Int J Clin Exp Med 2017;10(5):7743-7751
www.ijcem.com /ISSN:1940-5901/IJCEM0048404

Original Article

Effect of HSP70 on cardiomyocyte apoptosis induced by endoplasmic reticulum stress

Zhongdong Sun, Li Li, Yan Jiao

Department of Cardiovascular Surgery, Weifang People's Hospital, Weifang 261041, Shandong, China

Received November 5, 2016; Accepted March 20, 2017; Epub May 15, 2017; Published May 30, 2017

Abstract: We aimed to detect the role of HSP70 in endoplasmic reticulum (ER) stress-induced cell apoptosis, and to reveal the molecular mechanism underlying the HSP70 regulated ER stress induced-apoptosis, secretion and calcium signal transduction. The findings provide new intervention targets and theoretical basis of subcellular molecular application for myocardial protection. We used SD neonatal rat cardiomyocyte to establish a hypoxia cell model, and then we further detected the role of HSP70 in cell apoptosis, in production of ROS, in expression of casepase-12, CHOP, JNK pathway, CaSR and SERCA in ER caused by ER stress, as well as in the immunohistochemical staining of Bcl-xL, Bcl-2 and BAX, and Fas and FasL by overexpression or downexpression of HSP70. Results showed that hypoxia-induced cell apoptosis was inhibited by HSP70, but was promoted by HSP70 anti-sense oligonucleotide. Hypoxia-induced ROS production was also inhibited by HSP70, but was not inhibited by HSP70 anti-sense oligonucleotide. HSP70 significantly inhibited the expression of Caspase-12, CaSR and CHOP. HSP70 increased the expression of JNK, but did not influent the expression of SERCA. HSP70 anti-sense oligonucleotide significantly increased the expression of CHOP, but did not significantly influent other tested molecules. Bcl-xL and Bcl-2 were highly expressed in HSP70-treated cells, but were low expressed in HSP70 anti-sense oligonucleotide-treated cells. Bax, Fas, FasL were predominantly expressed in cell treated with HSP70 anti-sense oligonucleotide, but were not in HSP70-treated cells. Thus, HSP70 inhibited the cell apoptosis via inhibiting the expression of Caspase 12, CHOP, Bax, Fas and FasL, and inhibited the Ca^{2+} current via inhibiting the expression of CaSR, and that the JNK pathway might play important roles in cell apoptosis and ROS-induced Ca^{2+} current.

Keywords: HSP70, apoptosis, CaSR, hypoxia, cardiomyocytes

Introduction

Ischemic myocardial protection has always been a hot spot in the field of cardiovascular researches [1]. Since the beginning of the 21st century, research focused on myocardial protection has turned to cell mechanism which is to enhance the myocardial cell immunity of ischemia and hypoxia injury, to prevent cell damage from irreversible development and to improve cell survival rate [2]. It is commonly understood that heat shock protein 70 (HSP70) is a tissue-inducible protein, which has a significant protective effect on the myocardium [3]. HSP70 is the inducible (or stress) protein that has been well-studied up till now, but its detailed molecular mechanism of subcellular metabolic regulation for myocardial protection has not been completely elucidated yet.

Cell apoptosis is defined as autonomous and orderly death of the gene-controlled cell, so as to maintain the internal environment homeostasis [4]. In current, it was demonstrated that the signaling pathways involved in apoptosis mainly include: death receptor activation pathway (exogenous pathway), mitochondrial damage pathway (endogenous pathway) and endoplasmic reticulum (ER) stress activated apoptosis pathway [5]. Among them, the first two pathways are the classical apoptosis pathways, and the ER stress activated apoptosis pathway is a new and very important apoptosis pathway that has been discovered in recent years [6].

The ER is widely distributed in the cell, which is an important organelle in the cell [7]. According to whether the ribosomes are attached, the ER is divided into rough and smooth ER [8-11]. The inner membrane of the ER accounts for 50% of the total cell membrane structure and 10% of the total cell volume. ER membrane has very important physiological functions. It is not only the main site for the synthesis of protein fold-

ing, transportation as well as Ca^{2+} storage in the cell, but also the synthesis sites of cholesterol, steroids and many lipids [5]. The huge membrane structure of ER provides a wide platform for molecular packaging and reaction in the cell [12]. ER also plays a key role in multiple-signal activities. With its large membrane structure, ER induce apoptosis through special pathways such as Caspase-12, CHOP (C/EBP homologous protein), JNK (c-Junamino-terminal kinase), and so on [13, 14].

In this study, transgenic technology was applied to increase the expression of heat shock protein 70 (HSP70) gene in SD neonatal rat cardiomyocytes, and HSP70 antisense oligonucleotide technology was applied to inhibit the HSP70 expression. Then, the model of cardiomyocyte hypoxia/reoxygenation was established with the Webster method. After confirmed the expression of HSP70, ER was extracted to test the changes in expression of key signal molecular and pathway that related to apoptosis and mediated by ER stress. To study the molecular mechanism of HSP70 regulated ER stress-induced apoptosis, flow cytometry was used to detect apoptosis rate of neonatal rat myocardial cells, western blot was applied to test the expression of Caspase-12, CHOP, JNK signaling, sarco endoplasmic reticulum calcium adenosine triphosphatase (SERCA) and calcium sensitive receptor (CaSR). Also, changes of Bcl-2 family members on ER and Fas and FasL were detected by immunohistochemical staining. Thereby elucidates the HSP70 regulated changes of ER stress-activated Ca^{2+} signaling, and the subcellular molecular mechanism involved in HSP70 regulated apoptosis such as ER stress signaling transduction, protein synthesis and apoptotic signaling molecules. Our findings provide new intervention targets and molecular basis for myocardial subcellular protection.

Materials and methods

Cell isolation and establishment of hypoxia cell model

After anesthesia and heparin anticoagulation, quickly taken out the heart of neonatal rat into 4°C pre-cold PBS. After washed 3 times, the tissue minced and digested in PBS containing collagenase for 30 min in an orbital shaker at 37°C. Then, the supernatant was collected, and culture medium was added to neutralize the collagenase. Repeat the digestion and neutralization 7~8 times. All the collected supernatants were digested by collagenase for another 5 min and were neutralized by growth medium. After centrifugation, isolated cells were cultured at 37°C.

The neonatal rat cardiomyocyte monolayers were treated with high purity argon gas, pre argon saturated in sugar free serum medium for 3-4 days. Then, high purity argon gas (95% air/5% CO_2) was used to perfuse cells for 2 h to simulate hypoxia. Then, the culture medium containing sugar and serum was changed and continued to incubate the cells in 95% air/5% CO_2 for 4 h to simulate the reoxygenation.

Cell apoptosis

After treatment, cell apoptosis was detected by double staining of Annexin V-FITC and PI (Keygen, China). Cells were digested by trypsin without EDTA, and collected by centrifugation at 300 g, 4°C. Cells were suspended by 100 ul 1× Binding Buffer, mixed with 5 μl Annexin V-FITC and 5 μl PI Staining Solution, and then were incubated for 10 min in the dark at room temperature. Then, 400 μl 1× Binding Buffer was added, and cell apoptosis were detected by Accuri C6 flow cytometry (BD, Germany).

Western blot

The ER was isolated according to the Endoplasmic Reticulum Extraction Kit (ER0100, sigma, USA). Then, proteins in ER were further extracted by total protein extraction kit (Beyotime, China). The protein concertation was measured by Coomassie brilliant blue G-250. Proteins (40~60 μg) were isolated by SDS-PAGE and were transferred into PVDF membrane. After blocking with 5% fat-free milk for 60 min, the primary antibody (anti-caspase 12, anti-CasR, anti-CHOP, anti-JNK, anti-SERCA; 1:100; Abcam, USA) was incubated the membrane overnight at 4°C, and then HRP secondary antibody (1:1000, Beyotime, China) was incubated another 1 h at Room temperature. Finally, the blotting was observed in X light tablets by using the BeyoECLPlus chemiluminescence reagent (Beyotime, China).

Immunohistochemical staining

The samples were fixed in 4% PFA at room temperature, and washed with distilled water for 2

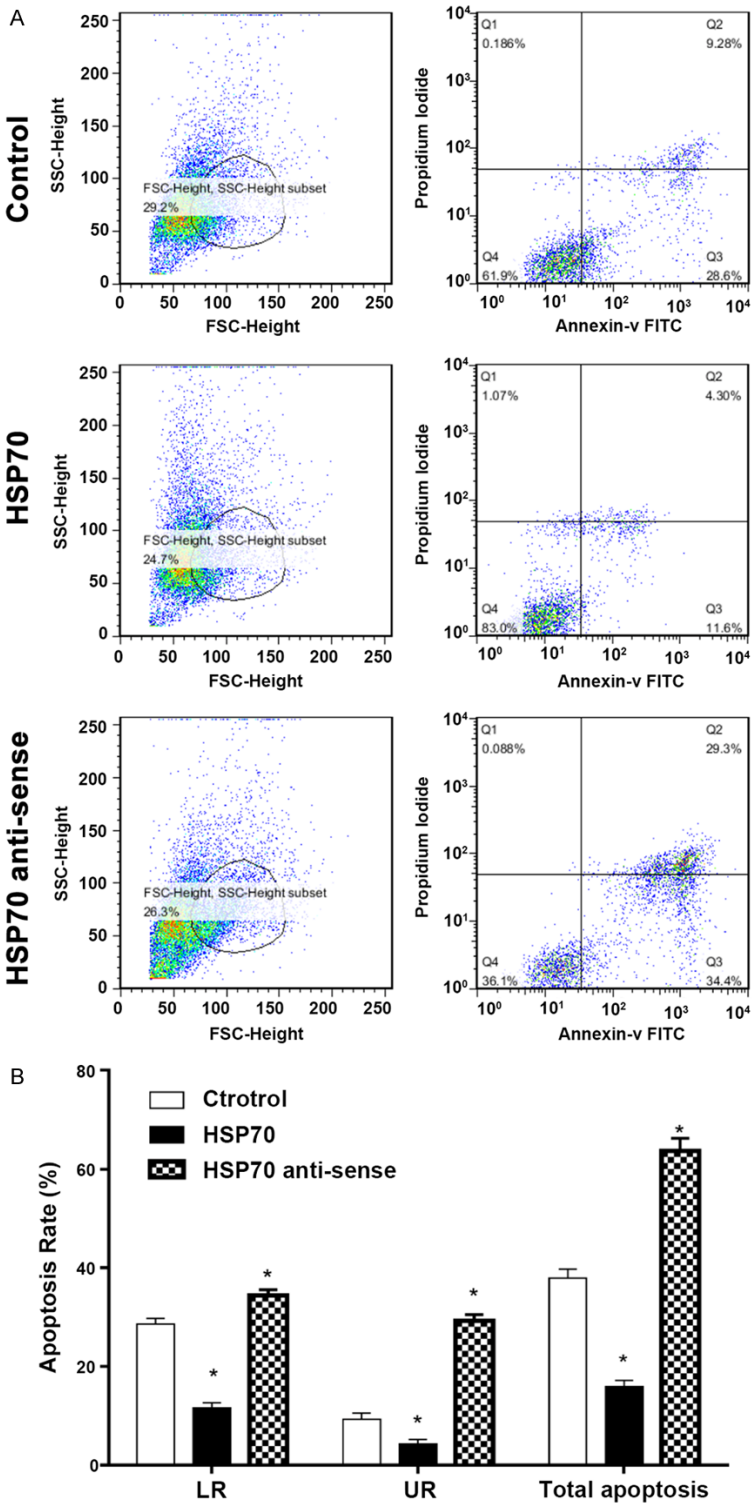

Figure 1. Cell apoptosis. A. Cell apoptosis in HSP70 and HSP70 anti-sense oligonucleotide groups were detected by flow cytometry. B. Quantification data of cell apoptosis. UR: later apoptosis; LR: early apoptosis; total apoptosis (UR+LR). *P<0.05 vs. control.

times, each time 5 min. The endogenous enzymes were inactivated by treatment of 3% hydrogen peroxide for 10 min at room temperature. After 3 times washing by distilled water, antigen was heat repaired by immersion in a citrate buffer solution. After high heat in a microwave oven for 4 minutes, cooled and washed with PBS for 5 min, samples were blocked by 5% BSA for 20 min at room temperature and were incubated with 50 μL primary antibodies (Bcl-2, 1:100; Bcl-Xl, 1:500; Bax, 1:50; Fas, 1:50; FasL, 1:50; Abcam, USA) overnight at 4°C. After washes, 30 μL biotinylated secondary antibodies were incubated the samples for 30 min in 37°C water bath. SABC reagent (BOSTER, China), DAB color rendering solution (BOSTER, China) and hematoxylin dye were added. After nuclear staining, the slide is followed by 70%, 80%, 90%, 100% alcohol gradient for each 5 minutes, and is mounted with neutral gum on dry slides. The images were taken out under the optical microscope.

To assess the cell-membrane staining, all slides were observed under a microscope. Immunohistochemical staining expression was graded using a 4-point scale, where 0 = No staining is observed in cells; 1+ = Weak, incomplete membrane staining in any proportion of cells, or weak, complete membrane staining in less than 10% of cells; 2+ = Complete membrane staining that is non-uniform or weak but with obvious circumferential distribution in at least 10% of cells, or intense complete membrane staining in 30%; 3+ = Uniform intense membrane staining of more than 30%. Investigators were blinded to the prognostic analysis data.

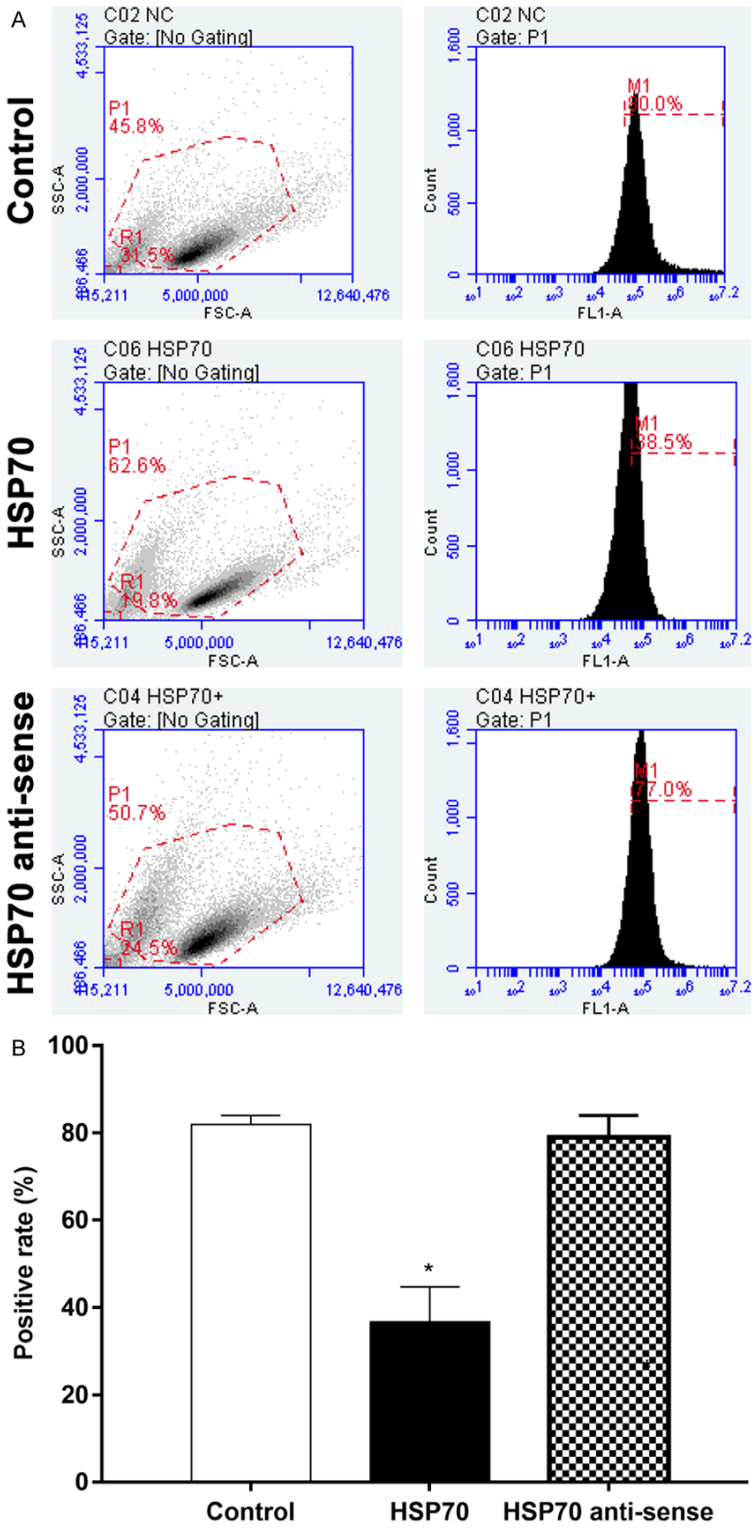

Figure 2. Production of ROS. ROS were labeled with DCFH-DA and analyzed by flow cytometry. A. ROS production was detected by flow cytometry. B. The positive rate was calculated. *P<0.05 vs. control. HSP70 significantly inhibited the production of ROS, but HSP70 anti-sense oligonucleotide not significantly influent the production of ROS.

Detection of ROS production

ROS was detected by using the ROS detection kit (Beyotime, China) according to manufacturer's instruction. In brief, formation of intracellular ROS was determined using a fluorescent probe 2', 7-dichlorofluorescein diacetate (DCFH-DA). After treatment, cells were incubated with 10 μM DCFH-DA for another 30 min incubation. Then, the cells were washed with PBS three times and the changes of fluorescence were observed using Accuri C6 flow cytometry (BD, Germany).

Statistical analysis

All results are expressed as the mean ± SD. Statistical significance between groups was analyzed using one-way analysis of variance (ANOVA) followed post-hoc test using SPSS 16.0 (SPSS, USA). P< 0.05 was considered statistically significant.

Results

HSP70 inhibited cell apoptosis, but HSP70 anti-sense oligonucleotide promoted

As shown in **Figure 1**, the apoptosis rate in the hypoxia cells was at very high level (UR+LR=9.28+28.6%, UR: later apoptosis; LR: early apoptosis). HSP70 inhibited hypoxia-induced cell apoptosis including both early and later apoptosis (UR+LR=4.3+11.6%). In contrast, HSP70 anti-sense oligonucleotide further promoted hypoxia-induced cell apoptosis including both early and later apoptosis (UR+LR=29.3+34.4%).

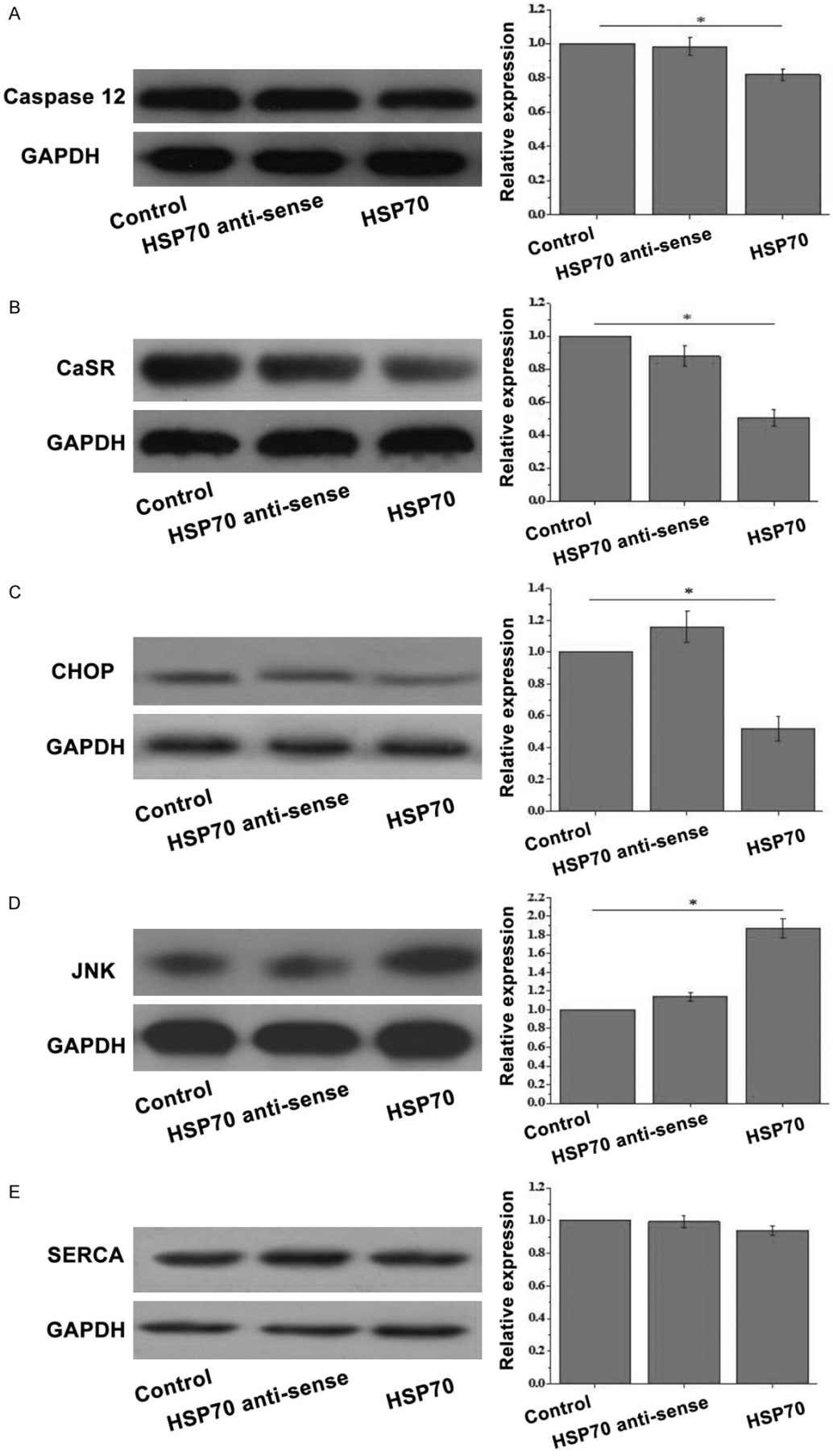
A
Caspase 12
GAPDH
Control
HSP70 anti-sense
HSP70
Relative expression
*
B
CaSR
GAPDH
Control
HSP70 anti-sense
HSP70
Relative expression
*
C
CHOP
GAPDH
Control
HSP70 anti-sense
HSP70
Relative expression
*
D
JNK
GAPDH
Control
HSP70 anti-sense
HSP70
Relative expression
*
E
SERCA
GAPDH
Control
HSP70 anti-sense
HSP70
Relative expression

Figure 3. Expression of (A) caspase 12, (B) CasR, (C) CHOP, (D) JNK, and (E) SERCA in endoplasmic reticulum in HSP70-transfected cells or HSP70 anti-sense oligonucleotide-treated cells were detected by western blot and quantification by using ImageJ. *P<0.05 HSP70 vs. control.

HSP70 significantly inhibited production of ROS, but HSP70 anti-sense oligonucleotide did not

Hypoxia induced high level of ROS. HSP70 significantly inhibited the production of ROS (38.5% of HSP70 vs. 80.0% of control; **Figure 2**). But HSP70 anti-sense oligonucleotide did not significantly influent the production of ROS (77.0% of HSP70 anti-sense oligonucleotide vs. 80.0% of control).

HSP70 inhibited the expression of caspase 12, CaSR and CHOP, increased the expression of JNK, and did not influent the expression of SERCA in endoplasmic reticulum

The expression levels of apoptosis-related molecules Caspase 12, CHOP and JNK, and Ca^{2+} current-related molecules CaSR and SERCA in ER were further investigated by western blot (**Figure 3**). HSP70 significantly inhibited the expression of Caspase 12 (**Figure 3A**), CaSR (**Figure 3B**) and CHOP (**Figure 3C**), increased the expression of JNK (**Figure 3D**), and did not influent the expression of SERCA (**Figure 3E**). HSP70 anti-sense oligonucleotide significantly increased the expression of CHOP, but did not significantly influent other tested molecules. It was suggested that HSP70 inhibited the cell apoptosis via inhibiting the expression of Caspase 12 and CHOP, and inhibited the Ca^{2+} current via inhibiting the expression of CaSR, and that JNK pathway might play important roles in cell apoptosis and ROS-induced Ca^{2+} current.

Effects of HSP70 and HSP70 anti-sense oligonucleotide in expression of Bcl-xL, Bcl-2 and BAX

Immunohistochemical staining of Bcl-xl, Bcl-2 and BAX was performed (**Figure 4**). This analysis indicated that Bcl-Xl and Bcl-2 were predominantly expressed by cells treated with HSP70, and BAX were predominantly expressed by cells treated with HSP70 anti-sense oligonucleotide. Very strong immunohistochemical staining (IHC3+) of Bcl-xL and Bcl-2 was observed in cells in HSP70 group, and that of BAX in cells in HSP70 anti-sense oligonucleotide group. It was further confirmed that HSP70 inhibited the hypoxia-induced cell apoptosis, and HSP70 anti-sense oligonucleotide promoted the hypoxia-induced cell apoptosis.

Effects of HSP70 and HSP70 anti-sense oligonucleotide in expression of Fas and FasL

Immunohistochemical staining of Fas and FasL were also detected (**Figure 5**). Fas and FasL were predominantly expressed in cells of HSP70 anti-sense oligonucleotide group. Lower staining levels of Fas and FasL detected in cells in HSP70 group were IHC 2+ and 0, respectively.

Discussion

At present, the time limitation of myocardial protection is still the main reason for restricting further development of cardiac surgery, and it is also one of the main death causes for the patients after cardiac surgery. The study of myocardium has been developed from the level of whole organ to the level of subcellular molecule [15, 16]. However, no new progress has been made in the protective methods in clinical practice up till now [17]. Therefore, it's necessary to explore the subcellular mechanisms and methods for myocardial protection, so as to strengthen the myocardial protection.

Apoptosis mediated by ER stress is different from the one mediated by death receptors and mitochondria. Apoptosis mediated by ER stress is a new apoptotic pathway. ER stress is a novel therapeutic target in heart diseases [18, 19]. The study of ER stress can deepen our understanding of cardiomyocyte biology. Molecular chaperone that is involved in ER stress and the protein specially induced by ER stress can be an effective target of myocardial protection.

We are trying to elucidate the subcellular mechanism of HSP70 prevent myocardial injury through inhibition of ER stress-induced cell apoptosis. We demonstrated that HSP70 participates in regulation of ER stress-mediated apoptosis in myocardial protection. In this study, transgenic technology was applied to increase the expression of HSP70 gene in SD neonatal rat cardiomyocytes, and HSP70 antisense oligo-

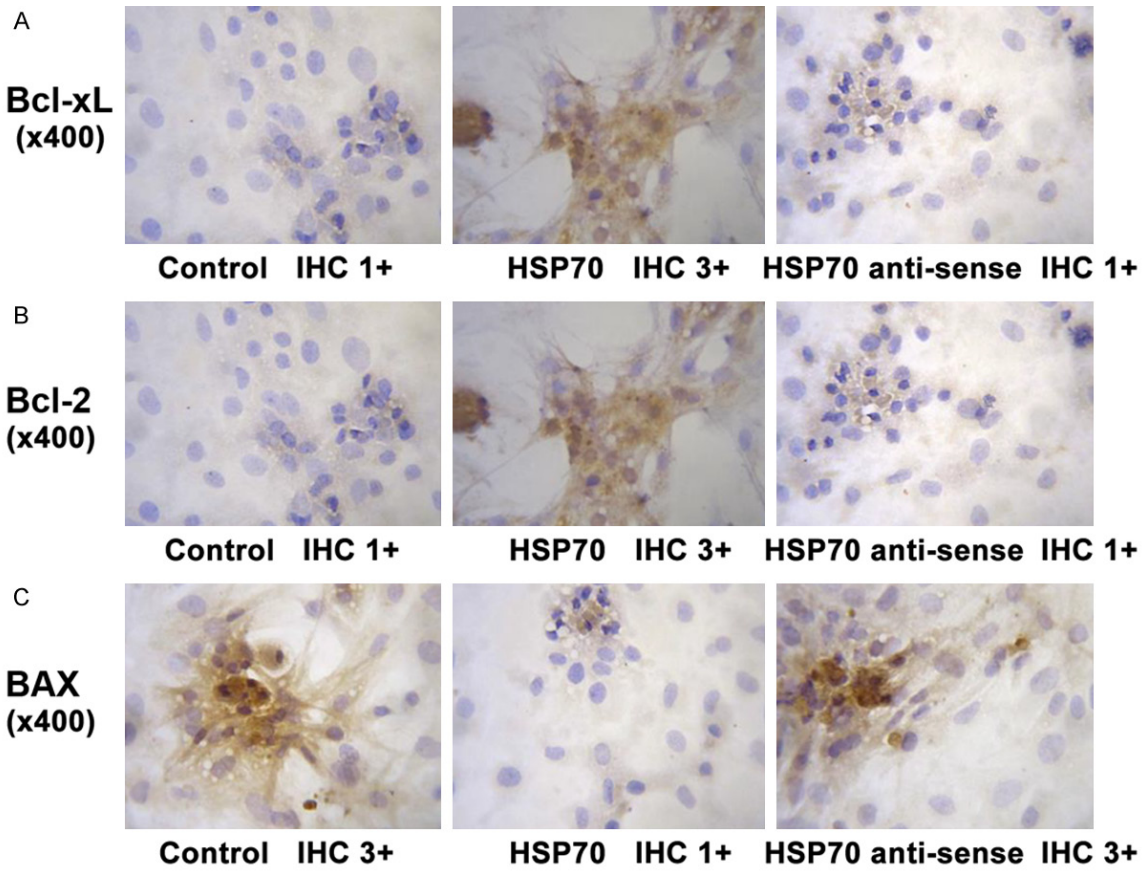

Figure 4. Immunohistochemical staining of Bcl-xL, Bcl-2, and BAX (×400). A. Bcl-xL immunohistochemical staining included very strong staining (IHC 3+) in cells in HSP70 group, but did not in cells in HSP70 anti-sense oligonucleotide group (IHC 1+). B. Bcl-2 immunohistochemical staining included very strong staining (IHC 3+) in cells in HSP70 group, but did not in cells in HSP70 anti-sense oligonucleotide group (IHC 1+). C. BAX immunohistochemical staining did not include strong staining (IHC 3+), but included very strong staining (IHC 3+) in cells in HSP70 anti-sense oligonucleotide group.

nucleotide technology was applied to inhibit HSP70 expression. Then, the model of cardiomyocyte hypoxia/reoxygenation was established with the webster method. After confirmed the expression of HSP70 and inhibition of HSP70 in cell apoptosis, ER was extracted to test the changes in expression of key signal molecular and pathway that related to apoptosis and mediated by ER stress, such as ROS, and casepase-12, CHOP, JNK pathway, CaSR, SERCA in ER, as well as Bcl-xL, Bcl-2 and BAX, and Fas and FasL.

It was demonstrated that hypoxia leads to Na, K-ATPase downregulation via Ca^{2+}-Release-Activated Ca^{2+} Channels and AMPK activation [20]. The production of ROS could induce the influx of Ca^{2+} and release of CytC, which further results in myocardial dysfunction [20]. Inhibition the production of ROS could inhibit the cell apoptosis. The deficient in Bax could inhibited the cell apoptosis, prevent the production of ROS. SERCA is a myocardial Ca^{2+}-ATP enzyme, plays an important role in calcium homeostasis. Heat-shock proteins attenuate SERCA inactivation via the anti-apoptotic protein Bcl-2, possible responsible for the ER Ca^{2+}-mediated apoptosis [13]. It was reported that PML regulates apoptosis at ER by modulating calcium release [21]. Exendin-4 prevents c-Jun N-terminal protein kinase activation via tumor necrosis factor alpha (TNF-α) and inhibits TNF-α-induced apoptosis in insulin-secreting cells [22]. This study further confirmed the HSP70 inhibited hypoxia-induced cell apoptosis via inhibition the production of ROS, expression of BAX, Caspase 12 and CHOP, and promotion of the Bcl-xL, Bcl-2 and JNK pathway. HSP70

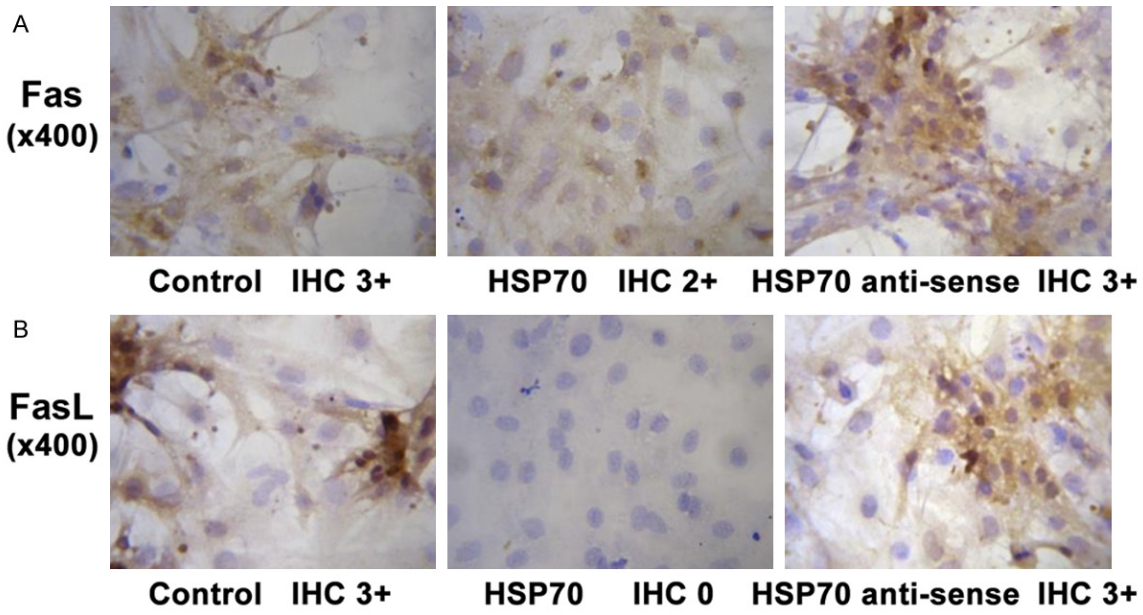

Figure 5. Immunohistochemical staining of Fas and FasL (×400). (A) Fas and (B) FasL. Fas and FasL were predominantly expressed in cells of HSP70 anti-sense oligonucleotide group (IHC 3+). Lower staining levels of Fas and FasL detected in cells in HSP70 group were IHC 2+ and 0, respectively.

might inhibited the hypoxia-induced influx of Ca^{2+} through decreasing ROS, and inhibition of CaSR and SERCA.

This project has been studied the regulation relevance of tissue-inducible protein HSP70 and ERS, which combines the myocardial protection with subcellular changes. This study is helpful to elucidate myocardial protection mechanism of HSP70 regulated ER stress-induced apoptosis from molecular levels, to lay the foundation of molecular theory for myocardial protection into subcellular levels, and to provide new intervention targets and theories of transformation application for myocardial subcellular protection.

Acknowledgements

Supported by Natural Science Foundation of Shandong Province (project number: ZR2013-HM008).

Disclosure of conflict of interest

None.

Address correspondence to: Yan Jiao, Department of Cardiovascular Surgery, Weifang People's Hospital, No. 151 Guangwen Street, Kuiwen District, Weifang 261041, Shandong, China. Tel: 05368192148; Fax: 05368232271; E-mail: jaeoth@sina.com

References

[1] Chambers DJ and Fallouh HB. Cardioplegia and cardiac surgery: pharmacological arrest and cardioprotection during global ischemia and reperfusion. Pharmacol Ther 2010; 127: 41-52.

[2] De Hert SG, Preckel B, Hollmann MW and Schlack WS. Drugs mediating myocardial protection. Eur J Anaesthesiol 2009; 26: 985-995.

[3] Bonomo J, Welsh JP, Manthiram K and Swartz JR. Comparing the functional properties of the Hsp70 chaperones, DnaK and BiP. Biophys Chem 2010; 149: 58-66.

[4] Eiras S, Fernandez P, Pineiro R, Iglesias MJ, Gonzalez-Juanatey JR and Lago F. Doxazosin induces activation of GADD153 and cleavage of focal adhesion kinase in cardiomyocytes en route to apoptosis. Cardiovasc Res 2006; 71: 118-128.

[5] Wang X, Eno CO, Altman BJ, Zhu Y, Zhao G, Olberding KE, Rathmell JC and Li C. ER stress modulates cellular metabolism. Biochem J 2011; 435: 285-296.

[6] Zhang K and Kaufman RJ. Identification and characterization of endoplasmic reticulum stress-induced apoptosis in vivo. Methods Enzymol 2008; 442: 395-419.

[7] Pincus D and Walter P. A first line of defense against ER stress. J Cell Biol 2012; 198: 277-279.

[8] Liu J, Ren F, Cheng Q, Bai L, Shen X, Gao F, Busuttil RW, Kupiec-Weglinski JW and Zhai Y.

Endoplasmic reticulum stress modulates liver inflammatory immune response in the pathogenesis of liver ischemia and reperfusion injury. Transplantation 2012; 94: 211-217.

[9] Qi W, Mu J, Luo ZF, Zeng W, Guo YH, Pang Q, Ye ZL, Liu L, Yuan FH and Feng B. Attenuation of diabetic nephropathy in diabetes rats induced by streptozotocin by regulating the endoplasmic reticulum stress inflammatory response. Metabolism 2011; 60: 594-603.

[10] Zhang K and Kaufman RJ. From endoplasmic-reticulum stress to the inflammatory response. Nature 2008; 454: 455-462.

[11] Tsutsumi S, Gotoh T, Tomisato W, Mima S, Hoshino T, Hwang HJ, Takenaka H, Tsuchiya T, Mori M and Mizushima T. Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis. Cell Death Differ 2004; 11: 1009-1016.

[12] Hsu CL, Prasad R, Blackman C and Ng DT. Endoplasmic reticulum stress regulation of the Kar2p/BiP chaperone alleviates proteotoxicity via dual degradation pathways. Mol Biol Cell 2012; 23: 630-641.

[13] Dremina ES, Sharov VS and Schoneich C. Heat-shock proteins attenuate SERCA inactivation by the anti-apoptotic protein Bcl-2: possible implications for the ER Ca^{2+}-mediated apoptosis. Biochem J 2012; 444: 127-139.

[14] Walter L and Hajnoczky G. Mitochondria and endoplasmic reticulum: the lethal interorganelle cross-talk. J Bioenerg Biomembr 2005; 37: 191-206.

[15] Zeng Y, Yao X, Chen L, Yan Z, Liu J, Zhang Y, Feng T, Wu J and Liu X. Sphingosine-1-phosphate induced epithelial-mesenchymal transition of hepatocellular carcinoma via an MMP-7/syndecan-1/TGF-β autocrine loop. Oncotarget 2016; 7: 63324-63337.

[16] Zeng Y and Liu J. Role of glypican-1 in endothelial NOS activation under various steady shear stress magnitudes. Exp Cell Res 2016; 348: 184-189.

[17] Kim I, Xu W and Reed JC. Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. Nat Rev Drug Discov 2008; 7: 1013-1030.

[18] Toth A, Nickson P, Mandl A, Bannister ML, Toth K and Erhardt P. Endoplasmic reticulum stress as a novel therapeutic target in heart diseases. Cardiovasc Hematol Disord Drug Targets 2007; 7: 205-218.

[19] Nickson P, Toth A and Erhardt P. PUMA is critical for neonatal cardiomyocyte apoptosis induced by endoplasmic reticulum stress. Cardiovasc Res 2007; 73: 48-56.

[20] Gusarova GA, Trejo HE, Dada LA, Briva A, Welch LC, Hamanaka RB, Mutlu GM, Chandel NS, Prakriya M and Sznajder JI. Hypoxia leads to Na,K-ATPase downregulation via Ca(2+) release-activated Ca(2+) channels and AMPK activation. Mol Cell Biol 2011; 31: 3546-3556.

[21] Giorgi C, Ito K, Lin HK, Santangelo C, Wieckowski MR, Lebiedzinska M, Bononi A, Bonora M, Duszynski J, Bernardi R, Rizzuto R, Tacchetti C, Pinton P and Pandolfi PP. PML regulates apoptosis at endoplasmic reticulum by modulating calcium release. Science 2010; 330: 1247-1251.

[22] Natalicchio A, De Stefano F, Orlando MR, Melchiorre M, Leonardini A, Cignarelli A, Labarbuta R, Marchetti P, Perrini S, Laviola L and Giorgino F. Exendin-4 prevents c-Jun N-terminal protein kinase activation by tumor necrosis factor-alpha (TNFalpha) and inhibits TNFalpha-induced apoptosis in insulin-secreting cells. Endocrinology 2010; 151: 2019-2029.