Endogenous Digitalis-Like Factor

Novel Digitalis-Like Factor, Marinobufotoxin, Isolated From Cultured Y-1 Cells, and Its Hypertensive Effect in Rats

Masamichi Yoshika, Yutaka Komiyama, Motomi Konishi, Toshifumi Akizawa, Takahisa Kobayashi, Mutshiro Date, Shinzo Kobatake, Midori Masuda, Hiroya Masaki, Hakuo Takahashi

Abstract—Marinobufagenin and telecinobufagin have been identified as digitalis-like factors in mammals. In toads, marinobufagenin-related compounds, such as marinobufotoxin (MBT), have been isolated in some tissues but not in mammals, and its biological action has not been elucidated. Herein, we aimed to explore the possible production and/or secretion of MBT and the biological action in rats. First, the MBT in culture supernatant of the adrenocortical-originated cell line Y-1 was analyzed by high-performance liquid chromatography and sensitive ELISA for marinobufagenin-like immunoreactivity. Moreover, the structural information was obtained by mass spectrometry. To determine the biological action, MBT (9.6 and 0.96 μg/kg per day) was intraperitoneally infused via an osmotic minipump for 1 week. Blood pressure and renal excretion of marinobufagenin-like immunoreactivity were measured. Marinobufagenin-like immunoreactivity was found in Y-1 cell culture media, and the concentration increased until 24 hours. The structural analysis suggested that marinobufagenin-like immunoreactivities were marinobufagenin and MBT, and tandem mass spectrum analysis revealed them with the specific daughter ions. The highest sensitive ELISA-positive peak of marinobufagenin-like immunoreactivity in the media was MBT. Continuous administration of MBT in rats for 1 week significantly increased systolic blood pressure and renal excretion of marinobufagenin-like immunoreactivity compared with control rats (135±3.0 versus 126±2.0 mm Hg and 1.41±0.286 versus 0.34±0.064 ng/day, respectively). These data suggest that MBT, arginine-suberoyl ester of marinobufagenin, can be a novel digitalis-like factor with hypertensive action and is secreted from the adrenocortical cells. (Hypertension. 2007;49:209-214.)

Key Words: marinobufotoxin ■ digitalis-like factor ■ adrenal gland ■ ELISA ■ mass spectrometry ■ hypertension

In the 1960s de Wardener1 proposed the concept of natriuretic factors. Since Haddy and Overbeck2 and Blaustein3 suggested the existence of circulating sodium pump inhibitors in volume-expanded hypertensive subjects in the 1970s, many investigators have searched for digitalis-like factors (DLFs) as the specific inhibitors of sodium pump.4 The potential candidates included ouabain, digoxin, marinobufagenin (MBG), and some other bufadienolides.

Recently, DLFs have been divided into 2 groups according to the difference in their structures. One group is composed of cardenolides, such as ouabain and its related compounds, which have an unsaturated 5-membered lactone ring, and the other group is composed of bufadienolides, which have an unsaturated 6-membered lactone ring. Hamlyn et al5 first identified ouabain as its isomer from a huge quantity of human plasma. Pitzalis et al6 Bauer et al,7 and our laboratory8 have recently postulated the pathophysiological meaning of cardenolide by some experimental and clinical studies. Bagrov et al9 identified MBG as a candidate of DLF, which belonged to bufadienolides, in the human urine, and later they found MBG-like immunoreactivity (MBGi) in the human plasma.10 Recently, they have reported that an antibody to MBG lowered blood pressure of pregnant rats on a high NaCl loading11 and also a central role in the pathogenesis of experimental uremic cardiomyopathy.12

Bufadienolides have been found not only in toads but also in some animal species and plants,13 but the series of studies by Bagrov et al9 on MBG is strikingly interesting, because bufadienolides were actually found in mammals. Moreover, Lichtstein et al14 and Li et al15 found 19-norbufalin in the human cataractous lenses and proscillaridin-like immunoreactivity in the human plasma, respectively. Very recently, we have identified telecinobufagin (TCB), a 2-hydrogen-added form of MBG, by liquid chromatography (LC)/mass spectrometry (MS), as well as MBG in the human plasma.16 Thus, the recent understanding is that various kinds of cardenolides and bufadienolides exist in the mammalian biological fluids and tissues.

The bufadienolides identified so far are all free hydroxyl-bond forms on the C3 site of steroid rings that are aglycone forms. On
the other hand, in the amphibian toads, there are more polar C3 conjugates than free hydroxyl-bound forms with C3. In the skin and venom of *Bufo marinus*, there are several C3 conjugate forms of bufadienolides; however, their physiological role has not been elucidated. In the same way, other unidentified bufadienolides may exist in mammals, such as humans or rats. If the authentic compounds and/or the specific antibodies were purchased and developed, these compounds in mammals were highly sensitively identified with use of recent technology of LC/MS and ELISA. In the present study, we found a novel DLF in Y-1 cells of adrenocortical origin, and the possible biological function was investigated.

**Methods**

**Cell Culture and Preparation of Cell Culture Supernatant for ELISA and LC/MS**

Y-1 cells were obtained from Dainippon Pharmaceutical Co, Ltd, and cultured according to the manufacturer’s instructions. Briefly, Y-1 cells were subcultured in nutrient mixture F-10 (HAMS F-10) (Sigma-Aldrich) with 15% horse serum and 2.5% FCS in a CO2 incubator.

For the ELISA, when Y-1 cells in 4-cm dishes were grown to semiconfluence with 2 mL of medium, the cells were washed twice with PBS and then incubated in HAMS F-10 with 0.4% BSA (fatty acid-free BSA, Sigma Chemicals) (F-10/BSA) for 3 hours to starve the cells from serum as described before. After washing cells with PBS again, the medium was changed to fresh F-10/BSA serum-free medium. Subsequently, 2 mL of the culture supernatant were collected at 0.5, 2, 4, and 24 hours. In the same way, 20 mL of medium for LC/MS were collected 24 hours after the culture with serum-free medium. The viability (>95%) of cells during the course of experiments was confirmed by trypan blue dye exclusion.

**Preparation of MBG and Marinobufotoxin**

MBG used in the present study was the same authentic MBG as described before. Marinobufotoxin (MBT) was extracted from the skin of *Bufo marinus* and purified by preparative high-performance liquid chromatography (HPLC) and then by analytical HPLC. Finally, MBT in the 50% acetonitrile fraction of analytical HPLC and then by analytical HPLC. Subsequently, MBT was shown at m/z 713.4126 (calculated for C38H57O9N4 713.4130). The result of NMR indicates nuclear magnetic resonance. Spectra obtained in CD3OD at 150 MHz in 13C NMR and at 600 MHz in 1H NMR. Chemical shifts are expressed in parts per million and 3J values are in hertz.

### Table 1. The Chemical Shift Values of MBT in 13C NMR and 1H NMR

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<tr>
<th>Position</th>
<th>13C NMR</th>
<th>1H NMR</th>
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<tr>
<td>1</td>
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<tr>
<td>2</td>
<td>25.53</td>
<td>1.74 (1H, m) 1.62 (1H, m)</td>
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<td>3</td>
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<td>5</td>
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<tr>
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<td>35.49</td>
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<td>7</td>
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<td>42.09</td>
<td>3.21 (2H, m)</td>
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**Production of an Anti-MBG Murine Monoclonal Antibody**

Immunogen was prepared as follows. The hydroxyl group of the MBG C3 site was treated by glutaric anhydride. The produced carboxyl derivative was coupled to an amino group of BSA by the N-succinimidyl ester method. The Balb/c mice were immunized 4 times, and their spleen cells were fused with mouse myeloma cell P3-X63Ag8-U1 by a modified procedure described by Köhler and Milstein. Then, the culture supernatant was used as a monoclonal antibody.

**Method for ELISA**

The MBGi was assayed by the ELISA system using monoclonal antibody to MBG. This ELISA was the modified system of our competitive ELISA for ouabain described below. The coating antigen was MBT-conjugated ovalbumin, which was more stably fixed on the ELISA plate than MBG. Twenty-five microliters of a sample or standard MBG were mixed with the same volume of anti-MBG antibody on an MBT-coated plate. Y-1 cell culture samples were precleared by Sep-Pak C18 cartridges (Waters) and eluted by 15% to 60% acetonitrile. The eluate was evaporated and reconstituted in dilution buffer, PBS with 0.1% gelatin, 0.1% BSA, 2% casein, and 0.02% Tween-20 (BCG buffer). An amplification system was biotinylated horseradish peroxidase (Pierce) and NeutAvidin (Pierce). The MBGi level was calculated from the standard
HPLC Analysis of Supernatant
Twenty milliliters of Y-1 cell culture samples were acidified and preclarified as described in the ELISA section and then analyzed by HPLC as described before.14 The column was equilibrated with 20% acetonitrile in H2O/0.01% trifluoroacetic acid at a flow rate of 0.5 mL/min and eluted with gradient elution over 90 minutes. The percentage of acetonitrile was raised to 35% at 20 minutes, 40% at 30 minutes, and 80% until 90 minutes in a linear mode and monitored by the ultraviolet absorbance at 230 nm.

Each fraction of 31 to 50 minutes was evaporated and reconstituted in 100 μL of BCG buffer for ELISA and reconstituted in 100 μL of 10% acetonitrile for LC/MS. Then, we confirmed the elution times of the authentic MBG and MBT.

Parameters for LC/MS Setting
The LC/MS assay was performed using an LC/MS ESI system (ion trap LC/MS system LCQ, Thermo Electron Co Ltd) in an ESI mode with HPLC system (LC-10 series; Shimadzu Inc) using a high-pressure C8 column (capsell pak, 0.39×15 cm, 5 μm, 300 A, Shiseido). In all of the fractions of HPLC, the LC/MS assay for MBT was performed by the following method.

The HPLC setting was as follows: solvent A was 0.5% acetic acid/water, and solvent B was acetonitrile. A linear gradient solvent B was performed from 20% to 40% at a flow rate of 0.3 mL/min. The details of LC/MS assay for MBG and the MS setting parameters were described before.16

Intraperitoneal Administration of MBT in Rats
This study was approved by our institutional review committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Thirty 8-week-old male Wistar rats were obtained from CLEA Japan Inc and were divided into 3 groups. All of the rats were implanted with intraperitoneal osmotic minibipumps (Alzet Model 2002, Durect Corp) under ether anesthesia for a few minutes. Each group was continuously infused with 0, 0.96, and 9.60 mg/kg h of pentobarbital (0.1 mg/kg).

Statistical Analysis
The data are expressed as mean±SEM. The means of the 2 groups were compared by the Student t test. A P value of <0.05 was considered significant.

Results
Specificity of Anti-MBG Monoclonal Antibody and ELISA for MBGi
The cross-reactivity of anti-MBG MoAb was 100% in MBG, 62.52% in MBT, 3.014% in TCB, 0.625% in telocinobufotoxin, 0.033% in bufalin, and 0.001% in proscillaridin. It was <0.001% in ouabain, digoxin, dehydroisoandrosterone, cortisol, corticosterone, and aldosterone. Therefore, the cross-reactivity of this MBG antibody was relatively high against MBT among the bufadienolides and relatively low against TCB, which is the epoxy open form of MBG. The cross-reactivity of this antibody with cardenolides was <0.001% and was also very low with other adrenocortical steroid hormones.

Measurement of MBGi of the Culture Supernatants by ELISA
The assay range of the ELISA system was from 1 to 1000 pg/mL. In the culture supernatants of Y-1 cells, MBGi was detected as early as half an hour, and the MBGi secretion continued until 24 hours even after the medium of subculture was replaced with serum-free culture medium (Figure 1).

Structural Characterization of MBGi
ELISA-Positive Fractions of the Culture Supernatants by LC/MS Analysis
To characterize the MBGi-positive fraction in which MBG-related molecules exist, each HPLC fraction of Y-1 culture supernatants was analyzed by ELISA. The high ELISA-positive peak was detected at tube number (TN) 37, and the low ELISA-positive peak was found at TN 46, which was the elution volume of the authentic MBG (Figure 2). Interestingly, this high peak fraction was the same elution time of the authentic MBT. Therefore, to characterize the molecular structure of the peak (TN 37) substance, LC/MS analysis was performed. The tandem mass spectrometric (MS/MS) analysis of the authentic MBT in the positive ion mode showed specific daughter ions of m/z 695.4, 331.2, and 278.1 from the parent ion (m/z 713.2). An m/z 695.4 ion was derived from a dehydrated form of the parent ion, and an m/z 331.2 ion was
derived from suberyl-arginine moiety. On the other hand, MS/MS analysis of the authentic MBG showed specific daughter ions as described before. The Y-1–derived TN. 37 fraction exhibited an MBT-specific daughter ion spectrum as shown in Figure 3A, and the TN 46 fraction showed an MBG-specific daughter ion spectrum as shown in Figure 3B. As the control study, the MBT and MBG signals in the medium (F-10/BSA) without cells were determined by LC/MS, and we confirmed no specific peak at the same elution time of authentic MBT and MBG (Figure 4).

Figure 3. Tandem mass spectrometric signals of MBT and MBG. A, Top, a mass chromatogram of m/z 331.2; bottom, a mass chromatogram of m/z 713.2. B, Right, a mass chromatogram of m/z 401.2; left, a mass chromatogram of m/z 365.2. Y-1–derived MBG: 0.5-mL fraction of TN 36 (46-minute fraction of high-pressure octadecyl-silica column) was evaporated and reconstituted as described above.

Figure 4. The control mass chromatogram of Y-1 medium with BSA (without cells). Right, a mass chromatogram of the highest daughter ion of MBG (m/z 331.2); left, a mass chromatogram of the highest daughter ion of MBT (m/z 365.2).
Effects of MBT on SBP and Urinary Excretion of MBGi

As shown in Table 2, baselines of SBP and urinary excretion of MBGi in 3 groups were not different from each other. SBP and urinary excretion of MBGi were not different between the control and the low-dose group at 1 week after the administration. The renal function of these rats was considered to be normal, because serum levels of creatinine and urea nitrogen in MBT-administered rats were the same as those of control rats. Administration of MBT did not affect heart rate. Interestingly, SBP was significantly increased in rats infused with a high dose of MBT, and urinary excretion of MBGi was actually increased in those rats as compared with the other 2 groups.

**Discussion**

The DLF secreted from Y-1 cells was confirmed to be MBT by LC/MS and MS/MS analysis. It was clearly different from MBG in terms of the retention time of HPLC and the mass spectrum. By using a newly established ELISA system for MBGi, we found that MBGi is secreted from adrenocortical originated cells. When the constituents were fractionated by HPLC, MBGi was found to be exclusively MBT. This was elucidated by LC/MS, MS/MS, and NMR analysis. Therefore, our finding, that MBT is secreted from mammalian adrenal cells, can be an important beginning discovery of bufadienolides production and/or the metabolic pathway.

On the physiological functions of DLF, such as ouabain, proscillaridin A-like substance, and MBG, they have been extensively studied to modulate sodium and water metabolism. However, the biological role of MBT has not been elucidated thus far. The MBT has Na⁺-, K⁺-ATPase inhibitory activity as potent as MBG. Therefore, we explored the possible vasopressor effect of MBT. Continuous administration of MBT for 1 week significantly increased SBP. MBT had been dissolved in ethanol solution and primed into osmotic minipumps. Because the pump may be damaged chemically with ethanol, they did not allow priming of the higher dose of MBT dissolved in ethanol solution versus the current dose. The lower dose of MBT did not affect blood pressure, because the dose was too low to affect blood pressure. It is apparent because the urinary excretion of MBGi was not significantly increased. Similar magnitude of hypertensive effects by cardiac glycosides was observed with ouabain infused for 2 weeks (118 ± 2 mm Hg; \( P < 0.05 \)). We further confirmed that the given MBT was excreted into urine. This means that MBT acts as a vasopressor substance in rats without any toxic effects on kidney function, because serum creatinine and urea nitrogen levels were not influenced by MBT. Therefore, we propose that MBT could be a novel biomarker of hypertension acting as DLF in mammals, although the origin of MBT is still not determined.

Regarding the limitations of this study, our ELISA system was not specific for MBT. However, MBT was analyzed by the HPLC fractionation and determination with ELISA, and the amount of MBT was obviously greater than that of MBG. Another point of argument is whether MBT is of an endogenous or exogenous origin. Because we used cow and horse sera in the subculture, the Y-1 cells would have taken up MBT of the exogenous origin when those sera contained MBT. Therefore, we made the most effort to exclude MBT of exogenous origin by serum starvation for 3 hours as described...
in the Methods section, although it may be enough to prove it. Because we did not use a tracer, such as $^{14}$C-cholesterol, as the substrate, we have no direct evidence to show the biosynthesis. Further study is definitely needed to confirm the MBT of endogenous origin.

Hypertensive response was not marked with the current dose. As described above, the dose of MBT (9.6 μg/kg per day) was the maximum dissolved in the maximum ethanol concentration. The effect may be at the minimum in normal rats, and we are now planning to repeat experiments with uninephrectomized and sodium-salt–loaded rats for a longer period of administration of MBT.

**Perspectives**

Our data showed a novel DLF, MBT, secreted in mammals, and administration of MBT significantly increased blood pressure. Further studies are needed to clarify the underlying mechanism for the release, the possible production of MBT, and the physiological/pathophysiological roles. Because both bufadienolide and cardenolidine are released similarly in the adrenal cells, those cardiotonic steroids may be working synergistically with bufadienolides to modulate the sodium pumps, although they may not be of endogenous origin. Because the concentration of MBT was far greater than that of MBG, MBT may be the most cardinal DLF candidate at present.

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**Disclosures**

None.

**References**


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