UltraRapid Communication

Reduced Myocardial Nerve Growth Factor Expression in Human and Experimental Heart Failure

David M. Kaye, Gautam Vaddadi, Sara L. Gruskin, Xiao-Jun Du, Murray D. Esler

Abstract—Maintenance of cardiac performance is tightly controlled by the autonomic nervous system. In congestive heart failure (CHF), although the adverse pathophysiological effects of cardiac sympathetic overactivity are increasingly recognized, the paradoxical finding of reduced sympathetic innervation density in the failing heart remains unexplained. Given these observations, we tested the hypothesis that a reduction in the myocardial production of nerve growth factor (NGF), which is important for the maintenance of sympathetic neuronal survival, could explain the conflicting neurochemical and neuroanatomical profile of CHF. In healthy humans (n=11), there was a significantly greater transcardiac venoarterial plasma NGF gradient than in CHF patients (n=11, P<0.05). In a rat model of CHF, a 40% reduction (P<0.05) NGF mRNA expression was apparent in association with a 24% reduction in tissue NGF content (P<0.05). In conjunction, evidence of reduced sympathetic innervation in the failing heart was apparent, as measured histologically by catecholamine fluorescence and by expression of the neuronal NGF receptor trkA. Norepinephrine (10 μmol/L) exposure reduced both NGF mRNA and protein expression in isolated cardiomyocytes, suggesting that myocardial NGF downregulation may represent an adaptive response to sympathetic overactivity. These data indicate that NGF expression in the heart is dynamic and may be altered in cardiovascular disease states. In CHF, reduced NGF expression may account for alterations in sympathetic neuronal function and neuroanatomy. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2000;86:e80-e84.)

Key Words: heart failure • nerve growth factor • sympathetic nervous system

The clinical features and adverse prognosis associated with congestive heart failure (CHF) represent the result of a complex interplay between impaired cardiac performance and neurohormonal activation. Sympathetic nervous system excitation has been well documented to exist in CHF, based on measurements of plasma catecholamines,1 radioisotope dilution determination of norepinephrine (NE) spillover rates to plasma,2 and measurement of multiunit sympathetic nerve discharge rates.3 Coupled with these data, recent observational and clinical trial data highlight the adverse effect of prolonged sympathetic overactivity in CHF patients.4–7

In addition to the documentation of a systemic increase in sympathetic tone in CHF, neurochemical studies of the rate of regional NE spillover indicate considerable regional heterogeneity in the pattern of sympathetic activation, this typically occurring earliest and being most elevated in the myocardium.5,8 Although the rate of NE spillover from cardiac sympathetic neurons to plasma is dependent on the nerve firing rate,9 other factors including the neuronal reuptake rate and innervation anatomy are of importance. Within this context, the paradoxical observation of reduced sympathetic nervous innervation density in the failing heart despite increased catecholamine overflow remains unexplained.10

Nerve growth factor (NGF) is the prototypic member of a family of proteins termed neurotrophins that play a pivotal role in the differentiation, maturation, and survival of innervating neurons.11,12 Specifically, in the periphery, NGF plays an important role in the maintenance and survival of both sympathetic and sensory neurons.13 Although it has been previously shown that the heart expresses NGF,14,15 the specific cellular source or its regulation has not been closely examined.

Given the critical role of the cardiac sympathetic nervous system in the pathophysiology of CHF, and the evidence for an apparent reduction in innervation density, we therefore aimed to characterize the expression of NGF in human and experimental cardiac failure. In addition, we aimed to identify whether cardiomyocytes express NGF and to investigate potentially relevant mechanisms for regulating expression of the neurotrophin.

Materials and Methods

Human Heart Failure and Transcardiac NGF Flux

Simultaneous arterial and coronary sinus blood sampling was performed in 11 healthy volunteers and 11 patients with severe CHF (mean age: 44±7 versus 54±3 years, P=NS), as previously described.4 The mean left ventricular ejection fraction for the CHF

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group was 17±2%, and the average New York Heart Association heart failure class was 3.1±0.1. The cause of heart failure was nonischemic in 7 patients and ischemic in 4 patients. All patients received angiotensin-converting enzyme (ACE) inhibitors, diuretics, and digoxin, and 1 patient received a β-blocker. The study was conducted with the approval of the Alfred Hospital Ethics Committee. Plasma NGF concentrations were determined by ELISA (Boehringer-Mannheim). All samples were assayed in triplicate.

 Coronary Artery Ligation Model of Heart Failure
Heart failure was induced in Sprague-Dawley rats (210 to 300 g) by coronary ligation, as described by Pfeffer et al.16 The study was conducted with the approval of the Baker Medical Research Institute Animal Ethics Committee. Eight weeks later, hemodynamic assessment of heart failure severity was performed, followed by excision of the heart. Left ventricular samples were obtained for subsequent reverse transcription–polymerase chain reaction (RT-PCR), tissue NGF content (by immunosassay; Boehringer-Mannheim), or catecholamine fluorescence.

 Myocardial Catecholamine Fluorescence
Histofluorescence specific for catecholamines was performed using the sucrose-potassium phosphate-glyoxylic acid method as previously described.10 This technique is a sensitive, well-developed method for studying sympathetic innervation.17 Sections were photographed at ×200 magnification using 35-mm film, under UV fluorescence. The number of stained catecholamine profiles was counted in 5 separate noninfarcted zones, and the result was averaged.

 Cardiomyocyte Cell Culture
Neonatal rat ventricular myocytes were isolated from D1-3 Sprague-Dawley rats, as previously described.18 Myocytes were plated on plastic culture dishes at a density of 1000 cells/mm².

 RNA Isolation and Semiquantitative RT-PCR
Total cellular RNA was isolated as described.19 Based on known sequences, primers were synthesized for NGF, the NGF receptor trkA, and the housekeeping gene L7.20–22 RNA (400 ng) was reverse-transcribed according to the manufacturer’s instructions (MuLV; Perkin-Elmer). Amplification of the resultant cDNA for NGF and L7 was performed by PCR (27 cycles; 94°C for 30 seconds, 63°C for 30 seconds, and 72°C for 1 minute) in a reaction that included 0.04 μCi/μL ³²P dCTP. TrkA PCR amplification was performed over 35 cycles. PCR reaction products were electrophoresed on a 5% polyacrylamide gel and analyzed by phosphorimaging.

 Western Blotting
Protein extracts were prepared from myocyte cultures collected in a radioimmunoprecipitation assay lysis buffer. Extracts (50 μg) were subjected to electrophoretic separation through a 10% SDS-polyacrylamide gel and subsequently transferred to nitrocellulose. Equal protein loading was confirmed by staining with Coomassie blue (see Figure 1 online; http://www.circresaha.org). Western blot analysis was performed by incubating the membranes with an anti–NGF monoclonal antibody (Santa Cruz), followed by a HRP-conjugated goat anti-rabbit antibody (1:5000). Membranes were then exposed to a chemiluminescent reagent (Renaissance; NEN) and autoradiographed using Kodak XOMat AR film.

 Statistical Analysis
Data are presented as mean±SEM. Between-group comparisons were performed by unpaired Student’s t test. Non–normally distributed data were compared by Mann-Whitney U test. A P value <0.05 was considered statistically significant.

 An expanded Materials and Methods section is available online at http://www.circresaha.org.

 Results

 NGF Release by the Human Heart
To determine whether the human heart released NGF and to examine the influence of heart failure on this phenomenon, we performed simultaneous arterial and coronary sinus blood sampling for the determination of the regional transcardiac NGF plasma concentration gradient. Both the arterial and coronary sinus plasma NGF concentrations were substantially greater in healthy control subjects than CHF patients (Figure 1). Furthermore, the transcardiac NGF concentration gradient was substantial and significantly higher than in CHF patients (221±134 versus 1.6±9.4 ng/mL, P<0.05), as shown in Figure 1.

 NGF Expression in Experimental Heart Failure
Eight weeks after the induction of heart failure by coronary artery ligation, typical markers of hemodynamic compromise were demonstrated by hemodynamic evaluation (Table). Rats were only selected if the myocardial infarct was of at least moderate size, defined as an infarct involving at least 20% of the LV. 

 Hemodynamic Parameters

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=8)</th>
<th>Infarct (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>452±9</td>
<td>438±16</td>
</tr>
<tr>
<td>LV, g</td>
<td>0.84±0.02*</td>
<td>0.9±0.02*</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>2.2±0.8</td>
<td>16.4±3.4†</td>
</tr>
<tr>
<td>dP/dt, mm Hg/s</td>
<td>5160±343</td>
<td>2880±227†</td>
</tr>
<tr>
<td>Lung weight, g</td>
<td>1.57±0.05</td>
<td>3.39±0.47†</td>
</tr>
</tbody>
</table>

BW indicates body weight; LV, left ventricle; and LVEDP, left ventricular end-diastolic pressure. *P<0.05, †P<0.01.
the left ventricular circumference by planimetry (data not shown). Increases in left ventricular end-diastolic pressure, left ventricular weight, and lung weight were noted as cardinal features of CHF (Table). After the induction of heart failure, there was a 40% reduction ($P<0.05$) in the myocardial expression of NGF mRNA as determined by semiquantitative RT-PCR. In parallel, NGF protein expression was significantly lower in myocardial tissue from rats with CHF (control versus CHF: 11.2 ± 0.7 versus 8.4 ± 0.8 pg/mg $P<0.05$).

**Indices of Myocardial Sympathetic Innervation**

To investigate whether loss of sympathetic neurons from the myocardium was apparent in our experimental model of CHF, we used two complementary approaches. Histological assessment (see Figure 2 online; http://www.circresaha.org) of fluorescence staining for catecholamines revealed a substantial reduction in the failing, noninfarcted myocardium (Figure 2A), and, in association, mRNA expression of the sympathetic neuronal NGF receptor trkA was largely undetectable in the failing heart compared with sham animals (Figure 2B).

**NGF Expression and Regulation in Cardiomyocytes**

To address the potential mechanism for the observed changes in NGF expression in heart failure, we studied the role of sympathetic tone per se, acting via NE, as a regulator of NGF expression in cardiomyocytes. NGF mRNA was readily detected in isolated neonatal rat ventricular cardiomyocytes, as assessed by RT-PCR using species-specific primers as described below. Sequencing of the PCR amplification product confirmed the identity of the product, with 100% homology at the predicted amino acid sequence level (data not shown). In subsequent experiments aimed at studying the regulation of NGF mRNA, we used a semiquantitative RT-PCR approach. To ensure that the PCR reaction had not reached a plateau phase of amplification, we examined the relationship between the number of amplification cycles and product generation. With 400 ng of total RNA, the amplification of both NGF and the housekeeping gene L7 was linear over the range of 21 to 29 cycles (data not shown), and, accordingly, 27 cycles of amplification were selected for both genes.

Exposure of neonatal cardiomyocytes to 10 μmol NE resulted in a consistent reduction in NGF mRNA to 56 ± 9% ($P<0.01$) of that observed in control cells (Figure 3A). This effect on NGF mRNA expression was also confirmed by Northern blot analysis (see Figure 3 online; http://www.circresaha.org). The action of NE was largely abolished by the $\alpha$-adrenoceptor antagonist prazosin, whereas the $\beta$-adrenoceptor antagonist propranolol was without effect. Furthermore, the role of protein kinase C (PKC) in mediating the actions of NE on NGF expression was examined using the PKC inhibitor bisindolylmaleimide. Consistent with the apparent role of $\alpha$-adrenoceptors in the regulation of NGF mRNA, exposure to 100 nmol/L bisindolylmaleimide also abolished the NGF mRNA downregulation elicited by 10 μmol/L NE (Figure 3A). In parallel studies, we also examined the influence of NE on NGF protein expression in neonatal cardiomyocytes. As with NGF mRNA expression, NE downregulated NGF protein levels, as assessed by Western blot analysis, and this effect was also blunted by prazosin (Figure 3B).

**Discussion**

The sympathetic nervous system plays a vital role in the regulation of myocardial performance, contributing to the maintenance of cardiovascular homeostasis under both acute and chronic challenges to circulatory stability. Target-organ responses to sympathetic nervous stimulation are directly related to the intrasynaptic concentration of the relevant neurotransmitter, NE. The local synaptic NE concentration is tightly controlled in both a spatial and temporal sense by a number of factors, including the innervation neuroanatomy, nerve discharge rate, catecholamine synthesis, and reuptake rate. Although the mechanisms for regulating some of these
processes are well understood, factors that regulate innervation neuroanatomy are less clear. NGF is the best-characterized member of a family of closely related peptide neurotrophic factors that contribute to the development and maintenance of sympathetic innervation.

Under certain clinical circumstances, most notably chronic CHF, activation of the sympathetic nervous system plays a key role in the pathophysiology of the disease process. Our group and others have identified the degree of cardiac sympathetic activation as being a highly relevant factor in determining outcome for CHF patients. Despite data indicating augmented release of NE from the failing heart, histological examination of the failing heart indicates that the density of sympathetic neurons is reduced. Moreover, the rate of extraction of NE by the failing heart is significantly reduced, perhaps amplifying the effect of increased nerve discharge rate on the local myocardial catecholamine concentration.

We tested the hypothesis that a component of the apparent cardiac sympathetic neurobiology of the failing heart could be explained by altered local production of NGF. Our study, for the first time, documents release of NGF from the healthy human heart, and, in association, we observed a marked reduction in the setting of heart failure. The plasma arterial concentration of NGF was also substantially lower in CHF patients. The mechanism for the latter observation is uncertain. To confirm these observations, in an experimental model of CHF, we examined the myocardial expression of NGF at both the mRNA and protein levels. Both techniques revealed a significant reduction in NGF synthesis in the failing heart, in noninfarcted tissue. In addition to the data indicating reduced NGF production, we confirmed the association between CHF and altered cardiac sympathetic innervation, as indicated by reduced catecholamine fluorescence and trkA mRNA expression.

Having identified the heart as a source of NGF in both humans and rodents, we investigated the cardiomyocyte as a potential cellular source and examined the role of NE as a potential regulator of NGF expression. NGF expression by cardiomyocytes was readily detectable at both the mRNA and protein levels, and the expression of NGF was significantly decreased by exposure to NE. This effect appeared to be mediated via α-adrenoceptors and involved the PKC signaling system. The findings of our study differ from previous studies of NGF regulation in some other cell types. Several groups have reported induction of NGF in response to catecholamines in glial cells, fibroblasts, and vascular smooth muscle cells, although others have suggested that the response may vary in a growth-dependent manner. Furthermore, in contrast to our study, previous investigators have suggested that the effects of catecholamines on NGF expression are mediated via β-adrenoceptor activation, with subsequent involvement of the cAMP/PKA second messenger signaling pathway. Involvement of PKC in NGF regulation has also been suggested in some cell types. Phorbol 12-myristate 13-acetate has been shown to upregulate NGF expression in fibroblasts. Given the directionally opposite effect of NE on NGF expression in cardiomyocytes observed in our study, these data suggest that this response may represent a cell-specific response to prolonged NE exposure. For example both experimental and clinical studies indicate that prolonged exposure to catecholamines exerts adverse effects on the myocardium. As a consequence, a reduction in NGF expression in the myocardium with a consequent reduction in sympathetic innervation density could represent an adaptive response to prolonged exposure to elevated sympathetic tone. However, it is also conceivable that alterations in tissue catecholamine levels do not provide the sole stimulus for the reduction in NGF expression.

In addition to the neurotrophic actions of NGF, other actions of importance to the myocardium have been recently suggested. Lockhart et al recently reported that NGF rapidly potentiated synaptic transmission between sympathetic neurons and cardiomyocytes in a coculture system. This action appeared to be mediated by neuronal tyrosine kinase receptors, although the specific intracellular mechanism responsible for this process remains uncertain. In a study by Wakade et al, it was demonstrated that both the expression of NE transporter mRNA and uptake-1 activity in chromaffin cells increased during exposure to NGF. This latter observation would be entirely in keeping with observations in the failing heart of reduced NGF synthesis and reduced NE reuptake.

**Study Limitations**

Although the data consistently indicate that NGF mRNA expression and protein expression are reduced in both human and experimental heart failure and in cardiomyocytes under the influence of NE, precise quantification of the magnitude of the effect on mRNA expression is not possible because of the semiquantitative nature of the RT-PCR approach used in the present study.

**Conclusions**

In the present study, we have comprehensively examined the regulation of NGF in the myocardium and highlighted its potential role in the pathophysiological changes that occur in the cardiac sympathetic nervous system in human and experimental heart failure. The data indicate that in the failing heart, a counterregulatory reduction in myocardial NGF expression occurs, providing an explanation for the previously unexplained finding of reduced sympathetic neuronal density.

**Acknowledgments**

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METHODS

*Human Heart Failure and Transcardiac NGF Flux*

To test the hypothesis that the release of NGF from the failing heart was diminished, simultaneous arterial and coronary sinus blood sampling was performed in 11 healthy volunteers and 11 patients with severe CHF (mean age: 44±7 vs 54±3 years, p=ns), as previously described \(^1\). In brief, arterial blood samples were obtained from an indwelling radial arterial cannula and coronary sinus blood samples were obtained via a coronary sinus catheter positioned under fluoroscopic guidance. The mean left ventricular ejection fraction for the CHF group was 17±2\%, and the average New York Heart Association functional heart failure class was 3.1±0.1. The cause of heart failure was non-ischemic in 7 patients and ischemic in 4 patients. All patients received ACE inhibitors, diuretics and digoxin, 1 patient received a β-blocker. The study was conducted with the approval of the Alfred Hospital Ethics Committee.

Blood samples were rapidly placed on ice in a tube containing EGTA, centrifuged and plasma was subsequently stored at −70°C. Plasma samples were concentrated by centrifugation in a microconcentrator tube (Microcon 10, Millipore), and the NGF concentration was determined using a commercially available immunoassay system (Boehringer-Mannheim). All samples were assayed in triplicate.

*Coronary artery ligation model of heart failure*

Male Sprague-Dawley rats (210-300gm) were obtained from the Biological Research Unit, Baker Medical Research Institute. Under anesthesia, the heart was exposed via a
left thoracotomy, and the proximal left coronary artery was ligated between the base of the left atrium and the pulmonary outflow tract \(^2\). Sham rats underwent an identical procedure, except that the suture was passed beneath the coronary artery and then removed. The study was conducted with the approval of the Baker Medical Research Institute Animal Ethics Committee.

Eight weeks after surgery a hemodynamic assessment of heart failure severity was performed followed by excision of the heart. Under anesthesia (pentobarbitone, 60mg/kg, i.p.) a micromanometer tipped catheter (Millar Instruments, Houston, TX, USA) was inserted into the right internal carotid artery and then advanced into the left ventricle for simultaneous measurement of the left ventricular pressure and its first derivative, dp/dt. Following left ventricular pressure measurement, ascending aortic pressure measurements were performed. At the conclusion of the hemodynamic monitoring procedure, the heart was rapidly excised and rinsed in ice cold saline. Following removal of the atria and right ventricle, samples were obtained for subsequent RT-PCR, tissue NGF content (by immunoassay, Boehringer-Mannheim) or catecholamine fluorescence.

*Myocardial Catecholamine Fluorescence*

Histofluorescence specific for catecholamines (Supplementary Figure 3) was performed using the sucrose-potassium phosphate-glyoxylic acid (SPG) method as previously described \(^3\). This technique is a sensitive, well developed method for studying sympathetic innervation \(^4\). Cross-sectional tissue blocks taken from sham and CHF rat hearts were placed in an embedding compound (OCT, Miles Inc) and
frozen in liquid N$_2$ cooled isopentane. Blocks were stored at -70°C and subsequently sectioned on a cryostat (-20°C). Sections were photographed at 200x magnification using 35mm film, under UV fluorescence. The number of stained catecholamine profiles were counted in 5 separate non-infarcted zones, and the result averaged.

*Cardiomyocyte Cell culture*

Neonatal rat ventricular myocytes were isolated from D1-3 Sprague Dawley rats, as previously described $^5$. Ventricles were excised and then rapidly immersed and minced in ice-cold Hanks buffer (Life Technologies Inc.). The ventricular tissue was then digested overnight in Hanks buffer containing 0.1% trypsin at 4°C. Ventricular cells were then isolated by further digestion in Hanks buffer containing 0.1% collagenase at 37°C. The myocytes were then re-suspended in DMEM supplemented with 7% FCS, and preplated twice to minimize the non-myocyte fraction. Cells were then plated on plastic culture dishes at a density of 1000 cells/mm$^2$.

*RNA Isolation and Semi-Quantitative RT-PCR*

Total cellular RNA was isolated from myocardial samples and cell cultures according to the method of Chomczynski and Saachi $^6$, quantified spectrophotometrically by its absorbance at 260nm, and subsequently stored at -70°C until use. With the use of published sequences, primers $^7$-$^9$ primers were synthesized for NGF (sense 5'-TCATCCACCCACCACAGTC-3' and anti-sense 5'-ACACGCAGGCTGTATCTA-3'), trkA (sense 5'-CACCCTCCCTGCGCTGGTTC-3' and anti-sense 5'-AAAGGAAGAGGCGCGGAG-3') and the housekeeping gene, L7 (sense 5'-CCTGAGGAAGAAGTTGCCC-3' and anti-sense 5'-
CTTGTGAGCTTCACAAAGGTGCC-3'). RNA (400ng) was reverse transcribed according to the manufacturers instructions (MuLV, Perkin Elmer, Branchburg, NJ, USA). Amplification of the resultant cDNA for NGF and L7 was performed by the polymerase chain reaction (27 cycles; 94°C denaturing for 30 sec; 63°C annealing for 30 sec; and 72°C extension for 1 min) and the reaction mixture included 0.04 uCi/ul $^{32}$P dCTP. In preliminary experiments, the amount of PCR product amplification for both NGF and L7 was shown to be within the linear range at 27 cycles (data not shown), allowing a semi-quantitative approach to the determination of mRNA abundance to be performed. TrkA PCR amplification was performed over 35 cycles. PCR reaction products were electrophoresed on a 5% polyacrylamide gel, which was then dried and subjected to phosphorimaging.

**Western blotting**

Protein extracts were prepared from cultures of neonatal rat ventricular myocytes by collection in a lysis buffer containing: 0.15M NaCl, 10mM tris (hydroxymethyl) aminomethane (Tris).HCl, 1% Triton X, 1% Nonidet-P40, 2mM EDTA, 2mM EGTA, 1mM phenylmethylsulfonyl fluoride (PMSF), 20 µM pepstatin, 20 µM leupeptin, pH 7.5. Samples were subsequently homogenized by sonication, and subjected to low speed centrifugation (1,000g, 5 min, 4°C). Protein extracts (50 µg) were subjected to electrophoretic separation through a 10% SDS-polyacrylamide gel and subsequently transferred to nitrocellulose by electroblotting. Equal loading was confirmed by Coomassie blue staining (Supplementary Figure 1). The membrane was blocked by overnight incubation with 5% milk powder in Tris-buffered saline containing 0.1% Tween 20. Western analysis was performed by incubating the membranes with an anti-
NGF monoclonal antibody (Santa Cruz, CA) overnight at 4°C at a final concentration of 1:2000. Membranes were then washed, and incubated for 1 hr at 4°C with a horseradish peroxidase-conjugated goat anti-rabbit antibody at a 1:5000 dilution. Membranes were washed again and exposed to a chemiluminescent reagent (Renaissance, NEN, Boston, MA), and autoradiographed using Kodak XOMat AR film.

Statistical Analysis

Data are presented as mean ±SEM. Between groups comparisons were performed by unpaired Students t-test. Non-normally distributed data were compared by Mann-Whitney U test. A p value less than 0.05 was considered statistically significant.

SUPPLEMENTARY FIGURE LEGEND

Figure 1. Coomassie blue stained SDS-PAGE gel, confirming equal loading.

Figure 2. Photoprints showing catecholamine histofluorescence in sham (A) and failing rat heart (B).

Figure 3. Northern blot showing effect of norepinephrine (NE) on NGF mRNA expression, and attenuation by prazosin.

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Supp Fig 1
- Control
- NE 10^{-5} \mu M
- NE + prazosin

NGF (1.3 kb)

18S