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Dietary Sodium Modulates Neonatal but Not Adult Cardiac Atrial Natriuretic Peptide in Rats

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ABSTRACT

After an initial postnatal diuresis, neonates are in positive sodium balance. Because atrial natriuretic peptide (ANP) contributes to sodium homeostasis, this study was designed to evaluate the maturational effects of increased dietary sodium intake on cardiac ANP production. Preweaned Sprague-Dawley rat pups were artificially reared by feeding them either a normal-sodium or high-sodium diet for 7 d and were compared with maternally reared rat pups. Adult rats were divided into three groups: the first group was given *ad libitum* rat food and 1% sodium chloride to drink, the second group was pair-fed with this group but given tap water to drink, and the third group was fed *ad libitum* rat food and water for 10 d. Atrial and ventricular pro-ANP and ANP contents and plasma ANP concentrations were measured by RIA. Steady state atrial and ventricular ANP mRNA expression was determined by Northern and dot-blot analysis. There was a 2-fold increase in atrial pro-ANP and ANP content and a 50% decrease in plasma ANP concentration in preweaned rat pups fed a high-salt diet. In contrast, atrial pro-ANP and ANP content and plasma ANP concentration were not affected by increased sodium intake in adult rats. Atrial and ventricular ANP mRNA levels and ventricular pro-ANP and ANP contents were not altered by dietary sodium at either age. We conclude that chronic increase in sodium intake in the preweaning period results in increased storage of atrial pro-ANP. The decrease in plasma ANP concentration in these preweaned rats may be due to reduced basal secretion or enhanced degradation of the peptide. (***Pediatr Res* 37: 310–315, 1995**)

Abbreviations

AH, adult high-sodium diet
AL, adult *ad libitum* diet
AN, adult normal-sodium diet
ANF, atrial natriuretic factor
ANP, atrial natriuretic peptide
GAPDH, glyceraldehyde 3-phosphate dehydrogenase
PWH, preweaned high-sodium diet
PWM, preweaned maternally reared
PWN, preweaned normal-sodium diet
C-receptor, clearance receptor

ANP, secreted primarily from atrial myocytes, exhibits natriuretic, diuretic, and vasorelaxant properties. Since 1981, when ANP was first described by de Bold *et al.* (1), the production, secretion, and actions of the peptide have been further elucidated. Although atrial stretch is generally recognized as the major stimulus for ANP release, recent studies suggest an important role for sodium ingestion in ANP regulation (2–4).

In term and preterm infants, there is an initial diuresis and natriuresis that occur in conjunction with an increase in plasma ANP concentration (5, 6). Thereafter, plasma ANP concentration falls to or below adult levels (7). After the initial postnatal diuresis, preweaned animals are in positive sodium balance, which is necessary for normal somatic growth (8). They have an attenuated response to acute volume expansion and do not excrete a sodium load as adults do (9–11). This finding has previously been attributed to the immaturity of the neonatal kidney (12).

The present studies were designed to test the hypothesis that in normal preweaned animals the activity of the ANP system is reduced as a consequence of the limited sodium intake provided by maternal milk. Preweaned rat pups were artificially reared by gastrostomy feeding with a formula containing either a normal or high sodium content. Adult rats were divided into normal- and high-sodium groups and received isocaloric diets (pair-fed). Atrial and ventricular pro-ANP and ANP content, plasma ANP concentration, and atrial and ventricular ANP

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mRNA levels were determined after 7–10 d of dietary manipulation.

METHODS

Animal preparation. Preweaned and adult male Sprague–Dawley rats (Hilltop, Scottsdale, PA) were used for the study. Gastrostomy feeding tubes were inserted into 7-d-old preweaned rat pups that were under methoxyflurane anesthesia (13). A Silastic (Dow Corning, Midland, MI) covered steel wire was orally inserted and passed into the stomach. After tactile confirmation of placement, the steel rod was pushed through the abdominal wall. A flanged polyethylene tube previously fitted with a plastic washer was attached to the oral end of the steel rod and gently drawn into the stomach. The flanged tubing and washer remained in the stomach, while the free end of the tube was fixed at the neck to avoid excessive tension on the intragastric tube. A specially formulated diet, similar in composition to rat milk, was fed by intermittent infusion for 12 20-min periods during each 24 h for 7 d. The diet, consisting of Enfamil Infant Formula (Mead Johnson, Evansville, IN) supplemented with soy protein 5.6 mg/mL (Protein Technologies International, St. Louis, MO), corn oil 7.2 mg/mL (Mazola, Best Foods, CPC International, Inc., Englewood Cliffs, NJ), multiple vitamins 50 μL/mL (Ross Laboratories, Columbus, OH), and copper 3.8 μg/mL, contained either normal sodium (25 mmol/L, PWN, $n = 18$) or high sodium (145 mmol/L, PWH, $n = 17$). The normal range of sodium content in rat milk has been determined in previous studies (14, 15). Intermittent infusion was by a microcontrolled multiple syringe pump (Harvard Apparatus, Inc., Boston, MA). Animals were housed in individual containers in a temperature (38–40°C)- and light-controlled (12 h light/dark) environment. Animals were weighed daily, and dietary volume was adjusted to provide 33% of the average body weight (16). Maternally reared rat pups were used as controls (PWM, $n = 19$).

Adult male rats weighing 200–255 g were studied for 10 d. Animals were placed in individual cages in a temperature- and light-controlled environment at the beginning of the study. Animals were fed either *ad libitum* Rat Chow (Ralston Purina, St. Louis, MO) containing 15.6 mmol sodium/100 g and tap water (AL, $n = 18$) or *ad libitum* Rat Chow and 1% NaCl solution to drink (AH, $n = 18$). In addition, a group of rats drinking tap water *ad libitum* were pair-fed with the AH group to control for caloric intake (AN, $n = 18$). Animals were weighed and the amount of Rat Chow and drink consumed was measured daily. Preweaned pups were treated for 7 d and adult rats were treated for 10 d to allow time for adults to adapt to their environment and diet.

Tissue preparation. At completion of the study, animals were anesthetized with intraperitoneal pentobarbital, 50 mg/kg intraperitoneally. After the peritoneum was opened, the abdominal aorta was cannulated and at least 1 mL of blood was withdrawn into an EDTA-coated syringe. The blood was immediately centrifuged at 4°C, and plasma was removed and stored at −70°C until assayed. The heart was excised, and atria and ventricles were separated, weighed, and frozen in liquid nitrogen. Tissue was stored at −70°C until used.

ANP and pro-ANP assay. ANP was extracted from combined right and left atria and combined ventricles by homogenization (Polytron, Brinkmann, Luzerne, Switzerland) in a 0.1 M acetic acid solution containing 1.0 μM aprotinin, 0.4% BSA, 0.1% bacitracin, 4.0 μM leupeptin, and 1.0 μM pepstatin A. The homogenate was centrifuged (10 000 × *g*) at 4°C for 10 min. Atrial samples were diluted 1:600 in assay diluent, and ventricular samples were diluted 1:4. ANP content in each sample was determined by RIA as described below. The protein content of the supernatant was determined by the Lowry assay using BSA (Sigma Chemical Co., St. Louis, MO) as the standard (17).

Plasma and tissue ANP concentration was measured by RIA as previously described (18). Briefly, frozen plasma samples were thawed and centrifuged (1700 × *g*) at 4°C for 20 min, then applied to Pre-Sep C18 (Fisher, Pittsburgh, PA) columns that were preconditioned with 100% methanol and water. Samples were then loaded on the columns, followed by a 1% trifluoroacetic acid wash. The samples were eluted with 1.5 mL of ethanol/water/acetic acid (90:6:4 vol), then dried with air in a 60°C water bath. The dried samples were reconstituted in assay diluent and placed on ice. RIA was performed with ^{125}I-labeled α-ANF-(1–28) and an NH_2-terminal sensitive antiserum (Amersham, Arlington Heights, IL). Tubes containing 25 μL of ^{125}I-labeled ANF, 25 μL of antiserum, and 50 μL of sample or rat α-ANF-(1–28) standard were incubated for 48–72 h at 4°C. Bound ANF was precipitated with goat anti-rabbit antiserum (Calbiochem, San Diego, CA) and separated from free tracer by centrifugation (1700 × *g*) for 30 min. Recovery of 20 pg of unlabeled rat α-ANF-(1–28) added to 0.5 mL of rat plasma was 66.3 ± 4.0% (mean ± SEM, $n = 4$). All ANP values were corrected for recovery. The mean r^2 for the log-logit regressions of 10 standard curves was 0.976 ± 0.005. The intraassay coefficient of variation was 6.67 ± 0.28%. The interassay coefficient of variation was 12.7%. The mean sensitivity as defined by 5% displacement of tracer was 0.35 ± 0.03 pg per tube, and the mean sensitivity as defined by 50% displacement was 7.51 ± 0.95 pg per tube.

One pro-ANP RIA was performed as for ANP; however, pro-ANP (prepared by J. K. Gierse, Monsanto Co., Chesterfield, MO) was used as a standard. The sensitivity of the assay as defined by 5% displacement was 6.26 pg per tube, and the sensitivity as defined by 50% displacement was 108.24 pg per tube. The intraassay coefficient of variation was 4.85 ± 0.89%.

ANP mRNA determination. Total RNA from combined right and left atria and combined ventricles was extracted using methods previously described by Chomczynski and Sacchi (19). The total RNA concentration was measured by spectrophotometry at 260 nm. ANP mRNA steady state levels were determined by Northern and dot-blot analyses (20). Five μg of total atrial mRNA or 10 μg of total ventricular mRNA was denatured at 65°C and resolved by gel electrophoresis. The RNA was transferred to a nitrocellulose membrane (Zeta Probe, Biorad, Melville, NY) by capillary action elution. Dot-blots were prepared using serial dilutions of atrial or ventricular total RNA spotted onto a nitrocellulose membrane using g a filtration manifold. RNA was cross-linked to the membrane with UV light (Stratlinker, Stratagene, La Jolla, CA). Blots

containing RNA was hybridized to full-length human ANP complementary DNA (gift of Cynthia T. Carilli, California Biotechnology, Inc., Mountain View, CA) labeled with ^{32}P by random prime (Boehringer Mannheim, Mannheim, Germany) labeling (21). Hybridization signals, detected by autoradiography using Kodak XAR-5 film and an intensifying screen, were quantified by scanning densitometry (LKB-2222–020 Ultra Scan XL Laser densitometer, Bromma, Sweden). Labeled ANP cDNA was removed from the membranes using 0.5% SDS and 0.1% standard saline citrate heated to 100°C. To compare the response of ANP with a control gene, the membranes were then hybridized to GAPDH cDNA labeled with ^{32}P and signals detected as described above. Results are expressed as a ratio of ANP to GAPDH.

Statistical analysis. Intergroup differences were evaluated by one-way analysis of variance with a Student-Newman-Keuls post hoc test or Mann-Whitney nonparametric analysis when appropriate. Results are expressed as mean ± SEM or median and range. Statistical significance was defined as $p < 0.05$.

RESULTS

Table 1 shows the final body weight and sodium intake of the experimental groups. There was no difference in final weight among groups of preweaned rat pups or groups of adult rats. Artificially reared pups fed the high-salt diet had a 6-fold increase in sodium intake compared with artificially reared rat pups fed a normal-salt diet. Adult rats drinking a 1% NaCl solution had a 4-fold increase in sodium intake compared with other adult groups. The sodium intake of the normal-salt adult groups was double that of the PWN group.

Figure 1*A* shows atrial and ventricular ANP content in preweaned rat pups. Atrial ANP content of artificially reared rat pups fed the high-salt diet was greater than that of artificially reared rat pups fed a normal-salt diet or maternally reared pups. Ventricular ANP content in high-sodium pups was greater than that of maternally reared rats. Atrial and ventricular ANP content was not different between artificially reared normal-sodium pups and maternally reared rats. Cardiac ANP content in adult rats (Fig. 1*B*) showed no difference among treatment groups for either atrial or ventricular ANP content. However, ventricular ANP content of the high-salt preweaned rats was higher than that of the high-salt adult group, whereas atrial ANP content in the maternally reared group was lower than that of the AL group (Fig. 1).

Table 1. *Final BW and sodium intake*

	Final BW (g)	Sodium intake (μmol/g BW)
Preweaned rat pups		
PWH (n = 17)	27 ± 1	47 ± 1*
PWN (n = 18)	28 ± 1	8 ± 1†
PWM (n = 19)	30 ± 1	ND
Adult rats		
AH (n = 18)	285 ± 7	53 ± 3‡
AN (n = 18)	275 ± 8	15 ± 1
AL (n = 18)	283 ± 8	15 ± 1

BW, body weight; ND, no data. Values are means ± SEM.
* $p < 0.05$ *vs* PWN.
† $p < 0.05$ *vs* AN.
‡ $p < 0.05$ *vs* AN or AL.

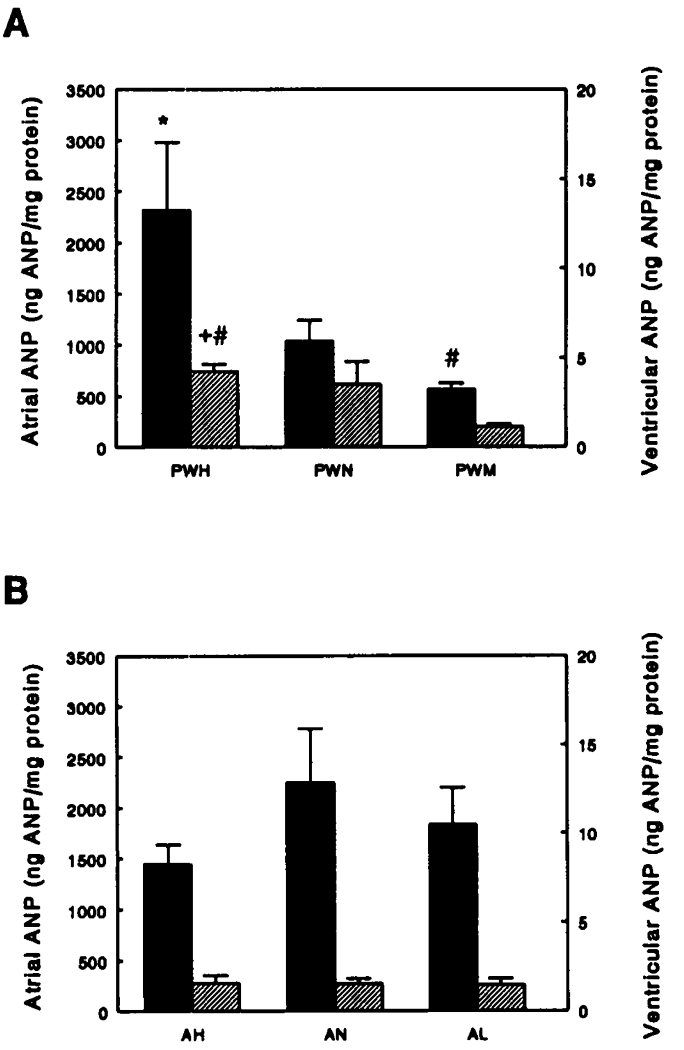

Figure 1. *A*, Atrial (*left axis*) and ventricular (*right axis*) ANP content in preweaned rat pups. *Bars* denote mean ± SEM. *Black bar*, atria; *hatched bar*, ventricles. *, $p < 0.05$ *vs* PWN and PWM; + $p < 0.05$ *vs* PWM; #, $p < 0.05$ *vs* same adult treatment group. *B*, Atrial (*left axis*) and ventricular (*right axis*) ANP content in adult rats.

Figure 2*A* shows atrial and ventricular pro-ANP content in preweaned rat pups. Pro-ANP was expressed as micrograms of pro-ANP per milligram of protein, whereas ANP was expressed as nanograms of ANP per milligram of protein. Preweaned rats fed a high-salt diet had higher atrial pro-ANP content compared with artificially reared pups fed a normal-salt diet or maternally reared rats. Ventricular pro-ANP was higher in both artificially reared groups of pups compared with maternally reared rats. Atrial and ventricular pro-ANP for adult rats is depicted in Figure 2*B*. There was no difference in atrial or ventricular pro-ANP among adult groups. PWH pups had increased atrial and ventricular pro-ANP compared with AH rats, and PWM pups had decreased atrial pro-ANP *versus* AL rats.

Table 2 shows the median plasma ANP concentration for adult and preweaned rat pups. Plasma ANP concentration in artificially reared high-salt rat pups was lower than in the

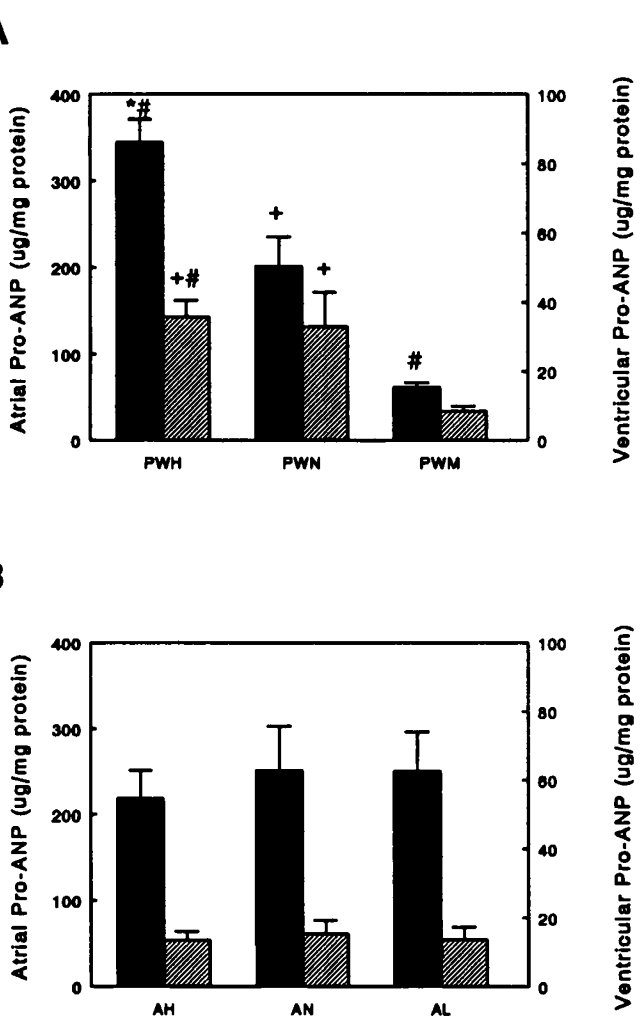

Figure 2. *A*, Atrial (*left axis*) and ventricular (*right axis*) pro-ANP content in preweaned rat pups. See Figure 1 for definition of symbols. *B*, Atrial (*left axis*) and ventricular (*right axis*) pro-ANP content in adult rats.

Table 2. *Plasma ANP (pmol/L)*

Preweaned rat pups	
PWH (n = 6)	29 (20–41)*
PWN (n = 6)	62 (38–453)†
PWM (n = 6)	59 (28–231)
Adult rats	
AH (n = 6)	27 (18–471)
AN (n = 6)	25 (5–49)
AL (n = 6)	27 (20–52)

Plasma ANP levels in experimental groups. Values are median (range).
* $p < 0.05$ *vs* PWM and PWN.
† $p < 0.05$ *vs* AN.

remaining groups of pups. There was no difference in plasma ANP concentration among adult rats. Plasma ANP concentration was higher in the PWN than in the AN group.

Representative atrial and ventricular ANP mRNA by Northern analysis are shown in Figure 3*A*. ANP cDNA hybridizes at 0.9 kB. The control gene, GAPDH, which hybridizes at 1.3 kB, is shown in the lower panel. The relative measurement of atrial and ventricular ANP mRNA compared with GAPDH mRNA from dot-blot analysis (Fig. 3*B*) showed no difference in atrial or ventricular ANP/GAPDH mRNA among groups of rat pups or adult rats (Table 3).

DISCUSSION

During neonatal life, mammals rely solely on maternal milk for nourishment. Human breast milk contains approximately 8 mEq of sodium per liter (22), whereas rat milk contains 28–33 mEq of sodium per liter (14, 15). Although the sodium content of rat milk exceeds that of human milk, the rate of growth of the neonatal rat greatly exceeds that of the human. In view of this limited sodium intake and the physiologic requirement for sodium conservation, we postulated that the activity of the ANP system is normally reduced during the preweaning period. Previous studies of the effects of increased sodium intake on ANP have involved postweaned animals (2–4, 23, 24). The experimental approach in the present study permitted precise manipulation of sodium intake as well as caloric intake in the artificially reared preweaned rat pups.

The artificial rearing procedure allowed normal growth of the preweaned rat pups. Although the sodium content of the rat formula was matched to that previously reported for rat milk (14, 15), the greater atrial and ventricular pro-ANP content of the artificially reared normal-sodium group suggests that the PWN animals actually received a sodium intake intermediate between PWH and PWM groups. The conclusions drawn by comparing only the artificially reared high- and normal-sodium groups may therefore underestimate the effects of chronic sodium loading on the variables measured. It should be noted, however, that even this level of sodium intake in the artificially reared normal-sodium preweaned pups was only half that of the AN group. Moreover, a lower sodium intake in the pups would impair somatic growth (25), which would further complicate the interpretation of the results.

The present study shows that a high-salt diet increases atrial pro-ANP and ANP content compared with the content in the normal-salt groups. The parallel changes of pro-ANP and ANP content suggest that sodium intake does not affect processing of the pro-ANP peptide. In addition, inasmuch as ANP mRNA levels did not change, it is likely that differences are caused by altered storage or release of the peptide rather than by increased transcription. The present experiments, however, do not rule out alterations in the stability of the mRNA. Previous studies of ANP mRNA levels in adult rats ingesting a high-salt diet showed no difference in atrial ANP mRNA levels compared with controls at 3 and 5 wk (3, 4).

Previous reports examining the effects of sodium intake on cardiac and plasma ANP levels in adult animals have shown varied responses. Lattion *et al.* (3) showed an increase in plasma ANP after 1 wk of 1% NaCl ingestion in rats. This effect was abrogated at 3 wk. Schwartz *et al.* (2) showed an increase in plasma ANP and a decrease in atrial ANP content after 2 wk of 8% NaCl ingestion, whereas Debinski *et al.* showed no change in plasma ANP after 3 wk of 8% NaCl ingestion and a decrease after 5 wk (4), and Widimsky *et al.* (24) showed a decline of plasma ANP after 5 wk of 8% NaCl intake. These studies suggest that cardiac ANP storage is decreased and plasma ANP levels are increased in the initial phase of chronic sodium ingestion in adult rats. This response appears to be abolished or even reversed with prolonged sodium ingestion. In the present studies using 1% NaCl inges-

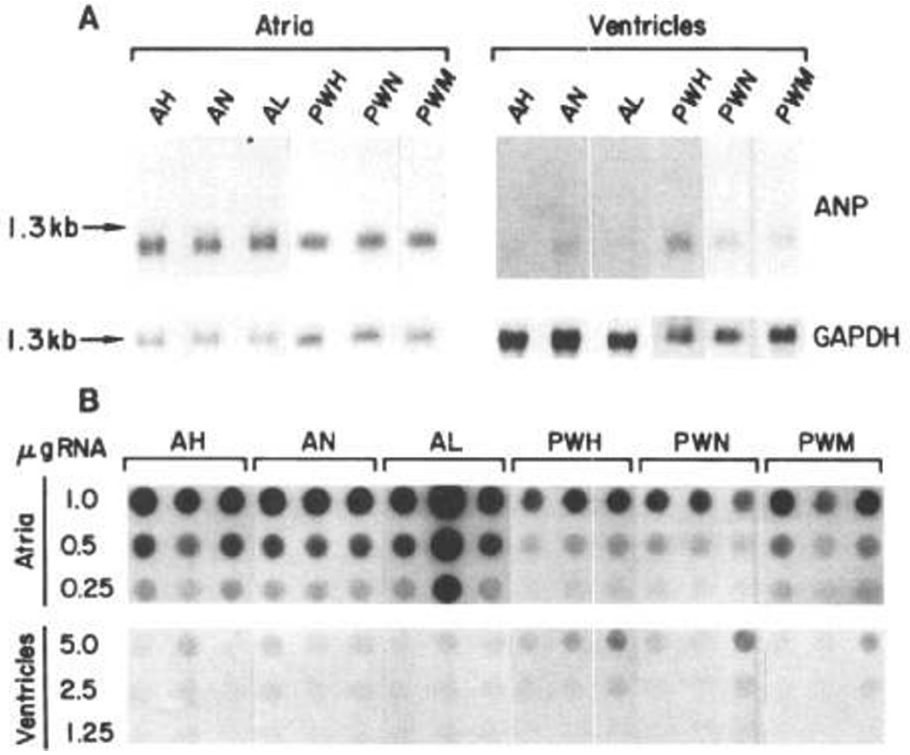

Figure 3. *A*, Autoradiogram of ANP mRNA (*top panel*) and GAPDH mRNA (*lower panel*) in atria and ventricles of preweaned and adult rats. Five μg of total atrial RNA and 10 μg of total ventricular RNA were subjected to Northern hybridization using ^{32}P-labeled ANP and GAPDH (sp act 8×10^8). Exposure time for atrial ANP samples, ventricular ANP samples, and GAPDH was 48 h, 7 d, and 8 h, respectively, at −70°C with an intensifying screen. *B*, Relative levels of ANP mRNA in preweaned and adult rats by dot-blot analysis. Each lane contains serial dilutions of total RNA from atria or ventricles from a single animal. The membrane was hybridized to ^{32}P-labeled ANP cDNA. Exposure time was 18 h for atria and 4 d for ventricles at −70°C with an intensifying screen.

Table 3. *ANP/GAPDH mRNA*

	Atria	Ventricles
Preweaned rat pups		
PWH (n = 6)	1.53 ± 0.14	2.14 ± 0.81
PWN (n = 6)	1.48 ± 0.13	1.07 ± 0.78
PWM (n = 6)	1.25 ± 0.14	1.14 ± 0.37
Adult rats		
AH (n = 6)	1.69 ± 0.15	0.74 ± 0.22
AN (n = 6)	1.42 ± 0.08	0.96 ± 0.16
AL (n = 6)	1.31 ± 0.13	1.51 ± 0.69

Relative intensity of ANP mRNA to GAPDH mRNA. There is no difference in ANP/GAPDH mRNA or ventricles among groups. Values are means ± SEM.

tion for 10 d in adult rats, no differences were seen in atrial and ventricular ANP content or plasma ANP levels. This may be due to the lower sodium intake compared with most of the previously published reports.

In the present study, atrial pro-ANP and ANP content were lower in preweaned maternally reared rat pups than in adults fed *ad libitum*. In contrast, atrial pro-ANP content was greater in preweaned high-salt pups than in high-salt adult rats. These results suggest that atrial storage of ANP is normally reduced in the preweaned pup but that, unlike in the adult, the storage can be significantly modulated by sodium intake.

Maturational changes in cardiac ANP content have been reported previously. Dolan *et al.* (26) reported significantly higher atrial ANP content in 15-d-old preweaned rat pups compared with adult rats. Inscho *et al.* (27) also found a greater atrial ANP content in 15-d-old preweaned rats than in postweaned or adult rats (when ANP content was factored for protein content). Wei *et al.* (28) reported a progressive increase in atrial ANP content during the preweaned period. Differences between these reports and the present study may be due to differences in volume status of the preweaned rats at the time of death.

The mechanism for secretion of ANP from the secretory granules in cardiac myocytes is thought to be exocytosis. Development of the atrial ANP secretory process in the hamster involves maturation of the atrial endothelial lining, which may alter activation of pro-ANP or secretion of active ANP into the blood (29). Greater sodium intake may lead to increased ANP production in the face of reduced cardiocyte secretion resulting from a normal developmental process.

In the present study, ventricular pro-ANP and ANP content were greater in artificially reared rat pups fed a high-salt diet than in the maternally reared normal-salt group. It has been shown that ventricular ANP content and mRNA are developmentally regulated and decrease at the time of weaning (28, 30). Atrial myocytes were found to store ANP in granules and to release it in a regulated manner, whereas ventricular myocytes store little ANP and secrete it in a constitutive fashion (31). Gu *et al.* (32) showed that after chronic left ventricular outflow obstruction the ventricles increase their production of ANP. In the cardiomyopathic hamster, more than 70% of ANP released by the heart is produced by the ventricles (33). An increase in left subendocardial ANP content was also reported in humans with dilated cardiomyopathy and valvular heart disease (34). Therefore, the ventricular contribution to the regulation of the ANP system may be important with certain

stimuli, including the preweaned rat pups receiving increased sodium intake. Processing of pro-ANP occurs either with constitutive ventricular release of ANP or with regulated atrial release (35).

In contrast to the increase in cardiac ANP content in preweaned rat pups receiving a high-sodium diet, plasma ANP concentration was decreased in this group of animals. This pattern is similar to that of hyperglycemic nonobese diabetic mice, which also have increased atrial ANP content in the face of reduced plasma ANP concentration (36). If secretion of ANP was not reduced, enhanced metabolic clearance of ANP must be considered. Removal of ANP from the circulation is achieved by its interaction with ANP C-receptors and by inactivation by neutral endopeptidase (37). The significant decrease in plasma ANP seen in the preweaned rat pups fed a high-salt diet suggests a possible role for increased numbers or enhanced action of C-receptors as demonstrated in adult rats (38, 39). However, others have described a down-regulation of C-receptors with salt loading in adult rats (40).

In summary, increased sodium intake in preweaned rat pups results in increased atrial pro-ANP and ANP content and decreased basal plasma ANP concentrations. These changes are not seen after similar dietary sodium manipulation in the adult rat. Moreover, atrial and ventricular ANP mRNA levels do not change in either preweaned rat pups or adult rats fed a high-sodium diet. The observed responses in the neonate receiving maternal milk may be caused by reduced basal ANP secretion by cardiac myocytes or increased systemic clearance of ANP. Either of these mechanisms would contribute to greater sodium retention in the neonate compared with the adult.

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