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Chronic Neuregulin-1 β Treatment Mitigates the Progression of Post-Myocardial Infarction Heart Failure in the Setting of Type 1 Diabetes Mellitus by Suppressing Myocardial Apoptosis, Fibrosis and Key Oxidant-Producing Enzymes

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Abstract

Type 1 diabetes mellitus (DM) patients surviving myocardial infarction (MI) have substantially higher cardiovascular morbidity and mortality compared to their non-diabetic counterparts owing to the more frequent development of subsequent heart failure (HF). Neuregulin (NRG)-1 β is released from cardiac microvascular endothelial cells and acts as a paracrine factor via the ErbB family of tyrosine kinase receptors expressed in cardiac myocytes to regulate cardiac development and stress responses. Since myocardial NRG-1/ErbB signaling has been documented to be impaired during HF associated with type 1 DM, we examined whether enhancement of NRG-1 β signaling via exogenous administration of recombinant NRG-1 β could exert beneficial effects against post-MI HF in the type 1 diabetic heart. Type 1 DM was induced in male Sprague Dawley rats by a single injection of streptozotocin (STZ) (65 mg/kg). Two weeks after induction of type 1 DM, rats underwent left coronary artery ligation to induce MI. STZ-diabetic rats were treated with saline or NRG-1 β (100 μ g/kg) twice a week for 7 weeks, starting two weeks prior to experimental MI. Residual left ventricular (LV) function was significantly greater in the NRG-1 β -treated STZ-diabetic MI group compared to the vehicle-treated STZ-diabetic MI group 5 weeks after MI as assessed by high-resolution echocardiography. NRG-1 β treatment of STZ-diabetic MI rats was associated with reduced myocardial fibrosis and apoptosis as well as decreased gene expression of key oxidant-producing enzymes. These results suggest that recombinant NRG-1 β may be a promising therapeutic for HF post-MI in the setting of type 1 DM.

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Keywords

Type 1 diabetes; Post-myocardial infarction heart failure; Neuregulin-1 β Fibrosis; Apoptosis; Oxidant-producing enzymes

Introduction

Ischemic heart disease, including myocardial infarction (MI), is the leading cause of death in patients with type 1 diabetes mellitus (DM) [22]. The excess mortality from ischemic heart disease in young adults with type 1 DM is between eight and 40 times that of the general population [22, 26], thereby producing a large survival gap between the two groups. Modern treatment strategies (percutaneous coronary intervention [PCI], thrombolytic agents, beta blockers, antiplatelets, angiotensin converting enzyme [ACE] inhibitors) have been unable to reduce the differences in survival after MI between DM and non-DM patients [1, 2, 28]. Type 1 DM patients surviving MI have substantially higher cardiovascular morbidity and mortality compared to those without type 1 DM owing to the more frequent development of subsequent heart failure (HF) [30]. Despite these observations, HF-sparing therapies in type 1 diabetic post-MI patients remain absent. Consequently, a “therapeutic deficit” exists in the treatment and prevention of post-MI HF in patients with type 1 DM.

Neuregulin-1 (NRG-1) is a growth and survival factor that is critical for heart development [15, 23] as well as maintenance of heart function [9]. In the adult heart, at least 3 different NRG-1 α isoforms and 8 NRG-1 β isoforms are expressed, with the β isoform being 10 to 100 times more bioactive. NRG-1's actions are mediated through a set of erythroblastic leukemia viral oncogene homolog (ErbB) receptors (ErbB2, ErbB3, and ErbB4) which stimulate survival of various organs, including the heart. NRG-1/ErbB signaling shares many features of insulin signaling and can regulate glucose uptake in skeletal and cardiac muscle [6-8, 32]. Endogenous NRG-1 has also been shown to play an important role in mediating cardiac repair after injury [18]. In light of the aforementioned protective roles of NRG-1, disruption of NRG-1/ErbB signaling may impair recovery of cardiac contractile function after an ischemic insult and thereby increase susceptibility to myocyte cell death and progression of HF. To that end, we recently reported that type 1 DM prevents compensatory up-regulation of the myocardial NRG-1 β /ErbB system in response to MI which is associated with a worsening of subsequent HF [29].

Enhancement of NRG-1 β signaling via exogenous administration of recombinant human (rh) NRG-1 β improves cardiac systolic function as well as survival in animal models of ischemic, dilated, and viral cardiomyopathy [27]. In rats or swine with MI-induced myocardial dysfunction, treatment with a second form of rhNRG-1 β , glial growth factor 2 (GGF2), improved systolic function and reduced progressive remodeling [13, 19]. Clinical trials with NRG-1 β have produced favorable effects on cardiac function of HF patients [14, 20]. However, whether NRG-1 β treatment can affect post-MI HF in type 1 DM remains to be investigated. Therefore, the primary objective of this study was to determine the effects of NRG-1 β treatment against MI-related HF in the setting of type 1 DM. Potential mechanisms for NRG-1 β 's effects on the viable myocardium were also explored.

Methods

Experimental Animals

Male Sprague Dawley rats (200-225g) were obtained from Harlan Laboratories (Indianapolis, IN). All experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center. All procedures conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health and the standards of the Association for Assessment and Accreditation of Laboratory Animal Care.

Induction of Type 1 DM

Type 1 DM was induced in male Sprague-Dawley rats via a single intraperitoneal injection of streptozotocin (STZ) (65 mg/kg body weight) prepared daily in citrate buffer pH 4.5 as previously described [10, 29]. Development of DM was confirmed 48 hours later by the presence of glycosuria (>2000 mg/dl) along with polyuria as described previously [10]. Two weeks after induction of type 1 DM, STZ-diabetic animals underwent coronary artery ligation to induce MI (Figure 1).

Measurement of Blood Glucose

Blood samples were collected from the tail vein for the determination of blood glucose levels prior to induction of MI and at the end of study using Accutrend Plus test strips and meter (Roche) as described previously [10, 29] to further verify type 1 DM.

Induction of MI

MI was produced in STZ-diabetic rats via ligation of the left anterior descending (LAD) coronary artery as described previously [10, 29]. Briefly, rats were anaesthetized intraperitoneally with Nembutal (40 mg/kg). Rats were then rapidly intubated and mechanically ventilated (tidal volume, 1 ml/100 g body weight; ventilation rate, 65 strokes/min) by a constant volume small animal ventilator (Model 683, Harvard Apparatus). A left thoracotomy was performed at the fourth intercostal space, and the LAD coronary artery was then ligated by irreversible tightening of a 6-0 suture loop. MI was confirmed by regional cyanosis of the myocardial surface distal to the suture, accompanied by S-T segment elevation on the electrocardiogram. Following successful induction of MI, the chest cavity was compressed to evacuate any air before being tightly sealed. Rats were given buprenorphine (0.05 mg/kg) pre-operatively, and then 8-12 hours post-operatively by subcutaneous injection for 72 h.

NRG-1 β Treatment

NRG-1 β (Recombinant Human NRG-1 β /HRG- β 1 Extracellular Domain; 377-HB/CF) was obtained from R&D Systems and reconstituted in sterile phosphate buffered saline. Rats were randomly administered NRG-1 β (100 ug/kg body weight) (n=10) or saline (n=10) via the right lateral tail vein twice a week for 7 weeks, starting 2 weeks prior to induction of experimental MI (Figure 1). During the post-MI period, two rats from the saline-treated group died while one rat from the NRG-1 β -treated group died. Thus, the number of

surviving animals that completed the study from the saline-treated group was n=8 while the number of surviving animals that completed the study from the NRG-1 β -treated group was n=9.

Assessment of *In Vivo* Global Residual LV Function by Echocardiography

Transthoracic echocardiographic images of hearts from all groups of rats were obtained at 1, 2, 3 and 5 weeks post-MI (Figure 1) using an ultra-high-resolution ultrasound scanner (Vevo 2100; VisualSonics) under isoflurane anesthesia. For M-mode recordings, the parasternal short-axis view was used to image the heart in two dimensions at the level of the papillary muscles. LV fractional shortening (FS) and ejection fraction (EF) were recorded along with LV cavity dimensions (end-diastolic and end-systolic) as described previously [10, 29]. All echocardiographic measurements were performed by sonographers in the Cardiovascular Pathophysiology and Complications Core (CPCC) at Vanderbilt University Medical Center. The sonographers in the CPCC were unaware of the study design as well as the identity of the respective animals with respect to the therapy that they were receiving and hence, were blinded for all echocardiographic measurements that they performed.

Measurement of Blood Pressure

At end of study, blood pressure was measured in saline-treated and NRG-1 β -treated diabetic MI rats by tail cuff using the Visitech BP-2000 system.

Tissue Harvest

Following the terminal echocardiographic assessment, hearts from all groups of rats were rapidly excised and perfused with ice-cold physiological saline and weighed. The atria and ventricles were dissected and the infarct scar and non-infarcted regions of the LV were separated. The remote, non-infarcted LV tissue was frozen in liquid nitrogen and used for all molecular analyses.

Pieces of tissues from the lungs and liver were removed and weighed. For the determination of dry weight, these were placed in an oven at 65°C until a constant weight was reached. Ratios of wet to dry weight were calculated for both lungs and liver.

Histology

A subset of hearts were frozen in O.C.T medium (Tissue-Tek) and 6-10 μ m sections of hearts from all groups were stained with Masson's trichrome for measurement of cardiac fibrosis. Collagen content was measured as a fraction of the entire section of the LV from remote, non-infarcted myocardium using NIH Image J software and was expressed in arbitrary units as a percentage of the total area of tissue section stained. Six fields were analyzed from each tissue section obtained.

Immunofluorescence Analysis of ErbB2 Expression

ErbB2 receptor expression was analyzed in LV sections (6 μ M) prepared with cryotome. Tissue sections were blocked with goat serum for 1 hour prior to incubation in anti-ErbB2 (Cat. SC-284 Santa Cruz) and β -Catenin (Cat. 610153, BD Biosciences). Secondary

antibody cy2- and cy3-conjugated donkey anti-rabbit and anti-mouse IgGs (1:500) were obtained from Jackson Immunoresearch Laboratories.

Western Blot Analysis of ErbB2 and MDM2 Expression

LV tissue was homogenized in 1× RIPA lysis buffer (Millipore), supplemented with protease inhibitor cocktail (Roche). 25 µg of protein was loaded onto a PAGE using precasted gradient gel 4-12% NuPage gels (Invitrogen). After electrophoresis, proteins were transferred to an Immun-Blot PVDF membrane using a Semi-Dry transfer apparatus. Membranes were blocked using PBST-5% BSA and then incubated overnight at 4°C with antibodies directed against murine double minute2 (Cat. SC-7918, Santa Cruz), ErbB2 (Cat. SC-284 Santa Cruz), and α -tubulin (Cat. SC-32293, Santa Cruz). Secondary horseradish peroxidase-conjugated antibody (Cell Signaling) was applied for 1 h at room temperature. Blots were visualized using the SuperSignal West Pico chemiluminiscent substrate (Thermo Scientific) and analyzed with NIH Image J densitometry software as described previously [29].

Measurement of Myocardial Apoptosis

To determine if exogenous NRG-1 β altered cell death, In situ Cell Death detection Kit, TMR red (Roche No. 12156792910) was used for terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL). LV sections were treated according to the manufacturer's instructions and co-stained for cardiomyocyte specific marker Troponin-I (Cat. 4002 Cell Signaling) and 4,6-diamidino-2-phenylindole (DAPI). Slides were applied with mounting media and covered with a glass cover. A Nikon Eclipse 80i fluorescence microscope was used to visualize the TUNEL-positive cardiomyocyte, and NIS Elements software was used to capture the images.

Measurement of Cardiomyocyte Proliferation

Cardiomyocyte proliferation was analyzed in LV sections (6 µM) prepared with cryotome from non-infarcted tissue. Tissue sections were blocked with goat serum for 1 hour prior to incubation in anti-ph3 (Cat. 06-570, Millipore) and α -actinin (Cat. 7811, Sigma). Cardiomyocyte proliferation was measured by counting 5 sections randomly from each animal.

Gene Expression Analysis

RNA Extraction and q PCR—LV tissues (30 mg) remote from the site of infarction were dissected and total RNA was extracted using RNA easy kit (Quiagen). Purified RNA was analyzed for integrity using an Agilent 2100 Analyzer. RNA with integrity of 6 or higher was reverse transcribed using cDNA synthesis kit (Bio-Rad). Quantitative gene expression was analyzed in duplicates using the CFX96 C-1000 (Bio-Rad, Hercules) using SYBR green PCR super mix (Bio-Rad). Primer sequences for q-PCR are listed in Table 1.

Protein Determination and Statistical Analysis

Protein concentration of LV tissue samples were determined using Bicinchoninic acid Protein Assay Kit (Pierce). Data were expressed as the mean $\pm$ S.E.M. and were statistically

analyzed using two-sample Student's t test unless specified otherwise. $P < 0.05$ was considered statistically significant.

Results

Animal Characteristics

No differences in body weight or blood glucose levels were observed between the NRG-1 β -treated STZ-diabetic MI group and vehicle-treated STZ-diabetic MI group at the end of study (Table 2). Similarly, no differences were observed between the NRG-1 β -treated STZ-diabetic MI group and vehicle-treated STZ-diabetic MI group with respect to the wet to dry weight ratios of lung and liver at the end of study (Table 2). With respect to blood pressure, no differences were observed between the NRG-1 β -treated STZ-diabetic MI and vehicle-treated STZ-diabetic MI groups of rats (Table 2).

Intravenous NRG-1 β Therapy Mitigates the Progression of Post-MI HF in STZ-Diabetic Rats

Serial echocardiographic measurements revealed differences in residual LV function between NRG-1 β -treated STZ-diabetic MI and vehicle-treated STZ-diabetic MI groups of rats. Representative echocardiographic images are shown in (Figure 2). Individual animal and summarized echocardiographic functional data are shown in Figure 3 and Figure 4, respectively. Echocardiographic assessment of LV systolic function one week after MI showed similar LV dysfunction and failure in both groups (Figure 4). STZ-diabetic MI rats treated with NRG-1 β showed higher LVFS and LVEF compared to the vehicle-treated STZ-diabetic MI rats starting at 2 weeks post-MI (Figure 4). LVFS and LVEF in the NRG-1 β -treated STZ-diabetic MI group was preserved throughout the remaining post-MI treatment period while in contrast, LVFS and LVEF in the vehicle-treated STZ-diabetic MI group progressively declined as the duration of the post-MI period increased (Figure 4). By 5 weeks post-MI, the difference in LVFS and LVEF between the vehicle-treated STZ-diabetic MI group and NRG-1 β -treated STZ-diabetic MI group reached statistical significance (Figure 4).

Intravenous NRG-1 β Therapy Reduces Adverse LV Remodeling in STZ-Diabetic Rats with MI

As shown in Figure 5, LV enlargement and dilatation occurred in both the NRG-1 β -treated STZ-diabetic MI and vehicle-treated STZ-diabetic MI groups of rats as indicated by progressive increases in left ventricular internal dimension, diastole (LVIDd) and left ventricular internal dimension, systole (LVIDs) during the post-MI periods examined. However, the extent of such increases in LVIDd and LVIDs were less pronounced in the NRG-1 β -treated STZ-diabetic MI group of rats compared to the vehicle-treated STZ-diabetic MI group of rats (Figure 5).

Exogenous NRG-1 β Reduces Myocardial Fibrosis in STZ-Diabetic MI Rats

Cardiac fibrosis was examined in OCT medium fixed sections of remote, noninfarcted LV myocardium from NRG-1 β -treated STZ-diabetic MI and vehicle-treated STZ-diabetic MI groups of rats. Compared to the hearts of saline-treated STZ-diabetic MI rats, the hearts

from the STZ-diabetic MI rats treated with NRG-1 β demonstrated significantly less fibrosis in LV myocardium remote from the site of infarction ($P<0.05$) (Figure 6).

Myocardial ErbB2 Receptor Expression is Up-regulated by NRG-1 β Treatment in STZ-Diabetic MI Rats

We examined the expression and localization of the NRG-1 β receptor, ErbB2, which is important for NRG-1 signaling in adult heart. ErbB2 receptors were localized primarily in the cellular membranes of myocytes and intercalated disks in both the saline-treated and NRG-1 β -treated STZ-diabetic MI rats (Figure 7A). ErbB2 expression was observed to be higher in the STZ-diabetic MI rats that received NRG-1 β treatment at end of study (Figure 7B and Figure 7C).

Exogenous NRG-1 β Does Not Alter MDM2 Expression in STZ-Diabetic MI Rats

Previously, we have demonstrated degradation and decreased protein expression of MDM2 in un-treated STZ-diabetic MI rats [29]. NRG-1 β treatment of STZ-diabetic MI rats did not alter myocardial MDM2 protein expression as no difference in MDM2 protein expression was observed between NRG-1 β -treated STZ-diabetic MI group of rats and vehicle-treated STZ-diabetic MI group of rats (Figure 8).

Effect of NRG-1 β on Myocardial Apoptosis and Phospho-H3 Expression

Myocardial apoptosis was analyzed using TUNEL stain in LV sections from saline and NRG-1 β -treated STZ-diabetic MI hearts. Compared to saline-treated hearts, we observed a reduced number of TUNEL positive cells in NRG-1 β -treated hearts (Figure 9A and 9B). Given the role of exogenous NRG-1 β in myocyte proliferation and repair after injury, we compared myocyte proliferation between saline-treated and NRG-1 β -treated STZ-diabetic hearts. Compared to saline-treated STZ-diabetic MI hearts, NRG-1 β -treated STZ-diabetic MI hearts had a higher number of phospho-H3 positive cells, a marker of mitosis. However, the increase did not reach statistical significance (Figure 9C and Figure 9D).

NRG-1 β Alters the Expression of Fibrotic and Apoptotic Genes in STZ-Diabetic MI Rats

To determine whether exogenous NRG-1 β therapy altered the expression of fibrotic and apoptotic genes in this model, we performed gene analysis using q-PCR. Representative electrophoresis run summary of RNA from LV samples used for determining gene expression using RT-qPCR is shown in Figure 10. As shown in Table 3, a reduction in the expression of fibrotic genes involving collagen type I and III was observed in the NRG-1 β -treated STZ-diabetic MI group of rats compared with the vehicle-treated STZ-diabetic MI group of rats. Compared to the vehicle-treated STZ-diabetic MI group of rats, NRG-1 β -treated STZ-diabetic MI rats exhibited reduced caspase-3 mRNA expression (Table 3).

NRG-1 β Suppresses Gene Expression of Key Oxidant-Producing Enzymes in STZ-Diabetic MI Rats

We have previously demonstrated an increase in the protein expression of the anti-oxidative enzyme, glutathione peroxidase, in non-diabetic post-MI rats treated with NRG-1 β [19]. To further explore the anti-oxidative effects of NRG-1 β in the setting of diabetic MI, we

examined the gene expression of key pro-oxidant enzymes. Gene expression of NADPH oxidase (NOX) 1 and NOX4 and myeloperoxidase (MPO) were all reduced in the non-infarcted LV of NRG-1 β -treated STZ-diabetic MI rats compared to vehicle-treated STZ-diabetic MI rats (Table 3).

Discussion

Although the ability of NRG-1 β to alleviate LV dysfunction and HF has been demonstrated in several small and large animal models [3, 4, 13, 16, 17, 19, 27] and in clinical trials [14, 20], no previous study has examined the therapeutic effects of NRG-1 β against post-MI HF in the setting of type 1 DM-- an issue of critical importance given the prevalence of MI-related HF in this patient population. Furthermore, no information is available on the pathophysiological responses to NRG-1 β in the type 1 diabetic infarcted heart, which hinders elucidation of its mechanism(s) of action in the diabetic milieu.

Through our pre-clinical studies with NRG-1 β in STZ-diabetic MI rats, we found that: i) NRG-1 β mitigates the progression of HF in STZ-diabetic rats with MI; ii) NRG-1 β therapy improves LV remodeling in STZ-diabetic MI rats as evidenced by a reduction in LV dilatation; iii) NRG-1 β treatment prevents loss of myocardial ErbB2 receptor expression in STZ-diabetic MI rats; iv) NRG-1 β therapy suppresses myocardial fibrosis and apoptosis in STZ-diabetic post-MI hearts, v) NRG-1 β therapy reduces the expression of pro-fibrotic and pro-apoptotic genes in STZ-diabetic post-MI hearts, and vi) NRG-1 β treatment reduces the gene expression of key oxidant-producing enzymes in STZ-diabetic MI hearts. Taken together, these results support NRG-1 β as a potential therapeutic to limit the progression of post-MI HF in type 1 DM and, in addition, offers insights into the potential mechanisms by which NRG-1 β attenuates HF induced by diabetic MI. Our pre-clinical data showing the ameliorative effect of NRG-1 β therapy on HF in type 1 diabetic post-MI rats offers a promising therapeutic strategy and warrants further studies examining whether NRG-1 β can have similar effects in type 1 diabetic patients with incident (first-time) MI.

Individuals with type 2 DM are also at greater risk for the development of HF. The pathogenesis of type 1 and type 2 DM are distinct and separate, with type 1 DM characterized by an absolute insulin deficiency arising from autoimmune destruction of insulin-producing pancreatic beta cells while type 2 DM results from an interplay of insulin resistance combined with relative insulin deficiency. To date, there has been no investigations into the cardioprotective effects of NRG-1 β therapy against post-MI HF in the setting of type 2 DM. Hence, it remains unclear as to whether insulin-insensitivity and type 2 DM affect the therapeutic response to NRG-1 β or if the present data may be taken as part representative for all diabetics. Important in this context is the observation from the current study that NRG-1 β had no influence on blood glucose levels. As such, this issue will require further investigation.

To identify the potential mechanisms underlying NRG-1 β 's effects on the type 1 diabetic infarcted heart, we first examined myocardial ErbB receptor protein expression. In a previous study, we showed that MI results in the up-regulation of ErbB2 receptor expression in non-DM rats while in contrast, expression of ErbB2 was markedly diminished in STZ-

diabetic post-MI rats concomitant with a worsening of subsequent HF [29]. In the present study, ErbB2 protein expression was found to be up-regulated in the STZ-diabetic MI rats that received NRG-1 β at the end of study. Therefore, it is likely that the increase in myocardial ErbB2 expression in STZ-diabetic MI rats treated with NRG-1 β increased the sensitivity of the diabetic myocardium to NRG-1 β sufficiently to prevent the disruption of NRG-1 β /ErbB signaling and consequent deterioration of residual cardiac function observed in our earlier study. However, we did not measure myocardial ErbB2 protein expression prior to the end of study and therefore it remains unknown as to the exact time course by which NRG-1 β increased myocardial ErbB2 protein expression in the STZ-diabetic MI rats in the present study. Given that the functional and geometrical parameters did not become evident until 5 weeks post-MI, we hypothesize that during the early post-MI period there may have still been repression of NRG-1 β receptor protein expression which may, at least in part, account for the delayed functional response of STZ-diabetic MI hearts to exogenous NRG-1 β treatment in the current study. Future studies aimed at investigating the temporal expression of ErbB2 in diabetic infarcted hearts treated with NRG-1 β are thus warranted.

Previous studies have shown that exogenous NRG-1 β can prevent anthracycline induced apoptosis via activation of PI3-kinase/AKT [12] via caspase-3 inhibition. Myocardial apoptosis has been implicated as one of the mechanisms underlying reduced cardiac function during HF arising from diabetic cardiomyopathy [24, 25]. In the present study, we observed fewer TUNEL positive cells in STZ-diabetic MI rat hearts that received NRG-1 β treatment, suggesting that reduced cardiac apoptosis may be a potential mechanism underlying NRG-1 β 's therapeutic effects in the diabetic infarcted heart. In a previous study from our laboratory, we found evidence for degradation and decreased protein expression of MDM2, a negative regulator of p53 mediated apoptosis induced by NRG-1, in STZ-diabetic hearts with MI [29]. In the current study, MDM2 expression in STZ-diabetic MI rat hearts was unaltered by NRG-1 β treatment, indicating that the reduction in myocardial apoptosis observed in NRG-1 β -treated STZ-diabetic MI hearts was independent of MDM2 modulation. MDM4, a homolog of MDM2, is also known to negatively regulate p53 mediated apoptosis in numerous cell types [33]. Further experimental work is needed to elucidate whether the observed anti-apoptotic effects of NRG-1 β therapy in diabetic infarcted hearts in the current study involves potentiation of MDM4-mediated inhibition of p53-mediated apoptosis.

NRG-1/ErbB signaling has been reported to be activated following ischemia-reperfusion in which reactive oxygen species (ROS) are also activated. ROS-mediated apoptosis has been documented to be reduced with NRG-1 treatment in a phosphatidylinositol-3-kinase-dependent manner, suggesting an inhibitory role for NRG-1 in ROS-mediated apoptosis [21]. *In vivo* studies in rats have demonstrated that improved cardiac function in non-diabetic post-MI rats treated with NRG-1 β is associated with an increase in the anti-oxidant enzyme, glutathione peroxidase [19]. Given that accentuated oxidative stress brought about by type 1 DM promotes HF after MI [31], we examined the gene expression of key oxidant-producing enzymes involving NOX1, NOX4, and MPO in the LV from saline-treated and NRG-1 β -treated STZ-diabetic MI rats. The LV myocardial gene expression of all of these pro-oxidative enzymes was reduced in the STZ-diabetic MI rats that received NRG-1 β

treatment. These results suggest that anti-oxidative effects may, at least in part, contribute to the beneficial actions of NRG-1 β in diabetic HF following MI.

The present study is one of a series that demonstrates that NRG-1 β can exert anti-remodeling and anti-fibrotic effects after cardiac injury in animals. In a large animal model of post-MI HF, treatment with GGF2, a NRG-1 β isoform that contains the Kringle domain, prevented adverse cardiac remodeling after MI in swine in part via anti-fibrotic effects in the heart [13]. Treatment with recombinant NRG-1 β has been reported to attenuate interstitial fibrosis and improve cardiac function in type 1 diabetic cardiomyopathy rats [24, 25]. Our results, however, are the first to demonstrate that the anti-remodeling and anti-fibrotic effects of NRG-1 β extend to the setting of diabetic MI.

NRG-1 β has been shown to increase glucose uptake in cardiac and skeletal muscle [6, 9]. Recent studies indicate a glucose lowering effect of NRG-1 β possibly via ErbB3 and AKT activation in liver in adult and old rats. In another study, the same group demonstrated an improvement in glucose tolerance in db/db mice possibly via reduction in hepatic gluconeogenesis [5, 11]. In the present study, we did not observe any appreciable effect of NRG-1 β on blood glucose levels. Hence, the attenuation of post-MI HF in STZ-diabetic rats that received NRG-1 β treatment appears to be independent of any blood glucose lowering effects.

Study Limitations

Remodeling processes can differ between males and females. In the present study, we utilized only male animals. Thus, our results only apply to males.

Conclusions

Pharmacological NRG-1 β treatment mitigates the progression of HF following diabetic MI by reducing adverse LV remodeling, fibrosis, apoptosis, and pro-oxidative enzymes. The attenuation of post-MI LV dysfunction in the type 1 diabetic heart elicited by NRG-1 β therapy was found to be independent of blood glucose lowering. Further clinical studies are needed to define the reproducibility and durability of these effects. The exact molecular pathways and molecular mechanisms underpinning NRG-1 β 's effects in the diabetic infarcted heart remain to be elucidated and warrant further exploration.

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Fig. 1.

Experimental design. STZ-diabetic MI rats were treated with NRG-1 β (100 μ g/kg) or vehicle (saline) for 7 weeks, beginning 2 weeks prior to experimental MI. Serial measurements of residual LV function were performed at 1, 2, 3, and 5 weeks post-MI using *in vivo* echocardiography

Fig. 2.
Representative echocardiographic images of STZ-diabetic MI hearts treated with saline or NRG-1 β at 5 weeks post-MI. Images consist of 2-dimensional echocardiography at parasternal short-axis plane (top portion) and M-mode short axis of LV (bottom portion)

Fig. 3.

Effects of NRG-1 β treatment on LV function in STZ-diabetic MI rats. **a** Serial measurements of LVFS in individual NRG-1 β -treated (n=9) and vehicle-treated (n=8) STZ-diabetic MI rats **b** Serial measurements of LVEF in individual NRG-1 β -treated (n=9) and vehicle-treated (n=8) STZ-diabetic MI rats

Fig. 4.

Summarized LVFS (a) and summarized LVEF (b) in STZ-diabetic post-MI rats that received saline (n=8) or NRG-1β (n=9). * P<0.05 vs. vehicle-treated STZ-diabetic MI rats. (a)

Normality Test: Passed (P = 0.853); (b) Normality Test: Passed (P = 0.855)

Fig. 5.

LVIDd (**a**) and LVIDs (**b**) measurements in NRG-1β-treated STZ-diabetic MI (n=9) and vehicle-treated STZ-diabetic MI (n=8) groups of rats. * $P < 0.05$ vs. vehicle-treated STZ-diabetic MI group of rats. (**a**) Normality Test: Passed ($P = 0.296$); (**b**) Normality Test: Passed ($P = 0.395$)

Fig. 6.

Effects of NRG-1 β on myocardial fibrosis in STZ-diabetic MI rats. Representative images of masson's trichrome staining from untreated (**a**) and NRG-1 β -treated (**b**) left ventricular tissues collected from STZ-diabetic post-MI rats, remote from the site of infarction. **c** Graphical representation of percent fibrosis in STZ-diabetic rat ventricular samples collected remote from the site of infarct. *P < 0.05 vs. vehicle-treated STZ-diabetic MI group of rats; Normality Test: Passed (P = 0.670)

Fig. 7.

Myocardial ErbB2 expression in vehicle and NRG-1 β treated STZ-diabetic MI rats **a** Immunofluorescence analysis of ErbB2 receptor expression in vehicle-treated and NRG-1 β -treated STZ-diabetic post-MI rat heart. Cross-sections of rat heart tissue were immunostained for ErbB2, β -Catenin, and DAPI and then visualized by fluorescent microscopy. Controls lacking primary anti-ErbB2 antibodies showed minimal background staining (data not shown). **b** Western blot for ErbB2, **c** Densitometric analysis of ErbB2 reactivity normalized to α -tubulin expression. * $P < 0.05$ vs. vehicle-treated STZ-diabetic MI group of rats; Normality Test: Passed ($P = 0.483$)

Fig. 8. Myocardial protein expression of MDM2 in vehicle-treated and NRG-1 β -treated STZ-diabetic MI rats. **a** Representative Western blot for MDM2, **b** Densitometric analysis of MDM2 immunoreactivity. In all samples, the densitometric measurement of MDM2 immunoreactivity was normalized to α -tubulin expression (*Normality Test: Passed ($P = 0.710$)*)

Fig. 9.

Effect of NRG-1 β treatment on myocardial apoptosis and proliferation in STZ-diabetic MI rats. **a** Representative immunohistochemical images of TUNEL positive LV sections from untreated and NRG-1 β -treated left ventricular tissues collected from STZ-diabetic post-MI rats, remote from the site of infarction, **b** Quantification shows significantly lower TUNEL positive cardiomyocytes in NRG-1 β treated hearts (* $P < 0.05$ vs. vehicle-treated STZ-diabetic MI group of rats [*Normality Test: Passed ($P = 0.414$)*], **c** Representative immunofluorescence analysis of pH3 from untreated and NRG-1 β -treated left ventricular tissues collected from STZ-diabetic post-MI rats, remote from the site of infarction. **d** Graphical representation of fold change in proliferation in STZ-diabetic rat ventricular samples collected remote from the site of infarct. Values are mean $\pm$ standard error of the mean and were statistically analyzed using the Mann-Whitney Rank Sum Test method.

Fig. 10.

Representative integrity of RNA extracted from LV tissue samples from untreated and NRG-1 β -treated STZ-diabetic MI rats used for determining the expression of genes listed in Table 3 using RT-qPCR **a** Representative gel run on an Agilent 2100 Bioanalyzer instrument including ladder (lane 1), standard (lane 2), and samples (lanes 3 and 4) from the LV of untreated and NRG-1 β -treated STZ-diabetic MI rats. **b** Electropherograms showing 18s and 28s rRNA peaks with RIN (RNA Integrity Number) from LV samples used for gene expression analysis. Y-axis, fluorescence intensity in fluorescence units (FU)

Table 1
Primers used for q-PCR

Gene	Forward sequence	Reverse sequence
collagen type I	GTT CGT GGT TCT CAG GGT AG	TTG TCG TAG CAG GGT TCT TT
collagen type III	TCC CCT GGA ATC TGT GAA TCA	CCT CCG ACT CCA GAC TTG ACA
caspase-3	AGT CTG ACT GGA AAG CCG AA	CGG GAT CTG TTT CTT TGC AT
Nox-1	TTC TGG GAA ACC TGC CTT TAG	ACA GGT GGG AGG GAA GAT TA
Nox-4	TTC TGG ACC TTT GTG CCT ATA C	CCA TGA CAT CTG AGG GAT GAT T
Myeloperoxidase	GGC ATC ACT ACC GTG TCT AAG	CCA GGA AGC CAG ATT CAG TT

Table 2
Characteristics of STZ-diabetic MI rats treated with saline or NRG-1 β at end of study

Parameters	Saline (n=8)	NRG-ip (n=9)
Body Weight, g	231 $\pm$ 7.8	228 $\pm$ 7.3
Blood Glucose, mg/dl	491 $\pm$ 24	472 $\pm$ 21
Lung Wet/Dry	5.0 $\pm$ 0.08	5.0 $\pm$ 0.1
Liver Wet/Dry	3.3 $\pm$ 0.0	3.3 $\pm$ 0.0
Systolic Blood Pressure, mm Hg	152 $\pm$ 17	149 $\pm$ 8

Values are mean $\pm$ standard error of the mean.

Table 3
Expression of selected genes in LV from diabetic post-MI hearts

Gene	Saline (n=5)	NRG-1 β (n=5)
Collagen type I	2.4 $\pm$ 1.0	1.1 $\pm$ 0.3
Collagen type III	1.8 $\pm$ 1.0	1.2 $\pm$ 0.2
Caspase-3	1.6 $\pm$ 0.7	1.0 $\pm$ 0.5
Nox-1	1.5 $\pm$ 0.2	0.9 $\pm$ 0.3
Nox-4	1.5 $\pm$ 0.3	0.8 $\pm$ 0.2
Myeloperoxidase	1.7 $\pm$ 0.4	0.9 $\pm$ 0.4

Values are mean $\pm$ standard error of the mean and were statistically analyzed using the δ - δ Ct method.