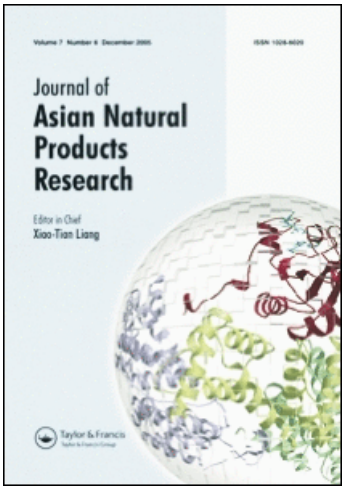

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Salvianolic acid A displays cardioprotective effects in *in vitro* models of heart hypoxia/reoxygenation injury

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ORIGINAL ARTICLE

Salvianolic acid A displays cardioprotective effects in *in vitro* models of heart hypoxia/reoxygenation injury

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Oxidative stress induced by overproduction of reactive oxygen species (ROS) plays an important role in hypoxia/reoxygenation (H/R) injury. In the present study, effects of salvianolic acid A (**1**) on heart H/R injury through its antioxidant activity were examined, using a molecule-based ROS scavenging system and cardiomyocyte model of H/R injury, as well as isolated rat heart model. As a result, **1** showed a potent antioxidant activity, scavenging all of the tested ROS and DPPH (2,2-diphenyl-1-picrylhydrazyl). The antioxidant effect of **1** was also observed in cardiomyocytes exposed to H/R. Compound **1** remarkably decreased dihydroethidium and dichlorofluorescein fluorescence and increased cell viability and mitochondrial membrane potential, $\Delta\Psi_m$, when compared to the H/R group. In isolated rat hearts exposed to H/R, **1** markedly increased the coronary flow, the peak of pressure development and the valley of pressure development, and significantly reduced the left ventricular end diastolic pressure when compared to the H/R group. These results suggested that **1** had significant protective effects against H/R-induced myocardial injury through its antioxidant activity.

Keywords: salvianolic acid A; hypoxia/reoxygenation injury; ROS scavenging; cardiomyocyte; isolated rat heart

1. Introduction

Ischemia/reperfusion (I/R) is one of the common causes of cardiovascular diseases. In this process, cardiomyocyte death is triggered, leading to complete or partial contractile dysfunction. To date, neither the mode nor the molecular mechanism of cardiomyocyte death is fully understood. It is generally assumed that cardiomyocyte death starts in the second phase (reperfusion) of I/R, while in the first phase (ischemia), early signaling events are translated into subsequent irreversible cardiomyocyte death (commitment phase of apoptosis signaling) [1]. Oxygen and nutrient insufficiency stress cells, including cardiomyocytes, responded by generating reactive oxygen species (ROS) such as superoxide anion radicals ($O_2^{\cdot-}$) [2].

Myocardial I/R is a highly lethal disease generally treated with thrombolysis as first aid [3]. However, blood flow reperfusion after thrombolysis also causes tissue damage due to the overproduction of

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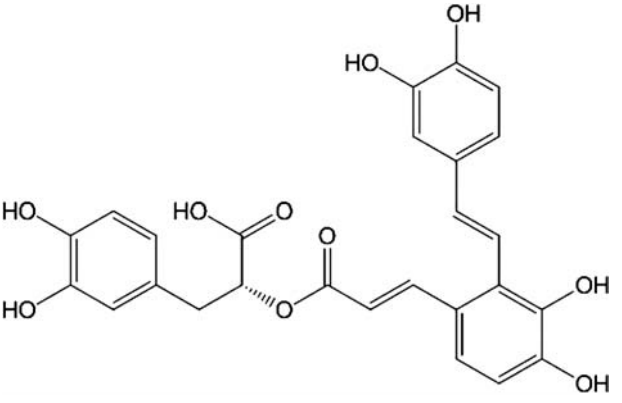

Figure 1. Chemical structure of Sal A.

ROS, and there is a lack of effective drugs in clinical practice. As a result, searching for a drug with resistance to reperfusion injury is a top priority. The dried root of *Salvia miltiorrhiza* Bunge (Danshen) is a well-known Chinese traditional herbal medicine that is widely used in Asian countries for the treatment of various diseases including cerebrovascular diseases, coronary artery diseases, and myocardial infarction [4]. Salvianolic acid A (Sal A, (2*R*)-3-(3,4-dihydroxyphenyl)-2-[(*E*)-3-[2-[(*E*)-2-(3,4-dihydroxyphenyl)ethenyl]-3,4-dihydroxyphenyl]-prop-2-enoyl]oxypropanoic acid, **1** (Figure 1)) is a main active, water-soluble constituent of *S. miltiorrhiza*. Compound **1** has a potent antioxidant activity against peroxidized damage to biomembranes [5], decreases lipid peroxidation, and scavenges hydroxyl radicals [6]. The beneficial effects of **1** seem to be partially due to its antioxidant activity, which protects vascular walls from oxidation, inflammation, and thrombus formation [7].

It is acknowledged that hypoxia/reoxygenation (H/R) is adopted to stimulate the I/R pathologic state [8]. To the best of our knowledge, the effect of **1** on the cardiac function after H/R injury has not previously been investigated. Therefore, in this study, a series of experiments were carried out to examine its *in vitro* ROS scavenging effect, intracellular antioxidant activity, cytoprotective action, and myocardial preservation on isolated perfused rat hearts suffering from H/R injury. The results showed that **1** significantly protected the cardiac function after H/R and had an obvious ROS scavenging effect and cytoprotective activity. Thus, **1** may be used to cure myocardial ischemia.

2. Results

2.1 Sal A scavenges free radicals in a molecule-based system

Compound **1** displayed a remarkable capacity to scavenge all the tested reactive species. The IC_{50} values (mean ± SD) for ROS and DPPH are shown in Table 1. IC_{50} values for $O_2^{\cdot-}$, H_2O_2, $HO^{\cdot-}$, and DPPH were 386.84 ± 17.8, 182.00 ± 13.7, 79.65 ± 9.63, and 24.26 ± 3.40 μM, respectively. Ascorbic acid exhibited IC_{50} values of 46.53 ± 8.43 μM for DPPH, 218.21 ± 14.00 μM for $O_2^{\cdot-}$, and 547.00 ± 35.30 μM for H_2O_2. Trolox displayed an IC_{50} value of 10.32 ± 1.39 μM in the $ROO^{\cdot}$ scavenging assay. Mannitol was the positive control for the $HO^{\cdot-}$ scavenging assay and demonstrated an IC_{50} value of 813.41 ± 12.42 μM.

Compound **1** promoted DPPH discoloration and prevented $O_2^{\cdot-}$-induced pyrogallic acid autoxidation, hydroxyl-induced

Table 1. DPPH, $O_2^{\cdot-}$, H_2O_2, and $HO^{\cdot-}$ scavenging activity of Sal A and positive control.

Compound	IC_{50} (μmol/l)					
	DPPH	$O_2^{\cdot-}$	H_2O_2	$HO^{\cdot-}$	ROO·	$ONOO^-$
Sal A	24.26 ± 3.40	386.84 ± 17.8	182.00 ± 13.7	160.17 ± 9.63	176.79 ± 12.55	216.26 ± 10.05
Trolox[a]	–	–	–	–	10.32 ± 1.39	–
Ascorbic acid[a]	46.53 ± 8.43	218.21 ± 14.00	547.00 ± 35.3	1300.05 ± 129	3.88 ± 0.95	5.45 ± 0.16
Mannitol[a]	–	–	–	813.41 ± 12.42	–	–

Notes: Data are expressed as mean ± SD values, $n = 5$.
[a] Ascorbic acid was used as the positive control for all the six free radical scavenging assays above, and Trolox and mannitol were used as positive controls for the peroxyl radical and hydrogen peroxide scavenging assays, respectively.

oxidation of orthophenanthroline-Fe^{2+} to orthophenanthroline-Fe^{3+}, and H_2O_2-induced oxidation of lucigenin (Table 1).

The oxygen radical absorbance capacity (ORAC) value was 1.76 ± 0.09 μmol for **1**, which was around 35% of the Trolox ORAC value. Ascorbic acid exhibited an ORAC value of 1.39 ± 0.61 μmol (Table 2). Compound **1** inhibited the ROO·-induced oxidation of fluorescein.

Compound **1** also exerted potent scavenging activity against reactive nitrogen species (RNS). The IC_{50} value for $ONOO^-$ was 216.26 ± 1.60 μM. Ascorbic acid had an IC_{50} value of 251.59 ± 5.00 μM for $ONOO^-$ (Table 1). Compound **1** inhibited $ONOO^-$-induced oxidation of dihydrorhodamine-123 (DHR-123).

2.2 Sal A protects cardiomyocytes against H/R-induced injury

As expected, H/R led to a marked decrease in cell viability (~54% decrease in MTT reduction; $p < 0.01$) for H9c2 cells (Figure 2). Moreover, during the reperfusion process, a series of concentrations of 0.001, 0.01, and 0.1 μM of **1** significantly increased cell viability (by 66.36, 77.19, and 90.62%, respectively) compared to the H/R group, and at the concentration of 0.1 μM, this phenolic acid seemed to perform a best cardioprotective effect.

2.3 Sal A inhibits ROS production induced by H/R in H9c2 cells

As oxygen stress injury caused by elevated ROS levels is one of the most common complications of myocardial ischemia, the cellular ROS level of cultured H9c2 cardiomyocytes incubated with H/R has been examined. H/R markedly increased dihydroethidium (DHE) fluorescence to 234.04% of blank control, reflecting an abnormal intracellular ROS level (Figure 3(A)). As shown in Figure 3(B), dramatic elevation of the intracellular ROS level was observed after reperfusion started.

Table 2. ROO˙ scavenging activity of Sal A and ascorbic acid evaluated by the ORAC value.

Compound	ORAC (μmol of Trolox/μmol of Sal A)
Sal A	1.76 ± 0.06
Ascorbic acid [1]	1.39 ± 0.61

Note: Data are mean ± SD values, $n = 5$.

Both 100 μM of tempol and 0.001, 0.01, and 0.1 μM of **1** significantly reduced ROS to 126.17, 201.37, 180.50, and 115.79% of blank control, respectively.

A similar result was observed using the dichlorofluorescein (DCF) fluorescence probe. The ROS level was indicated by increased ethidium fluorescence resulting from the oxidation of DCFH-DA. As shown in Figure 4(A), **1** significantly reduced H/R-stimulated DCF fluorescence intensity. Compound **1** at concentrations of 0.001, 0.1, and 1 μM significantly inhibited H/R-induced ROS production by 10.07, 21.87, and 35.54% (Figure 4(B)).

2.4 Protection of Sal A on Δ_m in H/R-induced cardiomyocytes

Mitochondrial function is traditionally expressed with the mitochondrial membrane potential (MMP, $\Delta\Psi_m$). As shown in Figure 5, $\Delta\Psi_m$ significantly decreased in response to H/R in H9c2 cells. Compound **1** at concentrations of 0.001, 0.01, and 0.1 μM significantly inhibited the H/R-induced $\Delta\Psi_m$ decrease, respectively (71.01, 82.61, and 96.69% of blank control, $n = 3$).

Furthermore, $\Delta\Psi_m$ under 0.1 μM of **1** (96.68% of blank control) was higher than $\Delta\Psi_m$ under 100 μM of tempol (82.82% of blank group), indicating that the ability of **1** to improve the mitochondrial function was better than the positive control drug.

2.5 Effects of Sal A on H/R injury in the isolated rat heart

To examine whether **1** can inhibit H/R-induced isolated heart injury, coronary flow (CF), the left ventricular end diastolic pressure (LVEDP), the peak of pressure

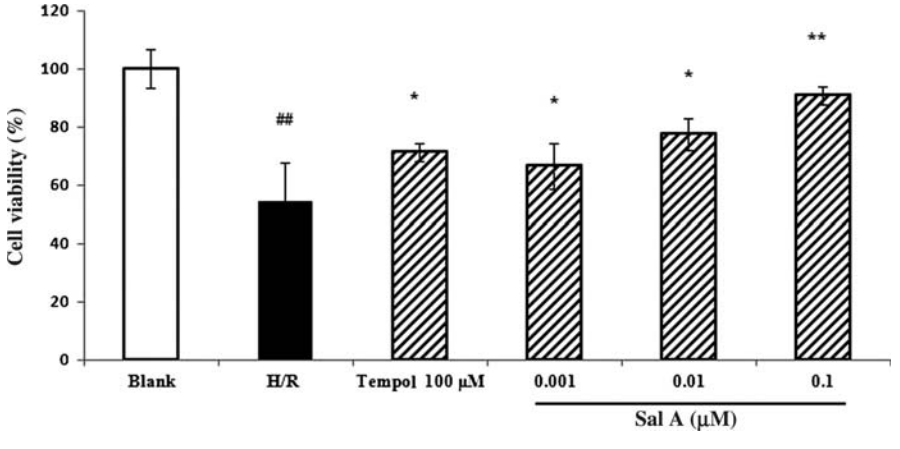

Figure 2. Effect of Sal A on H/R-induced H9c2 cell injuries. Cells were exposed to hypoxia for 12 h followed by reoxygenation for 8 h and treatment with Sal A at the concentrations of 0.001, 0.01, and 0.1 μM at the same time. The cell viability was determined using the MTT reduction assay. Cellular viability is expressed as percentage vs. blank control (100%). Results are expressed as mean ± SD, $n = 6$. $^{\#\#}p < 0.01$ vs. blank control; $*p < 005$, $**p < 0.01$ vs. H9c2 cells treated with H/R.

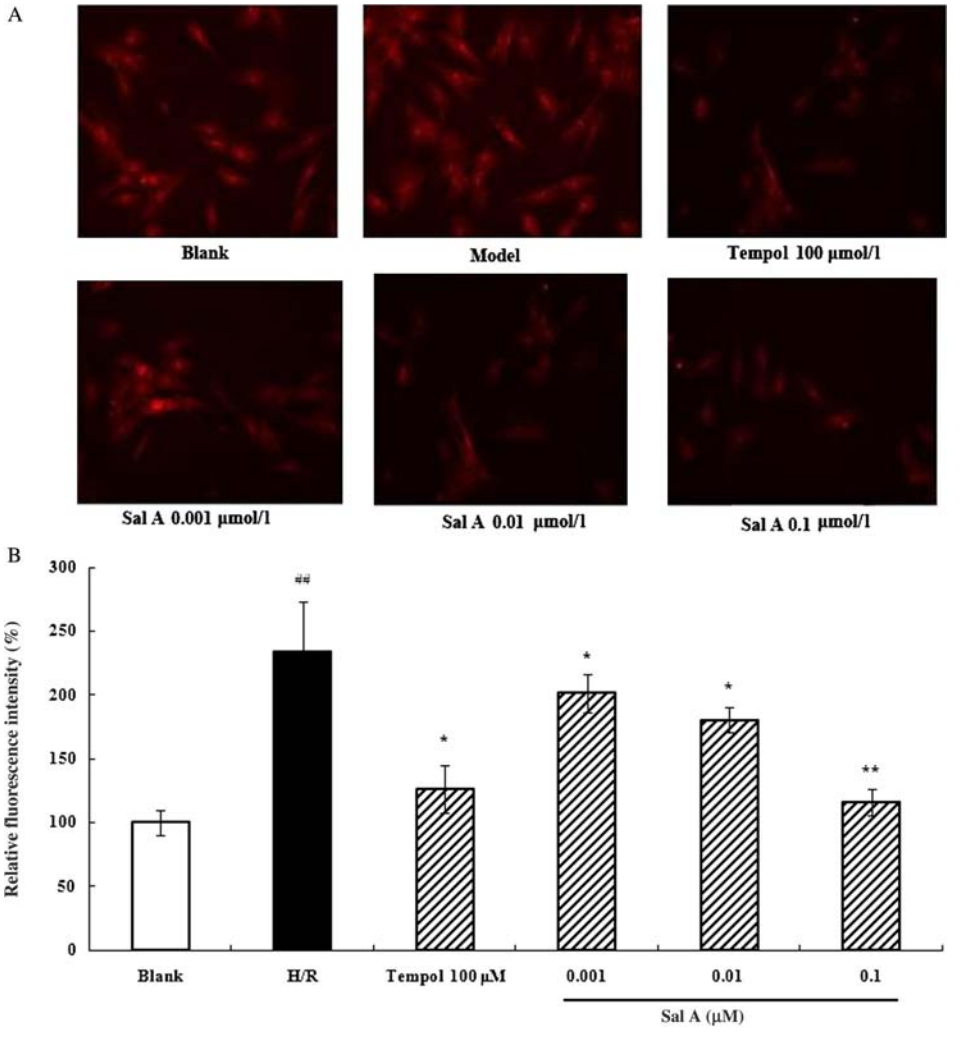

Figure 3. Effect of Sal A on H/R-stimulated H9c2 ROS production stained with DHE fluorescence probe. (A) H9c2 cells were seeded in a six-well plate. Cells were exposed to hypoxia for 12 h followed by reoxygenation for 8 h and treatment with Sal A at the concentrations of 0.001, 0.01, and 0.1 μM at the same time. After 30 min incubation with DHE (100 μmol/l), cells were rinsed with PBS three times. The representative images were from three independent experiments (200 ×). (B) The DHE fluorescence intensity was measured with IPP 6.0 software. $n = 4$, $^{\#\#}p < 0.01$ vs. blank control; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. H9c2 cells treated with H/R.

development [$(dP/dt)_{max}$], and the valley of pressure development [$(dP/dt)_{min}$] were measured.

H/R significantly decreased the CF during reperfusion (Figure 6). Compared with blank control, H/R decreased the CF from 10 to 8.2 ml/min. Administration with **1** at concentrations of 0.1, 0.2, and 0.4 μM significantly increased the CF from 8.2 to 8.30, 8.72, and 9.50 ml/min, respectively (Figure 7). Compound **1** at a concentration of 0.4 μM could maintain the CF at a stable level over the whole reperfusion period.

Similar results were also found in the LVEDP assay. As shown in Figure 8, LVEDP of the H/R group increased

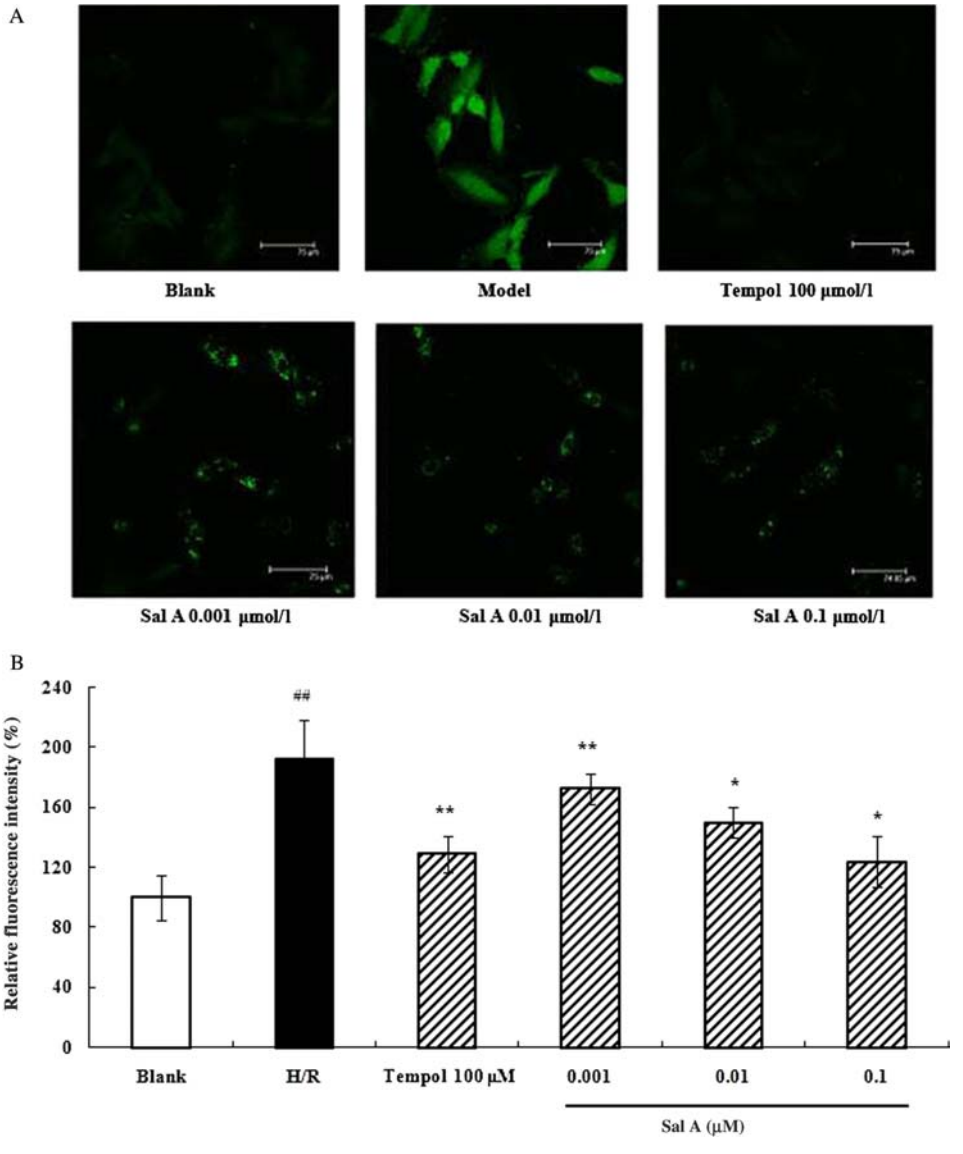

Figure 4. Effect of Sal A on H/R-stimulated H9c2 ROS production stained with the DCF fluorescence probe. (A) H9c2 cells were seeded in a six-well plate. Cells were exposed to hypoxia for 12 h followed by reoxygenation for 8 h and treatment with Sal A at the concentrations of 0.001, 0.01, and 0.1 μM at the same time. After 30 min incubation with DCFH-DA (10 μmol/l), cells were rinsed with PBS three times. The representative images were from three independent experiments (200 ×). (B) The DCF fluorescence intensity was measured with IPP 6.0 software. $n = 4$, $^{\#\#}p < 0.01$ vs. blank control; $*p < 0.05$, $**p < 0.01$ vs. H9c2 cells treated with H/R.

significantly after reperfusion, especially between 0 and 10 min, and then remained at a high level. H/R for 15 min increased the LVEDP by 117%. Compound **1** significantly attenuated H/R-stimulated LVEDP. Compound **1** at concentrations of 0.1, 0.2, and 0.4 μM significantly reduced LVEDPs of isolated rat hearts

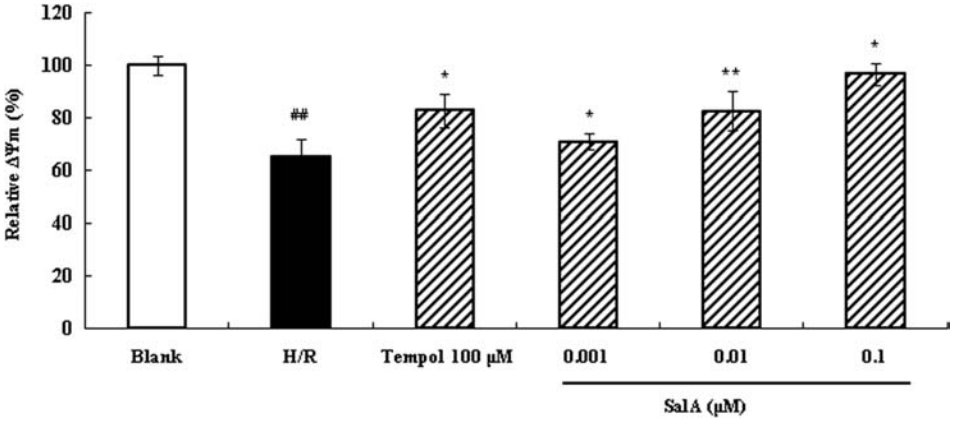

Figure 5. Effect of Sal A on H/R-induced changes in $\Delta\Psi_m$. H9c2 cells were exposed to hypoxia for 12 h followed by reoxygenation for 8 h and treatment with Sal A at the concentrations of 0.001, 0.01, and 0.1 μM at the same time. At the end of reoxygenation, the medium was removed, and about 1×10^6 cells were harvested by trypsinization. After washing twice with PBS, the cells were incubated with rhodamine-123 (5 μg/ml) for 30 min at 37°C in the dark. The cells were harvested and suspended in PBS. $\Delta\Psi_m$ was measured by the fluorescence intensity (FL-1, 530 nm) of 20,000 cells using flow cytometry. $n = 4$, $^{\#\#}p < 0.01$ vs. blank control; $*p < 0.05$, $**p < 0.01$ vs. H9c2 cells treated with H/R.

from 10.79 to 8.00, 5.63, and 2.87 mmHg, respectively (Figure 9).

As shown in Figures 10 and 11, H/R significantly decreased the $(dP/dt)_{max}$ level in the isolated rat heart; however, **1** at concentrations of 0.4, 0.8, and 1.6 μM as well as salvianolic acid B (Sal B, 14 μM) increased the $(dP/dt)_{max}$ levels from 249.00 mmHg/s to 263.33, 313.17, 424.67, and 282.83 mmHg/s, respectively.

As shown in Figures 12 and 13, $(dP/dt)_{min}$ significantly decreased in

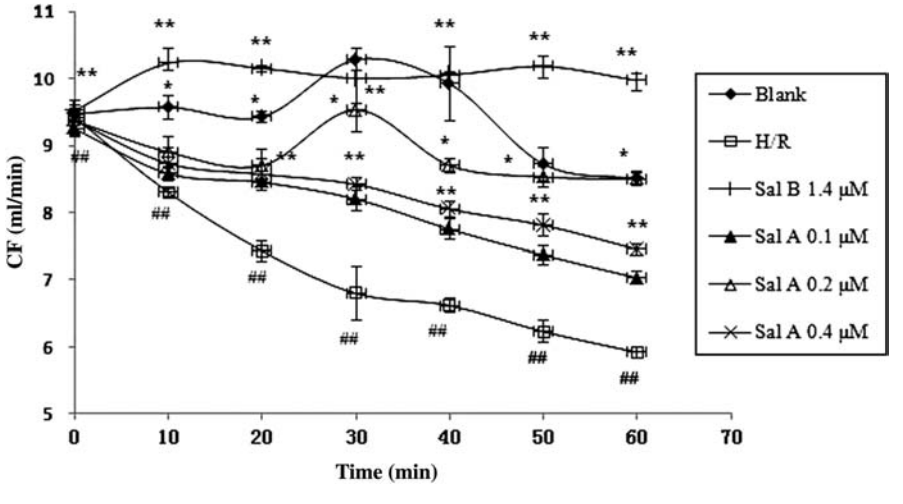

Figure 6. Sal A protects CF from H/R injury: time–effect relationship. Isolated rat hearts were stopped perfusing with K-H buffer for 15 min followed by reperfusion for 60 min and treatment with Sal A at the concentrations of 0.1, 0.2, and 0.4 μM at the same time. $n = 6$, $^{\#\#}p < 0.01$ vs. normal group; $*p < 0.05$, $**p < 0.01$ vs. isolated rat hearts treated with H/R.

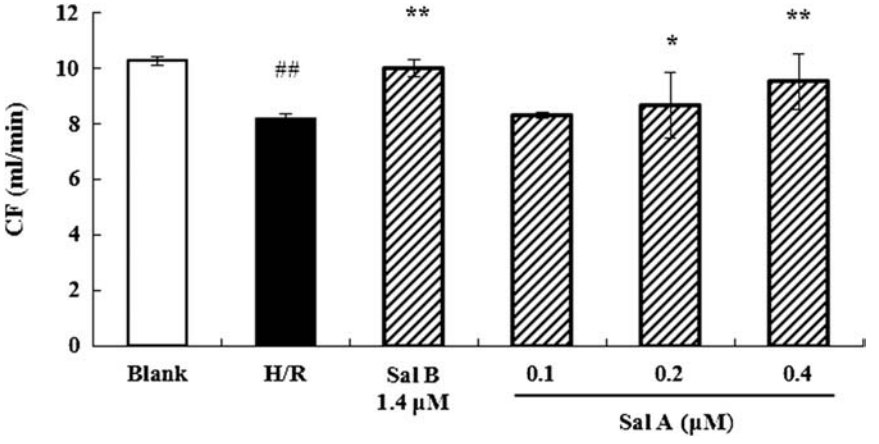

Figure 7. Sal A protects CF from H/R injury dose–effect relationship. Isolated rat hearts were stopped perfusing with K-H buffer for 15 min followed by reperfusion for 60 min, and treatment with Sal A at the concentrations of 0.1, 02, and 0.4 μM at the same time. $n = 6$, $^{\#\#}p < 0.01$ vs. normal group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. isolated rat hearts treated with H/R.

response to H/R. Compound **1** at concentrations of 0.4, 0.8, and 1.6 μM significantly increased $(dP/dt)_{min}$ from 229.33 mmHg/s to 239.00, 297.89, and 430.17 mmHg/s, respectively.

3. Discussion

The present study demonstrated that **1** had significant antioxidant properties and could effectively protect cardiomyocytes from acute oxidant injury, demonstrating further support of previous reports that the *Salvia miltiorrhiza* Bunge extract exhibited antioxidant effects in cardiomyocytes [9].

Antioxidants have an important role in lowering the oxidative stress caused by ROS. ROS, including superoxide anion radical, hydroxyl radical, and hydrogen peroxide, are generated under physiological and pathological stresses in the human

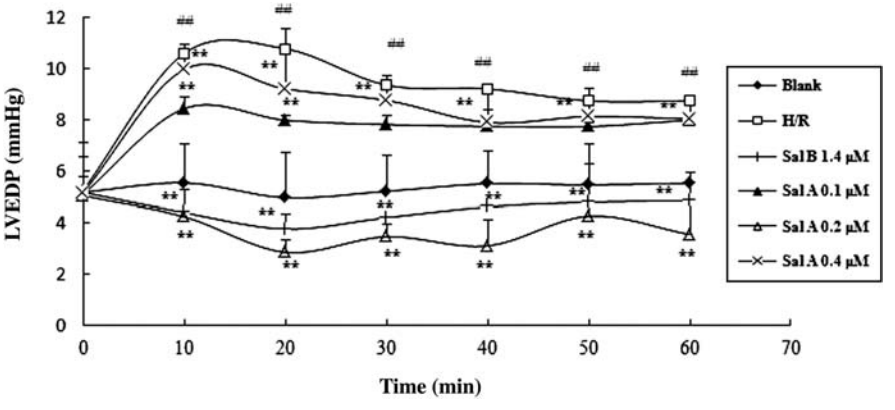

Figure 8. Sal A protects LVEDP from H/R injury: time–effect relationship. Isolated rat hearts were stopped perfusing with K-H buffer for 15 min followed by reperfusion for 60 min, and treatment with Sal A at the concentrations of 0.1, 0.2, and 0.4 μM at the same time. $n = 6$, $^{\#\#}p < 0.01$ vs. normal group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. isolated rat hearts treated with H/R.

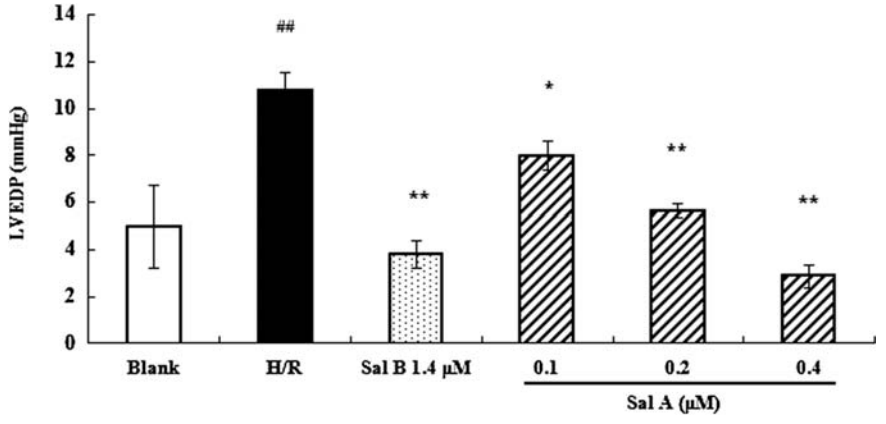

Figure 9. Sal A protects LVEDP from H/R injury: dose–effect relationship. Isolated rat hearts were stopped perfusing with K-H buffer for 15 min followed by reperfusion for 60 min, and treatment with Sal A at the concentrations of 0.1, 0.2, and 0.4 μM at the same time. $n = 6$, $^{\#\#}p < 0.01$ vs. normal group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. isolated rat hearts treated with H/R.

body [10]. Antioxidants act against these complex processes through scavenging free radicals and reducing hydrogen peroxide. Therefore, it is important to identify and study novel compounds characterized by antioxidant activity from natural sources in order to improve human health.

H/R is traditionally used to stimulate myocardial I/R [8]. The modeling method reflects pathological characteristics accurately and has similar molecular mechanisms as disease conditions, especially ROS production during reoxygenation and oxidative injury to cardiomyocytes [11], which has been acknowledged as a standard model to evaluate protective effects of tested drugs against myocardial damage. Therefore, this model in both cardiomyocyte assays and isolated perfused rat heart experiments was applied to assess whether **1** protects cardiomyocytes through ROS scavenging.

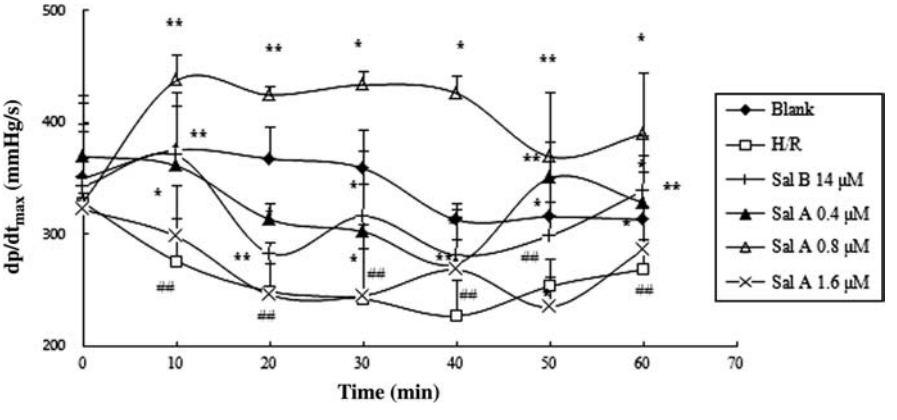

Figure 10. Sal A protects (dP/dt) from H/R injury: time–effect relationship. Isolated rat hearts were stopped perfusing with K-H buffer for 15 min followed by reperfusion for 60 min, and treatment with Sal A at the concentrations of 0.4, 0.8, and 1.6 μM at the same time. $n = 6$, $^{\#\#}p < 0.01$ vs. normal group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. isolated rat hearts treated with H/R.

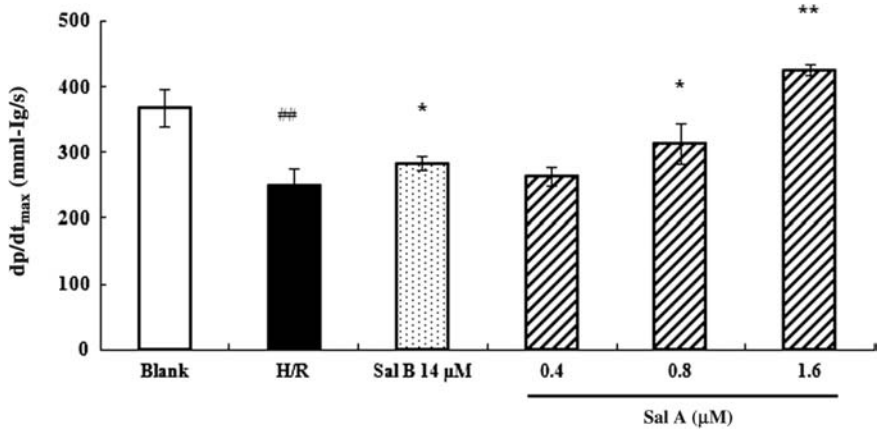

Figure 11. Sal A protects $(dP/dt)_{max}$ from H/R injury: dose–effect relationship. Isolated rat hearts were stopped perfusing with K-H buffer for 15 min followed by reperfusion for 60 min, and treatment with Sal A at the concentrations of 0.4, 0.8, and 1.6 μM at the same time. $n = 6$, $^{\#\#}p < 0.01$ vs. normal group; $*p < 0.05$, $**p < 0.01$ vs. isolated rat hearts treated with H/R.

Results obtained from the present *in vitro* studies clearly demonstrated that **1** was a very effective scavenger against all assayed ROS, RNS, and DPPH. This study corroborated and reinforced previous findings, which showed that plasma total antioxidant capacity (TCA) of **1**-treated diabetic rats recovered significantly compared to that of the control rats not treated with **1** [12]. Overall, the effects confirmed by our study may contribute to the therapeutic use of **1** when ROS and RNS overproduction are involved in a disease, especially ischemic disease. In an oxidative stressed microenvironment, the overproduction of $^{\cdot}NO$ in the presence of $O_2^{\cdot -}$ results in the formation of oxidizing RNS, such as $ONOO^-$ (due to the reaction of $^{\cdot}NO$ with $O_2^{\cdot -}$). This radical then leads to nitration of macromolecules and

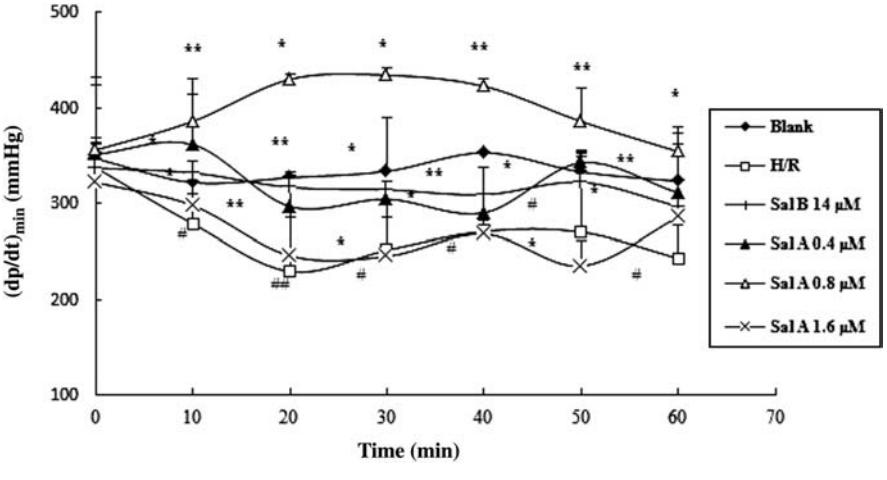

Figure 12. Sal A protects $(dP/dt)_{min}$ from H/R injury: time–effect relationship. Isolated rat hearts were stopped perfusing with K-H buffer for 15 min followed by reperfusion for 60 min, and treatment with Sal A at the concentrations of 0.4, 0.8, and 1.6 μM at the same time. $n = 6$, $^{\#\#}p < 0.01$ vs. normal group; $*p < 0.05$, $**p < 0.01$ vs. isolated rat hearts treated with H/R.

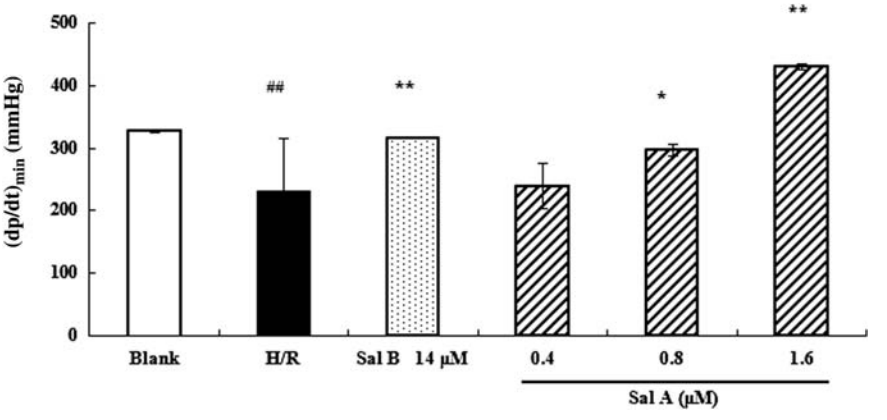

Figure 13. Sal A protects $(dP/dt)_{min}$ from H/R injury: dose–effect relationship. Isolated rat hearts were stopped perfusing with K-H buffer for 15 min followed by reperfusion for 60 min, and treatment with Sal A at the concentrations of 0.4, 0.8, and 1.6 μM at the same time. $n = 6$, $^{\#\#}p < 0.01$ vs. normal group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. isolated rat hearts treated with H/R.

consequent DNA damage, inhibition of mitochondrial respiration, protein dysfunction, and cell damage ('nitrosative stress') [13]. According to the results of our study, it may be concluded that **1** is an effective scavenger of $ONOO^-$.

To evaluate whether **1** protects cells through its antioxidant effect, the protection to cardiomyocytes from acute oxidant injury of **1** was tested. When the cells were exposed to H/R, the DCF fluorescence increased significantly. DCF fluorescence represents intracellular oxidative stress that is produced predominantly by the reaction of DCFH-DA with H_2O_2 and hydroxyl radical (which is formed by the Fenton reaction or Haber–Weiss reaction from H_2O_2) [14]. The principle enlightened us that exogenous H_2O_2 and hydroxyl radicals induced by H/R traversed the cell membrane to cause intracellular oxidative stress. Treatment with **1** significantly attenuated the increase in DCF fluorescence caused by H/R (Figure 4), with an approximately 35.54% reduction in response to 0.1 μM **1**. The corresponding DHE experiments also showed a decrease in DHE fluorescence by 50.53% at the same concentration (Figure 3). MMP ($\Delta\Psi_m$) is an indication of mitochondrial stability. $\Delta\Psi_m$ decreases when the cell is damaged [15]. Compound **1** increased the $\Delta\Psi_m$ of cardiomyocytes by 48.71%. The results suggested that **1** protected the cardiomyocytes from acute exogenous oxidative injury. Cellular-derived ROS are enzymatically produced by H/R and cardiomyocytes as part of oxidative stress responses to a pathogen or irritant [13]. Therefore, it could be postulated that the observed scavenging effects of **1** against $O_2^{\cdot-}$, H_2O_2, and $HO^{\cdot}$ contribute to the prevention of H/R-related adverse effects.

Compound **1** seemed to ameliorate the H/R injury in the isolated perfused rat heart. Oxidative stress is considered as one of the most common causes of H/R-induced injury in heart muscle [16]. Impairment of the heart function is initiated at the beginning of reperfusion because of the high production of ROS, leading to apoptosis of endothelial cells and cardiomyocytes. Compound **1** increased the CF by 16.22%. ROS has been shown to harm the artery diastolic function, while **1** ameliorated the coronary artery suffering from ROS. The optimal doses of **1** were 0.4 μM for CF and LVEDP, and 1.6 μM for $(dP/dt)_{max}$ and $(dP/dt)_{min}$. The effect of **1**

on LVEDP showed that the compound significantly improved heart diastolic action after ROS injury in cardiomyocytes. Compound **1** (0.1, 0.2, and 0.4 μM) exerted favorable protection from diastolic dysfunction, and the effect of 0.4 μM was better than the positive control Sal B, a commonly used drug to cure myocardial ischemia in clinical practice, which suggested that **1** had a promising application potential.

In conclusion, at the molecular-, cellular-, and isolated organ-based levels, **1** was a potent antioxidant that protected cardiomyocytes from oxidant-mediated injury. Such protection is, at least in part, mediated by its radical scavenging effect, especially against H_2O_2 and hydroxyl radicals. As a major constituent in the *Salvia miltiorrhiza* Bunge extract, **1** might play an important role in antioxidant actions to improve cardiomyocyte survival and contractile function during ischemia and reperfusion. The above results will enlighten further studies to assess whether **1** protects the heart through the same mechanism *in vivo* and might serve as a foundational study for *in vivo* studies on its cardioprotective effect.

4. Materials and methods

4.1 Preparation of the tested compound

Compound **1**, purity ≥ 99%, was provided by Shandong Target Drug Ltd Co. (Yantai, China). Compound **1** was dissolved in water to 100 μM as the stock solution preserved at −20°C, and then diluted with reaction buffer in free radical scavenging assay, with DMEM medium in the cell experiment, or with K-H buffer in the isolated rat heart experiment before use, respectively.

4.2 Chemical reagents

DHR-123, lucigenin, diethylenetriaminepentaacetic acid (DTPA), mannitol, ascorbic acid, manganese dioxide DHE, and tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) were purchased from Sigma-Aldrich (Trading Co., Ltd, St. Louis, MO, USA). Rhodamine-123 and dichlorofluorescein diacetate (H_2DCF-DA) were bought from Sigma-Aldrich. Azodiisobutyramidine dihydrochloride (AAPH) and Trolox were obtained from Sigma-Aldrich. Fluorescein sodium salt was obtained from Fluka Chemie GmbH (Steinheim, Germany). Hydrogen peroxide (30%) and ferric chloride anhydrous ($FeCl_3$) were obtained from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). Dimethylformamide (DMF), trichloroacetic acid, dimethyl sulfoxide (DMSO), sodium phosphate, potassium phosphate, potassium, sodium nitrite, sodium hydroxide, sodium chloride, potassium chloride, EDTA disodium salt, and hydrochloric acid were obtained from Beijing Chemical Reagent Co., Ltd (Beijing, China). Sal B salt (containing 80 mg salvia magnesium acetate per 100 mg) was purchased from Shanghai Lvgu Pharmaceutical Ltd Co. (Shanghai, China).

4.3 Animals

Adult male Sprague–Dawley rats weighing 250–280 g were used, provided by Beijing Weitonglihua Laboratory Animal Technology Ltd Co. (Beijing, China). Number of Certificate: SCXK 2007-0001.

4.4 Free radical scavenging assays

4.4.1 DPPH scavenging assay

The scavenging effect of the stable DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical was measured by monitoring its reduction at 515 nm, according to a described protocol with modifications [17]. Reaction mixtures contained 190 μl DPPH (200 μM, dissolved in ethanol) and 10 μl of **1** with a final volume of 200 μl. After 20 min, the absorbance was measured at 515 nm in a microplate reader (model Spectra Max 190, Molecular

Devices, Sunnyvale, CA, USA). The effects are expressed as the percentage reduction of DPPH. Ascorbic acid was used as the positive control. Each study corresponded to five independent experiments was performed in triplicate.

4.4.2 *Superoxide radical scavenging assay*

Superoxide radicals were generated by the pyrogallic acid autoxidation system, and the $O_2^{\cdot-}$ scavenging activity was determined spectrophotometrically in a microplate reader (model Spectra Max 190, Molecular Devices) by monitoring the effect of **1** on autoxidation rate (ΔA) at 325 nm for 2 min. The assay was performed at 25°C. Reaction mixtures contained 20 μl pyrogallic acid (8 mM, dissolved in 10 mM HCl),100 μl of **1** and 130 μl, 0.1 mol/l Tris–HCl buffer (pH 7.4, containing 2 mM EDTA) with a final volume of 250 μl. The effects are expressed as the percentage inhibition of autoxidation-induced increase of absorbance [18]. Trolox and ascorbic acid were used as positive controls. Each study corresponded to five independent experiments was performed in triplicate.

4.4.3 *Hydroxyl radical scavenging assay*

Hydroxyl radicals were generated by a Fenton system ($FeSO_4$–H_2O_2). Orthophenanthroline-Fe^{2+} was oxidized to orthophenanthroline-Fe^{3+} when exposed to hydroxyl radicals, which corresponds to a decreased absorbance at 536 nm. Reaction mixtures contained the following reagents at indicated final concentrations (with a final volume of 1 ml): 575 μl dibasic sodium phosphate–sodium dihydrogen phosphate buffer (150 mM, pH 7.4), 150 μl orthophenanthroline (0.75 mM), 50 μl $FeSO_4$ (0.75 mM), 100 μl H_2O_2 (0.1%), and 125 μl of **1**. After incubation at 37°C for 1 h, absorbance of the solution was measured at 536 nm. Ascorbic acid was used as the positive control. The effects are expressed as the percentage inhibition of orthophenanthroline-Fe^{2+} oxidized to orthophenanthroline-Fe^{3+}[19]. Each study corresponded to five independent experiments was performed in triplicate.

4.4.4 *Hydrogen peroxide scavenging assay*

Hydrogen peroxide scavenging activity was measured using a chemiluminescence methodology by monitoring the H_2O_2-induced oxidation of lucigenin, according to a previously described procedure [20]. Reaction mixtures contained 50 μl lucigenin (3 mM) and 100 μl of **1** and 100 μl potassium phosphate buffer (50 mM, pH 7.4) with a final volume of 250 μl. Blank values were measured in the absence of H_2O_2. Ascorbic acid was used as the positive control. H_2O_2-elicited lucigenin oxidation resulted in a chemiluminescent signal that was detected using a Synergy HT microplate reader. Chemiluminescence was monitored for 10 min. The effects are expressed as the percentage inhibition of H_2O_2-induced lucigenin oxidation. Each study corresponded to five independent experiments was performed in triplicate.

4.4.5 *Peroxyl radical scavenging assay*

Peroxyl radicals were generated by thermo-decomposition of AAPH in a microplate reader at 37°C. The $ROO^{\cdot}$ scavenging activity was measured by monitoring the decay of fluorescence due to the oxidation of fluorescein, termed as the ORAC, according to a previously described procedure [21]. Reaction mixtures contained the following reagents at the indicated final concentrations (with a final volume of 200 μl): 80 μl potassium phosphate buffer (75 mM, pH 7.4), 20 μl fluorescein (81.6 mM), 50 μl of **1**, and 50 μl AAPH (19.1 mM). Fluorescence measurements were performed by a

Synergy HT microplate reader, with excitation and emission wavelengths of 485 and 528 nm, respectively.

The effects are expressed as the relative Trolox equivalent ORAC value, which is calculated by the following equation:

$$R_{ORAC} = \frac{[(AUC_{sample} - AUC_{blank})/(AUC_{Trolox} - AUC_{blank})] \times M_{Trolox}}{C_{extract}}$$

where R_{ORAC} is the relative ORAC value, AUC represents the area under the curve, M_{Trolox} is the molarity of Trolox, and $C_{extract}$ is the concentration of **1** (mol/l).

Ascorbic acid was used as the positive control. Each study corresponded to five independent experiments was performed in triplicate.

4.4.6 Peroxynitrite scavenging assay

Synthesis of $ONOO^-$ was carried out according to a previously described procedure [22]. In brief, an acidic solution (0.7 mol/l HCl) of 0.6 mol/l H_2O_2 was mixed with 0.66 mol/l $NaNO_2$ on ice for 1 s, and the reaction was quenched with ice-cold 3 mol/l NaOH. Residual H_2O_2 was removed by mixing with granular MnO_2 prewashed with 3 mol/l NaOH. The stock solution of $ONOO^-$ was filtered and then frozen (−80°C), and the top settled layer of the solution was collected for the experiment. The concentration of $ONOO^-$ was determined by measuring the absorbance at 302 nm ($\varepsilon = 1670\,M^{-1}\,cm^{-1}$). The typical concentration of freshly prepared $ONOO^-$ ranged from 60 to 80 mM.

The $ONOO^-$ scavenging activity was measured by monitoring the oxidation of the non-fluorescent DHR-123 to the fluorescent rhodamine-123 by $ONOO^-$ using the method of Kooy et al. [23] with modifications. A stock solution of 2.89 mM DHR-123 in DMF was purged with nitrogen and stored at −20°C. Buffer (90 mM NaCl, 50 mM Na_3PO_4, and 5 mM KCl, pH adjusted to 7.4 with HCl) was purged with nitrogen and placed on ice before use. At the beginning of the experiments, 100 μM DTPA was added to the buffer. Working solutions of DHR-123 were diluted with the buffer from the stock solution immediately before the measurements and placed on ice in the dark. Reaction mixtures contained (with a final volume of 300 μl): 100 μl buffer, 40 μl DHR-123 (5 μM), 80 μl of **1**, and 80 μl $ONOO^-$ (600 nmol/l). The mixtures were incubated for 5 min at 37°C in a Synergy HT microplate reader. The fluorescent signal caused by the reaction of DHR-123 with $ONOO^-$ was measured using the microplate reader with excitation and emission wavelengths of 485 and 528 nm, respectively. The effects are expressed as the percentage inhibition of $ONOO^-$-induced DHR oxidation. Ascorbic acid was used as the positive control. Each study corresponded to five independent experiments was performed in triplicate.

4.5 Cell culture and induction of H/R

Embryonic rat heart-derived H9c2 cells (BCRC 60096) were obtained from the Culture Collection and Research Center of Institute of Basic Medical Sciences of Chinese Academy of Medical Sciences, Beijing, China. The H9c2 cells were maintained in growth medium composed of DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C in a 17.6% O_2 incubator. The medium was changed every two days. The growth medium was replaced by serum-free medium 24 h after cells were plated. After serum starvation

for 12 h, the medium was replaced with ischemic buffer (118 mM NaCl, 24 mM $NaHCO_3$, 1 mM $NaH_2PO_4{\cdot}H_2O$, 2.5 mM $CaCl_2{\cdot}2H_2O$, 0.5 mM sodium EDTA·$2H_2O$, 20 mM sodium lactate, and 16 mM KCl, pH 6.2), and cells were incubated in a 1% O_2 hypoxia incubator for 12 h to induce hypoxia. Finally, the buffer was replaced with the medium containing 10% FBS and **1** at different concentrations dissolved in DMEM, 100 μM of tempol as a positive control, and the same volume of the medium as a model control, and cells were incubated in a 17.6% O_2 normal incubator for 12 h to induce reoxygenation.

4.6 Assessment of cellular viability: MTT assays

A mitochondrial viability assay was performed using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. Briefly, after the reoxygenation period, cells were incubated in the medium containing 45 mg/ml MTT for 45 min at 37°C. Only viable cells were able to reduce MTT into a purple formazan product that was soluble in DMSO. The optical density was measured at 570 nm, and the absorbance was taken as an index of cell viability.

4.7 ROS assessment

The level of intracellular ROS was detected using the fluorescent probes DHE and H_2DCF-DA as per the manufacturer's instructions. Cells were loaded with 10 μM H_2DCF-DA in phosphate buffer solution (PBS) or 50 mM DHE in HEPES buffer at 37°C for 30 min. H_2DCF-DA is non-fluorescent until the acetate groups are removed by intracellular ROS. DHE fluorescence was imaged at room temperature under a fluorescence microscope (Olympus IX-70, Olympus America, NY, USA) with an excitation wavelength of 480 nm and an emission wavelength of 590 nm. DCF fluorescence was measured under a laser confocal scanning microscope (TCS SP2, Leica, Germany) at an excitation wavelength of 490 nm and an emission wavelength of 535 nm. Fluorescence intensity was measured using IPLab imaging software (Scanalytics, Inc., Fairfax, VA, USA). Results were displayed in a ratiometric fashion normalized by the control condition as previously described [24].

4.8 Mitochondrial membrane potential ($\Delta\Psi_m$) assessment

To evaluate $\Delta\Psi_m$, rhodamine-123 (Cell technology, Sigma) was employed. Rhodamine-123 dye exhibits potential-dependent accumulation in mitochondria, which can be detected at excitation/emission wavelengths of 485/530 nm. In our system, cells (1.5×10^5/ml) were plated in Φ100 mm dishes, grown for 24 h to reach 85% confluence followed by incubation in 1% O_2 for 12 h, and then recovered in 17.6% O_2 for 12 h. Cells were incubated with 5 μg/ml rhodamine-123 in the culture medium for 30 min in the dark in an incubator and then washed twice with PBS. Cells were read using a flow cytometer (EPICS XL, Beckman Coulter, Fullerton, CA, USA) at excitation/emission wavelengths of 485/530 nm. The $\Delta\Psi_m$ was measured by the fluorescence intensity (FL-1, 530 nm) of 20,000 cells using flow cytometry.

4.9 Isolated rat heart model of H/R in Langendorff perfusion experiments

Male Sprague–Dawley rats (250–300 g) were anesthetized by intraperitoneal injection of 2% Carbrital. Their hearts were removed and then rinsed in ice-cold K-H buffer (mM: 118 NaCl, 4.7 KCl, 1.25 $CaCl_2$, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 1.2 KH_2PO_4, and 7.0 glucose, pH 7.4). The aortas were cannulated, and hearts were perfused in a retrograde fashion according to Langendorff with the K-H buffer at

37°C and saturated with 95% O_2 and 5% CO_2. Hearts were then placed in a water-jacketed chamber at 37°C, and perfusion pressure was maintained at 60 mmHg. The left auricle was cut off, and an elastic sacculus was inserted into the left ventricle. Finally, contractive signals from a pressure transformer were inputted into a multi-lead physiological report system.

Isolated hearts were separated into three groups (six hearts per group) according to an established protocol [25] with modifications: the model group of hearts was exposed to global ischemia for 20 min followed by reperfusion for 60 min. The administration groups were supplied with the positive control drug, Sal B, at concentrations of 1.40 and 14.0 μM, or **1**, at doses of 0.1, 0.2, 0.4, 0.8, and 1.6 μM, from the injection hole at the beginning of reoxygenation. In control perfusions, the H/R period was replaced by an equal period of flow-through perfusion.

CF was measured by collecting coronary effluent every minute. Contractive functions, LVEDP, the peak of pressure development $((dP/dt)_{max})$, and the valley of pressure development $((dP/dt)_{min})$ were recorded with ACQ373 software.

4.10 Statistical analysis

The results are expressed as means ± SD for repeated measures, and one-way ANOVA was used for comparison of differences between groups. Differences were considered statistically significant when $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. blank group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. model group.

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