Neuron-Derived Neurotrophic Factor Ameliorates Adverse Cardiac Remodeling After Experimental Myocardial Infarction

Joki et al: NDNF and Myocardial Infarction

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Abstract

**Background**—Myocardial infarction (MI) is one of the major causes of death worldwide. Chronic heart failure is a serious complication of MI that leads to poor prognosis. We recently found that neuron-derived neurotrophic factor (NDNF) is a pro-angiogenic secretory protein that is upregulated in ischemic skeletal muscle. Here, we examined whether NDNF modulates cardiac remodeling in response to chronic ischemia.

**Methods and Results**—C57BL/6J wild-type mice were subjected to the permanent ligation of the left anterior descending coronary artery (LAD) to create MI. Adenoviral vectors expressing NDNF (Ad-NDNF) or β-galactosidase (control) were intramuscularly injected into mice 3 days prior to permanent LAD ligation. Intramuscular administration of Ad-NDNF to mice resulted in increased levels of circulating NDNF. Ad-NDNF administration improved left ventricular systolic dysfunction and dilatation following MI surgery. Moreover, Ad-NDNF enhanced capillary formation and reduced cardiomyocyte apoptosis and hypertrophy in the post-MI hearts. Treatment of cultured cardiomyocytes with recombinant NDNF protein led to reduced apoptosis under conditions of hypoxia. NDNF also promoted the phosphorylation of Akt and focal adhesion kinase (FAK) in cardiomyocytes. Blockade of FAK activation blocked the stimulatory effects of NDNF on cardiomyocyte survival and Akt phosphorylation. Similarly, treatment of cultured endothelial cells with NDNF protein led to enhancement of network formation and Akt phosphorylation, which was diminished by FAK inhibition.

**Conclusions**—These data suggest that NDNF ameliorates adverse myocardial remodeling after MI by its abilities to enhance myocyte survival and angiogenesis in the heart through FAK/Akt-dependent mechanisms.

**Key Words:** NDNF; cardiac remodeling; myocardial infarction; Akt; focal adhesion kinase
Cardiovascular disease is the major cause of morbidity and mortality in developed countries. Although percutaneous coronary intervention therapy for acute myocardial infarction (MI) is effective in reducing cardiovascular events and mortality, chronic heart failure after MI still remains as a serious complication, which is linked to poor prognosis. Adverse cardiac remodeling characterized by left ventricular dilatation, myocardial apoptosis and hypertrophy, and interstitial fibrosis, occurs after MI and contributes to the development of heart failure.

Neuron-derived neurotrophic factor (NDNF) is a secretory protein which has fibronectin type III domains. NDNF was initially identified as a neurotrophic factor that is produced by neurons. Recently, we reported that murine skeletal muscle abundantly expresses NDNF which is upregulated in response to ischemic insult. Intramuscular overexpression of NDNF promotes revascularization processes in ischemic hindlimb in a mouse model of peripheral artery disease. The in vitro experiments also showed that NDNF stimulates survival and angiogenic response of cultured endothelial cells via activation of Akt-endothelial nitric oxide synthase (eNOS) signaling pathways. Furthermore, NDNF modulates the endothelial cell response, at least in part, through integrin-dependent mechanisms. Thus, NDNF may act as a vascular protective factor that is secreted from skeletal muscle. However, it is unclear whether NDNF influences the pathological processes of the heart. Here, we investigated the impact of NDNF on cardiac remodeling after experimental MI.
Methods

An expanded Methods section can be found in the Data Supplement.

Mouse models of myocardial infarction, ischemia reperfusion injury and pressure overload

Male C57BL/6J (WT) mice at the age of 10 weeks were subjected to myocardial infarction surgery as previously described. Briefly, the left anterior descending coronary artery (LAD) was permanently ligated with 8-0 nylon suture after mice were intraperitoneally anesthetized with sodium pentobarbital (50 mg/kg).

In some experiments, WT mice were subjected to myocardial ischemia reperfusion (I/R) as previously described. Briefly, the LAD artery was ligated for 60 min with a suture using a snare occluder and then loosed. In some experiments, WT mice were subjected to cardiac pressure overload induced by transverse aortic constriction (TAC) operation. In brief, the chest was opened, and the thoracic aorta was identified after blunt dissection through the intercostal muscles. A 7-0 silk suture was placed around the transverse aorta and tied around a 24-gauge blunt needle, which was then removed as previously described. Ad-NDNF or Ad-β-gal (2×10⁹ plaque-forming units (pfu)/mouse) was injected into five different sites of adductor muscle in left hindlimb 3 days prior to the surgery. Study protocols were approved by the Institutional Animal Care and Use Committee at Nagoya University.

Statistical analysis

Data are shown as mean ± S.E. Differences between 2 groups were evaluated by the Student’s t test. Differences among more than 3 groups were evaluated by one-way analysis of variance.
(ANOVA) with Tukey Kramer’s test. A p value <0.05 denoted the presence of a statistically significant difference. All statistic calculations were performed by using SPSS for Windows.

Results

Intramuscular overexpression of NDNF ameliorates cardiac function after MI

To investigate the effect of skeletal muscle-derived NDNF on cardiac remodeling and ventricular function post-MI, Ad-NDNF or control Ad-β-gal was injected into adductor muscle of left hindlimb of WT mice 3 days before sham or MI surgery. Ad-NDNF increased plasma levels of NDNF of WT mice by a factor of 3.2 ± 0.1 at 2 weeks after sham operation compared with control (Figure 1A). Treatment of WT mice with Ad-NDNF also led to a 3.1 ± 0.2-fold increase in plasma NDNF level at 2 weeks after MI surgery. There were no differences in plasma NDNF levels between sham- and MI-operated mice after control adenoviral injection. Ad-NDNF-treated mice exhibited reduced heart weight (HW)/body weight (BW) ratio at 2 weeks after MI compared with control mice (Figure 1B). Ad-NDNF treatment had no effects on HW/BW ratio in sham-operated mice. Echocardiography was performed to assess cardiac dilatation and function in control and Ad-NDNF-treated mice at 2 weeks after MI or sham operation. Ad-NDNF-treated mice showed decreased LVDd and increased FS at 2 weeks post-MI compared with control mice (Figure 1C)(Supplemental Table 1). There were no statistically significant differences in LVDd and FS between control and Ad-NDNF-treated mice after sham operation (Supplemental Table 1). Ad-NDNF also increased circulating NDNF levels by a factor of 3.3 ± 0.1 at 4 weeks after MI compared with control (Supplemental Figure 1A). Ad-NDNF treatment reduced LVDd and increased FS in WT mice at 4 weeks post-MI (Supplemental Table 2). Thus, NDNF can improve myocardial function following permanent LAD ligation.
The effect of NDNF on cardiac function was also evaluated in a mouse model of myocardial ischemia reperfusion (I/R). Administration of Ad-NDNF to WT mice resulted in a 3.0 ± 0.3-fold increase in plasma NDNF level at 4 weeks after I/R compared with control treatment (Supplemental Figure 1B). Ad-NDNF-treated mice exhibited decreased LVDd and increased FS at 4 weeks after I/R compared with control mice (Supplemental Table 3). Furthermore, administration of Ad-NDNF to WT mice led to decreased LVDd and increased FS at 6 weeks after TAC with a 3.1 ± 0.1-fold increase in circulating NDNF as compared with control vector treatment (Supplemental Figure 1C) (Supplemental Table 4).

NDNF treatment enhances capillary density and reduces myocardial apoptosis and interstitial fibrosis

To evaluate the extent of myocardial infarct size after chronic ischemia, heart tissues were stained with Masson's trichrome. While Ad-NDNF treatment seems to attenuate LV dilatation at 2 weeks post-MI, there were no statistically significant differences in the ratio of total infarct length to total LV circumstance between control and Ad-NDNF-treated WT mice (Figure 2A).

Because an increase in coronary angiogenesis contributes to improvement of pathological cardiac remodeling 9-11, capillary density in peri-infarct areas was assessed by CD31 staining. Ad-NDNF administration increased the numbers of CD31-positive cells at the border zone of infarct hearts in WT mice at 2 weeks after MI, but not in sham-operated mice as compared with control treatment (Figure 2B).

Because apoptosis is a key feature during the progression of heart failure after MI 12, the extent of cardiomyocyte apoptosis was evaluated by double staining of histological sections from the remote zone of infarct hearts with TUNEL and the cardiomyocyte marker sarcomeric actinin. Ad-NDNF treatment reduced the frequencies of TUNEL-positive
cardiomyocytes in the remote area in WT mice at 2 weeks after MI compared with control treatment, whereas little or no TUNEL-positive cells were detected in the hearts of control and Ad-NDNF-treated mice after sham operation (Figure 2C). To further evaluate the fibrotic changes in the heart after MI, Picrosirius red staining was performed on sections from the remote zone of infarcted hearts. Ad-NDNF treatment reduced the area of interstitial fibrosis at the remote zone from infarct hearts of WT mice at 2 weeks after MI compared with control, whereas little fibrotic lesions were observed in the hearts of control and Ad-NDNF-treated mice after sham operation (Figure 2D).

To assess the extent of cardiomyocyte hypertrophy after MI, Wheat Germ Agglutinin staining was performed on sections from the remote zone of infarcted hearts. Ad-NDNF treatment reduced the cardiomyocyte cross-sectional area in the remote areas of post-MI hearts in WT mice compared with control treatment, whereas no statistically significant difference was observed in cardiomyocyte size between control and Ad-NDNF-treated mice after sham operation (Figure 2E). Ad-NDNF significantly suppressed the expression of markers of cardiac hypertrophy, including atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP), in post-MI hearts, but not in sham-operated hearts (Supplemental Figure 2). Furthermore, the expression of fibrotic makers, including transforming growth factor (TGF)-β1, collagen I and collagen III, were significantly reduced in the post-MI hearts of Ad-NDNF-treated mice compared with control mice, whereas Ad-NDNF did not affect the expression of these fibrotic makers in the hearts after sham operation (Supplemental Figure 2).

**NDNF reduces cardiomyocyte apoptosis through FAK/Akt signaling**

To examine the effects of NDNF on apoptosis at the cellular level, neonatal rat ventricular myocytes (NRVMs) and fibroblasts were cultured under conditions of normoxia or hypoxia
in the presence or absence of recombinant NDNF protein, and subjected to TUNEL staining. Hypoxia led to an increased frequency of TUNEL-positive NRVMs, which was suppressed by pretreatment with NDNF protein (Figure 3A). On the other hand, NDNF had no effect on cardiomyocyte apoptosis under normoxic conditions. NDNF treatment also significantly reduced the numbers of TUNEL-positive fibroblasts under conditions of hypoxia (Figure 3B).

Because Akt and AMP-activated protein kinase (AMPK) are important regulators that protect cardiomyocytes from apoptosis \(^{13-16}\), we evaluated phosphorylation levels of Akt and AMPK in NRVMs by Western blot analysis. Treatment of NRVMs with NDNF protein increased phosphorylation of Akt at Ser-473 in a time-dependent manner, whereas NDNF had no effects on phosphorylation of AMPK at Thr-172 (Figure 4A). Consistent with these in vitro data, Ad-NDNF administration augmented the phosphorylation of Akt in the post-MI heart tissues of mice (Figure 4B).

Because focal adhesion kinase (FAK) functions upstream of Akt signaling in several types of cells including cardiac cells \(^{17-20}\), we investigated the possible participation of FAK in NDNF-induced signaling in cardiac myocytes. Treatment of NRVMs with NDNF protein led to enhanced phosphorylation of FAK at Tyr-397 (Figure 4A). Moreover, Ad-NDNF administration promoted the FAK phosphorylation in the post-MI hearts of mice (Figure 4B).

To examine the contribution of Akt signaling to anti-apoptotic actions of NDNF, NRVMs were transduced with adenovirus expressing dominant negative mutant form of Akt (Ad-dn-Akt) or control Ad-β-gal, and incubated in the presence or absence of NDNF protein. Transduction of NRVMs with Ad-dn-Akt abolished NDNF-stimulated phosphorylation of GSK-3β, which is a downstream mediator of Akt (Figure 5A). Transduction with Ad-dn-Akt reversed the suppressive effects of NDNF on hypoxia-induced NRVM apoptosis (Figure 5B). Thus, Akt signaling is involved in anti-apoptotic function of NDNF in cardiomyocytes under conditions of hypoxia.
To investigate whether FAK is involved in NDNF-induced survival signal in cardiomyocytes, NRVMs were treated with the FAK inhibitor FAK-I14 or vehicle. Pretreatment of NRVMs with FAK-I14 abrogated NDNF-stimulated phosphorylation of Akt (Figure 5C). Furthermore, pretreatment with FAK-I14 reversed the inhibitory effects of NDNF on cardiomyocyte apoptosis under condition of hypoxia (Figure 5D). These results indicate that NDNF reduces myocyte apoptosis, at least in part, through FAK/Akt signaling pathways.

NDNF stimulates Akt activation and network formation of cultured endothelial cells via an integrin-dependent mechanism. Thus, to clarify the upstream mechanism of NDNF-induced Akt activation and survival of cardiomyocytes, NRVMs were cultured in the presence of integrin-blocking RGD-based peptides (GRGDSP) or control peptides (GRGESP). Pretreatment with GRGDSP peptides suppressed NDNF-stimulated increase in phosphorylation of Akt and FAK in NRVMs (Figure 6A). GRGDSP pretreatment also blocked the anti-apoptotic effect of NDNF in NRVMs under hypoxic conditions (Figure 6B).

**NDNF enhances endothelial cell network formation through FAK signaling**

Because NDNF promotes endothelial cell network formation via integrin-dependent activation of Akt and eNOS, we investigated the possible involvement of FAK in NDNF-induced angiogenic signaling in endothelial cells. Treatment of HUVECs with NDNF protein led to increased phosphorylation of Akt and eNOS (Figure 7A) in agreement with our previous findings. Likewise, Ad-NDNF administration augmented the phosphorylation of eNOS in the post-MI hearts (Figure 7B). Moreover, NDNF treatment increased the concentration of nitric oxide metabolites, nitrite/nitrate in cultured media of HUVECs (Figure 7C). NDNF treatment also stimulated FAK phosphorylation in HUVECs (Figure 7A).

Pretreatment of HUVECs with FAK-I14 blocked NDNF-stimulated phosphorylation of Akt
and eNOS (Figure 7D). Furthermore, NDNF stimulated network formation of HUVECs cultured on a Matrigel matrix consistent with our previous report 5, and pretreatment with FAK-I14 abrogated NDNF-induced network formation of HUVECs (Figure 7E). These data indicate that NDNF promotes endothelial cell function, at least in part, via FAK/Akt/eNOS signaling pathways.

**Discussion**

In the present study, we found, for the first time, that NDNF improves adverse remodeling of myocardium after chronic ischemia in vivo. Intramuscular administration of NDNF to mice led to elevation of circulating NDNF level and improvement of LV contractile dysfunction following MI, accompanied by reduction of myocyte apoptosis and hypertrophy, and interstitial fibrosis, and enhancement of capillary formation. Treatment of cultured cardiomyocytes with NDNF protein attenuated hypoxia-induced apoptotic activity. Furthermore, treatment with NDNF protein promoted angiogenic response of endothelial cells consistent with our previous report 5. Therapeutic approaches to attenuate myocardial apoptosis and promote revascularization under pathological conditions are shown to diminish the development of heart failure 21-23. Thus, NDNF can ameliorate post-MI remodeling and function through its ability to modulate apoptosis and angiogenesis in the heart by directly affecting the phenotypes of cardiomyocytes and endothelial cells. Moreover, administration of NDNF to mice led to improved LV function in response to I/R or pressure overload. These results indicate that NDNF represents a potential therapeutic target for treatment of various heart diseases.

Akt is reported to reduce cardiomyocyte apoptosis and cardiac ischemic injury 14. FAK acts as an upstream activator of Akt and functions to promote cardiomyocyte survival.
and protect the heart from ischemic injury. Our data showed that administration of NDNF to mice led to suppression of myocyte apoptosis in the post-MI heart, which was accompanied by increased phosphorylation of Akt and FAK. In addition, treatment of cardiomyocytes with NDNF protein resulted in reduction of hypoxia-induced apoptosis, which was reversed by blockade of Akt activation. Furthermore, pharmacological inhibition of FAK cancelled NDNF-induced increases in myocyte survival and Akt phosphorylation. Thus, it is likely that NDNF attenuates myocyte apoptosis post-MI through activation of FAK/Akt signaling pathway in cardiac myocytes.

Both FAK and Akt are important modulators of angiogenic response in vivo and in vitro. Recently, we reported that NDNF enhances the phosphorylation of Akt and its downstream molecule eNOS, and capillary formation in ischemic skeletal muscle of mice. We also demonstrated that NDNF promotes network formation of endothelial cells through its ability to stimulate the phosphorylation of Akt and eNOS. Here, we have extended these findings by showing that FAK is essential for activation of Akt and eNOS, and endothelial cell function in response to NDNF. Our present study also showed that NDNF enhanced capillary formation in the peri-infarct area after MI with an accompanying increase in eNOS phosphorylation. Therefore, NDNF-mediated activation of endothelial FAK/Akt/eNOS signaling pathways may contribute to enhancement of neovascularization in the post-MI heart, thereby leading to improvement of cardiac dysfunction. Recent our reports also demonstrated that NDNF promotes pro-angiogenic signaling and response of endothelial cells through integrin-dependent mechanism. Consistent with these findings, we found that an RGD-containing peptide cancelled NDNF-induced increases in cardiomyocyte survival signaling and response. Thus, NDNF can modulate the phenotypes of cardiomyocytes and endothelial cells, at least in part, through the integrin/FAK/Akt regulatory axis. Of interest, NDNF has no RGD motif in its amino acid sequence, and its effect on endothelial cells is
blocked by the RGD peptides. Similarly, some integrin ligands, including platelet endothelial cell adhesion molecule, matrix metalloproteinase-2 and cysteine-rich angiogenic inducer 61, have no RGD motif, and their associations with integrin are inhibited by the RGD peptides.

Accumulating evidence indicates that skeletal muscle produces various secretory factors, also known as myokines, which can directly act on neighboring and remote organs. We have shown that ischemic insult in hindlimb leads to an increase in muscle and circulating levels of NDNF. It has also been shown that NDNF is mainly expressed in endothelial cells of skeletal muscles and that NDNF secretion from cultured endothelial cells is enhanced in response to hypoxia. In addition, our data showed that NDNF expression was much higher in skeletal muscle than in heart in WT mice (Supplemental Figure 3). Collectively, these results suggest that NDNF can be designated as a myokine that modulates the development of ischemic heart disease in an endocrine manner.

In conclusion, we demonstrated that intramuscular administration of NDNF ameliorates post-MI remodeling and function in mice by its abilities to reduce myocyte apoptosis and promote neovascularization. Recently, we found that NDNF accelerates angiogenic repair of ischemic limbs in a model of peripheral artery disease. These results suggest that NDNF can exert favorable effects on various ischemic cardiovascular disorders. Thus, therapeutic approaches to increase NDNF production or enhance NDNF signaling pathways could be useful for prevention and treatment of cardiovascular diseases.

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Disclosures

None.

References


Figure Legends

Figure 1. Intramuscular injection of NDNF improves cardiac function after MI. A, Plasma level of NDNF in WT mice at 2 weeks after sham or MI operation. Intramuscular injection of Ad-NDNF (NDNF) or Ad-β-gal (control) was performed 3 days before sham or MI surgery. NDNF protein levels in plasma (2 μl) were evaluated by Western blot analysis. N=3 in each group. B, Heart weight (HW)/body weight (BW) ratio in control or NDNF-treated WT mice at 2 weeks after sham or MI surgery. Ad-NDNF or control Ad-β-gal was intramuscularly injected into WT mice 3 days prior to sham or MI operation. N=6 in sham groups. N=9 in MI groups. C, Echocardiographic analyses of control or NDNF-treated WT mice at 2 weeks after sham or MI operation. Left ventricular diastolic diameter (LVDd) and fractional shortening (FS) were analyzed. Ad-NDNF or control Ad-β-gal was injected into WT mice 3 days prior to sham or MI surgery. N=6 in sham groups. N=9 in MI groups.

Figure 2. NDNF increases capillary density and reduces cardiomyocyte apoptosis and interstitial fibrosis after MI. A, Myocardial infarct size of Ad-NDNF-treated (NDNF) or Ad-β-gal (control)-treated WT mice at 2 weeks after MI. Upper panels show representative images of heart sections stained with Masson’s trichrome. Lower panel shows quantitative analysis of relative infarct size. N=8 in each group. Scale bars = 1 mm. B, Capillary density in the hearts from control or NDNF-treated WT mice at 2 weeks after sham or MI operation. Upper panels show representative immunohistological images stained with CD31 (red). Lower panel shows quantitative analysis of CD31-positive capillaries. N=5 in sham groups. N=8 in MI groups. Scale bars = 50 μm. C, Cardiomyocyte apoptosis in the hearts of control and NDNF-treated WT mice at 2 weeks after sham or MI operation. Upper panels show representative photographs of heart sections stained with TUNEL (green), sarcomeric actinin...
(red) and DAPI (blue). Lower panel shows quantitative analysis of TUNEL-positive cardiomyocytes. N=5 in sham groups. N=8 in MI groups. Scale bars = 50 μm. **D**, Interstitial fibrosis in the hearts of control and NDNF-treated WT mice at 2 weeks following sham or MI operation. Heart sections were stained with Picosirius red, and myocardial interstitial fibrosis was quantified. N=5 in each group. Scale bars = 50 μm. **E**, Cardiomyocyte cross-sectional area in the hearts of control and NDNF-treated WT mice at 2 weeks after sham or MI operation. Upper panels show representative photographs of heart sections stained with Wheat germ agglutinin (green). Lower panel shows the quantitative analysis of cross-sectional area. N=6 in each group. Scale bars = 50 μm.

**Figure 3. Treatment of cultured cardiomyocytes and fibroblasts with NDNF protein attenuates hypoxia-induced apoptosis.** **A**, Neonatal rat ventricular myocytes (NRVMs) were treated with NDNF protein (200 ng/ml) or vehicle under conditions of normoxia or hypoxia for 24 hours. NRVMs were stained with TUNEL (green), sarcomeric actinin (red) and DAPI (blue), and quantitative analysis of TUNEL-positive myocytes was performed. N=4 in each group. **B**, Neonatal rat cardiac fibroblasts were treated with NDNF protein (200 ng/ml) or vehicle under conditions of normoxia or hypoxia for 24 hours. Quantitative analysis of TUNEL-positive fibroblasts was performed. N=4 in each group.

**Figure 4. NDNF stimulates phosphorylation of Akt and FAK in cardiomyocyte and post-MI heart.** **A**, Time course changes in phospho-Akt (P-Akt), Akt, phospho-focal adhesion kinase (FAK) (P-FAK), FAK, phospho-AMP activated protein kinase (AMPK) (P-AMPK), AMPK and α-tubulin (Tubulin) after NDNF treatment as assessed by Western blot analysis. Neonatal rat ventricular myocytes (NRVMs) were treated with NDNF protein (200 ng/ml) or vehicle for indicated lengths of time. Right panels show the quantitative
analyses of P-Akt and P-FAK levels relative to tubulin as evaluated by Image J program. N=3 in each group. B, Western blot analysis of P-Akt, Akt, P-FAK, FAK and Tubulin in heart tissues from Ad-NDNF-treated (NDNF) or Ad-β-gal (control)-treated WT mice at 2 weeks post-surgery. Right panels show quantitative analyses of P-Akt and P-FAK levels relative to tubulin as evaluated by Image J program. N=3 in each group.

Figure 5. NDNF attenuates cardiomyocyte apoptosis through FAK/Akt signaling pathways. A, Levels of phosphorylated glycogen synthase kinase (GSK)-3β (P-GSK-3β), GSK-3α/β, human influenza hemagglutinin (HA) and α-tubulin (Tubulin) as determined by Western blot analysis. Neonatal rat ventricular myocytes (NRVMs) were treated with an adenoviral vector expressing dominant negative mutant form of Akt tagged with HA (Ad-dn-Akt, 50 MOI) or control Ad-β-gal (50 MOI) for 24 hours followed by treatment with NDNF protein (200 ng/ml) or vehicle for 15 min. Lower panels show quantitative analysis of P-GSK-3β levels relative to tubulin as evaluated by Image J program. N=3 in each group. B, Involvement of Akt in NDNF-stimulated survival of NRVMs. After transduction with Ad-dn-Akt or Ad-β-gal for 24 hours, NRVMs were cultured in the presence of NDNF protein (200 ng/ml) or vehicle under hypoxic conditions for 24 hours. NRVMs were stained with TUNEL (green) and DAPI (blue), and quantitative analysis of TUNEL-positive myocytes was performed. N=4 in each group. C and D, Effect of FAK inhibitor on NDNF-stimulated increases in Akt phosphorylation and survival of NRVMs. NRVMs were pretreated with FAK inhibitor (FAK-I14)(2 μM) or vehicle for 60 min followed by treatment with NDNF protein (200 ng/ml) or vehicle for 15 min. C, Levels of P-Akt, Akt and α-tubulin (Tubulin) were assessed by Western blot analysis. Lower panels show quantitative analysis of P-Akt levels relative to tubulin as evaluated by Image J program. N=3 in each group. D, After treatment with FAK-I14 (2 μM) or vehicle for 60 min, NRVMs were cultured in the presence of NDNF.
protein (200 ng/ml) or vehicle under hypoxic conditions for 24 hours. NRVMs were stained with TUNEL and DAPI, and quantitative analysis of TUNEL-positive myocytes was performed. N=4 in each group.

**Figure 6. Integrin is involved in anti-apoptotic action of NDNF in cardiomyocytes.** A and B, Effect of integrin-blocking peptides on NDNF-stimulated increases in phosphorylation of Akt and focal adhesion kinase (FAK) and survival of neonatal rat ventricular myocytes (NRVMs). NRVMs were pretreated with integrin-blocking RGD-based peptides (GRGDSP) or control peptides (GRGESP)(100 μM each) for 60 min followed by treatment with NDNF protein (200 ng/ml) or vehicle for 15 min (A) or 24 hours (B). A, Protein levels of phospho-Akt (P-Akt), phospho-FAK (P-FAK), Akt, FAK and α-tubulin (Tubulin) were assessed by Western blot analysis. Right panels show quantitative analyses of P-Akt and P-FAK relative to tubulin as evaluated by Image J program. N=3 in each group. B, Apoptosis of NRVMs was evaluated by TUNEL staining. N=4 in each group.

**Figure 7. NDNF promotes network formation of endothelial cells through FAK signaling.** A, Western blot analysis of phospho-Akt (P-Akt), Akt, phospho-FAK (P-FAK), FAK and α-tubulin (Tubulin) in human umbilical vein endothelial cells (HUVECs) at 15 min after treatment with NDNF (200 ng/ml) or vehicle. Right panels show quantitative analyses of phosphorylation levels of eNOS (P-eNOS), Akt and FAK relative to tubulin as evaluated by Image J program. N=3 in each group. B, Western blot analysis of P-eNOS, eNOS and Tubulin in post-MI heart tissues from Ad-NDNF-treated (NDNF) or Ad-β-gal (control)-treated WT mice at 2 weeks post-surgery. Lower panel shows quantitative analysis of P-eNOS levels relative to tubulin as evaluated by Image J program. N=3 in each group. C, Nitrate/nitrite concentration in cultured media at 30 min after treatment with NDNF (200ng/ml) or vehicle.
N=6 in each group. **D,** Effect of FAK inhibitor on NDNF-stimulated phosphorylation signals in HUVECs. HUVECs were pretreated with FAK inhibitor (FAK-I14)(2 μM) or vehicle for 60 min and stimulated with NDNF (200 ng/ml) or vehicle for 15 min. Protein levels of P-Akt, Akt and Tubulin were assessed by Western blot analysis. Right panels show quantitative analyses of phosphorylation levels of eNOS and Akt relative to tubulin as evaluated by Image J program. N=3 in each group. **E,** Effect of FAK inhibitor on NDNF-stimulated network formation of HUVECs. HUVECs were pretreated with FAK-I14 (2 μM) or vehicle for 60 min and cultured on Matrigel in the presence or absence of NDNF (200 ng/ml) for 16 hours. Upper panels show representative photographs of network formation. Lower graph shows quantitative analysis of network area. N=4 in each group.
Figure 3

A

B

[Graphs and images showing TUNEL positive cells under different conditions with statistical significance indicated by P-values.]
Figure 4

A

P-Akt
Akt
P-FAK
FAK
P-AMPK
AMPK
Tubulin

0 15 30 60 120 (min)

(kD)

B

P-Akt
Akt
P-FAK
FAK
Tubulin

Control NDNF

(kD)

P-Act levels

P<0.01

P<0.01

0 15 30 60 120 (min)

P<0.05

P<0.05

0 15 30 60 120 (min)

P<0.05

P<0.05

Control NDNF
Figure 6

A

B
Neuron-Derived Neurotrophic Factor Ameliorates Adverse Cardiac Remodeling After Experimental Myocardial Infarction

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Expanded Methods

Materials
Mouse CD31 antibody was purchased from BD Pharmingen (San Jose, CA). Mouse sarcomeric actinin antibody was purchased from Sigma. Antibodies for phosphorylated Akt (Ser-473), phosphorylated AMP-activated protein kinase (AMPK)(Thr-172), phosphorylated focal adhesion kinase (FAK)(Tyr-397), phosphorylated glycogen synthase kinase (GSK)-3β (Ser-9), phosphorylated eNOS (Ser-1177), AMPK, Akt, FAK, eNOS, human influenza hemagglutinin (HA) and α-tubulin (Tubulin) were purchased from Cell Signaling Technology (Beverly, MA). Anti-NDNF antibody was purchased from ABGENT (San Diego, CA). GSK-3α/β antibody and FAK-114 were purchased from Santa Cruz (Texas, USA). The vendor names and catalog numbers for antibodies are listed in Supplemental table 5. GRGDSP and GRGESP peptides were purchased from AnaSpec (Fremont, CA). Adenoviral vectors expressing NDNF (Ad-NDNF) or HA-tagged dominant negative mutant Akt (Ad-dn-Akt) were constructed under the control of the CMV promoter as previously described 1-3. The adenoviral vectors expressing β-galactosidase (Ad-β-gal) were used as controls 4.

Histological analyses
Mice were sacrificed at 2 weeks after permanent LAD ligation. Heart samples were embedded in OCT compound (Sakura, Tokyo, JAPAN) and snap-frozen in
liquid nitrogen. Tissue slices (5 μm in thickness) were histologically analyzed. To detect the infarct area, heart sections were stained with Masson's trichrome. The extent of infarct size was calculated as total infarct circumference divided by total left ventricular (LV) circumference. Capillaries were assessed by immunohistological staining of histological sections from border zone of infarct heart tissue with CD31. Cardiomyocyte cross sectional area was evaluated by Wheat Germ Agglutinin staining. Apoptosis in the remote zone from infarct hearts was assessed by a terminal deoxynucleotidyltransferase-mediated dUTP-nick end labeling (TUNEL) staining using the In Situ Cell Death detection kit (Roche Diagnostics). DAPI was used for counter staining. Cardiomyocytes were determined by staining with sarcomeric actinin. The mean number of TUNEL-positive cells from five random fields (magnification of ×40) was calculated. Myocardial interstitial fibrosis in the remote zone was assessed by Picrosirius red staining and quantified under polarized light by using an image analysis system.

**Echocardiographic analysis**

Transthoracic echocardiography was performed to evaluate cardiac function of mice at 2 and 4 weeks after MI, at 4 weeks after I/R and at 6 weeks after TAC. Left ventricular diastolic diameter (LVDd) and LV systolic diameter (LVSd) were measured by M-mode images using an Acuson Sequioa C-256 machine with a 15-MHz probe. LV fractional shortening (FS) was calculated as (LVDd-LVSd)/LVDd X 100 (%).
Preparation of recombinant NDNF protein

COS-7 cells were transfected with the pShuttle vector expressing full-length mouse NDNF tagged with FLAG at the C terminus. The culture supernatants were collected and incubated with anti-FLAG M2 affinity gel (Sigma) for 16 hours. NDNF protein was eluted by incubation with 3×FLAG peptide (Sigma) and dialyzed with PBS.

Cell culture

Primary cultures of neonatal rat ventricular myocytes (NRVMs) were incubated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) as previously described. After 12 hours of serum starvation, cardiac myocytes were treated with NDNF protein at 200 ng/ml or vehicle for the indicated lengths of time. In some experiments, cells were exposed to 24 hours of hypoxia, and hypoxic conditions were generated using an AnaeroPack (Mitsubishi GAS Chemical). In some experiments, NRVMs were treated with Ad-dn-Akt or Ad-β-gal at 50 MOI for 24 hours. In some experiments, NRVMs were pretreated with FAK-I14 (2 μM) or vehicle (DMSO) for 60 min before NDNF treatment. In some experiments, NRVMs were pretreated with GRGDSP or GRGESP peptides at 100 μM before NDNF treatment.

Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial cell growth medium 2 (Lonza). HUVECs were cultured in the presence or absence of recombinant NDNF protein (200 ng/ml) for the indicated lengths of time.
Endothelial cell network formation assay

The formation of vascular-like structures of HUVECs cultured on growth factor-reduced Matrigel (BD Biosciences) was performed as previously described \(^3,4,12\). Network formation was quantified by measuring the area of the “tube-like” networks in each well. Each experiment was repeated three times.

Measurement of nitrate/nitrite concentrations

Nitrate/nitrite concentrations, which are stable metabolites of nitric oxide, were measured with a Nitrate/Nitrite Colorimetric Assay kit (Cayman Chemical), according to manufacturer’s protocol \(^13\).

Measurement of mRNA levels

Gene expression levels were determined by quantitative real-time PCR method. Total RNA was extracted with RNeasy-Mini Kit (Qiagen) according to the manufacture’s instruction \(^7\). Extracted RNA was reverse-transcribed using the Revatra Ace (Toyobo). PCR procedure was performed with a Bio-Rad real-time PCR detection system using THUNDERBIRD SYBR qPCR Mix as a double-standard DNA-specific dye. The primers are listed in Supplemental table 6.

Western blot analysis

Tissue and cell samples were prepared in lysis buffer (Cell Signaling Technology) containing 1mM PMSF (Sigma). Immunoblotting was performed
with antibodies at a 1:1000 dilution, followed by incubation with a secondary antibody conjugated with horseradish peroxidase at a 1:5000 dilution. An ECL system Western blotting detection kit (GE healthcare) was used. The protein expression level was determined by measurement of the band intensities by using Image J software and was expressed relative to tubulin signal.

References


muscle through a cyclooxygenase 2-dependent mechanism. *Mol Cell Biol.* 2009;29:3487-3499

Supplemental table 1

Echocardiographic data at 2 weeks after myocardial infarction (MI)

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>MI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>NDNF</td>
</tr>
<tr>
<td>IVS (mm)</td>
<td>0.87±0.49</td>
<td>0.77±0.56</td>
</tr>
<tr>
<td>PW (mm)</td>
<td>1.00±0.71</td>
<td>0.93±0.49</td>
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<tr>
<td>LVDd (mm)</td>
<td>2.32±0.06</td>
<td>2.23±0.06</td>
</tr>
<tr>
<td>FS (%)</td>
<td>73.0±0.5</td>
<td>73.6±1.0</td>
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</table>

IVS; interventricular septum thickness, PW; posterior wall thickness, LVDd; left ventricular end-diastolic dimension, FS; fractional shortening
**P<0.01 for Sham Control group, ##P<0.01 for MI Control group. N=6 in sham groups. N=9 in MI groups.
## Supplemental table 2

### Echocardiographic data at 4 weeks after myocardial infarction (MI)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NDNF</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS (mm)</td>
<td>0.46 ± 0.30</td>
<td>0.51 ± 0.26</td>
<td>0.17</td>
</tr>
<tr>
<td>PW (mm)</td>
<td>0.71 ± 0.46</td>
<td>0.91 ± 0.34</td>
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<tr>
<td>LVDd (mm)</td>
<td>6.29 ± 0.09</td>
<td>5.00 ± 0.17</td>
<td>&lt;0.01</td>
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<tr>
<td>FS (%)</td>
<td>10.7 ± 0.5</td>
<td>18.2 ± 1.4</td>
<td>&lt;0.01</td>
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</tbody>
</table>

IVS; interventricular septum thickness, PW; posterior wall thickness, LVDd; left ventricular end-diastolic dimension, FS; fractional shortening

N=7 in each group.
Supplemental table 3

Echocardiographic data at 4 weeks after cardiac ischemia reperfusion (I/R)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NDNF</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS (mm)</td>
<td>0.51 ± 0.05</td>
<td>0.66 ± 0.06</td>
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<tr>
<td>PW (mm)</td>
<td>0.84 ± 0.04</td>
<td>0.99 ± 0.05</td>
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<tr>
<td>LVDd (mm)</td>
<td>4.14 ± 0.15</td>
<td>3.36 ± 0.16</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FS (%)</td>
<td>24.7 ± 1.2</td>
<td>31.0 ± 2.3</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

IVS; interventricular septum thickness, PW; posterior wall thickness, LVDd; left ventricular end-diastolic dimension, FS; fractional shortening
N=7 in each group.
Table 4

Echocardiographic data at 6 weeks after transverse aortic constriction (TAC)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NDNF</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS (mm)</td>
<td>1.22±0.04</td>
<td>1.16±0.02</td>
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<tr>
<td>PW (mm)</td>
<td>1.18±0.06</td>
<td>1.18±0.02</td>
<td>0.97</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>3.26±0.1</td>
<td>2.54±0.08</td>
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<tr>
<td>FS (%)</td>
<td>49.4±2.7</td>
<td>63.7±0.7</td>
<td>&lt;0.01</td>
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</tbody>
</table>

IVS; interventricular septum thickness, PW; posterior wall thickness, LVDd; left ventricular end-diastolic dimension, FS; fractional shortening
N=5 in each group
Supplemental table 5

Vendor and catalog number of antibodies

<table>
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<tr>
<th>Antibody</th>
<th>Vendor</th>
<th>Cat. No.</th>
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<tr>
<td>NDNF</td>
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<td>AP5812a</td>
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<td>GSK-3α/β</td>
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<td>HA-Tag</td>
<td>Cell Signaling</td>
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<td>Phospho-AMPKα (Thr172)</td>
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<tr>
<td>AMPKα</td>
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<tr>
<td>Phospho-Akt (Ser473)</td>
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<tr>
<td>Akt</td>
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<tr>
<td>Phospho-FAK (Tyr397)</td>
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<td>FAK</td>
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<tr>
<td>Phospho-GSK-3β</td>
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<td>α-Tubulin</td>
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<td>2144</td>
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<td>anti-mouse IgG, HRP-linked Antibody</td>
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<td>7076</td>
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<tr>
<td>anti-rabbit IgG, HRP-linked Antibody</td>
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Supplemental table 6

Primers used for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>5'-TCACCACCATGGAGAAGGC-3'</td>
<td>5'-GCTAAGCAGTTGGGTGGCAG-3'</td>
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<tr>
<td>β-actin</td>
<td>5'-TCCTTCTTTGGGTATGGAATC-3'</td>
<td>5'-TAGAGGTCTTTACGGATGTC-3'</td>
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<tr>
<td>Collagen I</td>
<td>5'-GTCCCAACCCCAAGAC-3'</td>
<td>5'-CTGTCTCTGAGTTGAGTC-3'</td>
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<tr>
<td>Collagen III</td>
<td>5'-TGGTTTTCTTCTCACCTTCTT-3'</td>
<td>5'-TGCACTACAAACTTCAGCT-3'</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>5'-CACCGGAGAGCCCTTGTA-3'</td>
<td>5'-TTCCAAACCAGGCTCTTCT-3'</td>
</tr>
<tr>
<td>ANF</td>
<td>5'-AGGCCATATTGGAGAAATC-3'</td>
<td>5'-CAACGTCACAAAAGAAC-3'</td>
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<tr>
<td>BNP</td>
<td>5'-CAAGGCCTCACAAAAGAACA-3'</td>
<td>5'-ATCCGATCCCGCTCATCCT-3'</td>
</tr>
</tbody>
</table>

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, TGF-β1: Transforming growth factor-β1, ANF: Atrial natriuretic factor, BNP: Brain natriuretic peptide
Supplemental Figure 1

A

B

C

NDNF

NDNF

NDNF

(kD)

(kD)

(kD)

75

50

75

50

75

50

P<0.01

P<0.01

P<0.01

NDNF protein levels

NDNF protein levels

NDNF protein levels

Control

NDNF

Control

NDNF

Control

NDNF

MI

I/R

TAC

0

1

2

3

0

1

2

3

0

1

2

3
Supplemental Figure 2

- ANF mRNA levels
  - Control: P<0.01
  - NDNF: P<0.01

- BNP mRNA levels
  - Control: P<0.01
  - NDNF: P<0.01

- TGF-β1 mRNA levels
  - Control: P<0.01
  - NDNF: P<0.01

- Collagen I mRNA levels
  - Control: P<0.05
  - NDNF: P<0.01

- Collagen III mRNA levels
  - Control: P<0.05
  - NDNF: P<0.01

Legend:
- Control
- NDNF
Supplemental Figure 3

The figure shows a bar graph comparing NDNF mRNA levels in Skeletal Muscle and Heart. The bar for Skeletal Muscle is significantly higher than that for Heart, indicated by the notation P<0.05.
Supplemental Figure Legends

Supplemental Figure 1. Circulating NDNF levels after myocardial infarction (MI), cardiac ischemia reperfusion (I/R) or transverse aortic constriction (TAC). Plasma level of NDNF at 4 weeks after MI (A) or I/R (B) and at 6 weeks after TAC (C) were evaluated by Western blot analysis. Adenoviral vectors expressing NDNF (Ad-NDNF: NDNF) or Ad-β-gal (Control) were intramuscularly injected into WT mice 3 days before each operation.

Supplemental Figure 2. NDNF reduces the expression of cardiac hypertrophy and fibrosis markers in post-MI hearts. Quantitative real time PCR was performed to analyze mRNA levels of ANF, BNP, TGF-β1, Collagen I and Collagen III in the heart tissues from Ad-β-gal-treated (closed bars) or Ad-NDNF-treated (open bars) WT mice at 2 weeks after sham or MI. All results were normalized to GAPDH. N=6 in each group.

Supplemental Figure 3. Expression of NDNF in skeletal muscle and heart of WT mice. NDNF mRNA levels of adductor muscle and heart tissues were assessed by real time PCR methods. All results were normalized to β-actin. N=5 in each group.