The Effects of Urinary Digitalislike Factor on Cultured Vascular Smooth Muscle Cells

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SUMMARY We attempted to purify a digitalislike factor from human urine. On the assumption that a natural ligand for the digitalis receptor should be searched for on the basis of the effects on intact cells, we used an inhibitory effect on the binding of \([^3H]\)ouabain to human erythrocytes to determine digitalislike activity. A highly polar \([^3H]\)ouabain displacing activity was obtained by a combination of chromatographic procedures including reverse-phase high performance liquid chromatography. Urine-derived \([^3H]\)ouabain displacing activity, a competitive inhibitor of ouabain binding to human erythrocytes, acted on human lymphocytes in a similar manner. The dose-response curve of this compound was parallel to that of ouabain. Urine-derived \([^3H]\)ouabain displacing activity significantly inhibited monensin-stimulated increase in ouabain-sensitive \(^{86}\)Rb uptake, a parameter of Na\(^{+}\),K\(^{+}\)-adenosine triphosphatase (ATPase), by 95% (p<0.01) in cultured vascular smooth muscle cells (A10 cells). Furthermore, this compound enhanced net \(^{45}\)Ca influx by 30% (p<0.01) and reduced \(^{45}\)Ca efflux by 35% (p<0.01) in A10 cells. These results suggest that urine-derived \([^3H]\)ouabain displacing activity may be a regulator of Na\(^{+}\),K\(^{+}\)-ATPase and a modulator of vascular tone. (Hypertension 11: 645–650, 1988)

KEY WORDS  hypertension  •  Na\(^{+}\),K\(^{+}\)-adenosine triphosphatase inhibitor  •  rubidium-86 uptake  •  calcium-45 flux

A BODY of evidence has been accumulated that favors the existence of an endogenous digitalislike factor as a physiological regulator of the Na\(^{+}\)-K\(^{+}\) pump. This factor could play an important role in the regulation of sodium excretion and may be associated with human essential hypertension.1-3 Efforts by a number of laboratories to purify this factor have recently intensified. Several substances have been proposed as endogenous digitalislike factors, including unsaturated fatty acids, lysophosphatidylcholine, dehydroepiandrosterone sulfate, ascorbic acid, and mammalian lignans.4-8 However, of these compounds currently identified as having digitalislike activity, none appears to be the natural ligand of the digitalis receptor of Na\(^{+}\),K\(^{+}\)-adenosine triphosphatase (ATPase).

We assumed that a natural ligand for the digitalis receptor should be searched for on the basis of the effects on intact cells. In this study, we used an inhibitory effect on \([^3H]\)ouabain binding to human erythrocytes to monitor digitalislike activity and partially purified a digitalislike factor from human urine. Furthermore, we examined the effects of the purified material on ion fluxes of cultured rat vascular smooth muscle cells.

Materials and Methods
Preparation of Crude \([^3H]\)Ouabain Displacing Activity
Human urine, approximately 4000 L each, was collected five times from normal male volunteers. Batches of 4000 L were treated on the day of collection with 50 L of Amberlite XAD2 preequilibrated with distilled water. \([^3H]\)Ouabain displacing activity (ODA) was eluted from Amberlite XAD2 with 50 L of 100% methanol and evaporated to dryness. The residue was dissolved in 10 L of distilled water and filtered through Whatman no. 1 paper (Clifton, NJ, USA). The filtrate was successively applied to ion exchange resins. The pH of the sample was not adjusted, but it was maintained at approximately 4.

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The ODA was eluted into the void volume of both column (AG 50WX8) and anion (AG 3X4A) exchange column. The active fraction was chromatographed on a Sephadex G25 column (10 x 100 cm; void volume = 2.5 L) in distilled water. The ODA was eluted in a broad fraction at an elution volume between 6.5 and 7.5 L. The eluates from Sephadex G25 chromatography were pooled and lyophilized.

Further Purification of Urine-Derived \(^{3}H\)Ouabain Displacing Activity

In a preliminary experiment, the active fractions obtained by gel filtration were resolved into four discrete peaks inhibiting \(^{3}H\)ouabain binding to human erythrocytes by high performance liquid chromatography (HPLC) on an analytical R-ODS-5 column (YMC, Kyoto, Japan; 0.46 x 25 cm). The second peak of activity, eluted off the R-ODS-5 column by 18% acetonitrile, was further purified because it was the most potent. The first minor peak was not retained by reverse-phase HPLC and eluted in the solvent front.

The active materials coming from gel filtration were applied to a 5 x 20-cm column of preparative C\(_{18}\) resin (Waters, Tokyo, Japan) that had previously been washed with acetonitrile and distilled water. The ODA was eluted with 2 L of 20% acetonitrile. The eluates were resuspended in distilled water after lyophilization and centrifuged for 30 minutes at 18,000 g. The supernatant was filtered through a 0.45-\(\mu\)m nylon filter. The filtrate was chromatographed on a semipreparative D-ODS-5 column (YMC; 2.0 x 25 cm) eluted with a gradient of acetonitrile (0-20%) over 50 minutes at a flow rate of 10 ml/min. The active fractions eluted at a retention time of 44 minutes were collected and further purified by HPLC on a D-ODS-5 column eluted with 8% acetonitrile at 10 ml/min. An active fraction emerged at a retention time of 150 minutes. This fraction was further chromatographed by gel permeation mode HPLC on a GS-320H column (Asahikasei, Kawasaki, Japan; 0.4 x 25 cm) in distilled water at 1 ml/min. Only one active fraction emerged at a retention time of 9 minutes and was collected. The HPLCs were developed using an LC4A system (Shimadzu, Tokyo, Japan) and an M-600E system (Waters).

\(^{3}H\)Ouabain Binding to Human Erythrocytes and Lymphocytes

Blood was regularly collected from a healthy normotensive subject in heparin tubes, and the blood cells were washed three times in 5 ml of Tris HCl buffer containing 130 mM NaCl, 20 mM sucrose, 10 mM glucose, and 10 mM Tris, pH 7.4. Theuffy coat was removed, and the packed red blood cells were resuspended in Tris HCl buffer to a hematocrit of 15%.

The effects of the chromatographic fractions were tested by incubating 50-\(\mu\)l samples with the erythrocyte suspension (250 \(\mu\)l) for 2 hours at 37°C. Radioactive ouabain (15.6 Ci/mmol, 50 \(\mu\)l, to give a final concentration of 20 nM) then was added, and all tubes were reincubated for a further 2 hours. All samples were tested in duplicate.

The saturation experiments were performed by incubating 250 \(\mu\)l of the erythrocytes suspension with 50-\(\mu\)l aliquots of serially diluted (in Tris HCl buffer) \(^{3}H\)ouabain and 50-\(\mu\)l aliquots of the purified ODA or cold ouabain (0.1 mM) at 37°C for 4 hours.

At the end of the incubation, the erythrocytes were washed three times with 1 ml of ice-cold 0.1 M MgCl\(_{2}\) and 10% trichloroacetic acid (500 \(\mu\)l) was added to the final pellet of erythrocytes. The tubes then were vigorously mixed using a vortex and centrifuged to sediment the precipitated protein. Finally, 300-\(\mu\)l samples were used to measure the amount of bound radioactive ouabain in 7.5 ml of liquid scintillation fluid.

Human lymphocytes were prepared using Ficoll. Intact lymphocytes were washed three times and then resuspended in Tris-HCl buffer. The final cell density was adjusted to between 1.5 x 10\(^6\) and 3 x 10\(^6\) cells/ml. \(^{3}H\)Ouabain binding to human lymphocytes was assayed under the same conditions as just described.

Other Assay Techniques

The effects of various concentrations of ouabain were tested in three different biological activities in addition to the binding of \(^{3}H\)ouabain to human erythrocytes: 1) the activity of dog kidney Na\(^+\),K\(^+\)-ATPase,\(^9\) 2) the binding of \(^{3}H\)ouabain to rat brain synaptosome,\(^10\) and 3) the uptake of \(^{86}Rb\) into human erythrocytes.\(^11\) The effects of various concentrations of digoxin on \(^{3}H\)digoxin binding to antidigoxin antibody were also tested in radioimmunoassay.

Cell Culture

Rat aortic vascular smooth muscle cells (A10) were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco’s Modified Eagle Medium plus 10% fetal calf serum. It has been reported that A10 cells express vasopressin receptors, \(\beta\)-adrenergic receptors, and platelet-derived growth factor receptors.\(^12\)\(^13\) A10 cells were subcultured onto 24-well Falcon plates (Oxind, CA, USA) for transport experiments. Cells were used 5 to 7 days after subculture in confluent monolayers.

Net \(^{45}Ca\) Influx and Efflux of Cultured Vascular Smooth Muscle Cells

Before the assay, the medium was aspirated and the cells were rapidly washed three times with 0.5 ml of HEPES buffer (150 mM NaCl, 5 mM KCl, 1.8 mM CaCl\(_{2}\), 5 mM glucose, 10 mM HEPES; pH 7.4). The incubation was started by adding 0.5 ml of HEPES buffer containing 4 \(\mu\)Ci/ml of \(^{45}CaCl\(_{2}\) with samples. After a 15-minute incubation (during which net \(^{45}Ca\) influx was constant), the reaction was terminated by aspirating the assay medium and washing the cells six times with ice-cold isotonic MgCl\(_{2}\). Cells were extracted with 10% trichloroacetic acid. The incorporated radioactivity was measured as net \(^{45}Ca\) influx.

For \(^{45}Ca\) efflux measurement, the cells were prelabeled with 4 \(\mu\)Ci/ml of \(^{45}CaCl\(_{2}\) for 2 hours and the cells were washed three times with HEPES buffer. The cells
were then incubated with HEPES buffer containing samples. The released radioactivity was counted at the end of the incubation. Protein pellets were digested in 1 M NaOH and assayed for protein by the method of Bradford.\textsuperscript{14}

\textsuperscript{86}Rb Uptake of Cultured Vascular Smooth Muscle Cells

The medium was aspirated and the cells were rapidly rinsed three times with 0.5 ml of HEPES buffer (150 mM NaCl, 5 mM RbCl, 1.8 mM CaCl\textsubscript{2}, 5 mM glucose, 1 mM MgCl\textsubscript{2}, 10 mM HEPES, pH 7.4). The cells were then incubated with HEPES buffer containing samples in the presence or absence of 2 mM ouabain for 10 minutes at 37\textdegree C.

Assays were started by adding \textsuperscript{86}RbCl, 2 Ci/ml, in HEPES buffer. At the completion of the incubation period (20 minutes), during which uptake was linear with time, the cells were quickly washed six times with ice-cold isotonic MgCl\textsubscript{2} and extracted with 10\% trichloroacetic acid. The incorporated radioactivity was measured in a liquid scintillation counter. Ouabain-sensitive \textsuperscript{86}Rb uptake was the difference between \textsuperscript{86}Rb uptake in the absence and presence of ouabain and was taken as a measure of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity.

Chemicals

All of the reagents used were of analytical grade. The radioactive materials were all purchased from Amersham International (Buckinghamshire, England).

Statistics

Results were expressed as means ± SD. The differences were analyzed by Student's t test or analysis of variance. A p value of less than 0.05 was considered significant.

Results

Before the purification of a digitalislike factor from human urine, we compared the effects of digitalis (ouabain and digoxin) in five different procedures (Figure 1). Although the radioimmunoassay is clearly the most sensitive method, this assay is particularly susceptible to interference with many substances including steroids and lipids. The binding of \textsuperscript{3}H]ouabain to human red blood cells was considerably more sensitive to digitalis than were the other procedures examined.

On the assumption that the natural ligand for the digitalis receptor should have a sensitivity similar to digitalis in the different procedures, we chose an inhibitory activity on \textsuperscript{3}H]ouabain binding to human erythrocytes to monitor digitalislike activity. Furthermore, we found that unsaturated fatty acids, lysophosphatidylcholines, dehydroepiandrosterone sulfate, and ascobic acid have no appreciable effects on this assay system, although these substances have considerable effects on \textsuperscript{3}H]ouabain binding to rat brain synaptosome or isolated Na\textsuperscript{+},K\textsuperscript{+}-ATPase.

A crude ODA was obtained by a combination of adsorption chromatography, ion exchange chromatography, gel filtration, and reverse-phase chromatography as described in Materials and Methods. Figure 2 shows the chromatographic pattern of the separation of crude ODA on a semipreparative D-ODS-5 column using a linear gradient of 0 to 20\% acetone in water. The main peak with digitalislike activity was obtained at a retention time of 44 minutes. Figure 2B shows the chromatogram obtained when the active fraction from the previous step was further analyzed by HPLC on a D-ODS-5 column with an isocratic elution of 8\% acetone. The main active peak emerged at a retention time of 150 minutes. The active material was further purified by HPLC on a GS-320H column using an isocratic elution of distilled water (Figure 2C). A single active fraction with no apparent ultraviolet absorption at 214 nm was eluted at a retention time of 9 minutes, to yield less than 0.5 mg dry weight.

This final active material was tentatively termed urine-derived ODA. One unit of ODA was defined as that amount required to inhibit \textsuperscript{3}H]ouabain binding to human erythrocytes by 50\%. Radioactive ouabain could be displaced in a dose-dependent manner by urine-derived ODA as well as unlabeled ouabain. The dose-response curve of ODA was almost parallel to that of unlabeled ouabain (Figure 3).

The saturation experiments showed that the amount of radioactive ouabain specifically bound to human erythrocytes increased with an increasing concentration of total \textsuperscript{3}H]ouabain in a typical hyperbolic manner. Scatchard analysis indicated the presence of a single class of receptor sites (Figure 4). Urine-derived ODA did not affect the maximum binding capacity (8.2 ± 0.9 vs. 8.1 ± 1.2 pmol/ml RBC; p = NS), but the affinity of ouabain was decreased by urine-derived ODA. The affinity of ouabain for human erythrocytes was significantly decreased from a K\textsubscript{d} of 9.1 ± 1.0 nM in the absence of ODA to a K\textsubscript{d} of 23.2 ± 2.1 nM in the presence of ODA. Therefore, urine-derived ODA is a competitive inhibitor of ouabain binding to human red blood cells.
Human erythrocytes are unique nonnucleated cells with no intracellular organelles. We investigated the effects of urine-derived ODA on $[^{3}H]$ouabain binding to human lymphocytes. Five units of urine-derived ODA significantly inhibited $[^{3}H]$ouabain binding to human lymphocytes by 85% from 50.9 ± 6.3 to 8.2 ± 1.0 fpmol/10^6 cells ($p < 0.01$).

Figure 5 describes the effects of urine-derived ODA on 86Rb uptake into vascular smooth muscle cells. Ouabain-sensitive 86Rb uptake, as a parameter of Na⁺,K⁺-ATPase activity, accounted for about 50% of total 86Rb uptake in A10 cells under the basal condition. Na⁺-K⁺ pump activity of vascular smooth muscle cells is markedly influenced by the changes in sodium entry into cells. 15

To exclude secondary changes in Na⁺,K⁺-ATPase activity due to alterations in intracellular sodium concentration, the effects of urine-derived ODA were observed during maximal pump activation. The addition of the sodium ionophore monensin ($10^{-4}$ M) enhanced ouabain-sensitive 86Rb uptake by sixfold (from 0.7 ± 0.2 to 4.4 ± 0.5 pmol/µg protein). Five units of urine-derived ODA inhibited the monensin-induced increase in ouabain-sensitive 86Rb uptake by 95% (4.4 ± 0.5 vs 0.9 ± 0.5 pmol/µg protein; $p < 0.01$).

Figure 6 depicts the effects of urine-derived ODA on 45Ca fluxes of vascular smooth muscle cells. Urine-derived ODA, which inhibited the binding of $[^{3}H]$ouabain to human erythrocytes by 50%, significantly increased net 45Ca influx (from 32.1 ± 1.4 to 41.7 ± 2.8 fmol/µg protein; $p < 0.01$) and decreased 45Ca efflux.

![Figure 2](image-url)  
**Figure 2.** Elution profiles of $[^{3}H]$ouabain displacing activity from human urine on HPLC. The optical density (OD114) was monitored at 214 nm. The active fraction from preparative C_{18} resin was fractionated on a semipreparative D-ODS-5 column using a linear gradient of acetonitrile (0–20%) in water at 10 ml/min over 50 minutes (A). Fractions corresponding to a retention time of 44 minutes were isocratically purified on D-ODS-5 column (B). Mobile phase was 8% acetonitrile in water. The active fractions coming from the previous step were laid onto an analytical GS-320H column. The HPLC was run at 1 ml/min. Mobile phase was distilled water (C).
The effects of 1 unit of urine-derived [3H]ouabain displacing activity (ODA) on ouabain-sensitive 86Rb uptake as a parameter of Na+,K+-ATPase activity of cultured rat vascular smooth muscle cells. 86Rb was measured over a 20-minute period as described in Materials and Methods. Results are means ± SD of six experiments.

(from 35.2 ± 1.8 to 22.9 ± 1.0 fmol/µg protein; p < 0.01). In contrast, 2 mM ouabain, which completely inhibited [3H]ouabain binding to human erythrocytes, failed to stimulate net 45Ca influx and significantly reduced 45Ca efflux (from 35.2 ± 2.8 to 24.6 ± 1.1 fmol/µg protein; p < 0.01).

Discussion

Endogenous digitalislike factor, which is supposedly the natural ligand for the digitalis receptor of Na+,K+-ATPase, may participate in the disease mechanism of certain forms of hypertension, including volume-dependent or low renin forms. Isolation and identification of such a substance have been difficult, mainly because of the limited specificity of assays available to monitor digitalislike activity. In the current study, we used an inhibitory activity on the binding of labeled ouabain to human erythrocytes to determine digitalislike activity, taking into consideration its sensitivity and specificity. Actually, using a similar radioreceptor assay, Devysock et al.16 and Sagnella et al.17 found increased circulating ouabainlike factor in patients with essential hypertension as compared with normotensive subjects.

We partially purified a urinary ODA (urine-derived ODA) using several chromatographic steps from human urine. A few recent articles have identified compounds with digitalislike activity. These substances include linoleic acid, oleic acid, lysophosphatidylcholine, dehydroepiandrosterone, ascorbic acid, and mammalian lignans.4-8 The dose-response curves of these compounds were quite different from that of ouabain.18 On the other hand, urine-derived ODA was highly polar and the dose-response curve of ODA was parallel to that of ouabain. Furthermore, ODA exerted its potent effects on human lymphocytes. These properties of ODA may demonstrate that urine-derived ODA is different from the compounds purported to be endogenous digitalis. Two other unknown candidate substances, extracted from urine and hypothalamus, have been purified to a high degree. Cloix et al.19,20 purified an anionic endalin, and Haber and Haupert and colleagues3-21 purified a zwitterionic hypothalamic inhibitor. Urine-derived ODA was not retained by anion exchanger Mono Q and cation exchanger Mono S at neutral and acid pH, respectively (unpublished observations, 1987). Therefore, ODA may be different from these unknown compounds.

Endogenous digitalislike factor, as a physiological regulator of Na+,K+-ATPase, may control Na+,K+-ATPase activity in a variety of tissues. In fact, digitals like substances extracted from mammalian brain (hypothalamus) inhibit Na+,K+-ATPase activity stimulated by monensin, vasopressin, and cooling in chick embryo fibroblast, renal medulla, and cultured renal cells (LLC-PK1), respectively.21-23 In view of the known role of the Na+,K+-ATPase in modulating myocardial and vascular contractility, the possibility that endogenous digitalislike factor controls Na+,K+-ATPase activity in vascular tissue is of potential importance. In this study, urine-derived ODA completely inhibited monensin-stimulated ouabain-sensitive 86Rb uptake in A10 cells. This finding clearly indicates that urine-derived ODA is capable of inhibiting Na+,K+-ATPase activity in vascular smooth muscle cells.

Na+-K+ pump inhibition could lead to a rise in intracellular calcium and the subsequent development of increased tone and blood pressure.24 There are two possible mechanisms by which this may occur. Inhibition of the Na+-K+ pump may result in a small reduction of the membrane potential and consequently an increased calcium influx through a voltage-dependent channel. A rise in cellular sodium could increase cell calcium through its effects on the Na+-Ca2+ exchange system. Urine-derived ODA increased net 45Ca influx and reduced 45Ca efflux in A10 cells. These results tend to confirm the hypothesis of Blaustein et al.25 just...
described. However, the failure of ouabain to increase net \(^{40}\text{Ca}\) influx is in disagreement with previous works\(^{25,26}\) and is difficult to explain. The existence of the \(\text{Na}^+\text{-Ca}^2+\) exchange system in large vessels like the aorta is now established.\(^{26}\) On the other hand, the role of the \(\text{Na}^+\text{-Ca}^2+\) exchange system in the small resistance arteries is controversial. The effects of urine-derived ODA on vascular smooth muscle cells in the resistance vessels remain to be determined.

In preliminary experiments, urine-derived ODA directly inhibited dog kidney \(\text{Na}^+\text{-K}^+\text{-ATPase}\) activity and reduced ouabain-sensitive \(^{86}\text{Rb}\) uptake into human erythrocytes (unpublished observations, 1987). It was impossible to calculate accurately the purification factor and to assess the affinity for the \(\text{Na}^+\text{-K}^+\text{-ATPase}\) due to exceedingly small quantities of ODA. An attempt to obtain sufficient amounts of ODA for its complete chemical identification is now in progress in our laboratory.

In conclusion, we purified a highly polar ODA with potent effects on human erythrocytes and lymphocytes from human urine. This compound acted on vascular smooth muscle cells as an inhibitor of \(\text{Na}^+\text{-K}^+\text{-ATPase}\), increased net calcium influx, and decreased calcium efflux. Whether this compound is a circulating regulator of \(\text{Na}^+\text{-K}^+\text{-ATPase}\) and a modulator of vascular tone needs further investigation.

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