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Research paper

Isorhamnetin protects against hypoxia/reoxygenation-induced injure by attenuating apoptosis and oxidative stress in H9c2 cardiomyocytes

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ABSTRACT

To unveil the possible protective role of isorhamnetin, an immediate 3′-*O*-methylated metabolite of quercetin, in cardiomyocyte under hypoxia/reoxygenation (H/R) condition and the underlying mechanisms involved, H9c2 cardiomyocytes were exposed to the vehicle or H/R for 6 h (2 h of hypoxia following by 4 h of reoxygenation) with isorhamnetin (0, 3, 6, 12, 25, 50 μM for 4 h prior to H/R exposure). Apoptosis was evaluated by TUNEL staining, flow cytometry analysis and western blot assay for cleaved caspase-3. Myocardial injure in vivo was determined by infarct size using TTC staining, histological damage using H&E staining and myocardial apoptosis. Here, we found that isorhamnetin dose-dependently protected H9c2 cardiomyocytes against H/R-induced injure, as evidenced by the reduction in lactate dehydrogenase (LDH) levels, increases in cell viability, superoxide dismutase (SOD) and catalase (CAT) activity, with the maximal effects at 25 μM. In addition, isorhamnetin treatment significantly inhibited apoptosis in H/R-induced H9c2 cardiomyocytes and ameliorated H/R-induced myocardial injure in vivo, concomitant with the upregulation of sirtuin 1 (SIRT1) expression. Mechanism studies demonstrated that isorhamnetin pretreatment remarkably abolished H/R-induced downregulation of Nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) expressions and upregulation of NADPH oxidase-2/4 (NOX-2/4) expressions in cardiomyocytes. However, SIRT1 inhibition (Sirtinol) not only inhibited isorhamnetin-induced Nrf2/HO-1 upregulation and NOX-2/4 downregulation, but also alleviated its anti-apoptotic effects. Taken together, these data indicate that isorhamnetin can exhibit positive effect on H/R-induced injure by attenuating apoptosis and oxidative stress in H9c2 cardiomyocytes, which is partly attributable to the upregulation of SIRT1 and Nrf2/HO-1-mediated antioxidant signaling pathway.

1. Background

Myocardial ischemia/reperfusion (I/R) injury is characterized by deficient oxygen supply and subsequent restoration of blood flow (Lejay et al., 2016). The pathophysiological mechanisms responsible for the I/R injury are complicated, and is primarily mediated with a variety of factors (Liu et al., 2014; Zhang et al., 2014; Meng et al., 2015; Huang et al., 2016), such as the formation of reactive oxygen species (ROS) with more oxidants and less antioxidants, calcium overloading, oxidative stress and apoptosis during the processes of I/R injury. Isorhamnetin, a bioactive compound found in herbal medicinal plants, is a nature antioxidant with extensive pharmacological effects in the prevention and treatment of ischemic heart disease and circulatory disorders. However, the potential mechanisms responsible for the positive effects of isorhamnetin on cell apoptosis and oxidative stress induced by H/R insults remains enigmatic.

Silent information regulator 1 (SIRT1), a nicotinamide adenine dinucleotide (NAD)-dependent histone protein deacetylase, is a highly conserved pro-survival protein (Hu et al., 2010; Yang et al., 2013) and exhibits cytoprotective effects through regulation of antioxidants, downregulation of proapoptotic molecules and anti-inflammation effects (Li et al., 2017). It has been reported that SIRT1 protects the heart from I/R-induced injury through inhibiting the apoptosis of cardiomyocytes and exhibited protective effects on cardiomyocytes against the H/R-induced injury (Liu et al., 2010; Huang et al., 2016). Thus, it is suggested that SIRT1 may involve in the positive actions of isorhamnetin.

Yu et al. (2017) reported that SIRT1 serves as the upstream regulator of Nrf2/HO-1 pathway, which was found to protect against myocardial I/R injure. Nuclear factor erythroid 2-related factor 2

Abbreviations list: H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase; SOD, superoxide dismutase; CAT, catalase; HO-1, heme oxygenase-1; Nrf2, Nuclear factor erythroid 2-related factor 2; NOX-2/4, NADPH oxidase-2/4; SIRT1, Silent information regulator 1; NAD, nicotinamide adenine dinucleotide; CCK-8, cell counting kit-8; TUNEL, terminal deoxynucleotidyl-transferase mediated dUTP nick-end labeling

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(Nrf2), a transcriptional factor involved in cellular defenses against oxidative stress (Yuan et al., 2009; Pu et al., 2010; Chen et al., 2016), induces the endogenous antioxidant enzymes (Cheng et al., 2015), including Heme oxygenase-1 (HO-1), leading to Nrf2 dependent induction of the antioxidant genes HO-1 (He et al., 2011; Yang et al., 2014). HO-1, an endogenous cryoprotective enzyme, has recently attracted considerable attention due to their anti-apoptotic and anti-oxidative stress properties (Foresti et al., 2001; Luo et al., 2015). Oxidative stress could also lead to the liberation of Nrf2 from Keap1-Nrf2 complex and nucleus translocation (Zhang et al., 2016). HO-1 overexpression can decrease H/R-induced myocardial cell apoptosis induce by hypoxia/reoxygenation (Li et al., 2016). NADPH oxidase (NOX) 2 and 4, Nox4, a protective ROS generating vascular NAPDH oxidase (Zhang et al., 2010; Zhou et al., 2010; Guan et al., 2016), are upregulated during ischemia-reperfusion (I/R), thereby contributing to ROS production and consequent myocardial injury. Moreover, repression of either one of them can reduce ROS and I/R injury in the heart (Braunersreuther et al., 2013; Matsushima et al., 2014). Further investigations are needed to elucidate the underlying signaling mechanism.

Currently, the potential protective capacity of isorhamnetin in H/R-induced myocardial injure and the underlying mechanisms involved remains unclear. The present study is designed to investigate the role of isorhamnetin in regulating H/R-induced myocardial apoptosis and oxidative stress, and furtherly elucidate the involvement of SIRT1 and Nrf-2/HO-1 signaling pathway. Thus, isorhamnetin is a promising reagent for the treatment of myocardial I/R.

2. Materials and methods

2.1. Cell culture and experimental protocols

The rat H9c2 cardiomyocytes, purchased from (ATCC, Rockville, MD, USA), were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Tokyo, Japan) with 4.5 g/L glucose supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT, USA) and 1% (v/v) penicillin/streptomycin (Sigma, St. Louis, MO, USA) at 37 °C in a humidified atmosphere containing 5% CO_2 (Zhang et al., 2016).

H/R model was established in characterized H9c2 cardiomyocytes by exposure to hypoxia (5%CO_2, 1%O_2 and 94% N_2) for 2 h followed by reoxygenation (5%CO_2, 21%O_2 and 94% N_2) for 4 h. For further cell viability assay and determination of LDH levels, SOD and CAT activities, H9c2 cardiomyocytes were pretreated with vehicle (0.1% DMSO, sigma) or isorhamnetin (Shanghai Winherb Medical S&T Development, Shanghai, China) dissolved in DMSO to the final concentration of 3, 6, 12, 25 and 50 μM for 4 h, and then subjected to H/R. H9c2 cardiomyocytes without H/R exposure and isorhamnetin pretreatment served as control.

2.2. Cell viability assay

Cell viability were evaluated using cell counting kit-8 (CCK-8) (Dojindo, Mashiki-machi, Japan) method according to manufacture's instruction. Briefly, cells (1×10^4 per well) were plated in 96-well plates. The absorbance was measured at 450 nm using a microplate reader (MQX 200, BioTek Instruments, Winooski, VT, USA) following treatment with CCK-8 for 2 h at 37 °C, and represented as the percentage of control.

2.3. Determination of LDH levels, SOD and CAT activities

Lactate dehydrogenase (LDH) release, which served as a biochemical indicator of cellular damage, was investigated in the H/R − /Iso − (Con) and H/R + /Iso + (from 0 to 50 μM) group, respectively, according to the kit manufacture's instruction (Dojindo). Superoxide dismutase (SOD) and catalase (CAT) activities, two ROS-scavenging enzymes, were measured using a detection kit according to the manufacture's instruction. All experiments were repeated three times independently.

2.4. Apoptosis detection

Myocardial apoptosis was detected using terminal deoxynucleotidyl-transferase mediated dUTP nick-end labeling (TUNEL) assay (Roche Applied Science) and flow cytometry analysis in the H/R − /Iso − (Con), H/R + /Iso − (H/R) and H/R + /Iso + (H/R + Iso) and H/R − /Iso + (Iso) group, respectively. For quantification, the numbers of TUNEL-positive cells were counted in at least five randomly with three independent samples. The flow cytometry assay was then performed with BD FACSCalibur (Becton, Dickinson and Company, Lake Franklin, New Jersey, USA). Induced cell apoptosis was presented as percentile of apoptotic cells to total cells.

2.5. Western blot analysis

The expressions of SIRT1, Nrf-2, HO-1, Nox-2, Nox-4, caspase-3 and cleaved caspase-3 were measured using Western blot as described previously (Li et al., 2016) in the H/R − /Iso − (Con), H/R + /Iso − (H/R) and H/R + /Iso + (H/R + Iso) and H/R − /Iso + (Iso) group, respectively. The primary antibodies for SIRT1 (ab110304), Nrf2(ab62352), HO-1 (Santa Cruz, Calif., USA, sc-1796), Nox-2 (ab129068), Nox-4 (NB110–58849), caspase-3 (Sigma, C8487), cleaved caspase-3 (Sigma, SAB4503292) and β-actin (Sigma, A2228) were used. The corresponding secondary antibodies were obtained from Zhongshan biotechnology (Beijing, China). The bands were scanned and detected by chemiluminescence with a Tanon-5500 Imaging System (Tanon Science &Techology Ltd., Shanghai, China) and quantified with the ImageJ software.

2.6. Evaluation of myocardial injure in vivo

Myocardial injure in vivo was assessed by infarct size using triphenyltetrazolium chloride (TTC) staining, histological damage using hematoxylin and eosin (HE) staining examined under a light microscope (CKX41, 170 Olympus, Tokyo, Japan) and myocardial apoptosis (TUNEL staining and flow cytometry analysis). Myocardial infarct size was determined by Evans blue/TTC staining. The heart sections (1 mm thick) were immediately incubated in 1% TTC (Amresco, Solon, OH, USA) solution at 37 °C for 10 min in dark. The stained slices were photographed using a digital camera (S3100, Nikon, Japan). The size of infarct area in each section was calculated by using the image analyzer (Image-Pro Plus 6.0).

2.7. Statistical analysis

All values were represented as mean ± SEM from at least three repeated and independent experiments. Statistical analysis was performed using a one-way ANOVA. $P < 0.05$ was considered statistically significant. Statistical analysis is performed using SPSS Statistics software (SPSS16.0).

3. Results

3.1. Isorhamnetin inhibits cytotoxicity and improves antioxidant capacity in H/R-induced H9c2 cardiomyocytes

To investigate the impact of isorhamnetin on cytotoxic effect following H/R, H9c2 cardiomyocytes were pretreated with various concentration of isorhamnetin as indicated, cell viability and LDH release (a biochemical indicator of cellular damage) was assessed by CCK-8 and LDH assay (Fig. 1A and B). H/R led to a significant decrease in cell viability ($P < 0.05$) and increase in LDH release ($P < 0.05$) compared with that of the control group (Con), respectively. Whereas isorhamnetin pretreatment progressively increased cell viability and