Intralymphocytic Sodium and Free Calcium Concentration in Relation
to Salt Sensitivity in Patients with Essential Hypertension

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In order to clarify the relation between salt sensitivity and changes in intracellular sodium ([Na]i) and free calcium concentration ([Ca^{2+}]i) after salt loading, [Na]i and [Ca^{2+}]i were determined in lymphocytes of twenty patients with essential hypertension under a low salt diet (3 g/day) and a high salt diet (20 g/day) for seven days, respectively. They were classified as “salt-sensitive” (n = 10) or “nonsalt-sensitive” (n = 10) on the basis of the changes in blood pressure after salt loading.

Both lymphocytic [Na]i and [Ca^{2+}]i were significantly increased with salt loading in salt-sensitive patients (p < 0.05 for both), while they were not affected by salt loading in nonsalt-sensitive patients. Lymphocytic [Ca^{2+}]i showed a positive correlation with lymphocytic [Na]i under both low salt diet (r = 0.62, p < 0.01) and high salt diet (r = 0.70, p < 0.01) in all patients in both groups. In addition, a close and positive correlation was observed between the changes in lymphocytic [Na]i and those in lymphocytic [Ca^{2+}]i after salt loading in all patients in both groups (r = 0.80, p < 0.001).

These results suggest that the increase in [Ca^{2+}]i, possibly linked with the increase in [Na]i, may be involved in elevation of blood pressure in the salt-sensitive patients after salt loading.

There has been increasing evidence of cellular abnormalities in sodium transport and calcium handling associated with elevated intracellular sodium ([Na]i) and free calcium concentration ([Ca^{2+}]i) in patients with essential hypertension.1–5 In addition, it is likely that there are possible links between abnormalities of calcium handling and sodium transport. The Na+-Ca2+ exchange system has been demonstrated in vascular smooth muscle cells by Blaustein6 and in rabbit lymphocyte plasma membranes by Ueda.7 Recently, [Ca^{2+}]i has been reported to regulate sodium fluxes across the cell membrane.8 In a previous study, we also observed that [Ca^{2+}]i showed a positive correlation with [Na]i in lymphocytes and that both [Na]i and [Ca^{2+}]i were higher in hypertensive patients than in normotensive controls.9 Such findings may suggest that the increases in both [Na]i and [Ca^{2+}]i, at least in part, play an important role in the pathogenesis of essential hypertension.

The abnormality in sodium transport may be related to the state of sodium balance.10 For example, this abnormality has been reported to be exaggerated after salt loading11,12 and abolished after treatment with a low salt diet and

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It is now considered that the abnormality is associated with a rise in blood pressure after salt loading in essential hypertension. On the other hand, the relation of intracellular calcium to sodium balance is not well defined, although it is likely that \([Ca^{2+}]_i\) is closely related to \([Na]_i\). If \([Ca^{2+}]_i\) is also important in regulating blood pressure response after salt loading, it will be possible to demonstrate this. Thus in the present study, \([Na]_i\) and \([Ca^{2+}]_i\) in lymphocytes of patients with essential hypertension were measured under low and high salt diets in order to determine 1) whether changes in \([Ca^{2+}]_i\) as well as in \([Na]_i\) are detectable after salt loading and 2) whether such changes, if found, are associated with salt sensitivity.

**MATERIALS AND METHODS**

**Patients**

Twenty Japanese inpatients with essential hypertension composed of 13 males and 7 females with a mean age of 57 years (range: 34–70 years) were studied. No patients had received antihypertensive agents or any other drugs for four weeks before admission. All the patients had systolic blood pressures over 160 mmHg or diastolic blood pressure over 95 mmHg in the sitting position, or both, on at least three occasions in the outpatient clinic. Patients with secondary causes of hypertension and with extensive organ damages were excluded on the basis of appropriate clinical, biochemical and radiological evaluations.

**Study Protocol**

After a control period of one week when the patients were placed on a regular salt diet (10 g/day), all patients underwent a low salt diet (3 g/day) and a high salt diet (20 g/day) in order: each diet continued for one week. The high salt intake was achieved by adding Slow Sodium tablets (600 mg NaCl/tablet) to the diet. Potassium intake was kept constant during the study. On the 7th morning of each diet period, after the fasting patients had maintained a supine position for 30 minutes in a quiet and dark room, blood pressure was measured and then venous blood was obtained from the cubital vein for determinations of lymphocytic \([Na]_i\) and \([Ca^{2+}]_i\).

**TABLE 1** CHARACTERISTICS OF SALT-SENSITIVE AND NONSALT-SENSITIVE PATIENTS WITH ESSENTIAL HYPERTENSION UNDER A REGULAR SALT DIET

<table>
<thead>
<tr>
<th></th>
<th>Salt-Sensitive</th>
<th>Nonsalt-Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><strong>Sex (Male/Female)</strong></td>
<td>6/4</td>
<td>7/3</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>58 ± 9</td>
<td>55 ± 9</td>
</tr>
<tr>
<td><strong>Obesity Index (%)</strong></td>
<td>16 ± 19</td>
<td>16 ± 17</td>
</tr>
<tr>
<td><strong>Mean Blood Pressure (mmHg)</strong></td>
<td>114 ± 9</td>
<td>115 ± 11</td>
</tr>
<tr>
<td><strong>Heart Rate (beats/min)</strong></td>
<td>64 ± 3</td>
<td>65 ± 9</td>
</tr>
<tr>
<td><strong>Serum Creatinine (mg/dl)</strong></td>
<td>1.1 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
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</table>

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Blood pressure was measured with a mercury sphygmomanometer every minute for 10 minutes by the same observer throughout the three diet periods. The average of ten consecutive blood pressure readings was used for comparison of each diet period. Mean blood pressure was calculated as diastolic pressure plus one third of pulse pressure.

Methods

Lymphocytes were isolated with a modification of Boyum’s method. In short, heparinized blood was diluted 1:2 with RPMI-1640 medium (Gibco Laboratories, Ohio, USA), layered on a Ficoll-metrizoate mixture (Lymphoprep, Nyegaard Co, Oslo, Norway) having a density of 1077 g/L and centrifuged at 400g for 35 minutes. The isolated lymphocytes were washed twice in RPMI-1640 at 100g for 10 minutes and prepared for measurements of [Na]i and [Ca2+]i.

[Na]i in lymphocytes was measured by modified Ambrosioni’s method. In brief, the cells were washed twice with cold isotonic MgCl2 solution, transferred to a polycarbonate capillary tube (Hematolon; Kayagaki Irikakogyo, Tokyo, Japan) and centrifuged at 15000g for 5 minutes. The lymphocytes became packed at the bottom of the tube. The tube was cut at the boundary between the packed lymphocytes and the supernatant fluid. The percentage of extracellular fluid volume trapped within the lymphocyte pellet, determined by 131I-labelled human serum albumin, was about 26%. The cells were then weighed and dried at 80°C for 48 hours. Nitric acid (14 mol/l) was then added and the cellular pellet was completely dissolved. [Na]i was determined by flame photometry (Hitachi 775-A, Tokyo, Japan) and expressed in mmol/kg wet weight. The intraassay coefficient of variation was less than 5%, and the day-to-day intrasubject variation was less than 7%.

[Ca2+]i in lymphocytes was determined by modified Tsien’s method. Briefly, the lymphocytes were incubated at 37°C for 40 minutes with 50 μM of the fluorescent dye, quin 2 tetraacetoxyxymethylene (Dojindo Laboratories, Kumamoto, Japan). Then the cells were washed, transferred to fresh RPMI-1640 and left at room temperature for 60 minutes to allow complete hydrolysis of the ester. The evidence against gross toxicity of the quin 2 concentration in the present study was that loaded lymphocytes maintained high (>95%) eosin exclusion. The lymphocytes were centrifuged at 400g for 10 minutes and resuspended in a physiological saline containing (mM): NaCl 145, KCl 5, Na HEPES 10, Na2HPO4 1, CaCl2 1, MgSO4 0.5 and glucose 5, pH 7.4 at 37°C. Fluorescence was measured at 37°C with a Hitachi spectrofluorimeter 204 S (Tokyo, Japan). Excitation and emission wavelengths were 339 and 492 nm with bandwidth of 4 and 10 nm, respectively. Resting levels of lymphocytic [Ca2+]i were calculated by the equation: [Ca2+]i = 115 nM x (F-Min)/(Fmax-F), where 115 nM is the effective dissociation constant Kd of the Ca-quin 2 complex and F is the fluorescence of the intact cell suspension. Fmax was measured by releasing the dye from the cells with digitonin and Fmin was measured after adjusting pH to about 8.5 using enough Tris and setting the extracellular calcium concentration to

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1 mM by adding 2 mM EGTA. The intraassay coefficient of variation was less than 7%, and the day-to-day intrasubject variation was less than 8%.

Statistics

Data are presented as mean ± SD. Statistical analyses were performed using Student’s unpaired t test to compare the different groups and using Student’s paired t test to compare the intrasubject changes in blood pressure, [Na][i] and [Ca2+]i. Results were considered significant if the p value was less than 0.05. Correlations between parameters were tested by linear regression.

RESULTS

Since the median of the percent change in mean blood pressure (%ΔMBP) after salt loading of 50 inpatients with essential hypertension in our laboratory was about +5%, ten patients whose mean blood pressure in the high salt period exceeded by 5% that in the low salt period were classified as salt-sensitive. The remaining 10 patients were classified as nonsalt-sensitive as shown in Fig. 1. No significant differences could be demonstrated in sex, age, obesity index, mean blood pressure and heart rate in the regular salt diet between the two groups (Table I).

Mean values for lymphocytic [Na][i] and [Ca2+]i in salt-sensitive and nonsalt-sensitive patients at the end of low and high salt diets are shown in Fig. 2. As shown in Fig. 2a, [Na][i] was significantly increased in the high salt diet compared with the low salt diet in salt-sensitive patients (20.8 ± 1.6 to 22.3 ± 1.4 mmol/kg wet weight, p < 0.01), while it remained unchanged in nonsalt-sensitive patients (19.9 ± 1.0 to 20.0 ± 1.0 mmol/kg wet weight). As shown in Fig. 2b, [Ca2+]i was also increased with the high salt diet in salt-sensitive patients (134.9 ± 14.5 to 148.7 ± 18.5 nmol/l, p < 0.01), while it was unchanged in nonsalt-sensitive patients (124.3 ± 12.6 to 122.8 ± 11.2 nmol/l). Both [Na][i] and [Ca2+]i in salt-sensitive patients were higher than those in nonsalt-sensitive patients in the low and high salt diets. The differences were statistically significant in the high salt diet (p < 0.005 for [Na][i] and [Ca2+]i), but not significant in the low salt diet. The percent changes in [Na][i] (%Δ [Na][i]) and [Ca2+]i (%Δ [Ca2+]i) after salt loading were significantly higher in salt-sensitive patients than in nonsalt-sensitive patients (8.1 ± 5.2 vs 0.6 ±
Fig. 5. Correlation of percent change in mean blood pressure (%ΔMBP) with (a) percent change in intralymphocytic sodium (%Δ [Na]i) and (b) free calcium concentration (%Δ [Ca²⁺]i) in salt-sensitive and nonsalt-sensitive patients after salt loading.

4.8%, p < 0.005 for [Na]i; 10.2 ± 8.8 vs -0.9 ± 5.7%, p < 0.005 for [Ca²⁺]i, respectively.

In all patients in both groups, there was a positive correlation between [Na]i and [Ca]i under both low and high salt diets (Fig. 3). In addition, a close and positive correlation was also observed between %Δ [Na]i and %Δ [Ca²⁺]i in all patients in both groups of patients (Fig. 4). There was a positive correlation between %ΔMBP and %Δ [Ca²⁺]i as well as %Δ [Na]i (Fig. 5), while the baseline mean blood pressure was not correlated with [Na]i or [Ca²⁺]i under low and high salt diets.

**DISCUSSION**

There has been a widespread interest in the relation of dietary salt intake to blood pressure. A number of epidemiologic studies have shown a positive correlation between dietary salt intake of a population and the incidence of hypertension in that population. However, it is difficult to demonstrate a direct correlation between salt intake and blood pressure level within a population since a high salt intake raises blood pressure in only a small part of the population. Thus, blood pressure responses to salt loading were variable between individuals, although dietary salt intake is well known to be important in the pathogenesis of essential hypertension.

There is an exceedingly complex series of factors regulating blood pressure. These factors respond to changes of salt intake and interact with each other. Blood pressure response after salt loading is an end-product of the changes of such factors. Patients with essential hypertension were divided into the salt sensitive and the nonsalt-sensitive groups on the basis of their blood pressure responses to salt loading. Allopathy mechanisms underlying their differences in salt sensitivity have not been fully elucidated, several possible mechanisms have been reported. These include a greater sodium retention, impaired suppression of the renin-angiotensin-aldosterone system and enhanced vascular reactivity.

As another possible mechanism, the sodium transport hypothesis has been proposed by Dahl, Haddy, de Wardener, Blaustein, and many others to account for the involvement of the volume expansion after salt loading for an elevation of blood pressure through increased [Na]i and [Ca²⁺]i. A negative correlation between changes in blood pressure and changes in red cell Na efflux rate constant after salt loading in patients with essential hypertension was reported by Morgan et al. Ambrosioni et al reported that moderate salt restriction decreased both blood pressure and lymphocytic [Na]i in borderline hypertensives. The present study also showed a different effect of salt loading on %Δ [Na]i in lymphocytes of salt-sensitive and

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nonsalt-sensitive patients, and a positive correlation between $\% \Delta [\text{Na}]i$ and $\% \Delta \text{MBP}$ in all patients in both groups. These findings may be consistent with the responsibility of [Na]i for salt sensitivity.

The other important difference between salt-sensitive and nonsalt-sensitive patients was the changes of $[\text{Ca}^{2+}]i$ after salt loading. Lymphocytic $[\text{Ca}^{2+}]i$ was increased in salt-sensitive patients but not in nonsalt-sensitive patients. In addition, $\% \Delta [\text{Ca}^{2+}]i$ as well as $\% \Delta [\text{Na}]i$ had a positive correlation with $\% \Delta \text{MBP}$. We, therefore, confirmed that not only $[\text{Na}]i$ but also $[\text{Ca}^{2+}]i$ is one of the factors associated with salt sensitivity. However, it is unknown how salt loading influences $[\text{Ca}^{2+}]i$. The control of cellular calcium metabolism has not been fully understood and there is few reports concerning the relation of $[\text{Ca}^{2+}]i$ to the state of sodium balance. A positive correlation between $\% \Delta [\text{Na}]i$ and $\% \Delta [\text{Ca}^{2+}]i$ may indicate that the changes in $[\text{Ca}^{2+}]i$ after salt loading is secondary to the changes in $[\text{Na}]i$. This interpretation is consistent with the proposal of Blaustein that an elevation of $[\text{Na}]i$ may inhibit $\text{Na}^+ - \text{Ca}^{2+}$ exchange and thereby raise $[\text{Ca}^{2+}]i$. However, it is impossible to deny the possibility that $[\text{Ca}^{2+}]i$ regulates sodium transport across the cell membrane. Further studies are necessary to clarify the precise mechanism involved in the increase in $[\text{Ca}^{2+}]i$ after salt loading.

In summary, there were differences between the salt-sensitive and nonsalt-sensitive patients in the changes in both $[\text{Na}]i$ and $[\text{Ca}^{2+}]i$ after salt loading. In addition, the changes in $[\text{Na}]i$ showed a positive correlation with changes in $[\text{Ca}^{2+}]i$. These results suggest that the relationship between $[\text{Ca}^{2+}]i$ and $[\text{Na}]i$ after salt loading may be involved in salt sensitivity in patients with essential hypertension.

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