Endogenous sodium pump inhibitors and age-associated increases in salt sensitivity of blood pressure in normotensives


Laboratory on Cardiovascular Science and Clinical Research Branch, National Institute of Aging, Intramural Research Program, Gerontology Research Center and Loyola College, Mathematical Sciences Department, Baltimore, Maryland

Submitted 25 October 2007; accepted in final form 20 February 2008

Numerous studies have reported that the magnitude of the systolic blood pressure (SBP) response to acute changes in dietary NaCl intake, i.e., salt sensitivity of blood pressure, increases with advancing age (16, 20, 27, 32–35). Specific determinants of the greater blood pressure response of older persons to dietary NaCl, however, remain to be identified.

NaCl ingestion results in an increase in plasma volume and natriuresis. It has been postulated for some time that endogenous substances [sodium pump inhibitors (SPI)] are stimulated by increased Na intake and increase natriuresis by inhibiting renal tubular Na pumps to prevent renal reabsorption of filtered Na (6, 18, 30). However, such substances were not purified adequately in early studies, and their assays were nonspecific (17).

More recently, SPI assays have improved and become more specific (3, 14), and studies in various animal models and in humans have documented an increased elaboration of SPI in response to NaCl loading. This research has focused largely on two SPI: an endogenous ouabain (EO) and a bufadienolide, marinobufagenin (MBG). Differences in the kinetics and tissue actions of these substances in response to NaCl loading have been demonstrated in animal models and in human salt-sensitive hypertensive response (4, 7, 10, 19, 22, 28).

That age-associated differences in circulating endogenous Na pump inhibitors may be implicated in the age-associated increase in SBP and increased NaCl sensitivity of SBP in older humans has been suggested previously (21) but never tested. The goal of the present study was to investigate effects of a subacute change in dietary NaCl on urinary and plasma EO and MBG in middle-aged and older normotensive subjects, and to determine whether NaCl-induced individual differences in the levels of these substances are linked to variations in renal sodium excretion or salt sensitivity of SBP.

Methods

Subjects: inclusion and exclusion criteria. MBG and EO were measured in a series of healthy Caucasian women, ages 40–70 yr, who, as part of another study, were salt restricted and then salt loaded to determine interactions among breathing patterns, Pco2, and arterial pressure (2). Determination of subject eligibility involved telephone interview, physical examination, and informed consent. Inclusion and exclusion criteria also include the following: all qualified candidates were normotensive (resting SBP, <139 mmHg and resting diastolic blood pressure, <89 mmHg); had no history of respiratory, cardiovascular, liver or kidney disease, or diabetes; were free of cardiac organ damage determined by electrocardiogram; and were free of kidney dysfunction as determined by plasma creatinine >1.5. Subjects treated with estrogens or nonsteroidal anti-inflammatory agents, or medications affecting sodium, potassium, calcium, water, hemodynamic, or neural regulation (including diuretics, steroids, major tranquilizers, narcotics, or benzodiazepines) were also excluded, as were smokers or those with body mass index (BMI) of <19 or >30. The protocol of the study was approved by the Medstar Research Institute Institutional Review Board, and all study subjects signed informed consents.

Experimental design. The experiment consisted of a 12-day, outpatient, dietary intervention, including 6 days on a low-sodium (0.7 mmol·kg−1·day−1), low-potassium (0.7 mmol·kg−1·day−1) diet, followed immediately by 6 days on a high-sodium (4 mmol·kg−1·day−1), low-potassium (0.7 mmol·kg−1·day−1) diet. The average recommended dietary sodium intake in the American diet for an average-sized adult is

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
about 1.4 mmol·kg\(^{-1}\)·day\(^{-1}\) (2,300 mg/day), whereas the average sodium intake in the United States is \(-2.1\) mmol·kg\(^{-1}\)·day\(^{-1}\) (3,500 mg/day) (27).

The high-NaCl diet included supplementation with enteric-coated NaCl capsules (Slo-Sodium, CIBA-Geigy), ingested with each meal at bedtime. Dietary intake of calories, protein, fat, and percentage of calories from fat was standardized per body weight. Meals were provided to facilitate compliance with the dietary regimen. Participants were instructed to eat all of the food provided to them and nothing else and keep a food diary. Water was freely available, but participants were limited to one cup of a caffeinated beverage per day. Body weight was recorded at each session. Participants were instructed to maintain their normal daily activities during the 12-day period.

Blood pressure and heart rate were monitored during 25-min seated rest in the clinic setting immediately before and after each 6-day sodium diet condition. Blood pressure was recorded every 6 min during these sessions from an inflatable cuff attached to an automated oscillographic device (model 90207; Spacelabs, Redmond, WA). Venous blood was drawn following the low- and high-sodium diets. Plasma aliquots were frozen at \(-80{^\circ}\)C. Hematocrit, plasma sodium, potassium, calcium, magnesium, and creatinine were quantitated.

Twenty-four-hour urine samples were obtained for each subject during the last day on both diets. In addition, in a preliminary experiment with a group of eight subjects, 24-h urine samples were collected on a daily basis during the high-NaCl diet. Urine volume was determined, and aliquots were frozen at \(-80{^\circ}\)C. Sodium, potassium, and creatinine were quantitated.

Assays. Urinary and plasma SPI were measured using competitive fluoroimmunoassay as reported previously in detail (10).

Calculations. Creatinine clearance (CrC), SPI clearance, Na filtered, and fractional excretion of sodium (FENa) were calculated using the following equations:

\[
CrC (in \text{ml} \cdot \text{min}^{-1} \cdot 1.73 \text{m}^2) = \frac{uCr \times uVol \times 1.73}{(pCr \times T \times BSA)},
\]

where uCr is urine creatinine concentration (mg/ml), uVol is urine volume (ml), pCr is plasma creatinine concentration (mg/ml), and T is time (min). BSA (body surface area; \text{m}^2) = W^{0.425} \times H^{0.725} \times 0.007184, where W is weight (kg) and H is height (cm).

Clearances of MBG and EO were calculated with the same equation as creatinine clearance.

Na filtered = pNa \times CrC, where pNa is plasma sodium concentration (mmol/ml), CrC is creatinine clearance (in ml·min\(^{-1}\)·1.73 m\(^2\)). Na filtered is expressed as mmol/min.

FENA = uNa \times pCr \times 100/(pNa\timesCr), where uNa and pNa are urine and plasma sodium concentrations (mmol/l). FENA is expressed as percent.

STATISTICAL METHODS. Data are presented as means ± SE. The effect of dietary Na intake on measured variables was determined by a paired \(t\)-test. Pearson’s correlation coefficient was used to assess the significance of the associations between pairs of measured variables. Least squares linear regression analysis was used to obtain the best-fitting line. A linear mixed-effects model (24) was employed to estimate the best-fitting line. A linear mixed-effects model (24) was employed to estimate the best-fitting line. A linear mixed-effects model (24) was employed to estimate the best-fitting line.
describe the repeated-measures data and to determine independent predictors of SBP and MBG. Mixed-effects models provide a flexible way of modeling repeated-measures data. The model contains both fixed- and random-effects. The fixed-effects provide population-average regression coefficients that describe the mean effect of the explanatory variables on the response variable. Random-effects allow each subject to deviate from the average. These random effects impose a correlation structure that takes into account the association among the repeated observations within each subject. A backward elimination procedure was used to remove statistically nonsignificant variables until only statistically significant variables remained in the model.

RESULTS

Thirty-six subjects met the initial screening criteria, and 33 completed the study. Data from five subjects were excluded from the analysis on the basis of incomplete 24-h urine collection, resulting in a final total of 28 subjects. Mean age was 53 ± 1.6 yr, mean height was 163.4 ± 1.3 cm, and mean BMI was 25.2 ± 0.6.

Table 1 lists mean values of measured parameters following 6 days of dietary NaCl restriction and 6 days of NaCl loading for the entire sample. After 6 days on the high-sodium diet, systolic pressure, pulse pressure, and body weight were higher; diastolic blood pressure was unchanged; and heart rate was lower than subjects on the low-salt diet. High-dietary NaCl resulted in 35% increase in urine MBG (uMBG), sixfold increase in 24-h urinary Na and FENa, and 24% increase in urine volume. Plasma MBG and MBG clearance were both increased by 26%, while plasma EO and EO clearance were not different from low-salt levels. The relatively low creatinine clearance in this study may be attributed, at least in part, to the age and sex of the subjects. It is known that the creatinine clearance decreases with age, and is lower in women than in men (26). An “ideal” creatinine clearance calculation for our population, based on the formula from the study by Rowe et al. (26) using a “super-clean” population, is 91.7 ± 0.9 ml·min⁻¹·1.73m² only slightly higher than in our study (Table 1). Both plasma and uMBG and plasma and urine EO on a low-sodium intake were correlated with each other (r = 0.67, P = 0.0001; r = 0.53, P = 0.008, respectively). In response to high-NaCl intake (after 6 days), plasma MBG levels, urinary MBG levels, and MBG clearance increased compared with the low-NaCl diet (Table 1). In contrast, neither plasma levels, nor the urine levels of EO, nor the clearance of EO differed between low- and high-NaCl diets (Table 1); and there was no correlation between change in FENa and the urinary EO change (Table 2).

To detect day-to-day changes in SPI in humans in response to the NaCl loading protocol, we measured EO and MBG

Fig. 1. Renal excretion of sodium pump inhibitors. Renal endogenous ouabain (EO, open triangles; A) and marinobufagenin (MBG, closed circles; B) in a subset of 8 subjects during 6 days on a high-NaCl (HS) diet. Data are means ± SE. *P < 0.05 vs. day 0; mixed-effects analysis followed by the Bonferroni adjusted P value test.

Fig. 2. A: correlation of creatinine clearance and Na clearance on a low-NaCl (LS) and HS diets. B: correlation of change in fractional excretion of Na and change in urine Na from LS to HS diet.
production daily following the increase in dietary NaCl in eight subjects. Fig. 1A shows that EO increased transiently and then returned toward baseline 4 days after the initiation of the high-NaCl diet. In contrast, MBG increased progressively, and its levels remained elevated 6 days after the initiation of NaCl loading (Fig. 1B).

While neither the renal glomerular filtration rate, (i.e., 24-h creatinine clearance), nor filtered Na were affected by dietary NaCl (Table 1), the total Na clearance at a given creatinine clearance markedly increased during the high-NaCl diet (Fig. 2A). Neither total Na clearance on the high-Na diet nor the change in Na clearance from the low- to high-Na diet correlated with the change in filtered Na. However, on the high-NaCl diet, the increase in FENa (i.e., total urine Na/Na filtered) and the change in FENa elicited by the high-NaCl diet, were both highly correlated with the change in total urinary Na (Fig. 2B). Thus, the large increase in urinary Na excretion on high-Na diet was mediated by a mechanism distal to the glomerulus.

One renal tubular mechanism that affects the marked increase in the FENa during the high-NaCl diet (Fig. 2B) is a NaCl-induced elaboration of MBG (Table 1) that predominantly inhibits the renal α-1 Na pump isoform (8). The relationship of total urinary Na as a function of total urinary MBG on low- and high-Na intake is illustrated in Fig. 3A. During high-NaCl diet, urinary Na was directly related to urinary MBG, but no relationship between the two was present during NaCl restriction. Fig. 3B shows that the change in FENa among individuals in response to high-NaCl diet was significantly correlated with the change in MBG. As with the change in urine Na in response to high-NaCl diet, renal MBG excretion on the high-salt diet was inversely related to body weight (Fig. 3, C and D).

Next, we determined the association of NaCl loading with SBP and whether a sodium-induced change in SBP was related to the change in MBG. Figure 4A shows that SBP was inversely related to urinary Na, but only on the high-NaCl diet. Figure 4B shows that on the high-NaCl diet, the increased MBG was inversely related to SBP.

The interaction between SPI, age, and salt sensitivity of SBP is illustrated in Fig. 5. Figure 5A shows the NaCl-induced change in SBP as a function of age. Note that the response of SBP to NaCl loading varied among subjects: in most it increased; in some it decreased, and, still in others it did not change. This highly variable response to an increase in dietary NaCl is a pattern common in prior studies (32). More impor-
tantly, Figure 5A shows that the change in SBP to NaCl loading varied directly with age, as also previously demonstrated (32).

That salt sensitivity of SBP varied directly with age (Fig. 5A) and that salt sensitivity of SBP varied inversely with MBG on the high-NaCl diet (Fig. 4B), suggests that MBG production during NaCl loading might vary inversely with age. Fig. 5B shows that urinary MBG on the high-NaCl diet, indeed, was inversely related to age. Neither renal MBG clearance, nor renal EO clearance declined with age. Interestingly, the change in urine volume induced by the change in dietary NaCl tended to vary inversely with age ($r = -0.36$, $P > 0.06$).

In contrast to the relationship of MBG to urine Na, FENa, SBP, age (Figs. 3, 4, and 5B), EO was not related to any of these variables (Table 2).

Independent determinants of SBP. The bivariate correlations illustrated in Figs. 4 and 5 suggest that SBP is a function of dietary NaCl, age, and the natriuretic Na pump inhibitor MBG. We employed a linear mixed-effects model to determine the independent impact of a number of measured variables on SBP. The initial model contained the following main effects: NaCl intake (high vs. low), age, BMI, BMI$^2$, FENa, and uMBG, as well as a number of interactions of these terms. The final model is shown in Table 3. The high correlation of the observed and fitted values indicates that the model provided a good fit to the data. In the final model, NaCl intake (salt), age, BMI, and uMBG were significantly and independently corre-

Table 3. Final linear mixed-effects model of systolic blood pressure

<table>
<thead>
<tr>
<th>Variable</th>
<th>Parameter Estimate</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-118.04</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>4.43</td>
<td>0.013</td>
</tr>
<tr>
<td>Salt</td>
<td>8.63</td>
<td>0.027</td>
</tr>
<tr>
<td>BMI</td>
<td>7.20</td>
<td>0.038</td>
</tr>
<tr>
<td>FENa</td>
<td>4.70</td>
<td>0.211</td>
</tr>
<tr>
<td>uMBG</td>
<td>0.004</td>
<td>0.030</td>
</tr>
<tr>
<td>FENa $\times$ uMBG</td>
<td>-0.004</td>
<td>0.006</td>
</tr>
<tr>
<td>BMI $\times$ age</td>
<td>-0.14</td>
<td>0.035</td>
</tr>
<tr>
<td>Variance (error)</td>
<td>18.38</td>
<td></td>
</tr>
<tr>
<td>Variance (subjects)</td>
<td>52.04</td>
<td></td>
</tr>
<tr>
<td>Correlation ($y$, $\hat{y}$)</td>
<td>0.96</td>
<td></td>
</tr>
</tbody>
</table>

uMBG, urine marinobufagenin. *$y$* represents the $y$ values predicted from the mixed-effects model.
lated with SBP. Two significant interactions remained in the model: FENa × uMBG, and BMI × age. While the main effect of FENa was not statistically significant, it was retained in the model, since it had a statistically significant interaction (Table 4) (24). After adjusting for other terms in the model, SBP was, on average, 8.6 mmHg higher at high salt than at low salt. The coefficient of the BMI × age interaction indicates that the effect of BMI on SBP was linked to its effect on FENa. The effect of BMI on SBP decreased. Finally, the coefficient of the FENa × uMBG interaction indicates that the effect of uMBG on SBP was linked to its effect on FENa.

Independent determinants of uMBG. A second mixed-effects model for uMBG/weight (Table 4) contains main effects for NaCl intake, age, and urine EO/weight, and no interaction terms. The model showed that after accounting for age and urine EO/weight, the indexed uMBG averaged 7.8 pmol·kg⁻¹·24 h⁻¹ higher at high than at low salt. After accounting for salt level and urine EO/weight, the indexed uMBG decreased 0.77 pmol·kg⁻¹·24 h⁻¹ per year of age. Interestingly, after accounting for age and salt level, the indexed uMBG increased by 0.84 pmol·kg⁻¹·24 h⁻¹ with each unit of indexed urine EO.

DISCUSSION

The present results are the first to demonstrate in normotensive humans that following a change from a lower to a higher NaCl diet a sustained increase in MBG production occurs, and renal fractional NaCl excretion increases and correlates directly with increased MBG excretion. In addition, this study found that the salt-induced increase in MBG was inversely related to age and that the NaCl sensitivity of SBP was directly related to age, and inversely related to the NaCl-induced increase in MBG. A linear mixed effect model showed that, after accounting for age, dietary Na was an independent determinant of MBG, and that after accounting for the dietary NaCl, uMBG declined with age.

In contrast to the sustained increase in MBG on the high-NaCl diet, EO levels in the present study increased only transiently (Fig. 1). This finding is consistent with a previous study with normotensive men, which showed that high-NaCl intake increased plasma EO for 3 days followed by a decrease toward baseline on day 5 (22). We have reported a similar pattern of MBG and EO response in experimental studies following acute and chronic NaCl loading of Dahl-S rats (10). Using selective in vivo immunoneutralization of EO and MBG with specific antibodies and blockade of AT1 receptors, we found that following NaCl loading of Dahl-S, a peak response of EO, a neurohormone, via activation of renin-angiotensin system stimulated adrenocortical production of MBG, a natriuretic and a vasoconstrictor (11). Although in the present study, EO levels after 6 days of salt loading did not correlate with urinary Na, SBP, or age in accordance with our previous experimental findings, in the present study plasma EO were positively correlated with plasma MBG levels after salt loading. Also the renal excretion of MBG and EO were positively correlated with the high-NaCl diet (Table 2). Thus, in NaCl-loaded human subjects, a causal link may exist between EO and MBG production. Additional studies of the relationship between SPI in salt-loaded humans are warranted.

A prohypertensive effect of SPI in response to salt loading in hypertensive prone rats has been repeatedly observed (9, 10, 12, 13, 29). Our results show that a subacute response to an increase in MBG induced by 6 days of dietary NaCl in healthy, normotensive women is natriuretic. It should be noted that most of the participants in the present study were postmenopausal women. Menopause is associated with an increased salt-sensitivity of blood pressure and with a reduction in the levels of another steroid hormone with natriuretic and vasoactive substances that respond to NaCl ingestion. The vasoactive effects of SPI have been repeatedly documented with respect to NaCl-dependence of BP (6, 18, 30).

NaCl loading in salt-sensitive rodent models with abnormal renal function results in sustained, elevated levels of MBG that mediate sustained NaCl-dependent increases in arterial pressure (10). Specific antibodies against MBG prevent both the acute and sustained NaCl-induced increase in arterial pressure (10). The net effect of SPI actions (i.e., natriuresis vs. vasoconstriction) on blood pressure, however, depends upon the level of sustained concentrations that are achieved following NaCl ingestion; the response (potency, and sensitivity) of the renal and arterial Na pumps to these endogenous SPI; and the levels, potency, and sensitivity of other natriuretic and vasoactive substances that respond to NaCl ingestion.

The present results are the first to demonstrate in normotensive humans that following a change from a lower to a higher NaCl diet a sustained increase in MBG production occurs, and renal fractional NaCl excretion increases and correlates directly with increased MBG excretion. In addition, this study found that the salt-induced increase in MBG was inversely related to age and that the NaCl sensitivity of SBP was directly related to age, and inversely related to the NaCl-induced increase in MBG. A linear mixed effect model showed that, after accounting for age, dietary Na was an independent determinant of MBG, and that after accounting for the dietary NaCl, uMBG declined with age.

In contrast to the sustained increase in MBG on the high-NaCl diet, EO levels in the present study increased only transiently (Fig. 1). This finding is consistent with a previous study with normotensive men, which showed that high-NaCl intake increased plasma EO for 3 days followed by a decrease toward baseline on day 5 (22). We have reported a similar pattern of MBG and EO response in experimental studies following acute and chronic NaCl loading of Dahl-S rats (10). Using selective in vivo immunoneutralization of EO and MBG with specific antibodies and blockade of AT1 receptors, we found that following NaCl loading of Dahl-S, a peak response of EO, a neurohormone, via activation of renin-angiotensin system stimulated adrenocortical production of MBG, a natriuretic and a vasoconstrictor (11). Although in the present study, EO levels after 6 days of salt loading did not correlate with urinary Na, SBP, or age in accordance with our previous experimental findings, in the present study plasma EO were positively correlated with plasma MBG levels after salt loading. Also the renal excretion of MBG and EO were positively correlated with the high-NaCl diet (Table 2). Thus, in NaCl-loaded human subjects, a causal link may exist between EO and MBG production. Additional studies of the relationship between SPI in salt-loaded humans are warranted.

A prohypertensive effect of SPI in response to salt loading in hypertensive prone rats has been repeatedly observed (9, 10, 12, 13, 29). Our results show that a subacute response to an increase in MBG induced by 6 days of dietary NaCl in healthy, normotensive women is natriuretic. It should be noted that most of the participants in the present study were postmenopausal women. Menopause is associated with an increased salt-sensitivity of blood pressure and with a reduction in the levels of another steroid hormone with natriuretic action, i.e., progesterone (25). The impact of postmenopausal hormonal status on MBG production merits further study. Moreover, subjects in the present study were normotensive, and our findings do not necessarily reflect salt sensitivity or MBG production in hypertensive patients.

In accordance with the concept of a natriuretic hormone (30, 31), we can speculate that lower MBG levels during the first few days of NaCl loading in some subjects can add to the deficit (acquired or inherited) in sodium excretion, with commensurate increases in total blood volume, and, as a result, in greater elevation in BP. Furthermore, a lack of MBG production following NaCl loading could result in a relatively high

---

Table 4. Final linear mixed-effects model of urine marinobufagenin (adjusted for body weight)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Parameter Estimate</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>61.07</td>
<td></td>
</tr>
<tr>
<td>HS</td>
<td>7.81</td>
<td>0.005</td>
</tr>
<tr>
<td>Age</td>
<td>-0.76</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>uEO/Wt</td>
<td>0.84</td>
<td>0.001</td>
</tr>
<tr>
<td>Variance (Error)</td>
<td>85.06</td>
<td></td>
</tr>
<tr>
<td>Variance (Subjects)</td>
<td>3.76</td>
<td></td>
</tr>
<tr>
<td>Correlation (y, y*)</td>
<td>0.75</td>
<td></td>
</tr>
</tbody>
</table>

uEO, urine ouabain; Wt, body weight. *y* represents the y values predicted from the mixed-effects model.
renotubular sodium pump activity. Indeed, activation of renal Na\(^+\)-K\(^-\) -ATPase was shown to contribute to hypertension in Milan hypertensive rats, and was attributed to mutation of the \(\alpha\)-adducin gene (15). A polymorphism of the adducin gene has been detected in 20% of human hypertensive population (23). Whether or not these individuals exhibit reduced MBG production remains to be investigated.

**Perspectives and Significance**

A relative failure to increase natriuretic MBG levels in response to NaCl-loading with increasing age could be a factor involved in the increase in NaCl sensitivity of SBP with aging. A similar pattern has been described in a previous observational study showing that MBG excretion of more salt-sensitive African American participants was lower than that of less salt-sensitive non-African American subjects (1). The response to salt loading is complex, however, and includes the stimulation of other vasoactive and natriuretic substances (e.g., ANG II, ANP, endothelin, and vasopressin). To evaluate the specific role of MBG in the context of these other physiological factors in regulation of natriuresis and BP additional studies are required.

**ACKNOWLEDGMENTS**

We thank Beverly A. Parsons for her expert assistance with recruitment and care of the study subjects and Alexandra Newman, Danielle Joseph, and Chad Boily for outstanding technical support.

**GRANTS**

This study was supported by the Intramural Research Program of the National Institute on Aging.

**REFERENCES**