Na⁺, K⁺-ATPase Inhibitory Activity of Fractionated Urine during Changes in Dietary Sodium Intake in Man

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Abstract

Na⁺, K⁺-ATPase inhibitory activity in urine fractionated by HPLC was quantified in 7 normotensive male subjects during changes in dietary sodium intake. Subjects were studied on free sodium intake for 2 days, on low sodium intake (2 g/day) for 3 days, on high sodium intake (22 g/day) for 4 days and subsequently on normal sodium intake (6 g/day) for 2 days. Na⁺, K⁺-ATPase inhibitory activity in fraction 10 eluted with 17% acetonitrile by reverse-phase HPLC was $12.3 \pm 5.2\%$ (mean $\pm$ S.D.) on free sodium intake, $8.7 \pm 9.8\%$ on the 3rd day of low sodium intake, $61.2 \pm 6.6\%$ on the 4th day of high sodium intake, and $20.5 \pm 0.7\%$ on the 2nd day of the normal sodium intake. Changes in Na⁺, K⁺-ATPase inhibitory activity of fraction 10 were closely associated with those in urinary sodium excretion. These results suggest that an endogenous Na⁺, K⁺-ATPase inhibitor(s) which plays a physiological role in the control of sodium and water balance may exist in this particular fraction.

Numerous studies have indicated that plasma volume expansion or an increased intake of sodium causes a natriuresis and enhances vascular reactivity (de Wardener and Clarkson, 1985). These phenomena are thought to be due to an endogenous sodium-potassium activated adenosine 5'-triphosphatase (Na⁺, K⁺-ATPase) inhibitor

which inhibits the Na⁺-K⁺ pump in renal tubular cells and vascular smooth muscle cells (Haddy and Overbeck, 1976; Blaustein, 1977; de Wardener and MacGregor, 1980). Since an endogenous Na⁺, K⁺-ATPase inhibitor is postulated to play a physiological role in the control of sodium balance and blood pressure, a number of laboratories have made concerted efforts to isolate and identify this substance (Haber and Haupert, 1987). However, there is disagreement concerning the chemical nature, whether peptides (Gruber *et al.*, 1980; Kramer *et al.*, 1985; Morgan *et al.*, 1985),

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steroids (Cloix *et al.*, 1985; Vasdev *et al.*, 1985) or lipid compounds (Tamura *et al.*, 1985; Kelly *et al.*, 1986). The conflicting results may be due to various relevant and irrelevant substances. In order to elucidate the Na^+ , K^+ -ATPase inhibitor(s) which plays a physiological role in the control of sodium balance, the present study was designed to investigate the effect of changes in dietary sodium intake on daily Na^+ , K^+ -ATPase inhibitory activity in the urine of normal subjects, which was fractionated by high performance liquid chromatography (HPLC). We also investigated Na^+ , K^+ -ATPase inhibitory activity in pooled serum of normal subjects, which was fractionated by the same HPLC.

Materials and Methods

1. Subjects

Seven healthy male normotensive subjects (21~39 yrs old) were studied. All subjects were studied over a consecutive 11-day period. After a 2-day control period on their free sodium intake, they were placed on low sodium chloride intake (2g/day) for 3 days, high sodium intake (22 g/day) for the next 4 days and then normal sodium intake (6 g/day) for the next 2 days. The diets were provided by the metabolic ward kitchen. Fluid intake was *ad libitum*.

2. Urine preparation

Twenty-four-hour urine specimens were collected in clean plastic containers and no preservatives were used. The urine was refrigerated during collection. A 10% aliquot of 24-hour urine was centrifuged at $6,000 \times g$ for 15 min at 4°C . The supernatant was applied to a Sep-Pak C_{18} cartridge (Waters Associates Inc., Milford, Mass., USA) that had been preactivated by passing 2 ml of acetonitrile, followed by 10 ml of distilled water. Up to 100 ml of supernatant was passed through a single cartridge. The loaded cartridge was washed with 10 ml of distilled water and then eluted with 5 ml of 80% acetonitrile containing 0.1% trifluoroacetic acid. The eluate was evaporated below 40°C in a rotary evaporator, resuspended in 1.0 ml of distilled water and

centrifuged at $9,000 \times g$ for 10 min at 4°C . The supernatant was filtered through a disposable syringe filter unit.

3. HPLC

We used a gradient-capable HPLC system (CCLP type and CCLE type pump, Tosoh Co. Ltd., Tokyo, Japan) equipped with an ultra-violet detector (Soma Optical Co. Ltd., Tokyo, Japan) set at 260 nm. The chromatography was carried out on a Shimpak-PREODSP column (20×250 mm, Shimadzu Seisakusyo, Kyoto, Japan) equilibrated with distilled water using a linear elution gradient of 0-40% acetonitrile in distilled water at a flow rate of 5 ml/min. Fractions were collected every 5 min. Four-hundred μl of a sample was chromatographed twice under these conditions. Even numbered fractions were combined and evaporated in order to remove acetonitrile. Each fraction was reconstituted in 1.0 ml of distilled water before being assayed.

4. Determination of Na^+ , K^+ -ATPase activity

Na^+ , K^+ -ATPase activity was assayed by a coupled enzyme kinetic assay on a centrifugal analyzer (Cobas Bio, Hoffman-La Roche & Co. Ltd., Basel, Switzerland) with a canine kidney Na^+ , K^+ -ATPase (Sigma Chemical Co., St. Louis, MO, USA) preparation. Hog muscle lactate dehydrogenase (LDH), rabbit muscle pyruvate kinase (PK) in 50% glycerol solution, and Tris were purchased from Boehringer GmbH (Mannheim, F.R.G.), Phosphoenolpyruvate tricyclohexylammonium (PEP), ATP and NADH were from Oriental Yeast Co., Ltd. (Tokyo, Japan). Trimethylaminoethane sulfonic acid (TES), piperazine-*N*, *N'*-bis (2-ethane sulfonic acid) (PIPES) and EDTA were from Dojindo Laboratories (Kumamoto, Japan). Ethylene glycol bis (β -aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid (EGTA) was from Nakarai Chemicals, Ltd. (Kyoto, Japan). Other reagents used were of analytical grade. Reagents for Na^+ , K^+ -ATPase activity assay were divided into two separate sets, reagents A and B. Reagent A consisted of NaCl (143 mmol/l), KCl (29.8 mmol/l), MgSO_4 (6.4 mmol/l), EGTA (7.1 mmol/l), NADH (0.23 mmol/l), PEP (1.7 mmol/l), ATP (3.0 mmol/l), PK (1.7 U/ml) and LDH (1.7 U/ml). These reagents were dissolved in PIPES buffer (100 mmol/l, pH 7.4). Reagent B was prepared by dissolving Na^+ , K^+ -ATPase (490 μg protein/ml)

and EDTA (500 $\mu\text{mol/l}$) in TES-Tris buffer (10 mmol/l, pH 7.4).

Forty μl of a sample, 280 μl of reagent A and 50 μl of distilled water were pipetted into a cavity in the cuvette. The rotor was accelerated and spun for 30 seconds. Then 20 μl of reagent B and 20 μl of distilled water were added. For the blank, 40 μl of distilled water was added. While the rotor was accelerated and spun again, readings of the absorbance value at 340 nm were taken every 10 sec from 125 to 305 sec at 37°C. After all absorbance values were read, the analyzer found a linear regression portion of the data on each cavity and calculated the decrease in the absorbance value at 340 nm (ΔA_{340}) for 1 min from the slope of the regression line. We used distilled water as a control. Percent inhibition was calculated as:

$$100 - \frac{\text{Sample}(\Delta A_{340}/\text{min}) - \text{Blank}(\Delta A_{340}/\text{min})}{\text{Control}(\Delta A_{340}/\text{min}) - \text{Blank}(\Delta A_{340}/\text{min})} \times 100$$

The coefficient of variation on % inhibition against ouabain was below 2.0%.

5. Radioimmunoassay of digoxin-like activity

A solid-phase RIA system (Spac Digoxin RIA kit, Daiichi Radioisotope Co., Ltd., Tokyo, Japan) was used. Digoxin-like immunoreactivity was determined with 100 μl of a sample. The

values obtained were expressed as ng of digoxin equivalents per ml of reconstituted eluate.

6. Serum preparation

Five liters of pooled serum from normal individuals was dialyzed for 4 hours against 10 volumes of distilled water using seamless cellulose tubing (Union Carbide Co., USA). The dialysate was lyophilized and extracted with methanol and centrifuged at $9,000 \times g$ for 10 min. The supernatant was evaporated and dissolved in distilled water. Aliquots of the solution were filtered through the same disposable syringe filter unit and injected onto the same Shimpak-PREP-ODS column. The column was eluted with a linear 0–40% acetonitrile gradient in 40 min, at a flow rate of 5 ml/min. Fractions were collected every 5 min. Equal number fractions from different injections were combined and evaporated. Na^+ , K^+ -ATPase inhibitory activity of each fraction was tested.

7. Other assays

Nonesterified fatty acid and phospholipid were measured enzymatically with commercially available kits (NEFA-E, Iatron Laboratories, Inc., Tokyo, Japan and Sanassay PL, Sanko Junyaku Co., Ltd., Tokyo, Japan) using a centrifugal analyzer. The sodium concentration was determined by flame photometry.

Fig. 1. Elution profile of Na^+ , K^+ -ATPase inhibitory activity and digoxin-like immunoreactivity from urine on a Shimpak PREP-ODS column. Urine extract obtained from a Sep-Pak C_{18} cartridge was fractionated by reverse phase HPLC using a linear gradient of acetonitrile (0–40%) in water.

8. Statistical evaluation

Results were expressed as means $\pm$ S. D.. For statistical analysis of data, the paired Student's *t*-test was used.

Results

1. Na^+ , K^+ -ATPase inhibitory activity and digoxin-like immunoreactivity in fractionated urine

An elution profile of Na^+ , K^+ -ATPase inhibitory activity and digoxin-like immunoreactivity in urine extract obtained from by reverse phase HPLC is shown in Fig. 1. Three peaks (fractions 8, 10 and 12–13) which inhibited Na^+ , K^+ -ATPase and one

broad peak (Fraction 9–14) which cross-reacted with anti-digoxin antibody were observed. Although fraction 10 and fraction 12–13 inhibited Na^+ , K^+ -ATPase as well as crossreacting with anti-digoxin antibody, fraction 8 did not cross-react with anti-digoxin antibody.

2. Na^+ , K^+ -ATPase inhibitory activity of fractionated urine and urinary sodium excretion during changes in dietary sodium intake.

An example of chromatographic patterns in one subject during changes in dietary sodium intake is shown in Fig. 2. The Na^+ , K^+ -ATPase inhibitory activity of fraction 10 eluted with about 17% acetonitrile decreased with low sodium intake, increased with high sodium intake, and decreased again with low sodium intake, indicating that changes in the Na^+ , K^+ -ATPase inhibitory activity of fraction 10 were closely associated with the changes in dietary sodium intake. There was no significant correlation between Na^+ , K^+ -ATPase inhibitory activity in other fractions and dietary sodium intake.

The Na^+ , K^+ -ATPase inhibitory activity of fraction 10 and urinary sodium excretion in 7 normal subjects during changes in dietary sodium intake are shown in Fig. 3. With a reduction in sodium intake, urinary sodium excretion decreased to 31.2 ± 14.2 mEq/day, and the Na^+ , K^+ -ATPase inhibitory activity of fraction 10 declined gradually from $12.3 \pm 5.2\%$ (the average for 2 control days) to

Fig. 2. Na^+ , K^+ -ATPase inhibitory activity in urine fractionated by HPLC during changes in dietary sodium intake.

$8.7 \pm 9.8\%$ on the 3rd day of low sodium intake. In contrast to this, the Na^+ , K^+ -ATPase inhibitory activity of fraction 10 increased promptly from $15.0 \pm 12.2\%$ (the 1st day) to $61.2 \pm 6.6\%$ (the 4th day) during the period of high sodium intake. It remained elevated even on the 1st day of normal sodium intake ($70.5 \pm 23.3\%$), but returned to $20.5 \pm 0.7\%$ on the 2nd day of normal sodium intake (urinary sodium excretion; 76.8 ± 22.3 mEq/day). The Na^+ , K^+ -ATPase inhibitory activity of fraction 10 on the 3–4th day during the high

sodium diet and on the 1st day of the normal sodium diet was significantly higher than that of the control ($p < 0.01$).

3. Digoxin-like immunoreactivity in urine extract during changes in dietary sodium intake.

Digoxin-like immunoreactivity in urine extract of 7 normal subjects during alteration of dietary sodium intake is shown in Fig. 4. Although there was a small reduction in the digoxin-like immunoreactivity towards the end of the low sodium intake period and a small increase towards the end of the high sodium intake period, their changes were not statistically significant as compared with the control.

4. Na^+ , K^+ -ATPase inhibitory in fractionated serum

There were at least 4 peaks containing Na^+ , K^+ -ATPase inhibitory activity in serum extract obtained from reversephase HPLC, one of which consisted of fraction 10 eluted at about a 17% acetonitrile concentration (Fig. 5). Only fraction 17–19 contained either nonesterified fatty acid or phospholipid.

Fig. 3. Na^+ , K^+ -ATPase inhibitory activity of fraction 10 and urinary sodium excretion in 7 normal subjects during changes in dietary sodium intake. Results are means $\pm$ S.D. * $P < 0.01$ compared with the control.

Discussion

The present study clearly demonstrated that the Na^+ , K^+ -ATPase inhibitory activity of fraction 10 in the urine of normal subjects which was eluted with 17% acetonitrile concentration can be influenced by changes in dietary sodium intake. In particular, it de-

Fig. 4. Digoxin-like immunoreactivity in urine of 7 normal subjects during changes in dietary sodium intake. Urine was extracted through a Sep-Pak C₁₈ cartridge.

Fig. 5. Elution profile of Na⁺, K⁺-ATPase inhibitory activity from serum on a Shimpak PREP-ODS column. Serum extract obtained from dialysate was fractionated by reverse-phase HPLC using a linear gradient of acetonitrile (0–40%) in water.

monstrated that increases in Na⁺, K⁺-ATPase inhibitory activity in fraction 10 occurred following increases in dietary sodium intake. Additionally the Na⁺, K⁺-ATPase inhibitory activity in fraction 10 was closely associated with urinary sodium excretion throughout the whole study. Therefore, this close association between

changes in Na⁺, K⁺-ATPase inhibitory activity in fraction 10 and those in urinary sodium excretion clearly suggested that a Na⁺, K⁺-ATPase inhibitor(s) present in fraction 10 may play a physiological role in the control of sodium balance.

An endogenous Na⁺, K⁺-ATPase inhibitor is thought to possess digoxin-like properties

with respect to its ability to inhibit Na^+ , K^+ -ATPase. Gruber *et al.* (1980) showed immunological cross-reactivity with digitalis in the plasma of volume expanded dogs. Klingmüller *et al.* (1982) found digitalis-like immunoreactivity in the urine of sodium loaded normal human subjects. However, recent studies (Weiler *et al.*, 1985; Kramer *et al.*, 1985) have shown a lack of correlation between the digoxin-like immunoreactivity and either sodium intake or total natriuretic activity by bioassay. The present results showing dissociation of digoxin-like immunoreactivity from Na^+ , K^+ -ATPase inhibitory activity in fractionated urine and a lack of increased digoxin-like immunoreactivity in crude urine extract during high sodium intake are in agreement with these studies. The Na^+ , K^+ -ATPase inhibitory activity and the digoxin-like immunoreactivity in fraction 10 may not be the same moiety. Further analysis with different antibodies to digitalis derivatives will be needed to determine whether a Na^+ , K^+ -ATPase inhibitor possesses digoxin-like immunoreactivity.

We investigated Na^+ , K^+ -ATPase inhibitory activity in pooled serum from normal subjects by using the same HPLC. The serum extract was resolved into at least 4 peaks containing Na^+ , K^+ -ATPase inhibitory activity, one of which consisted of fraction 10. We could not determine which individual serum peaks with Na^+ , K^+ -ATPase inhibitory activity rose with salt loading, since it was difficult to obtain an adequate amount of serum to quantify from individual subjects. The peak consisting of fraction 10 might rise with salt loading, taking into account the result of urine. Further purification of serum and urine will be needed to determine whether the substance in fraction 10 of serum which inhibits Na^+ , K^+ -ATPase is identical with that in urine.

Tamura *et al.* (1985) reported that linoleic and oleic acids were detected as

Na^+ , K^+ -ATPase inhibitors in saline-infused hog plasma. Kelly *et al.* (1986) also reported that a major component of plasma Na^+ , K^+ -ATPase inhibitory activity is due to nonesterified fatty acids and lysophospholipids. However, they were not thought to be physiological regulators of Na^+ , K^+ -ATPase because of their limited affinity and specificity. To eliminate these lipid compounds liberated by boiling, we used serum extract obtained from dialysate for chromatographic procedure. Indeed, fraction 10 of serum contained neither nonesterified fatty acid nor phospholipid.

In summary, the present study clearly demonstrates that Na^+ , K^+ -ATPase inhibitory activity in the particular fraction can be modulated by changes in dietary sodium intake, suggesting that a Na^+ , K^+ -ATPase inhibitor(s) which might be important in salt and volume homeostasis may exist in man.

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