Dietary Salt Activates an Endothelial Proline-Rich Tyrosine Kinase 2/c-Src/Phosphatidylinositol 3-Kinase Complex to Promote Endothelial Nitric Oxide Synthase Phosphorylation

Wei-Zhong Ying, Kristal Aaron, Paul W. Sanders

Abstract—Although many laboratories have shown that dietary NaCl (salt) intake increases NO production in rodents and humans, the mechanism has not been uncovered. In the present study, pharmacological and dominant-negative strategies were used to show that feeding a formulated diet containing increased amounts of salt to young male Sprague-Dawley rats induced the formation of an endothelial cell-signaling complex that contained proline-rich tyrosine kinase 2, c-Src (also known as pp60c-src), and phosphatidylinositol 3-kinase. In the setting of a high-salt diet, proline-rich tyrosine kinase 2 served as the scaffold for c-Src–mediated phosphatidylinositol 3-kinase activation. Phosphatidylinositol 3-kinase was the upstream activator of protein kinase B (Akt), which was responsible for phosphorylation of the rat endothelial isoform of NO synthase at S1176 and thereby promoted the increase in NO production. The combined findings illustrated the crucial role for a proline-rich tyrosine kinase 2–signaling complex in the endothelial response to salt intake. (Hypertension. 2008;52:1134-1141.)

Key Words: NO ■ cell signaling ■ cell biology ■ animal models of human disease

Following the original description of the role of NO in the blood pressure responses to changes in dietary NaCl (termed “salt” in this article) intake,1 subsequent studies confirmed that increased salt intake increased NO production in rodents2–5 and healthy humans.6 NO plays an important role in the hemodynamic response to changes in salt intake. Salt-induced NO release promotes vasorelaxation of the afferent arteriole,7 augments the glomerular filtration rate,8 and improves the pressure-natriuresis curve, facilitating salt excretion.9 Inhibition of NO results in salt retention and salt-sensitive hypertension10 and, if protracted, leads to renal injury, particularly if the animals are maintained on a high-salt diet.11

The direct involvement of the endothelium in mediating NO production in response to a high-salt diet has been demonstrated.12 The mechanism by which salt intake increases endothelial NO production appears to be initiated through generation of shear forces.13–15 The endothelial isoform of NO synthase, termed “NOS3” in this article, is a highly regulated enzyme that is controlled by a variety of posttranslational events that include phosphorylation of multiple serine and threonine residues of NOS3. Although NOS3 can serve as a substrate for coupling to receptors, such as shear stress,23 but also by G protein–coupled receptors, such as the angiotensin type 1 receptor.22,24 Pyk2 has multiple binding partners that include c-Src, the 60-kDa protein of c-src (also known as pp60c-src), phosphatidylinositol 3-kinase (PI3-kinase), and Grb2.22,25–27 Binding to Pyk2 activates c-Src and PI3-kinase, and this signaling complex participates in a variety of intracellular processes.22,28 Because PI3-kinase is an upstream activator of Akt, the present study has, therefore, been designed to determine whether an increase in the phosphorylation state of S1176 of NOS3 accounts for the augmented endothelial NO production that occurs in the setting of increased salt intake and whether dietary salt intake induces a Pyk2/c-Src/PI3-kinase complex participates in a variety of intracellular processes.
complex that, in turn, increases NOS3 activity through activation of Akt.

Methods

Animal and Tissue Preparation

The institutional animal care and use committee at the University of Alabama at Birmingham approved the project. Studies were conducted using male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, Ind) that were 28 days of age at the start of study. The protocol that was followed has been standardized in our laboratory. The rats were housed under standard conditions and given formulated diets (AIN-76A, Dyets, Inc) that contained 0.3% and 8.0% (wt/wt) NaCl. These nitrate- and nitrate-free diets were prepared specifically to be identical in protein composition and differed only in NaCl and sucrose content. On the fourth day of analyses as performed previously. The primary antibodies under sterile conditions for incubation studies and immunoblotting analyses as performed previously.

The rats were anesthetized by intraperitoneal injection of pentobarbital sodium injection (Ovation Pharmaceuticals, Inc), 50 mg/kg body weight, and aorta and isolated glomeruli were obtained under sterile conditions for incubation studies and immunoblotting experiments or for in vitro incubation studies. At the time of tissue harvesting, urine was collected from the bladder to determine NO metabolites (NOx), which were assayed using a kit (QuantiChrom Nitric Oxide Assay kit, BioAssay Systems), and creatinine concentration, which was assayed using an autoanalyzer (Creatinine Analyzer 2, Beckman Coulter, Inc). In these studies, assays were performed in triplicate and averaged; NOx values were normalized using the creatinine concentration obtained in each sample. Isolated glomeruli and aortic ring segments were incubated in serum-free medium (DMEM; Invitrogen Corporation) at 37°C for 4 hours. The conditioned medium was harvested, centrifuged at 300g for 10 minutes at 4°C to remove cell debris, and then stored at −80°C until assayed for NOx; the results were factored by wet weight (for aortic tissue) or total protein (for glomeruli).

Coimmunoprecipitation Assays

Coimmunoprecipitation studies were performed to characterize the effect of dietary salt intake on the interactions between Pyk2 and PI3-kinase. Tissue lysates containing 500 µg of total protein were obtained from the rats used in the in vivo studies and were incubated with 2 µg of an anti-Pyk2 polyclonal antibody (Cell Signaling) at 4°C for 2 hours, followed by the addition of 30 µL of protein A-Sepharose and incubation. Immune pellets were washed 3 times with ice-cold radioimmunoprecipitation assay buffer and then boiled in SDS sample buffer containing dithiothreitol. The proteins were resolved on 7.5% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The membranes were probed with antibodies directed against 4 isoforms of the catalytic unit of PI3-kinase (p110α, p110β, p110γ, and p110δ) and the regulatory subunit p85 (Upstate Chemicon). Immunoreactive bands were visualized with the use of enhanced chemiluminescence.

Statistical Analysis

Data were expressed as means±SEs. Significant differences among data sets were determined by ANOVA with posthoc testing (Fisher’s protected least-significant difference; Statview 5.0, SAS Institute, Inc). P<0.05 assigned statistical significance.

Results

Dietary Salt Intake Increased NOS3 Phosphorylation Through an Akt-Dependent Mechanism

After 4 days on the 2 diets, lysates from aortic tissue and isolated glomeruli were obtained from the rats (n=6 in each group), and phosphorylation of NOS3 at S1176 was determined using an antibody that specifically recognized p-NOS3(S1176). Compared with samples from rats on the 0.3% NaCl diet, lysates from aorta and isolated glomeruli of rats on the 8.0% NaCl diet contained increased (P<0.05) amounts of p-NOS3(S1176), which was expressed as the ratio of the density of phosphorylated NOS3 and GAPDH in each sample (Figure 1A). When incubated in medium, aortic rings and isolated glomeruli from rats on the 8.0% NaCl diet produced increased amounts of NOx compared with samples from rats on the 0.3% NaCl diet. Mechanical removal of the endothelium reduced NOx production in both groups to levels that did not differ (Figure 1B). To determine whether PI3-kinase was involved in salt-induced phosphorylation of NOS3, on the day before study, rats on both diets received an intravenous bolus of LY294002 (Figure 2). Administration of LY294002 reduced p-NOS3(S1176) levels in both aorta and
glomeruli to those seen in corresponding samples obtained from rats on the 0.3% NaCl diet.

**Activation of NOS3 by Dietary Salt Occurred Through a Pyk2/c-Src/PI3-Kinase–Dependent Mechanism**

Previous studies showed that dietary salt induced the phosphorylation and activation of an endothelial Pyk2/c-Src complex. To test whether these enzymes were also involved in NO production during increased salt ingestion, in initial experiments, tissue samples from rats on both diets were incubated in medium that contained tyrphostin A9, a Pyk2 inhibitor, and PP2, a c-Src inhibitor, and NOx release into the medium was quantified. Both inhibitors decreased NOx production by aortic segments and by isolated glomeruli (Figure 3). A dominant-negative approach was then used to determine whether Pyk2 was involved directly in Akt activation. On the day before study, 1.25 nmol of Tat-AP, Tat-PBM, and Tat-GBM proteins were administered IV to groups of rats on both diets (Figure 4). Although Tat-GBM did not alter the phosphorylation state of Akt at T308 and S473 in aortic and glomerular lysates from rats on the 8.0% NaCl diet, both Tat-AP and Tat-PBM decreased (P<0.05) relative levels of p-Akt(T308) and p-Akt(S473) compared with correspond-

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

**Figure 1.** Effect of dietary salt intake on NOS3 phosphorylation at S1176, expressed as a ratio of the density of the phosphorylated NOS3 to GAPDH and NO production. A, Western analyses using lysates from aortic and isolated glomerular preparations. In vascular tissues obtained from rats on the 8.0% NaCl diet, the amount of p-NOS3(S1176) was greater (P<0.05) than levels of p-NOS3(S1176) in lyses from animals on the 0.3% NaCl diet. Each lane of the gels represented lysate obtained from a single rat (n=6 rats in each group). B, Aortic rings and isolated glomeruli from rats on the 8.0% NaCl diet released greater (P<0.05) amounts of NOx into the medium compared with tissues obtained from rats on the 0.3% NaCl diet. Mechanical removal of the endothelium reduced NOx production by aortic ring preparations to levels that did not differ between the 2 groups. *P<0.05 vs the 0.3% NaCl group.

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

**Figure 2.** Effect of intravenous administration of LY294002 on p-NOS3(S1176), expressed as a ratio of the density of the phosphorylated NOS3/GAPDH. LY294002 reduced the expression of p-NOS3(S1176) to levels that did not differ from levels of p-NOS3(S1176) in vascular tissues obtained from rats on the 0.3% NaCl diet. Each lane of the gels represented lysate obtained from a single rat (n=3 rats in each group). *P<0.05 vs the other 3 groups.
ing samples from animals that received the 8.0% NaCl diet and intravenous bolus of vehicle alone. None of the 3 Tat fusion proteins produced a significant effect on Akt phosphorylation at either amino acid residue in samples obtained from rats on the 0.3% NaCl diet. Initial coimmunoprecipitation experiments determined that the p110α isoform and not the p110β, p110γ, and p110δ isoforms served as the p85 partner in binding Pyk2 (data not shown). Intravenous administration of Tat-AP and Tat-PBM, but not Tat-GBM, resulted in diminished binding of p85 and p110α to Pyk2 in lysates from tissues obtained from rats on the 8.0% NaCl diet (Figure 5). Preadministration of the Tat fusion proteins produced no demonstrable effect on the binding of p85 and p110α to Pyk2 in lysates from tissues obtained from rats on the 0.3% NaCl diet. Together with previous studies, the combined experiments showed that Tat-AP and Tat-PBM

Figure 3. Effect of addition of tyrphostin A (Tyr) and PP2 on the release of NOx into the medium by aortic rings (top) and isolated glomeruli (bottom). The addition of Tyr and PP2 reduced (P<0.05) NOx production by both vascular tissues to levels that did not differ from production by corresponding tissues obtained from rats on the 0.3% NaCl diet. There were 6 rats in each group. *P<0.05 vs the other 3 groups.

Figure 4. Effect of intravenous administration of the Tat fusion proteins on expression of p-Akt(T308) and p-Akt(S473) relative to Akt in aortic and glomerular preparations. Total Akt levels did not differ among tissues obtained from the 8 groups of rats (n=4 rats in each group) on the 2 NaCl diets. Compared with rats that received vehicle (Veh), administration of Tat-AP and Tat-PBM, but not Tat-GBM, reduced (P<0.05) levels of p-Akt(T308) and p-Akt(S473) in lysates from aortic tissue and isolated glomeruli from rats on the 8.0% NaCl diet to levels that did not differ from the groups of rats that received the 0.3% NaCl diet. Each lane in the gel represents a single animal; 4 rats in each group were examined. *P<0.05 vs the other 6 groups.
disrupted a Pyk2/c-Src/PI3-kinase signaling complex that activated Akt. In other studies, both Tat-AP and Tat-PBM, but not Tat-GBM, disrupted the association of Pyk2 with p110α and p85 in samples obtained from rats on the 8.0% NaCl diet but did not alter binding to Pyk2 in lysates from rats on the 0.3% NaCl diet. Each lane represents data from a single rat (n=3 rats in each group).

Discussion

The current series of experiments used 2 different vascular tissues to confirm that endothelial NO production increased as dietary salt intake increased; the mechanism of this increase has now been clarified. Recent observations from this laboratory demonstrated a dose-dependent effect of salt intake on activation of endothelial Pyk2, which recruited and activated c-Src. Novel findings of the present study include the observation that dietary salt intake induced an intracellular signaling complex that not only contained Pyk2 and c-Src but also p85 and p110α; this complex was directly involved in the activation of Akt that, in turn, produced the posttranslational modification that increased NOS3 production of NO in the setting of a high-salt diet. By adding to the elegant studies of Matsui et al.,37 who showed that Pyk2 is involved in modulating NOS3 activity in angiogenesis and ischemia, the data further supported an integral role for Pyk2 in signal transduction events that regulate NOS3 function in normal and disease states. The present study is also not at variance with the recent findings of Fisslthaler et al.,38 who overexpressed Pyk2 to demonstrate that, in the setting of shear stress, Pyk2 can associate directly with NOS3 and promote the phosphorylation of Y657, which serves as an inhibitor of enzyme function and mitigates but does not prevent shear-induced augmentation of NO production.

The variety of posttranslational events that alter NOS3 activity has been reviewed.19,39 Serine phosphorylation of amino acid residue 1176 in the carboxyl terminal portion of NOS3 is a particularly important regulator of enzyme activity and sensitivity to calcium/calmodulin activation.20 Other than...
Akt, AMP kinase, protein kinase A, protein kinase G, and calcium/calmodulin–dependent protein kinase II have been implicated in the regulation of the state of phosphorylation of NOS3 at 1176.19,39 The evidence for the selective involvement of Akt in salt-mediated phosphorylation of NOS3 included the demonstration that Akt activation and NOS3 phosphorylation were prevented by the inhibition of recruitment and activation of PI3-kinase by Pyk2 after an increase in dietary salt intake, and LY294002 decreased p-NOS3(S1176) levels in animals receiving the 8.0% NaCl diet to those observed in rats on the 0.3% NaCl diet. Because the latter observation suggested that phosphorylation at 1176 was sufficient to explain the increase in NO production, other posttranslational modifications of NOS3, such as tyrosine phosphorylation of NOS3 at T83, which also increases NOS3 activity and occurs through c-Src,40 were not explored.

The data demonstrating increased phosphorylation of NOS3 at S1176 in glomeruli conflicted with the findings of Mount et al,5 who also showed that NOx production increased as dietary salt increased but did not demonstrate an increase in S1176 phosphorylation in kidney lysates. Their novel method for quantifying NOS3 initially used precipitation from whole-kidney lysates using 2′,5′-ADP Sepharose; aside from technical concerns related to this technique, their approach of using kidney lysates potentially obscured changes in regional or local expression of p-NOS3.

By permitting the intracellular delivery of our protein inhibitors without viral vectors,41,42 the use of Tat fusion proteins enabled additional testing of the hypothesis in the in vivo condition.21 The Tat fusion proteins (Tat-AP, Tat-PBM, and Tat-GBM) were designed to interfere specifically with binding of Pyk2 to c-Src, p85, and Grb2, respectively. Several laboratories independently demonstrated the efficacy of these inhibitors.21,43,44 Addition of both Tat-AP and Tat-PBM inhibited binding of p85 and p110 to Pyk2 and subsequent activation of Akt and phosphorylation of NOS3. One interpretation of these data is that binding of c-Src to Pyk2, which occurs through the SH3 domain on c-Src,45,46 was essential for binding to Pyk2 and activation of PI3-kinase. The data were consistent with the findings of Taniyama et al,28 who demonstrated that activated Pyk2 served as a scaffold to promote c-Src-dependent PI3-kinase activation. The data generated using Tat-GBM showed that there was no role for Grb2 in dietary salt-induced NO production and further permitted use of Tat-GBM as an additional control for the Tat fusion protein experiments.

**Perspectives**

In summary, the present series of experiments demonstrated that dietary salt intake directly promoted a complex endothelial cell-signaling cascade that induced Akt-mediated phosphorylation of NOS3 at S1176 and augmented NO production. It is relevant that salt-mediated vascular production of transforming growth factor-ß1 also occurs through a Pyk2-dependent process involving c-Src,21 and NO is an important regulator of vascular tone.

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**Figure 7.** Effect of intravenous administration of the Tat fusion proteins on production of NOx by aortic rings and isolated glomeruli (n=4 rats in each group). Administration of Tat-AP and Tat-PBM, but not Tat-GBM, reduced NOx production by both vascular tissues to levels that did not differ from production rates observed using vascular tissues from rats on the 0.3% NaCl diet. *P<0.05 vs the other 6 groups of rats.

**Figure 8.** Effect of intravenous administration of the Tat fusion proteins on the urinary excretion of NOx in vivo (n=4 rats in each group). Tat-AP and Tat-PBM decreased NOx/creatinine levels to those observed in the rats that received the 0.3% NaCl diet. *P<0.05 vs the remaining 6 groups; †P>0.05 vs the group that received the 0.3% NaCl diet and vehicle (Veh).
compensatory response that mitigates the effects of transforming growth factor-β.\textsuperscript{31} Pyk2, therefore, becomes a key signaling molecule in the vascular response to dietary salt intake. Although in the present study vascular reactivity was not examined, Atochin et al\textsuperscript{47} demonstrated the in vivo relevance of this posttranslational modification by showing that transgenic mice expressing NOS3 possessing a S1179D (phosphomimetic) mutation had increased vascular reactivity when compared with transgenic mice expressing NOS3 possessing an S1179A mutation. Although these findings suggest physiological benefit, in conditions that induce oxidative stress in the vessel wall, NOS3 can produce superoxide rather than NO, and phosphorylation of human NOS3 at S1177 is also pivotal in the regulation of superoxide production.\textsuperscript{8} Although the present findings were limited to rats, elucidating the mechanism of dietary salt-induced alterations in NOS3 function permits improved understanding of the nature of the interaction between the endothelium and dietary salt intake in physiological and pathological states that alter NO production, such as diabetes mellitus, hypertension, and aging.

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Disclosures
None.

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