Study on the Relationship Between Plasma Nitrite and Nitrate Level and Salt Sensitivity in Human Hypertension

Modulation of Nitric Oxide Synthesis by Salt Intake

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Background—High salt intake suppresses the effect of nitric oxide (NO) in the peripheral resistance vessels in animal models. We tested the hypothesis that the modulation of endogenous NO is related to salt sensitivity in human hypertension.

Methods and Results—Inpatients with essential hypertension (n=24) were maintained on a normal-salt diet (12 g/d NaCl) for 3 days, a low-salt diet (2 g), a high-salt diet (20 to 23 g), and a low-salt diet for 7 days. Normotensive subjects (n=16) were maintained on the first 2 salt diets. The hypertensive patients whose average 24-hour blood pressure was increased by >5% by salt loading were assigned to group 1 (n=8) and the others to group 2 (n=16). Nitrate plus nitrite (NO\(_x\)) was measured by the Griess method, and asymmetrical dimethylarginine (ADMA) by high-performance liquid chromatography. The plasma NO\(_x\) level during the normal-salt diet was lower in group 1 than in group 2 and the normotensive group. After salt loading, the plasma NO\(_x\) level was decreased and reversed after the second salt restriction. Plasma ADMA level was increased after salt loading and decreased after salt restriction. The change in plasma NO\(_x\) level was correlated inversely with those in blood pressure (\(r=−0.59\), \(P=0.0007\)) and plasma ADMA level (\(r=−0.64\), \(P=0.003\)) after salt loading and restriction.

Conclusions—Modulation of NO synthesis by salt intake may be involved in a mechanism for salt sensitivity in human hypertension, presumably via the change in ADMA.

Key Words: nitric oxide ■ sodium ■ hypertension

Nitric oxide (NO) contributes to vessel homeostasis by inhibiting vascular smooth muscle tone\(^1\) and growth,\(^2\) platelet aggregation,\(^3\) and leukocyte adhesion to the endothelium.\(^4\) Previous studies on the relationship between NO synthesis and salt intake showed that dietary salt loading had no effect on NO production in the peripheral hindquarter circulation in the rat,\(^5\) although it enhanced the urinary excretion of nitrite and nitrate (NO\(_x\)) as well as cGMP.\(^6\) In Dahl/Rapp salt-resistant rats, ingestion of a high-salt diet impaired the myogenic response of renal afferent arterioles\(^7\) and the dilator response of large cerebral collateral arterioles to cerebral occlusion.\(^8\) It is suggested that high salt intake suppresses NO production or the effect of NO in the peripheral resistance vessels and may affect systemic arterial blood pressure (BP). Even in humans, high salt intake was demonstrated to attenuate NO production.\(^9\) However, little attention has been paid to the relationship between endogenous NO level and the change in BP after salt loading, so-called salt sensitivity.

Analogues of L-arginine, such as asymmetrical dimethyl-L-arginine (ADMA) and N\(^\text{G}\)-monomethyl-L-arginine (L-NMMA), are competitive inhibitors of NO synthase (NOS). ADMA is produced by endothelial cells in culture\(^10\) and blood vessels\(^11\) and is present in plasma and urine of rats and human subjects,\(^12–14\) suggesting that ADMA may be an endogenous inhibitor of NOS in vivo. The renal excretion of ADMA has been shown to be increased in Dahl salt-sensitive rats compared with that in the salt-resistant rat fed a high-salt diet, and it is correlated with the level of BP.\(^15\) In the present study, we investigated the relationship between endogenous NO production and salt sensitivity in patients with essential hypertension (EH) and, in addition, the role of ADMA in salt sensitivity.

Methods

Subjects
Twenty-four patients with EH (15 men and 9 women; mean age, 50 years; range, 20 to 72 years) and 13 normotensive control subjects (11 men and 2 women; mean age, 47 years; range, 20 to 73 years) were investigated after informed consent was obtained from each subject. The study protocols, including salt loading and restriction in the patients with EH and salt restriction in the normotensive subjects, were approved by the Ethics Committee of the University Hospital.
The patients with EH had mild to moderate hypertension, with casual clinic systolic BP between 150 and 180 mm Hg and diastolic BP between 90 and 115 mm Hg. Patients with secondary hypertension were excluded by physical examination, urinalysis, adrenal CT scan, renal arteriography, and hormonal examinations, such as plasma renin activity (PRA), plasma aldosterone concentration, plasma catecholamine concentration, and 24-hour urinary excretions of 17-hydroxycorticosteroids and 17-ketosteroids. Normotensive control subjects were admitted for the evaluation of their atypical chest pain, and the coronary arteriographic examination revealed normal findings. The study was performed when each patient/subject was admitted to our hospital.

**Protocol**

In the patients with EH, all antihypertensive medications were discontinued ≥2 weeks before admission. They maintained a constant daily activity pattern, getting up at 6 AM and going to bed at 9 PM, and were subjected to specific diets arranged by the amount of salt and containing a constant amount of nitrate. They received a series of normal-salt diet (12 g/d NaCl and 500 μmol/d nitrate) for 3 days, low-salt diet (2 g/d NaCl and 500 μmol/d nitrate) for 7 days, high-salt diet (20 to 23 g/d NaCl and 600 μmol/d nitrate) for 7 days, and then low-salt diet for 7 days. Compliance with the prescribed diet was assessed by the measurements of 24-hour urinary sodium excretion on the last days of the low- and high-salt diets.

Blood was drawn from all subjects at 6 AM on the last day of the normal-salt diet for the determination of serum cholesterol, triglyceride, creatinine, sodium, potassium, and chloride concentrations; PRA, and plasma concentrations of aldosterone, norepinephrine (NE), epinephrine, and NOx. On the last day of each salt diet, every 30-minute noninvasive ambulatory BP monitoring was carried out for 24 hours with the use of ABPM-630 (Nippon Kohrin Co). The patients with EH were divided into 2 groups according to the response to salt loading. When the average of the mean BP [(systolic BP – diastolic BP)/3 + diastolic BP] measured for 24 hours on the last day of the high-salt diet exceeded by >5% the average on the last day of the low-salt diet, the patients were classified as group 1. When the increase in the average of mean BP was <5%, the patients were classified as group 2. Blood specimens for the subsequent determinations of PRA and plasma concentrations of aldosterone, ADMA, and NE were obtained from the brachial vein at 6 AM after overnight fasting on the last days of the low- and high-salt diets and for plasma concentration of NOx on the last days of the normal-, low-, and high-salt diets. All subjects were asked to record daily activities and their times and to refrain from smoking during the study period.

Normotensive control subjects were administered the normal-salt diet (12 g/d NaCl and 500 μmol/d nitrate) for 3 days, and then low-salt diet (2 g/d NaCl and 500 μmol/d nitrate) for 7 days. On the last days of the normal- and low-salt diets, every 30-minute noninvasive ambulatory BP monitoring was carried out for 24 hours with ABPM-630, and blood was sampled at 6 AM for the subsequent determination of serum cholesterol, triglyceride, creatinine, sodium, potassium, and chloride concentrations and plasma NOx concentration.

**NOx Analysis**

Plasma NOx levels were measured with the Griess method. Briefly, after being passed through 50-kD ultrafilters, 40 μL of the plasma was diluted with 240 μL assay buffer and mixed with 10 μL cofactor and 10 μL nitrate reductase (NOx colorimetric assay kit, Cayman Chemical Co). After the plasma had been kept at room temperature for 3 hours to convert nitrate to nitrite, total nitrite was measured at 540 nm absorbance by reaction with Griess reagent (sulfanilamide and naphthale–ethylene diamine dihydrochloride). Amounts of nitrite in the plasma were estimated by a standard curve obtained from enzymatic conversion of NaN3 to nitrite.

**ADMA Analysis**

Plasma (1 mL) was mixed with 2 mL of 10% trichloroacetic acid, put on ice for 10 minutes, and centrifuged at 2500g for 15 minutes. The resulting supernatant was evaporated under vacuum to dryness and was then loaded to a Bond Elut PRS column (Varian Associates Inc). After a washing with 10 mL of 1 mol/L pyridine, ADMA was eluted by 10 mL of 3 mol/L ammonia and was again evaporated under vacuum to dryness. The extract was incubated with 20 μL phenylthiocarbamoyl solution (ethanol:triethylamine:water:phenyl isothiocyanate 7:1:1:1, vol/vol) for 20 minutes at room temperature. The dried samples were applied on reverse-phase high-performance liquid chromatography (HPLC) with a YMC-Pack ODS-AM column (YMC Co) and 60 mmol/L acetic buffer (pH 6.6)/0.05% trifluoroacetic acid elution with a linear gradient of acetonitrile ranging from 6% to 60% over a period of 25 minutes at a flow rate of 1 mL/min. Amounts of ADMA in the plasma were estimated from a standard curve of synthetic ADMA (Sigma Chemical Co).

**Analysis of Other Variables**

Serum sodium, potassium, and chloride ion concentrations were measured with a flame photometer. Serum cholesterol, triglyceride, and creatinine concentrations were measured by an autoanalyzer method. Plasma NE and epinephrine concentrations were measured by HPLC. PRA and plasma aldosterone concentration were measured by radioimmunoassay.

**Statistics**

Values are shown as mean±SEM. Differences of mean values were assessed by a paired or unpaired Student’s t test for comparison of 2 variables and by ANOVA for comparison of multiple variables. Relationships between 2 continuous variables were assessed by a regression analysis using the Pearson correlation coefficient. Differences in sex and smoking habit between normotensive and hypertensive groups were analyzed by χ² test. A value of P<0.05 was considered statistically significant.

**Results**

**Patient Profiles**

Table 1 shows clinical characteristics for normotensive subjects and EH patients. Eight EH patients showed a response of the increase in mean BP by >5% after salt loading (group 1), whereas the other 16 showed no or little response (group 2).

**Table 1. Clinical Characteristics of Patients With EH and Normotensive Control Subjects**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients With EH</th>
<th>Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>53±4</td>
<td>48±4</td>
</tr>
<tr>
<td>Sex, M/F</td>
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<td>10/6</td>
</tr>
<tr>
<td>Height, cm</td>
<td>163±2</td>
<td>159±2</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>69±3</td>
<td>64±4</td>
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<tr>
<td>Smoking, yes/no</td>
<td>5/3</td>
<td>10/6</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>197±14</td>
<td>191±9</td>
</tr>
<tr>
<td>Triglyceride, mg/dL</td>
<td>122±24</td>
<td>120±13</td>
</tr>
<tr>
<td>Na, mEq/L</td>
<td>143±1</td>
<td>143±1</td>
</tr>
<tr>
<td>K, mEq/L</td>
<td>3.8±0.1</td>
<td>3.9±0.1</td>
</tr>
<tr>
<td>Cl, mEq/L</td>
<td>105±1</td>
<td>105±1</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.7±0.1</td>
<td>0.9±0.1</td>
</tr>
</tbody>
</table>
Figure 1. Plasma NOx concentrations on last day of each salt diet period in normotensive subjects and in hypertensive patients with EH. Hypertensive patients whose average 24-hour BP was increased by >5\% by salt loading were assigned to group 1 (n=8) and others to group 2 (n=16).

Figure 2. Relationship between percent change in mean BP and percent change in plasma NOx concentration after salt loading and restriction in patients with EH.

Figure 3. Relationship between percent change in mean BP and percent change in plasma NE concentration after salt loading and restriction in patients with EH.
Effect of Salt Loading on Plasma ADMA Concentration

Salt loading resulted in a significant increase in the plasma ADMA concentration, from $1.86 \pm 0.28$ to $2.23 \pm 0.24 \mu mol/L$, in the patients with EH ($P < 0.05$), whereas salt restriction elicited a significant decrease, to $1.76 \pm 0.24 \mu mol/L$ ($P < 0.05$). As shown in Figure 4, the percent change in plasma ADMA concentration was significantly inversely correlated with the percent change in plasma NO$_x$ concentration after salt loading and restriction ($r = -0.64, P = 0.003$).

Discussion

Modulation of NO Synthesis by Salt Intake

Previous studies showed that NO production in response to high salt intake may be impaired in EH patients. Higashi et al. demonstrated that salt loading attenuates the conversion of L-arginine to NO in the endothelium of the renal vasculature in salt-sensitive patients with EH, which was assessed by L-arginine–induced renovascular relaxation and the increase in plasma cGMP in response to L-arginine. Stein et al. showed that the administration of a high-salt diet to normotensive subjects does not alter NO-mediated vascular responsiveness, which was determined by methacholine-induced increase in forearm blood flow. However, these methods are not specific for the assessment of NO synthesis. Methacholine and L-arginine can cause vascular dilatation by mechanisms other than the generation of NO, and cGMP is also a second messenger of atrial natriuretic peptide. An increased breakdown of NO by free radicals may be another possible mechanism for salt-induced impairment of NO-mediated vasorelaxation. In experimental animals, such as spontaneously hypertensive rats, stroke-prone spontaneously hypertensive rats, and Dahl salt-sensitive rats, after a high-salt diet, superoxide production is enhanced in vessels, which was assessed by the effects of superoxide dismutase and direct measurement. Vitamin C has been shown to improve endothelium-dependent vasodilation in patients with EH suggesting that superoxide radicals function as a scavenger of NO in vivo in basal conditions.

NO is rapidly oxidized to nitrite and then to nitrate by oxygenated hemoglobin, molecular oxygen, and superoxide anions and is excreted as such into the urine. Thus, NO$_x$ measurement seems to be a direct method for measurement of NO production. The main drawback of the use of the total nitrate as a measure of NO synthesis is that nitrate may arise from sources other than the metabolism of NO. In mammalian cells, however, NO is synthesized from the guanidino nitrogen atoms of the amino acid L-arginine, and this is the only known route by which these nitrogen atoms may be incorporated into nitrate. Therefore, the measurement of total nitrate may be a specific indicator of total body NO synthesis. However, the measurement of endogenously generated nitrate may be confounded by several factors. The most important factor is the contribution of dietary nitrate to plasma NO$_x$. NO inhaled via tobacco smoke is another source of nitrate.

Campese et al. showed that high salt intake decreases plasma NO$_x$ concentration in black hypertensive patients but did not describe the nitrate content in salt diets. Because the half-life of nitrate was $\approx 8$ hours, $\approx 67\%$ of exogenously derived nitrate seems to be cleared from the plasma after overnight fasting. It is also possible that the patients failed to adhere to the high-salt diet. However, adherence to the diet was confirmed by the measurement of urinary sodium excretion in the present study. On the basis of the constant daily intake of nitrate and the stability of daily activities during the study period, our results indicate that plasma NO$_x$ concentration is decreased after salt loading, even though the high-salt diet included more nitrate than the low-salt diet. In contrast to salt loading, salt restriction significantly increased the plasma NO$_x$ concentration, further indicating that endogenous NO production is modulated by salt intake.

The main purpose of the present study was to elucidate whether endogenous NO production is related to salt sensitivity in human hypertension. The result showed that the plasma NO$_x$ concentration was significantly lower in group 1 EH patients than in group 2 during the high-salt diet, whereas it was similar between groups 1 and 2 during the low-salt diet. Furthermore, the changes in the plasma NO$_x$ concentration after salt loading and restriction were significantly inversely correlated with those in the mean BP. Thus, salt-induced modulation of endogenous NO may be related to salt sensitivity. To clarify whether the NO response to salt intake modulation contributes to the pathogenesis of hypertension or to that of salt sensitivity, we further examined the effect of salt intake modulation on the plasma NO$_x$ concentration in normotensive subjects. No responses of the plasma NO$_x$ concentration and BP after the change from the normal- to the low-salt diets were found in either the normotensive subjects or the group 2 EH patients, whereas there was a significant increase in the plasma NO$_x$ concentration level and a decrease in BP in the salt-sensitive group, indicating that the NO response to salt intake might contribute to the pathogenesis of salt sensitivity in human hypertension.

These lines of evidence raised another possibility, that the changes in plasma NO$_x$ concentration are in response to the changes in BP. Because in group 2 the plasma NO$_x$ concentration was significantly decreased and it increased without any changes in BP after the series of salt loading and
restriction, the changes in plasma NO\textsubscript{3} concentration may not be simply in response to the changes in BP. It has been well documented that the L-arginine–NO pathway is impaired in salt-sensitive experimental hypertension by reduced bioavailability of endothelial NO.\textsuperscript{21–25} In salt-sensitive Dahl rats, the perfusate for isolated kidney has been reported to contain less NO\textsubscript{3} than that in salt-resistant rats.\textsuperscript{26} However, to the best of our knowledge, this is the first report for the significant role of NO in salt sensitivity in human hypertension.

Whereas high salt intake resulted in a decrease in plasma NO\textsubscript{3} concentration, the plasma NE concentration also was reduced by salt loading. However, the mechanism for the change in plasma NO\textsubscript{3} concentration is independent of the changes in plasma NE, because no significant correlation was found between plasma NE and salt sensitivity.

Mechanism for NO Synthesis Suppression by Salt Loading

The suppressant effect of high salt intake on plasma NO\textsubscript{3} concentration could occur by several mechanisms, including altered transport of L-arginine through the endothelial membrane, decreased activity of the enzyme NOS, and an increased breakdown or excretion of NO.\textsuperscript{27} The present study was focused on the endogenous NO\textsubscript{3} antagonist ADMA, which also inhibits L-arginine uptake into endothelial cells, and showed that its plasma level was significantly increased after high salt intake and decreased after salt restriction. The normal plasma level of ADMA was shown to be \( \approx 1 \) to 2 \( \mu \text{mol/L} \).\textsuperscript{11} This level of ADMA alone is probably not sufficient to inhibit NOS activity. However, because methylarginines are concentrated within the cell,\textsuperscript{28} a moderate increase in plasma ADMA concentration, as observed in the present study, may reflect a higher increase of the compound in the vicinity of the NOS and thus result in a decrease in plasma NO\textsubscript{3} concentration. The significant inverse correlation of the percent change in plasma ADMA with the percent change in plasma NO\textsubscript{3} concentration after both salt loading and restriction may be consistent with this assumption. Although the origin of elevated plasma ADMA is unknown, the concentration of ADMA was shown to be increased substantially in the plasma of patients with uremia,\textsuperscript{13} hypertension,\textsuperscript{29} heart failure,\textsuperscript{30} and severe atherosclerosis\textsuperscript{31}; in hypercholesterolemic rabbits;\textsuperscript{32} in rats with heart failure;\textsuperscript{30} and in the urine of salt-loaded Dahl salt-sensitive rats.\textsuperscript{33} An increased production by methylation of L-arginine\textsuperscript{33} and degradation of methylated tissue protein\textsuperscript{34} and/or decreased metabolism to citrulline by dimethylarginine dimethylaminohydrolase\textsuperscript{33} and excretion from the kidney is likely to contribute to the elevation of plasma ADMA, but this issue still remains to be elucidated.

Limitations of the Study

The effects of both salt loading and restriction were examined only in the patients with EH. Therefore, it is not clear whether salt intake generally affects NO synthesis in humans. Because the BP of the present EH patients was elevated mildly to moderately, the finding may be applied to the normotensive subjects.

References

13. Kakimoto Y, Akazawa S. Isolation and identification of \( \text{NO}_2 \), \( \text{NO}_3 \)- and \( \text{NO}_2 \), \( \text{NO}_3 \)-dimethylarginine, \( \text{N}\text{-}\varepsilon\text{-}\text{mono-}, \text{di-}, \text{and trimethyllysine}, \) and glycosylgalactosyl- and galactosyl-delta-hydroxylysine from human urine. J Biol Chem. 1970;245:5751–5758.


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