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Original article

Corin protects H_2O_2-induced apoptosis through PI3K/AKT and NF-κB pathway in cardiomyocytes

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ABSTRACT

Background: The functional role of corin in H_2O_2-induced apoptosis is largely unexplored. The present study investigated the protective role of corin against cell injury by possible involvement of PI3K/AKT and NF-kB signaling pathways in cardiomyocytes.

Method: Cardiomyocytes H9c2 and HL-1 cells were used in the study. Cell viability was measured using CCK-8 assay; cell apoptosis was analyzed by flow cytometry, TUNEL assay, and western blot; and cell migration was measured using wound healing assay. The fluorescent intensities of reactive oxygen species (ROS) were measured using a flow cytometer. Quantitative RT-PCR was used to measure the mRNA expression of corin. Western blot was used to measure the protein expression of corin, apoptosis-related proteins (Bax, cleaved-Caspase-3 and -9), and PI3K/AKT and NF-κB signaling pathway proteins.

Results: Treatment with H_2O_2 (150 μM, 6 h) significantly decreased cell viability and relative migration, increased apoptosis, and decreased the expression of corin in H9c2 and HL-1 cells. Overexpression of corin alleviated the H_2O_2-induced cell injury by increasing cell viability and migration and decreasing apoptosis in the cardiomyocytes. Overexpression of corin also decreased the ROS level in the cardiomyocytes likely through upregulating HIF-1α. These effects of corin on the cell injury might be mediated via the corin-induced activations of PI3K/AKT and NF-κB signaling pathways.

Conclusion: Overexpression of corin protected cardiomyocytes from H_2O_2-induced injury by decreasing apoptosis and ROS level via activations of the PI3K/AKT and NF-κB signaling pathways and upregulating HIF-1α.

1. Introduction

Apoptosis is recognized to be involved in the pathological processes of various cardiovascular diseases. The role of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), in cardiomyocyte apoptosis is well established [1]. Cardiomyocyte apoptosis is known to contribute to myocardial ischemic injury, eventually resulting in heart failure. Progressive apoptosis also contributes in the cardiac remodeling, resulting in ventricular thinning and dilation [2]. In vitro studies show that cardiomyocytes, upon treatment with H_2O_2, cause free radical formation that in turn damages the biomembranes and filaments in the cardiomyocytes, eventually resulting in a FAS (Fas ligand/FasL)-mediated apoptosis. Other cardiomyocyte abnormalities due to H_2O_2-induced oxidative stress include membrane phospholipid peroxidation, thiol oxidation and ATP loss [2]. Constituents of the cardiomyocyte membrane increase many folds and a lethal disruption of the cardiomyocyte sarcolemma occurs post H_2O_2-induced oxidative stress. Cardiomyocytes treated with H_2O_2 in vitro also show various morphological and functional changes, such as pycnosis and karyorrhexis. Their cytoplasm retracts and they lose cell–cell contact causing a spontaneous disruption in beating [2].

The phosphoinositide 3-kinase (PI3K) is a lipid kinase that plays an important role in many aspects of cardiology, such as cell survival, myocardial hypertrophy, myocardial contractility, electropyhysiology and energy metabolism, making them a promising target for treatment and preventative measures in heart failure. PI3K generates phosphatidylinositol-3,4,5-phosphate membrane phospholipids that activate 3′-phosphoinositide-dependent kinase-1 (PDK-1), and AKT [3]. The PI3K/AKT cardioprotection pathway is activated by exercise, pressure overload, nutrient, and other stimulations. It is involved in insulin and insulin-like growth factor-1 (IGF-1)-dependent cardiomyocyte development. This pathway is known to inhibit apoptosis by increasing the

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mitochondrial membrane permeability, mediating protection against ischemia/reperfusion injury (IRI). LY294002 is a P13K inhibitor. It hinders the activation of the downstream regulators that include AKT [4]. Evidence indicates that elevated ROS regulates toll-like receptor-4-nuclear factor-κB (TLR-4-NF-κB) or mitogen-activated protein kinase (MAPK) pathways, and promote the secretion of inflammatory cytokines, which are highly detrimental to cardiomyocytes [1].

Corin is a trypsin-like serine protease that is highly expressed in cardiomyocytes. The primary function of corin is to activate pro-atrial natriuretic peptide (ANP), a precursor form of ANP that regulates salt-water balance and blood pressure. Under pathological conditions of heart failure, ANP along with B-type natriuretic peptide (BNP) are highly upregulated, serving a compensatory mechanism to low blood pressure and volume. They are therefore used as key biomarkers in heart failure assessments [5]. In experimental myocardial infarction studies, a significant relation between reduced levels of corin transcripts and protein levels in the heart was seen. In in vivo and in vitro studies, overexpression of corin significantly reduced infarct size and cardiomyocyte apoptosis. Corin plays a protective role by blocking cytochrome c-Caspase-mediated apoptotic pathway. Corin is also associated with hypertension, left ventricular hypertrophy, heart failure and other cardiovascular diseases [6,7]. In the present study, we examine the effect and mechanism of corin on myocardial injury induced by H_2O_2 and attempt to explore the role of PI3K/AKT and NF-κB signaling pathways in corin-mediated cardioprotective actions.

2. Material and methods

2.1. Cell culture and treatment

Cardiomyoblasts H9c2 and HL-1 cells, purchased from ATCC, were cultured in DMEM supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in an atmosphere of 95% air and 5% CO_2. To induce cell injury, 150 μM of H_2O_2 was used for a duration of 6 h. LY294002 and BAY 11-7082 at a concentration of 10 μM each were used as PI3K/AKT and NF-κB pathway inhibitor, respectively.

2.2. Transfection of plasmids

Corin expression vector, pcDNA3.1-corin was constructed by cloning full-length wild-type corin coding sequence into pcDNA3.1. The formation of clone constructs was confirmed by sequencing. An empty pcDNA3.1 construct was transfected as a control in the experiments.

2.3. Quantitative real-time PCR analysis

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) as per manufacturer's instructions. DNaseI (Promega) was added to the isolated RNA to remove DNA impurities. Reverse transcription was performed by MultiScribe RT kit (Applied Biosystems) using random hexamers or oligo (dT). The PCR conditions for the experiments were 10 min at 25 °C, 30 min at 48 °C, and 5 min at 95 °C.

2.4. Cell counting kit-8 assay

Cell viability was assessed by a cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). Cells were seeded in a 96-well plate with 5000 cells/well. After stimulation, the CCK-8 solution was added to the culture medium, and the cultures were incubated for 1 h at 37 °C in humidified 95% air and 5% CO_2. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

2.5. Annexin V/propidium iodide (PI) double staining assay

Cell apoptosis analysis was performed using PI and fluorescein isothiocynate (FITC)-conjugated Annexin V staining. Briefly, cells were washed in phosphate buffered saline (PBS) and fixed in 70% ethanol. Fixed cells were then washed twice in PBS and stained in PI/FITC-Annexin V in the presence of 50 μg/mL RNase A (Sigma-Aldrich), and then incubated for 1 h at room temperature in the dark. Flow cytometry analysis was done using a FACScan flow cytometer (Beckman Coulter, Fullerton, CA, USA) to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive). The data were analyzed using FlowJo software.

2.6. TUNEL assay

Cell apoptosis was also analyzed by TUNEL staining assay. Cells from different groups were fixed by 4% paraformaldehyde. TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

2.7. ROS assay

The total ROS was measured by flow cytometry using 2,7-dichlorofluorescein diacetate (DCFH-DA) (Nanjing Jiancheng, Nanjing, China). The cells seeded in a 6-well plate, were washed twice with PBS post treatment and co-incubated with serum-free culture medium containing 10 μM DCFH-DA (20 min, 37 °C, in dark). The cells were washed thrice with the medium post incubation with H_2O_2. All the samples were centrifuged and the supernatants were resuspended in 500 μL PBS. The fluorescent intensities were measured using a flow cytometer (488 nm excitation, 521 nm emission).

2.8. Wound healing assay

Cells were plated in 60 mM dishes until confluence. Post a 3-h cells pre-treatment with 50 μM mytomicin C, wounds were created by cell sheets with a sterile 200 μL pipette tip. The pictures of the scratched areas were taken by an inverted microscope (Leica, Germany) at specific positions every 24 h. The wound widths were measured and the relative wound widths were calculated. The results were expressed as mean ± standard deviation (SD) for three independent experiments.

2.9. Western blot assay

The proteins used for western blotting were extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Western blot system was established using Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1000. Primary antibody was incubated with the membrane at 4 °C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the polyvinylidene difluoride membrane-carried blots and antibodies were transferred into Bio-Rad ChemiDoc™ XRS system, and then 200 μL Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

2.10. Statistical analysis

The statistical analysis was performed using SPSS 19.0 statistical software. All the experiments were replicated at least thrice and the