Physiological roles of endogenous ouabain in normal rats

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Nesher M, Dvela M, Igbokwe VU, Rosen H, Lichtstein D. Physiological roles of endogenous ouabain in normal rats. Am J Physiol Heart Circ Physiol 297: H2026–H2034, 2009.—Endogenous ouabain (EO)-like compounds are synthesized in and released from the adrenal gland. Although EO has been implicated in several pathological states such as hypertension and heart and kidney failure, its physiological roles in normal animal have not been elucidated. To address this issue, we studied the effects of reduction in plasma EO resulting from antiouabain antibody administration. Normal rats were treated for 28 days with antiouabain antibodies or rabbit IgG as control. Infusions were delivered through a jugular vein cannula by osmotic pumps, and blood pressure was monitored by tail-cuff plethysmography. The animals were housed in metabolic cages to measure water and food consumption and urine excretion. After 28 days, the thoracic aorta was isolated and used to study phenylephrine-induced contraction and atrial natriuretic peptide (ANP)-induced vasorelaxation. The adrenal gland cortex was enlarged in the antiouabain antibody-treated rats. Moreover, on the second day of treatment, there was a significant transient reduction in natriuresis in the antiouabain antibody-treated rats, suggesting that EO is a natriuretic hormone. Reduction in natriuresis was also observed when EO levels were reduced by active immunization resulting from sequential injection of ouabain-albumin. Furthermore, following 28 days of treatment, the response to phenylephrine was significantly lowered and that to ANP was significantly increased in aortic rings from antiouabain antibody-treated rats. These findings show for the first time that circulatory ouabain plausibly originating in the adrenal has physiological roles controlling vasculature tone and sodium homeostasis in normal rats.

—vascular reactivity; sodium homeostasis; cardiac glycosides

DIGITALIS-LIKE COMPOUNDS (DLC) are a family of steroids synthesized in and released from the adrenal gland. DLC, the structure of which resembles that of plant cardiac glycosides, bind to the ubiquitous cell surface enzyme Na+/K+-ATPase (33). The binding results in inhibition of the ion transport by the enzyme and also the activation of various signal transduction cascades that induce changes in intracellular Ca2+ homeostasis and specific gene expression (33, 41).

DLC can be classified as cardenolides, compounds identified originally in plants, and bufadienolides, identified in toad venom. Two cardenolides, ouabain (20) and digoxin (18), have been identified in human plasma and in the hypothalamus. Several endogenous bufadienolides, including the 19-nor bufalin derivative (26), marinobufagenin (5), and, more recently, telocinobufagin (22), have been identified in human tissues.

In recent years, endogenous DLC are referred to as new steroidal hormones (33, 41). Endogenous ouabain (EO), the most frequently studied DLC, has been implicated in many cellular mechanisms and pathologies, including modulation of body or organ weight gain (15, 36, 43), hypertension (10, 29, 51), sodium homeostasis (28), vascular tone homeostasis (39, 45), mood disorders (17), and cancer (44) (for reviews, see Refs. 33 and 41). However, its physiological role in normal animal was not defined. The administration of exogenous cardiac glycosides to animals or cells is the main method used to study the roles of endogenous DLC (15, 36, 39, 51). Nevertheless, this method does not reveal the definite physiological roles of the endogenous compounds. Another approach addressing the possible physiological role of DLC was applied by Dostanic-Larson and coworkers (11) who showed that mutations in the ouabain-binding site of the Na+/K+-ATPase alter adrenocorticotropic hormone-induced hypertension (11, 29) and sodium homeostasis (28).

We now investigated by passive immunization of normal rats via chronic administration of antiouabain antibodies the short- and long-term effects of a reduction in circulating ouabain-like compounds. Growth gain, blood pressure (BP), renal function, and vascular reactivity were monitored during and after this treatment. We conclude that circulating EO, plausibly originating in the adrenal cortex, plays a physiological role in sodium homeostasis and vascular reactivity in the normal rat.

MATERIALS AND METHODS

Animals

The joint ethics committee (Institutional Animal Care and Use Committee) of the Hebrew University and Hadassah Medical Center approved the study protocol for animal welfare. The Hebrew University is an American Association for the Accreditation of Laboratory Animal Care international accredited institute. Male Wistar rats weighing 150–175 g were housed in the specific pathogen-free facility according to a 12:12-h light-dark cycle and were allowed a 5-day acclimation period with normal rat chow and tap water.

Experimental Procedures

Passive immunization of the rats. Following an acclimation period, the animals were subjected to a week of control period measurements. Next, for 28 days, the animals received a continuous infusion of antiouabain antibodies or rabbit nonspecific IgG-purified immunoglobulins (Sigma) as control (both 100 μg/day). Infusions were delivered through a jugular vein cannula by osmotic pumps (2ML4; Alzet) implanted subcutaneously in ketamine/xylazine anesthetized animals. Subsequently, BP was measured by tail-cuff plethysmography. Animals were housed in metabolic cages (Tecniplast) for 24 h to measure water and food consumption and urine excretion. Special care was taken to minimize any possible contamination of the urine samples with food or feces. Hence, the urine-collecting tube was
covered with a dense nylon net, allowing rapid collection of uncontaminated urine. Blood samples (300 μl) were withdrawn from the tail into cold lithium-heparin tubes (Vacutainer) and centrifuged (3,000 g, 5 min) for plasma separation. At the end of the experiment, the animals were killed by an overdose of pentobarbital sodium. Blood was then withdrawn from the abdominal aorta, organs were removed and weighed, and the thoracic aorta was used for smooth muscle contractility experiments as described below.

**Active immunization of the rats.** Ouabain-BSA conjugate, used to immunize the rats, was prepared as previously described (21, 27). Briefly, 215 mg BSA, 1g succinyl dihydrazide (Sigma) and 670 mg 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDAC; Sigma) were incubated overnight at 4°C in 14 ml H2O following overnight dialysis against NaOAc. In parallel, 670 mg ouabain were dissolved in H2O, and then sodium periodate (500 mg; Sigma) was added. After 1 h, 500 mg KH2PO4 were slowly added. Next, 56 mg mesoerythriol (Sigma) were added, and the mixture was incubated overnight at 4°C. Ouabain and BSA were mixed together and incubated overnight at 4°C. Sodium cyanoborohydride (24 mg; Sigma) and H2O were added to the reaction mix, which was then dialyzed against 25 mM Tris buffer. Six milliliters of ouabain-BSA conjugate in complete Freund’s adjuvant (1:1, vol/vol; Sigma) were injected subcutaneously in the rats. The animals were rechallenged as above at 2- and 3-wk intervals, with the same amount of antigen emulsified in incomplete Freund’s adjuvant (Sigma). The control group was challenged with BSA alone. Immediately after the third injection, the rats were housed in metabolic cages for 11 days to measure water and food consumption and urine excretion. The first 4 days were used for acclimation and to attain a sodium balance, and the results were not included.

**Vascular Reactivity Measurements**

Thoracic aortas were placed in gassed (95% O2 and 5% CO2), cold Krebs solution (KS) composed of (in mM) 118 NaCl, 2.05 CaCl2, 4.7 KCl, 1.18 KH2PO4, 1.64 MgSO4, 24.88 NaHCO3, and 5 glucose, and debrided of connective tissue under a microscope. Next, 3-mm-long segments were set up in a 10-ml chamber containing gassed KS (95% O2 and 5% CO2, pH 7.4, 37°C), under a resting tension of 1.5 g. Isometric tension was recorded using an isometric force transducer (Grass) connected to an amplifier (Laboratory linc V; Coulbourn Instruments, Allentown, PA). The analog signal was converted to a digital recording by a PowerLab 16SP integration system, and the data were stored on a personal computer by Chart 4.0 software (Adinstruments, Castle Hill, Australia). Following a 45-min equilibration period, the segments were exposed to 100 nM phenylephrine (PE) (Sigma) and 0.01 mM ACh (Sigma) to check viability and endothelial integrity, respectively. The segments were washed with 20 ml fresh KS (37°C) at 10-min intervals for 30 min until they reverted to resting tension. The maximal contraction response (MR) was then obtained by adding 0.1 mM norepinephrine (NE; Sigma) in 80 mM KCl to the chamber as previously described (23). Following a washout period of 90 min, the concentration-response relationship to PE (1 nM-0.1 mM) and vasorelaxing-response relationship to atrial natriuretic peptide (ANP, 0.3 nM-μM) were examined and analyzed. Finally, the rings were dried and weighed. ANP [synthesized as previously described (34)] was a gift from Yoram Shechter (Biological Chemistry and of Organic Chemistry, The Weizmann Institute of Science, Rehovot, Israel).

**Tail-Cuff BP Measurements**

Tail-cuff BP measurements were obtained using a LE5001 system (Panlab). Animals were placed in a restrainer and warmed to 32°C. For each measurement, preliminary BP measurements were obtained to acclimate the rat, and these were followed by systolic blood pressure (SBP) and heart rate (HR) measurements. The rats were carefully observed for movement artifacts during both preliminary and measurement cycles. The data were considered acceptable when a similar SBP was obtained in at least five constitutive measurements. Recordings not meeting these criteria were discarded.

**Partial Purification and Concentration of Rabbit Antiouabain Antibodies**

Antiouabain antibodies were prepared in rabbits by injection of ouabain-BSA conjugate as described previously, with minor modifications (17, 27). Protein A Sepharose CL-4B beads (Pharmacia Biotech, Uppsala, Sweden) and columns were prepared according to the manufacturer’s instructions. A total 2.5 ml of rabbit plasma containing ouabain antibodies was loaded onto each column. Unbound immunoglobulin was eluted with PBS. Bound immunoglobulin was then eluted with glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 3.0) in tubes containing 50 μl of 1 M NaOH. The presence and quantification of ouabain antibodies in the eluant were determined by enzyme-linked immunosorbent assay (ELISA), as described below. The partly purified antibody was concentrated by centrifugation (5,000 g, 15 min) using Centricron (Millipore/Amicon) centrifugal filters [3,000 nominal molecular weight limit (NMWL) membrane] to 10 mg IgG/ml. The concentrated ouabain antibodies and control rabbit IgG were dialyzed overnight against saline in a cellulose tubular dialysis membrane (6,000–50,000 cutoff, nominal pore size ~0.002 μm; Spectrapor Medical, Los Angeles, CA), which was preincubated in boiling water containing 1 mM EDTA and 2% Na2CO3 for 20 min. The amount of antibodies chosen to be injected in vivo (100 μg/day) was determined by ELISA as the lowest dilution of antiouabain antibodies needed to absorb 10 nM ouabain.

The antibodies used in this study are highly specific for ouabain and cross-react only with ouabagenin (53%), streptanthine (16.5%), digoxin (0.76%), and bufalin (0.6%). Other steroids, including cholesterol, testosterone, progesterone, corticosterone, 17-hydroxy pregnenolone, and 21-deoxycortisol do not cross-react with the antibodies, even at 10 μM.

**Extraction and Determination of EO**

The extraction and determination of EO were conducted as previously described, with minor modifications (27). Plasma samples (total 1.5 ml) were centrifuged (5 min, 15,000 g). The supernatant was first separated from high-molecular-weight compounds, using a 3,000 NMWL membrane centrifugal filter (Millipore/Amicon). The lower-molecular-weight fraction (3,000 g, 15 min, 28,500 g), the clear supernatant was loaded on a Sep-Pak C-18 column, which was then washed with 10 ml water containing 0.1% TFA, and the EO was eluted with 80% acetonitrile. The solvent was evaporated, the residue was dissolved in PBS, and aliquots from this solution were used to determine endogenous EO by a quantitative competitive ELISA based on antiouabain antibodies, as previously described (27).

**Determination of Rabbit IgG and Antiouabain Antibodies in Rat Serum by ELISA**

The presence of rabbit IgG in the serum was confirmed using a sandwich ELISA. Briefly, a 96-well plate was coated with 1 μg/well of goat antirabbit IgG (Sigma) and left overnight at room temperature. Following blocking of the wells with 50% FCS (1 h), dilutions of plasma were added to each well, and they were incubated for 1 h. The second antibody, goat antirabbit IgG alkaline-phosphatase conjugate (Sigma), was then added and IgG was quantified according to a standard curve of known dilutions of nonspecific rabbit IgG (Sigma). To confirm the presence of active rabbit antiouabain IgG in rat serum, a similar ELISA was performed using an ouabain-ovalbumin coated plate. Following minipump implantation, both groups showed the...
presence of rabbit IgG in their circulation, indicating successful implantation of the pumps. However, as expected, only plasma from the antiouabain antibody-treated rats showed the presence of antiouabain antibodies.

**Determination of Rat Antiouabain Self-Antibodies in Serum by ELISA**

To confirm the presence of active self antiouabain IgG in rat serum, a week after the second conjugate injection and upon termination of the experiment, an ELISA was performed using an ouabain-ovalbumin coated plate. Briefly, following blocking with FCS, dilutions of plasma with or without 1 mM ouabain were added to each well. The second antibody, goat antirat IgG alkaline-phosphatase conjugate (Enco), was then added. Both groups showed the presence of rat IgG in their circulation that cross-reacted with the ouabain-ovalbumin coated plate (the IgG against BSA from the control group cross-reacted with the ovalbumin), indicating successful immunization. However, as expected, only plasma from self-antiouabain-immunized rats showed ouabain-dependent cross-reactivity.

**Adrenal Histology**

Five representative adrenals of each group were fixed in 4% buffered formaldehyde and embedded in paraffin, and 5-μm-thick serial sections were stained with hematoxylin and eosin according to standard procedures. Stereological analysis of every fourth section of the midsagittal plane of the adrenal gland and its components (capsule, glomerulosa, fasciculata, reticularis, and medulla) was performed using the Photoshop (CS3; Adobe) measurements tool. The sphere volume of the adrenal zones was calculated from the number of pixels and the average radius of each zone in each section. The relative adrenal mass (and its components) was calculated as percent body mass.

**Plasma and Urine Measurements**

Electrolytes (Na⁺, K⁺) and creatinine determinations were performed at the Rambam Laboratories (Jerusalem, Israel). Urine osmolality measurements were made using a pressure osmometer (Vapro, Wescor, UT). Corticosterone and aldosterone were measured using commercial enzyme immunoassay kits according to the manufacturer’s instructions (Cayman).

**Statistics**

The results were analyzed using Student’s t-test, Mann-Whitney test, or analysis of variance for comparison between groups when appropriate. The values are expressed as means ± S.E. of the number of specimens used in each experiment. Differences were considered statistically significant at P < 0.05.

**RESULTS**

**Reduction in Plasma EO Levels in Rats Following Antiouabain Antibody Administration**

All control rats had measurable EO levels, ranging from 0.3 to 3.1 nM with an average value of 1.1 ± 0.3 nM (n = 10). This is in agreement with previous studies showing the presence of EO in rat circulation (12, 14, 19, 25, 43). As expected, on day 28, the EO levels did not change significantly in rats receiving nonspecific IgG antibodies (0.7 ± 0.1 nM, n = 5). However, the administration of antiouabain antibody resulted in a marked reduction in EO (0.2 ± 0.1 nM, n = 5, P < 0.01; Fig. 1). Thus experimental manipulation of the continuous administration of antiouabain antibody reduced the EO level and can be used for evaluating the physiological roles of these steroids.

**Effect of Antiouabain Antibody Administration on Body Weight Gain and BP**

As seen in Table 1, there were no significant differences between rats receiving antiouabain antibody and those receiving nonspecific IgG for 28 days, in body weight gain, water and food intake, and fecal output at any period during the experiment. Similarly, there were no significant differences between or within the groups in SBP or HR at any time throughout the experiment (Fig. 2).

**Effect of Antiouabain Antibody Administration on Organ Weight Gain**

There were no differences in heart or kidney weight between the experimental groups (Table 2). Interestingly, however, antiouabain antibody treatment induced a 25% increase in adrenal weight, the increase prevailing also when the weights were adjusted to the comparable tibia length (P < 0.05; Table 2). To evaluate which zone of the adrenals was enlarged, we calculated the absolute and relative mass compared with the body mass of the different zones. As seen in Table 3, the absolute and relative cortex mass were significantly increased in antiouabain antibody-treated rats, as a consequence of an increase in all cortical zones. Interestingly, the absolute and relative medulla mass did not differ between groups (P > 0.1).

**Plasma Corticosterone, Aldosterone, and Ouabain Levels in Rats Following Antiouabain Antibody Administration**

Enlargement of the adrenal cortex may result in greater concentrations of adrenal cortex steroid hormones in the plasma. As shown in Fig. 1, there was no change in plasma corticosterone or aldosterone concentrations upon termination of the experiment in either group.

As mentioned above, EO was reduced in antiouabain antibody-treated rats. Yet, there was a correlation between the adrenal mass and EO (but not corticosterone or aldosterone) concentration in the plasma of antiouabain antibody-treated rats (r² = 0.78; Fig. 3). A similar correlation was not found in the control group neither in ouabain plasma concentration nor in corticosterone or aldosterone concentrations (Fig. 3).
Table 1. Effect of antiouabain antibody administration on body weight gain, food and water intake, and fecal and urine output

<table>
<thead>
<tr>
<th></th>
<th>Day −5</th>
<th>Day 2</th>
<th>Day 9</th>
<th>Day 16</th>
<th>Day 23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
</tr>
<tr>
<td></td>
<td>242 ± 6</td>
<td>246 ± 8</td>
<td>259 ± 4</td>
<td>260 ± 6</td>
<td>289 ± 6</td>
</tr>
<tr>
<td>Food intake, g/24 h</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
</tr>
<tr>
<td></td>
<td>22.4 ± 06</td>
<td>23.0 ± 08</td>
<td>12.4 ± 1.8*</td>
<td>11.6 ± 1.7*</td>
<td>20.3 ± 1.1</td>
</tr>
<tr>
<td>Water intake, ml/24 h</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
</tr>
<tr>
<td></td>
<td>32.2 ± 1.7</td>
<td>31.0 ± 1.4</td>
<td>25.3 ± 2.2*</td>
<td>24.8 ± 2.2*</td>
<td>30.3 ± 2.3</td>
</tr>
<tr>
<td>Fecal wt, g/24 h</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
</tr>
<tr>
<td></td>
<td>11.1 ± 0.4</td>
<td>11.1 ± 0.9</td>
<td>6.3 ± 0.7*</td>
<td>6.4 ± 0.4*</td>
<td>9.7 ± 0.8</td>
</tr>
<tr>
<td>Urine output, g/24 h</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
</tr>
<tr>
<td></td>
<td>230 ± 10</td>
<td>252 ± 13</td>
<td>290 ± 6</td>
<td>290 ± 6</td>
<td>306 ± 8</td>
</tr>
<tr>
<td>Diuresis, ml/100 g h^{-1} 24 h</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
</tr>
<tr>
<td></td>
<td>5.7 ± 0.6</td>
<td>4.3 ± 0.2</td>
<td>5.4 ± 0.8</td>
<td>2.9 ± 0.2*</td>
<td>4.2 ± 0.2</td>
</tr>
<tr>
<td>Creatinine excretion, ml/min</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
</tr>
<tr>
<td></td>
<td>2.1 ± 0.2</td>
<td>2.1 ± 0.2</td>
<td>2.0 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Urine osmolality, mosmol/kgH2O</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
<td>Oua-Ab</td>
<td>IgG</td>
</tr>
<tr>
<td></td>
<td>1,446 ± 130</td>
<td>2,398 ± 96</td>
<td>1,252 ± 251</td>
<td>1,456 ± 198</td>
<td>1,522 ± 172</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8–9 rats in each group. Normal rats were implanted with osmotic pumps (Day 0) releasing nonspecific IgG (IgG) or antiouabain antibodies (Oua-Ab). Each week the animals were placed in metabolic cages for 24 h for water and food intake, fecal output, and urine collection. clr, Clearance measurements. *Lower than baseline values (day −5, 5 days before minipump implantation), P < 0.05.

Effect of Treatment with Antiouabain Antibodies on Aorta Vascular Reactivity

Following 28 days of antiouabain antibody or nonspecific IgG administration, the rats were killed, and PE-induced vasoconstriction and ANP-induced vasodilatation were studied. To assess the MR for each preparation, the rings were exposed to 0.1 mM NE in 80 mM KCl. There was no significant difference between the MR of rings from antiouabain antibody-treated rats and nonspecific IgG-treated rats (0.75 ± 0.07 and 0.84 ± 0.08 delta gram force (ΔgF) from baseline, respectively, P > 0.2). As expected, the addition of PE to the chamber induced a dose-dependent increase in aortic contraction in both groups. However, as seen in Fig. 4A, the PE-induced vasoconstriction response was reduced at an average 25% in aortic rings from antiouabain antibody-treated rats (P < 0.01). Similarly, the MR to PE was markedly attenuated in aortic rings from antiouabain antibody-treated rats vs. the nonspecific IgG-treated rats (0.47 ± 0.06 and 0.67 ± 0.06 ΔgF from baseline, respectively, P < 0.01). This reduction was probably not due to changes in α-adrenergic receptor affinity, as indicated by the similar EC_{50} values in the two groups (EC_{50} = −7.10 ± 0.07 and −7.02 ± 0.09 log[M] in the control IgG-treated rats and antiouabain antibody-treated rats, respectively). As seen in Fig. 4B, in contrast to PE, the response to ANP-induced vasodilatation was significantly elevated in aortic rings from the antiouabain antibody-treated rats compared with that in the control group (P < 0.01). Similarly, the maximal relaxation response to ANP-induced vasodilatation (0.1 μM ANP) was significantly higher in aorta rings from antiouabain antibody-treated rats, in spite of the lower PE-MR in this group (maximal relaxation response to ANP-induced vasodilatation = 68.7 ± 4.6 and 82.3 ± 6.6% of MR in the control IgG-treated rats and in the antiouabain antibody-treated rats, respectively, P < 0.05).

Effect of Antiouabain Antibody Administration on Renal Function

As described in MATERIALS AND METHODS, once a week, during antibody administration, the rats were housed in metabolic cages. A transient reduction in natriuresis was observed in the two experimental groups on the second day after the start of antibody administration (Fig. 5). Importantly, however, administration of the antiouabain antibody elicited a further significant reduction in natriuresis. On the second day of treatment, natriuresis, sodium clearance, and sodium fractional excretion dropped by 25% vs. that in the nonspecific IgG-treated control group (P < 0.05; Fig. 5). This reduction reverted to control values in the following week and was not accompanied by significant changes in diuresis or urine osmolarity (Table 1). There were no differences between the groups in potassium or creatinine clearance at any time during the experiment (Table 1).

Because the reduction in EO levels attenuated natriuresis, it may be concluded that EO is a natriuretic compound in the normal rat. However, the observed reduction in natriuresis at day 2 corresponded with instability in food intake and therefore questions this conclusion. Hence, to confirm it, EO levels were reduced by means of active immunization, and natriuresis of the autoimmune rats was determined. EO levels were reduced by means of active immunization, and natriuresis of the autoimmune rats was monitored. The basal natriuresis in rats that developed antibodies against ouabain were reduced compared with that in the control group, and no changes in kalriuresis, diuresis, and...
water and food intake throughout the experimental period were observed (Fig. 6 and Table 4). These results confirm the notion that EO is a natriuretic agent.

**DISCUSSION**

Endogenous DLC were identified in animal and human tissues and body fluids more than two centuries ago (18, 20, 26). Although a vast body of data has accumulated on the effects of these compounds at the cellular level (for review, see Refs. 33 and 41), their physiological role in normal animal was not proven. Several studies have demonstrated alteration in circulating EO levels following physiological perturbation and pathophysiological states in rats and human (3, 6, 19, 30, 31, 38). These observations, however, do not reveal the physiological process in which these hormones may be involved in.

**Table 2. Effect of antiouabain antibody administration on heart, kidney, and adrenal weight gain**

<table>
<thead>
<tr>
<th>Organ or Tissue</th>
<th>Organ Weight, g</th>
<th>Organ Weight/Tibia Length, g/cm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>Ouab-Ab</td>
</tr>
<tr>
<td>Heart</td>
<td>0.823 ± 0.025</td>
<td>0.854 ± 0.020</td>
</tr>
<tr>
<td>Left ventricle</td>
<td>0.616 ± 0.021</td>
<td>0.621 ± 0.018</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>0.155 ± 0.007</td>
<td>0.162 ± 0.004</td>
</tr>
<tr>
<td>Kidneys (both)</td>
<td>1.993 ± 0.080</td>
<td>1.989 ± 0.037</td>
</tr>
<tr>
<td>Adrenals (both)</td>
<td>0.060 ± 0.004</td>
<td>0.075 ± 0.005*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8-9 rats. Osmotic pumps releasing nonspecific IgG or antiouabain antibodies were implanted in normal rats (day 0). Upon termination of the experiment (day 28), the animals were killed, and the organs were weighed. *Values higher than those of the control group, P < 0.05.

One of the major obstacles in understanding the physiological roles of DLC in general and of EO in particular, is the lack of knowledge regarding their synthesis pathway. Consequently, knockout animals lacking DLC are not available. As mentioned above, administration of exogenous cardiac glycosides to animals or cells is the main method used to study the roles of DLC. However, other approaches, such as adrenalecnectomy (43), active immunization (48), digoxin antibody treatment (28), and genetic modulation of the DLC receptor, the Na⁺-K⁺-ATPase (11), were used to investigate DLC physiological roles. These approaches pointed to the possible involvements of DLC in vascular tone homeostasis (51), salt-sensitive hypertension (48), cardiac hypertrophy (43), and sodium homeostasis (28). The method applied in the present study was chronic administration of antiouabain antibodies to normal rats to reduce their EO levels. This method of passive immunization was successfully applied in elucidating the physiological roles of other hormones, such as ANP (32) and growth hormone (4), in the preknockout era. To the best of our knowledge, this is the first time that a passive immunization chronic study has been conducted in normal rats.

Several major conclusions may be drawn from the results of the present study: 1) the long-term reduction in EO induced the hypertrophy of the adrenal cortex, supporting the hypothesis that this is a major organ in which EO synthesis takes place; 2) the long-term reduction in EO did not affect heart or kidney growth, suggesting that EO does not participate in the growth control of these tissues in the mature rat; 3) the reduction in EO lowered urine sodium excretion, suggesting that it participates in the homeostasis of the sodium balance by acting as a natriuretic hormone; and 4) the long-term reduction in EO altered aorta smooth muscle reactivity to PE and ANP, suggesting that it contributes to vascular tone homeostasis by increasing the response of aorta smooth muscle to catecholamines and lowering the response to ANP.

**Body and Organ Growth**

In agreement with a previous study using active immunization of rats against ouabain (48), we did not observe any

**Table 3. Effect of antiouabain antibody administration on adrenal gland size**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Absolute Mass, mg</th>
<th>Relative Mass, mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>Ouab-Ab</td>
</tr>
<tr>
<td>Adrenal</td>
<td>57.86 ± 4.2</td>
<td>83.4 ± 6.1*</td>
</tr>
<tr>
<td>Cortex</td>
<td>54.0 ± 3.9</td>
<td>79.0 ± 6.0*</td>
</tr>
<tr>
<td>Capsule</td>
<td>1.9 ± 0.1</td>
<td>2.5 ± 0.2*</td>
</tr>
<tr>
<td>Glomerulosa</td>
<td>5.1 ± 0.5</td>
<td>7.0 ± 0.9*</td>
</tr>
<tr>
<td>Fasciculata</td>
<td>33.3 ± 2.0</td>
<td>44.6 ± 3.2*</td>
</tr>
<tr>
<td>Reticularis</td>
<td>13.7 ± 2.0</td>
<td>22.9 ± 2.9*</td>
</tr>
<tr>
<td>Medulla</td>
<td>3.9 ± 0.4</td>
<td>4.4 ± 0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 rats. Osmotic pumps releasing nonspecific IgG or antiouabain antibodies were implanted in normal rats (day 0). Upon termination of the experiment (day 28), the animals were killed, and the adrenals were removed, weighed, and analyzed as described in material and methods. Absolute mass was calculated as area relative size times the adrenal mass. Relative mass was calculated as the absolute mass (mg) divided by the body mass (kg). *Values higher than those of the control group, P < 0.05.
significant change in body, heart, or kidney weight gain after antiouabain antibody administration compared with the control group (Tables 1 and 2). However, there was a marked elevation in adrenal weight, in particular of the adrenal cortex, following this treatment (Tables 2 and 3). This elevation could be compensatory adrenal growth in response to the low EO levels. Such growth was also observed following reduction of other steroids, such as aldosterone and testosterone, using the active immunization model (35). The finding that there was a correlation between EO, but not corticosterone and aldosterone, plasma concentrations and adrenal mass supports this notion (Fig. 3). Previous studies suggested that EO is involved in cardiac hypertrophy (36, 43). Our results do not support this assumption. This apparent controversy may imply that EO participates in cardiac growth only under pathological hypertrophic conditions.

Sodium Homeostasis

Endogenous DLC were suggested many years ago to function as a natriuretic hormone (for review, see Refs. 8, 9, and 42). Indeed, administration of ouabain to sheep or to isolated rats kidneys resulted in marked natriuresis (16, 49). Recently, Loreaux and coworkers (28) showed that transgenic mice expressing ouabain-sensitive $\alpha_1$-Na$^+$-K$^+$-ATPase augmented the natriuretic response to acute salt load. The authors concluded that the ouabain-binding site of $\alpha_1$-Na$^+$-K$^+$-ATPase participates in the natriuretic response to salt load by responding to endogenous Na$^+$-K$^+$-ATPase ligands (28). The same conclusion may be drawn from the study of Fedorova and coworkers (12) who showed a reduction in natriuresis followed antiouabain antibody administration after acute salt loading in Dahl salt-sensitive rats. Our results showing a significant reduction in natriuresis 2 days following antiouabain antibody treatment (Fig. 5) are consistent with these conclusions. Importantly, the present study was conducted on normal rats without salt loading intervention, pointing to the putative action of EO in normal sodium balance. The transient nature of the reduction in natriuresis induced by antiouabain antibodies implies a strong negative feedback from other sodium balance regulators, similar to that seen in the mineralocorticoid escape phenomenon (50). A possible participant in such a feedback is aldosterone, which has been shown to be reduced in antiouabain active immunized rats on a low salt diet (47). We now

Fig. 3. Correlation between plasma ouabain, corticosterone, and aldosterone and adrenal weight in control and antiouabain antibody-treated rats. Correlation between plasma ouabain, corticosterone, and aldosterone and adrenal weight in nonspecific IgG (IgG) or antiouabain antibody-treated rats (Oua-Ab). Upon termination of the experiment (day 28), the animals were killed, blood samples were withdrawn, and the adrenals were weighed.

Fig. 4. Effect of antiouabain antibody administration on aortic vascular reactivity to phenylephrine (PE) and atrial natriuretic peptide (ANP). The response to PE and ANP was measured in aorta ring specimens of rats receiving antiouabain antibody (Oua-Ab) and nonspecific IgG (IgG) for 28 days. The contractile response to PE is expressed as the percentage of the maximal contraction response (MR) produced by norepinephrine and KCl (A). The relaxing response to ANP is expressed as the percentage of the MR produced by PE (0.1 mM) (B). *Lower than control value ($P < 0.01$); $n = 8–9$. 

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measured plasma aldosterone levels upon termination of the experiment and did not observe any differences between the groups (Fig. 1). However, at this point, there was also no difference in natriuresis.

Because the reduced natriuresis in the control rats raises some doubts as to the effect of antiouabain antibody administration on this parameter, we addressed this issue in an additional experimental system. The reduction in EO was obtained by active immunization via the administration of ouabain-BSA conjugate. Similar to the results of the passive immunization experiment, the basal natriuresis of rats that developed antiouabain antibodies was reduced compared with that of the control group that received BSA (Fig. 6 and Table 4). Thus both passive and active immunization experiments support the conclusion that EO is a natriuretic hormone. The finding that there were no changes in kaliuresis, diuresis, or water and food intake throughout the experimental period (Fig. 6 and Table 4) points to the specific effect of EO on sodium homeostasis.

Based on observations on Dahl salt-sensitive rats, Bagrov and coworkers (3, 12) recently suggested that endogenous marinobufagenin is the natriuretic DLC (12) and that its release is mediated by EO. Our results do not resolve the issue whether EO acts directly on the kidney to induce natriuresis or if this effect is mediated by marinobufagenin.

**Aorta Vascular Homeostasis and BP**

Long-term administration of exogenous ouabain to rats causes changes in vascular reactivity to many cardioactive substances, such as PE (39), NE (45) and nitric oxide (40), and in vasculature structure (7). Moreover, using the adrenocorticotrophic hormone-induced hypertension model, Lorenz et al. (29) recently showed changes in aortic ring reactivity to PE from mice with mutations in the ouabain binding site on the \( \alpha_2^\text{HL} \)-subunit of Na\(^{+}\)-K\(^{+}\)-ATPase. However, because these chronic treatments induce hypertension and heart remodeling,
the changes may be independent of EO. Here we show that chronic passive immunization against ouabain causes a reduction in aortic ring response to PE and in a heightened increased response to ANP (Fig. 4). Thus, our results suggest that EO participates in vascular tone homeostasis in the normal rat, contributing to the fine tuning of BP. However, we did not detect significant changes in SBP at any time in the experiment following antiouabain antibody treatment nor in the active immunization experiment (Fig. 2 and Nesher, unpublished observation). Furthermore, a similar lack of effect on BP following EO reduction was described by Yamada et al. (48) using the active immunization model. On the basis of our present study, the lack of change in BP may be due to the finding that EO has two opposing effects on BP. On the one hand, it induces natriuresis (Figs. 5 and 6), resulting in a reduction in SBP; on the other, it causes an elevation in vasculature tone (Fig. 4), resulting in an elevation in SBP. These two pathways counterbalance each other, resulting in a lack of change in BP (Fig. 2). Clearly, other regulatory mechanisms of BP regulation may blunt the effect of lowering EO concentration.

The molecular mechanisms underlying the role of EO in the regulation of natriuresis and vascular reactivity should be considered. EO circulating levels in the rat are in the nanomolar range (12, 14, 19, 25, 43 and Fig. 1). The canonical effect of these steroids is considered to be inhibition of sodium and potassium transport by the Na+/K+/ATPase (33, 41) but this inhibition is achieved only at relatively high (μM) concentrations. Thus, it is unlikely that the inhibition of ion transport by the Na+/K+/ATPase is the mechanism involved in the physiological roles of these compounds. Recent studies have shown that the binding of ouabain to Na+/K+/ATPase elicits numerous additional changes in cell function. These include activation of intracellular signal transduction (46), activation of cytoplasmic Ca2⁺ oscillation (2), stimulation of endocytosis, and inhibition of endocytosed membrane traffic (13, 37), as well as cell proliferation (1) and adhesion (24). Because all of these molecular mechanisms were induced by ouabain in the nanomolar range, any of them could be involved in the physiological processes described in this study.

In conclusion, the results of the present study demonstrate that circulating ouabain, probably originating in the adrenal cortex, plays a physiological role in sodium homeostasis and vascular reactivity in normal rats. Moreover, these findings point to possible cross talk between the DLC and the ANP hormonal systems.

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DISCLOSURES

None.

REFERENCES

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PHYSIOLOGICAL ROLES OF ENDOGENOUS OUABAIN


