Interaction of O$_2^-$ and NO in the Thick Ascending Limb

Pablo A. Ortiz, Jeffrey L. Garvin

Abstract—Nitric oxide (NO) is an important regulator of NaCl absorption by the thick ascending limb of the loop of Henle (THAL). The free radical superoxide (O$_2^-$) reacts with NO, decreasing its bioavailability. O$_2^-$ is produced by mitochondria and various oxidases, some of which are present in the THAL. However, the ability of the THAL to produce O$_2^-$ and its interaction with NO have not been studied. We hypothesized that NO bioavailability is decreased by O$_2^-$. THALs were isolated and perfused and NO production was measured with an NO-selective microelectrode. Addition of L-Arg (250 μmol/L), but not D-arginine, to the bath increased NO release by 34.8±11.8 pA (n=7). The response to L-Arg was completely abolished by the NO synthase inhibitor L-NAME (n=7). Scavenging THAL O$_2^-$ with the superoxide dismutase (SOD) mimetic Tempol (50 μmol/L) increased L-Arg-induced NO release. At all concentrations of L-Arg tested (50, 100, 250, 500, and 750 μmol/L), further addition of Tempol to the bath significantly increased NO release by THALs. Addition of SOD (300 U/mL) to the bath increased L-Arg-induced NO levels by 82% (n=5; P<0.02). Pretreatment of THALs with the SOD inhibitor diethyl-dithiocarbamate (250 μmol/L) blunted L-Arg-induced NO release by 63% compared with untreated tubules (n=5; P<0.05). Finally, we tested the effect of Tempol on NO-inhibited production of THAL chloride transport. Addition of L-Arg decreased THAL Cl$^-$ absorption by 35%. Subsequent addition of Tempol (50 μmol/L) to the bath further decreased Cl$^-$ absorption by 35% (n=6; P<0.05). We conclude that NO bioavailability in the THAL is decreased by O$_2^-$ in vivo. In addition, we believe our studies are the first to show that endogenous O$_2^-$ may act as a physiological regulator of nephron NaCl transport. (*Hypertension. 2002; 39[part 2]:591-596.*)

Key Words: nitric oxide • natriuresis

The thick ascending limb of the loop of Henle (THAL) plays an important role in the maintenance of NaCl homeostasis by reabsorbing 20% to 30% of filtered NaCl.$^1$ Nitric oxide (NO) is an important regulator of THAL NaCl transport. We have previously shown that endogenous NO inhibits THAL NaCl absorption,$^2,3$ consistent with the natriuretic and diuretic effects of NO observed in vivo.$^4,5$ Therefore, factors affecting THAL NO production as well as NO bioavailability are of physiological significance.

NO rapidly reacts with the free radical superoxide (O$_2^-$) (K=7×10$^9$ mol/L/sec) to produce peroxynitrite (OONO$^-$).$^6$ O$_2^-$ has been shown to decrease NO half-life in various tissues. Jones et al reported that O$_2^-$ produced by activated neutrophils decreased NO released by lung epithelial cells.$^7$ In the vasculature, O$_2^-$ decreases endothelium-dependent relaxation,$^8,9$ whereas superoxide dismutase (SOD) increases NO half-life and potentiates endothelium-dependent dilatation.$^{10}$ Because both NO and O$_2^-$ can exert physiological effects, the rate of production and the ratio of their concentrations are important in systems in which both radicals are produced.

O$_2^-$ is produced by the reduction of molecular oxygen. Virtually all aerobically metabolizing cells are able to produce O$_2^-$, because 1 to 5% of oxygen consumed in mitochondrial respiration is reduced to O$_2^{-}$. In addition, O$_2^-$ production can be catalyzed by different enzymes such as xanthine oxidase (XO),$^{12}$ nicotinamide adenine dinucleotide phosphate (NADPH) oxidase,$^{13}$ P450 monooxygenase,$^{14}$ and other systems.$^{15}$ The THAL contains large numbers of mitochondria. It also expresses some subunits of NADPH oxidase,$^{16}$ and XO activity has been reported as well.$^{17}$ However, it is not known whether the bioavailability of NO produced by the THAL is reduced by interaction with O$_2^-$, nor what effect this has on NaCl transport. We have found that (1) NO bioavailability in the THAL is decreased by O$_2^-$ and (2) scavenging O$_2^-$ enhances the inhibitory effect of NO on THAL NaCl absorption. To our knowledge, these are the first data to show that O$_2^-$ produced by a nephron segment can act as a physiological regulator of transport. Additionally, we report a new method for direct measurement of NO released by isolated, perfused tubules.

Methods

Isolation and Perfusion of Rat Thick Ascending Limbs
Male Sprague-Dawley rats weighing 120 to 150 g (Charles River, Wilmington, Mass) were fed a diet containing 0.22% sodium and...
1.1% potassium (Purina) for at least 5 days. On the day of the experiment, rats 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. Coronal slices were placed in oxygenated physiological saline. Cortical THALs were dissected from the medullary rays under a stereomicroscope at 4 to 10°C, then transferred to a temperature-regulated chamber and perfused using concentric glass pipettes at 37±1°C as described previously.14

NO Release by Isolated, Perfused THALs
L-arginine (L-Arg), the substrate for NOS (Sigma), was added to THALs and NO release measured using an amperometric microelectrode selective for NO (inNO measuring system, Harvard Apparatus). For details, see expanded Methods section.

Measurement of Chloride Absorption
THALs ranging from 0.6 to 1.0 mm were mounted on concentric glass pipettes and perfused at 37°C as described previously.18 The perfusion rate was set at 5 to 10 nL/min/mm. Chloride concentrations in the perfusate and collected fluid were measured by microfluorometry.19 For details, see expanded Methods section.

Results
To test whether NO bioavailability in the THAL is decreased by O2−, we first measured L-Arg-stimulated NO release by isolated, perfused THALs. We have shown that L-Arg decreases THAL transport via a nitric oxide synthase (NOS)-dependent pathway.2,3 However, we know of no direct measurements of NO produced by the THAL. An average increase in electrode output of 34.8 ±11.8 pA was observed when 250 μmol/L L-Arg was added to the bath (n=7) (Figure 1A). We found that the response to L-Arg was directly related to tubule length. When plotting NO response in pA versus THAL length in mm, a positive linear relationship was observed (r²=0.88) (Figure 1B). Consequently, all data are expressed as pA/mm of tubule length. The plot also indicates that the shortest THAL that can be used for these measurements is 0.55 mm.

To make sure the response produced by L-Arg was caused by NO released from THALs, we tested whether inhibition of NOS could prevent this response. Pretreatment of THALs with L-‘nitro-methylarginine (L-NAME) at 5 mmol/L completely abolished L-Arg-induced responses (n=6) (Figure 2). In addition, we tested whether the arginine isomer D-arginine could mimic the response caused by L-Arg; however, when D-arginine at 250 μmol/L was added to the bath, it did not significantly affect the baseline signal (n=6). Taken together, these data indicate that the response observed after adding L-Arg to the bath is caused by NO produced by NOS present in the THAL.

The bioavailability of NO can be reduced by O2−,7,10 and the THAL possesses a large number of mitochondria as well as O2−-producing oxidases.16,17 To study whether NO is degraded by O2−, we first tested whether decreasing the O2− concentration by scavenging with the SOD mimetic Tempol20 could increase the detectable amount of NO released after L-Arg stimulation. We found that adding 250 μmol/L L-Arg to the bath increased THAL NO by 46.1±8.4 pA/mm. Subsequent addition of 50 μmol/L Tempol to the bath significantly increased the signal to 75.5±10.1 pA/mm, a 64% increase in NO levels (n=5; P<0.01).

To test whether Tempol could increase NO levels over a wide range of L-Arg concentrations, we obtained a dose-response curve for L-Arg. Each L-Arg concentration (0, 50, 100, 250, 500, and 750 μmol/L) was tested in a separate

Statistics
Results are expressed as mean±SEM. Data were evaluated with Student’s paired t-test when possible. In the protocol in which the response to L-arginine was studied in THALs pretreated with either vehicle or diethyldithiocarbamate (DETC), one-way ANOVA was used.

Figure 1. Effects of L-arginine (L-Arg) on NO release by the isolated, perfused thick ascending limb (THAL). A, Original recording of the effect of L-Arg (250 μmol/L) on NO release by a perfused THAL as measured with an amperometric sensor. B, Correlation between the response to L-Arg (250 μmol/L) (pA) and THAL length (mm).

Figure 2. Effects of L-arginine (L-Arg) and D-arginine (D-Arg) on NO release by isolated, perfused THALs. Addition of L-Arg but not D-Arg to the bath increased the amount of NO released by isolated, perfused THALs (n=7; P<0.04). In the presence of the NOS inhibitor L-NAME (5 mmol/L), addition of L-Arg (250 μmol/L) to the bath did not increase NO release by THALs.
group of tubules, and the effect of Tempol was studied in each group. At all concentrations tested, Tempol significantly increased the detectable amount of NO in the THAL, whereas in the absence of L-Arg, adding Tempol to the bath did not increase NO (Figure 3).

The SOD mimetic Tempol scavenges both intracellular and extracellular \( \text{O}_2^- \). We tested whether scavenging only extracellular \( \text{O}_2^- \) could increase NO levels. For this we used SOD, which has a molecular weight of 32 kDa and does not diffuse across the cell membrane. Adding 250 \( \mu \text{mol/L} \) L-Arg to the bath increased THAL NO production by 40.8 \( \pm \)4.0 pA/mm. Subsequent addition of 300 U/mL SOD to the bath significantly increased the signal to 74.5 \( \pm \)11.4 pA/mm, an 82% increase in NO levels (n=5; \( P<0.02 \)) (Figure 4).

To make sure \( \text{O}_2^- \) was being produced by the THAL and was not present in the bath, we generated a response to an NO donor (spermine NONOate) and then added SOD. Without a perfused tubule in the chamber, adding 300 U/mL SOD to the bath did not increase NO levels generated by spermine NONOate (data not shown).

A decrease in \( \text{O}_2^- \) concentration caused by Tempol and SOD increased NO levels in the THAL. We tested whether increasing \( \text{O}_2^- \) concentration in the THAL by inhibiting endogenous SOD could blunt L-Arg-induced NO release. Under control conditions, adding 250 \( \mu \text{mol/L} \) L-Arg to the bath increased NO release to 64.6 \( \pm \)13.6 pA/mm (n=5). In contrast, when tubules were preincubated with the SOD inhibitor DETC at 250 \( \mu \text{mol/L} \) for 30 minutes, adding L-Arg only increased NO to 23.3 \( \pm \)6.3 pA/mm, a 63% decrease in NO production compared with untreated tubules (n=5; \( P<0.01 \)) (Figure 5). Taken together, these data indicate that NO bioavailability in the THAL is reduced by \( \text{O}_2^- \).

Because we found that endogenously produced \( \text{O}_2^- \) can decrease NO bioavailability in the THAL, we tested whether scavenging only \( \text{O}_2^- \) could decrease THAL chloride absorption after stimulating NO production with L-Arg. Under basal conditions, THALs absorbed chloride at a rate of 167.2 \( \pm \)16.6 pmol/min/mm. After adding 0.5 mmol/L L-Arg to the bath, chloride transport decreased to 106.5 \( \pm \)9.5 pmol/min/mm, a 35% decrease. In the presence of L-Arg, adding 50 \( \mu \text{mol/L} \) Tempol to the bath further decreased THAL chloride absorption to 68.7 \( \pm \)5.1 pmol/min/mm (n=6; \( P<0.03 \)) (Figure 6). Control experiments showed no significant change in chloride absorption over time in the presence of L-Arg (data not shown).

**Discussion**

Previous studies have shown that NO plays an important role in the regulation of sodium and fluid transport by the nephron. We have reported that endogenous NO inhibits THAL NaCl and bicarbonate absorption. However, no direct measurements of NO produced by the THAL have been made. Using an electrochemical method for detection of NO, we can now report for the first time that physiological concentrations of L-Arg induce NO production and release from isolated, perfused THALs. We found that NO release increased in a dose-dependent manner with higher concentrations of L-Arg. The minimum concentration of L-Arg that

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**Figure 3.** Effect of the superoxide scavenger Tempol on L-Arg-induced NO release by isolated, perfused THALs. Addition of L-Arg to the bath caused a dose-dependent increase in NO release by THALs. Each concentration of L-Arg was tested in a separate group of THALs (n=5 for each group, except n=3 for 750 \( \mu \text{mol/L} \)). At all concentrations of L-Arg tested (50, 100, 250, 500, and 750 \( \mu \text{mol/L} \)), addition of Tempol to the bath increased the detectable levels of NO produced by THALs (* \( P<0.05 \)).

**Figure 4.** Effect of superoxide dismutase (SOD) on L-Arg-induced increase in NO release by isolated, perfused THALs. Addition of L-Arg (250 \( \mu \text{mol/L} \)) to the bath increased NO levels by 40.8 \( \pm \)4.0 pA/mm. Subsequent addition of SOD (300 U/mL) to the bath increased detectable NO levels to 74.5 \( \pm \)11.4 pA/mm, an 82% increase in NO release (n=5; \( ^* P<0.02 \)).

**Figure 5.** Effect of the superoxide dismutase inhibitor diethyldithiocarbacmate (DETC) on L-Arg-stimulated NO release by isolated, perfused THALs. Addition of L-Arg to the bath increased NO release to 64.6 \( \pm \)13.6 pA/mm (n=5). In the presence of the SOD inhibitor DETC (250 \( \mu \text{mol/L} \)), addition of L-Arg increased NO release to 23.3 \( \pm \)6.3 pA/mm (n=5; \( ^* P<0.01 \)).
caused a measurable increase in NO release was 100 μmol/L, whereas the half-maximum response was 240 μmol/L, and saturation occurred at concentrations greater than 500 μmol/L.

Because this was the first time that an electrochemical method was used to measure NO release by isolated, perfused kidney tubules, we examined the response of the electrode to D-Arg and the ability of L-NAME to block the response to L-Arg. We found that in the presence of the NOS inhibitor L-NAME, addition of L-Arg did not increase the electrical signal. Similarly, exposing THALs to D-arginine, which is not a substrate for NOS, did not change the signal. These data indicate that the response caused by L-Arg is due to NO and verify that this method is suitable for the detection of NO released by THALs.

Electrochemical detection of NO has been used several times for in vitro detection of NO.23,24 The selectivity of the sensor is given by the redox potential of NO at which the anode is maintained, and a gas-permeable membrane that coats the electrode surface. We chose to use the inNO measuring system by Harvard Apparatus because it is highly stable (lasting ≈3 months) and highly sensitive (nmol/L range). Furthermore, only a small number of substances have been shown to interfere with electrochemical detection of NO.23 However, to obtain reproducible and accurate data one must isolate the electrode electrically and insulate it from temperature variations as described in detail in the expanded methods section. Without such precautions, detection of NO released from single renal tubules is impossible.

We hypothesized that NO bioavailability in the THAL is decreased by O2-. We first tested whether decreasing O2- concentration with Tempol, an SOD mimic, could increase NO levels in the THAL. We found that adding Tempol to the bath significantly enhanced the detectable amount of NO released by THALs at all concentrations of L-Arg tested. These data appear to indicate that O2- produced by THALs reduces the bioavailability of NO. Even though Tempol has been reported to have pharmacological effects unrelated to scavenging O2-, this is unlikely in our system because at the concentration we used, Tempol has only been reported to scavenge O2-.25-28 In addition, similar concentrations of Tempol have been shown to increase the detectable amount of NO released by endothelial cells by dismutation of O2-.25 These data indicate that the increase in NO release caused by Tempol is most likely due to selective removal of O2-.

To make sure that NO bioavailability in THALs is decreased by O2-, we investigated the effect of altering O2- levels by adding or inhibiting SOD. Adding SOD to the bath significantly increased the detectable amount of NO released by THALs after L-Arg stimulation. We found that SOD, which only scavenges extracellular O2-, increased NO levels more than Tempol, which scavenges both intra- and extracellular O2-. This may be explained on the basis of a greater efficacy of SOD (K=2×10^9 mol/L/sec) than Tempol (K=6×10^9 mol/L/sec) to react with O2-. We also tested whether blocking SOD with the irreversible inhibitor DETC could decrease NO release by THALs. In THALs treated with DETC, L-Arg-induced NO release was 60% smaller compared with untreated tubules. These data support the hypothesis that NO bioavailability is decreased by endogenously produced O2-. Additionally, they indicate that Tempol acts only by scavenging O2- in our preparation.

Similar to our results, there have been many reports showing that O2- decreases the bioavailability of NO. In the vasculature, O2- decreases endothelium-dependent relaxation,8,9 whereas SOD increases NO half-life and potentiates endothelium-dependent dilatation.10 Inhibiting SOD with DETC decreases muscle relaxation induced by gaseous NO in the gastric fundus29 and by NO donors in coronary arteries.30 In other cell types such as epithelial cells7 and astrocytes,31 endogenously produced O2- decreases extracellular NO availability. Finally, in both normal and spontaneously hypertensive rats, the effects of an NO donor on TGF were enhanced by scavenging O2- with Tempol.32

We have previously shown that the source of NO in the THAL is likely to be endothelial NO synthase (eNOS),33 which is bound to the plasma membrane.34 While the source(s) of O2- in the THAL have not been identified to our knowledge, three major sources include the mitochondria, xanthine oxidase, and NAD(P)H oxidase. Mitochondria, which are present in large numbers in the THAL, are known to produce O2- during aerobic respiration.11,35 Xanthine oxidase activity has been detected in membrane fractions of isolated THALs,15 although its presence has not been confirmed at the protein level. A novel isoform of NADPH oxidase has been found in the renal cortex,36 and the major subunits of NADPH oxidase have been visualized in the THAL by immunological techniques.16 We found that SOD, which only scavenges extracellular O2-, increased the detectable amount of NO produced by THALs. This indicates that at least one source of O2- is external, or that there is leakage of intracellular O2- across the plasma membrane. Due to the high reactivity and negative charge of O2-, diffusion across the plasma membrane is highly improbable. Therefore, our data suggest the existence of a phagocyte-like NADPH oxidase in the THAL, although at present we know of no definitive evidence of O2- production by NADPH oxidase in the THAL.

In vivo experiments have shown that infusion of substances like acetylcholine, which causes NO release, into the...
renal artery increased urinary volume and sodium excretion.\textsuperscript{5,37,38} In addition, decreased water and sodium excretion has been reported when inhibitors of endogenous NO production were infused into the kidney.\textsuperscript{3,39-41} In these studies, the natriuretic and diuretic effects of NO were not accompanied by proportional changes in glomerular filtration rate or renal blood flow. These data indicate that NO regulates nephron transport; however, the specific nephron segment(s) affected by NO were not identified. By studying isolated nephron segments, we have shown that endogenously produced NO inhibits chloride and bicarbonate absorption by the THAL.\textsuperscript{2,22} In agreement with in vivo data, we found that the effects of NO were reversible and could be abolished by L-NAME, suggesting that NO is a physiological regulator of THAL transport.\textsuperscript{3,22}

In vitro and in vivo data indicate that NO is an important regulator of THAL transport. Therefore, factors affecting NO bioavailability may be important for transport regulation in this segment. To determine the physiological role of endogenous O\textsubscript{2}\textsuperscript{−} in the THAL, we examined whether Tempol could enhance the inhibitory effect of endogenous NO on THAL chloride absorption. We found that adding Tempol to the bath decreased THAL chloride absorption by 35\% in the presence of L-Arg. Because Tempol increased L-Arg-stimulated NO levels in the THAL, the effects of Tempol on chloride absorption may be mediated by an increase in NO availability. However, preliminary data indicate that in the absence of L-Arg, Tempol decreased THAL chloride absorption (Ortiz and Garvin, unpublished observations), suggesting that O\textsubscript{2}\textsuperscript{−} levels stimulate transport in the absence of NO. Thus, the effect of Tempol on chloride absorption in the presence of NO is more likely due to both an increase in NO bioavailability and a direct effect of O\textsubscript{2}\textsuperscript{−}.

Little is known about the effect of interactions of NO and O\textsubscript{2}\textsuperscript{−} on transport in other nephron segments. Some reports have implicated O\textsubscript{2}\textsuperscript{−} in the regulation of physiological processes in the kidney. Lu and Wang\textsuperscript{42} found that the effect of NO on basolateral K\textsuperscript{+} channels in the collecting duct is modulated by O\textsubscript{2}\textsuperscript{−}. A role for O\textsubscript{2}\textsuperscript{−} in the regulation of tubuloglomerular feedback (TGF) has also been reported. Welch et al\textsuperscript{12} have shown that inhibition of TGF by NO in normal and spontaneously hypertensive rats is enhanced by scavenging O\textsubscript{2}\textsuperscript{−} with Tempol. This effect may be due to a reduction of Na/K/2 Cl cotransport, because TGF is initiated by changes in cotransport activity. The ability of O\textsubscript{2}\textsuperscript{−} to mitigate the actions of NO in both the vasculature and nephron segments may ultimately reduce urinary volume and sodium excretion. Recently, Zou et al\textsuperscript{43} reported that infusion of Tempol into the renal medulla enhanced urinary sodium excretion, and several investigators have reported that inhibition of NO production in this region has the opposite effect.\textsuperscript{44}

We conclude that NO bioavailability in the THAL is decreased by O\textsubscript{2}\textsuperscript{−}. In addition, we provide evidence showing for the first time that endogenous O\textsubscript{2}\textsuperscript{−} may act as a physiological regulator of tubular NaCl transport.

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References


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