Rat Adrenal Cortex Is a Source of a Circulating Ouabainlike Compound

James H. Ludens, Michael A. Clark, Franklin G. Robinson, and Donald W. DuCharme

To determine if the adrenal gland may be the source of plasma-borne ouabainlike compound (OLC) in rats, we 1) measured immunoreactivity expressed as OLC equivalents in extracts from a wide variety of central and peripheral tissues and, for adrenal cortex and medulla, chromatographed the extracts to determine to what extent immunoreactivity in the adrenal was OLC, and 2) measured OLC in the plasma of adrenalectomized and adrenal demedullectomized rats. The highest levels of immunoreactivity were found in adrenal cortex, adrenal medulla, atria, and the pituitary. Based on high-performance liquid chromatographic retention time, immunoreactivity in the adrenal cortex was almost exclusively immunoreactive OLC. Removal of this rich source of OLC from rats resulted in an approximate 50% decrease in circulating levels of OLC by 6 days after removal. Furthermore, although adrenal demedullectomy also caused a decrease in OLC 3 days after surgery, the decline was sustained only with total adrenalectomy, in that plasma levels of OLC in demedullectomized rats 6 days after surgery had returned to levels equal to those of sham controls. Taken together, these findings strongly suggest that the adrenal cortex is a major contributor to circulating OLC in the rat. (Hypertension 1992;19:721-724)

KEY WORDS  • adrenal glands • ouabainlike compounds • glycosides • rat studies

The chemical identity and source of an endogenous counterpart to the plant-derived digitalis glycosides have been pursued intently, ever since it was recognized that a high-affinity binding site for these substances existed on the membrane-bound enzyme Na⁺,K⁺-ATPase. Such substances, by virtue of inhibiting the membrane sodium pump (Na⁺,K⁺-ATPase), could play an important role in electrolyte homeostasis in mammalian cells and thereby modulate numerous aspects of cell biology.

The chemical identity of a potent inhibitor of the sodium pump isolated from human plasma recently was reported to be indistinguishable mass spectrometrically,1 biochemically,2 pharmacologically,3 and immunologically4 from the plant cardenolide ouabain. This purified material subsequently was referred to as ouabainlike compound (OLC),5 and several lines of evidence were presented which suggested that the OLC originated endogenously. Although the hypothalamus has been suggested to be a likely source of an endogenous inhibitor of the sodium pump,6,7 a variety of evidence implicates the adrenal gland as well. An inhibitor of Na⁺,K⁺-ATPase possessing biochemical properties strikingly similar to ouabain was extracted from bovine adrenals,8 and when a variety of tissue extracts were assayed with biochemical and immunological techniques, the level of digitalis-like or ouabainlike factors in adrenal extracts was among the highest.9,10 In addition, minced adrenal tissue was found to release an immunoreactive digitalis-like material into a serum-free incubation medium,11 and adrenalectomy was shown to diminish circulating levels of an immunoreactive digitalis-like factor.9,12

A highly sensitive, ouabain-specific enzyme-linked immunospecific assay (ELISA) that used a rabbit anti-ouabain antisera that cross-reacted completely with purified OLC was developed.4 Immunoactive OLC was evident in extracts of several tissues as well as plasma, with the adrenal gland extracts being particularly enriched.2 The present studies were carried out to evaluate further the contribution of the adrenal glands to circulating levels of OLC in the rat.

Methods

Preparation of Animals

Male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, Mass.) were maintained on Purina rodent lab chow (No. 5001) and tap water ad libitum before use. Animals were anesthetized with sodium pentobarbital (60 mg/kg i.p.) or methoxyflurane for the adrenalectomy and adrenal demedullectomy surgical procedures. The glands were approached either with the use of flank incisions or abdominally with a ventral midline incision. Adrenal demedullectomy was accomplished by cutting a small slit in the adrenal cortex in situ and applying pressure to extrude the adrenal medulla. After surgery, all animals including the sham-operated control rats were maintained on 0.9% saline ad libitum and 15 g Purina rodent chow (No. 5001). On this diet, adrenalectomized, demedullectomized, and sham rats consumed all of the rodent chow provided, and weight changes in all groups were similar (decreases of 0–20 g per rat). In one study, both adrenalectomized and sham-operated control rats were treated subcuta-
neously with 1 mg deoxycorticosterone acetate (DOCA) per day in corn oil or the corn oil vehicle for 6 days.

**Collection of Tissue and Blood**

Body weights ranged from 210 to 350 g at the time tissue and blood samples were obtained. Animals were anesthetized with methoxyflurane, and blood was drawn from the abdominal aorta with a 20-gauge hypodermic needle with a 10-ml syringe containing 1 ml of 3.8% sodium citrate to prevent coagulation. One milliliter of whole blood was saved, and plasma was separated from what was remaining by centrifugation at 1,500g for 10 minutes. Whole blood and plasma were frozen and stored at -20°C until used. When wanted, tissues were removed and immediately frozen by placement of the excised tissues in weighing boats on dry ice. Frozen tissues were weighed and stored at -20°C until extraction.

**Tissue Extraction**

Tissues, whole blood included, were thawed and homogenized in methanol (10 ml/g wet wt or a minimum of 2 ml if the tissue weighed less than 200 mg) with a polytron. The tissues were homogenized on ice for 40 seconds on setting 5 followed by 20 seconds on setting 5. The homogenates were centrifuged (4,000g) for 20 minutes at 4°C. The supernatants were retained, and the pellets were rehomogenized as described above except the homogenization times were changed to 30 and 15 seconds. The centrifugation process was repeated, and the supernatants were combined and dried under vacuum at room temperature. The solids were taken up in water containing 0.1% trifluoroacetic acid (5 ml/g wet wt or a minimum of 2 ml) and homogenized with the polytron for 30 seconds on setting 5 followed by 15 seconds on setting 5. The homogenate was centrifuged as before. The supernatants were retained, the water/trifluoroacetic acid extraction was repeated, and the supernatants were combined.

**Solid-Phase Extraction of Tissue and Plasma Samples**

Tissue samples (10 ml of 0.1% trifluoroacetic acid per gram wet wt with a minimum of 4 ml) and plasma samples (2 ml) were extracted on disposable Bond Elute solid-phase Si-C18 extraction columns (Analytichem International, Harbor City, Calif.). Unbound materials were washed off with water, and the columns were eluted with 2.8 ml of 25% acetonitrile.

**High-Performance Liquid Chromatographic Fractionation of Adrenal Extracts**

Solid-phase extracts of adrenal cortex and medulla were taken up in 3.0 ml water, filtered through 1.2-μm Acrodisc filters (Gelman Sciences Inc., Ann Arbor, Mich.), and pumped onto a C18 semiprep column (10 mm×25 cm) (Beckman Instruments, Inc., Fullerton, Calif.) preequilibrated with water containing 0.1% trifluoroacetic acid. The column was washed for 20 minutes under preequilibration conditions and eluted with acetonitrile containing 0.1% trifluoroacetic acid using a two-step gradient: to 10% acetonitrile in 10 minutes and from 10% to 30% over the next 50 minutes. The flow rate was 3 ml/min throughout, and 0.5-minute fractions were collected from 24.5 to 34.0 minutes into the gradient, i.e., from 15.5% to 19.5% acetonitrile.

**Assay of Samples for Ouabainlike Compound**

All tissue and plasma samples, i.e., solid-phase extracts and the high-performance liquid chromatographic fractions, were dried and the solids suspended in 90 μl buffer for assay in triplicate with the use of an indirect ELISA described previously. Briefly, 25 μl assay buffer containing sample or ouabain standard was added to half-area enzyme immunoassay plates (Costar Corp., Cambridge, Mass.) previously coated with a conjugate of ouabain linked to bovine serum albumin via a hexane diamine spacer. A second aliquot of assay buffer containing rabbit anti-ouabain antisera was added such that the final dilution of antisera was 1:2,000,000. After incubation, plates were washed, and 50 μl of goat anti-rabbit IgG-peroxidase diluted 1:1,000 was added to each well. Bound peroxidase, remaining after washing, was determined with reagent containing 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). The reaction was terminated with phosphoric acid, and absorbance at 450 nm was measured with a Vmax microtiter plate reader (Molecular Devices Corp., Palo Alto, Calif.). The ELISA as used on these samples had a working range of 20–2,000 fmol, with an IC50 of approximately 200 fmol per well.

**Table 1. Ouabainlike Compound in Tissues From Intact Rats**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ouabainlike compound (fmol/mg±SEM)</th>
<th>n</th>
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<tbody>
<tr>
<td>Blood</td>
<td>0.47±0.07</td>
<td>12</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.08±0.01</td>
<td>8</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.48±0.06</td>
<td>11</td>
</tr>
<tr>
<td>Ventricle</td>
<td>0.98±0.18</td>
<td>4</td>
</tr>
<tr>
<td>Atria</td>
<td>2.85±0.28</td>
<td>14</td>
</tr>
<tr>
<td>Adrenal</td>
<td>3.89±0.66</td>
<td>14</td>
</tr>
<tr>
<td>Adrenal cortex</td>
<td>6.10</td>
<td>P1</td>
</tr>
<tr>
<td>Adrenal medulla</td>
<td>2.50</td>
<td>P2</td>
</tr>
<tr>
<td>Thyroid</td>
<td>1.50±0.33</td>
<td>10</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>0.20±0.05</td>
<td>14</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.30±0.07</td>
<td>4</td>
</tr>
<tr>
<td>Midbrain</td>
<td>1.68±1.31</td>
<td>4</td>
</tr>
<tr>
<td>Pituitary</td>
<td>3.45±0.90</td>
<td>12</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>1.12±0.46</td>
<td>13</td>
</tr>
</tbody>
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P1, based on triplicate assays of pooled adrenal cortex obtained from five rats; P2, based on triplicate assays of pooled adrenal medulla obtained from 20 rats.

**Results**

**Immunoreactivity in Tissues of Intact Rats**

Table 1 shows tissue levels of immunoreactivity expressed as OLC equivalents in blood, plasma, and a variety of other tissues taken from intact rats on tap water and rodent chow ad libitum. The highest levels of immunoreactivity were found in adrenals, atria, and the pituitary. Less was apparent in the midbrain, hypothalamus, thyroid, and ventricle, with the least amount found in blood, plasma, kidney, cerebral cortex, and cerebellum. On a weight basis, the adrenal contained 50 times the amount found in plasma.

To determine if immunoreactivity in adrenal tissue was associated with OLC, we fractionated solid-phase...
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Source of Circulating Ouabainlike Compound

Figure 1. Chromatogram showing C18 reversed-phase chromatography of adrenal cortex. Solid line shows UV absorbance. Broken line shows linear acetonitrile gradient of 10% at 10 minutes to 22% at 40 minutes with which columns were developed. Bars show mean±SEM of immunoreactivity expressed as ouabainlike compound (OLC) equivalents per fraction based on triplicate assays. Extract from 83 mg of tissue was applied to the column. Flow rate was 3 ml/min; 0.5-minute fractions were collected. Arrow shows retention time for authentic ouabain or purified OLC in this system.

extracts of adrenal cortex and medulla with a high-performance liquid chromatographic system in which retention characteristics of OLC and ouabain were previously determined. With adrenal cortex (Figure 1), cross-reactivity was substantially greater to a fraction with a retention time identical to OLC (i.e., 28.5–29.5 minutes) than to all other fractions assayed. For adrenal cortex, extract from 83 mg of tissue was applied to the column. In contrast, with adrenal medulla (Figure 2), relative to the other fractions assayed, no appreciable amount of immunoreactivity was associated with the fraction that would contain OLC. For adrenal medulla, 127 mg of tissue was applied to the column.

Effect of Adrenalectomy or Adrenal Demedullectomy on Levels of Ouabainlike Compound

Figure 3 illustrates changes in plasma levels of OLC 3 and 6 days after removal of the adrenal gland or removal of only the medullary portion of the adrenal. Plasma OLC decreased approximately 25% in both groups after 3 days. After 6 days, however, OLC in the plasma of demedullectomized animals had returned to levels equal to or greater than that of sham controls, whereas the level of OLC in plasma of adrenalectomized rats continued to decline to approximately 50% of that in sham-operated controls.

In an attempt to prevent fluid volume loss associated with adrenalectomy, we conducted an additional experiment in which adrenalectomized rats were treated subcutaneously with 1 mg/day DOCA in corn oil for 6 days. The results are shown in Table 2. In both the adrenalectomized and the sham-operated control rats, circulating OLC levels were not significantly different in DOCA-treated rats compared with the respective control animals receiving corn oil. The adrenalectomized rats treated with corn oil lost 12±3 g of body weight, whereas DOCA-treated rats lost only 1±3 g over the course of the 6-day study, suggesting that the treatment was effective in preventing fluid loss in the adrenalectomized rats.

Discussion

A number of studies have suggested that the adrenal gland is enriched with digitalis-like or ouabainlike factors. In addition, it has been reported that cultured adrenal tissue releases digitalis-like or ouabainlike materials into the incubation media. Furthermore, it was reported that the circulating levels of an immunoreactive digitalis-like factor and an immunoreactive

Figure 2. Chromatogram showing C18 reversed-phase chromatography of adrenal medulla. Solid line shows UV absorbance. Broken line shows linear acetonitrile gradient of 10% at 10 minutes to 22% at 40 minutes with which column was developed. Bars show mean±SEM of immunoreactivity expressed as ouabainlike compound (OLC) equivalents per fraction based on triplicate assays. Extract from 127 mg of tissue was applied to the column. Flow rate was 3 ml/min; 0.5-minute fractions were collected. Arrow shows retention time for authentic ouabain or purified OLC in this system.

Figure 3. Graph showing effects of adrenalectomy (black circle) and adrenal demedullectomy (white circle) on plasma levels of ouabainlike compound (OLC) in rats. Values are mean±SEM from eight to 10 animals per point. *Significant difference (p<0.05) from sham-operated animals.
TABLE 2. Effect of Deoxycorticosterone Acetate on Plasma Ouabainlike Compound Levels of Sham-Operated and Adrenalectomized Rats

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Plasma OLC (fmol/ml±SEM)</th>
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<tr>
<td>Sham-operated rats</td>
<td>Corn oil (n=6) 232±41, DOCA (n=6) 175±22</td>
</tr>
<tr>
<td>Adrenalectomized rats</td>
<td>Corn oil (n=6) 139±15, DOCA (n=7) 121±31</td>
</tr>
</tbody>
</table>

OLC, ouabainlike compound; DOCA, deoxycorticosterone acetate.

ouabainlike compound were decreased in animals subjected to bilateral adrenalectomy. Other reports suggest that the hypothalamus is a likely source of the endogenous inhibitor of Na,K-ATPase. The present investigation was undertaken to determine if the adrenal gland contributes to the circulating levels of OLC.

Immunoreactive OLC was measured in a variety of rat tissues with a ouabain-specific ELISA. Extracts of the adrenal glands contained the highest level of immunoreactive OLC of the tissues examined. Furthermore, high-performance liquid chromatographic separation of extracts from the cortical portion of the adrenal gland demonstrated that immunoreactivity was associated with a substance that eluted from the column with a retention time identical to OLC. Extracts prepared from the medullary portion of the adrenal gland, on the other hand, did not appear to contain OLC; i.e., immunoreactivity was not associated with the chromatographic fraction that would contain OLC. These results demonstrate that the adrenal cortex is enriched with OLC but do not differentiate between a source of secretion or a site of storage.

Complete removal of the adrenal glands from the rat resulted in a progressive decrease in the circulating level of immunoreactive OLC. The level of OLC in plasma from rats 3 days after adrenalectomy was approximately 75% that of intact rats, and the level 6 days after adrenalectomy was only 50% that of intact rats. The plasma level of OLC in adrenal demedullectomized rats 3 days after surgery was equivalent to that of adrenalectomized animals; however, the level in plasma taken from animals 6 days after surgery was not different from that of intact animals. The initial decline observed after 3 days probably was related to a temporary, functional interruption of the cortical tissue that remained after the surgical procedure. By 6 days after surgery, it appears that the cortical tissue recovered functionally such that the plasma level at this time was similar to that of intact rats.

We were concerned that the decrease in OLC associated with adrenalectomy could be secondary to changes in fluid and electrolyte balance rather than to loss of the adrenal tissue per se. To test this possibility, plasma OLC was measured in adrenalectomized rats maintained on DOCA. Administration of DOCA prevented the weight loss associated with adrenalectomy but not the decrease in OLC. The circulating OLC level of the DOCA-treated animals was not significantly different from that of the respective control animals that received the corn oil vehicle. Thus, it appears that the effect of adrenalectomy on the plasma level of OLC was not secondary to changes in circulating fluid volume. Circulating OLC did not increase in the sham-operated animals treated with DOCA as has been reported. In the present study, however, DOCA was administered at a dose of 1 mg/day with the intent of maintaining steroid levels after adrenalectomy rather than at the large doses required to induce DOCA hypertension.

In summary, the present studies demonstrate that extracts of rat adrenal glands contain high levels of immunoreactive OLC compared with most other tissue extracts and that the adrenal OLC is associated with cortical rather than medullary tissue. Removal of the adrenal glands resulted in an approximate 50% decrease in the plasma OLC level within 6 days after surgery, whereas the level of OLC in plasma of rats subjected to adrenal demedullectomy was not different from intact control rats at this time. These findings strongly suggest that the adrenal cortex is a major contributor to circulating OLC in the rat.

References
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