Is Ouabain an Authentic Endogenous Mammalian Substance Derived From the Adrenal?

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Abstract Ouabain has recently been reported to be an endogenous mammalian substance released by the adrenal cortex and present in normal plasma. We have attempted to confirm and extend this observation. Using a ouabain radioimmunoassay developed in this laboratory, we fractionated by high-performance liquid chromatography (HPLC) normal human plasma from healthy volunteers to determine the presence of ouabain immunoreactivity and compare this immunoreactivity with authentic ouabain. In most subjects no ouabain immunoreactivity that coeluted with authentic ouabain was observed. Some subjects had ouabain-immunoreactive material present at low levels, but it was largely attributable to cross-reactivity with diverse substances found not to be ouabain. Similar results were obtained after analysis of plasma collected from 10 patients entering a medical intensive care unit. Studies of serum-free medium conditioned by bovine adrenocortical cells showed some ouabain immunoreactivity. To determine whether this material might be a steroid product of cholesterol side-chain cleavage, we performed chemical blockade of steroidogenesis, which effectively suppressed progesterone production by these cells but had no consistent effect on ouabain immunoreactivity in this medium. Stimulation of steroidogenesis with 22-R-OH-cholesterol in bovine adrenocortical cells did not produce any increase in the ouabain immunoreactivity present in conditioned medium. Subsequent HPLC studies of ouabain immunoreactivity in bovine adrenocortical cell-conditioned medium indicated that authentic ouabain did not account for most of the ouabain immunoreactivity in serum-free medium. Studies with bovine adrenocortical cells incubated in a minimal salt and glucose medium indicated a small peak of immunoreactivity that may correspond to authentic ouabain. Examination of ouabain immunoreactivity in serum-supplemented medium conditioned by the murine adrenocortical tumor cell line Y1 indicated much higher levels of immunoreactivity; however, the major portion of this immunoreactivity eluted during reversed-phase HPLC with much lower polarity than authentic ouabain. These studies indicate only a weak likelihood that ouabain is a biologically relevant product of the mammalian adrenal cortex.

Key Words: adrenal cortex • cardiac glycosides • tissue culture • chromatography, high-performance liquid • radioimmunoassay • ouabain

Numerous studies have attempted to identify and evaluate potential endogenous regulators of sodium-potassium ATPase activity that function via the cardiac glycoside binding site on this enzyme.1-3 Of the candidate materials identified, none met the necessary criteria of specificity, selectivity, effective concentration range in plasma, and evidence for physiologically meaningful alterations in plasma levels until recent studies indicating that ouabain, or a stereoisomer of ouabain, was an endogenous mammalian material, apparently derived from the adrenal cortex.4-6

This observation is surprising for a variety of reasons. Ouabain previously has been known only as a plant-derived material. The sugar rhamnose, which is attached to the C3 position of the A ring of the ouabain steroid nucleus, has previously been considered a plant sugar, and there is only sparse evidence for its utilization in mammalian metabolism.7-9 Furthermore, ouabain is a highly polar steroid because of multiple hydroxylations of the steroid nucleus. The finding that ouabain was released by adrenocortical cells4 implied that such multiple hydroxylation of the steroid nucleus occurred in the adrenal cortex. Expression of such a broad array of cytochrome P-450 oxidases in the adrenal cortex is without precedent. This is particularly true of 14α-hydroxylase, which is presumably required to place the C and D rings of the steroid nucleus in the cis configuration typical of plant-derived cardiac glycosides, but it is without precedent in studies of mammalian adrenal steroid metabolism. There is also the problem of how secretion of such a polar steroid across the cell membrane might be accomplished. It is further surprising in that the group identifying ouabain6 had previously indicated that the major cardiac glycoside-like material they were purifying from human plasma had some properties that distinguished it from ouabain. However, none of these concerns is sufficient to discount the strong evidence produced that ouabain was an authentic mammalian compound and that cultured adrenocortical...
cells release material that is cardiac glycosidelike by a number of criteria, we have examined the adrenal production of ouabain in further detail.

Methods

Human subjects

Healthy volunteers

Whole blood from six subjects was collected from an antecubital vein into heparinized vacuum tubes. All subjects were healthy and not being treated for any illness at the time of sampling. They were aged between 23 and 30 years; five were male and one was female. Between 30 and 40 mL of blood was collected into heparinized tubes and rapidly centrifuged to collect plasma. The plasma was stored frozen until extraction for high-performance liquid chromatography (HPLC) and immunoassay.

Medical Intensive Care Unit Patients

Blood samples (15 to 20 mL) were similarly drawn from 10 patients being admitted to the MICU/CCU for evaluation and treatment of a range of conditions. Five patients were female, five male. Ages ranged from 30 to 71 years. Four patients had congestive heart failure (CHF, grades II through IV on the New York Heart Association scale). Two patients were hypertensive when samples were drawn (230/130 and 180/110 mm Hg, respectively); one of these was comatose after cerebrovascular hemorrhage (prior medications were unknown). Three patients had a history of hypertension but were not hypertensive when samples were drawn. Three patients were known to be taking digoxin when samples were collected. Two patients were in renal failure, one in association with diabetes and coronary artery disease and the other with CHF. The use of material from human subjects in these studies was approved by the Institutional Review Board.

Tissue Culture Studies

Studies were performed on two types of cultured cells. Primary bovine adrenocortical cells (BAC) and murine adrenocortical tumor cells (Y1) were both obtained from American Type Culture Collection and maintained and passaged according to the supplier's recommendations. Cells were grown in T-75 flasks. Studies with Y1 cells involved collection of growth medium conditioned by cells as they grew from seeding to confluence. Medium was replaced every 2 or 3 days. Studies with BAC involved conditioning serum-free medium of Dulbecco's modified Eagle medium (DMEM)/F12 or Krebs-Ringer bicarbonate solution. Some studies with BAC involved inhibition of cholesterol side-chain cleavage (SCC) and pregnenolone metabolism using previously described agents and doses. In another series of studies we attempted to elevate biosynthesis of adrenal steroids by incubating BAC in the presence of 22-R-OH-cholesterol, a substrate of cholesterol SCC that is rapidly converted to steroid hormone products by the adrenals. Progesterone is a major steroid product of BAC, and production of this steroid by BAC was measured by radioimmunoassay. Cells were incubated in 5 mL serum-free DMEM/F12 to which 25 μmol/L 22-R-OH-cholesterol had been added.

Sample Preparation

Before HPLC or radioimmunoassay, samples of plasma or medium were prepared by solid-phase extraction using disposable octadecyl silica-packed cartridges (Bond Elut, Varian Associates). After the cartridges were wetted with methanol, followed by a water rinse, samples were slowly drawn over the cartridges, which were then rinsed with Tris base (10 mmol/L, pH 7.4). Elution was performed using 25% acetonitrile in water or 80% methanol in water. Both of these elution systems yield greater than 95% recovery of tritiated ouabain extracted from normal plasma or tissue culture medium. These procedures and materials are similar to those reported by Hamlyn and colleagues.

High-Performance Liquid Chromatography

HPLC studies used a Rabbit gradient HPLC system and a Microsorb MV (25 cm×4.6 mm) C18 column (Rainin Instrument Co Inc) (clinical subjects) or a µBondapak (25 cm×4.6 mm) C18 column (Waters Chromatography Division) (all other samples). A gradient elution profile was developed with a linear increase of acetonitrile concentration in 0.1% trifluoroacetic acid. Eluted materials were collected into 12×75-mm disposable glass tubes as 1- or 2-minute fractions, and the solvent was evaporated from the fractions under an air stream. Dried fractions were reconstituted in buffer before being assayed for ouabain content. Blank injections (100 μL starting solvent) were routinely performed to evaluate carryover from prior analyses.

Radioimmunoassays

Anti-ouabain antiserum was raised in a rabbit by subcutaneous inoculation in Freund's adjuvant of an antigen composed of ouabain coupled to bovine serum albumin via reductive amination essentially as described by Butler and Tseng. After several boosts, antiserum was obtained that was used to develop a radioimmunoassay. This assay used commercial tritiated ouabain (NEN-Du Pont) and was performed in 250 μmol/L Tris-HCl buffer, pH 7.4. The assay was incubated at room temperature for 1 to 2 hours. The antiserum was used at a final dilution of 1:20 000. At this concentration approximately 25% of total radioactivity added to the assay tube is bound by the antiserum in the absence of unlabeled ouabain. Separation of bound from free label was performed with goat anti-rabbit γ-globulin and 25% polyethylene glycol. Supernatant was aspirated and the pellet solubilized in 100 μL of 1 mol/L NaOH, which was then neutralized with 1 mol/L HCl. The solubilized pellet was then added to scintillation fluid and counted on an LS6800 counter (Beckman Instruments). The displacement curve produced by increasing doses of unlabeled ouabain was fitted to a four-parameter logistic model by the least-squares method; typical correlation coefficients exceeded 0.98. The typical estimated dose at 50% displacement of bound label (ED50) for the standard curve was 350 pg. The antiserum was highly specific and unable to recognize steroids unrelated to cardiac glycosides. Cross-reactivity with progesterone, corticosterone, and aldosterone was less than 0.01% and with pregnenolone less than 0.025%. Cross-reactivity with digoxin was 4.5% and with ouabagenin was 56.2%.

Progesterone was measured by a radioimmunoassay fully described previously.

Results

A typical standard curve from the ouabain immunonassay is shown in Fig 1. Measurements of ouabain immunoreactivity (OI) in 2 mL plasma from six healthy subjects extracted from C18 Bond-Elut columns with 25% acetonitrile indicated that OI was below the limits of detection in these subjects (this corresponds to a plasma concentration of less than 76.4 pmol/L).

Fig 2 shows the HPLC elution profile of ouabain-immunoreactive material in extracts of 10 mL plasma from each of six subjects and compares this elution with the elution of authentic ouabain (Sigma Chemical Co). As can be seen, there is little concordance between authentic ouabain and the ouabainlike immunoreactivity present, indicating that little, if any, authentic ouabain is normally present in human plasma.

In the patient population results were similar to those in the healthy population. Only two patients had OI in...
trifluoroacetic acid for 40 minutes, and 20-minute fractions were collected. In the bottom panel, standard ouabain was the HPLC system with standard ouabain had occurred: blank system was not occurring. Immunoreactive materials previously introduced into the HPLC system was not occurring. Care was taken to ensure that no contamination of the HPLC system with standard ouabain had occurred. Blank injections (100 µL water) were performed, and fractions were collected and assayed to ensure that ghosting of ouabain-immunoreactive materials previously introduced into the HPLC system was not occurring.

plasma, which coeluted with authentic ouabain on HPLC. One individual had a plasma level of authentic ouabain (determined as coelution on HPLC with authentic ouabain) of 29.3 pg/mL (50.1 pmol/L); another had a level of 12.2 pg/mL. The first patient was diagnosed with tricuspid regurgitation and supraventricular tachycardia. This patient had a history of hypertension but was normotensive when studied. The second patient also had a history of hypertension and was admitted with fever and chest pain and subsequently diagnosed with influenza and musculoskeletal chest pain. Only one other patient showed any detectable OI; this patient had a single OI peak that represented a material much less polar than ouabain and was equivalent to 29.3 pg/mL ouabain. This patient was taking digoxin, although two other patients taking this drug did not show OI in any fraction.

Experiments on cultured BAC were performed to attempt to replicate the report that OI is released into serum-free DMEM/F12 medium by these cultures. Extracts of medium conditioned for 2 hours were found to contain 96.4±28.9 pg/mL of OI.

Cholesterol SCC is a step common to adrenal steroidogenic pathways, and because ouabain is a steroid and other vertebrate cardiotonic steroids (bufodienolides) have been shown to be synthesized from cholesterol, experiments were performed to determine whether inhibition of cholesterol SCC and further metabolism of pregnenolone would alter the OI appearing in conditioned medium. These experiments were performed in a double crossover design. One set of cultures was incubated first in the absence of inhibitors of cholesterol SCC (aminogluthethimide) and pregnenolone metabolism (SU 10603 and cyanoketone). Another set of cultures was treated with these agents. After 2 hours of incubation, medium was collected, the cultures were rinsed, and the treatments were reversed for another 2-hour period. As can be seen in Fig 3, progesterone levels in BAC-conditioned medium were readily inhibited by this treatment; however, no significant effects on OI in conditioned medium were observed.

In experiments performed to determine whether the biosynthesis of OI could be increased by culturing BAC in the presence of the cholesterol SCC substrate 22-R- OH-cholesterol, no significant effect on the presence of OI in extracts of conditioned medium was observed. However, large increases in progesterone production were measured, indicating that cholesterol SCC and resulting steroidogenesis were highly amplified by this treatment (Fig 4).

To determine whether the material responsible for the OI in BAC-conditioned medium was authentic ouabain, we analyzed 200 mL of conditioned medium by Bond-Elut extraction and reversed-phase HPLC. Fig 5 shows the elution profile of OI in this system. Elution of authentic ouabain occurs in fraction 6 in this system. A small amount of OI was observed in this peak; however, the pattern of all OI appeared to correlate with the elution of phenol red indicator derived from the culture medium, and similar peaks were observed in unconditioned medium (data not shown). To eliminate problems caused by possible interference from complex molecules in serum-free DMEM/F12, we repeated these experiments using a minimal salt medium (Kreb-Ringer bicarbonate glucose solution). Again, small peaks of OI were observed (Fig 6), and the largest peak corresponded to authentic ouabain. However, the
amount of material present was low, and there were other adjacent peaks that could not be attributed to ouabain.

Finally, we compared OI in extracts derived from a pool of serum-supplemented medium that had been conditioned by the murine adrenocortical tumor cell line Y1 with immunoreactivity present in a similar volume of unconditioned, serum-supplemented medium. Previous studies in this laboratory have indicated the presence of cardiac glycoside–like material in this medium, but the major active fractions were not found to correspond to ouabain in these earlier studies. After extraction and HPLC purification of a large pool of conditioned medium, OI was found at different levels dispersed across the elution profile (Fig 7). Essentially no ouabain was observed in the fractions corresponding to authentic ouabain. There appeared to be a major peak of immunoreactivity that eluted later with the less polar material. This peak may correspond to the peak of digoxin immunoreactivity that has been previously described in this medium but that is not attributable to authentic digoxin.

Discussion

The recent publication of a series of studies identifying ouabain, or a substance differing only in stereoisomerism of the rhamnose moiety, as an apparently endogenous mammalian material represented an important observation in the long search for biologically relevant endogenous inhibitors of the sodium pump that operate through the cardiac glycoside receptor. Although the result was surprising, the purification procedures were quite rigorous, and there is little reason to expect that the experiments might have produced a false-positive result. Nevertheless, so surprising a result requires the independent confirmation and validation that was the anticipated outcome of the present study. However, significant differences have been found in the present study, requiring that the conclusion that ouabain is a biologically relevant endogenous mammalian compound be treated with caution.

Ouabain has been previously identified in a large pool of human plasma–derived material. This pool was subject to dialysis, amberlite resin extraction, preliminary preparative-scale HPLC, affinity chromatography on purified...
lamb kidney–derived sodium-potassium ATPase, and final purification through further HPLC.\(^5\) The material so purified was identified as ouabain, or a material differing only in isomerism of the sugar residue, on the basis of fast atom bombardment mass spectroscopy.\(^4,6\) Less stringent purification methods using a one-step solid-phase extraction method have revealed the presence of OI in human plasma from healthy and diseased subjects.\(^3\) The possibility that this material was due to cross-reaction with nonouabain material or materials appears to have been discounted largely because of the partial selectivity of the solid-phase extraction step.\(^3\) A dietary source of this OI was discounted because of the low oral bioavailability of ouabain\(^2\) and because subjects on total parenteral nutrition did not appear to have lower levels of plasma OI.\(^4\)

In view of these findings we have sought to confirm whether OI can routinely be shown to be present in normal human plasma using a different antiserum and immunoassay method and to determine whether any OI present corresponds to authentic ouabain by extraction and HPLC purification of plasma. A relatively sensitive and robust assay system has been developed and the antiserum evaluated for cross-reactivity. Our results indicate that although small amounts of OI may be present in plasma, little of this immunoreactivity can be accounted for by authentic ouabain either in healthy young subjects or in patients receiving care in a medical intensive care unit. Although the sensitivity of our assay is limited by the use of a tritium tracer, the levels of plasma ouabain reported in assays using the same extraction technique in conjunction with an enzyme-linked immunosorbent assay (ELISA) have produced normal plasma values readily detectable using the sample volumes employed in the present study.\(^2\) Thus, in our measurements of 2-mL aliquots of plasma our assay should be able to detect any levels greater than 80 pmol/L. This value is approximately half the level of plasma OI initially reported in healthy human subjects,\(^4\) although subsequent reports from the same group have shown a surprising upward drift in the basal plasma ouabain levels observed.\(^2\) Therefore, we do not believe our results can be attributed to false-negative error.

Our studies of clinical subjects reveal a pattern very similar to that obtained in the healthy volunteers. Authentic ouabain was present in only 2 of 10 subjects, and the levels were much lower than those reported previously from CHF patients.\(^2\) Indeed, no authentic ouabain was found in plasma from any of the CHF patients in this study. These findings indicate that measurements of plasma OI in simple plasma extracts using some immunoassays can lead to falsely elevated measures of ouabain that are not attributable to authentic ouabain. To determine whether plasma ouabain-immunoreactive materials are attributable to authentic ouabain, the results of immunoassays must be validated by further purification of plasma after simple solid-phase extraction. Although material behaving as authentic ouabain was occasionally found in plasma on HPLC, its level is sufficiently low to make its biologic significance as well as endogenous origin subject to question.

Previous reports that the most highly enriched tissue source of ouabain was the adrenal cortex\(^2,22\) were consonant with our own previous observation that adrenal tissue is an enriched source of material that is cardiac glycosidelike in a variety of assay systems and that appears to be released from explanted glandular tissue and cultured adrenocortical cells.\(^11,17,26,22\) Our present studies examining ouabain in culture medium conditioned by BAC indicate that a small amount of cross-reactive material is present in extracts of this medium, but this material does not appear to derive from cholesterol metabolism and therefore may not be steroidal. Furthermore, HPLC analysis of this material failed to confirm that the majority of the immunoreactivity could be attributed to authentic ouabain. Conditioned medium from the murine adrenocortical tumor cell line was a more enriched source of OI; however, in this case also authentic ouabain could not account for the large majority of this immunoreactivity. These findings are problematic for the conclusion that ouabain, or a closely
producing very large increases in progesterone levels in medium provides two possible conclusions: first, that ouabain is not synthesized in these adrenocortical cells via cholesterol SCC or, alternatively, that ouabain is not synthesized in these adrenocortical cells at all.

This attempt to independently verify the presence of ouabain in normal human plasma and in plasma from patients with cardiovascular disease and to demonstrate its synthesis in serum-free conditions by adrenocortical cells in culture leads us to conclude that ouabain is unlikely to be an endogenous mammalian cardiotonic steroid.

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