Endogenous Ligand of α1 Sodium Pump, Marinobufagenin, Is a Novel Mediator of Sodium Chloride–Dependent Hypertension

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Background—Digitalis-like sodium pump ligands (SPLs) effect natriuresis via inhibition of renal tubular Na\(^{+}\),K\(^{+}\)-ATPase but may induce vasoconstriction. The present study investigated the potential roles of 2 putative endogenous SPLs, an ouabain-like compound (OLC) and an α1 Na\(^{+}\),K\(^{+}\)-ATPase inhibitor, marinobufagenin (MBG), in regulating natriuresis and blood pressure (BP) responses to sustained and acute NaCl loading in Dahl salt-sensitive rats (DS).

Methods and Results—During 4 weeks of an 8% NaCl diet, DS exhibited a progressive increase in MBG renal excretion (66±13 pmol/24 hours at week 4 versus 11±1 pmol/24 hours at baseline, n=48), which paralleled an increase in systolic BP (174±10 mm Hg at week 4 versus 110±2 mm Hg at baseline). By contrast, OLC excretion peaked at week 1 and returned to baseline levels. Administration of an anti-MBG, but not anti-ouabain antibody, to DS after 3 weeks of a high NaCl diet lowered BP (139±7 versus 175±5 mm Hg, P<0.001, n=5). Acute NaCl loading (2 hours) of DS (n=5) increased MBG and OLC excretion and natriuresis. Pretreatment of acutely NaCl-loaded DS with an anti-MBG antibody (n=5) reduced the excretion of sodium and MBG but not that of OLC. An anti-ouabain antibody (n=5) reduced sodium excretion and both OLC and MBG.

Conclusions—An initial transient stimulation of OLC induced by NaCl loading of DS precedes an MBG response. A sustained increase in MBG production in DS contributes to the chronic BP elevation induced by a sustained high NaCl intake. (Circulation. 2002;105:1122-1127.)

Key Words: hypertension ■ Na\(^{+}\)-K\(^{+}\)-exchanging ATPase ■ sodium ■ bufadienolides ■ ouabain

The role of endogenous digitalis-like sodium pump ligands (SPLs) in the pathogenesis of hypertension has been disputed for almost 3 decades.\(^{1}\) Endogenous SPLs are believed to promote natriuresis via renal Na\(^{+}\) pump inhibition and may link the Na\(^{+}\) retention that occurs during NaCl loading to an increase in arterial pressure via inhibition of the Na\(^{+}\),K\(^{+}\)-ATPase in cardiovascular tissues.\(^{2-4}\) An endogenous ouabain-like compound (OLC) was the first mammalian SPL to be purified.\(^{5}\) Subsequent studies showed that mammals also produce another class of SPLs, ie, bufadienolides.\(^{6-8}\) Bufadienolides, which were discovered in Amphibia, inhibit Na\(^{+}\),K\(^{+}\)-ATPase and cross-react with digitalis antibodies.\(^{9}\) In Amphibia, skin bufadienolides are responsible for Na\(^{+}\) excretion,\(^{9}\) and bufadienolide levels in toads vary with changes in environmental salinity.\(^{10}\)

Several bufadienolides have been suggested as candidate SPLs in mammals, including marinobufagenin (MBG),\(^{7}\) which acts in vitro as a vasoconstrictor\(^{11,12}\) and exhibits greater affinity to rodent ouabain-resistant α1 isoform of the Na\(^{+}\),K\(^{+}\)-ATPase than to an α1 isoform.\(^{11,13,14}\) MBG immuno-reactive material purified from human and rat urine was found to be identical to MBG from the toad, Bufo marinus, by mass spectral analysis\(^{15}\) and by its ability to inhibit rat kidney Na\(^{+}\),K\(^{+}\)-ATPase.\(^{14}\) Enhanced MBG production occurs in volume-expanded dogs\(^{16}\) and rats\(^{17}\) and in pathological states associated with fluid retention, including preeclampsia,\(^{12}\) hypertension in endstage renal disease,\(^{18}\) and salt-sensitive hypertension in Dahl rats.\(^{14}\)

In NaCl-induced hypertension in Dahl salt-sensitive rats (DS), which exhibit a mutation of the α1 Na\(^{+}\),K\(^{+}\)-ATPase isoform,\(^{19,20}\) we have demonstrated that in response to acute NaCl loading, the production of both types of endogenous SPL, OLC and MBG, increases\(^{13}\) and that during chronic NaCl loading the sustained increase in arterial blood pressure (BP) is associated with increased renal MBG excretion.\(^{14}\) However, the time course of changes in plasma levels of SPL and their specific roles in renal NaCl excretion and arterial BP modulation remained unclear. In the present study, we used specific antibodies against both OLC and MBG in an attempt to define the specific roles of both SPL in the natriuretic response to acute NaCl loading of DS and in the sustained arterial pressure increase in these rats during a sustained high NaCl intake.

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Experimental Protocol

The protocol of study was approved by the Animal Care and Use Committee of Gerontology Research Center, National Institute on Aging. Six-week-old male DS (SS/JrHsd) (255±10 g) (n=73) and Dahl-resistant rats (DR) (SR/JrHsd) (256±7 g) (n=48) were obtained from Harlan Sprague-Dawley Inc (Indianapolis, Ind). Animals were individually caged in metabolic chambers throughout the experiment.

In the first experiment, 16 DS and 16 DR were fed a normal low (0.2%) (LS) or high (8%) (HS) NaCl diet (ICN Biochemicals) and water ad libitum (n=8 in each group) for 4 weeks. Systolic BP (SBP) was measured weekly by tail-cuff plethysmography (ITTC model 31, ITTC Life Science). Twenty-four hour urine samples were collected to establish the profile of renal excretion of MBG and OLC and electrolytes (Beckman Synchon, EL/ISE).

Additional DS and DR (8 rats of each strain at each period) were anesthetized with Ketamine (10 mg/kg) and killed by bleeding before and after 1, 2, or 4 weeks of HS diet. Plasma and the pituitary and adrenal glands were collected for measurements of MBG and OLC.

Tissue Preparation

Pituitary and adrenal glands were homogenized in 5 volumes of distilled water and centrifuged at 1000 x g. Pituitary and adrenal glands were homogenized in 5 volumes of distilled water and centrifuged at 1000 x g. The supernatants, plasma, and urine (by 0.5 mL) were applied to Sep-Pak C18 cartridges. MBG and OLC were eluted (7.5 mL 20% acetonitrile followed by 7.5 mL of 80% acetonitrile), and the eluate was evaporated and reconstituted in the initial volume of assay buffer (Immunossays, below).

Effects of Anti-MBG and Anti-Ouabain Antibody

The in vitro effects of varying concentrations of anti-MBG and anti-ouabain antibody on erythrocyte Na+/K+-ATPase were studied in red blood cells from DS during low NaCl intake (n=5) and after 3 weeks of HS diet (n=6). Aliquots of the whole blood (0.5 mL) were preincubated at room temperature for 30 minutes in the presence and absence of different concentrations of rabbit polyclonal MBG or ouabain antisera (0.1 to 10 µg/mL of blood; a 5-µM/mL dilution corresponds to the concentrations of anti-MBG and anti-ouabain antibodies, which, in vitro, block the IC50 of MBG-induced inhibition of Na+,K+-ATPase from the outer medulla of rat kidney and ouabain inhibition of Na+,K+-ATPase from rat fetal brain). Erythrocytes were washed 3 times in an isotonic medium (145 mmol/L NaCl in 20 mmol/L Tris buffer; pH 7.6 at 4°C). Activity of Na+,K+-ATPase was determined, as reported previously in detail.5 Erythrocytes were preincubated with Tween-20 (0.5%) in sucrose (250 mmol/L) and Tris buffer (20 mmol/L; pH 7.4, 37°C) for 30 minutes and were incubated for 30 minutes in the medium containing (in mmol/L) NaCl 100, KCl 10, MgCl2, 3, EDTA 0.5, Tris 50, and ATP 2 (pH 7.4, 37°C) in the final dilution 1:40. The reaction was stopped by the addition of trichloroacetic acid to final concentration 7%. Total ATPase activity was measured by the production of inorganic phosphate (P(i)), and Na+,K+-ATPase activity was estimated as the difference between ATPase activity in the presence and absence of 5 mmol/L ouabain.

The effects of anti-MBG and anti-ouabain antibodies on SBP were compared in a subset of hypertensive DS after the sustained administration of a HS diet for 3 weeks. Animals were anesthetized with ketamine (10 mg/kg). Polypropylene catheters were inserted into left jugular veins (for antibody administration), femoral veins (for blood collection), and abdominal aorta (for direct BP measurement) (Stoelting). After 30 minutes of equilibration, venous blood samples were obtained and rats received intravenous bolus injections of MBG antibody (n=5) or ouabain antibody (n=5) (250 µL/kg, both antibodies were given at concentrations which in vitro reverse MBG-induced inhibition of rat kidney Na+,K+-ATPase and ouabain inhibition of Na+,K+-ATPase from rat fetal brain). Sixty minutes after antibody administration, a 0.5-mL sample of venous blood was collected and animals were killed by bleeding.

Next, the effects of intravenous administration of anti-MBG or anti-ouabain antibodies were examined in DS (6-week-old males, n=15, LS) after acute NaCl loading via a single intraperitoneal injection of 20% NaCl solution (0.8 g/kg of body weight in 2 mL), as reported recently in detail.13 Thirty minutes before NaCl loading, animals were anesthetized with ketamine (10 mg/kg). Polypropylene catheters were inserted into left jugular veins for blood collection and an acute antibody administration of vehicle (nonimmune rabbit serum, n=5), anti-MBG (n=5), or anti-ouabain (n=5) antibody. Animals were placed in metabolic chambers, and renal excretion of Na+, MBG, and OLC was monitored for 2 hours.

The ouabain antibody was the same as used for measurement of OLC (Immunossays, below). The MBG antibody was raised in rabbits immunized by MBG-glycoside-RAase conjugate.

The cross-reactivity of MBG antibody is (in %) MBG 100, ouabain <0.01, digoxin <0.01, digitoxin <0.01, bufalin <0.02, cinobufagin <1, cinobufotalin <10, prednisone <0.01, spirolactone <0.01, proscillaridin <0.01, progesterone <0.25, and mixture of bufadienolides from Bufo marinus venom except MBG = 10.

Immunossays

The MBG competitive fluoroimmunoassay, based on a polyclonal rabbit antibody raised against MBG-glycoside BSA, was performed as recently described.15 The cross-reactivity of this MBG antibody is (in %) MBG = 100, ouabain = 0.1, digoxin = 1.0, digitoxin = 3.0, bufalin = 1.0, cinobufagin = 1.0, prednisone <0.1, spirolactone <0.1, proscillaridin <1.0, progesterone <0.1, and mixture of bufadienolides from Bufo marinus venom except MBG = 5. The OLC assay was based on a similar principle using an ouabain-ovalbumin conjugate and a rabbit ouabain antibody (1:150,000, Chemicon International Inc, Temecula, Calif). The cross-reactivity of this ouabain antibody is (in %) ouabain = 100, digoxin = 7.4, progesterone <0.01, 5-beta cholanic acid, prednisone, and canrenonic acid <0.01, proscillaridin = 0.2, MBG-free mixture of bufadienolides from Bufo marinus venom total venom <0.26, bufalin <0.03, aldosterone <0.09, and MBG = 0.5.

Plasma concentrations of free (unbound by administered antibodies) MBG and OLC were measured after in vivo administration of anti-MBG or anti-ouabain antibodies. Because the organic extraction of plasma (as described above) would result in the destruction of an SPL-antibody complex, the antigen-antibody complexes were sedimented by treatment of plasma with saturated (NH4)2SO4 (1:1, overnight, 4°C). Then samples were centrifuged at 4000g for 15 minutes (4°C), and supernatants were collected and assayed for MBG and ouabain immunoreactivity as described above.

Statistics

Results are reported as mean±SEM. Statistical differences among the measured variables were assessed by one-way ANOVA or repeated-measures ANOVA followed by Newman-Keuls tests or a 2-tailed t test, when appropriate, using GraphPad Prism software (GraphPad Inc). P<0.05 was considered statistically significant.

Results

Figure 1 illustrates the SBP and renal excretion of MBG and OLC in DS and DR during LS and during a sustained HS. There were no differences between DS and DR in SBP or either SPL on LS. DS exhibited a progressive, sustained increase in SBP during HS (Figure 1A); in contrast, DR developed only a modest SBP elevation, and this did not reach statistical significance (Figure 1B). The renal excretion of OLC increased 6-fold in DS during the first week of HS and then decreased to the baseline levels (Figure 1C); in DR, no change in renal OLC excretion occurred, and the renal excretion of MBG increased by only 1.6-fold (Figure 1D). In contrast to OLC, a progressive increase in the excretion of MBG (up to 6-fold) occurred in DS during HS, and this
increase in MBG paralleled the SBP elevation (Figures 1A and 1C).

Figure 2 illustrates the average concentrations of OLC and MBG in pituitary and adrenals glands and plasma in DS and DR before and during HS. The pituitary level of OLC in DS increased 4-fold within 1 week of the high NaCl diet and then decreased to the baseline levels, whereas no change in pituitary OLC occurred in DR. Neither DS nor DR exhibited a significant change in pituitary MBG concentrations during HS (Figure 2A).

In DS, the adrenal OLC increased within the first week of HS and decreased to the baseline levels by week 4 (Figure 2B). In contrast to OLC, adrenal MBG in DS exhibited a progressive increase at 4 weeks of HS. During HS in DR, the adrenal OLC level had doubled the baseline level value at week 2 only, and MBG did not change.

The plasma OLC level in DS doubled within 1 week of HS and then decreased to the baseline level (Figure 2C). In contrast, the plasma concentration of MBG in DS during HS progressively increased and tripled within 4 weeks of the experiment. Interestingly, as in the adrenals, DR exhibited an elevation of plasma OLC only during the second week of HS. In DR, the plasma level of MBG in DR did not change during HS.

Thus, the pattern of OLC responses to HS was similar for urine excretion, plasma, and adrenal and pituitary levels: a transient early increase followed by a decline to the baseline level. The same pattern of MBG responses to HS, ie, sustained increases paralleling the increase in BP, was observed for urine excretion and plasma and adrenals levels but not for pituitary MBG, which did not increase during HS.

Figure 3 displays the average values of 24-hour NaCl intake, diuresis, renal NaCl excretion, hematocrit, and plasma Na⁺ and K⁺ in DS and DR before and during HS. After 4 weeks of HS, greater diuresis, less natriuresis, lower hematocrit, and higher concentration of plasma Na⁺ was observed in DS versus DR.

We next used anti-SPL antibodies to establish the cause and effect relationship of SPL to BP elevation or natriuresis. Before the in vivo administration of SPL antibody, the in vitro dose response of erythrocyte Na⁺,K⁺-ATPase to OLC or
MBG antibody was established. As illustrated in Figure 4A, the activity of erythrocyte Na⁺,K⁺-ATPase of DS after 3 weeks of HS was lower than that of LS. The in vitro administration of MBG antibody restored the erythrocyte Na⁺,K⁺-ATPase activity in a concentration-dependent manner, whereas the ouabain antibody did not affect the Na⁺,K⁺-ATPase activity. Neither the MBG nor ouabain antibody affected the activity of Na⁺,K⁺-ATPase in erythrocytes from DS on LS (data not shown).

To determine the role of the sustained increase in MBG level in the chronic increase in BP during HS, antibodies to MBG or ouabain were administered to a subset of 10 DS after 3 weeks of HS (Figures 4B through 4D). Administration of the anti-MBG antibody resulted in a reduction in the level of free MBG but did not affect the level of free OLC. Conversely, the anti-ouabain antibody decreased the concentration of free OLC but did not affect that of MBG (Figure 4B). SBP decreased within 15 minutes after the bolus administration of the anti-MBG antibody in all animals and did not increase within the next 60 minutes of observation (Figure 4C). In contrast, no antihypertensive effect was observed after administration of the anti-ouabain antibody to acutely NaCl-loaded DS (Figure 4D).

Figure 5 illustrates the effect of the MBG or ouabain antibody on renal excretion of Na, MBG, and OLC of DS after acute NaCl loading. Acute NaCl loading in vehicle-treated rats was associated with a marked natriuresis and stimulation of renal excretion of MBG and OLC. Pretreatment of the animals with the anti-MBG antibody reduced Na excretion by 42% and reduced MBG excretion by 85%, but OLC excretion did not change. Surprisingly, the administration of the anti-ouabain antibody not only reduced OLC excretion but that of MBG as well. Na⁺,K⁺-ATPase excretion was reduced by the anti-ouabain antibody to the same extent as that effected by the MBG antibody.

Discussion

The present results define different patterns of endogenous OLC and MBG in response to sustained high NaCl intake in DS. The results establish a direct link of MBG to the acutely increased natriuresis and to the sustained BP elevation and suggest a relationship between OLC and MBG in the response to high NaCl intake.

In the present and our prior experiments in NaCl-loaded DS, concomitant increases in the pituitary level and renal excretion of OLC preceded a sustained increase in MBG excretion that paralleled sustained hypertension. The administration of anti-ouabain antibody to acutely NaCl-loaded DS blunted the MBG response. These results suggest that there may be a causative link between OLC and increased MBG excretion.

During a sustained high NaCl intake, the pituitary level and renal excretion of OLC exhibited a marked increase, peaking within 1 week; adrenals and plasma OLC also exhibited a similar biphasic pattern, but the transient increase was less
than that in the pituitary and urine. The initial increase of OLC we observed is in agreement with the results of prior experiments in which the importance of brain OLC in the onset of NaCl-induced hypertension in DS has been noted. Leenen and coworkers demonstrated that the blockade of brain OLC with digoxin antibody alleviates the NaCl-induced hypertension in DS. Furthermore, Gomes-Sanchez et al. have shown that active immunization of DS against ouabain prevents the acute phase of NaCl loading of DS, an acute increase in OLC. The latter evokes a natriuretic response. During sustained high NaCl intake, OLC level decreases but MBG level continues to increase as does MBG.

Our present finding that the blockade of MBG after the administration of MBG antibody to acutely NaCl-loaded DS attenuates the acute natriuretic response supports previous evidence that MBG exerts a natriuretic action. That the ouabain antibody also reduced Na⁺ excretion after acute NaCl loading follows from its effect to suppress MBG production. During sustained NaCl loading, which produced sustained hypertension in DS despite the fact that urinary MBG excretion and diuresis rate increased to higher levels in DS than in DR, the urinary excretion of Na⁺ was lower and the hematocrit was higher in DS than in DR. This pattern suggests that both pressure natriuresis and natriuretic response to MBG are defective in DS. Our interpretation of this result is that an exaggerated production of an α, Na⁺,K⁺-ATPase ligand, MBG, in response to high NaCl intake in DS is a compensatory response to the inability of the renal Na⁺ pump to accommodate the excess of Na⁺ because of the defect in the Na⁺,K⁺-ATPase α₁ subunit of the DS. However, the increased production of MBG does not fully compensate for the impaired properties of Na⁺,K⁺-ATPase.

The results of the present study also provide additional support for a role of MBG in the sustained BP elevation that occurs in DS during sustained NaCl loading. Our previous experiments have demonstrated that in vitro, 1 nmol/L MBG inhibited the Na⁺,K⁺-ATPase from rat kidney outer medulla and aortic sarcolemma by 25%. In the present study, plasma concentration of MBG increased 3.5-fold and reached the level of 1.25 nmol/L. Thus, the plasma levels of MBG observed in vivo in DS may be sufficient to significantly alter the vascular tone.

A role of MBG in the maintenance of NaCl-induced hypertension in DS is confirmed by the results of the present experiment using an MBG antibody in vivo. The intravenous administration of the MBG antibody to hypertensive DS substantially lowered the BP. The anti-MBG antibody used in the present study exhibits extremely low cross-reactivity with cardenolides and has substantial cross-reactivity only with cinobufotalin, which is an epoxybufodeinolide, differing from MBG only in having one extra hydroxyl group. No effect on BP was observed in response to ouabain antibody. The lack of an effect of the ouabain antibody on BP not only serves as an important negative control for the MBG antibody experiment but also indicates that MBG does not modulate BP in DS during sustained NaCl loading at least in the same manner as does MBG.

In summary, we interpret our results as follows: in the acute phase of NaCl loading of DS, an acute increase in OLC precedes an increase in MBG. The latter evokes a natriuretic response. During sustained high NaCl intake, OLC level decreases but MBG level continues to increase in a graded manner with an increase in BP. The BP increase occurs, in part at least, because even the acutely elevated MBG level cannot effect sufficient natriuresis to reduce plasma volume. The sustained high level of MBG inhibits vascular Na⁺,K⁺-ATPase, leading to an increase in vascular smooth muscle cells, Na⁺, and, subsequently, Ca²⁺ concentration via Na⁺/Ca²⁺ exchange. Thus, a blunted kidney response to MBG (reduced natriuresis and diuresis) is associated with excessive MBG production (increased vascular tone), and both mechanisms contribute to a sustained elevation of BP.

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References


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