Atrial Natriuretic Peptide Dose-Dependently Inhibits Pressure Overload–Induced Cardiac Remodeling

Veronica Franco, Yiu-Fai Chen, Suzanne Oparil, Ji An Feng, Dajun Wang, Fadi Hage, Gilbert Perry

Abstract—We hypothesized that a single copy of the proatrial natriuretic peptide gene (Nppa+/−) would not be adequate to protect heterozygous mice against exaggerated cardiac hypertrophy and remodeling after pressure-overload stress. Nppa+/−, Nppa+/+, and Nppa−/− mice were subjected to sham surgery or transverse aortic constriction and fed a basal salt diet. Heart weight varied inversely with Nppa gene load by 1 week after either surgery. Fractional shortening did not differ among genotypes at baseline and fell in Nppa−/− mice only after transverse aortic constriction. There was a graded response in collagen deposition related to atrial natriuretic peptide (ANP) expression after either surgery. A robust interstitial and perivascular fibrosis was noted in Nppa−/− and Nppa+/− but not in Nppa+/+ mice after transverse aortic constriction. Our findings are consistent with a growing body of evidence that ANP is an important modulator of cardiac hypertrophy and remodeling in response to hemodynamic stress. The observation that partial ANP deficiency results in exaggerated hypertrophy and remodeling after pressure overload suggests that genetic or environmental variation in ANP levels may play a role in the development of cardiac hypertrophy, remodeling, and failure in humans. (Hypertension. 2004;44:746-750.)

Key Words: atrial natriuretic factor ■ natriuretic peptides ■ receptors, atrial natriuretic factor ■ hypertrophy, cardiac ■ remodeling ■ extracellular matrix ■ collagen

Recent studies suggest that atrial natriuretic factor (ANP) is an autocrine/paracrine modulator of cardiac hypertrophy and remodeling in response to pathologic stimuli.1–10 Mice with homozygous deletion of the pro-ANP gene (Nppa−/−) or the natriuretic peptide receptor-A gene (Npr1−/−) exhibit cardiac hypertrophy under resting conditions3,5,11–13 and develop exaggerated hypertrophy after volume or pressure overload.6,12,13 Furthermore, these studies raise the question as to whether variation in ANP response to hemodynamic stress is an important mediator of cardiac hypertrophy and remodeling in human hypertension and heart failure. Although studies in Nppa−/− mice have clearly demonstrated the adverse effect of ANP deletion on cardiac hypertrophy and remodeling, the effect of a modest ANP deficiency on the development of cardiac hypertrophy, remodeling, and failure remains unknown.

ANP-heterozygous mice (Nppa+/−) have normal blood pressure on either a normal- (0.5% NaCl) or an intermediate-salt (2% NaCl) diet,6,14 in contrast to the hypertension observed in Nppa−/− mice under these conditions.11 On a very-high-salt (8% NaCl) diet, however, Nppa−/− mice develop hypertension.6 The cardiac phenotype of the heterozygous ANP-knockout has not been rigorously studied under either basal or stress conditions. However, in their original report of this model, John et al6 did not find a significant difference in cardiac weight between Nppa+/+ and Nppa+/− mice. The relatively normal cardiac phenotype in the heterozygous ANP-knockout might indicate that the quantity of ANP is not critical under nonstressful conditions. In a different murine model, we demonstrated that variation in angiotensin-converting enzyme levels does not importantly affect either angiotensin II levels or the hypertrophic response to volume overload.15 Thus, effects of the complete absence or pharmacological blockade of a gene product does not predict whether or not a modest variation in that gene product will have important effects on phenotype.

The effect of varying levels of endogenous ANP expression on cardiac structure and the development of heart failure is unknown and difficult to study in humans, owing to a confounding effect of increased ANP expression with worsening severity of failure. The Nppa−/− mouse provides an ideal model to study the effect of a modest reduction in ANP expression on cardiac remodeling and function in response to hemodynamic stress. We hypothesized that ANP-heterozygous mice would demonstrate a normal cardiac phenotype under nonstressful conditions but an exaggerated cardiac hypertrophy and remodeling in response to pressure-overload stress.

Methods

Animal Preparation

Heterozygous Nppa+/− mice were obtained by breeding female Nppa+/+ and male Nppa−/− mice in our resident colonies. Wild-type...
Nppa+/+ mice of the C57BL/6 strain and Nppa−/− mice were originally generated by Dr Oliver Smithies. Genotypes were identified by polymerase chain reaction. Mice were housed 3 or 4 per cage; maintained at constant humidity (60±5%), temperature (24±1°C), and light cycle (6 AM to 6 PM); and fed a standard diet (0.55% NaCl, Harlan-Teklad). 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 published by the US National Institutes of Health (NIH publication No. 96-01, revised 1996).

**Surgical Procedure**
Male Nppa+/+, Nppa−/+ , and Nppa−/− mice, 9 to 12 weeks of age, were anesthetized with an intraperitoneally administered mixture of ketamine (8 mg/100 g) and xylazine (1.2 mg/100 g), and transverse aortic constriction (TAC) was performed as described previously. Pressure gradients across the TAC were similar among genotypes (53±6.5 mm Hg in Nppa+/+, and 55±6.8 mm Hg in Nppa−/− mice). Sham-operated mice served as controls.

**Echocardiographic Study**
One week after TAC or sham surgery, echocardiography was performed as previously described with a 6–15-MHz transducer (Philips) and a commercially available ultrasound system (Phillips Sonos 5500). Left ventricular (LV) end-diastolic dimension (EDD), LV end-systolic dimension (ESD), and septal (interventricular septal [IVS]) and posterior wall (PW) diastolic thicknesses were measured by 2D-guided M-mode echocardiography from the parasternal long-axis view. Wall thickness was calculated as the average of IVS and PW. Fractional shortening (FS) was calculated by the formula (LVEDD − LVESD)/LVEDD. A single examiner, blinded to genotype and treatment, performed all studies.

**Plasma ANP**
Blood (0.5 mL) was collected via retro-ocular approach from conscious mice the day after the echocardiographic study. ANP measurement was performed as previously described with the use of radioimmunoassay kits (Peninsula Laboratories).

**Tissue Collection**
Mice were humanely killed after blood collection by cervical dislocation. Hearts were removed, and the LV, right ventricle (RV), and atria were weighed. The LV was divided into 2 portions: the apical portion was fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned for histologic analysis; the basal portion was immediately frozen in LN2 for RNA isolation. Lung, kidney, brain, liver, and spleen were also weighed.

**Collagen Volume**
LV myocardial collagen (interstitial and perivascular) volume, at the level below the mitral valve, was measured in picrosirius red (0.1%)–stained cross sections on a microscopic system with a green (540-nm) filter to enhance contrast for computer imaging analysis (Image-1 Software). Only collagen fibers appear green; green regions are counted by the aforementioned software and are given as a percentage of the total area per field. A minimum of 8 randomly selected images were counted from each LV. A single examiner, blinded to the experimental group, performed all histologic analyses.

**Northern Blot Analysis**
Northern blot analysis was performed as described previously with 32P-labeled selective cDNA probes for ANP (generated in our laboratory by reverse transcription–polymerase chain reaction with mouse heart RNA as the template). 18S rRNA was used as a control to account for variation in RNA loading.

**Statistical Analysis**
Results are expressed as mean±SEM. Tissue weights and echocardiographic measurements were normalized by ANCOVA with body weight as the covariate. Our primary statistical test was ANOVA, 1-way ANOVA to evaluate the differences in mean values due to main effects (genotype or TAC), and 2-way ANOVA to test their interactions. P<0.05 was considered significant.

**Results**
There was no difference in plasma ANP level or LV ANP mRNA expression between sham Nppa−/− mice, and Nppa−/− mice after TAC or sham surgery. G×TAC indicates genotype by TAC interactions. Results are mean±SEM, where n=number of mice. *P<0.05 vs respective sham mice; †P<0.05 vs respective Nppa+/+ mice; ‡P<0.05 vs respective Nppa−/− mice. Other abbreviations are as defined in text.

**Plasma ANP and Cardiac ANP mRNA Expression**
There was no difference in plasma ANP level or LV ANP mRNA expression between sham Nppa−/− and Nppa+/+ mice (Figure 1). Nppa−/− mice had disrupted pro-ANP mRNA, and plasma ANP and LV ANP mRNA levels were undetectable. TAC increased plasma ANP and LV ANP mRNA levels in Nppa−/− and Nppa+/+ mice compared with sham controls. There was a genotypic effect on ANP response to TAC: Nppa−/− mice had less robust responses to TAC than did Nppa−/− mice (2-way ANOVA, P<0.05).

**Tissue Weight**
Both genotype and TAC affected whole-heart, LV, and RV weight, and there was an interaction between these 2 variables (Table 1). Whole-heart and LV weight followed ANP gene expression in Nppa−/− versus both Nppa+/+ and Nppa+/+ mice, as in Nppa+/+ and Nppa+/+ mice, after sham surgery. Whole-heart, LV, and RV weight differed among all 3 genotypes in a graded fashion (Nppa−/−>Nppa+/+>Nppa+/+) after TAC.

**Echocardiography**
Normalized echocardiographic measurements are shown in Table 2. LVEDD was increased by TAC and by decreasing...
the number of ANP genes. Pairwise multiple comparison revealed that LVEDD did not differ among the 3 genotypes after sham surgery. After TAC, LVEDD was greater in \(Nppa^{-/-}\) versus \(Nppa^{+/+}\) and in \(Nppa^{-/-}\) versus \(Nppa^{+/+}\) mice but did not differ between \(Nppa^{+/+}\) and \(Nppa^{-/-}\) mice. LVFS did not differ among the 3 genotypes after sham surgery and was reduced only in \(Nppa^{-/-}\) mice subjected to TAC.

Collagen Volume

Collagen volume differed significantly among the 3 genotypes after sham surgery (\(Nppa^{+/+}\), 0.2±0.0%; \(Nppa^{-/-}\), 1.1±0.2%; and \(Nppa^{-/-}\), 3.5±0.2%; \(P<0.05, 1\)-way ANOVA) and after TAC (\(Nppa^{+/+}\), 0.1±0.0%; \(Nppa^{-/-}\), 6.4±1.3%; and \(Nppa^{-/-}\), 24.3±5.9%; \(P<0.01, 1\)-way ANOVA). Collagen deposition occurred predominantly in the interstitium and perivascular area after TAC and was inversely related to ANP gene load (Figure 2A and 2B). There was robust interstitial and perivascular fibrosis in 1-week TAC-\(Nppa^{-/-}\) mice and a significant but less severe response in 1-week TAC-\(Nppa^{-/-}\) mice (2-way ANOVA, \(P<0.01\), interaction of genotype×TAC). Collagen deposition increased by \(\approx 6\)-fold in 1-week TAC-\(Nppa^{-/-}\) and TAC-\(Nppa^{+/+}\) mice when compared with their sham controls but did not differ between sham and TAC-\(Nppa^{+/+}\) mice.

Discussion

The major finding of this study is that the mouse heterozygous for ANP expression displays an intermediate phenotype between that of the \(Nppa^{+/+}\) and \(Nppa^{-/-}\) mouse both at baseline and in response to hemodynamic stress. Cardiac hypertrophy and remodeling were exaggerated after pressure-overload stress in a graded response related to ANP expression, with even partial ANP deficiency resulting in adverse cardiac remodeling under hemodynamic stress. Our results emphasize the importance of ANP as a modulator of cardiac growth and collagen content under both basal and stressful conditions and suggest that the gene encoding ANP is a good candidate for investigation with regard to the development of cardiac hypertrophy, remodeling, and failure in humans.

Previous studies have documented that the complete absence of ANP or its receptor results in the development of salt-sensitive hypertension, cardiac hypertrophy, and remodeling.\(^5,6,21,22\) Partial ANP deficiency does not affect blood pressure on a normal-salt diet\(^5\) but has not been rigorously studied with regard to cardiac phenotype. John et al.\(^6\) reported cardiac hypertrophy only in \(Nppa^{+/+}\) mice fed 2% NaCl and found a statistically nonsignificant increase in the \(Nppa^{-/-}\) genotype, similar in magnitude to that observed in sham-operated animals in the current study. We also observed a modest increase in both collagen volume and heart weight in the heterozygotes versus wild types under basal conditions, despite apparently similar ANP levels. Because of limitations in the sensitivity (10 to 20 pg/mL of plasma) of the radioimmunoassay for ANP and Northern blot analysis of ANP mRNA, it is possible that there are small (<20%) differences in these parameters between \(Nppa^{+/+}\) and \(Nppa^{-/-}\) mice that were undetectable by our methods. Clearly, major differences in ANP levels between \(Nppa^{-/-}\) and \(Nppa^{+/+}\) were manifested only under stress conditions. It is unlikely that higher blood pressure explains these increases in heart weight and collagen.

**Table 1. Normalized Heart and Tissue Weights of \(Nppa^{+/+}\), \(Nppa^{-/-}\), and \(Nppa^{-/-}\) Mice 1 Week After TAC or Sham Surgery**

<table>
<thead>
<tr>
<th>Tissue Weight (g)</th>
<th>Sham (Nppa^{+/+}) (13)</th>
<th>Sham (Nppa^{-/-}) (12)</th>
<th>TAC (Nppa^{+/+}) (10)</th>
<th>TAC (Nppa^{-/-}) (7)</th>
<th>2-Way ANCOVA, (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WH</td>
<td>132±2</td>
<td>148±6†</td>
<td>182±3†‡</td>
<td>145±4</td>
<td>0.0001</td>
</tr>
<tr>
<td>LV</td>
<td>99±2</td>
<td>115±5†</td>
<td>123±3†</td>
<td>113±3*</td>
<td>0.0001</td>
</tr>
<tr>
<td>RV</td>
<td>23±1</td>
<td>25±1</td>
<td>45±2‡</td>
<td>22±1</td>
<td>0.0001</td>
</tr>
<tr>
<td>AT</td>
<td>10±0</td>
<td>9±0</td>
<td>12±0†</td>
<td>10±0</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

**Table 2. Normalized Echocardiographic Measurements of \(Nppa^{+/+}\), \(Nppa^{-/-}\), and \(Nppa^{-/-}\) Mice 1 Week After TAC or Sham Surgery**

<table>
<thead>
<tr>
<th>Echo Measurement</th>
<th>Sham (Nppa^{+/+}) (5)</th>
<th>Sham (Nppa^{-/-}) (7)</th>
<th>TAC (Nppa^{+/+}) (14)</th>
<th>TAC (Nppa^{-/-}) (8)</th>
<th>2-Way ANCOVA, (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDD</td>
<td>3.7±0.1</td>
<td>3.6±0.0</td>
<td>3.8±0.1</td>
<td>3.7±0.1</td>
<td>0.0029</td>
</tr>
<tr>
<td>LVESD</td>
<td>2.2±0.1</td>
<td>2.1±0.0</td>
<td>2.2±0.0</td>
<td>2.2±0.1</td>
<td>0.0132</td>
</tr>
<tr>
<td>Wall thickness</td>
<td>0.4±0.0†</td>
<td>0.7±0.0†</td>
<td>0.7±0.0†</td>
<td>0.7±0.0°</td>
<td>0.0001</td>
</tr>
<tr>
<td>FS</td>
<td>41±1.6</td>
<td>44±1.7</td>
<td>42±1.3</td>
<td>42±1.7</td>
<td>0.1917</td>
</tr>
</tbody>
</table>

**Abbreviations are as defined in text. Results are mean±SEM, where \(n=\) number of mice. The normalized chamber sizes were determined by ANCOVA with body weight as a covariate.**

*\(P<0.05\) vs respective sham mice; †\(P<0.05\) vs respective \(Nppa^{+/+}\) mice; ‡\(P<0.05\) vs respective \(Nppa^{-/-}\) mice.
volume in heterozygous versus wild-type mice, because previous reports have documented no difference in blood pressure between Nppa+/+ and Nppa−/− mice on either a normal- (0.5%) or an intermediate- (2.0% NaCl) salt diet.6,14 In addition, correction of hypertension in Nppa−/− mice by a very-low-salt diet (0.05% NaCl) does not prevent cardiac hypertrophy, either at baseline or in response to hemodynamic stress, suggesting that alternative mechanisms are operative in ANP-induced hypertrophy and fibrosis.11

In the current study, partial ANP deficiency clearly resulted in excess cardiac hypertrophy and remodeling in response to hemodynamic stress compared with wild-type mice. This was accompanied by a 6-fold increase in collagen in TAC-Nppa−/− and TAC-Nppa+/− versus the respective sham-operated mice compared with no change in collagen volume in Nppa−/− mice after TAC. Importantly, whereas collagen volume increased after TAC in Nppa−/− relative to sham and to TAC-Nppa+/− mice, it was still substantially less than that seen in TAC-Nppa−/− mice. Consistent with those findings, the presence of 1 copy of the ANP gene was sufficient to protect against the development of LV dysfunction compared with mice with a complete absence of ANP. In contrast to ANP, B-type natriuretic peptide (BNP) appears not to modulate cardiac enlargement. BNP−/− mice do not develop cardiac hypertrophy; circulating BNP is not increased in Nppa−/− mice; and BNP is unable to compensate for the lack of ANP in Nppa−/− mice under stress conditions.13,16,24 These findings suggest that ANP deficiency alone is sufficient to generate cardiac hypertrophy/remodeling, particularly under conditions of hemodynamic stress. Our findings are consistent with a growing body of evidence that remodeling of the cardiac interstitium is a major determinant of pathologic hypertrophy, leading to cardiac dysfunction and failure, and underscore the importance of ANP as a modulator of that interstitial remodeling.12,13

Perspectives
The observation in the current study that cardiac weight and collagen content vary with ANP gene load both at baseline and after 1 week of pressure-overload hemodynamic stress underscores the critical role of ANP in modulating cardiac growth and structure under both physiologic and pathophysiologic conditions. We chose heterozygous ANP-knockout mice for the current study because humans are more likely to have partial rather than an absolute deficiency of ANP. Both environmental and genetic variations in ANP have been described in humans. Blunted secretion of ANP has been observed in black salt-sensitive hypertensives in response to high salt intake,30 and a polymorphism of the ANP gene has been observed more frequently in black salt-sensitive hypertensives compared with normotensives or white hypertensives.31–33 Obese individuals demonstrate decreased levels of both ANP and BNP, possibly related to more rapid clearance by adipocyte NPR-C receptors.34 Our findings suggest that these observed variations in ANP levels maybe a fruitful area for study with regard to the development of cardiac hypertrophy, remodeling, and failure in response to hemodynamic stress in humans.

Acknowledgments
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References


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