Protective Effects of Salidroside on Endothelial Cell Apoptosis Induced by Cobalt Chloride

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Salidroside is a major constituent of Rhodiola rosea L. that elicits beneficial effects for ischemic cardiovascular diseases. The aim of this study was to investigate the protective effects of salidroside on endothelial cells apoptosis induced by the hypoxia mimicking agent, cobalt chloride. After challenge with cobalt chloride for 24 h, loss of cell viability and excessive apoptotic cell death were observed in EA.hy926 endothelial cells, and the level of intracellular reactive oxygen species (ROS) increased concentration-dependently. However, the endothelial cell apoptosis and excessive ROS generation were attenuated markedly by salidroside pretreatment. In addition, salidroside inhibited activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase (PARP) induced by cobalt chloride, decreased expression of Bax and rescued the balance of pro- and anti-apoptotic proteins. These findings suggest that salidroside protects endothelial cells from cobalt chloride-induced apoptosis as an antioxidant and by regulating Bcl-2 family. Salidroside may represent a novel therapeutic agent for the treatment and prevention of hypoxia and oxidative stress-related diseases.

Key words salidroside; cobalt chloride; apoptosis; endothelial cell

Mounting evidence strongly suggests that vascular endothelial cells are impaired through apoptosis, contributing to overall endothelial dysfunction in a range of clinical settings including hypoxic and ischemic injury. Hypoxia and ischemia can lead to increase of intracellular reactive oxygen species (ROS) and activation of several transcription factors, and initiate the complex apoptotic cascade in endothelial cells. Many molecules are involved in the apoptotic cascade, and the caspase and Bcl-2 families are especially important among these molecules. Caspases are a family of cysteine proteases that are essential for apoptosis in cells, and thus have been termed “executioner” proteins for their roles in the cell. The Bcl-2 family includes both pro- and anti-apoptotic proteins that largely determine whether a cell is living or dead.

The hypoxia-responsive pathway can be specifically stimulated by exposure to cobalt chloride, which has been widely used to simulate hypoxic conditions in vitro and in vivo. Several reports have demonstrated that cobalt chloride can induce apoptosis in different type of cells such as PC12 cells, rat C6 glioma cells, human alveolar macrophages, HeLa cells, skeletal L6C5 and C2C12 cells.

Rhodiola rosea L. (Crassulaceae) has been used for a very long time in traditional medicines in China. There are many studies showing that extracts from these plants have diverse pharmacological effects including anti-aging, antidepressant, anticancer, cardioprotective, hepatoprotective, anti-hypoxic insults, and central nervous system system enhancement. One of the major biologically active compounds extracted from these medicinal plants is salidroside (Fig. 1). Clinical and experimental studies have demonstrated that Salidroside and R. rosea L. can protect against ischemic cardiovascular and cerebrovascular injuries, however, the mechanisms of these effects are still elusive.

Our previous studies demonstrated protective effects of salidroside on glutamate-induced PC12 injury and sodium azide-induced mitochondria damage. The aim of this study was to investigate the effects of salidroside on endothelial cell apoptosis induced by cobalt chloride.

MATERIALS AND METHODS

MaterialsSalidroside (purity>99%) was purchased from the National Institute of Pharmaceutical and Biological Products (Beijing, China). Cobalt chloride, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were from Sigma-Aldrich (St. Louis, U.S.A.). Vybrant® apoptosis kit #4 was purchased from Molecular Probes, Inc. (Eugene, U.S.A.). Anti-β-actin, anti-hypoxia-inducible factor-1 alpha, anti-Bcl-2 and anti-Bax antibodies were purchased from Santa Cruz Biotechnology, Inc. (U.S.A.). Anti-poly(ADP-ribose) polymerase (PARP) and anti-caspase-3 antibodies were from Boster Biological Technology Ltd. (Wuhan, China). All of the other chemicals and reagents were standard commercially available biochemical quality.

Cell CultureEA.hy926 endothelial cells (ECs) were cultivated in RPMI-1640 medium supplemented with 15% heat-inactivated fetal bovine serum. All of the cells were grown at 37 °C in a humidified atmosphere of 5% CO₂.

Cell Viability AssayECs were seeded at a density of 1.0×10⁴ per well in 96-well plates. MTT reduction assay was used to evaluate the cell viability. Briefly, ECs were treated with different concentrations of salidroside (0.1, 1.10 μg/ml) for 2 h, then exposed to 300 μM cobalt chloride for another 24 h in the presence of salidroside. At the setting time points, MTT was added to the medium at a final concentration of 0.5 mg/ml and incubated at 37 °C for 4 h. The medium was

Fig. 1. Chemical Structure of Salidroside

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removed carefully and dimethyl sulfoxide added to resolve the formazan dye crystals. The absorbance was measured by SPECTRA-Max M5 microplate reader at 540 nm (Molecular Device, U.S.A.).

**Nuclear Staining** ECs were plated into 96-well black plates and treated with salidroside and cobalt chloride as described above. After cobalt chloride challenge for 24 h, the ECs were incubated with Hoechst33258 (final concentration 1 μg/ml) at 37 °C for 10 min, washed, then examined with inverted fluorescence microscope (Olympus IX71, Japan) to distinguish the apoptotic cells by their fragmented and condensed nuclei.

**Flow Cytometric Assay** The percentage of apoptotic cells was measured with Vybrant® apoptosis assay kit #4 (YO-PRO®-1/propidium iodide (PI)), and the ECs samples for flow cytometric analysis were prepared according to the manufacturer’s instruction. In brief, after treatment with salidroside (0.1, 1, 10 μg/ml) for 2 h and incubation with 300 μM cobalt chloride for another 24 h in the presence of salidroside, the ECs were collected and washed with PBS. The ECs were resuspended in PBS buffer containing YO-PRO®-1 and propidium iodide each 1 μl for 30 min on ice in the dark, and fluorescence intensity was determined by flow cytometry (Coulter EPICS® XI™, Beckman-Coulter Inc., U.S.A.).

**Measurement of Intracellular ROS** Intracellular ROS generation was determined with the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as described previously. Briefly, after stimulation with different concentrations of cobalt chloride for 24 h, the ECs were incubated with DCFH-DA (final concentration 10 μM) at 37 °C for 30 min in the dark. The nonfluorescent agent DCFH-DA diffused into cells and was rapidly oxidized into highly fluorescent dichlorofluorescein (DCF) in the presence of ROS. Hence the intensity of fluorescence reflects the level of intracellular ROS.

The quality of DCF fluorescence was measured at an emission wavelength of 530 nm and an excitation wavelength of 485 nm using microplate reader (SPECTRA-Max M5, Molecular Device, U.S.A.). To evaluate the effect of salidroside on intracellular ROS production, ECs were treated with salidroside (0.1, 1, 10 μg/ml) for 2 h followed by cobalt chloride challenge for another 24 h in the presence of salidroside, then fluorescence intensity was measured as described above. Results were expressed as percentage of control fluorescence intensity.

**Western Blotting** After pretreatment with salidroside (0.1, 1, 10 μg/ml) for 2 h followed by cobalt chloride treatment for another 24 h in the presence of salidroside, ECs were collected and resuspended in lysis buffer (0.05 M Tris–HCl pH 7.6, containing 1% NP40, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 10 μg/ml each of leupeptin, pepstatin and aprotinin). After 30 min on ice, lysates were centrifuged at 12000 g at 4 °C for 20 min and protein concentration was determined using Bicinchoninic acid method (BCA kit, Applygen Technologies Inc., Beijing, China). Protein samples containing 50 μg total protein were separated on 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham). The membranes were incubated for 4 h with 5% skim milk in TBST buffer (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20) to block nonspecific binding. Subsequently, the membranes were probed with specific primary antibodies (anti caspase-3, PARP, HIF-1α, Bel-2, Bax, and β-actin antibodies) overnight, followed by incubation with horseradish peroxidase-coupled secondary antibodies for 1 h. Then the blot was developed with enhanced chemiluminescence reagents and scanned by LAS3000 imaging system (Fujifilm, Japan).

**Data Analysis** All data are expressed as mean±S.E.M., and ANOVA was used for statistical analysis. p<0.05 was considered as significant.

**RESULTS**

**Protective Effects of Salidroside against Cobalt Chloride-Induced Cytotoxicity** After exposure to cobalt chloride for 24 h, dramatic morphologic changes were observed in ECs. Adherent ECs showed typical cobblestone morphology in the control group, however, with increased cobalt chloride concentrations (10—1000 μM), some cells became spindle-shaped or even round. At the highest concentration (1000 μM), the adherent cell numbers decreased sharply. Compared with cells treated with the same dose (300 μM) of cobalt chloride alone, salidroside could ameliorate these damages (Fig. 2A). MTT assay suggested that cobalt chloride (10—1000 μM) resulted in cell viability loss in a dose-dependent manner, and the viability of ECs decreased to 43.1±7.8% of the control value after 300 μM cobalt chloride challenge for 24 h (Fig. 2B). Salidroside at concentrations 0.1—10 μg/ml could significantly prevent loss of cell viability. Cell viability in the group pretreated with salidroside at 1 and 10 μg/ml was up to 54.2±8.4% and 59.5±6.7% of the control value respectively (Fig. 2C). The results of MTT assay suggested that salidroside at these concentrations did not result in apparent cytotoxicity (data not shown).

**Salidroside Protected Endothelial Cells from Apoptosis Induced by Cobalt Chloride** Cobalt chloride has been shown to induce apoptosis in human alveolar macrophages, HeLa cells, and skeletal L6C5 and C2C12 cells. In this study, typical apoptotic morphological changes such as condensed chromatin and shrinkage of nuclei were observed in ECs exposed to cobalt chloride for 24 h. However, the cell number characterized as undergoing apoptosis decreased remarkably in the groups pretreated with salidroside 1 and 30 μM cobalt chloride (300 μM) for 24 h. However, the percent-age was significantly reduced to 13.5% and 10.1%, respectively by salidroside 1 and 1000 μM (Fig. 3A). Further to confirm the apoptotic inhibitory effect of salidroside, we stained ECs with YO-PRO®-1 and propidium iodide dye, and counted the stained cells by flow cytometry. As shown in Fig. 3B, the percentage of apoptotic ECs increased from 4.7 to 20.5% after challenge with cobalt chloride (300 μM) for 24 h. However, the percentage was significantly reduced to 13.5% and 10.1%, respectively by salidroside 1 and 10 μg/ml (Fig. 3C).

**Generation of Intracellular ROS** The level of intracellular ROS was measured with fluorescence probe DCFH-DA as described in Materials and Methods. Compared with control, the intracellular ROS manifested as fluorescence intensity increased remarkably in a concentration-dependent manner after cobalt chloride challenge for 24 h (Fig. 4A). After exposure of ECs to cobalt chloride (300 μM) for 24 h, the intracellular ROS level increased to 167.9±19.8% of control, and decreased to 121.3±22.4% and 108.6±22.4% in the 1 μg/ml and 10 μg/ml salidroside treatment group respec-
Fig. 2. Protective Effects of Salidroside on Cobalt Chloride-Induced Loss of Cell Viability in EA.hy926 ECs
(A) Morphological changes of ECs after cobalt chloride (300 μM) treatment alone (b) or pretreatment with salidroside (c, d). (B) ECs were challenged with different concentrations (10, 30, 100, 300, 1000 μM) of cobalt chloride for 24 h, and cell viability was measured by MTT assay. **p < 0.01 vs. control. (C) Protective effect of salidroside on cobalt chloride-induced ECs injury. ECs were treated with salidroside (0.1, 1, 10 μg/ml) for 2 h followed by 300 μM cobalt chloride for another 24 h in the presence of salidroside. *p < 0.05, **p < 0.01 vs. vehicle. Data are expressed as mean±S.E.M. of five experiments.

Fig. 3. Effects of Salidroside on Cobalt Chloride-Induced Endothelial Cell Apoptosis
(A) Nuclear staining of ECs apoptosis. (B) ECs apoptosis determined by flow cytometric assay. X and Y axis represent YO-PRO-1® and PI fluorescence, respectively. (C) The mean percentage of apoptotic ECs measured by flow cytometric assay (n=5). ECs were treated with or without salidroside for 2 h followed by cobalt chloride (300 μM) for another 24 h. Data are expressed as mean±S.E.M. *p<0.05 vs. control.
tively (Fig. 4B). Meanwhile, the level of HIF-1α, a key mediator related to hypoxic effects and participating in the regulation of many genes and proteins expression after hypoxia stimulation, increased markedly after cobalt chloride challenge for 24 h as assayed by Western blotting (Fig. 5A).

**Expression and Activation of Caspase-3** Caspase-3 is known as an important executioner in apoptosis, it hydrolyzes a number of structural and signaling proteins involved in apoptosis, including the DNA repair enzyme PARP. As illustrated in Fig. 5B, Western blot analysis showed that the level of cleaved caspase-3 increased remarkably after challenge with cobalt chloride for 24 h. As a result, the cleavage of PARP, an intrinsic substrate of caspase-3, increased markedly. However, the excessive activation of caspase-3 and cleavage of PARP were ameliorated in the salidroside (1, 10 mg/ml) treatment groups. The action of salidroside on these molecular events is paralleled with its effects on apoptosis.

**Modulation of Expression of Bcl-2/Bax** Bcl-2, a key protein contributing to maintain cell survival, was present at a relatively high level in the normal endothelial cells, and decreased in endothelial cells exposed to cobalt chloride for 24 h (Fig. 5C). On the other hand, the level of Bax, an important pro-apoptotic protein, increased markedly after challenge with cobalt chloride for 24 h. As a result, the ratio of Bcl-2/Bax decreased significantly, and these events might be involved in the apoptotic death induced by cobalt chloride. At the concentration range of 0.1—10 μg/ml, salidroside could reduce the upregulation of Bax protein, but affected little the level of Bcl-2. Therefore, salidroside treatment is helpful to rescue the balance of Bcl-2 and Bax.

**DISCUSSION**

In this study, we found that treatment with cobalt chloride, an extensively used hypoxia mimicking agent, resulted in loss of endothelial cells viability. Moreover, the results of nuclear staining also suggested apoptotic death after cobalt chloride challenge in EA.hy926 endothelial cells. This was similar with the results in other cellular systems induced by hypoxia and cobalt chloride injury. Furthermore, our results demonstrated that salidroside (0.1, 1, 10 μg/ml) significantly decreased the level of ROS and ameliorated apoptotic cascade and endothelial cells apoptosis induced by cobalt chloride.

Hypoxia and/or ischemia can promote production of ROS, resulting in endothelial dysfunction and apoptosis, and is a major concern in various clinical entities including ischemic...
cardiovascular diseases. Clinical evidence and in vivo experiments have suggested that *R. rosea* L. elicits beneficial effects for ischemic cardiovascular diseases, and the present results show that salidroside, the major component of *R. rosea* L., protects endothelial cells from apoptosis induced by cobalt chloride. One of the possible protective mechanisms of salidroside might be its direct scavenging of ROS produced by cobalt chloride, since salidroside can scavenge different kinds of organic free radicals such as superoxide anion and hydroxyl radicals, and ROS can initiate apoptotic cascades in endothelial cells. Taken together, the protective effects of salidroside on ischemic myocardial injuries might benefit from its protective action on hypoxia-induced endothelial cell apoptosis.

HIF-1α accumulates under hypoxic conditions and mediates diverse subsequent molecular and cellular events as a cellular oxygen sensor. As an extensively used hypoxic mimic agent, cobalt chloride can result in accumulation of HIF-1α, and the mechanisms may involve ROS generation and operation of the ERK1/ERK2 and PI3K signaling pathways. Previous studies have shown that excessive ROS was involved in cell apoptosis and necrosis and implicated in hypoxia-related pathologies such as ischemic injury and tumors. Our results suggest that cobalt chloride resulted in excessive intracellular ROS formation and accumulation of HIF-1α in endothelial cells, however, the excessive accumulation of ROS and HIF-1α was attenuated by pretreatment with salidroside in the concentration ranges from 0.1 to 10 μg/ml. It can be speculated that salidroside may interfere with genes transcription and downstream molecular events through its inhibitory effects on production of ROS and accumulation of HIF-1α, but further investigation is needed.

Caspases are a large proteinase family and play pivotal roles in the apoptotic process. Caspase-3 is a convergence point of various apoptosis-regulating signal pathways and an important executioner in apoptosis. Caspase-3 has been implicated in cobalt chloride-induced cell death. Similarly, the level of caspase-3 increased markedly in endothelial cells after exposure to cobalt chloride in this study. At the same time, our results showed that cleavage of PARP, a major intrinsic substrate of caspase-3, increased markedly after cobalt chloride exposure. It has been demonstrated that cleavage of PARP by caspase-3 was involved in endothelial cell apoptosis triggered by oxidative stress. Taken together, it can be speculated that inhibition of caspase-3 activation and PARP cleavage by salidroside might be important factors mediating the protective effects against cobalt chloride-induced endothelial cells apoptosis.

The Bcl-2 family of anti-apoptotic proteins (*e.g.*, Bcl-2) and pro-apoptotic proteins (*e.g.*, Bax) are the central regulators of caspase activation and cellular life-and-death switch. Elevation of Bax expression is a common occurrence during ischemia, and alteration of the ratio of Bcl-2 to Bax is significant in determining whether apoptosis occurs. In the present study, Western blot analysis showed that Bax was upregulated and Bcl-2 downregulated after cobalt chloride challenge. Salidroside treatment can reduce the level of Bax and rescue the decreased Bcl-2/Bax ratio. Bax promotes apoptosis by inducing loss of the electrochemical gradient across the inner membrane of mitochondria and formation of the mitochondrial pores, which influence the efflux of apoptogenic factors and mediators. Hence modulation of Bax and Bcl-2/Bax ratio might be one of the major mechanisms whereby salidroside protects against endothelial cell apoptosis induced by cobalt chloride.

In summary, our results suggest that salidroside may protect endothelial cells from cobalt chloride-induced apoptosis as an antioxidant. Modulation of Bcl-2 family and inhibition of caspase-3 activation might contribute to the protective effects of salidroside.

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**REFERENCES**