Salidroside Inhibits Endogenous Hydrogen Peroxide Induced Cytotoxicity of Endothelial Cells

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Salidroside, a phenylpropanoid glycoside isolated from Rhodiola rosea L., shows potent antioxidant property. Herein, we investigated the protective effects of salidroside against hydrogen peroxide (H₂O₂)-induced oxidative damage in human endothelial cells (EVC-304). EVC-304 cells were incubated in the presence or absence of low steady states of H₂O₂ (3–4 µM) generated by glucose oxidase (GOX) with or without salidroside. 3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH) assays were performed, together with Hoechst 33258 staining and flow cytometric analysis using Annexin-V and propidium iodide (PI) label. The results indicated that salidroside pretreatment attenuated endogenous H₂O₂ induced apoptotic cell death in EVC-304 cells in a dose-dependent pattern. Furthermore, Western blot data revealed that salidroside inhibited activation of caspase-3, 9 and cleavage of poly(ADP-ribose) polymerase (PARP) induced by endogenous H₂O₂. It also decreased the expression of Bax and rescued the balance of pro- and anti-apoptotic proteins. All these results demonstrated that salidroside may present a potential therapy for oxidative stress in cardiovascular and cerebrovascular diseases.

Key words endothelial injury model; salidroside; oxidative stress; apoptosis; hydrogen peroxide

Vascular endothelial cells serve as a barrier between tissue and blood stream, which plays an important role in maintaining vascular homeostasis. Oxidative stress has been implicated as a major cause of endothelial injuries in a variety of clinical abnormalities including atherosclerosis, ischemia reperfusion injury, and diabetes. 1,2,3

Rhodiola rosea L. (Crassulaceae) has been used for a long time as folk medicine in Russia and China.4 Its claimed benefits include anti-depress, anticancer, anti-stress, anti-oxidation and immune function improvement.5 Salidroside (p-hydroxyphenethyl-β-D-glucoside, chemical structure shown in Fig. 1), a major active ingredient isolated from the plant R. rosea, has been used in the treatment of diabetes, hypertension, fatigue and hypoxia.5,6 Recent study showed that salidroside had been found to exhibit marked antioxidant effects and its activity in scavenging superoxide radicals is concentration- and time-dependent.8 Salidroside was also reported to be capable of protecting SH-SY5Y cells against hydrogen peroxide-induced cell apoptosis in a dose-dependent manner9 and protecting the PC12 cells against hypoglycemia/serum limitation-induced cytotoxicity.10 Although salidroside has been known to have significant anti-oxidative and neuroprotective properties, there are no reports on its effects on the vascular endothelial cells.

Fig. 1. Salidroside Inhibition of Endogenous H₂O₂-Induced Cell Damage

Cells were incubated with FBS-free medium, 10 mU GOX, different concentration of salidroside, or 10 mU GOX combined with different concentration of salidroside for 24h. Cell proliferation was determined by MTT assay. Values are means±S.D. of five experiments. *p<0.05 compared with 10 mU GOX group and #p<0.05 vs. untreated cells group.

The authors declare no conflict of interest.

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This study was to investigate whether salidroside could inhibit endogenous H₂O₂-induced toxicity in endothelial cells and the possible mechanism for protection.

MATERIALS AND METHODS

Chemicals and Reagents  Salidroside was provided by Tianjin Jianfeng Natural Product R&D Co., Ltd. (Tianjin, China), and the content of salidroside was about 2.0%, and the purity was above 98.0% (HPLC).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco Chemical Co., Ltd. EVC-304 cells were obtained from the Jilin Medical University. Dulbecco’s modified Eagle’s medium (DMEM) high glucose Medium was purchased from Gibco BRL (Grand Island, NY, U.S.A.). Fetal bovine serum (FBS) was purchased from Hangzhou Sijijing Co., Ltd. (Hangzhou, China). Aspergillus niger (100000–250000 U/g solid) was purchased from the Institute of Jiancheng Biology Engineering (Nanjing, Jiangsu, China).

Cell Culture  EVC-304 cells were grown in DMEM low glucose, supplemented with 10% fetal calf serum. The culture maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. For experiments, cells were plated in 60 mm dishes and reached confluence within 2 to 3 d.

MTT Assay  EVC-304 cells (ca. 1×10⁴) were seeded in 96 well cell culture plates. After treatment with 10 µM GOX, different concentration salidroside, or 10 µM GOX cultured with different concentration salidroside for 24 h, and 20 µL MTT (5 mg/mL) were added to the media and incubated for 4 h at 37°C. The MTT solution was replaced by 150 µL dimethyl sulfoxide (DMSO) and agitated for 10 min. The absorbance was then read at 570 nm using an ELISA reader.

Western Blot Analysis  Cells were seeded in 6-well plate. After treatment for 8h, the cells were harvested and lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris–HCl pH 7.4) for 30 min on ice. After centrifugation (13000×g 4°C, 15 min), the supernatant was loaded on to 12% polyacrylamide SDS gel. Each lane was loaded with 60 µg of cell lysate protein. After electrophoresis, the gels were blotted onto a polyvinylidene difluoride (PVDF) membrane, blocked with 5% (w/v) milk for 1 h on a shaker at room temperature, washed third with TBS-T for 10 min each, and then incubated with primary antibody at one of the following: Bcl-2, Bax, PARP, pro caspase-3, pro caspase-9 at a dilution of 1 : 1000 with primary antibody at one of the following: Bcl-2, Bax, PARP, pro caspase-3, pro caspase-9 at a dilution of 1 : 1000 overnight at 4°C. Primary antibody was detected with anti-rabbit immunoglobulin G (IgG) conjugated to horse-radish peroxidase (HRP), antibody dilutions were 1 : 5000, and visualized using ECL enhanced chemiluminescence.

Statistical Analysis  Data was represented as means±S.D. for at least three independent experiments. The differences between treatment groups were assessed by one-way ANOVA followed by unpaired Student’s t-test. Statistical significance was defined as p<0.05 to reject a null hypothesis.

RESULTS

Effects of Salidroside on EVC-304 Cells Exposed to Endogenous H₂O₂  Glucose oxidase catalyzes the oxidation of d-glucose in the presence of oxygen to d-gluconic acid and H₂O₂. The initial rates of H₂O₂ production by 10 µM GOX in HBSS was 1.5 µM H₂O₂/min. Under culture conditions, extracellular hydrogen peroxide (H₂O₂) concentration was maintained at approximately 7 to 9 µM with 10 µM GOX.

In order to measure the effects of salidroside on endothelial damage induced by H₂O₂, the MTT reduction assay was used. As show in Fig. 1, 10 µM GOX induced significant decrease in cell viability, while the cells treated with 100 µM salidroside did not show visible changes in cell number (Fig. 1), indicating that salidroside protects against H₂O₂-induced cell damage.

Salidroside Represses MDA Production, and Restores the Activities of Total SOD and GSH-Px in EVC-304 Cells Under disease conditions, oxygen free radical is the most lively and damaging free radical which is produced through enzymatic and non-enzyme system. It may promote lipid per-
oxidation of polyunsaturated fatty acids (PUFA) of biomembranes, leading to formation of lipid peroxides such as MDA and hydroxyl, therefore resulting in tissue or cells damage. The elimination of free radical is dependent on the preventive or interrupted regulations of antioxidant defense system. SOD serves as an important member of the antioxidant system, which removes superoxides and protects cells from damage. GSH-Px protects the structural integrity of cell membrane and function. Therefore the changes of MDA content, SOD and GSH-Px activity not only reflect the ability of scavenging oxygen free radical, but also indirectly reflect the extents of cell membrane damage attacked by free radical. The effects of salidroside on lipid peroxidation, and endogenous antioxidant preservation were evaluated as described in Materials and Methods. As shown in Fig. 2B, GOX alone increased MDA equivalents (thiobarbituric acid-reactive substances) in the control groups \( p < 0.01 \). In contrast, 100 µM salidroside significantly reduced MDA equivalents.

Additionally, treatment with GOX alone decreased the activities of total SOD and GSH-Px, whereas treatment with 100 µM salidroside markedly attenuated the changes in total SOD (Fig. 2A) and GSH-Px (Fig. 2C) activities.

**Salidroside Inhibited Endogenous H\textsubscript{2}O\textsubscript{2}-Induced Cell Apoptosis**

Hoechst 33258 is a cell permeable blue fluorescent DNA dye to detect nuclear condensation, fragmentation and characteristics of apoptosis. As shown in Fig. 3A, apoptotic bodies containing nuclear fragments were significantly generated in 10 mU GOX-treated cells (Fig. 3A). However, treatment of 100 µM salidroside reduced cell apoptosis (Fig. 3A). Cells showed a normal Hoechst staining similar to that of control cells. To confirm this result, we stained the cells with Annexin-V, an early apoptotic marker, and PI for detection of late apoptosis. Flow cytometry was used to quantify fluorescent cells, and the results showed that salidroside significantly reduced the percentage of apoptotic cells (Figs. 3B, C).

**Effect of Salidroside on Expression of Bcl-2 Family Proteins in Endogenous H\textsubscript{2}O\textsubscript{2} Treated Cells**

Members of the Bcl-2 family proteins such as Bcl-2 and Bax are critical regulators of the apoptotic pathway.\(^1\) We examined the effect of salidroside on the expression of anti-apoptotic Bcl-2 protein and pro-apoptotic Bax protein. As shown in Fig. 4, the protein level of pro-apoptotic protein, Bax, was decreased, whereas the protein level of anti-apoptotic protein, Bcl-2, was increased in the cells treated with both 10 mU GOX and 100 µM salidroside when compared with 10 mU GOX group.

**DISCUSSION**

It has been well established that the release of reactive oxygen species (ROS) from vessel tissue was elevated under pathological conditions. ROS, such as hydrogen peroxide, superoxide anion and hydroxyl radicals, readily damage biological molecules, which could ultimately lead to apoptosis or cell death.\(^1\) Thus, protection of endothelial cells from ROS-induced injury may therefore provide beneficial therapeutic intervention for successful treatment of cardiovascular diseases.\(^1\) Salidroside, a major active ingredient existing naturally in *R. rosea* L., has been reported to be a strong antioxidant. In the present study, we demonstrated for the first time that salidroside was capable of protecting EVC-304 cells from oxidative stress and the accompanied apoptosis caused...
by endogenous H$_2$O$_2$. Then we further explored the possible molecular mechanisms underlying the antioxidative effects of salidroside on protecting EVC-304 cells against endogenous H$_2$O$_2$-induced cell apoptosis.

H$_2$O$_2$ is continuously produced in vivo and remains in a quasi steady state: its concentration changes in a time scale slower than its turnover.$^{15}$ Hence, exposing cells to steady state concentrations of H$_2$O$_2$, instead of bolus additions, constitutes a superior method for oxidant delivery that mimics the physiological setting.$^{18}$ Enzymatic generation of low and steady H$_2$O$_2$ fluxes may also be appropriate in cell culture studies, because the bolus addition of H$_2$O$_2$ may not adequately reflect its production or delivery in vivo.$^{11,19-21}$ In this regard, the GOX system may be useful in mimicking the mode of extracellular oxidant production from physiological sources such as xanthine oxidase and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.$^{22,23}$ Our present studies confirmed that treating cells with 10 mU GOX resulted in a cell viability loss. However, when incubated with 100 µM and 200 µM salidroside, the cell viability loss was greatly decreased. These results indicated that salidroside did significantly protect EVC-304 cells from H$_2$O$_2$-induced cytotoxicity.

H$_2$O$_2$ ultimately lead to apoptotic or necrotic cell death. We further explored whether salidroside has a protective effect against EVC-304 cell apoptosis induced by H$_2$O$_2$. 10mU GOX treated cells stained with fluorescent DNA binding dye, Hoechst 33258, displayed typical morphological features of apoptosis with sickle shaped-nuclei. Salidroside could mitt-
gate these morphological changes. To confirm this result, we stained the cells with Annexin-V, an early apoptotic marker, and PI for detection of late apoptosis. Flow cytometry was used to quantify fluorescent cells, and the result showed that salidroside significantly reduced the percentage of apoptotic cells. All these results signified that salidroside undoubtedly plays a key role in preventing cells from undergoing H2O2-induced apoptosis.

Membrane lipid is a major target for intracellular ROS. Lipid peroxidation, generally assessed by MDA formation, may disrupt the integrity of plasma membrane. On the contrary, endogenous antioxidant enzymes including SOD and GSH-Px can compromise the excessive ROS in vivo to regulate intracellular redox status. Therefore, abrogating occurrence of lipid peroxidation and increasing levels of endogenous antioxidants may provide a repairing mechanism for ROS-mediated cell damage. The present study suggests that 100 µM salidroside significantly increased SOD and GSH-Px activities in endogenous H2O2 treated EVC-304 cells, along with an attenuated MDA production. These results suggested that enhancement of endogenous antioxidant preservation and subsequent attenuation of lipid peroxidation may represent a primary mechanism of cellular protection for salidroside.

Mitochondria are thought to be a pathway for apoptosis and its function is regulated through Bcl-2 family proteins. To examine further mechanism of anti-apoptosis of salidroside, we investigated the expression of some Bcl-2 family members including a wide variety of anti-apoptotic proteins as well as pro-apoptotic proteins, such as Bax. Bcl-2 inhibits apoptosis by negatively regulating the apoptotic activity of Bax and forming Bcl-2/Bax heterodimers. The Bcl-2/Bax ratio is a measure of the cell death switch, which determines whether a cell will live or die upon being exposed to an apoptotic stimulus. Thus, the expression of the Bcl-2 and Bax proteins in the cells of each group were measured using western blots. Our current study demonstrated a remarkable increase of the Bax and decrease of Bcl-2 at protein levels after salidroside treatment.

PARP is the substrate for effective caspases during apoptosis, which is involved in DNA repair, genome surveillance,
and maintenance of genomic integrity in response to environmental stress. The cleavage of PARP is the hallmark of apoptosis. The result showed that caspase-mediated PARP cleavage induced by 10 mU GOX apoptosis was decreased by incubation with salidroside. We further examined the involvement of Caspases in H₂O₂-mediated apoptosis. Compared with the control group, caspases-3, 9-were inactivated after combination with salidroside.

In summary, salidroside could ameliorate H₂O₂-induced oxidative stress and apoptosis in EVC-304 cells. These data strongly support that salidroside plays a protective role in H₂O₂-induced endothelial injury as a potent antioxidant. Due to its efficacy, salidroside might be a potential therapy for oxidative stress in cardiovascular and cerebrovascular diseases.

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REFERENCES AND NOTE