Increased Transcripts for B-Type Natriuretic Peptide in Spontaneously Hypertensive Rats
Quantitative Polymerase Chain Reaction for Atrial and Brain Natriuretic Peptide Transcripts
Lina Dagnino, Jean-Pierre Lavigne, and Mona Nemer

The cardiac natriuretic peptide family includes atrial natriuretic factor and brain or B-type natriuretic peptide, also known as iso-atrial natriuretic factor (isoANF). Although these peptides contribute to cardiovascular homeostasis, their respective roles remain unclear. To study regulation of atrial natriuretic factor and isoANF gene expression during progression of hypertension, we developed a quantitative polymerase chain reaction protocol to measure their transcript level in spontaneously hypertensive rat (SHR) hearts. At the onset of hypertension, atrial natriuretic factor transcripts in 5-week-old SHR were 50% of those of age-matched Wistar-Kyoto (WKY) rats, whereas the level of isoANF transcripts was similar in atria and twofold higher in ventricles. Because atria are the major sites of atrial natriuretic factor gene expression and ventricles contribute predominantly to cardiac isoANF synthesis, total atrial natriuretic factor messenger RNA (mRNA) in the hearts of 5-week-old SHR was about 50% of that in WKY rats, and total isoANF mRNA content was already higher than in control rats. In left ventricles and ventricular septa, progression of hypertension led to a maximal increase of twofold and fourfold in atrial natriuretic factor and isoANF mRNA levels, respectively, with no detectable change in right ventricles. In the atria of older SHR, atrial natriuretic factor and isoANF mRNA levels were comparable to those of age-matched controls. These data indicate that, although increased blood pressure stimulates both atrial natriuretic factor and isoANF gene expression, regulation of the two natriuretic peptide genes is not temporally coordinated in all cardiac compartments. Furthermore, isoANF mRNA is already induced in the ventricles at the onset of the hypertensive stage, and in older SHR, the isoANF gene is hyperresponsive to progression of hypertension compared with atrial natriuretic factor. Thus, isoANF might represent a very sensitive marker of cardiac changes in hypertension. (Hypertension 1992;20:690-700)

KEY WORDS • natriuretic hormones • gene expression • polymerase chain reaction • heart • rats, inbred SHR

Cardiovascular function and homeostasis result from complex interaction among multiple regulatory factors. These factors include, on the one hand, vasoconstrictor agents such as endothelin and the renin-angiotensin system, and, on the other hand, vasodilatory and hypotensive agents such as cardiac natriuretic peptides.

The first cardiac peptide hormone identified was atrial natriuretic factor (ANF). This peptide exhibits potent natriuretic, diuretic, and vasodilatory activities and may be a functional antagonist of the renin-angiotensin system. ANF appears to elicit its biological effects by interaction with specific receptors with guanylate cyclase activity. More recently, other members of the natriuretic peptide family have been identified, including brain or B-type natriuretic peptide and its rat homologue, hereby termed iso-atrial natriuretic factor (isoANF). These peptides also bind to ANF receptors with high affinity, triggering the formation of cyclic GMP and eliciting natriuretic, diuretic, and vasodilatory responses similar to those observed with ANF. Despite the apparently similar biological properties of ANF and isoANF, different mechanisms regulating their expression could reflect distinct physiological or pathophysiological roles for these peptides. Although plasma levels of these peptides and cardiac ANF messenger RNA (mRNA) content have been measured in patients with heart disease, after myocardial infarction, and with other cardiovascular disorders, the pathways that lead to alterations in natriuretic peptide gene expression in different physiological and pathophysiological conditions are poorly understood. Furthermore, the scarcity of brain natriuretic peptide and isoANF mRNA has added difficulty to the study of the mechanisms governing natriuretic peptide gene expression.

Previous studies have shown that ANF gene expression is induced in spontaneously hypertensive rats...
(SHR) after the onset of hypertension.16-18 Since isoANF also exhibits hypotensive activity, we investigated regulation of the isoANF gene at the mRNA level in SHR during the development of the hypertensive state and compared it directly with changes in ANF mRNA levels. To this end, we developed a quantitative polymerase chain reaction (PCR) assay that allowed measurement of changes in both ANF and isoANF transcripts, which represent about 1% and 0.01-0.001%, respectively, of total mRNA in cardiac tissues.19

Methods

Animals

Male SHR and normotensive Wistar-Kyoto (WKY) rats, 5, 11, or 16 weeks old, were purchased from Taconic Farms, Germantown, N.Y. The animals, housed at 22°C with a 12-hour light/dark cycle, were fed pelleted chow (Purina, Richmond, Va.) and had access to water ad libitum. Systolic blood pressure (SBP) measurements were conducted by the tail-cuff method in conscious animals. The animals were decapitated and hearts were removed and weighed. Atria and ventricles were carefully dissected and weighed separately. Samples were then frozen in liquid nitrogen and maintained at −80°C until RNA extraction.

RNA Extraction

Tissue samples from age- and weight-matched animals (n=5–10) were pooled and split in two batches for RNA extraction. Total RNA was extracted by the guanidinium thiocyanate method, as previously described.20 The integrity of RNA preparations was assessed after electrophoresis on agarose gels; RNA samples were quantified by measuring UV absorbance at 260 nm, and total mRNA was measured by dot blot hybridization to end-labeled oligonucleotide (OL16) corresponding to sequences in the isoANF gene located between the two PCR primers and quantified by laser densitometry. For each cDNA sample, α-tubulin and isoANF sequences were coamplified to correct for possible variations in the initial amounts of the cDNA. In these experiments, cDNA samples corresponding to 150 ng total RNA were subjected to 20 cycles of PCR amplification using the following primers: OL30/OL18 and, for tubulin amplification, a sense and an antisense primer with sequences 5'-TCCATCCACGTCGGGCCAGGCT-3' and 5'-GTAGGGCTCAAACCACAGCAGT-3', respectively. Amplification of cDNA samples with the latter two primers gave rise to a 550-bp fragment. The tubulin and isoANF DNA fragments were separated by electrophoresis on agarose gels and transferred to a membrane. The PCR fragments were hybridized to OL16 and to an oligonucleotide probe corresponding to tubulin sequences downstream from the sense primer and then were quantified by laser densitometry.

Polymerase Chain Reaction Amplification of Atrial Natriuretic Factor Sequences

Samples of cDNA equivalent to 3, 14, and 28 ng, respectively, of total atrial and left and right ventricular RNA were mixed with 700 ng each of a sense primer (5'-CTGCTAGACCCACGTGGAGGAG-3') and an antisense primer (5'-CCAGGGAGGTATTTCCACACCCAGCT-3') corresponding to sequences in the second and third exon of the rat ANF gene.22 Amplification of ANF sequences was conducted as described above for isoANF. Products arising from amplification of cDNA or genomic DNA templates yielded 436-bp or 828-bp fragments, respectively. After separation on agarose gels and transfer to membranes, ANF mRNA was also measured by Northern blot analysis, in which case hybridization to a rat β-actin cDNA as internal control. The ANF/actin mRNA ratios were calculated after densitometric scanning of the autoradiographs.

Complementary DNA Synthesis

Samples of 1–2 μg total RNA were mixed with p(dT)11-18 (2-4 μg, Sigma Chemical Co., Saint Louis, Mo.), 20 units of plasmid RNase inhibitor (Pharmacia, Montréal, Canada) and 25 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.). After incubation at 37°C for 1.5 hour, the cDNA samples prepared were used directly for PCR amplification, as described below. The yield of the cDNA syntheses was consistently 50-60%.

Polymerase Chain Reaction Amplification of Iso-Atrial Natriuretic Factor Sequences

Portions of atrial or ventricular cDNA samples equivalent to 150 ng total RNA were mixed with 700 ng each of a sense primer corresponding to sequences in the 5'-untranslated region of the isoANF cDNA (5'-CCATCGGACGTGCGGGCCCATCACCCTCTG-3', OL30)23 and an antisense primer corresponding to sequences in the second exon of the isoANF gene (5'-GACTGCGCGATCCGGTG-3', OL18). The cDNA was subjected to 15-25 cycles of PCR amplification using 2.5 units of Thermus aquaticus (Tag) DNA polymerase (Perkin-Elmer-Cetus, Montreal, Canada). As an internal control for the amplification process, 200 ng rat genomic DNA were added to each sample. Amplification of the cDNA and the genomic DNA templates yielded, respectively, a 347-bp and a 546-bp fragment. Amplification products were separated by agarose-gel electrophoresis and transferred to nylon membranes (Dupont, Mississauga, Canada). The DNA was hybridized to an end-labeled oligonucleotide corresponding to sequences in the isoANF gene located between the two PCR primers and quantified by laser densitometry. For each cDNA sample, α-tubulin and isoANF sequences were coamplified to correct for possible variations in the initial amounts of the cDNA. In these experiments, cDNA samples corresponding to 150 ng total RNA were subjected to 20 cycles of PCR amplification using the following primers: OL30/OL18 and, for tubulin amplification, a sense and an antisense primer with sequences 5'-ATCCATCCACGTCGGGCCAGGCT-3' and 5'-GTAGGGCTCAAACCACACAGCAGT-3', respectively. Amplification of cDNA samples with the latter two primers gave rise to a 550-bp fragment. The tubulin and isoANF DNA fragments were separated by electrophoresis on agarose gels and transferred to a membrane. The PCR fragments were hybridized to OL16 and to an oligonucleotide probe corresponding to tubulin sequences downstream from the sense primer and then were quantified by laser densitometry.

Polymerase Chain Reaction Amplification of Atrial Natriuretic Factor Sequences

Samples of cDNA equivalent to 3, 14, and 28 ng, respectively, of total atrial and left and right ventricular RNA were mixed with 700 ng each of a sense primer (5'-CTGCTAGACCCACGTGGAGGAG-3') and an antisense primer (5'-CCAGGGAGGTATTTCCACACCCAGCT-3') corresponding to sequences in the second and third exon of the rat ANF gene.22 Amplification of ANF sequences was conducted as described above for isoANF. Products arising from amplification of cDNA or genomic DNA templates yielded 436-bp or 828-bp fragments, respectively. After separation on agarose gels and transfer to membranes, ANF products were hybridized to a fragment containing the entire ANF cDNA sequence and were quantified by laser densitometry. ANF mRNA was also measured by Northern blot analysis, in which case hybridization to a β-actin probe was used to correct for differences in the amounts of RNA loaded, as previously described.20 In all experiments, the initial amounts of cDNA templates and number of PCR cycles were appropriate to maintain exponential amplification of the fragments of interest. PCR experiments were conducted using two different cDNA preparations and were repeated three to four times for each cDNA sample. The statistical significance of differences in transcript levels in each heart compartment between age-matched WKY rats and SHR was calculated using unpaired t tests.
Results

Quantitative Amplification of Iso-Atrial Natriuretic Factor Sequences

The amplification of DNA sequences by PCR will reflect the initial amounts of template present only during the exponential phase of the amplification. Once a critical amount of DNA has been produced, the efficiency of the amplification process decreases. This phenomenon may occur because of insufficient primer molecules relative to the amplified quantities of template, insufficient enzyme molecules to copy all the existing DNA, or both. Thus, exponential amplification may be achieved by optimization of the initial amount of DNA template and of the number of PCR cycles. To determine the optimal conditions for quantitative PCR amplification of cardiac isoANF sequences, cDNA samples corresponding to 50–200 ng total atrial or ventricular RNA were subjected to 16–25 PCR cycles. Under these amplification conditions, the yield of DNA products was directly proportional to the initial template levels, in both atria (Figure 1A) and ventricle (Figure 1B). Because the signal obtained was optimal when cDNA samples corresponding to 150 ng total RNA were used, all further experiments were conducted with that amount of cDNA template.

During the PCR process, the efficacy of amplification between samples may differ because of factors such as variation in well temperature in the thermal cycler or the presence of inhibitors in cDNA preparations. Thus, an internal control for the amplification process is necessary. In these experiments, rat genomic DNA (200 ng/tube) was added to each cDNA template as an internal control of amplification efficacy. Because the primers used for PCR corresponded to sequences in the first and second exon of the isoANF gene, the amplification product of the genomic template was larger than that generated from the cDNA template and, therefore, readily resolvable by electrophoresis. Amplification of genomic and cDNA isoANF sequences gave rise, respectively, to 546- and 346-bp fragments (Figures 1 and 2). In addition, the number of cycles appropriate for exponential amplification of samples containing both cDNA and genomic templates was determined. Samples containing 150 ng atrial RNA and 200 ng genomic DNA were subjected to 16–25 PCR cycles. Plots of the log of the amounts of PCR products (expressed as UV absorbance units measured from Southern blots) versus number of cycles were linear between 16 and 25 cycles, indicating that the amplification of both cDNA and genomic DNA templates was exponential under these conditions (Figure 2). Thus, PCR amplification of cardiac isoANF sequences was conducted with cDNA samples equivalent to 150 ng RNA for 20 cycles. Amplification of isoANF cDNA templates in the absence of genomic DNA only gave rise to the 346-bp fragment, confirming the absence of contaminating DNA and detectable unspliced RNA in the cDNA preparations (data not shown). Similar experiments using tubulin-specific primers indicated that an initial amount of cDNA template equivalent to 150 ng RNA subjected to 20 PCR cycles was adequate for quantitative PCR amplification of cardiac tubulin sequences (data not shown).

FIGURE 1. Scatterplots show quantitative amplification of iso–atrial natriuretic factor (isoANF) sequences. Portions of complementary DNA (cDNA) preparations corresponding to 40–200 ng total RNA from right atria (panel A) or 50–180 ng total RNA from right ventricle (panel B) from 16-week-old Wistar-Kyoto rats were subjected to 20 polymerase chain reaction (PCR) cycles, as described in “Methods.” Genomic DNA (200 ng) was added as internal control for amplification. PCR products from cDNA and genomic templates yielded, respectively, 346-bp (lower band in insets) and 546-bp (upper band in insets) fragments, which were separated by electrophoresis on agarose gels. PCR fragments were hybridized to the probe OL16, a 16-mer corresponding to isoANF sequences located between the two PCR primers, and quantified by laser densitometry of Southern blots. Results are shown after correction with respect to the genomic DNA control. Bands at the top of the inset in panel B show a longer exposure of the genomic template products in this experiment. mRNA, messenger RNA.

Quantitative Amplification of Atrial Natriuretic Factor Sequences

Although ANF transcripts are readily detected by Northern blot analysis, simultaneous detection of both ANF and isoANF mRNA by the same technique allows direct comparative studies of natriuretic peptide gene regulation. Thus, conditions necessary for exponential amplification of ANF sequences were determined in a manner similar to that described for isoANF. However, since previous experiments indicated that cardiac ANF transcripts are substantially more abundant than those of isoANF, the amounts of cDNA template used were lowered proportionally. When samples of atrial cDNA
corresponding to 1–5 ng total RNA were subjected to 20 PCR cycles using ANF-specific primers, the amounts of amplified products were directly proportional to the initial cDNA levels present (Figure 3A). Similar results were obtained when cDNA samples corresponding to 7–28 ng ventricular RNA were amplified (Figure 3B).

To ensure that cDNA and genomic templates could be simultaneously amplified exponentially, reaction mixtures containing cDNA template equivalent to 3 ng atrial RNA and 200 ng genomic DNA were subjected to 16–25 PCR cycles. Plots of the log₂ of the DNA product versus number of PCR cycles yielded straight lines for both templates, indicating a geometric amplification of ANF sequences under these experimental conditions (Figure 4). Thus, in further experiments, the amplification of ANF sequences was conducted using cDNA samples equivalent to 3 ng and 14–28 ng, respectively, of atrial and ventricular RNA and 20 PCR cycles.

Iso—Attrial Natriuretic Factor Transcript Levels During Development of Hypertension

To investigate whether increases in blood pressure are associated with changes in isoANF gene expression, cardiac isoANF transcript levels were measured in SHR and WKY rats 5, 11, or 16 weeks of age. The development of the hypertensive state in SHR occurs at or shortly after birth. In the present study, the SBP of 5-week-old SHR was already higher than that of age-matched WKY rats (Table 1). The largest difference in SBP between SHR and WKY rats was observed at 11 weeks of age, and SBP did not increase further in 16-week-old SHR. Within the SHR strain, the largest increase in SBP was observed between 5- and 11-week-old animals. Interestingly, the ratio of heart to body weight was already 23% higher in 5-week-old SHR compared with that in WKY rats; this ratio remained about 20% higher in older SHR relative to WKY rats, and thus, in the SHR animals studied, there was no evidence of increased cardiac hypertrophy.

Atrial and ventricular isoANF mRNA levels were measured using the quantitative PCR method described above. IsoANF mRNA levels in the right atria of 5-week-old SHR were about 40% of those in WKY rats (p<0.025) and increased with age to levels comparable to those found in WKY rats (Figure 5). In right ventri-
FIGURE 4. Scatterplot shows kinetics of polymerase chain reaction (PCR) amplification of atrial natriuretic factor (ANF) sequences. Portions of complementary DNA (cDNA) corresponding to 3 ng total RNA from right atria of 16-week-old Wistar-Kyoto rats were subjected to 17-24 PCR cycles, as described in "Methods." Genomic DNA (200 ng) was also added to each sample. PCR products generated from cDNA templates (440 bp, lower band in inset) or genomic templates (828 bp, upper band in inset) were quantified by laser densitometry of Southern blots, as described in the legend to Figure 3. The number of PCR cycles performed on each sample is indicated above the corresponding lane in the inset. Data represent a linear regression analysis of log₂ O.D. mRNA, messenger RNA.

Changes in Cardiac Atrial Natriuretic Factor Messenger RNA Levels in Spontaneously Hypertensive Rats

To examine whether the isoANF and ANF genes were coordinately regulated, cardiac ANF mRNA levels were simultaneously measured using PCR amplification techniques. The levels of ANF mRNA in right atria of 5-, 11-, or 16-week-old SHR were similar to those in age-matched WKY rats (Figure 5). Likewise, ANF mRNA levels in the left atria of 11- and 16-week-old SHR were not significantly different from those in WKY rats (Figure 5). However, ANF mRNA levels were higher in the left atria of 5-week-old SHR than in WKY rats (p<0.025). Thus, atrial levels of both ANF and isoANF mRNA were lower in SHR compared with WKY rats during the early hypertensive stage (see Figure 8).

The levels of ANF transcripts in right ventricles from SHR were comparable to those in WKY rats (Figure 6). In contrast, ANF mRNA levels were substantially increased in the left ventricle and the ventricular septum of SHR at 11 and 16 weeks of age (Figure 6). Furthermore, within the SHR, the largest increase in ANF mRNA is observed between 5- and 11-week-old animals, which also corresponds to the largest difference in

TABLE 1. Changes in Mean Arterial Blood Pressure and Heart Weight in Spontaneously Hypertensive Rats During Progression of Hypertension

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Body weight (g)</th>
<th>Heart weight (mg)</th>
<th>Atrial weight (mg)</th>
<th>Ventricular weight (mg)</th>
<th>SBP (mm Hg)</th>
<th>n</th>
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<tbody>
<tr>
<td>SHR</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>5</td>
<td>82±2*</td>
<td>521±12</td>
<td>37±4†</td>
<td>354±7</td>
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<td>990±29</td>
<td>56±2†</td>
<td>878±25</td>
<td>154±3*</td>
<td>9</td>
</tr>
<tr>
<td>16</td>
<td>283±8*</td>
<td>1,106±16</td>
<td>66±3</td>
<td>1,005±15</td>
<td>154±3*</td>
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</tr>
<tr>
<td>WKY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>5</td>
<td>92±2</td>
<td>460±10</td>
<td>56±5</td>
<td>334±17</td>
<td>91±5</td>
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<td>283±7</td>
<td>920±18</td>
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<td>77±7</td>
<td>939±27</td>
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</table>

Note that when ventricular weight is expressed per 100 grams body weight, ventricles appear to be 15–20% larger in spontaneously hypertensive rats (SHR) than in Wistar-Kyoto (WKY) rats at all ages (p<0.001). Values are mean±SEM. SBP, systolic blood pressure.

Statistical significance: *p<0.001, †p<0.01 SHR vs. WKY rats.

icles, isoANF mRNA levels did not differ significantly between SHR and age-matched WKY controls (Figure 6).

The largest changes in isoANF mRNA content in SHR relative to WKY rats were observed in left ventricle and ventricular septum. In left ventricle, isoANF mRNA levels were, respectively, twofold, fourfold, and fivefold higher in 5-, 11-, and 16-week-old SHR than in age-matched WKY rats. In ventricular septum, which appears to contain isoANF mRNA levels similar to those in left ventricle, isoANF mRNA levels were comparable in 5-week-old SHR and WKY rats. However, isoANF mRNA levels increased in the ventricular septum with increases in blood pressure to levels threefold higher in 11- and 16-week-old SHR relative to control WKY rats (Figure 6, p<0.025). Thus, in SHR, the most pronounced increases in isoANF mRNA levels occurred in right atrium, left ventricle, and ventricular septum in animals between 5 and 11 weeks of age simultaneously with the largest increases in SBP recorded (Table 1). The stabilization of SBP during the period between 11 and 16 weeks of age in SHR was accompanied by little change in cardiac isoANF mRNA levels.

Changes in Cardiac Atrial Natriuretic Factor Messenger RNA Levels in Spontaneously Hypertensive Rats

To examine whether the isoANF and ANF genes were coordinately regulated, cardiac ANF mRNA levels were simultaneously measured using PCR amplification techniques. The levels of ANF mRNA in right atria of 5-, 11-, or 16-week-old SHR were similar to those in age-matched WKY rats (Figure 5). Likewise, ANF mRNA levels in the left atria of 11- and 16-week-old SHR were not significantly different from those in WKY rats (Figure 5). However, ANF mRNA levels were higher in the left atria of 5-week-old SHR than in WKY rats (p<0.025). Thus, atrial levels of both ANF and isoANF mRNA were lower in SHR compared with WKY rats during the early hypertensive stage (see Figure 8).

The levels of ANF transcripts in right ventricles from SHR were comparable to those in WKY rats (Figure 6). In contrast, ANF mRNA levels were substantially increased in the left ventricle and the ventricular septum of SHR at 11 and 16 weeks of age (Figure 6). Furthermore, within the SHR, the largest increase in ANF mRNA is observed between 5- and 11-week-old animals, which also corresponds to the largest difference in
blood pressure. These results were in agreement with data obtained from Northern blot quantification of cardiac ANF mRNA in SHR and WKY rats as shown in Figures 7A and 7B and summarized in Table 2.

**Total Cardiac Iso-Atrial Natriuretic Factor and Atrial Natriuretic Factor Messenger RNA Content**

Based on the mean abundance of atrial and ventricular isoANF and ANF transcripts, as well as the mean heart weight of each group of animals (Table 1), the relative contribution of atria and ventricles to total cardiac ANF and isoANF mRNA content was estimated (Figure 8A). In all groups of animals, ventricular isoANF mRNA constituted over 90% of total cardiac isoANF mRNA, indicating that isoANF is predominantly a ventricular hormone. Total isoANF mRNA levels were already twofold higher in 5-week-old SHR than in age-matched WKY rats and increased further...
FIGURE 6. Ventricular iso-atrial natriuretic factor (isoANF) and ANF messenger RNA (mRNA) levels in spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Panel A: Polymerase chain reaction (PCR) amplification of isoANF and tubulin (top) and ANF (bottom) sequences. IsoANF and tubulin sequences were amplified using complementary DNA (cDNA) corresponding to 150 ng total ventricular RNA and 20 PCR cycles. IsoANF sequences (150 ng) were also amplified in the presence of 200 ng genomic DNA. To amplify ANF sequences, samples containing 200 ng genomic DNA and cDNA corresponding to 14 ng (left ventricle and ventricular septum) or 28 ng (right ventricle) total RNA were subjected to 20 PCR cycles as described in "Methods." For simplicity, the products arising from amplification of genomic sequences are not shown. Panel B: Bar graph shows relative abundance of ventricular ANF transcripts in SHR and WKY rats. Results are expressed as mean±SEM of four measurements. IsoANF transcripts in the left ventricle of 11-week-old WKY rats constituted about 0.003% of total ventricular mRNA. Panel C: Bar graph shows relative abundance of ventricular isoANF transcripts in SHR or WKY rats, expressed as mean±SEM of four measurements. IsoANF transcripts in the left ventricles of 11-week-old WKY rats constituted about 0.0007% of total ventricular mRNA. *p<0.05, **p<0.025 relative to the corresponding compartment in age-matched WKY rats. S, SHR; W, WKY rats.

with age to reach levels threefold higher in 11- or 16-week-old SHR, relative to age-matched WKY rats. In contrast, ANF levels in 5-week-old SHR were about 50% of those in the WKY controls (Figure 8B), suggesting a deficiency in ANF gene expression during the early stages of hypertension in SHR. Cardiac ANF mRNA content increased modestly with progression of hypertension, reaching levels 1.4-fold higher in 16-week-old SHR than in age-matched WKY rats.

Discussion

Cardiac myocytes synthesize two peptide hormones, ANF and isoANF, which share structural and functional properties. ANF and isoANF are encoded by two distinct genes that may be coordinately or differentially regulated. We have investigated changes in cardiac ANF and isoANF mRNA levels in both normotensive rats and SHR at the early stages and during progression of hypertension, using quantitative PCR amplification. Our results show that increases in blood pressure are accompanied by differential changes of both ANF and isoANF mRNAs in different cardiac compartments.

Quantitative Amplification of Cardiac Atrial Natriuretic Factor and Iso-Atrial Natriuretic Factor Transcripts

Although the abundance of cardiac ANF transcripts is high enough to allow their quantification by several methods, the scarcity of cardiac isoANF transcripts complicates their accurate quantification by standard RNA assays such as Northern blot or RNase protection analysis. To measure changes in cardiac ANF and isoANF gene expression at the mRNA level, we developed a quantitative PCR assay that allows simultaneous
measurements of various cardiac transcripts. Quantitative amplification of transcript sequences by PCR is a powerful technique that may have important clinical applications, especially when transcript abundance falls below the detection limits of conventional protocols or when examining gene expression in small samples such as biopsy specimens. However, the yield of PCR products reflects initial template levels only under conditions in which the templates are amplified exponentially. Because of the exponential character of the PCR process, even small differences in any of the variables that control the amplification may greatly affect the amount of DNA produced. Such variables include initial amounts of template, number of PCR cycles, quality of initial RNA preparations, concentration of thermostable DNA polymerase and other reagents, and even small inter-well variation in the thermal cycler block. The optimal initial template quantities and number of PCR cycles must be experimentally defined for each system, and any variation in amplification efficiency

![Image](https://example.com/image.png)

**Figure 7.** Northern blot analysis of cardiac atrial natriuretic factor (ANF) messenger RNA (mRNA). Panel A: Representative Northern blot showing ANF and β-actin mRNA in 1 µg of total atrial RNA. Panel B: Representative Northern blot detection of the same mRNAs in ventricular total RNA. Note the difference in the amount of total RNA used in right and left ventricles and the much greater amount of total RNA required for detection of ANF mRNA in ventricles. S, spontaneously hypertensive rats; W, Wistar-Kyoto rats.

**Table 2.** Percentage of Atrial Natriuretic Factor Messenger RNA in Spontaneously Hypertensive Rats Relative to Age-Matched Wistar-Kyoto Rats As Determined by Polymerase Chain Reaction Amplification and Northern Blot Analysis

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>PCR</th>
<th>Northern blot</th>
<th>PCR</th>
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<td>11</td>
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<tr>
<td>Left atria</td>
<td>50</td>
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<td>120</td>
<td>100</td>
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<tr>
<td>Right atria</td>
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<td>100</td>
<td>150</td>
<td>120</td>
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<tr>
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</tr>
<tr>
<td>Right ventricles</td>
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<td>20</td>
<td>110</td>
<td>100</td>
<td>150</td>
<td>180</td>
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</table>

The data are expressed as the percentage of mean (n=4) of atrial natriuretic factor (ANF) messenger RNA (mRNA) in spontaneously hypertensive rats (SHR) relative to age-matched Wistar-Kyoto (WKY) rats for individual heart compartments. Cardiac ANF mRNA levels in SHR and WKY rats were measured by polymerase chain reaction (PCR) or by Northern blot analysis in the same total RNA preparations. PCR assays were conducted as described in "Methods" using 3 ng total atrial RNA or 15–30 ng total ventricular RNA. Tubulin sequences were coamplified to correct for variations in the initial amounts of complementary DNA (cDNA) present for amplification. For Northern blot analysis, 250 ng total atrial RNA or 15–60 µg total ventricular RNA were used. Blots were sequentially hybridized to a labeled 600-bp fragment containing rat ANF cDNA sequences and to a β-actin cDNA probe to correct for variations in the amounts of samples loaded on gel as previously described.18
between samples should be determined using appropriate internal controls.

Because of the wide differences in cardiac ANF and isoANF mRNA levels, optimal amounts of initial template for each transcript were determined in both atria and ventricles. Exponential amplification of isoANF sequences was attained with initial amounts of template equivalent to 40–200 ng total RNA (Figure 1). The sensitivity of the quantitative PCR method under these conditions was demonstrated, since a 25% difference in the initial amount of cDNA template was easily detected (Figure 1). Since genomic sequences were coamplified as internal control, the dependence of PCR product yield on the number of cycles was examined in samples containing both genomic and cDNA templates. Genomic isoANF sequences were deemed appropriate internal controls because of their similarity to the cDNA sequences and because primer efficiency is identical for both templates.

The RNA samples used for these experiments were quantified spectrophotometrically; total mRNA was quantitated by hybridization to end-labeled p(dT),12-18 and cytoplasmic actin transcripts were measured by Northern blots (data not shown). However, to account for differences in the efficiency of cDNA synthesis, tubulin sequences were also coamplified with isoANF templates. Comparison between the initial estimates of RNA levels and the yields of tubulin sequences amplified failed to reveal any substantial changes in tubulin transcription with age or with progression of hypertension, confirming that tubulin is an appropriate control to correct for variations in cDNA template levels in this system (Figures 5 and 6).

Results of the PCR amplifications were compared with mRNA measurements obtained independently using Northern blot analysis for ANF (Table 2) and primer extension assays for isoANF, which were carried out on selected samples (not shown). In all cases, the data obtained by PCR were in agreement with those generated by the more conventional RNA assays. The quantitative PCR approach offers the advantage of higher sensitivity and therefore requires much less initial tissue sample.

**Differential Expression of Natriuretic Peptide Genes During the Early Stage of Hypertension**

Given the diuretic, natriuretic, and vasodilatory activities of cardiac natriuretic peptides, these hormones are candidates to play an important role in cardiovascular homeostasis. In the present study, the hypothesis that alterations in cardiac natriuretic peptide gene expression accompany the development of hypertension was tested.

During the early stages of the hypertensive state in SHR, ANF mRNA concentrations in left atria of SHR were significantly lower ($p<0.025$) as compared with those in age-matched WKY rats. In the other heart compartments (right atria, left ventricle, and ventricular septum) ANF mRNA levels were similar in SHR and WKY rats. These results were consistently obtained using Northern blot analysis and PCR amplification (Figures 6 and 7 and Table 2). In contrast, isoANF mRNA concentrations were similar in left atria, right ventricles, and ventricular septum of 5-week-old SHR and WKY rats. IsoANF mRNA concentrations were lower in right atria and twofold higher in left ventricles of young SHR compared with those in age-matched WKY rats. These data clearly point to a differential spatial regulation of the two natriuretic hormone genes within the heart during the early stages of hypertension. The differential regulation of ANF and isoANF genes leads to a striking difference in the total amount of ANF and isoANF mRNA per heart between SHR and WKY rats (Figure 8). Indeed, hearts from 5-week-old SHR...
isoANF synthesis, the total amount of isoANF tran-
tests than ANF. Since ventricles are the major sites of
isoANF gene expression was induced in heart ventricles
by stimulation of both ANF and isoANF mRNA levels,
individuals with mild hypertension.

The deficiency in ANF and isoANF gene expression in several heart compartments raises the possibility that these peptide hormones may be involved in the patho-
gensis of hypertension. Although several reports have
described changes in ANF mRNA and peptide levels in
SHR,17-19,24 most of these studies have focused on older
animals with well-developed high blood pressure. These
animals had compensatory increases in ANF mRNA
levels, which is not surprising since ANF synthesis is
augmented in response to increases in blood pressure.3,4
Our data (discussed below) indicate that the isoANF
gene is also upregulated in parallel to increased blood
pressure. Considering that SBP in 5-week-old SHR was
already enhanced, ANF and isoANF mRNA levels
might already reflect a stimulated state. This in turn
raises the intriguing possibility that ANF and isoANF
gene expression might be deficient in younger prehyper-
tensive animals and that this deficiency in natriuretic
peptide gene expression might contribute to develop-
ment of genetic hypertension in SHR and possibly in
humans.

Hyperresponsiveness of the Iso-Attrial
Natriuretic Factor Gene to Hypertension

Previous studies on changes in ANF gene expression
in old SHR had revealed a preferential increase in ANF
mRNA and peptides in heart ventricle.24-25 Our results
(Figure 6 and Table 2) are in perfect agreement with
these studies. Similar increases in ANF gene expression
that accompany the development of hypertension have
been observed in other animal models, such as Dahl
salt-sensitive rats.26 Although there is general agree-
ment on a positive correlation between hypertension
and stimulation of ventricular ANF mRNA levels, the
association of increased blood pressure with changes in
plasma ANF levels is still controversial. For example,
with progression of hypertension in SHR from 3 to 16
weeks, plasma immunoreactive ANF (irANF) levels
increased fourfold relative to those in WKY rats.17
However, in several studies there was no significant
increase in irANF plasma levels in animal27,28 or hu-
mant29,30 hypertension. Our results showing the total
amount of ANF transcripts per heart (Figure 8) re-
vealed little difference between hypertensive SHR and
age-matched WKY rats despite increased ventricular
ANF gene expression. This finding is in perfect agree-
ment with measurements of plasma irANF, which failed
to reveal any significant difference between 10- or
17-week-old SHR and WKY rats (Lavigne J.-P. and
Nemer M., unpublished results). These results suggest
that total cardiac ANF production is not significantly
enhanced in hypertension and may be significant to
understanding the inconsistent plasma irANF data in
individuals with mild hypertension.

Although increased blood pressure was accompanied
by stimulation of both ANF and isoANF mRNA levels,
isoANF gene expression was induced in heart ventricles
during progression of hypertension to far greater ex-
tents than ANF. Since ventricles are the major sites of
isoANF synthesis, the total amount of isoANF tran-
scripts in SHR hearts is significantly higher than in
WKY rats in all age groups (Figure 8). Since hormone
secretion from ventricles follows the constitutitive path-
way, these findings suggest that isoANF plasma levels
would be increased to a greater extent than those of
ANF in SHR. This prediction is in line with recent
findings showing greater increases in the levels of plasma
isoANF than ANF in deoxycorticosterone ace-
tate–salt hypertensive rats, another animal model of
hypertension,31 and in human hypertension.13 Further-
more, while this article was in preparation increased
ventricular isoANF synthesis and secretion were re-
ported in 12-week-old stroke-prone SHR relative to
age-matched WKY rats,32 and these increases were
accompanied by enhanced isoANF mRNA levels. Thus,
our data, together with these observations, suggest that
the isoANF gene is hyperresponsive to pressure and
that it may represent a sensitive marker of cardiac
changes associated with early manifestations of
hypertension.

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