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Neuregulin-1 protects cardiac electrical conduction through downregulating matrix metalloproteinase-9 and upregulating connexin 43 in a rat myocardial infarction model

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Aims: Neuregulin-1 (NRG-1) is a member of the epidermal growth factor family, and has an important role in cardiomyocyte development and myocardial regeneration. The aim of this study was to determine the protective effect of NRG-1 on cardiac electrical conduction in a rat myocardial infarction (MI) model. *Methods:* Thirty-three adult male SPF SD rats were randomized into three groups: sham-operated (n=9), acute myocardial infarction (AMI, n=12), and the NRG-1-treated (NRG-1, n=12) groups. All rats were sacrificed on day 8 after inducing MI. The 6-lead electrocardiograms (ECG) were recorded pre-operatively and eight days after operation, and analyzed. The expression levels of matrix metalloproteinase (MMP)-9 and gap junction protein connexin 43 (Cx43) in the infarcted myocardium were measured by Western blotting, and its *in-situ* distribution was evaluated using immunohistochemistry. *Results:* The PR, QRS and QT intervals were significantly prolonged in the AMI group compared to the sham operated animals ($P<0.05$, $P<0.01$ and $P<0.01$ respectively), and the PR and QRS intervals were partially restored in the NRG-1-treated rats ($P<0.01$ and $P<0.01$ compared to AMI group). Similarly, the increased levels of MMP-9 in the AMI group was restored upon NRG-1 treatment. The myocardial expression of Cx43 was decreased significantly in the AMI group, and was upregulated by NRG-1 treatment. *Conclusions:* NRG-1 attenuates MI-induced dysfunctional cardiac electrical conduction by downregulating MMP-9 and upregulating Cx43.

1. Introduction

Around 80 % of sudden cardiac deaths are caused by persistent ventricular tachycardia and ventricular fibrillation secondary to acute myocardial infarction (AMI) (Rubart 2005). The arrhythmias can be a result of MI-induced slower cardiac electrical conduction, disordered distribution of the gap junction protein Cx43 in the infarcted area (Akar and Akar 2007; Yao 2003), alteration in Na^+ and K^+ levels (Luqman et al. 2007), reduction in action potential duration (Macianskiene et al. 2018), structural defects, metabolic imbalances and inflammation, in addition to the prescribed drugs as well as genetic susceptibility. Cx43 mediates the cardiac electrical flow and coordinates the excitation of the entire myocardium and the subsequent diffusion of contraction (Zhang and Shaw 2013). MI can trigger complex structural changes in the three dimensional structure of gap junction proteins (Rivera-Torres et al. 2016; Zhang, et al. 2018). In addition, MI-induced downregulation of Cx43 results in a significant decrease in conduction velocity and an increase in the incidence of ventricular tachyarrhythmias in mice (Ando, et al. 2005). Taken together, Cx43 dysfunction in cardiomyocytes could be the underlying mechanism of the cardiac electrical conduction changes caused by AMI.

MMP-9 levels increase in the early stages after MI (Ramirez et al. 2014). The MMPs hydrolyze the extracellular matrix proteins (Lindsey 2004), including Cx43, around the cardiomyocytes which breaks down the cell-to-cell junctions, and impairs inter-cellular signaling. Myocardial hypoxia-induced Cx43 downregulation can be attenuated by an inhibition of MMP (Wu et al. 2013). Therefore, changes in cardiac electrical conduction and ventricular arrhythmia after AMI are likely related to the increased MMP activity, which destroys the connection between cardiomyocytes, as well as the number and distribution of Cx43. NRG-1, a transmembrane polypeptide growth factor, is a member of the epidermal growth factor family. It acts through the tyrosine kinase transmembrane receptors ErbB2, ErbB3 and ErbB4. In the myocardial tissue, NRG-1 is secreted by microvascular endothelial cells (Cote et al. 2005; Lemmens et al. 2006). Previous studies have shown that NRG-1 can significantly affect the excitability of cardiomyocytes by inhibiting the expression of GIRK and acetylcholine receptor genes, and regulating the balance between sympathetic and parasympathetic responses, ultimately leading to arrhythmias (Ford et al. 2003). In NRG-1 knockout heterozygous mice, muscarinic receptor stimulation does not counteract β-adrenergic activation (Okoshi 2004). In addition, after stimulation with NRG-1, embryonic cardiomyocytes can differentiate into cardiac conduction system cells, and fetal cardiomyocytes can be transformed into cardiac pacemaker-like cells (Odiete et al. 2012).

However, little is known about the role of the NRG1/ErbB signaling pathway on cardiac electrical conduction after AMI. To elucidate this, we established a rat model of AMI by ligating the left anterior descending artery, and assessed the PR, QRS and QT intervals, along with the myocardial expression of MMP-9 and Cx43.

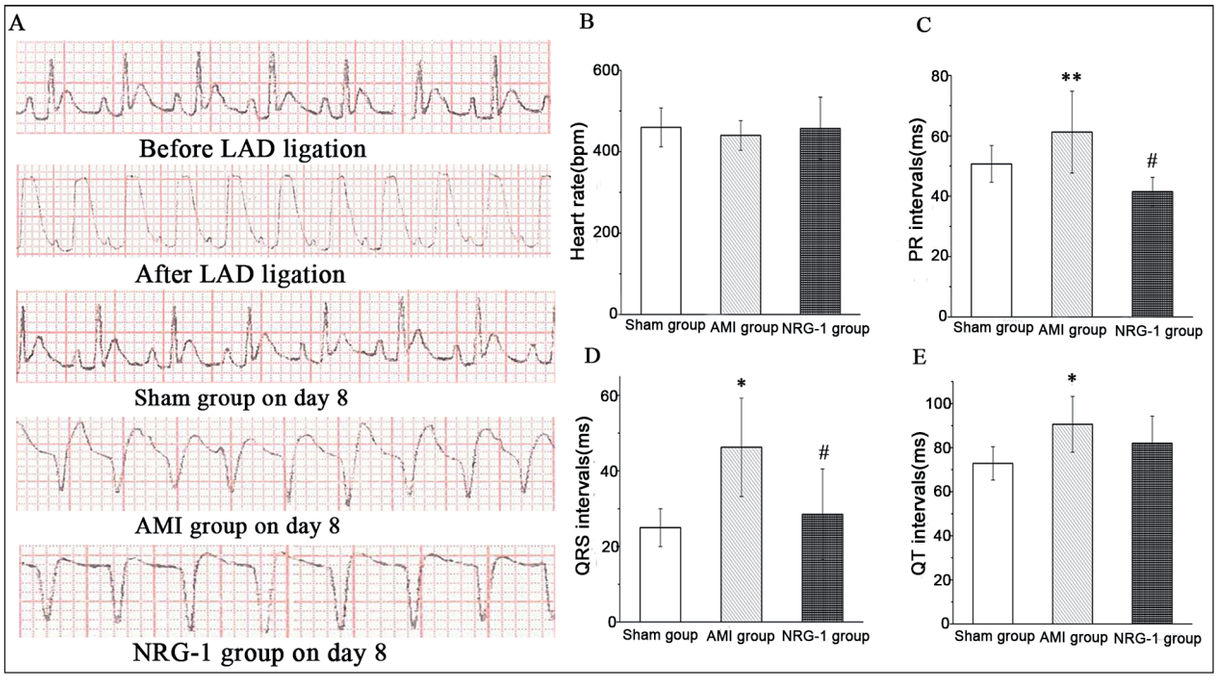

Fig. 1: Changes of ECG parameters on day 8 after ligation, n=8-9. **P<0.05 VS Sham group; *P<0.01 VS Sham group; #P<0.01 VS AMI group.

2. Investigations and results

2.1. Assessment ECG

Out of the 33 operated rats, 3 died in the AMI and 4 in the NRG-1 group. No significant differences were seen in the pre-operative and post-operative heart rate (day 1 and day 8) across the three groups. However, the PR, QRS and QT intervals were significantly increased in the AMI group compared to the sham-operated rats ($p<0.05$, $p<0.01$ and $p<0.01$). These parameters were restored by NRG-1 treatment, with significant reduction in the PR and QRS intervals ($p<0.01$ and $p<0.01$ compared to AMI), and only a slight reduction in the QT interval (Fig. 1).

2.2. The expression of MMP-9 and Cx43

Compared to the Sham group, the expression of MMP-9 in the core infarcted region of the left ventricle was significantly increased after AMI ($P<0.05$), while that of Cx43 was significantly decreased ($P<0.05$). NRG-1 treatment decreased the expression of MMP-9 ($P<0.05$), and increased that of Cx43 ($P<0.05$) compared to the untreated AMI group (Fig. 2).

2.3. In situ levels of Cx43 in the left ventricular infarct area

In the Sham group, the majority of Cx43 protein was distributed in the intercalated disk, with less expression in the cytoplasm and more towards the cellular boundaries. After AMI, Cx43 levels were significantly reduced and showed a disordered arrangement, which was accompanied by extensive myocardial cell thickening and dissolution. However, NRG-1treatment increased the levels of Cx43 protein ($P<0.05$), and also reduced its lateral distribution (Fig. 3).

3. Discussion

This study is the first to report the effect of NRG-1 on the aberrant cardiac electrical conduction induced by AMI. NRG-1 improved several conduction parameters after AMI, in addition to downregulating MMP-9 and upregulating Cx43 in the myocardium.

Any structural change induced by AMI in the cardiomyocytes can potentially alter cell-cell pulse conduction through gap junctions, interfere with cardiac electrical activity, and increase the likelihood of ectopic impulse formation (Saffitz and Corradi 2016). AMI resulted in prolonged PR, QRS and QT intervals, which were ameliorated to some extent in the rats treated with NRG-1. Electrical coupling between ventricular cardiomyocytes in the healthy myocardium cells depends on Cx43 (Fernandes et al. 2009), and any disruption of this protein impairs cardiac conduction (Kalcheva et al. 2007; Roell et al. 2018). Nattel et al. (2007) reported that MI causes irregular distribution of Cx43 in the myocardial tissue near the infarcted area, which leads to altered ion channels and increases the risk of ventricular arrhythmia. Osbourne et al. (2014) found that downregulation of Cx43 induced by overexpression of miRNA130a resulted in a prolonged PR interval and a significant increase in persistent VT. Ng et al (2016) found that the conduction velocity in the infarct border zone improved, and the arrhythmia susceptibility was significantly reduced after the Cx43 gap junction coupling was improved. The post-AMI rats in our study showed prolonged PR and QRS intervals, reduced Cx43 levels in the myocardium, which was restored by NRG-1.These are analogous to the findings of Rentschler et al. (2002) who showed that NRG-1 promoted formation of murine cardiac conduction system, and another study that found an association of a common missense mutation of the NRG-1 gene with sudden cardiac death (Huertas-Vazquez et al. 2013).

In addition, Li et al. (2008) found that NRG-1 improved neuron survival and delayed irreversible neuron necrosis in rat models by inactivating MMP-9 and thus preventing inflammation. MMP-9 cleaves various extracellular matrix and inflammatory proteins (Ramirez et al. 2014), thereby affecting the function of gap proteins. Lindsey et al. (2006) showed that MMPs may cause arrhythmias by affecting Cx43. Consistent with this, MMP-9 levels were significantly upregulated in the myocardia of the AMI rats in our study, and inhibited upon NRG-1 treatment. Our study shows for the first time that NRG-1 protects cardiac electrical conduction after AMI via its action on MMP-9.

In terms of study limitations, we did not conduct a more in-depth electrophysiological examination, and did not investigate the mechanism underlying NRG-1-mediated regulation of MMP-9. Nevertheless, we can conclude that NRG-1 restores normal cardiac electrical conduction after AMI by downregulating MMP-9 and upregulating Cx43. Our findings lay the foundation for developing novel approaches to treat arrhythmias.

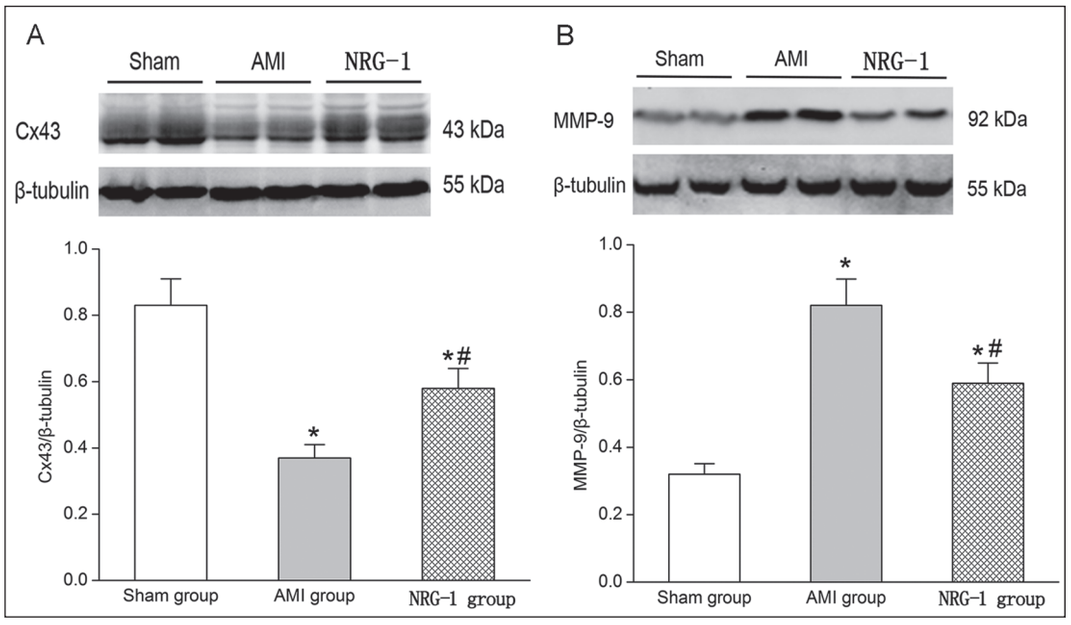

Fig. 2: The Cx43 and MMP-9 protein expression levels in the core region of left ventricular infarction (three group, n=4). *P<0.05 VS Sham group; #P<0.05 VS AMI group

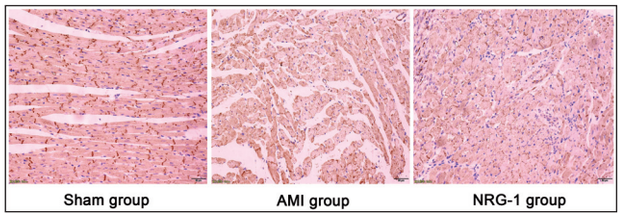

Fig. 3: Representative immunohistochemistry pictures showing Cx43 expression in the left ventricle infarct area (X200).

4. Experimental

4.1. Materials

NRG-1 (cat no. #5218SF) and Cx43 antibody (1:1000; cat no. #3512) and anti-rabbit secondary antibody (1:2000; cat no. #7074) were obtained from Cell Signaling Technology Inc, the anti-MMP-9 rabbit polyclonal antibody (1:1000; cat no. W680) from Bioworld technology Inc, and the BCA Kit (cat no. AR0146) from Boster Biological Technology co.ltd. ALC-V9A animal respirator and MPA-CFS animal heart function analysis system were purchased from Shanghai Aokete Biotechnology Co. Ltd (Shanghai, China), the ECG-9130P electrocardiograph from Japan Optoelectronic Industry Corporation (Tokyo, Japan), YJ12001 electronic balance from Minqiao Precision Science Co. Ltd. (Shanghai, China), and the Nikon Eclipse mounted optical microscope from Nikon Corporation (Tokyo, Japan).

4.2. Establishment of rat AMI model

All animals were treated humanely and in accordance with the "Guidelines for the Care and Use of Laboratory Animals" published by the National Institutes of Health (8th edition, 2011). The experimental protocol was approved by the Animal Care and Use Committee of Guangxi Medical Research Institute. Thirty-three male Sprague Dawley rats (7-8 weeks old with average body weight 298.56±38.73 g) were obtained from the Animal Center of Guangxi Medical University (Nannin, China). After anesthetizing the rats by an intra-peritoneal injection of sodium pentobarbital (1 %, 35 mg/kg), they were intubated at 4 ml/100 g and ventilated at 80 breaths/min. A left-side thoracotomy was performed, and the fourth and fifth ribs were removed. The rats were then randomized into the sham-operated, AMI and NRG-1-treatedgroups.The sham-operated rats only underwent thoracotomy without left anterior descending ligation. For the other two groups, the left coronary artery was ligated approximately 2 mm below the left atrial appendage with 6-0 polypropylene suture after pericardial incision. Successful MI was determined by ST segment elevation and Q wave changes in Leads II and III in ECG, and pallor appearance in the LV anterior wall. The chest was closed until a stable cycle was obtained. The rats were then accordingly injected with either 10 μg/kg NRG-1 or normal saline (sham-operated and AMI groups) via the tail vein 2 h after the operation, and continued once daily for 7 days.

4.3. ECG recording

The ECGs of the animals were recorded before the coronary artery ligation and on the 8th day after ligation. Briefly, the rats were anesthetized and ventilated as described above, and 4 stainless steel electrodes were subcutaneously fixed to the right pectoral muscle and the lower left side of the chest wall. A 6-lead surface ECG was then obtained, and the artifacts were manually excluded. The PR, QRS and QT intervals were measured manually by 3 individuals in a blinded fashion, and the average was calculated.

4.4. Immunohistochemistry

The heart tissues were fixed in 4 % formalin, embedded in paraffin, and cut into 5-μm sections. After incubating with 3 % hydrogen peroxide for 5-10 min at room temperature to quench endogenous peroxidase, the cardiac cross-sections were immuno-stained with Cx43 antibody (50 μl) overnight at 4 °C. After re-warming the slides for 1 h, the secondary antibody was added dropwise. The sections were stained with DAB solution and counterstained with hematoxylin.

4.5. Western blotting

As previously described (Gui et al. 2012). The myocardial tissues were collected on ice and weighed, and the left ventricle was homogenized in liquid nitrogen. RIPA buffer containing PMSF was added immediately for protein extraction, and the lysates were quantified using a BCA kit. Equal amount of proteins per sample; were separated by SDS-PAGE and transferred to a PVDF membrane. After blocking the membranes with a blocking solution at 4 °C for 2 h, the primary antibody was added and the blots were incubated overnight at 4 °C. The positive bands were detected using horseradishperoxidase-labeled secondary antibody and a chemiluminescence detection system, and densitometrically quantified using image analysis software.

4.6. Statistical analysis

All data are expressed as mean±standard deviation, and compared by one-way analysis of variance. P values less than 0.05 were considered statistically significant. All statistical analyses were performed with the SPSS 16.0 software package (IBM Corp., Armonk, NY, USA).

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Conflict of interests: We have no financial interest or conflict of interest in association with this work.

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