Salt-sensitive hypertension in ANP knockout mice is prevented by AT₁ receptor antagonist losartan

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Melo, Luis G., Anthony T. Veress, Chee K. Chong, Uwe Ackermann, and Harald Sonnenberg. Salt-sensitive hypertension in ANP knockout mice is prevented by AT₁ receptor antagonist losartan. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R624–R630, 1999.—Mice harboring a functional deletion of the pro-atrial natriuretic peptide (ANP) gene (−/−) develop salt-sensitive hypertension relative to their wild-type (+/+ ) counterparts after prolonged (>1 wk) maintenance on high-salt (HS, 8% NaCl) diet. We reported recently that the sensitization of arterial blood pressure (ABP) to dietary salt in the −/− mice is associated with failure to downregulate plasma renin activity. To further characterize the role and mechanism of ANG II in the sensitization of ABP to salt in the ANP ''knockout'' mice, we measured ABP, heart rate (HR), and plasma catecholamine and aldosterone concentrations in −/− and +/+ mice maintained on HS for 4 wk and treated with daily injections of AT₁ receptor antagonist DuP-753 (losartan) or distilled water (control). Daily food and water intake and fluid and electrolyte excretion were also measured during the first and last weeks of the dietary regimen. Cumulative urinary excretion of fluid and electrolytes did not differ significantly between genotypes and was not altered by chronic treatment with losartan. Basal ABP and HR were significantly elevated in control −/− mice compared with control +/+ mice. Losartan did not affect ABP or HR in +/+ mice, but reduced ABP and HR in the −/− mice to the levels in the +/+ mice. Total plasma catecholamine was elevated by approximately tenfold in control −/− mice compared with control +/+ mice. Losartan reduced plasma catecholamine concentration significantly in −/− mice and abrogated the differences in plasma catecholamine between −/− and +/+ mice on HS diet. Plasma aldosterone did not differ significantly between genotypes and was not altered by losartan. We conclude that salt sensitivity of ABP in ANP knockout mice is mediated, at least in part, by a synergistic interaction between ANG II and sympathetic nerve activity.

aldosterone; angiotensin; catecholamines; sympathetic nerve activity; urinary salt excretion

ACUTE ADMINISTRATION of atrial natriuretic peptide (ANP), a peptide hormone synthesized and released by the heart (10), is accompanied by pronounced natriuresis and diuresis (8). These renal excretory effects of ANP are due to a direct inhibition of sodium reabsorption in the medullary collecting duct (35) and suppression of the major hormonal and neural salt-conserving mechanisms, including inhibition of renin (38) and aldoste-

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time-dependent retention of salt and whether this is corrected by AT₁ receptor inhibition. At termination of the experiment, total plasma catecholamine, taken as an indirect index of SNA, and aldosterone concentrations were measured in all groups to assess their potential role in the development of salt sensitivity of ABP in /−/− mice. This work describes our findings, which indicate that development of salt sensitivity of ABP in mice lacking endogenous ANP activity may be due to ANG II-induced increase in sympathetic activity.

METHODS

Animals. The production and molecular analysis of ANP knockout mice and the housing conditions have previously been described (18, 26). F2 homozygous mutant (−/−) and wild-type (+/+) mice of both sexes, 20–24 wk old, and weighing 30–42 g, were used in all studies. The animals were obtained from our resident colony, which was founded with pathogen-free heterozygous breeding pairs. All experimental procedures complied with preapproved protocols and with the Canadian guidelines for animal research.

Materials. All materials for the catecholamine radioenzymatic assay were supplied with the kit (Amersham, Oakville, Ontario, Canada), with the exception of tolune and isoamyl alcohol, which were purchased from Sigma Chemical (St. Louis, MO), and Liquifluor, which was purchased from Canberra Packard (Mississauga, Ontario, Canada). Pentolinium, hexamethonium, and norepinephrine were also from Sigma. All other chemicals were from VWR (Mississauga, Ontario, Canada).

Dietary regimen and losartan treatment. Two groups each of +/+ and −/− mice were maintained for 4 wk on a powdered Purina diet containing 8% NaCl. One group each of +/+ (n = 7) and −/− (n = 5) mice received daily injections of losartan (20 mg/kg body wt ip, courtesy of DuPont-Merck) for the duration of the dietary regimen. The antagonist was dissolved in distilled deionized water (10 mg/ml) and kept at 4°C shielded from light in an aluminum foil-wrapped dark bottle. The remaining two groups (+/+, n = 6; −/−, n = 6) received equivalent daily injections of vehicle.

Surgical preparation for blood pressure measurement. At the end of the dietary regimen, mice were anesthetized with inactin (150 µg/g body wt ip) and kept at a body temperature near 38°C with a heat lamp. After tracheostomy, a jugular vein and carotid artery were cannulated with catheters (300–400 µm diameter) fashioned from PE-50 tubing for intravenous infusion and measurement of blood pressure, respectively. On completion of surgery, 0.12 ml of isotonic saline containing 2.25% bovine serum albumin and 1% glucose were infused over 15 min as a priming dose, followed by constant infusion of the same solution at 0.12 ml/h. Measurements of blood pressure were begun after an additional 30-min equilibration period.

Measurement of ABP and HR. ABP and HR were monitored continuously using a small volume displacement pressure transducer (model RP 1500, Narco Systems) connected to a MacLab/4e data-acquisition system. HR was calculated instantaneously from the pressure pulses. Four consecutive measurements of ABP and HR were taken at 15-min intervals after the equilibration period. Because the values of ABP and HR for the individual measurements did not differ significantly from one another, the average of the four measurements was used for statistical comparisons.

Blood sample collection. At termination of the experiment, blood for catecholamine and aldosterone analysis was collected by exsanguination through the carotid artery and chilled tubes containing a final concentration of 1.8 mg EDTA:1.2 mg glutathione per ml. The tubes were gently inverted several times to mix the blood with the preservatives and centrifuged at 3,000 revolutions/min (rpm) for 15 min at 3°C. The plasma samples were then stored at −70°C until assayed.

Total plasma catecholamine concentration. Total plasma catecholamine (norepinephrine and epinephrine) concentration was measured by a modified radioenzymatic method of Peuler and Johnson (31) with a commercially available kit (Amersham) in accordance with the manufacturer’s instructions. Briefly, 50 µl of plasma were mixed in duplicate with 40 µl of a reaction mixture consisting of Tris-EGTA-MgCl₂ buffer, pH 8.5, tritiated S-adenosyl-L-methionine, and catechol-O-methyltransferase in disposable glass tubes. A norepinephrine plus epinephrine standard was added to one of the plasma samples to a final concentration of 400 pg/ml in a final volume of 100 µl, and an equivalent volume of stabilizing buffer was added to the duplicate sample. All samples were incubated at 37°C for 1 h. At the end of the incubation, the contents of each tube were mixed vigorously with 50 µl of a 4 mM mixture of metanephrine and normetanephrine for termination of the methylation reaction. Each sample was mixed with 2 ml of tolune-isoamyl alcohol (3:2 vol/vol) for catecholamine extraction, centrifuged at 100 rpm, and frozen for 15 s in a dry ice:ethanol bath. The upper organic phase was decanted into a second set of tubes and mixed vigorously with 100 µl of 0.1 M acetic acid. The mixture was frozen in the dry ice:ethanol bath as before, and the organic phase was aspirated. The acetic acid residue was dried under a stream of air for 2 h and mixed vigorously with 1 ml of 0.05 M ammonium hydroxide. Periodate oxidation of the samples was initiated by addition of 50 µl of 4% (wt/vol) of sodium metaperiodate and terminated after 5 min by addition of 50 µl of 10% (vol/vol) glycerol and 1 ml of 0.1 M acetic acid. Each sample was mixed vigorously for 20 s with 10 ml of toluene-Liquifluor (1,000:50, vol/vol) and frozen in dry ice-ethanol. The upper organic phase was decanted into separate scintillation vials containing 2 ml of 0.1 M acetic acid and counted in a liquid scintillation counter for 2 min. The sensitivity of the assay is in the range of 2–5 pg/50 µl of sample.

Plasma aldosterone concentration. Aldosterone was measured in unextracted plasma samples with the Coat-A-Count Aldosterone radioimmunoassay kit (Diagnostics Products, Los Angeles, CA), according to the instructions provided by the manufacturer. All reactions were prepared in an ice bath (−4°C). Briefly, 200 µl of plasma were incubated in duplicate with 1 ml of 125I-labeled aldosterone in aldosterone antibody-coated polypropylene tubes. The mixture was incubated at 37°C for 3 h. At the end of the incubation, the tubes were decanted thoroughly and the bound radioactivity was counted for 2 min in a gamma counter. The concentration of aldosterone in the plasma samples was obtained from a standard curve prepared with human serum-based calibrators having aldosterone concentrations in the range of 25–1,200 pg/ml.

Under these conditions, the sensitivity of the assay was 19 pg/ml, the 50% displacement was at 340 pg/ml, and the intrassay coefficient of variation was 5.4%.

Urinary electrolyte concentrations. Daily urinary concentrations of sodium, potassium, and chloride were measured by flame photometry and electrometric titration, respectively, as previously described (30).
Table 1. Body weight changes in control and losartan-treated −/− and +/+ mice on high-salt diet for 4 wk

<table>
<thead>
<tr>
<th>Control</th>
<th>−/− (n = 7)</th>
<th>+/+ (n = 7)</th>
<th>End of Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>41.7 ± 2.9*†</td>
<td>33.9 ± 2.3*</td>
<td>18.5 ± 2.3</td>
</tr>
<tr>
<td>Day 7</td>
<td>31.4 ± 1.4†</td>
<td>28.3 ± 1.2</td>
<td>14.2 ± 3.5</td>
</tr>
<tr>
<td>Δ, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Losartan</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−/− (n = 5)</td>
<td>36.7 ± 3.0†</td>
<td>31.5 ± 2.9</td>
<td>−9.4 ± 2.8</td>
</tr>
<tr>
<td>+/+ (n = 6)</td>
<td>32.2 ± 1.7†</td>
<td>26.3 ± 1.9</td>
<td>−18.1 ± 4.7</td>
</tr>
<tr>
<td>2-Way ANOVA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P, genotypes</td>
<td>0.005</td>
<td>0.016</td>
<td>NS</td>
</tr>
<tr>
<td>P, treatments</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>P, interaction</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE, except for change (Δ), −/−, +/+, ANP knockout, wild-type mice, respectively; NS, not significant. *P < 0.05 between −/− and +/+ mice (unpaired t-test); †P < 0.05 between day 1 and day 7 (paired t-test).

Statistical analysis. All results are presented as means ± SE. The data were analyzed by two-way ANOVA to test for separate and combined effects of genotype and treatment (losartan vs. distilled water) on food and water intake and urinary excretion of fluid and electrolytes, ABP, HR, and total plasma catecholamine and aldosterone concentrations. Statistical differences between groups were isolated with a multiple comparison test using the Bonferroni method. Differences in food and water intake and urinary excretion of fluid and electrolytes between weeks 1 and 4 of the metabolic study were analyzed by t-test for each group. ANOVA of <0.05 was considered to indicate statistically significant difference.

RESULTS

Average body weight changes during the dietary regimen are shown for all groups in Table 1. Initial (day 1) body weights were significantly greater (P = 0.005) in −/− mice than in +/+ mice. All groups lost weight significantly by a similar relative amount during the first week, possibly due to a reduction in food intake consequent to changing from regular pelleted maintenance diet to the powdered HS treatment diet. However, body weight losses had stabilized afterward, and by the end of the dietary regimen, the body weights did not differ significantly from day 7 in all groups.

The cumulative food and water intake and urinary excretion of fluid and electrolytes for the first and fourth week of the dietary regimen are shown for all groups in Table 2. Food and water intake was significantly higher in +/+ mice compared with the −/− mice during the first week (P = 0.02), resulting in higher absolute urinary excretion of water and electrolytes. However, the relative urinary excretion of fluid and electrolytes, as represented by the ratio of urinary output to dietary intake, did not differ significantly between genotypes. In agreement with this, the hematocrits, measured at the end of the experiment, did not differ significantly between genotypes (−/− = 39.2 ± 2.3, +/+ = 38.5 ± 1.9). These genotype-related differences in food and water intake and urinary excretion of fluid and electrolytes were absent by the last week of the dietary regimen. There were no statistical differences (paired t-test) between week 1 and 4 in relative urinary excretion of water and electrolytes in any of the groups.

Losartan treatment did not significantly affect food and water intake or urinary excretion of fluid and electrolytes or hematocrit on either genotype during the period of the metabolic study.

Figure 1 shows the effect of chronic treatment with the AT1 antagonist losartan on ABP and HR of −/− and +/+ mice maintained on high-salt diet for 4 wk.

Table 2. Cumulative dietary intake of food and water and urinary output of water, sodium, chloride, and potassium for first and last weeks in control and losartan-treated −/− and +/+ mice maintained on high-salt diet for 4 wk

<table>
<thead>
<tr>
<th>Food</th>
<th>Water, ml/7 days</th>
<th>Na, mmol/7 days</th>
<th>Cl, mmol/7 days</th>
<th>K, mmol/7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>In, g</td>
<td>In</td>
<td>Out</td>
<td>Δ, %</td>
<td>In</td>
</tr>
<tr>
<td>Control</td>
<td>−/− (n = 6)</td>
<td>Week 1</td>
<td>16 ± 3</td>
<td>80 ± 8</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>16 ± 3</td>
<td>115 ± 8</td>
<td>81 ± 18</td>
</tr>
<tr>
<td></td>
<td>+/+ (n = 6)</td>
<td>Week 1</td>
<td>24 ± 5</td>
<td>108 ± 8</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>25 ± 2</td>
<td>125 ± 9</td>
<td>95 ± 9</td>
</tr>
<tr>
<td>Losartan</td>
<td>−/− (n = 5)</td>
<td>Week 1</td>
<td>16 ± 3</td>
<td>90 ± 10</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>17 ± 4</td>
<td>78 ± 16</td>
<td>76 ± 17</td>
</tr>
<tr>
<td></td>
<td>+/+ (n = 7)</td>
<td>Week 1</td>
<td>23 ± 4</td>
<td>112 ± 11</td>
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<tr>
<td></td>
<td>Week 4</td>
<td>17 ± 2</td>
<td>98 ± 8</td>
<td>80 ± 8</td>
</tr>
<tr>
<td>2-Way ANOVA</td>
<td>P, genotypes</td>
<td>Week 1</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>P, treatment</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Week 1</td>
<td>P, treatment</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SE. *P < 0.05 between weeks 1 and 4 (paired t-test).
+/+ mice maintained on 8% NaCl for 4 wk. Basal ABP (Fig. 1A) and HR (Fig. 1B) were significantly elevated in control −/− mice compared with control +/+ mice (ABP, −/− = 139 ± 5, +/+ = 82 ± 1 mmHg; HR, −/− = 504 ± 20, +/+ = 425 ± 25 beats/min P < 0.05). The antagonist had no effect on ABP and HR in +/+ mice, but reduced ABP and HR significantly (P, 0.05) in the −/− mice to the levels in the +/+ mice.

Total plasma catecholamine concentration (pg/ml) in control and losartan-treated −/− and +/+ mice is shown in Fig. 2. The control −/− mice had a significant 10-fold elevation in basal plasma catecholamine concentration compared with the control +/+ mice (−/− = 8,633 ± 1,769, +/+ = 827 ± 133; P < 0.05). Chronic treatment with losartan lowered plasma catecholamine significantly (P < 0.05) in the −/− mice approximately to the levels in control +/+ mice and tended to increase plasma catecholamine concentration in the +/+ mice.

Plasma aldosterone concentrations (pg/ml) in the same control and losartan-treated mice are shown in Fig. 3. Plasma aldosterone did not differ significantly between control −/− and +/+ mice (−/− = 137 ± 23, +/+ = 156 ± 37). Losartan treatment did not significantly alter plasma aldosterone in either genotype. However, there was a trend toward lower aldosterone concentration in the losartan-treated −/− mice relative to the control −/− mice.

DISCUSSION
Chronic increase in dietary salt intake is normally accompanied by homeostatic deactivation of salt-conserving mechanisms, such as the renin-angiotensin system (RAS; Ref. 7), aldosterone (14), and SNA (7, 9). There is evidence that ANP is, at least partially, involved in suppressing the activity of antinatriuretic mechanisms during HS intake (19, 39). The present study shows that chronic inhibition of AT1 receptor function abrogates the difference in ABP between +/+ mice and genetically matched ANP-deficient mice (−/−)
fed on HS diet, while having no effect on +/+ mice. Thus, in conjunction with our previous finding that the salt sensitivity of ABP (18, 26) in −/− mice fed the same diet is associated with failure to downregulate PRA (26), the current study establishes conclusively that the inability to suppress the activity of the RAS plays a causal role in the sensitization of ABP to dietary salt in these mice. This strengthens the premise that ANP-dependent antagonism of RAS activity is essential for the chronic adaptation to high salt intake.

In analogy with the hemodynamic, hormonal, and renal changes seen in experimental ANG II-dependent salt-sensitive hypertension (20), we hypothesized that the salt-fed −/− mice would require an increase in ABP and renal perfusion pressure to achieve long-term salt balance against the background of antinatriuresis imposed by the tonically elevated ANG II levels (26) and by the absence of direct natriuretic action of ANP in the inner medullary collecting duct (15). The current observation that the losartan-treated −/− mice maintain salt balance despite the marked fall in ABP, supports the contention that the primary role of elevation of ABP in the salt-fed −/− mice is to overcome the reduced sensitivity of the pressure natriuresis mechanism brought about by the persistent antinatriuretic action of ANG II in the proximal tubule. Indeed, if this were not the case, then the decline in ABP that occurs in the −/− mice with chronic losartan treatment would have been expected to lead to relative salt retention. In this regard, it is noted that ANP has been reported to increase the sensitivity of the pressure natriuresis mechanism, in part, by inhibiting ANG II-mediated sodium reabsorption in the proximal tubule (13, 27). On this basis, we suggest that the salt-induced elevation of ABP in the −/− mice is essential for maintenance of salt balance as compensation for the absence of ANP-dependent antagonism of the antinatriuretic action of ANG II. On the other hand, the +/+ control mice display normal ANP bioactivity and are capable of deactivating antinatriuretic mechanisms during high dietary salt intake, thus precluding the need for a rise in ABP.

The question remains how does the derangement in ANG II synthesis lead to sensitization of ABP to salt in the −/− mice? Chronic infusion of ANG II, even at subpressor doses, leads to hypertension, which is exacerbated by high dietary salt (20). Although the direct renal and vascular effects of ANG II contribute to the hypertension (33), there is evidence that the widespread sympathoexcitatory activity of ANG II (32) plays a major role in the maintenance of hypertension (4). On the basis of this evidence, we anticipated that the increased level of ANG II in the salt-fed −/− mice could provide continuous stimulatory input to the sympathetic nervous system and that the resultant increase in SNA would, at least in part, account for the pressor effect of salt. The present study shows that the salt-fed −/− mice have inappropriately elevated sympathetic tone, as indicated by the 10-fold elevation in total plasma catecholamine concentration and by the higher basal HR in relation to the similarly maintained +/+ mice. Interestingly, the differences in plasma catecholamine concentration and HR between the −/− and +/+ mice are abolished by losartan, thus showing the dependency of the elevated basal sympathetic tone on ANG II activity. This implies that the sensitization of ABP to salt in the −/− mice is, at least in part, due to the tonic potentiation of SNA by ANG II. Unexpectedly, losartan tends to increase plasma catecholamine concentration in +/+ mice. This apparently paradoxical effect may reflect a compensatory response aimed at counterbalancing a possible hypotensive effect of losartan in these mice. The mechanism and site(s) of ANG II-induced sympathoexcitation in the salt-loaded −/− mice, however, remain to be elucidated.

In conclusion, the current study shows that chronic inhibition of AT₁ receptor function prevents the development of salt sensitivity of ABP in −/− mice, thereby implicating a causal role of inappropriately elevated activity of the RAS in mediating the sensitization of ABP to salt in these mice. We postulate that the resultant tonic potentiation of sympathetic tone by ANG II contributes, at least in part, to the hypertensive effect of salt. The ensuing increase in ABP overcomes the background of antinatriuresis associated with elevated ANG II and permits long-term maintenance of salt and volume balance by pressure natriuresis.

Perspectives

Previous studies from our laboratory showed that PRA fails to decrease during prolonged high salt intake in ANP gene knockout mice (26), leading to salt-sensitive hypertension. The current investigation extends these findings and provides evidence that the
manifestation of salt sensitivity in the --/-- mice is associated with, presumably, synergistic interactions between ANG II and the sympathetic nervous system, likely leading to a reduction in the sensitivity of the pressure-natriuresis mechanism. These findings suggest that ANP-mediated antagonism of RAS is an essential component of the adaptation to chronic high salt intake.

Deficiencies in ANP activity and/or target organ responsiveness have been reported in several experimental and natural variants of salt-sensitive hypertension (6, 17, 28), as well as in other sodium-retaining disorders such as congestive heart failure and cirrhosis (24). A dysfunction in PRA regulation strikingly similar to that of ANP knockout mice has been reported in Dahl salt-sensitive rats. These rats also fail to suppress PRA (3) and SNA (11) when fed on HS, and, consequently, they develop salt-sensitive hypertension. Interestingly, the development of salt-sensitive hypertension in these rats is prevented by exogenous ANP gene delivery (23), implying that a deficiency in ANP activity may be an underlying cause of the salt sensitivity in this hypertensive model. In congestive heart failure, there is a marked hyperresponsiveness to ANP, which can be corrected by inhibition of RAS (25), suggesting that in this condition, the ANP-mediated antagonism of RAS is attenuated, and this could partly account for the elevated sympathetic tone characteristic of this disease. In their totality, these observations indicate that weakened or reduced effectiveness of ANP-dependent inhibition of RAS may be an underlying causative factor in these diseases.

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