High Salt Intake Impairs Vascular Nitric Oxide/Cyclic Guanosine Monophosphate System in Spontaneously Hypertensive Rats

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ABSTRACT
In aortas of spontaneously hypertensive rats (SHRs), excessive dietary salt causes down-regulation of soluble guanylate cyclase (sGC) followed by decreased cyclic GMP production, which leads to impairment of the vascular relaxation response to nitric oxide (NO). The present study aimed to elucidate whether this impaired NO/cyclic GMP system results secondarily from increased blood pressure or from an effect of the salt itself. The antihypertensive drug nifedipine was used on 4-week-old SHRs that received a normal-salt diet or a high-salt diet for 4 weeks. Treatment with nifedipine (30 mg/kg/day, p.o.) reduced the increased blood pressure of SHRs fed the high-salt diet to the level of SHRs fed the normal-salt diet. In aortic rings from SHRs fed the high-salt diet, not only endothelium-dependent relaxations but also endothelium-independent relaxations were significantly impaired. However, these impairments were not alleviated by treatment with nifedipine. Furthermore, nifedipine did not prevent the increase in protein levels of endothelial NO synthase and the decrease in the protein levels of sGC in aortas from SHRs fed the high-salt diet. These alterations by high salt intake were restored after replacement with the normal-salt diet for 4 additional weeks. These results indicate that in SHRs given excessive dietary salt, normalization of salt intake but not blood pressure reduction can ameliorate alterations in the NO/cyclic GMP system. High salt intake may directly affect the vascular smooth muscle and cause impairment of the relaxation response to NO.

Nitric oxide (NO), known as an endothelium-derived relaxing factor, is usually generated in the circulation via stimulation of vascular endothelial NO synthase (eNOS) by physical stimuli, such as increase in transmural pressure or blood flow (shear stress) (Rubanyi et al., 1990; Fleming and Busse, 1999). NO, which is released from the endothelium to smooth muscle cells, activates the soluble isofrm of guanylate cyclase (sGC) to form cyclic GMP (Lucas et al., 2000). The increased cyclic GMP level causes vascular smooth muscle relaxation. Such a NO/cyclic GMP system regulates vascular tone in various vascular beds (Fürstermann et al., 1986; Köseling and Friebe, 1999). Thus, it is widely accepted that NO plays an important role in the pathogenesis of hypertension (Boulanger, 1999).

Several vasodilators, including acetylcholine, are known to produce endothelium-dependent relaxations through stimulation/release of NO in the endothelium of various arteries. Many studies have documented that endothelium-dependent relaxations in response to acetylcholine are impaired in the aortas from experimental hypertensive rats, such as aged spontaneously hypertensive rats (SHRs) (Konishi and Su, 1983) and Dahl salt-sensitive rats (Lüscher et al., 1987). Furthermore, treatment with calcium antagonists, drugs commonly used to control hypertension, can prevent endothelial dysfunction in the aortas of adult SHRs (Gray et al., 1993) and Dahl salt-sensitive rats (Boulanger et al., 1994). These findings assume that the endothelial dysfunction is a consequence of the sustained high blood pressure. However, there are few accounts of work on smooth muscle dysfunction, e.g., a reduction of sGC activity or a decrease in cyclic GMP level in adult SHRs (Shirasaki et al., 1988; Kojda et al., 1998). Recently, attenuated expression of sGC has been reported in SHRs (Ruetten et al., 1999). However, there was a contrary report that messenger RNA levels of the sGC increase in SHRs (Papapetropoulos et al., 1994).

We have previously demonstrated that in young SHRs, excessive dietary salt causes impairment of the relaxation response in the aortas, and this impairment is mediated by a decrease in cyclic GMP production following a decrease in the protein level of sGC in the smooth muscle cells, despite enhanced NO production/release in the endothelium.

ABBREVIATIONS: NO, nitric oxide; eNOS, endothelial NO synthase; sGC, soluble guanylate cyclase; SHR, spontaneously hypertensive rat; NOx, NO2 plus NO3; pEC50, negative logarithm molar concentration of agonist required to produce 50% of the maximal response; Rmax, maximum relaxation response; ANOVA, analysis of variance; PLSD, protected least significant difference.
O2/5% CO2, and then the rings were allowed to equilibrate for 90 min. The bath solution was continuously aerated with a gas mixture of 95% C, pH 7.4) described above. In some rings, the endothelium was cleaned of adherent tissue and cut into 3-mm rings, taking care not to damage the endothelium (Ding and Vaziri, 2000; Brovkovych et al., 2001). Therefore, an additional aim of this study was to find whether treatment with nifedipine affects the sGC/cyclic GMP pathway in vascular smooth muscle cells.

**Materials and Methods**

**Experimental Animals.** Male 4-week-old SHRs (SHR/Izm) (Dis-ease Model Cooperative Research Association, Kyoto, Japan) were used. Diets were purchased from Japan SLC (Hamamatsu, Japan). The animals received a normal-salt diet containing 0.3% NaCl (control group, n = 6) or a high-salt diet containing 8% NaCl (high salt group, n = 6) for 4 weeks. The other components of each diet were described above, and the diet was switched to a normal-salt diet, which was given to the rats for 4 more weeks (high/low salt group, n = 6). The rats in the control and high salt groups were orally given a 0.5% carboxymethylcellulose solution alone (0.2 ml/100 g body weight). The dose of nifedipine used had been demonstrated to be an effective one in our preliminary study.

In a different experiment, after male 4-week-old SHRs had received the high-salt diet for 4 weeks in a manner similar to that described above, the diet was switched to a normal-salt diet, which was given to the rats for 4 more weeks (high/low salt group, n = 5). The control rats were maintained on the normal-salt diet alone for 8 weeks (control group, n = 5).

The rats were kept in an air-conditioned room (23 ± 1°C and 60 ± 10% humidity) under an artificial 12-h light/dark cycle (7:00 AM to 7:00 PM). Diet and water were available ad libitum during the experimental period. The systolic blood pressure was determined once a week in conscious rats by the indirect tail-cuff method. On this day, nifedipine was administered after the blood pressure measurement. The study protocols were performed according to the Guideline Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

**Aortic Preparations.** At the end of the experiment, blood was taken from the abdominal aorta of rats under anesthesia with pentobarbital sodium (40 mg/kg, i.p.). The thoracic aorta was then removed and immediately placed in Krebs-Henseleit solution (118.4 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25.0 mM NaHCO₃, and 11.1 mM glucose). The aorta was cleaned of adherent tissue and cut into 3-mm rings, taking care not to damage the endothelium. Each ring was fixed vertically under a resting tension of 1.0 g in a 10-ml organ bath filled with the solution (37°C, pH 7.4) described above. In some rings, the endothelium was mechanically removed by gentle rubbing with moistened cotton. The bath solution was continuously aerated with a gas mixture of 95% O₂/5% CO₂, and then the rings were allowed to equilibrate for 90 min before the start of the experiments. Isometric tension change was measured with a force-displacement transducer (model t-7; NEC San-Ei, Tokyo, Japan) coupled to a dual-channel chart recorder (model SK21; NEC San-Ei).

**Vascular Relaxation Studies.** Aortic rings with intact endothelium were preconstricted with 0.1 μM noradrenaline to generate approximately 80% maximal contraction to noradrenaline. This concentration of noradrenaline could produce a sustained contraction, against which agonist-induced relaxations could be satisfactorily obtained. When the contractile response reached a plateau, acetylcholine (0.1 nM to 1 μM) or adenosine diphosphate (0.1 nM to 1 μM) was added cumulatively to the bath solution. Similarly, endotheli-um-denuded aortic rings were preconstricted with noradrenaline (0.1 μM), and then sodium nitroprusside (0.1 nM to 0.1 μM) or nitroglyc-erin (1 nM to 1 μM) was added cumulatively to the bath solution. Denudation of the endothelium was confirmed pharmacologically by the disappearance of the 1 μM acetylcholine-induced relaxation response. The relaxation responses obtained were expressed as a percentage of the maximal relaxation evoked by papaverine (100 μM). Indomethacin (10 μM), a cyclooxygenase inhibitor, was present in the Krebs-Henseleit solution during all experiments.

**Western Blot Analysis.** The amounts of eNOS and sGC protein in the aortas were measured by Western blot analysis as described in a previous paper (Kagota et al., 2001). The thoracic aortas of SHRs were homogenized in a glass/glass homogenizer in a lysis buffer. An equivalent amount of total protein (20 μg) was loaded on each lane and electrophoresed by 7.5% SDS-polyacrylamide gel by elec-trophoresis, and then transferred to nitrocellulose membranes. The membrane was blocked with 5% blocking reagent (Amersham Biosciences, Piscataway, NJ) supplied in phosphate-buffered saline containing 0.1% Tween 20 for 1 h at room temperature. The membrane was then incubated overnight at 4°C with mouse monoclonal anti-eNOS antibody (1:2000 dilution; Transduction Laboratories, Lexington, KY) or mouse monoclonal anti-sGC antibody (B4) (1:2500 dilution, a generous gift from Prof. Ferid Murad, University of Texas, Houston, TX). Thereafter, it was washed and finally incubated with a goat anti-mouse IgG conjugated to horseradish peroxidase (1:2500 dilution; Transduction Laboratories) for 2 h at room temperature. Subsequent detection of the specific proteins was performed with an enhanced chemiluminescence (ECL Western blotting analysis sys-tem; Amersham Biosciences) on X-ray film. The X-ray film was scanned into an Adobe PhotoShop program (version 3.0; Adobe Sys-tems, Mountain View, CA) with an Epson scanner (GT-9000; Epson, Kyoto, Japan) or mouse monoclonal anti-sGC antibody (B4) (1:2500 dilution, a generous gift from Prof. Ferid Murad, University of Texas, Houston, TX). Thereafter, it was washed and finally incubated with a goat anti-mouse IgG conjugated to horseradish peroxidase (1:2500 dilution; Transduction Laboratories) for 2 h at room temperature. Subsequent detection of the specific proteins was performed with an enhanced chemiluminescence (ECL Western blotting analysis sys-tem; Amersham Biosciences) on X-ray film. The X-ray film was scanned into an Adobe PhotoShop program (version 3.0; Adobe Sys-tems, Mountain View, CA) with an Epson scanner (GT-9000; Epson, America, Torrance, CA) and transferred to the Macintosh NIH Im-age program (version 1.61; http://rsb.info.nih.gov/nih-image/). The density of the bands was measured using NIH Image gel macros. The eNOS and sGC protein signals were normalized to the respective signals of β-actin, a constituent in a wide variety of tissues, and α-actin, a specific smooth muscle cell marker, respectively. For this purpose, a parallel gel with identical signals was also subjected to the electrophoresis, blotted onto nitrocellulose, and subjected to Western blot analysis with monoclonal antibody against β-actin (1: 5,000 dilution; Sigma-Aldrich, St. Louis, MO) or α-actin (1:10,000 dilution; Fogen, Heidelberg, Germany). The protein signals obtained were expressed as eNOS/β-actin and sGC/α-actin ratios.

**Determination of NOx Levels in Serum.** At the end of the experiment, the serum was separated from the blood by centrifugation at 3,000 g for 10 min. The serum NOx (NO₂⁻ plus NO₃⁻) levels were determined using a commercial kit (NOX/NO₃ NOx Assay Kit-C; Doiding Laboratories, Kumamoto, Japan) based on the Griess reac-tion. In this case, the deproteinization of serum by addition of meth-anol/diethyl ether (3:1, v/v) (Guevara et al., 1998) was essential for quantitative measurement. The serum NOx level was expressed as micromoles per liter (μM).

**Drugs.** Drugs used in the present experiments were as follows: acetylsalicylic acid (Daiichi Pharmaceutical Co., Ltd., Tokyo, Ja-pan) and indomethacin (Sigma-Aldrich); sodium nitroprusside and papaverine hydrochloride (Nacalai Tesque Inc., Kyoto, Japan); and...
noradrenaline (Sankyo Co., Ltd., Tokyo, Japan); adenosine diphosphate (Kohjin Co., Ltd., Tokyo, Japan) and nitroglycerin (Millisrol; Nihon Kayaku Co., Ltd., Tokyo, Japan). Other chemicals of analytical reagent grade were purchased from Nacalai Tesque Inc.

Indomethacin was dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide in the Krebs-Henseleit solution was 0.05% (v/v), which did not influence vascular responses. All other compounds were dissolved in distilled water.

Data Analysis. Data are expressed as means ± S.E.M. Individual concentration-response curves were characterized by determining the pEC50 (negative logarithm molar concentration of agonist required to produce 50% of the maximal response) and the Rmax (maximum relaxation response). The pEC50 values and Rmax were calculated using GraphPad Prism software (version 3.0; GraphPad Software, San Diego, CA). In the antihypertensive therapy experiment (three groups), statistical analysis was based on analysis of variance (ANOVA) followed by Fisher’s PLSD test using StatView software (version 5.0; SAS Institute Inc., Cary, NC). In the salt restriction experiment (two groups), the F test was used to compare the variance of data between the high/low salt and control groups, and then statistical analysis was performed using the unpaired Student’s t test. Differences were considered statistically significant at P < 0.05.

Results

Effect of Nifedipine on SHRs Fed a High-salt Diet

Body Weight, Blood Pressure, and Heart Rate. As shown in Table 1, the body weight was not significantly different among the three groups during the experimental period. The systolic blood pressure of every SHR gradually increased, particularly with feeding of the high-salt diet. At the end of the experiment, the blood pressure in the high salt group significantly increased by 1.3-fold compared with that in the control group. This increased blood pressure was significantly depressed by treatment with nifedipine to the level in the control group. No significant differences in the heart rate were noted among the three groups during the experimental period.

Endothelium-Dependent Relaxations. In endothelium-intact aortic rings, the contractile responses to noradrenaline were almost the same in the three groups (high salt + nifedipine group, 0.938 ± 0.075; high salt group, 0.900 ± 0.058; and control group, 0.902 ± 0.093 g). As shown in Fig. 1 and Table 2, the endothelium-dependent relaxations in response to acetylcholine and adenosine diphosphate were significantly attenuated in the high salt group compared with the control group. However, there was no significant difference between the relaxation responses in the high salt + nifedipine group and the high salt group.

Endothelium-Independent Relaxations. In endothelium-denuded aortic rings, the contractile responses to noradrenaline were also almost the same in the three groups (high salt + nifedipine group, 1.00 ± 0.05; high salt group, 0.947 ± 0.040; and control group, 0.902 ± 0.093 g). As shown in Fig. 2 and Table 2, the endothelium-independent relaxations in response to sodium nitroprusside and nitroglycerin, NO donors, were significantly attenuated in the high salt group compared with the control group. However, there was no significant difference between the relaxation responses in the high salt + nifedipine group and the high salt group.

Protein Levels of eNOS and sGC in Aortas. Figure 3 shows a typical pattern of expression and the relative protein levels of eNOS and sGC in aortas obtained by Western blot analysis. The high salt group showed a significant increase in the protein level of eNOS, but a significant decrease in that of sGC compared with the control group. These changes by high salt intake were not affected by treatment with nifedipine.

Serum NOx Levels. The serum NOx level was determined as an index of NO production in the whole body. As shown in Fig. 4, the serum NOx level significantly increased in the high salt group compared with the control group. This change by high salt intake was not affected by treatment with nifedipine.

Effect of Salt Restriction on SHRs Fed a High-Salt Diet

Body Weight, Blood Pressure, and Heart Rate. Throughout the 8-week experimental period, the body weight was not significantly different between the control group and the high/low salt group, which was given the normal diet in place of the high-salt diet from the middle of the experimental period. At the end of the experiment, the body weights of the high/low salt and control groups were 239 ± 5 and 250 ± 6 g, respectively.

At the midpoint of the experimental period (fourth week), i.e., the high-salt diet period, the systolic blood pressure of the high/low salt group was significantly higher than that of the control group (high/low salt group, 251 ± 4; control group, 202 ± 6 mm Hg; P < 0.05), whereas at the end of the experimental period (eighth week), after replacement with the normal-salt diet, it returned to the level of the control group (high/low salt group, 217 ± 6; control group, 223 ± 3 mm Hg). The heart rate was not significantly different between the two groups throughout the experimental period. At the end of the experiment, the heart rates of the high/low salt and control groups were 391 ± 10 and 396 ± 8 beats/min, respectively.

TABLE 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Animals</th>
<th>Body Weight</th>
<th>Systolic Blood Pressure</th>
<th>Heart Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Initial</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>67.0 ± 2.2</td>
<td>182 ± 3</td>
<td>109 ± 3</td>
</tr>
<tr>
<td>High salt</td>
<td>6</td>
<td>68.5 ± 2.6</td>
<td>179 ± 5</td>
<td>108 ± 4</td>
</tr>
<tr>
<td>High salt + nifedipine</td>
<td>6</td>
<td>67.4 ± 1.1</td>
<td>179 ± 3</td>
<td>107 ± 4</td>
</tr>
</tbody>
</table>

* P < 0.05, high salt vs. control; #P < 0.05, high salt + nifedipine vs. high salt.

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Endothelium-Dependent and Endothelium-Independent Relaxations. Four weeks after replacement of the high-salt diet with the normal-salt diet, the relaxations in response to acetylcholine in the high/low group returned to the levels of those in the control group (high/low salt group, pEC<sub>50</sub> = 7.63 ± 0.04, R<sub>max</sub> = 96.3 ± 1.3%; control group, pEC<sub>50</sub> = 7.73 ± 0.06, R<sub>max</sub> = 93.8 ± 2.3%) (Fig. 5). Similarly, the sodium nitroprusside-induced relaxations were not significantly different between the two groups (high/low salt group, pEC<sub>50</sub> = 8.41 ± 0.03, R<sub>max</sub> = 96.7 ± 0.6%; control group, pEC<sub>50</sub> = 8.53 ± 0.09, R<sub>max</sub> = 94.4 ± 1.6%) (Fig. 5).

Protein Levels of eNOS and sGC in Aortas. As shown in Fig. 6, the protein levels of both eNOS and sGC in the high/low group were almost restored to the levels in the control group at 4 weeks after replacement with the normal-salt diet. Serum NOx Levels. The serum NOx level in the high/low group was almost the same as that in the control group at 4 weeks after replacement with the normal-salt diet (high/low group, 9.82 ± 0.72; control group, 11.62 ± 1.00 μM).

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Acetylcholine</th>
<th>Adenosine Diphosphate</th>
<th>Sodium Nitroprusside</th>
<th>Nitroglycerin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>R&lt;sub&gt;max&lt;/sub&gt;</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>R&lt;sub&gt;max&lt;/sub&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>7.78 ± 0.03</td>
<td>93.0 ± 1.2</td>
<td>7.52 ± 0.1</td>
<td>77.2 ± 5.0</td>
</tr>
<tr>
<td>High salt</td>
<td>7.52 ± 0.11*</td>
<td>84.0 ± 2.3*</td>
<td>7.18 ± 0.09*</td>
<td>56.0 ± 5.4*</td>
</tr>
<tr>
<td>High salt + nifedipine</td>
<td>7.59 ± 0.08</td>
<td>80.2 ± 3.7*</td>
<td>7.17 ± 0.07*</td>
<td>60.0 ± 3.8*</td>
</tr>
</tbody>
</table>

* P < 0.05, high salt vs. control.
Cardiovascular events occur more frequently in patients and experimental animals with sodium-sensitive hypertension (Morimoto et al., 1997; Raij, 1999). High salt intake has been recognized to be closely correlated with blood pressure elevation, but the mechanism of how it is involved in hypertension is not yet clearly understood. High salt intake has been reported to cause an abnormal increase in sympathetic nerve activity, leading to an increase in peripheral vascular resistance in hypertensive patients and in salt-loaded SHRs (Fujita et al., 1980; Brooks et al., 2001). We have previously reported that in young SHRs, excessive dietary salt causes down-regulation of sGC followed by a decrease in cyclic GMP production in the aorta, which leads to impairment of vascular relaxation in response to NO, despite increases in NO production and eNOS protein expression (Kagota et al., 2001). In the present study, we demonstrated that such changes in the NO/cyclic GMP system are restored by dietary salt restriction, but not by antihypertensive therapy. These results indicate that in SHR aortas, neither up-regulation of the eNOS/NO pathway nor down-regulation of the sGC/cyclic GMP pathway is due to the blood pressure elevation induced by high salt intake. Thus, increased dietary salt may directly impair the NO/cyclic GMP system in the vessel wall independently of the rising arterial pressure.

In aortas from SHRs, Vaziri et al. (1998) have shown increased eNOS activity and eNOS protein expression compared with those of normotensive Wistar-Kyoto rats. Ruetten et al. (1999) have also demonstrated that protein expression of sGC and mRNA expression of cyclic GMP-dependent protein kinase-I are reduced in aortas from SHRs compared with those in age-matched Wistar-Kyoto rats. Therefore, these alterations in the NO/cyclic GMP system, namely, the enhanced eNOS/NO pathway and reduced sGC/cyclic GMP pathway, seem to be phenotypic characteristics of the arteries of SHRs. Similarly, in the present study, we observed potent alterations in the NO/cyclic GMP system in aortas from young SHRs fed a high-salt diet compared with a normal-salt diet. Thus, excessive dietary salt should accelerate the alteration in the NO/cyclic GMP system in the SHR aorta, because in normotensive Wistar-Kyoto rats, excessive salt intake has little effect on the aortic vasodilator response as well as blood pressure (Kagota et al., 2001). In preliminary work, we have also found that a high-salt diet exerts little influence on the protein levels of sGC in aorta of Wistar-Kyoto rats (data not shown). With regard to the endothelial eNOS/NO pathway, the effects of excessive salt intake on SHRs seem to be quite the opposite of those of salt-sensitive Dahl rats (Lüscher et al., 1987) and stroke-prone SHRs (Volpe et al., 1996).

Nifedipine, a calcium antagonist, was used in the antihypertensive therapy experiment, because it is known to be effective for lowering increased blood pressure in salt-loaded SHRs (Leenen and Yuan, 1992; Fujiwara et al., 1998) and in patients with essential hypertension (MacGregor et al., 1987). Chronic antihypertensive therapy with calcium antagonists has been reported to prevent endothelial dysfunction in hypertensive salt-sensitive Dahl rats (Boulanger et al., 1990).
1994) and in salt-loaded stroke-prone SHRs (Krenek et al., 2001). Furthermore, chronic nifedipine treatment causes an increase in expression of eNOS messenger RNA in the aorta from stroke-prone SHRs (Naruse et al., 1999). Nifedipine can up-regulate the eNOS expression and activity in cultured endothelial cells from the human coronary artery (Ding and Vaziri, 2000). However, to the best of our knowledge, no study has been reported on the effects of nifedipine on the sGC/cyclic GMP pathway in the arterial smooth muscle cells of SHRs, although nifedipine improves endothelial dysfunction in SHRs (Tschudi et al., 1994). In the present study, treatment with nifedipine, as expected, could sufficiently reduce the elevated blood pressure of SHRs fed the high-salt diet to the level of SHRs fed the normal-salt diet. Nonetheless, treatment with nifedipine could not prevent all the vascular events developing in SHRs fed the high-salt diet, including reduced endothelium-dependent and -independent relaxations, increased eNOS, and reduced sGC protein levels in the aorta, as well as increased serum NOx levels. In this case, the reduction in renal function does not seem to develop in the SHR, because we had confirmed that the plasma creatinine level and the urinary excretion of creatinine and protein are unaffected by high salt intake (data not shown). Therefore, serum NOx levels may represent a generalized NO production. Millgard et al. (1998) have also reported that treatment with nifedipine cannot ameliorate the attenuated endothelium-dependent vasodilation in the forearm of hypertensive patients. Thus, our findings emphasize that in SHR aortas, both up-regulation of the eNOS/NO pathway in endothelial cells and down-regulation of the sGC/cyclic GMP pathway in smooth muscle cells do not appear to be due to the increased blood pressure caused by high salt intake.

The effects of salt restriction on blood pressure are heterogeneous and remain to be precisely established (Muntzel and Drueke, 1992). In SHRs, a low-salt diet lowers systolic blood pressure when given at 6 weeks of age (Gradin et al., 1986), but not when given after the hypertension becomes more established (Meldrum and Glenton, 1992; Iyer et al., 2000). In the salt-loaded SHR, we found that dietary salt restriction in the middle of the experiment can completely...
reverse not only the blood pressure elevation but also the impairment of the aortic relaxing function and the NO/cyclic GMP system to the levels in the salt-unloaded SHR. The serum NOx levels were also restored after the second salt restriction. In our preliminary studies, we have found that the degree of impairment of relaxation responses in aortas of SHRs is greater when maintained on a 4% NaCl diet than on an 8% NaCl diet for 4 weeks, despite the comparable blood pressure elevation (Tamashiro et al., 1997). These findings suggest that in SHRs, high salt intake exerts an impairment effect on the vascular NO/cyclic GMP system independent of blood pressure elevation, and such changes are reversible.

The exact mechanism by which high salt intake induces alteration of the NO/cyclic GMP system in the SHR aorta is not yet known; e.g., whether it occurs directly or indirectly through humoral factors. However, it is certain that the reduction of vascular relaxation responses by high salt intake is due to a far stronger decrease in sGC expression beyond the increase in eNOS expression in the endothelium. The relaxing function downstream of cyclic GMP in vascular smooth muscle is normal, because the relaxation in response to 8-bromo-cyclic GMP, a stable cyclic GMP analog, remains unchanged by high salt intake (Kagota et al., 2001). In vascular smooth muscle, high salt intake has been postulated to inhibit Na⁺,K⁺-ATPase through volume expansion, thereby increasing the intracellular sodium concentration, which results in depolarization and increased calcium influx and, hence, contraction (MacGregor et al., 1987; Haddy, 1990). Therefore, the blood pressure-lowering effect of calcium antagonists seems to be enhanced when sodium intake is increased (MacGregor et al., 1987; Shimosawa et al., 1995). Furthermore, calcium appears to be a negative allosteric modulator of sGC (Levine et al., 1979). Indeed, Ca²⁺ and cyclic GMP have antagonistic functions in vascular smooth muscle, as contraction is mediated by an increase in intracellular calcium ion concentration, whereas relaxation occurs as a result of an increase in the intracellular cyclic GMP level. These findings might explain the down-regulation of sGC in the aorta when SHRs are maintained on a high-salt diet. However, in the present study, treatment with nifedipine could not prevent the down-regulation of sGC by high salt intake. Thus, mechanisms other than an increase in intracellular calcium concentration seem to be involved in the impaired sGC protein expression. Recently, Vaziri and Wang (1999) have reported that NO serves as a negative-feedback regulator of eNOS expression via a cyclic GMP-mediated process in cultured endothelial cells from the human coronary artery. This finding and our data led us to postulate that some cyclic GMP-mediated feedback regulation exists between eNOS and sGC expressions in aorta of salt-loaded SHRs. Additional studies are needed to clarify the mechanisms of the salt-induced alteration of the NO/cyclic GMP system in SHR aortas.

In summary, the present study demonstrated that in SHR aortas, high salt intake impairs the vascular relaxing function regardless of blood pressure elevation, and this is exerted by a decrease in cyclic GMP production following a reduced protein level of sGC in smooth muscle cells, despite enhanced NO production in the endothelium. Damage of the vascular smooth muscle might partly contribute to the aggravation of hypertension by excessive salt intake.

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