

# Injection of Nerve Growth Factor Into Stellate Ganglia Improves Norepinephrine Reuptake Into Failing Hearts

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**Abstract**—An impairment of cardiac norepinephrine reuptake through the neuronal norepinephrine transporter promotes depletion of cardiac norepinephrine stores and local cardiac sympathetic activation in heart failure. Nerve growth factor regulates differentiation and survival of adult sympathetic cells and is decreased in failing hearts. We hypothesized that injection of nerve growth factor into stellate ganglia normalizes cardiac norepinephrine homeostasis in experimental heart failure. Rats with transverse aortic constriction characterized by heart failure, depleted cardiac norepinephrine stores, and impaired cardiac norepinephrine reuptake were used as an experimental model. Nerve growth factor (20  $\mu\text{g}$ ) or saline was directly injected into left stellate ganglia 4 weeks after transverse aortic constriction. Thirty-two hours after injection, determinants of cardiac norepinephrine homeostasis were measured. As compared with saline, nerve growth factor refilled depleted cardiac norepinephrine stores and improved cardiac [ $^3\text{H}$ ]-norepinephrine uptake into isolated perfused hearts of transverse aortic constricted rats. In addition, pharmacological blockade of the norepinephrine transporter led to a higher increase in the overflow of endogenous norepinephrine from hearts of nerve growth factor-injected than saline-injected transverse aortic constricted rats. Norepinephrine transporter mRNA levels and the density of cardiac sympathetic nerves were not changed. Thirty-two hours after nerve growth factor injection, echocardiography revealed an increase in fractional shortening as compared with 2 days before injection. In conclusion, nerve growth factor attenuates local cardiac sympathetic overdrive of hypertrophic hearts by improving cardiac norepinephrine reuptake and might represent a novel therapeutic principle in the treatment of heart failure. (*Hypertension*. 2006;47:209-215.)

**Key Words:** heart failure ■ neuroregulators ■ norepinephrine ■ sympathetic nervous system

The degree of activation of the sympathetic nervous system correlates with the severity of left ventricular dysfunction of patients with congestive heart failure (CHF).<sup>1</sup> It has been demonstrated that an impaired neuronal norepinephrine (NE) reuptake via the NE transporter (NET) contributes to an enhanced NE net release.<sup>2-5</sup> As a consequence, depletion of cardiac NE stores and chronic overstimulation of adrenergic receptors leads to worsening of heart failure and sudden death, as well as to profound alterations of postreceptor signal coupling and cardiac remodeling.<sup>6,7</sup> Whereas some studies reported that an impaired NE reuptake was the result of a reduced NET density on cardiac sympathetic nerve endings,<sup>5</sup> other studies demonstrated a loss of sympathetic nerve endings, which occurred mainly in advanced stages of experimental CHF.<sup>8</sup> Likewise, it has been implied that an impaired NE reuptake precedes a loss of sympathetic nerves.<sup>9</sup> As a consequence of both processes, NE stores within the sympathetic nerve endings of failing hearts might be depleted, resulting in an attenuated noradrenergic response to stress and exercise.<sup>10</sup> However, it remains unclear which mediators are involved in these processes.

The sympathetic nerve function is not only regulated by the central nervous system but also by its target organ. The innervated organs secrete neurotrophins, which are essential for development, differentiation, and survival of peripheral neurons.<sup>11</sup> The major neurotrophin that is secreted from cardiomyocytes is nerve growth factor (NGF). NGF-null mice lack sympathetic ganglia.<sup>12</sup> In adult animals, the density of sympathetic innervation correlates with NGF expression of the corresponding organ.<sup>13</sup> In both experimental and clinical CHF, myocardial NGF expression is diminished.<sup>14,15</sup> To test the hypothesis of whether NGF improves the cardiac sympathetic nerve function in experimental CHF, NGF was directly injected into stellate ganglia of rats with transverse aortic constriction (TAC), which have been characterized previously by overt heart failure, depleted NE stores, and an impaired NE reuptake.<sup>5,16</sup>

## Methods

### Experimental Animals

The investigation conforms with the guide for care and use of laboratory animals published by the National Institutes of Health

Received August 15, 2005; first decision September 7, 2005; revision accepted November 9, 2005.

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*Hypertension* is available at <http://www.hypertensionaha.org>

DOI: 10.1161/01.HYP.0000200157.25792.26

(NIH Publication No. 85-23, revised 1996) and was approved by the authorities of the Regierungspräsidium Karlsruhe, Germany. The experiments were performed in 8-week-old male Wistar rats (Charles River Wiga, Sulzfeld, Germany), caged individually at 26°C with a 12-hour light regimen.

### Experimental Protocol

CHF was induced by TAC as described previously.<sup>5</sup> A group of sham-operated rats (surgery without insertion of a clip) served as controls. After 4 weeks, the measurements were performed as described below. Thirty-two hours before the final evaluation, the TAC rats underwent a second surgery for application of NGF. TAC rats were anesthetized with pentobarbital (50 g/kg IP; Merial, Hallbergmoos), and left pectoral and intercostal muscles were partially dissected between the first and second rib to get access to the left stellate ganglion, which is the localized dorsal of the left subclavian artery and vein. Using a microsyringe (Model 810, Hamilton), 20 µg of NGF (Sigma Aldrich) or the vehicle saline were directly injected into the stellate ganglia. Thirty-two hours later, the rats were anesthetized with thiopental (100 mg/kg IP; Byk Gulden). Left stellate ganglia were quickly removed, immediately frozen in liquid nitrogen (LN<sub>2</sub>), and maintained at -80°C until used for RNA extraction. The hearts were excised, weighed, and used for isolated heart perfusion, frozen in LN<sub>2</sub> and maintained at -80°C until NE concentration was determined, or frozen as a block in isopentane cooled with LN<sub>2</sub> and stored at -80°C until used for histochemistry. In addition, lungs were excised for measuring the lung wet weight.

### Isolated Heart Perfusion

Wistar rats were anesthetized with thiopental. The hearts were rapidly cut out, rinsed in ice-cold buffer, and the aorta was cannulated for perfusion according to Langendorff.<sup>17</sup>

Cardiac [<sup>3</sup>H]-NE uptake was determined as described previously.<sup>5</sup> Briefly, a bolus of [<sup>3</sup>H]-NE (1 mL, 3 µCi, 100 pmol NE; Amersham-Buchler) was injected into the perfusion system and proportionally distributed to the hearts and blank channels. Radioactivity was measured in the effluent. The amount of [<sup>3</sup>H]-NE, which was extracted by the hearts (uptake), is expressed as the percentage of radioactivity measured in the blank channels.

Overflow of endogenous NE was evoked by electrical field stimulation: 2 subsequent electrical stimulations (S1 and S2; stimulation frequency 4 Hz; voltage 5 V), each of 1 minute duration, were applied with an interval of 10 minutes in the presence of atropine (1 µmol/L). Five minutes before S2, desipramine (DMI; 1 µmol/L), an inhibitor of NET, was added. S1 served as intraindividual control. The effect of DMI was characterized by calculating the S2/S1 ratio of NE concentration in the coronary venous effluent for each heart.

### Measurement of NE Concentration

NE in the heart perfusion effluent and left and right ventricular NE was determined by high-performance liquid chromatography and electrochemical detection as described previously.<sup>18,19</sup>

### Histology

Eight-micrometer cross-sections of apical and basal parts of frozen left ventricles were cut on a cryostat and then processed for glyoxylic acid-induced histofluorescence<sup>8</sup> or for immunohistochemistry<sup>20</sup> using a polyclonal sheep anti-tyrosine hydroxylase (TH) and a secondary biotinylated anti-sheep antibody (both purchased from Chemicon International, Hofheim, Germany). Morphometric analysis was performed as described previously.<sup>20</sup>

### RT-PCR and Northern Blotting

Total cellular RNA was isolated from frozen left stellate ganglia by acid guanidium thiocyanate/phenol/chloroform extraction.<sup>21</sup> Reverse transcription was performed using First-Strand cDNA Synthesis kit for RT-PCR (Roche Diagnostics) according to the manufacturer's instructions. For real-time PCR, specific primer and defined concentrations of cRNA standards for NET and 18S-rRNA were used as described previously.<sup>5</sup> Real-time PCR was performed in a LightCycler

with DNA Master Hybridization Probes (labeled with fluorescein and LC-red 640) according to the manufacturer's instructions (Roche Diagnostics). TH Northern blot analysis was performed as described previously.<sup>5</sup> The blot membrane was hybridized with a TH full-length antisense riboprobe labeled with [<sup>32</sup>P]-UTP (Amersham Buchler) analyzed by phosphoimaging and normalized to 18S-rRNA.

### Echocardiography

Two days before and 32 hours after injection of NGF or saline, intraindividual effects on cardiac function were determined by transthoracic echocardiography as described previously in detail.<sup>22</sup> The investigator who conducted the echocardiography was blinded for the treatment status.

### Statistical Methods

Results are expressed as mean ± SEM. Differences between groups were tested for statistical significance by the unpaired Student *t* test (2 groups) or ANOVA (3 groups). Intraindividual differences, as measured by echocardiography, were tested for statistical significance by the paired Student *t* test. A *P* value of <0.05 was considered significant.

## Results

### Baseline Characteristics of TAC Rats

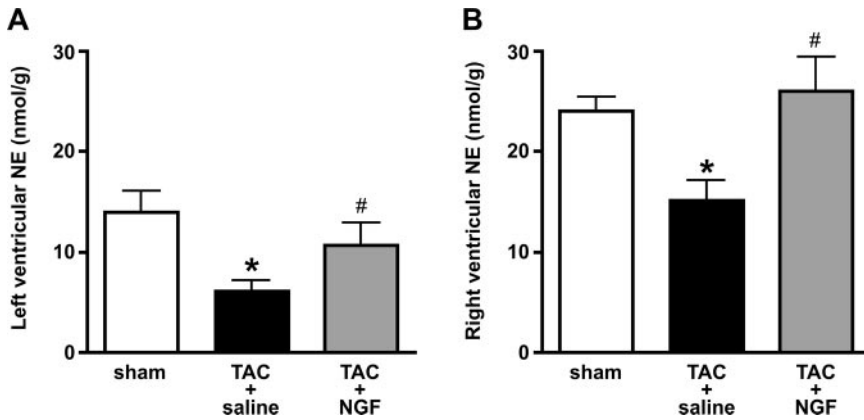
As a model for cardiac hypertrophy and CHF, we used Wistar rats 4 weeks after TAC. As shown previously, these rats develop biventricular hypertrophy and overt heart failure with elevated left ventricular end-diastolic pressures and increased plasma pro-atrial natriuretic peptide levels.<sup>5,16</sup> Consistently, we observed in the present study compared with our previous studies similar increased heart weight/body weight and lung wet weight/body weight ratios, indicating cardiac hypertrophy and pulmonary congestion (supplemental Table I, available online at <http://hyper.ahajournals.org>). As expected, 32 hours after injection of NGF, organ weights were not changed as compared with saline treatment (supplement Table I).

### Cardiac NE Stores

To investigate potential effects on cardiac NE stores, we measured the left and right ventricular NE concentration. Consistent with previous findings,<sup>5</sup> cardiac NE stores were diminished in both ventricles of TAC rats as compared with sham-operated controls (Figure 1A and 1B). Remarkably, NGF injection into the left stellate ganglion restored left and right ventricular NE stores as compared with saline injection (Figure 1A and 1B).

### NE Synthesis

A possible explanation for cardiac NE restoration may be an increased NE de novo synthesis. To address this possibility, we determined the expression of TH, a key enzyme for NE synthesis, by Northern blot analysis. As demonstrated previously,<sup>5</sup> TAC rats showed an increase in TH mRNA expression in stellate ganglia compared with sham (Figure 2). Here, we show that NGF treatment normalized the mRNA concentration of TH in stellate ganglia to the same level as observed in sham-operated rats (Figure 2). Although TH mRNA concentrations do not necessarily allow us to directly extrapolate on TH activity, the decrease in TH mRNA makes a substantial increase of cardiac catecholamine synthesis rather unlikely.



**Figure 1.** Cardiac NE stores of sham-operated rats (n=10) and rats with TAC. NGF (20 μg; n=5) or the vehicle saline (n=11) was injected into the left stellate ganglion of TAC rats 32 hours before. (A) Left ventricular NE concentration. (B) Right ventricular NE concentration. \*P<0.05 vs sham; #P<0.05 vs TAC+saline.

**Cardiac Sympathetic Nerve Density**

Another reason for elevated cardiac NE stores in response to NGF might be an increased density of sympathetic nerve endings within the hearts. Using glyoxylic acid-induced histofluorescence to visualize NE, we found no difference in the area density of noradrenergic nerve terminals (Figure 3) or in the number of noradrenergic nerve profiles (data not shown) in TAC rats after injection of NGF as compared with saline injection. The same result was obtained by TH immunofluorescence, an approach that measures sympathetic nerve profiles independent of the NE concentration. Thirty-two hours after injection of NGF, the number of TH-labeled nerve profiles was unchanged at both the basis (NGF: 38.9±7.1; saline: 52.1±3.2 profiles/mm<sup>2</sup>; n=4) and the apex of TAC hearts (NGF: 47.4±6.9; saline: 44.5±4.4 profiles/mm<sup>2</sup>; n=4) as compared with saline injection.

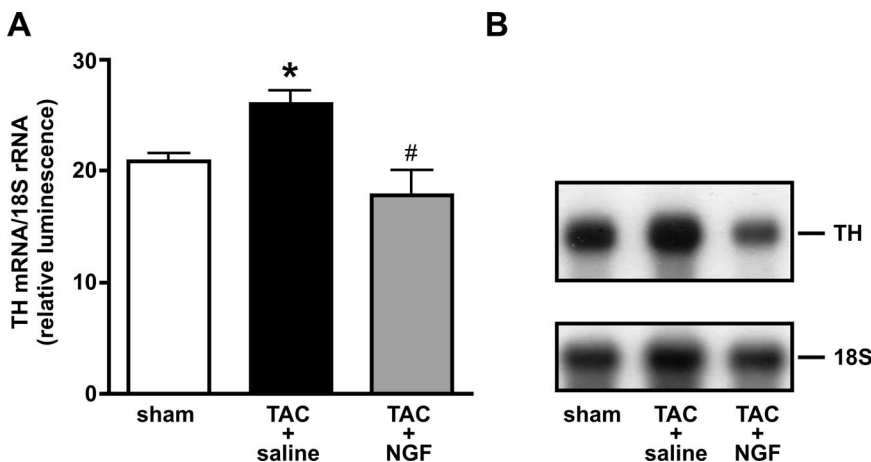
**Cardiac NE Reuptake**

To test the possibility that NGF injection restored cardiac NE by improving NE reuptake, 2 different approaches were used. First, consistent with lower cardiac NE stores, electrical field stimulation (S1) resulted in a smaller net overflow of endogenous NE from isolated perfused hearts of TAC rats as compared with those of sham controls (Figure 4A). NGF treatment significantly increased this releasable NE pool but did not completely normalize it (Figure 4A). The stimulated NE overflow after NET blockade with DMI (S2, exocytotic NE release) was also reduced in saline-treated TAC but

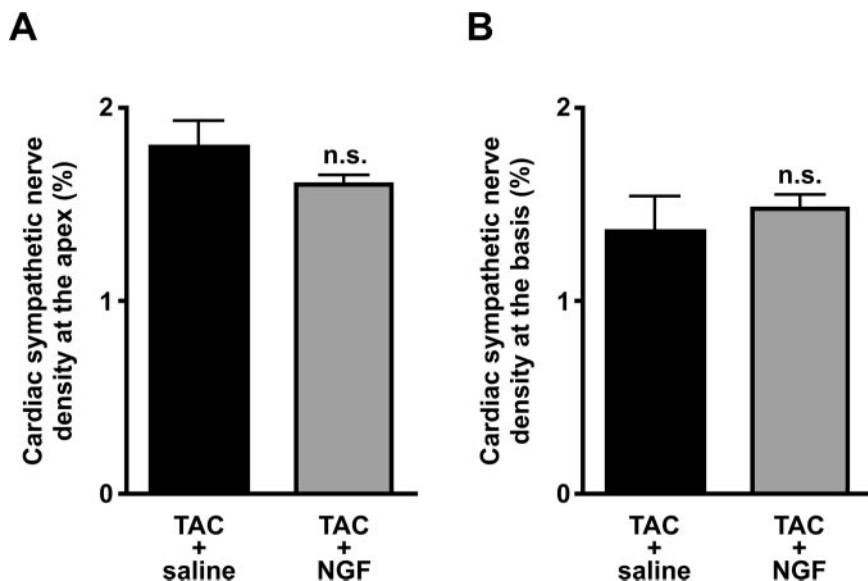
normalized in NGF-treated TAC (Figure 4B). The ratio between S2 and S1 reflects the fraction of NE that is taken up by the NET. Blockade of NET resulted in a significantly smaller increase in stimulated overflow of endogenous NE from the hearts of TAC rats as compared with sham (Figure 4C), indicating an impaired NE reuptake in TAC animals. NGF treatment normalized the increase in stimulated NE overflow in response to DMI, indicating an improved function of the NET (Figure 4C). Second, to confirm this finding, an independent approach was used that does not depend on releasable endogenous NE pools of the sympathetic nerve ending. In hearts of TAC rats, the uptake of exogenous NE (ie, bolus injection of [<sup>3</sup>H]-NE) was reduced (Figure 4D). There was no difference between TAC and sham in the residual uptake of [<sup>3</sup>H]-NE after specific blockade of NET with DMI (5.1±1.6 versus 4.0±1.9%; n≥7), indicating that the diminished cardiac elimination of [<sup>3</sup>H]-NE was entirely the result of a reduced uptake via NET and not, for example, via extraneuronal uptake. Injection of NGF into left stellate ganglia normalized cardiac uptake of exogenous [<sup>3</sup>H]-NE compared with saline-treated TAC rats (Figure 4D). As assessed by quantitative RT-PCR, there was no significant effect on NET mRNA (normalized to 18S rRNA) expression in TAC rats treated with NGF (2.08±0.35; n=6) as compared with saline (2.88±0.28; n=6).

**Echocardiographic Parameters Before and After NGF Treatment**

Echocardiography was performed 2 days before and 32 hours after application of NGF or saline. NGF improved the



**Figure 2.** TH mRNA concentration in the left stellate ganglion of sham-operated rats (n=5) and rats with TAC. NGF (20 μg; n=6) or the vehicle saline (n=13) was injected into the left stellate ganglion of TAC rats 32 hours before RNA isolation. (A) TH mRNA quantified by Northern Blot normalized to 18S rRNA. \*P<0.05 vs sham; #P<0.05 vs TAC+saline. (B) Representative Northern blot.



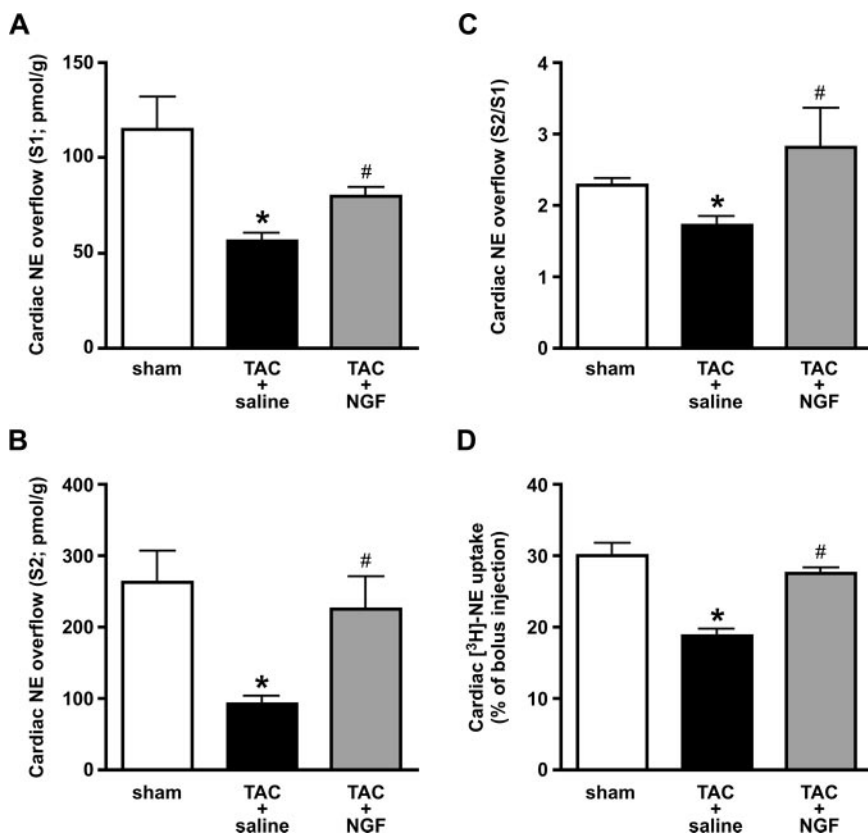
**Figure 3.** Cardiac sympathetic nerves stained by glyoxylic acid-induced histo-fluorescence in rats with TAC. NGF (n=4) or the vehicle saline (n=4) was injected into the left stellate ganglion of TAC rats 32 hours before sample preparation. Shown is the quantitative analysis of the sympathetic nerve density per analyzed myocardial area at the apex (A) and the basis (B) of the heart. n.s.=not significant.

fractional shortening intraindividually in all of the animals that received NGF (Figure 5A and 5B). In contrast, rats treated with saline did not show any changes after 32 hours (Figure 5B). Heart rate, anterior and posterior wall thicknesses, and left ventricular end-systolic and end-diastolic diameters remained unaltered after NGF injection (supplemental Table II).

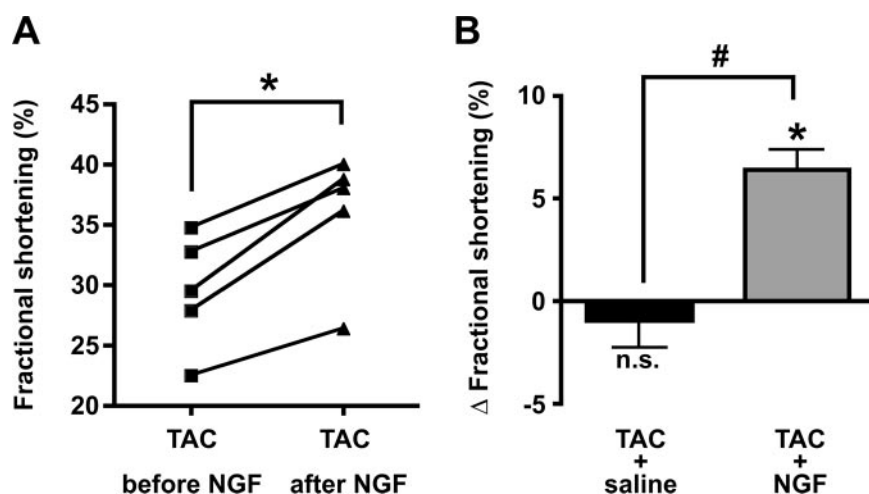
### Discussion

In the present study, we demonstrate that injection of NGF into stellate ganglia of animals with experimental CHF refills

depleted cardiac NE stores, normalizes cardiac NET function, and improves left ventricular contractility. Transgenic mice overexpressing NGF in the heart show elevated cardiac catecholamine levels and sympathetic hyperinnervation.<sup>23,24</sup> Cao et al<sup>25</sup> reported that a prolonged (5 weeks) infusion of NGF into the left stellate ganglion of dogs after myocardial infarction induces hyperinnervation, which results in ventricular tachyarrhythmias and sudden cardiac death. However, in vivo, nerve sprouting was not observed as early as 1 week after the beginning of NGF application.<sup>26</sup> In accordance, we did not find an increased sympathetic nerve density 32 hours



**Figure 4.** Cardiac release and uptake of NE in isolated perfused hearts from sham-operated rats and rats with TAC. NGF (20  $\mu$ g) or the vehicle saline was injected into the left stellate ganglion of TAC rats 32 hours before heart perfusion. Stimulated overflow of endogenous NE, evoked by electrical field stimulation before (S1) and after (S2) specific blockade of NET with DMI (1  $\mu$ mol/L). Shown is the absolute NE release after S1 (A), after S2 (B), and the S2/S1 ratio (C). Sham (n=5), TAC+saline (n=8), TAC+NGF (n=3). \* $P$ <0.05 vs sham; # $P$ <0.05 vs TAC+saline. (D) Cardiac uptake of a bolus injection of exogenous [<sup>3</sup>H]-NE. Sham (n=9), TAC+saline (n=11), TAC+NGF (n=4). \* $P$ <0.05 vs sham; # $P$ <0.05 vs TAC+saline.



**Figure 5.** Left ventricular fractional shortening. (A) Fractional shortening in 5 rats with TAC 32 hours after injection of NGF (20  $\mu$ g) into the left stellate ganglion as compared with 2 days before NGF injection (TAC after NGF vs TAC before NGF). (B) Mean change from baseline fractional shortening (TAC rats 2 days before saline or NGF injection) as compared with fractional shortening 32 hour after saline (n=4) or NGF (n=5;  $\Delta$  fractional shortening). \* $P$ <0.005 vs 2 days before NGF treatment. n.s.=not significant vs 2 days before saline treatment. # $P$ <0.005 vs  $\Delta$  fractional shortening of TAC+saline.

after injection of NGF into stellate ganglia of TAC rats. In addition to the morphological long-term effects of NGF on nerve sprouting, it has also been shown that NGF modulates the function of sympathoadrenergic neurotransmission in cocultures of cardiomyocytes with neurons.<sup>27</sup> No data are available about the specific role of NGF on the disturbed local cardiac sympathetic nerve function in CHF.

In a previous study,<sup>5</sup> we reported that TAC rats develop overt CHF, markedly depleted NE stores, a reduced NET activity, and increased TH-mRNA levels but not an absolute or relative loss of cardiac sympathetic nerves. We used this well-characterized model to investigate the short-term effects of NGF on determinants of sympathetic nerve function. NGF was directly injected into the stellate ganglion that mainly innervates the heart. Of note, the neurotrophin receptors TrkA and TrkC are expressed not only at the axon terminals but also on the cell surface of the perikarya in the sympathetic ganglia.<sup>28</sup> Only the left stellate ganglion was injected, because it contributes more to the cardiac sympathetic innervation than the right ganglion.<sup>29</sup> Our results show that injection of NGF markedly increased cardiac [<sup>3</sup>H]-NE uptake. Because myocardial hypertrophy may affect the diffusion distance between the coronary circulation and the synaptic cleft, we chose a second approach to assess NET function that neutralized any potential relevance of diffusion barriers. The stimulated basal release of endogenous NE from each heart served as intraindividual control and was related to the release after specific blockade of the NET with DMI. Indeed, the latter approach confirmed the result obtained by measurement of cardiac [<sup>3</sup>H]-NE uptake. The latter approach also eliminates possible side effects that are the consequence of an initial distributive phase of bolus-injected [<sup>3</sup>H]-NE and that might be unrelated to real neuronal uptake.

The observation that the density of sympathetic nerve endings in the hypertrophic myocardium of TAC rats was unchanged as compared with sham<sup>5</sup> and to NGF-treated TAC supports that the observed effects on [<sup>3</sup>H]-NE uptake were not simply because of hypoinnervation as a consequence of cardiac hypertrophy or because of hyperinnervation in response to NGF treatment. Therefore, these findings identified NGF as an acute positive regulator of NET function. Kiriazis et al<sup>23</sup> reported that mice overexpressing NGF in the heart

show a doubled NE reuptake via NET as compared with healthy wild-type mice. However, in healthy mice, >95% of the released NE is taken up by the NET.<sup>2</sup> Therefore, a doubled NE reuptake in the latter study is rather caused by the previously reported cardiac hyperinnervation in these mice.<sup>24</sup>

How does NGF improve NET function? We did not observe a change of NET mRNA levels. In accordance, NET impairment in TAC rats has been shown to be mediated by a posttranscriptional mechanism.<sup>5</sup> Interestingly, protein kinase C (PKC) leads to a translocation of NET from the plasma membrane to the cytosol and subsequent to its degradation.<sup>30</sup> Recently, we demonstrated that endothelin 1 (ET-1) inhibits NET through an endothelin A receptor- and PKC-dependent pathway.<sup>16</sup> Additional studies are warranted to test the possibility of whether NGF interferes with the ET-1 signaling cascade. Ieda et al<sup>31</sup> reported, vice versa, that ET-1 regulates cardiac sympathetic innervation by controlling NGF expression through an endothelin A receptor- and PKC-dependent pathway. In this regard, the NGF-mediated improvement of cardiac sympathetic function in TAC rats might represent a negative feedback loop because of an activated ET-1 system.

In theory, an increased NE reuptake would enhance the concentration of NE within the sympathetic nerve terminal reflecting an improved NE recycling. In fact, NGF increased cardiac NE stores and the releasable cardiac NE pool of TAC rats. TH gene expression, which was enhanced in saline-treated TAC rats, decreased after NGF treatment to normal levels, implicating that the restoration of cardiac NE was unlikely because of an enhanced NE de novo synthesis. From these data, we cannot distinguish whether TH is a direct or indirect target of NGF signaling. The available data obtained by others in cell culture experiments are controversial.<sup>32,33</sup> However, one could speculate that the effect on TH gene expression is rather a reactive process because of an improved NET activity or because of other potentially NGF-affected determinants of sympathetic nerve function, such as exocytotic NE release or sympathetic nerve activity, which were not evaluated in detail in this study.

An intriguing finding of this study is that NGF leads to an improvement of left ventricular contractility 32 hours after injection into the left stellate ganglion of TAC rats. We cannot rule out the possibility of a systemic effect, such as

that a part of the injected NGF diffused away from the stellate ganglion, so that it reached the circulatory system and eventually affected cardiac myocytes directly. However, taken together with the effects of NGF that we described above, we rather suggest that this effect depends on the local cardiac sympathetic nerve function. Assuming that the NGF-mediated NET improvement results in an attenuated activation of adrenergic receptors as observed under therapy of heart failure patients with  $\beta$ -blockers, it may appear surprising that the positive effect of cardiac contractility occurs already after 32 hours, whereas  $\beta$ -blockers exert their positive effects after months of treatment. A possible explanation for this controversy may be that an improvement of NE reuptake leads to, other than a decreased activation of postsynaptic adrenergic receptors, a restoration of NE in cardiac sympathetic nerve endings and to an increase of the releasable NE pool. As a consequence, NGF might restore the responsiveness of cardiomyocytes to NE. In accordance, depleted NE stores have been shown to reduce cardiac contractility,<sup>34</sup> and refilled NE stores have been implicated to improve exercise tolerance.<sup>10</sup>

In the present study, we investigated pressure overload-induced heart failure, which is a model for some entities of heart failure, such as aortic stenosis or, in part, arterial hypertension. However, similar studies are also needed that investigate the effects in other models, such as myocardial infarction or volume overload, because the changes in cardiac sympathetic functions may differ depending on different heart failure entities (Kristen AV, Kreusser MM, Lehmann L, Kinscherf R, Katus HA, Haass M, Backs J, unpublished observations, 2005).

### Perspectives

Functional impairment of NET contributes to depletion of cardiac NE stores and potentiates sympathetic overstimulation of failing hearts.<sup>2-5,35,36</sup> As a consequence, there may be a higher incidence of life-threatening tachyarrhythmias and cardiac remodeling. Likewise, it has been shown that an impairment of NE reuptake negatively correlates with the prognosis of patients with CHF.<sup>37,38</sup> In the present study, we demonstrate that a single injection of NGF into stellate ganglia of rats with experimental CHF improves NET function and restores cardiac NE. Moreover, evidence is provided that these NGF effects lead to an improvement of cardiac contractility. Therefore, we postulate that NGF has the potency to counteract the local sympathetic overdrive in failing hearts within days. On the other hand, it was reported that a continuous 5-week NGF treatment after myocardial infarction in dogs induces hyperinnervation and ventricular tachyarrhythmias.<sup>25</sup> Consequently, it will be necessary to clarify the dosage, duration, and application form of a potential beneficial versus detrimental therapeutic NGF approach. Despite these and other open questions, NGF treatment might become a novel therapeutic principle to treat patients with acute and/or chronic CHF.

### Acknowledgments

The expert technical assistance of Silvia Harrack, Jutta Krebs, and Michaela Oestringer is gratefully acknowledged. J.B. was supported

by a grant of the Young Investigator Program of the University of Heidelberg.

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*Hypertension*. 2006;47:209-215; originally published online December 27, 2005;  
doi: 10.1161/01.HYP.0000200157.25792.26

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231  
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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