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

Olga V. Fedorova, PhD; Mark I. Talan, MD, PhD; Natalia I. Agalakova, PhD; Edward G. Lakatta, MD; Alexei Y. Bagrov, MD, PhD

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.

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) 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).

Key Words: hypertension ■ Na⁺-K⁺-exchanging ATPase ■ sodium ■ bufadienolides ■ ouabain

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From the Laboratory of Cardiovascular Science, Intramural Research Program, National Institute on Aging, National Institutes of Health, Baltimore, Md.
Correspondence to Dr Alexei Bagrov, Laboratory of Cardiovascular Science, National Institute on Aging, 5600 Nathan Shock Dr, Baltimore, MD 21224. E-mail BagrovA@grc.nia.nih.gov
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**Methods**

**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, IITC Life Science). Twenty-four-hour urine samples were collected to establish the profile of renal excretion of MBG and OLC and electrolytes (Beckman Synchron, 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 1000g for 10 minutes. The tissue 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 (Immunoassays, 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 2 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 

**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.
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 by week 4 (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, i.e., 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 (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 results in a reduced BP response to the sustained administration of a high NaCl diet.

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 \(\alpha\) 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 \(\alpha\) 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 mmol/L MBG inhibited the Na\(^+\),K\(^+\)-ATPase from rat kidney outer medulla and aortic sarcolemmma by 25%. In the present study, plasma concentration of MBG increased 3.5-fold and reached the level of 1.25 mmol/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 OLC 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, Ca\(^{2+}\) concentration via Na\(^+\)/Ca\(^{2+}\) 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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