Ouabainlike Na\textsuperscript{+},K\textsuperscript{+}-ATPase Inhibitor in the Plasma of Normotensive and Hypertensive Humans and Rats

HUGH E. DE WARDENER, JACQUELINE MILLETT, SHARON HOLLAND, GRAHAM A. MACGREGOR, AND JAMSHID ALAGHBAND-ZADEH

SUMMARY Acute volume expansion, increased sodium intake, and restraint on sodium excretion endow the plasma with an increased capacity to inhibit sodium transport. Cytochemical techniques can detect the presence of Na\textsuperscript{+}K\textsuperscript{+}-adenosine triphosphatase (ATPase) inhibitor in the plasma of normal humans and rats, the concentration of which is controlled by salt intake. The substance responsible appears to originate in the hypothalamus, where the concentration is also controlled by salt intake. The plasma concentration of the cytochemically detectable Na\textsuperscript{+},K\textsuperscript{+}-ATPase inhibitor is substantially raised in the plasma of patients with essential hypertension, spontaneously hypertensive rats (SHR) and of Milan hypertensive rats. The concentration of activity in the hypothalamus of SHR is also considerably raised. These findings demonstrate that these forms of hypertension are associated with a rise in the concentration of a cytochemically detectable circulating Na\textsuperscript{+},K\textsuperscript{+}-ATPase inhibitor that under normal circumstances is controlled by salt intake.

(Key Words: cytochemical bioassay • sodium intake • hypothalamus)

It has been known for about 15 years that the plasma and urine of normal humans and animals have the ability to inhibit sodium transport, and that the potency of this effect is regulated by the state of fluid volume. Various methods have been used to demonstrate that this is due, in part at least, to a Na\textsuperscript{+},K\textsuperscript{+}-adenosine triphosphatase (ATPase) inhibitor. We have used direct and indirect cytochemical bioassay techniques to measure changes in Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity of live cells because their sensitivity is such that assays are performed with dilutions of plasma and they have a certain selectivity.

\textbf{Na\textsuperscript{+},K\textsuperscript{+}-ATPase Inhibition}

Cytochemical bioassays are in use for several known hormones such as thyroid-stimulating hormone, parathyroid hormone, and gastrin. Such assays are based on the principle that when a hormone binds to its receptor it produces a transient, rapidly reversible effect on an enzyme that can be revealed by means of a colored end product at the site of enzymatic activity. The density of the color is then measured with a scanning and integrating microdensitometer. Usually a cytochemical bioassay is developed after a protracted search for a suitable enzymatic effect using a pure preparation of the hormone. The specificity of the assay is determined in a variety of ways, including demonstration that it is competent to distinguish between plasmas with raised and low circulating levels, and more precisely by demonstrating that the assay is blocked by an antibody to the hormone.

In our attempt to obtain a cytochemical assay for an endogenous Na\textsuperscript{+},K\textsuperscript{+}-ATPase inhibitor the search was reversed. We were starting with a predetermined known enzymatic effect, that is, inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity, to look for a Na\textsuperscript{+},K\textsuperscript{+}-ATPase inhibitor of unknown structure, the concentration of which in plasma was controlled by the state of fluid volume. We used a technique developed by Chayen et al. to measure Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in fresh tissue. The selectivity of our assay was determined in several ways. In the absence of an antibody raised specifically against the endogenous Na\textsuperscript{+},K\textsuperscript{+}-ATPase inhibitor, an antibody to digoxin was used.
It was found that ouabain and normal human plasma both induced a reversible inhibition of Na\(^+\), K\(^+\)-ATPase of fresh guinea pig kidney proximal tubule that is maximal at 4 to 6 minutes (Figure 1). In cytochemical assays of known hormones the time at which the enzymatic change is maximal is one of the features that confers specificity. The plasma had no effect on ouabain-insensitive Na\(^+\), K\(^+\)-ATPase activity. These observations demonstrate that plasma not only inhibits Na\(^+\), K\(^+\)-ATPase but that the nature of this inhibition resembles that of ouabain.

Using this technique we found that dilutions of plasma from salt-loaded humans inhibit Na\(^+\), K\(^+\)-ATPase about 25 times more than those of persons who are on a low salt diet.\(^6\) This suggests that the technique is measuring something of physiological importance related to volume, or at any rate to sodium intake.

**Glucose-6-Phosphate Dehydrogenase Stimulation**

The cytochemical technique with which Na\(^+\), K\(^+\)-ATPase is measured is semiquantitative in that, although it is possible to obtain some information from the time course, the small amount of baseline Na\(^+\), K\(^+\)-ATPase activity remaining in the kidney section has made it difficult to obtain a dose-inhibitory response curve of sufficient steepness for a reproducible quantitative assay. Inhibition of Na\(^+\), K\(^+\)-ATPase, however, is associated with stimulation of glucose-6-phosphate dehydrogenase (G6PD) activity,\(^7\) and it has been found that the steepness of the rise in G6PD activity gives a satisfactory dose-response curve. This has made it possible to develop and validate a precise quantitative assay that measures the capacity of body fluids or extracts to stimulate G6PD as an index of their ability to inhibit Na\(^+\), K\(^+\)-ATPase.\(^4\)

Figure 1 shows that normal human plasma and ouabain both induce a reversible rise in G6PD activity, which is maximal at 2 minutes. A plasma of high potency is used as a standard. In established cytochemical bioassays for known hormones, the quantitative effect induced by a pure hormone when measured at a predetermined time of exposure is only evident over a specific range of low concentrations of the hormone. Similarly, with increasing dilutions of the high potency plasma, stimulation of G6PD activity, measured at the predetermined time of exposure (2 minutes), first becomes evident and then reaches a peak of activity before returning to baseline in a log-linear relationship (Figure 2). A biphasic phenomenon of the dose response is seen in all established cytochemical bioassays\(^3\) for known hormones. It is attributed to the rapid effect of the hormone on enzyme activity, so that at high concentrations of the hormone the induced effect on the enzyme activity, after a 2-minute exposure in this instance, is in the recovery phase and thus appears to be less than at a lower concentration. It is the linearity and reproducibility of the return of G6PD-stimulating activity to zero that endows the assay with precision. The amount of G6PD stimulation that produces maximum G6PD stimulation at 2 minutes is defined as 1 unit of G6PD-stimulating activity. To be quantitative, the dose-response curve of the unknown must be parallel to that of the standard.

Statistical assessment of assay validity and potency ratio has been made with reference to the *European Pharmacopoeia*.\(^8\) The intra-assay variation is within 10% and interassay variation is 25%, with a mean index of precision for nine consecutive assays of 0.07 ± 0.04 (SD), which is well within the accepted limits for bioassays. The sensitivity of the assay is such that, with normal human plasma a rise in G6PD can be detected at dilutions as great as 1:10,000. As with the
ability of plasma to inhibit Na\(^+\),K\(^+\)-ATPase activity at 4 to 6 minutes, the ability of plasma to increase G6PD activity at 2 minutes appears to be due to a substance that has several characteristics of ouabain in that the time course of the reversible rise in G6PD activity is like that of ouabain. Recently we found that the rise in G6PD activity and fall in Na\(^+\),K\(^+\)-ATPase induced by plasma is reduced by more than 80% by the addition of digitoxin antibody to the plasma.\(^3\) These findings also demonstrate that the activity of plasma to stimulate G6PD is closely linked to its capacity to inhibit Na\(^+\),K\(^+\)-ATPase. Up to the present, all the materials so far tested that have stimulated G6PD activity maximally at 2 minutes, including ouabain; plasma from normal subjects and rats\(^5\),\(^9\) and from hypertensive patients\(^10\) and rats\(^11\),\(^12\); and extracts of hypothalami from normal\(^6\) and hypertensive rats\(^11\) and human urine,\(^13\) have also inhibited Na\(^+\),K\(^+\)-ATPase activity maximally at 4 to 6 minutes, and their dose-response curves in the G6PD assay have been parallel.

Tests of specificity have been performed by assay- ing many substances in physiological and pharmacological concentrations,\(^4\),\(^5\) many of which are known to have metabolic effects on the proximal tubule: epinephrine, aldosterone, angiotensin, arginine vasopressin, calcitonin, 1,25-dihydroxycholecalciferol, dopamine, luteinizing hormone–releasing hormone, 3-methoxy-4-hydroxyphenyl ethylamine, norepinephrine, parathyroid hormone, prolactin, sodium orthovanadate, thyrotropin-releasing hormone, 3,3',5-triiodo-L-thyronine, atrial peptin III, sodium vanadate, and recently, linoleic acid, oleic acid, dehydroepiandrosterone sulfate, lysophosphatidyl choline, and atrial natriuretic peptide, which are known inhibitors of purified preparations of Na\(^+\),K\(^+\)-ATPase. Only ouabain has shown any cross-reactivity. So far, therefore, the only connection that we have been able to find with the substance in the plasma that inhibits Na\(^+\),K\(^+\)-ATPase at 4 to 6 minutes and stimulates G6PD at 2 minutes are the glycosides ouabain and digoxin.

Acetone extracts of the following organs were assayed: pituitary, hypothalamus, cerebral cortex, muscle, heart, adrenal, liver, spleen, pancreas, kidney, stomach, duodenum, jejunum, and ileum.\(^4\) The only tissue that yielded extracts of high activity was the hypothalamus. The time course of the effect of the hypothalamic extract was the same as that obtained with ouabain and plasma, while the dose-response curve of the hypothalamic material was parallel to that of plasma. In addition, like plasma, the ability of hypothalamic extract to stimulate G6PD activity was depressed by more than 80% by incubating the extract with digoxin antibody. Purification and isolation of the cytochemically detectable active material from the hypothalamus is being attempted by acetone extraction, chloroform wash, silica gel electrophoresis, and reverse-phase high-performance liquid chromatography when the activity appears in a discrete fraction that shows no ultraviolet absorption.\(^5\) Using the same procedures, we found that the Na\(^+\),K\(^+\)-ATPase-inhibiting, G6PD-stimulating activity of plasma supernate was also insoluble in chloroform and appeared in the same fractions as the hypothalamic activity. The activity of both hypothalamic extract and plasma supernate survived boiling and 6 N HCl at 105°C for 18 hours under nitrogen and passed through an Amicon UM05 ultrafiltration membrane (Danvers, MA, USA). These several similarities between plasma and hypothalamic extract suggest that the active substance in plasma and the hypothalamus may be closely related structurally.

The following additional information has been obtained about the activity of the substance in whole plasma.\(^5\) The activity of whole plasma was increased by more than 100% by incubation at 37°C for 45 minutes, an effect that was not influenced by apronitin (Trasylol), and plasma activity was severely depressed by freezing and thawing on more than one occasion. Figure 3 shows that the ability of the plasma of humans and rats to stimulate G6PD is controlled by salt intake.\(^4\) The plasma obtained when 12 subjects were on a high sodium diet for 5 days stimulated G6PD activity about 20 times more than when they were on a low sodium diet for the same time. In addition, it was found that the hypothalamus of the rats on a high sodium diet contained approximately 100 times more G6PD-stimulating activity than extracts obtained from

![Figure 3. The effect on glucose-6-phosphate dehydrogenase (G6PD) activity of guinea pig kidney at 2 minutes, of plasma from normal humans and rats on low and high sodium intake: and the content of G6PD-stimulating activity of extracts from the hypothalami of normal rats on low and high sodium intake. The ordinate for the plasma results represents G6PD-stimulating units per milliliter, and for the hypothalamic extract \(10^6\) G6PD-stimulating units per hypothalamic.]
rats on a low sodium diet (see Figure 3). These observations, in addition to those mentioned above, which suggest that the substance responsible for the Na\(^{+}\),K\(^{+}\)-ATPase-inhibiting, G6PD-stimulating activity in the plasma and the hypothalamus may be the same, suggest that the active substance in the plasma may originate from the hypothalamus.

**Hypertension**

**Humans**

The plasma of patients with essential hypertension has a significantly greater ability to stimulate G6PD than that of normal controls, and the plasma of patients with low plasma renin (i.e., less than 0.5 ng/hr/ml) is significantly greater than that of persons with a normal plasma renin. A significant correlation was noted between systolic, mean, and diastolic arterial pressures and the capacity of plasma to stimulate G6PD activity.

**Rats**

We measured the capacity of plasma and hypothalamic extract from Okamoto spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) control rats to inhibit Na\(^{+}\),K\(^{+}\)-ATPase and stimulate G6PD. The ability of plasma from control WKY to stimulate G6PD is similar to that of the normal Wistar rat, while the ability of plasma from the Okamoto SHR to do so is much greater. The capacity of the hypothalamic extract from control WKY to stimulate G6PD is again similar to that of the normal Wistar rat, while the extract obtained from the Okamoto SHR has a greatly raised ability to stimulate G6PD: approximately 100 times greater than the high levels previously obtained from the hypothalamus of salt-loaded normal rats.

To confirm that these large increases in the ability of the plasma and hypothalamus of the Okamoto SHR to stimulate G6PD are an indication of an increased ability to inhibit Na\(^{+}\),K\(^{+}\)-ATPase, time courses of the reversible effect of plasma and hypothalamic extract on Na\(^{+}\),K\(^{+}\)-ATPase activity were measured. Using this semiquantitative technique, it was found that the ability of the plasma and the hypothalamus from the Okamoto SHR to inhibit Na\(^{+}\),K\(^{+}\)-ATPase activity was substantially greater than the plasma of the control WKY (Figure 4).

More recently we studied the plasma of 3- and 9-week-old Milan hypertensive rats (MHS) and their normotensive controls. The blood pressure of the MHS at 3 weeks was normal, whereas it was raised at 9 weeks. The ability of the plasma from the 9-week-old rat to inhibit Na\(^{+}\),K\(^{+}\)-ATPase and to stimulate G6PD was greatly raised. At 3 weeks the plasma level of G6PD-stimulating activity was significantly raised, but it was considerably less than at 9 weeks. This finding in the young MHS before the main rise in blood pressure had taken place suggests that the rise in Na\(^{+}\),K\(^{+}\)-ATPase-inhibiting, G6PD-stimulating ability of the plasma at 9 weeks, when the rat is hypertensive, is not a secondary phenomenon related to either the rise in arterial pressure or the effect of the hypertension on renal function. It also suggests that the substance responsible for the inhibition of Na\(^{+}\),K\(^{+}\)-ATPase activity may be involved in the rise in arterial pressure.

Overall, these findings in normal humans and rats, in patients with essential hypertension, and in two strains of hypertensive rats demonstrate that these hereditary forms of hypertension are associated with a rise in the concentration of a cytochemically detectable circulating Na\(^{+}\),K\(^{+}\)-ATPase inhibitor that, under normal circumstances, is controlled by salt intake. The findings are consistent, therefore, with the hypothesis that the rise in the ability of plasma to inhibit Na\(^{+}\),K\(^{+}\)-ATPase in hereditary forms of hypertension is due to an inherited renal difficulty in excreting sodium.
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H E de Wardener, J Millett, S Holland, G A MacGregor and J Alaghband-Zadeh

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