Mechanical Unloading Versus Neurohumoral Stimulation on Myocardial Structure and Endocrine Function In Vivo

Ondrej Lisy, MD; Margaret M. Redfield, MD; Sofija Jovanovic, DVM, PhD; Michihisa Jougasaki, MD, PhD; Aleksandar Jovanovic, MD, PhD; Hanna Leskinen, MD, PhD; Andre Terzic, MD, PhD; John C. Burnett, Jr, MD

Background—Mechanical load and humoral stimuli such as endothelin (ET) and angiotensin II (Ang II) are potent modulators of cardiac structure and endocrine function, specifically gene expression and production and release of atrial natriuretic peptide (ANP). We define the contribution of mechanical load compared with neurohumoral stimulation in vivo with specific focus on myocardial and circulating ANP during chronic myocardial unloading produced by thoracic inferior vena caval constriction (TIVCC).

Methods and Results—TIVCC was produced by banding the IVC for 10 days in 7 dogs, whereas in the 6 control dogs, the band was not constricted. TIVCC was characterized by a decrease in cardiac output, right atrial pressure, and left ventricular (LV) end-diastolic diameter and marked activation of ET and Ang II in plasma and atrial and ventricular myocardium. Despite neurohumoral stimulation, LV mass index and myocyte diameters in unloaded hearts decreased, reflecting myocyte atrophy. The total number of myocytes in the LV remained unchanged. Atrial stores of ANP increased, but plasma ANP did not change, in association with a trend toward ANP gene expression to decrease in unloaded hearts.

Conclusions—Chronic mechanical unloading of the heart results in myocardial atrophy and lack of activation of ANP synthesis despite marked neurohumoral stimulation by the growth promoters ET and Ang II. (Circulation. 2000;102:338-343.)

Key Words: myocardium ■ heart-assist device ■ atrial natriuretic peptide ■ endothelin ■ angiotensin

Elucidation of physiological and biochemical mechanisms that contribute to the control of myocardial structure and function continues to evolve as this relates to congestive heart failure (CHF). Investigations have established mechanical load as a modulator of cardiac structure, including cardiomyocyte size and shape.1,2 Studies have provided evidence that the humoral factors endothelin (ET) and angiotensin II (Ang II) promote myocardial cell growth.3–6 Thus, receptor antagonists to ET and Ang II have been developed to interrupt their growth-promoting actions in CHF, in which activation of myocardial and circulating ET and Ang II is a hallmark.7,8

Both mechanical and humoral stimuli possess properties that modify myocardial endocrine function, specifically gene expression and production and release of atrial natriuretic peptide (ANP). ANP possesses growth-inhibiting, antifibrotic, vasodilatory, and natriuretic properties.9–11 The importance of understanding mechanisms that control ANP synthesis is underscored by genetic or pharmacological blockade of ANP receptors and studies in which exogenous ANP is administered or ANP is overexpressed in vivo.9–13

Such studies established that ANP suppresses myocardial growth and participates in arterial pressure and volume homeostasis. With regard to ANP production, myocardial stretch as well as ET and Ang II activate ANP gene expression and release from both intact hearts and cultured cardiomyocytes.14–18

Although mechanical load and neurohumoral stimulation contribute to cardiac remodeling and myocardial endocrine function, it is unclear which stimuli predominate when the heart is unloaded. This question has clinical relevance, because diuretics reduce preload and activate neurohumoral factors, including the renin-angiotensin-aldosterone system (RAAS) and ET. Another paradigm to myocardial unloading is the use of left ventricular assist devices (LVADs) in CHF. LVADs reduce cardiac volumes, but with variable actions on biochemical mediators. Furthermore, growth-promoting properties of ET and Ang II are difficult to discern, because their activation occurs in states, such as CHF, in which mechanical load to the heart is increased.

Our study defined the relative primacy of mechanical unloading compared with neurohumoral stimulation by en-
Endogenous ET and Ang II in vivo on myocardial structure and endocrine function. We used a unique model of chronic myocardial unloading produced by thoracic inferior vena caval constriction (TIVCC) that results in reduced myocardial load and marked activation of endogenous ET and Ang II.\textsuperscript{19–21} We found that chronic mechanical unloading of the heart resulted in myocardial atrophy despite marked increases in circulating and myocardial ET and Ang II. Despite activation of ET and Ang II, myocardial ANP storage was enhanced but ANP synthesis was not. These studies advance our understanding of the physical and biochemical mechanisms that modulate myocardial structure and endocrine function in vivo, underscoring the primacy of myocardial load compared with neurohumoral mechanisms during chronic cardiac unloading.

Methods

Surgical Procedure

Studies were performed in male mongrel dogs (20 to 26 kg); they conformed to the guidelines of the American Physiological Society and were approved by the Mayo Clinic Animal Care and Use Committee. Thirteen dogs were randomly divided into 2 groups. After they had received prophylactic antibiotic treatment, the surgery was performed, and an adjustable band was placed around the thoracic IVC. Group 1, sham-operated (Sham group), had the band placed without constriction (n = 6). Group 2, with TIVCC, had the band constricted (n = 7) to create ~50% reduction in the IVC weight at the time of TIVCC or sham surgery was used for this estimation. Left atrial and ventricular sections were taken from the myocardial unloading produced by thoracic inferior vena caval constriction (TIVCC) that results in reduced myocardial load and marked activation of endogenous ET and Ang II.\textsuperscript{19–21} We found that chronic mechanical unloading of the heart resulted in myocardial atrophy despite marked increases in circulating and myocardial ET and Ang II. Despite activation of ET and Ang II, myocardial ANP storage was enhanced but ANP synthesis was not. These studies advance our understanding of the physical and biochemical mechanisms that modulate myocardial structure and endocrine function in vivo, underscoring the primacy of myocardial load compared with neurohumoral mechanisms during chronic cardiac unloading.

Echocardiography

On day 11, an M-mode echocardiogram was performed with the dogs in the conscious state. LV end-diastolic (LVEDD) and end-systolic (LVESD) diameters were determined from 3 repeated M-mode tracings, and ejection fraction was calculated.\textsuperscript{23}

Acute Experiment

After the echocardiogram, dogs were anesthetized with pentobarbital sodium (30 mg/kg) and ventilated on room air supplemented with oxygen. The right jugular vein was exposed, and a thermodilution catheter was advanced into the pulmonary artery. The right femoral artery was cannulated for blood pressure measurement and blood sampling. Measurements were obtained after 60 minutes of equilibration. Brains were euthanized with KCl. Hearts were removed, and all chambers were dissected and weighed. The LV weight was used for calculating LV mass index (LV weight/body weight). Because dogs with TIVCC developed fluid retention and ascites, the body weight at the time of TIVCC or sham surgery was used for this calculation. Left atrial and ventricular sections were taken from the full thickness of the free wall and fixed in 10% buffered formalin. The tissue samples were frozen in liquid nitrogen and stored at −80°C until processing.

Histology

After fixation, LV sections were paraffin-embedded, cut, and mounted on glass slides. Slides were stained with hematoxylin-eosin and Masson’s trichrome according to the standard procedure.

Radioimmunoassays

Arterial blood for hormone analysis was collected into sodium-EDTA tubes, placed on ice, and centrifuged at 2500 rpm at 4°C. Plasma and tissue (left atrial and ventricular) ET were determined with an ET-1 assay (Amersham International) as described previously.\textsuperscript{21} Tissue homogenates were ultracentrifuged for 30 minutes at 15 000 rpm and 4°C, and the supernatant was stored at −20°C until radioimmunoassay, with a sample taken for protein measurement. Ang II was measured by radioimmunoassay. The plasma for Ang II measurements was collected in tubes containing protease inhibitors. Plasmas were extracted, washed and eluted with 1 mL methanol, lyophilized, and reconstituted. Standards or samples were incubated in glass tubes with a commercially available polyclonal rabbit antibody (Phoenix Pharmaceuticals, Inc) at 4°C for 18 hours.\textsuperscript{12} Labeled Ang II was added to each tube, and incubation was continued for another 18 hours at 4°C. Bound-antigen-antibody was separated with goat anti-rabbit γ-globulin, and the precipitate was counted on a γ-counter. The lower limit of detection of this assay is 0.5 pg/tube, with a standard range of 0.5 to 128 pg/tube. The intra-assay coefficient of variation is 9%, interassay coefficient of variation is 13%, and recovery is 71%. The cross-reactivity is 100% with Ang III and 0.5% with Ang I. Plasma and tissue ANP were determined by a sensitive radioimmunoassay as previously described.\textsuperscript{24}

Northern Blot Analysis

For analysis of myocardial ANP gene expression, mRNA was extracted from left atrial and ventricular samples (Fast-track Kit, Invitrogen). Four micrograms mRNA per extract was loaded on 1.2% agarose-formaldehyde gel and electrophoresed for 2 to 3 hours at 75 V. For amplification of the canine ANP cDNA fragment, the following primers were selected: sense, 5' -CAACGAGACCTGATGATT-3'; anti-sense, 5' -AGTCCGCTCTGGCTCCTCAAAT-3'. ANP probe was random-primed with [\textsuperscript{32}P], (random-primer DNA labeling kit, Boehringer-Mannheim Biochemical) and column-purified. To control for loading conditions and mRNA transfer onto the membranes, we rehybridized blots with a GAPDH probe. The respective autoradiographic bands for ANP and GAPDH were quantified with a scanning spectrophotometer, and ANP mRNA was expressed in arbitrary units as the ratio of autodigraphic density of the ANP band to that of the GAPDH band.

Immunohistochemistry

Immunohistochemical staining (IHCS) for ANP was performed in the left atrial tissue. IHCS studies were performed by the indirect immunoperoxidase method as described previously.\textsuperscript{25}

Morphometric and Stereological Analysis

To visualize single cardiomyocytes within myocardium, cardiac tissue slices were stained with fluorescein-conjugated wheat germ agglutinin (WGA-FITC; Vector Laboratories). Sections were examined on laser confocal microscopy and interfused with a computerized image analysis system (IBAS, Carl Zeiss Inc.).\textsuperscript{26} Morphometric and stereological parameters that define size and number of cardiomyocytes in the LV were determined.\textsuperscript{26} The maximal diameter, minimal diameter, and cross-sectional area of cardiomyocytes were determined directly by the IBAS image analyzer system,\textsuperscript{26} and the volume of cardiomyocytes was calculated according to the equation \(V_{myoc} = N_{myoc} \times R_{myoc} \times R_{myoc} \), where \(R_{myoc} \) is longitudinal cross-sectional area and \(R_{myoc} \) is transverse radius.\textsuperscript{26} The total number of cardiomyocytes in the LV was calculated with the equation \(N_{myoc} = \frac{N_{myoc} \times R_{myoc} \times R_{myoc}}{\text{LV vol}} \), where \(N_{myoc} \) is the number of cardiomyocytes per unit of volume (mm\(^3\)) of LV tissue and LV vol is LV volume, determined according to Astorri et al\textsuperscript{27} and Anversa et al.\textsuperscript{28}

Statistical Analysis

Results of quantitative studies are expressed as mean ± SEM. Statistical comparisons between groups were performed by unpaired Student’s t test. Statistical significance was accepted at a value of \(P<0.05\).

Results

Characteristics of the Model

Myocardial unloading produced by TIVCC was characterized by a significant decrease in cardiac output and right atrial pressure compared with the Sham group (Table 1). No
significant difference was observed in mean arterial pressure or pulmonary capillary wedge pressure between groups, whereas systemic vascular resistance was significantly higher in the unloaded heart group (Table 1). Echocardiography revealed a significant decrease in the LVEDD in the unloaded heart group, with no difference in systolic function (ie, ejection fraction) between groups (Table 1). The endogenous ET system in the unloaded heart group was markedly activated in plasma and atrial and ventricular myocardium compared with the Sham group (Table 2). We also observed marked increases in plasma Ang II in the unloaded heart group, with parallel increases in cardiac Ang II concentrations (Table 2).

**Myocardial Structure in Unloaded and Sham-Operated Hearts**

Hematoxylin-eosin staining of LV sections revealed an absence of ventricular hypertrophy in both the unloaded and sham groups (Figure 1). LV myocardial sections stained with Masson’s trichrome to evaluate the presence of myocardial fibrosis were scored as 0, absent; 1, mild; 2, moderate; and 3, severe fibrosis by a pathologist blinded to the study. The fibrosis score (Sham, 0.25±0.17 versus TIVCC, 0.71±0.29) was not significantly different between the 2 groups (Figure 1). LV mass index obtained at autopsy was significantly lower. The total numbers of cardiomyocytes in the LV myocardium were similar in the Sham and TIVCC groups (Sham: 319±65×10² myocytes; TIVCC: 343±72×10² myocytes, P>0.05; Figure 2C).

**Cardiac ANP in Unloaded and Sham-Operated Hearts**

There was no difference in plasma ANP between groups (Figure 3A). Northern blot analysis of ANP gene expression revealed a trend toward decreased ANP gene expression in unloaded hearts, particularly in the left atria (Figures 3B and 4). In association with this downward trend for ANP gene expression in the unloaded hearts, a significant increase in left atrial ANP concentrations occurred (Figure 3C). Furthermore, IHCS revealed marked staining for ANP in the atria of unloaded hearts, with localization of ANP in granules within the cardiomyocyte cytoplasm (Figure 5).

**Discussion**

The major finding of this study was that chronic unloading of the heart results in myocardial atrophy despite the presence of intense activation of the prohypertrophic humoral factors ET and Ang II. ANP myocardial gene expression was not enhanced in the presence of ET and Ang II activation, although storage of ANP in the atria was increased. These findings advance our understanding of the mechanisms that modulate myocardial structure and endocrine function during chronic cardiac unloading and provide evidence for primacy of myocardial load.

Myocardial hypertrophy is mediated by both mechanical and humoral stimuli, including such prohypertrophic factors as ET and Ang II³⁵,⁷,²⁹,³⁰ In vivo, these mediators are
activated in conditions associated with ventricular dilatation and/or pressure overload. Specifically with regard to Ang II, Dostal and Baker reported that the infusion of subdepressor doses of Ang II in adult rats for 7 days results in significant LV hypertrophy. Rockman et al. also reported that Ang II receptor blockade prevents ventricular hypertrophy and ANP gene expression with pressure overload in mice. However, concomitant effects on load are difficult to exclude in these types of experiments. More recently, Hamawaki et al. reported that mice devoid of Ang II type 1A receptors had a normal hypertrophic response to chronic pressure overload, suggesting that this component of the RAAS should not be necessary in the process of cardiac hypertrophy.

Our study allowed us to investigate the contribution of ET and Ang II on myocardial structure and endocrine function during chronic myocardial unloading. Despite marked activation of endogenous ET and Ang II, mechanical unloading resulted in cardiac atrophy. The presence of myocardial unloading and atrophy was demonstrated by echocardiographic findings of decreased LVEDD, right atrial pressure, and LV mass index, with reductions in cardiomyocyte dimensions and volumes. We observed no evidence for increased ANP gene expression, recognizing ANP to be a marker for myocardial hypertrophy. Our findings support previous studies in feline myocardium in which unloading produced by severing chordae tendineae resulted in cardiac atrophy. Furthermore, Kent et al. reported in the feline model that unloaded myocardium lost structural and functional integrity through loss of myofibrils and contractile activity.

Plasma ANP concentrations are elevated in CHF and other volume-overloaded states as a compensatory response to preserve volume homeostasis. In addition, ventricular ANP gene expression is enhanced during the process of...
cardiac hypertrophy and contributes to an increase in plasma ANP concentration in cardiovascular disorders. The modulating action of ANP on the hypertrophic process has been underscored by the development of ventricular hypertrophy in genetically altered models in which the natriuretic peptide-A receptor to which ANP binds is genetically deleted. Thus, augmented production of ANP in hypertrophied myocardium can be considered a compensatory mechanism against ventricular overload, because ANP serves to reduce cardiac preload and afterload by natriuretic, diuretic, and vasodilatory actions. This concept is consistent with a recent report by Masciotra et al that suggests a protective role for ANP against ventricular hypertrophy, reporting that low ventricular ANP gene expression is linked genetically to increased cardiac mass independent of blood pressure.

The mechanisms controlling ANP gene expression and production have been linked to mechanical stretch and humoral stimulation. It is known that a key determinant of ANP production is cardiomyocyte stretch. A major role for ET and Ang II in mediating ANP gene expression and production has been advanced. The role of intense neurohumoral stimulation by ET and Ang II on ANP gene expression and production independent of cardiac volume and/or pressure overload has not been addressed to date. Chronic myocardial unloading in our study resulted in the absence of ANP gene activation and even a tendency to decrease ANP gene expression in the atria. However, atrial storage of ANP was markedly increased. The present finding reinforces the importance of mechanical load and not neurohumoral stimulation as the principal regulator of ANP cardiac production.

Physiological and Clinical Speculations
On the basis of the present investigations and others, a unifying concept emerges regarding the regulation of myocardial cell growth in vivo. As has been demonstrated, a hypertrophic response occurs in response to myocardial overload, whether by increased volume or pressure, which may be independent of such growth factors as Ang II, as shown in cats in which the RAAS was pharmacologically blocked or in mice in which the Ang II receptors were genetically deleted. Our report complements these previous reports but focuses on cardiac unloading and supports the conclusion that mechanical unloading results in cardiac atrophy and a quiescence of cardiac ANP synthesis. The present investigation also supports the interpretation that regression of cardiac mass and reduction in ANP production with chronic unloading occurs despite intense stimulation by ET and Ang II. Thus, the traditional Frank-Starling relationship, which dictates that myocardial function operates in parallel with load, can be extended to myocardial structure and endocrine function of the heart.

Reduction in cardiac load is a fundamental therapeutic objective for CHF and may be achieved with diuretics, vasodilators, or LVADs. The present investigation supports the concept that chronic unloading of the heart results in cardiac atrophy and reduced myocardial humoral endocrine function. This study may be relevant to the use of LVADs for the treatment of CHF as a “bridge to recovery.” Specifically, partial ventricular loading may be required rather than complete unloading so as to prevent possible cardiac atrophy. Further studies should address this issue in humans. Finally, this study complements a recent investigation that provided insight into additional complex adaptations by the heart during chronic unloading. If our investigation is taken to its logical conclusion, one could speculate that therapeutic strategies, which reduce cardiac preload and thereby unload the heart, could result in a decrease in ventricular mass irrespective of neurohumoral stimulation.

Acknowledgments
This research was supported by Grant-in-Aid 9806361X from the American Heart Association, Minnesota Affiliate; NIH grant HL-36634; the Miami Heart Research Institute; the Mayo Foundation; and the Bestor Funds. The authors acknowledge the technical assistance of Gail Harty, Denise Heublein, and Sharon Sandberg.

References


Mechanical Unloading Versus Neurohumoral Stimulation on Myocardial Structure and Endocrine Function In Vivo
Ondrej Lisy, Margaret M. Redfield, Sofija Jovanovic, Michihisa Jougasaki, Aleksandar Jovanovic, Hanna Leskinen, Andre Terzic and John C. Burnett, Jr

_Circulation_. 2000;102:338-343
doi: 10.1161/01.CIR.102.3.338

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/102/3/338