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CIRCULATING FACTOR WITH OUABAIN-LIKE IMMUNOREACTIVITY IN PATIENTS WITH PRIMARY ALDOSTERONISM

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Summary. Circulating factor with ouabain-like immunoreactivity was studied in patients with primary aldosteronism. Anti-ouabain antibody was prepared from specific pathogen-free rabbits. In the plasma of patients with primary aldosteronism, the level of a factor with ouabain-like immunoreactivity was 2.59 ± 1.39 pmol ouabain equivalent/ml plasma. This value was significantly ($p < 0.05$) higher than that of age-matched normotensive subjects, 1.06 ± 0.86 pmol ouabain equivalent/ml plasma. The plasma level of ouabain-like immunoreactivity correlated significantly ($p < 0.05$) with blood pressure. These results indicate that the factor with ouabain-like immunoreactivity may play a pathophysiological role in the maintenance of the high blood pressure observed in patients with primary aldosteronism.

Circulating inhibitor of Na^+,K^+-ATPase is thought to be important in the origin and development of essential hypertension (1) and to be secreted from unknown tissue leading to an increase in circulating blood volume. Because this inhibitor behaves like cardiac glycosides and cross-reacts with antidigoxin antibody, the term digitalis-like substance or endoxin was used by Gruber et al to describe the molecule (2). The molecular weight of circulating inhibitor has been reported to be less than 1,000 daltons (3) and is highly polar (4). Because the polarity of digoxin is less than ouabain, it is considered more appropriate to assay the circulating Na^+,K^+-ATPase inhibitor with anti-ouabain antibody rather than anti-digoxin antibody.

In this study, rabbit anti-ouabain antibody was used to study the presence of polar circulating inhibitor and the plasma level of ouabain-like immunoreactivity in patients with primary aldosteronism.

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MATERIALS AND METHODS

Chemicals: Ouabain, bovine serum albumin and Na^+,K^+-ATPase were obtained from Sigma Chemical Co., St. Louis, MO. Tritiated ouabain (specific radioactivity, 2-50 Ci/mmole) was purchased from Amersham Japan Co., Tokyo. Sep-Pak silica cartridge was purchased from Waters Associates, Milford, MA. Sodium periodate, sodium cyanoborohydride, Freund's complete adjuvant and other reagents were purchased from Wako Chemical Co., Osaka, Japan.

Animals: Specific pathogen-free (SPF) New Zealand white rabbits were obtained from Keari Co., Osaka.

Preparation of ouabain-bound bovine serum albumin. Ouabain (0.2 mmol) and sodium periodate (0.25 mmol) were dissolved in 5 ml of distilled water. After standing 24 hours at 4°C, 10 ml of ethanol was added and the pH was adjusted to 8.35 by the addition of NaOH. Bovine serum albumin (2 μmol) was added and the pH was readjusted to 9.35 with NaOH. After standing overnight at 4°C, sodium cyanoborohydride (0.8 mmol) was added and the solution was dialyzed against flowing tap water for 48 hours. The solution was lyophylized after re-dialyzation (three times) against distilled water. By this method, 33 mol of ouabain were bound to one mol of bovine serum albumin as measured by spectrophotometric assay at 370 nm in 50% sulfuric acid solution.

Preparation of anti-ouabain antibody: To prepare the antibody, 100 μl of ouabain-bound bovine serum albumin in Freund's complete adjuvant was injected subcutaneously into the foot pads and abdomen of female SPF rabbits. Two weeks later the same amount of antigen in Freund's incomplete adjuvant was injected. Booster injections were given twice, with an interval between injections of two weeks. Two weeks after the last injection serum was obtained and heated at 56°C for 30 min. The IgG fraction was obtained by ammonium sulfate fractionation as described by Heide and Schwick (5). The lyophylized IgG fraction was stored at -20°C until use.

Treatment of plasma samples: Five ml of blood from seven patients with primary aldosteronism (mean age = 43.1 ± 6.4 years) receiving no medication and seven age-matched normotensive subjects (mean age = 42.8 ± 8.5 years) were obtained in EDTA-containing tubes between 8:00 a.m. and 9:00 a.m. during fasting. Two ml of plasma and four ml of ethanol were mixed and incubated at 90°C for three min. After cooling to 4°C the precipitate was centrifuged at 10,000 x g for 10 min and removed. The supernatant was evaporated to dryness under vacuum and was dissolved in one ml of re-distilled water. The solution was applied to a Sep-Pak silica column and washed with 15 ml of re-distilled water. By this procedure, the contaminating sodium and potassium ions were completely removed. The sample was eluted with 5 ml of 52.2% ethanol. This fraction was re-evaporated to dryness and dissolved in one ml of distilled water and used immediately in the assay procedure.

Radioimmunoassay: The reaction mixture (total 500 μl) consisted of 200 μl of phosphate buffered saline (pH 7.4) (containing 0.1% gelatin and 0.1% sodium azide), 30 μl of ^{3}H-ouabain solution, 100 μl of antibody solution and plasma sample or unlabeled ouabain. The binding reaction was started by the addition of labeled ouabain and incubated at 25°C. After 15 min, 500 μl of charcoal suspension (containing 9 g of Wako SX, 0.62 g of dextran T-40 and 1 g of sodium azide in 1 liter of 0.1 M Tris-HCl buffer, pH 7.8) was added and the suspension was centrifuged at 3,000 rpm for 10 min. The supernatant was assayed by liquid scintigraphy.

Statistical analysis: Statistical analyses were done by the Student's paired t test. The significance of the dependence of ouabain-like immunoreactivity on blood pressure was determined by correlation analysis.

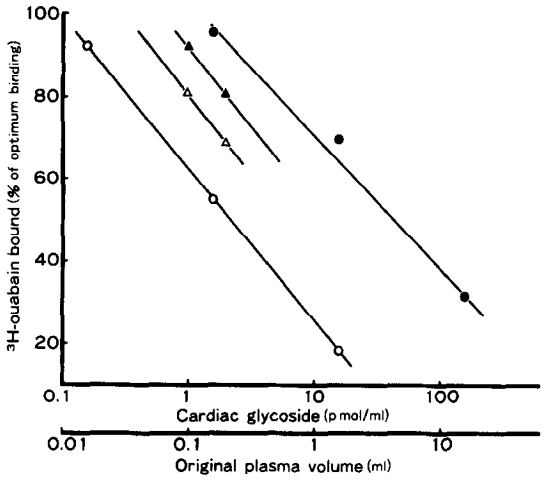

Fig. 1. Standard curve obtained from the radioimmunoassay of ouabain. The assay was performed in the presence of ouabain (open circle), digoxin (closed circle) and plasma samples from patients with primary aldosteronism (open triangle) and normotensive subjects (closed triangle).

RESULTS

Anti-ouabain antibody had a high affinity to a standard of ouabain, yielding an EC_{50} of 2.5 ± 0.59 pmol/ml, whereas the affinity of this antibody to a standard of digoxin was 18 times less potent. The data obtained from the plasma samples showed a curve parallel to the standard curve of ouabain (Fig. 1). The mean blood pressure in patients with primary aldosteronism was significantly higher ($p<0.01$) than that in normotensive subjects. The mean plasma level of ouabain-like immunoreactivity in the patients with primary aldosteronism was 2.59 ± 1.39 pmol ouabain equivalent/ml of plasma, which was significantly ($p < 0.05$) higher than the 1.06 ± 0.86 pmol ouabain equivalent/ml obtained from the plasma of normotensive subjects (Table 1). The plasma level of ouabain-like immunoreactivity correlated significantly ($p < 0.01$) with mean blood pressure (Fig. 2).

DISCUSSION

Digoxin-like immunoreactiveity has been studied in plasma (2,6-9), urine (3,8,10,11) and some tissues (12-14). This factor has been

Table 1. Comparison of ouabain-like immunoreactivity of plasma from patients with primary aldosteronism and normotensive subjects

Subjects	Number	MBP[a] (mm Hg)	OLI[b] (pmol/ml)
Primary aldosteronism	7	111.9 ± 12.2**	2.59 ± 1.39*
Normotensive	7	87.2 ± 3.7	1.06 ± 0.86

Data are shown as mean ± S.D.
[a]MBP, mean blood pressure. [b]OLI, ouabain-like immunoreactivity.
*$p < 0.05$. **$p < 0.01$.

thought to cross-react with circulating Na^+,K^+-ATPase inhibitor(s), a putative natriuretic hormone and highly polar molecule(4). Among cardiac glycosides, ouabain has been reported to be more polar than digoxin, but there have appeared as yet no reportes on ouabain-like immunoreactivity in the plasma of patients with hypertension. Natriuretic hormone levels are increased by treatments that increase circulating blood volume. To study the ouabain-like immunoreactivity in human plasma, patients with primary aldosteronism were chosen as a representative of volume-dependent hypertension. By the methods used in this study, a factor with ouabain-like immunoreactivity was detected in human plasma. The plasma level of the ouabain-like immunoreactivity was significantly higher in patients with

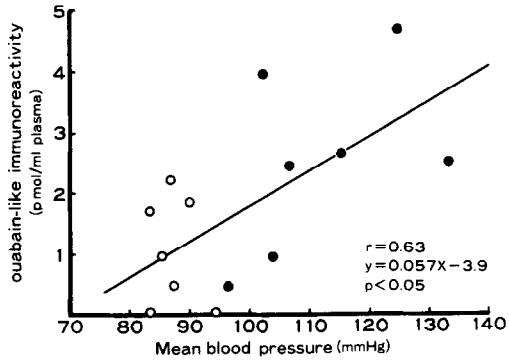

Fig. 2. Correlation between the plasma level of ouabain-like immunoreactivity and blood pressure. Closed circles and open circles represent the values obtained from patients with primary aldosteronism and normotensive subjects, respectively.

primary aldosteronism than in age-matched normotensive subjects. The plasma level of ouabain-like immunoreactivity correlated significantly with blood pressure. These results indicate that the circulating factor with ouabain-like immunoreactivity is present in plasma and participates in the pathophysiological regulation of blood pressure in patients with primary aldosteronism.

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