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Original Research

Effect of Cdc42 on myocardial ischemia-reperfusion of rats

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Abstract: To investigate the effects and their possible mechanisms of cell division cycle 42 (Cdc42) to neonatal rat myocardial cells subjected to the ischemia-repefusion. Neonatal rat cardiomyocytes were cultured and then subjected to the ischemia-reperfusion. Experimental groups 1. Control group; 2. Ischemia-repefusion group (I/R group); 3. Oligofectamine group (Oli group); 4. Oligofectamine and antisense oligodeoxynucleotide (AS-ODN) group (As group); 5. Oligofectamine and missense oligodeoxynucleotide (MS-ODN) group (Ms group); 6. SP600125 and Oligofectamine and AS-ODN group (SP600125/As group); 7. SP600125 and Oligofectamine and MS-ODN group (SP600125/Ms group). The cardiacmyocyte apoptosis rate was detected by AnnexinV/PI with flow cytometry. Cdc42, JNK, p-JNK, Bax and Bcl-2 were detected by western blot. In comparison with control group, Cdc42, the cardiacmyocyte apoptosis rate and phosphorylation of JNK were increased and the ratio of Bcl-2/Bax was reduced in the I/R group; Cdc42, the cardiacmyocyte apoptosis rate and phosphorylation of JNK in As group was lower than the I/R group, Oli group and the Ms group, and the ratio of Bcl-2/Bax was the highest in the four groups; Cdc42, cardiacmyocyte apoptosis rate, phosphorylation of JNK and the ratio of Bcl-2/Bax showed no differences in the I/R group, Oli group and the Ms group. Compared with As group, phosphorylation of JNK was lower in the SP600125/As group, phosphorylation of JNK in SP600125/Ms group was lower than the Ms group, and it showed no differences between the SP600125 & As group and the SP600125 & Ms group. Cdc42 in myocardial I/R can promote cardiacmyocyte apoptosis rate. AS-ODN of Cdc42 can decrease the cardiacmyocyte apoptosis rate in I/R. Cdc42 may played a role in myocardial I/R via JNK , Bcl-2 and Bax signal pathway.

Key words: Cell division cycle 42; Antisense oligodeoxynucleotide; Myocardial cells; Ischemia-repefusion; Apoptosis.

Introduction

Apoptosis is one of the important mechanisms of myocardial ischemia-reperfusion injury, thus the study on signal pathway of apoptosis in myocardial ischemia-reperfusion and the efforts for looking for targeted interventions play important roles on the prevention and treatment of myocardial ischemia-reperfusion injury. Cell division cycle 42 (Cdc42) is one kind of small G protein, which is involved in a variety of cell signal transduction, and activated G protein affects a series of important biological phenomena, such as cell growth, differentiation, apoptosis and cell cycle through acting on a series of substrate, which has a wide range of biological effects. Studies have shown that (1), in cerebral ischemia-reperfusion, Cdc42 can mediate apoptosis of brain cells through activation of c-Jun N-terminal kinase (JNK) in mitogen activated protein kinases (MAPKs). SP600125, which is involved in this study, is a reversible ATP competitive inhibitor with >20-fold selectivity and a range of kinases and enzymes tested. It can dose dependently inhibited the phosphorylation of c-Jun as well as expression lots of inflammatory genes such as COX-2, IL-2, IFN-, TNF-β (5). In this study, rat myocardial cells were used to establish the model of ischemia-reperfusion, and study the role and mechanism of small G protein Cdc42 in myocardial ischemia-reperfusion.

Materials and Methods

Experimental animals and materials

1-3 days newborn SD rats (provided by Xuzhou Medical College Animal Center); Collagenase I, pancreatic enzyme bought from Gibco Company; High sugar DMEM bought from Hyclone Company; Fetal bovine serum bought from Hangzhou Sijiqing Company; Cdc42 antisense oligonucleotide (ACTTAATTGTCTGCA), Cdc42 missense oligonucleotide (TGACTATTCCATATG)compounded by Shanghai Sangon; Bax (Sc526), Bcl-2 (Sc492), p-JNK (Sc6254) resistance bought from Beijing Zhongshan Jinqiao Biological Technology co., LTD.; SP600125 (s1876), JNK/SAPK (AJ518-1) bought from Blue Skies Biotechnology Research Institute; Cdc42 (AB4201) resistance bought from Millipore Company; Annexin V/PI apoptosis kit (AP101-60, Cluster technology limited biological packing) bought from Bivision Company; Oligofectamine Reagent bought from Invitrogen Company. This study was approved by the ethic committee of Department of internal medicine, Nanjing Medical University.

Rats myocardial cell culture

Fixed 1-3 days newborn Sprague-Dawley (SD) rats

were disinfected with iodine volts, then the chest were opened and hearts were taken, following with the hearts being put into iced bathed phosphate buffer saline (PBS) liquid, and washed twice. Then the myocardial was put into serum bottle with small rotor, and the myocardial tissue was cut into 0.5 mm x 0.5 mm x 0.5 mm size, washed with PBS liquid until the color of red blood cells were invisible, and was added to 0.08% trypsin and 0. 08% collagenase I solution. 4-5 min later, 37 ℃ water bath was dumped onto the magnetic stirrer, supernatant was assimilated and digestion was terminated in 20% fetal bovine serum, and all the steps above were repeated until most of myocardial tissue digestion was finished. Then the residue was filtered with 200 purpose filter, collected into centrifuge tube. Supernatant was abandoned after centrifugation. Weight dropped cells were added with 20% fetal bovine serum shaken for 2min, and vaccinated on culture plate. Transfection was performed after 1h (differential sticking wall) and cells were put into new cultivating orifice, added with bromine deoxidization uracil nucleoside (Brdu) (0.1mol/L), and cultured in cultivating box of 37 ℃, 5 %CO_2 (3,4).

Liposome transfection Cdc42 antisense oligonucleotide (missense)

The double antibody was removed and was transfected by antisense oligonucleotide (missense) with concentration of 200 nmol/L (reagent recommended concentration), then was put into one hole of the 96-well plates. 10 μL 20 μmol/L antisense (missense) oligonucleotides deoxyribonucleotide was added into 175 μL serum-free Opti - MEM I medium, gently blended. And 4μL Oligofectamine liposomes was collected and put into 11 μL serum-free Opti - MEM I medium, then incubated for 5 ~ 10 min with indoor temperature. Then the mixtures were incubated for 15 ~ 20 min with indoor temperature; and cell culture medium was discarded, washed with serum-free medium twice. 4h after adding 60% fetal bovine serum culture medium, the incubation was conducted for 0h to 72h follwing with simulation of ischemia-reperfusion.

Experiment grouping and SP600125 joining

7 groups were invole in this study, Control group (control group): normal training; I/R group: no transfection but simulation of myocyte ischemia-reperfusion; Oli group: simulation of myocyte ischemia-reperfusion with only Oligofectamine liposomes; AS group: simulation of myocyte ischemia-reperfusion with antisense Oligonucleotides deoxyribonucleotide after liposome transfection; MS group: simulation of myocyte ischemia-reperfusion with missense Oligonucleotides deoxyribonucleotide after liposome transfection; SP600125/As group: simulation of myocyte ischemia-reperfusion for half a hour with SP600125(5) after adding antisense Oligonucleotides deoxyribonucleotide with liposome transfection, to the final concentration of 10μmol/L; SP600125/Ms group: simulation of myocyte ischemia-reperfusion for half a hour with SP600125(5) after adding missense Oligonucleotides deoxyribonucleotide with liposome transfection, to the final concentration of 10 μmol/L.

Simulation of myocyte ischemia-reperfusion model

Saturated D - Hanks liquid (6) was used with high purity nitrogen gas in advance, cell culture medium replaced with D - Hanks liquid, then myocardial cells were put into incubator with 5%CO_2, 95%N_2 for 4h; myocardial cells ischemia was simulated, then D - Hanks liquid was replaced with DMEM culture containing 20% fetal bovine serum, and cultured in incubator in 37℃, 5% CO_2, following with simulation of myocyte ischemia-reperfusion. 4h after reperfusion, cells were collected and apoptosis rate were measured with flow cytometry instrument, and the western blot detection was conducted 24h later after the collection of protein.

Annexin V/PI double dye cell apoptosis rate

Cells were digested and collected with 0.25% trypsin 4h after reperfusion, then were weight dropped and trypsin was washed with PBS, and the weight was dropped with 1X annexin-binding buffer after centrifugation; 5μL Annexin V and 10μL PI was added and mixed, and apoptosis rate was measured with flow cytometry instrument after 5min incubation at room temperature away from light.

Western blot determination of protein concentration of the AS group

Cells were collected and protein was extracted 24h after reperfusion, and expression of Cdc42, JNK, p-JNK, Bax, Bcl 2 protein was determined with western blot determination for the AS group. The procedure of Western blot was according to the kit instruction.

Statistical processing

Measurement data were expressed with mean standard deviation($\overline{x} \pm s$), and SPSS13.0 statistical software was used for analysis. Differences between groups was compared using single factor analysis of variance (One - way ANOVA), while q test was used for comparison between multiple samples, for which statistical significance was considered when $P < 0.05$.

Results

Cdc42 expression with Cdc42 antisense oligonucleotides deoxyribonucleotide transfection of different times

For the AS group, the transfection times were from 0-72h, Cdc42 protein expression was bimodal sample, and the strongest inhibitory effect of Cdc42 was 36h after transfection ($P < 0.05$), as shown in figure 1.

Myocardial cell apoptosis rate

Myocardial cell apoptosis rate of every group was as shown in figure 2 and table 1, myocardial cell apoptosis rate of I/R group increased compared with the control

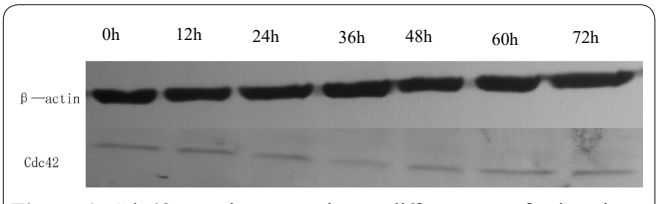

Figure 1. Cdc42 protein expression at different transfection times of the AS group.

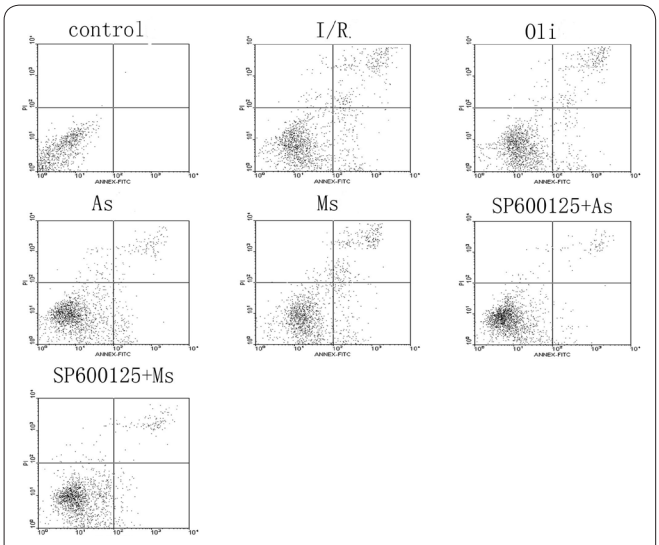

Figure 2. AnnexinV/PI double dye flow cytometry.

Table 1. Annexin V/PI double dye determination of myocardial apoptosis rate with flow cytometry instrument.

	Apoptosis rate (%)
Control group	0.05 ±0.03
Oli group	36.77±1.81
I/R group	34.64±1.99[a]
As group	17.76±3.03[b]
Ms group	33.40±2.84
SP600125/As group	11.76±1.58[c]
SP600125/Ms group	10.89±1.67[d]

[a]vs As group $P<0.05$; [b] vs I/R group $P<0.05$; [c] vs As group $P<0.05$;[-d] vs Ms group $P<0.05$.

group ((34.64±1.99)% *vs* (0.05 ±0.03)%) ($P<0.05$); and myocardial cell apoptosis rate of AS group decreased compared with the I/R group ((17.76±3.03)% *vs* (34.64±1.99)%) ($P<0.05$), while no statistical significance was observed comparing myocardial cell apoptosis rate in all groups (I/R group (34.64±1.99)%,, Oli group (36.77±1.81)%, Ms group((33.40±2.84)%) ($P>0.05$); myocardial cell apoptosis rate of SP600125/As group was lower than the AS group ((11.76±1.58)% *vs* (17.76±3.03)%) ($P<0.05$), and myocardial cell apoptosis rate of SP600125/Ms group was lower than the MS group ((10.89±1.67)% *vs* (33.40±2.84)%) ($P<0.05$), and there was no statistical significance among SP600125/As group and SP600125/Ms group ((11.76±1.58)%vs(10.89±1.67)%) (P>0.05).

Western blot results

Western blot results before adding SP600125

As shown in figure 3, Cdc42 protein expression of I/R group increased obviously compared with the control group ($P<0.05$), while Cdc42 protein expression of AS group decreased compared with the I/R group ($P<0.05$), however there was no statistical significance in I/R group, Oli group and Ms group ($P>0.05$). Meanwhile, JNK phosphorylation level of I/R group was higher than the control group ($P<0.05$), and JNK phosphorylation level of As group was lower than that of I/R group ($P<0.05$), and there was no statistical significance in I/R group, Oli group and Ms group ($P>0.05$). On the contrary, Bcl-2/Bax of I/R group was lower than that of control group ($P<0.05$), and Bcl-2/Bax of As group was higher than that of I/R group($P<0.05$); there was no statistical significance in I/R group, Oli group and Ms group (P>0.05).

Western blot results after adding SP600125

As shown in figure 4, JNK phosphorylation level of SP600125/As group was lower than that of As group as well as SP600125/Ms group compared with Ms group ($P<0.05$), while there was no statistical significance between SP600125/As group and SP600125/Ms group ($P>0.05$).

Discussion

Cdc42 is a member of Rho protein family, and its activation level is decided by counterbalance of GTPase activating protein (GAP) and guanosine exchange (GEF). Rho protein family includes Rho, Rac, Cdc42, Rnd and so on, with the study of RhoA, Rac and Cdc42 most clearly. For the reason that their most distinctive feature is to adjust the activation of actin dynamics, researches on themmainly focused on terms of tumor before. But recently, they were found to play an important role in development process of cardiovascular disease. Experiments have shown that Rho kinase (ROCK) can promote coronary artery spasm (7-8) which was related to atherosclerosis (9-10). It can also participate in myocardial remodeling after myocardial infarction and heart failure (11-12) , and be seen as intervention target for cardiovascular disease. Moreover, researches of Zhao et

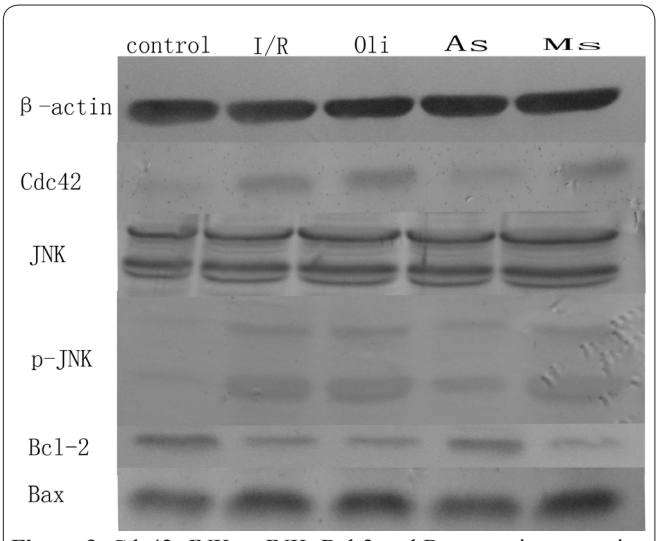

Figure 3. Cdc42, JNK, p-JNK, Bcl-2 and Bax protein expression before adding SP600125.

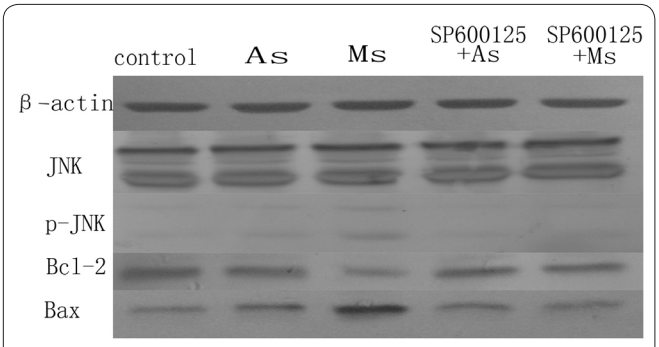

Figure 4. JNK, p-JNK, Bcl-2 and Bax protein expression after adding SP600125.

al. (1) have shown that Cdc42 can promote brain cell apoptosis in ischemia-reperfusion, and this experiment further studied the role of that in myocyte ischemia-reperfusion of rats.

In this experiment, Cdc42 gene expression in myocyte ischemia-reperfusion was interdicted by Cdc42 antisense phosphorthuoate oligodeoxynucleotides to study the role of Cdc42 in myocyte ischemia-reperfusion. Antisense technology is a new rising technology in the field of molecular biology, which mainly contains antisense oligonucleotide technology, antisense RNA technology and nuclear enzyme technology. The most common use of antisense technology is antisense oligonucleotide technology, although it has disadvantages such as unsteadiness compared with other myocardial cell protection medicine in ischemia-reperfusion, such as Lithium chloride (13). However it also has advantages of high target specificity (complementary base), easy design, diversity, simple synthesis and high localization and pertinence., which are better than conventional medicine design, production and function.

This study showed that protein expression of Cdc42 was bimodal sample at all time points, and was highest at 0h and 72h, while lowest at 36h, and increased protein expression may be caused by the degradation of antisense oligonucleotides deoxyribonucleotide after 36h which lead to decrease of blocking effect on Cdc42, and the transfection time was 36h. Protein expression of Cdc42 and myocardial cell apoptosis rate of I/R group increased obviously compared with that of control group, which indicated that myocardial cell apoptosis rate increased in ischemia-reperfusion, and in this process, Cdc42 was activated. Protein expression of Cdc42, myocardial cell apoptosis rate and JNK phosphorylation level of As group decreased compared with I/R group, and the ratio of suppression apoptosis protein Bcl-2 and promotion of apoptosis proteins Bax increased, and there was no statistical significance of these four indicators in I/R group, Oli group and Ms group, which indicated that Cdc42 antisense oligonucleotides deoxyribonucleotide can block Cdc42 gene expression, and then decrease myocardial cell apoptosis rate in ischemia-reperfusion. And for the reason that Cdc42 expression was blocked as well as JNK phosphorylation level decreased, we can speculated that JNK was in the downstream of Cdc42 signaling pathways. However, JNK phosphorylation level of SP600125/As group decreased compared with As group, as well as SP600125/Ms group compared with Ms group, and there was no statistical significance of JNK phosphorylation level between SP600125/As group and SP600125/Ms group, which indicated that activation of JNK was in the upstream of other signaling pathways during myocyte ischemia-reperfusion.

In conclusion, Cdc42 can promote myocardial apoptosis during myocyte ischemia-reperfusion through JNK, Bcl-2 and Bax signaling pathways, and its antisense oligonucleotides deoxyribonucleotide can block Cdc42 expression and decrease myocardial cell apoptosis rate. It is worth for further studying the effect of Cdc42 on protein in downstream and whether it can effect JNK directly or other pathways, as well as whether Cdc42 mediate myocardial apoptosis in ischemia-reperfusion through other pathways. Moreover, activation of JNK in ischemia-reperfusion is not only just through the pathway of Cdc42 (14). JNK family is a big family which can be divided into JNK1, JNK2 and JNK3, distributed in all myocardial cells, and further discussion is needed to research which kind or kinds of subtypes (15) can be activated by Cdc42, as well as the specific mechanism of these subtypes in myocyte ischemia-reperfusion.

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