Nerve Growth Factor Is Critical for Cardiac Sensory Innervation and Rescues Neuropathy in Diabetic Hearts

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Background—Molecular mechanisms regulating the cardiac sensory nervous system remain poorly understood. Cardiac sensory nerve impairment causes silent myocardial ischemia, a main cause of sudden death in diabetes mellitus (DM). The present study focused on the roles of nerve growth factor (NGF) in the regulation of the cardiac sensory nervous system and analyzed the mechanism of silent myocardial ischemia in DM.

Methods and Results—We screened neurotrophic factors and found that cardiac sensory nerves developed in parallel with NGF synthesized in the heart. Cardiac nociceptive sensory nerves that were immunopositive for calcitonin gene-related peptide, dorsal root ganglia (DRG), and the dorsal horn were markedly retarded in NGF-deficient mice, whereas cardiac-specific overexpression of NGF rescued these deficits. DM was induced with streptozotocin in wild-type and transgenic mice overexpressing NGF in the heart. Downregulation of NGF, calcitonin gene-related peptide–immunopositive cardiac sensory denervation, and atrophic changes in DRG were observed in DM-induced wild-type mice, whereas these deteriorations were reversed in DM-induced NGF transgenic mice. Cardiac sensory function, measured by myocardial ischemia–induced c-Fos expression in DRG, was also downregulated by DM in the wild-type mice but not in NGF transgenic mice. Direct gene transfer of NGF in the diabetic rat hearts improved impaired cardiac sensory innervation and function, determined by electrophysiological activity of cardiac afferent nerves during myocardial ischemia.

Conclusions—These findings demonstrate that the development and regulation of the cardiac sensory nervous system are dependent on NGF synthesized in the heart and that DM-induced NGF reduction causes cardiac sensory neuropathy.

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Key Words: diabetes mellitus ■ gene therapy ■ ischemia ■ nervous system

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The cardiac sensory nervous system is responsible for pain perception and for initiating a protective cardiovascular response during myocardial ischemia. The sensory signals are conducted through cardiac afferents, primarily thinly myelinated Aδ-fibers and unmyelinated C-fibers that project to the upper thoracic dorsal horn (DH) via dorsal root ganglia (DRG).1,2 Silent myocardial ischemia, characterized by loss of pain perception during myocardial ischemia, is a frequent and major complication of diabetes mellitus (DM) that places these patients at high risk of sudden cardiac death.3 Despite the severity of this complication, the alterations of cardiac sensory innervation and the molecular mechanism that underlies sensory neuropathy in diabetic hearts are poorly understood. Moreover, little is known about the regulation of cardiac sensory nerve development under physiological conditions. Addressing these questions requires a better understanding of the anatomical distribution of cardiac sensory nerves, the molecular mechanism of its innervation during development, and the pathophysiological changes of the cardiac sensory nerves, DRG, and DH in DM.

Nerve growth factor (NGF) is a prototypic member of the neurotrophin family that is pivotal in the differentiation, survival, and synaptic activity of the cardiac sympathetic nervous system.4,5 Nociceptive sensory nerves are subclassified into NGF and glial cell line–derived neurotrophin factor (GDNF)–dependent nerves in somatic tissues.6 The neurons in the DRG were found to be poorly differentiated in
NGF-deficient (NGF−/−) mice, and these mice lacked the pain reflex to tail pinch. The exact roles of neurotrophic factors on cardiac sensory nerves and the constitution of nociceptive sensory nerves in the heart remain largely unknown, however. In the skin of diabetic rats, deteriorated sensory function and decreases in calcitonin gene-related peptide (CGRP)– and substance P–immunopositive nerves, markers of NGF-dependent nociceptive sensory nerves, correlated with a reduced NGF expression. Although NGF supplementation improved diabetic neuropathy in animal models, a clinical trial of systemic NGF administration did not show beneficial effects in diabetic polynuropathy. The lack of beneficial effects seen in the clinical trial could possibly be due to inadequate dosage and route of administration. Therefore, uncertainty remains regarding the use of NGF treatment in diabetic neuropathy.

In this study, the development of the cardiac sensory nervous system and DM-induced cardiac sensory neuropathy were dependent on NGF synthesized in hearts, and NGF gene therapy in diabetic hearts rescued silent myocardial ischemia.

### Methods

#### Gene-Modified Animals

NGF−/− mice (The Jackson Laboratory, Bar Harbor, Me) and transgenic mice overexpressing NGF under the control of an α-myosin heavy chain promoter (NGFTG) (Howard J. Federoff, Rochester, NY) were generated as described previously. NGF−/− mice were crossed with NGFTG mice to generate NGF−/−/TG mice, which were crossed with NGF+/- to generate NGF+/-/TG mice.

#### Construction of Plasmids

To produce the NGF expression vector (p-NGF), mouse NGF cDNA was inserted into an expression plasmid that uses the chicken β-actin promoter/enhancer. The chicken β-actin expression vector plasmid (p-con), which does not contain NGF cDNA, was used as a control.

#### In Vivo Gene Transfer

Hemagglutinating virus of Japan–envelope vector kit (Ishihara Sangyo Kaisha, Osaka, Japan) was used as described previously. Hemagglutinating virus of Japan–liposome complex containing the plasmids (5 or 50 μg) was injected directly into the hearts with a 30-gauge needle after thoracotomy in 8-week-old DM-induced rats (CLEA Japan, Japan).

#### Electrophysiology

Rats were anesthetized and intubated during cardiac sympathetic afferent nerve recordings. For cardiac nerve recordings, left stellate ganglia were exposed through a midline sternotomy, and recordings of sympathetic afferent nerves were performed. Polytetrafluoroethylene-coated multistrand stainless steel electrodes (A-M Systems, Inc, Carlsborg, Wash) were placed on the nerves, and the nerves and electrodes were covered with silicon gel (Wacker-Chemie, Munich, Germany). Neural recording electrodes were connected to a high-impedance probe (JB101J, Nihon Kohden Corp, Tokyo, Japan), which was connected to a differential amplifier with a band-pass filter of 50 to 1000 Hz. The filtered neurogram was integrated by a resistance-capacitance circuit (time constant, 20 ms). Only the spontaneously active nerves with receptive fields in the heart were studied. The response during myocardial ischemia induced by coronary artery occlusion was analyzed.

#### Statistical Analysis

Values are presented as mean±SEM. Differences between groups were examined for statistical significance with the use of Student t test or ANOVA with the Fisher protected least significant difference test. Probability values of <0.05 were regarded as significant. All other experimental procedures are described in the online-only Data Supplement.

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

### Results

#### Cardiac Sensory Innervation Increases With Development Concordantly With NGF Expression in the Heart

We analyzed the time course of cardiac sensory innervation and neurotrophic factor levels in developing hearts. To determine cardiac sensory nerve density in mice, immunostaining for CGRP, a well-known marker for nociceptive sensory nerves, was performed (Figure 1A and 1B). Nerve endings were barely detectable at E15.5, appeared at E18.5, and peaked at postnatal day (P) 14. CGRP-immunopositive nerves were abundant in the ventricular myocardium and more so in epicardial sites. Among several neurotrophic factors to be involved in somatic sensory innervation, only NGF increased linearly as innervation progressed (Figure 1C to 1E). The concentration of cardiac CGRP and substance P also significantly increased with development (Figure 1F and 1G). The cardiac sensory fibers project to the DH of the upper thoracic (T) spinal cord, mediated through DRG neurons. The number of neurons immunopositive for CGRP and the intensity of staining increased as development proceeded in both the DRG and DH (Figure 1H and 1I). These results revealed that NGF and cardiac sensory nervous system increased coincidentally with the developmental stages.

#### Cardiac Sensory Nervous System Is Retarded in NGF-Deficient Mice

To investigate whether NGF is critical for cardiac sensory nerve development, we analyzed NGF-deficient mice at P4. CGRP-immunopositive nerves showed a significant decrease to 20% in NGF+/- hearts compared with NGF+/-, with the NGF−/− hearts showing no CGRP-immunopositive nerves (Figure 2A and 2C). Immunostaining for tyrosine hydroxylase, a marker of sympathetic nerves, was performed as a control experiment (Figure 2A). To confirm the differentiation of sensory and sympathetic nerves, immunostaining was performed on DRG and sympathetic ganglia. Only CGRP labeling was detected in the DRG, and tyrosine hydroxylase staining demarcated the sympathetic ganglia (Figure 2B). The concentrations of CGRP and substance P were significantly lower in the NGF−/− and NGF−/− hearts compared with their NGF+/- littermates (Figure 2D and 2E), as were the levels of NGF mRNA (Figure 2F). Hematoxylin-eosin staining of the T1-T4 DRG and DH revealed that NGF−/− DRG contained fewer neurons by 72% than NGF+/- littermates (Figure 2G and 2H). The CGRP-immunopositive neurons in NGF+/- and NGF−/− DRG were decreased to 49% and 0% of NGF+/- littermates, respectively (Figure 2G and 2I). CGRP immunostaining of DH also reduced in NGF-deficient mice (Figure 2J). Together, these results indicated that the cardiac sensory nervous system is retarded in NGF-deficient mice, suggesting a dependence on this factor for development of this system.
Figure 1. Cardiac sensory innervation is increased with development coincident with NGF expression in the heart. A, Representative immunostaining for CGRP in murine hearts at E15.5, 18.5, P4, and P14. Boxed areas correspond to the higher-power photomicrographs in the insets. Sensory innervation was increased with development. B, The immunopositive nerve areas for CGRP were determined with the use of NIH Image software (n=4). C to E, Time courses of cardiac NGF, neurotrophin-3, and GDNF expression were determined by quantitative RT-PCR (n=6). F, G, Cardiac CGRP and substance P (SP) concentrations were measured by enzyme-linked immunosorbent assay (n=6). H, CGRP immunostaining of murine DRG and DH at E15.5, 18.5, P4, and P14 at the T2 level of section. The boundary of DRG is demarcated by the arrows. I, Time course of CGRP+ cells per total neurons in T1-T4 DRG is shown (n=5). *P<0.01; **P<0.05 vs E15.5. Bar=100 µm.
Figure 2. The cardiac sensory nervous system is retarded in NGF-deficient mice. A, Immunostaining for CGRP and tyrosine hydroxylase in NGF+/+/, NGF+/-, and NGF−/− hearts at P4. Note that CGRP+ nerve endings were reduced in an NGF gene dosage-dependent manner. Boxed areas correspond to the higher-power photomicrographs in the insets. B, NGF+/+ DRG and sympathetic ganglia (SG) were immunostained with anti-CGRP and anti-tyrosine hydroxylase antibodies. Note that different structures were immunopositive with each antibody. C, The CGRP+ nerve areas were determined with the use of NIH Image (n=4). D, E, Cardiac CGRP and substance P (SP) concentrations were analyzed by enzyme-linked immunosorbent assay (n=5). F, Cardiac NGF expression was measured by quantitative RT-PCR (n=5). G, CGRP immunostaining and hematoxylin-eosin (H&E) staining (low- and high-power fields) of NGF+/+, NGF+/-, and NGF−/− DRG at the T2 level. The boundary of DRG is demarcated by the arrows. Note that NGF−/− DRG contained fewer small to medium neurons, but large neurons are present. H, I, Numbers of total neurons and CGRP+ neurons were reduced in T1-T4 NGF-deficient DRG (n=5). J, CGRP immunoreactivity was reduced in NGF-deficient DH. Representative data are shown. *P<0.01; **P<0.05 vs NGF+/+. Bar=100 μm.
Cardiac-Specific Overexpression of NGF Rescues the Cardiac Sensory Nervous System Defects in NGF-Deficient Mice

It is possible that the low sensory nerve density in the heart is an indirect effect of reduced CGRP-positive DRG neurons in NGF-deficient mice and that the immature development of the DRG is due to the depletion of NGF in DRG. We therefore performed a genetic rescue of cardiac NGF expression in the NGF−/− mice to ascertain whether the defects could be ascribed specifically to cardiac NGF expression. The NGF+/−/WT, NGF+/−/TG, and NGF−/−/TG hearts were hyperinnervated compared with NGF−/−/WT littermates (Figure 3A and 3B). In contrast, there was no difference in skin denervation (Figure 3A). Quantitative reverse transcription–polymerase chain reaction (RT-PCR) revealed strong NGF expression, 22-fold over WT in the NGF−/−/TG hearts (Figure 3C). The CGRP and substance P concentrations were increased in the NGF−/−/TG hearts (Figure 3D and 3E). The defects of DRG and DH in NGF−/− mice were also completely overcome in NGF+/−/TG at the T1-T4 level (Figure 3F to 3H). In contrast, the proportion of CGRP-positive neurons was low, as was the intensity of staining, in NGF+/−/TG lumbar DRG (Figure 3I and 3J). These results indicated that NGF synthesized in the heart is critical for development of the cardiac sensory nervous system.

NGF Secreted From Cardiomyocytes Is Required for Axonal Extension and CGRP Expression in DRG Neurons

To resolve whether the alteration of cardiac sensory nervous system in the NGF+/−/WT or NGF+/−/TG was due to cell-autonomous effects within the DRG or rather to a change in the endogenous NGF secreted from cardiomyocytes, we performed DRG culture experiments. NGF induced axonal growth in a dose-dependent manner (Figure 4A and 4B), consistent with previous findings. We stimulated the DRG explants with conditioned media from the NGF+/−/WT, NGF+/−/TG, and NGF−/−/TG cardiomyocytes cultures. The NGF+/−/TG media significantly induced axonal extension in DRG neurons, and this growth was strongly suppressed by pretreatment with anti-NGF blocking antibody. In contrast, conditioned media from the NGF+/−/WT cardiomyocytes did not induce axonal growth (Figure 4C and 4D). To determine whether the NGF+/−/WT or NGF+/−/TG DRG development was due to cell-autonomous effects, we stimulated E12.5 NGF+/−/WT, NGF+/−/TG, and NGF−/−/TG DRG explants with conditioned media from NGF+/− cardiomyocytes. Axonal growth was not different among these DRG cultures (Figure 4E and 4F), indicating that the difference between NGF+/−/WT and NGF+/−/TG DRG axonal extension was not dependent on cell-autonomous effects but was due to NGF secreted from cardiomyocytes.

To investigate whether NGF secreted from cardiomyocytes is critical for CGRP expression in DRG, NGF+/−/WT, and NGF+/−/TG cardiomyocytes were dissociated and stimulated with conditioned media from NGF+/−/WT, NGF+/−/WT, or NGF−/−/TG cardiomyocytes (Figure 4G and 4H). Immunostaining for neurofilament-M and CGRP showed different proportions of CGRP-positive DRG neurons in the explants (27%, 7%, and 38% in the cultured media from NGF+/−/WT, NGF+/−/WT, and NGF−/−/TG, respectively). These results indicated that NGF secreted from cardiomyocytes is also critical for CGRP expression in DRG neurons.

Cardiac Sensory Innervation and NGF Production Are Reduced in Streptozotocin-Induced Diabetic Mice

Although silent myocardial ischemia is a well-known phenomenon in diabetes, alterations to cardiac sensory innervation and the relevance of NGF and sensory neuropathy remain unknown. Streptozotocin (STZ) was used to induce diabetes in Jcl : ICR mice, and the mice were analyzed after 8 (8 weeks-STZ) and 16 (16 weeks-STZ) weeks. Body weights were decreased and blood glucose was elevated in STZ-treated mice (Figure 5A). CGRP-immunopositive nerves were not altered in 8 weeks-STZ but were significantly decreased to 53% in 16 weeks-STZ hearts (Figure 5B and 5C). The concentration of cardiac CGRP was reduced to 60% in 16 weeks-STZ hearts (Figure 5D). The levels of NGF mRNA did not change in 8 weeks-STZ but were significantly downregulated to 57% in 16 weeks-STZ hearts (Figure 5E). Immunostaining for CGRP in T1-T4 DRG and DH revealed a significant decrease in CGRP-immunopositive neurons and nerves in 16 weeks-STZ (Figure 1 in the online-only Data Supplement). These results indicated that long-term diabetes results in CGRP-immunopositive sensory denervation and reduced NGF production in the heart.

Some nociceptive nerves are GDNF-dependent in other tissues such as skin. To analyze GDNF-dependent sensory nerves in the heart, we performed immunostaining for P2X3, the purinoreceptor subtype (Figure 5F). Although CGRP-immunopositive nerves were abundant in both epicardium and ventricular myocardium, fewer P2X3-immunopositive nerves were detected, and only at epicardial sites. Differentiation of GCP and P2X3-immunopositive nerves was confirmed by immunostaining of DH (Figure 5G). Diabetic hearts showed a greater reduction of CGRP-immunopositive nerves compared with P2X3-immunopositive nerves (Figure 5H), and NGF, expressed more in hearts than skin in control mice, was significantly reduced in both tissues with DM. In contrast, GDNF, synthesized abundantly in skin, was specifically downregulated in diabetic skin but not in hearts (Figure 5I and 5J). These results indicated that neurotrophin synthesis is regulated in a tissue type–specific manner and that P2X3-immunopositive sensory nerves are slightly reduced in diabetic hearts irrespective of GDNF expression.

Cardiac-Specific Overexpression of NGF Rescues the Sensory Denervation in Diabetic Hearts

To determine the contribution of cardiac NGF to diabetic neuropathy, diabetes was induced in the NGF+/− mice (NGFTG-control) and their wild-type littermates (WT-control) by STZ, and the animals were analyzed after 16 weeks. Both DM-induced wild-type (WT-DM) and NGFTG (NGFTG-DM) mice had similar hyperglycemia and lost body weight (Figure 6A). Immunostaining for cardiac CGRP...
Figure 3. Cardiac-specific overexpression of NGF rescues the defects of the cardiac sensory nervous system in NGF-deficient mice. A, Immunostaining for CGRP in the hearts and limb skin of NGF^{+/+}/WT, NGF^{-/-}/WT, and NGF^{-/-}/TG mice. B, The immunopositive nerve areas for CGRP were quantified (n=5). C, NGF expression in the NGF^{+/+}/WT, NGF^{-/-}/WT, and NGF^{-/-}/TG hearts is shown (n=5). The reduced NGF expression in NGF^{-/-}/WT heart was completely overcome by the cardiac-specific overexpression of NGF. D, E, The cardiac CGRP and substance P (SP) concentrations were increased in the NGF^{-/-}/TG compared with NGF^{-/-}/WT mice (n=5). F, CGRP immunostaining and hematoxylin-eosin (H&E) staining (low- and high-power fields) of NGF^{+/+}/WT, NGF^{-/-}/WT, and NGF^{-/-}/TG DRG at the T2 level of section. The boundary of DRG is demarcated by the arrows. Note that reduction in size and loss of CGRP immunoreactivity in NGF^{-/-}/WT DRG was completely restored in NGF^{-/-}/TG DRG. G, H, The number of neurons and CGRP^+ proportion in each T1-T4 DRG are shown (n=4 to 6). I, J, CGRP immunostaining and CGRP^+ proportion in NGF^{-/-}/TG DRG at T and lumbar (L) levels are shown (n=3). Note that NGF^{-/-}/TG did not rescue loss of CGRP immunoreactivity in L1-L4 DRG. The arrows in I indicate the boundary of DRG. K, CGRP immunoreactivity was restored in NGF^{-/-}/TG DH. Representative data are shown in each panel. *P<0.01 vs NGF^{-/-}/WT. Bar=100 μm.
Figure 4. NGF secreted from cardiomyocytes is required for axonal extension and CGRP expression in DRG neurons. A, B, NGF promoted axonal extension in DRG explants in a dose-dependent manner. Nerve fibers were immunostained with anti-neurofilament-M (NF-M) antibody. Bar=300 μm. C, DRG explants cultured in NGF+/+ WT, NGF−/− WT, and NGF−/−/TG cardiomyocyte-conditioned media are shown on the left. DRG explants pretreated with anti-NGF blocking antibody for 30 minutes and then incubated with each conditioned media are shown on the right. Bar=400 μm. D, Axon length in C (n=4). E, F, NGF+/+ WT DRG, NGF−/− WT DRG, and NGF−/−/TG DRG explants are cultured in NGF+/+ cardiomyocyte-conditioned media (n=5). Bar=200 μm. G, H, Immunostaining for NF-M and CGRP in dissociated DRG explants treated with NGF+/+ WT, NGF+/− WT, and NGF+/−/TG cardiomyocyte-conditioned media. Bar=100 μm. Note that the reduction of CGRP immunoreactivity in DRG cultured in NGF−/−/WT cardiomyocyte-conditioned media was completely restored in DRG cultured in NGF−/−/TG cardiomyocyte-conditioned media (n=5). Representative data are shown in each panel. *P<0.01; NS indicates not significant vs relative control.
Figure 5. Cardiac sensory innervation and NGF production are reduced in STZ-induced diabetic mice. A, Body weight and blood glucose in control (C) and STZ-treated mice. B, C, Immunostaining for CGRP in the hearts of control, 8 weeks-STZ, and 16 weeks-STZ mice. Epicardial (epi) sites are shown in the upper panels, and midventricular (mid) sites are shown in the lower panels. Sensory innervation was significantly reduced in 16 weeks-STZ hearts (n=5). D, The cardiac CGRP concentration was reduced in 16 weeks-STZ hearts (n=4). E, NGF mRNA expression was downregulated in 16 weeks-STZ hearts but not in 8 weeks-STZ hearts (n=5). F, H, Double-immunofluorescence staining for CGRP (red) or P2X3 (green) and TOTO-3 (blue) in control and DM (16 weeks-STZ) hearts. The reduction in CGRP nerves was greater than that of P2X3 nerves in DM hearts (n=5). G, DH was immunostained with anti-CGRP and anti-P2X3 antibodies. I, NGF was reduced in diabetic hearts and skin (n=4). J, GDNF was abundantly expressed in skin compared with hearts. GDNF was significantly reduced in diabetic skin but not in hearts (n=5). Representative data are shown in each panel. Bar=100 μm. *P<0.01; **P<0.05; NS indicates not significant vs relative control.
Figure 6. Cardiac-specific overexpression of NGF rescues the sensory denervation in the diabetic heart. A, Body weight and blood glucose are shown. B, C, Immunostaining for CGRP in the hearts of control and diabetic WT and NGFTG mice. Epicardial (epi) sites and midventricular (mid) sites are shown. Sensory hyperinnervation was observed in both NGFTG-control and NGFTG-DM hearts (n=5). D, The cardiac CGRP concentration was not different between NGFTG-control and NGFTG-DM hearts (n=3). E, NGF mRNA expression was downregulated in WT-DM hearts but not in NGFTG-DM compared with the relative control (n=4). F to H, CGRP immunostaining of control and diabetic WT and NGFTG DRG and DH at the T2 level of section. The CGRP* proportions in T1-T4 DRG are shown in H (n=5). Representative data are shown in each panel. Bar=100 µm. *P<0.01; **P<0.05; NS indicates not significant vs relative control.
Cardiac-Specific Overexpression of NGF Rescues the DM-Induced Loss of Pain Perception in the Heart

To investigate whether cardiac-specific overexpression of NGF affects sensory nerve function, c-Fos mRNA induction was measured after 0.5 hour of CAO in the WT-control and NGFTG-control mice. Basal levels in DRG were not different between these mice, but c-Fos expression was significantly increased in NGFTG-control mice after CAO (Figure 7C). The same experiment with WT-DM and NGFTG-DM mice showed that, although c-Fos induction after CAO was significantly downregulated by 54% in WT-DM, no significant difference was observed between NGFTG-DM and NGFTG-control mice (Figure 7D). Cardiac sensory nerves not only conduct pain perception but also release CGRP and substance P, which have protective effects on cardiac ischemia.16 To determine whether NGF rescues ischemic myocardial damage in diabetes via neuropeptide restoration, we analyzed infarct size and apoptotic cardiomyocytes in WT- and NGFTG-DM mice using the ischemia/reperfusion injury model (Figure II in the online-only Data Supplement). Cardiac-specific overexpression of NGF rescued the increased infarct size and myocardial apoptosis in diabetes. Collectively, long-term diabetes induced cardiac sensory functional deficits, and cardiac-specific overexpression of NGF rescued the sensory impairment in diabetic hearts.

Gene Transfer of NGF Improves Impaired Sensory Innervation in Diabetic Hearts

To address whether NGF could improve sensory function in diabetes, we investigated the efficacy of NGF gene therapy in diabetic rat hearts (Figure 8A). First, we analyzed NGF expression in cardiomyocytes transfected with p-con or p-NGF. Quantitative RT-PCR revealed abundant expression of NGF in p-NGF–transfected cardiomyocytes (Figure 8B; 100-fold increase). To determine whether NGF synthesized by p-NGF–transfected cardiomyocytes had biological effects, we stimulated PC12 cells with the cardiomyocyte-conditioned media. The p-NGF–transfected cardiomyocyte-conditioned media significantly induced neurite extension in PC12 cells (Figure 8C and 8D).

We next transfected p-NGF or p-con directly into the rat hearts after 8 weeks of STZ injection. Body weights were decreased and blood glucose was elevated in STZ-treated rats (data not shown). Rats were analyzed after 4 weeks of gene transfer. NGF was significantly increased in p-NGF–treated hearts in a dose-dependent manner (Figure 8E). The reduction of CGRP-immunopositive nerves and CGRP concentration in diabetic hearts was almost completely rescued in p-NGF–treated rats (Figure 8F to 8H). To analyze sensory function, rats were subjected to myocardial ischemia, and cardiac sympathetic afferent activities were measured. Although the baseline activity was not different, the response to myocardial ischemia was significantly reduced by 60% in diabetic rats. In contrast, NGF gene therapy prevented the impairment of cardiac afferent discharge to ischemia in diabetes (Figure 8I and 8J). These results indicated that NGF gene therapy could rescue neuropathy in diabetic hearts.

Discussion

Unlike somatic tissues, visceral organs such as the heart are believed to have rich autonomic efferent innervation but few

Figure 7. Cardiac-specific overexpression of NGF rescues the DM-induced cardiac sensory impairment. A, Induction of c-Fos mRNA expression in left T1-T6 DRG by CAO for a specified time interval (n=5). B, The c-Fos mRNA expression by 0.5-hour CAO in T1-T6 control, 8 weeks-STZ, and 16 weeks-STZ DRG was measured by quantitative RT-PCR (n=5) (sham, black bars; post-CAO, white bars). C, The c-Fos mRNA expression in T1-T6 DRG by CAO for 0.5 hour in WT-control and NGFTG-control (n=5). D, The c-Fos mRNA induction after CAO was reduced in WT-DM DRG but not in NGFTG-DM DRG compared with the relative control (n=4). *P<0.01; **P<0.05; NS indicates not significant vs relative control.

showed a 9-fold hyperinnervation in NGFTG-control and NGFTG-DM hearts compared with WT-control and no difference in CGRP concentration between NGFTG-control and NGFTG-DM hearts (Figure 6B to 6D). Although cardiac NGF mRNA was downregulated in WT-DM compared with WT-control, strong NGF mRNA expression was detected in NGFTG-control and NGFTG-DM hearts (Figure 6E). Reduced CGRP immunoreactivities observed in WT-DM DRG and DH were also rescued in NGFTG-DM (Figure 6F to 6H). These results indicated that cardiac-specific overexpression of NGF rescued sensory denervation and that downregulation of cardiac NGF led to sensory neuropathy in diabetic hearts.
Figure 8. NGF gene transfer restores impaired sensory innervation in diabetic hearts. A, Schematic representation of the plasmid-containing chicken β-actin promoter, mouse NGF cDNA, and human growth hormone (hGH) polyadenylation signal (pA). B, RT-PCR analysis of NGF in plasmid-transfected cardiomyocytes. C, D, PC12 cells stimulated with cardiomyocyte-conditioned media. Note that p-NGF-CM strongly induced neurite extension (n=5). E, Gene transfer of p-con or p-NGF into the diabetic rat (DM) heart. NGF expression was significantly increased in the p-NGF-injected group (n=5). F, G, CGRP-immunopositive sensory denervation in DM was completely rescued by 50 μg p-NGF gene transfer (n=5). H, Cardiac CGRP concentration measured by enzyme-linked immunosorbent assay in these animals (n=5). I, Recording of impulse activity from cardiac sympathetic afferent nerves. Myocardial ischemia (CAO) was induced at the time point indicated by arrows. Note that the response of cardiac afferent nerves was reduced in DM injected with p-con. p-NGF gene transfer (50 μg) preserved cardiac sensory nerve function in DM. J, The nerve responses evoked by CAO were measured as the percent changes from baseline activities (n=5). Representative data are shown in each panel. Bar=100 μm. *P<0.01; **P<0.05.
nociceptive afferent nerves. One of the difficulties in analyzing sensory innervation of the heart is the lack of markers. In the present study, we identified for the first time, using an anti-CGRP antibody, that cardiac sensory innervation is rich not only in epicardial sites but also in the ventricular myocardium and that sensory innervation increases with development, dependent on NGF in hearts. We also identified fewer GDNF-dependent, P2X3-immunopositive sensory nerves, and we only detected them at epicardial sites. These results are consistent with previous findings that vanilloid receptor-1-immunopositive sensory nerves (NGF- or GDNF-dependent nerves) are enriched in the epicardium. The constitution of nociceptive sensory innervation varies considerably among tissues. As we and others revealed, although NGF-dependent nerves abundantly innervate skin and hearts, GDNF-dependent nerves preferentially innervate cutaneous regions. We have also shown that NGF is more highly expressed in heart than skin, although GDNF was exclusively synthesized in skin. These findings are consistent with the neurotrophin hypothesis that innervation density roughly corresponds to local neurotrophin expression and demonstrate the critical roles of NGF- and GDNF-immunopositive sensory nerves in the heart.

Although our genetic approaches clearly demonstrated that NGF synthesized in the heart is necessary and sufficient for development of the cardiac sensory nervous system, other factors regulated by NGF or the cell-autonomous effects within DRG might affect cardiac sensory innervation. However, the present in vitro findings that pretreatment with anti-NGF blocking antibody inhibited the cardiomycocyte-conditioned media-induced axonal extension and the axonal extension was not cell-autonomous in DRG neurons, indicated that cardiomycocyte-derived NGF specifically determines cardiac sensory innervation.

The diabetic neuropathy could be explained by several mechanisms, such as depletion of the neurotrophic factors, microvascular complications, generation of reactive oxygen species, activation of the polyol pathway, and formation of advanced glycation end products. We found that CGRP-immunopositive sensory nerves, sensory function, and NGF production were downregulated in diabetic hearts, whereas these defects were reversed by NGF restoration. On the basis of these findings, we proposed that the decrease of NGF production in diabetic hearts causes cardiac sensory denervation and deterioration of sensory function. Consistent with our present results, NGF synthesis and sympathetic innervation are reduced in STZ-induced diabetic hearts. Reduced tissue perfusion through the vasa nervosum is associated with diabetic neuropathy, and vascular endothelial growth factor administration prevents the impairment of sensory function via restoration of neural vascularity. Given that NGF promotes angiogenesis in ischemic hindlimb via the release of vascular endothelial growth factor, we suspect that supplementation of NGF in diabetic hearts would directly and indirectly prevent functional sensory deficits via improvement of microvascular ischemia.

In contrast to our findings, Christianson et al reported that intrathecal administration of NGF failed to improve cutaneous innervation, but GDNF rescued diabetes-induced cutaneous axon loss. Our findings that the sensory nerve constitution is different among tissues and that GDNF was markedly downregulated in diabetic skin could explain this difference. We also found that P2X3-immunopositive nerve area but not GDNF expression was reduced in diabetic hearts. Given that retrograde transport of neurotrophic factors is impaired in diabetic nerves, deteriorated axonal transport of GDNF in diabetic hearts might cause P2X3-immunopositive nerve reduction. It will be intriguing to determine the regulatory mechanisms of neurotrophic factor expression and to investigate whether GDNF treatment improves sensory neuropathy in diabetic hearts. Phase I and phase II clinical trials of systemic administration of recombinant NGF revealed safety and potential efficacy in diabetic polyneuropathy; a phase III trial, however, did not show beneficial effects because the dosage and route of administration may have been suboptimal. The dosage of NGF was restricted by side effects, and development of an anti-NGF antibody may have contributed to the lack of beneficial effects in the phase III clinical trial. These complications could be avoided by administering NGF directly to the cells that require the factor. NGF and CGRP-immunopositive nerves were proportionally reduced in diabetic hearts, and thus we successfully treated cardiac sensory neuropathy by direct NGF gene transfer. Consistent with our findings, the efficacy of NGF gene therapy has been reported in diabetic cystopathy and neuropathy of footpad. Further studies on the reliable and efficient method of this gene therapy are required before clinical trials can proceed. In conclusion, these findings indicate that NGF plays critical roles in sensory nerve development and cardiac sensory impairment in diabetic hearts.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

The cardiac sensory nervous system is responsible for pain perception and for initiating protective cardiovascular responses during myocardial ischemia. Silent myocardial ischemia, characterized by loss of pain perception during myocardial ischemia, is a major complication of diabetes mellitus and is of prognostic importance. Despite its clinical importance, the alterations of cardiac sensory innervation and the molecular mechanism that underlies sensory neuropathy in diabetic hearts are poorly understood. Moreover, little is known about the regulation of cardiac sensory nerve development under physiological conditions. Nerve growth factor (NGF), a member of the neurotrophin family, among others, plays pivotal roles in the regulation of somatic sensory nerves. Phase I and II clinical trials of systemic administration of recombinant NGF revealed safety and potential efficacy in diabetic polyneuropathy; a phase III trial did not show beneficial effects, however, because the dosage and route of administration may have been suboptimal. The roles of NGF on cardiac sensory innervation and the efficacy of NGF treatment in diabetic neuropathy remain understudied. In the present study, we show that the development and regulation of the cardiac sensory nervous system are dependent on NGF synthesized in the heart and that diabetes mellitus-induced NGF reduction in the heart causes cardiac sensory denervation and functional deficits. Furthermore, direct gene transfer of NGF in the diabetic rat hearts improves silent myocardial ischemia. Although further studies on the reliable and efficient method of the gene therapy are required before clinical trials, NGF may be a potential therapeutic target for sensory neuropathy in diabetic hearts.
Nerve Growth Factor Is Critical for Cardiac Sensory Innervation and Rescues Neuropathy in Diabetic Hearts

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Supplementary Figure 1

Panel A: Images of DRG (left) and DH (right) from control (C) and 16wk-STZ groups. The images show differences in the staining patterns.

Panel B: Graph showing the percentage of CGRP+ neurons. The bar for 16wk-STZ is significantly lower than that for control (C), indicated by **.