Exogenous nerve growth factor supplementation elevates myocardial immunoreactivity and attenuates cardiac remodeling in pressure-overload rats

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It is postulated that supplementation of exogenous nerve growth factor (NGF) might mediate improvement of the cardiac sympathetic nerve function in heart failure (HF). Local intramuscular injection of NGF near the cardiac sympathetic ganglia could influence the innervation pattern, norepinephrine transporter (NET) gene expression, and improve the cardiac remodeling in experimental HF animals. In this study, we injected NGF into the scalenus medius muscles of Sprague–Dawley rats with abdominal aortic constriction (AC). The nerve innervated pattern, left ventricular morphology, and function following injection in rats with AC were investigated respectively by immunohistochemistry and echocardiography. Levels of mRNA expression of NET, growth associated protein 43 (GAP 43), NGF and its receptors TrkA and p75NTR, and brain natriuretic peptide (BNP) were measured by real-time polymerase chain reaction. The results showed that myocardial NGF mRNA levels were comparable in rats with AC. Short-term supplementation of exogenous NGF raised the myocardial NGF immunoreactivity, but did not cause hyperinnervation and NET mRNA upregulation in the AC rats. Furthermore, myocardial TrkA mRNA was found to be remarkably decreased and p75NTR mRNA was increased. Myocardial TrkA downregulation may play a beneficial effect for avoiding the hyperinnervation, and it is reasonable to postulate that p75NTR can function as an NGF receptor in the absence of TrkA. Interestingly, local NGF administration into the neck muscles near the ganglia could attenuate cardiac remodeling and downregulate BNP mRNA. These results suggest that exogenous NGF can reach the target tissue along the axons anterogradely, and improve the cardiac remodeling.

Keywords nerve growth factor; sympathetic nerve; cardiac remodeling; pressure overload

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Introduction

It is well known that the function of cardiac sympathetic nerve is abnormal in heart failure (HF), which is characterized by augmented release, reduced density of heart innervation, and uptake of the transmitter norepinephrine (NE) from the nerve terminals. Dysfunction of the NE uptake perhaps amplifies the effect of increased nerve discharge rate on the local myocardial NE concentration, which contributes to progressive deterioration of cardiac function [1,2].

The sympathetic nerve function is not only regulated by the central nervous system but also by its target organs. The innervated organs secrete neurotrophins that are essential for development, differentiation, survival of peripheral neurons, and synaptic activity of the cardiac sympathetic nervous system. The major neurotrophin secreted by cardiomyocytes is nerve growth factor (NGF). In adult animals, the density of sympathetic innervation correlates with NGF expression of the corresponding organ [3,4]. In both clinical [5] and experimental HF [6–10], myocardial NGF level is diminished. Thus, it is postulated that supplementation of exogenous NGF might improve the nerve dysfunction and density. Recently, Kreussler et al. have reported that by left thoracotomy direct injection of NGF into stellate ganglia of animals with HF refills depleted cardiac NE stores, normalizes cardiac NE transporter (NET) function, and improves left ventricular contractility [11]. Obviously, supplementation of NGF by left thoracotomy is not a convenient and eligible way. Two studies showed that supplementation of exogenous NGF by local injection or infusion could induce distant neuronal plasticity and the effect is highly compatible with the anatomical connections known for theses neurons, possibly...
mediated by the neurotrophin axonal transport [12,13]. Therefore, we postulated that local intramuscular injection of NGF near the cardiac sympathetic ganglia—middle cervical—stellate ganglion complex (MC–SG complex) [14,15] might have an impact on the pattern of sympathetic innervation in myocardium of ventricle and improve the cardiac function in HF animals. In this study, we injected NGF into the scalenus medius muscle of rats with abdominal aortic constriction (AC) to examine effect of short-term supplementation of exogenous NGF on profile of the sympathetic nerve, NET expression and cardiac remodeling.

Materials and Methods

**Rat model of abdominal aortic constriction**

On the day of surgery, the male Sprague–Dawley rats (Vital River Laboratory Animal Technology, Beijing, China) aged 60 days, weighing 240–310 g were anesthetized with pentobarbital sodium (80 mg/kg). The abdominal AC was performed as described previously [16]. Sham-operated control animals were prepared in a similar way, except that the aorta was not constricted.

The MC–SG of the rat is ventral to the scalenus medius muscle and just located immediately beneath the first and second ribs [15]. Four weeks after AC, the different experimental procedures were used in four groups with five rats in each group. The skin of the neck was carefully shaved and the injection was performed in conscious animals. In the sham group, needles were only inserted into the scalenus medius muscle without any injection. In the AC control group, 20 µl of sterile phosphate buffered saline (PBS) solution was injected in the muscles. In the NGF injection groups 20 µg NGF in PBS solution (100 µg/100 µl sterile PBS) (NGF-7S from murine submaxillary gland; Sigma-Aldrich, St Louis, USA) was injected in the muscles for 1 or 5 days successively.

**Echocardiographic measurements**

One day before, 1 and 5 days after the injection, cardiac function was determined by transthoracic echocardiography. One day before, 1 and 5 days after the injection, cardiac function was determined by transthoracic echocardiography One day before, 1 and 5 days after the injection, cardiac function was determined by transthoracic echocardiography.

**Histological analysis**

Two hours after the measurement of echocardiographic data, the rats were sacrificed to remove the heart to perform the following experiments. The hearts (n = 5, each group) were rinsed with isotonic saline, then dissected and weighed. The weights of the total heart were normalized to the body weight and used as an index of ventricular hypertrophy.

The ventricles were excised, weighed, and dissected into the left and right ventricles. The LV was separated into two equivalent sections along its long axis. The upper sections of left ventricular myocardium were quickly frozen in liquid nitrogen (LN2) until the extraction of RNA and histochemistry analysis. The lower sections were immersed and fixed with 0.04 g/ml paraformaldehyde at 4°C and processed through graded ethanol and xylene prior to embedding in paraffin. The MC–SG complex was quickly removed, immediately frozen in LN2, and maintained at −80°C until RNA extraction.

**Measurement of cardiomyocyte cross-sectional area**

The lower sections of left ventricular myocardium embedded in paraffin were sectioned at 5 µm for histological analysis. The mean cardiomyocyte cross-sectional area was determined in the LV septal region and stained with fluorescein isothiocyanate (FITC)-labeled wheat germ agglutinin (1: 100 dilution; Invitrogen, Grand Island, USA). The nuclei were counterstained with 4’,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, USA). Morphometric analysis was performed with Image pro-plus 6.0. A value from each heart was calculated by use of the measurements of 40–50 cells from an individual heart. The results were expressed as mean ± SEM from each experimental group.

**Immunohistochemistry and western blot analysis**

Sympathetic fibers were identified by staining the upper sections for tyrosine hydroxylase (TH) [2]. The embedded tissues were serially sectioned at 18 µm for immunohistochemical staining for adrenergic innervation of the heart. The immunohistochemical method for TH was used as described previously [18–20]. Cross-sections (8 µm) of the optimal cutting temperature (OCT) compound-embedded frozen myocardium were cut on a cryostat for immunofluorescent analysis of NGF expression. Briefly, sections were rinsed with PBS containing 0.3% Triton X-100 (PBST), blocked (1% bovine serum albumin plus 5% goat serum in PBST), and incubated overnight at room temperature with rabbit anti-NGF antibody (Santa Cruz Biotechnology, Santa Cruz, USA) (1 : 200), rinsed in PBS-NGS (normal
goat serum), placed in biotinylated goat anti-rabbit IgG in PBS for 30 min, and then in avidin-FITC for 45 min (1:200; Vector Laboratories). Following several washes in PBS (pH 7.8), the slides were coveredslipped using Vectashield (Vector Laboratories) and viewed by microscopy. Additionally, specific staining was also performed in negative control sections processed without the addition of the primary antibodies. Micrographs were captured on a fluorescence microscope at 400x magnification (Nikon Eclipse E600; Nikon, Tokyo, Japan) using Image pro-plus 6.0. Three tissue sections were counted for each animal, and for each side. Three fields that had the highest number of NGF immunoreactive positive points were used for analysis.

Western blot was performed to determine NGF protein abundance. The LV was homogenized individually in 5 ml of ice-cold extraction buffer (200 mM NaCl, 1% Triton X-100, 0.1% NaN3, 20 mM Tris–HCl, and 2 μg/ml aprotinin). The ratio of tissue to buffer is 1:4 (wt/vol). The homogenates were then agitated at 4°C. Samples were centrifuged at 4°C for 10 min at 14,000 g, and the supernatant was aliquoted after determination of protein concentration by the bicinchoninic acid method. Samples (40 μg) were loaded onto a 10% sodium dodecyl sulphate polyacrylamide gel, separated under reducing conditions, and then transferred to a polyvinylidene fluoride membrane. Membranes were incubated with specific rabbit anti-NGF (1:400; Santa Cruz Biotechnology, Santa Cruz, USA) antibodies and with secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5,000) and detected with an enhanced chemiluminescent kit (Amersham Biosciences, Piscataway, USA) following the manufacturer’s instruction.

Real-time polymerase chain reaction
RNA was isolated from individual MC–SG and myocardium using the RNAeasy Mini kit (Qiagen, Valencia, USA). Total RNA was quantified by optical density at 260 nm, and 25 ng of total RNA was treated with DNase and reverse transcribed. Each RT reaction was tested by regular polymerase chain reaction (PCR) to confirm reverse transcription, and a RNA-alone control was included for each sample to test for genomic DNA contamination. Real-time PCR was performed with the ABI Power SYBR Green PCR Master Mix kit (ABI, Foster City, USA) in the 7300 Sequence Detection System (ABI). For the PCR amplification, 2 μl of cDNA sample was used in a total volume of 25 μl, and each sample was assayed in duplicate. Controls lacking template were included to determine the level of primer dimer formation and/or contamination. Each 25-μl reaction mixture included 3.0 mM MgCl2, 0.5 μM primer each, and 2 μl of DNA master. The primers used were 5'-TCCACCCAACCGATCTTC-3' and 5'-GCCCTTCC TGCTAGCACA-3' for NGF, 5'-ATCTCTACGGCA AGTTCAGCA-3' and 5'-ATCGCTCTAGTGAGGAG CT-3' for tropomyosin-related kinase A (Trk A), 5'-ACCCG CAACCTCTTCCC-3' and 5'-CAGTCTCGTGTGCGTTTC-3' for p75 neurotrophic receptor (p75NTR), 5'-TGT TCCCCGGTTTGGATCTG-3' and 5'-CAGAACGGAA CATTGACACA-3' for growth-associated protein (GAP43), 5'-CTCAAGGACCAGCC-3' and 5'-GTCCGTAAAGTAG GAGGC-3' for brain natriuretic peptide (BNP) [10], and 5'-TCTGTGTGGATGGTGCTC-3' and 5'-AGAAGCA TTGTGGGTGCAC-3' for β-actin as an external control. Annealing temperatures were 55°C–60°C for the different primers, and the PCR parameters were as follows: denaturation at 95°C for 10 min followed by 45 cycles of 95°C for 15 s, 55°C–60°C for 1 min, and 72°C for 20 s. One fluorescence reading was taken after each cycle at the end of the 72°C elongation time. Fluorescence was plotted as a function of cycle number to determine when reactions were in the linear phase of amplification. Fluorescence data were processed and analyzed with the ABI PRISM Sequence Detection software. The threshold cycle number (Ct) for each tested gene was used to quantify the relative abundance of the gene; arbitrary units were calculated as relative expression levels were normalized with β-actin and calculated with the 2^-ΔΔCT method [21]. The expression levels of target genes were shown as mean for duplicate determinations (the gene/β-actin mRNA × 10^-6) (mean ± SD).

Statistical analysis
Values are presented as mean ± SEM. Differences between groups were examined by using the Student t test or analysis of variance with the Fisher protected least significant difference test. Probability value of <0.05 was regarded as significant.

Results

Morphologic data and BNP mRNA in left ventricular myocardium
There was no significant difference in body weight among the four groups. The heart weight and body weight (BW)normalized heart weight were significantly increased in AC group compared with sham and 5d NGF injection rats, whereas the left ventricular weight (LVW)/BW ratio was significantly decreased in 5d NGF injection group (Table 1). Cardiomyocyte cross-sectional area was significantly increased in AC rats except 5d NGF injection rats compared with sham rats (Fig. 1). Cardiac hypertrophy was accompanied, as expected, by a significant increase in BNP mRNA by about 2 folds compared with the sham rats. NGF injection for 5 days attenuated cardiac hypertrophy and reduced myocardial BNP expression significantly (Fig. 2).
Echocardiographic evaluation

Echocardiographic results showed that the thickness of interventricular septum and left ventricular posterior wall was increased (P < 0.05) was decreased significantly without apparent alteration of FS in AC and NGF groups (Table 2).

NGF expression and sympathetic nerve density in LV

There was an increase of myocardial NGF immunoreactivity in AC and NGF injection rats compared with sham group (P < 0.05) [Fig. 3(A)]. It was significantly higher in 5d NGF injection rats than that of AC control and 1d NGF injection rats (P < 0.05). Western blot analysis also showed similar alteration of myocardial NGF protein levels [Fig. 3(B)]. No difference was found in the density of TH-labeled nerves counts between sham, AC, and NGF injection rats (Fig. 4).

Expression of GAP43, NGF, TrkA, and p75NTR mRNA in myocardium and NET in cardiac ganglion

The membrane phosphoprotein GAP43 expressed in the growth cones of sprouting axons is a marker for nerve sprouting [22]. TrkA is a high-affinity receptor, through which NGF mediates neutrophic effects [23]. Although myocardial NGF mRNA expression was increased in both of AC and NGF injection groups compared with sham rats [Fig. 5(A)], real-time PCR results showed that no difference in GAP43 mRNA levels was observed between the sham, AC and NGF injection rats (data not shown). TrkA mRNA expression was significantly decreased in AC and NGF injection rats compared with sham rats (P < 0.05) [Fig. 5(B)], but p75NTR mRNA significantly was increased among AC groups [Fig. 5(C)], suggesting that p75NTR, a pleiotropic signaling molecule, may play an alternative or a compensative role when trkA is down-regulated. Real-time PCR results showed no difference in NET mRNA levels in the ganglia derived from sham, AC, and NGF injection rats. Also, no significant differences in NET mRNA could be detected in 1d and 5d NGF injection rats (data not shown).

Discussion

In this study, we investigated the effects of local administration of NGF near cardiac sympathetic ganglia on myocardial NGF immunoreactivity, profiles of sympathetic nerve, cardiac remodeling, and functional parameters in the pressure overload rats. A number of studies showed that the level of NGF in myocardium is down-regulated in rodents and in humans with HF [5–10]. In cultured cells, NGF has been demonstrated to increase NET mRNA levels and the
neuronal reuptake of NE [24]. A single injection of NGF into the ganglion restored the reduced neuronal reuptake of NE, replenished the depleted NE content and led to an improvement of left ventricular contractility [11]. Local over-expression of NET in the myocardium resulted in marked structural and functional improvement of HF [25]. Additionally, NGF might be transported antegradely from its site of synthesis in neuronal cell bodies to peripheral tissues via sympathetic axons in NGF transgenic mice [26].

We demonstrated herein that AC-induced elevation of NGF mRNA and protein in the myocardium of the LV, but TrkA mRNA expression was decreased significantly in AC rats. We also found that there was no difference between AC and NGF injection rats in NGF mRNA levels. However, short-term supplementation of exogenous NGF raised the myocardial NGF immunoreactivity further, but the upregulation might not certainly lead to cardiac remodeling.

Table 2 Parameters of M-mode echocardiography

<table>
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<th>Sham (n = 5)</th>
<th>AC control (n = 5)</th>
<th>AC + NGF 1d (n = 5)</th>
<th>AC + NGF 5d (n = 5)</th>
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<tr>
<td>IVSd (cm)</td>
<td>0.17 ± 0.04</td>
<td>0.28 ± 0.07*</td>
<td>0.29 ± 0.04*</td>
<td>0.24 ± 0.02*</td>
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<tr>
<td>IVSs (cm)</td>
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<td>0.34 ± 0.06*</td>
<td>0.35 ± 0.02*</td>
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<tr>
<td>LVIDd (cm)</td>
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<td>0.47 ± 0.03</td>
<td>0.51 ± 0.11</td>
<td>0.45 ± 0.05</td>
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<td>LVIDs (cm)</td>
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<td>0.31 ± 0.04</td>
<td>0.35 ± 0.03</td>
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<tr>
<td>LVPWd (cm)</td>
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<td>0.28 ± 0.01*</td>
<td>0.25 ± 0.02*</td>
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<td>FS (%)</td>
<td>0.35 ± 0.03</td>
<td>0.40 ± 0.03*</td>
<td>0.37 ± 0.03</td>
<td>0.39 ± 0.01</td>
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Values are mean ± SEM. IVSd, interventricular septal thickness during diastole; IVSs, interventricular septal thickness during systole; LVIDd, diastolic left ventricular internal diameter; LVIDs, systolic left ventricular internal diameter; LVPWd, diastolic LV posterior wall thickness. *P < 0.05 vs. Sham.

Figure 3 Myocardial NGF protein levels after intramuscular injection of NGF in the vicinity of MC–SG (A) Immunofluorescent analysis of NGF expression in sham, AC, NGF injection for 1 day and 5 days rats. Bar = 200 μm. (B) Western blot analysis of NGF protein in sham, AC, NGF injection for 1 day and 5 days rats. Data are expressed as mean ± SEM. n = 5. *P < 0.05.

Figure 4 Density of TH-positive nerve of rats from four experimental groups TH-positive nerves were detected by immunohistochemistry in myocardia of rats of different groups. Sympathetic fiber pattern and density are similar in myocardium of the groups. Data are expressed as mean ± SEM. n = 5. Bar = 200 μm.
sympathetic hyperinnervation and did not enhance NET mRNA levels in the ganglion. Surprisingly, NGF injection into the neck muscle near the ganglia could attenuate cardiac remodeling induced by AC.

NGF is the best characterized member of the neurotrophin family responsible for survival, differentiation, and neurite outgrowth of sympathetic neurons by targeting the TrkA receptor [3,25]. The endogenous NGF not only controls the sympathetic innervation [25], but is involved in reparative angiogenesis and suppression of endothelial apoptosis [3,27,28]. In general, NGF selectively activates its receptor TrkA and then is transported retrogradely along the axons through the receptor-mediated endocytosis [3,27]. A previous study showed that NGF levels in dopamine β-hydroxylase (DBH)-NGF transgenic mice were significantly elevated in the superior cervical ganglion and adrenal gland, and that NGF was transported from its synthesis site in neuronal cell bodies to peripheral tissues via sympathetic axons [26]. In this study, after the neck injection of NGF near the ganglia there was a substantial increase of detectable NGF immunopositivity in the LV myocardium in AC rats as compared with control rats. However, myocardial NGF mRNA levels were similar in AC rats, which suggested that the neurotrophin bound to its receptors on the neurons, and then was transported anterogradely along axons and released in the target tissue, rather than produced by the cardiomyocytes.

There is evidence that NGF infused into left stellate ganglion and myocardium over several weeks elicits pronounced sympathetic neuronal growth and nerve sprouting in the heart [28]. However, a single NGF injection into the ganglia could not change TH nerve profiles in the heart of transverse AC rat [11]. In accordance with the finding of Kreusser et al. [11], we did not find changes in TH-immunopositive sympathetic nerve density, GAP43 and NET mRNA expression after NGF injection into the neck muscles of AC rats once and for consecutive 5 days, despite the significant increase of NGF-immunopositive protein in the myocardium. It may be deduced that short-term injection of NGF may not have an apparent effect on nerve sprouting in the heart. In consistence with the others [11,29], we did not observe any change in NET mRNA levels in this study.

The pattern of NGF expression in HF is controversial. Most of the studies showed that myocardial NGF levels decreased in clinical and experimental HF induced by pressure overload [4–10,30]. The reasons for this controversy may lie in a heterogeneous patient medication or in different CHF etiologies, models, and the duration of HF. Furthermore, there were also mixed results regarding myocardial NGF levels and sympathetic nerve density in the pressure overload rat model [31–33]. Endothelin-1(ET-1)/NGF signaling is critical for sympathetic nerve development [34]. Endogenous cardiac production of ET-1 may play a pivotal regulatory role in mechanical load-induced cardiac NGF expression. The production of ET-1 has also been shown to increase in the hypertrophied heart in various models of pressure overload [35–39]. Thus, it is reasonable to postulate that upregulating ET mediates upregulation of NGF expression at mRNA level in cardiomyocytes in the pressure overload rats. We found no difference in the density of TH-positive nerve in AC alone rats and AC rats after injection of NGF, although myocardial NGF protein levels were increased when compared with sham rats.

Like NGF, TrkA may modulate the growth of the sympathetic nerves. Deletion of TrkA gene in mice has resulted in extensivne neuronal cell loss in sympathetic ganglia [3]. Activated Trk receptors have been shown to function as rapid retrograde signal carriers in response to target-derived neurotrophins [3,4,26]. There is evidence that sympathetic nerve innervation density and TrkA expression are reduced in HF [4–6]. In this study, TrkA mRNA level was found to be decreased in myocardium of AC rats. Although potential posttranscriptional modification of TrkA does not necessarily allow direct extrapolation from TrkA mRNA level to TrkA protein, the decrease in TrkA mRNA level makes a substantial consistency or increment of the protein synthesis rather unlikely. Thus, it is postulated that TrkA downregulation in HF may contribute to loss of the nerve terminals.
and dysfunction of NE reuptake. In contrast, the reduction in the hyperinnervation could be beneficial for alleviation of arrhythmogenesis. In this study, we observed that despite elevation of NGF protein in the myocardium of AC and NGF injection rats, there was no change in the nerve density, which might be attributed to a scarcity of their receptors.

An intriguing and unexpected finding of this study is that injection of NGF in the vicinity of the ganglia in rats can attenuate cardiac hypertrophy induced by AC. Expression of BNP gene is one of the most reliable markers for activation of the hypertrophic program in clinical states and experimental models associated with hypertrophy [40]. The elevated BNP supports the presence of cardiac hypertrophy. In this study, cardiac BNP mRNA was measured to confirm the presence of cardiac hypertrophy in AC rats. We found that myocardial BNP level in the NGF injection for 5d rats was lower than that in control AC and NGF for 1d rats, indicating a reduction of hypertrophy. In addition, quantification of cardiomyocyte size confirmed the observation.

Nevertheless, the possibility of a systemic effect cannot be ruled out, such as that a part of the injected NGF diffused away from the scalenus medius muscle, reached the circulatory system and eventually affected cardiac myocytes directly. There were little effects on the cardiac performance, which may be resulted from LV endocardial FS as endocardial contractility index, a less sensitive performance parameter [41] used in this study. The results are similar to those of many previous studies of hypertrophied rats [10,42,43].

NGF is an autocrine prosurvival factor for cardiomyocytes, and an increased level of this neurotrophin protects cardiomyocytes from apoptosis. The prosurvival signal of NGF in cardiac myocytes is mediated by TrkA and downstream Akt phosphorylation. In response to Akt activation by NGF, Forkhead transcription factors Foxo are phosphorylated and excluded from the nucleus, thus resulting in increased cardiomyocyte survival [3,44]. It is well known that neurotrophins, including NGF, act through two distinct types of receptors: the high affinity Trk receptors, and the lower affinity p75 receptor (p75NTR) [3]. In contrast to the specificity of the Trk receptors, each of the mature neurotrophins binds to p75NTR with equivalent affinity, but with unique kinetics. Previous studies showed that in the absence of TrkA, p75NTR is capable of internalizing NGF, and transporting NGF retrogradely from the retina to the isthmo-optic nucleus [45]. Nyquist-Battie et al. [46] previously showed that in Dahl salt-sensitive rats with cardiac hypertrophy induced by high-salt diet, NGF and p75NTR were elevated in the LV. Our findings that among AC groups p75NTR mRNA was augmented while simultaneously TrkA decreased suggest that p75NTR may mediate the biological effects of NGF when TrkA is insufficient.

To our knowledge, this is the first report that exogenous NGF supplementation attenuates the cardiac remodeling. The potential mechanisms underlying this phenomenon may be due to (i) NGF-induced angiogenesis to prevent the myocardial ischemia from cardiac hypertrophy; (ii) NGF-induced protection of myocardiocytes from apoptosis; and (iii) NGF-induced improvement of NET reuptake. Further studies are required to understand this phenomenon and its precise mechanisms.

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