K⁺-2Cl⁻ cotransport activity is likely to be mediated by reducing cotransporter levels at the apical membrane. However, such a mechanism has not yet been elucidated to our knowledge.

The effects of NO on the other basolateral transporters involved directly or indirectly with NaCl absorption have not been studied. These include basolateral K⁺ channels, Cl⁻ channels, and K⁺-Cl⁻ cotransporters.

TRANSPORTERS INVOLVED IN ACID-BASE BALANCE

The thick ascending limb absorbs most of the HCO₃⁻ that escapes the proximal tubule (21). HCO₃⁻ absorption is due to secretion of H⁺ via luminal Na⁺/H⁺ exchange. Consequently, HCO₃⁻ reabsorption is regulated primarily through this apical exchanger (21, 22, 24). HCO₃⁻ transport across the basolateral membrane occurs via basolateral Na⁺/HCO₃⁻ (NBCn1) (65), Cl⁻/HCO₃⁻ (AE2) (79), and K⁺/HCO₃⁻ exchangers (8).

Direct measurements of HCO₃⁻ flux in the isolated, perfused tubule demonstrated that stimulation of endogenous NO production by addition of the NOS substrate l-arginine inhibited thick ascending limb HCO₃⁻ transport (69). This effect could be explained by direct inhibition of apical Na⁺/H⁺ exchanger activity, blockade of the basolateral HCO₃⁻ cotransporter or a decrease in basolateral Na⁺-K⁺-ATPase activity, leading to a diminished electrochemical gradient across the membrane. Measurements of the effects of NO on steady-state and intracellular pH (pHi) recovery after an acid load demonstrated that the NO donors spermine-NO NONOate and nitroglycerin inhibited both steady-state pHi and proton efflux as calculated from pHi recovery experiments in isolated, perfused thick ascending limbs (20). Because NO has no direct acute effect on Na⁺-K⁺-ATPase (72), a decrease in Na⁺/H⁺ exchange can only be explained by a direct effect of NO on the exchanger. In addition, by selectively inhibiting the basolateral Na⁺/H⁺ exchanger with dimethyl amiloride, it was found that NO directly inhibited luminal Na⁺/H⁺ exchange. Although the inhibitory effect of NO on thick ascending limb HCO₃⁻ could also be explained by inhibition of basolateral transporters, the direct effects of NO on basolateral HCO₃⁻ transporters have not been investigated to our knowledge.

The signaling cascade whereby NO inhibits thick ascending limb HCO₃⁻ absorption is different from that responsible for inhibition of NaCl absorption. The inhibitory effect of endogenous NO can be mimicked by the cell-permeant cGMP analogs, dibutyryl cGMP and 8-bromoadenosine-cGMP. Addition of l-arginine did not change the rate of HCO₃⁻ absorption after pretreatment with dibutyryl cGMP. These data indicate that cGMP mediates all of the effects of NO on HCO₃⁻ reabsorption by this segment. In addition, the inhibitory effect of l-arginine on HCO₃⁻ transport was completely blocked by a cGMP-dependent protein kinase inhibitor (69).

In contrast to these data, Good et al. (23) described a stimulatory effect of l-arginine and NO donors on thick ascending limb HCO₃⁻ transport. Although the explanation for such disparate results is unclear, it may be due to the age of the animals or different diets. The rats Good et al. used were 2–3 wk younger than those in the study showing NO-induced inhibition (18) and were fed a different diet. Both age and diet could alter endogenous cyclic nucleotide levels and thus the effect of NO (19, 56).

In addition to absorbing HCO₃⁻, the thick ascending limb has been reported to secrete this ion via a recently identified K⁺-dependent cotransporter at the apical membrane (92). Although we could find no reports of the effects of NO on this transporter, its stimulation would appear as inhibition of net absorption, and differential expression of the K⁺/HCO₃⁻ cotransporter could explain the controversy over whether NO stimulates or inhibits HCO₃⁻ absorption.

The thick ascending limb expresses at least two other transporters involved in acid-base balance: the basolateral Na⁺/H⁺ exchanger (11, 66) and a putative K⁺/NH₄⁺ exchanger (3). NO has been shown to inhibit basolateral Na⁺/H⁺ exchange (20), but whether NO inhibits or stimulates K⁺/NH₄⁺ exchange is unknown.

Besides the acute effects of NO on Na⁺/H⁺ exchange, very little is known about its chronic effects. Turban et al. (88) reported that systemic blockade of NOS by infusion of l-NAME decreased renal abundance of Na⁺/H⁺ exchanger NHE3 during aldosterone escape. These data suggest that NO stimulates thick ascending limb exchanger expression. However, this observation could have been the result of increased blood pressure rather than a direct effect of NO on cotransporter expression.

OTHER IONS

The direct effects of NO on thick ascending limb Mg²⁺, Ca²⁺, and NH₄⁺ transport have not been investigated to our knowledge. In this segment, Mg²⁺ and Ca²⁺ are reabsorbed via the paracellular pathway due to the lumen-positive potential (25). Thus one would predict that NO would inhibit divalent cation absorption due to its inhibitory effect on Na⁺-K⁺-2Cl⁻ cotransport. Similarly, one would expect that NO would diminish NH₄⁺ absorption, because NH₄⁺ is absorbed primarily via Na⁺-K⁺-2Cl⁻ cotransport. However, there is no direct evidence in the literature addressing either issue.

ACUTE AND CHRONIC REGULATION OF NO PRODUCTION

As discussed above, NO produced by the thick ascending limb acts as an autacoid to inhibit NaCl absorption. Thus a discussion of the factors that regulate NO production is relevant when one is discussing how NO regulates thick ascending limb transport. The thick ascending limb expresses all three NOS isoforms: NOS1, NOS2, and NOS3 (4, 36, 52, 61, 67, 84, 89). However, studies using knockout mice demonstrated that l-arginine inhibited NaCl reabsorption in thick ascending limbs isolated from NOS1 and NOS2 but not NOS3 knockouts. Furthermore, in vivo gene transfer of NOS3 in the thick ascending limbs of NOS3 knockout mice restored the inhibitory effect of l-arginine on NaCl reabsorption. These data indicate that NOS3-derived NO is responsible for inhibition of NaCl absorption in the thick ascending limb (74, 77). However, they do not rule out the possibility that other NOS isoforms regulate other transport processes in the thick ascending limb, or that they play a role in NaCl absorption under different experimental conditions (i.e., placing animals on a high-salt diet or inducing hypertension or diabetes).

The fact that NOS3 is responsible for the inhibition of thick ascending limb NaCl absorption begs the question of whether
NOS3 in the thick ascending limb is regulated by the same factors that stimulate its activity in endothelial cells. In endothelial cells, endothelin-1 (ET-1), α2-adrenergic activation (2), and ATP (47) all stimulate NOS3 to produce NO. In the isolated, perfused thick ascending limb, we (76) and others (42) found that endothelin-1 inhibited NaCl reabsorption. This inhibitory effect was blunted by the NOS inhibitor L-NAME and depended on α1-NAME. Endothelin-1-dependent inhibition of NaCl absorption was abolished in the presence of an ETB-receptor antagonist and mimicked by an ETB-receptor agonist. In contrast, an ETA antagonist had no effect. Thus endothelin-1 regulates NO production in the thick ascending limb (76). The α2-adrenergic agonist clonidine also reduced NaCl reabsorption in the thick ascending limb. Similar to endothelin-1, this inhibition was abolished by the NOS inhibitor L-NAME or removal of L-arginine. These data suggest that α2-adrenergic receptor activation also regulates NOS in the thick ascending limb (75). Recent data show that ATP can acutely stimulate NO production in isolated thick ascending limbs by activating P2X receptors (85). These data suggest that extracellular ATP may regulate NOS3 activity in the thick ascending limb; however, the effects of ATP on NaCl transport have not been reported to our knowledge. In addition to the physiological regulators described above, the antimalarial drug sodium artesunate inhibits NaCl reabsorption in isolated perfused thick ascending limbs (10). The inhibitory effect on transport was blocked by L-NAME, suggesting that the effect of this drug on renal function may be due to enhanced NO levels in the thick ascending limb.

In addition to humoral factors, mechanical stimuli can also activate NOS3. In endothelial cells, flow activates NOS3 (16, 17, 81). In isolated, perfused thick ascending limbs, increasing luminal flow from 0 to 20 nl/min stimulated NOS3 activity and NO production (73). Although blockade of phosphatidylinositol 3-kinase (PI3-kinase) prevented flow-induced NOS3 activation in thick ascending limbs, similar to endothelial cells, activation of NOS3 in the thick ascending limb causes its translocation from intracellular compartments to the luminal membrane. This does not occur in endothelial cells. Given that flow through the thick ascending limb changes both acutely (13, 41, 53–55, 83) and chronically (2, 15, 54), flow-induced NOS3 activation is likely to play a role in the regulation of NO levels and thick ascending limb transport. However, how NO regulates thick ascending limb NaCl transport under different flow conditions remains to be explored, and our laboratory is currently investigating this.

Although acute regulation of NOS3 activity in the thick ascending limb is similar to endothelial cells in many respects, chronic NOS3 regulation is not. A high-salt diet was shown to augment NOS3 expression in the thick ascending limb. However, this increase in expression was transient, reaching a maximum at 3 days and returning to control levels by 28 days (38). The effects of high salt on NOS3 expression are mediated by activation of ETB receptors and PI3-kinase (35) (Fig. 2). A dual ETA/ETB receptor antagonist blocked the effect of high salt in vivo, and the ability of endothelin to enhance expression was blocked by an ETB-receptor antagonist (36). Additionally, an inhibitor of PI3-kinase blocked the effects of endothelin on NOS3 expression in culture (35). An increase in medullary osmolality is also involved. High salt increased outer medullary osmolality, which, in turn, enhanced endothelin release from thick ascending limb cells and stimulated NOS3 expression in primary cultures (36). The signaling cascade responsible for the decline in NOS3 expression after longer exposure to a high-salt diet has not been investigated to our knowledge.

Although the effects of high salt on both NOS3 expression and activity are biphasic, they are not temporally correlated. Expression peaks at 3 days, whereas NO production peaks at 1 day and returns to control levels by 3 days. This dissociation is a result of differential NOS3 phosphorylation (38). Interestingly, despite unchanged NO levels, inhibition of thick ascending limb NaCl absorption by a given amount of NO was greater in rats fed high salt for 7 days (67). Thus it appears that the signaling cascade beyond NO is sensitized by high salt, so that equal amounts of NO will have a greater effect. From these data, one can speculate that during the early phase of a high-salt diet (day 1), NOS3 expression and NO production will be elevated and thus NaCl reabsorption will be inhibited. By 7 days, the NO signaling cascade becomes sensitized, so that enhanced NO levels are no longer needed. This could be a protective mechanism against the detrimental effects caused by high levels of NO. Our laboratory is currently investigating the mechanism involved.

NOS3 activity in the thick ascending limb also appears to change as a result of diabetes (52). Renal medullary NOS3 activity is increased in diabetic rats. Changes in activity were found to correlate with changes in NOS3 phosphorylation (95). In summary, endogenous and exogenous NO inhibit thick ascending limb transport by directly acting on individual transporters, and thus NO plays a role in the regulation of Na+, water, and acid-base homeostasis. NO production by the thick ascending limb can be acutely regulated by humoral and mechanical stimuli. Changes in the effects and production of NO occur during physiological stress and pathophysiological conditions. Our understanding of how NO production is regulated in the thick ascending limb, as well as how that process affects transport, is still at a rudimentary level.
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