Loss of Cardiac Sympathetic Neurotransmitters in Heart Failure and NE Infusion Is Associated with Reduced NGF

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Abbreviated title: Cardiac NGF in CHF

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Sympathetic neurotransmitters are diminished in cardiac sympathetic efferent nerve endings in congestive heart failure (CHF). Similar changes occur after exogenous NE infusion. Recently, NE has been shown to reduce nerve growth factor (NGF) in cultured myocytes. Since NGF plays an important role in the survival of sympathetic neurons through high-affinity neurotrophic receptor tyrosine kinase (TrKA), we speculate that the loss of noradrenergic transmitters in the failing heart may be caused by the NE-mediated reduction of NGF or TrKA. Adult mongrel dogs were assigned to receive either a rapid ventricular pacing (225 beats/min) or NE (0.5 µg/kg/min) minipump for 8 weeks. The control animals received either a cardiac pacing of 100 beats/min or saline infusion. We measured NGF and TrKA protein expression by Western blot and immunocytochemistry, NGF and TrkA mRNA expression by RT-PCR, neuronal catecholaminergic histofluorescence and tyrosine hydroxylase immunostained profiles, and plasma NE. Rapid ventricular pacing produced CHF with increased plasma NE, decreased myocardial protein expression of NGF (0.61±0.07 vs. 1.04±0.04, p<0.05), and TrKA (0.75±0.08 vs. 0.98±0.06, p<0.05), mRNA expression of NGF and TrKA, and reduced neuronal catecholaminergic (197±23 vs. 485±43, p<0.05) and tyrosine hydroxylase immunostained (360±51 vs. 773±76, p<0.05) profiles. A decrease in tissue NGF or TrKA protein was also found 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 the decreases of cardiac catecholaminergic and tyrosine hydroxylase profiles. The findings indicate that the decrease of cardiac sympathetic neurotransmitters in heart failure is associated with NE-mediated reduction of NGF and TrKA.
**Key words:** Heart failure, nerve growth factor, norepinephrine, sympathetic efferent nerve fibers.
INTRODUCTION

We have shown previously 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 (14, 16, 31). 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, since 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 the protein and mRNA expression of nerve growth factor (NGF) in cultured neonatal rat myocytes, suggesting a potential linkage of NE and NGF to the 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 (TrKA) (13).

The purpose of this study was to determine whether the 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 the 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 the 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 guiding principles approved by the Council of American Physiological Society and the National Institutes of Health Guide on the humane care and use of laboratory animals. Two different experimental protocols were used in this study.

1. **Chronic heart failure**

   Adult mongrel dogs weighing 20.3 to 25.6 kg were anesthetized with intravenous sodium pentobarbital (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 Konigsberg micromanometer (Konigsberg Instrument, Pasadena, CA) into the left ventricle through a stab wound in the apex (14). A Medtronic pulse generator (Medtronic, Minneapolis, MN) which had been 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, one week 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 weeks. CHF animals developed clinical heart failure as determined
by tachypnea, ascites, and elevated left atrial pressure.

2. 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 Konigsberg transducer (Konigsberg Instrument, Houston, TX) into the left ventricle, as
described under “Chronic heart failure”. One week later, an Alzet model 2ML4 osmotic
minipump (Alza Corp., Palo Alto, CA) was implanted aseptically at the nape of the neck. The
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 weeks later to maintain constant NE delivery for 8 weeks.

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 weeks 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 Spectramed
P23XL (Spectramed, Oxnard, CA) transducers and an eight-channel Brush model 480 recorder
(Gould, Inc, Instruments System Division, 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
differentiater. Cardiac output was measured by the indocyanine green (Cardio-Green; Hynson,
Wectcott & Dunning Inc., Baltimore, MD) dye dilution technique, using a Gilford model 140
cardiac output system (Gilford Instrument Laboratories, Inc, Oberlin, OH). These hemodynamic data were obtained in triplicate at 5-min intervals over a 15-min period after the animal had been resting comfortable 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. The blood was centrifuged, and the plasma was stored at -70 °C for subsequent radioenzymatic NE assay (26), using Cat-A-Kit assay system (Amersham Corp., Arlington Heights, IL).

**Animal sacrifice and tissue preparation**

After hemodynamic studies, the animal was given a lethal dose (>100 mg/kg) of sodium pentobarbital. 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 phenylmethanesulfonyl fluoride, 0.5 µg/ml aprotinin, 1 µg/ml leupeptin and 0.05 mM dithiothreitol. The 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 Laboratories, Hercules, CA) and the bands were quantified using Quantity One Program (Bio-Rad Laboratories). 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 pre-incubated tissue with blocking peptides for NGF and TrKA (Santa Cruz Biotechnology) in order 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 deparaffined 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). The sections were incubated with secondary antibody goat anti-rabbit IgG-conjugated fluorescein (Vector Laboratory, Burlingame, CA). To identify cardiomyocytes, the tissue sections were incubated with mouse anti-myosin heavy chain monoclonal antibody (Chemicon International Inc., Temecula, CA). Goat anti-mouse IgG conjugated TRITC (Sigma Chemical Co., 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. The slides were examined under an Olympus BX40 fluorescence microscope (Olympus America, Inc., Melville, NY).

For immunocytochemical analysis of NGF and TrKA expression, frozen tissue sections were fixed in pre-cold acetone in phosphate buffered saline 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. The sections were incubated with biotin-conjugated anti-rabbit IgG (Vector Laboratory) and then incubated with avidin and biotinylated horseradish peroxidase macromolecular complex (ABC, Vector Laboratory). 3-Amino-9-ethylcarbazole (AEC, Vector Laboratory) was used as a chromagen and hematoxylin (Vector Laboratory) as a counterstain. For negative control, primary antibodies for NGF or TrKA were omitted. The 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 Chemical Co.). According to the published sequences (37, 38), primers were synthesized for NGF (sense, 5'-'TCATCCACCCACCCAGTC-3' and anti-sense, 5'-ACACGCAGGTGTATCTA-3'), TrKA (sense 5'-ATGAGACCAGCTTCATC-3' and antisense 5'-CATTCTCAAGTGGAAGC-3') and GAPDH (sense, 5'-GCCAAAAGGGTCATCTC-3' and antisense 5'-GGCCATCCACAGTCTCTC-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). The cDNA was then amplified in a Programmable Thermal Controller (PTC-100, MJ Research, Inc, 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 MgCl$_2$, 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 $\alpha$-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. The gels were dried, and exposed to Kodak X-OMAT film at -70 °C and autoradiographs quantified using a GS-700 imaging densitometer. The optical densities of the NGF and TrKA bands were subsequently normalized to that of the housekeeping gene GAPDH. The optical density readings were expressed as ratios to a reference sample taken from a control pacing or saline infused dog, which was assigned an arbitrary value of 1.

Noradrenergic nerve terminal transmitter profiles
The tissue block preparation for noradrenergic nerve terminal transmitter profiles was described previously (14). Catecholaminergic histofluorescence was measured using the sucrose-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. The blocks were mounted on a cryostat (-20 °C) for either longitudinal or cross section at 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 hour 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 hours at 4 °C and then frozen on dry ice and stored at -80 °C. Frozen tissue sections were cut at 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 anti-tyrosine hydroxylase primary antibody (Eugenetech, Eugene, OR) diluted 1:60,000 in 0.15% normal goat serum in phosphate buffer containing 0.4% Triton X-100 for 24 hours at 4 °C with gentle agitation. On the following day, sections were rinsed and incubated with secondary antibody goat anti-rabbit IgG diluted 1:1000 in 0.15% normal goat serum in buffer for 2 hours, followed by the incubation in avidin-biotin-peroxidase complex (Vector laboratories, Burlingame, CA).

Tissue preparation with longitudinally sectioned myofibers were examined under the microscope using a 20x objective, and photographed at the same magnification (X50) 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-mm$^3$ field. The results of five fields were averaged for each ventricle. The 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-infusion groups. A difference with $p<0.05$ was considered significant. The Pearson product-moment correlation coefficient analysis was used to determine the relationship between the number of sympathetic 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 to the control animals. CHF animals also showed a 5-fold increase in plasma NE compared to 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 infusion groups.
Table 1 also shows that left ventricular weight increased slightly in CHF dogs compared to 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 infusion 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 Figures 1 and 2. PC12 cells were used as a source for positive control for TrKA (Figure 2). The group data on Figure 3 show that the myocardial contents of NGF and TrKA were reduced in both CHF and NE-infused dogs compared to 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 (Figure 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 co-localized in the same cells, indicating that NGF was expressed in the cytoplasm of cardiomyocytes.

In addition, using immunocytochemical analysis (Figure 5), we showed that NGF protein expression was markedly decreased in CHF or NE-treated animals compared to the control pacing or saline infusion dog. However, unlike NGF which is present in cardiomyocytes, TrKA expression was abundant only around intramyocardial blood vessels (Figure 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, as compared to the control and saline-treated animals.

**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, when compared to 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 (Figure 10) and TrKA protein (Figure 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, since 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 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 is 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 binds to the TrKA (p140) receptor to elicit a cascade of intracellular events involved in neuronal differentiation, maturation and survival (13, 18, 35). The expression of NGF correlates with the density of sympathetic innervation in effector organs, including the heart (30), and the amount of NGF can affect the sympathetic nerve survival and synaptic transmission between neurons and cardiac myocytes (21, 22). Our present study showed that the cardiomyocyte was a potential cellular source, 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 following 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 dog, 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 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 $\alpha$-adrenergic receptor antagonist prazosin, while propranolol, a $\beta$-adrenergic receptor blocker, had no effect. The findings indicate that this effect of NE on NGF is mediated by an $\alpha$-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 phentolamine, an $\alpha$-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.

Conclusions

Our present findings indicate that the heightened sympathetic state of CHF is associated with decreased expression of myocardial NGF and 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 neuronal catecholamines and tyrosine hydroxylase in the failing heart.

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LEGENDS TO ILLUSTRATIONS

Figure 1. Representative Western blots showing reductions of left ventricular NGF protein in CHF (A) and NE-treated dogs (B) compared to sham-operated control (Control) and saline-treated animals.

Figure 2. Representative Western blots showing reductions of left ventricular TrKA protein in CHF (A) and NE-treated dogs (B) compared to sham-operated control (Control) and saline-treated animals. PC12 cell lysates were used as a positive control for TrKA in panel B.

Figure 3. Left ventricular NGF and TrKA (by Western blot analysis) were reduced significantly in the CHF and NE infusion dogs. N=6-8 in each group. Bars indicate SE of the mean. *p<0.001, †p<0.01, and ¶p<0.0001, compared to either the sham-operated control (Control) or Saline infusion group.

Figure 4. Photomicrographs showing NGF expression using rabbit anti-NGF antibody in cardiomyocytes of dog left ventricular tissue sections. Panel A shows negative control with normal rabbit IgG instead of rabbit anti-NGF antibody. Panel B shows the distribution of anti-myosin heavy chain antibody labeling of myocyte cytoplasm (red fluorescence) and the nuclei (propidium iodide staining) in the same field as the panel A. Panel C shows NGF expression stained by green fluorescence. Panel D shows the distribution of anti-myosin heavy chain antibody labeling of myocyte cytoplasm by red fluorescence in the same field as the panel C.

Figure 5. Photomicrographs demonstrating decreases of NGF protein expression (by
immunohistochemistry) in the left ventricles of CHF and NE infusion animals compared to the Control and saline infusion animals. The left panels show negative control tissue treated with normal rabbit IgG instead of rabbit anti-NGF antibody. The middle and right panels show myocardial NGF expression in CHF, sham-operated control (Control), NE-infusion and saline-infusion animals.

Figure 6. Photomicrographs illustrating TrKA expression (arrows) in the area of intramyocardial blood vessels and decrease of TrKA protein expression in CHF and NE-treated animals compared to the Control and saline-treated animals. The left panels show negative control with normal rabbit IgG instead of rabbit anti-TrKA antibody. The middle and right panels show myocardial TrKA expression in CHF, sham-operated control (Control), NE-infused and saline-infused animals.

Figure 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 to sham-operated control (Control) and saline-infused animals. The vertical bars separate the two different groups of animals in each panel. NGF and TrKA mRNAs were normalized against GAPDH for the purpose of statistical comparisons.

Figure 8. Left ventricular NGF and TrKA mRNA levels (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<0.01, †p<0.05, and ¶p<0.001, compared to
either the Control or saline infusion group.

Figure 9. Left ventricular catecholaminergic histofluorescence and tyrosine hydroxylase immunostained profiles were reduced in the CHF and NE infusion animals. N=8-10 in each group. Bars indicate SE of the mean. *p<0.0001, and †p<0.001, compared to either the sham-operated control (Control) or saline infusion group.

Figure 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.

Figure 11. Correlation between cardiac noradrenergic terminal transmitter profiles and myocardial TrKA proteins in dogs. Each data point represents a single dog. r=coefficient of correlation.
Table 1. Body weight, resting hemodynamics, plasma NE, and left ventricular weight in four experimental groups

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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.
Figure 1

A

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<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF</td>
<td></td>
<td></td>
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</tbody>
</table>
Figure 2

A

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CHF</th>
</tr>
</thead>
<tbody>
<tr>
<td>TrkA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>PC12</th>
<th>Saline</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TrkA</td>
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<td></td>
</tr>
</tbody>
</table>
Figure 3

Nerve growth factor

Protein expression (arbitrary units)

Control CHF

Saline NE

Pacing Infusion

TrkA

*†
Figure 5

<table>
<thead>
<tr>
<th>Normal IgG</th>
<th>Anti-NGF antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>Control</td>
</tr>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>Negative control</td>
<td>Saline</td>
</tr>
<tr>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 6

<table>
<thead>
<tr>
<th>Normal IgG</th>
<th>Anti-TrKA antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>CHF</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Negative control</th>
<th>Saline</th>
<th>NE</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 7

A

<table>
<thead>
<tr>
<th>Control</th>
<th>CHF</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF</td>
<td></td>
</tr>
<tr>
<td>TrKA</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Saline</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF</td>
<td></td>
</tr>
<tr>
<td>TrKA</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
</tr>
</tbody>
</table>
Figure 8

**Nerve growth factor**

- Control CHF
- Pacing

**TrKA**

- Saline NE
- Infusion

mRNA expression (arbitrary units)
Figure 9

Catecholaminergic histofluorescence

Tyrosine hydroxylase immunocytochemistry

Noradrenergic terminal profiles per field

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CHF</th>
<th>Saline</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pacing</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant difference
† Highly significant difference
Figure 10

- **Catecholaminergic histoimmunofluorescence**
  - $r=0.858$
  - $p<0.001$

- **Tyrosine hydroxylase immunocytochemistry**
  - $r=0.879$
  - $p<0.001$

- Graphs showing the relationship between NGF protein (arbitrary units) and noradrenergic terminal profiles per field.
Figure 11

**Catecholaminergic histofluorescence**

Noradrenergic terminal profiles per field

- $r = 0.782$
- $p < 0.001$

**Tyrosine hydroxylase immunocytochemistry**

Noradrenergic terminal profiles per field

- $r = 0.813$
- $p < 0.001$