Characterization of a Urinary Bufodienolide Na\(^{+}\),K\(^{+}\)-ATPase Inhibitor in Patients After Acute Myocardial Infarction

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*Abstract*—Recent evidence suggests the existence of several endogenous Na\(^{+}\),K\(^{+}\)-ATPase inhibitors in mammals. Previously, we have shown that the amphibian Na\(^{+}\),K\(^{+}\)-ATPase inhibitor marinobufagenin (3,5-dihydroxy-14,15-epoxy bufodienolide) acts as a vasoconstrictor in isolated rat and human arteries. Mammalian plasma was shown to contain marinobufagenin-like immunoreactive material, which is responsive to saline volume expansion. The present study describes purification of a bufodienolide, which is similar to marinobufagenin, from the urine of patients after acute myocardial infarction with the use of thin-layer chromatography and reverse-phase high-performance liquid chromatography (HPLC). The purified substance cross-reacted with marinobufagenin antibody, demonstrated maximal UV absorbance at 300 nm characteristic of bufodienolides, and eluted from HPLC columns with the same retention time as marinobufagenin. Mass spectrometry of purified material revealed the presence of a substance indistinguishable from amphibian marinobufagenin and having molecular mass of 400 D. The present studies show that one of the human digitalis-like factors may have a bufodienolide structure and is likely to represent marinobufagenin or its isomer, and they suggest a role for this substance in the pathogenesis of myocardial ischemia. *(Hypertension. 1998;31:1097-1103.)*

**Key Words:** Na\(^{+}\)-K\(^{+}\)-ATPase ■ bufodienolides ■ myocardial infarction ■ digitalis-like factors

Endogenous inhibitors of Na\(^{+}\),K\(^{+}\)-ATPase, DLFs, were previously found to be involved in water and electrolyte homeostasis and in the genesis of vasoconstriction in volume-dependent forms of hypertension.\(^1,3\) Endogenous ouabain (or a structurally very similar compound) was the first DLF to be purified from human plasma\(^4\) and bovine brain.\(^5\) However, several studies indicate that mammalian tissues contain other Na\(^{+}\),K\(^{+}\)-ATPase inhibitors that resemble digoxin\(^6,7\) and that sodium pump inhibition observed in hypertension cannot be fully attributed to the action of a ouabain-like compound.\(^8,9\)

Some amphibians, especially several species of toad, are known to have high levels of endogenous steroidal Na\(^{+}\),K\(^{+}\)-ATPase inhibitors with bufodienolide structure.\(^10,11\) Bufodienolides differ from cardenolides in having a doubly unsaturated six-membered lactone ring (Fig 1A).\(^10\) Previously, we have shown that one of the toad bufodienolides, marinobufagenin (3,5-dihydroxy-14,15-epoxy bufodienolide may also be referred to as marinobufagin), acts as a potent vasoconstrictor in isolated rat aorta and in human pulmonary artery, inhibits the sodium pump, and cross-reacts with digoxin (but not ouabain) antibody.\(^12-14\) Later, marinobufagenin-like immunoreactive material was detected in human\(^15\) and canine plasma\(^16\) and urine. When chloroform-extracted human urine was fractionated on HPLC columns, marinobufagenin-like immunoreactive substance eluted as a single peak separated from the ouabain-like immunoreactive material.\(^14,15\)

Plasma marinobufagenin-like immunoreactivity was increased in saline-induced plasma volume expansion in dogs,\(^16\) during pressor response to hypoventilation in healthy humans\(^15\) and unanesthetized micropigs,\(^17\) and in patients after AMI.\(^18\)

In several previous studies it has been shown that acute coronary ligation of laboratory animals is associated with inhibition of myocardial Na\(^{+}\),K\(^{+}\)-ATPase and with a parallel loss of high-affinity digitalis receptor sites in the ventricular myocardium.\(^19-21\) Inhibition of Na\(^{+}\),K\(^{+}\)-ATPase in ventricular myocardium, in turn, is believed to contribute to the genesis of myocardial ischemia–induced arrhythmias.\(^21,22\) Based on these observations, we hypothesized that ischemia-induced inhibition of myocardial Na\(^{+}\),K\(^{+}\)-ATPase may be due at least in part to the action of DLF. In support of this hypothesis, it has been demonstrated that in coronary-ligated rats and in patients with AMI, plasma levels of digoxin-like immunoreactivity were increased and Na\(^{+}\),K\(^{+}\)-ATPase levels in erythrocytes\(^23-25\) and in myocardium\(^26\) were inhibited. Pretreatment of the coronary-ligated rats with digoxin antibody prevented inhibition of erythrocyte and myocardial Na\(^{+}\),K\(^{+}\)-ATPase and was associated with an antiarrhythmic effect.\(^21,26\) When the
antiarrhythmic potency of several antibodies was compared in coronary-ligated rats, antibody against the mixture of bufodienolides possessed greater effect than rabbit and ovine (DIGIBIND) digoxin antibody.27

Considering the sensitivity of endogenous marinobufagenin-like immunoreactive factor to plasma volume expansion16 and the vasoconstrictor properties of marinobufagenin,13,14 we decided to further characterize the human marinobufagenin-like substance. Since increased DLF has been shown to be involved in the genesis of sodium pump inhibition in acute myocardial ischemia23–27 and increased urinary excretion of involved in the genesis of sodium pump inhibition in acute myocardial ischemia23–27 and increased urinary excretion of

Figure 1. A. Structure of marinobufagenin. Elution pattern of ouabain-like (B) and marinobufagenin-like (C) immunoreactive material from semipurified material (3 mL 1-minute fractions from a total of 150 mL of urine) when chromatographed on semipreparative Beckman Ultrapore RPMC in the linear gradient of acetonitrile.

Methods

Urine Collection, Extraction, and Prepurification

The protocol of the study was approved by the Research Council of Dzhanelidze Institute of Emergency Medicine, St Petersburg, Russia. We collected 24-hour urine samples and obtained venous blood samples from 12 patients (8 men and 4 women; mean age, 56.2±2.8 years) during the first 24 hours after the onset of a first transmural AMI (Minnesota codes 1–1-1, 1–2-5, 1–2-6, and 1–2-7). Since patients with unstable angina pectoris were shown to have normal plasma levels of DLF previously,24,25 10 patients with unstable angina pectoris (8 men and 2 women; mean age, 55±4 years) served as the controls.

The procedure of purification and characterization of DLF included (1) prepurification with the use of thin-layer chromatography, (2) repeated reverse-phase HPLC fractionation and separation of fraction(s) with marinobufagenin-like properties, and (3) comparison of mass-spectral characteristics of endogenous material with marinobufagenin.

A total of 10 L of urine was extracted with 15 L of chloroform, and the chloroform phase was dried under vacuum. The resulting material was prepurified by thin-layer chromatography (Silica-gel 60 F254, 60 g, 20×250 mm), as recently reported for purification of marinobufagenin from the venom of Bufo marinus toad.13 The material with Rf (ratio of fronts) similar to that of marinobufagenin was separated.

High-Performance Liquid Chromatography

The purpose of the first HPLC fractionation was to determine the elution patterns of marinobufagenin-like and ouabain-like immunoreactive material. Approximately 20% of the material semipurified by thin-layer chromatography was fractionated on a semipreparative reverse-phase column (Beckman Ultrapure RPMC, 5 μm, 10×250 mm, 3 mL/min, detection at 220 and 300 nm, elution in a linear gradient of acetonitrile [0% to 80%] and 0.1% TFA) with the use of a Gilson HPLC system (model 303, detector model 116). Sixty-minute fractions were collected and analyzed for the presence of marinobufagenin-like and ouabain-like immunoreactive material.

The remaining part of partially purified material was dissolved in 20% acetonitrile and 0.1% TFA and was fractionated on the preparative-scale reverse-phase HPLC column (DIASORB 130 C18, 300 A, 3.9×150 mm, 5 μm, isocratic elution with 32% acetonitrile). This was followed by subsequent injection and elution of an identical sample in the presence of marinobufagenin standard (20 μL of 100 μmol/L solution).

The material was then further fractionated on a semipreparative reverse-phase column (Beckman Ultrapure RPMC, 5 μm, 10×250 mm, 3 mL/min, detection at 220 and 300 nm, isocratic elution with 32% acetonitrile). Individual peaks were collected and analyzed on an analytical HPLC column (see above), lyophilized, tested for marinobufagenin-like immunoreactivity, and submitted to mass-spectral analysis.

To confirm that the HPLC system was not contaminated by marinobufagenin standard, after fractionation of the urinary extract in the presence of marinobufagenin standard, a control sample (20% acetonitrile and 0.1% TFA) was run through the DeltaPak C18 column, and the resulting HPLC fractions were analyzed for marinobufagenin-like immunoreactivity.

Mass Spectrometry

Sample Preparation

Samples were dissolved in 200 μL of acetonitrile/water (1:1). For ES-MS analysis, 5 mL of each of these stock solutions was further diluted to a concentration of approximately 25 pmol/μL (based on

Selected Abbreviations and Acronyms

AMI = acute myocardial infarction
DLF = digitalis-like factor
HPLC = high-performance liquid chromatography
ES = electrospray ionization
MS = mass spectrometry
TFA = trifluoroacetic acid
the presumption that the molecular weight of DLF is similar to that of marinobufagenin; \(M_r=400\) by the addition of this solvent. For high-resolution liquid secondary ionization MS providing accurate mass measurement, 1 to 2 \(\mu\)L of the stock solution was used for each measurement.

**Electrospray Ionization MS**

ES-MS analyses were performed with the use of a VG Quattro II (tandem quadrupole) mass spectrometer (Micromass Ltd) operating at an electrospray voltage of 4.0 kV fitted with a Shimadzu LC-10AD solvent delivery module (Shimadzu Corp). Data acquisition and processing were controlled by the PC-based Micromass MassLynx (version 2.0) data system. The electrospray ionization–MS interface was operated at a flow rate of 20 \(\mu\)L/min with the use of a mobile phase of water/acetonitrile/formic acid (49.5%/50%/0.5%) with \(N_2\) as nebulizing and drying gas. Sample injections of 10 \(\mu\)L were used for each analysis.

**Accurate Mass Measurement**

Accurate mass measurement was accomplished by liquid secondary ionization MS with the use of a Micromass 70SEQ Tandem Hybrid mass spectrometer (Micromass Ltd) equipped with a Fast Atom Bombardment gun (Ion Tech Ltd). Data acquisition and processing were controlled by a VG 11 to 250J data system. Measurements were made at a resolving power of 10 000 by computer peak matching of analyte peaks with reference peaks of polyethylene glycol dissolved in 3-nitrobenzyl alcohol matrix.

**Immunassays**

Marinobufagenin-like immunoreactivity was measured by solid-phase fluoroimmunoassay (DELFIA Arcus Fluorometer, Wallac OY) in HPLC fractions in nonextracted urine on day 1 of AMI and in extracted plasma (0.5 mL, C18 reverse-phase cartridges [Waters Inc], elution with 7.5 mL 32% acetonitrile followed by 7.5 mL 80% acetonitrile) on days 1 and 5 of AMI, as recently reported in detail.\(^{13,16}\) The method is based on competition of plasma DLF and immobilized conjugated ligand (marinobufagenin-glycoside–bovine serum albumin conjugate) with sample marinobufagenin antibody. Cross-reactivity of marinobufagenin antibody has been recently reported.\(^{16}\)

Ouabain-like immunoreactivity in HPLC fractions was measured by the DELFIA method, based on the competition between immobilized ouabain-ovalbumin conjugate and sample ouabain for europium-labeled ouabain antibody. Ouabain antibody was obtained from Chemicon International Inc and labeled with nonradioactive europium with the Europium-Labeling Kit (Wallac OY). Cross-reactivity of ouabain antibody was as follows: marinobufagenin, 3.5%; digifor, 10%; digifoxigenin, 0.5%; ouabagenin, 40%; bufalin, 1%; cinobufagin, <0.1%; proscillaridin, 0.2%; cortisol, <0.1%; progesterone, <0.1%; prednisone, <0.1%; and spironolactone <0.1%.

The sensitivity of both immunosassays was 0.01 nmol/L (0.0012 pmol per well). Marinobufagenin was purified from the venom of *Bufo marinus* toads by thin-layer chromatography as reported recently in detail.\(^{13}\) Results were analyzed statistically (unpaired and paired Student’s \(t\) tests) with the use of GraphPad Instat and GraphPad Prism.

**Results**

The 24-hour urinary excretion values of marinobufagenin-like immunoreactive material by patients on the first day of AMI and by patients with angina pectoris were 12.2±1.77 and 4.1±0.8 nmol, respectively \((P<0.01, \text{unpaired} \ t \text{test})\). The 24-hour urinary volumes in patients with AMI and in the control group were 1544±229 and 1386±136 mL, respectively. Plasma concentration of marinobufagenin-like immunoreactivity in patients during the first day after AMI was significantly higher \((1.85±0.38 \text{nmol/L}; P<0.01, \text{unpaired} \ t \text{test})\) than in patients with unstable angina pectoris

Figure 2. A, Elution pattern of marinobufagenin-like immunoreactive material (10 mL 2-minute fractions, equivalent of approximately 8 L of urine) from DIASORB 130 C reverse-phase HPLC column in the gradient of acetonitrile. B, UV absorbance pattern \((\lambda = 220 \text{ nm [solid line]}, \lambda = 250 \text{ nm [dotted line]}\) isocratic elution with 32% acetonitrile, DeltaPak C18 reverse-phase analytical HPLC) of partially purified material (10 \(\mu\)L sample from pooled fractions 28 and 29) (Fig 2A) in the absence and in the presence (C) of marinobufagenin standard.

\((0.50±0.07 \text{ nmol/L})\). On day 5 after the onset of AMI, plasma levels of marinobufagenin-like immunoreactivity decreased and did not differ from the control values \((0.37±0.1 \text{ nmol/L}; P<0.01, \text{paired} \ t \text{test})\).

Fig 1B and 1C demonstrates the pattern of elution of ouabain-like and marinobufagenin-like material from Beckman Ultrapore RPMC reverse-phase columns. More than 80% of ouabain-like material eluted at 23 minutes (28% acetonitrile). Marinobufagenin-like immunoreactivity eluted at 31 minutes (38% acetonitrile).

Fig 2A demonstrates distribution of marinobufagenin-like immunoreactivity among 45 two-minute fractions eluting from preparative reverse-phase HPLC columns. The presence
of marinobufagenin-like immunoreactive material was detected in fractions 20 to 33; the highest concentrations of marinobufagenin-like immunoreactivity were detected in fractions 28 and 29. When fractions 28 and 29 were put together and analyzed on an analytical HPLC column (Fig 2B and 2C), the retention time of the material showing maximal absorbance at 300 nm was similar to that of the marinobufagenin standard. The total amount of marinobufagenin-like immunoreactivity eluted from the analytical HPLC column when the urinary extract was fractionated in the presence of marinobufagenin standard was 2.52 nmol. The column was loaded with a control sample immediately after this fractionation, and the total amount was <10 pmol when resulting fractions were analyzed for marinobufagenin-like immunoreactivity.

Fig 3 demonstrates the results of further fractionation of material from fractions 28 and 29 on a semipreparative reverse-phase column. More than 90% of total marinobufagenin-like immunoreactivity was eluted in fraction 24 (Fig 3B). This fraction contained a sharp peak of absorbance at 300 nm (Fig 3A). Spectrophotometric scans of 20 μL material from fraction 24 and of marinobufagenin standard (20 μL, 100 μmol/L) are presented in Fig 4 (insets).

Eighteen micrograms of the material from fraction 24 was studied by MS. Fig 4 provides the direct comparison of ES-MS from HPLC fraction isolated from the urine and that of the marinobufagenin from the Bufo marinus toad (M_r=400) reference, respectively. Both spectra exhibited predominant peaks at Da/e 401 and 423 corresponding to the protonated [M+H]^+ and the cation adduct, [M+Na]^+, of the molecular species. The observed consecutive neutral losses of water from the [M+H]^+ giving rise to the ions of Da/e 383 and 365 are consistent with the dihydroxy functionality of the molecule. Accurate mass measurement data were as follows: calculated for the [M+H]^+ of marinobufagenin C_{32}H_{30}O_5, 401.233; found, 401.233.

Discussion

The results of our study show that human urine is likely to contain marinobufagenin or its isomer and are consistent with previous reports suggesting that one of the mammalian sodium pump inhibitors may have a bufodienolide nature. These observations include demonstration of bufalin-like immunoreactive material in human bile, urine, and plasma; purification of bufalin derivatives from human eye lenses; detection of proscillaridin A–immunoreactive substance in human plasma and murine adrenocortical cell culture, and purification of a bufodienolide derivative from human placenta. In addition, our results provide further evidence that a marinobufagenin-like immunoreactive substance may be involved in the pathogenesis of AMI.

Previously, marinobufagenin has been found only in amphibian species. Along with our present data, several functional features make marinobufagenin a realistic candidate for a role of one of the endogenous ligands of digitalis receptors. First, in isolated human arteries the dose response of vasoconstriction to marinobufagenin is in the same range as the circulating concentrations of marinobufagenin-like immunoreactivity. Second, marinobufagenin is characterized by a relatively low toxicity. For example, the LD_{50} values of marinobufagenin and another bufodienolide, bufalin, in cats are 1.49 and 0.14 mg/kg, respectively. Finally, although the mechanism of biosynthesis of bufodienolides in mammals has not been described, some vertebrates, such as amphibians, are capable of synthesizing bufodienolides from cholesterol. Furthermore, cultured murine adrenocortical cells recently were found to produce material that cross-reacts with an antibody to a plant-derived bufodienolide, proscillaridin A. However, the origin of the described human urinary compound remains to be identified.

The demonstration of similarity of human DLF and amphibian marinobufagenin in our experiments was based on the identical retention time of two compounds on reverse-phase HPLC columns; similar maximal absorbance at 300 nm, which is typical for bufodienolides; identical MS fragmentation patterns; and cross-reactivity with marinobufagenin antibody. Previously, Lichtstein and coworkers reported the presence of Na^+/K^-ATPase inhibitor showing maximal absorbance at 300 nm in human cerebrospinal fluid and eye lenses.

In accord with our previous results, ouabain-like immunoreactive material from human urine eluted from reverse-phase HPLC columns earlier than marinobufagenin-like immunoreactivity. This pattern is consistent with the previous observations demonstrating the presence of less and more polar Na^+,K^-ATPase inhibitors in HPLC fractions from human urine. The more polar fraction has been identified as a ouabain-like compound. A less polar substance, which eluted from HPLC columns at 31% acetonitrile and which is similar to an adrenal factor described by Shaikh et al, may represent an endogenous bufodienolide.

Previously, Cloix et al demonstrated the presence of a steroidal DLF with molecular mass of approximately 400 in...
human urine. Recently, Kramer et al. have shown the presence of a digitalis-like Na\(^+\),K\(^+\)-ATPase inhibitor with the molecular mass of approximately 400 in HPLC-fractionated urine from salt-loaded humans. As shown previously, marinobufagenin cross-reacts with digoxin antibody. In the studies of Cloix et al. and of Kramer et al., purified factors also reacted with digoxin antibody.

Several observations indicate that the presence of a substance with a molecular weight of 400 in our study was not due to possible contamination of the HPLC system with marinobufagenin standard. First, after fractionation of urinary extract in the presence of marinobufagenin standard, the system was loaded with vehicle, and only trace (picomolar) amounts of marinobufagenin-like immunoreactivity were detected in the eluant. Second, the total amount of marinobufagenin standard applied to the HPLC system was 1 mg. This is considerably less than the amount of material purified from urine (18 \(\mu g\) from 8 L of urine). When we considered the average diuresis (1500 mL/d), average release of marinobufagenin-like immunoreactive material (12.2 nmol), and molecular weight of marinobufagenin (400), the expected amount of material to be purified was 26 \(\mu g\).

Marinobufagenin standards elute from HPLC columns as a single peak. After semipreparative-scale HPLC, 95% of marinobufagenin-like immunoreactive material also eluted as a single peak. At the same time as when urinary extract was fractionated on preparative-scale HPLC columns, several fractions demonstrated the ability to react with marinobufagenin antibody (Fig 2A). We hypothesize that this dissociation could have occurred because of several factors. First, dissociation may be due to different fractionation regimens: 60 minutes on Ultrapore RPMC columns and 90 minutes on DIAJOB columns. Second, it may be due to the instability of marinobufagenin-like material (fractionation on preparative-scale HPLC was performed 3 months later than the first fractionation presented in Fig 1). In addition, since at least four times more material was fractionated by preparative-scale HPLC compared with the first fractionation, the extract could contain significantly higher concentrations of marinobufagenin derivatives. Plasma of Bufo marinus toad, along with marinobufagenin, contains marinobufagin-3-sulfate, 11\(\alpha\),19-hydroxymarinobufagin, and marinobufotoxin (conjugate with suberylarginine). Although all of these compounds have demonstrated the ability to inhibit Na\(^+\),K\(^+\)-ATPase and to displace \(^3\)H-ouabain, they have demonstrated different retention times on HPLC columns. The presence of marinobufagenin derivatives in humans could explain in part...
the fact that several HPLC fractions in our study reacted with marinobufagenin antibody.

Tao et al have recently purified a sodium pump inhibitor from the peritoneal dialysate of patients with renal failure. This inhibitor (unlike ouabain) displayed remarkable ability from the peritoneal dialysate of patients with renal failure.

Interestingly, in our experiments marinobufagenin also acted as a potent inhibitor of the α-1 subunit of the sodium pump from rat aorta and plasma levels of marinobufagenin-like immunoreactive material in patients with renal failure were significantly increased. The results of the present study confirm earlier data demonstrating an increase in plasma concentration and urinary excretion of marinobufagenin-like immunoreactive factor in acute myocardial ischemia. It is believed that inhibition of the Na\(^+\)-K\(^+\) pump in cardiomyocytes plays an important role in the genesis of ischemia-induced arrhythmias. Previously we showed that this inhibition is due at least in part to the action of increased plasma digoxin-like immunoreactive factor and that pretreatment of the coronary-ligated rats with the activity in the course of a period of myocardial ischemia during transcutaneous coronary angioplasty in a group of patients with ischemic heart disease.

In the present study urinary release and plasma levels of marinobufagenin-like material in patients after AMI were increased threefold compared with control subjects. In the nanomolar range of concentrations, marinobufagenin produced substantial inhibition of Na\(^+\),K\(^+\)-ATPase from human pulmonary artery sarcolemma. Therefore, levels of marinobufagenin in plasma of patients with AMI may be sufficient to cause functionally significant inhibition of Na\(^+\),K\(^+\)-ATPase in human cardiovascular tissues.

Acknowledgments

The studies in the Laboratory of Pharmacology, Sechenov Institute of Evolutionary Physiology and Biochemistry were supported in part by Biomedical Sciences Research Laboratories, Millersville, Md.

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_Hypertension._ 1998;31:1097-1103
doi: 10.1161/01.HYP.31.5.1097

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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