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Protective effect of salidroside on cardiac apoptosis in mice with chronic intermittent hypoxia

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ABSTRACT

Background: The goal of this study is to determine if salidroside has protective effects on hypoxia-induced cardiac widely dispersed apoptosis in mice with severe sleep apnea model.
Methods: Sixty-four C57BL/6 J mice 5–6 months of age were divided into four groups, i.e. Control group (21% O_2, 24 h per day, 8 weeks, $n = 16$); Hypoxia group (Hypoxia: 7% O_2 60 s, 20% O_2 alternating 60 s, 8 h per day, 8 weeks, $n = 16$); and Hypoxia + S10 and Hypoxia + S30 groups (Hypoxia for 1st 4 weeks, hypoxia pretreated 10 mg/kg and 30 mg/kg salidroside by oral gavage per day for 2nd 4 weeks, $n = 16$ and 16). The excised hearts from four groups were measured by the heart weight index, H&E staining, TUNEL-positive assays and Western blotting.
Results: TUNEL-positive apoptotic cells in mice heart were less in Hypoxia + S10 and Hypoxia + S30 than those in the Hypoxia group. Compared with Hypoxia, the protein levels of Fas ligand, Fas death receptors, Fas-Associated Death Domain (FADD), activated caspase 8, and activated caspase 3 (Fas pathways) were decreased in Hypoxia + S10 and Hypoxia + S30. In the mitochondria pathway, the protein levels of BcLx, Bcl2, and Bid (anti-apoptotic Bcl2 family) in Hypoxia + S10 and Hypoxia + S30 were more than those in Hypoxia. The protein levels of Bax, t-Bid, activated caspase 9, and activated caspase 3 were less in Hypoxia + S10 and Hypoxia + S30 than those in hypoxia.
Conclusions: Our findings suggest that salidroside has protective effects on chronic intermittent hypoxia-induced Fas-dependent and mitochondria-dependent apoptotic pathways in mice hearts.

1. Introduction

Obstructive sleep apnea (OSA), a sleep breathing disorder, is associated with nocturnal airflow disruption in humans [1]. OSA is a high risk factor of cardiovascular diseases [2] and could increase the chance of heart failure by 140%, stroke by 60%, and coronary heart diseases by 30% [3,4]. Chronic intermittent hypoxia (CIH) led to multiple long-term cardiovascular pathophysiologic consequences similar to what we observed in OSA [2,5]. One study showed that CIH leads to left ventrical myocardial dysfunction [6] and our previous study showed that 8 week CIH induced cardiac abnormalities and apoptosis [7].

Salidroside [2-(4-hydroxyphenyl)ethyl beta-D-glucopyranoside], active ingredients of *Rhodiola rosea*, was used for high mountain sickness to protect erythrocytes against oxidative stress and improve resistance to stress and fatigue [8]. Salidroside was found to reduce cell apoptosis, improved cardiomyocyte glucose uptake, and reduced ischemia/reperfusion-induced cardiomyocyte damage [9]. However, the effect of salidroside on cardiovascular health is still not totally understood.

Apoptosis, a cell death program, has long been recognized to be involved in cardiovascular diseases [10,11]. The cardiomyocyte apoptosis

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is one of the predictors of cardiac diseases or heart failure [12]. Cardiac widely dispersed apoptosis was found by our laboratory in chronic cardiometabolic or stressful conditions such as obesity [13–15], hypertension [11,16,17], diabetes [18,19], ovariectomy [20], long-term hypoxia [7,21,22], and smoke [23]. The Fas receptor-dependent apoptotic (Type I) pathway is a major pathway triggering cardiac apoptosis [10,24] and initiates binding the Fas ligand to the Fas receptor [24–27]. This binding, followed by Fas-receptor oligomerization leading to the death-inducing signal complex, starts with recruitment of the Fas-Associated Death Domain (FADD) adaptor protein [24]. The activated caspase 8 cleaves pro-caspase 3 then undergoes autocatalysis to form active caspase 3, an effector caspase of apoptosis [24,25]. The mitochondria-dependent (Type II) apoptotic pathway starts with apoptosis-regulating protein family exemplified by Bcl-2 family, such as anti-apoptotic Bcl-2 and pro-apoptotic Bad [24,26,27]. Pro-apoptotic Bcl2 family will enhance cytochrome *c* release from mitochondria [24,26–28]. Cytochrome *c* release into cytosol activates caspase-9, then caspase-3 executes the apoptotic program [24,27]. Besides, t-Bid was regarded as a main intracellular molecule signaling mediator from Fas to mitochondrial pathway because activated caspase 8 can cleave Bid to t-Bid then release cytochrome *c* to activate mitochondria-dependent apoptosis [24,25]. In our previous study, the 8-week CIH was found to activate the Fas-dependent and mitochondria-dependent apoptotic pathways in rat hearts [7]. Salidroside protects H9c2 cells from ischemia/reperfusion-induced apoptosis through reduced cytochrome *c* release and caspase-3 activity [29] and attenuates apoptosis in ischemic hearts [30].

The effect of salidroside on CIH-induced cardiac apoptosis in mice hearts is not understood. We hypothesized that salidroside may prevent CIH-activated Fas-mediated and mitochondria-mediated cardiac apoptosis in mice hearts.

2. Materials and methods

2.1. Animal model and salidroside

The studies were performed on sixty-four C57BL/6 J 5–6 month old male mice. Sixty-four C57BL/6 J mice 5–6 months of age were divided into four groups, the Control group (21% O_2, 24 h per day, 8 weeks, $n = 16$); Hypoxia group (Hypoxia: 7% O_2 60 s, 20% O_2 alternating 60 s, 8 h per day, 8 weeks, $n = 16$); and Hypoxia + S10 group and Hypoxia +S30 group (Hypoxia for the first 4 weeks, Hypoxia pretreated 10 mg/kg and 30 mg/kg salidroside by oral gavage per day for the second 4 weeks, $n = 16$ and $n = 16$). Salidroside in the current study was purchased from Venter International Co., Ltd. The salidroside was extracted from Rhodiola and was tested over 98% purity in high performance liquid chromatography fingerprinting analyses. Ambient temperature was maintained at 25 °C. All mice were kept on an artificial 12-h light–dark cycle and the light period began at 7:00 A.M. The mice were provided with standard laboratory chow (Lab Diet 5001; PMI Nutrition International Inc., Brentwood, MO, USA) and unlimited water. All procedures were reviewed by the Institutional Animal Care and Use Committee, China Medical University, Taichung, Taiwan, and the principles of laboratory animal care (NIH publication) were followed.

2.2. Cardiac characteristics

The hearts of mice in the four groups (Control, Hypoxia, Hypoxia + S10, Hypoxia + S30) were excised and PBS was used to clean them. The weights of the left ventricles and the whole heart were measured. Once weighed, 8 mice hearts in each group were soaked in formalin and then were analyzed by hematoxylin–eosin, Masson trichrome staining, DAPI staining and TUNEL assay. The other 8 mice hearts of each group were cleaned, frozen, and were analyzed with Western blotting. The right tibias were separated and measured using a digital caliper to calculate the whole heart weight. The ratios of the whole heart weight (WHW) to body weight (BW), the left ventricle weight (LVW) to body weight (BW), the left ventricle weight (LVW) to the whole heart weight (WHW), the whole heart weight to tibia length and the left ventricle weight to tibia length were measured.

2.3. Echocardiography

Transthoracic echocardiographic images of all mice were gained by Philips M2424A ultrasound systems (Andover, MA) in anesthesia with 1% isoflurane via a nose cone-shaped device. M-mode echocardiographic inspection was performed with a 6–15 MHz linear transducer (15–6 L) via a parasternal long axis method. Left ventricular M-mode dimensions at the volume of the papillary muscles integrated interventricular septum (IVS), left ventricular internal end-diastolic dimensions (LVIDd), left ventricular internal end-systolic dimensions (LVIDs), posterior wall thicknesses (LVPW), and fractional shortening (FS). FS% was designed by the equation FS% = [(LVIDd − LVIDs) / LVIDd] / 100.

2.4. Tissue extraction

The left ventricle samples of cardiac tissue extracts were homogenized into a lysis buffer in a ratio of 100 mg tissue/1 ml buffer for 1 min to obtain cardiac tissue extracts. The homogenates were place on ice for 10 min then centrifuged on 12,000 *g* used for 40 min twice. The supernatant was collected and stored at −70 °C.

2.5. Electrophoresis and Western blot

The Bradford method was used to determine the protein concentration of cardiac tissue extracts (Bio-Rad Protein Assay, Hercules, CA, USA). Protein samples (50 μg/lane) were separated on a SDS polyacrylamide gel (10%) electrophoresis (SDS-PAGE) with a controlled voltage of 75 V. Electrophoresed proteins were applied to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, 0.45 μm pore size) with a Bio-Rad transfer apparatus. PVDF membranes were incubated in 5% milk with a TBS buffer. Primary antibodies used in the current study including Bcl-2 (BD Biosciences, San Jose, California, USA), Fas ligand, Fas receptor, FADD, Bcl-xL, Bax, Bid, t-Bid, caspase 8, caspase-9, caspase-3 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA), and α-tubulin (Neo Markers, Fremont, CA, USA) were diluted to a 1:500 ratio in an antibody binding buffer overnight at 4 °C. The immunoblots were washed three times in TBS buffer for 10 min and then immersed in the second antibody solution containing goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, or donkey anti goat IgG-HRP (Santa Cruz) for 1 h and diluted 500-fold in TBS buffer. The immunoblots were then washed in TBS buffer for 10 min three times. The immunoblotted proteins were visualized using an enhanced chemiluminescence ECL Western Blotting luminal Reagent (Santa Cruz, CA, USA) and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Tokyo, Japan).

Table 1
Values are mean ± SD. WHW/BW, whole heart weight normalized by body weight; LVW/BW, left ventricular weight normalized by body weight; LVW/WHW, left ventricular weight normalized by whole heart weight; WHW/Tibia, whole heart weight normalized by tibia length; LVW/Tibia, left ventricular weight normalized by tibia length; IVSd, interventricular septum at diastole; LVPWd, left ventricular posterior wall thickness at diastole; LVIDd, internal dimension at diastole of left ventricle; LVIDs, internal dimension at systole of left ventricle; FS, fractional shortening (LVIDd − LVIDs / LVIDd × l00). $^{*}P < 0.05$, significant differences from the Control group. $^{\#}P < 0.05$, significant differences from the Hypoxia group.

	Control	Hypoxia + saline	Hypoxia + S10	Hypoxia + S30
Number of mice	10	7	8	8
Body weight (BW), g	21.5 + 1	22 + 0.5	216 + 1.9	21.7 + 0.9
Whole heart weight (WHW), g	0.106 + 0.010	0.112 + 0.009	0.107 + 0.015	0.108 + 0.005
Left ventricular weight (LVW), g	0.082 + 0.011	0.078 + 0.008	0.080 + 0.008	0.085 + 0.006
WHW/BW($\times 10^4$)	49.4 + 14.7	50.6 + 4.7	50.5 + 3.6	50.6 + 4.8
LVW/BW($\times 10^4$)	36.6 + 5.2	35.1 + 4.6	38.7 + 7.5	37.9 + 5.2
LVW/WHW	0.72 + 0.05	0.69 + 0.05	0.71 + 0.07	0.76 + 0.04
WHW/Tibia, g/mm ($\times 10^4$)	58.9 + 5.1	58.8 + 2.1	61.3 + 8.9	61.7 + 4.4
LVW/Tibia, g/mm ($\times 10^4$)	44.9 + 5.8	42.7 + 4.8	46.6 + 6.7	48.0 + 5.8
(IVSd), mm	0.74 + 0.06	0 64 + 0.07	0.64 + 0.06	0.73 + 0.08
(LVPWd), mm	0.74 + 0.12	0.64 + 0.10	0.70 + 0.08	0.71 + 0.08
(LVIDd), mm	3.77 + 0.23	3.69 + 0.26	3.35 + 0.32	3.47 + 0.17
(LVIDs), mm	2.50 + 0.42	2.63 + 0.30	2.55 + 0.34	2.34 + 0.21
Fractional shortening (FS),%	33.91 + 9.77	28.93 + 6.42	34.43 + 4.61#	32.40 + 3.47