Inhibition of Na-K-ATPase in thick ascending limbs by NO depends on $O_2^-$ and is diminished by a high-salt diet

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Varela, Marisela, Marcela Herrera, and Jeffrey L. Garvin. Inhibition of Na-K-ATPase in thick ascending limbs by NO depends on $O_2^-$ and is diminished by a high-salt diet. Am J Physiol Renal Physiol 287: F224–F230, 2004. First published April 27, 2004; 10.1152/ajprenal.00427.2003.—A high-salt diet enhances nitric oxide (NO)-induced inhibition of transport in the thick ascending limb (THAL). Long exposures to NO inhibit Na-K-ATPase in cultured cells. We hypothesized that NO inhibits THAL Na-K-ATPase after long exposures and a high-salt diet would augment this effect. Rats drank either tap water or 1% NaCl for 7–10 days. Na-K-ATPase activity was assessed by measuring ouabain-sensitive ATP hydrolysis (23.7 ± 2.0 nmol P i/mg protein · min in THALs from rats on a normal diet (P < 0.05). Nitroglycerin also reduced Na-K-ATPase activity (P < 0.04). After 20 min, SPM had no effect (change −0.07 ± 0.05 nmol P i/mg protein · min). When rats were fed high salt, SPM did not inhibit Na-K-ATPase after 120 min. To investigate whether ONOO− formed by NO reacting with $O_2^-$ was involved, we measured $O_2^-$ production. THALs from rats on normal and high salt produced 35.8 ± 0.3 and 23.7 ± 0.8 nmol $O_2^-$/min · mg protein −1, respectively (P < 0.01). Because $O_2^-$ production differed, we studied the effects of the $O_2^-$ scavenger tempol. In the presence of 50 μM tempol, SPM did not inhibit Na-K-ATPase after 120 min (0.50 ± 0.05 vs. 0.52 ± 0.07 nmol P i/mg protein · min −1). Propyl gallate, another $O_2^-$ scavenger, also prevented SPM-induced inhibition of Na-K-ATPase activity.

METHODS

Animals. Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were fed chow containing 1.1% K and 0.2% Na and given either tap water or 1% NaCl in drinking water for 7–10 days. Rats were anesthetized with ketamine (100 mg/kg body wt ip) and xylazine (20 mg/kg body wt ip). All protocols were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

Isolation of thick ascending limbs. Suspensions of medullary thick ascending limbs were prepared as described previously (29). Briefly, kidneys were perfused retrograde at 6 ml/min for 6.6 min via the aorta with a physiological solution (see Solutions) containing 0.1% collagenase (Sigma, St. Louis, MO) and 2.5 U/ml heparin. The inner stripe of the outer medulla was cut from coronal slices of the kidneys, minced, and incubated at 37°C for 30 min in 0.1% collagenase. Tissue was pelleted by centrifugation at 114 g, resuspended in cold physiological solution, and stirred on ice for 30 min to release the tubules. The suspension was filtered through 250-μm nylon mesh and centrifuged at 114 g. The tubules were washed, pelleted again, and finally resuspended in cold physiological solution.

Solutions. The physiological solution contained (in mM) 114 NaCl, 4.0 KCl, 25 NaHCO 3, 2.5 NaH 2 PO 4, 1.2 MgSO 4, 2.0 Ca lactate, 5.5
glucose, 6.0 alanine, and 1.0 Na₃ citrate, and it was gassed with 95% O₂-5% CO₂. Arginine was omitted to prevent endogenous NO production. Osmolality was 290 ± 3 mosmol/kg H₂O as measured by freezing-point depression. Solution A contained (in mM) 1 Na₂EGTA, 5 MgCl₂, 100 mmidazole, 6 Na₂ATP, 45 NaOH, and 5 KOH, pH 7.0. Solution B contained (in mM) 1 Na₂EGTA, 5 MgCl₂, 100 mmidazole, 6 Na₂ATP, 50 NaOH, and 2 ouabain, pH 7.0.

Na-K-ATPase assay. Thick ascending limb suspensions were resuspended in physiological solution and divided into Eppendorf tubes (see Protocols). The tubes were spun, and the pellet was resuspended in physiological saline. Thick ascending limbs were incubated for 20 min or 2 h with vehicle, a NO donor, and/or a O₂ scavenger as indicated in Protocols. Tubes were gassed with 95% air-5% CO₂ at 37°C. At the end of the incubation period, tubules were rinsed three times with 500 µl of cold 150 mM NaCl. Between rinses, they were spun at 148 g in a Sorvall centrifuge for 2 min at 4°C. After the last rinse, they were resuspended in 50 µl of distilled H₂O and then frozen and thawed three times on dry ice to lyse the cells. After the last freeze, 6 mM ATP and 350 µl of solution A or B (prewarmed to 37°C) were added (see Solutions) and the lysates were incubated at 37°C for 10 min. To stop the reaction, a single dose of 350 µl of 5% trichloroacetic acid was added and the tubes were centrifuged at 16,000 g for 2 min. Aliquots of 100 µl were used to assay inorganic phosphate (Pi). To dissolve the protein pellet, 200 µl of 0.04% SDS and 0.05 M NaOH were added to the tubes. Protein concentration in 25-µl aliquots was measured using Coomassie protein assay reagent (Pierce, Rockford, IL). P_i was determined by a method similar to that of Fiske and Subbarrow (40). Two hundred microliters of 5% trichloroacetic acid, 300 µl of incubation buffer, and 150 µl of acid-molybdate solution were added to the 100-µl aliquots, followed by 40 µl of Fiske and Subbarrow’s reducing agent (Sigma). After 10 min, absorbance was read at 660 nm.

Protocol 1. Thick ascending limb suspensions were divided into four tubes. After spinning to pellet, tubes 1 and 2 were resuspended in physiological solution plus vehicle and tubes 3 and 4 in physiological solution plus 5 µM spermine NONOate (SPM) for 120 min. Rats were placed on either a normal- or high-salt diet.

Protocol 2. Thick ascending limb suspensions were divided into four tubes. After spinning to pellet, tubes 1 and 2 were resuspended in physiological solution plus vehicle and tubes 3 and 4 in physiological solution plus 5 µM SPM for 20 min. All rats were placed on a normal diet.

Protocol 3. Thick ascending limb suspensions were divided into four tubes. After spinning to pellet, tubes 1 and 2 were resuspended in physiological solution plus vehicle and tubes 3 and 4 in physiological solution plus 10 µM nitroglycerin for 120 min. All rats were placed on a normal diet.

Protocol 4. Thick ascending limb suspensions were divided into six tubes. Tubes 1 and 2 were resuspended in physiological solution plus vehicle, tubes 3 and 4 in physiological solution plus 50 µM 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol) or 10 µM n-propyl gallate, and tubes 5 and 6 in physiological solution plus 5 µM SPM and either tempol or propyl gallate. All tubes were incubated for 120 min. All rats were placed on a normal diet.

Protocol 5. Thick ascending limb suspensions were divided into four tubes. After spinning to pellet, tubes 1 and 2 were resuspended in physiological solution plus 5 µM SPM and tubes 3 and 4 in physiological solution plus 5 µM SPM and 0.5 μM xanthine oxidase, and 0.5 mM hypoxanthine (to increase O₂ levels) for 120 min. All rats were placed on a high-salt diet.

Protocol 6. Thick ascending limb suspensions were divided into four tubes. After spinning to pellet, tubes 1 and 2 were resuspended in physiological solution plus 5 µM SPM and tubes 3 and 4 in physiological solution plus 5 µM SPM and 1 µM SQ-29548 (an endoperoxide receptor antagonist) for 120 min. All rats were placed on a normal diet.

Superoxide measurement. Suspensions of medullary thick ascending limbs were prepared as described previously (29). Tubules were resuspended in 1 ml HEPES-buffered perfusion solution containing (in mM) 125 NaCl, 4.0 KCl, 10 HEPES, 2.5 NaH₄PO₄, 1.2 MgSO₄, 2.0 Ca lactate, 5.5 glucose, 6.0 alanine, and 1.0 Na₃ citrate. pH was increased to 7.4 with NaOH, and the solution was gassed with 100% O₂. Aliquots (100 µl) were added to 1.6-ml polypropylene tubes, diluted in HEPES-buffered perfusion solution to a final volume of 900 µl, and placed on ice. Lucigenin (100 µl; final concentration 5 µM) was added to the diluted suspensions, which were then incubated for 30 min at 37°C. Tubes were placed in a luminometer chamber (model 20c, Turner Designs, Mountain View, CA) maintained at 37°C. The average of 10 consecutive 30-s measurements was recorded for each sample. The metal-dependent O₂ scavenger 4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt (Tiron; 10 µl) was added to the sample for a final concentration of 10 mM, and 10 consecutive 30-s measurements were made, taking the average of the last three. The difference in average luminescence between samples with and without Tiron was used to quantify O₂. Measurements were normalized to protein content. The average luminescence of 10 consecutive measurements was calculated for a blank containing PBS and lucigenin. Arbitrary luminescence units were converted to nanomoles per minute per milligram of protein by means of a calibration curve (31). Tubules were resuspended in a solution lacking l-arginine, the substrate for NOS, so that NO production did not confound measurement of O₂.

Statistics. Results are expressed as means ± SE. Na-K-ATPase activity was evaluated by a Student’s paired t-test. O₂ production was evaluated by an unpaired t-test, taking P < 0.05 as significant.

RESULTS

We previously reported that NO had no effect on Na-K-ATPase activity of freshly isolated renal tubules when exposures were less than 20 min (29). In contrast, others showed that NO appeared to inhibit pump activity in cultured cells when the exposure was significantly longer (5, 15, 16). To examine whether NO-induced inhibition of Na-K-ATPase activity in thick ascending limbs was time dependent, we first tested the ability of 5 µM SPM, a NO donor, to reduce Na-K-ATPase activity after 20 or 120 min. In freshly isolated suspensions of thick ascending limbs from rats on a normal diet, Na-K-ATPase activity in tubules treated with vehicle for 20 min was 0.53 ± 0.05 nmol P_i·µg protein⁻¹·min⁻¹. Na-K-ATPase activity of thick ascending limbs exposed to SPM for 20 min was not significantly different (change −0.07 ± 0.05 nmol P_i·µg protein⁻¹·min⁻¹). In contrast, in tubules exposed to 5 µM SPM for 120 min, Na-K-ATPase activity decreased by 32% compared with vehicle (from 0.44 ± 0.03 to 0.30 ± 0.04 nmol P_i·µg protein⁻¹·min⁻¹; P < 0.03; n = 7; Fig. 1).

To make sure the effect of SPM we observed was due to release of NO, we treated tubules with a different NO donor, nitroglycerin (10 µM), for 120 min. In vehicle-treated suspensions, Na-K-ATPase activity was 0.40 ± 0.01 nmol P_i·µg protein⁻¹·min⁻¹. In nitroglycerin-treated tubules, it was 0.31 ± 0.03 nmol P_i·µg protein⁻¹·min⁻¹ (P < 0.04), a decrease of 23%. Taken together, these data suggest that NO-induced inhibition of Na-K-ATPase activity is time dependent.

In cultured cells, NO-induced reductions in pump activity require O₂⁻ (5). We therefore examined whether SPM-induced inhibition depends on O₂ by testing the ability of O₂ scavengers to block NO-induced inhibition of thick ascending limb Na-K-ATPase activity in rats on a normal diet. In the presence of 50 µM tempol, Na-K-ATPase activity was 0.50 ± 0.05

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nmol P$_i$·µg protein$^{-1}$·min$^{-1}$ ($n = 6$) after 120 min of vehicle treatment. In the presence of SPM and tempol, pump activity was $0.52 \pm 0.07$ nmol P$_i$·µg protein$^{-1}$·min$^{-1}$ (n = 6) after 120 min, not significantly different (Fig. 2). Tempol alone had no significant effect on Na-K-ATPase activity ($0.44 \pm 0.06$ vs. $0.50 \pm 0.05$ nmol P$_i$·µg protein$^{-1}$·min$^{-1}$; n = 6). To make sure the tempol results were due to scavenging of O$_2^-$, we tested another O$_2^-$ scavenger, N-propyl gallate. In the presence of 10 µM propyl gallate, SPM had no significant effect on Na-K-ATPase activity after 120 min of treatment (SPM + propyl gallate: $0.44 \pm 0.09$ nmol P$_i$·µg protein$^{-1}$·min$^{-1}$ vs. vehicle: $0.39 \pm 0.02$ nmol P$_i$·µg protein$^{-1}$·min$^{-1}$; n = 5). Propyl gallate alone had no significant effect on Na-K-ATPase activity compared with vehicle. Taken together, these data indicate that NO-induced inhibition of pump activity requires the formation of a reactive nitrogen species from NO and O$_2^-$, probably OONO$^-$.

Previously, we reported that a high-salt diet enhances the ability of NO to inhibit transport in the thick ascending limb (28). Those data suggest that a high-salt diet enhances the ability of NO to inhibit Na-K-ATPase activity. Thus we tested whether the effect of SPM on pump activity would be augmented by a high-salt diet. Na-K-ATPase activity of thick ascending limbs from rats on a high-salt diet treated with vehicle for 120 min was $0.40 \pm 0.03$ nmol P$_i$·µg protein$^{-1}$·min$^{-1}$. In tubules treated with SPM for 120 min, Na-K-ATPase activity was $0.35 \pm 0.04$ nmol P$_i$·µg protein$^{-1}$·min$^{-1}$, not statistically different (Fig. 3). Thus a high-salt diet reduced the ability of NO to inhibit pump activity, the opposite of what we expected.

Given that 1) NO-induced inhibition of Na-K-ATPase in thick ascending limbs from rats on a normal diet required O$_2^-$ and 2) NO had no effect on pump activity when rats were fed a high-salt diet, we studied the effect of SPM on Na-K-ATPase activity in the presence of an exogenous source of O$_2^-$ and measured O$_2^-$ production by thick ascending limbs from rats on a high-salt diet. After 120 min of SPM alone, Na-K-ATPase activity was $0.64 \pm 0.01$ nmol P$_i$·µg protein$^{-1}$·min$^{-1}$. Treatment with SPM plus 0.5 mU/ml xanthine oxidase and 0.5 mM hypoxanthine reduced Na-K-ATPase activity by 24% to $0.49 \pm 0.03$ nmol P$_i$·µg protein$^{-1}$·min$^{-1}$ ($P < 0.05$; Fig. 4). Next, we investigated whether a high-salt diet alters O$_2^-$ production by suspensions of thick ascending limbs. In thick ascending limbs from rats on a normal diet, O$_2^-$ production was $35.8 \pm 0.3$ nmol O$_2^-$·min$^{-1}$·mg protein$^{-1}$; however, in thick ascending limbs from rats on a high-salt diet it was $23.7 \pm 0.8$ nmol O$_2^-$·min$^{-1}$·mg protein$^{-1}$, a decrease of 34% ($P < 0.01$;
NO inhibits Na-K-ATPase

The effects of NO on Na-K-ATPase activity in renal epithelial cells have been controversial, with reports of both inhibition (5, 15, 16) and no effect (29, 40). We set out to investigate 1) the time dependency of Na pump inhibition in thick ascending limbs, 2) the role of \( \text{O}_2^- \) in this process, and 3) how the effects of NO are altered by a high-salt diet. Our data show that the NO donors SPM and nitroglycerin both inhibited Na-K-ATPase activity when thick ascending limbs from rats on a normal diet were treated for 120 min but not for only 20 min. The ability of NO to reduce pump activity was prevented by treating tubules with tempol and propyl gallate, both \( \text{O}_2^- \) scavengers, but not by an endoperoxide receptor antagonist. NO did not inhibit pump activity in tubules from rats on a high-salt diet, a treatment that reduced \( \text{O}_2^- \) levels. When we artificially enhanced \( \text{O}_2^- \) in thick ascending limbs from the high-salt group, NO-induced inhibition was restored.

Time course studies showed that the NO donor SPM reduced Na-K-ATPase activity by \( \approx 30\% \) when tubules from rats on a normal diet were treated for 120 min but not for only 20 min. This indicates that the ability of NO to inhibit Na-K-ATPase is time dependent and also confirms earlier reports showing that NO donors had no effect on Na-K-ATPase activity in freshly isolated thick ascending limbs (29) or cortical collecting ducts (40) after 20 min, whereas NO inhibits pump activity in renal epithelial cells at longer incubation times (5, 16). To make sure the inhibition caused by SPM was due to NO rather than some other degradation product, we tested nitroglycerin and found that it also inhibited pump activity. Although SPM and nitroglycerin yield chemically distinct degradation products, both produce NO; thus these data indicate that NO is responsible for the reduction in Na-K-ATPase activity.

In cultured proximal tubule cells, NO-induced inhibition of Na-K-ATPase activity has been reported to require \( \text{O}_2^- \) (5). For this reason, we studied the ability of tempol and propyl gallate to block SPM inhibition of Na pump activity in thick ascending limbs from rats on a normal diet. SPM by itself reduced Na-K-ATPase activity by \( \approx 30\% \), but it had no effect on pump activity when tubules were also incubated with either tempol or propyl gallate, confirming that NO-induced reduction of Na-K-ATPase activity in thick ascending limbs from rats on a normal diet requires \( \text{O}_2^- \). Thus NO per se is probably not responsible for the inhibition, but rather another nitrogen-containing reactive oxygen species such as \( \text{OONO}^- \). It is unlikely that the data could be explained by tempol or propyl gallate directly interacting with NO or directly affecting Na-K-ATPase activity, because the scavengers are chemically distinct and had no effect on the Na pump in the absence of NO.

Given that we previously showed that thick ascending limbs produce NO, one might reasonably ask why tempol or propyl gallate alone did not alter Na-K-ATPase activity in the absence of SPM. The answer is that these experiments were performed in the absence of L-arginine, the substrate for NOS, so that...
endogenously produced NO would not confound interpretation of the results. Parenthetically, the fact that neither tempol nor propyl gallate by itself altered Na-K-ATPase also indicates that endogenously produced \(\text{O}_2\) does not affect activity.

We previously observed that a high-salt diet enhances the ability of a given concentration of NO to inhibit net NaCl absorption by isolated, perfused thick ascending limbs (28). Therefore, we thought it likely that a high-salt diet would enhance the effect of NO on Na-K-ATPase. Instead, we found that NO no longer caused a significant decline in Na-K-ATPase activity after 2 h. Thus we needed to explain the varying NO-induced inhibition we observed in rats on different diets. Because \(\text{O}_2\) was required for NO to inhibit Na-K-ATPase in thick ascending limbs of rats on a normal diet, we measured \(\text{O}_2\) production with both a normal diet and a high-salt diet and found that it was significantly lower in the high-salt group. To see whether the reduced \(\text{O}_2\) levels prevented NO from lowering pump activity, we applied an NO donor in the presence of an exogenous \(\text{O}_2\)-generating system, xanthine oxidase/hypoxanthine, and found that SPM now inhibited Na pump activity by \(\approx 25\%\). These data indicate that reduced \(\text{O}_2\) production eliminates the ability of NO to inhibit Na-K-ATPase activity in thick ascending limbs from rats on a high-salt diet, thereby supporting the theory that NO per se is not responsible for inhibition, but rather a product of the NO/\(\text{O}_2\) reaction such as \(\text{ONO}^-\).

Our results show that inhibition of Na-K-ATPase activity by NO in freshly isolated thick ascending limbs depends on \(\text{O}_2\). NO and \(\text{O}_2\) may react to form \(\text{ONO}^-\) (7). Thus it is likely that \(\text{ONO}^-\) mediates these effects, confirming similar reported data involving proximal tubule cells. Guzman et al. (5) reported that NO produced by inducible NOS inhibited Na-K-ATPase in cultured mouse proximal tubule cells treated with interferon-\(\gamma\) and lipopolysaccharide, although it took 4 h to do so. Inhibition was prevented by prior treatment with either \(N^\text{-nitro-l-arginine, a nonselective NOS inhibitor, or superoxide dismutase. NO donors mimicked the effects of interferon-\(\gamma\) and lipopolysaccharide, but notably a cell-permeant analog of cGMP did not. These authors concluded that NO inhibits pump activity by generating \(\text{ONO}^-\). More recently, the inhibitory phase of the biphasic dose-response relationship between angiotensin and proximal nephron transport has been shown to be due to \(\text{ONO}^-\) (43). In suspensions of rat proximal tubules, inhibition of Na-K-ATPase activity by angiotensin concentrations of \(10^{-8} \text{ M or greater was completely blocked by l-NMMA, a nonselective NOS inhibitor, indicating that the process is dependent on NO. Inhibition was also blocked by superoxide dismutase. Thus these authors concluded that O}_2 was involved and that NO reacted with O}_2 to form \(\text{ONO}^-, which mediated the effect. Finally, there are reports that \(\text{ONO}^-\) inhibits Na-K-ATPase in hepatocytes (25), erythrocytes (41), and cells of the cerebral cortex (36).

Because \(\text{ONO}^-\) appeared to be responsible for Na pump inhibition, we tested the ability of an endothoperoxide receptor antagonist, SQ-29548, to block the effects of NO on thick ascending limb Na-K-ATPase in rats on a normal diet. \(\text{ONO}^-\) formed from NO and \(\text{O}_2\) can cause the formation of isoprostanes through nonenzymatic oxidation of arachidonic acid. Often isoprostanes exert their effects by activating the endothoperoxide receptor. However, we found that SQ-29548 did not reduce NO-induced inactivation of Na-K-ATPase. Thus either isoprostanes are not involved in such inhibition or else their effects are mediated via some other pathway.

Although we showed that \(\text{ONO}^-\) is responsible for the inhibition of Na-K-ATPase activity after long exposures in the thick ascending limb, it is not clear why the effect takes so long to develop. Assuming inhibition does not require de novo protein synthesis, the actions of \(\text{ONO}^-\) could be mediated by either a signaling pathway or a direct effect due to nitration/nitrosation/nitrosylation of one of the subunits of Na-K-ATPase. \(\text{ONO}^-\) probably inhibits pump activity via covalent modification by nitrogen-containing groups (25, 36). Generally, the effects of activating signaling pathways that do not involve de novo protein synthesis occur within 20–30 min. Additionally, nitration/nitrosation/nitrosylation of Na-K-ATPase would essentially be expected to occur immediately after \(\text{ONO}^-\) was formed, due to the fact that it is highly reactive. However, it may be that a number of nitration/nitrosations/nitrosylations must accumulate or that an intracellular buffer of the process must be overwhelmed before an effect is apparent. In either case, the rate at which these occur would depend on the rate of \(\text{O}_2\) generation. In the final analysis, however, the mechanism by which \(\text{ONO}^-\) reduces Na-K-ATPase activity remains undefined.

Although the inhibitory effect of NO on Na-K-ATPase depends on \(\text{O}_2\) in many cells, other mechanisms have also been implicated. Liang and Knox (15) reported that NO inhibited Na-K-ATPase activity in opossum kidney (OK) cells, a proximal nephron cell line, acting via generation of cGMP and activation of protein kinase C. They also reported that antioxidants did not alter this inhibitory effect. Thus in OK cells \(\text{O}_2\), and consequently \(\text{ONO}^-\) formation, is not required for NO-induced inhibition of pump activity. In hepatocytes, the reduction of Na-K-ATPase activity caused by NO is mediated by at least two different mechanisms, one involving \(\text{O}_2/\text{ONO}^-\) and the other not (25). The explanation for this discrepancy is unclear, but it may involve differences in \(\text{O}_2\) production and NO concentrations or species differences.

Previously, we reported that a high-salt diet enhances the ability of NO to inhibit transport in the thick ascending limb (28). Thus we thought it likely that a high-salt diet would enhance the effect of NO on Na-K-ATPase. Instead, we found that NO did not reduce Na-K-ATPase activity in thick ascending limbs from rats on a high-salt diet. We concluded that this was due to a reduction in endogenously produced \(\text{O}_2\), because inhibition could be restored by adding an exogenous source of \(\text{O}_2\). Given that NO could scavenge \(\text{O}_2\), one might argue that the decrease in \(\text{O}_2\) levels was simply due to heightened NO production resulting from the increase in endothelial NOS expression caused by high salt (28). However, this cannot be the case, because all experiments presented here, including \(\text{O}_2\) measurements, were performed in the absence of \(l\)-arginine, the substrate for NO synthesis. We previously showed that in the absence of exogenously added \(l\)-arginine, thick ascending limbs produce negligible amounts of NO (35).

Although we did not identify the source of \(\text{O}_2\) in our study, NAD(P)H oxidase appears to be the most likely candidate. Several proteins produce \(\text{O}_2\), including NAD(P)H oxidase (32), xanthine oxidase (33), NO synthase (42), and cyclooxygenase (10). The thick ascending limb expresses all of these, although NAD(P)H oxidase is likely to be a major source of \(\text{O}_2\) in this segment. NAD(P)H oxidase expression is stimulated
by angiotensin (30). When angiotensin concentrations fall, as they do when animals are placed on a high-salt diet, expression and assembly of NAD(P)H oxidase are also reduced. Thus changes in NAD(P)H oxidase expression are likely to explain the observed decrease in O$_2^*$ production caused by high salt. However, catalobolization of O$_2^*$ may also be involved as discussed below.

The fact that O$_2^*$ production in the thick ascending limb falls when rats are placed on a high-salt diet is consistent with the finding that oxidative stress is increased in renovascular hypertension (14) and in ANG II-induced hypertension (26, 27, 37). Given that Sprague-Dawley rats do not develop hypertension when placed on a high-salt diet, it is also consistent with findings in the Dahl salt-resistant rat. In this strain, a high-salt diet increases medullary manganese superoxide dismutase expression, which catalobolizes O$_2^*$, and total medullary O$_2^*$ production is not increased (24). Furthermore, if one mitigates oxidative stress in the salt-sensitive strain, a high-salt diet no longer produces hypertension (23). These data appear to contrast with the reported finding that a high-salt diet increases oxidative stress in the renal cortex (8). Although the explanation for this discrepancy is unclear, it may be that isoprostane production does not precisely mimic O$_2^*$ production (24), and/or superoxide dismutase mRNA levels may not correlate with protein expression. Alternatively, it may be because cortical proximal tubules respond to high salt differently from medullary thick ascending limbs. Precedence for opposite effects of high salt in cells of the cortex vs. the medulla is shown by the fact that a high-salt diet decreases cyclooxygenase-2 expression in the macula densa but increases cyclooxygenase-2 in the medullary interstitial cells (6).

In summary, we have shown that NO inhibits Na-K-ATPase in freshly isolated thick ascending limbs from rats on a normal diet after long incubations. This inhibition depends on O$_2^*$ and/or inhibition is likely mediated by OONO$^-$. Nitric oxide-induced inhibition of transport by thick ascending limbs from Dahl salt-sensitive THALs.

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**GRANTS**

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