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# Downregulation of vascular soluble guanylate cyclase induced by high salt intake in spontaneously hypertensive rats

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**1** Cyclic guanosine monophosphate (cyclic GMP)-mediated mechanism plays an important role in vasodilatation and blood pressure regulation. We investigated the effects of high salt intake on the nitric oxide (NO)–cyclic GMP signal transduction pathway regulating relaxation in aortas of spontaneously hypertensive rats (SHR).

**2** Four-week-old SHR and normotensive Wistar-Kyoto rats (WKY) received a normal salt diet (0.3% NaCl) or a high salt diet (8% NaCl) for 4 weeks.

**3** In aortic rings from SHR, endothelium-dependent relaxations in response to acetylcholine (ACh), adenosine diphosphate (ADP) and calcium ionophore A23187 were significantly impaired by the high salt intake. The endothelium-independent relaxations in response to sodium nitroprusside (SNP) and nitroglycerin were also impaired, but that to 8-bromo-cyclic GMP remained unchanged. On the other hand, high salt diet had no significant effects on the relaxations of aortic rings from WKY.

**4** In aortas from SHR, the release of NO stimulated by ACh was significantly enhanced, whereas the production of cyclic GMP induced by either ACh or SNP was decreased by the high salt intake.

**5** Western blot analysis showed that the protein level of endothelial NO synthase (eNOS) was slightly increased, whereas that of soluble guanylate cyclase (sGC) was dramatically reduced by the high salt intake.

**6** These results indicate that in SHR, excessive dietary salt can result in downregulation of sGC followed by decreased cyclic GMP production, which leads to impairment of vascular relaxation in responses to NO. It is notable that chronic high salt intake impairs the sGC/cyclic GMP pathway but not the eNOS/NO pathway.

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**Keywords:** Endothelium; guanylate cyclase; nitric oxide synthase; salt; smooth muscle; spontaneously hypertensive rats

**Abbreviations:** ACh, acetylcholine; ADP, adenosine diphosphate; 8-bromo-cyclic GMP, 8-bromo-cyclic guanosine monophosphate; A23187, calcium ionophore A23187; eNOS, endothelial NO synthase; EDRF, endothelium-derived relaxing factors; IND, indomethacin; NO, nitric oxide; L-NAME, $N^G$-nitro-L-arginine methyl ester; sGC, soluble guanylate cyclase; SNP, sodium nitroprusside; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats

## Introduction

A large proportion of individuals with essential hypertension are salt-sensitive (Kawasaki *et al*., 1978; Fujita *et al*., 1980; Weinberger, 1996) and this salt sensitivity is considered to be a marker of the potential for cardiovascular disease (Morimoto *et al*., 1997; Raij, 1999). A high salt intake has been recognized to be closely correlated with blood pressure elevation (Dahl *et al*., 1967; Tobian, 1991). However, the underlying mechanism of how salt intake induces hypertension is not yet clearly understood. It has been demonstrated that a high salt intake in hypertensive patients enhances sympathetic nerve activity and disturbs the renin-angiotensin system, leading to an increase in peripheral vascular resistance (Fujita *et al*., 1980; Williams & Hollenberg, 1991). In Dahl salt-sensitive hypertensive rats, a high salt diet has been shown to impair endothelium-dependent relaxations induced by a variety of vasodilators (Lüscher *et al*., 1987; Raij *et al*., 1988). This impairment is considered to be due to a decreased release of nitric oxide (NO) from the endothelium (Nava & Lüscher, 1995; Boegehold, 1992), which results from reduced endothelial NO synthase (eNOS) activity or increased endogenously synthesized NOS inhibitor (Hayakawa & Raij, 1997; Matsuoka *et al*., 1997). Similar impairment of NO synthesis and release by a high salt intake has been observed as a pathogenic factor in the development of hypertension in healthy subjects (Facchini *et al*., 1999) or normal Sprague-Dawley rats (Tolins & Shultz, 1994). These findings seem to favour the view that the endothelium plays an important role in peripheral vascular tonus and thereby in blood pressure regulation (Lüscher & Barton, 1997; Fleming & Busse, 1999).

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As an animal model of essential hypertension (Okamoto & Aoki, 1963), spontaneously hypertensive rats (SHR) are known to be susceptible to high dietary salt (Dietz *et al*., 1980; Karr-Dullien & Bloomquist, 1979). However, there is little information regarding the impairment of cyclic GMP-mediated vasodilation by salt loading in SHR (Mervaala *et al*., 1997; Matrougui *et al*., 1998). The present study was designed to elucidate the role of the NO–cyclic GMP system in the pathogenesis of vascular dysfunction in SHR fed a high salt diet.

## Methods

### *Experimental animals*

Male 4-week-old SHR (SHR/Izm) and normotensive WKY (WKY/Izm) (Disease Model Cooperative Research Association, Kyoto, Japan) were used. The animals received a basal diet supplemented with 0.3% NaCl (control group) or a high salt diet supplemented with 8% NaCl (high salt group). They were maintained in an air-conditioned room ($23\pm1^{\circ}$C and $60\pm10$% humidity) under an artificial 12-h light/dark cycle (0700–1900) for 4 weeks. Each diet and water were given *ad libitum* during the experimental period. The systolic blood pressure was determined in conscious rats by the indirect tail-cuff method once a week. The study protocols were performed according to the guidelines of the Laboratory Animal Care and Use of Mukogawa Women's University.

### *Aortic preparations*

At the end of the experiment, the rats were anaesthetized with pentobarbitone sodium (40 mg $kg^{-1}$, i.p.). The thoracic aortas were then removed and immediately placed in Krebs-Henseleit solution (mM: NaCl 118.4. KCl 4.7, $CaCl_2$ 2.5, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 11.1). The aortas were 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% $O_2$ and 5% $CO_2$ 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 8K21, NEC San-Ei).

### *Vascular relaxation studies*

Aortic rings with intact endothelium were preconstricted with 0.1 $\mu$M noradrenaline to generate approximately 80% maximal contraction to noradrenaline. This concentration of noradrenaline produced a sustained contraction against which agonist-induced relaxations could be satisfactorily obtained. When the contractile response reached a plateau, ACh (0.1 nM–10 $\mu$M), adenosine diphosphate (ADP, 1 nM–1 $\mu$M) or calcium ionophore A23187 (A23187, 0.1 nM–0.1 $\mu$M) was cumulatively added to the bath solution. The rings were then repeatedly washed and equilibrated for 30 min before starting the next protocol. In some experiments, the rings were preincubated with indomethacin (IND, 10 $\mu$M), $N^G$-nitro-L-arginine methyl ester (L-NAME, 100 $\mu$M), or superoxide dismutase (150 U $ML^{-1}$) plus catalase (1200 U $ML^{-1}$) for 30 min and then the contraction–relaxation procedure described above was repeated. Endothelium-denuded aortic rings were preconstricted with noradrenaline (0.1 $\mu$M), and sodium nitroprusside (SNP, 0.1 nM–1 $\mu$M), nitroglycerin (NG, 0.1 nM–1 $\mu$M) or 8-bromo-cyclic GMP (1–100 $\mu$M) was cumulatively added to the bath medium. Denudation of the endothelium was confirmed pharmacologically by the disappearance of the 1 $\mu$M ACh-induced relaxation response. The relaxation responses obtained were expressed as a percentage of the maximal relaxation evoked by papaverine (100 $\mu$M).

### *Sandwich bioassay studies*

The amount of endothelium-derived relaxing factors (EDRF), namely NO, was measured by a modified bioassay method using a 'sandwich' preparation, as described in a previous paper (Kagota *et al*., 1998). Briefly, helical strips (5 mm long and 3 mm wide) were prepared from aortas of both SHR and Wistar rats (male, 7 weeks old). The endothelium-intact strip from two groups of SHR was employed as a donor strip, and the endothelium-denuded strip from Wistar rats as a detector strip. Denudation of endothelium in the detector strip was confirmed pharmacologically by the disappearance of the 1 $\mu$M ACh-induced relaxation response. A donor strip was sandwiched (layered) with its intimal surface against the intimal surface of a detector strip, and one end of the sandwich was fastened with a clip. The other end of each strip was separately suspended on a hook in a 10-ml organ bath filled with the Krebs-Henseleit solution. The donor strip was kept in as close a contact as possible with the detector strip to maximize the effect of the relaxing factors released from the endothelium of the donor strip. A pair of donor and detector strip segments was preconstricted with noradrenaline (0.01 $\mu$M). When the noradrenaline-induced contraction reached a plateau, ACh (0.1 nM–10 $\mu$M) was cumulatively added to the bath medium. The relaxation responses obtained were expressed as a percentage of the maximal relaxation evoked by papaverine (100 $\mu$M).

### *Determination of cyclic GMP level*

Endothelium-intact rings from SHR were placed in Krebs-Henseleit solution bubbled with a gas mixture of 95% $O_2$ and 5% $CO_2$. After 30-min incubation in the presence of IND (10 $\mu$M), the rings were preconstricted with noradrenaline (0.1 $\mu$M) for 5 min and then stimulated with ACh (0.1 $\mu$M) or SNP (0.01 $\mu$M) for 1 or 3 min, respectively. This stimulation time and concentration allowed the generation of approximately 80% of the maximal relaxation. The tissues were immediately frozen in liquid nitrogen and then homogenized in a glass/glass homogenizer in ice-cold 6% trichloroacetic acid. The homogenates were centrifuged at $3000\times g$ for 15 min at 4°C. The supernatants were extracted three times in five volumes of ether, and the aqueous phase was lyophilized. The cyclic GMP content was determined

with an enzyme immunoassay kit (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). Protein in the precipitates was determined by the method of Lowry *et al*. (1951), with bovine serum albumin as the standard. The amount of cyclic GMP increased by ACh or SNP was calculated from the difference between the cyclic GMP levels in the presence and absence of ACh or SNP, respectively, and this value was expressed in picomoles cyclic GMP per milligram protein of the sample.

### *Immunoblot analysis*

The thoracic aortas of SHR were homogenized in a glass/glass homogenizer in a lysis buffer (50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 10 mM EDTA, 0.1% Tween-20, 0.01% (v $v^{-1}$) protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 1 mM dithiothreitol. Protein concentration was determined for each sample using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, U.S.A.). An equivalent amount of total aortic protein (20 $\mu$g) was loaded on each lane and electrophoresed in a 7.5% SDS-polyacrylamide gel (Biomate Co., Ltd., Tokyo, Japan) by electrophoresis. The proteins were then transferred to Hybond ECL membranes (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) by semi-dry electroblotting (Owl Scientific Inc., Woburn, MA, U.S.A.) for 60 min. The membrane was blocked with 5% blocking reagent (Amersham Pharmacia Biotech) supplied in PBS containing 0.1% Tween-20 for 1.5 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, U.S.A.) or mouse monoclonal anti-sGC antibody (B4) (1 : 2500 dilution, generous gift from Prof Ferid Murad) in PBS containing 0.1% Tween-20. Thereafter, it was washed and finally incubated with a goat anti-mouse IgG conjugated to horseradish peroxidase (1 : 2500 dilution, Transduction Laboratories) for 1 h at room temperature. Subsequent detection of the specific proteins was detected by enhanced chemiluminescence (ECL Western blotting analysis system, Amersham Pharmacia Biotech) on X-ray film (Amersham Pharmacia Biotech). The X-ray film was scanned into an Adobe Photoshop program (Ver. 3.0) with an Epson scanner (GT-9000) and transferred to the Macintosh NIH-Image program (Ver. 1.61). The density of the bands was measured using NIH-Image gel macros. Proteins extracted from human aortic endothelial cells (Transduction Laboratories) and those from rat lung were used as positive controls for eNOS and sGC, respectively. We took the density of the band representing the control group as 100% to calculate the relative density of other bands on the same gel. Also, the eNOS and sGC protein signals were normalized to the respective signals of beta-actin, a constituent in a wide variety of tissues, and alpha-actin, a specific smooth muscle cell marker, respectively. For this purpose, a parallel gel with identical samples was also subjected to the electrophoresis, blotted onto nitrocellulose, and subjected to Western blot analysis with monoclonal antibody against beta-actin (1 : 5000 dilution, Sigma, St. Louis, MO, U.S.A.) or alpha-actin (1 : 10,000 dilution, Progen, Heidelberg, Germany). The signals were obtained as eNOS/beta-actin and sGC/alpha-actin ratios, respectively.

### *Drugs*

Drugs used in the present experiments were as follows: ACh chloride (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan); A23187, 8-bromo-cyclic GMP, IND and L-NAME (Sigma Chemical Co., St. Louis, MO, U.S.A.); SNP and papaverine hydrochloride (Nacalai Tesque Inc., Kyoto, Japan); noradrenaline (Sankyo Co., Ltd., Tokyo, Japan); ADP (Kohjin Co., Ltd., Tokyo, Japan); NG (Millisrol, Nihon Kayaku Co., Ltd., Tokyo, Japan); superoxide dismutase (Toyobo Co., Ltd., Osaka, Japan) and catalase (PL Biochemicals Inc., Milwaukee, WI, U.S.A.). Other chemicals of analytical reagent grade were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

IND was dissolved in dimethylsulphoxide, and the final concentration of dimethylsulphoxide in the Krebs-Henseleit solution was 0.01% (v $v^{-1}$), which did not influence vascular responses. Superoxide dismutase and catalase were dissolved in Dulbecco's phosphate-buffered saline (pH 7.4). All other compounds were dissolved in distilled water.

### *Data analysis*

Data are expressed as means ± s.e.mean. Individual concentration–response curve was characterized by determining the $pEC_{50}$ (negative logarithm molar concentration of agonist required to produce 50% of the maximal response) and the $R$ (maximum relaxation response at the highest concentration of agonist). $pEC_{50}$ values were calculated using the computer program PHARM/PCS v. 4.0 (Tallarida & Murray, 1987). To compare variances of groups of measurements, the $F$-test was used. Statistical analysis was performed using unpaired Student's $t$-test between the data from the control and high salt groups. Differences were considered statistically significant at $P<0.05$.

## Results

### *Systolic blood pressure and heart rate*

The mean systolic blood pressure of SHR increased with feeding of the high salt diet. At the end of the experiment, the blood pressure markedly increased in the high salt group compared to the control group (the high salt group, 259 ± 9 *versus* the control group, 218 ± 6 mmHg; $P<0.05$), while the blood pressure of WKY only slightly increased by the same treatment (the high salt group, 156 ± 4 *versus* the control group, 143 ± 4 mmHg; $P<0.05$). The heart rate tended to be increased by high salt intake, but the difference was not statistically significant in SHR (the high salt group, 385 ± 8 *versus* the control group, 366 ± 7 beats/min; $P=0.08$) and WKY (the high salt group, 388 ± 14 *versus* the control group, 364 ± 9 beats/min; $P=0.08$).

### *Endothelium-dependent relaxations*

There was no significant difference between the high salt group and the control group in the contractile responses induced by noradrenaline in endothelium-intact aortic rings from SHR (the high salt group, 84.7 ± 4.5 *versus* the control group, 90.4 ± 2.6 g/g wet weight of tissue; $P=0.35$) and WKY (the high salt group, 91.4 ± 4.8 versus the control

group, 103±7 g/g wet weight of tissue; $P=0.20$). Figure 1 shows the endothelium-dependent relaxations in response to ACh, ADP and calcium ionophore A23187 in endothelium-intact aortic rings from SHR and WKY. Table 1 also shows the values of vasodilator potency ($pEC_{50}$ and $R$) estimated from concentration–response curves of these agonists. In aortic rings from SHR, the relaxations in response to ACh, ADP and calcium ionophore A23187 were significantly attenuated in the high salt group compared to the control group. However, these effects of the high salt diet were not significant in aortas from WKY. Furthermore, when the animals were fed the normal salt diet, the relaxations in response to these agonists were not significantly different between SHR and WKY.

These relaxations in both groups were completely inhibited by L-NAME, a NO synthase inhibitor, but not affected by neither IND, a cyclo-oxygenase inhibitor, nor superoxide dismutase plus catalase, scavengers of superoxide anions (data not shown).

### *Endothelium-independent relaxations*

There was no significant difference between the high salt group and the control group in the contractile response induced by noradrenaline in endothelium-denuded aortic rings from SHR (the high salt group, 81.8±16.0 *versus* the control group, 111±10 g/g wet weight of tissue; $P=0.15$) and WKY (the high salt group, 98.5±7.5 *versus* the control group, 112±8 g/g wet weight of tissue; $P=0.26$).

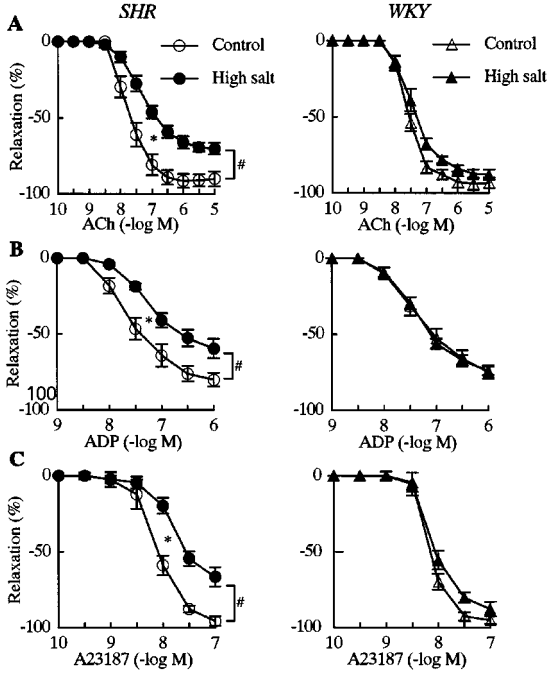


**Figure 1** Endothelium-dependent relaxations in response to acetylcholine (ACh) (A), adenosine diphosphate (ADP) (B) and calcium ionophore A23187 (A23187) (C) in thoracic aortas from spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) fed a normal salt diet (0.3% NaCl, control group, $n=6$) or a high salt diet (8% NaCl, high salt group, $n=6$) for 4 weeks. Results are expressed as the mean± s.e.mean. Comparisons between the data of $pEC_{50}$ and $R$ from the high salt group and the control group were done by unpaired Student's $t$-test; * and # statistically different with $P<0.05$, respectively.

Figure 2 presents the endothelium-independent relaxations in response to SNP and NG, which are NO donors, in endothelium-denuded aortic rings from SHR and WKY. Table 1 also shows the values of vasodilator potency ($pEC_{50}$ and $R$). In endothelium-denuded aortic rings from SHR, the relaxation in response to SNP was significantly attenuated in the high salt group compared to the control group, but the maximum response remained unchanged. The NG-induced relaxation was also significantly attenuated. However, these effects of high salt diet were not observed in aortas from WKY on the relaxations.

On the other hand, as shown in Figure 3 and Table 1, the relaxation in response to 8-bromo-cyclic GMP, a stable cyclic GMP analogue, was not affected by high salt diet in endothelium-denuded aortic rings from SHR.

### *NO release from endothelium*

The 'sandwich' preparations were constituted of a donor strip (endothelium-intact strip from SHR) and a detector strip (endothelium-denuded strip from Wistar rats). In the preparations, ACh could induce relaxations in the detector strip without endothelium. These relaxations are considered to reflect bioactive NO release and transfer from the donor strip with endothelium. As shown in Figure 4, ACh relaxed the detector strip by stimulating the donor strips from both SHR groups, and the degree of relaxation was slightly but significantly greater in the high salt group than the control group ($pEC_{50}$: the high salt group, 7.51±0.09 *versus* the control group, 7.21±0.09; $P<0.05$), but the maximum response remained unchanged ($R$: the high salt group, 78.8±2.3 *versus* the control group, 86.7±3.6%; $P=0.09$).

At the end of the experiments, a thin cellophane sheet was inserted between donor and detector strip segments, and it was confirmed that ACh causes relaxation in the donor strip but not in the detector strip.

### *Production of cyclic GMP*

Figure 5 shows that cyclic GMP levels increased in SHR aortic rings stimulated by ACh (0.1 $\mu$M, 1 min) or SNP (0.01 $\mu$M, 3 min) at the end of the experiment. Under each condition, approximately 80% of the maximal relaxation was obtained. The increased cyclic GMP levels induced by both stimulants were significantly lower in the high salt group than the control group.

### *Protein levels of eNOS and sGC*

Figure 6 presents the protein levels of eNOS and sGC in aortas from SHR fed the normal or high salt diet for 4 weeks. The high salt group showed a significant increase in protein level of eNOS compared to the control group, but eNOS/beta-actin ratio did not change significantly (Figure 6A and C). In contrast, the protein level of sGC was dramatically reduced in the high salt group compared to the control group (Figure 6B and D).

## Discussion

The mechanism underlying the relationship between excessive salt intake and hypertension is not fully understood. The

**Table 1** Values of $pEC_{50}$ and $R$ of relaxation responses to acetylcholine (ACh), adenosine disphosphate (ADP), calcium ionophore A23187 (A23817), sodium nitroprusside (SNP), nitroglycerin (NG) and 8-bromo-cyclic GMP in thoracic aortas from spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) fed a normal salt diet (0.3% NaCl, control group, $n=6$) or a high salt diet (8% NaCl, high salt group, $n=6$) for 4 weeks

| | *Control* | | *High salt* | |
|---|---|---|---|---|
| *Agonist* | *$pEC_{50}$* | R (%) | *$pEC_{50}$* | R (%) |
| SHR | | | | |
| ACh | 7.66±0.09 | 90.0±4.9 | 7.26±0.11* | 71.1±3.7* |
| ADP | 7.53±0.10 | 80.0±4.5 | 7.23±0.05* | 58.5±6.0* |
| A23187 | 8.00±0.10 | 95.5±2.3 | 7.82±0.07* | 66.3±4.8* |
| SNP | 8.37±0.04 | 98.6±0.5 | 8.05±0.03* | 93.7±1.2 |
| NG | 7.84±0.06 | 92.5±1.8 | 7.42±0.08* | 78.1±3.7* |
| 8-bromo-cyclic GMP | 5.03±0.05 | 59.9±2.9 | 4.98±0.05 | 52.6±0.8 |
| WKY | | | | |
| ACh | 7.54±0.06 | 93.4±3.4 | 7.42±0.10 | 87.9±3.5 |
| ADP | 7.32±0.11 | 74.8±4.3 | 7.38±0.08 | 74.0±2.7 |
| A23187 | 8.07±0.05 | 95.1±2.6 | 7.97±0.05 | 87.7±3.4 |
| SNP | 8.24±0.06 | 97.5±1.4 | 8.10±0.02 | 96.0±1.3 |
| NG | 7.79±0.05 | 96.3±1.5 | 7.68±0.02 | 91.8±0.5 |

Results are expressed as the mean±s.e.mean. $pEC_{50}$; negative logarithm molar concentration required to produce 50% of the maximal response. $R$; relaxation responses at the maximum concentration used. Comparisons between data from the high salt group and the control group were done by unpaired Student's $t$-test; *statistically different with $P>0.05$, respectively.

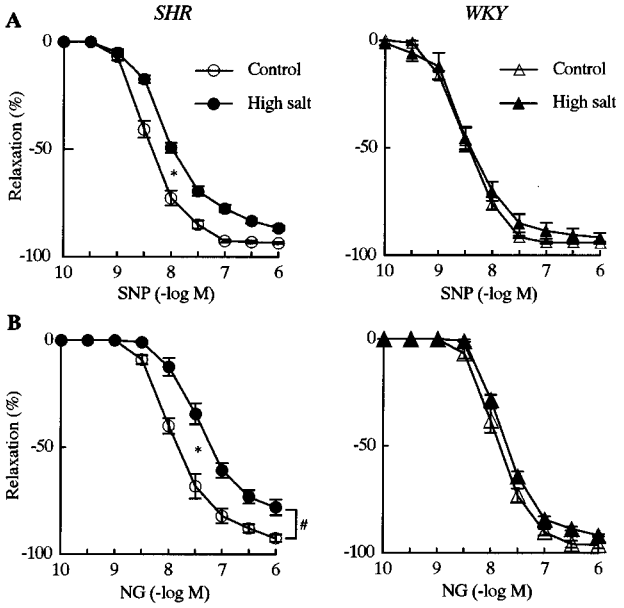


**Figure 2** Endothelium-independent relaxations in response to sodium nitroprusside (SNP) (A) and nitroglycerin (NG) (B) in thoracic aortas from spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) fed a normal salt diet (0.3% NaCl, control group, $n=6$) or a high salt diet (8% NaCl, high salt group, $n=6$) for 4 weeks. Results are expressed as the mean±s.e.mean. Comparisons between the data of $pEC_{50}$ and $R$ from the high salt group and the control group were done by unpaired Student's $t$-test; * and # statistically different with $P<0.05$, respectively.

present study shows that SHR receiving excessive salt displayed significantly elevated blood pressure and vasodilator dysfunction in the aortic preparation. In this study, we demonstrated that aortas from SHR fed the high salt diet show: (1) impairment of both endothelium-dependent and -independent relaxations; (2) no change in cyclic GMP-induced relaxation; (3) a slight increase in bioactive NO release from endothelium; (4) a decrease in cyclic GMP level produced by NO; and (5) a slight increase in eNOS expression and a decrease in sGC expression. These findings indicate that in aortas of SHR, the decreased relaxation due to excessive dietary salt results from a greater decrease in the sGC expression in smooth muscle cells beyond an increase in the eNOS expression in the endothelium. These effects of excessive dietary salt seem likely to be events occurring in SHR with genetic hypertension, because we also were able to demonstrate that excessive salt intake produces little effect on either blood pressure or vasodilator response in normotensive Wistar-Kyoto rats.

Impaired endothelium-dependent relaxations to ACh and other agonists are recognized in many animal models of hypertension. However, this dysfunction presents different characteristics depending on the model studied (Boulanger, 1999). In Dahl-salt sensitive rats, impaired eNOS activity (Hayakawa & Raij, 1997) and increased endogenous NOS inhibitor (Matsuoka *et al*., 1997) are associated with a decrease in endothelium-dependent relaxations. Furthermore, in large arteries from aged SHR, endothelium-dependent relaxations are impaired because of the concomitant augmented release of a contractile factor (Lüscher & Vanhoutte, 1986) and superoxide anions (Bauersachs *et al*., 1998). On the other hand, it has been reported that ACh-induced relaxations in aorta from 17-week-old SHR are almost the same as those in age-matched WKY (Kitagawa *et al*., 1995). In the present study, similar results were observed in 8-week-old SHR fed the normal salt diet. However, we found that the excessive salt intake impairs endothelium-dependent relaxations in response to ACh, ADP (receptor-mediated each) and A23187 (nonreceptor-mediated) in aortas from young SHR. These relaxations seem to be mediated mainly by NO, because they almost disappear in the presence of L-NAME, an inhibitor of NOS. To assess the association between NO release and impaired relaxation due to excessive salt intake, we measured the amount of bioactive NO released from the endothelium by the sandwich bioassay together with the protein expression of eNOS by Western blot study. Surprisingly, the results showed significant

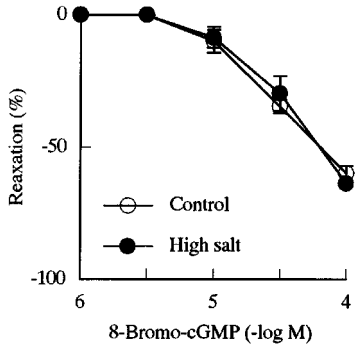


**Figure 3** Endothelium-independent relaxations in response to 8-bromo-cyclic GMP in thoracic aortas from spontaneously hypertensive rats (SHR) fed a normal salt diet (0.3% NaCl, control group, $n=6$) or a high salt diet (8% NaCl, high salt group, $n=6$) for 4 weeks. Results are expressed as the mean ± s.e.mean. Comparisons between the data of $pEC_{50}$ and $R$ from the high salt group and the control group were done by unpaired Student's $t$-test.

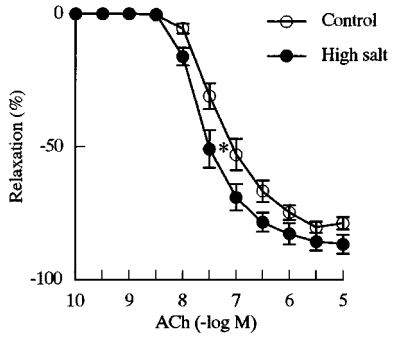


**Figure 4** Relaxation responses in detector strips without endothelium to nitric oxide (NO) released from donor strips with endothelium induced by acetylcholine (ACh). A donor strip was prepared from thoracic aortas of spontaneously hypertensive rats (SHR) fed a normal salt diet (0.3% NaCl, control group, $n=6$) or a high salt diet (8% NaCl, high salt group, $n=6$) for 4 weeks. A detector strip was prepared from thoracic aortas of Wistar rats. Results are expressed as the mean ± s.e.mean. Comparisons between the data of $pEC_{50}$ and $R$ from the high salt group and the control group were done by unpaired Student's $t$-test; * statistically different with $P<0.05$.

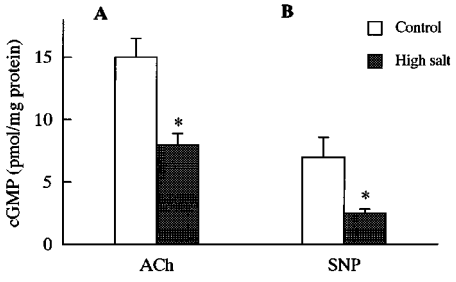


**Figure 5** Cyclic-GMP levels induced by acetylcholine (ACh, 0.1 $\mu$M, 1 min) (A) and sodium nitroprusside (SNP, 0.01 $\mu$M, 3 min) (B) in thoracic aortas from spontaneously hypertensive rats (SHR) fed a normal salt diet (0.3% NaCl, control group, $n=6$) or a high salt diet (8% NaCl, high salt group, $n=6$) for 4 weeks. Results are expressed as the mean ± s.e.mean. Comparisons between the data from the high salt group and the control group were done by unpaired Student's $t$-test; * statistically different with $P<0.05$.

increases in either NO release or eNOS expression in aortas of SHR fed the high salt diet. Therefore, it is unlikely that excessive salt-induced vasodilator dysfunction arises from an impaired eNOS/NO pathway followed by decreased NO production and release. Alteration in the smooth muscle seems likely to be involved in the pathogenesis of the excessive salt-induced vascular dysfunction.

It is well-known that NO activates the soluble isoform of GC to form cyclic GMP. The increased cyclic GMP level causes vascular smooth muscle relaxation and regulates vascular tone in various vascular beds (Förstermann *et al*., 1986; Koesling & Friebe, 1999). In aged SHR, altered vascular activity of the NO-cyclic GMP pathway, such as reduced sGC activity and decreased cyclic GMP accumulation, has been reported (Kojda *et al*., 1998; Shirasaki *et al*., 1988). In the present study, the endothelium-independent relaxations in aortas induced by a NO-generator, such as SNP and NG, were impaired by excessive salt intake. Similar results have been reported for the mesenteric artery of SHR fed a high salt diet (Mervaala *et al*., 1997). We also confirmed that the increased cyclic GMP level in response to ACh or SNP was significantly suppressed by excessive salt loading despite the fact that the relaxation in response to 8-bromo-cyclic GMP, a stable cyclic GMP analogue, remained unchanged. These findings suggest that in SHR, excessive salt intake primarily causes a decrease in sGC activity or protein level followed by a decrease in cyclic GMP formation, leading to impairment of the smooth muscle relaxation, beyond the rise in eNOS activity and expression. In the present study, we demonstrated that salt loading markedly reduces the sGC protein level in SHR aortas. Thus, the vasodilator dysfunction must be associated with suppression of the sGC/cyclic GMP pathway. Recently, decreased sGC expression has been documented in aged SHR aorta (Ruetten *et al*., 1999). These findings support our view that impairment of the sGC/cyclic GMP pathway, not the eNOS/NO pathway, contributes to the vascular dysfunction in salt-induced hypertension of SHR. In young SHR, excessive dietary salt may accelerate these changes caused by ageing. It has been shown that reduced expression of sGC in the kidney of Dahl salt-sensitive rats is associated with elevated blood pressure by dietary salt (Azam *et al*., 1998). Further studies, such as control of blood pressure by a drug, are necessary to demonstrate that changes in sGC/cyclic GMP pathway are due to the increase of blood pressure by high salt intake or the effect of salt itself.

Superoxide production has been reported to trigger desensitization of vascular sGC in hypertension (Kojda *et al*., 1998). However, this can not explain the present findings given that impaired relaxation in response to ACh in aortas from SHR fed the high salt diet could not be restored by pretreatment with superoxide dismutase plus catalase. Similar data have been reported for the aorta of SHR (Auch-Schwelk *et al*., 1989) and the forearm vasculature of patients with essential hypertension (Garcia *et al*., 1995). Furthermore, it is possible that sGC expression may be inhibited by overproduction of NO, namely the presence of a negative autoregulatory mechanism (Scott & Nakayama, 1998; Filippov *et al*., 1997; Papapetropoulos *et al*., 1996). This might be plausible in the case of salt-loaded SHR, because NO synthesis increases with a high salt diet as described above. Thus, we speculate that excessive salt intake results in

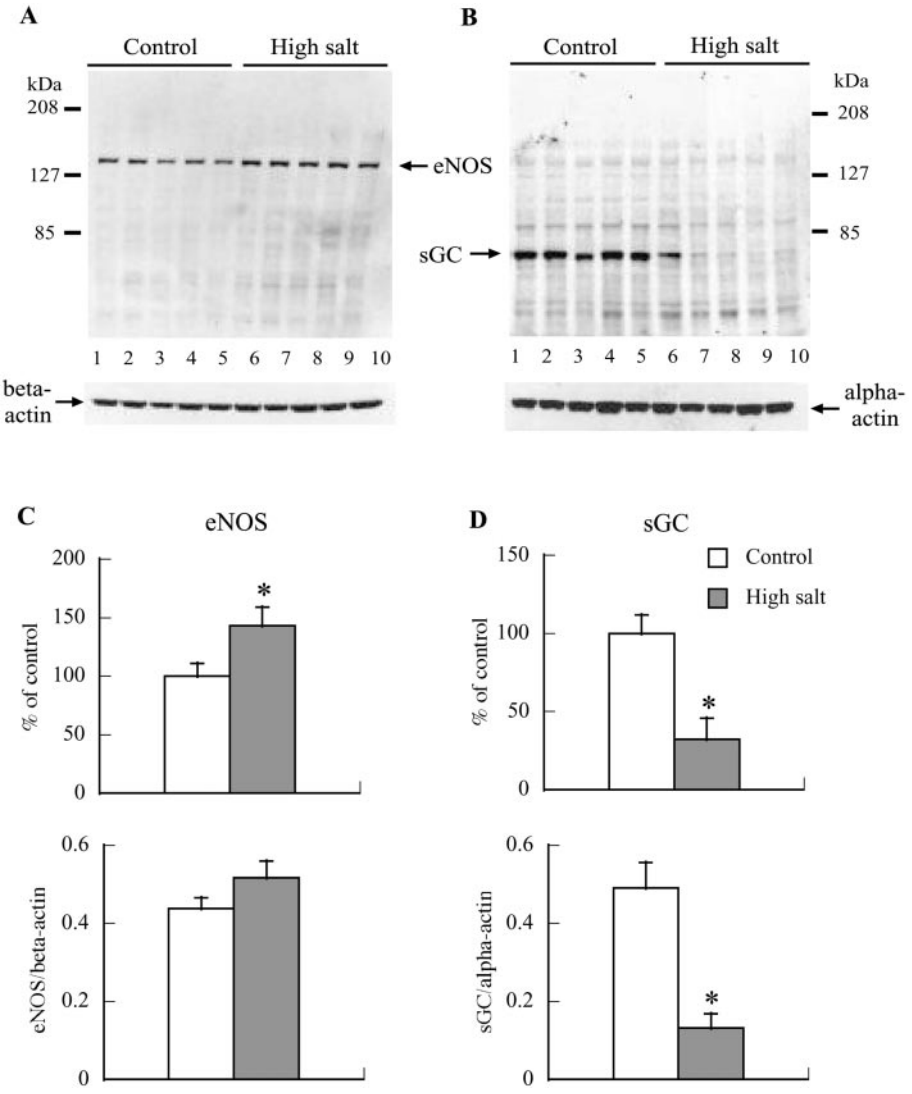


**Figure 6** Immunoblot analyses of endothelial nitric oxide synthase (eNOS) (A, C) and soluble guanylyl cyclase (sGC) (B, D). Thoracic aorta from spontaneously hypertensive rats (SHR) fed a normal salt diet (0.3% NaCl, control group, $n=5$) (lanes 1–5) or a high salt diet (8% NaCl, high salt group, $n=5$) (lanes 6–10) for 4 weeks was isolated and blot analysed separately. The eNOS and sGC protein signals were normalized to the corresponding signals of beta- and alpha-actin for each lane. Quantitative results are expressed as the mean ± s.e.mean. Comparisons between the data from the high salt group and the control group were done by unpaired Student's *t*-test; * statistically different with $P<0.05$.

progressive downregulation of sGC directly or through an increase in NO production and that conversely, the increased eNOS expression contributes to downregulate sGC. Further studies are needed to clarify the mechanisms underlying the downregulation of sGC by excessive salt intake.

In summary, the present study reveals that in SHR aorta, excessive dietary salt impairs vascular relaxations, and this impairment is mediated by reduced cyclic GMP production as a result of a reduced protein level of sGC in smooth muscle in spite of enhanced NO production in the endothelium. These changes may be partly associated with the development of hypertension due to excessive salt intake.

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