Transforming Growth Factor-β Regulates Endothelial Function During High Salt Intake in Rats

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

Abstract—Previous studies have demonstrated that an increase in dietary NaCl (salt) intake stimulated endothelial cells to produce transforming growth factor-β (TGF-β). The intent of the present study was to determine the functional significance of increased TGF-β on endothelial cell function. Young Sprague-Dawley rats were fed diets containing 0.3 or 8.0% NaCl for 2 days before treatment with a specific inhibitor of the TGF-β receptor I/activin receptor-like kinase 5 kinase, or vehicle for another 2 days. At day 4 of study, endothelial phosphorylated Smad2 (S465/467) increased and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) levels decreased in the high-salt–treated rats. In addition, phosphorylated Akt (S473) and phosphorylation of the endothelial isoform of NO synthase (NOS3) at S1177 increased. Treatment with the TGF-β receptor I/activin receptor-like kinase 5 inhibitor reduced Smad2 phosphorylation to levels observed in rats on the low-salt diet and prevented the downstream signaling events induced by the high-salt diet. In human umbilical vein endothelial cells, reduction in PTEN levels increased phosphorylated Akt and NOS3. Treatment of macrovascular endothelial cells with TGF-β1 increased phosphorylated NOS3 and the concentration of NO metabolites in the medium but had no effect on either of these variables in cells pretreated with small interfering RNA directed against PTEN. Thus, during high salt intake, an increase in TGF-β directly promoted a reduction in endothelial PTEN levels, which in turn regulated Akt activation and NOS3 phosphorylation. This effect closes a feedback loop that potentially mitigates the effect of TGF-β on the vasculature. (Hypertension. 2013;62:951-956.)

Key Words: Akt ■ dietary sodium ■ endothelium ■ nitric oxide synthase type III ■ PTEN protein

The endothelium serves as an integral component of arterial function through maintenance of vascular tone and smooth muscle function. Regulation of endothelial function is complex and may be modified by environmental stress. Findings from several studies developed an evolving paradigm that suggests the vasoactive function of endothelium is modified by dietary NaCl (termed salt in this article) content. These prior studies demonstrated that an increase in dietary salt intake promotes the endothelial cell production of active transforming growth factor-β (TGF-β). The mechanism of endothelial cell activation by salt intake required a signaling cascade involving the large-conductance Ca2+-activated potassium (BKCa) channel, phosphatidylinositol 3-kinase (PI3K), and protein kinase B (also known as Akt).4,7,8

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a lipid phosphatase that directly antagonizes the 3-kinase activity of the class I PI3K family. Levels of PTEN, therefore, directly participate in the regulation of cellular levels of phosphatidylinositol 3,4,5-triphosphate (PIP3). Among other effects, increased PIP3 levels promote Akt activation.13 One target of activated Akt is S1177 on the endothelial isoform of NO synthase (NOS3).14 This important post-translational phosphorylation event provides more prolonged NO release by NOS3 at baseline and after stimulation.15–16 Because this regulatory mechanism is especially significant in the setting of changes in dietary salt intake,7 endothelial PTEN levels might directly regulate NO production through control of Akt activation. The hypothesis tested in this article is that dietary salt–induced production of TGF-β promoted alterations in endothelial cell function that facilitated NO production, and the primary control mechanism of this process involved PTEN and the TGF-β receptor I/activin receptor-like kinase 5 and the Smad signaling pathway.17

Materials and Methods

Animal and Tissue Preparation

This study was performed in accordance with the recommendations in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved the project. Studies were conducted using 24 one-month-old male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN). The rats were housed under standard conditions and given 0.3% NaCl.
diet (AIN-76A with 0.3% NaCl; Dyets, Inc., Bethlehem, PA) and water ad libitum for 4 days before receiving diet containing either 0.3% NaCl or 8.0% NaCl (AIN-76A with 8.0% NaCl; Dyets, Inc., Bethlehem, PA) for 4 days. Two days before the end of the experiment, vehicle or 6-2-tetra-Butyl-5-(6-methyl-pyridin-2-yl)-1H-imidazol-4-yl)-quinoxaline (SB525334, Selleck Chemicals, Houston, TX), which is a specific inhibitor of the kinase activity of TGF-β receptor I/activin receptor-like kinase 5 (TIRI/ALK5) and has been shown to prevent renal fibrosis in vivo in pyrocinin-induced nephritis, was added to the drinking water to achieve a dose of 10 mg/kg per day. Four groups of rats were studied. Group 1 rats received the 0.3% NaCl diet and vehicle; group 2 rats were fed the 0.3% NaCl diet and given SB525334. Group 3 rats received 8.0% NaCl and vehicle, whereas group 4 rats received 8.0% NaCl and SB525334. On the final day of study, rats were anesthetized with 2% isoflurane. Aortae were harvested under sterile conditions, and aortic endothelial cell lysates were obtained as described previously. To validate further the experimental approach used to isolate aortic endothelial cells, endothelial lysates from 12 animals were probed for NOS3, SM22α (transgelin), which is a protein abundantly expressed in smooth muscle cells, and GAPDH using Western blot analyses (Figure 1).

**Silencing Endothelial PTEN and In Vitro Incubation Studies**

RNA interference was accomplished using SignalSilence PTEN small interfering RNA (siRNA) II (No. 6538, Cell Signaling Technology, Danvers, MA); nontargeting siRNA No. 1 (D-001810, Dharmacon RNA interference was accomplished using SignalSilence PTEN small interfering RNA (siRNA) II (No. 6538, Cell Signaling Technology, Danvers, MA); nontargeting siRNA No. 1 (D-001810, Dharmacon RNA Technologies, Lafayette, CO) served as a control in these experiments. HUVECs at 80% confluence were transfected using siRNA transfection reagent (DharmaFECT4, Thermo Fisher Scientific, Waltham, MA) containing the siRNA. Preliminary experiments using siTOX transfection control (Thermo Fisher Scientific) determined the optimum exposure conditions that maximized transfection efficiency and minimized toxicity. PTEN siRNA (100 nmol/L) was complexed with 2 μL of DharmaFECT4 in 200-μL total volume and then added to complete medium in a final volume of 1 mL for each well in a 12-well plate. After incubation in the transfection solution for 12 hours, the medium was replaced and incubation continued up to 72 hours. In some experiments, cells were then incubated in medium that contained vehicle alone or human recombinant TGF-β1 (R&D Systems, Minneapolis, MN). 1 μmol/L, for an additional 16 hours.

After incubation, 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 NO metabolites (NOx). Cell lysates were obtained for analysis of PTEN, Akt, NOS3, GAPDH, and total protein concentration.

**NO Metabolites**

In samples of the conditioned medium, NOx was assayed using the optimized VCI, reagent-based kit (QuantiChrom Nitric Oxide Assay Kit; D2NO-100, BioAssay Systems, Hayward, CA), which determines nitrite concentrations using Griess methodology after reduction of nitrate to nitrite. The time required for the reduction is 10 minutes at 60°C. In these studies, cell culture media samples were deproteinated, and assays were performed in duplicate and averaged.

**Western Blot Analyses**

Cell lysates were produced using modified radioimmunoprecipitation assay buffer that contained 10 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 0.1% SDS, 20 mmol/L sodium pyrophosphate, 2 mmol/L Na3VO4, 1 mmol/L NaF, 1 mmol/L PMSF; and a protease inhibitor cocktail (Complete, EDTA-free, Roche Applied Science, Indianapolis, IN). Total protein concentration was determined using a kit (BCA Protein Assay Reagent Kit; Thermo Fisher Scientific Pierce Protein Research Products, Rockford, IL), and the samples were processed for Western blotting. Protein extracts (10–60 μg) were boiled for 3 minutes in Laemmli buffer and separated by 7% to 12% SDS-PAGE (Bio-Rad Laboratories, Hercules, CA) before electrophoretic transfer onto polyvinylidene difluoride membranes. The membranes were blocked in 5% nonfat milk and then probed with an antibody (diluted 1:1000) that recognized specifically NOS3 (BD Biosciences, BD Transduction Laboratories, San Jose, CA), PTEN, phospho-NOS3 (S1177), phospho-Akt (S473), total Akt (all from Cell Signaling Technology), SM22α (Novus Biologicals, Littleton, CO), and GAPDH (Abcam, Inc., Cambridge, MA), which served as the loading control. The published cDNA sequence of rat NOS3 (accession No. NM_021838) showed that the serine residue that corresponded to S1177 in the human NOS3 sequence was S1176. In this article, phosphorylation of this serine residue was referred to as phospho-NOS3 (S1177). The membranes were developed in standard fashion (SuperSignal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific Pierce Protein Research Products); density of the bands was quantitated using Quantity One software (Bio-Rad Laboratories).

**Statistical Analyses**

Data are expressed as the mean±SEM. Significant differences were determined by ANOVA with post hoc testing. A P value <0.05 assigned statistical significance.

**Results**

An increase in dietary salt intake promoted activation of the endothelial Smad signaling pathway, which decreased PTEN levels. Four groups of rats were studied. The first 2 groups were fed the 0.3% NaCl diet and given either vehicle or SB525334, which inhibits TGF-β receptor I/activin receptor-like kinase 5,2 days before the end of the experiment. The second 2 groups received the 8.0% NaCl diet and either vehicle or SB525334 2 days before the end of the experiment. An increase in dietary...
salt intake increased p-Smad2 (S465/467) in endothelial lysates; this increase was inhibited by treatment with SB525334 (Figure 2). Total Smad2/3 levels did not change. Increased salt intake also decreased endothelial PTEN levels and increased p-Akt (S473) without changing total Akt levels; treatment with SB525334 prevented these salt-induced changes in PTEN and p-Akt (S473). The dietary salt–induced relative increase in p-NOS3 (S1177), which increases NO production,14–16 was abrogated by treatment with SB525334 (Figure 2). As shown previously,1,5,6 a high-salt diet increased NOS3 protein levels in young rats. In the present study, compared with rats on the 0.3% NaCl diet, the 8.0% NaCl diet increased relative NOS3 (NOS3/GAPDH) levels (0.11±0.004 versus 0.27±0.02; P<0.001). Addition of SB525334 did not inhibit NOS3 protein expression during high salt intake (0.28±0.01) and did not change NOS3 protein expression during low salt intake (0.14±0.01).

Endothelial PTEN levels determined Akt activity and NOS3 phosphorylation in vitro. PTEN levels fell in HUVECs treated with siRNA directed against PTEN. A concomitant increase in p-Akt (S473) without a change in total Akt levels was observed. In addition, p-NOS3 (S1177) increased without a change in NOS3 when endothelial PTEN levels were reduced (Figure 3).

The effect of TGF-β on p-NOS3 and NO production was dependent on PTEN. After treatment with siRNA directed against PTEN, cells were incubated with 12 pmoL/L of active TGF-β1 to determine whether TGF-β regulated p-NOS3 levels independent of PTEN. Addition of TGF-β1 promoted an increase in p-NOS3 (S1177) in the vehicle-treated cells, whereas TGF-β1 produced no additional effect on p-NOS3 (S1177) in HUVECs lacking PTEN from treatment with siRNA (Figure 4). NOx in the medium increased with TGF-β1 treatment, but no changes in NOx levels were observed after TGF-β1 treatment in the siRNA-pretreated cells (Figure 5).

**Discussion**

Work performed over the past 15 years has shown that the endothelium acts as a dietary salt sensor that responds to changes in salt intake. This effect is mediated through endothelial BKCa channel activity, which regulates not only the development of a signalosome complex composed of Pyk2, c-Src, and PI3K but also promotes a decrease in endothelial PTEN levels.1,3–8,21–24 PTEN is a phosphatase that counteracts the production of PIP3 by PI3K. Decreasing PTEN levels, therefore, facilitates the activity of PI3K, which regulates Akt activation through the generation of intracellular PIP3.9–12 One net effect of increased dietary salt intake is augmented endothelial production of TGF-β and potentially bioavailable NO.3 Building on these findings, the data in the present study demonstrated that (1) dietary salt induced endothelial cell production of TGF-β, which promoted an autocrine function on endothelial cells mediated through TGF-β receptor I/activin receptor-like kinase 5 (TβRI/ALK5) kinase inhibitor, SB525334, prevented the salt-induced increases in Smad2 phosphorylation and the downstream signaling events that included the reduction in PTEN levels and increases in phosphorylated Akt (p-Akt) and phosphorylated NOS3 (p-NOS3; S1177). The graphs on the right represent the pertinent relative protein levels of all the animals in the study (n=6 rats in each group). *P<0.05 compared with the other 3 groups.

Figure 2. Increased dietary salt intake activates the Smad2 signaling pathway to promote a decrease in endothelial phosphatase and tensin homologue deleted on chromosome 10 (PTEN) levels, phosphorylation of Akt at S473, and phosphorylation of NOS3 at S1177. The transforming growth factor-β (TGF-β) receptor I/activin receptor-like kinase 5 (TβRI/ALK5) kinase inhibitor, SB525334, prevented the salt-induced increases in Smad2 phosphorylation and the downstream signaling events that included the reduction in PTEN levels and increases in phosphorylated Akt (p-Akt; S473) and phosphorylated NOS3 (p-NOS3; S1177). The graphs on the right represent the pertinent relative protein levels of all the animals in the study (n=6 rats in each group). *P<0.05 compared with the other 3 groups.
The PI3K/PTEN/Akt/NOS3 pathway is gaining increased attention in endothelial cell biology. Findings from the present study complemented prior studies that demonstrated the critical inter-relationship between enzymatic activities of both PI3K and PTEN in the regulation of intracellular PIP3 levels.9–12 PIP3 activates pleckstrin-homology domain-containing enzymes that include Akt13; in turn, activated Akt facilitates NOS3 phosphorylation at S1177 and thereby increases NO production.26 NO production through Akt activation has been shown to increase during high dietary salt intake in young animals,7 and this pathway is also directly involved in the regulation of vascular permeability27 and vasodilation induced by vascular endothelial growth factor.28 Several pharmaceutical agents also interact with this pathway. Statins activate endothelial Akt, which results in phosphorylation of NOS3,29,30 Nitroglycerin seems to increase endothelial NO production by directly inhibiting PTEN, promoting Akt activation and NOS3 phosphorylation.31 Hyperglycemia directly inhibits this pathway via reactive oxygen species–mediated dephosphorylation of Akt12 and direct O-linked N-acetylglucosamine modification of NOS3 at S1177.33 Thus, the PI3K/PTEN/Akt/NOS3 pathway is a critical regulator of endothelial NO production and may be altered by changes in the extracellular environment, such as increased salt intake, cardiovascular drugs, and disease states that include diabetes mellitus.

Other factors participate in the endothelial cell response to changes in dietary salt intake. Aging promotes a reduction in NO produced by NOS3 in response to increased salt intake. The effect of aging seems to be related to endothelial cell PTEN levels, which increase with age and become refractory to changes in dietary salt intake in rats.6 An age-related increase in PTEN levels in cultured human microvascular endothelial cells has also been demonstrated.34 Shear-induced generation of NO by NOS3 seems to be optimized by the concomitant
expression of GTP cyclohydrolase I, the rate-limiting enzyme in tetrahydrobiopterin biosynthesis.\textsuperscript{35} Generation of oxidative stress in the endothelium independently mediates reduction in bioavailable NO and enhances peroxynitrite formation particularly in advanced age.\textsuperscript{36–39}

**Perspectives**

Vascular pathology (excluding atherosclerosis) includes remodeling not only of resistance vessels but also compliance vessels. The disease burden—myocardial infarction, stroke, left ventricular hypertrophy, heart failure, kidney failure, and death—associated with decreased arterial compliance is very high.\textsuperscript{40–45} As an independent predictor of cardiovascular morbidity and mortality, reductions in conduit artery compliance represent an important health problem for the nation as a whole. The fibrogenic growth factor, TGF-β, seems to be involved in this process.\textsuperscript{46} The present study uncovered a direct effect of dietary salt–induced production of TGF-β on macrovascular endothelial cell function. This effect, mediated through the Smad signaling pathway, reduced intracellular PTEN and thereby facilitated Akt activation, which was be involved in this process.\textsuperscript{47} The present study uncovered a potential inhibitor of the effects of TGF-β on vascular function.\textsuperscript{1} Although presently untested, this inhibitory feedback system may be critically important in determining the changes in arterial compliance related to excess salt intake.\textsuperscript{48–49}

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**Disclosures**

None.

**References**

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Novelty and Significance

What Is New?

Novel findings demonstrated in the present study include the following:

- An increase in dietary salt stimulated endothelial cell production of transforming growth factor-β (TGF-β), an important growth factor that is now shown to have a direct action on endothelial cells mediated through the classical signaling pathway activated by TGF-β;

- TGF-β directly regulated an important phosphatase (phosphatase and tensin homologue deleted on chromosome 10 [PTEN]) in vascular endothelium; and

- The inhibitory effect of TGF-β on intracellular levels of PTEN promoted endothelial cell production of NO by increasing the phosphorylation of the endothelial isoform of NO synthase.

What Is Relevant?

- Work from our laboratory has demonstrated that the endothelium acts as a salt sensor that responds to changes in dietary salt intake. One net effect of increased dietary salt intake is augmented endothelial production of TGF-β and potentially bioavailable NO. Building on these findings, the data in the present study demonstrated that by directly promoting a decrease in endothelial PTEN levels, active TGF-β produced during high salt intake can facilitate the generation of endothelium-derived NO, which in turn has been shown to mitigate the production of TGF-β in the vasculature as well as promote other endothelial cell functions that are dependent on Akt. This newly identified autocrine action of salt-induced TGF-β is therefore important in arterial function.
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