A Plasma Inhibitor of Sodium and Potassium Activated Adenosine Triphosphatase in Patients with Essential Hypertension

SHUNICHI TORIYABE, YUKIO MIURA, SHINOBU KIMURA, YUKI MEGURO, TAKASHI SUGAWARA, TAKAO NOSHIRO, MASAKI TAKAHASHI, HIROFUMI OHASHI, NAOKI SANO, HIROSHI WATANABE and KAORU YOSHINAGA

The Second Department of Internal Medicine, Tohoku University School of Medicine, Sendai 980


The purpose of this study is to evaluate the plasma Na, K-ATPase inhibitor (NKI) in patients with essential hypertension and to compare the mode of its biochemical actions on the Na, K-ATPase with that of ouabain. Plasma NKI was extracted through a reversed-phase cartridge column and its inhibitory action on hog brain Na, K-ATPase was measured in vitro. Plasma NKI activity was significantly greater in patients with essential hypertension (44±2.8°c (SE.), n = 28, p <0.01) than in normotensive controls (25±2.4°c, n = 21). No significant correlation was demonstrated between the values of plasma NKI and mean arterial pressure in either group. Both plasma NKI and ouabain showed a dose-dependent inhibition on the Na, K-ATPase reaction. An action of ouabain was competitively antagonized by increased concentration of potassium in the reaction mixture, while plasma NKI showed a constant inhibition on the Na, K-ATPase independently of potassium concentrations. The action of plasma NKI was of rapid onset and linear with time, while ouabain showed a delayed onset of the reaction over 30 sec, followed by a progressively increasing inhibition on the enzyme reaction. Finally, the inhibitory action of plasma NKI on Na, K-ATPase was completely abolished in the presence of bovine serum albumin even at the concentration of 500 μg/ml in the reaction mixture, which did not have any influence on the actions of ouabain. To sum up, the results showed a markedly different nature of plasma NKI from ouabain in the mode of biochemical actions on the Na, K-ATPase in vitro. This study may also raise a question whether plasma free NKI, supposedly an active form of NKI, is actually working as a
physiological regulator in vivo. It seems thus premature to assume it as a pathogenic factor in essential hypertension.

Studies over the last two decades have indicated that an unknown natriuretic factor is released in mammalian blood following an expansion of extracellular fluid volume (Clarkson et al. 1976, 1979). Some investigators have recently postulated that this factor is an inhibitor of sodium, potassium activated adenosine triphosphatase (Na, K-ATPase), which probably interacts with the ouabain binding site on the enzyme (de Wardener and MacGregor 1982; Blaustein and Hamlyn 1983; Haddy and Pamnani 1983). This factor is thereby called digitalis-like substance or ouabain-like substance. According to the hypothesis proposed by de Wardener and MacGregor (1982), Na, K-ATPase inhibitor might be a causative factor of some forms of hypertension. The inhibition of Na, K-ATPase in the vascular cell membrane increases the contractile force of vascular smooth muscle, elevates peripheral vascular resistance, and thereby results in a raised arterial blood pressure.

Although this hypothesis has been supported by many lines of evidence so far (Gruber et al. 1980, 1982; MacGregor et al. 1981; Poston et al. 1981a, b; Hamlyn et al. 1982, 1987; Devynck et al. 1983; Haddy and Pamnani 1983; de The et al. 1984; Boschi et al. 1985; Tamura et al. 1985, 1987), the properties of endogenous sodium transport inhibitors are widely controversial. In the present study, we tried to evaluate the plasma Na, K-ATPase inhibitor (NKI) in patients with essential hypertension. The mode of its biochemical actions on the hog brain Na, K-ATPase preparation was also studied and compared with that of ouabain.

Materials and Methods

Patients and controls

Subjects in this study consisted of 28 patients with essential hypertension (16 men and 12 women, mean age 43±2.6 (s.e.) years, range 18–64) and 21 normal subjects (14 men and 7 women, mean age 35±2.6 years, range 16–60) with no history of hypertension. All hypertensive patients had been proved to have blood pressures of 140/90 mmHg or greater during three consecutive visits to our clinic. Clinical evaluations were performed to exclude secondary forms of hypertension. Patients with evidence of ischemic heart disease, cerebrovascular disease, diabetes mellitus or with abnormal serum concentrations of urea, creatinine or potassium were excluded from the study. An informed consent was obtained from each subject after the outline of this study was explained. All subjects were on their ordinary sodium intake, and all medications, if any, were discontinued for at least 2–4 weeks prior to the study. After 30 min in the supine position, systolic and diastolic pressures were measured by a sphygmomanometer. Blood samples (10–12 ml) were then collected for measuring plasma’s ability to inhibit Na, K-ATPase in vitro. Plasma was immediately separated at 4°C and stored at −20°C until assayed.

Extraction of Na, K-ATPase inhibitor (NKI) from plasma

NKI was extracted from plasma specimens according to the method reported by Boschi et al. (1985). Two ml of plasma was acidified to pH 3.0 with 5% formic acid and applied
to C8 disposable column (BondElut™, Analytichem International, Harborcity, CA, USA), which was previously washed with methanol and then with formate solution (distilled water adjusted to pH 3.0 with formic acid). The column was then washed twice with 4.0 ml of formate solution and eluted with 2.0 ml of absolute ethanol adjusted to pH 1.0 with HCl. The eluate was evaporated under vacuum and reconstituted with 2.0 ml of imidazole-EGTA buffer.

Na, K-ATPase assay by a colorimetric method

A hog brain Na, K-ATPase preparation was purchased from Sigma Chemical Co., St. Louis, MO, USA (specific activity 0.36 μmole/min/mg protein). This enzyme preparation contained an ouabain-insensitive Mg2+-ATPase activity which was less than 5% of the total ATPase activity. Each reaction tube contained 30 μg protein of Na, K-ATPase and 200 μl of reconstituted plasma extract or control imidazole-EGTA buffer. Final volume of the reaction mixture was 1.0 ml. The assay buffer (Boschi et al. 1985) consisted of NaCl 150 mmole/liter; KCl 1.0 mmole/liter; MgCl2 3.0 mmole/liter; EGTA 1.0 mmole/liter; imidazole-HCl 50 mmole/liter, pH 7.4. After 10 min preincubation at 37°C, the reaction was started by adding disodium ATP 10 mmole/liter to give a final concentration of 0.5 mmole/liter. The reaction was stopped after 30 min incubation by adding Phosphor C-test solution (Wako Pure Chemical Industries, Ltd., Osaka), a clinical kit for measuring plasma inorganic phosphorus containing 15% sulfuric acid, and the amount of phosphate liberated was measured by colorimetry. In this condition, ATP hydrolysis was directly proportional to the time of incubation. Each assay was performed in duplicate. Intra-assay and inter-assay variations were 2.5% and 3.5%, respectively.

Na, K-ATPase assay by a coupled enzyme assay

Na, K-ATPase activity was also tested by a coupled enzyme assay (Hamlyn et al. 1982) to observe the time course of the action of NKI on the enzyme. In this assay, the regeneration of enzymatically hydrolyzed ATP was coupled to the oxidation of NADH, the rate constant of ATP hydrolysis was immediately measured by continuously recording the absorbance of NADH at 340 nm by spectrophotometer (Hitachi 220A, Tokyo). The assay solution contained in a total volume of 2.0 ml the following concentrations of chemicals: NaCl 150 mmole/liter; KCl 1.0 mmole/liter; MgCl2 3.0 mmole/liter; EGTA 1.0 mmole/liter; trimethylaminomethanesulfonic acid (TES)-Tris 40 mmole/liter; pH 7.4; phosphoenolpyruvate tricyclohexyammonium (PEP) 1.2 mmole/liter; disodium ATP 0.5 mmole/liter; NADH 0.25 mmole/liter; pyruvate kinase (PK) 5.0 U/ml; lactic dehydrogenase (LDH) 5.0 U/ml; hog brain Na, K-ATPase 30 μg protein/ml. ATP, PEP, NADH, LDH and PK were from Boehringer Mannheim Biochemicals (Mannheim, FRG).

Statistical analysis

The results are expressed as mean ± s.e. To compare the effect of one parameter on two populations, Student’s t-test was used. Each variable was regressed on the others to compute correlation coefficients between them.

RESULTS

Fig. 1 shows the effects of increasing concentrations of plasma extracts or ouabain on Na, K-ATPase activity. Both ouabain and plasma extract inhibited Na, K-ATPase activity in a dose-dependent manner. The slopes for plasma extract paralleled that for ouabain. Plasma extract showed a decreased inhibition in the presence of a larger amount of the enzyme (60 μg protein/ml). NKI in plasma extract was heatstable, and did not decrease at −20°C for at least two
weeks. The inhibitory effect of NKI on Na, K-ATPase was completely abolished in the presence of bovine serum albumin at the concentration of 500 μg/ml in the reaction mixture, while an action of ouabain was not affected by the same condition.

The interaction of potassium and the inhibitory effect of ouabain and plasma extract on the Na, K-ATPase was kinetically studied. Double reciprocal plots for the enzyme reactions of Na, K-ATPase (Michal 1983) are illustrated in Fig. 2.

Fig. 1. Percent inhibition of Na, K-ATPase activity by ouabain (•—○) or plasma extracts in the presence of 30 μg/ml (●—●) or 60 μg/ml (□—□) protein of enzyme.

Fig. 2. In vitro inhibition of Na, K-ATPase by ouabain or plasma extract at various concentrations of potassium in the reaction medium. Na, K-ATPase activity was assayed by the rate of ATP hydrolysis by a colorimetric method in the control medium (————) in the presence of ouabain 0.2 (μmole/liter) (●—●) or plasma extract (—□—□).
Ouabain shifted the slope of the plots upward but the intercept, Vmax, was unchanged. The Km value was 1.4 mM for 1.0 μM ouabain. At higher concentrations of potassium, the action of ouabain was competitively antagonized by potassium. On the other hand, plasma extract showed a constant inhibition on Na-K-ATPase activity independently of potassium concentration. The slope and the intercept of the plots were altered by plasma extract but Km remained unchanged, indicating a noncompetitive mode of the action. Fig. 3B shows the kinetic behavior of Na-K-ATPase assayed in the presence of a control solution or plasma extracts. In all experiments, the rates of hydrolysis were linear with time both in the presence and absence of plasma extracts. In contrast, the action of ouabain on the enzyme reaction was of delayed onset over 30 sec, followed by a progressively increasing inhibition (Fig. 3A).

The clinical profiles of patients and normal subjects are shown in Table 1. Plasma extracts from hypertensive patients caused a significantly greater inhibition on Na-K-ATPase than those from control subjects (p < 0.01). The patients tended to be of higher ages than the controls (p < 0.01), while body weight, height

| Table 1. Characteristics and NKI of normotensive subjects and patients with essential hypertension |
|-------------|-----------|-------|-----|--------|
|             | n | Age (years) | BMI | MBP   | NKI (%) |
| Normotensive subjects | 21 | 35 ± 2.6 | 35.2 ± 1.2 | 88 ± 1.7 | 25 ± 2.4 |
| Patients with essential hypertension | 28 | 43 ± 2.6** | 37.4 ± 1.0 | 110 ± 2.2** | 44 ± 2.8** |

MBP, mean blood pressure (mmHg);
BMI, body mass index: weight (Kg)/height² (m).
All values indicate means ± s.e.
**p < 0.01, when compared with normotensive subjects.
DISCUSSION

The present study confirmed the previous findings that an inhibitory activity of Na, K-ATPase was detectable in human plasma and was increased in patients with essential hypertension as compared with that in normotensive subjects (MacGregor et al. 1981; Poston et al. 1981a, b; Hamlyn et al. 1982). Despite the significant difference of the NKI between the two groups, no significant correlation was shown between the values of NKI and mean blood pressure. Although the present study failed to match the ages of each group, the difference of the NKI values between the two groups is not likely due to the mismatch of the age because no correlation was found between NKI values and the age in each of subjects studied.

Previous reports indicated that plasma NKI is active i) to inhibit Na, K-ATPase (Gruber et al. 1980; Hamlyn et al. 1982; Boschi et al. 1985), ii) to depress Na transport in white blood cells (MacGregor et al. 1981; Poston et al. 1981a, b) or in vascular smooth muscle cells (Haddy and Pamnani 1983), iii) to prevent ouabain from binding to its cellular receptor (Devynck et al. 1983; de The et al. 1984), and iv) to cross-react with antidigoxin antibody (Gruber et al. 1980, 1982).

Despite all the similarities to digitalis, the plasma sodium transport inhibitors are known to show some distinct features from digitalis. Digitalis has a low rate constant of binding on the receptor site so that its inhibitory effect appears slowly

![Fig. 4. Relationship between mean arterial blood pressure and percent inhibition of Na, K-ATPase by plasma extracts from normotensive (●) and hypertensive (○) subjects.](image-url)
and increases progressively. The inhibitory effect is competitively antagonized by potassium that causes allosteric change of the receptor site. In contrast, the inhibitory effect of plasma NKI on Na, K-ATPase in vitro appeared promptly, reached rapidly its maximum, and remained constant for at least 15 min in this study. Moreover, its inhibitory action was independent of potassium concentration, even a concentration of 20 mmole/liter, failed to affect the NKI action.

These findings are fairly comparable with some previous reports from others. Human deproteinized plasma is known to show an inhibitory action on Na, K-ATPase (Hamlyn et al. 1982). The rate of its inhibitory action was constant throughout the whole reaction period (15 min) and an excessive potassium of 100 mmole/liter still failed to antagonize the action effectively (Hamlyn et al. 1985). An extract from the bovine hypothamus, another endogenous NKI, has been reported to show a high affinity to Na, K-ATPase (Hauert et al. 1984). This substance requires magnesium for maximal binding to the enzyme, whereas ouabain requires magnesium, sodium and ATP. The rate of the reaction mediated by Na, K-ATPase was linear with time either in the presence or absence of the hypothalamic extract (Hauert et al. 1984). These attributes of endogenous NKI(s) are well consistent with our present findings on plasma extract, but clearly inconsistent with those of ouabain.

An action of plasma NKI on Na, K-ATPase was completely abolished in the presence of bovine serum albumin even at the concentration as low as 500 μg/ml in this study. This finding may suggest the possibility that NKI, if released into the blood stream, is readily bound to serum proteins and circulates in the biologically inactive form. The consequence would prompt us to raise the question whether plasma free NKI, supposedly an active form of NKI, is actually working as a physiological regulator in vivo, as suggested by recent works (Hamlyn et al. 1987). It is also necessary to keep in mind that the findings on extracted substances, even if they are proved to have an inhibitory action on the Na, K-ATPase preparations in vitro, do not necessarily translate the physiological sequence of cellular sodium transport.

Of the recent studies on the chemical nature of NKI, Clarkson and de Wardener (1985) reported a fraction with natriuretic activity from human urine, which showed an inhibitory activity to Na, K-ATPase, a digoxin-like immuno-reactivity, and an ability to prevent ouabain binding to cellular receptors, well satisfying the biochemical properties of the endogenous digitalis-like substances. With successive purifications, however, each of these activities was separately proved in the different fractions, suggesting a multiplicity of the related substances rather than a single compound. Moreover, Tamura et al. (1985, 1987) have purified NKIs from the plasma of hog acutely infused with saline. The inhibitors have been identified as unsaturated free fatty acids, linoleic and oleic acids, and lysophosphatidylecholines. Hamlyn et al. (1987) have also reported two amphipathic materials isolated from the plasma of volume-expanded humans.
Structural analysis by mass spectrometry could identify one material as lysophosphatidylcholine-γ-palmitoyl. Although some properties and actions of these materials showed a similarity to the cardiotonic steroids, their detailed studies (Hamlyn et al. 1987) suggested that the actions of these materials were not affected by alterations of assay conditions known to modify the actions of ouabain or vanadate. Finally, they concluded that these materials most likely inhibit the Na, K-ATPase by their detergent-like properties rather than by a direct interaction with ouabain-binding site. Our present findings on plasma NK.I may be thus in line with their conclusions. It seems premature to assume plasma NK.I as a pathogenic factor in essential hypertension.

References


