Mass Spectral Characterization of an Endogenous Digitalislike Factor From Human Plasma

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A sodium pump inhibitor has been isolated from human plasma and extensively purified. This material, endogenous digitalislike factor, was examined by a variety of mass spectrometric techniques. A low-resolution fast atom bombardment mass spectrometric analysis of a sample of purified endogenous digitalislike factor revealed a single unique molecular ion in the mass range 100–2,500. The accurate mass was determined to be 585.295 Da in a second high-resolution fast atom bombardment mass spectrometric experiment. Based on this accurate mass, the elemental composition of endogenous digitalislike factor was determined and found to be identical to the elemental composition of the known cardenolide ouabain. Direct comparison of ouabain and endogenous digitalislike factor by linked scan tandem mass spectrometry, derivatization with acetic anhydride coupled with fast atom bombardment mass spectrometry, and analytical high-performance liquid chromatography failed to reveal any differences. We conclude that the endogenous digitalislike factor isolated from human plasma is ouabain or a closely related isomer. (Hypertension 1991;17:930–935)

A variety of experimental evidence suggests that an endogenous inhibitor of Na⁺,K⁺-ATPase with properties similar to the cardiac glycosides exists in mammals. Such an inhibitor or endogenous digitalislike factor (EDLF) has been suggested to play a role in regulation of plasma volume and arterial pressure. The existence of an EDLF is based on two general arguments. First, Na⁺,K⁺-ATPase has a highly specific receptor for the cardiac glycosides, which suggests that an endogenous ligand for the receptor may exist. This argument is analogous to the one that led to the discovery of the endogenous opiate receptor ligands. Second, a variety of physiological experiments provide evidence for the existence of a circulating inhibitor of Na⁺,K⁺-ATPase. Based on cross-circulation experiments with dogs, de Wardener et al. first suggested in 1961 that an inhibitor of Na⁺,K⁺-ATPase was involved in natriuresis. Dahl et al. later suggested that an EDLF was important in hypertension. Since then, a number of studies showing elevated levels of Na⁺,K⁺-ATPase inhibitors in experimentally hypertensive animals, clinically hypertensive humans, and volume-expanded or sodium-loaded animals have appeared in the literature. Based on these arguments, a number of investigators have tried to isolate and characterize EDLF. Although a few compounds have been purified and characterized, none of the previously identified compounds appears to satisfy all of the criteria for an EDLF; that is, it should be a specific, competitive inhibitor of the transport, receptor, and ATPase activities of Na⁺,K⁺-ATPase.

We recently described the purification and biochemical characterization of an EDLF isolated from volume-expanded human plasma. This EDLF had the characteristics expected for an endogenous Na⁺,K⁺-ATPase inhibitor. This material now has been purified in sufficient amounts to allow structural analysis by modifying the purification step to include an Na⁺,K⁺-ATPase affinity step. We report here the characterization of this highly purified EDLF using a variety of mass spectrometric techniques.

Methods

Materials
Glycerol and 4-dimethylaminopyridine (DMAP) were obtained from Aldrich Chemical Co., Milwaukee, Wis. Pyridine and trifluoroacetic acid (TFA)
Purification of Endogenous Digitalislike Factor

EDLF was purified from volume-expanded human plasma as described. Approximately 10 μg EDLF purified from two different batches of human plasma was used for structure determination. EDLF concentrations were estimated based on UV absorbance (220 nm) and enzyme titrations with Na\(^+\), K\(^+\)-ATPase.

Mass Spectrometry

Fast atom bombardment mass spectrometry (FABMS) was carried out using a VG 70SE (VG Analytical Ltd., Manchester, UK) instrument equipped with a Cs\(^+\) ion gun. Low-resolution spectra were recorded using a VG 11-250J data system and were obtained by scanning the magnetic field from m/z 2,500 to m/z 100 at a resolution of 2,500. Samples were prepared in a glycerol matrix. This typically was carried out by dissolving the sample in 100-250 μl of an aqueous solution containing 2% glycerol and 1% acetic acid, followed by removal of the excess water in a vacuum centrifuge. High-resolution spectra were obtained while operating the instrument in the "multichannel analyzer" mode and scanning the accelerating voltage at a resolution of 4,000. Daughter ion spectra were obtained by scanning the electric (E) and magnetic (B) fields such that the ratio B/E remained constant under the control of the 11-250 data system.

O-Acetylation

For the first O-acetylation experiment, approximately 3.5 μg ouabain and approximately 2 μg EDLF were taken to dryness in a vacuum centrifuge and then reacted with 25 μl acetic anhydride/pyridine (1:1) for 35 minutes at room temperature. After excess reagent was removed under vacuum, the samples were dis-
solved in glycerol for FABMS. A second set of samples, approximately 1 μg each of EDLF and ouabain, were acetylated with 50 μl of a 1:1 mixture of acetic anhydride and DMAP (15 mg/ml in pyridine) for 2 hours at room temperature. After excess reagent was removed under vacuum, the samples were dissolved in glycerol/thioglycerol for FABMS.

High-Performance Liquid Chromatography

HPLC was carried out on a Series 4 system (The Perkin-Elmer Corp., Norwalk, Conn.) equipped with a Model 783A UV detector (Applied Biosystems, Foster City, Calif.). Samples were chromatographed using a 4.6x250 mm Bakerbond octadecyl column (J.T. Baker Inc., Phillipsburg, N.J.) eluted isocratically with water/acetonitrile/TFA (90:10:0.1) at a flow rate of 1 ml/min and a 4.6x250 mm ASTEC Cyclobond II (gamma) column (Advanced Separation Technologies, Inc., Whippany, N.J.) eluted with water/methanol/t-butanol (94:5:1) at a flow rate of 1 ml/min.

Results

EDLF was purified from human plasma as described. Material from the final purification step, reversed-phase HPLC, was used for mass spectral characterization. The active fraction and the two adjacent fractions were concentrated, dissolved in glycerol matrix, and analyzed by FABMS. FAB mass spectra were acquired over a mass range of \( m/z \) 100–2,500. This mass range bracketed the molecular weight of EDLF, 300–900 Da, that had been estimated based on gel filtration chromatography. The FAB mass spectra of the three fractions from \( m/z \) 200 to 800 are shown in Figure 1. A single unique protonated molecular ion at \( m/z \) 585.2 was seen in the active fraction (Figure 1B) and not in the adjacent fractions (Figures 1A and 1C). A small ion at \( m/z \) 607.2 corresponding to the sodium adduct ion also was seen in the active fraction. No other unique ions were seen in the mass range 100–2,500.

A second sample of purified EDLF was used to obtain elemental composition information. The accu-
rate mass of EDLF was determined to be 585.295 Da by matching the peak at m/z 585 to glycerol cluster ion peaks at m/z 553.292 and 645.339 at a resolution of 4,000. The elemental composition, C_{29}H_{45}O_{12}, of the protonated form of EDLF was determined based on the accurate mass. The elemental composition suggested a cardiac glycoside–type structure, and inspection of the cardenolide literature revealed that the experimentally determined molecular weight and elemental composition of EDLF matched those of the cardenolide ouabain. The theoretical accurate mass of ouabain is 585.291 Da (protonated form). Subsequent experiments were designed to compare EDLF and ouabain.

To obtain additional structural information, linked scan tandem mass spectrometry was carried out on the protonated molecular ions of EDLF and ouabain. Linked scan tandem mass spectrometry is a technique that can be used to obtain fragments (or daughter ions) from molecular ions (or parent ions) of compounds ionized by FABMS and other soft ionization methods in which fragmentation usually is not observed. As shown in Figure 2, the daughter ion spectra of EDLF and ouabain are identical. Both exhibit a major fragment ion at m/z 439.2, corresponding to the aglycone portion of the molecule with addition of 2H⁺. Ions corresponding to successive losses of H₂O from the intact species (m/z 567, 549, 531, 513, 495) and the aglycone peak (m/z 421, 403, 385) also are seen. The aglycone peak results from cleavage of the glycoside bond and loss of the sugar moiety and indicates that sugar in EDLF, like ouabain, is a deoxyhexose. These daughter ion spectra are very similar to the FAB spectra of cardiac glycosides reported by Isobe et al.

Further comparison of EDLF and ouabain is shown in Figure 3. Ouabain and EDLF were acetylated with acetic anhydride/pyridine, and the products were analyzed by FABMS. After acetylation, EDLF and ouabain gave nearly identical spectra. In each case, ions corresponding to partial acetylation products, fragments due to loss of H₂O, and adduct ions were observed. The ions were assigned as follows (where M is the parent molecule, Ac is the acetyl group, and R is a cyclohexylamine contaminant): m/z 669 [M(584) +2Ac+H]^+, m/z 693 [M+3Ac−H₂O+H]^+, m/z 711 [M+3Ac+H]^+, m/z 735 [M+4Ac−H₂O+H]^+, m/z 753

FIGURE 3. Mass spectra of ouabain (panel A) and endogenous digitalislike factor (panel B) after acetylation with acetic anhydride/pyridine. Ions are labeled as described in text. Ac, acetyl group; R, cyclohexylamine contaminant, introduced by the glassware used for the acetylation reaction.
Comparison of endogenous digitalis-like factor (EDLF) and ouabain by analytical high-performance liquid chromatography using a reversed-phase column (panels A–C) and a Cyclobond column (panels D–F). A, absorbance.

\[ [M + 4\text{Ac} + H]^+, \text{m/z 777} \]
\[ [M + 5\text{Ac} - \text{H}_2\text{O} + H]^+, \text{m/z 795} \]
\[ [M + 5\text{Ac} + H]^+, \text{m/z 810} \]
\[ [M + 3\text{Ac} + \text{R} + H]^+, \text{m/z 852} \]
\[ [M + 4\text{Ac} + \text{R} + H]^+, \text{m/z 894} \]

Sodium adduct ions at \text{m/z 733} \[ [M + 3\text{Ac} + \text{Na}]^+ \] and \text{m/z 775} \[ [M + 4\text{Ac} + \text{Na}]^+ \] were observed in the EDLF sample but not the ouabain sample, consistent with the presence of sodium in the EDLF sample (see Figure 1). Because these acetylation conditions were not sufficient to drive the reaction to completion, a second set of samples was acetylated using more rigorous reaction conditions. Acetylation of EDLF and ouabain with acetic anhydride/pyridine/DMAP resulted in identical spectra, with major ions corresponding to the addition of six acetyl groups and minor ions corresponding to the addition of seven and eight acetyl groups, respectively (data not shown). The acetylation results are consistent with the structure of ouabain, which has six reactive primary or secondary OH groups and two hindered tertiary OH groups.

As a final comparison, the chromatographic behavior of EDLF and ouabain was investigated. Two different supports were selected as being likely to be able to resolve ouabain isomers. A C18 reversed-phase column was used, because Cassels reported that ouabain and acalongifloroside K, isomers that differ only in the stereochemistry of a single sugar OH, could be resolved on this support. Cyclobond columns have unique cyclo-dextrin solid supports that have been reported to be very useful for resolving a wide variety of geometric, stereo, and structural isomers. EDLF and ouabain were analyzed on a Bakerbond C18 reversed-phase column (Figures 4A–C) and a Cyclobond II gamma column (Figures 4D–F). In each case, a single peak was obtained when the samples were coinjected.

Discussion

EDLF purified from plasma from volume-expanded human patients has been extensively characterized. After initial mass spectrometric data had indicated that EDLF shared the same elemental composition and accurate mass as the known cardiac glycoside ouabain, we compared the two compounds to see if we could discover any differences. We were not able to detect any physicochemical differences between EDLF and
ouabain. In addition to the low-resolution and high-resolution mass spectral data, the linked scan tandem mass spectra were identical. Acetylation with acetic anhydride/pyridine or acetic anhydride/pyridine/DMAP resulted in identical products as determined by FABMS. Finally, EDLF and ouabain cochromatographed on two different analytical HPLC columns. Together, these results strongly suggest that EDLF either is identical to ouabain or is a closely related isomer of ouabain.

Although EDLF clearly is very similar to ouabain, the present data do not rule out the possibility that EDLF may be a closely related isomer. However, several observations argue against this possibility. First, the acetylation experiments indicate that both compounds have OH groups of similar reactivity, because both gave the same distribution of partial acetylation products. Acetylation using the DMAP catalyst indicates that both have two hindered OH groups and six more reactive OH groups. Second, EDLF and ouabain cochromatograph on two different HPLC columns. Third, no significant biochemical differences between EDLF and ouabain have been detected (unpublished observations). Finally, a specific polyclonal antibody to ouabain cross-reacts strongly with EDLF.18

Until now we have referred to the inhibitor of the sodium pump we isolated from human plasma as EDLF. We used this general nomenclature because we found our inhibitor to possess many of the properties of the cardiac glycosides. Our current findings that the biochemical, physicochemical, and immunological properties of this substance are identical to those of ouabain suggest that more specific nomenclature such as ouabainlike compound (OLC) be used.

The identification of OLC as a known cardenolide raises the issue of the origin of this compound. Ouabain is found in Strophanthus gratus, Acanthacurrus ouabaoa, and possibly other plants. Ouabain is very polar and is not orally active.19 Therefore, it is not likely that diet is a source of OLC. It also is not likely that ouabain was introduced as a contaminant during the sample purification.13 Ouabain has been used clinically as an intravenous solution but is no longer in common usage. None of the patients from whom volume-expanded plasma was obtained for this study was taking ouabain or other cardiac glycosides. Furthermore, using a specific antibody to ouabain, we have shown that plasma OLC levels in the rat decrease after adrenalectomy and increase with deoxycorticosterone acetate salt treatment (unpublished observations), results that argue for an endogenous source of OLC.

The steroid nature of OLC suggests that the adrenal may be a likely site of synthesis or secretion. Several groups89 have shown that an EDLF with chromatographic properties similar to the one identified here is derived from the adrenal gland. Preliminary data based on the immunological studies indicate that the adrenal is a rich source of immunoreactivity and that adrenalectomy reduces plasma OLC levels (unpublished observations). Proof of the endogenous nature of OLC will require the demonstration that humans have the biosynthetic capability to produce the material.

References


Key Words • digitalislike factors • ouabain cardiac glycosides • spectrum analysis, mass
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