Does Plasma Immunoreactive Ouabain Originate From the Adrenal Gland?

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Abstract
It was reported recently that the endogenous digitalis-like factor ouabain may mainly originate from the adrenal gland. To ascertain the pathophysiological significance of endogenous ouabain and to examine if it originates in the adrenal gland, we determined plasma immunoreactive ouabain levels in patients with various cardiovascular and endocrine diseases. Plasma immunoreactive ouabain levels were determined in the renal venous blood by adrenal venous sampling. Plasma immunoreactive ouabain levels were significantly increased in patients with essential hypertension, primary aldosteronism, Cushing’s syndrome, pheochromocytoma, acromegaly, and chronic renal failure. Plasma immunoreactive ouabain levels were decreased in patients with primary aldosteronism after unilateral adrenalectomy, acromegaly after pituitary adenomectomy, and chronic renal failure after hemodialysis. Plasma immunoreactive ouabain is a major source of plasma ouabain, although a possible additional source of plasma ouabain is the peripheral circulation.

Methods
Subjects
Plasma immunoreactive ouabain levels were determined in 17 healthy subjects and patients with various diseases as follows: essential hypertension without (n=42) and with (n=15) antihypertensive agents, primary aldosteronism (n=20), Cushing’s syndrome (n=2), Cushing’s disease after bilateral adrenalectomy (n=2), pheochromocytoma (n=7), acromegaly (n=16), chronic renal failure (without hemodialysis, n=4; with hemodialysis, n=10), and hypertension with adrenal tumor without any known adrenal hormone excess (n=7, patients 1 to 7, Table). Plasma immunoreactive ouabain was determined after unilateral adrenalectomy in patients with primary aldosteronism and pituitary adenoma by transsphenoidal surgery in patients with acromegaly, respectively. Blood samples were collected from a peripheral vein between 8:30 AM and 10:00 AM after an overnight fast and after 30 minutes of recumbency. In patients under chronic hemodialysis, plasma levels were determined before and after 5 hours of hemodialysis.

Extraction Procedure
Blood samples collected in chilled tubes containing Na$_2$EDTA (4 mmol/L) were centrifuged at 4°C, and plasma was stored at −70°C until assayed. One milliliter of each plasma was applied on a Sep-Pak C18 cartridge (Waters Associates, Milford, Mass). The adsorbed material was eluted with 3 mL of 75% ethanol, evaporated, and lyophilized, dissolved in 300 µL of the assay buffer, and subjected to radioimmunoassay. Recovery of the ouabain (Sigma Chemical Co, St Louis, Mo) added to 1 mL of plasma was approximately 95%.
Plasma Immunoreactive Ouabain Levels in Patients With Hypertension and Adrenal Tumor Demonstrated by Adrenal Computed Tomographic Scan

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Sex</th>
<th>SBP, mm Hg</th>
<th>DBP, mm Hg</th>
<th>PRA, nmol · L⁻¹ · h⁻¹</th>
<th>Aldo, pmol/L</th>
<th>F, nmol/L</th>
<th>E, μmol/L</th>
<th>NE, μmol/L</th>
<th>Immunoreactive Ouabain, pmol/L</th>
<th>Adrenal Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65</td>
<td>M</td>
<td>206</td>
<td>94</td>
<td>0.31</td>
<td>342</td>
<td>588</td>
<td>0.17</td>
<td>0.94</td>
<td>31.5</td>
<td>Left</td>
</tr>
<tr>
<td>2</td>
<td>43</td>
<td>M</td>
<td>172</td>
<td>102</td>
<td>0.85</td>
<td>680</td>
<td>364</td>
<td>0.17</td>
<td>1.88</td>
<td>41.6</td>
<td>Right 3 and 6</td>
</tr>
<tr>
<td>3</td>
<td>85</td>
<td>F</td>
<td>178</td>
<td>98</td>
<td>2.31</td>
<td>192</td>
<td>...</td>
<td>0.94</td>
<td>5.66</td>
<td>22.9</td>
<td>Left 25x10</td>
</tr>
<tr>
<td>4</td>
<td>58</td>
<td>M</td>
<td>158</td>
<td>108</td>
<td>1.00</td>
<td>398</td>
<td>...</td>
<td>0.11</td>
<td>3.07</td>
<td>52.1</td>
<td>Left 13x12</td>
</tr>
<tr>
<td>5</td>
<td>43</td>
<td>M</td>
<td>128*</td>
<td>80*</td>
<td>1.08</td>
<td>319</td>
<td>462</td>
<td>0.06</td>
<td>1.95</td>
<td>8.1</td>
<td>Right 17x15</td>
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<tr>
<td>6</td>
<td>72</td>
<td>F</td>
<td>164</td>
<td>74</td>
<td>0.92</td>
<td>269</td>
<td>330</td>
<td>0.22</td>
<td>4.54</td>
<td>30.4</td>
<td>Left 25x17</td>
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<td>F</td>
<td>172</td>
<td>92</td>
<td>0.46</td>
<td>512</td>
<td>417</td>
<td>...</td>
<td>...</td>
<td>75.9</td>
<td>Right 10x10</td>
</tr>
</tbody>
</table>

Normal range: 0.39-2.3 pmol/L

SBP indicates systolic blood pressure; DBP, diastolic blood pressure; PRA, plasma renin activity; Aldo, aldosterone; F, cortisol; E, epinephrine; and NE, norepinephrine.

*With nifedipine.

Immunoreactive digoxin was detected by the commercially available radioimmunoassay kits for digoxin in the plasma extracts of healthy subjects.

**Radioimmunoassay**

Antiserum to ouabain was prepared by the method described previously. In brief, ouabain pretreated with sodium periodate was conjugated to bovine serum albumin (BSA) using cyanoborohydride. Conjugated ouabain was emulsified with Freund's complete adjuvant (Difco) and used for immunizing New Zealand White rabbits. They were boosted with the same amount of the conjugate emulsified with Freund's incomplete adjuvant and bled at 2-week intervals. Although the cross-reactivity of the antiserum obtained was 9.2% with digoxin, that with BSA, rhamnose, hydrocortisone, and aldosterone was less than 0.007%.

The radioimmunoassay incubation mixture consisted of 100 μL of standard ouabain, 100 μL of antiserum (final dilution of 1:10), and 200 μL of the assay buffer (0.1 mol/L phosphate buffer, pH 7.4, containing 0.1% BSA [fraction V, Sigma] and 0.01% NaN₃). After incubation for 15 minutes at 4°C, 100 μL of [21,22-³H]ouabain (approximately 10 000 cpm, Amersham International plc, Buckinghamshire, England) was added and incubated for 30 minutes at 4°C. The antibody-bound and -free tracer ouabain were separated by adding 100 μL each of goat anti-rabbit IgG antiserum (1:25) and normal rabbit serum (1:100) and incubating overnight at 4°C. The sensitivity of the radioimmunoassay was 6 pg per tube with 50% displacement at 140 pg per tube.

The dilution curves of plasma extracts were parallel to the standard curve. The plasma immunoreactive ouabain was further characterized by reversed-phase high-performance liquid chromatography on a Zorbax ODS column (0.46×25 cm, Du Pont, Wilmington, Del) with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid at a rate of 1 mL/min. Immunoreactive ouabain in plasma samples was composed of a major peak with a retention time corresponding to that of ouabain.

**Statistical Analysis**

Data were analyzed by the Student's t test, Mann-Whitney U test, or Wilcoxon signed rank test, as appropriate. A value of P<.05 was considered statistically significant.

**Results**

Plasma immunoreactive ouabain levels were significantly higher in patients with essential hypertension (mean±SD, 62.1±43.8 pmol/L), primary aldosteronism (74.0±65.2 pmol/L), Cushing's syndrome (102.8±58.1 pmol/L), pheochromocytoma (76.2±64.7 pmol/L), acromegaly (77.3±57.1 pmol/L), and chronic renal failure (103.3±80.6 pmol/L) than in the healthy subjects (40.4±9.0 pmol/L) (Fig 1). In essential hypertension, plasma levels were significantly lower in patients receiving antihypertensive agents (49.2±15.1 pmol/L) than those without treatments. There was no significant correlation between plasma immunoreactive ouabain and blood pressure. After unilateral adrenalectomy in primary aldosteronism (P<.01), pituitary adenomectomy in acromegaly (P<.01), and hemodialysis in chronic renal failure (P<.05), plasma immunoreactive ouabain decreased significantly (Fig 2). Plasma immunoreactive ouabain levels in two patients after bilateral adrenalectomy were 30.3 and 31.0 pmol/L, respectively.

Clinical features of seven patients with hypertension and adrenal tumors but without definitive evidence for any excess of adrenal hormones are summarized in the Table. Plasma immunoreactive ouabain levels in six patients did not differ from those of healthy subjects, whereas patient 7 showed an increased plasma level.

In patient 8, plasma immunoreactive ouabain levels in the right adrenal vein were lower than in the left adrenal vein. There was no significant step-up of immunoreactive ouabain levels in the adrenal vein from the inferior vena cava (Fig 3a). The right adrenal, which was surgically removed, contained four small adenocortical nodules. Also in patient 9, there was no step-up of plasma immunoreactive ouabain levels in the adrenal vein from the inferior vena cava (Fig 3b). Because plasma aldosterone levels in the right adrenal vein were significantly higher than those in the left adrenal vein and the inferior vena cava, whereas there was no significant change in the plasma aldosterone and cortisol levels in either side of the adrenal vein (Fig 3c).
Discussion

Plasma immunoreactive ouabain levels were significantly elevated in patients with various diseases associated with hypertension and/or fluid overload, suggesting an important role in the pathophysiology of the cardiovascular system. Although the plasma levels were significantly lower than the known ED$_{50}$ value of ouabain in inhibiting Na$^+$,K$^+$-ATPase activity, details of the source, regulatory mechanism, mode of action, and clearance from the circulation are not known presently. It may act through a paracrine/autocrine mechanism rather than an endocrine mechanism. Therefore, the pathophysiological significance of the plasma immunoreactive ouabain may not be simply discussed only from the plasma level.

One of the critical issues concerning the pathophysiological significance of the endogenous digitalis-like factor is whether digitalis glycosides cause hypertension. Recently, it was demonstrated that chronic infusion of ouabain provokes chronic hypertension in rats. Thus, the increased plasma immunoreactive ouabain levels in essential hypertension suggest that endogenous ouabain may be a causative factor. Interestingly, it was found that plasma immunoreactive ouabain levels were almost normal after therapy. It is therefore suggested that ouabain may be a part of a compensatory mechanism through its natriuretic effect, which in turn elevates the blood pressure.

Increased plasma immunoreactive ouabain levels in acromegaly coincide with a previous finding of a digitalis-like factor. Hypertension is one of the representative features of the disease. The mechanism underlying the hypertension, however, is not known. The increased plasma immunoreactive ouabain can be a candidate for the cause of hypertension. Decrease in the plasma levels after pituitary surgery suggests a pituitary origin of the plasma immunoreactive ouabain. However, no evidence has been available to support the presence of ouabain in the anterior pituitary. In contrast, we have recently shown the presence of immunoreactive ouabain in the hypothalamus, suggesting a hypothalamic origin of endogenous ouabain. It is not known, however, whether the hypothalamic ouabain is released into the circulation, and if so, how the pituitary tumor affects the hypothalamic ouabain. Increased plasma levels also could be a result of a compensatory mechanism for the increased plasma volume in acromegaly and may participate in the hypertension.

Plasma immunoreactive ouabain was significantly elevated and showed a slight but significant decrease after hemodialysis in patients with chronic renal failure. The changes in plasma levels may relate to changes in body...
fluid volume, although possible removal from the circulation by hemodialysis remains to be investigated.

Manunta et al reported that plasma ouabain may originate largely from the adrenal gland. Increased plasma immunoreactive ouabain in primary aldosteronism and the decrease after adrenalectomy agree with this hypothesis. However, plasma immunoreactive ouabain in two patients after bilateral adrenalectomy was in the normal range. In addition, although possible episodic secretion from the adrenal was not ruled out, there was no definite step-up of plasma immunoreactive ouabain levels in the adrenal vein from levels in the inferior vena cava. Therefore, it is unlikely that the adrenal is a major source.

The same group also reported a new syndrome with elevated plasma ouabain and hypertension secondary to an adrenal tumor/primary aldosteronism. In the present study, there was no significant increase in plasma immunoreactive ouabain in six patients with hypertension and adrenal tumors. However, in one patient, plasma immunoreactive ouabain was elevated both in the right adrenal and peripheral veins, suggesting the right adenocortical tumor as a source of ouabain. Therefore, the issue of adrenal primary aldosteronism as a cause of hypertension deserves further investigation.

The role of the diet as a source of endogenous ouabain is a major source.

The possible plant origin of plasma immunoreactive ouabain was not ruled out, and adrenal and peripheral veins. However, in one patient, plasma immunoreactive ouabain in six patients with hypertension originated largely from the adrenal gland. Increased plasma immunoreactive ouabain was elevated both in the right adrenal and peripheral veins, suggesting the right adenocortical tumor as a source of ouabain. Therefore, the issue of adrenal primary aldosteronism as a cause of hypertension deserves further investigation.

The role of the diet as a source of endogenous ouabain has been suggested. In addition, Will et al showed that ouabain bound to the Na⁺,K⁺-ATPase on the cell membrane could be internalized and then released very slowly without structural modification. The possible plant origin of plasma immunoreactive ouabain, therefore, cannot be completely excluded at the present time. Whatever the source of ouabain, the presence of immunoreactive ouabain in the plasma and dynamic changes of plasma levels in various disease states suggest its pathophysiological significance.

References

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