Loss of cardiac sympathetic neurotransmitters in heart failure and NE infusion is associated with reduced NGF

FUZHONG QIN, RAJU S. VULAPALLI, SUZANNE Y. STEVENS, AND CHANG-SENG LIANG

Departments of Medicine and Neurobiology and Anatomy, University of Rochester Medical Center, Rochester, New York 14642

Received 19 April 2001; accepted in final form 24 September 2001

Qin, Fuzhong, Raju S. Vulapalli, Suzanne Y. Stevens, and Chang-Seng Liang. Loss of cardiac sympathetic neurotransmitters in heart failure and NE infusion is associated with reduced NGF. Am J Physiol Heart Circ Physiol 282: H363–H371, 2002. First published September 27, 2001, 10.1152/ajpheart.00319.2001.—Sympathetic neurotransmitters are diminished in cardiac efferent nerve endings in congestive heart failure (CHF). Similar changes occur after exogenous norepinephrine (NE) infusion. Since NE reduces nerve growth factor (NGF) in cultured cardiomyocytes, we proposed to determine whether the loss of noradrenergic transmitters in the failing heart is caused by the NE-mediated reduction of NGF or its neurotrophic receptor tyrosine kinase A (TrKA). Dogs were assigned to receive either rapid ventricular pacing (225 beats/min) or NE infusion (0.5 μg/kg/min) for 8 wk. Control animals received either cardiac pacing of 100 beats/min or saline infusion. We measured NGF and TrKA proteins by Western blot and immunocytochemistry and measured NGF and TrKA mRNAs by reverse transcription polymerase chain reaction, neuron specific catecholaminergic histofluorescence, tyrosine hydroxylase-immunostained profiles, and plasma NE. Rapid ventricular pacing produced CHF with increased plasma NE, decreased myocardial NGF protein (0.61 ± 0.07 vs. 1.04 ± 0.04, P < 0.05), TrKA protein (0.75 ± 0.08 vs. 0.98 ± 0.06, P < 0.05), NGF and TrKA mRNAs and reduced catecholaminergic histofluorescence (197 ± 23 vs. 485 ± 43, P < 0.05), and tyrosine hydroxylase profiles (360 ± 51 vs. 773 ± 36, P < 0.05). Decreases in tissue NGF and TrKA protein were also noted by immunocytochemistry. Similar changes occurred in NE-treated animals. Tissue NGF and TrKA levels correlated closely with the noradrenergic transmitter profiles. We conclude that cardiac NGF and TrKA are reduced by rapid ventricular pacing and NE infusion, and that these changes correlate with decreases of cardiac catecholaminergic and tyrosine hydroxylase profiles. Findings indicate that decrease of cardiac sympathetic transmitters in heart failure is associated with NE-mediated reduction of NGF and TrKA.

nerve growth factor; norepinephrine; sympathetic efferent nerve fibers

We have shown previously (14, 16, 31) that the cardiac sympathetic efferent postganglionic nerve terminal function is abnormal in chronic heart failure (CHF) as evidenced by the decrease of norepinephrine (NE) uptake activity, NE uptake-1 site density, neuronal catecholaminergic histofluorescence profiles, tyrosine hydroxylase-immunostained profiles, and neuropeptide Y immunostained profiles. This is believed to be a response to increased cardiac interstitial NE concentration, because similar changes occur after NE infusion in animals (14, 20). Furthermore, because the effects of NE were prevented by antioxidant vitamins or free radical scavengers (20), the changes on sympathetic nerve endings are thought to be caused by formation of NE-derived oxygen free radicals. However, the exact mechanism by which NE causes the changes is not known. Recently, NE has been shown to reduce protein and mRNA expression of nerve growth factor (NGF) in cultured neonatal rat myocytes, suggesting a potential linkage of NE and NGF to cardiac sympathetic nerve terminal dysfunction in heart failure (17).

NGF protein and mRNA are produced in target tissues of the peripheral sympathetic and sensory nervous system and PC12 cells (15, 18, 29). NGF plays an important role in neuronal differentiation, maturation, and survival (15, 19). These effects of NGF are mediated largely via the high-affinity neurotrophic receptor tyrosine kinase A (TrKA) (13).

The purpose of this study was to determine whether changes in cardiac sympathetic nerve terminal function produced by NE infusion or in CHF were associated with a reduction of myocardial NGF or TrKA. We measured protein and mRNA expressions of NGF and TrKA using Western blot analysis and reverse transcription polymerase chain reaction (RT-PCR), respectively. Tissue distributions of the NGF or TrKA in the heart were determined using immunocytochemistry. Finally, we measured the profiles of neuronal catecholaminergic histofluorescence and tyrosine hydroxylase in the heart. Results of the our study indicate that cardiac NGF and TrKA levels correlated with the density of sympathetic nerve transmitters.

METHODS

Animal Preparation

The present study was approved by the University of Rochester Committee Animal Resources and conformed to the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: Chang-seng Liang, Univ. of Rochester Medical Center, Cardiology Unit, Box 679, 601 Elmwood Ave., Rochester, NY 14642 (E-mail: chang-seng_liang@urmc.rochester.edu).

http://www.ajpheart.org 0363-6135/02 $5.00 Copyright © 2002 the American Physiological Society H363

the guiding principles approved by the Council of American Physiological Society and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). Two different experimental protocols were used in this study.

**CHF.** Adult mongrel dogs weighing 20.3 to 25.6 kg were anesthetized with intravenous pentobarbital sodium (25 mg/kg) and artificially ventilated using a Harvard ventilator. A sterile left thoracotomy was then performed for insertion of heparin-filled Tygon catheters into the main pulmonary artery, left atrium, and thoracic aorta and a micromanometer (Konigsberg Instruments; Pasadena, CA) into the left ventricle through a stab wound in the apex (14). A Medtronic pulse generator (Medtronic; Minneapolis, MN) modified for rapid pacing was implanted in a cervical pocket and connected to a bipolar transvenous ventricular pacing lead positioned in the apex of the right ventricle through an external jugular vein. The chest was then closed.

Animals were assigned, 1 wk after thoracotomy, to receive either rapid ventricular pacing at a rate of 225 beats/min (CHF group, n = 9) or control pacing at a rate of 100 beats/min (control group, n = 8) for 8 wk. CHF animals developed clinical heart failure as determined by tachypnea, ascites, and elevated left atrial pressure.

**Chronic NE administration.** Adult mongrel dogs weighing 21.0 to 25.6 kg were anesthetized and instrumented with chronic indwelling catheters in the left atrium, main pulmonary artery, and descending aorta and a transducer (Konigsberg Instruments) into the left ventricle, as described under CHF. One week later, an osmotic minipump (model 2ML4, Alza; Palo Alto, CA) was implanted aseptically at the nape of the neck. Animals received either NE at a rate of 0.5 μg/kg/min (n = 8) or normal saline (n = 10). A second minipump was implanted 4 wk later to maintain constant NE delivery for 8 wk.

**Resting Hemodynamic Measurements**

Animals were acclimatized to the laboratory and trained to lie quietly on a table with minimal restraint during the study period. At the end of 8 wk of pacing or infusion, the animals were placed in a lateral decubitus position on the table for resting hemodynamic studies under a conscious state. The previously implanted catheters were attached to transducers (model P23XL, SpectraMed, Oxnard, CA) and an eight-channel Brush recorder (model 480, Gould; Cleveland, OH) for measuring heart rate, left atrial pressure, and aortic pressure. The Konigsberg transducer was connected to the Brush recorder for measuring left ventricular pressure and its first derivative (dP/dt) using an electronic differentiator. Cardiac output was measured by the indocyanine green (CardioGreen, Hynson, Westcott & Dunning; Baltimore, MD) dye dilution technique, using a cardiac output system (model 140, Gilford Instrument Laboratories; Oberlin, OH). These hemodynamic data were obtained in triplicate at 5-min intervals over a 15-min period after the animal had been resting comfortably on the table for at least half an hour. Averages of the triplicates were used for statistical analysis.

**Plasma NE Concentration**

Arterial blood was collected into ice-cold tubes containing reduced glutathione. Blood was centrifuged, and plasma was stored at −70°C for subsequent radioenzymatic NE assay (26), using Cat-A-Kit assay system (Amersham; Arlington Heights, IL).

**Animal Death and Tissue Preparation**

After hemodynamic studies, the animal was given a lethal dose (≥100 mg/kg) of pentobarbital sodium. The heart was removed and weighed. The left ventricular free wall was either quickly frozen in liquid nitrogen and stored for subsequent studies or processed immediately for measuring 1) myocardial NGF and NGF receptor TrKA proteins using Western blot analysis and immunocytochemistry, 2) cardiac total RNA isolation and NGF and TrKA mRNAs using RT-PCR, and 3) cardiac sympathetic nerve terminal profiles of NE by histofluorescence and tyrosine hydroxylase by immunocytochemistry.

**Western Blot Analysis for NGF and Its Receptor TrKA Proteins**

Left ventricular tissue was homogenized in a lysis buffer containing 100 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, 1 μg/ml leupeptin and 0.05 mM dithiothreitol. Homogenate was centrifuged at 18,000 g for 30 min at 4°C. Protein concentration of the supernatant was determined using a bicinchoninic acid protein assay reagent (Pierce; Rockford, IL) and bovine serum albumin as a standard. Aliquots containing 50 μg of protein were loaded onto a 10–12% SDS-polyacrylamide gel for electrophoresis. Equal loading of protein was confirmed by Coomassie blue staining. Proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% dry fat-free milk in Tris-buffered saline containing 0.1% Tween 20 (TBST). Rabbit anti-NGF polyclonal antibody (Santa Cruz Biotechnology; Santa Cruz, CA) and rabbit anti-TrKA polyclonal antibody (Santa Cruz Biotechnology) were used for NGF and TrKA detection, respectively. Blots were washed in TBST and incubated with goat anti-rabbit peroxidase-conjugated antibody. Finally, the Phototope-HRP Western blot detection kit (New England BioLab; Beverly, MA) was used to visualize the bands. The autoradiograms were scanned by a GS-700 imaging densitometer (Bio-Rad; Hercules, CA), and the bands were quantified using Quantity One program (Bio-Rad). A value of 1 was arbitrarily assigned to the optical density reading of a randomly selected control sample for calculation. To determine the specificity of the primary antibodies for NGF and TrKA, we used PC12 cell lysates (Santa Cruz Biotechnology) as a positive control and nonimmune rabbit serum (Oncogene Research Products; Cambridge, MA) as negative control. We also preincubated tissue with blocking peptides for NGF and TrKA (Santa Cruz Biotechnology) to document the specificity of the anti-NGF and anti-TrKA antibodies.

**Immunocytochemistry for the Location of NGF and Its Receptor TrKA Expression**

To determine localization of NGF within the myocardium, myocardial muscle samples were fixed in 10% neutral buffered formalin, embedded with paraffin, and then sectioned. The paraffin sections were deparaffinated and rehydrated with xylene and graded alcohol series. The tissue sections were washed, blocked with 10% normal goat serum, and subsequently incubated with rabbit anti-NGF polyclonal antibody (Santa Cruz Biotechnology). Sections were incubated with secondary antibody goat anti-rabbit IgG-conjugated fluorescein (Vector Laboratories; Burlingame, CA). To identify cardiomyocytes, tissue sections were incubated with mouse anti-myosin heavy chain monoclonal antibody (Chemicon International; Temecula, CA). Goat anti-mouse IgG conjugated
TRITC (Sigma; St. Louis, MO) was used as the secondary antibody. Finally, the tissue sections were stained with propidium iodide to visualize nuclei. For negative control, the primary anti-NGF antibody was omitted in the assay. Slides were examined under an Olympus B ×40 fluorescence microscope.

For immunocytochemical analysis of NGF and TrKA expression, frozen tissue sections were fixed in precooled acetone at −20°C for 10 min. After rinsing, the sections were treated with 0.3% H2O2 in methanol to quench endogenous peroxidase activity, blocked with 10% goat serum, and incubated with either rabbit anti-NGF or anti-TrKA polyclonal antibody. Sections were incubated with biotin-conjugated anti-rabbit IgG (Vector Laboratories) and then incubated with avidin and biotinylated horseradish peroxidase macromolecular complex (ABC, Vector Laboratories). 3-Amino-9-ethylcarbazole (Vector Laboratories) was used as a chromagen and hematoxylin (Vector Laboratories) as a counterstain. For negative control, primary antibodies for NGF or TrKA were omitted. Samples were examined under light microscopy.

RNA Isolation and RT-PCR for NGF mRNA Expression

Total RNA was isolated from left ventricular tissue using Trisolv reagents (Life Technologies; Grand Island, NY), as we described previously (27). RT-PCR was used to measure NGF and TrKA mRNAs, using an enhanced Avian RT-PCR kit (Sigma). According to the published sequences (37, 38), primers were synthesized for NGF (sense, 5′-TATCATCACCCACCGTCTC-3′ and anti-sense, 5′-ACACGCAGGCTGTATCTA-3′), TrKA (sense 5′-ATGAGACCGCTCTCATC-3′ and antisense 5′-CATTCTCAAGTGGAGGACG-3′), and GAPDH (sense 5′-GGCCATCCACAGTCTA-3′ and antisense 5′-GGCCATCCACAGTCTA-3′) genes. First-strand cDNA was synthesized by reverse transcription of total RNA using random primers according to the manufacturer’s instructions (Invitrogen; San Diego, CA). cDNA was then amplified in a programmable thermal controller (PTC-100, MJ Research; Watertown, MA) with 2.5 units of Thermus aquaticus DNA polymerase (United States Biomedical; Cleveland, OH) and 50 μl of 10 mmol/l Tris·HCl containing 50 mmol/l KCl, 2.5 mmol/l MgCl2, 0.001% (wt/vol) gelatin, 200 mmol/l of each dNTP and 1.0 mmol/l of specific 5′ and 3′ primers and 0.25 μCi of [α-32P]dCTP (New England Nuclear; Boston, MA). The number of PCR cycles used was based on pilot studies to allow for optimal amplification of each cDNA in a linear range (data not shown). We used 30 cycles of 94°C denaturing for 30 sec, 58°C annealing for 1 min, and 72°C extension for 2 min. The PCR products were resolved on a 5% polyacrylamide native gel with a buffer containing 45 mM Tris-borate and 1 mM EDTA. Gels were dried and exposed to Kodak X-OMAT film at −70°C, and autoradiographs were quantitated using a GS-700 imaging densitometer. Optical densities of the NGF and TrKA bands were subsequently normalized to that of the housekeeping gene GAPDH. Optical density readings were expressed as ratios to a reference sample taken from a control pacing or saline-infused dog assigned an arbitrary value of 1.

Noradrenergic Nerve Terminal Transmitter Profiles

Tissue block preparation for noradrenergic nerve terminal transmitter profiles was described previously (14). Cathecholaminergic histofluorescence was measured using the succrose-potassium phosphate-glyoxylic acid (SPG) condensation method (3) and tyrosine hydroxylase detected by immunocytochemistry. To measure NE histofluorescence, left ventricular tissue block was rapidly frozen on dry ice and stored in liquid nitrogen. Blocks were mounted on a cryostat (−20°C) either longitudinally or by cross section at a thickness of 16 μm. Sections were picked up on glass slides, dipped in SPG, dried, heated under oil at 95°C for 2.5 min, coverslipped, and viewed under ultraviolet light using a Nikon fluorescence microscope equipped with epi-illumination accessories.

For immunocytochemical visualization of tyrosine hydroxylase, ventricular muscle blocks were fixed for 24 h in 4% paraformaldehyde in 0.15 M phosphate buffer (pH 7.4) at 4°C. Blocks were transferred to 25% sucrose in 0.15 M phosphate (pH 7.4) for an additional 24 h at 4°C and then frozen on dry ice and stored at −80°C. Frozen tissue sections were cut at a thickness of 40 μm and picked up on gelatin-subbed glass slides. Sections were rinsed, blocked in 10% normal goat serum for 10 min, and incubated with antityrosine hydroxylase primary antibody (Eugenetech; Eugene, OR) diluted 1:50,000 in 0.15% normal goat serum in phosphate buffer containing 0.4% Triton X-100 for 24 h at 4°C with gentle agitation. On the following day, sections were rinsed and incubated with secondary antibody goat anti-rabbit IgG diluted 1:1,000 in 0.15% normal goat serum in buffer for 2 h followed by the incubation in avidin-biotin-peroxidase complex (Vector Laboratories).

Tissue preparation with longitudinally sectioned myocardium was examined under the microscope using a ×20 objective and photographed at the same magnification (×50) onto 35-mm slides. The SPG-induced catecholaminergic fluorescence and immunostained tyrosine hydroxylase profiles were measured as the number of intersections of the stained nerves with fixed horizontal lines (10) in a 0.00885-mm² field. The results of five fields were averaged for each ventricle. Averages were used for statistical analysis.

Statistical Analysis

Results are presented as means ± SE. Student’s t-test for unpaired data was used to determine the statistical significance of differences between the CHF and control groups, as well as differences between the NE- and saline-infused groups. A difference of P < 0.05 was considered significant. The Pearson product-moment correlation coefficient analysis was used to determine the relationship between the number of sympathtic nerve transmitter profiles and NGF or TrKA protein contents.

RESULTS

Resting Hemodynamics, Plasma NE, and Heart Weight

Table 1 shows that the CHF dogs exhibited elevated heart rate, left atrial pressure, and left ventricular end-diastolic pressure, and lower mean aortic pressure, left ventricular peak dP/dt and cardiac output compared with the control animals. CHF animals also showed a fivefold increase in plasma NE compared with the control animals.

NE infusion caused a marked increase in plasma NE and a slight reduction of heart rate. However, there were no significant changes in mean aorta pressure, left atrial pressure, left ventricular end-diastolic pressure, left ventricular peak dP/dt, and cardiac output between the NE- and saline-infused groups.
Table 1 also shows that left ventricular weight increased slightly in CHF dogs compared with the control animals without CHF. Left ventricular weight was higher in dogs with NE infusion, but the difference in left ventricular weight between NE- and saline-infused groups did not reach statistical significance.

**Myocardial NGF and TrKA Protein Expression by Western Blot and Immunocytochemistry**

Representative Western blots for myocardial NGF and TrKA proteins are shown on Figs. 1 and 2. PC12 cells were used as a source for positive control for TrKA (Fig. 2). The group data on Fig. 3 show that the myocardial contents of NGF and TrKA were reduced in both CHF and NE-infused dogs compared with the control and saline-infused animals.

The primary antibody chosen for the study was specific for NGF. No protein expression was seen when normal rabbit IgG was used in place of the primary antibody in the reaction (Fig. 4). The protein expression also was not seen when the blocking peptide for NGF was used along with the primary antibody (data not shown). To determine the cellular localization of NGF, we carried out the immune fluorescence staining in a control dog heart muscle with double labeling for myosin β-heavy chain and NGF. Figure 4 shows that NGF and myosin β-heavy chain were colocalized in the same cells, indicating that NGF was expressed in the cytoplasm of cardiomyocytes.

In addition, using immunocytochemical analysis (Fig. 5), we showed that NGF protein expression was markedly decreased in CHF or NE-treated animals compared with the control pacing or saline-infused dog. However, unlike NGF, which is present in cardiomyocytes, TrKA expression was abundant only around intramyocardial blood vessels (Fig. 6). Little TrKA immunoreactivity was noted within cardiomyocytes. It is evident from both the Western blots and immunocytochemical staining that NGF and TrKA protein was both reduced in the CHF and NE-treated animals compared with the control and saline-treated animals.

Table 1. **Body weight, resting hemodynamics, plasma NE, and left ventricular weight in 4 experimental groups**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pacing Protocol</th>
<th>Infusion Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control CHF</td>
<td>Saline NE</td>
</tr>
<tr>
<td>No. of animals</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>23 ± 0.5</td>
<td>23 ± 0.6</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>98 ± 7</td>
<td>129 ± 6‡</td>
</tr>
<tr>
<td>Mean aortic pressure, mmHg</td>
<td>107 ± 2</td>
<td>97 ± 3*</td>
</tr>
<tr>
<td>Left atrial pressure, mmHg</td>
<td>8 ± 1</td>
<td>27 ± 3†</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mmHg</td>
<td>9 ± 1</td>
<td>31 ± 4†</td>
</tr>
<tr>
<td>LV dP/dt, mmHg/s</td>
<td>3,151 ± 154</td>
<td>1,457 ± 72‡</td>
</tr>
<tr>
<td>Cardiac output, l/min</td>
<td>3.74 ± 0.26</td>
<td>2.69 ± 0.27*</td>
</tr>
<tr>
<td>Plasma NE, ng/ml</td>
<td>0.25 ± 0.06</td>
<td>1.34 ± 0.26†</td>
</tr>
<tr>
<td>LV weight, g</td>
<td>92 ± 5</td>
<td>111 ± 5†</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05, ‡P < 0.01, and †P < 0.001 vs. control or saline group; CHF, congestive heart failure; LV, left ventricular; NE, norepinephrine.

**Fig. 1.** Representative Western blots showing reductions of left ventricular nerve growth factor (NGF) protein in chronic heart failure (CHF) (A) and norepinephrine (NE)-treated dogs (B) compared with sham-operated control and saline-treated animals.

**Fig. 2.** Representative Western blots showing reductions of left ventricular tyrosine kinase A (TrKA) protein in CHF (A) and NE-treated dogs (B) compared with sham-operated control and saline-treated animals. PC12 cell lysates were used as a positive control for TrKA shown in B.
NGF and TrKA mRNA Expression by RT-PCR

Figure 7 shows that representative gel electrophoresis of the RT-PCR products of NGF and TrKA mRNAs. The NGF and TrKA mRNA levels were normalized to that of GAPDH. Figure 8 shows that NGF and TrKA mRNA levels were both reduced in the CHF and NE-treated animals compared with their respective control groups.

Catecholaminergic Histofluorescence and Tyrosine Hydroxylase-Immunostained Nerve Profiles

Figure 9 shows that the cardiac noradrenergic terminal transmitter (catecholaminergic and tyrosine hydroxylase) profiles were reduced in both CHF and NE-infused animals. We also plotted the noradrenergic terminal transmitter profiles against the tissue NGF (Fig. 10) and TrKA protein (Fig. 11). The coefficients of correlation (r) were statistically significant. The quantities $r^2$ indicate that 60–77% of the variances of catecholaminergic and tyrosine hydroxylase profiles are associated with linear regressions on NGF or TrKA content.

DISCUSSION

Our present study is the first one to indicate close correlations between NGF or TrKA levels with cardiac sympathetic nerve terminal transmitter profiles in intact animals with CHF and NE infusion. Although a direct causal relationship has not been established, the data ($r^2$) support that NGF and TrKA levels may account for 60–77% of the changes in cardiac sympathetic neurotransmitter profiles. Furthermore, because both experimental models have been shown to cause an elevation in cardiac interstitial NE (4), results of our present study suggest that elevated NE may play a role in the reduction of cardiac NGF and TrKA and thus contributes to the abnormalities in cardiac sympathetic nerve terminal function in CHF. Tissue interstitial NE content may be increased by either an increased release of NE, a decreased clearance of NE, or both. It is speculated that the cardiac interstitial NE is increased in early CHF because of heightened cardiac sympathetic activity resulting in increased NE release.

Fig. 3. Left ventricular NGF (A) and TrKA (B) (by Western blot analysis) were reduced significantly in the CHF and NE-infused dogs. n = 6–8 in each group. Bars indicate SE of the mean. $^*P < 0.001$, $^\dagger P < 0.01$, and $\mathcal{\Pi} P < 0.0001$, compared with either the sham-operated control or saline-infused group.

Fig. 4. Photomicrographs showing NGF expression using rabbit anti-NGF antibody in cardiomyocytes of dog left ventricular tissue sections. A: negative control with normal rabbit IgG instead of rabbit anti-NGF antibody is shown. B: distribution is shown of anti-myosin heavy chain antibody labeling of myocyte cytoplasm (red fluorescence) and the nuclei (propidium iodide staining) in the same field as in A. C: NGF expression stained by green fluorescence is shown. D: distribution is shown of anti-myosin heavy chain antibody labeling of myocyte cytoplasm by red fluorescence in the same field as in C.
Fig. 5. Photomicrographs demonstrating decreases of NGF protein expression (by immunohistochemistry) in the left ventricles of CHF and NE-infused animals compared with control and saline-infused animals. Left, negative control tissue treated with normal rabbit IgG instead of rabbit anti-NGF antibody; middle and right, myocardial NGF expression in CHF, sham-operated control, NE-, and saline-infused animals.

Fig. 6. Photomicrographs illustrating decreases of TrKA protein expression (arrows) in the area of intramyocardial blood vessels and decreases of TrKA protein expression in CHF and NE-treated animals compared with the control and saline-treated animals. Left, negative control with normal rabbit IgG instead of rabbit anti-TrKA antibody; middle and right, myocardial TrKA expression in CHF, sham-operated control, NE-, and saline-infused animals.
from the sympathetic postganglionic nerve endings, but as the number of functioning sympathetic nerve terminals decreases in advanced heart failure, decreased NE uptake may play an increasingly important role for the increased cardiac interstitial NE concentration.

**Involvement of NGF Reduction in Sympathetic Nerve Terminal Dysfunction**

NGF was the first discovered and best-known member of the neurotrophic factor family (34). Its mature active form is a dimer of two 118 amino acid polypeptides. NGF is produced in target tissues of the peripheral sympathetic and sensory nervous systems and

**Fig. 7.** Representative gel electrophoresis showing the reductions of left ventricular NGF, and TrKA mRNA expression in the CHF (A) and NE-infused dogs (B) compared with sham-operated control and saline-infused animals. Vertical bars separate the two different groups of animals. NGF and TrKA mRNAs were normalized against GAPDH for the purpose of statistical comparisons.

**Fig. 8.** Left ventricular NGF (A) and TrKA (B) mRNA levels (reverse transcription polymerase chain reaction, RT-PCR) were reduced in the CHF and NE-treated animals. The values were normalized against GAPDH to correct for variations in mRNA loading. A value of 1 was assigned to the value obtained with a control pacing and saline-infused animal. n = 7–9 in each group. Bars indicate SE of the mean. *P < 0.0001 and †P < 0.001, compared with either the sham-operated control or saline-infused group.

**Fig. 9.** Left ventricular catecholaminergic histofluorescence (A) and tyrosine hydroxylase (B)-immunostained profiles were reduced in the CHF and NE-infused animals. n = 8–10 in each group. Bars indicate SE of the mean. *P < 0.0001 and †P < 0.001, compared with either the sham-operated control or saline-infused group.

**Fig. 10.** Correlation between cardiac noradrenergic terminal transmitter profiles and cardiac NGF protein in dogs. Each data point represents a single dog; r = coefficient of correlation. A: catecholaminergic histofluorescence; B: tyrosine hydroxylase immunocytochemistry.
decrease of sympathetic neurotransmitter production of cardiac NGF production was associated with a in pacing-induced dog heart failure and that the reduction of NGF protein and mRNA were decreased in cardiomyocytes of myocardial tissue. Our study also showed that NGF expression in cells, as evidenced by NGF expression in cardiomyocytes of myocardial tissue. Our study also showed that NGF protein and mRNA were decreased in pacing-induced dog heart failure and that the reduction of cardiac NGF production was associated with a decrease of sympathetic neurotransmitter profiles in CHF. Likewise, NGF levels and catecholaminergic fluorescence were reduced in the failing rat heart after coronary artery occlusion (17). In addition, a marked reduction of cardiac release of NGF (difference between coronary sinus and artery plasma NGF) has been reported in heart failure patients (17). Other studies have demonstrated that decreased availability of NGF is responsible for the pathogenesis of diabetic polyneuropathy (12, 24). Heterogeneous cardiac sympathetic denervation in streptozotocin-induced diabetes also has been shown to be associated with regional depletion of myocardial NGF protein (28). These findings indicate a potential correlation exists between NGF reduction and sympathetic nerve abnormalities in a variety of cardiovascular disease.

Like NGF, TrKA may modulate the growth of the sympathetic nerves; deletion of TrKA gene in mice has resulted in extensive neuronal cell loss in sympathetic ganglia (32). Activated TrK receptors have been shown to function as rapid retrograde signal carriers in response to target-derived neurotrophins (1). In our present study, tissue TrKA protein and mRNA decreased in CHF and NE-infused dogs, a finding also reported in rats with ischemic cardiomyopathy (17). Our immunocytochemistry also revealed that TrKA was expressed predominantly in the perivascular region, presumably in the neuronal plexus. This is consistent with the prior findings that TrKA is localized on sensory and sympathetic neurons and PC12 cells.

Effects of NE on NGF Production

Arterial circulating and cardiac interstitial NE concentrations are increased in CHF (4, 36). Evidence has accumulated that NE regulates NGF synthesis and secretion in sympathetic target organs (5, 6, 33). However, the effects of NE on NGF production vary from tissue to tissue (25). β-Adrenergic receptor agonist stimulation has been shown to increase NGF production in neuronal cells (7, 8) and vascular smooth muscle cells (2). In contrast, NE decreased NGF and mRNA levels in a dose-dependent manner in the iris (11) and brown adipose tissue (23). Our present study has extended the prior observations in cultured cardiac cells (17) to intact animals that NE infusion decreases myocardial NGF. The findings suggest that NE-induced NGF reduction contributes to cardiac sympathetic nerve terminal dysfunction in CHF.

Possible Mechanisms of NE-Mediated NGF Reduction

Kaye et al. (17) reported that the NE-induced reduction of NGF in cultured myocytes was abolished by the α-adrenergic receptor antagonist prazosin, whereas propranolol, a β-adrenergic receptor blocker, had no effect. The findings indicate that this effect of NE on NGF is mediated by an α-adrenergic receptor-coupled protein kinase C signal pathway. However, conflicting data exist. Hellweg et al. (11) showed the effect of NE on cultured rat iris NGF was not abolished by phenolamine, an α-adrenergic receptor blocker. In addition, studies have shown that NGF mRNA expression is reduced by oxidative stress and that the reduction of NGF is abolished by antioxidants (9). However, whether the effect of NE on cardiac NGF reduction is related to the increased production of oxygen free radicals remains to be investigated.

In conclusion, our present findings indicate that the heightened sympathetic state of CHF is associated with decreased expression of myocardial NGF, TrKA protein and mRNA and reduced cardiac sympathetic neurotransmitters. Similar changes occur in dogs after chronic NE administration. The significant correlation between the NGF or TrKA protein and noradrenergic terminal transmitter density suggests a potential functional linkage from NE elevation to reduced NGF production and loss of noradrenergic transmitters in cardiac sympathetic nerve endings. Additional studies are needed to establish a cause and effect relationship between the reduction of NGF and decrease of neuro-
nalar catecholamines and tyrosine hydroxylase in the failing heart.

The authors thank Sherry Steinmetz and Amy Mohan for excellent technical support.

The study was supported, in part, by the National Heart, Lung, and Blood Institute Grants HL-35174 and HL-39260, a postdoctoral fellowship from the American Heart Association New York State Affiliate, and a Paul N. Yu fellowship from the University of Rochester Cardiology Unit.

The study was presented, in part, before the 73rd Annual Scientific Sessions of the American Heart Association, New Orleans, LA, November 14, 2000.

REFERENCES


