Endogenous Na,K Pump Ligands Are Differentially Regulated During Acute NaCl Loading of Dahl Rats

Olga V. Fedorova, PhD; Edward G. Lakatta, MD; Alexei Y. Bagrov, MD, PhD

Background—Two mammalian digitalis-like factors, an ouabain-like compound (OLC) and marinobufagenin (MBG), exhibit specificity to α-3 and α-1 Na\(^{+},K^{+}\)-ATPase isoforms, respectively. We compared regulation of MBG and OLC by acute NaCl loading in Dahl salt-sensitive (DS) and salt-resistant (DR) rats.

Methods and Results—An intraperitoneal NaCl load (0.8 g/kg) was given to adult male rats (24 DS and 24 DR). Diuresis, natriuresis, renal excretion, and tissue levels of MBG and OLC were measured. Inhibition of renal Na\(^{+},K^{+}\)-ATPase by MBG and ouabain was compared in DS, DR, and Wistar rats. DS (versus DR) exhibited a smaller peak (2 hours) natriuretic response (1.34±0.10 versus 2.08±0.14 mmol · kg\(^{-1}\)· h\(^{-1}\); P<0.01), despite a greater plasma Na\(^{+}\) (153±2 versus 145±1 mmol/L; P<0.01). In DS and DR, pituitary, adrenal, and plasma OLC exhibited transient 2-fold to 3-fold increases, followed by a decrease to baseline levels. Plasma and adrenal MBG doubled in both strains within 1 hour of NaCl loading and remained elevated. Eight-hour MBG excretion in DS was 4-fold greater than in DR (15.8±0.8 versus 3.6±0.4 pmol; P<0.01), whereas OLC excretion in DS was only 30% greater than in DR (16.1±1.1 and 11.9±0.8 pmol; P<0.05). Kidney Na\(^{+},K^{+}\)-ATPase (α-1 isoform) from Wistar rats and DS exhibited greater sensitivity to MBG than to ouabain.

Conclusions—NaCl loading of DS causes transient increase in OLC but sustained increases in MBG tissue levels and excretion. We hypothesize that increased MBG production occurs in an attempt to compensate for genetically impaired pressure-natriuresis mechanisms. (Circulation. 2000;102:3009-3014.)

Key Words: hypertension ■ rats, inbred Dahl ■ sodium, dietary ■ Na(+)-K(+)-exchanging ATPase ■ steroids ■ bufanolides ■ ouabain

Endogenous cardiotonic steroids (CS): ie, “digitalis-like” factors, are thought to contribute to the pathogenesis of NaCl-sensitive hypertension.\(^{1,2}\) The primary role of CS is believed to be to facilitate natriuresis through inhibition of the sodium pump in renal basolateral membranes.\(^{3}\) However, excessive production of CS may also inhibit the sodium pump in vascular smooth muscle and result in vasoconstriction.\(^{1}\) An endogenous cardenolid (ouabain-like compound [OLC] from human plasma\(^{3}\) and bovine hypothalamus\(^{4}\)) was the first mammalian CS to be purified. Recent studies have shown that mammalian tissues also contain bufadienolide CS.\(^{5-7}\) Bufadienolides were discovered in amphibians and differ from cardenolides in having a 6-membered doubly unsaturated lactone ring.\(^{5}\) One CS that was purified from human urine by high-performance liquid chromatography mass spectrometrically was identical to an amphibian bufadienolide, marinobufagenin (MBG).\(^{6}\) In vitro, MBG is a potent vasconstrictor and Na\(^{+},K^{+}\)-ATPase inhibitor and, in rat aorta, exhibits greater affinity to the α-1 subunit of Na\(^{+},K^{+}\)-ATPase than does ouabain.\(^{9-11}\) Plasma levels of MBG immunoreactivity have been found to be elevated in several volume-expanded hypertensive states.\(^{11-13}\)

Hypertension that develops in Dahl salt-sensitive rats (DS) on a high-NaCl diet is associated with plasma volume expansion that results from renal sodium retention due to blunted pressure natriuresis and is accompanied by dysregulation of the biosynthesis of Na\(^{+},K^{+}\)-ATPase in various tissues, including kidney.\(^{14}\) Impaired pressure natriuresis in DS with high-sodium intake appears to be due several factors, which include activation of a Cl\(^{-}\) transport in the loop of Henle\(^{15}\) and abnormal pumping properties of Na\(^{+},K^{+}\)-ATPase in the proximal tubules.\(^{16}\) Because the α-1 isoform of Na\(^{+},K^{+}\)-ATPase represents main sodium transporting system in the basolateral membrane\(^{17}\) and exhibits altered sodium transport properties in DS,\(^{18}\) α-1 gene has been hypothesized to be a candidate hypertension gene. Indeed, an altered transport ratio of Na\(^{+}\) to K\(^{+}\) in DS was found to be associated with a mutation in the α-1 Na\(^{+},K^{+}\)-ATPase gene.\(^{18}\) Subsequently, transgenic DS rats that expressed wild-type α-1 Na\(^{+},K^{+}\)-ATPase while on an 8% NaCl diet exhibited less
blood pressure increases, less renal injury, and a longer life span than nontransgenic DS on the same high-NaCl diet.\(^3\)

One could postulate that in the presence of deficient renal 
Na\(^+\) excretion, NaCl loading of the DS rat would elicit exaggerated production of endogenous natriuretic substance that inhibits renal Na\(^+\),K\(^+\)-ATPase (α-1 isofrom); ie, MBG. Therefore, we determined whether NaCl loading elicits response in MBG as well as OLC. We investigated effects of NaCl loading on natriuresis and central and peripheral OLC and MBG in DS and DR. Our results demonstrate that acute NaCl loading stimulates MBG in addition to OLC, that the OLC and MBG responses occur with different time courses, and that the magnitude of MBG excretion in DS is 3-fold greater than in DR. Our data also show that in Wistar rats and DS, MBG inhibits Na\(^+\),K\(^+\)-ATPase α-1 isofrom at the level of high-affinity (nanomolar) binding sites.

**Methods**

**Experimental Protocol**

The protocol of the study was approved by the ACUC of the Gerontology Research Center (GRC), National Institute on Aging, NIH. Twenty-four 10-week-old male DS (SS/JrHsd; 524±4 g) and 24 DR (SR/JHsd; 463±4 g) were obtained from Harlan Sprague-Dawley Inc and were fed normal chow (0.5% NaCl) and water ad libitum. One hour before the experiment, blood pressure was measured by tail-cuff plethysmography (model 31, IITC Life Science). Acute NaCl loading was achieved by a single intraperitoneal injection of 20% NaCl solution (0.8 g/kg body wt in 2 mL). Rats were individually caged in metabolic chambers with free access to drinking water. At 1 and 8 hours after injection, groups of rats (n=8 each) were anesthetized with ketamine (10 mg/kg) and killed by bleeding. Blood was collected in heparin tubes for measurement of electrolytes, MBG, and OLC. Urine samples were filtered through Millipore filters (0.45 m, Milipore Products Division), and kept frozen (−80°C) for measurements of Na\(^+\), K\(^+\), OLC, and MBG. Plasma and urinary sodium and potassium were analyzed (Beckman Instruments, Synchron, EL/ISE), and excretion rates of these ions were expressed in millimoles per hour per kilogram of body weight.

**Tissue Preparation**

Tissue samples were homogenized in 5 vol of distilled water centrifuged at 1000g for 10 minutes. Tissue supernatants or plasma (0.5 mL) were applied to Sep-Pak C18 cartridges that had been activated with acetonitrile. After washing with 10 mL distilled water, OLC and MBG were eluted with acetonitrile (7.5 mL of 20% acetonitrile followed by 7.5 mL of 80% acetonitrile). Eluate was evaporated and reconstituted in the initial volume of assay buffer.

**Immunoaassays**

MBG immunoaassay was performed as recently described.\(^13\) The assay is based on competition between immobilized antigen (MBG-glycosidase-RNAse) and CS within the sample for a limited number of binding sites on polyclonal rabbit MBG (against MBG-glycoside-RNAse) and CS. Secondary (goat anti-rabbit) antibody binding sites on polyclonal rabbit MBG (against MBG-glycoside-RNAse) and CS within the sample for a limited number of binding sites on polyclonal rabbit MBG (against MBG-glycoside-RNAse) and CS.

**Immunoblotting of Na\(^+\),K\(^+\)-ATPase**

Solubilized membrane protein from renal medulla and rat kidney and brain as controls (10 μg of protein per lane) were separated by 8% Tris–glycine PAGE in SDS-buffer (Novel Experimental Technology), and protein was transferred to a nitrocellulose membrane (0.45-μm pore size) as described previously.\(^24,25\) Blots were blocked in 5% dried nonfat milk (wt/vol) and 0.1% Tween 20 in TBS overnight (14°C) and incubated for 1 hour with isofrom-specific anti-α-1 mouse monoclonal (1:1000; Upstate Biotechnology) and an anti-α-3 rabbit monoclonal (MA3-915; 1:450; Affinity BioReagents Inc) antibodies. After being washed (TBS and 0.1% Tween 20), blots were incubated with a 1:1000 dilution of HRP-conjugated anti-rabbit IgG and visualized by chemiluminescence (ECL plus, Amersham). Bands were quantified using a Molecular Dynamics GS 690 Imaging System.
were incubated with corresponding peroxidase-conjugated affinity-purified anti-mouse antiserum (Amersham Corp) at 1:1000 for 1 hour. Immunoreactivity was detected by enhanced chemiluminescence (Hyperfilm-ECL; Amersham).

Statistics
Results are reported as mean±SEM. Statistical differences among the means studied were assessed by 1-way ANOVA followed by Bonferroni tests (intragroup comparisons), 2-way ANOVA (intergroup comparisons), 2-tailed t test (when appropriate), or nonlinear regression with GraphPad Prism software (GraphPad Inc). P<0.05 was considered significant.

Results
Blood Pressure and Plasma Electrolytes
Baseline systolic blood pressure in DS and DR was 121±3 and 113±3 mm Hg, respectively (P=0.08). Within 8 hours after intraperitoneal administration of hypertonic saline, systolic blood pressure rose to 170±7 and 158±6 mm Hg, respectively, in DS and DR (P<0.01). Values of plasma Na⁺ and K⁺ concentrations are listed in Table 1. Within 1 hour of saline administration, plasma concentrations of Na⁺ and K⁺ significantly rose in both DS and DR, and after 8 hours, concentrations returned to baseline levels in DR but remained elevated in DS.

Ouabain-Like Compound
Baseline OLC concentrations in the pituitary, adrenals, and plasma did not differ between DS and DR (Figure 1). Within 1 hour after administration of hypertonic saline, pituitary OLC exhibited a 3-fold increase and adrenal and plasma OLC showed 40% increases in both DS and DR. At 8 hours after saline administration, concentrations of OLC in all 3 tissues decreased to levels not significantly different from baseline.

Baseline urinary OLC excretion in DS and DR did not differ (Figure 2A). Within 1 hour after saline loading, excretion of OLC in DS and DR rose 5-fold and 3.8-fold, respectively. In both strains, OLC excretion gradually decreased to baseline values. Total 8-hour OLC excretion by DS and DR was 16.1±1.1 and 11.9±0.8 pmol, respectively (P<0.05).

Marinobufagenin
Effects of acute NaCl loading on tissue and plasma MBG are presented in Figure 1. No differences were seen between DS and DR in baseline pituitary, adrenal, or plasma MBG levels. Acute NaCl loading caused a nonsignificant increase in MBG immunoreactivity in the pituitary in both strains (Figure 1A). However, adrenal (Figure 1B) and plasma (Figure 1C) MBG concentrations in both DS and DR doubled within 1 hour after saline administration. Eight hours after NaCl loading, the concentration of MBG remained elevated in plasma of both DS and DR and in adrenal tissue of DS.

Baseline urinary excretion of MBG was 0.32±0.11 and 0.46±0.04 pmol/h (P>0.5) in DS and DR, respectively. As demonstrated in Figure 2B, urinary excretion of MBG increased by 12-fold in DS within 4 hours after saline loading and then decreased to a level that remained significantly elevated above baseline levels for duration of experiment. In
DR, MBG excretion increased 3-fold within the first 2 hours of NaCl loading and did not change for duration of experiment. Total MBG excretion was 5-fold greater in DS than DR (15.8 ± 0.8 and 3.6 ± 0.4 pmol, respectively; \( P < 0.01 \)).

**Diuresis and Natriuresis**

As illustrated in Figure 3, maximal diuretic and natriuretic responses peaked within 2 hours after administration of hypertonic saline in both strains and subsequently decreased to baseline levels. Although DS had a greater diuretic response than DR during first 2 hours after NaCl loading (Figure 3A), natriuretic response was 40% greater in DR than in DS (Figure 3B). Urinary K⁺ excretion exhibited a similar time course and strain differences as natriuretic response (Figure 3C).

**Na⁺,K⁺-ATPase Inhibitory Effects of MBG and Ouabain**

As demonstrated in Figure 4A, \( \alpha-1 \) monoclonal antibody detected the presence of \( \alpha-1 \) immunoreactivity in renal membranes from Wistar rats, DS, and DR, whereas \( \alpha-3 \) immunoreactivity was undetectable in all 3 strains of rats. Values of Na⁺,K⁺-ATPase activity are given in Table 2. Concentration-response curves of Na⁺,K⁺-ATPase inhibitory effects of MBG and ouabain in renal membranes from DS, DR, and Wistar rats are presented in Figure 4B and 4C. Curves of Na⁺,K⁺-ATPase inhibition were analyzed by use of 2- and 1-site competition models. IC₅₀ values corresponding to Na⁺,K⁺-ATPase inhibition occurring at the levels of high-affinity and low-affinity sites are listed in Table 2. Compared with ouabain, MBG exhibited a greater Na⁺,K⁺-ATPase inhibitory effect in the membranes from all 3 strains of rats. In DS, Na⁺,K⁺-ATPase inhibitory action of MBG was weaker than that in Wistar rats but greater than that in the DR. In all 3 strains of rats, MBG-induced Na⁺,K⁺-ATPase inhibition has occurred at the levels of both higher-affinity and lower-affinity receptor sites. Analyses of the effect of ouabain in the renal membranes did not detect a high-affinity component in the Na⁺,K⁺-ATPase inhibitory action of ouabain in any of the 3 strains.

**Discussion**

The main novel observations of the present study are (1) that acute NaCl loading through intraperitoneal administration of
hypertonic saline to Dahl rats stimulates both MBG and OLC; (2) that responses of OLC and MBG exhibit different time courses: the former transiently increases, whereas the increase in the latter is sustained; (3) that DS exhibit a greater increase in MBG excretion than DR; and (4) that renal Na⁺,K⁺-ATPase (α-1 isoform) from DS and from Wistar rats but not from DR exhibits greater sensitivity to MBG than to ouabain.

In our present experiment, MBG acted as a potent in vitro inhibitor of renal Na⁺,K⁺-ATPase from Wistar rats and DS. This confirms our previous observations that demonstrated natriuretic properties of MBG12 and its greater (versus ouabain) affinity to the α-1 Na⁺,K⁺-ATPase isoform,9 the exclusive sodium pump isoform of the renal tubules. In membranes from DS and Wistar rats, MBG, unlike ouabain, produced Na⁺,K⁺-ATPase inhibition at the level of high-affinity binding sites. Analysis of Na⁺,K⁺-ATPase inhibition by ouabain by use of a 2-site competition model did not reveal a high-affinity component in DS, DR, and Wistar rats. This dissociation between the sensitivity of renal Na⁺,K⁺-ATPase to MBG and ouabain is similar to that observed by Ferrandi et al23 for digitalis and hypothalamic Na⁺,K⁺-ATPase inhibitory factor.

Within 1 hour of NaCl loading, concentrations of OLC rose proportionally in plasma and adrenal glands in both DS and DR. At the same time, peak increases in OLC pituitary levels and urinary excretion in DS exceeded those in DR by 33% and 70%, respectively. This observation is consistent with the earlier studies that demonstrated greater reactivity of OLC in DS (versus DR) to acute air stress and administration of a high-NaCl diet.26 The time course of urinary OLC excretion reflected changes in OLC levels in the pituitary, adrenals, and plasma. Eight hours after administration of hypertonic saline, urinary excretion of OLC, in addition to pituitary, plasma, and adrenal OLC concentrations, had decreased to baseline levels. Unlike OLC, MBG exhibited more sustained increases after acute NaCl loading. Thus, concentrations of MBG in plasma and adrenals increased substantially at 1 hour after NaCl loading but remained elevated 8 hours after saline administration.

In our previous experiment,12 neutralization of MBG activity by administration of a digoxin antibody in anesthetized, volume-expanded dogs resulted in a decreased natriuretic response. In the present study, despite the fact that urinary MBG excretion and rate of diuresis in NaCl-loaded DS was significantly higher than that in DR, the urinary excretion of sodium in DS during the first 2 hours after saline loading was significantly lower in DS than in DR. These observations seem to be in accordance with previously observed blunted pressure natriuresis in DS, believed to be due, at least in part, to a mutation in the α-1 subunit of Na⁺,K⁺-ATPase.19 Thus, an exaggerated renal production of an α-1 ligand, MBG, in DS could be interpreted to be a compensatory response to the inability of the sodium pump in basolateral membranes of the former to fully accommodate the excess of sodium. However, even increased production of MBG does not seem to fully compensate for the impaired properties of Na⁺,K⁺-ATPase in the DS basolateral membrane.

In the present experiment, the increase in MBG excretion exceeded concomitant changes in MBG concentrations in plasma and adrenals in either DS or DR: after the first hour of saline loading, plasma and adrenal concentrations of MBG doubled in both strains, whereas urinary excretion of MBG increased 12-fold in DS and 3-fold in DR over baseline levels. Several possibilities could explain the dissociation between observed patterns of adrenal and plasma levels and renal excretion of MBG in NaCl-loaded DS. First, the kidneys may participate in the production or storage of MBG; renal production of Na⁺,K⁺-ATPase inhibitory, natriuretic, and digoxin-like immunoreactive material, including isolated perfused kidneys and cultured proximal tubular cells, of saline volume-expanded rats has been demonstrated.27 Second, it is possible that increased urinary MBG excretion by DS may reflect an altered renal metabolism of adrenocorticosteroids. Kidney is known to play an active role in metabolism of corticosteroids, and significant differences in renal handling of adrenocorticosteroids have been detected between DS and DR.28 Third, it is possible that a urine-concentrating mechanism occurs in the DS kidney that is absent in the DR kidney, which may account for enhanced MBG excretion. In our experiment, plasma level of MBG in NaCl-loaded DS was ≈0.5 nmol/L. IC₅₀ of MBG inhibition of kidney Na⁺,K⁺-ATPase in DS at the level of high-affinity binding sites is 3.3 nmol/L. Therefore, MBG would be able to inhibit ≤10% of Na⁺,K⁺-ATPase receptor sites in the renal tubules. The above arguments suggest that local levels of MBG in kidney might be higher than in the other tissues and cause the relevant inhibition of the renal tubular sodium pumps.

In conclusion, our results demonstrate that acute NaCl loading of DS and DR is associated with transient increases in OLC but sustained increases in MBG. Although urinary OLC excretion in DS during the first 2 hours after saline administration exceeded that in DR by only 30%, excretion of MBG in DS was 3-fold higher than that in DR. Peak (2-hour) natriuretic response to NaCl loading was 30% less in DS,

### TABLE 2. Interactions of MBG and Ouabain With Renal Na,K-ATPase

<table>
<thead>
<tr>
<th></th>
<th>Na⁺,K⁺-ATPase Activity, μmol P/mg protein per h</th>
<th>% Na⁺,K⁺-ATPase Activity of Total ATPase Activity</th>
<th>MBG</th>
<th>Ouabain</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>High-Affinity, nmol/L</td>
<td>Low-Affinity, μmol/L</td>
<td>High-Affinity, nmol/L</td>
<td>Low-Affinity, μmol/L</td>
</tr>
<tr>
<td>DS</td>
<td>84±1</td>
<td>79</td>
<td>3.3 (1.3–8.3)</td>
<td>74 (44–124)</td>
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<tr>
<td>DR</td>
<td>80±3</td>
<td>81</td>
<td>77 (26–225)</td>
<td>126 (71–224)</td>
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<tr>
<td>Wistar</td>
<td>50±2</td>
<td>83</td>
<td>7.2 (3.4–15)</td>
<td>99 (28–340)</td>
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</tbody>
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Two-site competition model. Data are mean±SEM from 6 to 9 experiments; 95% confidence values are given in parentheses.
despite greater diuresis. We hypothesize that increased MBG production occurs after NaCl loading in DS in an attempt to compensate for genetically impaired pressure-natriuresis mechanisms.

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