Salt, endogenous ouabain and blood pressure interactions in the general population
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\textbf{Objective} Experimental data show that ouabain is a modulator of the sodium–potassium pump, which plays an important role in sodium homeostasis and blood pressure regulation. We investigated the distribution of plasma ouabain in the general population in relation to blood pressure and other determinants of sodium homeostasis.

\textbf{Methods} In 379 subjects enrolled in a Belgian population study, we measured plasma ouabain, clinical characteristics including blood pressure, serum and urinary electrolytes, urinary aldosterone excretion, various lifestyle factors, and the Gly460Trp polymorphism of the \(\alpha\)-adducin gene. Our statistical methods included analysis of covariance and multiple linear regression.

\textbf{Results} Plasma ouabain (median, 140 pmol/l) correlated independently and positively with male gender (\(n = 182, P = 0.002\)), smoking (\(n = 116, P = 0.05\)), urinary potassium excretion (mean 69 mmol/day, \(P < 0.0001\)), and mutation of the \(\alpha\)-adducin gene (\(n = 161, P < 0.0001\)). Both before and after adjustment for covariables, continuous as well as categorical analyses revealed a significant interaction (\(P < 0.02\)) between plasma ouabain and urinary sodium excretion (mean 194 mmol/day) in relation to blood pressure (mean systolic blood pressure/diastolic blood pressure, 123/76 mmHg). In individuals with plasma ouabain values below the median, blood pressure increased by 2.2 mmHg systolic and 1.4 mmHg diastolic for each 50 mmol/day increment in urinary sodium excretion (\(P < 0.01\)). No association between blood pressure and urinary sodium excretion was found when plasma ouabain exceeded the median.

\textbf{Conclusions} Plasma ouabain behaves as a blood pressure modulating factor, possibly released in response to potassium, either inhibiting the pressor effect of an excessive salt intake or counteracting the depressor action of sodium depletion. *J Hypertens* 21:1475–1481 © 2003 Lippincott Williams & Wilkins.

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\section*{Introduction}
Endogenous ouabain is released from the adrenal gland and possibly from the hypothalamus [1–5]. The hypothesis gaining ground is that endogenous ouabain behaves as a versatile modulator of the ubiquitously expressed Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (sodium–potassium pump) [6], which plays an important role in sodium homeostasis and regulation of blood pressure [7]. In rats, chronic low-dose infusion of exogenous ouabain produces hypertension [8]. In humans, hypertension [9], pre-eclampsia [10] and left ventricular dysfunction [11] are associated with increased plasma concentration of endogenous ouabain.

In addition to endogenous ouabain, many other genetic [12,13] and lifestyle [14] factors potentially interfere with Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity. For example, substitution of glycine by tryptophan at position 460 of the \(\alpha\)-subunit of the heterodimeric cytoskeleton protein adducin (Gly460Trp polymorphism) leads to increased Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity [12,13] and induces sodium retention [15,16]. Potassium supplementation may up-regulate Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity [14].

At present, little is known about the plasma concentration of endogenous ouabain in the population at large and on the association of this hormone with blood pressure or other factors influencing sodium homeostasis. We therefore investigated these issues in Caucasian subjects randomly selected from a Belgian population.
Methods

Study population

The protocol of the Flemish Study on Environment, Genes and Health Outcomes (FLEMENGHO) was approved by the Ethics Committee of the University of Leuven. All subjects gave informed consent. From August 1985 to December 2000, a random sample of the households living in a geographically defined area of Northern Belgium was investigated [17]. The present cross-sectional study involved 452 subjects recruited in the year 2000. The participation rate among the subjects contacted was 66.1%. Fifty-seven subjects declined to collect a 24-h urine sample, and 16 had incomplete measurements including urinary volume (n = 1), endogenous ouabain concentration (n = 14) or α-adducin genotype (n = 1). Thus, the number of subjects included in the present analysis totalled 379.

Field work

Study nurses repeatedly visited the participants in their homes. The same observer measured a subject’s blood pressure five times consecutively at each of two home visits 4–6 weeks apart. Conventional sphygmomanometry was performed according to the guidelines of the British Hypertension Society after the subjects had rested for 5 min in the sitting position. For analysis, we averaged the 10 blood pressure readings. Hypertension was diagnosed if the average blood pressure was at least 140 mmHg systolic or 90 mmHg diastolic, or when the patients were on antihypertensive medication.

We used a validated [18] questionnaire to collect information on medical history, smoking habits, alcohol intake and use of medications. In the interval between the two home visits, the participants collected a 24-h urine sample in a wide neck plastic container for the measurement of sodium, potassium, aldosterone and creatinine. Venous blood was sampled, usually within 2 weeks after the urine collection, for measurement of plasma ouabain and the serum concentration of sodium and potassium.

Laboratory methods

Endogenous ouabain was extracted from plasma and measured by a specific radio-immunoassay as previously described [3]. The immuno-crossreactivity of the antiserum was 100% for ouabain; for digoxin it was only 0.42 and 0.04% before and after extraction of plasma on C18 columns, respectively. The threshold sensitivity of the antiserum was 20 pmol/l. In ouabain dilution experiments, the EC₅₀ displacement was at 200–300 pmol/l. The intra-assay and inter-assay coefficients of variations were 7 and 10%, respectively [3]. In the context of this article, the term ‘plasma ouabain’ refers to the concentration of the endogenous compound. Urinary aldosterone was also measured by radio-immunoassay. The serum and urinary concentrations of sodium and potassium were determined by flame photometry.

Genomic DNA was extracted from peripheral blood. Allelic discrimination of the Gly460Trp α-adducin polymorphism was carried out as previously described [17] using the 5’ nuclease assay on an ABI Prism 7700 apparatus (Perkin Elmer, Foster City, California, USA).

Statistical analysis

For database management and statistical analysis, we used SAS software, version 8.1 (SAS Institute, Cary, North Carolina, USA). Measurements with a skewed distribution were normalized by logarithmic transformation. Means and proportions were compared with the standard normal z test and Fisher’s exact test, respectively. We searched for possible confounders using stepwise multiple regression with the P value for covariables to enter and stay in the model set at 0.15. We used analysis of covariance and multiple linear regression to test associations of interest, while controlling for covariables.

Results

Characteristics of the participants

The 182 male and 197 female participants had similar mean age (Table 1). Men had slightly higher body mass index than women (P = 0.02). Among men, 33.5% (n = 62) were current smokers and 48.4% (n = 88) reported intake of alcohol. In women, these proportions were 27.4% (n = 54) and 21.8% (n = 43), respectively. Fifty-seven women (28.9%) used oral contraceptives and eight (4.1%) took hormonal replacement therapy. The study sample included 82 hypertensive patients, of whom 44 took antihypertensive drugs (β-blockers, n = 26; diuretics, n = 10; calcium-channel blockers, n = 7; centrally acting agents, n = 3; angiotensin-converting enzyme inhibitors, n = 2; and α-blockers n = 2).

In stepwise multiple regression, we considered gender, age, body mass index, smoking, alcohol intake, serum total cholesterol, and use of β-blockers, diuretics or oral contraceptives as potential correlates of plasma ouabain. Of these variables, only gender (P = 0.002) and smoking (P = 0.05) entered the model. Plasma ouabain was higher in men than women (Table 1), and in smokers than non-smokers (157.8 versus 147.5 pmol/l), but it was neither dependent on age nor body mass index (P > 0.80). In men (r = 0.26, P = 0.0004) and women (r = 0.14, P = 0.05), haematocrit increased with higher plasma ouabain concentration. In all further analyses with plasma ouabain as the dependent variable, we allowed for gender and smoking.
Association between plasma ouabain and the α-adducin Gly460Trp polymorphism

The frequencies of the α-adducin genotypes did not deviate from Hardy–Weinberg equilibrium (GlyGly, 0.58; GlyTrp, 0.36; TrpTrp, 0.06; \( P = 0.85 \)). Characteristics of the subjects were similar across the α-adducin genotypes. Both before and after (Fig. 1) adjustment for gender and smoking, plasma ouabain was significantly (\( P < 0.0001 \) for trend) associated with the number of copies of the α-adducin Trp allele. Compared with GlyGly homozygotes, plasma ouabain was 14.4 pmol/l higher (95% confidence interval (CI), 4.9–23.9 pmol/l; \( P = 0.003 \)) in GlyTrp heterozygotes and 29.5 pmol/l higher (95% CI, 10.7–48.4 pmol/l; \( P = 0.002 \)) in TrpTrp homozygotes.

Association between plasma ouabain and urinary sodium and potassium excretion

After adjustment for gender, smoking and the α-adducin Gly460Trp polymorphism, plasma ouabain was significantly (\( P < 0.0001 \) and 0.001) and positively associated with urinary potassium excretion (Fig. 2), but it was neither dependent on urinary sodium excretion nor on the serum concentration of sodium or potassium (\( P > 0.11 \)). Each 25 mmol/day increase in urinary potassium excretion was associated with a 9.1 pmol/l increment in plasma ouabain (95% CI, 4.7–13.5 pmol/l). This association was independent of the serum concentration of potassium. As expected, a linear and positive association between urinary aldosterone excretion and urinary potassium excretion was also observed (\( P < 0.0001 \)) (Fig. 2). However, with similar adjustments as before, there was only a weak positive relationship between plasma ouabain and urinary aldosterone excretion (\( r = 0.07, P = 0.20 \)).

Association between plasma ouabain and blood pressure

After adjustment for gender, age, age\(^2\), body mass index, smoking, alcohol intake, use of antihypertensive drugs or oral contraceptives, and the α-adducin Gly460Trp polymorphism, the systolic and diastolic blood pressures were not significantly associated with

Table 1. Characteristics of the participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Men</th>
<th>Women</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>40.0 ± 17.3</td>
<td>40.4 ± 16.9</td>
<td>0.80</td>
</tr>
<tr>
<td>Body mass index (kg/m(^2))</td>
<td>25.2 ± 3.8</td>
<td>24.2 ± 4.5</td>
<td>0.02</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>126.9 ± 13.5</td>
<td>120.3 ± 14.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>78.6 ± 10.8</td>
<td>74.5 ± 9.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pulse rate (beat/min)</td>
<td>67.1 ± 7.5</td>
<td>69.8 ± 7.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hypertensive patients (%)</td>
<td>51 (28.0)</td>
<td>31 (15.7)</td>
<td>0.004</td>
</tr>
<tr>
<td>Treated hypertensive patients (%)</td>
<td>25 (13.7)</td>
<td>19 (9.6)</td>
<td>0.28</td>
</tr>
<tr>
<td>Plasma ouabain concentration (mmol/l)</td>
<td>141.9 ± 2.6</td>
<td>141.3 ± 2.4</td>
<td>0.04</td>
</tr>
<tr>
<td>Serum K(^+) concentration (mmol/l)</td>
<td>4.17 ± 0.31</td>
<td>4.11 ± 0.32</td>
<td>0.07</td>
</tr>
<tr>
<td>Urinary volume (l/day)</td>
<td>1.49 ± 0.68</td>
<td>1.54 ± 0.75</td>
<td>0.51</td>
</tr>
<tr>
<td>Creatinine excretion (mmol/day)</td>
<td>13.2 ± 3.5</td>
<td>8.9 ± 2.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sodium excretion (mmol/day)</td>
<td>212 ± 64</td>
<td>176 ± 52</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Potassium excretion (mmol/day)</td>
<td>76 ± 28</td>
<td>62 ± 22</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Urinary Na(^+)/K(^+) ratio</td>
<td>3.00 ± 1.02</td>
<td>3.05 ± 1.03</td>
<td>0.63</td>
</tr>
<tr>
<td>Aldosterone excretion (nmol/day)</td>
<td>18.5 (16.3–20.9)</td>
<td>20.8 (18.3–23.7)</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Values are arithmetic means ± standard deviation, geometric means (95% confidence interval) or number of subjects (% of column total). * For gender difference.
plasma ouabain or urinary sodium excretion ($P > 0.23$) when the latter two measurements were considered in isolation. However, both before and after adjustment, we observed a statistically significant interaction between plasma ouabain and urinary sodium excretion in relation to systolic blood pressure and diastolic blood pressure, irrespective of whether plasma ouabain and urinary sodium excretion were analysed as continuous (Fig. 3) ($P < 0.02$) or categorical variables (Fig. 4) ($P < 0.02$).

We derived quantitative estimates of the blood pressure effects from the categorical analysis (Fig. 4). In 190 subjects whose plasma ouabain was equal to or less than 140 pmol/l (median), each 50 mmol/day increment in urinary sodium excretion was associated with an increase in blood pressure averaging 2.2 mmHg (95% CI, 0.7–3.6 mmHg; $P = 0.004$) systolic and 1.4 mmHg (95% CI, 0.3–2.5 mmHg; $P = 0.01$) diastolic. In contrast, in subjects whose plasma ouabain was higher than 140 pmol/l, the association between blood pressure and urinary sodium excretion was not statistically significant ($P > 0.10$).

### Sensitivity analysis
We repeated our analyses after exclusion of subjects on antihypertensive drugs, oral contraceptives or hormonal replacement therapy. In these 273 untreated subjects, our findings remained unaltered. The plasma ouabain concentration (± standard error) was 146.2 ± 3.6, 157.2 ± 4.5 and 181.2 ± 11.5 pmol/l in GlyGly, GlyTrp
and TrpTrp subjects ($P = 0.002$ for trend), respectively, and it was 8.2 pmol/l higher (95% CI, 2.9–13.4 pmol/l; $P = 0.002$) for each 25 mmol/day increment in urinary potassium. In 132 untreated subjects whose plasma ouabain was equal to or less than 140 pmol/l, each 50 mmol/day increment in urinary sodium excretion was associated with an increase in blood pressure averaging 1.9 mmHg (95% CI, 0.2–3.6 mmHg, $P = 0.03$) systolic and 1.6 mmHg (95% CI, 0.4–2.8 mmHg; $P = 0.01$) diastolic.

**Discussion**

Our study is the first population-based investigation of endogenous ouabain. We demonstrated that carriers of the mutated α-adducin Trp allele had higher plasma ouabain concentration than GlyGly homozygotes. Second, there was a linear and positive association between plasma ouabain and urinary potassium excretion. Third, blood pressure increased with higher urinary sodium excretion in subjects with low plasma ouabain, but not in those with high levels. The observation that smoking is associated with higher plasma ouabain suggests that our measurements of plasma ouabain were accurate. Indeed, decreased oxygen saturation of the blood in experimental animals [19] or in patients with obstructive sleep apnoea [20] has been linked with higher plasma concentration of the endogenous inhibitor of the sodium–potassium pump. The positive relationship between the urinary excretion of aldosterone and potassium also constitutes an internal validation of our study.

One possible interpretation of our findings is that endogenous ouabain might play a central role in the homeostatic regulation of blood pressure in response to changes in sodium intake. Several mechanisms may explain why the relationship between blood pressure and ouabain is positive. First, in sodium-depleted subjects, endogenous ouabain may enhance sodium retention. Several studies [21,22] have shown that low subnanomolar concentrations of ouabain may stimulate, not inhibit, renal Na\(^+\)/K\(^+\)-ATPase. In addition, low concentrations of ouabain may raise the intracellular concentration of free and stored calcium ions, enhance excitation–contraction coupling in vascular smooth muscle cells, and increase peripheral vascular resistance [23]. We also observed a positive association between haematocrit and plasma ouabain. Thus, an increase in blood viscosity may also raise blood pressure [24]. However, whether the relationship between haematocrit and ouabain is causal remains to be elucidated.

During elevated salt intakes, endogenous ouabain may act as a compensatory factor protecting against the development of sodium-sensitive high blood pressure, a proposal consistent with the commonly held view that this substance promotes natriuresis [1]. Carriers of the mutated α-adducin Trp allele have a genetically determined higher Na\(^+\)/K\(^+\)-ATPase activity [13], which may induce a volume-dependent hypertension [15] via sodium retention [15,16]. This pathogenic mechanism may be counteracted by a feedback increment in circulating ouabain (Fig. 1). In the absence of the compensation by endogenous ouabain, hypertension may develop with increasing body sodium [25,26].

Failure to account for endogenous ouabain as a player in the relationship between blood pressure and salt intake may have confounded the results of earlier studies [15,16,27–33]. The blood pressure-lowering effect of salt restriction differed between trials or subgroups of study subjects [27–33]. For instance, in the D ietary Approaches to Stop Hypertension (DASH) trial, compared with men, women showed a more pronounced blood pressure reduction in response to a low sodium diet [32]. Endogenous ouabain might play
a part in the determination of this gender difference, because we noticed that women had a lower plasma ouabain concentration than men and that only at low levels of ouabain was blood pressure positively associated with sodium excretion. Furthermore, there is clear experimental evidence that mutation of the α-adducin gene enhances proximal renal tubular sodium reabsorption and increases salt sensitivity [15,16]. Nevertheless, the results of observational and epidemiological studies were more equivocal [34] with the exception of those in homogeneous groups of low-renin hypertensive patients [35–37]. Our study now supports that endogenous ouabain might act as a hormone compensating for the volume-dependent increase in blood pressure in carriers of the mutated α-adducin Trp allele.

The finding that plasma ouabain rises with higher urinary potassium excretion also supports the hypothesis of the participation of the steroid in blood pressure regulation and homeostasis of electrolytes. Blood pressure is inversely correlated with plasma concentration of potassium, exchangeable potassium and total body potassium [26]. Potassium restriction increases blood pressure in normotensive subjects [38] and aggravates hypertension in patients with this disease [39]. A meta-analysis of randomized trials showed that the blood pressure-lowering effect of potassium supplementation occurred when the average urinary sodium excretion was 165 mmol/day or more [40]. Similarly, the DASH trial revealed that a potassium-enriched diet (mean urinary potassium excretion, 80 mmol/day versus 40 mmol/day) produced a greater blood pressure reduction on high and intermediate salt intake (mean urinary sodium excretion, 144 mmol/day and 107 mmol/day, respectively) than on low salt intake (mean urinary sodium excretion, 67 mmol/day) [41]. The difference in blood pressure-lowering effects averaged approximately 3 mmHg systolic and 2 mmHg diastolic.

In conclusion, endogenous ouabain may be a blood pressure modulating factor, possibly released in response to potassium, either inhibiting the pressor effect of an excessive salt intake or counteracting the depressor action of sodium depletion. Our study was observational in nature not designed to reveal causal association, and should therefore be considered as hypothesis generating. Nevertheless, if confirmed by experimental studies and other epidemiological investigations, our findings might have clinical implications for the management of human hypertension and the prevention of cardiovascular disorders.

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