Biological Activity of Partially Purified Digitalis-like Substance and Na-K-ATPase Inhibitor in Rats

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In order to study the biological activity of endogenous digitalis-like substance (DLS) and Na-K-ATPase inhibitor (ATPI), human urine was partially purified and administered to rats, and its effects on the urinary volume, urinary Na excretion and blood pressure (BP) were determined. In addition, the effect on myocardial Na-K-ATPase activity was also measured.

After the extraction of 40L of urine with a reversed phase cartridge column (S-fraction), 20 ml of chloroform was added and extraction was repeated. The chloroform layer was applied to an open silica gel column, and at a fraction with ethylacetate: methanol (60: 40, T-1 fraction), DLS and ATPI were eluted at the highest concentration. The water layer was treated with charcoal (D-1 fraction). The acute administration of K-1, T-1 fraction to rats in vivo caused significant rises in urinary volume, urinary Na excretion and BP. In chronic administration of K-1 fraction, urinary Na excretion was significantly elevated and myocardial Na-K-ATPase activity was also significantly suppressed.

These results suggest that DLS and ATPI cause increase in the urinary volume and urine Na excretion and also possess a hypertensive action; and moreover, these substance may affect the heart like cardiotonic steroids and regulate BP by increasing cardiac contractility.

SEVERAL studies have been performed on changes in the endogenous digitalis-like substance (DLS) and in the Na-K-ATPase inhibitor (ATPI) found in human blood and urine in various disease. The authors1,2 and other workers3,4 have reported that DLS and ATPI have a circadian rhythm, are increased by salt loads, cardiac failure, hyperthyroidism and hypertension, and are decreased by the administration of mineralocorticoid. Although a number of studies on the regulation of DLS and ATPI have been conducted, there have been few studies on what effects they have on blood pressure and natriuresis when administered in vivo because complete purification of these substances has not been successful. The physiological activity of these substances and their role in vivo, however, are important points which must be investigated. Therefore, to clarify these points, we administered partially purified DLS and ATPI obtained from human urine samples in vivo to rats and

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investigated their biological activity. Since few reports have surfaced concerning the effects of these substances on target organs, the effects on Na-K-ATPase activity in the myocardium, considered to be one of the target organs, were also evaluated.

MATERIALS AND METHODS

Partial purification of DLS and ATPI

Forty liters of human urine were adsorbed on SEPAK C18 and eluted with 80% acetonitrile after washing with 0.1% trifluoroacetic acid and then freeze-dried (S-fraction). After the eluant was redissolved in 5 ml of 50% methanol (MeOH), 20 ml of chloroform (CHCl3) was added and re-extracted. The chloroform layer was applied to an open silica gel column (1.2 x 10 cm) and elution was performed with 10 ml each of 100% CHCl3, ethyl acetate (AcOEt)/CHCl3 (10/90), AcOEt/CHCl3 (50/50), 100% AcOEt, AcOEt/MeOH (90/40), 100% MeOH and H2O. About 5 g of charcoal was added to the water layer and DLS and ATPI were eluted with 100% ethanol (K-1 fraction, Fig. 1). In each of these fractions, the contents of DLS, ATPI, epinephrine (E), norepinephrine (NE), dopamine (DP), angiotensin II (A II) and atrial natriuretic peptide (ANP) were measured.

Acute administration

Cannulation was performed in the femoral vein, carotid artery and urinary bladder of male Wistar rats weighing 300g under pentobarbital anesthesia. The arterial blood pressure was measured directly by a cannula inserted into the carotid artery using a pressure transducer (Nihon Koden, Japan) and femoral vein was used for sample administration, and urine was collected via cannula inserted into the bladder. Physiological saline was administered continuously at a rate of 3 ml/h until completion of the experiment. The experiment was started after the arterial pressure had become stable (after about 15 min)
after an intramuscular injection of 0.1 mg of pentolinium tartrate. At the start of the experiment, 300 μl of physiological saline was injected over 1 min, urine was collected for 30 min, and was used as the control. Thereafter 200 μl of partially purified DLS and ATPI dissolved in physiological saline at a concentration of 100 mg/ml was injected over 40 sec and flushing was performed for 20 sec with 100 μl of physiological saline. Urine was collected for 30 min, and the urinary volume and urinary Na, K and creatinine (Cr) were measured (Fig. 2-a).

Chronic administration
Ten male Wistar rats weighing 300g underwent preliminary rearing for 3 days in metabolic cages. After a control period over the 4th and 5th days, we embedded mini-osmotic pump (ALZA Corporation USA, model 2001) subcutaneously in their backs under pentobarbital anesthesia on the 6th day. Urine was collected every 2 days from the 1st day of administration, and the urinary volume and urinary Na, K, creatinine, DLS and ATPI were measured, and observed for 6 days. Blood pressure was measured by the tail cuff method before and on the 6th day of administration. After the blood pressure measurement on the 6th day of administration, the animals were sacrificed by decapitation, blood was collected and serum Na, K, and Cr were measured. The heart was removed immediately after decapitation and prepared for measurement of Na-K-ATPase activity.

The partially purified DLS and ATPI were prepared at a concentration of 300 mg/ml in physiological saline, each osmotic minipump was filled with 0.2 ml and they were administered subcutaneously for about 1 week continuously (Fig. 2-a).

Preparation of Na-K-ATPase in the cell walls of rat myocardium
After removal, the hearts were rinsed in physiological saline, and homogenized in a solution of
Fig. 3. Effects of the acute administration of partially purified DLS and ATPI on urinary volume (UV), urinary Na (UNaV), K (UKV) and Cr (UCrV) excretion, and systolic blood pressure (BPs).

0.25M sucrose, 5 mM EDTA, 5 mM histidine and 0.15% deoxycholate (pH 6.8). The homogenate was centrifuged at 1,100g for 10 min and then at 12,300g for 20 min, and the nucleus and mitochondria fractions were removed. The supernatant was subjected to gel filtration using sepharose 2B (Pharmacia) gel. The column was 1.0 x 2.5 cm and the flow rate was 30 ml/hr. For the elution, 0.05M Tris-HCl buffer (pH 7.5) was used. The eluant was collected every 5 min and used for ouabain-sensitive Na-K-ATPase activity. Ouabain-sensitive Na-K-ATPase was measured using the amount of inorganic phosphate formed in the cocktail containing ATP.

Measurement

DLS was measured by radioimmunoassay (RIA) and ATPI was measured by changes in absorbancy as previously reported.

ANP was measured by RIA after extraction with SEP-PAK C₁₈, washing with 0.1% TFA, eluting with 80% acetonitrile. A commercial kit (Amer sham International plc, England) was used for the RIA. The recovery rates when 50 and 200 pg of synthetic h-αANP was added to ANP-free plasma were 85.2 ± 3.6 and 86.3 ± 4.2%, respectively, and the intrassay and interassay variations were 4.2% and 8.9%, respectively. A II was measured by RIA using the antibody produced by IgG Corporation, USA, and Ⅱ angiotensin II (NEN, USA). The samples for A II using all were measured by the same assay. E, NE and DP were measured by high-pressure liquid chromatography as previously reported.

Statistical testing was performed using the

Fig. 4. Effects of the chronic administration of partially purified DLS and ATPI on urinary volume (UV), urinary Na (UNaV) and K (UKV) excretion.

(Mean ± S.E, *: p < 0.05, **: p < 0.005 vs control)
paired or unpaired t-test and differences in multigroup average values were tested by Dunnett’s method. Differences were considered to be significant at p < 0.05.

RESULTS

Results of partial purification

As shown in Fig. 1, DLS and ATPI purified by a silica gel column showed the highest values in AcOEt/MeOH (60/40) fraction (T-1 fraction). This fraction and the K-1 and S fractions were used in the subsequent administration experiments. Table I shows the contents of DLS, ATPI and other physiological active substances. Also shown in Table I, as reference (Ref), are the results of an investigation of the minimum amounts of substances other than DLS and ATPI required to cause changes in the blood pressure, urinary volume and urinary Na excretion when administered under the same conditions as in the acute administration experiment in the present study. Among these 3 fractions, the S and K-1 fractions were used in the chronic administration experiment and the S, K-1 and T-1 fractions in the acute administration experiment.

Acute administration

During acute administration of the 3 fractions, the urinary volume and urinary Na excretion increased significantly, while the urinary Cr excretion showed no significant change. The BP increased significantly by the administration of K-1 and T-1 fraction and urinary K excretion showed also significant increase by T-1 fraction (Fig. 3).

Chronic administration

The urinary volume did not show any significant differences compared with the control period in the S and K-1 administered groups. The urinary Na excretion did not change significantly in the S-fraction administered group. In the K-1 fraction administered group, there was a
significant increase (p<0.01) of about 125% on the 3rd and 4th days of administration. The urinary K excretion did not show any significant changes in the S and K-1 groups (Fig. 4).

The urinary excretion of DLS increased markedly on the 1st and 2nd days (p<0.01) and on the 3rd and 4th days (p<0.05) in the S-fraction administered group. In the K-1 administered group, there was a significant increase (p<0.01) on the 3rd and 4th days. The urinary excretion of ATPI increased significantly (p<0.01) on the 3rd and 4th days in the K-1 fraction group (Fig. 5).

Blood pressure and pulse rate showed no remarkable changes in either group.

Changes in myocardial Na-K-ATPase activity

The myocardial Na-K-ATPase activity obtained with the gel filtration method was 1.58 ± 0.17 μmoles Pi/mg of protein/hr in the K-1 fraction administration group. This value was significantly (p<0.05) low compared with control value of 2.07 ± 0.15.

DISCUSSION

Although purification of DLS and ATPI is underway in many institutions, several reports suspect that these are not single substances. In our study, however, good correlation was seen in the kinetics of these 2 substances, and it appears that some relationship exists between the 2. Several substances inhibit Na-K-ATPase in vivo, and there is a possibility that DLS is 1 of these. DLS and ATPI obtained by partial purification in the present study show parallel gel filtration patterns and it was accordingly assumed that 1 of ATPI (s) may be DLS.

Several substances including dopamine, peptide, steroid and fatty acids have been reported as candidates for DLS and ATPI, and studies have been performed on such materials using cross reactivity with digoxin antibody, inhibition of H-ouabain binding, inhibition of Na-K-ATPase activity, and so on. On the other hand, very few reports have been published on general studies of natriuretic and hypertensive actions when these substances are administered in vivo. There are only reports on BP and ion reflux of red blood cells due to the administration of linoleic acids. Since it is very important to confirm these points when discussing the pathophysiological significance of ATPI and DLS, we investigated some of these points using partially purified ATPI and DLS.

The K-1 and T-1 fractions used in the present study show comparatively high purities of DLS and ATPI. Although other physiologically active substances are present, their levels are low enough to be disregarded except for A II. There was a significant increase in urinary Na excretion upon acute infusion of the K-1 and T-1 fractions and chronic administration of K-1 fraction, and also significant increase of BP by acute infusion of the T-1 and K-1 fractions. These fractions did not contain enough of the endogenous substances to cause an increased sodium excretion. Although ANP is the most potent endogenous natriuretic substance among the contamination of these fractions, the minimum dose of ANP required to cause natriuretic action in rats was about 3 ng/min by continuous administration and 10 ng by 1 bolus (Table I). The maximum amount of contamination of ANP is 1.4 ng/20 mg (the dose of 1 bolus), so it is difficult to explain the natriuresis of the S, K-1 and T-1 fraction.
solely by ANP contained in these fractions.

The possibility that the BP increase due to acute administration of these fractions was caused by the contamination of A II cannot be overlooked. In our studies, however, the BP increases due to A II were limited to about 10–12 mmHg when 1 bolus of 2 ng/min was administered i.v. Also the fact that the BP increased by more than 20 mmHg in this case and that in the fraction S and T-1, the amount of A II in these fractions cannot cause natriuresis suggested that substance(s) other than A II were involved. Therefore, it was suggested that unknown natriuretic and hypertensinogenic factor(s) are present and that ATPi and/or DLS in particular were assumed most likely to be such factor(s).

Two reasons can be considered to explain why chronic administration caused no BP increase seen with acute administration. The first is that the chronic dose might have been too small because only 70 mg/7 days was administered chronically compared with 20 mg/min in the acute administration. The second reason is that, judging from the urinary excretion of DLS and ATPi, it is assumed that substances administered exogenously require 3 to 4 days to reach peak concentrations in vivo, and BP was measured under conditions where there already tended to be a decrease on the 6th day. This point must be investigated again by changing the dose and the timing of the BP measurements.

From the results of the above experiments on acute and chronic administration, it was assumed that partially purified DLS and ATPi have natriuretic and hypertensive actions which are difficult to explain by known physiologically active substances.

With respect to the effects on Na-K-ATPase activity in the tissues, there have been no reports of studies on the effects of partially purified substances administered in vivo. Therefore, we investigated the in vivo effects of partially purified ATPi and DLS on the myocardial Na-K-ATPase activity. The reason for selecting the myocardium was that the action of cardiotonics such as digitalis or ouabain is against Na-K-ATPase. These drugs strengthen the contractility of the myocardium by inhibiting Na-K-ATPase, and if endogenous ATPi and/or DLS play some role, the heart appears to be an important target organ together with the kidneys and the vascular walls. The results of a study using the K-I fraction, which can be considered partially purified ATPi, showed that Na-K-ATPase activity significantly decreased in the rat myocardium. This results suggests that, in addition to natriuresis by action on the kidneys and hypertensive action by action on the vascular walls which were pointed out previously for ATPi, a third action of ATPi might be indirect BP control by action on the heart to strengthen cardiac contractility similar to that seen with cardiotonics.

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