

Effects of neuregulin-1 on autonomic nervous system remodeling post-myocardial infarction in a rat model

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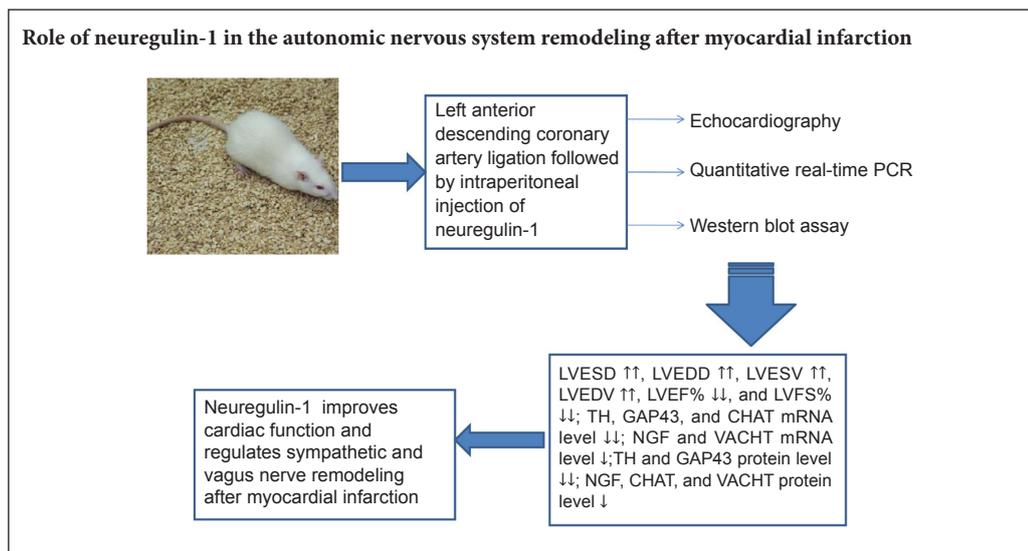
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Graphical Abstract



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Abstract

Sympathetic nerve and vagus nerve remodeling play an important part in cardiac function post-myocardial infarction (MI). Increasing evidence indicates that neuregulin-1 (NRG-1) improves cardiac function following heart failure. Since its impact on cardiac function and neural remodeling post-MI is poorly understood, we aimed to investigate the role of NRG-1 in autonomic nervous system remodeling post-MI. Forty-five Sprague-Dawley rats were equally randomized into three groups: sham (with the left anterior descending coronary artery exposed but without ligation), MI (left anterior descending coronary artery ligation), and MI plus NRG-1 (left anterior descending coronary artery ligation followed by intraperitoneal injection of NRG-1 (10 µg/kg, once daily for 7 days)). At 4 weeks after MI, echocardiography was used to detect the rat cardiac function by measuring the left ventricular end-systolic inner diameter, left ventricular diastolic diameter, left ventricular end-systolic volume, left ventricular end-diastolic volume, left ventricular ejection fraction, and left ventricular fractional shortening. mRNA and protein expression levels of tyrosine hydroxylase, growth associated protein-43 (neuronal specific protein), nerve growth factor, choline acetyltransferase (vagus nerve marker), and vesicular acetylcholine transporter (cardiac vagal nerve fiber marker) in ischemic myocardia were detected by real-time PCR and western blot assay to assess autonomous nervous remodeling. After MI, the rat cardiac function deteriorated significantly, and it was significantly improved after NRG-1 injection. Compared with the MI group, mRNA and protein levels of tyrosine hydroxylase and growth associated protein-43, as well as choline acetyltransferase mRNA level significantly decreased in the MI plus NRG-1 group, while mRNA and protein levels of nerve growth factor and vesicular acetylcholine transporters, as well as choline acetyltransferase protein level slightly decreased. Our results indicate that NRG-1 can improve cardiac function and regulate sympathetic and vagus nerve remodeling post-MI, thus reaching a new balance of the autonomic nervous system to protect the heart from injury.

Key Words: nerve remodeling; myocardial infarction; neuregulin-1; sympathetic nerve; vagus nerve; animal model; real-time PCR; western blot assay; cardiac function; echocardiography

Introduction

Neuregulin-1 (NRG-1), a member of the epidermal growth factor family, plays an important regulatory role in the development and repair of the nervous system (Murphy et al., 2002; Lemmens et al., 2007; Melenhorst et al., 2008; Ding et al., 2014; Yamada et al., 2014). Moreover, some scholars believe that NRG-1 and its receptors (ErbB2, ErbB3, and ErbB4) are crucial in cardiac development (Mei and Xiong, 2008; Pasca et al., 2014). NRG-1 has been found to be able to induce hypertrophy and inhibit cell apoptosis in rat ventricular myocytes (Zhao et al., 1998; Baliga et al., 1999; Rohrbach et al., 1999). Recent studies also found that NRG-1 activates tyrosine kinase, thus causing various cardiovascular biological effects, including regulating the structure and function of cardiomyocytes and its apoptosis and proliferation, and promoting angiogenesis (Odiete et al., 2012; Mendes-Ferreira et al., 2013). Experimental studies *in vivo* have found that NRG-1 can significantly improve acute and chronic ischemic cardiomyopathy and myocarditis, and phase II clinical trials revealed that the short-term administration of recombinant human NRG-1 can improve chronic cardiac function in patients with heart failure (Gao et al., 2010; Jabbour et al., 2011). However, phase III clinical trials of recombinant human NRG-1 for heart failure are ongoing. Notably, in patients with breast cancer accepting anti-ErbB2 monoclonal antibody trastuzumab treatment, heart failure occurred partially (Feldman et al., 2000; Schneider et al., 2001). Therefore, NRG-1 and its receptors are being considered gradually for the cardiovascular system.

A large amount of norepinephrine is released from sympathetic nerves during acute myocardial ischemia, and sympathetic sprouting and sympathetic remodeling reach a peak at 1 week after myocardial infarction (MI) (Wu et al., 2012; Holmes et al., 2016; Prabhu and Frangogiannis, 2016). At the same time, sympathetic nerve reinnervated by budding results in sympathetic hyperinnervation, and this imbalanced innervation plays an important role in the occurrence of ventricular arrhythmias (Li et al., 2004; Zhou et al., 2004; Boogers et al., 2010; Alston et al., 2011). Meanwhile, vagus nerve injury, necrosis, regeneration, and reconstruction also occur post-MI (Suo et al., 2013).

Most studies focused on the roles of NRG-1 in nerve repair and neurohumoral regulation of heart injuries (Monje et al., 2006; Calvo et al., 2010; Cahill et al., 2013), but the effects of NRG-1 on the neural remodeling post-MI remain unclear. We hypothesized that NRG-1 might play a role in neural remodeling following MI. Thus, we prepared a rat model of MI to investigate how NRG-1 impacts cardiac function and neural remodeling after MI.

Materials and Methods

Animals

Forty-five adult male Sprague-Dawley rats aged 12 weeks and weighing 250–300 g were provided by the Animal Experiment Center of Wuhan University in China (No. 42000500005679). Rats were housed in temperature-con-

trolled and humidity-controlled large cages with sawdust bedding and given access to tap water and food *ad libitum* for 7 days. All animal care and experimental procedures were performed in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (publication no. 85-23, revised 1986). This research was approved by the Administration Committee of Experimental Animals, Hubei Province, China. Rats were randomized into three groups ($n = 15$ per group): sham operation group, MI group, and MI plus NRG-1 group.

Establishment of the MI model

The left anterior descending coronary artery of the rats in the MI and MI plus NRG-1 groups was ligated to establish the MI model, as previously described (Wang et al., 2014). In brief, after anesthesia with 3.6% chloral hydrate (1 mL/100 g, Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) was administered, the rats were connected to the electrocardiograph and then disinfected. We cut the skin along the left side of the sternum; separated fascia and muscle with hemostatic forceps; performed thoracotomy at the third and fourth ribs to expose the mediastinum and pericardium; found the left atrial appendage, pulmonary artery cones, and veins; identified the left anterior descending coronary artery and ligated it using a 5-0 (or 6-0) suture; and closed the chest and skin layer by layer. A successful MI model was confirmed when the color of the ischemic area became pale and ST-segment elevation was detected in leads I, II, and aVL by electrocardiography. In the sham group, rats received the same procedures, except left anterior descending coronary artery ligation.

Experimental interventions

Rats in the MI plus NRG-1 group were given an intraperitoneal injection of NRG-1 (10 $\mu\text{g}/\text{kg}$) after MI, once daily for 7 consecutive days (Lemmens et al., 2011). At 4 weeks after MI, echocardiography was performed to evaluate cardiac function, and myocardial tissues from the infarct border zone were collected to detect the levels of tyrosine hydroxylase (TH), growth associated protein-43 (GAP43), nerve growth factor (NGF), choline acetyltransferase (CHAT), and vesicular acetylcholine transporter (VACHT) in the assessment of neural remodeling (Ajjijola et al., 2015).

Assessment of heart function

Heart function was tested using the Philips IE33 ultrasound system (GE Healthcare, Milwaukee, WI, USA) at 4 weeks post-MI. Each rat was measured under anesthesia, and the indexes included the left ventricular end-systolic inner diameter (LVESD), left ventricular diastolic diameter (LVEDD), left ventricular end-systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV), left ventricular ejection fraction (LVEF%), and left ventricular fractional shortening (LVFS%) were recorded and measured. The following equation was used: $\text{LVEF}\% = (\text{LVEDD}^3 - \text{LVESD}^3) / \text{LVEDD}^3 \times 100\%$ and $\text{LVFS}\% = (\text{LVEDD} - \text{LVESD}) / \text{LVEDD} \times 100\%$.

Table 1 Gene sequences

Gene	Primer sequence (5' to 3')
TH	Sense: CGT GTT TCA ATG CAC CCA GTA T Antisense: CTG GGA GAA CTG GGC AAA TG
GAP43	Sense: GAG CCT AAA CAA GCC GAT GTG Antisense: CTC ATC CTG TCG GGC ACT TT
NGF	Sense: GAT AAG ACC ACA GCC ACG GAC Antisense: TGA GTC GTG GTG CAG TAT GAG TT
CHAT	Sense: GAA GGC TGA GGT GGA AAT GTT C Antisense: TGA TGT TGT CCA CCC GAC CT
VACHT	Sense: CGC TCA CCA CTT GTA ACA TTC C Antisense: CAG AGT CCG CAG AGC GAG AC
GAPDH	Sense: CGC TAA CAT CAA ATG GGG TG Antisense: TTG CTG ACA ATC TTG AGG GAG

TH: Tyrosine hydroxylase; GAP43: growth associated protein-43; NGF: nerve growth factor; CHAT: choline acetyltransferase; VACHT: vesicular acetylcholine transporter; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Measurements of the neural remodeling markers (TH, GAP43, NGF, CHAT, and VACHT)

Quantitative real-time polymerase chain reaction (qRT-PCR)

Approximately 100 mg of myocardial tissues from the infarct border zone were collected at 4 weeks after MI. Total RNA was extracted by Trizol reagent (15596-026; Invitrogen, Carlsbad, CA, USA), cDNA was obtained with the First Strand cDNA Synthesis Kit (FSK-100; Toyobo, Kita-ku, Osaka, Japan), and then the fragments were amplified with the SYBR Green-based assays kit (Invitrogen) according to the manufacturer's instructions. Next, qRT-PCR was conducted using the StepOne™ Real-Time PCR System (ABI, Life Technologies, Rockville, MD, USA). RT-PCR conditions were 42°C/30 minutes and 80°C/5 minutes for reverse transcription; 95°C/60 seconds for pre-denature; and 95°C/15 seconds, 58°C/20 seconds, and 72°C/20 seconds. All steps were performed for 40 cycles. The gene sequences are shown in **Table 1**. The mRNA expression levels of TH, GAP43, NGF, CHAT, VACHT, and GAPDH were detected, and GAPDH was used for normalization. The relative gene expression in the sample was calculated as $2^{-\Delta\Delta CT}$. Experiments were performed in triplicate.

Western blot assay

Fifty µg of protein was extracted from the ischemic myocardial tissues at 4 weeks after MI. The tissues were lysed in radioimmunoprecipitation assay (AS1004, Aspen, Wuhan, China), and lysis buffer and protein concentrations were determined using the BCA kit (AS1086, Aspen). The proteins were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were blocked by incubation with 5% bovine serum albumin in a TBS-Tween buffer (10 mM Tris-HCl, 150 mM NaCl, and 0.5% Tween-20) for 1 hour at room temperature, and subsequently, incubated with different primary antibodies: rabbit anti-TH (Santa, Shanghai, China), anti-GAP43 (1:1,000; Abcam, Cambridge, UK), anti-NGF

(1:500; Abcam), anti-CHAT (1:500; Bioss, Beijing, China), anti-VACHT (1:500; Abcam), or anti-GAPDH (1:10,000; Abcam) overnight at 4°C. After the membrane was washed three times, the blots were incubated with secondary HRP conjugated goat anti-rabbit antibody (1:10,000; Pierce, Rockford, IL, USA) for 30 minutes at room temperature. Membranes were detected by enhanced chemiluminescence (Beyotime Biotechnology, Jiangsu, China) and exposed to film in the dark. The optical density intensity of each band was measured using AlphaEaseFC software (Alpha Innotech Corp., San Leandro, CA, USA). Results are shown as the optical density ratio to GAPDH.

Statistical analysis

All data were analyzed by SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Values are presented as the mean ± SD. The statistical significance of differences was analyzed using one-way analysis of variance with the Student-Newman-Keuls *post hoc* test for comparisons among groups. Values of $P < 0.05$ were considered statistically significant.

Results

Effect of NRG-1 on cardiac function

At 4 weeks after MI, echocardiography results showed significant differences in LVESD, LVEDD, LVESV, LVEDV, LVEF%, and LVFS% among groups. Compared with the sham group, the MI group showed a significant deterioration in cardiac function; that is, LVESD, LVEDD, LVESV, and LVEDV were significantly increased, and LVEF% and LVFS% were significantly decreased ($P < 0.05$). After NRG-1 injection, LVESD, LVEDD, LVESV, and LVEDV significantly decreased; whereas, LVEF%, and LVFS% significantly increased ($P < 0.05$), suggesting a significant improvement in cardiac function (**Table 2**).

Effect of NRG-1 on the mRNA levels of neural remodeling markers

The mRNA expression levels of TH, GAP43, NGF, CHAT, and VACHT were significantly increased in the MI and MI plus NRG-1 groups compared with the sham group ($P < 0.05$). Compared with the MI group, there was a significant decrease in mRNA expression levels of TH, GAP43, and CHAT in the MI plus NRG-1 group ($P < 0.05$). Simultaneously, there was a decreasing trend in NGF and VACHT mRNA expression levels ($P > 0.05$; **Table 3**).

Effect of NRG-1 on the protein levels of neural remodeling markers

The protein expression levels of TH, GAP43, NGF, CHAT, and VACHT were significantly increased in the MI and MI plus NRG-1 groups compared with the sham group ($P < 0.05$). The protein expression levels of TH and GAP43 in the ischemic myocardia in the MI plus NRG-1 group were significantly lower than those in the MI group ($P < 0.05$); the protein expression levels of NGF, CHAT, and VACHT were slightly lower in the MI plus NRG-1 group than those in the

Table 2 Effect of NRG-1 on cardiac function in rats after MI

Group	LVESD (cm)	LVEDD (cm)	LVESV (mL)	LVEDV (mL)	LVEF (%)	LVFS (%)
Sham	0.35±0.015	0.62±0.021	0.11±0.015	0.55±0.047	80.46±4.35	43.97±4.52
MI	0.56±0.04*	0.77±0.023*	0.41±0.08*	0.92±0.12*	53.28±0.48*	23.8±0.21*
MI+N	0.42±0.017†	0.6±0.035†	0.18±0.02†	0.70±0.02†	73.8±4.19†	38.0±3.72†

All values are expressed as the mean ± SD ($n = 15$ rats in each group). * $P < 0.05$ vs. sham group; † $P < 0.05$, vs. MI group (one-way analysis of variance with the Student-Newman-Keuls *post hoc* test). MI: Myocardial infarction; N or NRG-1: neuregulin-1; LVESD: left ventricular end-systolic inner diameter; LVEDD: left ventricular diastolic diameter; LVESV: left ventricular end-systolic volume; LVEDV: left ventricular end-diastolic volume; LVEF: left ventricular ejection fraction; LVFS: left ventricular fractional shortening.

Table 3 Effect of NRG-1 on relative mRNA levels (/GAPDH) of neural remodeling markers in rat ischemic myocardia

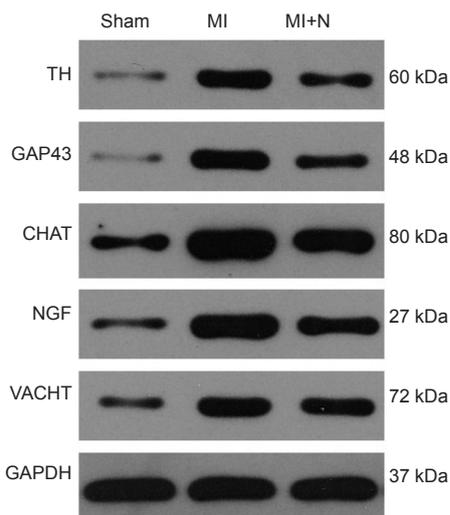
Group	TH	GAP43	NGF	CHAT	VACHT
Sham	1.04±0.252	1.21±0.453	1.00±0.095	0.91±0.121	1.00±0.205
MI	4.78±0.505*	4.94±0.452*	4.85±0.450*	3.60±0.797*	4.13±0.890*
MI+N	3.59±0.280†	3.95±0.165†	4.22±0.382	2.14±0.466†	2.97±0.424

All values determined by real-time polymerase chain reaction are presented as the mean ± SD ($n = 15$ in each group). * $P < 0.05$, vs. sham group; † $P < 0.05$, vs. MI group (one-way analysis of variance with Student-Newman-Keuls *post hoc* test). MI: Myocardial infarction; N or NRG-1: neuregulin-1; TH: tyrosine hydroxylase; GAP43: growth associated protein-43; NGF: nerve growth factor; CHAT: choline acetyltransferase; VACHT: vesicular acetylcholine transporter; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Table 4 Effect of NRG-1 on protein levels (/GAPDH) of neural remodeling markers in rat ischemic myocardia

Group	TH	GAP43	NGF	CHAT	VACHT
Sham	0.185±0.076	0.124±0.062	0.237±0.092	0.409±0.149	0.231±0.096
MI	0.678±0.102*	0.837±0.043*	0.981±0.174*	1.129±0.450*	0.749±0.176*
MI+N	0.364±0.141†	0.445±0.043†	0.642±0.156	0.812±0.231	0.693±0.141

All values determined by western blot assay are presented as the mean ± SD of optical density of target protein relative to GAPDH ($n = 15$ per group). * $P < 0.05$, vs. sham group; † $P < 0.05$, vs. MI group (one-way analysis of variance with Student-Newman-Keuls *post hoc* test). MI: Myocardial infarction; N or NRG-1: neuregulin-1; TH: tyrosine hydroxylase; GAP43: growth associated protein-43; NGF: nerve growth factor; CHAT: choline acetyltransferase; VACHT: vesicular acetylcholine transporter; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

**Figure 1 NRG-1 injection regulated protein levels of neural remodeling markers in rat ischemic myocardia.**

Target protein expression was analyzed by western blot assay with antibodies against TH, GAP43, NGF, CHAT, and VACHT relative to GAPDH. MI: Myocardial infarction; N: neuregulin-1; TH: tyrosine hydroxylase; GAP43: growth associated protein-43; NGF: nerve growth factor; CHAT: choline acetyltransferase; VACHT: vesicular acetylcholine transporter; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

MI group ($P > 0.05$; Table 4 and Figure 1).

Discussion

Some studies have shown that in the NRG-1 knockout mouse model of MI, cardiac systolic function was significantly impaired, but it was improved significantly after NRG-1 intervention (McCormick et al., 2015; Vandekerckhove et al., 2016; Yasui et al., 2016; Zhang et al., 2016). In an MI model, mitochondrial dysfunction and apoptosis were obviously reduced after intravenous administration of NRG-1, thereby reducing left ventricular remodeling post-injury (Guo and Wang, 2012). Gu et al. (2010) found that NRG-1 significantly up-regulated cardiac myosin light chain kinase and myosin light chain phosphorylation post-MI; the values of LVEF%, LVFS%, LVEDD, and LVESD were significantly increased, and cardiac function was significantly improved. In the current study, we observed that NRG-1 improved cardiac function post-MI.

NRG-1 can promote the proliferation of Schwann cells and increase the density and area of dendritic spines of neurons (Limpert and Carter, 2010). For peripheral nerves, NRG-1 promotes myelination and plays a significant role in axonal degeneration, axonal regeneration, remyelination, and innervation (Gambarotta et al., 2013; Shin et al., 2014).

It has been reported that sympathetic nerve hyperinnervation and denervation result in neural remodeling, but generally, sympathetic nerve remodeling is also accompanied with vagus nerve remodeling (Yu et al., 2010). Additionally, our study found sympathetic and vagus nerve remodeling after MI, which was expressed as a significantly increased GAP43 level, as well as increased CHAT and VACHT levels.

TH was generated by norepinephrine and located in the cytoplasm of adrenergic nerve fibers. GAP43, a kind of neuronal specific protein, exists in axons, which marks neuronal growth by neuronal synthesis. NGF can promote the growth of neuritis and induce cardiac sympathetic hyperinnervation (Lu et al., 2012). Chen et al. (2014) reported that TH, GAP43, and NGF significantly increased post-MI as confirmed by western blot analysis and immunohistochemistry, and aerobic exercise inhibited the cardiac sympathetic nerve sprouting and restored B1-AR/B3-AR balance. Our study's results also showed that the expression levels of TH, GAP43, and NGF were significantly increased post-MI, but after the NRG-1 intervention, their expression levels decreased, suggesting that NRG-1 can inhibit sympathetic remodeling post-MI. CHAT is a vagus nerve marker. Suo et al. (2013) found that CHAT-positive nerve fiber density and its mRNA expression level were higher post-MI. Besides, VACHT is a cardiac vagal nerve fiber marker. Xi et al. (2004) reported that vagal innervation density appeared to significantly increase after MI. In the current study, after the NRG-1 intervention, the expression levels of CHAT and VACHT were not significantly different when compared with those in the MI group post-MI, except the mRNA level of CHAT, implying that NRG-1 may not inhibit vagus nerve regeneration and repair following MI.

In summary, NRG-1 intervention effectively down-regulates sympathetic nerve mRNA and protein expression levels, thus inhibiting the sympathetic nerve remodeling post-MI, which reaches a new equilibrium of the autonomic nervous system to protect cardiac function by reducing sympathetic nerve tension. But the further mechanism of nerve remodeling is not involved in the current study, and we will explore the possible mechanisms in the further research.

Author contributions: XW, LW and XL participated in study conception and design. GKF, LZ and XL collected and analyzed data, and ensured the integrity of the data. HXF, SD and XL wrote the paper. XW, NZ, ZQL and XL served as principle investigators, and were in charge of paper authorization. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Research ethics: The study protocol was approved by Administration Committee of Experimental Animals of Wuhan University of China. All animal care and animal surgeries were in accordance with the Guide for Care and Use of Laboratory Animals (Public Health Service, 1996, NIH Publication No. 85-23).

Data sharing statement: Datasets analyzed during the current study are available from the corresponding author on reasonable request.

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