High-Salt Diet Increases Sensitivity to NO and eNOS Expression But Not NO Production in THALs

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Abstract—L-Arginine inhibits thick ascending limb (THAL) NaCl absorption by activating endothelial NO synthase (eNOS) and increasing NO production. Inhibition of renal NO production combined with a high-salt diet produces hypertension, and the THAL has been implicated in salt-sensitive hypertension. We hypothesized that a high-salt diet enhances the inhibitory action of L-arginine on NaCl absorption by THALs because of increased eNOS expression and NO production. To test this, we used isolated THALs from rats on a normal-salt (NS) or high-salt diet (HS) for 7 to 10 days. L-Arginine (1 mmol/L) decreased chloride absorption by 56±10% in THALs from rats on a HS diet, but only 29±3% in THALs from rats on a NS diet. eNOS expression in isolated THALs from rats on a HS diet was increased by 3.9-fold compared with NS (P<0.03). However, L-arginine increased NO levels to the same extent in THALs from both groups, as measured with DAF-2 DA or a NO-sensitive electrode. To determine whether a HS diet increases the sensitivity of the THAL to NO, we tested the effects of the NO donor spermine NONOate on chloride absorption. In THALs from rats on a HS diet, 1 and 5 μmol/L spermine NONOate reduced chloride absorption by 35±5% and 58±6%, respectively. In contrast, these same concentrations of spermine NONOate reduced chloride absorption by 4±4% (P<0.03 versus HS diet) and 43±9% in THALs from rats on a NS diet. We conclude that a HS diet enhances the effect of NO in the THAL. L-Arginine–stimulated NO production was not enhanced by a HS diet, despite increased eNOS protein. (Hypertension. 2003;41[part 2]:682-687.)

Key Words: urine ■ hypertension, sodium dependent ■ Na/K/Cl cotransport ■ nitric oxide synthase

It is known that NO is an important regulator of urinary volume and urinary sodium excretion via actions on both the renal vasculature and nephron segments.1-10 Defects in the response to or production of NO have been implicated in salt-sensitive hypertension.9,11-13 It has been shown that when rats are fed a high-salt diet, both outer- and inner-medullary expression of the neuronal, inducible (i), and endothelial isoforms increases, whereas expression in the cortex does not change significantly.14,15 However, conclusions about the expression of a specific NO synthase (NOS) isoform in a specific nephron segment based on data from whole kidneys may be misleading. For instance, in the macula densa, which is located in the cortex, expression of nNOS actually decreases in response to a high-salt diet rather than remaining unchanged.16

The thick ascending limb (THAL) of the loop of Henle absorbs 20% to 30% of the filtered load of NaCl and creates the corticomedullary gradient necessary for water absorption.17,18 We have previously shown that NO is an important regulator of NaCl transport in this nephron segment. NO is generated from L-arginine by at least 3 enzymes: neuronal (n), inducible (i), and endothelial (e) NOS.5,14,19-23 The THAL expresses all 3 NOS isoforms, but eNOS is responsible for the inhibition of transport caused by L-arginine.3 NO produced by this isoform acts as an autacoid, inhibiting NaCl absorption.7,24 However, it is still not known whether a high-salt diet affects eNOS expression, NO production, or the effects of NO in the THAL.

We hypothesized that a high-salt diet would enhance eNOS expression and NO production by the THAL. Consequently, inhibition of NaCl transport by L-arginine would be enhanced by a high-salt diet.

Methods

Isolation and Perfusion of THALs

THALs were obtained from male Sprague-Dawley rats weighing 120 to 150 g (Charles River Breeding Laboratories, Wilmington, Mass) fed a diet containing 0.22% sodium and 1.1% potassium (Purina), with (high-salt diet) or without (normal salt diet) 1% NaCl in their drinking water for 7 to 10 days. Animals had free access to food and drinking water with or without salt. On the day of the experiment, rats were anesthetized with ketamine (100 mg/kg body weight IP) and xylazine (20 mg/kg body weight 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. THALs were dissected from the renal medulla at 4°C to 10°C with a stereomicroscope. Medullary THALs (length, 0.5 to 1.0 mm) were transferred to a temperature-regulated chamber and perfused with concentric glass pipettes at 37±1°C, as described previously.25 The composition of the basolateral bath and perfusate (physiological...
Chloride Absorption

Chloride concentration was measured in samples of the bath, perfusate, and collected fluid by ultra-microfluorometric assay. Net chloride absorption \( J_{Cl} \) was calculated according to the following equation:

\[
J_{Cl} = C.R(C_{0 Cl} - C_{1 Cl})
\]

where \( C.R \) is the collection rate normalized per tubule length, \( C_{0 Cl} \) is the chloride concentration in the perfusion solution, and \( C_{1 Cl} \) is the chloride concentration in the collected fluid. The composition of the bath and perfusion solution for the set of experiments in which the effect of L-arginine was studied was (in mmol/L) NaCl 114, NaHCO3 25, NaH2PO4 2.5, KCl 4, MgSO4 1.2, alanine 6, trisodium citrate 1, glucose 5.5, and calcium dillactate 2.

Western Blot

Medullary THALs were lysed in buffer containing 20 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 0.3 mol/L sucrose, 1.0% NP-40, 0.1% sodium dodecyl sulfate, 5 μg/mL antipain, 10 μg/mL aprotinin, 5 μg/mL leupeptin, 4 mmol/L benzamidine, 5 μg/mL chymostatin, 5 μg/mL pepstatin A, and 0.105 mol/L pf-block (Sigma). Debris was removed by centrifugation. Twenty to 100 μg protein was loaded in each lane of an 8% polyacrylamide gel, separated by electrophoresis, and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was incubated in blocking buffer containing 50 mmol/L Tris, 500 mmol/L NaCl, 5% nonfat dried milk, and 0.1% Tween-20 for 60 minutes, and then with a 1:1000 dilution of an eNOS-specific monoclonal antibody (BD Transduction Laboratories) in blocking buffer for 60 minutes at room temperature. The membrane was washed in blocking buffer containing 50 mmol/L Tris, 500 mmol/L NaCl, and 0.1% Tween-20 and incubated with a 1:1000 dilution of secondary antibody against the appropriate IgG conjugated to hors eradish peroxidase (Amersham). The reaction products were detected with a chemiluminescence kit (Amersham) and quantified by densitometry with the Molecular Analyst Program (Biorad Laboratories). Because it was not always possible to dissect the same number of tubules, THALs were measured before lysing, and optical density units were normalized per total tubule length loaded.

Measurement of Intracellular NO by DAF-2 DA

Isolated perfused THALs were loaded by adding 2 μmol/L of the fluorescent dye DAF-2 DA (Calbiochem) to the bath for 45 minutes. Then tubules were washed for 30 minutes with physiological saline. The dye was excited with an argon laser set at 488 nm, and fluorescence emitted by NO-bound dye was measured with a scanning laser confocal microscope (Noran Instruments). Measurements were recorded every minute for a 10-minute control period. Then 1 mmol/L L-arginine was added to the bath, and 10 minutes later, fluorescence was measured during a 10-minute experimental period. In situ calibration of DAF-2 DA was performed using 3 concentrations of the NO donor spermine NONOate to check for linearity of dye fluorescence versus NO concentration.

Results

First, we investigated whether the effect of the substrate for NO synthase, L-arginine, on NaCl absorption by the THAL is enhanced by a high-salt diet. In isolated perfused THALs from rats on a normal diet, 1 mmol/L L-arginine reduced chloride absorption by 28.7 ± 3.3% (from 117.2 ± 19.4 to 82.5 ± 11.7 pmol/min per mm). In contrast, 1 mmol/L L-arginine reduced chloride absorption by 56.0 ± 9.5% when rats were placed on a high-salt diet (Figure 1) \( (P < 0.04) \). Thus, 1 mmol/L L-arginine had a greater effect on NaCl transport in the high-salt group.

The simplest explanation for the enhanced effect of L-arginine in THALs from rats on high salt was increased eNOS expression and, hence, increased NO production. Therefore, we next investigated whether a high-salt diet augments eNOS expression in THALs from rats on normal and high salt. Figure 2 (bottom) shows the densitometric data. A high-salt diet dramatically increased eNOS expression in isolated THALs from 0.30 ± 0.11 to 1.16 ± 0.23 optical density units per millimeter of tubule, a 3.9-fold increase \( (P < 0.03) \). Thus, a high-salt diet enhanced eNOS expression in the THAL.

To investigate whether the enhanced effect of L-arginine was due to increased NO levels, we next measured NO production using the fluorescent dye DAF-2 DA. In THALs from rats fed a normal diet, 1 mmol/L L-arginine increased fluorescence by 9.4 ± 1.1 arbitrary units. In THALs from rats fed a high-salt diet, 1 mmol/L L-arginine increased fluorescence by 9.4 ± 1.0 arbitrary units \( (n=5 \text{ for each group}) \) (Figure 3, top). In situ calibration of DAF-2 DA resulted in a

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linear response to concentrations of the NO donor spermine NONOate, which bracketed the L-arginine–induced increases in fluorescence (Figure 3, bottom). Thus, THALs from rats on normal- and high-salt diets produced the same amount of NO in response to 1 mmol/L L-arginine.

To make sure the results with DAF-2 DA were accurate, we examined NO production with a NO-sensitive electrode. In THALs from rats fed a normal diet, 1 mmol/L L-arginine increased electrode current by 81±6 pA/mm. Similarly, in THALs from rats fed a high-salt diet, 1 mmol/L L-arginine increased electrode current by 77±22 pA/mm (n=6 for each group; data not shown). These data also indicate that NO production induced by 1 mmol/L L-arginine did not differ between THALs from rats on a normal- or high-salt diet.

Because NO production was not different in the 2 groups, we next hypothesized that the inhibition of NaCl transport produced by L-arginine was due to increased sensitivity of the THAL to a given concentration of NO. Consequently, we next measured the effect of 2 different concentrations of spermine NONOate on chloride absorption by THALs from rats on a normal- or high-salt diet (Figure 4). In THALs from rats on a normal diet, 1 and 5 μmol/L spermine NONOate reduced chloride absorption from 140.5±9.7 to 132.5±7.6 and 75.6±8.0 pmol/min per mm, respectively. However, in THALs from rats on a high-salt diet, these same concentrations of the NO donor reduced chloride absorption from
tubules from rats on high salt. The higher concentration inhibited transport to the same extent in THALs from both groups. (43±9% normal versus 59±6% high salt).

Discussion

Our data show that a high-salt diet increases the inhibitory effect of L-arginine on NaCl absorption and also increases eNOS expression in medullary THALs. Even though eNOS expression was increased almost 4-fold, L-arginine–induced NO production was not enhanced by hypertonic NaCl administration. However, the response to a NO donor was augmented in THALs from rats on high salt compared with normal salt.

We have previously shown that adding L-arginine, the substrate for NO synthase (NOS), to the bath of isolated perfused THALs inhibits NaCl absorption via a NO-dependent mechanism. Inhibition of renal NO production combined with a high-salt diet produces hypertension, and the THAL has been implicated in salt-sensitive hypertension. Consequently, we examined whether the effect of L-arginine is enhanced by a high-salt diet. In THALs from rats on high salt, L-arginine reduced NaCl absorption by 56%. In contrast, L-arginine only reduced chloride absorption by 29% when rats were on a normal diet. Thus the inhibitory effect of L-arginine was enhanced in the high-salt group.

Other investigators have shown that expression of all 3 NOS isoforms—iNOS, nNOS, and eNOS—is enhanced in both the outer and inner medulla of the kidney when rats are fed a high-salt diet. However, the effect of high salt on individual nephron segments has not been examined previously to our knowledge. Consequently, we investigated whether a high-salt diet enhances eNOS expression in medullary THALs. We found that in THALs from rats fed normal chow and given 1% NaCl in their drinking water for 7 to 10 days, eNOS expression was increased by nearly 4-fold compared with a the level for a normal diet.

Other investigators have assumed that increased expression of the various NOS isoforms in the kidney necessarily results in enhanced NO production. We also assumed this was the case, and so we next measured NO production using the fluorescent dye DAF-2 DA. Contrary to our hypothesis, we found that NO levels induced by the same concentration of L-arginine were the same in THALs taken from rats on normal- and high-salt diets. To make sure our inability to observe a difference was not due to a limitation of the dye, we obtained in situ calibration curves using a NO donor. These curves showed that the increase in fluorescence caused by NO was linear over a range of responses that bracketed the NO response we obtained with L-arginine. Thus, our inability to detect a difference between the 2 groups was not due to failure of the dye to respond to greater concentrations of NO. As a further measure and to make sure the results obtained with DAF-2 DA were correct, we examined NO production by a completely different method, a NO-sensitive electrode. Once again, the increase in NO levels caused by L-arginine was the same in THALs taken from rats on a normal- or high-salt diet. We concluded that although eNOS expression is enhanced, NO production is not.

We found that despite increased eNOS expression, NO production was not enhanced by a high-salt diet. This may be due to an inadequate supply of cofactors required for NO synthesis such as tetrahydrobiopterin, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), or nicotinamide adenine dinucleotide (NADH). Under some circumstances, it has been reported that NO production by iNOS can become limited. However, this primarily occurs during rapid bursts of NO production when iNOS expression increases 10-fold in macrophages. It is unlikely that a 10-fold increase in eNOS expression and activity could significantly reduce the intracellular concentration of cofactors, unless a high-salt diet by itself reduces their production or enhances their degradation. This could be the case because in some forms of hypertension tetrahydrobiopterin levels become limiting, and this decreases NO production by eNOS in the vasculature. Currently, it is not known whether a high-salt diet affects intracellular concentrations of necessary cofactors.

Alternatively, the supply of the substrate, L-arginine, may be limiting. The intracellular concentration of L-arginine is determined by both consumption and transport across the plasma membrane by several transporters, primarily the cationic amino acid transporter y+ activity. Because we used a saturating concentration of L-arginine in our experiments, reduced extracellular L-arginine cannot be the explanation for diminished NO production. Additionally, measurements of plasma L-arginine in rats on a high-salt diet revealed no significant changes compared with rats on normal salt. Currently it is not known how L-arginine transport in the THAL is altered by a high-salt diet. However, transport may be diminished because angiotensin stimulates y+ activity, and circulating angiotensin levels would be expected to fall because of high salt. Thus, the intracellular levels of L-arginine could be diminished during a high-salt diet.

Another possibility is that NO itself inhibits eNOS activity via a short loop feedback system. Such an effect of NO on NOS activity has been reported in endothelial cells. Thus, high salt would lead to an increase in eNOS expression. This in turn would lead to enhanced NO production and elevated intracellular NO concentrations. The elevated NO concentration would then inhibit NOS activity. Ultimately, there would be more eNOS protein, but a proportional amount of NO would not be synthesized.

The mechanism by which high salt could stimulate eNOS gene expression is currently unknown. When animals are placed on a high-salt diet, plasma levels of endothelin increase. We have previously shown that endothelin acutely inhibits THAL transport via an NO-dependent pathway. Thus, endothelin may mediate the effects of high salt on eNOS expression. Plasma levels of vasopressin are also increased during salt loading. However, it has been shown that chronic vasopressin infusion does not increase eNOS expression in the renal medulla. In addition, chronic infusion of a V2-receptor antagonist did not alter eNOS expression in the renal medulla of water-restricted rats. These data suggest that vasopressin may not regulate eNOS expression in the THAL during salt loading. We are currently studying which mechanism mediates eNOS expression during a high-salt diet.
Because NO production did not differ between the 2 groups, we next hypothesized that the inhibition of NaCl transport produced by a given concentration of NO must be enhanced to account for such data. Consequently, we next measured the effect of 2 different concentrations of spermine NONOate on chloride absorption by THALs taken from rats on normal- and high-salt diets. We found that 1 μmol/L had no significant effect on transport by THALs from rats on a normal diet, but this concentration inhibited NaCl absorption by 35% in tubules from rats on high salt. In contrast, a higher concentration inhibited transport to the same extent in both groups. Thus, our data indicate that the enhanced effect of L-arginine on NaCl transport in THALs from rats on high salt is due to enhanced sensitivity of tubules to NO rather than to augmented NO generation.

We have previously reported that the signaling cascade by which NO inhibits NaCl absorption involves activation of soluble guanylate cyclase, an increase in cGMP, stimulation of cGMP-stimulated phosphodiesterase, and a subsequent decrease in cAMP levels. The decrease in cAMP, which stimulates Na/K/Cl\(^{-}\) cotransport via activation of protein kinase A, is finally responsible for the decrease in NaCl absorption. Thus, increasing the NaCl content of the diet may enhance either the sensitivity or expression of soluble guanylate cyclase. In addition, the effect of a given concentration of cGMP on cGMP-stimulated phosphodiesterase could be increased by high salt, which could alter the activation of protein kinase A by cAMP or affect cAMP levels. However, because a high-salt diet may affect the expression of several proteins, NO may activate some other signaling cascade in addition to or instead of the cGMP cascade. There is some evidence in the literature that NO can activate other second-messenger cascades in addition to cGMP. However, the nature of the signal that activates either cGMP or an alternate cascade is unknown. We are currently investigating these issues.

NO produced by the kidney is important for maintenance of blood pressure in the face of elevated salt intake. If renal NO production is inhibited, animals develop hypertension when placed on a high-salt diet. The THAL absorbs 20% to 30% of the filtered load of NaCl and creates the corticomedullary gradient necessary for water absorption. Inappropriate salt absorption by this segment has been implicated in salt-sensitive hypertension. The importance of the THAL in the regulation of blood pressure is perhaps best evidenced by the fact that the diuretic furosemide acts in this segment and dramatically increases natriuresis and diuresis, while lowering blood pressure. Increasing the sensitivity of tubules to NO rather than increasing its production could prevent NO concentrations from becoming high enough to become cytotoxic.

**Perspectives**

We conclude that a high-salt diet enhances the inhibitory effect of L-arginine on NaCl absorption by the THAL. Despite increased immunoreactive eNOS during salt loading, the enhanced effect of L-arginine is due to increased sensitivity of this nephron segment to NO rather than to increased NO production.

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