Research Paper

Carvedilol alleviates myocardial cell hypoxia/reoxygenation injury via PI3K/AKT/GSK3β pathway

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ABSTRACT

This study was conducted to investigated the protective mechanisms of carvedilol (C) treatment on H9c2 cell during hypoxia/reoxygenation (I/R) injury. H9c2 cell was performed for 6 h hypoxia and 12 h reoxygenation. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, annexin V-FITC/PI was conducted to detect cell apoptosis, and protein levels of p-AKT and p-GSK3β were determined by western blot. C increased viability and decreased apoptosis in H9c2 cell which was exposed to hypoxia/reoxygenation. Furthermore, C increased AKT and GSK3β phosphorylation, an effect that was immediately eliminated by LY294002. In conclusion, C could protect the cardiomyocyte from hypoxia/reoxygenation induced injury. Its mechanism is involved in the activation of PI3K/AKT/GSK3β survival pathway via increasing the phosphorylation of AKT and GSK3β, resulting in increasing cell viability and reducing cell apoptosis.

Key words: Carvedilol, hypoxia/reoxygenation, H9c2, myocardial protection.

INTRODUCTION

AMI is a severe cardiovascular disease that has been a key contributor to death and disability worldwide (O’Gara et al., 2013). In patients with AMI, timely reopening of the occluded coronary artery can reduce the extent of necrosis thereby lowering mortality. Nevertheless, the process still cause damage, called myocardial ischemia/reperfusion (I/R) injury, which implicates reactive oxygen species–induced oxidative stress, calcium overload, and calpain activation (Moens et al., 2005). A number of studies have indicated that myocardial apoptosis is initiated shortly after the onset of ischemia, and strongly associated with heart failure (Zhao et al., 2001; Larose et al., 2010).

A variety of signaling molecules, including survival kinases (Hausenloy and Yellon, 2006), contribute to ischemic preconditioning and pharmacological preconditioning response (Cao et al., 2010; Ban et al., 2008; Uchiyama et al., 2004; Vigneron et al., 2011; Crawford et al., 2003). Such survival kinases include PI3K/AKT and the downstream component glycogen synthase kinase 3β (GSK3β), ERK 1/2, mito k+ ATP channels, mitochondrial permeability transition pore (mPTP), and caveolin-3 (GαV3). AKT is a critical regulator of PI3 kinase–mediated cell survival. Constitutive activation of AKT signaling is sufficient to block cell death induced by a variety of apoptotic stimuli (Datta et al., 1999). GSK3β, a downstream target of AKT, is important in glycogen metabolism, as well as in cell proliferation, growth, and death. In contrast with other protein kinases, GSK3β is inactivated by phosphorylation. Many studies have shown that GSK3β inhibition during I/R is an important mechanism of myocardial adaptation (Juhaszova et al., 2009; Tong et al., 2002); GSK3β phosphorylation/inactivation prevents the opening of mPTP, thereby preventing myocardial apoptosis and injury from I/R (Nishihara et al., 2007).

At present, encouraging results have been obtained from clinical studies on β1-blockers that can reduce infarction size (Lund, 2010; Zhang et al., 2015; Seo et al., 2015). For example, one of β1-selective antagonist, metoprolol, is
known to increase myocardial salvage, as shown by magnetic resonance imaging (Ibanez et al., 2007). Carvedilol (CAR) is a third-generation β receptor blocker. It is used for the treatment of hypertension, angina, and cardiac dysfunction in clinical (Ripley and Saseen, 2014).

At present, many clinical and animal experiments have confirmed that carvedilol plays an important role in the prevention and treatment of myocardial ischemia-reperfusion injury and anti-myocardial cell apoptosis (Hayashi et al., 2010; Lai, 2010). However, the mechanism of myocardial ischemia reperfusion has not been elucidated. Based on this, in this study, we explore the relationship between the protective effect of carvedilol and the activation of PI3K/AKT/GSK3 signaling pathway.

**MATERIALS AND METHODS**

Rat embryonic cardiomyoblast-derived H9c2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, NY, USA) supplemented with 10% fetal calf serum at 37°C with CO2 incubation. In this study we subjected the cells to hypoxia in an “hypoxia buffer” (mM) [ NaCl 137, KCl 12, MgCl2 0.49, CaCl2 2, H2O 0.9, HEPES 4 and sodium lactate 20 (pH 6.2)] followed by reperfusion to simulate myocardial A/R injury in vitro. Briefly, H9c2 cells were incubated with the “hypoxia buffer” and exposed to a hypoxic environment of 95% N2 and 5% CO2 in air tight gas chambers at 37°C for 6 h (Thermo scientific, USA). Cells were then removed from the gas chamber, and the “hypoxia buffer” was replaced with normal culture medium for 12 h (reoxgenation) in a CO2 incubator at 37°C. The cells were then used in subsequent experiments. In all experiments, cells were plated at an appropriate density according to the experimental design and used under 70 to 80% confluence. The cells were divided into following groups: i) control, ii) H/R, iii) Carvedilol (2 μM), and iv) PI3K/AKT inhibitor, LY294002 (25 μM). In the control group, H9c2 cells were cultured under normal condition with 5% CO2.

**Cell viability assay**

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (Yu et al., 2015). H9c2 cells were seeded at a density of 1 × 10⁴ cells per well in 96-well plates. After different treatments, viable cells were stained with MTT (5 mg/ml) at 37°C for 4 h. The medium was removed, and DMSO (150 µl) was added to each well to dissolve the formazan crystals. The optical density (OD) was determined at 570 nm using a microplate reader (SpectraMax M5; Molecular Devices, California). The survival ratio of H9c2 cells was expressed as a percentage of the control.

**Determination of reactive oxygen species (ROS) production**

Intracellular ROS generation was monitored by flow cytometry using the peroxide-sensitive fluorescent probe, 2',7'-dichlorofluorescein diacetate, as described previously (Jiang et al., 2016). Intracellular ROS generation was monitored by flow cytometry using the peroxide-sensitive fluorescent probe, according to the manufacturer’s instructions (Beyotime biotechnology, Shanghai, China). Briefly, H9c2 cells were cultured in 6-well plates for 24 h. After treatment, cells were washed with the washing buffer and then incubated with ROS detection solution at 37°C in the dark for 30 min. We also harvested and resuspended the cells in the washing buffer at approximately 1 × 10⁶ cells/ml. After staining with the ROS detection solution, the cells were analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ).

**Cell death assay**

Dual staining with Annexin V-FITC/PI was conducted to detect cell apoptosis (Calderon-Sanchez et al., 2016). Flow cytometric analysis was performed 12 h after reoxgenation. The procedures were carried out in accordance with the manufacturers’ instructions. Cells were harvested by trypsinization, washed twice with phosphate-buffered saline (PBS) and resuspended in binding buffer prior to the addition of Annexin V-FITC/PI. The mixture was incubated in the dark at room temperature for 10 min. Subsequently, cellular fluorescence was measured by bivariate flow cytometry using a FACScan system (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with CellQuest™ software (BD Biosciences). Annexin V-FITC/PI dual staining discriminated between intact cells (Annexin V- /PI-), apoptotic/early apoptotic cells (Annexin V+/PI-) and necrotic/late apoptotic cells (AnnexinV+/PI+).

**Western blot analysis**

20 μg of total protein was loaded on 10% SDS-PAGE and then transferred onto PVDF membranes (Millipore Corporation, USA) in a Tris-glycine buffer at 300 mA for 90 min. The membranes were blocked by incubation with 10% (w/v) non-fat milk in Tris buffer containing 0.1% Tween-20 (TBST) by incubation for 1 h at room temperature. Subsequently, the respective membrane was incubated overnight at 4°C with the following primary antibodies (Cell Signaling Technology): p-AKT (Ser 473; 1:1000), AKT (1 : 1000), p-GSK3β (Ser9; 1 :1000), GSK3β (1 : 1000), or GAPDH (Bioworld Technology, St. Louis Park) (1:10000).
Figure 1: Cell viability was measured by MTT assays. C (2µM) treatment protected against H/R-induced cell injury. H/R decreased cell viability compared with control group, which was reversed by C (2µM). The PI3K/AKT inhibitor LY294002 eliminated the effect of C. Results represent the mean±SD from three independent experiments (n=3). *p<0.05. CTL: control; H/R: hypoxia/reoxygenation; C: carvedilol; LY: LY294002.

The membrane was washed twice with TBST, incubated with the respective secondary antibodies for 1 h at room temperature, and again washed twice with TBST. Protein blots were developed using an enhanced chemiluminescence solution. The relative band intensity was visualized and analyzed with the UVP image software (Bio-Rad, USA). Pixel densities of bands were quantified by means of UVP program and were normalized to loading control on the same blot. All experiments were performed at least three times. The mean ± SEM values of the independent experiments were calculated.

**Statistical analysis**

Significant differences between sham and I/R groups were determined using ANOVA, followed by Newman-Keuls multiple comparison test. The statistical analyses were performed using a 2-tailed Student’s t-test and represented as mean ± SEM. * P<0.05 and ** P<0.01 were considered statistically significant.

**RESULTS**

**Effect of Carvedilol on survival of H9c2 cells**

To evaluate the effect of Carvedilol on the survival of cardiomyocytes subjected to H/R, H9c2 cells were treated with different concentrations (20, 2, and 0.2 µM) of Carvedilol. As shown in Figure 1, 6 h of hypoxia followed by 12 h of reoxygenation resulted in decrease of cell survival rate to 23.78%±3.5% (versus control); in contrast, the survival rates were elevated to 73.20%±4.2, 78.38%±2.7, and 74.25%±4.6% in the presence of 20, 2, and 0.2 µmol/L of Carvedilol, respectively. We used the optimal concentration of Carvedilol (2 µmol/L) for further study. Carvedilol (2 µmol/L) treatment strongly increased cell viability. Furthermore, LY294002 abolished the protective effect of Carvedilol on H/R induced cell injury.

**Effect of Carvedilol on cell apoptosis**

To quantify the protective effect of Carvedilol on myocardial cells, we assessed the cell apoptosis by Annexin V/PI staining and flow cytometry. As shown in Figure 2, H/R induced more than 30% of H9c2 cells apoptosis; whereas, Carvedilol (2 µmol/L) treatment strongly suppressed H/R induced cell apoptosis. Furthermore, LY294002 abolished the protective effect of Carvedilol on H/R induced cell apoptosis.

**Effect of Carvedilol on the expression of AKT and its downstream protein in H9c2 cells**

Western blotting was conducted to measure AKT activity and the expression of its target genes. As shown in Figure 3, H/R induced a moderate increase of p-AKT, and Carvedilol treatment further increased AKT phosphorylation. We then examined the levels of phosphorylated GSK3β, which is a known substrate of AKT. In accordance with the change in p-AKT, the p-GSK3β level was moderately increased with H/R exposure and further increased in the cells pretreated with Carvedilol (H/R+C group). However, in the presence of LY294004, the increases of p-AKT and p-GSK3β were both abolished.
DISCUSSION

H9c2 cells in Carvedilol treatment group presented higher viability and lower apoptosis. Myocardial I/R injury are a complex process which ultimately leads to cell damage and organ dysfunction. The occurrence of apoptosis has been confirmed in the ischemia reperfusion process, according to experimental studies and clinical observations (Saraste et al., 1997; Fliss and Gattinger, 1996; Gottlieb et al., 1994). Therapies targeting apoptosis may be useful in the prevention of myocardial I/R injury, as suggested by the observation in the present study that Carvedilol was protective for H9c2 cells during H/R.

It has been shown that oxidative stress induces the PI3K/Akt dependent apoptosis in cardiac cells (Zhu et al., 2011; Angeloni et al., 2007). PI3K produces two lipid products as PIP3 contributes predominantly to Thr308 (T308) phosphorylation and membrane-associated activation of Akt (Zhu et al., 2011; Angeloni et al., 2007; Verdouw et al., 1998), while PIP2 contributes mostly to Ser473 (S473) phosphorylation (Burgoyne et al., 2012; Scheid et al., 2002) and cytoplasmic activation of Akt (Scheid et al., 2002). Recent studies have shown that AKT/GSK3β signaling plays a critical role in cardioprotection. The activation of AKT and inactivation of GSK3β are observed in preconditioning (Yin et al., 2012). In this study, Carvedilol increased phosphorylated Akt (S473) and phosphorylated GSK-3 β in the H9c2 cells. This result suggests that Carvedilol may protect against I/R induced injury by activating cytosolic Akt through phosphorylation at S473, consequently leading to inhibition of the downstream target, GSK-3β, in the H9c2 cells. This is in line
with other studies that showed that GSK-3β inhibition limits myocardial IR injury (Omar et al., 2010).

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