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Salidroside inhibits H_2O_2-induced apoptosis in PC12 cells by preventing cytochrome *c* release and inactivating of caspase cascade

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We used a rat pheochromocytoma (PC12) cell line to study the effects of salidroside on hydrogen peroxide (H_2O_2)-induced apoptosis. In PC12 cells, H_2O_2-induced apoptosis was accompanied by the down-regulation of Bcl-2, the up-regulation of Bax, the release of mitochondrial cytochrome *c* to cytosol, and the activation of caspase-3, -8 and -9. However, salidroside suppressed the down-regulation of Bcl-2, the up-regulation of Bax and the release of mitochondrial cytochrome *c* to cytosol. Moreover, salidroside attenuated caspase-3, -8 and -9 activation, and eventually protected cells against H_2O_2-induced apoptosis. Taken together, these results suggest that treatment of PC12 cells with salidroside can block H_2O_2-induced apoptosis by regulating Bcl-2 family members and by suppressing cytochrome *c* release and caspase cascade activation.

Keywords salidroside; hydrogen peroxide; apoptosis; PC12 cells

Alzheimer's disease (AD) is a multifaceted neurodegenerative disorder characterized by the progressive deterioration of cognition and memory in association with widespread neuronal loss and the deposit of senile plaques. To date, the cause and the mechanism by which neurons die as a result of AD still remain unclear, yet several lines of evidence support the involvement of apoptosis. Studies on post-mortem tissues have provided direct morphological and biochemical evidence that some neurons in the brains of AD patients degenerate via an apoptotic mechanism relating to the presence of DNA damage, nuclear apoptotic bodies, and other markers of apoptosis [1,2]. These results suggest therapeutic strategies aimed at preventing and delaying apoptosis might be a reasonable choice for the treatment of the disease.

Hydrogen peroxide (H_2O_2), a major source of reactive oxygen species, destroys neurons by inducing apoptosis, which has implications for several biological and pathological processes, including AD. H_2O_2 has been used in many studies to trigger cell apoptosis [3,4]. Therefore, we used H_2O_2 to induce apoptosis in PC12 cells in present study.

Considerable efforts have been made to find natural substances with neuroprotective potential, and attention has been focused particularly on Chinese medicinal plants with nootropic effects. Some plants have been used for thousands of years in China to improve cognition or as anti-aging remedies. In our search for new ingredients from traditional Chinese medicinal herbs, salidroside, a phenolic glycoside involved in cell anti-apoptosis processes [5], was isolated from the rhizome of *Rhodiola rosea* L. (Crassulaceae). However, the neuroprotective role of salidroside is unclear. The present study's aim was to explore whether salidroside could inhibit H_2O_2-induced toxicity in PC12 cells and the possible mechanism.

Materials and Methods

Materials

Salidroside was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). MTT, fluorescent DNA-binding dye Hoechst 33258, and propidium iodide were purchased from Sigma-Aldrich (St. Louis, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum were obtained from Gibco Life Technologies (Grand Island, USA). Lactic dehydrogenase (LDH) activity assay kit was obtained from Jiancheng Institute of Biotechnology (Nanjing, China).

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Antibody of cytochrome *c* was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). DNA extraction kit and caspase-3, -8 and -9 activity kits were from Beyotime Institute of Biotechnology (Nantong, China). All other chemicals and reagents were of analytical grade.

Cell culture and treatment and analysis of cell viability

Cells were cultured and treated as described by Qian *et al* [6]. Briefly, PC12 cells were maintained in DMEM supplemented with heat-inactivated 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin in a water-saturated 5% CO_2 atmosphere at 37 ºC. Experiments were carried out 48 h after cells were seeded into 24-well plates. To produce oxidative stress, H_2O_2 was freshly prepared from 30% stock solution prior to each experiment, and after 12 h exposure, the level of cellular MTT was quantified as described by Chen *et al* [7]. Cells in 24-well plates were briefly rinsed with phosphate-buffered saline (PBS), and 0.5 mg/ml MTT was added to each well. The microplate was incubated at 37 ºC for an additional 4 h. At the end of the incubation, the medium with MTT was removed and 500 ml dimethyl sulfoxide was added to each well. The plate was shaken on a microplate shaker to dissolve the blue MTT-formazan. The absorbance was read at 570 nm on a microplate reader. When the effects of salidroside on the PC12 cells were studied, different concentrations of salidroside were added simultaneous to the medium just before the H_2O_2 was added.

Measurement LDH release

LDH release was measured according to the method of Kruman *et al* [8]. Cells were cultured in 24-well culture plates at a density of 1×10^4 cells/well for LDH assay. After 12 h exposure to H_2O_2, LDH activities in the medium were measured using an assay kit according to the manufacturer's instructions.

Hoechst staining

To quantify and assess nuclear morphology, PC12 cells were fixed for 10 min with 4% paraformaldehyde in PBS. The cells were then stained for 10 min with 10 μg/ml fluorescent DNA-binding dye Hoechst 33258 to reveal nuclear condensation [9]. Hoechst-stained cells were visualized and photographed under a Leica DMIL microscope (Nussloch, Germany).

Analysis of DNA fragmentation

Fragmented DNA was isolated using a DNA extraction kit according to the manufacturer's instructions. The elutriants containing DNA pellets were electrophoresed on a 1.8% agarose gel at 80 V for 1.5 h. The gel was examined and photographed using an ultraviolet gel documentation system.

Flow cytometric analysis of DNA content

DNA content was measured according to the methods of Weinmann *et al* [10]. Briefly, cells were collected and washed with ice-cold PBS and fixed with 70% ethanol. The fixed cells were harvested by centrifugation at 1000 *g* for 5 min; dissolved in 100 ml PBS containing 50 mg/ml RNase A, 50 mg/ml propidium iodide, 0.1% Triton X-100 and 0.1 mM EDTA (pH 7.4); and then incubated at 37 ºC for 30 min. The fluorescence of cell was measured by flow cytometer (FACSCalibur; Becton Dickinson, San Jose, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from PC12 cells, and the potential residual genomic DNA was eliminated with RNase-free-DNase I for 30 min at 37 ºC. First-strand complementary DNA was synthesized as follows: 1 h at 42 ºC with 100 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, USA), 15 U ribonuclease inhibitor (Promega), 500 μM dNTP, 0.5 μg oligo(dT)18 and 2 μg total RNA in a final volume 25 μl, and then 5 min at 95 ºC. For PCR amplification, the specific primers included the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 213 bp): 5′-ATTCAACGGCACAGTCAAGG-3′ (forward) and 3′-AGTAGAGGCGGGGAAGACG-5′ (reverse); Bcl-2 (303 bp): 5′-GATGACTTCTCTCGTCGC-TA-3′ (forward) and 3′-TACGGAAACACCTTGATATA-5′ (reverse); Bax (331 bp): 5′-GAACTGGACAATAATATG-GA-3′ (forward) and 3′-TCACTGGTAGAAACACCGAC-5′ (reverse). The PCR mixture contained 0.8 pM forward and reverse primers of the *Bax* or *Bcl-2*, 0.4 pM forward and reverse primers of the *GAPDH*, 2.0 mM $MgCl_2$, 200 μM deoxyribonucleotide triphosphate, and 1.5 U Taq DNA polymerase. The PCR procedure was performed at 94 ºC for 5 min, followed by 28 cycles at 94 ºC for 1 min, 51 ºC for 30 s, 72 ºC for 45 s and extension at 72 ºC for 10 min. Next, 10 μl PCR products was mixed with 2 μl loading solution, and electrophoresed on agarose-ethidium bromide gel at 100 V for 1 h. The gels were examined and analyzed by an ultraviolet gel documentation system.

Analysis of caspase-3, -8, and -9 activities

Caspase-3, -8, and -9 activities were measured using assay kits according to the manufacturer's instructions. Supernatant was mixed with buffer containing the recognition sequence for caspase attached to p-nitroanilide. The

absorbance of p-nitroanilide was determined at 405 nm. The caspase activities were expressed as percentage compared to control.

Western blot analysis of cytochrome *c*

Cell lysates were prepared as described by Jia *et al* [11]. To ensure equal loading of the protein samples, protein concentrations of the cell lysates were determined by Bradford assay. Equal amounts of protein (30 μg in total) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in 1×Tris-buffered saline containing 0.05% Tween-20 (TBST) for 1 h. After blocking, the membrane was incubated with 1% skim milk in TBST, containing the primary mouse monoclonal antibody against cytochrome *c* (1:500) overnight. The membranes were then washed three times with 1×TBST and then incubated with 1% skim milk in TBST, containing a peroxidase-conjugated goat anti-mouse immunoglobulin G secondary antibody (1:5000) (ZSGB-BIO, Beijing, China). The detection of protein bands was performed using the 3,3′-diaminobenzidine tetrahydrochloride substrate kit (ZSGB-BIO).

Statistical analysis

All experiments were performed in triplicate. Data are presented as mean±SD. The Duncan test and one-way ANOVA were used for multiple comparisons using SPSS 12.0 software (SPSS, Chicago, USA).

Results

Inhibition of H_2O_2-induced cytotoxicity by salidroside

In PC12 cells, the protective effect on H_2O_2-induced cytotoxicity was assessed by MTT assay after 12 h incubation. As shown in **Table 1**, when the cells were pre-incubated with salidroside (10 and 100 μM), H_2O_2-induced cell toxicity was significantly reduced in comparison with the control. Necrosis results in a disruption of the cytoplasmic membrane, and the necrotic cells release cytoplasmic LDH and other cytotoxic substances into the medium. We therefore examined the existence of LDH in the cells' culture medium. The LDH index was significantly reduced at doses of 10 and 100 μM in comparison with the control (**Table 2**). The results of MTT and LDH assays showed that salidroside could have a protective effect against H_2O_2-induced cytotoxicity.

Table 1 Protective effect of salidroside on PC12 cells with H_2O_2-induced injury assessed by MTT assay

Group	A570	Survival (%)
Normal cells	1.82±0.00	100.0
Injured cells	1.37±0.07##	75.3
Injured cells+100 μM sal	1.80±0.03**	98.9
Injured cells+10 μM sal	1.54±0.17**	84.6
Injured cells+1 μM sal	1.37±0.03	75.3

Data are represented as mean±SD. ***P*<0.01 versus injured cells group; ##*P*<0.01 versus control group. sal, salidroside.

Table 2 Inhibitory effect of salidroside on LDH release of PC12 cells under H_2O_2 treatment

Group	LDH activity (U/ml)	Increment (%)
Normal cells	0.313±0.001	
Injured cells	0.514±0.001##	64.2
Injured cells+100 μM sal	0.377±0.001**	20.4
Injured cells+10 μM sal	0.451±0.002**	44.1
Injured cells+1 μM sal	0.511±0.001	63.3

Data are represented as mean±SD. ***P*<0.01 versus injured cells group; ##*P*<0.01 versus control group. sal, salidroside.

Salidroside suppresses H_2O_2-induced apoptosis

Hoechst 33258 assay revealed the appearance of a collection of multiple chromatin and fragmented apoptotic nuclei after treatment with 0.5 mM H_2O_2 for 12 h. However, the apoptotic nuclei were significantly reduced when cells were treated with 100 μM salidroside and 0.5 mM H_2O_2 [**Fig. 1(A)**]. After the PC12 cells were treated with 0.5 mM H_2O_2 for 12 h, DNA ladder pattern was detected, but salidroside was able to reduce the ladder pattern in a dose-dependent manner [**Fig. 1(B)**]. When the apoptotic cells were analyzed quantitatively by flow cytometry, a significant increase in the apoptotic rate (from 9.78%±0.2% to 32.23%±4.0%) was found after PC12 cells were treated with 0.5 mM H_2O_2 for 12 h. When PC12 cells were treated with 100 μM salidroside and 0.5 mM H_2O_2 for 12 h, the percentage of apoptotic cells decreased from 32.23%±4.0% to 18.61%±1.5% [**Fig. 1(C)**].

Regulation of mRNA expression of *Bax* or *Bcl-2* by salidroside

As shown in **Fig. 2**, after H_2O_2 treatment for 6 h, mRNA expressions of *Bax* and *Bcl-2* analyzed by RT-PCR analysis showed *Bcl*-2 expression began to decrease and *Bax*

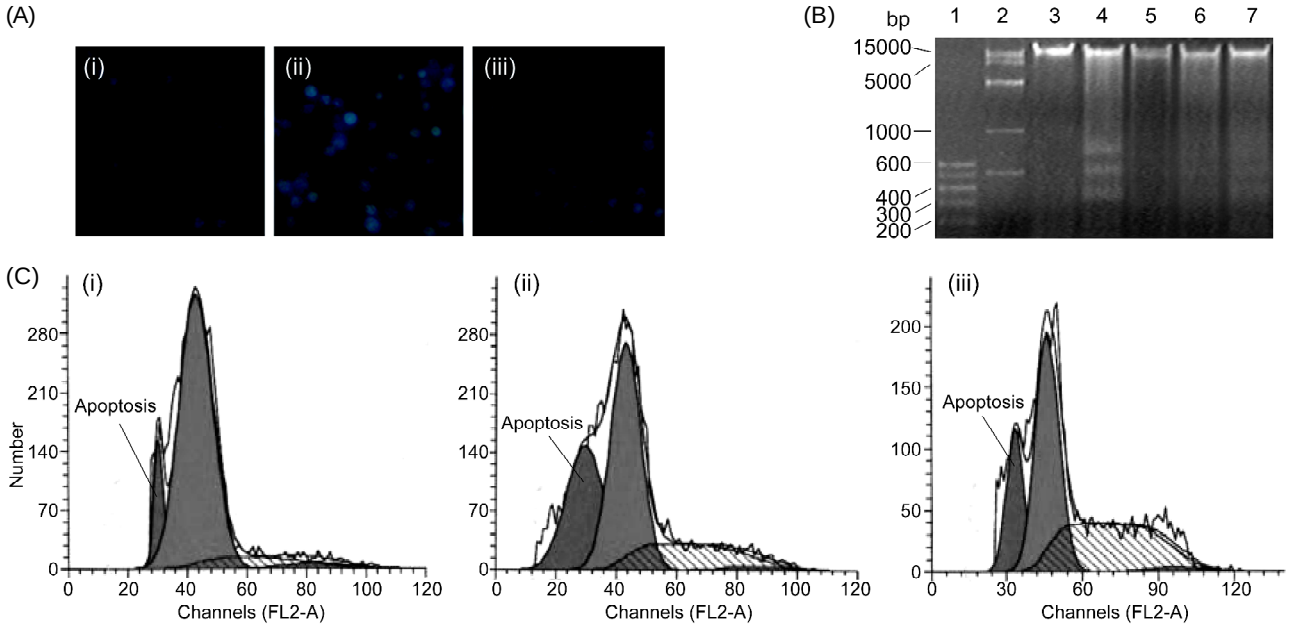

Fig. 1 Salidroside inhibited H_2O_2-induced injury in PC12 cells (A) Morphological analysis of nuclear chromatin in by Hoechst 33258. (i) Control. (ii) After exposure to 0.5 mM H_2O_2 for 12 h, cells displayed condensed chromatin and apoptotic nuclei. (iii) After exposure to 0.5 mM H_2O_2 and 100 μM salidroside for 12 h. (B) Agarose gel electrophoresis of DNA fragmentation. Lane 1, DNA ladder marker 1; lane 2, DNA ladder marker 2; lane 3, control; lane 4, 0.5 mM H_2O_2 for 12 h; lanes 5–7, salidroside (100, 10 and 1 μM, respectively) and 0.5 mM H_2O_2 treatment for 12 h. (C) Flow cytometric histograms. (i) Control PC12 cells (apoptotic rate is 9.78%±0.2%). (ii) PC12 cells treated with 0.5 mM H_2O_2 (apoptotic rate is 32.23%±4.0%). (iii) PC12 cells treated simultaneously with 0.5 mM H_2O_2 and 100 μM salidroside for 12 h (apoptotic rate is 18.61%±1.5%).

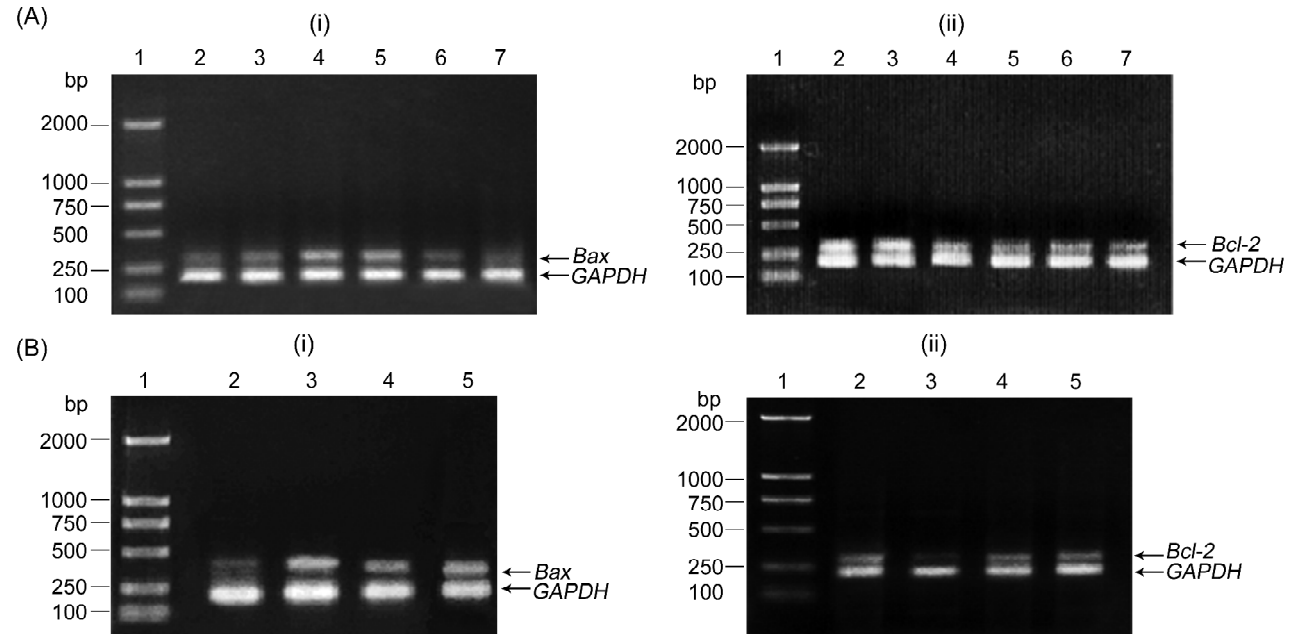

Fig. 2 RT-PCR analysis of mRNA expression of *Bax* and *Bcl-2* in PC12 cells (A) mRNA expressions of *Bax* (i) and *Bcl-2* (ii) in PC12 cells treated with 0.05 mM H_2O_2 for indicated time. Lane 1, DNA marker; lane 2, untreated PC12 cells; lanes 3–7, PC12 cells treated with 0.5 mM H_2O_2 for 3, 6, 9, 12 and 24 h, respectively. (B) After co-treatment with 0.5 mM H_2O_2 and salidroside (100 and 1 μM, respectively) for 6 h, total RNA was extracted for RT-PCR analysis of *Bax* (i) and *Bcl-2* (ii) expressions. Lane 1, DNA marker; lane 2, untreated cells; lane 3, PC12 cells treated with 0.5 mM H_2O_2 for 6 h; lanes 4–5, PC12 cells treated with 0.5 mM H_2O_2 and salidroside (100 and 1 μM, respectively) for 6 h. GAPDH, the internal control.

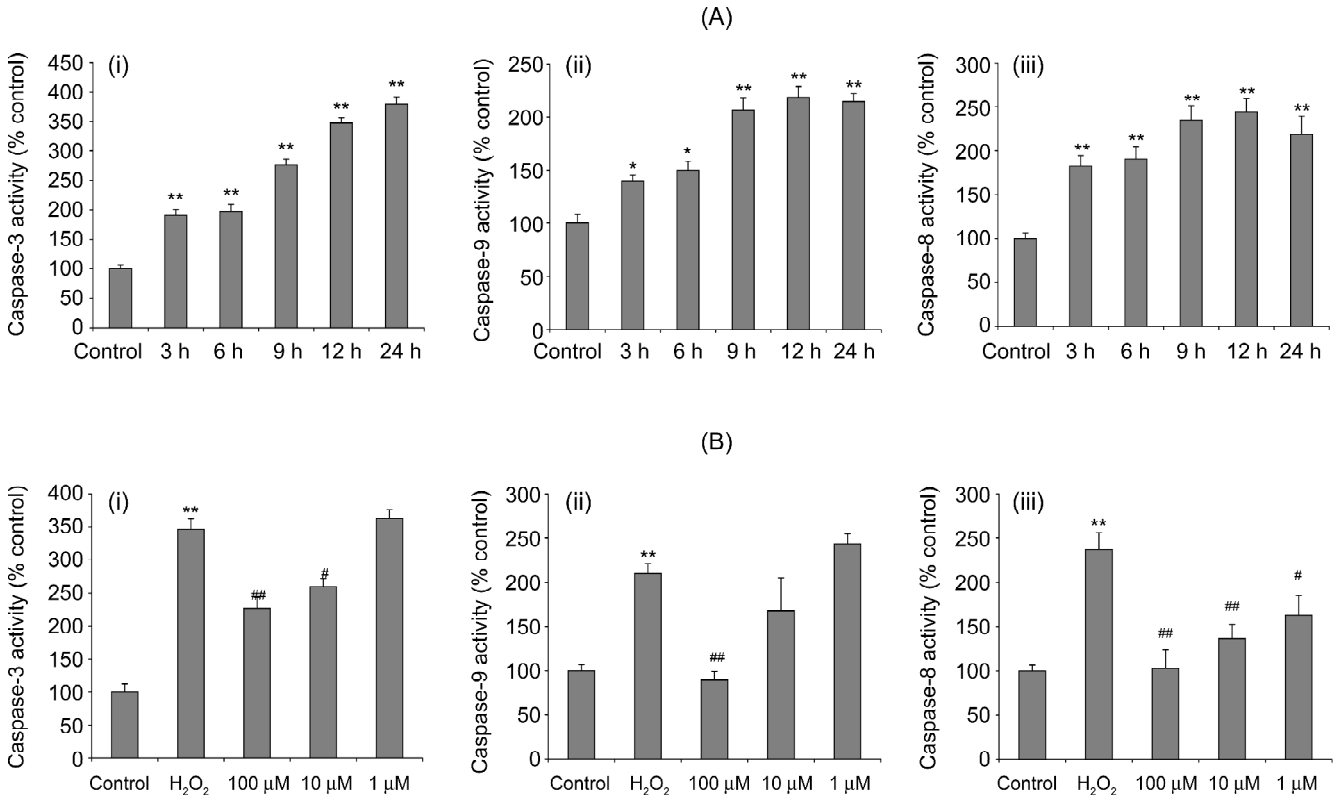

Fig. 3 Detection of caspase-3, -8 and -9 activities in PC12 cells (A) Treatment with 0.05 mM H_2O_2 causes a time-dependent increase in caspase-3 (i), -8 (ii) and -9 (iii) activities in PC12 cells. Control, untreated cells. (B) PC12 cells were co-treated with 0.05 mM H_2O_2 and salidroside (100, 10 and 1 μM) for 9 h. The activities of caspase-3 (i), -8 (ii) and -9 (iii) were detected. Control, untreated cells. **P*<0.05, ***P*<0.01 versus control; $^{\#}P$<0.05, $^{\#\#}P$<0.01 versus H_2O_2 alone.

expression began to increase. The effects of salidroside on mRNA expression were investigated at the same indicated time. The results showed salidroside (100 and 1 μM) significantly raised *Bcl-2* expression and reduced *Bax* in PC12 cells treated with 0.5 mM H_2O_2 (**Fig. 2**).

Salidroside inhibits the activities of caspase-3, -8 and -9

To gain insight into the molecular effector pathway of H_2O_2-induced apoptosis, we first examined whether caspases were downstream effectors in H_2O_2-mediated apoptosis. H_2O_2 treatment caused a time-dependent increase in caspase-3, -8 and -9 proteolytic activities. However, when salidroside and H_2O_2 were added simultaneously to the medium, decreases in the activity of caspase-3, -8 and -9 were detected (**Fig. 3**).

Salidroside reduced cytochrome *c* in the cytosol

As indicated in **Fig. 4**, Western blot analysis revealed that H_2O_2 treatment caused a progressive accumulation of cytochrome *c* in the cytosol. This was reduced when PC12 cells were treated with salidroside.

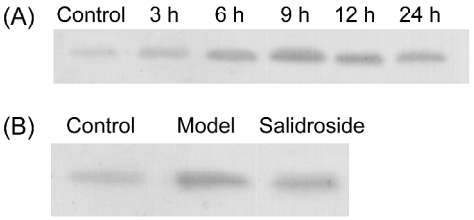

Fig. 4 Total cell lysates extracted from untreated PC12 cells or PC12 cells treated with salidroside and hydrogen peroxide (H_2O_2), and analyzed for cytochrome *c* content by Western blot analysis (A) Cytochrome *c* expression in cytosol of PC12 cells treated with 0.5 mM H_2O_2 for indicated times. (B) Effect of 100 μM salidroside on cytochrome *c* release in PC12 cells treated with 0.5 mM H_2O_2 for 9 h. Control, untreated cells. Model, cells treated with 0.5 mM H_2O_2 for 9 h.

Discussion

Recently, researchers have made considerable efforts to search for natural substances with neuroprotective potential, and particular attention has been paid to Chinese

medicinal plants with nootropic effect. The rhizome of *Rhodiola rosea* L. has been used in East Asia as a tonic and anti-aging agent since ancient times. There has been mounting evidence that the extract from the rhizome of *Rhodiola rosea* L. possesses significant neuroprotective activity and antioxidative effects [12,13], although little is known about its pharmacological effects or active ingredients. In a previous study, salidroside was isolated from the rhizome of *Rhodiola rosea* L. and could significantly inhibite O_2^-- or H_2O_2-induced neurotoxicity in rat cortical cultures [14]. Earlier results showed that 100 μM salidroside has little effect on PC12 cells, and there was no significant difference compared with control group. The present findings demonstrated that, in PC12 cells, salidroside reduced H_2O_2-induced apoptotic death caused by oxidative stress. Treatment with salidroside significantly attenuated increased LDH leakage and decreased viability in differentiated PC12 cells exposed to H_2O_2. In these instances, the amount of H_2O_2 was greater than that of salidroside, and the decrease in cell survival caused by H_2O_2 was nearly suppressed in the presence of 0.1 mM salidroside. Therefore, we have speculated that antioxidation is just one of salidroside's pathways in this model. Inhibition of relative targets in apoptosis might be a possible mechanism involved in the protective effects of salidroside.

It has been well documented that some pathological neuronal loss in AD occurs through apoptosis. The results of this present study showed that salidroside protected PC12 cells against H_2O_2-induced apoptosis. Exposure to 0.5 mM H_2O_2 induced typical apoptosis in PC12 cells. These results were in accordance with previous studies that found oxidative stress to be a common cause of apoptosis [15, 16]. When cells were pre-incubated with salidroside, H_2O_2-induced cell injury was significantly attenuated. For these reasons, salidroside could be a useful neuroprotective agent to ameliorate oxidative stress-induced apoptosis, which may be used in the treatment of AD.

Apoptosis is a type of cell death that represents the culmination of naturally occurring or highly programmed mechanisms. Elucidating the expression patterns of those factors during apoptotic cell death may be critical to our understanding of the underlying mechanisms. Caspase-3 is a key executioner caspase involved in neuronal apoptosis, and its activity is controlled by upstream regulators, such as caspase-8 or caspase-9, which modulate the mitochondria- and death receptor-dependent pathway, respectively [17]. The present study showed that caspase-3 activity was up-regulated in H_2O_2-treated cells. We also detected enhanced caspase-9 activity in H_2O_2-treated cells and the release of cytochrome *c* from mitochondria into cytosol. Taken together, these results suggested that H_2O_2-induced apoptosis in PC12 cells is associated with the release of cytochrome *c* and the activation of caspases, probably via the mitochondria-mediated apoptosis pathway. We further demonstrated the down-regulation of Bcl-2 or up-regulation of Bax in H_2O_2-treated cells. Increased Bax and lowered Bcl-2 expression have been shown to reduce mitochondrial membrane potential and increase reactive oxygen species production in neurons [18], both of which are early events in the process of apoptosis [19]. Our results suggested that the down-regulation of Bcl-2 or up-regulation of Bax alters mitochondrial membrane permeability, triggers mitochondrial cytochrome *c* release to cytosol and activates caspase cascade.

Caspase-8 is a key initiating caspase involved in neuronal apoptosis and that modulates the death receptor-dependent pathway. We detected enhanced caspase-8 activity in H_2O_2-treated cells. The results suggested that the death receptor-mediated pathway is involved in H_2O_2-induced apoptosis. However, recent studies have suggested that caspase-8 is not always activated early in the context of Fas signaling. In some cells, caspase-9 initiates the processing of caspase-3, which in turn activates caspase-2 and -6. Caspase-6 was found to be required for the activation of downstream caspase-8 [20]. In summary, our study suggested that H_2O_2-induced apoptosis in PC12 cells is mediated by at least one pathway through mitochondria that regulates the Bcl-2 family and caspase-3 and -9. However, future studies are required to determine whether the death receptor-mediated pathway is involved in H_2O_2-induced apoptosis.

Apoptosis is closely associated with the progression of AD and other neurological diseases. In searching for anti-apoptosis agents, this study examined the possible role of salidroside. Salidroside is an invaluable source for the development of effective neuroprotective agents to protect against apoptosis in PC12 cells in the treatment of age-related neurological diseases.

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