Original article

Neuregulin-1 attenuates mitochondrial dysfunction in a rat model of heart failure

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Keywords: *neuregulin-1; heart failure; mitochondria; cardioprotection*

Background Mitochondrial dysfunction plays a pivotal role in the progression of left ventricular (LV) remodeling and heart failure (HF). Recombinant human neuregulin-1 (rhNRG-1) improves cardiac function in models of experimental HF and in clinical trials; however, its impact on mitochondrial function during chronic HF remains largely unknown. The purpose of this study was to investigate whether rhNRG-1 could attenuate the functional and structural changes that occur in cardiac mitochondria in a rat model of HF induced by myocardial infarction.

Methods Sixty adult rats underwent sham or coronary ligation to induce HF. Four weeks after ligation, 29 animals with LV ejective fraction ≤50% were randomized to receive either vehicle or rhNRG-1 (10 $\mu g \cdot kg^{-1} \cdot d^{-1}$, I.V.) for 10 days, another 12 sham-operated animals were given no treatment. Echocardiography was used to determine physiological changes. Mitochondrial membrane potential (MMP), respiratory function and tissue adenosine triphosphate (ATP) production were analyzed. Cytochrome *c* expression and cardiomyocyte apoptosis were determined. Oxidative stress was evaluated by reactive oxygen species production using fluorescence assays and gene expression of glutathione peroxidase measured by real-time quantitative PCR.

Results Compared with sham-operated animals, vehicle treated HF rats exhibited severe LV remodeling and dysfunction, significant mitochondrial dysfunction, increased mitochondrial cytochrome *c* release, increased myocyte apoptosis and enhanced oxidative stress. Short-term treatment with rhNRG-1 significantly attenuated LV remodeling and cardiac function. Concomitant with this change, mitochondrial dysfunction was significantly attenuated; with ATP production, MMP and respiratory function restored, cytochrome *c* release and apoptosis inhibited, and oxidative stress reduced.

Conclusion The present study demonstrated that rhNRG-1 can significantly improve LV remodeling and cardiac function in the failing heart, this beneficial effect is related to reducing mitochondrial dysfunction, myocyte apoptosis and oxidative stress.

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Heart failure (HF) is a major and growing public health concern in modern societies worldwide. Although previous basic, clinical and epidemiological research have advanced the treatment of HF, the prognosis remains poor, with a 3-year mortality of 35%. The challenge remains to develop novel drugs with new mechanisms of activity for the treatment of HF. Recombinant human neuregulin-1 (rhNRG-1) is a new prospective drug which has recently been shown to prevent left ventricular (LV) remodeling and improve cardiac function in models of experimental HF and in a clinical trial.[1,2] however the underlying mechanisms of its activity are yet to be fully determined.

Neuregulin-1 (NRG-1), a member of the neuregulin family found in the adult heart, is expressed in the endocardium and cardiac microvascular endothelial cells. It activates both erbB2 and erbB4 receptors, stimulating proliferation, differentiation and cardiomyocyte survival.[3] Targeted disruption of the NRG-1 gene, or of either erbB2 or erbB4 in mice, leads to death during midembryogenesis.[4-6] Conditional null mutant mice for erbB2 localized to cardiomyocytes develop severe dilated cardiomyopathy by the second postnatal month.[7] Furthermore, administration of the erbB2 receptor inhibitor as a co-therapy with chemotherapeutics increased the development of HF.[8] These findings clearly implicate NRG-1/erbB signaling as having an indispensable role in cardiac development and maintenance of adult heart structural and functional integrity. Because of these previous findings it is hypothesized that NRG-1/erbB signaling may provide a potentially novel therapeutic target in the treatment of

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HF.[3,9] In a very elegant study, Liu et al[1] showed that short-term intravenous administration of rhNRG-1 improved cardiac function and survival in different animal models of cardiomyopathy. Recently, a phase II clinical trial reported that rhNRG-1 improved cardiac function in congestive HF patients and exhibited anti-remodeling capabilities. Taken together, rhNRG-1 presents a potentially useful therapy for HF treatment.

The mechanisms underlying the cardioprotective effects exhibited by NRG-1 are currently unclear. Mitochondrial dysfunction, oxidative stress and apoptosis have all been implicated as contributing to the progression of HF.[10,11] Attenuation of NRG-1/erbB signaling in cultured rat cardiomyocytes by pharmacological tyrosine kinase inhibition, or by reduction of erbB receptor expression, is shown to disturb mitochondrial function (reducing membrane potential, adenosine triphosphate (ATP) formation and redox capacity) and enhance spontaneous mitochondrial dependent apoptosis, increasing cytochrome *c* release, as well as caspase-9 and caspase-3 activation and cleavage of nuclear DNA.[12] In skeletal muscle cells, NRG-1 increased mitochondrial oxidative capacity, mitochondrial membrane potential (MMP) and attenuated mitochondrial dysfunction.[13] These findings indicate that NRG-1 has a significant role in regulating mitochondrial function. The purpose of this study was to investigate whether rhNRG-1 could attenuate the functional changes in cardiac mitochondria during CHF.

METHODS

Animal model of myocardial infarction (MI)

The current study was approved by the Animal Research Ethics Committee of Capital Medical University and performed in accordance with the Animal Management Rules of the Ministry of Health of the People's Republic of China. Sixty male Wister rats (200 to 250 g) obtained from the Animal Center of the Chinese Academy of Medical Sciences, Beijing, China, were anesthetized using 10% chloral hydrate (0.35–0.4 ml/100 mg) and the left anterior descending coronary artery (LAD) was ligated as previously described.[14] Sham-operated animals underwent the same procedure except the suture placed around the coronary artery was left untied.

Echocardiography

Echocardiography was performed four weeks after surgery and after 10 days of rhNRG-1 treatment by a subset of investigators who were blinded from the treatment groups. The LV was imaged in the short-axis view at the midpapillary muscle and M-mode measurements of left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were recorded. LV ejection fraction (LVEF) and fractional shortening (FS) were measured using the Teichholtz method.

Experimental protocol

Four weeks after coronary artery ligation, 29 rats displaying an EF of ≤50% were randomly assigned to receive either 10 $\mu g \cdot kg^{-1} \cdot d^{-1}$ rhNRG-1 (Zensun Science and Technology Co. Ltd., Shanghai, China) I.V. for 10 days, or vehicle (water). The dose of rhNRG-1 was chosen based on a previous study of its efficacy,[1] 12 sham-operated animals received no treatment.

Myocardial tissue preparation

Myocardial tissue having sustained an infarct were carefully dissected into two sections, one consisting of the infarcted LV including the peri-infarct rim (0.5–1 mm rim of normal appearing tissue), and the remaining section comprising the non-infarcted LV. In all subsequent assays, the comparison was made between the non-infarcted LV myocardium from MI animals and control LV myocardium from sham-operated animals.

Isolation of mitochondria

Myocardial tissue was homogenized at 4°C in a solution of 0.22 mol/L mannitol, 0.07 mol/L sucrose, 1 mmol/L ethylenediamine tetraacetic acid (EDTA) and 10 mmol/L Tris-HCl, pH 7.4. After centrifugation of the homogenates at 600 ×*g* for 10 minutes, the pellet was discarded and the supernatant was centrifuged at 8000 ×*g* for 15 minutes. The pelleted mitochondria were washed twice and then resuspended in the same buffer solution. Protein content was assayed by the microbiuret method. Isolated mitochondria were kept at 4°C and used within 4 hours after isolation.

Mitochondrial respiration assay

Mitochondrial oxygen consumption was determined in a water-jacketed vessel fitted with a Clark electrode and a stirring apparatus. The mitochondrial State 4 rate of O_2 uptake (resting or controlled respiration) was determined in a solution containing 0.225 mol/L mannitol, 0.07 mol/L sucrose, 1 mmol/L EDTA, 10 mmol/L potassium phosphate, 0.1% (w/v) bovine serum albumin (BSA), 2 mmol/L succinate and 20 mmol/L Tris-HCl, pH 7.4 at 25°C. Subsequently, 100 μmol/L adenosine diphosphate (ADP) was added for the determination of State 3 rates of O_2 uptake (active respiration). The respiratory control ratio was calculated as the rate of O_2 uptake in State 3 to the rate of O_2 uptake in State 4.

Reactive oxygen species (ROS) measurement

ROS production within the mitochondria was assessed using the fluorescent probe 5-(and-6)-carboxy-2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) by fluorometry at 499 nm excitation and 520 nm emission wavelengths. The reaction medium was 0.25 mol/L sucrose, 2 mmol/L hepes, 0.5 mmol/L KH_2PO_4, 2 μmol/L rotenone and 4.2 mmol/L succinate, pH 7.4, at 25°C, with 0.5 mg of mitochondrial protein/ml added. The fluorescence intensity over time provided a measurement of ROS production speed.

MMP

The MMP was measured by fluorescence using Rhodamin123 (molecular probe) as previously

described.[15] The reaction medium was 0.25 mol/L sucrose, 2 mmol/L hepes, 0.5 mmol/L KH_2PO_4, 2 μmol/L rotenone and 4.2 mmol/L succinate, pH 7.4, at 25°C, with 0.5 mg mitochondrial protein/ml added. Excitation and emission wavelengths were 505 nm and 534 nm respectively, at 25°C.

Heart tissue ATP production

ATP production was measured using an ATP colorimetric assay kit. The kit was dependent on the principal that ATP can be resolved by ATP into ADP and Pi, with the interaction between Pi and the dye producing a green Pi–dye complex, capable of being assayed by fluorescence. The ATP level is determined by measuring the expression of Pi. The optical density was read at 636 nm.

Western blotting

SDS-PAGE and Western blotting were performed as described previously.[16] Briefly, tissue from rat hearts was homogenized and lysed in NP-40 lysis buffer, 10 mmol/L Hepes, pH 7.4, 2 mmol/L egta ethylene glycol bis-amino tetmacetate (EGTA), 0.5% NP-40, plus protease inhibitors. Equivalent samples (20 μg protein) were subjected to SDS-PAGE and then transferred onto nitrocellulose membranes. Membranes were probed with specific antibodies followed by appropriate HRP-conjugated secondary antibodies (KPL, Gaithersburg, MD, USA). Immunoreactive bands were visualized with a chemiluminescence kit (Pierce, USA).

Apoptosis

Apoptosis was measured using the terminal deoxynucleotide transferase-mediated nick-end labeling (TUNEL) assay. The TUNEL assay was performed *in situ* using a cell death detection kit according to the manufacturer's instructions (Roche Biochemical; Shanghai, China). Terminal deoxynucleotidyl transferase catalyzes the polymerization of fluorescein dUTP to free 3′-OH DNA ends in a template independent manner. TUNEL positive cells were identified directly by fluorescence from incorporated dUTP. Apoptosis of cardiomyocytes was quantified by dividing the number of apoptotic nuclei by total nuclei from 10 continuous microscopic fields under 200 × magnification, and multiplying by 100 to provide values as apoptosis index = (apoptotic nuclei/total nuclei) × 100.

Real-time quantitative PCR

Glutathione peroxidase (GSHPx) mRNA was determined by a quantitative real-time PCR. The following are the gene-specific primers: GSHPx primers, forward 5′-aggagaatggcaagaatgaagag-3′ and reverse 5′-aggaaggta aagagcgggtga-3′, generate a 136 bp fragment. β-actin primers, forward 5′-gagaccttcaacaccccagcc-3′ and reverse 5′-aatgtcacgcacgatttccc-3′, generate a 264 bp fragment. Standard PCR was conducted with these primers using single-strand cDNA from the sample as a template. Gel electrophoresis confirmed that each PCR product was a single, correct size band. The purified products were quantitated spectrophotometrically for real-time PCR, using an ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems). To compare the number of mRNA encoding GSHPx in different samples, values were normalized as a ratio of GSHPx/β-actin.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed with the SPSS13.0 software (SPSS Inc., USA) by one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis. For all statistical tests, a *P* value less than 0.05 was considered significant.

RESULTS

Animal characteristics and effect of rhNRG-1 treatment on attenuating cardiac dysfunction and LV remodeling

Sixty rats (12 with the sham-operation and 48 with the LAD ligation) were used for the present study. At four weeks post-surgery 12 sham-operated and 29 MI rats survived. The MI rats exhibited a significantly enlarged LV chamber and reduced contractile function compared to sham animals. This time point was used as the baseline prior to rhNRG-1 or vehicle treatment. After 10 days of rhNRG-1, vehicle treatment or no treatment, the LV chamber dilation, as measured by LVEDD and LVESD, in animals receiving rhNRG-1 was substantially reduced compared with vehicle-treated HF rats; LVEDD (8.22±0.41) mm vs. (9.15±0.41) mm (P <0.05), LVESD (5.86±0.46) mm vs. (7.20±0.55) mm (P <0.01). Furthermore, LV function, as assessed by LVEF, (59.6±7.10)% vs. (41.6±6.71)% (P <0.01), and FS, (28.7±4.20)% vs. (21.3±4.17)% (P <0.01), were also markedly improved with rhNRG-1 treatment (Table 1).

rhNRG-1 significantly protected mitochondrial integrity and attenuated mitochondrial dysfunction in MI-induced HF

The respiration rate of mitochondria isolated from sham,

Table 1. Cardiac structure and function at baseline and after vehicle or rhNRG-1 treatment in sham, HF and NRG groups

Parameters	Sham	HF	NRG
Baseline			
n	12	15	14
LVEDD (mm)	5.64±0.37	8.85±0.87*	8.89±0.70*
LVESD (mm)	2.76±0.38	6.84±0.64*	6.91±0.45*
LVEF (%)	84.2±5.14	43.0±3.14*	42.7±3.10*
FS (%)	51.1±4.81	22.7±4.23*	22.2±4.10*
After treatment			
n	12	14	14
LVEDD (mm)	5.82±0.35	9.15±0.41*	8.22±0.41*†
LVESD (mm)	2.64±0.40	7.20±0.55*	5.86±0.46*‡
LVEF (%)	87.9±6.04	41.6±6.71*	59.6±7.10*‡
FS (%)	54.6±7.71	21.3±4.17*	28.7±4.20*‡

Values are mean±SD. LVEDD: left ventricular end-diastolic diameter. LVESD: left ventricular end-systolic diameter. LVEF: left ventricular ejection fraction. FS: fractional shortening. *P <0.01 vs. sham, †P <0.05, ‡P <0.01 vs. HF.

vehicle-treated and rhNRG-1-treated rats was compared. In cardiac mitochondria from HF animals, the respiratory control ratio (RCR, State 3 divided by State 4) was significantly lower than that of sham hearts, 2.00 vs. 2.71 (P <0.01). Treating animals with rhNRG-1 rescued RCR levels following MI; 2.62 vs. 2.00 (P <0.01) (Figure 1A). MMP was notably reduced in HF animals compared to sham, 221.8 mV vs. 246.4 mV (P <0.05). Again, administration of rhNRG-1 restored MMP and improved mitochondrial integrity following MI; 256.1 mV vs. 221.8 mV (P <0.05) (Figure 1B). ATP levels in heart tissue were significantly reduced in the HF group compared to the sham animals, (7.76±3.55) μmol/mg vs. (17.09±2.89) μmol/mg protein (P <0.01), while rhNRG-1 treatment significantly attenuated the reduction of ATP, restoring ATP production to levels approaching levels in sham animals, (14.55±4.69) μmol/mg vs. (17.09±2.89) μmol/mg protein (P >0.05) (Figure 1C). MMP and RCR were used as indicators of mitochondrial integrity and respiratory coupling. Our data suggest that rhNRG-1 treatment significantly protects mitochondrial structure and integrity, maintains energy-coupling efficiency and ATP production.

rhNRG-1 reduces cytochrome *c* release and myocyte apoptosis

Cytochrome *c* is an essential pro-apoptotic protein located within the mitochondria. Upon mitochondrial injury, cytochrome *c* is released into the cytosol, activating caspase-3 and the apoptotic cascade. Compared with sham-operated animals, vehicle-treated HF animals showed an increased cytochrome *c* level in the cytosolic fraction (Figure 2). This response was associated with a significant increase in apoptosis as determined by the number of TUNEL-positive nuclei, (18.1±3.0)% vs. (2.4±0.5)% (P <0.01). rhNRG-1 significantly attenuated the release of cytochrome *c* from the mitochondria and inhibited apoptosis; (11.9±1.4)% vs. (18.1±3.0)% (P <0.01) (Figure 3). Taken together, these data suggest that rhNRG-1 improves mitochondrial integrity and prevents myocyte apoptosis.

RhNRG-1 treatment prevents ROS production and upregulates GSHPx mRNA level

Mitochondrial ROS production following MI was found to be almost 2.47 times higher than that of the sham group. Treatment with rhNRG-1 significantly reduced ROS production in cardiomyocyte mitochondria of HF animals (Figure 4A). To explore the potential mechanism, we have checked mRNA level of an important endogenous antioxidant enzyme, GSHPx, by real time-PCR, and found that the GSHPx mRNA level were dramatically upregulated by rhNRG-1 treatment (Figure 5). Taken together these data suggest that rhNRG-1 can function *in vivo* to attenuate oxidative stress associated with HF.

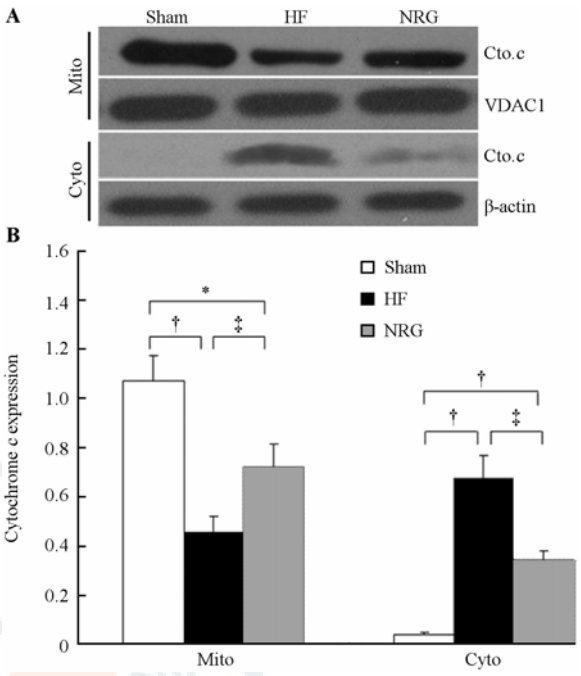

Figure 2. The effects of NRG-1 on release of cytochrome *c*. **A:** Release of cytochrome *c* from mitochondria to cytosol among sham, HF and NRG groups was checked by Western blotting. Voltage dependent anion selective channel protein 1 (VDAC1) and β-actin were used as loading controls. **B:** Bar graph showed results of densitometric analysis of intensities of cytochrome *c* relative to that of VDAC1 and β-actin (right, n=5). $^{*}P$ <0.05, $^{\dagger}P$ <0.01 vs. sham, $^{\ddagger}P$ <0.05 vs. HF.

DISCUSSION

rhNRG-1 has been proven to improve cardiac function in models of experimental HF and in clinical trials, and is thought to be a potentially useful therapy for HF

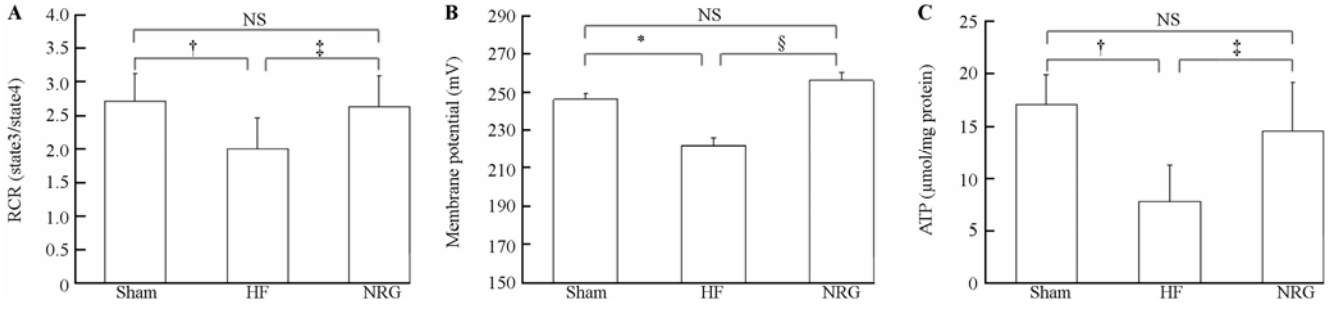

Figure 1. Physiological function analysis of cardiac mitochondria including RCR (**A**), membrane potential (**B**) and cardiac tissue ATP levels (**C**) from sham, HF and NRG rats, n=8 each. $^{*}P$ <0.05, $^{\dagger}P$ <0.01 vs. sham, $^{\ddagger}P$ <0.01, $^{\S}P$ <0.05 vs. HF.

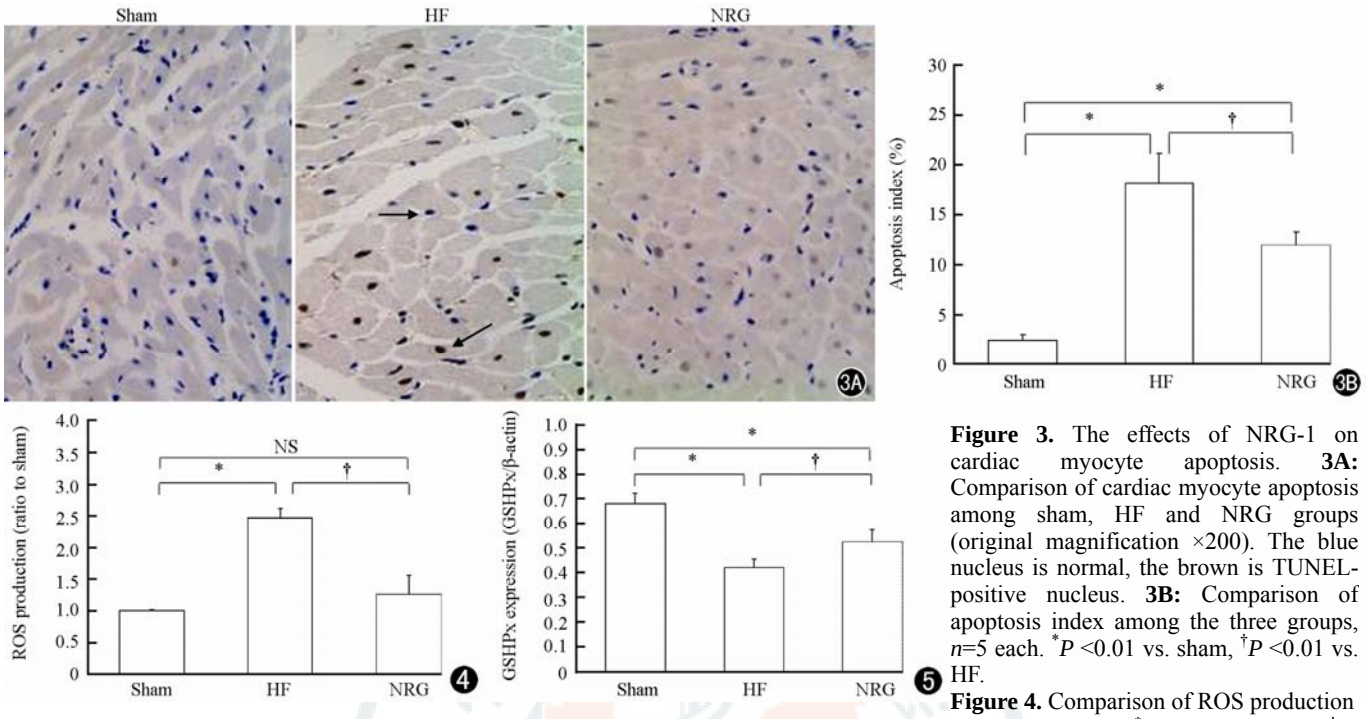

Figure 3. The effects of NRG-1 on cardiac myocyte apoptosis. **3A:** Comparison of cardiac myocyte apoptosis among sham, HF and NRG groups (original magnification ×200). The blue nucleus is normal, the brown is TUNEL-positive nucleus. **3B:** Comparison of apoptosis index among the three groups, n=5 each. $^{*}P$ <0.01 vs. sham, $^{\dagger}P$ <0.01 vs. HF.

Figure 4. Comparison of ROS production among sham, HF and NRG groups. Data are expressed as ratio to sham values, n=8 each. Values are mean±SD. $^{*}P$ <0.01 vs. sham, $^{\dagger}P$ <0.01 vs. HF.

Figure 5. GSHPx mRNA expression was assessed with real-time PCR in tissue collected from sham, HF and NRG groups (relative to β-actin), n=5 each. $^{*}P$ <0.01 vs. sham, $^{\dagger}P$ <0.05 vs. HF.

treatment; however the underlying mechanisms responsible for its activity remain largely unknown. Previous studies have found that NRG-1 could significantly reduce anthracycline induced disarray in adult rat ventricular myocytes in culture,[17] regulate cardiac parasympathetic activity,[18] up-regulate cardiac myosin light chain kinase (cMLCK) in monkeys with HF,[19] and modulate nitric oxide synthesis and calcium handling in rat cardiomyocytes.[20] But its effect on mitochondrial function has not been reported. The present study, using MI-induced CHF, found that short-term administration of rhNRG-1 attenuated LV remodeling and significantly improved LV systolic function. This beneficial effect is related to reducing mitochondrial dysfunction, oxidative stress and cardiomyocyte apoptosis.

Mitochondria are one of the most important subcellular organelles; in healthy heart tissue their primary function is to meet the high energy demands of the beating heart by providing ATP through oxidative phosphorylation. Low ATP content in cardiac tissue was observed in patients with chronic HF despite the absence of myocardial ischemia, and cardiac tissue from the failing heart has a reduced mitochondrial capacity for oxygen consumption and oxidative phosphorylation compared to the normal heart.[21] The shortage of bioenergy can induce mechanical and metabolic dysfunction of the myocardium and impair ion homeostasis, resulting in reduced cardiac function. Several mechanisms can impair energy production in cardiac mitochondria, including damage to the electron transport chain and phosphorylation apparatus, mtDNA injury or loss of membrane potential. Previous *in vitro* studies reported that inhibition of NRG-1/erbB signaling impaired mitochondrial function and integrity, including a reduction in membrane potential, ATP formation and redox capacity.[22,23] In skeletal muscle cells, experimental administration of NRG-1 increased the abundance of oxidative phosphorylation subunits and enhanced MMP.[13] These prior reports strongly suggest that NRG-1 plays a significant role in mitochondrial regulation, capable of increasing mitochondrial integrity and function with elevated levels of NRG-1. The current study supports these findings as rhNRG-1 administered to live rats markedly improved mitochondrial structure and integrity, restored membrane potential and increased respiratory rate and energy production in a model of MI-induced HF. This indicates that rhNRG-1 can significantly attenuate mitochondrial dysfunction.

Damage to the MMP induces the release of pro-apoptotic proteins, including cytochrome *c*. Cytochrome *c* is a key component of the electron transport chain. When released into the cytosol it activates caspase-3 and a series of proteases, eventually resulting in cellular breakdown and programmed cell death. Although the number of apoptotic cardiomyocytes is low at any given time point in HF, the accumulated number of apoptotic cells over time may be high enough to cause myocardial dysfunction. Observations from prior studies indicated that NRG-1 can promote survival and protect cultured cardiomyocytes from apoptotic cell death induced by oxidative stress or anthracycline.[1,24-26] In cultured adult rat ventricular

cardiomyocytes, rhNRG-1 prevented β-adrenergic receptor (β-AR) stimulated apoptosis, suppressed mitochondrial cytochrome *c* release and cytosolic caspase-3 activity.[27] Results from the present study further confirm that rhNRG-1 treatment can markedly suppress cytochrome *c* release from the mitochondria in the setting of HF, and this is associated with a significant reduction of myocyte apoptosis. Grazette et al reported that inhibition of NRG-1/erbB signaling induced cytochrome *c* release and mitochondrial apoptosis through an increased Bcl-xS/Bcl-xL protein ratio.[22] Therefore, it could be suggested that the inhibitory effect of rhNRG-1 on cytochrome *c* release is mediated via a reduction of the Bcl-xS/Bcl-xL protein ratio, or second, due to rhNRG-1 induced amelioration of mitochondrial membrane damage. Further investigations are required to reveal the mechanisms by which NRG-1 inhibits apoptosis.

Mitochondrial oxidative stress is another pathology associated with mitochondrial dysfunction. ROS is the byproduct of mitochondrial electron transfer activity. In the failing heart, mtDNA damage or reduced expression of co-factors involved in the respiratory chain both increase electron leak and elevate ROS production. As the mitochondria are the primary organelles producing ROS, improved mitochondrial function is synonymous with lower ROS production. NRG-1 is known to inhibit H_2O_2 induced ROS production in PC12 cells, and can provide antioxidant effects, reducing peroxide or doxo induced oxidative stress and ROS production. Our current study revealed that rhNRG-1 treatment can significantly reduce ROS production in an experimental model of HF. Under the setting of HF, ROS expression is chronically elevated, damaging not only mitochondrial proteins, lipids and DNA, but also inducing hypertrophy and interstitial fibrosis via the activation of MMP. In sum, the reduction in ROS production caused by rhNRG-1 treatment significantly attenuates detrimental cardiac remodeling via several mechanisms.

The mechanism by which rhNRG-1 elicits its mitochondrial protective effect is not clear. In our model of the failing heart, ROS formation was significantly increased. As the mitochondria is both the major ROS producer and ROS target, the chronic increase in ROS production within the mitochondria can lead to a catastrophic feed-forward cycle of mtDNA damage, further oxygen radical generation and enhanced cellular injury.[28,29] Reducing ROS production by rhNRG-1 treatment removes the agonist, and thus prevents the feed-forward mechanism continuing, attenuating ROS-induced cellular damage. In addition, development of HF is shown to be associated with decreased antioxidant capacity and increased oxidative stress; for example, the activities of superoxide dismutase, GSHPx and catalase were significantly reduced in animal models of HF.[30,31] One previous study[32] reported that NRG-1 can elevate several factors involved in redox regulation, including thioredoxin, glutathione S-transferase, hioredoxin-reductase-1, catalase, CuZn-superoxide dismutase, GSHPx, and glutathione reductase. Our findings further support rhNRG-1 as a stimulant of antioxidant activity, up-regulating GSHPx expression in our model of HF. Many studies using animal models and human trials now clearly indicate that the administration of antioxidants protect against age and ischemia-associated mitochondrial damage, including oxidant damage to mtDNA, glutathione oxidation, increased peroxide generation and the impairment of both mitochondrial function and morphology.[33-35] These findings suggest that the beneficial effects of rhNRG-1 on mitochondrial integrity and function are mediated, at least in part, by reducing oxidative stress in the failing heart via increased antioxidant activity.

Furthermore, NRG-1 has been shown mimic the antiadrenergic effects of muscarinic cholinergic signaling and regulate the cardiac sympathovagal balance.[18] In cultured adult rat ventricular cardiomyocytes, chronic β-AR stimulation induced oxidative stress, cytochrome *c* release, caspase-3 activation and mitochondrial apoptosis.[36] Angiotensin II significantly increased mitochondrial H_2O_2 production, decreased MMP, decreased respiratory coupling and the efficiency of oxidative phosphorylation within the mitochondria.[37] Application of rhNRG-1 was found to abolish or attenuate β-AR induced mitochondrial injury and sustain mitochondrial function.[36] We speculate that rhNRG-1, via its effects on the cholinergic system, can prevent mitochondrial dysfunction following chronic β-AR or angiotensin II stimulation, as observed in hypertension and HF.

In summary, the present study demonstrated that rhNRG-1 could attenuate mitochondrial dysfunction, oxidative stress and apoptosis in the failing heart. This beneficial effect on cardiac mitochondria may afford an alternative mechanism to explain the cardiac-protective effects of NRG-1.

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