Demonstration of a Ouabainlike Plasma Compound in Hypertension Prone and Hypertension Resistant Rats

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SUMMARY Material extracted and partially purified from plasma of the Sabra hypertension prone rats was found to be capable of 1) inhibiting the binding of $^3$H-ouabain to rat brain synaptosomes, 2) inhibiting the activity of rat brain microsomal Na, K activated adenosine triphosphatase, and 3) increasing the contractile force of rat heart muscle. The results demonstrate the presence of a ouabainlike compound in the plasma of these rats. The plasma concentration of this compound in Sabra hypertension prone rats was 698 ± 199 nmol/ml in ouabain equivalents (SEM; n = 11) versus 2543 ± 1140 nmol/ml (n = 9) in the Sabra normotensive strain. The presence of ouabainlike compound in the plasma is consistent with the hypothesis that this compound functions as a hormone that regulates Na, K activated adenosine triphosphatase activity and the physiological processes in which this enzyme is involved. (Hypertension 7: 729-733, 1985)

KEY WORDS • digitalis • ouabainlike compounds • Na, K activated adenosine triphosphatase • genetic hypertensive rats • heart contractility

O VER the past 20 years numerous studies have demonstrated the presence of a natriuretic factor in mammalian blood and urine that is activated or released by volume expansion.1-2 Recent evidence suggests that this factor is an inhibitor of Na, K activated adenosine triphosphatase (Na, K-ATPase),3-4 which probably interacts with the ouabain binding site on the enzyme.6,7

Theoretical considerations and experimental evidence suggest that inhibition of Na, K-ATPase can increase the contractile force of vascular smooth muscle.9-11 Since this action may elevate peripheral vascular resistance and induce a rise in blood pressure,9,11 a link between the natriuretic factor and the etiological process of some forms of hypertension was postulated.14-17 This hypothesis is supported by the identification of a circulating endogenous inhibitor of the Na, K-ATPase in hypertensive animals14-16 and humans.17,18

Moreover, the concentration of the Na, K-ATPase inhibitor has been reported to be linearly related to the level of blood pressure in normal and hypertensive individuals.17

The presence of a digitalislike or ouabainlike compound (OLC) also has been reported in animal tissue.19-25 These studies were prompted by the demonstration of specific receptors for cardiac glycosides on the Na, K-ATPase, which suggested the existence of an endogenous ligand. Since compounds that can inhibit Na, K-ATPase activity by interacting with the digitalis binding site on the enzyme were indeed extracted from mammalian brain19-23 and from toad skin and blood,24,25 we thought it reasonable to assume that the natriuretic substance and the ouabainlike compound are identical.

The availability of the Sabra hypertension prone rats (SBH) and Sabra normotensive rats (SBN) prompted a search for a circulating ouabainlike inhibitor of the Na, K-ATPase in this model. The two strains initially were selected from the Hebrew University Sabra rat for their respective sensitivity or resistance to deoxyxorticosterone acetate salt hypertension.26 (For a detailed description of the main characteristics of these animals, see refs. 27 and 28.) We also sought to determine whether the disparate susceptibility to hypertension of these rats could be related to the presence of a circulating Na, K-ATPase inhibitor in the plasma.
Materials and Methods

Partial Purification of Ouabainlike Compound

Studies were performed on male SBH and SBN, aged 2 to 5 months, maintained since weaning on regular laboratory chow (Ambar Food Mills, Hadera, Israel) containing 0.6 to 0.8% sodium and tap water. The blood pressure (mean ± SE) measured in prewarmed, unanesthetized animals was 151 ± 2.4 and 127 ± 1.3 mm Hg respectively ($p < 0.01$). The animals were bled from the abdominal aorta under ether anesthesia. The plasma of 3 to 10 rats was pooled, and methanol was added (v/v). Proteins were precipitated by high speed centrifugation (100,000 g), and the supernatant decanted. The methanol was evaporated, and the samples were centrifuged again at 100,000 g. The aqueous supernatant was separated and lyophilized. The residue was dissolved in 1.2 ml of methanol, and aliquots of 20 to 200 /µl were fractionated by high-performance liquid chromatography (model 985, Tracor Instruments, Austin, TX, USA) using an Alltech amino column. The column had been equilibrated with 90% acetonitrile and eluted with a linear 90% to 20% acetonitrile gradient in 40 minutes at a flow rate of 1 ml/min. The elution of the compounds was monitored at 300 nm with a Tracor 970A detector. Fractions of 2 ml were collected and evaporated, and the residue was dissolved in 1 ml of distilled water. Aliquots from this solution were used for measurements of biological activity.

The method used for the extraction and partial purification of the OLC is highly reproducible. When aliquots of pooled plasma extract were processed by this method values of OLC obtained did not vary by more than 5%.

Measurements of 3H-Ouabain Binding to Rat Brain Synaptosomes

Rat brain synaptosomes were prepared as previously described.21 Binding of 3H-ouabain was assayed by a conventional filtration technique. The reaction mixture (500 /µl final volume) contained the following constituents (in final concentrations): 50 mM tris(hydroxymethyl) aminomethane hydrochloride buffer (pH 7.4), 0.5 mM ethylenediaminetetraacetic acid, 80 mM NaCl, 4 mM MgSO₄, 2 mM adenosine triphosphate (Tris, vanadium-free; Sigma Chemical Co., St. Louis, MO, USA), 32 nM [3H]ouabain (19.5 Ci/ mmol; New England Nuclear Corp., Boston, MA, USA), and other additions as indicated. Reactions were initiated by the addition of 200 /µl of a crude synaptosomal fraction, which resulted in a final protein concentration of 200 /µg per reaction. Binding equilibrium was achieved after incubation for 1 hour at 37 °C. The reactions were terminated by adding 3 ml of ice-cold 50 mM Tris-hydrochloride (pH 7.4), and the suspension was filtered over Whatman GF/F filters (Whatman, Inc., Clifton, NJ, USA). Filters were washed three times with 3 ml of the same Tris buffer, dried, and assayed for radioactivity in a Packard liquid scintillation counter (Packard Instrument Co., Inc., Rockville, MD, USA) at a counting efficiency of approximately 35%. Specific binding was calculated by subtracting the binding observed in the presence of 100 nM unlabeled ouabain from that observed in the absence of unlabeled ouabain. Specific binding represented 93% of the total binding under control conditions. Binding of [3H]ouabain was a linear function of membrane protein concentration in the range of 20 to 1000 nM per assay. Binding to the filters was negligible when synaptosomes were omitted from the incubation.

Measurements of Na, K Activated ATPase Activity

Enzyme activity in rat brain microsomal fraction was measured by the colorimetric determination of inorganic phosphate after the incubation of synaptosomes (200 /µg of protein per reaction) at 37 °C in a solution containing (in final concentrations): 54 mM Tris-hydrochloride (pH 7.4), 100 mM NaCl, 20 mM KCl, 4 mM MgCl₂, and 4 mM adenosine triphosphate (Tris, vanadium-free). The adenosine triphosphate was added after a 10-minute preincubation to initiate the reaction. Reactions were terminated by adding 5% tricarboxylic acid, and the precipitate was removed by centrifugation. The Na, K-independent ATPase activity was determined by omitting Na⁺ and K⁺ from the reaction mixture or by adding 1 mM ouabain to the complete assay mixture.

Measurements of Cardiac Muscle Contraction

Tension recordings were done from rat atrial strips placed in a 2-ml chamber and perfused with control solution containing 140 mM NaCl, 5.4 mM KCl, 12 mM NaHCO₃, 2 mM CaCl₂, 1 mM MgCl₂, 0.4 mM Na₂HPO₄, and 5 mM glucose, continuously bubbled with 95% O₂, 5% CO₂ with a pH of 7.4 at 36 °C. One end was attached to an AKERS force transducer (Aks-jeselskapet mikro-elektronikk, Horten, Norway) for measuring the strength of contraction. Constant stimulation was applied at a rate of 0.5 Hz, and twitch magnitude was monitored on a pen recorder and oscilloscope and stored on magnetic tape.

Results

An example of a chromatographic pattern is shown in Figure 1. The biological activity (i.e., inhibition of 3H-ouabain binding) was found in the peaks at a retention time of 12 to 20 minutes with a maximum in the fraction at 15 minutes.

In the first set of experiments approximately 300 ml of pooled plasma from SBH was used. The OLC was extracted and partially purified as described in Methods. The material present in the fractions with a retention time of 12 to 20 minutes was collected, and aliquots were tested. As can be seen in Figure 2, the endogenous material was capable of inhibiting both ouabain binding and Na, K-ATPase activity. Both effects appeared to depend on the concentration of the material in the assay. Like ouabain, the endogenous compound inhibited Na, K-ATPase activity at concentrations almost 100 times higher than those need-
High-performance liquid chromatography of methanol extract of plasma obtained from Sabra hypertensive rats. Methanol extract of 20 ml of plasma obtained from these rats was processed as described in Methods. The optical density is plotted (left ordinate) against retention time (abscissa). On the right ordinate, the relative biological activity is represented by hatched columns. This activity was measured by testing the capability of each fraction to inhibit \(^3\)H-ouabain binding to rat brain synaptosomes (see Figure 2).

**Figure 1.**

The positive inotropic effect of the partially purified material on rat atrium. The ouabainlike compound was extracted from pooled rat plasma and partially purified as described in Methods. Top tracing: Tension traces are shown from left to right in a control solution and after exposure to 0.5 \(\mu\)M of the compound for 9 minutes and 13 minutes, and after 20 minutes of washout with control solution. Bottom tracing: Superposition of tension traces in control and after 13 minutes of exposure to the ouabainlike compound.

**Figure 3.**

Inhibition of \(^3\)H-ouabain binding and Na, K-activated adenosine triphosphatase (Na, K-ATPase) activity by plasma extracts separated from Sabra hypertensive rats. The methods for synaptosome and microsome preparations from rat brain and measurements of \(^3\)H-ouabain binding and Na, K-ATPase have been described previously.\(^{21,22}\) The control binding in the absence of extract was 12.5 pmol \(^3\)H-ouabain per milligram of protein and was taken as 100%. Closed circles represent the inhibition of binding by partially purified ouabainlike compound. The ouabainlike compound obtained from pooled plasma was diluted, and the equivalent initial plasma value was calculated. Control ATPase activities were 45.0, 19.3, and 25.7 \(\mu\)mol P/mg protein/hr for total, Na, K-dependent, and Mg-dependent enzymes respectively. Open circles show the inhibition of Na, K-ATPase at different concentrations of plasma.

**Figure 2.**

In a second set of experiments, the compound partially purified from plasma of several groups of SBH and SBN (with the use of the chromatographic system described in Methods) was compared for its ability to inhibit \(^3\)H-ouabain binding.\(^8\) Furthermore, at concentrations that caused 50% inhibition of Na, K-ATPase activity, no change in Mg-ATPase activity was detected.

Since cardiac glycosides increase the contractile force of heart muscle, demonstration of such an effect by the endogenous compound might provide further indication of its ouabainlike activity. As can be seen in Figure 3, the addition of the partially purified material to a preparation of rat atrial muscle resulted in a marked increase (30%) in the contractile force that was completely reversible on washout. In two other experiments, 20 \(\mu\)M ouabain resulted in an increase in the force of contraction by 70% and 100% (data not shown). The results of this set of experiments are consistent with the contention that the endogenous compound is ouabainlike in nature.

As shown in Table 1, the plasma levels of the \(^3\)H-ouabain displacing material were extremely variable, averaging 2543 ± 1140 and 698 ± 199 pmol/ml of plasma (mean ± SEM) for SBN and SBH respectively. The ability of the compound to inhibit Na, K-ATPase...
activity was not assessed in this study since, as previously stated, large volumes of plasma are needed for this assay.

Discussion

This study provides evidence for the presence of an endogenous inhibitor of Na-K-ATPase in the plasma of hypertension prone Sabra rats. The compound also appears to be capable of inhibiting 3H-ouabain binding to its specific receptors on the plasma membrane. These findings indicate that the inhibition of the enzyme activity by the endogenous compound is due to its interaction with the digitalis binding sites of Na-K-ATPase.

In addition, our results demonstrate that the endogenous plasma compound, like digitalis, increases the contractile force of heart muscle. The increase in the positive inotropic effect amounted to 30% and was observed at a concentration of 0.5 μM ouabain equivalents. The concentration of the OLC was approximated from the binding experiments under the assumption that the endogenous material and ouabain have the same affinity for their common binding site. Evidently, this is a crude estimation, and the actual plasma concentration of the material remains to be determined. Moreover, it is premature to compare the relative potencies of OLC to cardiac glycosides in their ionotropic effect. Atrial contractility may be influenced by other factors including local release of endogenous catecholamines and alterations in calcium metabolism. However, the observation that the same material that inhibited 3H-ouabain binding and Na-K-ATPase activity also increased the contractile force of the myocardium strongly supports the contention that the endogenous compound is ouabainlike in nature.

The finding of an increase in the contractile force of heart muscle by material isolated from rat blood is in accordance with our recent report of a similar effect of compounds partially purified from toad skin and sheep brain. Due to the small amount of OLC obtained from the rat plasma, we were unable to characterize the inotropic effect with respect to its dose dependence, sensitivity to α-adrenergic and β-adrenergic blockers, extracellular ion concentration, and rate of stimulation. It is noteworthy, however, that the compounds partially purified from toad skin, human cerebrospinal fluid, and sheep brain also increase the contractile force of heart muscle. The inotropic effect of the toad skin OLC was characterized in detail and was found to be almost identical to that obtained with cardiac glycosides (unpublished observation). Since all the compounds in these studies were separated using almost identical methods, the similarity of the toad skin compound to digitalis may also apply to the OLCs obtained from other sources. The presence of such compounds in mammalian blood raises the possibility that they might participate in the regulation of cardiac contractility under physiological conditions.

In spite of extensive investigation, the structure of these compounds is unknown. Partial characterization of the OLC obtained from different sources indicates that it is of low molecular weight (< 1000 daltons). The elucidation of its structure is clearly an important challenge, and studies are in progress to achieve this goal.

In the present study, while the difference in blood pressure between SBH and SBN was highly significant, no difference in OLC levels was detected. The moderate difference in systolic blood pressure reported here was invariably associated with a measurable degree of cardiac hypertrophy in the SBH. For example, the heart weight of adult rats corrected for 100 g body weight averaged 268 ± 8 mg in SBH compared with 247 ± 4 mg in SBN (p < 0.01). Previous studies have shown that the two strains are dissimilar in several other physiological parameters. For example, compared with SBN, the SBH are heavier, have lower fluid intake and urinary output, and have markedly elevated urine osmolality. These important differences have no apparent effect on the activity of the renal Na-K-ATPase, which is comparable in isolated nephron segments. The lack of difference in OLC levels (as estimated by inhibition of ouabain binding in rat brain synaptosomes), in conjunction with results in isolated nephron, does not suggest a correlation between high blood pressure and alterations in pump activity in the SBH. These results are at variance with two recent studies in humans in which a significant difference between hypertensive and normotensive subjects was observed.

The large variation of OLC level in our rats may reflect individual variability of undefined physiological parameters that affect the metabolism of the compound. Alternatively, it may be due to technical prob-

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**Table 1.** 3H-Ouabain Displacing Material in Plasma of Sabra Normotensive (SBN) and Hypertensive (SBH) Rats

<table>
<thead>
<tr>
<th>Experiment</th>
<th>SBN (pmol/ml plasma)</th>
<th>SBH (pmol/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>287</td>
<td>282</td>
</tr>
<tr>
<td>2</td>
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<td>1122</td>
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<td>10</td>
<td>—</td>
<td>439</td>
</tr>
<tr>
<td>11</td>
<td>—</td>
<td>70</td>
</tr>
</tbody>
</table>

Mean ± SEM: 2543 ± 1140 (SBN) 698 ± 199 (SBH)

The concentration of the compound in pooled plasma of 3 to 10 rats was estimated by testing the ability of extracted and separated material to inhibit 3H-ouabain binding to rat brain synaptosomes. Each experiment represents a different pool. The difference in the concentration of the compound between SBH and SBN was not statistically significant.
lems such as variation in the recovery of the chromatographic system used for the separation of the compound. Another factor could be the time interval from the extraction of the material until its determination, which ranged from 1 week up to 3 months in this study. Since the long-term stability of the compound is still unknown, the length of storage may have affected the results.

Finally, since the OLC was determined on deproteinized plasmas, the effect of denaturation of plasma proteins should be considered. It is possible that a large portion of the compound, like steroid hormones, is bound to plasma proteins. Assuming that only the free fraction is physiologically active and relevant to hypertension, a putative strain difference would be obliterated by the current method in which the total plasma OLC values were determined.

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