A High-Salt Diet Dissociates NO Synthase-3 Expression and NO Production by the Thick Ascending Limb

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Abstract—NO produced by endothelial NO synthase (NOS3) decreases sodium transport by the thick ascending limb (THAL). We found previously that 7 days of high salt (HS) increased THAL-NOS3 expression but not NO production. NOS3 phosphorylation regulates enzyme activity. We hypothesized that HS acutely increases NOS3 expression and NO production, and, over time, changes in NOS3 phosphorylation dissociate NO production from expression. NOS3 expression increased by 71±13%, 127±24%, and 69±16% at days 1, 3, and 7 of HS, respectively. At days 14 and 28, expression was back to normal salt. After 1 day of HS, NO production in response to 250 μmol/L L-arginine was elevated by 146% and, by day 3, returned to normal salt. Similar increases were found in response to endothelin-1. Inhibitors of NOS1/2 did not blunt the salt-induced increase in NO. Phosphorylation at Thr495, an inhibitory site, decreased by 39±8% at day 1 of HS and then increased by 116±18% at day 3. Phosphorylation at Ser1177 (stimulatory sites) decreased by ~25% at day 1 and remained depressed at day 3. Superoxide production increased by 71% at day 1, decreased by 57% at day 3, and decreased by 55% at day 7. The NOS inhibitor L-Nω-nitroarginine methyl ester did not alter superoxide levels at any time point. The addition of reduced nicotinamide adenine dinucleotide phosphate and tetrahydrobiopterin had no effect on NO release after 3 days of HS. We conclude the following: (1) HS transiently increases NO production and NOS3 expression; (2) NOS3 expression and NO production are dissociated by HS; and (3) changes in phosphorylation explain how THAL NOS3 activity and expression are dissociated by HS.

Key Words: nitric oxide synthase • kidney • phosphorylation
Methods

Animals
Male Sprague-Dawley rats 6 to 7 weeks of age and weighing 101 to 125 g (Charles River, Kalamazoo, MI) were fed a diet containing 0.22% sodium and 1.1% potassium (Purina). At this age, a rat consumes ~10 g of food and 20 mL of water per day, so that such a diet provides a salt intake of ~0.1 g salt/kg per day. Animals on a “normal” diet drank tap water, whereas animals on the HS diet drank 1% NaCl, resulting in a salt intake of ~0.2 g salt per day (1 g/kg per day) or 10 times the amount of sodium consumed by rats on a regular diet (tap water). In contrast, a HS diet (food) that contains ≥4% Na will result in 0.4 g salt per day (2 g/kg per day). We used this diet because salt intake is better controlled when salt is given in the water.

Medullary THAL Suspensions
Sprague-Dawley 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 kidneys flushed with 40 mL of ice-cold 0.1% collagenase in a perfusion solution containing 130 mmol/L NaCl, 2.5 mmol/L NaH2PO4, 4 mmol/L KCl, 1.2 mmol/L MgSO4, 6 mmol/L alanine, 1 mmol/L Na2 citrate, 5.5 mmol/L glucose, 2 mmol/L Ca (lactate), and 10 mmol/L HEPES (pH 7.4) via retrograde perfusion of the aorta. Solutions were gassed with compressed air. Kidneys were removed and coronal slices cut. The inner stripe of the outer medulla was minced into 1-mm fragments at 4°C and digested in 0.1 mg/mL collagenase at 37°C for 30 minutes. During each 5-minute period, the tissue was gently agitated and gassed with compressed air. After continuous agitation for 30 minutes in cold solution, the tissue was filtered through a 250-μm nylon mesh and rinsed twice with the same solution. This procedure yielded a 92% pure suspension of THALs when this preparation was immunostained for Tamm-Horsfall protein (which is exclusively expressed in this nephron segment), and, therefore, the contribution of other cell types present in our preparation (if any) would be minimal. When suspensions were prepared for measurement of phosphorylated eNOS, a 1/1000 dilution of phosphatase inhibitor mixture III (Calbiochem) was added to the perfusion solution.

Western Blot
Medullary THAL suspensions were centrifuged and tubules lysed by vortexing them in 300 μL of a buffer containing 20 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 0.3 mol/L sucrose, 1.0% Nonidet P-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 4-(2-aminoethyl)benzene sulfonic fluoride (Sigma). For phosphorylated NOS3, a 1/1000 dilution of phosphatase inhibitor mixture III (Calbiochem) was added to the buffer. Samples were centrifuged at 6000g for 5 minutes at 4°C and protein content in the supernatant determined. For total and phosphorylated NOS3, 10 and 100 μg of total protein, respectively, were loaded into each lane of an 8% sodium dodecyl sulfate-polyacrylamide gel, separated by electrophoresis, and trans-ferred to a polyvinylidene fluoride membrane (Millipore). Equal amounts of protein obtained from THALs of animals fed a normal and HS diet were run in the same gel. Fresh samples were always used, because we found that freezing degrades NOS3 and leads to multiple bands on Western blotting. 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 a NOS3-specific monoclonal antibody (BD Transduction Laboratories), a 1/5000 dilution of a monoclonal antibody against NOS3 phosphorylated at Ser1177 (BD Transduction Laboratories), a 1/1000 dilution of a polyclonal antibody against NOS3 phosphorylated at Ser1177 (Upstate), and a 1/2000 dilution of a polyclonal antibody against NOS3 phosphorylated at Thr495 (Upstate) in blocking buffer for 60 minutes at room temperature. The membrane was washed in a buffer containing 50 mmol/L Tris, 500 mmol/L NaCl, and 0.1% Tween 20 and incubated with a 1/1000 dilution of a secondary antibody against the appropriate IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). The reaction products were detected with a chemiluminescence kit (Amersham Pharmacia Biotech). Chemiluminescence was detected by exposure to Fuji RX film and quantified by densitometry. Because NOS3 expression changes with HS, we normalized phosphorylated NOS3 expression by total NOS3 expression for both HS and normal-salt experiments. We then expressed changes caused by HS (phosphorylated NOS3/total NOS3) as a percentage of normal salt (phosphorylated NOS3/total NOS3). Thus, for normal salt, the ratio between each phosphorylated form and total NOS3 equals 1.

Protein Content Determination
Total protein content was determined using Coomassie Plus reagent (Pierce), based on Bradford’s colorimetric method.

Measurements of NO Release
Medullary THAL suspensions were placed in a buffer containing 130 mmol/L NaCl, 2.5 mmol/L NaH2PO4, 4 mmol/L KCl, 1.2 mmol/L MgSO4, 6 mmol/L alanine, 1 mmol/L Na2 citrate, 5.5 mmol/L glucose, 2 mmol/L Ca (lactate), and 10 mmol/L HEPES (pH 7.4) and maintained in a temperature-regulated chamber at 37°C with continuous agitation. NO in the bath was monitored using a precalibrated amperometric sensor selective for NO (amiNO-700, Innovative Systems) and its corresponding software (inNO measuring system, Harvard Apparatus). After a baseline was obtained, 250 μmol/L/L-arginine, when NO release was measured continuously. When endothelin-1 was used to stimulate NO release, 1 mmol/L was added in the presence of l-arginine. NO release was measured continuously for 7 minutes after adding l-arginine, when the signal usually reached a plateau. As a control for selectivity, NO release in response to 250 μmol/L L-arginine was measured in the presence of the nonselective NOS inhibitor L-NH2-nitroarginine methyl ester (L-NAMe, Sigma). We found that 100 μmol/L L-NAMe inhibited l-arginine–stimulated NO release by 85±2% (P<0.01; n=3), indicating that the sensor was selectively responding to changes in NO concentration. Animals on the HS diet and their respective age-matched controls (normal salt) were processed the same day. At the end of the experiment, medullary THAL suspensions were homogenized as indicated for Western blots and total protein content determined. Results are expressed in pmol/NO per milligram of protein. When 1400W hydrochloride (NOS2 inhibitor, Cayman) and 7-nitroindazole (7-NI; NOS1 inhibitor, Cayman) were used, they were added to the working solution to a final concentration of 100 mmol/L and 10 μmol/L, respectively. 7-NI was dissolved by sonication in 98% ethanol. The final concentration of ethanol in the THAL suspension was 0.016%. When experiments were performed in the presence and absence of cofactors, 5 μmol/L sepiapterin (the precursor of tetrahydrobiopterin, Sigma) and 1 mmol/L reduced β-nicotinamide-adenine dinucleotide phosphate (β-NADPH, Sigma) were added to the tubule suspension 30 minutes before measurements.

Measurements of Superoxide Production
THAL suspensions (100 μL) were placed in 1.6-mL polypropylene tubes. Then, 800 μL of perfusion solution was added, and tubes were placed on ice. Lucigenin (Sigma) was added to the suspensions to a final concentration of 5 μmol/L, and then the tubes were incubated for 30 minutes at 37°C. Tubes were placed in a luminometer (model 20e, Turner Devices) maintained at 37°C. The average of the last 3 of 10 consecutive recorded 30-s measurements was calculated for each sample. The superoxide (O2·−) scavenger 4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt monohydrate (Tiron, Sigma) was added to the sample to a final concentration of 10 mmol/L, and measurements were repeated. The difference in average luminescence between samples with and without Tiron was used to calculate the luminescence produced by O2·−. Measurements were normalized to protein content. Samples without tubules were processed identically and used as blanks. When l-NAME (Sigma) was used, it was
added to the tubule suspension at the beginning of the experiment at a final concentration of 100 μmol/L.

Statistics
Data are reported as mean±SEM. They were evaluated by nonparametric Mann-Whitney test, 1 sample t test, or paired t test as appropriate. All of the statistical analyses were performed by the Biostatistics Department at Henry Ford Hospital. *P<0.05 was considered significant.

Results
Our previous data showed increased THAL NOS3 expression at 7 days of a HS diet. To characterize the time course of this increase, we first measured THAL NOS3 expression by Western blot in age-matched animals fed a normal or HS diet for 1, 3, 7, 14, and 28 days. We found that THAL NOS3 expression was significantly increased by 71±13% (*P<0.001; n=7) after 1 day of HS. After 3 days, it was increased by 127±24% of normal salt (*P<0.001; n=8). Thereafter, expression declined so that after 7 days it was only 69±16% greater than normal salt (*P<0.003; n=8). After 14 and 28 days of HS, NOS3 expression returned to baseline (change of 39±34% [n=6] and 1±9% [n=7], respectively; not significant; Figure 1). Thus, a HS diet increased THAL NOS3 expression in a biphasic manner.

To investigate whether a HS diet acutely increases NO production by the THAL, we measured NO release in medullary THAL suspensions from animals fed a normal or HS diet. Because NOS3 expression peaked by day 3 and we found previously that NO production returned to baseline by 7 days, we measured NO release after 1, 3, and 7 days of a HS diet using a precalibrated NO-selective sensor. At day 1, adding 250 μmol/L L-arginine (the substrate for NOS) stimulated NO release by 5.8±1.3 pmol/mg in THALs from rats fed a normal diet and 14.4±2.7 pmol/mg in THALs from rats fed HS, a 148% increase (*P<0.01 versus NS; n=7). However, there were no differences in NO release by THALs from rats on a normal diet or HS at day 3 or 7 (Figure 2). In the presence of L-arginine, the addition of 1 nmol/L endothelin-1 produced similar differences between HS and normal salt in terms of NO release. These data suggest that a HS diet has a biphasic effect on NO release, but the time course is different from expression.

To make sure the increased NO release caused by 1 day of HS was produced by NOS3, we measured NO release in the presence and absence of the selective NOS1 and NOS2 inhibitors 7-NI (50 μmol/L) and 1400 W hydrochloride (100 nmol/L), respectively. In medullary THAL suspensions from animals fed a HS diet for 1 day, 7-NI and 1400 W had no effect on HS-induced NO release by the THAL (difference, 6±1%), indicating that the increase in THAL NO release caused by 1 day of HS was produced mainly by NOS3.

Because NO decreased at day 3 and 7 after the initiation of HS compared with normal salt, we investigated the possibility that the decline was due to increased O2 production. We
found that O₂⁻ production by THALs isolated from rats fed HS was increased by 71% at day 1 (P<0.02) but reduced by 57% at day 3 (P<0.05) and by 55% at day 7 (P<0.04). These data suggest that the decline in NO production observed at 3 and 7 days is not because of the scavenging effects of O₂⁻.

Dissociation between THAL NOS3 activity and expression by HS was maximally evident after 3 days. To examine whether this was because of allosteric modifications of enzyme activity, we measured NOS3 phosphorylation. In medullary THAL suspensions, phosphorylation at Thr⁴⁹⁵ was reduced by 39±8% at day 1 (P<0.002 versus normal salt; n=8) and increased to 216±18% at day 3 (P<0.005 versus day 1; n=8; Figure 3). Thus, a HS diet acutely (1 day) decreased phosphorylation of NOS3 at Thr⁴⁹⁵, predicting increased NOS3 activity. Chronically (3 days), HS increased phosphorylation of NOS3 at Thr⁴⁹⁵, predicting decreased NOS3 activity. In contrast, phosphorylation at Ser⁶³³ was reduced to 73±9% at day 1 and remained depressed at day 3 (71±10%; P<0.03 versus normal salt; n=7 for each; Figure 4). In addition, phosphorylation at Ser¹¹⁷⁷ was reduced to 77±8% at day 1 and 78±5% at day 3 (P<0.03 versus normal salt; n=7 for each; Figure 5). Thus, phosphorylation of the 2 positive regulatory sites decreased at day 1 and 3 of HS, predicting reduced enzyme activity. Together these data suggest that phosphorylation of NOS3 at Thr⁴⁹⁵ predominates in the regulation of enzyme activity, because changes in phosphorylation at this regulatory site may explain the changes in NO release caused by HS.

Because it has been suggested that in endothelial cells Thr⁴⁹⁵ is a phosphorylation site that functions as a switch that may regulate whether NOS3 produces O₂⁻ and/or NO,¹¹ we investigated whether the changes in phosphorylation at Thr⁴⁹⁵ induced by HS resulted in NOS3-derived O₂⁻ production. To do this, we measured O₂⁻ production by THALs in the presence and absence of the NOS inhibitor L-NAME at day 1 and 3 of HS. Measurements were performed in the absence of the substrate for NOS, L-arginine, to favor NOS3 uncoupling.

Figure 3. Effect of a HS diet on NOS3 phosphorylated at Thr⁴⁹⁵ [pNOS3 (Thr⁴⁹⁵)] by medullary THAL suspensions at day 1 and 3. Top: representative individual experiment. Bottom: mean densitometry values from Western blots (n=8 for each group). *P<0.002 vs NS. **P<0.005 vs 1 day.

Figure 4. Effect of a HS diet on NOS3 phosphorylated at Ser⁶³³ [pNOS3 (Ser⁶³³)] by medullary THAL suspensions at day 1 and 3. Top: representative individual experiment. Bottom: mean densitometry values from Western blots (n=7 for each group). **P<0.03 vs NS.

Figure 5. Effect of a HS diet on NOS3 phosphorylated at Ser¹¹⁷⁷ [pNOS3 (Ser¹¹⁷⁷)] by medullary THAL suspensions at day 1 and 3. Top: Representative individual experiment. Bottom: mean densitometry values from Western blots (n=7 for each group). **P<0.03 vs NS.
We found no differences between O$_2$ production in the presence and absence of L-NAME at day 1 (2.5±0.5 versus 2.8±0.8 luminescence units/min per milligram of protein) and day 3 (2.4±0.5 versus 2.2±0.4 luminescence units/min per milligram of protein), indicating that NOS does not contribute to the production of O$_2$ in the THAL during HS.

ROS activity can also be influenced by its cofactors, tetrahydrobiopterin and NADPH, in addition to phosphorylation. To determine whether the decline in THAL NOS3 activity by day 3 of HS was because of a lack of cofactors, we measured THAL NO release after 3 days of HS in the presence and absence of 5 μmol/L sepiapterin (the precursor of tetrahydrobiopterin) and 1 mmol/L β-NADPH. In medullary THAL suspensions, adding these cofactors did not restore the increase in NO release observed after 1 day of HS, suggesting that cofactor availability does not limit NOS3 activity.

Discussion

We reported previously that a HS diet increased NOS3 expression in medullary THALs but did not enhance NO production. However, these measurements were performed at a single time point, 7 days after the initiation of HS. To investigate whether this finding was the result of measurements being made at a single time point, in the present study we measured the effect of a HS diet on NOS3 expression at 1, 3, 7, 14, and 28 days. We found that NOS3 expression increased at day 1 and peaked at day 3. On day 7, expression was less than at 3 days but still higher than with normal salt. By 14 and 28 days, NOS3 expression by THALs from rats on HS was not different from normal salt. Because expression peaked at 3 days and we reported previously that NO production returned to baseline by 7 days, we next measured the effect of HS on NO production by THALs at 1, 3, and 7 days and compared it to normal salt. We found that NO release in response to either L-arginine or endothelin-1 peaked at day 1 and by day 3 had already returned to basal values. Taken together, these data indicate that NO release by THALs is dissociated from NOS3 expression when animals are fed a HS diet.

NOS3 is the dominant NOS isoform expressed by the THAL. Using transgenic mice, our laboratory has demonstrated that NOS3 is the isoform responsible for NO-dependent inhibition of transport in this segment. However, NOS1 and NOS2 mRNA and protein are also present in the THAL. Therefore, to make sure the increased NO release caused by 1 day of HS was produced by NOS3, we measured NO release in the presence and absence of the specific NOS1 and NOS2 inhibitors, 7-NI and 1400 W hydrochloride, respectively. At the concentrations we used, 7-NI and 1400 W have been shown to specifically inhibit NOS1 without affecting NOS3. We found that these inhibitors did not affect the increase in NO induced by HS at day 1, suggesting that this increase was produced by NOS3.

Our finding that THAL NOS3 expression is modulated by HS is similar to the report by Ni and Vaziri. These authors found that NOS expression increased in the outer medulla of rats after 2 days of HS intake, but this increase was reversed after 1 day of HS, suggesting that cofactor availability does not limit NOS3 activity.
activity beyond the larger changes caused by phosphorylation at Thr495.

Our data suggest that changes in NO production during HS are mainly attributable to changes in enzyme activity due to NOS3 phosphorylation at Thr495 and that O2− does not play a role in the decline of bioavailable NO. However, because in endothelial cells phosphorylation at Thr495 has been suggested to function as a switch determining whether NOS3 produces NO or O2− attributed to NOS3 uncoupling,11 we investigated whether production of O2− by THALs was L-NAME inhibited. We found no significant differences in O2− production when the NOS inhibitor was present. These data indicate that NOS does not contribute significantly to the production of O2− in the THAL when rats are on a HS diet. Therefore, we concluded that THAL NOS3 is not uncoupled by phosphorylation at Thr495.

We do not know how NOS3 phosphorylation in the THAL is regulated by HS. Phosphorylation and dephosphorylation of NOS3 are known to occur via protein kinase A, C, and G, phosphatidylinositol 3-kinase/Akt, AMP-activated kinase, and protein phosphatases (PP1 and PP2).3,24–26 Most of these kinases and phosphatases are present in the THAL.3,26–30 Therefore, HS intake may directly influence kinases and/or phosphatases, which could affect NOS3. NO itself may inhibit NOS3 activity via a short-loop feedback system, triggering changes in NOS3 phosphorylation. Such an inhibitory effect of NO on NOS has been reported in endothelial cells.31,32 Thus, acutely, HS would lead to dephosphorylation of NOS3 at Thr495, enhanced NOS3 activity, NO production, and NO concentration. Chronically, the elevated NO would then inhibit NOS3 activity by rephosphorylation at Thr495 without affecting expression. Ultimately, there would be more NOS3 protein, but a proportional amount of NO would not be synthesized. The existence of such a short feedback loop in the THAL has not been investigated to our knowledge.

The regulation of NOS3 activity involves many mechanisms. These include phosphorylation, trafficking,7,8 and interactions with scaffolding proteins, such as heat shock protein 90, calmodulin, dynamin-2, caveolin, NOSIP, and NOSTRIN.5 All of these mechanisms function as an integrated complex in the regulation of enzyme activity. Previous reports from this group, as well as the present study, implicate NOS3 translocation and phosphorylation9 at different regulatory sites as important regulators of THAL NOS3 activity. In a recent study, Ortiz et al33 showed that NOS3 is activated at which step of the signaling cascade NOS3 phosphorylation is modulated, and our laboratory is currently investigating this complex mechanism. Understanding how THAL NOS3 is regulated by HS could contribute to the elucidation of the defective mechanisms responsible for lack of adaptation to elevated salt intake and the resulting salt-sensitive hypertension, which affects a great many people around the world.

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References

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