Transforming growth factor-β mediates endothelial dysfunction in rats during high salt intake

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1Division of Nephrology, Department of Medicine, Nephrology Research and Training Center, University of Alabama at Birmingham, Birmingham, Alabama; 2Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, Alabama; and 3Department of Veterans Affairs Medical Center, Birmingham, Alabama

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Feng W, Ying W-Z, Aaron KJ, Sanders PW. Transforming growth factor-β mediates endothelial dysfunction in rats during high salt intake. Am J Physiol Renal Physiol 309: F1018–F1025, 2015. First published October 7, 2015; doi:10.1152/ajprenal.00328.2015.—Endothelial dysfunction has been shown to be predictive of subsequent cardiovascular events and death. Through a mechanism that is incompletely understood, increased dietary salt intake promotes endothelial dysfunction in healthy, salt-resistant humans. The present study tested the hypothesis that dietary salt-induced transforming growth factor (TGF)-β promoted endothelial dysfunction and salt-dependent changes in blood pressure (BP). Sprague-Dawley rats that received diets containing 0.3% NaCl (low salt [LS]) or 8.0% NaCl (high salt [HS]) were treated with vehicle or SB-525334, a specific inhibitor of TGF-β receptor I/activin receptor-like kinase 5, beginning on day 5. BP was monitored using radiotelemetry in four groups of rats (LS, LS + SB-525334, HS, and HS + SB-525334) for up to 14 days. By day 14 of the study, mean daytime systolic BP and mean pulse pressure of the HS group treated with vehicle was greater than those in the other three groups; mean daytime systolic BP and pulse pressure of the HS + SB-525334 group did not differ from the LS and LS + SB-525334-treated groups. Whereas mean systolic BP, mean diastolic BP, and mean arterial pressure did not differ among the groups on the seventh day of the study, endothelium-dependent vasorelaxation was impaired specifically in the HS group; treatment with the activin receptor-like kinase 5 inhibitor prevented the dietary HS intake-induced increases in phospho-Smad2 (Ser465/467) and NADPH oxidase-4 in endothelial lysates and normalized endothelial function. These findings suggest that HS-induced endothelial dysfunction and the development of salt-dependent increases in BP were related to endothelial TGF-β signaling.

dietary salt; endothelial dysfunction; endothelium; hypertension; transforming growth factor-β

DIETARY NaCl (termed “salt” in the present article) content directly impacts endothelial cell function, regulating, for example, the production of nitric oxide (NO) in rodents (4, 6, 26) and humans (1). Increased salt intake induces the formation of an endothelial cell signaling complex that contains proline-rich tyrosine kinase 2, c-Src, and phosphatidylinositol 3-kinase (PI3K). Phosphatidylinositol 3-kinase is an upstream activator of PKB (Akt), which phosphorylates the endothelial isoform of NO synthase (NOS3) to increase NO production. This signaling cascade also promotes the endothelial cell production of transforming growth factor (TGF)-β, which has recently been shown to serve an autocrine role in endothelial cell biology during high salt (HS) intake (35). Additional studies have demonstrated a functional interaction between TGF-β and NO (33, 35, 38). However, TGF-β also promotes vascular NO bioavailability and regulates vascular responses to dietary salt intake.

Endothelial dysfunction (ED), defined as a reduction in arterial endothelium-dependent vasodilation, has been shown to be predictive of subsequent cardiovascular events and death (3, 21, 32). While a number of underlying pathogenetic processes may produce ED, attention has focused on the potential role that a diet high in salt content may play in this process. Recent studies have clearly demonstrated that 7 days on a HS diet impaired forearm endothelium-dependent vasodilation independently of blood pressure (BP) and serum Na+ concentration; however, endothelium-independent vasodilation did not change (7, 10). These findings highlight the need to define the underlying molecular mechanisms by which dietary salt intake affects endothelial cell biology. The hypothesis tested in the present study was that dietary salt-induced TGF-β directly promotes ED and increases salt-dependent effects on BP.

MATERIALS AND METHODS

Animal and tissue preparation. The present study was performed in accordance with recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of the University of Alabama at Birmingham approved the project. Experiments were conducted using 1.5-mo-old male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN).

Rats were housed under standard conditions and given a 0.3% NaCl diet [low-salt (LS) diet, AIN-76A with 0.3% NaCl, Dyets, Bethlehem, PA] and water ad libitum for 4 days before initiation of the study. Rats received diets containing either 0.3% NaCl or 8.0% NaCl (HS diet; AIN-76A with 8.0% NaCl, Dyets). At day 5 of the experiment, vehicle or 6-[2-tert-butyl-5-[(6-methylpyridin-2-yl)-1H-imidazol-4-yl]-quinoxaline (SB-525334, Selleck Chemicals, Houston, TX), which is a specific inhibitor of the kinase activity of TGF-β receptor I/activin receptor-like kinase (ALK5), was added to the drinking water to achieve a dose of 10 mg·kg⁻¹·day⁻¹. SB-525334 prevented renal fibrosis in vivo and ameliorated ischemia-reperfusion injury (11, 17, 20) and has previously been used at this dose to demonstrate a functional role for TGF-β signaling in endothelial function (36). Four groups of rats were studied. The first group of rats received the 0.3% NaCl diet and vehicle (LS group), the second group of rats received the 0.3% NaCl diet and SB-525334 (LS + SB-525334), the third group of rats received 8.0% NaCl and vehicle (HS group), and the fourth group of rats received 8.0% NaCl and SB-525334 (HS + SB-525334 group). On the seventh day of the study, rats were anesthetized with 2% isoflurane. Aortae were harvested under sterile conditions, and aortic endothelial cell lysates were obtained as previ-
GAPDH (1:10,000 dilution, Abcam, Cambridge, MA). Density of the bands was quantified using an Odyssey CLx Imager system and Image Studio 4 software (Li-COR Biotechnology, Lincoln, NE).

Statistical analyses. Data are expressed as means ± SE. To analyze BP responses to dietary salt intake (LS or HS) and ALK5 inhibition (treatment with vehicle or SB-525334), a repeated-measures analysis using linear mixed models was used (Proc Glimmix). Holm–Tukey’s adjustment for multiple comparisons of the group by timepoint simple effects was used in post hoc analyses. For the remaining data, two-way ANOVA was performed using dietary salt intake (LS or HS) and ALK5 inhibition (treatment with vehicle or SB-525334) as independent variables. If the difference in mean values among the different levels of dietary salt intake was greater than what would be expected by chance, all pairwise multiple-comparison post hoc testing (Tukey’s honestly significant difference test) was performed (Proc GLM). SAS 9.4 (Cary, NC) was used for the statistical analyses, and P values of ≤0.05 were assigned statistical significance.

RESULTS

Increased dietary salt intake elevated SBP by day 14 of the study but was prevented by ALK5 inhibition. Mean body weights and mean concentrations of serum electrolytes (Na⁺, K⁺, and Cl⁻), which were determined on day 14 in a subset (n = 3 animals/group) of the total animals in the study, did not differ among the four groups of rats (Table 1). BP was monitored using radiotelemetry in the four groups of rats (LS, LS + SB-525334, HS, and HS + SB-525334) for 14 days. Differences in BP responses among the groups under study emerged over the experimental time period (Fig. 1). Compared with BP parameters of rats in the HS group at day 14, rats in the LS group had a lower mean daytime SBP (125.7 ± 1.2 vs. 133.6 ± 1.2 mmHg, P = 0.0038), daytime mean arterial pressure (MAP; 103.8 ± 1 vs. 109.6 ± 1.3 mmHg, P = 0.023), daytime pulse pressure (PP; 41.1 ± 1.2 vs. 44.8 ± 0.7 mmHg, P = 0.0543), and nighttime PP (40.2 ± 1.3 vs. 44.6 ± 0.9 mmHg, P = 0.0364) and higher nighttime average heart rates (49.1 ± 6.5 vs. 404 ± 5.7 beats/min, P = 0.0023).

By day 14 of the experimental time period, compared with BP parameters in rats in the HS group, rats in the HS + SB-525334 group had lower mean daytime SBP (127.7 ± 1.2 vs. 133.6 ± 1.2 mmHg, P = 0.0477) and did not differ from rats in the LS + SB-525334 group (127.7 ± 1.2 vs. 124.1 ± 2.4 mmHg, P = 0.4049) or rats in the LS group (127.7 ± 1.2 vs. 125.7 ± 1.2 mmHg, P = 0.7978). Rats in the HS + SB-525334 group also had lower mean daytime MAP (106.9 ± 1.1 vs. 109.6 ± 1.3 mmHg, P = 0.0288), mean daytime PP (37.8 ± 0.8 vs. 44.8 ± 0.7 mmHg, P = 0.0003), and nighttime PP (38.3 ± 0.7 vs. 44.6 ± 0.9 mmHg, P = 0.003) compared with rats in the HS group. These parameters did not differ with those observed in rats in the LS group or rats in the LS + SB-525334 group.

Table 1. Mean body weight and serum electrolytes of each of the four groups on day 7 of the experiment

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>Serum Na⁺ Concentration, mmol/l</th>
<th>Serum K⁺ Concentration, mmol/l</th>
<th>Serum Cl⁻ Concentration, mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS group</td>
<td>225.0 ± 15.4</td>
<td>146.4 ± 1.8</td>
<td>5.1 ± 0.2</td>
<td>106.1 ± 1.3</td>
</tr>
<tr>
<td>LS + SB-525334</td>
<td>237.0 ± 10.9</td>
<td>143.8 ± 0.1</td>
<td>5.4 ± 0.3</td>
<td>105.1 ± 0.5</td>
</tr>
<tr>
<td>HS group</td>
<td>215.0 ± 15.5</td>
<td>144.8 ± 1.0</td>
<td>4.9 ± 0.2</td>
<td>103.5 ± 1.1</td>
</tr>
<tr>
<td>HS + SB-525334</td>
<td>222.8 ± 11.0</td>
<td>144.7 ± 1.4</td>
<td>5.1 ± 0.2</td>
<td>104.0 ± 0.8</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 9 rats/group. LS, low-salt diet; HS, high-salt diet.
Representative tracings of SBP and DBP throughout 24 h of four rats in the LS, LS/SB-525334, HS, and HS/SB-525334 groups are shown in Fig. 2. BP increased during the nighttime period in each animal in every group and was consistent with an intact circadian rhythm of a nocturnal animal. At day 14, the differences between daytime and nighttime BP, termed ΔSBP (LS group: 6.2 ± 0.7 mmHg, LS + SB-525334 group: 6.2 ± 0.9 mmHg, HS group: 6.4 ± 1.6 mmHg, and HS + SB-525334 group: 5.4 ± 1.0 mmHg) and ΔDBP (LS group: 7.2 ± 0.5 mmHg, LS + SB-525334 group: 6.1 ± 0.4 mmHg, HS group: 6.7 ± 1.6 mmHg, and HS + SB-525334 group: 5.7 ± 2.1 mmHg), did not differ among the groups, indicating that the circadian rhythm was not affected by dietary salt intake or by treatment with the ALK5 inhibitor. In addition, the mean times at which the animals manifested nighttime peak SBP and peak DBP, daytime trough SBP and trough DBP did not differ among the four groups at day 14 of the study (data not shown).
Increased dietary salt intake impaired endothelium-dependent relaxation but was prevented by ALK5 inhibition. Because mean SBP, DBP, and MAP did not differ among the four groups at day 7, tests of endothelial function were performed at that time. Endothelium-dependent vasorelaxation was impaired specifically in the HS group; treatment with SB-525334 normalized this effect (Fig. 3). L-NAME pretreatment completely abolished the ACh-induced relaxation among the four groups of rats. Endothelium-independent vasorelaxation, as assessed by the addition of sodium nitroprusside, did not differ among the four groups. In addition, the contractile responses of the aortae to Phe did not differ among the four groups of rats (Fig. 3).

Increased dietary salt intake increased endothelial phospho-Smad2 (Ser465/467) and NOX4. Aortic endothelial cell lysates obtained from the four groups of rats on day 7 of the experiment demonstrated activation of ALK5 by HS intake as well as the anticipated inhibition of ALK5 by SB-525334 (Fig. 4A). As a consequence, an increase in dietary salt intake increased phospho-Smad2 (Ser465/467) in endothelial lysates; this increase was also inhibited by treatment with SB-525334 (Fig. 4B). Total Smad2/3 levels did not change.

The findings further showed a stimulatory effect of dietary salt intake and an inhibitory effect of treatment with SB-525334 on endothelial NOX4 levels, indicating the involvement of ALK5 in the salt-induced increase in NOX4 (Fig. 4C).

**DISCUSSION**

A previous study (37) has shown that dietary salt modulated the endothelial production of TGF-β and NO through a common signaling pathway, but these mediators may produce countervailing influences on vascular function. To define the dynamic functional significance of these molecular changes during HS diet intake, the present study demonstrated that 14 days on a HS diet increased SBP and PP. Importantly, the present study clearly demonstrated that treatment with the ALK5 inhibitor prevented this elevation in SBP and PP. Furthermore, ALK5 inhibition also prevented salt-induced phosphorylation of Smad2 and increases in NOX4 and reversed the ED observed before the development of the increase in BP. The observation that dietary salt intake promoted ED in the absence of changes in BP was consistent with prior findings...
reported by Durand et al. (8) and Raffai et al. (24) and with recent reports (7, 10) in which high dietary Na\(^+\) intake impaired endothelial dysfunction in Sprague-Dawley rats. Rats were fed with a LS or HS diet for 7 days and received either Veh or the ALK5 inhibitor SB beginning on day 5. Vascular responses were tested using aortae from the four groups of rats. A, left: contractile responses of aortic ring preparations to phenylephrine (Phe) did not differ among the four groups. Right, pEC\(_{50}\) values derived from the cumulative concentration-response curves did not differ among the four groups of rats in the study. B: cumulative concentration-response curves (left) were generated using ACh (0.001–10 \(\mu\)M) and used to calculate pEC\(_{50}\) values (right). HS intake significantly impaired endothelium-dependent relaxation, and ALK5 inhibition normalized this effect. Data are presented as means ± SE; \(n = 9\) rats/group. *\(P < 0.05\) vs. the HS SB group. C, left: pretreatment of ring preparations with \(N^\circ\)-nitro-L-arginine methyl ester (L-NAME) completely abolished ACh-dependent vasorelaxation. Right, responses of aortic ring preparations to sodium nitroprusside (SNP) did not differ among the four groups of rats.

Fig. 3. HS intake impaired endothelium-dependent relaxation and transforming growth factor (TGF)-\(\beta\) receptor inhibition normalizes HS-induced endothelial dysfunction in Sprague-Dawley rats. Rats were fed with a LS or HS diet for 7 days and received either Veh or the ALK5 inhibitor SB beginning on \(\textit{day 5}\). Vascular responses were tested using aortae from the four groups of rats. A, left: contractile responses of aortic ring preparations to phenylephrine (Phe) did not differ among the four groups. Right, pEC\(_{50}\) values derived from the cumulative concentration-response curves did not differ among the four groups of rats in the study. B: cumulative concentration-response curves (left) were generated using ACh (0.001–10 \(\mu\)M) and used to calculate pEC\(_{50}\) values (right). HS intake significantly impaired endothelium-dependent relaxation, and ALK5 inhibition normalized this effect. Data are presented as means ± SE; \(n = 9\) rats/group. *\(P < 0.05\) vs. the HS SB group. C, left: pretreatment of ring preparations with \(N^\circ\)-nitro-L-arginine methyl ester (L-NAME) completely abolished ACh-dependent vasorelaxation. Right, responses of aortic ring preparations to sodium nitroprusside (SNP) did not differ among the four groups of rats.

An increase in ROS in response to HS intake has been shown to play an important role in reducing endothelium-dependent dilation in humans (10), mice (23), and rats (24). Using a model similar to that used in the present study, previous studies have demonstrated that a HS diet promoted ED in middle cerebral arteries (8) and mesenteric arteries.
These studies further showed an increase in vascular ROS (24). Stimulation of the G protein-coupled receptor, Mas, by infusion of either ANG-(1–7) or AVE-0991, corrected salt-induced ED (8, 24). While these studies did not demonstrate the cellular mechanism of improvement in ED, Sampaio et al. (25) showed that Mas receptor activation increased endothelial NO production by activating NOS3 through posttranslational modifications (serine and threonine phosphorylation).

The present study also found that HS intake promoted an ALK5-dependent increase in endothelial NOX4. These data are consistent with Thannickal et al. (30), who found that TGF-β1 increased production of H₂O₂ and reduced cellular glutathione stores in endothelial cells. These investigators further demonstrated that the TGF-β1-induced increases in H₂O₂ were dependent on activation of ALK5 and the Smad2/3 pathway, which increased the activity of NOX4 (13). Additionally, the findings were consistent with Yan et al. (31), who also showed a TGF-β-mediated induction of NOX4 in the endothelium, and Boulden et al. (2), who demonstrated that H₂O₂, which is the principal product of constitutively active NOX4 (22), promoted ED by decreasing tetrahydrobiopterin and bioavailable NO. By demonstrating that ED developed during HS intake through the activation of ALK5 and the Smad2 pathway, our results suggested the pathophysiological significance of this TGF-β-dependent mechanism in the alteration of endothelial function.

Previous studies have suggested a role for TGF-β in the development of hypertension. For example, genetic disruption of elastin microfibril interfacer 1, which is involved in the vascular regulation of TGF-β, promoted the development of arterial hypertension by increasing the activity of TGF-β (39). Terrell and colleagues (29) administered recombinant human TGF-β2 at varying doses (20, 100, and 400 g/kg) chronically to rats and rabbits. They observed that after 9 wk, TGF-β2 caused modest increases in SBP; however, renal hemodynamic end points were not significantly altered. Proteinuria and minimal tubular interstitial fibrosis developed after 6 wk of dosing at the high dose (400 g/kg) (16).

Using dietary salt intake as the stimulus for the vascular production of TGF-β, radiotelemetry monitoring of BP identified subtle but significant increases in SBP after 2 wk. Because the increase in BP was abrogated by the addition of the ALK5 inhibitor, the present findings are consistent with a role for this growth factor in the development of salt-dependent increases in BP.

Recent Framingham data have shown that large artery ED antedated the development of hypertension (15). This central pathophysiological link has been implicated in the initiation of increased arterial stiffness, hypertension, ath-
erosclerosis, chronic kidney disease, restenosis after percutaneous coronary intervention, and stenosis of venous bypasses of coronary arteries (5, 9, 12, 18, 27, 28). Dietary salt intake promotes a dose-dependent decrease in survival in rats, related to the acceleration of cardiovascular and renal disease as salt intake exceeds 2% (19). The present study unveils the role of TGF-β in this complex interaction of the endothelium with dietary salt intake and improves understanding of the mechanism of ED in the development of hypertension.

In summary, the findings demonstrated the dynamic function of the endothelium and subsequent changes in BP that occurred during HS intake. Our results showed that salt-induced ED preceded changes in BP and further identified TGF-β as the upstream mechanism of salt-induced ED. While the cellular events that modulate bioavailable NO are complicated, the present study supports the involvement of endothelial ALK5 activation and Smad2 signaling in salt-induced ED.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES
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