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Original Article

Effect of RIP3 gene silencing on myocardial injury in acute myocardial infarction rats

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Abstract: This study aimed to investigate the effect of RIP3 gene silencing on myocardial injury in acute myocardial infarction rats. 30 AMI rat models were established by ligating left anterior descending coronary arteries. They were randomly divided into the model group and RIP3 silencing group. Another 15 rats were selected to establish the sham group as the control. The rats in RIP3 silencing group were injected with the adenovirus packaged with RIP3 silence plasmid via the heart ventricle. The rats in the control group and model group were injected with adenovirus packaged with empty vector. The levels of RIP3, RIP1 mRNA and protein in rat myocardial tissue in three groups were analyzed using RT-PCR and Western blot. The phosphorylation levels of RIP1 and RIP3 proteins were analyzed. The levels of TNF-α mRNA and protein in rat myocardial tissue were analyzed using RT-PCR and ELISA. The myocardial infarction sizes were analyzed using TTC staining. The levels of RIP3 mRNA and protein in rat myocardial tissue were significantly lower than those of control group and model group ($P<0.05$). Compared with the control group, the phosphorylation levels of RIP3 and RIP3 proteins in the model group were significantly increased, while the phosphorylation levels of RIP3 and RIP3 proteins in RIP3 group were significantly decreased ($P<0.05$). Compared with the control group, the levels of TNF-α mRNA and protein were significantly decreased in the model group, while the levels of TNF-α mRNA and protein were significantly decreased in RIP3 silencing group ($P<0.05$). Compared with the control group, the myocardial infarct size was significantly increased in the model group and RIP3 silencing group, while the infarct size was significantly decreased in RIP3 silencing group ($P<0.05$). In conclusion, RIP3 gene silencing mediated by adenovirus can significantly inhibit the cell necrosis pathway and reduce myocardial infarction tissue, which has a protective effect on myocardium.

Keywords: RIP3, acute myocardial infarction, gene silence, myocardial protection

Introduction

Acute myocardial infarction is on the basis of coronary atherosclerosis stenosis, coronary atherosclerotic plaque rupture due to some inducements, platelet aggregation on ruptured plaque surface, blood clot (thrombus) formation, so that the coronary artery lumen is suddenly blocked, resulting in myocardial ischemic necrosis [1, 2]. There are two forms of cell deaths in the acute myocardial infarction process, namely apoptosis and necrosis [3]. Study showed that myocardial apoptosis occurred in the early stage of myocardial infarction, and myocardial necrosis mainly in the middle and late stage of myocardial infarction [4]. Many patients did not receive the timely treatment after the attack of myocardial infarction, resulting in a large area of myocardial necrosis and irreversible myocardial function damage. Therefore, to effectively inhibit myocardial necrosis is of great significance to save the myocardial function of patients. The present study showed that cell necrosis was a process of gene regulation [5, 6]. Among them, RIP3 was a member of serine/threonine protein kinase families. It was a major cell stress sensing molecule, which played a major role in the regulation of cell survival, cell apoptosis and cell necrosis pathway [7, 8]. RIP3 was involved in the biological process of programmed cell death induced by tumor necrosis factor TNF-α, and was the key molecular switch of TNF-α induced cell apoptosis and necrosis different death pathways [9, 10]. After acute myocardial infarction, with the release of a large number of inflammatory factors, such as TNF-α, RIP3 pathway was activated [11]. Therefore, the pro-

tective mechanism of RIP3 on myocardial cells in acute myocardial infarction rats was analyzed through RIP3 silencing mediated by adenovirus in the study.

Materials and methods

Model establishment and grouping

SD rats, with the average weight of (200±17) g, were purchased from Henan Experimental Animal Center, Zhengzhou, China. 45 experimental rats were injected with 1 ml 100 g/L 10% chloral hydrate solution via intraperitoneal cavity. After anesthesia, the rats were in supine and fixed on the test bench. 24 G trocar as tracheal intubation was inserted into the trachea to assist breathing; the left chest median incision was made. The tissues were separated layer by layer. The thoracic cavity was cut open along the fourth intercostal space. The pericardium was separated to expose the left anterior descending coronary artery. The 6.0 needle with non-invasive thread was used to traverse the heart wall and ligate (The myocardial tissue below the ligation site color appeared shallower, which indicated the ligation was successful). The heart was quickly put back into the thoracic cavity [12]. The rats in the control group were treated with thoracotomy, the left coronary artery was not ligated. 30 model rats were randomly divided into the model group and RIP3 silencing group. The rats in the control group and model group were injected with adenovirus packaged with empty vector; the rats in RIP3 silencing group were injected with adenovirus packaged with RIP3 silencing plasmid via left ventricle. The rats were killed after treated for 96 h. The heart tissue was taken for the following experiments. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). Eighth Edition, 2010. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Huaihe Hospital of Henan University.

Real-time fluorescence quantitative PCR

0.1 g rat myocardial tissue in each group was removed, placed in liquid nitrogen and ground to powder in the mortar. Then 1 ml Trizol solution (TaKaRa, Dalian, China) was added. The RNA was extracted according to the kit instruction after mixing. Briefly, 200 μl chloroform solution was added in the above solution, mixed, quietly placed, delaminated and centrifuged at 12000 rpm for 10 min. The supernatant was transferred to isometric isopropanol solution and centrifuged at 15000 rpm for 10 min. The sediment was washed with 70% ethanol once and dissolved in DEPC treated double distilled water. RNA was reversely transcribed into cDNA using the reverse transcription kit (TaKaRa, Dalian, China).

According to TNF-α, RIP1 and RIP3 cDNA sequences, the real-time fluorescence quantitative PCR primers of RIP1 and RIP3 were designed. GAPDH was taken as the internal reference. The primer sequence was as follows: TNF-α-F: 5'-TCCCAACAAGGAGGAGAAGTTCC-3', TNF-α-R: 5'-GGCAGCCTTGTCCCTTGAAGAGA-3'; RIP1-F: 5'-GGGTGTTCATCCATTCTC-3', RIP1-R: 5'-CCCAGCATCTTGTGTTTC-3'; RIP3-F: 5'-GTGGGATGATGACGACG-3', RIP3-R: 5'-TACGACCAGAGGCATACAGG-3'; GAPDH-F: 5'-GCGGGAAATCGTGCGTGAC-3', GAPDH-R: CGTCATACTCCTGCTTGCTG-3'. Then PCR reaction system was designed as follows: 2*SYBR Green fluorescence quantitative PCR mix (Roche, Basel, Switzerland) 10 μl, upstream primers 1 μl and downstream primer 1 μl, cDNA template (1:200 dilution) 8.8 μl, total volume was 20 μl. PCR reaction was conducted according to the following conditions: pre-degeneration at 94°C for 30 s; degeneration at 94°C for 5 s; annealing at 94°C for 5 s; extension at 72°C for 40 s; 35 cycles, terminal extension at 72°C for 5 min. The reaction was terminated. The solubility curve was built. Finally, the data were read directly from the real-time fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA).

Western blot

The rats were killed. 0.1 g myocardial tissue was removed. The tissue lysate was added and ground using the grinding rod, quietly placed on ice so as to fully lyse cells. The protease inhibitor was added in the lysate and centrifuged for 15 min at 15000 rpm. 1*SDS loading buffer was added in the supernatant and boiled in the metal bath for 20 min. After high speed centrifugation of the sediment, SDS-PAGE was conducted. The protein was transferred to PVDF film by horizontal electrophoresis apparatus,

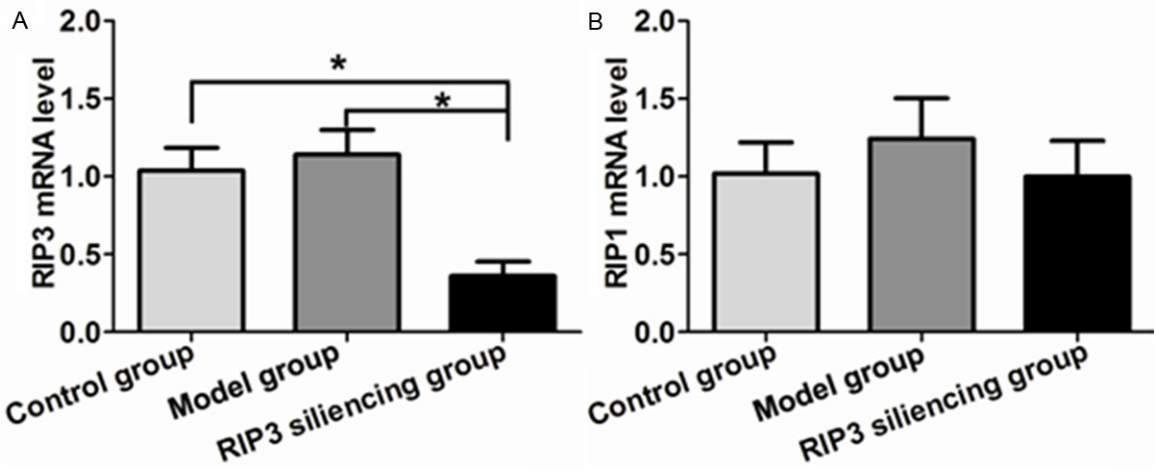

Figure 1. Comparison of RIP3 and RIP1 mRNA in myocardial tissue of rats in three groups. A. RIP3 mRNA expression level; B. RIP1 mRNA expression level.

closed for 30 min with 5% skim milk, coated with the primary antibody (Abcam, Cambridge, UK) overnight, washed with PBS for three times, 5 min/once, coated for 1 h with HRP conjugated sheep anti mouse secondary antibody (ZSGB-BIO, Beijing, China), washed for three times with PBS, 5 min/once. The luminescence solution was coated on PVDF film for developing. GAPDH was taken as the internal reference.

ELISA analysis

The rats were killed. 0.1 g myocardial tissue was removed. The tissue lysate was added and ground using the grinding rod, quietly placed on ice so as to fully lyse cells. The protease inhibitor was added in the lysate. The tissue was centrifuged for 15 min at 15000 rpm. The supernatant was collected for ELISA test. The experimental operation was strictly according to the kit instruction. 3 complex holes were set for each sample and standard substance. The OD value was measured at 492 nm using the microplate reader.

TTC staining

The rats in each group were killed. The heart was quickly removed and washed with PBS. The atrium and right ventricle were removed. The left ventricle was retained, quick-frozen at -20°C for 20 min. Then the tissue was cut into pieces using the freezing microtome. The thickness of each section was 2 mm. The sections were placed in preheated 37°C TTC staining fluid and incubated at 37°C away from light for 15 min. The myocardial tissue was turned over so as to ensure homogeneous staining, washed with PBS for three times. At this time, the infarction zone was white and non-infarct zone was deep red. The different staining areas were measured using the image processing software. Myocardial infarct size = (infarct size/myocardial area * weight of myocardial section)/left ventricular mass *100.

Statistical analysis

All data were analyzed using SPSS13.0 statistical software (SPSS Inc, Chicago, IL, USA). The enumeration data were expressed with X±S. The measurement data were compared using independent samples t-test. $P<0.05$ indicated that the difference was statistically significant.

Results

Comparison of RIP3 and RIP1 mRNA in myocardial tissue of rats in three groups

The effect of RIP3 silencing plasmid mediated by adenovirus on RIP3 mRNA in myocardial tissue was analyzed in the experiment. And its silencing specificity was analyzed with RIP1 as the control. As shown in **Figure 1A**, the level of RIP3 mRNA in myocardial tissue of rats in RIP3 silencing group was significantly lower than that of the control group and model group, the difference was statistically significant ($P<0.05$). The levels of RIP1 mRNA in myocardial tissue of

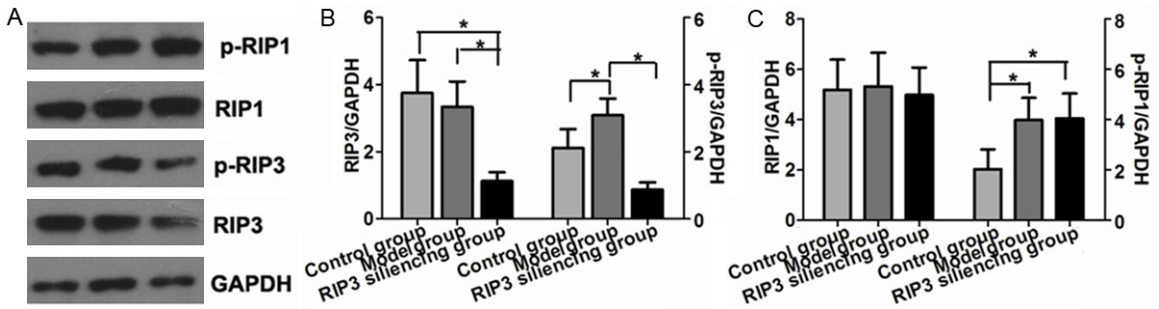

Figure 2. Comparison of RIP3 and RIP1 proteins in myocardial tissue of rats in three groups. A. Western blot show RIP3 and RIP1 protein level; B. Quantitative analysis show RIP3 protein expression level; C. Quantitative analysis show RIP1 protein expression level.

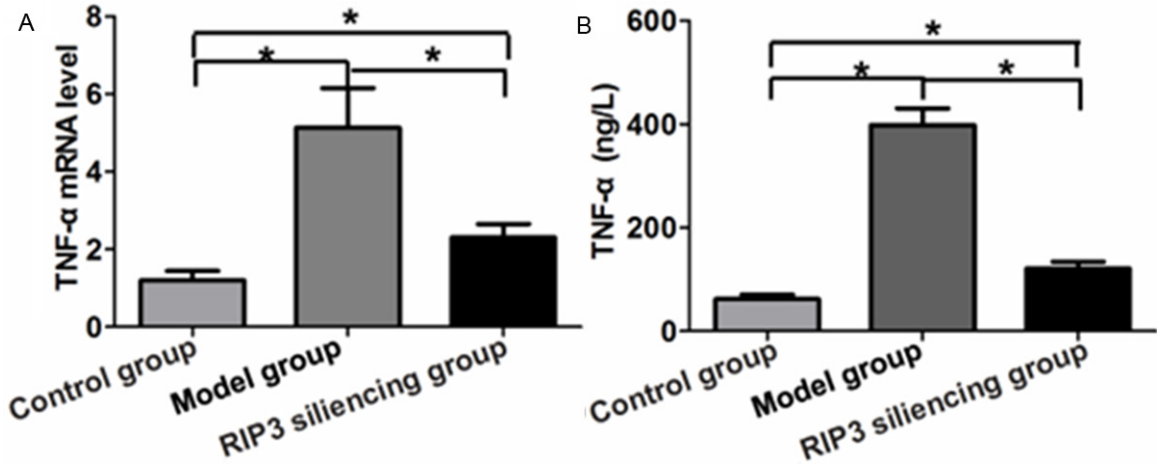

Figure 3. Comparison of TNF-α mRNA and protein in myocardial tissue of rats in three groups. A. TNF-α mRNA level; B. TNF-α protein level.

rats in three groups showed no statistical significance (P>0.05) (**Figure 1B**).

Comparison of RIP3 and RIP1 protein in myocardial tissue of rats in three groups

The levels of RIP3 and RIP1 protein in myocardium of rats in three groups were further analyzed in the experiment. The results were shown in **Figure 2A**, **2B**. Compared with the model group and control group, the level of RIP3 protein in myocardium of rats was significantly decreased in RIP3 silencing group. And RIP3 phosphorylation level was also significantly decreased (P<0.05). The level of total RIP3 protein was not increased in the model group, but its phosphorylation level was significantly higher than that of the control group (P<0.05) (**Figure 2A**, **2B**). There was no difference in total RIP1 protein level between the control group, model group and RIP3 group (P>0.05), while the expression level of RIP1 protein was significantly increased in the model group and RIP3 group (P<0.05) (**Figure 2A**, **2C**).

Comparison of TNF-α mRNA and protein in myocardium of rats in three groups

Compared with the control group, the levels of TNF-α mRNA and protein were significantly increased in the model group (P<0.05). The levels of TNF-α mRNA and protein in myocardial tissue of rats in RIP3 silencing group were significantly increased, but significantly lower than those of the model group (P<0.05) (**Figure 3**).

Comparison of infarct size in myocardial tissue of rats in three groups

The myocardial tissue manifested red in the control group after TTC staining. A large area of white ischemic area appeared in rats in the

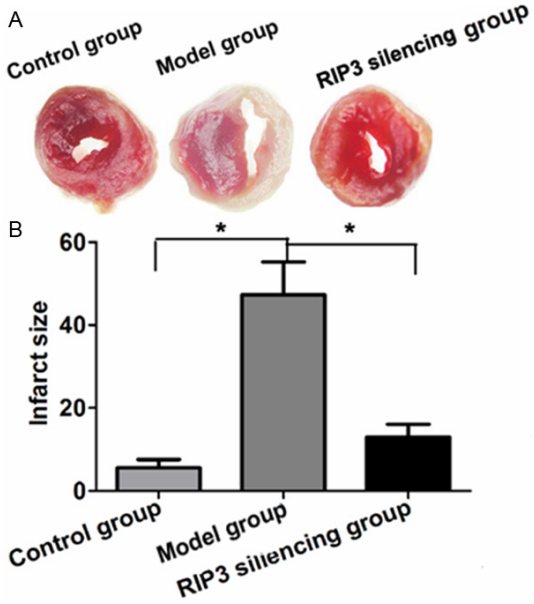

Figure 4. Comparison of infarct size in myocardial tissues of three groups. A. TTC show infarct size in myocardial tissues of three groups; B. Quantitative analysis show infarct size in myocardial tissues of three groups.

model group, while little white ischemic area appeared in myocardial tissue of rats in RIP3 silencing group. The quantitative analysis showed that the infarct size in the model group was significantly higher than that of the control group, and the infarct size in myocardial tissue of rats in RIP3 silencing group was significantly lower than that of the model group ($P<0.05$) (**Figure 4**).

Discussion

Cell necrosis is cell damage and death caused by extremely physical, chemical factors or severe pathological stimulation. It is considered that cell necrosis is a passive process for a long time. But in recent years, studies have shown that cell necrosis is regulated by related genes, which is known as programmed cell necrosis [13, 14]. Programmed cell necrosis was a series of sequential signal regulating processes triggered by death receptors such as tumor necrosis factor receptor (TNFR), Fas etc, and then mediated by receptor interaction of protease-1/3 (RIPl/RIP3) kinase. It appeared the morphology of cell necrosis, such as an increase in cell size, organelles swelling and absence of plasma membrane integrity [15]. Programmed cell necrosis could cause significant inflammatory response with a large number of inflammatory cells infiltration [16]. Acute myocardial infarction is a common cardiovascular and cerebrovascular disease. It manifested cell necrosis in the early stage of disease and a large area of cell necrosis in the middle and later periods. Once the myocardial tissue occurred to necrosis, the myocardial function would appear irreversible phenomenon, resulting in myocardial function dysfunction. Therefore, it is of great significance to effectively inhibit programmed cell necrosis for prolonging and protecting myocardial function.

RIP3 is a molecular switch of programmed cell necrosis and apoptosis. If stimulated by programmed necrosis, RIP1 and RIP3 could form programmed necrosis compounds. Their mutual phosphorylation could trigger programmed necrosis signal transduction pathway [17, 18]. Therefore, RIP3 gene silencing in myocardial tissue was mediated by adeno-associated virus as the vector in the study. The real-time fluorescence quantification PCR showed that the levels of RIP3 mRNA and protein in myocardial tissue of rats in RIP1 silencing group were significantly decreased compared with the control group and model group. The RIP3 gene silencing could not affect the levels of RIP1 gene mRNA and protein. This result laid the foundation for the following experiments. RIP3 and RIP1 formed RIPl-RIP3 programmed necrosis compounds and phosphorylated in the process of programmed cell necrosis [19]. The phosphorylation level of RIP3 protein was analyzed after RIP3 silencing using Western blot. The result showed that the phosphorylation level of RIP3 protein in RIP3 protein silencing group was significantly lower than that of the model group. The down-regulation of RIP3 protein level and phosphorylation would result in the inhibition of programmed cell necrosis pathway, thereby reducing the process of myocardial cell necrosis.

In addition, cell necrosis could release a large number of cellular contents, resulting in significant inflammatory response [20]. Our study showed that the levels of mRNA TNF-α mRNA and protein in myocardial tissue of rats in RIP3 silencing group were significantly decreased compared with the model group, which was consistent with the down-regulation of RIP3 protein. The myocardial infarction size was the

ultimate manifestation of severe myocardial infarction. TTC staining was a common method to identify myocardial infarct size [21]. Our study showed that showed that myocardial infarction size was significantly lower than that of the model group after RIP3 gene silencing using TTC staining method, indicating that RIP3 gene silencing significantly reduced myocardial infarction.

In conclusion, RIP3 gene silencing mediated by adenovirus can significantly reduce the levels of RIP3 protein and phosphorylation of rat myocardial tissue, and decrease the release of the inflammatory factors and myocardial infarction, which has a protective effect on myocardium.

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Disclosure of conflict of interest

None.

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