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Original Article

Influence of silencing toll-like receptor 4 with RNA interference on cardiomyocytes apoptosis induced by isoproterenol

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Abstract: In this study, we investigated the influence of silencing TLR4 with RNA interference (RNAi) on cardiomyocyte apoptosis induced by isoproterenol (ISO). Two plasmids containing specific to TLR4 siRNA sequences were constructed and transfected into primary cultured mice cardiomyocytes. The mRNA and protein expressions of TLR4 were analyzed by real-time polymerase chain reaction (PCR) and Western blotting respectively, and the plasmids that silenced TLR4 most significantly were selected, and renamed TL-R. The plasmids carrying a non-specific siRNA coding sequence (PCN) served as the negative control. Apoptosis of cardiomyocyte was induced by ISO at a concentration of 1 umol/L. Rates of cardiomyocyte apoptosis were evaluated by flow cytometery. The protein expression levels of Bcl-2 and Bax were detected by Western blotting. The plasmids contained siRNA sequences specific of TLR4 were constructed and transfected into the primary cultured cardiomyocytes successfully. Rates of cardiomyocyte apoptosis was obviously increased in three ISO-treat group. The results demonstrated that silencing TLR4 with RNA interference can significantly decreased cardiomyocyte apoptosis, accompanied by an increased expression of the antiapoptotic protein Bcl-2 and a decreased expression of the proapoptotic protein Bax. It was concluded that the recombinant plasmids could be successfully constructed, and transfected into the primary cultured cardiomyocytes. They could ameliorate the cardiomyocyte apoptosis induced by ISO efficiently.

Keywords: Cardiomyocyte, isoproterenol, toll-like receptor 4, RNAi, apoptosis

Introduction

Cardiovascular disease is the leading cause of morbidity and mortality in the world [1], and the mechanism of cardiovascular disease has caused wide attention. Toll-like receptor 4 (TLR4) is a pivotal signaling receptor responsible for triggering innate immune response and is expressed on the surface of variety of cardiac cells, including cardiomyocytes, endothelial cells and smooth muscle cells. In recent years, Riad et al. [2] found that TLR4 contributes to cardiac inflammation, oxidative stress, and apoptosis as well as LV function in experimental. Inhibition of TLR4 is therefore a promising therapeutic strategy for cardiovascular disease.

As a beta agonist, Isoproterenol can be used to treat the bronchial asthma and cardiac atrioventricular block, but it also can cause different degrees of myocardial damage. Isoproterenol stimulation gave rise to a hypertrophic morphology and resulted in the early activation of key signaling pathways including ROCK [3], and Chowdhury et al. [4] demonstrated that isoproterenol induced cardiac hypertrophy effects at the molecular level, affecting the cardiac protein expression profiles in SD rats. At the same time, a large number of studies found that Isoproterenol are associated with Ca^{2+} leak when it had an effect on the cardiac [5-7].

RNA interference (RNAi) is a post-transcriptional regulatory pathway that can result in efficient and specific silencing gene functions, which is a major advantage of targeted therapy [8]. In present study, in order to evaluate the influence of silencing TLR4 with RNAi on cardiomyocyte apoptosis induced by isoproterenol (ISO), we

designed and constructed recombinant plasmids target to TLR4, transferred them into the primary cultured mice cardiomyocytes, and then delineated whether TLR4 in ISO-treat group could be down-regulated with RNAi and whether siRNA-TLR4 could alleviate ISO-induced cardiomyocyte apoptosis.

Methods

Animals and regents

The neonatal BALB/c mice born within 24 to 72 h were obtained from the Experimental Animal Center of Medical College of Shihezi University. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Shihezi University. ISO was provided by Sigma Company. pSUPER.retro.neo+GFP (Oligo Engine). Collagenasel, pancreatin, foetal bovine serum (FBS) and DMEM high-glucose medium was provided the services by Gibco Co. USA. The plasmids carrying a nonspecific siRNA coding sequence (PCN), and Trizol were purchased from Invitrogen company, USA. FuGENE HD was provided by Roche Company. M-MLV reverse transcription kit and PCR master mix were obtained from TOYOBO Co. EZNA Plasmid Kit was provided from Omega company. Annexin V-PE/7-AAD Apoptosis Detection Kit was provided from Nanjing KeyGen Biotech. Co. Ltd. The PCR primers for TLR4 and GAPDH were provided the services by Invitrogen company.

Recombinant plasmid amplification and purification

Target sequences were selected on the strength of the TLR4 mRNA nucleotide sequences published on GenBank (NM_021297). Two optimal siRNA specific sequences were obtained: TL-1: 5'-GAA CAA ATG ACA TGT GCAA-3', TL-2: 5'-GAA ATG AGC TGG TAA AGAA-3'. The recombinant plasmids TL-1 and TL-2 were created by us in our Laboratory. Steps for cloning oligonucleotides into pSUPER.retro plasmid were formulated strictly on the base of pSUPER RNAi system protocol. A duplex was formed by annealling forward and reverse oligonucleotides. With the same restriction enzymes, the annealed oligos were then ligated into the Bgl II-Hind III cleavage-site within the pSUPER.retro vector prelinearised. Competent E.coli cells which recombinant plasmid containing inserts was transformed into cultured at 37°C overnight in a rocking bed after selection in ampicillin-containing medium. The plasmids were extracted on the base of manufacturer's instructions. The plasmids carrying a nonspecific siRNA coding sequence (named PCN) was served as the negative control.

Cardiomyocyte culture and transfection

Neonatal cardiomyocytes were minced in a Ca^{2+} and Mg^{2+} free Hanks' balanced solution. Cardiomyocytes were digested with 0.125% trypsin and 0.1% Collagenase I at 37°C and gentle shaked. The isolated cells were plated in DMEM (Dulbecco's modified Eagle medium) containing 15% FBS in 6-well polystyrene plates and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Bromodeoxyuridine (Brdu), 0.1 mmol/L, was present for 48 hours from the time of plating to prevent non-cardiomyocyte proliferation. Immunohistochemistry staining of α-actin was used to identify cardiomyocytes.

Myocardial cells were divided into four groups: 1. Control group (normal control); 2. PCN group; 3. TL-1 group; 4. TL-2 group in which the myocardial cells were treated with negative-siRNA plasmids (PCN), TL-1 plasmids and TL-2 plasmids respectively for 48 h. Then the plasmids were transfected into primary cultured mouse cardiomyocytes by using FuGENE HD transfection agents on the base of the manufacturer's protocol. An inverse fluorescence microscope was used to observe the expression of green fluorescent protein (GFP) in the cardiomyocytes and the transfection efficiency was figured up a total 48 h after the transfection.

RNA extraction and real-time PCR

Total RNA from cardiomyocytes was extracted with Trizol (Invitrogen, Carlsbad, Calif., USA) after transfection which sustained for forty-eight hours. DNA was degraded using RQ1 RNase-Free DNae (Promega, Madison, Wisc., USA) in strict accordance with the manufacturer's instructions to avoid genomic DNA contamination. For first-strand cDNA synthesis, 2.5 μg total RNA was dissolved in 25 μl DEPC-treated

ddH_2O. Each sample was added five microlitres of OligdT (200 ng/μL) before incubation at 70°C for 10 min and cooled on ice. Then samples were subjected to a mixture consisting of 5'M-MLV RT-buffer (10 μl/sample; Promega, Madison, Wisc., USA), dNTP (10 mM, 5 μl/sample), RNAsin (40 U/μl, 1 μl/sample; Promega), M-MLV RT (200 U/μl, 1 μl/sample; Promega) and DEPC-treated ddH_2O (3 μl/sample). This mixture of 20 μl was added to each RNA/OligdT solution. Then samples were scored 60 min at 42°C and finally 15 min at 70°C after sufficiently mixed.

Relative quantification of specific gene expression was done by two-step real-time PCR using cDNA as a template after conversion of RNA into cDNA with reverse transcriptase. The SYBR Premix Ex TaqTM (Takara, Dalian, China) on a LightCycler system (Roche Molecular Biochemicals, Indianapolis, Ind., USA) was used to monitor the amount of PCR. The sequences of the primers were as follows: TLR4 forward: 5'ACCTGGCTGGTTTACACGTC3' and reverse: 5'-CTGCCAGAGACATTGCAGAA-3'. GAPDH forward: 5'-GCC AAA AGG GTC ATC ATC TC-3' and reverse: 5'-GTA GAG GCA GGG ATG ATG TTC-3'. The PCR mixture contained 20 μl reaction solution including 2.0 μl cDNA, 10 μl 2× SYBR Premix Ex TaqTM and 0.2 μM of primers (0.4 μl forward primer and reverse primer, respectively). Amplification was in accordance with the following temperature profile: 95°C for 2 min, followed by 40 cycles at 94°C for 15 s, annealing and extension at 65°C for 30 s after optimization of PCR conditions. And the efficiency of amplification was tested in standard curves using serial cDNA dilution. Correlation coefficient had to be more than 0.9 and slope around -3.5. Melting curves was used to check amplification specificity. The Light-Cycler software was for data analysis. All samples were amplified in triplicate from the same RNA preparation and the mean value was well considered. The mRNA of GAPDH, which was independent of cardiac hypertrophy and was used as a housekeeping gene, was used to normalize the expression of gene. Calculation of expression was by the $2^{-\Delta\Delta CT}$ method in accordance with Pfaffl [9].

Western blot analysis

Transfected cardiomyocytes were harvested, centrifuged, and total protein was extracted. The BCA assay was used to determine protein concentrations. Protein samples (20 μg) were separated by 10% SDS-PAGE and then transferred onto PVDF membranes. These membranes were blocked in Tris buffered saline (TBS) containing 5% non-fat dry milk for 3 h at room temperature, and 1% between 100 followed by incubation with primary antibodies (anti-TLR4 1:500) overnight at 4°C. With anti-mouse horseradish peroxidase-conjugated secondary antibody (1:1000) for 3 h at room temperature, blots were then incubated. Immunoreactive proteins were visualized by means of enhanced chemiluminescence reagent by ECL Hyperfilm. The protein band intensity was evaluated by the Quantity One software, calculated the inhibition ratio of TLR4 expression, and selected the plasmids having the best silence effect to TLR4, renamed it as TL-R plasmids.

Experimental grouping (effect of silencing TLR4 on cardiomyocyte apoptosis induced by ISO)

As shown in **Figure 5**, cardiomyocytes were divided into four groups: 1. Control group; 2. ISO group in which the cardiomyocytes were treated with ISO of 1 umol/L; 3. PCN-ISO group; 4. TL-R+ISO group in which the cells were pretreated with PCN or TL-R plasmids for 48 hours, then treatment with ISO at a concentration of 1 umol/L for another 24 hours. Apoptotic cell death was analyzed by assays of Annexin V-PE and 7-aminoactinomycin D (7-AAD) with the use of flow cytometry, on the base of the manufacturer's instructions of Annexin V-PE/7-AAD Apoptosis Detection Kit. After double staining, flow cytometry (BD Biosciences, USA) was used to evaluate the ratio of apoptosis. Cardiomyocytes of the 4 groups were harvested and centrifuged, and the expression of Bcl-2 and Bax proteins was detected by Western Blotting, as described previously.

Statistical analysis

The data are expressed as $\bar{x}\pm s$. Differences between groups were assessed with one-way analysis of variance (ANOVA), with the aid of SPSS11.5 software package. A value of $P < 0.05$ was considered statistically significant.

Results

Identification of primary cardiomyocytes

Over 95% of the cells thus obtained were cardiomyoeytes. Immunohistochemistry staining

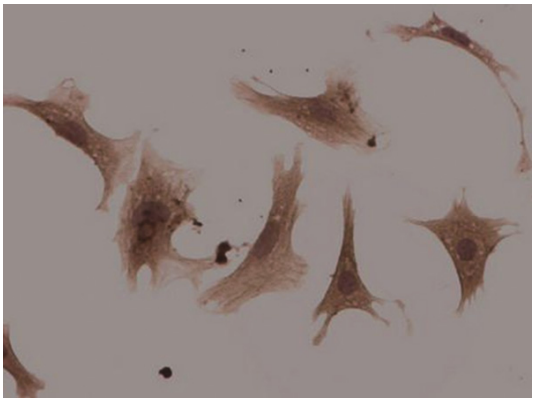

Figure 1. Cardiomyocytes identified by anti-α-actin antibody immunostaining (×400).

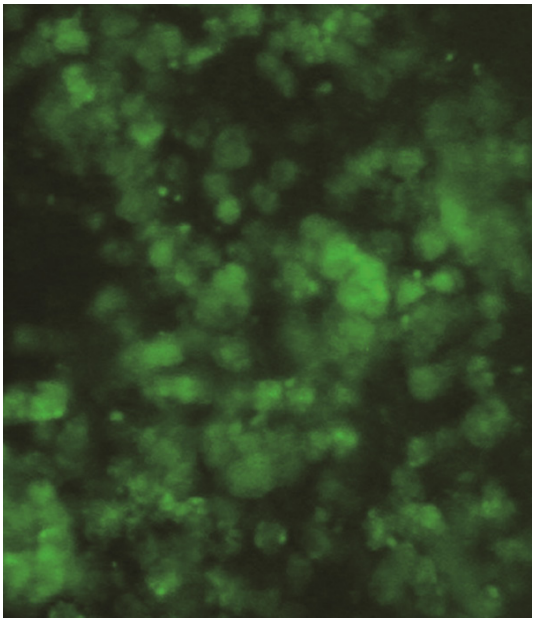

Figure 2. GFP expressions in the cardiomyocytes detected by fluorescence microscopy 48 h after transfection (×200).

of α-actin was used to identify cardiomyocytes (**Figure 1**).

Evaluation of transfection efficiency by expression of GFP

Transfection efficiency was examined under a fluorescence microscope to check the green fluorescent protein (GFP) expression. After 48 h transfection, emitting green fluorescence cells were obvious (**Figure 2**), indicating that cardiomyocytes had a high transfection efficiency with FuGENE HD, about 80%.

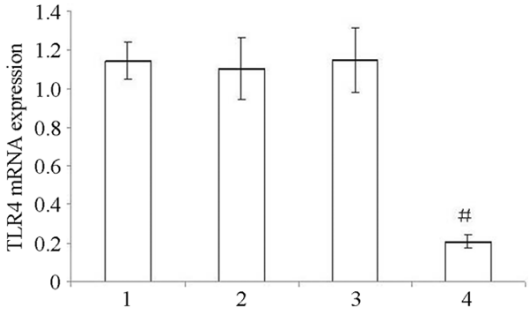

Figure 3. Bar graph of quantitative analysis of TLR4 mRNA expression of cardiomyocytes after transfection by real-time PCR. Values shown are mean ± SD. Lande 1, control group; Lande 2, PCN group; Lande 3, TL-1 group; and lande 4, TL-2 group. $^{\#}P < 0.05$, TL-2 group vs. control group, PCN group or TL-1 group.

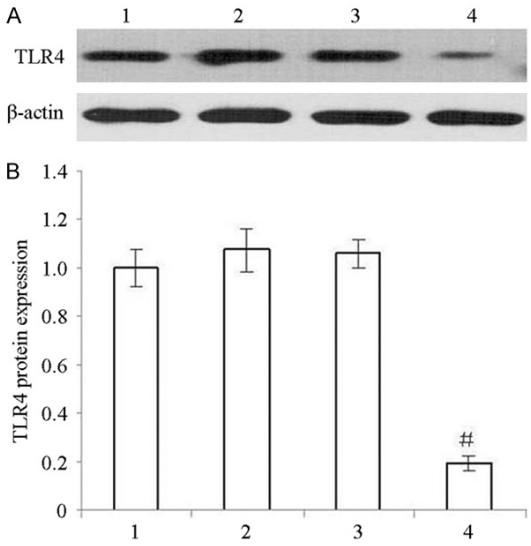

Figure 4. A. Representative Western blot analysis of TLR4 protein relative expression of cardiomyocytes after transfection. B. Quantitative analysis of TLR4 phosphorylation by Western blot. Values are shown as mean ± SD. Lande 1, control group; Lande 2, PCN group; Lande 3, TL-1 group; and Lande 4, TL-2 group. $^{\#}P < 0.05$, TL-2 group vs. control group, PCN group or TL-1 group.

TLR4 expression of cardiomyocytes after transfection

Compared with the normal control cardiomyocytes, TLR4 mRNA and protein were significantly reduced 81% and 80% ($P < 0.01$) by TL-2 plasmid (**Figures 3**, **4**). On the other hand, PCN and TLR4 transfected cardiomyocytes showed no difference in the expression as compared to the untreated cells (**Figures 3**, **4**). Obviously the

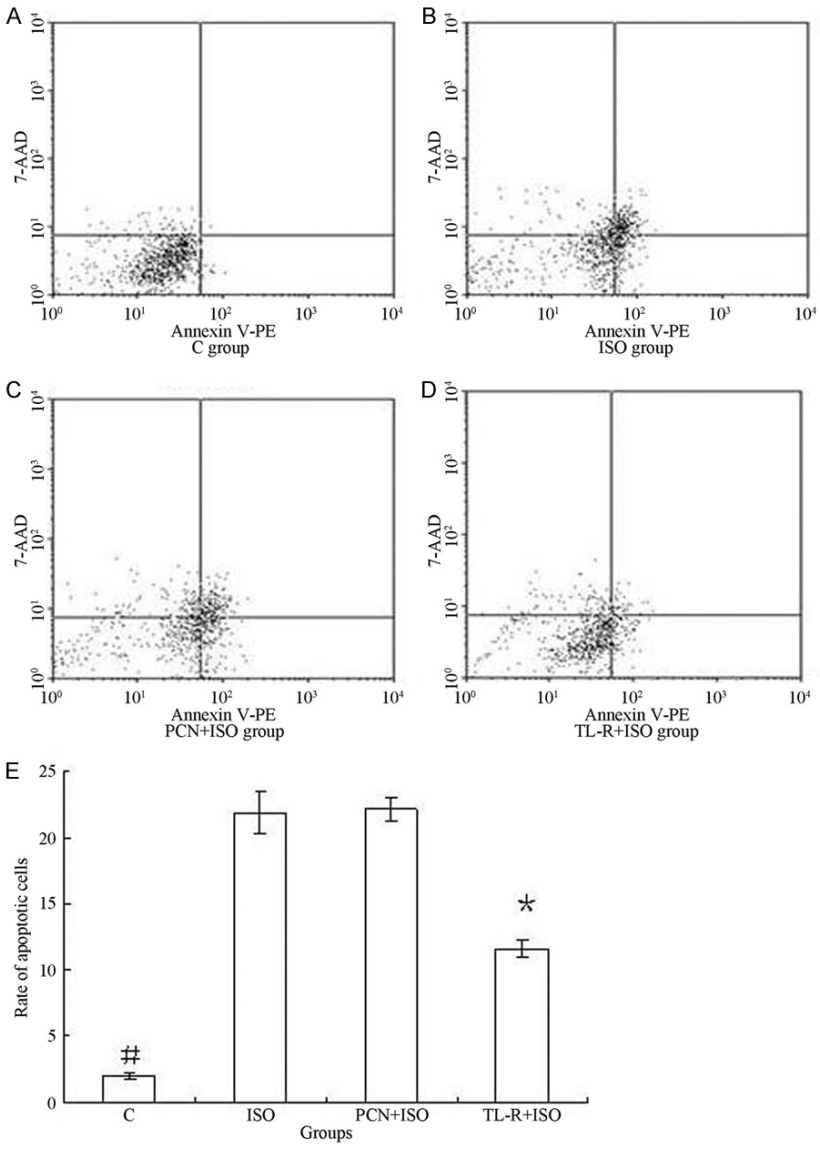

Figure 5. Effect of silencing TLR4 on cardiomyocytes apoptosis induced by ISO texted by flow cytometery. $^{\#}P < 0.05$, control group vs. ISO group, PCN+ISO group or TL-R+ISO. $^{*}P < 0.05$, TL-R+ISO group vs. ISO group or PCN+ISO group.

TL-2 plasmids had the best silence effect to TLR4, and it was renamed TL-R.

Effect of silencing TLR4 on cardiomyocyte apoptosis induced by ISO

Analysis of Annexin V-PE/7-AAD staining, our data demonstrated that apoptotic cells were obviously increased after ISO treatment. Pretreatment with TL-R plasmids significantly reduced the rate of apoptotic cardiomyocytes to near control levels (**Figure 5**).

Effect of silencing TLR4 on Bcl-2 and Bax expression of cardiomyocytes after ISO treatment

Western blotting revealed that as compared with the control group, the expression levels of Bax were increased, and those of Bcl-2 decreased in the ISO group ($P < 0.01$). However, the expression levels of Bax were decreased, and those of Bcl-2 increased in the TL-R+ISO group as compared with ISO group and PCN+ISO group ($P < 0.01$). The ratio of Bcl-2/Bax was higher in TL-R+ISO group than in ISO group and PCN+ISO group ($P < 0.01$) (**Figure 6**).

Discussion

With the change of life style, the physical and economic loss caused by cardiovascular diseases are getting more and more attention. Several lines of evidence proved that toll-like receptors (TLRs) play a great role in the process of cardiovascular disease [10-12]. TLRs, expecially TLR4 can combine with endogenous ligands and relays the signal via MyD88, a commom signal adaptor molecule, then triggering nuclear translocation and activation of NF-κB, transcription of inflammatory cytokine genes and activation of mitogen-activated protein kinases (MAPKs), leading to cardiomyocyte apoptosis [13]. A recent study suggested that the low expression of TLR4 can suppress the rates of cardiomyocyte apoptosis, thus may ameliorate the cardiac function following myocardial infarction [14]. In agreement with the previous studies, we found that ISO-induced

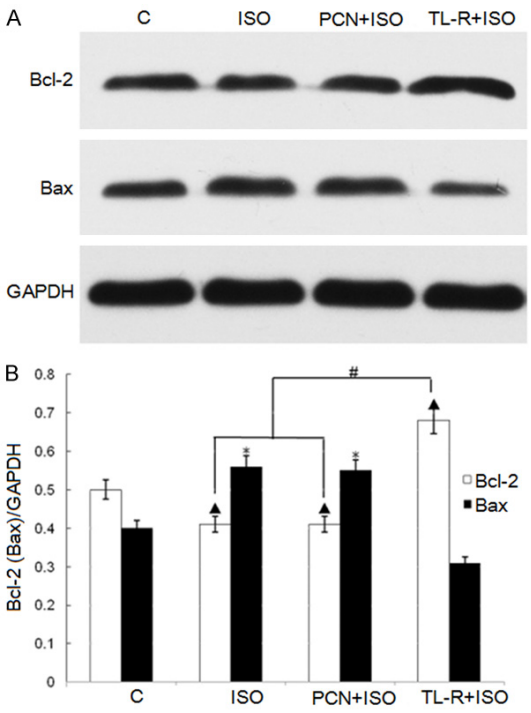

Figure 6. Effect of silencing TLR4 on Bcl-2 and Bax expression of cardiomyocytes after ISO treatment. Data were expressed as mean ± SD (n = 3). $^{*}P$ < 0.01 vs. control group or TL-R+ISO group, $^{\blacktriangle}P$ < 0.01 vs. control group, $^{\#}P$ < 0.01, TL-R+ISO group vs. ISO group or PCN+ISO.

group was observed with increased mRNA and protein levels of TLR4 compare to the control group. Inhibition of TLR4 therefore is a therapeutic method for ISO-induced cardiotoxicity.

ISO, a kind of beta receptor agonist, has important implications for the treatment of bronchial asthma and the treatment of cardiac atrioventricular block, especially for patients with cardiac arrest. What's more, echocardiography measurements showed that ISO [15]. ISO is a classic tool agent in ischemic heart diseases and heart failure investigations because of strong inotropic and chronotropic effect on cardiac muscle cells, depression effect on coronary microcirculation, as well as direct myocardial toxicity [16]. Recent studies suggest that apoptosis plays an undisputed role in ISO-induced cardiotoxicity and ISO leads to cardiomyocyte apoptosis is a major cause of myocardial ischemic necrosis [17, 18]. In this report, we found that rates of cardiomyocyte apoptosis evaluated by flow cytometery were obviously increased in three ISO-treat group compared with the control group, which suggested that the ISO-induced cardiomyocyte apoptosis model was established successfully and so was Moreau et al. [3] report.

RNAi, in which small interfering RNAs (siRNAs) can trigger specific gene transcriptional and post-transcriptional silencing, is a sequence-specific gene silencing phenomenon [19]. RNAi, focused on RNAi as a crucial role in studies on gene therapeutics recently, can disclose gene function quickly and easily result in silencing of target gene expression and disclose gene-related function in the end [20-22]. As shown in our study, the plasmids containing siRNA sequences specific of TLR4 were constructed and transfected into the primary cultured cardiomyocytes successfully. It was found that the expressions of mRNA and protein of TLR4 in TL-R group were significantly decreased than the other groups (P < 0.01), which proved that RNAi could selectively silence the expression of TLR4 in the cultured cardiomyocytes.

Furthermore, our studies had identified that inhibition of TLR4 with RNAi can lead to a cardio-protective influence on the apoptosis of cardiomyocyte induced by ISO. As we have observed that the rates of cardiomyocyte apoptosis was obviously increased in the three ISO-induced group compared with the control group (P < 0.01). In addition, compared with the ISO group and PCN-ISO group, the rate of apoptotic cardiomyocytes in TL-R group was significantly reduced (P < 0.01). From what has been discussed above, it is clear that the silience of TLR4 exerted a significant decrease in the rate of cardiomyocyte apoptosis induced by ISO. Apoptosis is regulated by a series of regulating proteins. Members of the Bcl-2 family are key regulators of apoptosis. Previous studies have showed that Bcl-2 is antiapoptotic member and Bax is proapoptotic member [23-25]. Our results found that the expression levels of Bax were increased, and those of Bcl-2 decreased in the ISO-treated groups as compared with the control group (P < 0.01). However, the expression levels of Bax were decreased, and those of Bcl-2 increased in the TL-R+ISO group in comparison to the ISO group and PCN+ISO group (P < 0.01). The results demonstrated that inhibition of TLR4 exerted a cardioprotective effect on ISO-induced apoptosis of cardiomyocytes, at least to some extent, by increasing

the antiapoptotic protein Bcl-2 and decreasing the proapoptotic protein Bax.

In summary, the present study provides preliminary evidence that RNAi can inhibit the expressions of mRNA and protein of TLR4, which exert a cardio-protective effect on cardiomyocytes, especially those apoptosis cardiomyocytes induced by ISO. Moreover, the expression of TLR4 down regulated can directly decrease the rates of apoptotic of cardiomyocytes and may ameliorated the injury of cardiomyocytes induced by ISO via interference apoptotic pathway. Thus, the expression of TLR4 silenced by RNAi may serve as a potential target for gene therapeutics in cardiomyocyte apoptosis.

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Disclosure of conflict of interest

None.

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References

[1] Nguyen MD, Tinney JP, Ye F, Elnakib AA, Yuan F, El-Baz A, Sethu P, Keller BB and Giridharan GA. Effects of physiologic mechanical stimulation on embryonic chick cardiomyocytes using a microfluidic cardiac cell culture model. Anal Chem 2015; 87: 2107-2113.

[2] Riad A, Bien S, Gratz M, Escher F, Westermann D, Heimesaat MM, Bereswill S, Krieg T, Felix SB, Schultheiss HP, Kroemer HK and Tschope C. Toll-like receptor-4 deficiency attenuates doxorubicin-induced cardiomyopathy in mice. Eur J Heart Fail 2008; 10: 233-243.

[3] Moreau S, DaSilva JN, Valdivia A and Fernando P. N-[(11)C]-methyl-hydroxyfasudil is a potential biomarker of cardiac hypertrophy. Nucl Med Biol 2015; 42: 192-197.

[4] Chowdhury D, Tangutur AD, Khatua TN, Saxena P, Banerjee SK and Bhadra MP. A proteomic view of isoproterenol induced cardiac hypertrophy: prohibitin identified as a potential biomarker in rats. J Transl Med 2013; 11: 130.

[5] Chen G, Li S, Karakikes I, Ren L, Chow MZ, Chopra A, Keung W, Yan B, Chan CW, Costa KD, Kong CW, Hajjar RJ, Chen CS and Li RA. Phospholamban as a crucial determinant of the inotropic response of human pluripotent stem cell-derived ventricular cardiomyocytes and engineered 3-dimensional tissue constructs. Circ Arrhythm Electrophysiol 2015; 8: 193-202.

[6] Roy J, Oger C, Thireau J, Roussel J, Mercier-Touzet O, Faure D, Pinot E, Farah C, Taber DF, Cristol JP, Lee JC, Lacampagne A, Galano JM, Durand T and Le Guennec JY. Nonenzymatic lipid mediators, neuroprostanes, exert the antiarrhythmic properties of docosahexaenoic acid. Free Radic Biol Med 2015; 86: 269-278.

[7] Szentandrassy N, Kistamas K, Hegyi B, Horvath B, Ruzsnavszky F, Vaczi K, Magyar J, Banyasz T, Varro A and Nanasi PP. Contribution of ion currents to beat-to-beat variability of action potential duration in canine ventricular myocytes. Pflugers Arch 2015; 467: 1431-1443.

[8] Chen XY, Tang T and Wong KS. RNA interference therapy: a new solution for intracranial atherosclerosis? Ann Transl Med 2014; 2: 79.

[9] Wen Y, Zhang XJ, Ma YX, Xu XJ, Hong LF and Lu ZH. Erythropoietin attenuates hypertrophy of neonatal rat cardiac myocytes induced by angiotensin-II in vitro. Scand J Clin Lab Invest 2009; 69: 518-525.

[10] Zhai Y, Ao L, Cleveland JC, Zeng Q, Reece TB, Fullerton DA and Meng X. Toll-like receptor 4 mediates the inflammatory responses and matrix protein remodeling in remote non-ischemic myocardium in a mouse model of myocardial ischemia and reperfusion. PLoS One 2015; 10: e0121853.

[11] Wang E, Feng Y, Zhang M, Zou L, Li Y, Buys ES, Huang P, Brouckaert P and Chao W. Toll-like receptor 4 signaling confers cardiac protection against ischemic injury via inducible nitric oxide synthase- and soluble guanylate cyclase-dependent mechanisms. Anesthesiology 2011; 114: 603-613.

[12] Arslan F, Keogh B, McGuirk P and Parker AE. TLR2 and TLR4 in ischemia reperfusion injury. Mediators Inflamm 2010; 2010: 704202.

[13] Liu ZW, Zhu HT, Chen KL, Qiu C, Tang KF and Niu XL. Selenium attenuates high glucose-induced ROS/TLR-4 involved apoptosis of rat cardiomyocyte. Biol Trace Elem Res 2013; 156: 262-270.

[14] Liu Q, Zhang J, Xu Y, Huang Y and Wu C. Effect of carvedilol on cardiomyocyte apoptosis in a rat model of myocardial infarction: a role for toll-like receptor 4. Indian J Pharmacol 2013; 45: 458-463.

[15] Kuo IY, Kwaczala AT, Nguyen L, Russell KS, Campbell SG and Ehrlich BE. Decreased poly-

cystin 2 expression alters calcium-contraction coupling and changes beta-adrenergic signaling pathways. Proc Natl Acad Sci U S A 2014; 111: 16604-16609.
[16] Rona G. Catecholamine cardiotoxicity. J Mol Cell Cardiol 1985; 17: 291-306.
[17] Sahu BD, Putcha UK, Kuncha M, Rachamalla SS and Sistla R. Carnosic acid promotes myocardial antioxidant response and prevents isoproterenol-induced myocardial oxidative stress and apoptosis in mice. Mol Cell Biochem 2014; 394: 163-176.
[18] Grisanti LA, Talarico JA, Carter RL, Yu JE, Repas AA, Radcliffe SW, Tang HA, Makarewich CA, Houser SR and Tilley DG. beta-Adrenergic receptor-mediated transactivation of epidermal growth factor receptor decreases cardiomyocyte apoptosis through differential subcellular activation of ERK1/2 and Akt. J Mol Cell Cardiol 2014; 72: 39-51.
[19] Borna H, Imani S, Iman M and Azimzadeh Jamalkandi S. Therapeutic face of RNAi: in vivo challenges. Expert Opin Biol Ther 2015; 15: 269-285.
[20] Du G, Lv J, He L and Ma Y. Influence of silencing soluble epoxide hydrolase with RNA interference on cardiomyocytes apoptosis induced by doxorubicin. J Huazhong Univ Sci Technolog Med Sci 2011; 31: 324-328.
[21] Nordlie MA, Wold LE, Simkhovich BZ, Sesti C and Kloner RA. Molecular aspects of ischemic heart disease: ischemia/reperfusion-induced genetic changes and potential applications of gene and RNA interference therapy. J Cardiovasc Pharmacol Ther 2006; 11: 17-30.
[22] Fitzgerald K, Frank-Kamenetsky M, Shulga-Morskaya S, Liebow A, Bettencourt BR, Sutherland JE, Hutabarat RM, Clausen VA, Karsten V, Cehelsky J, Nochur SV, Kotelianski V, Horton J, Mant T, Chiesa J, Ritter J, Munisamy M, Vaishnaw AK, Gollob JA and Simon A. Effect of an RNA interference drug on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum LDL cholesterol in healthy volunteers: a randomised, single-blind, placebo-controlled, phase 1 trial. Lancet 2014; 383: 60-68.
[23] Ola MS, Nawaz M and Ahsan H. Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. Mol Cell Biochem 2011; 351: 41-58.
[24] Westphal D, Dewson G, Czabotar PE and Kluck RM. Molecular biology of Bax and Bak activation and action. Biochim Biophys Acta 2011; 1813: 521-531.
[25] Garcia-Saez AJ. The secrets of the Bcl-2 family. Cell Death Differ 2012; 19: 1733-1740.