Dietary salt supplementation selectively downregulates NPR-C receptor expression in kidney independently of ANP

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Sun, Ju-Zhong, Shi-Juan Chen, Erum Majid-Hasan, Suzanne Oparil, and Yiu-Fai Chen. Dietary salt supplementation selectively downregulates NPR-C receptor expression in kidney independently of ANP. Am J Physiol Renal Physiol 282: F220–F227, 2002. First published August 30, 2001; 10.1152/ajprenal.00166.2001.—Atrial natriuretic peptide (ANP) is a peptide hormone that has potent natriuretic, diuretic, vasodilator, sympatholytic, and renin- and aldosterone-suppressing activities and is involved in the regulation of volume and electrolyte balance and blood pressure (3–5, 25). The biological functions of ANP are mediated through activation of the NPR-A receptor and increases in intracellular cGMP (6). Circulating levels of ANP are regulated by binding to and inactivation by NPR-C, a membrane-bound receptor that functions as a clearance receptor to eliminate ANP from the circulation (16, 19), as well as hydrolysis by neutral endopeptidase 24.11 (NEP) (7).

Several lines of evidence indicate that ANP is involved in the pathogenesis of salt-sensitive hypertension (29). Chronic blockade of endogenous ANP with a monoclonal antibody accelerates the development and exacerbates the severity of hypertension in stroke-prone, spontaneously hypertensive rats (SHR-SP) and DOCA-salt hypertensive rats (8). Dietary salt supplementation in normotensive salt-resistant rats is associated with increased plasma ANP levels (11), whereas salt-sensitive SHR do not increase plasma ANP levels appropriately in response to dietary NaCl supplementation, thus failing to mount a natriuretic response and normalize blood pressure in the presence of dietary NaCl stress (11, 13). Furthermore, administration of either exogenous ANP or the NEP inhibitor SCH-34826, which protects endogenous ANP from hydrolysis, prevents salt-sensitive hypertension in SHR (12).

NPR-C accounts for the overwhelming majority of ANP binding sites in most tissues, including kidney, lung, and brain (16), and plays an important role in ANP clearance. Blocking NPR-C with the selective antagonist C-ANP4–23 markedly increases (∼3-fold) circulating ANP levels (19) and attenuates hypoxia-induced pulmonary hypertension in the rat (10). Furthermore, the half-life of ANP in the circulation has been shown to be two-thirds longer in mice with homologous deletion of the NPR-C gene (Npr3−/−) compared with wild-type mice (21).

We have previously demonstrated that NPR-C, but not NPR-A, gene expression is selectively downregulated in the lungs of rats and mice in response to the stress of hypoxia (17, 27). This phenomenon was found to be mediated by overexpression of tyrosine kinase-activating growth factors, such as acidic fibroblast...
growth factor (FGF-1), and was seen even in the absence of ANP gene expression (28). We hypothesize that selective downregulation of NPR-C expression in lung in the setting of hypoxia may contribute to the increase in circulating ANP levels seen under hypoxic conditions and may enhance the vasodilator effects of ANP in lung, thus modulating hypoxic pulmonary vasoconstriction/hypertension.

The present study tested the hypothesis that, under the stress of dietary salt supplementation, NPR-C gene expression is selectively downregulated in kidneys of wild-type (ANP+/+) mice and mice with insertion inactivation of the ANP gene (ANP−/−). We hypothesize that local potentiation of ANP [and possibly other natriuretic hormone(s)] protects against the development of salt-sensitive hypertension by this mechanism.

METHODS

Transgenic mice. Mice with a mutated pro-ANP gene (Nppa), ANP−/−, generated by John et al. (14), were used (Jackson Laboratory, Bar Harbor, ME). The animals were raised from our resident colony, which was founded with pathogen-free breeding pairs. The genotypes were identified by PCR assay of genomic DNA from tail snips after the mice were weaned. All mice were housed in groups of three to four per cage and maintained at a constant humidity (60 ± 5%), temperature (24 ± 1°C), and light cycle (6 AM–6 PM). All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and were consistent with the Guide for the Care and Use of Laboratory Animals [DHEW Publication No. (NIH) 85–23, revised 1985].

In ANP−/− mice, a disruptive neomycin resistance gene (Neo) inserted into exon 2 of the pro-ANP gene, interrupts the expression of ANP mRNA (14). In the present study, we applied RT-PCR and DNA sequencing methods to test whether the mutated Nppa gene in ANP−/− mice was functional. Our RT-PCR/DNA sequencing data indicated that the mutated Nppa gene was still capable of functioning as a template to produce mRNA in vivo. However, the mutated mRNA contained a truncated exon 2 of the Nppa gene and a 44-bp fragment of the Neo gene. These data confirmed that ANP−/− mice have disrupted pro-ANP mRNA and therefore cannot generate intact and functional ANP protein (Fig. 1).

High-salt dietary regimen, hemodynamic measurement, and tissue collection. Male ANP+/+ and ANP−/− mice (9–10 wk old), weighing 25–30 g, were used in this study. Half of the mice in each genotypic group (ANP+/+ and ANP−/−) were placed at random on a high-salt diet (3.2% Na+, 4.8% Cl−, and 0.68% Ca2+, Teklad diet 92012, Harlan-Teklad, Madison, WI) for 5 wk, whereas the other half remained on the standard salt diet (0.27% Na+, 0.28% Cl−, and 0.77% Ca2+, Teklad diet 8746, Harlan-Teklad). Food and water were available ad libitum throughout the study. Five weeks after initiation of the special diet, mice were weighed and anesthetized with a mixture of ketamine (10 mg/100 g im) and xylazine (1.5 mg/100 g im). A PE-10 catheter was implanted into the left common carotid artery through the left external carotid artery. Twenty-four hours after surgery, the arterial catheter was connected to a pressure transducer coupled to a Biopac model MP100 data-acquisition unit with output to a PC sampling at 100 Hz and using Acknowledge Software (Biopac Systems, Santa Barbara, CA). MAP and heart rate (HR) were measured simultaneously in conscious mice.

After hemodynamic measurements were taken, mice were killed by cervical dislocation and blood for ANP assay was withdrawn from the right ventricle (RV) and placed in iced tubes containing 2.25 mg of EDTA and 1.5 trypsin inhibitor units of aprotinin. Plasma was separated by centrifugation and stored at −80°C until used for RIA for ANP. The hearts, kidneys, lungs, and brains were removed quickly and

Fig. 1. DNA sequences of mutant and wild-type alleles. RT-PCR analysis of intact and mutated atrial natriuretic peptide (ANP) mRNA were carried out on 5 μg of total RNA from lungs of wild-type (ANP+/+) and homozygous mutant (ANP−/−) mice as a template. Twenty micrometers of the DNA from RT-PCR were used for DNA sequencing. A: the same pair of primers amplified a 462- and 313-bp cDNA fragment from lung RNA of ANP+/+ and ANP−/− mice, respectively, during RT-PCR. B and C: DNA sequencing indicated that the 462-bp fragment from ANP+/+ mice matched the authentic sequence of ANP mRNA and the 313-bp fragment from ANP−/− mice contained a truncated exon 2 of ANP gene and a 44-bp fragment of neomycin gene (Neo). D: the selective sense and antisense primers of ANP gene used in RT-PCR.
weighed. The atria and the RV-free wall were dissected from the left ventricle (LV) and septum, and each chamber was weighed. The RV and/or LV weight-to-body weight ratios were used as indexes of cardiac hypertrophy. Tissues were then frozen in liquid N₂ and stored at −80°C until RNA extraction.

RNA isolation and Northern blot analysis. Total RNA was extracted by the guanidine thiocyanate method, and Northern analysis was performed as previously described (17), using 3²P-labeled selective cDNA probes for ANP (courtesy of Dr. R. Wiegand at Monsanto, St. Louis, MO) and cDNA probes for NPR-A and NPR-C that had been generated in our laboratory by RT-PCR, using lung RNA as the template as previously described (17). Between each reprobing, 3²P-labeled cDNA was stripped off the membrane by pouring boiling 0.1% standard sodium citrate, 0.1% SDS onto the membrane and shaking for 20 min at room temperature. To quantitate the amount of RNA loaded, blots were stripped as above and rehybridized with a control probe, a 3²P-labeled GAPDH probe. Autoradiographic signals were scanned with an optical densitometer (GS-670 Imaging Densitometer, Bio-Rad, Hercules, CA). The results were expressed as the ratios of specific mRNA to GAPDH mRNA.

ANP and cGMP measurements. Plasma ANP content was measured by RIA with ANP RIA kits (Peninsula Laboratories, Belmont, CA) after extraction with Sep-Pak C₁₈ cartridges (Waters Associates, Milford, MA) as previously described (27). Tissue cGMP content was measured by RIA with cGMP RIA kits (DuPont NEN Research Products, Boston, MA) as previously described (27). The sensitivities of the ANP and cGMP RIAs are 2 pg and 25 fmol/assay tube, respectively.

Statistical analysis. Results are expressed as means ± SE. Statistical analyses were carried out using the CRUNCH statistical package (CRUNCH Software, Oakland, CA) on an IBM PC-compatible computer. The data were analyzed by 2-way ANOVA to test the effects of genotypes or high-salt diet on the above variables. Differences were reported as significant if the P value was <0.05.

RESULTS

Body weights in the normal-salt groups did not differ between genotypes (Table 1). ANP+/+, but not ANP−/−, mice fed the high-salt diet had slightly but significantly lower body weights than their normal salt controls. MAP of the ANP−/− mice was significantly greater than that of ANP+/+ mice on the normal-salt diet (Table 1, Fig. 2A), and dietary salt supplementation produced significant increases in MAP in ANP−/− (22.2 ± 4.3 mmHg or 20.6 ± 4.0% increase, P < 0.05, compared with normal-salt ANP−/−) but not in ANP+/+ mice (7.6 ± 3.8 mmHg or 8.3 ± 4.2% increase, P > 0.1, compared with normal-salt ANP+/+). No differences in HR between genotypes or diet groups were observed (Table 1).

As anticipated, plasma ANP and steady-state ventricular (LV and RV) ANP mRNA levels were barely detectable in either normal- or high-salt-fed ANP−/− mice (Fig. 2, B, D, and F). Salt supplementation significantly increased plasma ANP and LV, but not RV, ANP mRNA levels in ANP+/+ mice. ANP−/− mice on the normal-salt diet had heavier atria (Table 1), LV (Fig. 2C), RV (Fig. 2E), and LV+RV (Table 1) than ANP+/+ mice. The high-salt diet was not associated with significant enlargement of atria, LV, or RV in ANP+/+ mice but was associated with significant increases in atrial, LV, RV, and LV+RV weights in ANP−/− mice. The increases in atrial, LV, RV, and LV+RV weights were significantly greater in ANP−/− mice than in ANP+/+ mice on the high-salt diet (P < 0.05; 2-way ANOVA showed a significant interaction between salt and ANP genotype on atrial, LV, RV, and LV+RV weights). Kidney, brain, and lung weights did not differ between the two mouse genotypic groups on the normal-salt diet. The high-salt diet increased kidney, but not brain or lung, weights in ANP−/− mice. Steady-state NPR-C mRNA levels were significantly lower in both kidneys (Fig. 3A) and lungs (Fig. 3C) of ANP−/− mice compared with ANP+/+ mice on the normal-salt diet. Dietary salt supplementation was associated with major (68 ± 9 and 60 ± 7% in ANP+/+ and ANP−/− mice, respectively) reductions in NPR-C expression in kidney only (Fig. 3A). NPR-C expression in lung was not sensitive to salt intake (Fig. 3C). NPR-C expression in heart (LV and RV) (Fig. 3, E and G) and brain (Fig. 3I) did not differ between genotypes or diet groups. Unlike NPR-C, steady-state NPR-A mRNA levels in all organs examined did not differ between genotypes or dietary regimens (Fig. 3, B, D, F, H, and J).

Kidney cGMP content in the normal-salt groups did not differ between genotypes (Fig. 4). Dietary salt supplementation was associated with significant increases in kidney cGMP content in both groups. The increases

Table 1. Effects of 5-wk high-salt dietary supplementation on body weight, blood pressure, and tissue weight of ANP+/+ and ANP−/− mice

<table>
<thead>
<tr>
<th>Genotype/Condition</th>
<th>BW, g</th>
<th>MAP, mmHg</th>
<th>HR, bpm</th>
<th>Atria/BW, mg/g</th>
<th>(RV + LV)/BW, mg/g</th>
<th>Kidney/BW, mg/g</th>
<th>Brain/BW, mg/g</th>
<th>Lung/BW, mg/g</th>
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<tr>
<td>ANP+/+</td>
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<tr>
<td>Normal salt (10)</td>
<td>33.5 ± 0.9</td>
<td>90.6 ± 3.9</td>
<td>422 ± 11</td>
<td>0.29 ± 0.02</td>
<td>4.19 ± 0.22</td>
<td>11.7 ± 0.6</td>
<td>14.7 ± 0.4</td>
<td>5.53 ± 0.18</td>
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<tr>
<td>High salt (8)</td>
<td>26.5 ± 1.1*</td>
<td>98.2 ± 5.7</td>
<td>389 ± 25</td>
<td>0.27 ± 0.01</td>
<td>4.47 ± 0.11</td>
<td>13.0 ± 0.7</td>
<td>15.3 ± 0.7</td>
<td>5.79 ± 0.32</td>
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<tr>
<td>ANP−/−</td>
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<tr>
<td>Normal salt (10)</td>
<td>31.6 ± 3.0</td>
<td>107.6 ± 3.8†</td>
<td>429 ± 21</td>
<td>0.44 ± 0.05†</td>
<td>5.39 ± 0.39†</td>
<td>13.5 ± 1.3</td>
<td>13.9 ± 0.5</td>
<td>5.43 ± 0.36</td>
</tr>
<tr>
<td>High salt (7)</td>
<td>30.8 ± 1.0</td>
<td>129.8 ± 9.9‡</td>
<td>386 ± 28</td>
<td>0.67 ± 0.02‡</td>
<td>7.10 ± 0.31‡</td>
<td>16.7 ± 0.4‡</td>
<td>14.0 ± 0.6</td>
<td>5.42 ± 0.30</td>
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Values are means ± SE with no. of animals/group in parentheses. BW, body weight; MAP, mean arterial pressure; HR, heart rate; †, ratio; RV, right ventricle; LV, left ventricle. *P < 0.05 vs. respective normal-salt-diet groups; †P < 0.05 vs. respective ANP+/+ groups.
in kidney cGMP content were significantly greater in ANP+/+ mice than ANP−/− mice fed the high-salt diet (P < 0.05; 2-way ANOVA showed a significant interaction between salt and ANP genotype) (Fig. 4A). cGMP content in lung (Fig. 4B) and brain (Fig. 4C) did not differ between genotypes or diet groups.

DISCUSSION

The major finding of the present study was that dietary salt supplementation was associated with a selective reduction in steady-state NPR-C mRNA levels in kidney of ANP+/+ and ANP−/− mice. Major (> 60%) reductions in renal NPR-C mRNA were seen in both genotypes despite major differences in circulating ANP levels, steady-state ANP mRNA expression, and MAP, indicating that the high-salt-induced down-regulation of NPR-C expression in kidney is independent of endogenous ANP and blood pressure levels.

The finding that expression of NPR-C is selectively reduced in kidneys of both ANP+/+ and ANP−/− mice fed a high-NaCl diet is in agreement with previous reports by Nagase et al. (23). They demonstrated that NPR-C gene expression was selectively reduced in the kidney by chronic salt loading (8% NaCl diet for 4 wk) in both Dahl salt-sensitive (DS) and salt-resistant (DR) rats, whereas expression of NPR-A and NPR-B was not altered. The reduction in renal NPR-C mRNA levels in response to salt supplementation was greater in DS (−53%) than in DR rats (−28%). However, because the plasma ANP levels were significantly increased in salt-loaded DS rats, but not in salt-loaded DR rats, the above-mentioned investigators could not determine whether the downregulation of NPR-C gene expression was caused by a ligand-mediated mechanism. The present study demonstrated that the selective down-regulation of NPR-C expression in kidney of salt-supplemented subjects is independent of endogenous ANP levels.

Our present findings are consistent with previous observations in animal models and humans that ANP modulates blood pressure and protects against the development of salt-sensitive hypertension. Studies from our own laboratory (11–13) demonstrated that high-salt-diet-induced ANP secretion induces vasodilation and natriuresis that modulate the development of salt-sensitive hypertension in SHR. John et al. (14) and Melo et al. (22) have demonstrated that ANP−/− mice manifest increases in blood pressure in response to dietary salt supplementation. Furthermore, NPR-A null mice fed either low (0.008% NaCl)-, normal (0.7% NaCl)-, or high (8% NaCl)-salt diets have elevated blood pressures compared with wild-type controls (18). Conversely, transgenic mice that overexpress ANP are characterized by markedly decreased arterial pressure (26), whereas NPR-C null mice exhibit a prolonged half-life of circulating ANP and decreased blood pres-
The relevance of these observations to human hypertension is demonstrated by our previous finding that, in NaCl-sensitive hypertensive African-Americans, a high-NaCl diet causes an increase in arterial pressure and, paradoxically, a decrease in plasma ANP (1). Thus a deficiency in circulating ANP under conditions of dietary NaCl stress likely contributes to salt-sensitive hypertension in humans due to the lack of this important natriuretic/vasodilatory factor.

Approximately 99% of all ANP receptors in kidney are NPR-C, and these are localized mainly in the vascular and glomerular structures of the cortex, particularly in the podocytes of glomerular cells (16, 19, 23).
The direct renal actions of ANP to promote natriuresis and diuresis are mediated through relaxation of preconstricted renal vasculature and increases in renal blood flow and glomerular filtration rate (2). The present observation of selective reductions in NPR-C in kidney under conditions of dietary salt supplementation suggests a mechanism of locally regulating ANP concentrations to facilitate natriuresis/diuresis and the maintenance of volume homeostasis. Reductions in NPR-C density on the surface of ANP target cells, such as kidney vessels and glomeruli, would tend to retard the clearance of ANP from the renal circulation and thus increase the local availability of ANP to the biologically active NPR-A receptor, which is not downregulated during salt supplementation, leading to augmented vasodilator and natriuretic/diuretic effects. Guanylate cyclase-coupled NPR-A receptors are expressed in renal vessels, glomeruli, and papillae (20, 23), so the ANP that is potentiated by local downregulation of NPR-C can stimulate natriuresis/diuresis at both vascular and tubular levels.

The present finding that the cGMP content of the kidney, but not of the lung and brain, of high-salt-fed ANP+/+ mice was significantly increased (>5-fold) compared with ANP+/+ mice on the normal-salt diet provides further evidence that the downregulation of NPR-C potentiates the biological effects of ANP in kidney. This inhomogeneity of the cGMP response among target organs suggests that the increase in renal cGMP levels was not simply a consequence of the doubling of plasma ANP seen during dietary salt supplementation. Interestingly, the cGMP content of kidneys of high-salt-fed ANP−/− mice was also slightly but significantly increased. Our interpretation is that the dietary salt supplementation-induced downregulation of NPR-C may also potentiate the stimulatory effect of B-type natriuretic peptide (BNP) on cGMP production in the kidney in both genotypes. BNP has cardiovascular effects similar to those of ANP and, like ANP, acts through the NPR-A and NPR-B receptors to enhance intracellular cGMP (4–6). It has been postulated that ANP and BNP participate in a complementary “dual-peptide system” to modulate vascular responses and intravascular fluid homeostasis and that BNP may act as an emergency molecule against ventricular overload (4–6). If this were the case, it might be expected that when ANP is deficient or lacking, BNP synthesis would be increased and compensate for the lack of ANP in the present model. A limitation of the present study is the inability to measure BNP.

Mouse BNP antigen could not be measured because of the unavailability of a selective antibody for mouse BNP. Future studies in our laboratory are needed to address this deficiency.

The present findings and our previous studies in hypoxia-adapted animals have shown that ANP levels can be regulated locally in the target organs, thereby allowing the function of the natriuretic peptide system to be tailored to specific local needs. We have previously demonstrated that steady-state NPR-C mRNA expression is selectively downregulated in the lungs of hypoxia-adapted rats (17) and mice (27) in association with increased circulating levels of ANP. In contrast, mRNA levels of NPR-A and NPR-B are unchanged or increased in lungs under these conditions. The reduction in NPR-C in the hypoxic lung retards the clearance of ANP and allows more ANP to bind to biologically active NPR-A in the pulmonary circulation, thereby activating guanylate cyclase, increasing cGMP production, relaxing preconstricted pulmonary vessels, reducing pulmonary arterial pressure, and attenuating the development of hypoxia-induced pulmonary hypertension and vascular remodeling.

The cellular/molecular mechanisms by which NPR-C is regulated have received considerable attention in our laboratory and others. Downregulation of NPR-C in tissues has been attributed to elevated ANP levels in the circulation, which stimulate increased cGMP production through the activation of NPR-A and guanylate cyclase (16). However, our experience with the ANP−/− mouse indicates that NPR-C expression is reduced selectively in critical target organs of mice
subjected to stress conditions (e.g., hypoxia, dietary salt supplementation) in the complete absence of circulating ANP (27). Furthermore, we recently demonstrated that neither ANP nor cGMP in high concentrations reduced NPR-C gene expression in pulmonary arterial smooth muscle cells (PASMCs) in vitro (28).

In contrast, a variety of growth factors, including fibroblast growth factors (FGF-1 and FGF-2), protein kinase A, protein kinase C, and β-adrenergic agonists (9, 16, 24, 27), have been shown to regulate ANP receptors in tissues. We have recently shown that FGF-1, FGF-2, and platelet-derived growth factor (PDGF)-BB induce rapid dose- and time-dependent reductions in NPR-C mRNA expression in PASMCs in vitro (28). The rapid (within 1 h) reduction in NPR-C mRNA levels observed in this study in response to very low concentrations (1 ng/ml to 40–60 pM) of FGF-1, FGF-2, and PDGF-BB suggests that these growth factors could be physiological regulators of NPR-C gene expression in vivo. Studies using inhibitors of signal transduction pathways indicate that the inhibitory effects of FGF and PDGF on NPR-C mRNA expression are mediated through activation of membrane tyrosine kinase receptors and intracellular Ras-Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase-MAPK signaling transduction pathways. Whether these regulators are involved in the high-salt-loading-induced downregulation of NPR-C gene expression in kidney deserves further investigation.

Other mechanisms adduced to explain the selective downregulation of NPR-C in kidneys of mice fed a high-salt diet include the direct effect of NaCl on NPR-C gene expression. Katafuchi et al. (15) reported that the NPR-C levels in cultured bovine carotid artery endothelial cells are sensitive to changes in the salt concentration of the culture medium. High-NaCl (50 mM) medium induced a marked reduction in the number of NPR-C, whereas NPR-A density was not affected by the treatment. However, these authors did not carry out further investigations to reveal the signaling mechanism(s) by which NaCl-mediated alterations in NPR-C expression occur.

In summary, this study provides evidence that chronic oral salt supplementation can selectively downregulate renal NPR-C gene expression independently of changes of ANP levels and expression of NPR-A. Downregulation of NPR-C likely represents an adaptation aimed at reducing ANP clearance from the circulation, thus enhancing the vasodilatory and natriuretic effects of ANP in the kidney and mitigating the severity of salt-sensitive hypertension. These findings support the interpretation that NPR-C plays an important role in the regulation of NaCl balance and the pathophysiology of salt-sensitive hypertension.

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