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Effect and mechanism of Irbesartan on occurrence of ventricular arrhythmias in rats with myocardial ischemia through connexin43 (cx43)

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ABSTRACT

Objective: To explore the effect and mechanism of angiotensin II receptor blockers – Irbesartan on occurrence of ventricular arrhythmias in rats with myocardial ischemia.
Methods: Rats with embryonic cardiomyocytes-H9c2 were randomly divided into control group, ischemia group, Irbesartan group and Irbesartan + ischemia group. The cell viability of rats in each group was tested using MTT. Real-time PCR was employed to detect the expression of connexin43 (Cx43) mRNA and western blot to detect the expression of Cx43 and phosphorylated Cx43. SD rats were randomly divided into the sham-operation group (SO), myocardial infarction group (MI), Irbesartan group and MI + Irbesartan group, with 10 rats in each group. HE staining was employed to observe the change in the pathomorphology of left ventricular tissue and TUNEL method to analyze the cell apoptosis in the tissue. The immunofluorescence was adopted to observe the expression and distribution of Cx43 in the left ventricular myocardium and study the change in the expression of Cx43 in the cardiac muscular tissue at mRNA and protein level.
Results: The intervention of Irbesartan in the condition of ischemia indicated the significant decrease in the number of necrotic cells. The expression of Cx43 was significantly decreased under the culture of ischemia ($P < 0.05$), but in the presence of Irbesartan, the expression of Cx43 was increased compared with the ischemia group ($P < 0.01$). The results of WB assay showed the similar trend of change at mRNA level. There was the significant difference in the score of ventricular arrhythmia between MI group and SO group ($P < 0.01$). The incidence of ventricular tachycardia or ventricular fibrillation was significantly increased compared with the one in SO group ($P < 0.05$). There was the significant difference in the overall score between MI + Irbesartan group and MI group ($P < 0.05$). The expression of Cx43 in the cardiac muscular tissue in MI group was significantly decreased ($P < 0.01$ *vs* SO group). But the expression of Cx43 was increased after the treatment with Irbesartan.
Conclusions: Irbesartan can inhibit the injury of H9c2 cardiomyocytes and the decreased expression of Cx43 that are induced by the ischemic myocardial infarction. Irbesartan can also improve the reconstruction of Cx43 in rats with ischemic myocardium to inhibit the myocardial infarction-induced arrhythmias.

1. Introduction

The ventricular arrhythmias refer to the cardiac arrhythmia that is rooted in the ventricle, as the common arrhythmia. Arrhythmias can be found in patients without the organic heart diseases and with the severe heart diseases because of myocardial ischemia. The previous research indicated that, in the condition of myocardial ischemia and hypoxia, the incidence of ventricular arrhythmias was significantly increased, which might even cause the death of cardiomyocytes. The death of cardiomyocytes because of myocardial ischemia was also the significant cause for the death of patients with cardiovascular diseases [1].

The connexin (Cx) is the family of membrane proteins that form the intercellular gap junction to play the role of signal communication. Cx was also of critical significance in regulating

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the growth, differentiation, apoptosis and tumor of cells [2]. According to the previous researches, there were abundant gap junctions between the cardiomyocytes and smooth muscle cells and the abnormal change in the gap connection could cause the cardiovascular diseases [3,4]. The connexin43 (Cx43) is one of connexins in the gap connection of interdiac structure of cardiomyocytes. The normal expression and distribution of Cx43 were of critical importance to maintain the normal function of myocardial connection and heart [5–7].

The Irbesartan is the receptor inhibitor of Angiotensin II (Ang II), which can inhibit the transformation of Ang I into Ang II. As the new long-acting angiotensin antagonist, it could be used in the clinical treatment of hypertension and congestive heart failure [8], which could increase the threshold of ventricular fibrillation during the ischemia and reduce the occurrence times and incidence of ventricular tachycardia and ventricular fibrillation because of myocardial ischemia [9,10]. The research also indicated that, Angiotensin II could induce the hypertrophy of cardiomyocytes and significantly reduce the expression of Cx43, which might be related to the change in the cell cycle during the myocardial hypertrophy. But there have been limited studies on the issue whether Irbesartan can affect the expression and distribution of Cx43 and thus improve the injury of cardiac muscular tissue so far. Therefore, it has the certain clinical significance to study the effect of Irbesartan on the expression of Cx43 and the mechanism of ventricular arrhythmias that is caused by myocardial ischemia.

In this study, based on the serum-free culture of rat cardiomyocytes-H9c2 and the modeling of myocardial ischemia, the expression of Cx43 in rats in the condition of myocardial ischemia was explored at the cellular and animal level. Besides, the effect of Irbesartan on the expression of Cx43 in the cardiac muscular tissue and its possible mechanism were discussed, in order to provide the reference for the prevention and reduction of ventricular tachycardia.

2. Materials and methods

2.1. Reagents and instruments

Rats with cardiomyocytes-H9c2 was purchased from ATCC and conserved in this laboratory; SPF (4–8) week male SD rats, with the weight of (200–230) g, were provided by Laboratory Animal Center of Nanchang University and approved by Medical Research Ethics Committee of The Second Affiliated Hospital to Nanchang University; Irbesartan tablets were purchased from Xiuzheng Pharmaceutical Group Co., Ltd.; DMEM medium from Hyclone (USA); the fetal bovine serum from GIBCO (USA); RNA extraction kit from QIAGEN (Germany); the reverse transcription kit from Applied Biosystems (USA); Real-time PCR fluorescence quantitative kit from Applied Biosystems (USA); ReadyPrep protein extraction kit from Bio-Rad (USA); MTT cell proliferation assay kit from Beyotime Biotechnology; protein assay kit from Vazyme Biotech (China); horseradish peroxidase (HRP) labeled secondary antibodies from Beijing Zhongshan Jinqiao Biotechnology; 0.22 μm PVDF membrane from Millipore (USA); ECL chemiluminescence assay kit from Millipore (USA); the immunofluorescence kit from Beyotime Biotechnology; Tunel assay kit from Vazyma (USA); rabbit anti-rat CX43, p-CX43 and β-actin monoclonal antibody from Abcam (UK).

The animal ECG measurement and analysis system was ECGenie; the fluorescence inverse microscope was Beckman; the quantitative protein and nucleic acid analyzer was Nanodrop 2000; the fluorescence quantitative PCR was Applied Biosystems 7500; XD-2A heart diagnosis and treatment instrument was purchased from Suzhou Dongfang Instrument Factory.

2.2. Methods

2.2.1. Cell culture and grouping

After the recovery of rat cardiomyocytes-H9c2, the cells were resuspended in DMEM medium and then put in the incubator at 37 °C and with 5% CO_2 for the culture. Cells were randomly divided into the control group, ischemia group, Irbesartan group and Irbesartan + ischemia group. Forty-eight hours after the serum-free culture, MTT was employed to detect the cell viability. After extracting the total RNA, Real-time PCR was used to detect the expression of Cx43 at mRNA level. BCA assay was adopted to measure the concentration of total protein and detect the expression of Cx43 and phosphorylated Cx43.

2.2.2. Real-time PCR

The total RNA was extracted by RNA extraction kit and then transcripted reversely into cDNA. Real-time PCR was employed to detect the expression of related genes. The mRNA sequence of gene could be referred to NCBI database and then the Real-time PCR primers could be designed. All primers were synthesized by Shanghai Sangon Biotech, with the specific sequence shown as follows: Cx43 (NM_010288.3): For: ACAAGGTCCAAGCCTACTCCA; Rev: CCGGGTTGTTGAGTGTTACAG, GAPDH (NM_008085): For: AATGGATTTGGACGCATTGGT; Rev: TTTGCACTGGTACGTGTTGAT. The double $^{\triangle\triangle}$Ct method was adopted to calculate the relative expression of target gene: the mean of three repeats was treated as the CT value of each sample, $^{\triangle}$CT = CT (Target Gene) – $^{\triangle}$CT (reference), $^{\triangle\triangle}$CT = $^{\triangle}$CT (sample) – $^{\triangle}$CT (contro1). Therefore, the relative expression of target gene = $2^{-\triangle\triangle CT}$ and the relative expression of control group was 2^0 = 1 [11]. The PCR system (20 μL) was: 2×SYBR Fast qPCR Mix: 10 μL; PCR Forward/Reverse Primer (10 mM): 1 μL; cDNA template: 2 μL. The reaction parameters were: Holding Stage 95 °C for 33 s, Cycling Stage 95 °C for 3 s, 60 °C for 12 s, Cycles = 40.

2.2.3. Western blot

ReadyPrep protein extraction kit was used to extract the total protein and BCA kit to detect the concentration of protein. After SDS-PAGE electrophoresis for 20 μg total protein samples, the gel was transferred into membrane. The primary antibody with the appropriate degree of dilution was added for the incubation at the room temperature for 2 h. The membrane was incubated with the secondary antibody that was diluted with TBS/T (1:10 000, HRP-labeled) at the room temperature for 1 h. It was exposed and then photographed to save the experimental results. Quantity one v4.62 was used to measure the gray value of molecular band (trace tracking). The semi-quantitative value of target protein/reference protein was treated as the quantitative foundation. The statistical analysis was performed as well.

2.2.4. Modeling and grouping of rats with chronic myocardial ischemia [12]

The laboratory animals were fed in the standard animal cage, with 5 rats in each cage. Rats were given the diet and drinking

freely during the experiment, with the good ventilation in the feeding room and natural lighting day and night. The room temperature was maintained at (18–25) °C, with the humidity of 50%–60%. There were randomly divided into the sham-operation group (SO) (10 rats), with the threading below the left anterior descending (LAD) and without ligation; myocardial ischemia group (MI) (10 rats); Irbesartan group (10 rats); and myocardial ischemia + Irbesartan group (MI + Irbesartan) (10 rats). Rats were given the intraperitoneal injection of 2% chloral hydrate for the anesthesia and then the endotracheal intubation was connected to the ventilator [tidal volume of (4–8) mL, respiratory ratio of 5:4 and respiratory frequency of 90 times/min]. After finding the 3rd and 4th ribs at the left side of breastbone, the chest was opened to expose the heart. The middle and upper 1/3 part of left coronary artery was sutured. The change in ECG characteristics was taken as the index to confirm the successful replication of model. After the operation, rats were given the irrigation of Irbesartan at the dose of 30 mg/kg/d. Rats in the control group were given the irrigation of double distilled water at the same dose and fed with the normal diet for 12 weeks.

The heart tissue was separated and the vessels at the atrioventricular junction were cut off, with the free wall of atrial myocytes. Only the cardiac muscular tissue of left ventricular and left ventricular septum was remained. The cardiac muscular tissue was cut along the longitudinal direction of muscle fiber. Part of cardiac muscular tissue was fixed with formaldehyde, embedded in paraffin and stained with HE. Part of cardiac muscular tissue was stored in −80 °C refrigerator. The immunofluorescence assay was performed according to the instruction manual of assay kit. The fluorescence microscope was employed to observe the specific fluorescence intensity of samples. The preparation and staining of TUNEL detection liquid were performed according to the instruction manual of assay kit. Image-Pro Plus 6.0 was employed to count the positive cells.

2.2.5. Echocardiographic assessment

Before the intervention and 12 weeks after the intervention of Irbesartan, 10% chloral hydrate was used for the anesthesia. The color Doppler ultrasonic diagnostic apparatus (high-frequency probe) was employed to detect the Left ventricular posterior wall thickness, interventricular septum thickness at end-systole, left ventricular posterior wall thickness at end-diastole and interventricular septum thickness at end-diastole. The mean of five continuous cardiac cycles was taken. The occurrence times of ventricular arrhythmias for rats in three groups were monitored 15, 30, 45, 60, 75 and 90 d after the drug administration. The rats with myocardial ischemia were selected and then they were monitored by the continuous electrocardiogram to observe the occurrence times of ventricular arrhythmias after the modeling of myocardial ischemia, including the ventricular premature beat (VP), ventricular tachycardia (VT) and ventricular fibrillation (VF). The ventricular tachycardia was defined as the continuous 4 VPs or more. The arrhythmia was scored [13]: ventricular premature beat (VP) 0 point: VP = 0 time; 1 point: 0 < VP ≤ 10 times; 2 points: 10 < VP ≤ 50 times; 3 points: VP > 50 times; ventricular tachycardia (VT) −4 points: VT = 1 time; 5 points: 2 ≤ VT ≤ 5 times; 6 points: VT > 5 times; ventricular fibrillation (VF) −7 points: VF = 1 time; 8 points: 2 ≤ VF ≤ 5 times; 9 points: VF > 5 times.

2.3. Data analysis

The data was treated with SPSS 11.5 and the results were expressed by mean ± SD. The t test was employed for the comparison between groups and $P < 0.05$ indicated the statistical difference.

3. Results

3.1. Effect of Irbesartan on expression of Cx43 in ischemic cardiomyocytes

H9c2 cardiomyocytes were randomly divided into the control group, ischemia group, Irbesartan group and Irbesartan + ischemia group. Eight hours after the serum-free culture, the phase-contrast microscope was used to observe cells. It could be indicated that, due to the deficiency of serum, the cellular morphology was changed and the intercellular adhesion was disappeared, showing the cell vacuolation. But the cells in the control group had the clear boundary and normal morphology. After the intervention of Irbesartan in the condition of ischemia, the number of necrotic cells was significantly decreased. The results of MTT assay also indicated that the cell viability in the ischemia group was lowest, while the cell viability in the Irbesartan group was increased by 44.19%. Real-time PCR was employed to detect the expression of Cx43 in mRNA. In the condition of ischemia culture, the expression of Cx43 was significantly decreased ($P < 0.05$); while in the presence of Irbesartan, the expression of Cx43 was increased compared with the ischemia group ($P < 0.01$). The western blot was used to detect the expression of Cx43 and phosphorylated Cx43. The results showed the similar trend of change at mRNA level, namely the expression of Cx43 could be increased in the presence of Irbesartan. Besides, the expression was also increased to the certain extent at the phosphorylation level, with results shown in Table 1.

Table 1
Effect of Irbesartan on expression of Cx43 in ischemic cardiomyocytes (mean ± SD).

Group	OD value	CX43/GAPDH (mRNA)	CX43/GAPDH (protein)	p-CX43/CX43
Control	0.78 ± 0.08	1.00 ± 0.10	0.81 ± 0.08	0.78 ± 0.07
Irbesartan	0.80 ± 0.08	1.18 ± 0.11	0.87 ± 0.08	0.83 ± 0.08
Ischemia	0.43 ± 0.04**	0.28 ± 0.02**	0.42 ± 0.04**	0.40 ± 0.04**
Irbesartan + Ischemia	0.62 ± 0.06##	0.63 ± 0.06##	0.70 ± 0.07##	0.67 ± 0.07##

**$P < 0.01$ compared with control group; ##$P < 0.01$ compared with ischemia group.

3.2. Irbesartan to improve the ventricular arrhythmias and expression of Cx43 in rats with ischemic myocardium

A total of 40 male SD rats were randomly divided into the SO group, MI group, Irbesartan group and MI + Irbesartan group, with 10 rats in each group. The change in ECG characteristics was taken as the index to confirm the model of myocardial ischemia. The ECG characteristics of rat model of chronic myocardial ischemia had the abnormal decrease according to the results of conductive poles of I, II, III, aVR, aVL

and aVF ≥0.05 mV, which could confirm the successful modeling of myocardial ischemia.

After 12 weeks of treatment with Irbesartan, HE staining was employed to observe the change in the pathological morphology of left ventricular tissue. SO group showed the orderly arrangement of cardiac muscle fibers, with the clear horizontal stripes. But MI group showed the patchy necrosis of cardiomyocytes, karyopyknosis and karyorrhexis, unclear or disappeared horizontal stripes and diffuse infiltration of polymorphonuclear neutrophils. After 12 weeks of treatment with Irbesartan, the cardiac muscle fibers tended to be orderly and the infiltration of polymorphonuclear neutrophils was decreased.

The cardiac muscular tissue was separated in each group and the tissue sections were prepared. TUNEL method was employed to analyze the myocardial necrosis after the myocardial ischemia. The staining was performed according to the instruction of kit and Image-Pro Plus 6.0 was used to count the number of positive cells, where SO group: 18.24 ± 0.15; Irbesartan group: 16.27 ± 1.59; MI group: 103.66 ± 12.35; MI + Irbesartan group: 53.64 ± 7.06. There was the significant difference between MI group and SO group, MI + Irbesartan group and MI group ($P < 0.05$), which could indicate that Irbesartan could significantly inhibit the myocardial necrosis of rats with MI.

The echocardiography was employed to measure the cardiac function. The rats with myocardial ischemia were selected and then they were monitored by the continuous electrocardiogram to observe the occurrence times of ventricular arrhythmias after the modeling of myocardial ischemia, including VP, VT and VF. The ventricular tachycardia was defined as the continuous 3 VPs or more, with results shown in Table 1. There was the significant difference in the score of ventricular arrhythmia between MI group and SO group ($P < 0.01$). The incidence of ventricular tachycardia or ventricular fibrillation in MI group was up to 80% (8/10), which was significantly increased compared with SO group ($P < 0.05$). There was the significant difference in the total score between MI + Irbesartan group and MI group ($P < 0.05$), with results shown in Table 2.

Table 2
Score of ventricular arrhythmia.

Group	VP	VT	VF	Total
SO	1.28 ± 0.68	3.28 ± 0.38	5.66 ± 0.58	10.09 ± 0.93
Irbesartan	0.34 ± 0.01	2.19 ± 0.28	4.57 ± 0.68	6.93 ± 0.61
MI	3.03 ± 0.36**	6.09 ± 0.27**	9.03 ± 0.31**	18.01 ± 0.23**
MI + Irbesartan	3.01 ± 0.15##	4.14 ± 0.35##	7.65 ± 0.61##	14.78 ± 0.21##

*$P < 0.05$ compared with SO group; **$P < 0.01$ compared with SO group; ##$P < 0.05$ compared with MI group.

The immunofluorescence was employed to detect the expression of Cx43 in the cardiac muscular tissue of left ventricle. The results indicated that the distribution of Cx43 in SO group showed the end-to-end connection of cardiomyocytes that was perpendicular to the long axis of cardiac muscle fibers. In the ischemia group, the discontinuous distribution of Cx43 showed the side-to-side connection that was parallel to the long axis of cardiac muscle fibers. The myocardial ischemia could lead to the change in the distribution of Cx43. As shown in Table 3, the expression of Cx43 in the cardiac muscular tissue was detected at mRNA and protein level respectively. The expression of Cx43 in MI group was significantly decreased. But the expression of Cx43 was increased after the treatment with Irbesartan, with the results shown in Table 3.

Table 3
Effect of Irbesartan on expression of Cx43 in ischemic cardiac muscular tissue (mean ± SD).

Group	CX43/GAPDH (mRNA)	CX43/GAPDH (protein)
SO	0.72 ± 0.07	0.89 ± 0.09
Irbesartan	0.79 ± 0.08	0.32 ± 0.03
MI	0.29 ± 0.03**	0.93 ± 0.09**
MI + Irbesartan	0.67 ± 0.06##	0.60 ± 0.06##

*$P < 0.05$ compared with SO group; *$P < 0.01$ compared with SO group; ##$P < 0.05$ compared with MI group.

4. Discussion

The cardiovascular diseases refer to the major diseases that threaten the health of human life. The injury of cardiomyocytes that is caused by the myocardial ischemia and hypoxia is recognized as the focus in the cardiovascular diseases. The animal model of myocardial ischemia is the key material to study the cardiovascular diseases. The most common method in the animal model of myocardial ischemia is the ligation of coronary artery, generally the ligation of left anterior descending. Such method has been widely applied because of the easy operation. But there is the high mortality due to the ligation of coronary artery during the modeling. For instance, the success rate of modeling in this study was about 90%. Rats were suddenly died of the surgical infection and heart arrest.

Ang II is a key enzyme in the renin-angiotensin system, with the strong activity of vasoconstriction. It could be bound with the receptor of Ang II to promote the vasoconstriction and secretion of aldosterone to cause a great number of physiological responses [14,15], which plays a key role in the pathogenesis of hypertension, arterial diseases, cardiac hypertrophy, heart failure, diabetes and kidney diseases. The Ang II receptor inhibitors such as Irbesartan could inhibit the transformation of Ang I into Ang II, specifically antagonize the conversion of angiotensin into AT1 receptor and stop the bounding between Ang II and ATl receptor to mitigate the myocardial injury [16,17]. It has the functions of resisting the ischemia arrhythmias, relieve the myocardial stunning and reduce the infarction size. According to the results of pathomorphology in this study, the sham-operation group showed the orderly arrangement of cardiac muscle fibers, with the clear horizontal stripes. But the ischemia model group showed the severe injury of tissue, unclear cell boundaries, broken muscle fiber, disappeared horizontal stripes and infiltration of polymorphonuclear neutrophils. After 12 weeks of treatment with Irbesartan, the cardiac muscle fibers tended to be orderly with the clear boundaries and the infiltration of polymorphonuclear neutrophils was decreased. The TUNEL method was employed to analyze the myocardial necrosis after the myocardial ischemia. The results indicated that the apoptosis of cardiomyocytes was significantly increased in rats with myocardial ischemia ($P < 0.05$). The intervention of Irbesartan could inhibit

the injury of cardiac muscular tissue that was caused by the ischemia. The pathways that were involved in the process of Irbesartan to inhibit the apoptosis of cardiomyocytes included Bcl-2, bax, Fas/FasL and p-53 [18].

The previous researches indicated the change in the gap junctions in many pathological cardiac tissues and such change was related to the susceptibility of arrhythmias. The myocardial gap junction is also named as the communication junction, which is composed of Cx. There are mainly three kinds of Cx in human body, namely Cx43, Cx40 and Cx45 [19,20]. In this study, it also found the significant decrease in the expression of Cx43 in the condition of ischemia culture ($P < 0.05$); while the expression of Cx43 was significantly increased in the presence of Irbesartan compared with the ischemia group ($P < 0.01$). It indicated the significant change in the expression of Cx43 in the process of cardiomyocytes injury because of ischemia. The expression of Cx43 in the cardiac muscular tissue was detected at mRNA and protein level respectively. Where, the expression of Cx43 in MI group was significantly decreased ($P < 0.01$ *vs* SO). After the treatment with Irbesartan, the expression of Cx43 was increased. Cx43 may be involved in the inhibition of injury because of myocardial ischemia by Irbesartan. The immunofluorescence was employed to detect the expression of Cx43 in the cardiac muscular tissue of left ventricle. The results indicated that the distribution of Cx43 in SO group showed the end-to-end connection of cardiomyocytes that was perpendicular to the long axis of cardiac muscle fibers. In the ischemia group, the discontinuous distribution of Cx43 showed the side-to-side connection that was parallel to the long axis of cardiac muscle fibers. The myocardial ischemia could lead to the change in the distribution of Cx43. According to the study at the cellular level, the expression of Cx43 was increased in the presence of Irbesartan. Besides, the expression of Cx43 was also increased to the certain extent at the phosphorylated level, which indicated that in the condition of myocardial ischemia, the activity of Cx43 was inhibited and thus its subcellular localization was changed. In the process that Irbesartan inhibited the injury of cardiomyocytes through Cx43, it plays the key role of regulation in the expression of Cx43 and its activation. But the specific molecular mechanism has not been clear yet.

In the study of arrhythmia, the echocardiography was employed to measure the cardiac function. The rats with myocardial ischemia were selected and then they were monitored by the continuous electrocardiogram to observe the occurrence times of ventricular arrhythmias after the modeling of myocardial ischemia. The results indicated that there was the significant difference in the score of ventricular arrhythmia between MI group and SO group ($P < 0.01$). The incidence of ventricular tachycardia or ventricular fibrillation in MI group was up to 80%, which was significantly increased compared with SO group ($P < 0.05$). There was the significant difference in the total score between MI + Irbesartan group and MI group ($P < 0.05$). It indicated that the intervention treatment of Irbesartan can reduce the occurrence of arrhythmia.

Conflict of interest statement

We declare that we have no conflict of interest.

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