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

Marcela Herrera, Guillermo Silva, Jeffrey L. Garvin

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 \(71 \pm 13\)% at day 1, \(127 \pm 24\)% 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 \(\mu\)mol/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 Thr\(^{348}\), an inhibitory site, decreased by \(39 \pm 8\)% at day 1 of HS and then increased by \(116 \pm 18\)% at day 3. Phosphorylation at Ser\(^{353}\) and Ser\(^{1177}\) (stimulatory sites) decreased by \(\approx 25\)% 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-N\(^{4}\)-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

Nitric oxide (NO) produced by endothelial NO synthase (NOS3) in the thick ascending limb (THAL) inhibits THAL sodium reabsorption by inhibiting Na/K/2Cl cointransporter activity.\(^1\) Seven days of a high-salt (HS) diet increased NOS3 expression in the THAL,\(^2\) suggesting that this enzyme may be important in chronic adaptation to HS intake. HS heightens NOS3 expression in the THAL by increasing medullary osmolality, enhancing release of endothelin-1, and activating endothelin B receptors.\(^2,3\) However, NO production was not increased after 7 days of HS.\(^4\) There are at least 3 possible reasons for the lack of correlation between enzyme expression and NO production: (1) NO bioavailability may be reduced; (2) availability of cofactors may be limited; or (3) NOS3 activity may be suppressed by allosteric modifications, which may or may not induce NOS3 uncoupling. Because superoxide (\(O_2^-\)) levels in the THAL are decreased by \(33.8\)% at day 7 of a HS diet,\(^5\) it does not appear likely that NO bioavailability is reduced. Similarly, whereas NO production by NOS3 can be limited by cofactor availability under some circumstances, this occurs primarily during rapid bursts of NO production accompanied by robust NOS expression in macrophages,\(^6\) making it unlikely that a 2- to 3-fold increase in NOS3 expression and activity could significantly reduce intracellular cofactor concentrations. Thus, allosteric modulation of enzyme activity seems to be the most likely explanation.

NOS3 activity can be allosterically regulated by posttranscriptional modifications, such as protein–protein interactions, translocation, and phosphorylation.\(^7\) Phosphorylation of NOS3 is perhaps the best understood modulator of enzyme activity. In endothelial cells, phosphorylation of NOS3 at Ser\(^{1177}\) and Ser\(^{353}\) positively regulates enzyme activity, whereas phosphorylation at Thr\(^{348}\) inhibits it\(^8\) (human amino acid sequence numbering). Data from recent studies suggest that phosphorylation is an important modulator of NOS3 in the renal medulla. Ortiz et al\(^9\) found recently that acute flow–induced increases in NOS3 activity in the THAL were because of phosphorylation at Ser\(^{1177}\). Lee et al\(^10\) reported that increased NOS3 activity in the renal medulla of diabetic rats correlated with dephosphorylation of the negative regulatory site Thr\(^{348}\). Based on these findings, we hypothesized that a HS diet modifies THAL NOS3 expression and activity in a time-dependent manner and that enzyme activity depends on the phosphorylation state.
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 Na3 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-mm3 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 30-μm nylon mesh and rinsed twice with the same solution. This procedure yielded a 92% pure suspension of THALs when this 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 sulfonyl 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 transferred 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 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 Na3 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 (L-NAME) was added, and 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 NO inhibitor L-N(ω)-nitroarginine methyl ester (L-NAME, Sigma). We found that 100 μmol/L L-NAME inhibited L-arginine–stimulated NO release by 86±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, Cayman) 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 polypyrrole 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 Designs) 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.2 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,4 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

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Effects of a HS diet on NOS3 expression by medullary THAL suspensions from animals fed a normal (NS) or HS diet for 1, 3, 7, 14, or 28 days (n=7 to 8 for each group). *P<0.001 vs NS; #P<0.003 vs NS.

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Effect of L-arginine (250 μmol/L) on NO release by medullary THAL suspensions from rats fed a normal (NS) or HS diet for (A) 1 day, (B) 3 days, or (C) 7 days (n=7 for each group). ¥P<0.002 vs 1 day NS for all time points.
found that O$_2$ 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 O$_2$.

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 Thr$^{655}$ 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 Thr$^{655}$, predicting increased NOS3 activity. Chronically (3 days), HS increased phosphorylation of NOS3 at Thr$^{655}$, predicting decreased NOS3 activity. In contrast, phosphorylation at Ser$^{633}$ 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 Ser$^{1177}$ 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 Thr$^{655}$ 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 Thr$^{655}$ is a phosphorylation site that functions as a switch that may regulate whether NOS3 produces O$_2$ and/or NO, we investigated whether the changes in phosphorylation at Thr$^{655}$ induced by HS resulted in NOS3-derived O$_2$ production. To do this, we measured O$_2$ 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 Thr$^{655}$ [pNOS3 (Thr$^{655}$)] 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 Ser$^{633}$ [pNOS3 (Ser$^{633}$)] 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 Ser$^{1177}$ [pNOS3 (Ser$^{1177}$)] 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.

NO 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 increased at day 1 and peaked at day 3 of HS, we might predict that NO production would first increase and then decline, as we found for NO release. In contrast, phosphorylation of NOS3 at Ser1177 and Ser1176, 2 positive regulatory sites, was reduced at both day 1 and 3, suggesting that NO production would be reduced by HS monotonously, in contrast to our NO release data. Therefore, phosphorylation of Thr495 appears to predominate over the long-term regulation of NOS3 activity by HS (hours to days), because NO release was increased despite decreased phosphorylation at Ser1177 and Ser1176.

Phosphorylation as a regulator of NOS3 activity has been studied for the past decade. Phosphorylation at Thr495 was first shown to be an important negative regulator of NOS3 activity in endothelial cells. More recently, Lee et al reported increased NOS3 activity in the cytosolic fraction of the renal medulla of diabetic rats, correlated with dephosphorylation of NOS3 at Thr495 along with Ser413. Such measurements of enzyme activity were performed in tissue homogenates using conversion of l-arginine to l-citrulline at optimal substrate and cofactor levels. These data correlate with ours, because we found increased NO release at day 1 after initiation of the HS diet in parallel with decreased phosphorylation of NOS3 at both Thr495 and Ser413; however, those authors found no changes in NOS3 expression, suggesting an important role for phosphorylation in regulating NOS3 activity independent of changes in expression. Thus, phosphorylation of Thr495 appears to play a dominant role in regulating NOS3 activity long-term. In contrast, phosphorylation of NOS3 at Ser1177 and Ser413 occurs rapidly in response to different stimuli that lead to increased enzyme activity and NO production.

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 chronic salt loading (3 weeks). However, because the renal medulla is composed of other cell types besides THALs that may also express NOS3, such as the vasa recta, interstitial cells, and medullary collecting ducts, the contribution of THAL NOS3 to the findings of Ni and Vaziri is not clear.

Despite increased levels of NOS3 expression, our data show that NO release was stimulated at day 1 and returned to baseline by day 3. This dissociation between expression and production implies that enzyme expression does not always correlate with activity, and, therefore, NOS3 activity must be regulated independent of protein levels. This has been demonstrated to be the case in other cell types and tissues. To study the mechanism involved in this dissociation, we first investigated whether increased $O_2$ production could be responsible for the decline in bioavailable NO. We found that THAL $O_2$ production is decreased at day 3 and 7 of HS compared with normal salt, and, therefore, we excluded the possibility of $O_2$ scavenging NO.
activity beyond the larger changes caused by phosphorylation at Thr^495.

Our data suggest that changes in NO production during HS are mainly attributable to changes in enzyme activity due to NOS3 phosphorylation at Thr^495 and that O_3^- does not play a role in the decline of bioavailable NO. However, because in endothelial cells phosphorylation at Thr^495 has been suggested to function as a switch determining whether NOS3 produces NO or O_3^- attributed to NOS3 uncoupling, we investigated whether production of O_3^- by THALs was l-NNAME inhibit- able. We found no significant differences in O_3^- production when the NOS inhibitor was present. These data indicate that NOS does not contribute significantly to the production of O_3^- in the THAL when rats are on a HS diet. Therefore, we concluded that THAL NOS3 is not uncoupled by phosphorylation at Thr^495.

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). Most of these kinases and phosphatases are present in the THAL. 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. Thus, acutely, HS would lead to dephosphorylation of NOS3 at Thr^495, enhanced NOS3 activity, NO production, and NO concentration. Chronicly, the elevated NO would then inhibit NOS3 activity by rephosphorylation at Thr^495 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, and interactions with scaffolding proteins, such as heat shock protein 90, calmodulin, dynamin-2, caveolin, NOSIP, and NOSTRIN. 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 phosphorylation at different regulatory sites as important regulators of THAL NOS3 activity. In a recent study, Ortiz et al showed that NOS3 is activated when it localizes to the apical membrane of the THAL. Whether changes in NOS3 phosphorylation and enzyme expression are cytosolic intermediates necessary for the trafficking process is still unknown. It is possible that phosphorylation is part of the mechanism whereby NOS3 localizes to the apical membrane without a direct relationship with membrane-bound NOS3. We believe that future studies addressing total and phosphorylated NOS3 localization will contribute to our understanding of NOS3 activation in the THAL. Some of the proteins that interact with NOS have been localized to the kidney; however, its role in the regulation of THAL NOS3 has not been thoroughly investigated except for the interaction between NOS3 and heat shock protein 90. Our laboratory is currently attempting to establish which other components of this mechanism are involved in regulation of THAL NOS3 activity.

**Perspectives**

We conclude that phosphorylation is an important regulatory mechanism of NOS3 activity in the THAL that acts independently of protein expression. Although advances have been made in our understanding of NOS3 regulation within the kidney, clearly much remains to be addressed. NOS3 activation is a complex process involving protein translocation and protein–protein interactions other than phosphorylation. Whether protein expression and phosphorylation are directly related to NOS3 localized to the apical membrane of the THAL remains to be elucidated. It is important to determine 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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