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SYK protects cardiocytes against anoxia and hypoglycemia-induced injury in ischemic heart failure

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ABSTRACT

Spleen tyrosine kinase (SYK), a non-receptor protein tyrosine kinase, is reported to be related to cell survival after A/H (anoxia/hypoglycemia) insult. However, the role of SYK in cardiocyte survival under A/H injury remains unclear. In this study, we aimed to gain insight into the role and molecular mechanism of SYK in cardiocytes exposed to A/H stress. The mRNA and protein expressions of SYK in H9c2 cardiocytes exposed to A/H injury, separately detected by real-time quantitative PCR and Western blot, were both robustly up-regulated. Then we overexpressed SYK in H9c2 with A/H injury, and found that cell viability was significantly increased and LDH leakage was decreased. Moreover, apoptosis measured by annexin V–fluorescein isothiocyanate/propidium iodide and reactive oxygen species (ROS) identified by 2′, 7′-dichlorofluorescin diacetate were markedly inhibited in H9c2 with A/H injury following SYK overexpression. Furthermore, we observed that SYK could induce HO-1 expression by regulating the Akt phosphorylation level in H9c2 with A/H injury, protecting H9c2 from the injury induced by A/H treatment.

1. Introduction

Ischemic heart disease (IHD) is caused by coronary artery atherosclerotic lesions with vascular stenosis or obstruction, in turn leading to myocardial ischemia, hypoxia or necrosis (Brondum-Jacobsen et al., 2012; Darby et al., 2013). The clinical symptoms of IHD are mainly stethalgia, palpitation, nausea and vomiting (Bunch et al., 2014; Nyrnes et al., 2013). The incidence of IHD in urban areas is higher than in rural areas, and higher in men than women (Teo et al., 2013). End-stage IHD can develop into ischemic heart failure (IHF), and the 5-year mortality rate can be higher than 50.0% (Gandelman et al., 2012). It has been shown that myocardial ischemia plays a key role in IHF (Swirski and Nahrendorf, 2013; Matsumoto et al., 2013). Ischemia always gives rise to oxidative stress. A better understanding of the molecular mechanism of myocardial ischemia is of benefit for the treatment for IHF.

Spleen tyrosine kinase (SYK), a non-receptor protein tyrosine kinase, is widely expressed in a variety of cells including fibroblasts, epithelial cells, nerve cells and myocardial cells (Brazeau and Rosse, 2014; Matsuda et al., 2016; Duta et al., 2006; Hou et al., 2016). Studies have shown that SYK is closely related to survival of a variety of cells, such as human eosinophils and breast cancer cells (Blancato et al., 2014; Hong et al., 2014). SYK has the function of preventing apoptosis in human eosinophils, chronic lymphocytic leukemia B cells and neurons, and the inhibition of SYK can induce apoptosis in T-cell non-Hodgkin lymphoma cell lines (Scheib et al., 2012; Hoellenriegel et al., 2012; Larose et al., 2014; Wilcox et al., 2010). SYK can be activated by oxidative stress and plays a key role in the activation of the serine-threonine kinase Akt, which is recognized to be an important factor in the process of cell survival and apoptosis prevention in various cells including cancer cells, neurons and myocardial cells (Chen et al., 2013; Lin et al., 2015). Studies have demonstrated that SYK is crucial for activating Akt and facilitates the cellular prevention of apoptosis induced by oxidative stress in B cells (Chen et al., 2013). However, the role of SYK in cardiocyte survival in oxidative stress-induced injury remains unclear. With this in focus, we investigated the role and molecular mechanism of SYK in protecting cardiocytes against injury induced by oxidative stress.

Heme oxygenase-1 (HO-1) is a rate-limiting enzyme of the catabolism of heme, and can antagonize the oxidative stress response, thereby protecting cells from oxidative stress-induced injury (Zhang et al., 2014). HO-1 degrades heme into carbon monoxide, iron and bilirubin (Nikam et al., 2016). The protective effect of HO-1 on cells and tissues

Abbreviations: IHD, ischemic heart disease; IHF, ischemic heart failure; SYK, spleen tyrosine kinase; HO-1, heme oxygenase-1; ROS, reactive oxygen species; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; A/H, anoxia/hypoglycemic; TBS, tris-buffered saline; RT-PCR, reverse transcription PCR; Annexin V-FITC/PI, annexin V–fluorescein isothiocyanate/propidium iodide; DLBCLs, diffuse large B cell lymphomas; DADS, diallyl disulfide

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is closely related to the increase of those degradation products. HO-1 is indicated to play an important role in the resistance of oxidative stress in cardiocytes (Chien et al., 2015). It has been demonstrated that activation of Akt can lead to the induction of HO-1 (Hsu et al., 2007). Thus, we proposed the hypothesis that SYK activates Akt to protect cardiocytes against oxidative stress by controlling the expression of HO-1.

To sum up, we aimed to detect the role of SYK in the survival of cardiocytes under oxidative stress, and measured the expression of SYK in ischemic cardiocytes. The reactive oxygen species (ROS) level, apoptosis, cell growth and viability were measured in cardiocytes with SYK overexpression. Additionally, we further investigated the underlying protective molecular mechanism of SYK in ischemic cardiocytes. Therefore, the potential role of SYK in the treatment of IHF was proposed.

2. Materials and methods

2.1. Cell culture

H9c2 cardiocyte cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 80 μg/mL G418 and 80 μg/mL hygromycin B in an incubator with 5% CO_2 at 37 °C.

2.2. Anoxia/hypoglycemic (A/H) injury

Cell culture medium was exchanged for Earle's balanced salt solution followed by incubation under hypoxic conditions (95% N_2, 5% CO_2) for 8.5 h. Thereafter, the medium was replaced by serum-free DMEM and cultured in 5% CO_2 at 37 °C for 14 h. Additionally, the culture medium of cells without A/H injury treatment was Earle's balanced salt solution supplemented with sugar, maintained under non-hypoxic conditions (95% air, 5% CO_2). All other operations were the same as the A/H injury group.

2.3. Real-time quantitative polymerase chain reaction (RT-qPCR) assay

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reversed into cDNA in accordance with the standard instructions in the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was then used as the template in the RT-qPCR. RT-qPCR was performed in a 20 μL reaction volume containing SYBR Premix Ex Taq II (TaKaRa Biotechnology, Dalian, China). The cycling parameters were: 94 °C for 30 s; 40 cycles at 94 °C for 30 s, 58 °C (SYK) or 60 °C (HO-1) for 30 s and 72 °C for 30 s; 72 °C for 10 min. The primers used were: SYK, sense primer: 5′-TGT CAA GGA TAA GAA CAT CAT AG-3′, anti-sense primer: 5′-CAC CAC GTC ATA GTA GTA ATT G-3′; HO-1, sense primer: 5′-CGG CCC TGG AAG AGG AGA TAG −3′, anti-sense primer: 5′-GGT GGG GTT GTC GAT GCT CGG-3′; GAPDH, sense primer: 5′-GGA AGA TGG TGA TGG GAT T-3′, anti-sense primer: 5′-GGA TTT GGT CGT ATT GGG-3′. The relative levels of gene expression were estimated by the $2^{-D\Delta Ct}$ method and normalized to GAPDH.

2.4. Western blot analysis

Protein was extracted following the treatment of cells with lysate (Beyotime, Nantong, China) and then quantified by a BCA kit (Beyotime). Next, protein (25 μg) was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The PVDF membrane was blocked in Tris-buffered saline (TBS) containing 5% (w/v) skim milk powder at room temperature for 2 h. Primary antibodies (SYK, HO-1, GAPDH (1:500), Abcam Inc., Cambridge, MA, USA; Akt, phospho-Akt, (1:800), Cell Signaling Technology, Beverley, MA, USA) were separatively incubated with the membrane overnight at 4 °C. The membrane was then incubated with secondary antibody ((1:1000), Abcam Inc) at 37 °C for 1.5 h. Finally, the protein was detected using an enhanced chemiluminescence method. GAPDH was the normalized protein.

2.5. Construction of recombinant vectors and cell transfection

The full length cDNA of SYK (Accession number L28824) and HO-1 (Accession number NM_012580.2) were amplified by using reverse transcription PCR (RT-PCR) followed by separative insertion into the plasmid pcDNA.3.1 (Invitrogen) with *Eco*RI and *Bam*HI restriction sites. The recombinant vectors were transformed into DH5 *Escherichia coli*-competent cells (TaKaRa) to be amplified. Finally, the amplified plasmids were extracted and sequenced, and the correct ones were named as pcDNA.3.1-SYK or pcDNA.3.1-HO-1.

Cells (1×10^4 cells/well) were plated in 12-well plates and cultured in a humid atmosphere of CO_2 at 37 °C until cell fusion reached 70%–80%. Next, the transfection was conducted using TurboFect (Thermo Fisher Scientific) as per the manufacturer's instructions. Briefly, pcDNA.3.1-SYK (5 μg), pcDNA.3.1-HO-1 (5 μg) or pcDNA.3.1 (5 μg) was diluted in 200 μL FBS-free DMEM with 6 μL TurboFect and mixed. The mixture was then added to wells followed by incubation in an incubator (Thermo Fisher Scientific) with 5% CO_2 at 37 °C for 24 h. Thereafter, the transfected cells were treated with the anoxia/hypoglycemia (A/H) injury. The transfection efficiency was measured by Western blot.

2.6. Cell growth and viability and LDH assays

Transfected or non-transfected cells (1×10^5 cells/well) were plated in a 96-well plate and treated with A/H injury. Cell growth and viability were measured by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The culture medium was replaced by 23 μL of MTT (5 g/L) diluted in phosphate-buffered saline (PBS) and incubated at 37 °C for 5 h. After that, the formation was dissolved in 150 μL of dimethyl sulfoxide (DMSO). Finally, the results were measured at a wavelength of 490 nm by using an ELISA reader (Titertek Plus MS 212, ICN, Eschwege, Germany). LDH leakage measurement, which tests cell membrane integrity, was tested by detecting the LDH activity in the culture medium supernatant using an LDH kit (Beyotime, Nantong, China) in accordance with the manufacturer's specifications.

2.7. Annexin V–fluorescein isothiocyanate/propidium iodide (annexin V-FITC/PI) and Caspase-3 activity assay

Cell apoptosis was tested by using an annexin V-FITC/PI apoptosis detection kit (Invitrogen) referring to the standard instructions. The cells were suspended in binding buffer, and 8 μL of annexin V-FITC solution was incubated with the cell suspension at 4 °C for 30 min. Cells were washed with PBS and incubated with PI (10 μL) for 8 min. The results were measured by a FACS analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

Caspase-3 activity in the cells was assessed with a caspase-3 activity assay kit (Beyotime, Nantong, China) according to the manufacturer's instructions.

2.8. ROS detection

The levels of ROS were detected following the incubation of cells with carboxy-H_2DCF-DA (Calbiochem, San Diego, CA, USA) (4 μM) for 50 min at 37 °C. Thereafter, cells were resuspended in PBS. Fluorescence-positive cells were measured by a FACSCalibur flow cytometer (BD Biosciences) at the excitation and emission filters of 488 and 530 nm.