

Original Article

Irbesartan reduces chronic ischemic myocardial injury by up-regulating connexin 43 expression in rats

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Abstract: This study was conducted to investigate the impact and possible mechanisms of irbesartan on the expression of gap junction protein 43 (connexin, Cx43) in rats with myocardial injury. Fifty rats (8-12 week old, weighting 300 ± 50 g) were used as model of chronic myocardial infarction. The rats were divided into sham (thoracotomy only), infarcted models without or with irbesartan treatment for two weeks. The survived rats were measured for heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), the maximal rate of rise of left ventricular pressure ($+dP/dt_{max}$) and the maximal rate of decrease of left ventricular pressure ($-dP/dt_{max}$), apoptosis assay using the TUNEL method and Cx43 level using immunofluorescence microscopy, qRT-PCR and Western blot. The levels of cardiac troponin I (cTnI) were also determined. Compared with sham group, infarction group had significantly more apoptotic cells ($P < 0.05$) and reduced levels of Cx43 at both protein and mRNA levels ($P < 0.05$). Compared with infarction group, irbesartan reduced the downregulation of Cx43 expression. cTnI contents were 6.37 ± 0.98 ng/ml in sham group, 10.97 ± 1.28 ng/ml in infarction model and 7.65 ± 0.41 ng/ml in irbesartan group, suggesting that irbesartan has protective effect on myocardial tissue. It is concluded that Irbesartan protects myocardial cells from chronic ischemic injury by increasing Cx43 expression in myocardial cells.

Keywords: Irbesartan, myocardial ischemia, arrhythmia, Cx43

Introduction

Cardiovascular disease is one of the most important causes of death in the world and the death of myocardial cells caused by myocardial ischemia is an important cause of death in patients with the diseases. Studies have shown that a variety of cardiovascular diseases are almost all associated with two complications, namely gap junction re-distribution and change in the level of gap connexins (Cxs) [1, 2], which result in changed anisotropy of electrical conductivity and reduced velocity of electric conduction, leading to more frequent reentry and damage of myocardial cells. Gap junction is an intracellular channel that connects the cytoplasm between adjacent cells and has been identified in various tissues and cells, such as the epithelial cells of renal vessel, connective tissues, macrophages, liver cells, astrocytes, myocardial cells, endothelial cells and smooth

muscle cells. Different Cxs have been found in different tissues [3, 4]. There are three major Cxs (Cx43, Cx40 and Cx45) in human [5] and Cx43 is the major Cx in myocardial cells on the intercalated disk and it transmits signals between the myocardial cells [6-8]. The normal expression and distribution of Cx43 are important for electrical coupling in cardiac gap channels, cardiac electrical activity and the coordination of the cardiac contraction.

Angiotensin II receptor blocker (ARB) is a class of compounds that modulate the renin-angiotensin system. ARB drugs are used clinically for treatment of various hypertension and congestive heart failures [9]. Studies have shown that ARB prevents the decrease in the threshold of ventricular fibrillation during ischemia and remarkably reduces the occurrence of ventricular tachycardia and fibrillation during ischemic reperfusion. For example, earlier studies

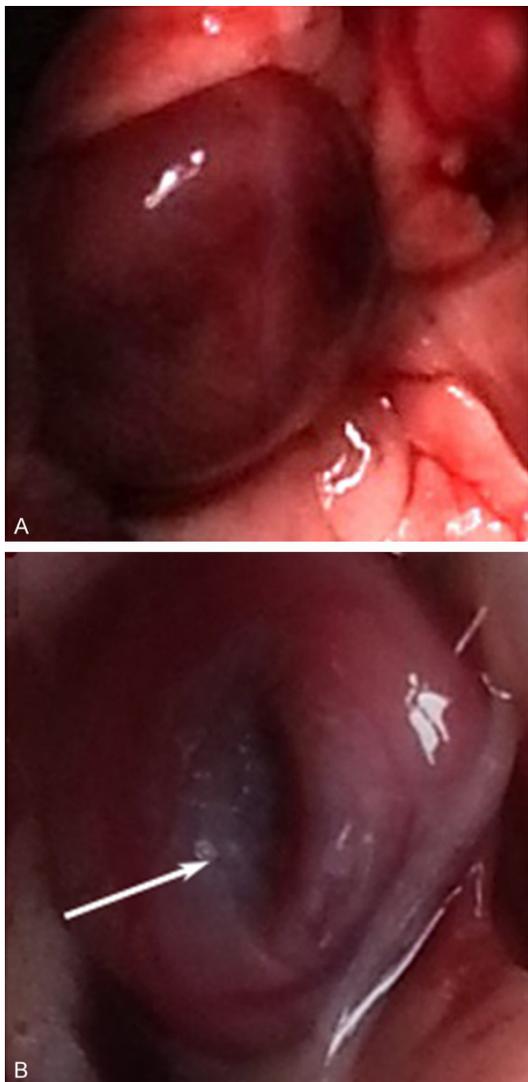


Figure 1. Representative myocardial tissues of rats in sham (A) and infarction group (B). Arrow indicates infarcted myocardial tissue.

found that there were much less patients with ventricular arrhythmia when ARB was used to treat hypertensive ventricular arrhythmia [10, 11]. So far, spontaneous hypertension patients are most frequently used models for ARB-related study, little is known about the effect of ARB on the expression of Cx43 during myocardial infarction.

In this study, we used rat myocardial infarction models to study the expression of Cx43 under myocardial ischemia condition to gain insights into the effect of ARB drug irbesartan on Cx43 expression and possible mechanisms underlying the effect. The findings would be of significance for the prevention and treatment of ventricular tachycardia.

Materials and methods

Infarction models and treatments

Sprague Dawley® rats (specific pathogen-free, aged 8-12 weeks and weighting 18 ± 2 g) were purchased from the Experimental Animal Center, Nanchang University, and were housed in standard animal cages (5 per cage). The animals had free access to food and drinking water in well vented rooms at 18-25°C and humidity of 50-60% under natural lighting.

Infarction models were constructed as described [12]. Briefly, the rats were anesthetized with 5% chloral hydrate and the chests were cut open between the 3 and 4th left sternums to expose the hearts. The upper middle part of left coronary artery at 1/3 position was sutured. The animals were divided into sham (underwent thoracotomy only), infarcted without (model) or with irbesartan. Untreated rats were used as control. Irbesartan was orally administered at a dose of 20 mg/kg for 2 weeks. After the treatments, the heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), the maximal rate of rise of left ventricular pressure ($+dp/dt_{max}$) and the maximal rate of decrease of left ventricular pressure ($-dp/dt_{max}$) were measured using PowerLab data acquisition device (ADInstruments, Australia). The animal experimental protocols were approved by the ethics committee of Nanchang University.

qRT-PCR

qRT-PCR was preformed as described [13]. Briefly, myocardial tissue was isolated, rinsed in pre-chilled PBS and total RNA extracted using a RNA extraction kit (Qiagen, USA) following the supplier's instructions. RNA was quantified using Qubit fluorometer and used for reverse transcription using a reverse transcription kit (Applied Biosystems, USA) following the supplier's instructions. The PCR reaction was performed on Applied Biosystems 7500 (Applied Biosystems, USA) using 100 ng cDNA as template with 300-450 nM each of primers. The PCR reaction parameters included pre-denaturing at 95°C for 10 min, denaturing at 95°C for 10 s, followed by 40 cycles of annealing at melting temperature for 20 s and extension at 72°C for 33 s, using SYBR Green reverse transcription PCR kit (Applied Biosystems, USA)

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Table 1. Comparison of cardiac functions between sham and infarction models with chronic myocardial ischemia

Group	n	HR (time/min)	LVSP (kPa)	LVEDP (kPa)	+dp/dt _{max} (kPa/s)	-dp/d _{tmax} (kPa/s)
Sham	10	351 ± 14	12.6 ± 0.9	0.83 ± 0.9	429 ± 20	376 ± 13
Model	48	394 ± 10	7.6 ± 1.2*	1.26 ± 0.3*	207 ± 24**	234 ± 19*

* and ** denote significant or highly significant difference vs sham.

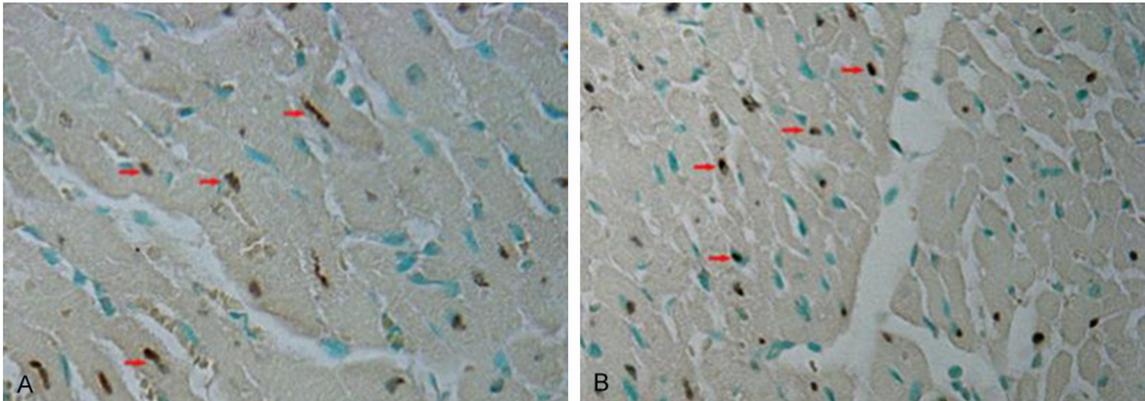


Figure 2. Apoptosis of myocardial cells in normal and model rats. Arrows indicate apoptotic cells.

according to the manufacturer's protocol. The primer sequences for metalloproteinase inhibitor 1 (TIMP-1, Genbank accession no. nmNM_010288) were: forward, ACAAGGTCCAAGCCTACTCCA; and reverse, CCGGGTTGTTGAGTGTTACAG). Glyceraldehyde-3-phosphate dehydrogenase (GADPH, Genbank accession no. nmNM_008085) was amplified as internal reference using forward primer: AATGGATTGGACGCATTGGT and reverse primer: TTTGCACTGGTACGTGTTGAT. The $\Delta\Delta C_t$ method was used to calculate the relative mRNA level [14].

Immunofluorescence microscopy and TUNEL assay

For immunofluorescence microscopy, myocardial tissue was embedded in paraffin, sectioned transversely and incubated with fluorescein-labeled antibody (rabbit anti-mouse Cx43, Abcam, UK) for 30 min. The sections were rinsed several times with PBS (0.01 mol/L, pH7) and viewed using a fluorescence microscope. For the TUNEL assay, muscle was isolated from the middle left ventricle, fixed in 4% paraformaldehyde (pH 7.4) at 4°C for 24 h, washed twice with xylene and anhydrous alcohol, followed by washing with 95% and 75% ethanol each for 3 min. The samples were then incubated with DNase-free protease K at 37°C

for 15 min, rinsed three times with PBS, stained and detected using a TUNEL assay kit (Vazyma, USA) according to the manufacturer's instructions. Under the light microscope, the nuclei of normal (negative) myocardium stained blue while apoptotic cells were dark-brown colored.

Western blot

Tissues were grounded in liquid nitrogen and extracted for total proteins using ReadyPrep protein extraction kit (Bio-Rad, USA) according to the manufacturer's instructions. Extracted proteins were quantified using BCA quantification kit (Vazyme Biotech, China) according to the manufacturer's instructions.

Proteins were then separated using SDS-PAGE electrophoresis and transferred to PVDF membranes (Millipore, USA). The membranes were washed with TBST buffer and incubated with diluted primary antibodies (rabbit anti-mouse Cx43 and anti β -actin monoclonal antibodies, Abcam, UK) for 2 h at room temperature. After rinsing, the membranes were incubated in the secondary antibody solution (horseradish peroxidase-labeled goat anti rabbit Ig (H+L), 1:1000 dilution, Beyotime Biotechnology Co. Ltd., Shanghai) at room temperature for 1-2 h. The membranes were then washed, added with ECL developer solution and exposed in Chemi-

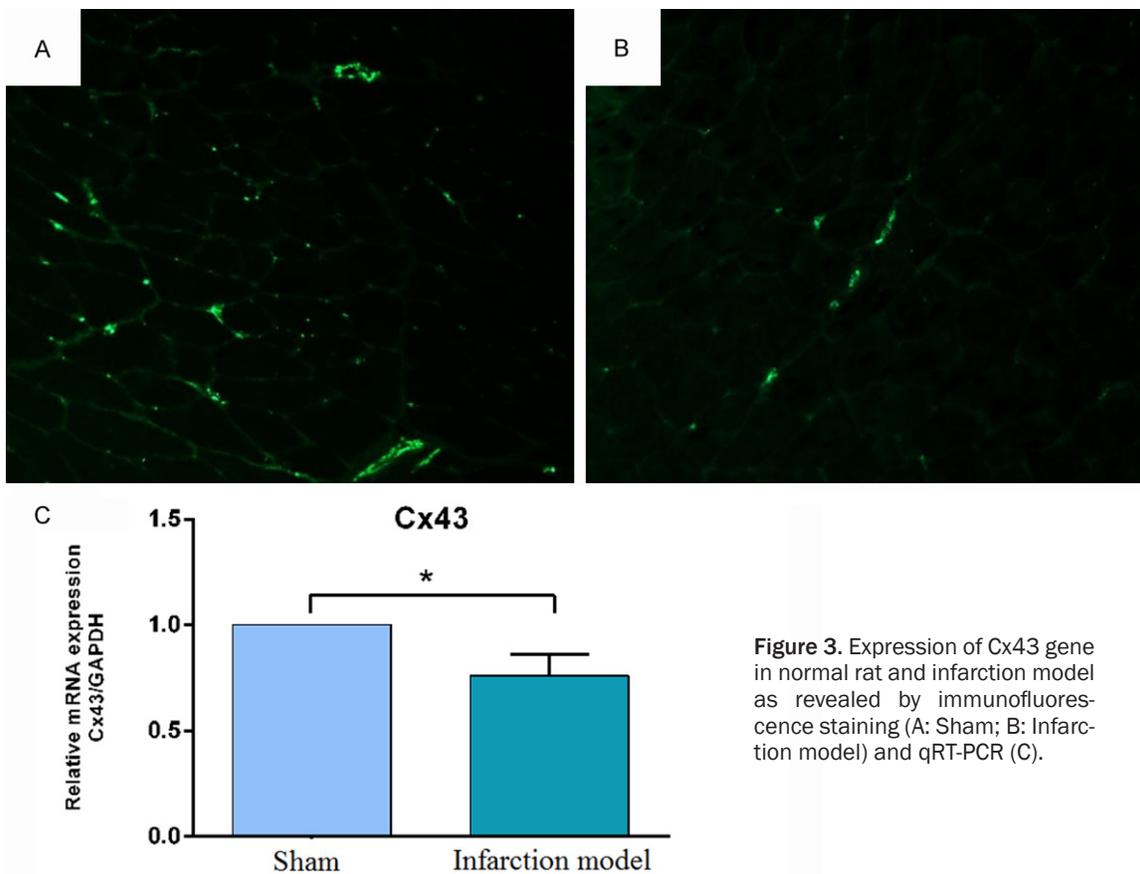


Figure 3. Expression of Cx43 gene in normal rat and infarction model as revealed by immunofluorescence staining (A: Sham; B: Infarction model) and qRT-PCR (C).

DocTM XRS gel imaging system (Bio-Rad, USA). The gray values of band were measured using the Quantity One software.

Statistical analysis

Data were analyzed using statistical software SPSS13.0 and were expressed as mean \pm SD (standard deviation). Differences between the groups were compared using Student's *t*-test and were considered significant if *P* was < 0.05 or highly significant if *P* was < 0.01 .

Results

Myocardial infarction model

Two weeks after modelling, examinations showed that myocardial infarction modelling was successful in 48 rats (**Figure 1**). In compared with rats in sham group, the models had significantly reduced LVSP, LVEDP, $+dp/dt_{max}$, and $-dp/dt_{max}$ (**Table 1**). The remaining two rats died after the surgery for unknown reason.

Apoptosis in myocardial infarction model

We then assessed the apoptosis in the myocardial tissues using the TUNEL method. The

results showed that there were significantly more apoptotic cells in the models as compared with sham (**Figure 2**, 90.75 ± 4.8 vs 43.83 ± 3.1 , $P < 0.05$).

Cx43 expression in myocardial infarction model

Immunofluorescence staining was performed to examine the expression of Cx43 and the results showed that there was less fluorescence from the myocardial tissues in the infarction model as compared to normal rats (**Figure 3A and 3B**); qRT-PCR showed that expression of Cx43 was significantly down-regulated at mRNA level in the models as compared to sham (**Figure 3C**).

Protection of myocardial tissue against chronic ischemic injury by irbesartan

We then evaluated the cardiac functions of rats and found that LVSP, LVEDP, $+dp/dt_{max}$, and $-dp/dt_{max}$ were significantly lower in infarction models than in sham group and significantly higher in irbesartan group than in infarction models except for VEDP (**Table 2**). Furthermore, mea-

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Table 2. Comparison of cardiac functions in rats receiving sham, infarction and irbesartan treatments

Group	n	HR (time/min)	LVSP (kPa)	LVEDP (kPa)	+dp/dt _{max} (kPa/s)	-dp/dt _{max} (kPa/s)
Sham	30	334 ± 13	12.8 ± 1.3	0.90 ± 0.9	437 ± 16	366 ± 26
Infarction model	30	402 ± 17	8.4 ± 0.8*	1.57 ± 0.3	198 ± 27**	247 ± 12*
Irbesartan	30	320 ± 7	10.4 ± 0.9 ^Δ	1.16 ± 1.3	224 ± 45 ^Δ	308 ± 19 ^Δ

*, ** and ^Δ denote significant or highly significant difference vs sham or infarction model, respectively.

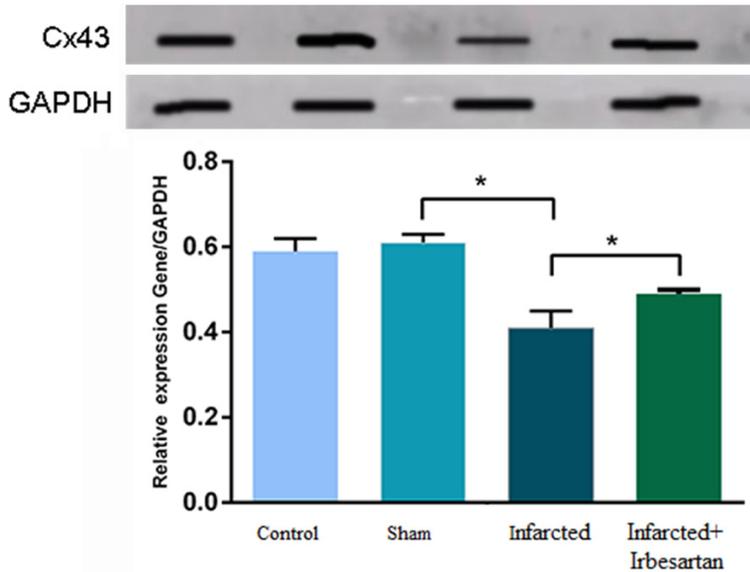


Figure 4. Effect of irbesartan on the expression of CX43 in cardiac tissue.

Measurements showed that cTnI content was higher in infarction model than in sham group (10.97 ± 1.28 ng/ml vs 6.37 ± 0.98 ng/ml). After irbesartan treatment, the content reduced to 7.65 ± 0.41 ng/ml, suggesting that irbesartan could protect myocardial tissue against chronic ischemic injury. We further examined the expression of Cx43 protein in the myocardial tissues and the results revealed that Cx43 expression was significantly lower in infarction models as compared with sham and the down-regulation was significantly less in irbesartan group than in infarction models (Figure 4).

Discussion

Gap junction connects the cytoplasm between adjacent cells and is an important channel for intercellular material and information exchange. It plays an important role in coordinating the biological processes in multicellular organisms. Studies have demonstrated that in diseased cardiac tissues gap junction is altered, leading to increased susceptibility to cardiac arrhythmia.

Cx43 is a 43 kD protein that exists in the intercalated discs in myocardial tissue and is associated with signal transduction between the myocardial cells. It is abundantly expressed in the heart tissue in mammals. In rat embryogenesis, it is expressed in the primitive atrium, ventricle and cardiac neural cells [15]. Davis et al showed that Cx43 is not expressed in sinoatrial node, but abundantly expressed in atrioventricular node and specifically in ventricular myocytes [16]. Thomas et al found that in the ventricular cells, Cx43 is the primary current conductor. A 50% reduction in Cx43 expression could result

in significant reduction in signal transduction. Meanwhile, in the atrium, no association has been found between Cx43 expression level and signal transduction [17], suggesting that Cx43 is the major Cx in the ventricle but not in atrium.

Our results show that the expression of Cx43 was significantly down-regulated in infarcted rats, as compared with control and sham rats. This is inconsistent with the early study [18]. Cx43 plays a role in regulating cardiac function by mediating the contraction of myocardial cells. During infarction, Cxs may undergo a number of changes in expression level, structure and function and these changes are influenced by the receptors in nerve, endocrine systems, inflammatory mediators, electrophysiological factors, leading to the reconstruction of gap junction proteins [19, 20].

Irbesartan is a common ARB drug clinically used to treat hypertension and congestive heart failure, and also shown to have anti-arrhythmia effect [10]. In this study, we treated infarcted rats with irbesartan to examine if the

inhibition of renin, angiotensin and aldosterone synthesis and up-regulation of Cx43 would prevent the myocardial tissue from ischemic injury. Although Cx43 expression was down-regulated in the infarction models as compared with the sham group, irbesartan intervention significantly reduced the down-regulation. Meanwhile, improved cardiac functions were observed after irbesartan treatment with reduced content of cTnI, suggesting that the improved cardiac functions are likely attributed to the alleviated down-regulation of Cx43, although further studies are needed to elucidate the molecular mechanisms underlying the effect.

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Disclosure of conflict of interest

None.

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