Increased Dietary Salt Activates Rat Aortic Endothelium

Wei-Zhong Ying, Paul W. Sanders

Abstract—The function of vascular endothelium as a biomechanical sensor permits alterations in gene expression in the vascular tree in response to wall stress. The present study explored the mechanism by which the arterial endothelium responds to changes in dietary salt. Normotensive rats were fed diets containing varying amounts of NaCl for 4 days. At that time, levels of phosphorylated p38 MAP kinase, p42/44 MAP kinase, and p46/54 JNK/SAPK kinase increased when the diet contained ≥3.0% NaCl. Kinase assays demonstrated dose-response relationships between dietary salt intake and the activities of p38 MAP kinase and p42/44 MAP kinase. Aortic segments from animals on the 8.0% NaCl diet produced greater amounts of total and active transforming growth factor-beta 1 (TGF-β1) and nitric oxide. The MEK1 inhibitor, PD-098059, and the p38 MAP kinase inhibitor, SB-203580, decreased production of these bioactive compounds to background levels. Intravenous injection of tetraethylammonium chloride (TEA) into rats on the 8.0% NaCl diet decreased the activities of p38 MAP kinase and p42/44 MAP kinase, compared with rats on the same diet and given vehicle intravenously. These findings provided direct evidence that dietary salt modulated gene expression in the arterial wall through a tetraethylammonium-sensitive mechanism and activation of the p38 and p42/44 MAP kinase pathways. (Hypertension. 2002;39:239-244.)

Key Words: protein kinases ■ transforming growth factors ■ nitric oxide

As the cellular monolayer that lines the lumen of blood vessels, the endothelium performs not only a barrier function but also serves as a biomechanical sensor that maintains vascular integrity. Blood coursing through a vessel creates fluid shear stress from the friction of blood against the vessel wall. This force, which acts in parallel to the vessel wall, activates the endothelium.1 As reviewed by Davies,2 vessel wall. This force, which acts in parallel to the vessel surface, activates the endothelium.1 As reviewed by Davies,2 multiple signal transduction events occur in endothelial cells in response to changes in shear stress. The mitogen-activated protein kinase (MAPK) pathways are integral components of these events.3–6 These pathways serve as major signaling systems that transduce extracellular cues into intracellular responses that alter the phenotype of the endothelium and the release of vasoactive substances.

Details regarding the activation and function of the MAPK pathways have been reviewed.7–9 Most of the knowledge concerning the activation and function of MAPKs in endothelial cells has come from in vitro studies using cells in culture. MAPK pathways are evolutionarily conserved among eukaryotes and play key roles in gene expression. There are at least three broad families: extracellular signal-regulated kinases (ERK, or p42/44 MAPK), p38α-δ MAPKs, and c-jun NH2-terminal kinases or stress-activated protein kinases (p46/54 JNK/SAPK). Each MAPK is activated by specific serine/threonine phosphorylation events that occur in parallel cascades; once activated, MAPKs phosphorylate specific transcription factors that alter gene expression. All three MAPK families are present in endothelium and may be affected by shear forces.3–6

Recent studies demonstrated that dietary salt plays a direct role in expression of the fibrogenic growth factor, transforming growth factor-β1 (TGF-β1), and endothelial nitric oxide synthase (NOS3) in glomeruli and arterial endothelium of rats; this effect occurred independently of blood pressure.10–12 These studies further suggested a role for shear stress in this process for the following reasons. An increase in dietary salt increases blood volume and thus arterial flow, which enhances shear stress. For example, an increase in daily salt intake by healthy humans from 5 g (~80 mmol) to 10 g (~160 mmol) expands extracellular fluid volume by approximately 1.0 to 1.5 L.13 In addition, human subjects on a 5.8-g Na+ diet demonstrated an increase in renal plasma flow, compared with subjects on a low-salt (1.8-g Na+) diet.14 Secondly, shear stress opens on endothelial cells a tetraethylammonium ion (TEA)-inhibitable potassium channel, which is directly involved in altering gene expression in endothelial cells.15,16 The increased expression of TGF-β1 and NOS3 by dietary salt in vivo was inhibited by addition of TEA.10–12 Augmented expression of TGF-β1, which in turn increased expression of NOS3, occurred specifically in the endothelial cell monolayer of the aorta.12 The present study examined this process further and determined the relationship...
between dietary salt intake, MAPK activation, and production of TGF-β1 and NO by the aortic endothelium.

Methods

Animal Preparation

Studies were conducted using 40 male Sprague-Dawley rats, 28 days of age, obtained from Charles River Laboratories (Wilmington, Mass). Animals were chosen at this age because of our previous experience that showed normal renal function and blood pressure responses to dietary salt for up to 2 weeks of observation. The rats were given 0.3% NaCl diet (AIN-76A; Dyets, Inc) and water ad libitum for 4 days before initiating the experiment. The rats were then continued on a diet (AIN-76A; Dyets, Inc) that contained 0.3%, 1.0%, 3.0%, or 8.0% NaCl. These formulated diets were identical in protein and electrolyte composition, except for NaCl content. On the fourth day of study, rats were anesthetized with pentobarbital sodium, 50 mg/kg, intraperitoneally. The aortas were perfused in situ with a cold isotonic heparinized perfusion solution that contained 90 mmol/L NaCl, 50 mmol/L sodium fluoride, 1 mmol/L Na3VO4, and 10 mmol/L sodium pyrophosphate. Fifty milliliters of solution was perfused over 2 minutes. In some experiments, 5 minutes before harvesting the aorta, a 1-mL bolus of either TEA (Sigma Chemical Co), 15 mmol/L in isotonic saline, or saline alone was injected intravenously in the tail vein over 5 minutes. This dose is less than the amount (2–6 mg/kg) administered intravenously to human volunteers with congestive heart failure; no toxic effects were reported in that study. The aorta was perfused in situ with the perfusion solution that also contained 3 mmol/L TEA in those animals that received TEA intravenously. The aorta was harvested under sterile conditions.

Western Blot Analysis (p38 MAPK, p42/44 MAPK, p46/54 JNK/SAPK, ATF-2, and Elk-1)

Harvesting of aortic tissue, generation of protein lysates, and Western blotting proceeded as described,10–12 with slight modifications (see online Methods). The lysis buffer contained sodium pyrophosphate, 2.5 mmol/L, and Na3VO4, 1 mmol/L. The primary antibodies were diluted 1:1000 and recognized specifically total and phosphorylated forms of p38 MAPK, p42/44 MAPK, and p46/54 JNK/SAPK (Cell Signaling Tech, Inc.). Total and phosphorylated forms of the activating transcription factor, ATF-2, were detected using a kit (PhosphoPlus® ATF-2 (Thr71) Antibody Kit; Cell Signaling Tech, Inc). Phosphorylated Elk-1 was detected using a monoclonal antibody (Phospho-Elk-1 [Ser383] 2B1 monoclonal antibody, Cell Signaling Tech, Inc).

Activity Assays for p38 MAPK and p42/44 MAPK

Activities of p38 MAPK and p42/44 MAPK were determined in vitro in standard fashion using kits (Cell Signaling Tech, Inc), after immunoprecipitation of the MAPK of interest (see online Methods).

Immunohistochemical Staining for Phospho-p38 MAPK and Phospho-p42/44 MAPK

Immunohistochemical analysis proceeded in standard fashion (see online Methods), using affinity-purified rabbit polyclonal anti-phospho-p38 MAPK (1:100 dilution in TBS/Triton/BSA buffer) or anti-phospho-p42/44 MAPK (1:100 dilution in TBS/Triton/BSA buffer) (both from Cell Signaling Tech, Inc).

In Vitro Incubation Studies (See Online Methods)

Aortic ring segments were incubated for 24 hours at 37° C with serum-free medium (RPMI 1640; Life Technologies) that contained 0.1% (vol/vol) DMSO or medium containing 50 μmol/L PD-098059, a potent and specific cell-permeable inhibitor of activation of MAPK kinase-1 (MEK1),19 10 μmol/L SB-203580, a highly specific and cell-permeable inhibitor of p38 MAPK,20,21 or both inhibitors. Medium was assayed for total and active TGF-β1 using enzyme-linked immunoassay (TGF-β1 Emax TM ImmunoAssay System; Promega, Inc)10–12 and for nitrite and nitrate (NOx) using

![Figure 1](http://hyper.ahajournals.org/) Western analyses demonstrating the effect of dietary salt intake on phosphorylated and total p42/44 mitogen-activated protein kinase (MAPK) (left), p38 MAPK (middle), and p46/54 JNK/SAPK (right). Each lane represents a sample obtained from a single animal (n=4 in each group) and containing 60 μg total protein. An increase in dietary salt produced sustained increases (P<0.05) in the relative amounts of phosphorylated forms of all of the MAPKs (graph at bottom).
Statistical Analysis

Data were presented as mean ± standard error. Significant differences among data sets were determined using either unpaired t test or one-way analysis of variance using multiple comparisons by Fisher’s protected least significant difference method, where appropriate. A P value less than 0.05 assigned statistical significance.

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

Results

Activities of p38 MAPK, p42/44 MAPK, and JNK/SAPK were Increased in Aortas of Rats on 3.0% and 8.0% NaCl Diets

Four days after initiation of the formulated diets that contained 0.3%, 1.0%, 3.0%, or 8.0% NaCl, cytoplasmic extracts of aorta were obtained as described and examined. Our published experience with this experimental protocol demonstrated that mean blood pressures of rats was not influenced by intake of salt over this time frame of study (127 ± 4 mm Hg on 8.0% NaCl diet versus 130 ± 2 mm Hg on 0.3% NaCl diet). The animals are healthy, their intake of diet among the groups is similar, and they demonstrate no renal abnormalities.12,17,24 While total levels of p38 MAPK, p42/44 MAPK, and p46/p54 JNK/SAPK did not change, the relative amounts of the phosphorylated forms of all of these MAPK enzymes were increased (P < 0.05) with an increase in dietary salt (Figure 1). Subsequent experiments focused on p38 MAPK and p42/44 MAPK. To confirm the functional significance of the increase in relative phosphorylation states of p38 MAPK and p42/44 MAPK, activity assays were performed in standard fashion by immunoprecipitating the MAPK of interest from cytoplasmic extracts of aortic tissue and then determining phosphorylation of substrate in vitro (Figure 2). As dietary salt was increased to 3.0% and higher, activities of both p38 MAPK and p42/44 MAPK increased (P < 0.05), compared with findings obtained from rats on 0.3% and 1.0% NaCl diets for the same duration. Immunohistochemistry using antibodies that recognized specifically the phosphorylated forms of p38 MAPK and p42/44 MAPK demonstrated nuclear localization primarily in aortic endothelial cells of aortic segments of rats on 8.0% NaCl (Figure 3) and sporadically in endothelial cells of aortic segments from rats on the 0.3% NaCl diet (not shown). Expression was also observed using this technique in occasional nuclei in the medial and adventitial layers of the aorta. Control experiments that omitted the primary antibody did not demonstrate positive staining of the cells (not shown). Western analysis (Figure 4) demonstrated increased levels of the phosphorylated forms of ATF-2, a nuclear target of p38 MAPK,23 and Elk-1, a nuclear target of p42/44 MAPK,29 in samples from the aorta of rats on the 8.0% NaCl diet, compared with samples from rats on the 0.3% NaCl diet.

Dietary Salt Increased Endothelial Expression of TGF-β1 and Production of NO Through Both p38 and p42/44 MAPK Pathways

This laboratory demonstrated previously that dietary salt increased aortic endothelial cell production of NOx by increased expression of NOS3.12 Consistent with these findings, in the present study aortic segments from rats on 8.0% NaCl diet produced more (P < 0.05) total and active TGF-β1 and NOx, compared with aortic segments from rats on the 0.3% NaCl diet (Figure 5). There were direct correlations between total and active TGF-β1 (r²=0.84, P < 0.05) and between active TGF-β1 and NOx (r²=0.691, P < 0.05). Removal of the endothelium decreased production of both TGF-β1 and NOx to background levels, indicating also that
the NOS responsible for NO production was present in the endothelium. Addition of the MEK inhibitor (PD-098059), a p38 MAPK inhibitor (SB-203580), or both inhibitors to the medium decreased production of both TGF-β1 and NOx to levels close to background. Thus, activation of both pathways by dietary salt was required to increase production of TGF-β1 and NOx.

Dietary Salt Increased MAPK Activities Through a TEA-Sensitive Potassium Channel

Previous experiments demonstrated that addition of TEA to the medium prevented the increase in expression of TGF-β1 and NOS3 that occurs in the aortic endothelium and glomerulus in response to an increase in dietary salt. Based on these experiments, 5 minutes before harvesting the aorta, rats on 8.0% NaCl diet were injected intravenously with a 1-mL bolus of TEA; the findings were compared with rats that were on the same diet and received vehicle alone. Administration of TEA decreased (P<0.05) activities of both p38 MAPK and p42/44 MAPK, which were obtained from the protein extracts of aortic tissue, compared with findings from animals that received vehicle alone (Figure 6).

Discussion

The present study determined that the MAPK pathways were integrally involved in the signal transduction mechanism through which dietary salt controls the endothelium. Aortic segments from rats on 8.0% NaCl diet for 4 days demonstrated persistent increases in phosphorylated p38 MAPK, p42/44 MAPK, and p46/54 MAPK, compared with aortic segments from rats on 0.3% NaCl diet. Activity assays documented a dose-dependent activation of p38 MAPK and p42/44 MAPK by dietary salt. Nuclear localization of these MAPK enzymes in the endothelial cells was demonstrated using immunohistochemistry (Figure 4) and is required to phosphorylate transcription factors, such as ATF-2 and Elk-1. Experiments confirmed the effect of dietary salt on endothelial cell expression of TGF-β1 and production of NOx. Addition of either the MEK1/2 inhibitor (PD-098059) or the p38 MAPK inhibitor (SB-203580) decreased production of both TGF-β1 and NOx to levels close to background measurements. Thus, activation of both p38 MAPK and p42/44 MAPK were involved directly in the physiological response of the endothelium to increased dietary salt and promoted increased production of TGF-β1 and NO. Finally, addition of TEA inhibited activities of both p38 MAPK and p42/44 MAPK in rats on the 8.0% NaCl diet. These studies were consistent with previous experiments that showed TEA inhibited the increase in production of TGF-β1 and NOx by dietary salt. The findings provided evidence of activation of signal transduction pathways through which dietary salt stimulates enhanced gene expression in the arterial wall.

The pathways activated by dietary salt are reminiscent of the described effect of shear on endothelial cells in culture. Over the time period of the study, the rat model used in the present studies does not show changes in blood pressure with an increase in dietary salt. Therefore, strain forces were not involved in this process. Both shear stress in vitro and...
increased dietary salt intake in vivo (present study) led to the activation of p42/44 MAPK, p38 MAPK, and p46/54 JNK/SAPK in the endothelium. In addition, kinase activation occurred by a mechanism that included a TEA-sensitive potassium channel. Finally, both shear and dietary salt intake act principally on endothelial cells in the intact aorta.

Previous studies demonstrated that endothelial cell and glomerular production of TGF-β1 and NO in vivo is modulated by dietary salt. Aortic endothelium from rats on 8.0% NaCl diet showed increased expression of both NOS3 and TGF-β1. Addition of a neutralizing antibody to TGF-β1 decreased NO production. This interrelationship between TGF-β1 and NOS3 was also demonstrated to occur in glomeruli. Taken together, the studies showed a coordinated expression of TGF-β1 and NOS3 and involvement of TGF-β1 in NO production by aortic endothelium. The functional significance of an increase in NO production in blood pressure regulation as dietary salt intake increased has been shown in rats and humans. By stimulating NO production, TGF-β1 facilitates this process. TGF-β also promotes vascular smooth muscle hypertrophy and facilitates the synthesis of matrix proteins necessary for arterial stiffening. However, the fibrogenic effect of sustained expression of TGF-β1 is potentially detrimental. Work from the 1950s demonstrated that an increase in dietary salt shortened the life span of rats and seemed to be related to development of renal arteriolonephrosclerosis. The mechanism by which dietary NaCl produced end-organ damage was assumed to be related to hypertension. The present study showed that, independently of blood pressure, dietary salt increased production of TGF-β1; it is tempting to speculate that this process may contribute to the arteriolonephrosclerosis that developed in rats chronically fed a diet high in salt content.

In summary, the present findings demonstrated the signal transduction mechanism by which dietary salt intake altered gene expression by vascular endothelium. This pathway seemed to involve a TEA-sensitive mechanism and stimulation of the MAPK pathways. One consequence of activation of the p38 MAPK and p42/44 MAPK pathways was a coordinated, augmented production of TGF-β1 and NOx. From a therapeutic point of view, understanding the molecular mechanisms regulating MAPK activities by dietary salt in normal and pathological conditions could lead to new strategies for the effective prevention and control of end-organ damage by dietary salt.

Acknowledgments

This work was supported by a National Institutes of Health grant (R01 DK46199) and the Office of Research and Development, Medical Research Service, Department of Veterans Affairs.

References


Increased Dietary Salt Activates Rat Aortic Endothelium
Wei-Zhong Ying and Paul W. Sanders

_Hypertension_. 2002;39:239-244
doi: 10.1161/hy0202.104142

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/39/2/239

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2002/02/03/39.2.239.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org//subscriptions/