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The Effects of Rho-associated Kinases in Rats with Acute Myocardial Infarction by Fasudil and its Protection of the Myocytes*

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ABSTRACT Objective: To analyze the expression of Rho kinases and cardiac myocyte apoptosis status on rats of AMI, to Explore the effect of fasudil on the Rho kinase expression, try to find the protective effect on myocardia of Fasudil after AMI. **Methods:** Male Wistar rats were randomly divided into three groups: the treatment group, the AMI group and the sham-operated group. The treatment group and the AMI group clamped left anterior descending branch of the coronary artery. The sham group consisted of rats undergone a suture below the left anterior descending branch without clamping of the vessel. Rats in the treatment group received intra-peritoneal injections of 5mg/kg of Fasudil twice a day, Both the AMI group and The sham-operated groups received an equivalent normal saline injections only. All rats were sacrificed one week after. Tests were performed as follows: confirmation of areas of myocardial infarction and ischemia by double staining with EvensBlue and NBT, analysis of mRNA expression of Rho kinases by RT-PCR, TUNEL assay to determine the AI in ischemia region, measurement of changes of the expression of apoptosis related proteins, bcl-2 and bax, by immunohistochemistry. **Results:** One week after,compared to the sham-operated group, in the AMI group, there was significant increase in the expression of Rho kinase mRNA and apoptosis related protein bax and The AI, whereas the expression of bcl-2 was decreased ($P<0.01$). Compared to the AMI group, in treatment group ,the area of infarction was significantly smaller($P<0.05$), there was significant decrease in the expression of Rho kinase mRNA and bax and The AI, while the expression of bcl-2 was increased ($P<0.01$). **Conclusion:** After AMI of Rats, the expression of Rho kinase increased accompanied with the AI. Administration of Fasudil after AMI for 1 week could decrease myocyte apoptosis by inhibit the Rho/Rock signaling pathway, and therefore protect the myocardia.

Key words: Acute myocardial infarctio; Fasudil; Rho kinase; Apoptosis

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Introduction

Research confirmed that both have myocardial cell necrosis and apoptosis after the AMI, therefore apoptosis mechanism involved in the myocardial cell injury of myocardial infarction [1]. Myocardial protection after AMI and prevention of left ventricular remodeling and heart failure are still a major research interest in the field of cardiovascular diseases. A number of studies have found that Rho kinase activity was elevated after AMI. Fasudil can reduce the incidence of apoptosis by reducing the activity of JNK kinase and AIF factor [2]. Fasudil is a specific inhibitor of Rho kinase. Studies revealed the relationship between apoptosis and AMI, currently, it is considered that post-AMI apoptosis is an important factor for left ventricular remodeling[3]. The animal experiments also have proved that 4 weeks after AMI, intraperitoneal injection of Fasudil 5 mg/ kg seems to protect the ischemic myocardia, and reduce the size of infarction[4]. Our current study aimed to find whether administration of Fasudil after AMI for 1 week could provide the similar effects.

1 Materials and Methods

1.1 Materials

92 Male Wistar rats were provided by Drug Testing Qingdao. Main reagents: Fasudil is provided by Pharmaceutical Co., Ltd. Tianjin Hongri, China. RNA extraction and RT-PCR kits are bought from Biological Engineering Co., Ltd. Hangzhou Bori, China. Tunel apoptotic monitoring kit, Antibodies of both Bax and Bcl-2 are bought from Biological Engineering Co., Ltd. Wuhan Boster, China.

1.2 Methods

1.2.1 Animal Models To establish the AMI model, male Wistar rats with body weights between 220 g-270 g were subjected to coronary artery ligation using a modified method [1]. AMI were considered when there was a significant ST elevation in the ECG. The myocardia became pale in the distal part of the ligation, and the ventricular contraction was decreased. If the criteria were not met, coronary ligation was repeated. The sham-operated group consisted of rats undergone a suture below the left anterior de-

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scending branch without clamping of the vessel.

1.2.2 Groups Rats that survived the first 24 hours were randomly assigned to the treatment group (n=36) or the AMI group (n=36). Another 20 rats were recruited to the sham-operated group. Rats in the treatment group received intra-peritoneal injections of 5 mg/kg of Fasudil, while both the AMI group and the sham-operated group received equal volumes of normal saline injections only for 1 week.

1.2.3 Determination the size of myocardial infarction After 1 week, to make the heart arrest in diastole with potassium chloride. Confirmation of areas of myocardial infarction and ischemia by double staining with EvensBlue and NBT in accordance with the method of literature [5]. NIH pmgram image analysis software is used to measure the infarction area, and calculated the ischemic area and infarct size according to the formula [6]. The remaining myocardium was divided into two parts. One part was stored in -70℃ refrigerator, using for extracting RNA. The other part was put into 10% formaldehyde for immunohistochemistry.

1.2.4 Determination the mRNA expression of Rho kinase 50mg of cardiac tissue was used Total RNA was extracted according to the instructions of kit. By RT-PCR, the extracted RNA was reversely transcribed into cDNA, using AMV reverse transcriptase. Then PCR amplification was undergone with the Taq enzyme with the template of cDNA. PCR specific primers were designed and synthesized by the Shanghai Biological Engineering Technology Co.Ltd.

① Amplification of β-actin gene (493 bp). The upstream primer sequence was 5-TGTTTGAGACCTTCAACACCCC-3, the downstream primer sequence was 5-ACGTCACACTTCATGATGGAATTGA-3. ② Amplification of Rho kinase gene (297 bp). The upstream primer sequence was 5-GGTGATGGCTATTATGGACGAGA -3, while the downstream primer sequence was 5 - TCGGAGCGTTTCCCAAGC -3. Amplified the genes in the following conditions: pre-denatured 3min at 94℃, denatured 30 s at 94℃, annealed 30 s (Temperature of Rho kinase was 58 ℃, β-actin 55 ℃), extended 45 s at 72℃, cycled 35 times, undergoing terminal extension 5min at 72℃ to terminate the reaction. Electrophoresis 10μl PCR products in the 1% agarose gel, and then analyzed the results by gel imaging system to calculate the Rho kinase mRNA expression, with the β-actin as an internal reference.

1.2.5 The detection of myocardial cell apoptosis Application of TUNEL in situ tag apoptotic cell nucleus. Each specimen taked three different parts of the slice, to count the positive apoptosis cell nucleus of 5 horizons at high magnification of each slice /the total number of nuclei, the AI is the average .

1.2.6 Observe the expression of the apoptosis-related protein Bax and Bcl-2 by immunohistochemistry All specimen were fixed in formalin, embedded in paraffin, and cut into 2μm slicesThe. immunohistochemical staining used the SP method according to the kit instructions. The Bax and Bcl-2 protein appeared as yellow or brown granules of diffuse distribution, located in the membrane or cytoplasm under the microscope. Each slice was randomly selected five high power fields, and the Image Pro Plus analysis system was used for computer image analysis. The total area of the positive points of each slice vision and the integrated optical density were calculated. The average optical density by the semi-quantitative analysis indicated the protein expression intensity of Bax, Bcl-2.

2 Statistical analysis

The SPSS 19 statistical software was used for data processing. Differences in qualitative data were compared using the Chi square test. Quantitative parameters between groups were assessed using Student's t test. The multiple comparisons among groups used q test, which was one of the single factor analysis of variance. A p value of less than 0.05 was considered statistically significant.

3 Results

3.1 Living conditions of rats

After AMI surgery, 7 of these rats died in the subsequent 1-week intervention period, of which 3 rats were from the treatment group, 4 rats from the AMI group. The mortality rates were 8.3% and 11%, respectively. There was no statistical difference. In the sham-operated group, no rats died. Finally, the number of rats in the treatment group was 33, the AMI group was 32 and the sham-operated group was 20, which was used for the data analysis.

3.2 Comparison of myocardial ischemia area and infarct size and AI

Compared with the AMI group, there was no significant difference in ischemic area, but the infarct size reduced significantly in the treatment group. The difference was significant ($P<0.05$). The AI was significant decreased ($P<0.01$).

Table 1 Comparison of ischemic area, infarct area and AI between the treatment group and AMI group(n=33, $\bar{x}\pm$ s, %)

Group	AI	Ischemic area	Infarct area
Treatment group	16.67± 2.23*	38.53± 2.34	27.32± 2.14*
AMI group	29.42± 3.86	39.97± 3.01	33.18± 3.27

Note: * Compared with the AMI group, $P<0.05$

3.3 The expression of Rho kinase mRNA and Bcl-2, Bax protein of Myocardial cells

Compared with the sham group, the expression of Rho kinase mRNA and apoptosis-related protein bax was significantly in-

creased in AMI group, while the bcl-2 expression was significantly decreased (P<0.01). Compared with the AMI group, the expression of Rho kinase mRNA and apoptosis-related protein bax decreased (P<0.01)in the treatment group, while the bcl-2 expression increased (P<0.01).

Table 2 Comparison of the expression of Rho kinase mRNA and Bcl-2, Bax protein in the myocardial ischemia area of three groups($\bar{x}\pm s$)

Group	Rho kinase	Bax	Bcl-2
Treatment group	0.31± 0.027*#	0.91± 0.14*#	1.91± 0.29*#
AMI group	0.51± 0.045*	2.46± 0.37*	0.63± 0.22*
Sham group	0.42± 0.024	0.36± 0.05	3.02± 0.12

Note: compared with the sham group, P <0.01; # and the AMI group, P<0.01

4 Discussion

Rho is a Ras-related monomeric GTP enzyme. As a downstream effector of Rho, Rho kinase and Rho participate in a variety of biological activities of regulating cells[7]. Infarction size and myocardial remodeling is the two key factors influencing the prognosis of myocardial infarction [5]. Research confirmed that both have cardiomyocytes necrosis and apoptosis after the AMI [1], while the death of cardiomyocytes in the relative ischemic area surrounding the necrotic cells is related to the apoptosis. This indicates that apoptosis is also involved in the pathogenesis of infarction, affecting the infarct size [8]. This is an important factor of the myocardial infarction expansion, the reduced systolic function and an early remodeling of left ventricular [9]. Animal experiments confirmed that Rho kinase activity was elevated when myocardial remodeling occurs after myocardial infarction [10], which directly involved in the pathological process of cardiovascular disease and closely related to the development of cardiovascular disease [11]. This study confirmed that the level of the expression of Rho kinase mRNA elevated in the myocardial tissue surrounding the infarct area in AMI group, while application of fasudil for 1 week could reduce the expresssion of Rho kinase mRNA. Fasudil is a specific inhibitor of Rho kinase, Fasudil mainly competes with ATP the Rho kinase catalytic domain ATP binding site, thereby blocking the activation of Rho kinase [12], while Rho kinase can promote the expression of its mRNA [13]. This may be associated with that fasudil can inhibit the expression of Rho kinase mRNA.

Compared to the AMI group, this study found that ischemic cardiomyocytes apoptosis reduced and myocardial infarction area significantly decreased in treatment group, and the results consistent with literature reports [14]. Therefore fasudil protects the myocardial by decrease cardiomyocytes apoptosis [15]. Bcl-2 and Bax are recognized as the regulatory proteins closely related with apoptosis. Studies have shown that the low expression of Bcl-2 gene is closely related to the apoptosis of ischemic myocardial cells [16]. The immunohistochemistry showed that compared with sham-operated group the expression of bcl-2 decreased in the ischemic myocardium tissue of AMI group, while the expression of bax increased. Administration of Fasudil after AMI for 1 week, there was significant decrease in the expression of bax and The AI, while the expression of bcl-2 was increased, indicating that fasudil can reduce apoptosis and protect cardiomyocytes by affecting the expression of bcl-2 and bax. The results consistent with the reports of PAN Nana[4]. Studies also confirmed that the expression of bcl-2 was increased by inhibiting activity of Rho kinase during acute ischemia-reperfusion injury, and significantly reduce the apoptosis of cardiomyocytes[17].

The results showed that Rho kinase involves in various stages of apoptosis, and plays a key role in the occurrence and development of apoptosis[18]. Fasudil can reduce the incidence of apoptosis by reducing the activity of JNK kinase and AIF factor [2]. Recent studies have shown that down-regulation of ROCK1 and ROCK2 expression could suppresses the apoptosis of rat cardiomyocytes induced by hypoxia, the mechanism is associated with the inhibition of caspase-3 activation and the up-regulation of p-PI3K expression [19]. Caspase plays a central role in process of apoptosis, ROCK1 is substrate of direct cracking of activated Caspase-3, which have positive feedback effect to the apoptosis, PI3K be activated in ischemic preconditioning and ischemia-reperfusion injury, thereby inhibit apoptosis of cardiomyocytes, p - PI3K is the state of the activation of PI3K[14]. While the specific mechanisms of Rho kinase involves in apoptosis remain unclear. Fasudil could reduce the effect of myocardial apoptosis inducing factors on the myocardium by inhibiting the activation of neuroendocrine and powerful antioxidant[20], for the purpose of protecting the ischemic myocardium and reducing the infarct size.

This study confirmed that Fasudil could decrease the expression of Rho kinase mRNA and reduce apoptosis of cardiomyocytes in the ischemic myocardium tissue of MI, the infarct size reduced significantly, therefore Fasudil could prevent the remodeling and reduced systolic function of left ventricular. Fasudil is expected to become the new drug to improve the prognosis of MI.

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法舒地尔对急性心肌梗死大鼠心肌组织中 Rho 激酶的影响及心肌保护作用*

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摘要 目的：分析急性心肌梗死（AMI）后大鼠心肌组织 Rho 激酶表达的变化及心肌细胞凋亡情况，观察法舒地尔对急性心肌梗死（AMI）后大鼠心肌组织 Rho 激酶表达的影响，探讨法舒地尔对心梗后心肌的保护作用。**方法：**选取雄性 Wistar 大鼠，随机分为三组：治疗组、AMI 组、假手术组。治疗组及 AMI 组均结扎左前降支（LAD）制作 AMI 模型；假手术组只在其 LAD 下穿线不结扎。治疗组给予法舒地尔 5mg/kg，腹腔注射，每日两次；对照组和假手术组给予等量生理盐水。1 周后，EvensBlue 及 NBT 双染色确定缺血面积及梗死面积，RT-PCR 法测定 rho 激酶 mRNA 的表达，DNA 断裂的原位末端标记法（T UNEL 法）检测缺血区心肌细胞凋亡指数（AI），免疫组化测定凋亡相关蛋白 bcl-2 及 bax 表达的变化。**结果：**1 周后，AMI 组与假手术组相比，AMI 组大鼠 Rho 激酶 mRNA 表达增加（P<0.01），凋亡相关蛋白 bax 表达增加（P<0.01），bcl-2 表达减少（P<0.01），AI 明显增加（P<0.01）。治疗组与 AMI 组相比，梗死面积显著减小（P<0.05），Rho 激酶 mRNA 及 bax 表达显著减少，AI 显著降低，bcl-2 表达显著增加（均 P<0.01）。**结论：**大鼠 AMI 后，心肌组织中 Rho 激酶的表达增加，心肌细胞凋亡指数增加，连续应用法舒地尔 1 周能有效减少心肌细胞凋亡指数，起到心肌保护的作用。

关键词：急性心肌梗死；法舒地尔；Rho 激酶；细胞凋亡

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