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Fas/FasL induces myocardial cell apoptosis in myocardial ischemia-reperfusion rat model

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Abstract. – OBJECTIVE: Myocardium ischemia reperfusion is easy to induce myocardial injury. Fas/FasL is an important signaling pathway mediating cell apoptosis. This study aims to analyze the cell apoptosis and Fas/FasL expression in myocardial cell ischemia reperfusion rat model.

MATERIALS AND METHODS: Coronary artery ligation method was used to establish myocardial ischemia reperfusion model. Rats were grouped according to different ischemia and reperfusion time: Group A, myocardial ischemia for 30 min and reperfusion for 24 h; Group B, myocardial ischemia for 30 min and reperfusion for 48 h; Group C, myocardial ischemia for 1 h and reperfusion for 24 h. Myocardial i... indicators were tested. Myocardial cell a...sis was detected by transferase-mediate...e-oxyuridine triphosphate-biotin nick end lab...g (TUNEL) assay. Fas and FasL mRNA and pro... expressions were evaluated by Real-time P... (RT-PCR) and Western blot.

RESULTS: Creatine kinas...K), ...c dehydrogenase (LDH), and m...dialdeh...e (MDA) significantly elevated, ... supe...dismutase (SOD) obviou...y d... ...d ... mental group comp... wit...l and blank group (p<0.05). C...H, and M...adually up-regulated, wher...OD was red...in experimental group... ...the time e...nsion of ischemia and ...eperfu... (p<0.05). Apoptosis cell numb...as marked...her in the experimental ...up compared w...ntrol and blank group... ...0.05). ...poptosis ce...number gradually i...ased i... ...e experimental groups following ...mi... ...nd rep...fusion time extension (p<0.0... ...FasL m...A and protein markedly ...qual... the ...rimental group compared ...contro...k group (p<0.05). Fas/FasL mR... and pr... expressions enhanced in experimental groups following the time extension ...d reperfusion (p<0.05).

CONCLUSIONS: Fas/FasL induces myocardial cell apoptosis in the process of myocardium ...mia reperfusion in rat model.

Key Words:
Fas, FasL, Ischemia reperfusion, Myocardium, Apoptosis.

Introduction

Numerous ...arches sug...th...metabolic disorder ma...or aggrava...chemic tissue even...f ti...blood reper...fusion, named ischemia-reperfusion ...ury[1]. Ischemia-reperfusi...y is based ... ischemic damage. R...fusion aggravates th... reversible ischemic d...age, leading ... the injury irreversible[2]. The l...t is the firs... discovered organ for ischemi...perfusion ...ury. Further in-depth investigati...owe...t the whole body organ, such as bra...y, intestine, lung, and skin, can ...uffer from ischemia-reperfusion injury[3,4]. Pre...studies[3,4] pointed out that the process of ...che...-reperfusion injury can cause myocardial cell death, which includes necrosis and apoptosis. Among them, cell apoptosis is an important process in myocardial cell ischemia-reperfusion injury. Inhibiting apoptosis can reduce the area of myocardial infarction[5]. A basic research proposed that during ischemia-reperfusion injury, a large number of oxygen free radicals appear, intracellular calcium level significantly increases, numerous neutrophils infiltrate, and mitochondria is damaged. All of these pathological changes promote the myocardial cell apoptosis[6]. The occurrence and development of cell apoptosis are regulated by multiple signaling pathways. Fas/FasL signaling pathway is close related to myocardial cell apoptosis. Fas, known as Apo1 or CD95, is a transmembrane glycoprotein that is widely distributed in a variety of cell surfaces. It is homologous with tumor necrosis factor receptor and nerve growth factor receptor. FasL, the ligand of Fas, is a kind of type II transmembrane glycoprotein[7]. It is proved that Fas/FasL system participates in the occurrence and development of various cardiovascular diseases[8]. This study analyzed cell apoptosis and Fas/FasL expression in myocardial cell ischemia reperfusion via constructing rat myocardial ischemia-reperfusion model.

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Materials and Methods

Experimental Animals

A total of 50 male Wistar rats at 8-week old and 180±20 g were provided by Chinese Academy of Military Medical Sciences Laboratory Animal Center. Rats were raised under specific pathogen free (SPF) with standard fodder and water. Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Tangshan Workers' Hospital.

Reagents and Instruments

Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) kit was purchased from Roche (Catalogue No. 11684817910; Basel, Switzerland). Rabbit anti-rat Fas polyclonal antibody (Catalogue No. sc-7886; 1:2000) and rabbit anti-rat FasL polyclonal antibody (Catalogue No. sc-6237; 1:2000), horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Catalogue No. sc-2004; 1:2000), and RT-PCR kit (Catalogue No. sc-43500) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Super-clean worktable was from Formal (Shanghai, China). Centrifugal machine was from Flying (Tianjin, China). Inverted microscope was from Olympus (Tokyo, Japan). Desktop thermos oscillator was from Jinghong (Shanghai, China).

Rat Myocardial Ischemia Reperfusion Model Establishment

Coronary artery ligation method to establish myocardial ischemia reperfusion model [9]. The rat was anesthetized by urethane 800 mg/kg intraperitoneal injection. Next, rat received endotracheal intubation and artificial ventilation under electrocardiograph monitoring. The chest was opened to expose the heart and the thread was passed through the left coronary artery. Another two threads were drawn from the knot to loose ligature. After ligating the left coronary artery, cardiogram (ECG) showed ST-segment elevation and myocardium appeared cyanosis. The thread was loosed after a specified period to restore coronary permeability, leading to reperfusion. At last, the chest was closed to restore spontaneous breathing.

Grouping

Rats were randomly divided into 5 groups with 10 in each group. Experimental group: rats were divided into 3 groups according to different ischemia and reperfusion time, Group A, myocardial ischemia for 30 min and reperfusion for 24 h, Group B, myocardial ischemia for 30 min and reperfusion for 48 h, and Group C, myocardial ischemia for 1 h and reperfusion for 24 h. Control group: rat was anesthetized by urethane to expose coronary. The thread was passed through the coronary artery without ligation. Blank group: the healthy rat received conventional feeding.

Myocardial Damage Indicators Detection

Serum creatine kinase (CK) and lactic dehydrogenase (LDH) contents were detected by colorimetry assay on UV-visible violet visible spectrophotometer. Superoxide dismutase (SOD) activity was evaluated by xanthine oxidase method. Malondialdehyde (MDA) content was determined by barbituric acid method.

TUNEL Assay

The rat was sacrificed and the heart was taken out. The tissue between infarction and non-infarction areas was fixed and cut into 1 mm × 1 mm × 1 mm. After dehydration, paraffin embedding, and section, tissue was stained by TUNEL and rinsed by PBS. After ethanol gradient washing, the tissue was treated by proteinase K. Next, the tissue was added to 50 µl TUNEL reaction mixture to obtain apoptotic cell. Then the tissue was added to50 µl converter-POD, 50-100 µl diaminobenzidine (DAB) substrate, and hematoxylin or methyl green. Cells were photographed and counted.

Western blot

A total of 40 µg protein was separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blocked at room temperature for 1 h. Then the membrane was incubated with Fas and FasL antibodies (1:1200) at 4°C overnight. After a secondary antibody was added for 1 h, and the membrane was developed.

RT-PCR

Total RNA was extracted using TRIzol and quantified by D260 nm/D280 nm. The RNA was synthetized for the poly A tail of miRNA and reverse transcribed to cDNA. The primers used for PCR were listed in Table I. PCR reaction was composed of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. U6 was selected as internal reference.

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was adopted for data analysis. Measurement

Table I. Primer sequence.

Gene		Sequence (5′-3′)
Fas mRNA	Forward	5’-AGCCGTCAAGAGCAATAACC
	Reverse	5’-GTGCAGGGTCCGAGGT-3’
FasL mRNA	Forward	5’-TGGTGGGCAGTATGTTC
	Reverse	5’-GCTATTGGCATTGGTC 3’
U6	Forward	5’-CTCGCTTCGGCAGCA
	Reverse	5’-AACGCTTCACGA TTTG

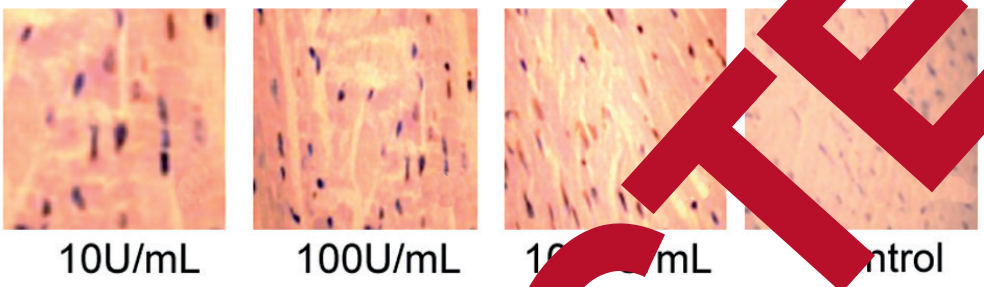

Figure 1. Myocardial apoptosis.

data was depicted as mean ± standard deviation and compared by analysis of variance (ANOVA). meration data was compared by χ^2 test. was considered as statistical significance.

Results

Myocardial Injury Indi rs Ch ges

CK, LDH, and MDA ifican while SOD obviously slin expe tal group compared contr blank group (p<0.05). CK, LD d MDA gr y upregulated, whereas ually reduc n expe rimental grou follow he time extension of ischemia eperfusion 05) (Table II).

My dial Apoptosis

A to 000 cells were counted in each roup. Apoptosis cell number was significantly higher in experimental group compared with control and blank group (p<0.05). Apoptosis cell number gradually increased in experimental groups following ischemia and reperfusion time extension (p<0.05) (Table III, Figure 1).

Fas and FasL Protein Expressions

Fas/FasL protein markedly upregulated in the experimental group compared with control and blank group (p<0.05). Fas/FasL protein expression enhanced in experimental groups following the time extension of ischemia and reperfusion (p<0.05) (Table IV, Figure 2).

Table dial inju icators changes.

Group	Cases	CK (U/L)	LDH (U/L)	SOD (U/g)	MDA (nmol/g)
oup	30				
oup A	10	3011±589$^{*\#}$	2142±313$^{*\#}$	11285±3054$^{*\#}$	1962±336$^{*\#}$
Group B	10	3568±612$^{*\#\&}$	2562±405$^{*\#\&}$	10024±2096$^{*\#\&}$	2204±341$^{*\#\&}$
up C	10	3923±708$^{*\#\&@}$	2962±412$^{*\#\&@}$	9867±924$^{*\#\&@}$	2652±383$^{*\#\&@}$
ol	10	2026±783	1036±348	19242±3425	1785±235
B k group	10	1912±205	924±134	19785±3156	1512±202

$^{*}p$<0.05, compared with control. $^{\#}p$<0.05, compared with blank group. $^{\&}p$<0.05, compared with group A. $^{@}p$<0.05, compared with group B. CK: Creatine kinase, LDH: lactic dehydrogenase, MDA: malondialdehyde, SODl superoxide dismutase.

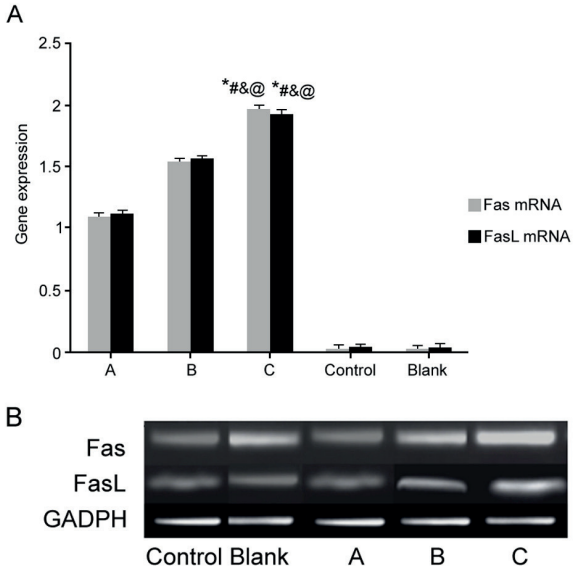

Figure 3. Fas and FasL mRNA expressions in myocardial cells. *p<0.05, compared with control. #p<0.05, compared with blank group. &p<0.05, compared with group A. @p<0.05, compared with. with group B.

Fas and FasL mRNA Expressions

Fas/FasL mRNA markedly up-regulated e experimental group compared with control l blank group (p<0.05). Fas/FasL mRNA expr sion enhanced in experimental following the time extension of ischer and erfusion (p<0.05) (Figure 3).

Discussion

Ischemia-re injury refers he more serious and ger ar mage of myocardial cells afte chemia an od reperfusion[10]. Apopto or programmed c eath, is a type of physiolog phe na during body develop-ment. Insufficient o nced apoptosis under som logical cond may result in tissue d ge and dysfunction[11]. Fas/FasL signaling way is one the signaling pathways that ctly trigger ptosis. It is closely related to t currence, elopment, treatment, and prog diseases[13]. This study explored the imp s/FasL system on myocardial cell apoptosis via constructing rat myocardial ischemia reperfusion model. Coronary artery ligation was used to establish myocardial ischemia reperfusion model. Rats were grouped according to different ischemia and reperfusion time: Group A, myocardial ischemia for 30 min and reperfusion for 24 h; Group B, myocardial ischemia for 30 min and reperfusion for 48 h; Group C, myocardial ischemia for 1 h and reperfusion for 24 h. Myocardial injury indicators were tested. CK, LDH, and MDA significantly elevated, while SOD obviously declined in the experimental group compared with control and blank group. CK, LDH, and MDA gradually upregulated, whereas SOD gradually reduced in experimental

Table III. Myocardial cell apoptosis detected by TUNEL assay.

Group	Cases	Apoptotic cell num
Experimental group		
Group A	10	±1.02*#
Group B	10	.44*#&
Group C	10	21.2 *#&@
Control	10	3.0
Blank group	10	3.12±1.

*p<0.05, compared with ol. #p<0 ompared w n blank group. & p<0.05 d w oup A. <0.05, compared with group TU erase-m ed de-oxyuridine triphos biotin l labeli

Table IV. d FasL n expressions in myocardial cells.

Group	Cases	Fas	FasL
l group			
oup	10	5.02±1.13*#	4.62±1.69*#
Group B	10	7.36±1.57*#&	6.23±2.08*#&
roup C	10	9.15±2.89*#&@	8.76±2.54*#&@
rol	10	1.13±0.15	1.12±0.48
k group	10	1.07±0.25	1.08±0.67

*p<0.05, compared with control. #p<0.05, compared with blank group. &p<0.05, compared with group A. @p<0.05, compared with group B.

groups, following the time extension of ischemia and reperfusion. It suggested that myocardial injury indicators obviously increased in ischemia-reperfusion rat. The heart tissue was obtained to test myocardial cell apoptosis. Apoptosis cell number was markedly higher in the experimental group compared with control and blank group. Apoptosis cell number gradually increased in experimental groups following ischemia and reperfusion time extension. Myocardial cell apoptosis is rare in normal rat. Myocardial cell apoptosis increased in ischemia-reperfusion rat, and mainly distributed in apex cordis. Numerous myocardial cell apoptosis led to cell loss, thus affecting myocardial contraction and resulting in cardiac dysfunction[14,15]. Our results indicated that myocardial cell apoptosis participated in the occurrence and development of ischemia-reperfusion heart injury. *In vitro* cardiac ischemia-reperfusion model revealed that myocardial cells appeared apoptosis at 10 min after ischemia and reached peak at 30 min. It suggested that myocardial ischemia may induce cell apoptosis [16]. *In vivo* cardiac ischemia-reperfusion model demonstrated that both sustained ischemia for 2 h and i- mia for 45 min followed by reperfusion can lead to cell apoptosis. Apoptotic cell nu- er increased following reperfusion time extens- which was similar to our results. To explore role of Fas/FasL signaling pat- myoca- dial ischemia-reperfusion, t- vo- ted Fas and FasL expressions in r- ardium as/FasL protein markedly upreg- d in - mental group compa- wi- - and - k group. Fas/FasL pr- expre- enhanced in experimental gr- following t- e exten- sion of ischem- erfusion. - er gene analysis show- that - L mRNA markedly upregulat- the experi- l group compared with c- and blank grou- as/FasL mRNA expr- n enha- d in experimental groups follow- he ti- xtensi-n of ischemia and reperfusio- med th- as/FasL upregulated in myoc- l cell- er ischemia-reperfusion. - sL ov- ed in myocardial cells of *in vitr-* chemia- erfusion model[18]. It was found th- otic cells accounted for 90% when - arction sustained for 2 h, and their - el and Fas protein expression gradually incre- following time extension[19]. Apoptotic index (A- evated in ischemia-reperfusion rat compared with in lymphedema rats. Apoptotic cells mainly exist in the marginal zone of infarction. Fas plays a critical role in the process of myocardium ischemia-reperfusion[20-23], which is in accordance with our study.

Conclusions

Myocardial cell apoptosis enha- in cardiac ischemia-reperfusion following isc- and reperfusion time extension. Ce- popto- Fas/FasL system involved in t- athogenes- - diac ischemia-reperfusi- Blocki- cell ap- sis or Fas/FasL syste- ay be- ew approach for the prevention - t- - cardi- che-mia-reperfusion.

Conflict - nter-

The authors declare no- icts of interest.

References

- -guez M, Do- , Feijoo E, Goni S, Prieto J, Berasain - -a M. -ovel pharmacologic strategies to p- -er from ischemia-reperfusion injury. Recent Pat Cardiovasc Drug Discov 2008; 3: 9-18.
- Wu C, Zhang Y, Sun Z, Li P. Molecular evolution - family proteins: novel domain formation in - vertebrates and the subsequent divergence. BMC Evol Biol 2008; 8: 159.
- Li J, Li R, Lv GY, Liu HQ. The mechanisms and strategies to protect from hepatic ischemia-reperfusion injury. Eur Rev Med Pharmacol Sci 2015; 19: 2036-2047.
- Pchejetski D, Kunduzova O, Dayon A, Calise D, Seguelas MH, Leduco N, Seif I, Parini A, Cuvillier O. Oxidative stress-dependent sphingosine kinase-1 inhibition mediates monoamine oxidase A-associated cardiac cell apoptosis. Circ Res 2007; 100: 41-49.
- Varfolomeev E, Goncharov T, Fedorova AV, Dynek JN, Zobel K, Deshayes K, Fairbrother WJ, Vucic D. c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation. J Biol Chem 2008; 283: 24295-24299.
- Rauert H, Wicovsky A, Muller N, Siegmund D, Spindler V, Waschke J, Kneitz C, Wajant H. Membrane tumor necrosis factor (TNF) induces p100 processing via TNF receptor-2 (TNFR2). J Biol Chem 2010; 285: 7394-7404.
- Yao YW, Zhang GH, Zhang YY, Li WD, Wang CH, Yin CY, Zhang FM. Lipopolysaccharide pretreatment protects against ischemia/reperfusion injury via increase of HSP70 and inhibition of NF-kappaB. Cell Stress Chaperones 2011; 16: 287-296.
- Baban B, Liu JY, Mozaffari MS. Pressure overload regulates expression of cytokines, gammaH2AX, and growth arrest- and DNA-damage inducible

protein 153 via glycogen synthase kinase-3beta in ischemic-reperfused hearts. Hypertension 2013; 61: 95-104.

9) Dhanasekaran DN, Reddy EP. JNK signaling in apoptosis. Oncogene 2008; 27: 6245-6251.

10) Jones EV, Dickman MJ, Whitmarsh AJ. Regulation of p73-mediated apoptosis by c-Jun N-terminal kinase. Biochem J 2007; 405: 617-623.

11) Chinda K, Sanit J, Chattipakorn S, Chattipakorn N. Dipeptidyl peptidase-4 inhibitor reduces infarct size and preserves cardiac function via mitochondrial protection in ischaemia-reperfusion rat heart. Diab Vasc Dis Res 2014; 11: 75-83.

12) Sun WZ, Li MH, Chu M, Wei LL, Bi MY, He Y, Yu LB. Id1 knockdown induces the apoptosis and inhibits the proliferation and invasion of ovarian cancer cells. Eur Rev Med Pharmacol Sci 2016; 20: 2812-2818.

13) Wesche-Soldato DE, Swan RZ, Chung CS, Ayala A. The apoptotic pathway as a therapeutic target in sepsis. Curr Drug Targets 2007; 8: 493-500.

14) Wu X, Xu T, Li D, Zhu S, Chen Q, Hu W, Pan D, Zhu H, Sun H. ERK/PP1a/PLB/SERCA2a and JNK pathways are involved in luteolin-mediated protection of rat hearts and cardiomyocytes following ischemia/reperfusion. PLoS One 2013; 8: e82957.

15) Ghosh J, Das J, Manna P, Sil PC. The protective role of arjunolic acid against doxorubicin ind ed intracellular ROS dependent JNK-p38 a mediated cardiac apoptosis. Biomaterial 11; 32: 4857-4866.

16) Kuhla A, Eipel C, Siebert N, Abshagen K, Menger Vollmar B. Hepatocellular apoptosis is mediated TNFalpha-dependent Fas/Fas cytotoxicl in a murine model of acute l apoptosis 2008; 13: 1427-1438.

17) Andera L. Signaling activated by the death receptors of the TNFR family. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2009; 153: 173-180.

18) Jeremias I, Kupatt C, Martin-Villalba A, H Schenkel J, Boekstegers P, Debatin KM ven of CD95/Apo1/Fas in cell death a myocardial ischemia. Circulation 2000; 102 920.

19) Das J, Ghosh J, Manna P, Sil PC. Tau presses doxorubicin-triggered oxida stress ardiac apoptosis in rat via up-re ion of PI3 nd inhibition of p53, p38 . Biochem Pha 2011; 81: 891-909.

20) Chen YH, Wu XD, Y Sun S XH. Cal ineurin is involved i ca on in d by ischemic po ndition rough nuating endoplasm ticulum stre i d J (Engl) 2011; 12 340.

21) Zhan un L, Han X, Zh ng B, Lu N, Shi Y, Tan W, Zhou D, Zhang X, Guo Y, Lin D tional polym sms in FAS and FASL ute to increas apoptosis of tumor infiltration lymphocytes and risk of breast cancer. Carcinogenes 2007; 28: 1067-1073.

2 Komamura K, T, Hanatani A, Kim J, Hashimura Ishida Y, O u Y, Asayama K, Tanaka T, Ogai A, ani T ra S, Kangawa K, Miyatake K, Kitakaze M fatty acid binding protein is a novel prognostic marker in patients with non-ischaemic dilated cardiomyopathy. Heart 2006; 92: 615-618.

A, Cure MC, Kalkan Y, Balik MS, Guvercin aprak E, Yuce S, Sehitoglu I, Cure E. Protective effects of thymoguinone and alpha-tocopherol on the sciatic nerve and femoral muscle due to lower limb ischemia-reperfusion injury. Eur Rev Med Pharmacol Sci 2016; 20: 1192-1202.