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Salt intake and depletion increase circulating levels of endogenous ouabain in normal men

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Manunta, Paolo, Bruce P. Hamilton, and John M. Hamlyn. Salt intake and depletion increase circulating levels of endogenous ouabain in normal men. Am J Physiol Regul Integr Comp Physiol 290: R553–R559, 2006; doi:10.1152/ajpregu.00648.2005.—High-salt diets elevate circulating Na⁺ pump inhibitors, vascular resistance, and blood pressure. Ouabain induces a form of hypertension mediated via the α₂-Na⁺ pump isoform and the calcium influx mode of the vascular sodium calcium exchanger (NCX). Whereas elevated levels of an endogenous ouabain (EO) and NCX have been implicated in salt-sensitive hypertension, acute changes in sodium balance do not affect plasma EO. This study investigated the impact of longer-term alterations in sodium balance on the circulating levels and renal clearance of EO in normal humans. Thirteen normal men consumed a normal diet, high-salt diet, and hydrochlorothiazide (HCTZ), each for 5-day periods to alter sodium balance. EO and other humoral and urinary variables were determined daily. On a normal diet, urinary sodium excretion (140 ± 16 meq/day), plasma EO (0.43 ± 0.08 nmol/l) and urinary EO excretion (1.04 ± 0.13 nmol/day) were at steady state. On the 3rd day of a high-salt diet, urine sodium excretion (315 ± 28 meq/day), plasma EO (5.8 ± 2.2 nmol/l), and the urinary EO excretion (1.69 ± 0.27 nmol/day) were significantly increased, while plasma renin activity and aldosterone levels were suppressed. The salt-evoked increase in plasma EO was greater in older individuals, in subjects whose baseline circulating EO was higher, and in those with low renal clearance. During HCTZ, body weight decreased and plasma renin activity, aldosterone, and EO (1.71 ± 0.77 nmol/l) rose, while urinary EO excretion remained within the normal range (1.44 ± 0.31 nmol/day). Blood pressure fell in one subject during HCTZ. HPLC of the plasma extracts showed one primary peak of EO immunoreactivity with a retention time equivalent to ouabain. High-salt diets and HCTZ raise plasma EO by stimulating EO secretion, and a J-shaped curve relates sodium balance and EO in healthy men. Under normal dietary conditions, ~98% of the filtered load of EO is reabsorbed by the kidney, and differences in the circulating levels of EO are strongly influenced by secretion and urinary excretion of EO. The dramatic impact of high-salt diets on plasma EO is consistent with its proposed role as a humoral vasoconstrictor that links salt intake with vascular function in hypertension.

cardiac glycosides; sodium; sodium pump; inhibitor; circulation; hypertension

Epidemiological and interventional observations suggest that dietary sodium intake is a key factor in the etiology of human hypertension (39, 50). The mechanism by which dietary sodium influences blood pressure is debated but is linked with an increase in the mean filling pressure of the circulation (15). Such a condition may arise initially from increased intravascular volume, while subsequently, it is associated with increased total peripheral vascular resistance that reflects increased vascular contractility and/or altered structure. Elevated circulating levels of Na⁺ pump inhibitors have been described in experimental low renin forms of hypertension and in essential hypertension (19, 30, 37, 38, 40, 42). Attempts to identify Na⁺ pump inhibitors from the human circulation led to the isolation and identification of ouabain or a closely related isomer by mass spectrometry (17, 36). In addition, other steroids that cross-react with antibodies raised against digitalis glycosides and bufadienolides have also been described in the mammalian circulation (e.g., 1, 10, 14). The endogenous ouabain (EO) and related materials are increased in the circulation of normal men.

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METHODS

Thirteen normotensive healthy subjects living in the Baltimore City area (white, male, age 35 ± 2 years, range 21–55) were studied during three periods each of 5 days duration: normal salt diet (baseline), normal salt diet plus 10 g (171 meq) NaCl/day (high-salt diet) and, following a minimum 10-day washout period (range 10–28 days), a normal salt diet plus one 25 mg tablet of hydrochlorothiazide (HCTZ) daily (diuretic). For the high-salt period, individuals took 10 g NaCl tablets over the course of the day with their normal food. Some individuals dissolved the NaCl tablets in water and drank the solution at lunch and dinner. The diuretic was taken typically with breakfast; thus values listed for days 1 and 2 of diuretic treatment refer to measurements on samples collected 20–24 h after drug administration.

Blood pressure, pulse rate (HR), body weight (BW), antecubital venous blood samples, and 24-h urine samples were collected every day, for the determination of plasma and urinary EO, plasma renin activity (PRA), plasma aldosterone, urinary sodium excretion (UNaV), and urinary creatinine. Blood pressure and HR were measured after 10-min rest in the sitting position with a mercury manometer used by the same trained observer. Korotkoff phase V was taken as diastolic blood pressure. Mean arterial blood pressure (MBP) was calculated by adding one-third of the pulse pressure to the diastolic blood pressure. Compliance with the dietary and diuretic regimen was determined by interviews, pill counts (NaCl and HCTZ), and by measurements of 24-h urinary sodium and creatinine excretion. The protocol used was approved by the University of Maryland at Baltimore Institutional Review Board, and informed written consent was obtained in each case.

Urinary sodium was measured by flame photometry and urinary creatinine by automated methods. PRA and aldosterone were measured by radioimmunoassay using kits from DuPont and Diagnostic Products, respectively.

Preparation of plasma and urine for EO determination. Venous blood was collected in vacutainer (Cat-a-Kit, Amersham, Arlington Heights, IL) tubes containing EGTA and reduced glutathione. Plasma was obtained by immediate centrifugation and stored at −20°C. EO was extracted from 2 ml plasma or 10 ml urine by first mixing each sample with one volume of water containing 0.1% trifluoroacetic acid. The acidified samples were passed over prewashed 200 mg C-18 disposable Bond Elut columns (Analytichem International, Harbor City, CA). Unbound materials were washed off the columns with 12 ml water, and EO was eluted with 4 ml of 25% acetonitrile in water. The eluate was dried with a vacuum centrifuge, and dried extracts were reconstituted in immunoassay buffer. EO was measured by immunoassay by methods similar to those described by Harris and colleagues (20). As noted previously, the antiserum (R7) has no significant crossreactivity (<0.01%) for a wide variety of adrenal, testicular, and ovarian steroids, and the immunoreactivity detected by this antiserum correlates well with the biological potency of the sample extracts (12). In addition, under the elution conditions used, digitalis and bufadienolide steroids typically remain column bound so that the final assay shows minimal cross-reactivity for digoxin (<0.5%) and other steroids that do not closely resemble ouabain structurally. No subjects had any known intake of cardiac glycosides. The intra-assay and interassay coefficients of variation were 4.8% and 9.2%, respectively.

In other experiments, pooled plasma extracts obtained during the baseline, high-salt, and diuretic periods were individually chromatographed using a HPLC system using a Beckman semipreparative 5 μm C-18 column. Bound materials were eluted with a gradient program of acidified acetoni ter 0.25N, 10 min; 10–20N, 40 min; 20–95N, 15 min) similar to that used previously (17). Fractions were collected and dried by vacuum centrifuge. The dried samples were reconstituted in assay buffer.

The renal clearance of EO was determined from the 24 h urinary excretion divided by plasma levels.

RESULTS

Figure 1 shows the mean plasma level of EO (0.43 ± 0.08 nmol/l) over a 5-day baseline period in the 13 individuals on their normal diet. In response to the high-salt diet, plasma EO increased >13-fold reaching 5.8 ± 2.2 nmol/l (P < 0.05) on the 3rd day of the diet. On the 5th day of the diet, plasma EO levels were 3.2 ± 1.4 nmol/l. The 24-h urinary sodium excretion increased progressively from a baseline value of 156 ± 9 to 315 ± 28 meq/l on the 3rd day of the high-salt diet and remained elevated (Fig. 1). Figure 2A presents the individual values for plasma EO and their range (0.093 to 1.05 nmol/l) during the baseline period. In response to the high-salt diet, plasma EO rose in all subjects, but the magnitude of the change differed markedly among individuals. In general, the increase in plasma EO in response to the high-salt diet was related to the baseline plasma EO. For example, Fig. 2A shows that among subjects whose baseline plasma EO was <0.4 nmol/l (n = 8), the increase of plasma EO in response to the high-salt stimulus was often significantly less (P < 0.05) than in those individuals with higher (>0.4 nmol/l, n = 5) baseline EO. Figure 2B shows the individual responses of plasma EO during treatment with HCTZ. The diuretic raised plasma EO in all subjects except one. In both the high-salt and diuretic phases, plasma renin activity and aldosterone changed appropriately in all individuals, while blood pressure remained unchanged (Table 1). Urinary EO excretion was ~1.6-fold above baseline during the high-salt period, whereas during HCTZ administration, the urinary EO excretion was similar to baseline. There were no significant changes in heart rate or urinary creatinine during the high-salt or the diuretic periods, with the exception of one individual in whom blood pressure fell and heart rate increased

Statistics. Data were subjected to ANOVA and tested for distribution normality. Means for normally distributed data were compared with Tukey’s honestly significant difference test. Means for other distributions were compared with nonparametric tests (Wilcoxon signed rank or Mann-Whitney U-test) for independent or related samples, respectively. A value of P < 0.05 was considered to be significant. Group data are expressed as means ± SE. All correlations were determined from linear regression analysis. Statistics were computed using SYSTAT (Evanston, IL).
HYDROCHLORTHAZIDE DAILY. The
period. The mean (±SE) diuretic value was calculated from values
over the entire 5-day period, during which each subject received 25 mg
hydrochlorothiazide daily. The mean (±SE) value for the high-salt
diet (B) was from the third study day. The mean (±SE) diuretic value was obtained from the individual means over the 5-day baseline
period. The mean (±SE) value for the high-salt diet in (A) was from the third
study day. The mean (±SE) diuretic value was calculated from values
over the entire 5-day period, during which each subject received 25 mg
hydrochlorothiazide daily. The y-axes are discontinuous. *P < 0.05; **P <
0.01, vs. normal diet).

during HCTZ. In this individual, the baseline 5-day average for
plasma EO was 0.2 ± 0.02 nmol/l and rose to 1.8 ± 0.1 nmol/l
with HCTZ. With all subjects included, the grouped mean body
weight did not change significantly in the high-salt or diuretic
periods. However, when the specific weight change was looked at in a model in which each individual served as his own
control from the baseline period, a different pattern was found. During the diuretic phase, eight individuals lost weight (range
0.4–1.5 kg) and four showed no change [i.e., within the
standard error of the weight change (±0.23 kg)]. Weight rose
1.5 kg in one individual on the diuretic, even though PRA and
aldosterone increased substantially. Nevertheless, during the
diuretic phase, the mean specific change in weight, when all
subjects were considered, was −0.48 kg.

Figure 3 shows the typical temporal changes observed for
plasma EO levels in two subjects during each day of the three
study periods. During the baseline period the plasma levels of
EO were steady. During the first 2 days of the high-salt period,
there was typically no significant increase in circulating EO
while plasma levels peaked on day 3 and then declined by day
5 to values that remained seven- to eightfold above baseline. In
some other subjects (not shown), the peak plasma EO occurred
on the 1st or 2nd day of the high-salt diet. However, in most
subjects, the maximal plasma EO occurred on the 3rd day of
the high-salt diet. No prominent or consistent peaks or troughs
of plasma EO were observed in individuals during the 5 days of
HCTZ treatment.

The results from the HPLC of the C-18 plasma extracts used
for EO immunoassay during the three study periods are shown
in Fig. 4. B–D. In each case, one major peak of EO immuno-
reactivity was detected at 23 min (~14% acetonitrile), corre-
sponding also to the characteristic retention time for commer-
cial ouabain (Fig. 4A). The EO peak at 23 min was specifically
increased in samples from the high-salt period. Other small
peaks of immunoactivity in the fractions adjacent to EO were
not changed significantly, and these additional peaks were
repeatedly detected in only 2 (one of which is shown in Fig. 4)
of the 13 subjects. In the remaining subjects, only a single peak
of EO immunoactivity was seen. No immunoactivity was
found when C-18 extracts of water/trifluoroacetic acid were
subjected to HPLC immediately before the samples. Moreover,
ouabain standards were injected after the plasma extracts to
rule out the possibility that ouabain contamination of the HPLC
could compromise the results. Thus the HPLC results reinforce
the notion that the primary immunoreactive species in human

Table 1. Characteristics of the Study Individuals

<table>
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<tr>
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<th>Normal Diet</th>
<th>High Salt</th>
<th>Diuretic</th>
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<tr>
<td>MBP, mm Hg</td>
<td>94±2</td>
<td>93±2</td>
<td>94±2</td>
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<tr>
<td>PRA, ng/ml·h⁻¹</td>
<td>2.02±0.3</td>
<td>0.88±0.15†</td>
<td>3.9±0.4†</td>
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<tr>
<td>Aldosterone, ng/dl</td>
<td>19±2</td>
<td>7.6±1.2‡</td>
<td>29±1.7‡</td>
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<td>UNaV, mEq/day</td>
<td>156±9</td>
<td>288±182†</td>
<td>199±11</td>
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<td>UEOV, nmol/day</td>
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<td>1.69±0.27*</td>
<td>1.44±0.31</td>
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<td>Ur. Vol., ml/day</td>
<td>1.427±127</td>
<td>2.228±303*</td>
<td>1.741±120</td>
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<td>78±5</td>
<td>77±4</td>
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<tr>
<td>Weight change, kg</td>
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<td>ND</td>
<td>−0.49±0.23*</td>
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</tbody>
</table>

The means ± SE for the 5th day (steady state) of each study period are shown. *P < 0.05; †P < 0.001; ‡P < 0.0001 vs. normal diet, n = 13 subjects. MBP, mean arterial blood pressure; PRA, plasma renin activity; UNaV, urinary sodium excretion; UEOV, urinary endogenous ouabain excretion; Ur, urine. HR, heart rate; BW, body weight; ND, not determined. Body weight change during the diuretic phase is with reference to the normal diet (set as zero) for each individual.

Fig. 3. Daily plasma levels of EO in two normal subjects on normal and high-salt intake and during diuretic treatment.

Fig. 2. Individual plasma EO in subjects on a normal diet, during a high-salt
diet (A) and during diuretic administration (B). Mean values ± SE for the
normal diet were obtained from the individual means over the 5-day baseline
period. The mean (±SE) value for the high-salt diet in (A) was from the third
study day. The mean (±SE) diuretic value was calculated from values
over the entire 5-day period, during which each subject received 25 mg
hydrochlorothiazide daily. The y-axes are discontinuous. *P < 0.05; **P <
0.01, vs. normal diet).
plasma extracts during the dietary and diuretic periods is isopolaric with ouabain and confirm the overall impression from the regular immunoassay of the C-18 plasma extracts.

To examine the relationship between plasma EO and its calculated urinary clearance, the daily measured values for these two parameters were averaged for each of the three 5-day periods in the 13 subjects. As shown in Fig. 5A, the plasma EO and the urinary clearance of this steroid were inversely related ($r = -0.73$, $P < 0.01$, slope = $-0.84$) over an ~100-fold range for each parameter. Figure 5B shows that a significant inverse relationship was present in the baseline ($r = -0.57$, $P < 0.01$, slope = $-0.74$) and high-salt ($r = -0.88$, $P < 0.001$, slope = $-1.2$) periods. A similar relationship was found in the diuretic period ($r = -0.57$, $P < 0.01$, slope = $-0.45$, not shown). All three relationships were well fit by a simple linear regression model. The increased slope ($P < 0.05$) and the marked increase in plasma EO at any given clearance in the high-salt phase likely reflects increased secretion of this steroid.

Baseline plasma EO correlated significantly with age ($r = 0.64$, $P < 0.02$, not shown), whereas no other significant correlations were found with plasma and urinary EO and the other parameters measured.

**DISCUSSION**

The major results of this study are that the plasma levels and urinary excretion of EO are sensitive to sustained increases in salt intake in most normotensive subjects. This is the first direct evidence that a high-salt diet can elevate EO in normal individuals and, as such, provides support for the hypothesis that increased circulating EO can, in principle, be an early event linking salt intake with hypertension. The data show also that low-dose HCTZ provides a rapid and modest stimulation to plasma EO and that this effect is especially surprising given the stimulatory effect of the high-salt diet. Previous studies have shown increased digitalis-like activity in the plasma and urine of hypertensive patients after salt loading using bioassays (14, 30, 41). This study used an antiserum that cross-reacts fully to the pure human EO (20), and we confirm that the retention time of the primary peak of EO immunoreactivity is indistinguishable from that of ouabain.

![Fig. 4. Reverse-phase HPLC of C-18 extracts used for immunoassay. A: ouabain standard; plasma extracts from an individual on either a normal (B) or high-salt (C) diet, or diuretic (D), respectively. Each plasma pool was 5 ml. For the high-salt period, equal-sized sample volumes from days 3 and 5 were combined.](image)

![Fig. 5. Relationship between urinary ouabain clearance and plasma EO levels. A: overall relationship from all subjects studied during the baseline, high-salt diet, and diuretic study periods. Each data point is a 5-day mean value. B: specific relationship between plasma EO and urinary clearance during the normal diet (○) and high-salt periods (●). The solid and dashed lines are the linear regressions and the 95th percentile confidence limits, respectively.](image)
able from commercial ouabain in an HPLC system capable of resolving other polar cardiac glycosides (7).

The design of the study involved adjacent 5-day periods of normal and sodium chloride-supplemented diets followed by a minimum 10-day interval between the end of the high-salt diet and the commencement of diuretic treatment. This interval has been found, on the basis of earlier studies (24), to be more than sufficient to return sodium balance to normal. Throughout the study, the compliance of all individuals with the various regimens was excellent. For example, on the baseline diet, the average daily urinary sodium excretion was 156 ± 9 meq. (refer to Table 1), and during voluntary supplementation of this diet with 171 meq of NaCl/day, the measured excretion of sodium rose to 310 ± 28 meq on the 3rd day of the high-salt diet, indicating that a new and more positive sodium balance had been obtained. Moreover, plasma renin and aldosterone changed appropriately during the high-salt and diuretic periods in every individual, indicating that positive and negative changes in sodium balance occurred (Table 1). When compared with loop diuretics such as furosemide, thiazide diuretics such as HCTZ are weaker because they have a distal tubular mechanism of action that limits their maximal impact to only 5–8% of the filtered load of sodium (43). Accordingly, large increases in urinary sodium excretion are not typically observed with HCTZ. Nevertheless, during the diuretic phase, there was a significant decline in body weight in most subjects. The mean specific weight change for the entire group was −0.5 kg, and this likely corresponds to the net loss of ~72 mmol of sodium and ~0.5 l of fluid. Most of this salt and water loss occurred within the first 24 h after diuretic administration (not shown). Thus the modest weight loss and elevated renin and aldosterone are all consistent with the view that HCTZ induced mild sodium depletion in these subjects.

The net result of the above-mentioned maneuvers was that the relationship between sodium balance and EO was J-shaped (Fig. 6). This result confirms that in another recent study that the relationship between sodium balance and EO was J-shaped, although this was not mentioned (32). Nevertheless, the similarity of the phenomenon in two different clinical and geographic populations using different methods of dietary sodium manipulation is encouraging and also because it raises the possibility that high-salt diets may raise EO in otherwise normotensive individuals at risk for future hypertension. From a physiological perspective, we suggest that the increase in renin, aldosterone, and EO with sodium depletion likely reflects their teleological role in maintaining overall circulatory volume and pressure homeostasis in sodium-poor environments. Our view is consistent also with recent observations concerning the physiological role of EO in the general population (52). However, another corollary of the J relationship is that sustained increases in EO evoked by high-salt diets are not only unwarranted but may predispose to hypertension. Accordingly, it might be expected that interference with the biosynthetic pathway and/or the target receptor for EO may lower blood pressure (e.g., 12).

As shown in Fig. 1 and Fig. 3, plasma EO levels typically peaked on the 3rd day of the high-salt diet, and this is a time when a new state of sodium balance is attained in most individuals (24). The result demonstrates that EO is not sensitive to acute (i.e., hour) changes in salt balance in normal men but that it is influenced by longer-range changes in sodium balance. Plasma EO declined somewhat by the 5th day of the high-salt period, yet the overall result shows clearly that circulating EO is raised during sustained increases in sodium balance. In view of the aforementioned relationship, it was surprising to us that the diuretic, which diminishes total body sodium, also proved to be a stimulus to plasma EO. The effect is neither explained by cross-reactivity of HCTZ in the immunnoassay nor changes in other immunoreactive species (Fig. 4). The urinary clearance of EO was well maintained during HCTZ, and as ANG II stimulates EO secretion from adrenocortical cells (25), the stimulus to plasma EO was likely secondary to the diuretic-induced increase in plasma renin activity.

As shown in Fig. 2, in four subjects, the increase of plasma EO was dramatic (>10-fold) during the high-salt diet, and the concentration exceeded 10 nmol/l in three of these individuals. Therapeutic levels for plasma digoxin in patients with congestive heart failure range from 2–5 nmol/l (36), and yet no adverse effects of the high EO were noted during the high-salt diet. Several factors may be pertinent to this issue. First, the peak plasma concentrations of EO may be tolerated because they are clearly transient (Fig. 3). Second, normal individuals may be less sensitive to cardiac glycosides than patients with congestive heart failure because several neurohumoral and calcium-mobilizing systems are activated in the latter state (9). Third, the measured EO may not be biologically active; this can be excluded because previous work showed that the primary ouabain immunoreactive compound in human plasma is functionally equivalent to commercial ouabain in most respects (6, 12, 17, 36). Fourth, in vivo, EO may circulate in a different form, either complexed, conjugated with other entities, or in a different intramolecular configuration (e.g., an 11–19 hemiketal) that is sensitive to acidic conditions (e.g., 18). Some support for this notion arises from our unpublished observations that much lower values for immunoreassayable EO are obtained when the extraction process is acid (TFA) free.

\[9 meq \text{ on the 3rd day of the high-salt diet.} \]

\[310 \text{ mmol of sodium}\]

\[10-fold\] during the high-salt diet, and the EO was dramatic (\(|>10\text{-fold}\)| during the high-salt diet, and the concentration exceeded 10 nmol/l in three of these individuals. Therapeutic levels for plasma digoxin in patients with congestive heart failure range from 2–5 nmol/l (36), and yet no adverse effects of the high EO were noted during the high-salt diet. Several factors may be pertinent to this issue. First, the peak plasma concentrations of EO may be tolerated because they are clearly transient (Fig. 3). Second, normal individuals may be less sensitive to cardiac glycosides than patients with congestive heart failure because several neurohumoral and calcium-mobilizing systems are activated in the latter state (9). Third, the measured EO may not be biologically active; this can be excluded because previous work showed that the primary ouabain immunoreactive compound in human plasma is functionally equivalent to commercial ouabain in most respects (6, 12, 17, 36). Fourth, in vivo, EO may circulate in a different form, either complexed, conjugated with other entities, or in a different intramolecular configuration (e.g., an 11–19 hemiketal) that is sensitive to acidic conditions (e.g., 18). Some support for this notion arises from our unpublished observations that much lower values for immunoreassayable EO are obtained when the extraction process is acid (TFA) free.

\[\text{J-shaped relationship between sodium balance and circulating EO in normal men.} \]

\[\text{Plasma EO (nmol/L)} \]

\[\text{SODIUM BALANCE} \]

\[\text{Fig. 6.} \]

\[\text{J-shaped relationship between sodium balance and circulating EO in normal men. The plasma EO is reported as the average for days 1–5 for both the "normal" and "negative" sodium balance categories (the latter because there were no obvious transients in daily circulating EO for subjects on the diuretic). For the positive sodium balance, the peak EO values on day 3 were used. Values are expressed as means ± SE. *P < 0.05; **P < 0.01 vs. normal sodium balance (n = 13 subjects).} \]
The plasma levels and the urinary excretion of EO exhibited an inverse steady-state relationship (Fig. 5). The slope of the relationship shows that the urinary clearance is an important determinant of the circulating EO, and this interpretation is consistent also with elevated EO in patients with chronic renal failure (46) and with the key role of the kidney in the disposition of intravenous ouabain (47, 51). However, although the urinary excretion of EO is significant, the present studies reveal also that the bulk of the filtered load of EO is likely subject to extensive renal reabsorption. For example, at a circulating EO concentration of 0.5 nmol/l, and given a glomerular filtration rate (GFR) of 120 ml·min⁻¹·1.73 M², the calculated filtered load is ~86 nmol/day. The urinary excretion of EO (Table 1) was ~1 nmol/day, indicating that >98% of the daily filtered load of EO is reabsorbed, perhaps by specific tubular epithelial pathways (21) and likely returned to the circulation. Moreover, the well-known age-related decline in GFR (45) may contribute to the higher baseline EO, as well as the exaggerated response of EO to the high-salt diet among the older individuals in this study.

EO is a highly water-soluble steroid, so that the extent of its renal reabsorption seems surprising; increasing the water solubility of steroids by attaching polar groups often increases their urinary excretion (3). The renal conservation of EO may confer advantages. For example, the strong bias to resorption points to a large underlying capacity to raise urinary excretion, which may limit the potential for self-poisoning from adrenocortical tumors that oversecrete EO (31). Second, EO may be the most highly oxygenated mammalian steroid, and renal conservation would minimize the metabolic cost of maintaining its circulating concentrations and may obviate the need for prominent biosynthetic and secretory mechanisms.

A key question arising from the present studies concerns the genesis of the increased plasma levels of EO during the high-salt diet. The slope relating the urinary clearance with circulating EO was well fit by a linear regression model in the double log plot, consistent with a first-order mechanism (Fig. 5A). When the data were analyzed according to the different study periods, the slope was clearly significantly increased by the high-salt diet (Fig. 5B), and the high correlation (r = 0.86) reveals that urinary excretion explains at least 75% of the variance in plasma EO. The increased slope and convergence of the lines on the clearance axis also suggest that the high-salt diet stimulated EO secretion 5- to 10-fold. The additional EO likely originates from the adrenal cortex (5, 17, 27), and a plausible mediator of the high-salt diet may be the atrial peptides (49).

In view of the dramatic increase in plasma EO during the high-salt diet, it was of some interest that the HPLC of the C-18 plasma extracts used for assay (Fig. 4 B-D) revealed only one major peak of EO immunoreactivity. We had expected that multiple immunocross-reactive polar materials would circulate when EO secretion was stimulated. Indeed, although other polar peaks of immunoreactivity were detected in 2 of the 13 subjects, they did not change significantly with the high-salt diet, and no new peaks appeared in the other subjects. The former observation implies that they may be part of another biosynthetic pathway, whereas the latter suggest that EO biosynthesis is highly compartmentalized within the cell so that polar intermediates are not available for secretion. Nevertheless, the HPLC results substantiate the impression given from the assay of the C-18 extracts and indicate that EO can usually be assayed with reasonable confidence under those conditions.

The age-related rise of blood pressure has been linked with the dietary intake of salt (50). The molecular mediators of this relationship remain unclear. In normal men, the renin-angiotensin-aldosterone axis and sympathetic nervous system activity usually decline when salt intake is increased (24, 29). Therefore, it is of considerable interest that EO is increased by salt loading in humans because elevated circulating levels of ouabain-like factors have been described in hypertensive patients, and the prolonged administration of ouabain induces chronic hypertension in rats (14, 31, 33, 35, 53) and mice (8). In addition, both high-salt diets and ouabain attenuate the potassium-induced vasodilation of the forearm vasculature of normal man (13). In our study group of normal individuals, the blood pressures were not salt-sensitive (28), perhaps because the high-salt phase lasted only 5 days. In addition, elevated plasma levels of cardiac glycosides do not ordinarily raise blood pressure unless short-term cardiovascular reflexes are compromised (23), and in the rat, ouabain infusions typically do not raise blood pressure for ~7–10 days (33). In addition, although reduced renal mass can also accelerate the hypertensinogenic activity of ouabain (53), all of the individuals we studied had normal renal function. Thus it appears that long-term studies, as well as novel pharmacological tools, will be needed to uncover the steady-state relationships between sodium intake, circulating EO, and blood pressure (32).

In conclusion, the present study has shown that high-salt diets raise the plasma and urinary levels of EO in normal men. These observations, when taken with other data, add additional support for the hypothesis that EO is a humoral link between salt intake and high blood pressure in humans.

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