Hypertrophy, Pathology, and Molecular Markers of Cardiac Pathogenesis

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Abstract—Increased ventricular expression of several genes, including atrial natriuretic factor (ANF), has been documented in experimental models of cardiac hypertrophy. It remains to be clarified whether altered expression of these genes is a consistent marker of the hypertrophy itself or a marker of some parallel pathogenetic process. Using a transgenic mouse model of hypertrophic cardiomyopathy as a tool, we assessed the relationship between the amount of ventricular ANF gene expression and the degree of hypertrophy as well as the relationship between the cells expressing ANF and tissue pathology. We determined that hypertrophy is not always associated with increased ventricular expression of ANF and that cells expressing ANF are found in regions of tissue pathology. We propose that alteration in the ventricular expression of this gene is a sensitive indicator of cardiac pathogenesis and may result from a number of different stimuli that include, among others, abnormal tissue architecture and hemodynamic load. (Circ Res. 1998;82:773-778.)

Key Words: hypertrophy • gene expression • pathogenesis • atrial natriuretic factor

Hypertrophy is a compensatory response that allows the heart to cope with the pathogenic stimuli found with many cardiovascular diseases, including the primary myocardial dysfunction associated with cardiomyopathies. The normal and hypertrophied heart exhibit qualitative as well as quantitative differences in gene expression. This observation has spurred efforts over the past decade to understand the molecular mechanisms underlying the hypertrophic process, and a number of genes have been identified that exhibit altered expression patterns in cardiac hypertrophy. Among others, these include transient expression of proto-oncogenes and increased expression of α-skeletal actin and β-myosin heavy chain. It is thought that these changes may reflect a shift toward an embryonic program of gene expression (for a review see Reference 9).

Changes in the expression of ANF in patient populations and in experimental models of cardiac hypertrophy have been well documented. ANF, a peptide hormone with diuretic, natriuretic, and vasorelaxant properties (for reviews see References 17 and 18), was first identified in extracts of rat atria. Under normal conditions in the mammalian heart, atrial levels of ANF are about 100-fold greater than ventricular levels, whereas ventricular expression of ANF increases with conditions of increased hemodynamic load, often concomitant with increases in ventricular mass (for examples see References 4, 10, 11, and 12).

However, it remains to be clarified whether altered expression of ANF is a marker of the hypertrophic process or whether it is a marker of a parallel process during cardiac pathogenesis. For example, is there a quantitative relationship between the amount of gene expression and the degree of hypertrophy, such that a threshold level of gene expression is indicative of hypertrophy? Through analysis of a transgenic mouse model of HCM, we show that hypertrophy can occur in the absence of increased ventricular levels of ANF message and that increased levels of this mRNA can also occur in the absence of detectable cardiac hypertrophy. In this genetic model, increases in ventricular levels of the ANF gene product reflect local changes in gene expression that correlate with areas of tissue pathology, prompting the conclusion that to some extent increased ventricular expression of ANF reflects part of a cellular pathological response.

Materials and Methods

Animal Husbandry

A previously described transgenic mouse model for HCM was used for these studies. These animals express a mutant α-myosin heavy chain with expression driven by a rat α-myosin heavy chain promoter. HCM transgenic mice, line 140, were maintained under specific pathogen-free conditions with free access to water and food. Heterozygously transgenic lines were maintained with backcrossing to C57/Bl6 mice obtained from the Institute for Behavioral Genetics, University of Colorado at Boulder. At the time of these studies, the transgenic line had been backcrossed to C57/016 for >14 generations. All animal protocols were approved by the Institutional Animal Use and Care Committee at the University of Colorado at Boulder.
Selected Abbreviations and Acronyms

ANF = atrial natriuretic factor
HCM = hypertrophic cardiomyopathy
LV = left ventricle
RV = right ventricle

Slot Blot Analysis
Total RNA was purified from LV and RV tissue using the guanidinium–acid phenol method.\textsuperscript{[2]} Total RNA (3 μg) in denaturing buffer (50% formamide, 6% formaldehyde, 20 mmol/L MOPS, pH 7.0, 50 mmol/L sodium acetate, and 1.0 mmol/L EDTA) was heated to 65°C for 15 minutes and then rapidly cooled on ice. Two volumes of 20× SSC (3 mol/L NaCl and 0.3 mol/L) was added, and the mixture was applied to positively charged nylon membrane using a vacuum slot-blott apparatus. Random-primed \textsuperscript{32}P-labeled probes were generated with the Ready-to-Go DNA labeling kit (Pharmacia Biotech) using a PstI fragment from a rat ANF cDNA,\textsuperscript{[23]} a PstI fragment from a rat GAPDH cDNA,\textsuperscript{[24]} or a Sac–HindIII fragment corresponding to the 5′ untranslated region of murine α-skeletal actin mRNA\textsuperscript{[25]} as templates. Membranes were hybridized overnight at 37°C in 50% formamide, 5× SSPE (0.9 mol/L NaCl, 0.05 mol/L sodium phosphate, and 5 mmol/L EDTA, pH 7.5), \texttimes 1 Denhardt’s solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 0.2% SDS, 0.2 mg/mL sheared denatured salmon sperm DNA, and 1\times 10\textsuperscript{6} cpm/mL of \textsuperscript{32}P-labeled probe. Afterward, the membranes were washed for 10 minutes at 65°C in each of the following buffers: 2× SSC and 0.2% SDS; 1× SSC, 0.2% SDS; 0.5× SSC, and 0.2% SDS; and 0.1× SSC and 0.2% SDS; the membranes were then exposed to PhosphorImager plates (Molecular Dynamics). Images were acquired using a STORM PhosphorImager (Molecular Dynamics, and the intensity of the signals was measured using ImageQuant software (Molecular Dynamics). Hybridization to yeast tRNA was used to determine background levels.

In Situ Hybridization
A 580-bp PstI fragment from the rat ANF cDNA\textsuperscript{[23]} was subcloned into pBluescriptKS (Stratagene) and used as a template to generate sense and anti-sense \textsuperscript{35}S-labeled RNA probes. A 364-bp region of the SV40 large T antigen that corresponds to the 3′ end of the HCM transgene\textsuperscript{[21]} was subcloned into pTZ19R (United States Biochemical) and used to generate a transgene-specific anti-sense RNA probe. All RNA probes were synthesized by using the Promega Riboprobe kit and following the manufacturer’s instructions.

Hearts were removed from 12-week-old female HCM mice and fixed overnight at 4°C in PBS containing 4% formaldehyde. After they were dehydrated through a graded ethanol series and cleared in cedarwood oil, tissues were embedded in paraffin. Sections (7 μm) were cut on a microtome and adhered to SectionLock slides (Polysciences Inc). Subsequent processing of the specimens followed the procedure described by Sassoon and Rosenthal\textsuperscript{[26]} with the addition of an N-ethylmaleimide blocking step.\textsuperscript{[27]} After hybridization and washing, slides were dried and then coated with Kodak NTB2 autoradiography emulsion (Eastman Kodak). Slides were developed using D-19 developer (Kodak) and photographed with bright-field optics.

Electron Microscopy
Hearts were removed, rinsed in saline, fixed with a mixture of formaldehyde and glutaraldehyde,\textsuperscript{[28]} and then embedded. Abnormal regions within the myocardium were identified in thick sections. Thin sections were then cut from these regions, stained with uranyl acetate and lead citrate, and examined on the electron microscope.

Statistical Analysis
Data are expressed as the mean±SD. Differences between groups were assessed using an unpaired Student $t$ test, and the correlation between various parameters was determined by linear regression analysis. In all cases, differences were considered significant at $P<.05$.

Results
Increased Ventricular Expression of ANF mRNA Does Not Correlate With Hypertrophy in a Murine Model of HCM
To determine the relationship between global hypertrophy, tissue pathology, and the induction of ventricular expression of ANF, we examined the ventricular levels of this message in a transgenic mouse model of HCM.\textsuperscript{[21]} Previous characterization of this transgenic mouse had shown that 12-week-old animals exhibit significant LV and RV hypertrophy.\textsuperscript{[21]} When Northern blot analysis was used to assess the mRNA expression of ANF, a disproportionate increase in ANF mRNA levels was detected in the LV samples (Figure 1A), although transgene expression is similar in both the LV and RV. LV and RV RNA from 12-week-old female transgenic animals,
with nontransgenic littermates serving as controls, was also subjected to slot blot analysis using probes for ANF and α-skeletal actin. GAPDH mRNA levels were also determined for the same blots and used to correct for small variations in sample loading. Under the hybridization conditions used, these probes detect single bands on Northern blots with the extent of LV hypertrophy ($R = 0.09; P > .05$). However, the abundance of ANF mRNA in the RV samples was indistinguishable from control samples ($R = 0.11$ for LV and RV comparisons, respectively). Similar results were seen in male HCM mice at this age as well (data not shown).

**Ventricular Expression of ANF mRNA Does Not Result From Focal Transgene Expression**

The discordance between hypertrophy and ANF mRNA levels in the RV suggests that some trigger other than hypertrophy may induce the increased ANF mRNA levels in the LVs of the HCM mice. We had previously noted that tissue pathology in these mice is focal and is more extensive in the LV than the RV, implying that the greater incidence of abnormal tissue architecture in the LV might be responsible for the increased LV ANF mRNA levels. However, it has been noted that transgene expression under the α-myosin heavy chain promoter sometimes results in patchy expression in the heart. Patchy expression of the mutant myosin mRNA in these HCM mice could also contribute to differential gene expression between the RV and LV. To determine the relationship between transgene expression and ANF expression, we performed in situ hybridization analysis with probes recognizing either ANF or transgene mRNAs. In HCM mouse hearts, transgene expression was found uniformly throughout the LV and RV, with lower expression seen in the atria (Figure 3A and 3C). As expected, ANF gene expression was very strong in the atria (Figure 3B). Intensely positive cells were also found in foci throughout the LV (Figure 3B and 3D). A small number of ANF mRNA–positive cells were also seen in control hearts, especially near the endomyocardial surface and at the fibrous base of the cardiac valves (not shown). However, in control animals, the number of ANF-positive cells in the ventricular myocardium was much less than in the HCM hearts, and they were found in scattered groups containing one to three positive cells. These staining patterns do not result from nonspecific hybridization, since the transgene-specific probe was negative in control mouse hearts and a sense-ANF probe was negative in both control and HCM hearts (data not shown).

**Foci of ANF-Positive Ventricular Myocytes Are Found in Regions With Tissue Pathology and Fibrosis**

The pattern of ANF mRNA–positive cells in the HCM mouse hearts was suggestive of the pattern of tissue pathology seen in the hearts of these mice. To determine whether ANF mRNA expression occurred in regions of tissue pathology, serial sections were obtained from 12-week-old female HCM mice and processed for in situ hybridization with the antisense ANF probe or stained with Masson’s trichrome to visualize areas of fibrosis (Figure 4). Regions of the ventricular myocardium with foci of intensely positive cells were identified by in situ hybridization (Figure 4A), and the corresponding region in the adjacent section was located (Figure 4B). Foci of ANF-expressing ventricular myocytes were found in regions of the heart with tissue pathology, especially in regions with fibrosis but also including severe disarray. Interestingly, many of these foci of ANF expression and tissue pathology were found adjacent to small intramural coronary vessels.

**ANF Secretory Granules Are Found in Areas of Abnormal Tissue Architecture**

The colocalization of foci of ANF expression and regions of tissue pathology described above is limited by the distance between adjacent paraffin sections (~7 μm) and by one’s ability to match landmarks in two adjacent sections. Therefore, we wished to confirm our findings by using a higher resolution. In atrial myocytes, ANF is stored in distinctive secretory granules that are often called atrial particles. Although these particles are scarce in the normal rodent ventricle, they are found more readily in pathological states (for example see Reference 30). We predicted that if areas containing tissue pathology were identified in HCM mouse hearts and then examined by electron microscopy, we would find ventricular myocytes containing atrial particles. Regions of ventricular myocardium with significant cellular disarray or fibrosis were identified in thick sections of embedded...
tissue, and thin sections of these regions were examined by electron microscopy. Areas with abnormal ultrastructure were readily apparent in these sections, and cells with prominent secretory granules similar in appearance to atrial particles were identified (Figure 5). These cells typically were surrounded by matrix accumulations with prominent collagen fibrils (Figure 5, see asterisks). Although more extensive characterization would be required to conclude that these secretory granules are in fact ANF particles, these morphological data are consistent with our observation that increased ANF gene expression in this mouse model of cardiomyopathy is strongly associated with the presence of tissue pathology.

Discussion

The development of cardiac hypertrophy is accompanied by changes in cardiac gene expression that are thought to provide the heart with a means to compensate for increased hemodynamic load. During cardiac hypertrophy in rodents, increased ventricular expression of genes, such as ANF, α-skeletal actin, and β-myosin heavy chain, normally expressed during development may reflect the reinduction of an embryonic pattern of gene expression. Increases in ventricular expression of ANF have been documented in numerous experimental models of cardiac hypertrophy and failure as well as in human heart failure. In these pathological states, increased ANF levels may serve to reduce preload through the natriuretic and vasodilatory properties of the secreted peptide (for a review see Reference 17), leading to the hypothesis that increased ventricular levels of ANF may be a molecular marker of cardiac hypertrophy.

It is clear that increased ventricular expression of ANF can occur in response to a number of different stimuli. Investigators have shown significant correlation between the extent of LV hypertrophy and LV ANF mRNA content in experimental models of cardiac hypertrophy resulting from volume or pressure overload or isoproterenol infusion. It has been suggested that changes in gene expression accompanying cardiac hypertrophy are the result of a multifactorial process. In the case of ventricular expression of ANF, this is clearly the case. Careful monitoring of the induction of ANF message after the imposition of hemodynamic overload or isoproterenol infusion demonstrates a biphasic pattern of induction with the greatest increases in ventricular ANF levels occurring within 3 to 4 days after the imposition of the stimulus. Both load-dependent and load-independent mechanisms and the renin-angiotensin system have been implicated in this process.

We have presented data suggesting that cardiac hypertrophy and increased ventricular expression of ANF are not necessarily correlated. Through analysis of a transgenic mouse model of HCM we have shown that hypertrophy can occur in the absence of increased ventricular levels of ANF message and that increased levels of this mRNA can also occur in the absence of detectable cardiac hypertrophy. In this genetic model, increases in ventricular levels of the ANF
gene product reflect local changes in gene expression that appear to correlate with areas of tissue pathology. Foci of cells positive for ANF expression were often found near small intramural vessels in the cardiomyopathic hearts (Figure 4). In normal rat heart, solitary myocytes immunopositive for ANF have been detected, albeit rarely, in the vicinity of small intramural vessels. In the HCM mice, increased incidence of ANF-positive myocytes near small vessels may reflect a regional pathogenetic process. We had previously reported the presence of abnormal small vessels in the hearts of these transgenic mice, a feature that is also seen in most patients with HCM. Local changes in ANF gene expression may reflect the response of the surrounding myocardium to alterations in vessel structure and/or function or may occur concomitant with increasing fibrosis in the vessel vicinity.

Regional changes in cardiac gene expression have been noted in other model systems. Induction of ventricular ANF mRNA levels is greater in the septum of young cardiomyopathic Syrian hamsters than in the RV or LV free wall. During the early phases of cardiac hypertrophy in a rat model of pressure overload, β-myosin heavy chain gene expression exhibits transmural differences as well as increases around large coronary arteries. In addition, in endomyocardial biopsies taken from HCM patients, increased ANF levels are seen in specimens that also exhibit significant fibrosis and cellular disarray.

In our experimental model, we detected relatively small changes in ANF gene expression. It should be noted that much greater increases in ANF gene expression have been reported in experimental models of acute pressure or volume overload (10- to 20-fold increases), which may represent a different pathogenetic response. The relatively small increases in ventricular ANF mRNA that we have seen in young HCM mice may reflect an early phase in the pathogenesis of this murine cardiomyopathy. In any case, it is clear that increased ventricular expression of ANF is not always associated with cardiac hypertrophy and thus should not be considered a stable marker of cardiac hypertrophy. We propose that alteration in the ventricular expression of this gene is a sensitive indicator of cardiac pathogenesis and may result from a number of different stimuli that include, among others, abnormal tissue architecture and hemodynamic load.

Figure 4. Serial sections from 12-week-old female HCM mice were processed for in situ hybridization with the ANF probe (A) or stained with Masson’s trichrome (B). Foci of ANF-positive ventricular myocytes (for examples, see arrows in panel A) were found in regions of the ventricle that in the adjacent section (B) exhibited significant tissue pathology and fibrosis. Bar=250 μm.

Figure 5. Granules resembling ANF particles were found in ventricular myocytes bordering regions of collagen accumulation. AP, indicates ANP granules; asterisk, collagen fibrils. Bar=0.1 μm.
Molecular Marker of Cardiac Pathogenesis

Acknowledgments

This study was supported by a grant to Dr Leinwand from the Colorado/Wyoming Affiliate of the American Heart Association. The authors would like to thank Amy Whilde for cloning the ANF riboprobe template during an Undergraduate Research Opportunity fellowship supported by the Undergraduate Research Initiative of the Howard Hughes Medical Institute at the University of Colorado, Boulder.

References


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Circ Res. 1998;82:773-778
doi: 10.1161/01.RES.82.7.773

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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