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Salidroside induces cell-cycle arrest and apoptosis in human breast cancer cells

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ABSTRACT

Recently, salidroside (*p*-hydroxyphenethyl-β-D-glucoside) has been identified as one of the most potent compounds isolated from plants of the *Rhodiola* genus used widely in traditional Chinese medicine, but pharmacokinetic data on the compound are unavailable. We were the first to report the cytotoxic effects of salidroside on cancer cell lines derived from different tissues, and we found that human breast cancer MDA-MB-231 cells (estrogen receptor negative) were sensitive to the inhibitory action of low-concentration salidroside. To further investigate the cytotoxic effects of salidroside on breast cancer cells and reveal possible ER-related differences in response to salidroside, we used MDA-MB-231 cells and MCF-7 cells (estrogen receptor-positive) as models to study possible molecular mechanisms; we evaluated the effects of salidroside on cell growth characteristics, such as proliferation, cell cycle duration, and apoptosis, and on the expression of apoptosis-related molecules. Our results demonstrated for the first time that salidroside induces cell-cycle arrest and apoptosis in human breast cancer cells and may be a promising candidate for breast cancer treatment.

1. Introduction

Breast cancer is one of the most prevalent cancers worldwide, especially in Western societies. The mammary cell proliferation induced by estrogen is considered the first step of breast cancer development. However, hormone-independent growth is observed in later stages [1]. The main challenge for breast cancer treatment is resistance of estrogen receptor- (ER-) negative tumors to anti-hormone therapy [2]. In recent years, phytochemicals have been recognized as a new prevention and therapeutic approach for breast cancer [3,4]. Animal studies have shown that different phytochemicals may prevent breast cancer incidence and mortality [5,6]. Plants from the genus *Rhodiola* have been important herbs used for thousands of years in Asia and Eastern Europe to treat various diseases. Another research group had reported the anti-cancer action of *Rhodiola* extracts in animal models in a series of studies many years ago [7], and a few studies have emerged recently about the anti-cancer effect of *Rhodiola* extracts [8,9]; however, the role of these extracts as an anti-cancer agent has not been established.

Preclinical investigations regarding Chinese herbal medicine are usually fragmentary and are often not comparable due to different extracts and administration, but it is important to explore the mechanism for the active ingredient in the herbs [10]. Salidroside (*p*-hydroxyphenethyl-β-D-glucoside, chemical structure shown in Fig. 1A), one of the most potent ingredients extracted from plants of the genus *Rhodiola* [11], has been shown to have neuroprotective [12], cardiovascular protective [13], and antiviral effects [14]. Recent studies have shown that salidroside may prevent the growth of leukemia, stomach adenocarcinoma and parotid carcinoma in vitro (published in Chinese) and may also significantly decrease neovascular reactions to all doses applied [15], but pharmacokinetic data on the compound are unavailable.

We previously reported that purified salidroside could inhibit the growth of human cancer cell lines derived from different tissues [16], and we found that ER-negative human breast cancer MDA-MB-231 cells were sensitive to the inhibitory action of low-concentration salidroside, but the mechanism was not clear. Therefore, in this study, we further investigated the possible molecular mechanism of salidroside's cytotoxic effects on human breast cancer MDA-MB-231 (hormone-resistant) and MCF-7 cells (hormone-sensitive), and we found the first evidence that salidroside may induce cell-cycle arrest and apoptosis in human breast cancer cells.

2. Materials and methods

2.1. Materials

Salidroside with a purity of 99% was ordered from National Institute for the Control of Pharmaceutical and Biological Products

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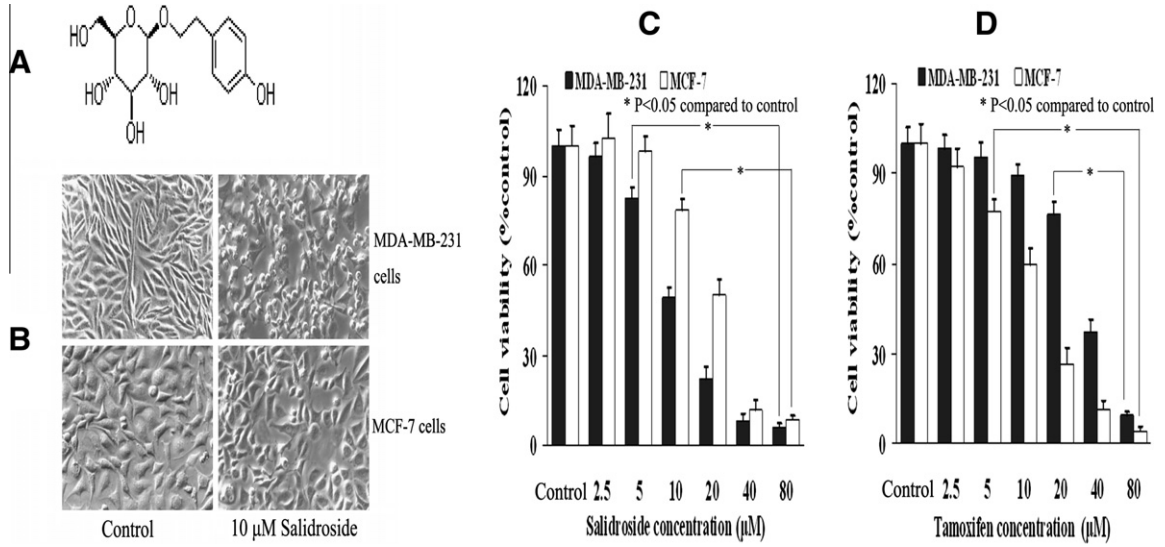

Fig. 1. Effect of salidroside on the growth of MDA-MB-231 and MCF-7 cells. (A) Chemical structure of salidroside. (B) Morphological changes of the MDA-MB-231 and MCF-7 cells after salidroside treatment. Cells were treated with 10-μM salidroside for 24 h, and the morphological changes were observed using an inverted microscope (200×). (C) MDA-MB-231 and MCF-7 cells were treated with salidroside (0–80 μM) for 48 h. (D) Tamoxifen (0–80 μM) was used as a positive control. Viability was determined by the MTT assay. The data are presented as the percentages relative to the control (defined as 100%). The data are expressed as the mean ± standard deviation of triplicate samples from three independent experiments. $^{*}p < 0.05$ compared to control using the Student–Newman–Keuls test.

(Beijing, China). Salidroside was dissolved in water and filtered through a 0.22-μm filter before use. All culture media and serum were obtained from GIBCO/BRL Life Technologies, Inc. Antibodies to cleaved caspase 9, Bcl-2, Bax and β-actin were purchased from Cell Signaling Technology, Inc. All other reagents and plastic wares were obtained from commercial sources. Culture dishes were purchased from Falcon Plastics.

2.2. Cell lines and cell culture

Human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from American Type Culture Collection (ATCC, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or RMPI-1640 supplemented with 10% fetal bovine serum (FBS) in a humidified incubator at 37 °C and 5% CO_2. The cells were harvested following trypsinization (0.025% trypsin and 0.02% EDTA) and washed twice with phosphate buffered saline (PBS). When the cell density reached approximately 80% confluence, the cells were subcultured, and salidroside was then added to the cells after a 24-h incubation.

2.3. Cell viability assay

Cells were plated in 96-well culture plates at an initial density of 1×10^4 cells/well and allowed to attach to the plates. The culture medium was replaced by fresh medium containing salidroside and tamoxifen at concentrations ranging from 0 to 80 μM and incubated for 48 h. The cell proliferation Kit I (MTT) from Roche Applied Science was used to measure cell viability. Briefly, 10 μl of the labeling solution was added to each well of the 96-well plates. After 2 h in the CO_2 incubator, 100 μl of the solubilization solution was added to dissolve the purple crystals, which were the products of the MTT substrates. The absorbance was measured at 570 nm by a plate reader (Perkin-Elmer). Absorbance measured in the MTT assays are expressed as a percentage of the control (defined as 100%).

2.4. Cell cycle analysis

Cells were seeded in 25-cm^2 flasks and incubated overnight to allow cells to attach to the plate. MDA-MB-231 cells were treated with 10-μM salidroside for 24 h, and MCF-7 cells were treated with 20-μM salidroside for 24 h. After treatment, control (untreated) and treated floating and adherent cells were collected by trypsinization. The cells (1×10^6 cells/ml) were washed twice with cold PBS and fixed in 70% ethanol. Immediately before the analysis, the cells were washed with PBS and stained with a solution containing PI (0.2 mg/ml) for 1 h at 4 °C and with RNase A (0.1 mg/ml) for 30 min at 37 °C. The distribution of cells in the cell cycle was measured by flow cytometry (Becton Dickinson, FACSCaliburTM). Percentages of cells in the cell cycle phases were calculated using the CellQuest software (Becton Dickinson).

2.5. Annexin V-FITC and PI staining

Cells were treated without salidroside (control) and with 5- and 10-μM salidroside for 48 h. Cells were then detached by exposure to trypsin/EDTA. Both suspended and adherent cells were collected and rinsed in PBS. After washing, cells at a concentration of 2×10^6 cells/ml were incubated with Annexin V-FITC and PI in the dark for 15 min at room temperature, and then, the cells were pelleted and analyzed by flow cytometry (Becton Dickinson).

2.6. Detection of apoptosis by ELISA

Cytoplasmic histone-associated DNA fragmentation as a marker for apoptosis was detected by a Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics) according to the manufacturer's instructions. Briefly, after 24 h of stimulation with various concentrations of salidroside (0–20 μM), floating cells and adherent cells were collected for analysis, and cells were lysed according to the manufacturer's manual. A mixture of anti-histone-biotin and anti-DNA-POD was added and incubated for 2 h at 15–25 °C. After removal of unbound antibodies using a washing step, POD was determined photometrically at 405 nm with ABTS as a substrate. Data were calculated as the ratio of values obtained for the salidroside-treated cells to those for the untreated controls.

2.7. Western blot

Cells were treated without (control) and with different concentrations of salidroside (0–10 μM). After 24 h of stimulation, floating

cells and adherent cells were collected and washed three times with ice-cold PBS and harvested in lysis buffer. The lysate was centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatant was collected. The Bradford protein assay method was used to determine the protein concentrations. Samples (30–50 μg) were subjected to electrophoresis on 12–18% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The membrane was then blocked with 10% (v/v) dried fat-free milk in PBS with 0.1% Tween 20 prior to immunoblotting with primary antibodies against Bax (1:1000), Bcl-2 (1:1000), cleaved caspase 9 (1:1000), and β-actin (1:3000). Membranes were incubated with primary antibodies overnight at 4 °C, washed for 1 h with PBST. After washing, membranes were incubated with HRP-conjugated anti-rabbit or mouse IgG diluted 1:4000 in PBS for 1 h at room temperature, washed for 1 h with PBST, and analyzed for detection using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

2.8. Statistical analysis

The results were expressed as the mean ± standard deviation, and statistical differences were evaluated by one-way analysis of variance between groups (ANOVA) followed by the Student's *t*-test. A statistically significant difference was considered to be at $p < 0.05$.

3. Results and discussion

3.1. Salidroside inhibited the growth of human breast cancer cells

One of the main challenges in breast cancer treatment is the occurrence of ER-negative tumors that are resistant to anti-hormone therapy [2]. In view of these limitations, there is a need to develop a better drug for breast cancer. In our previous paper, we showed that human breast cancer MDA-MB-231 cells were more sensitive to the inhibitory action of salidroside than cancer cells derived from different tissues [16]. Here, the MTT assay was applied to evaluate the anti-proliferative activities of salidroside on ER-negative MDA-MB-231 cells and ER-positive MCF-7 cells.

MDA-MB-231 and MCF-7 cells were treated with increasing concentrations of salidroside (0–80 μM) for 48 h (Fig. 1C). As a comparison, tamoxifen was used as a positive control (Fig. 1D). Results on cell viability showed that salidroside was cytotoxic for both estrogen-sensitive MCF-7 and estrogen-insensitive MDA-MB-231 cells in a concentration-dependent manner ($p < 0.05$). Salidroside showed a cytotoxic effect on MCF-7 cells, with a 50% inhibitory concentration (IC_{50}) of 20 μM, and the IC_{50} for MDA-MB-231 cells was 10 μM at 48 h. The morphological cell changes also showed that MDA-MB-231 cells were more sensitive to the inhibitory action of 10-μM salidroside than MCF-7 cells (Fig. 1B). These results suggested that ER-negative MDA-MB-231 cells may be more susceptible to low-concentration salidroside-mediated cytotoxicity than ER-positive MCF-7 cells and that the cytotoxic action induced by salidroside may be independent of the estrogen receptor.

The IC_{50} of salidroside and tamoxifen on MCF-7 cells were 20 and 30 μM, respectively, at 48 h, indicating that the cytotoxic effect of salidroside on MCF-7 cells was greater than that of tamoxifen. Tamoxifen, a nonsteroidal estrogen antagonist, has been widely used to treat estrogen-positive breast cancer by interfering with the estrogen-dependent proliferation of breast cancer cells [17,18]. We suspected that salidroside may act as an estrogen antagonist to induce the cell death of ER-positive MCF-7 cells. However, the receptor-binding assay indicated that salidroside was not an estrogen antagonist like tamoxifen (data not shown). Therefore, salidroside may exert cytotoxic action through some other mechanism independent of the estrogen receptor in human breast cancer cells.

3.2. Salidroside induced cell-cycle arrest of human breast cancer cells

Several therapeutic drugs currently in use for breast cancer include drugs that inhibit specific hormone receptors, inhibit growth factor receptors, and induce cell-cycle arrest [19–21]. To determine whether the growth inhibition may be the result of cell-cycle arrest, we analyzed the DNA content by propidium iodide staining followed by flow cytometry. The stained DNA of MCF-7 cells after treatment with 20-μM salidroside demonstrated that 67% of the cells were in the G1/G0 phase, 21% in the S phase, and 12% in the G2/M phase (Fig. 2). Stained DNA of MDA-MB-231 cells following treatment with 10-μM salidroside demonstrated that 48% of the cells were in the G1/G0 phase, 18% in the S phase, and 34% in the G2/M phase (Fig. 2). These results suggested that that 20-μM salidroside caused the G0/G1 arrest of MCF-7 cells and that 10-μM salidroside induced the G2/M arrest of MDA-MB-231 cells; the inhibition of cell cycle progression may be one of the molecular events associated with the selective anti-cancer efficacy of salidroside in breast cancer cells.

Several different cyclin-dependent kinases (CDKS) regulate the cell cycle progression by binding to different types of cyclins. Cyclin D1 is known to bind to and activate CDK4, which is largely involved in controlling the G1/S restriction point, and the G2/M transition is positively regulated by the CDC2 and cyclin B complex [22,23]. CDK–cyclin complexes are negatively controlled by the Kip/Cip family of cyclin-dependent kinase inhibitors (CDKIs), namely $p27^{Kip1}$ and $p21^{Cip1}$ [24]. The G0/G1 arrest induced by 20-μM salidroside in MCF-7 cells may be related to the downregulation of CDK4 and cyclin D1 (data not shown), and the G2/M arrest of MDA-MB-231 cells may be related to the downregulation of cyclin B1 and Cdc2 (data not shown). In addition, the increased levels of $p21^{Cip1}$ and $p27^{Kip1}$ induced by salidroside may be part of the mechanisms inhibiting CDK-Cyclin kinase activity [16]; this suggested that the inhibition of cell cycle progression may be one of the molecular events associated with the selective anti-cancer efficacy of salidroside in breast cancer cells.

3.3. Salidroside induced apoptosis of human breast cancer cell lines

Since cell apoptosis may be one of the consequences of cell-cycle arrest [25], we examined using flow cytometry whether salidroside induced apoptosis in MCF-7 and MDA-MB-231 cells. We stained the cells with Annexin V-FITC and PI, and we conducted internucleosomal DNA fragmentation assays. In addition, we evaluated the expression of apoptosis-related molecules by Western blot analysis.

Annexin V staining detects early apoptosis by binding to membrane phospholipid, the phosphatidylserines translocated from the inner to the outer leaflet of the plasma membrane during apoptosis [26]. As shown in Fig. 3A, treatment of MDA-MB-231 cells with 5-μM salidroside resulted in 25% of the cells categorized as apoptotic, treatment with 10-μM salidroside resulted in 57%, and no treatment for the control resulted in 3%. Not only did the early apoptotic cells increase to 13% and 19% for the 5- and 10-μM treatments, respectively, but the late apoptotic cells also increased significantly. Only 10-μM salidroside significantly affected apoptosis of MCF-7 cells, increasing the total apoptotic cells to 15% (Fig. 3A).

In search of further evidence of apoptosis, we conducted an anti-histone/DNA monoclonal antibody ELISA assay, which examined double-strand DNA breaks occurring at an early stage. As shown in Fig. 3B, a significant increase in nucleosome formation was found in both MCF-7 and MDA-MB-231 cells compared with

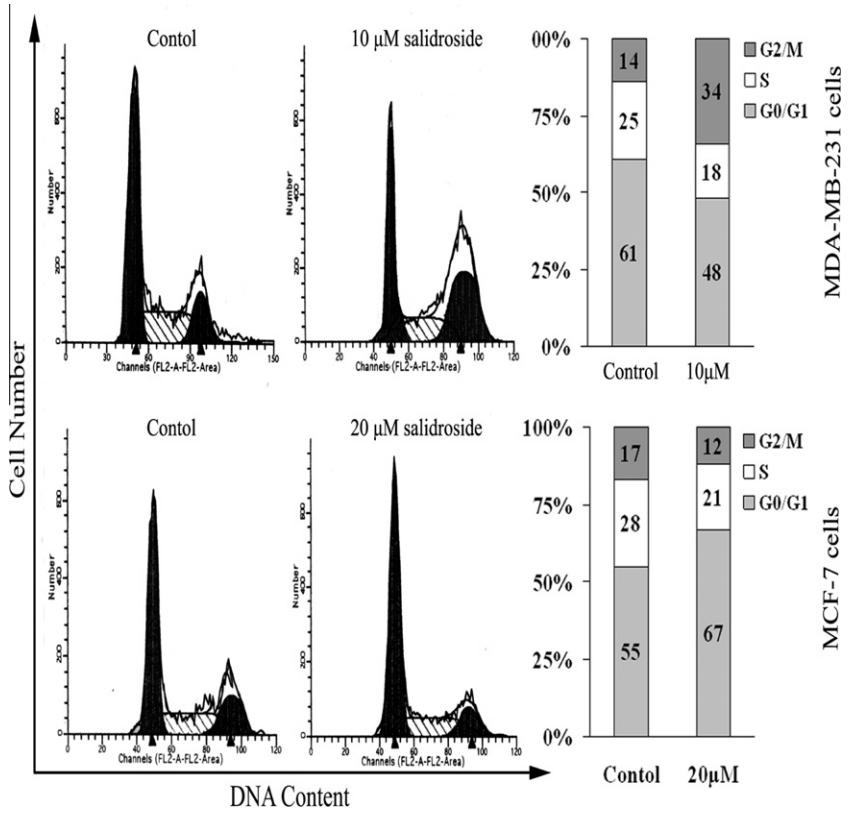

Fig. 2. Effect of salidroside on the cell cycle of MDA-MB-231 and MCF-7 cells. MDA-MB-231 cells were cultured without (control) or with 10-μM salidroside for 24 h, and MCF-7 cells were cultured without or with 20-μM salidroside for 24 h. The percentage of cells at each stage of the cell cycle was analyzed by a Becton–Dickinson flow cytometer after DNA staining with propidium iodide. These values were from a representative result of three independent experiments.

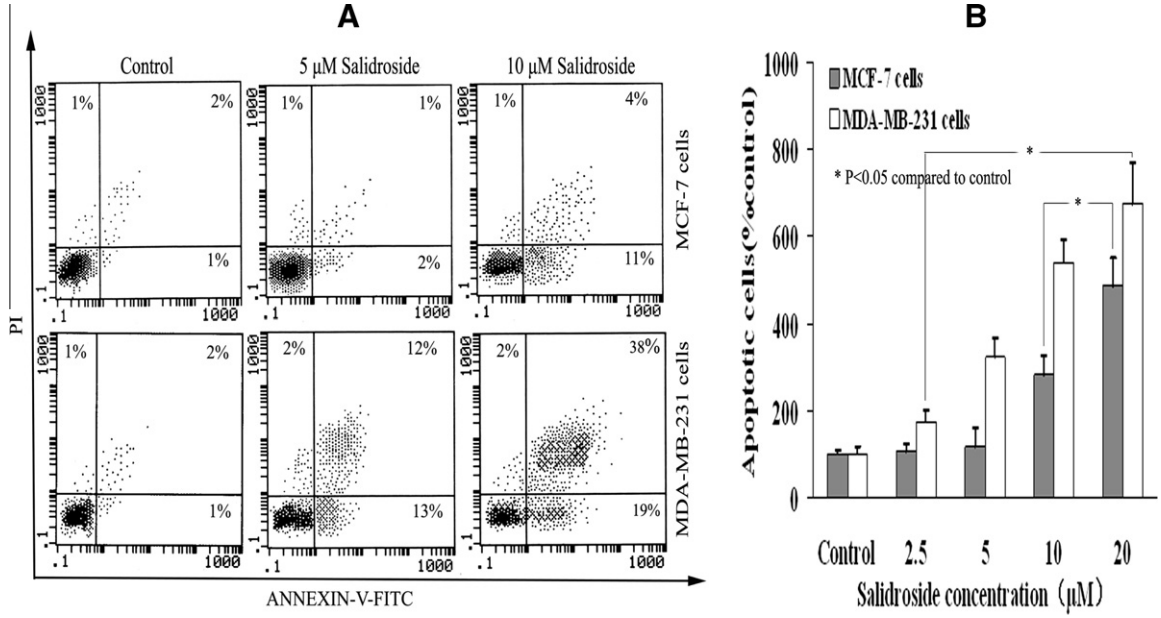

Fig. 3. Effect of salidroside on the apoptosis of MDA-MB-231 and MCF-7 cells. (A) Five- and 10-μM salidroside treatment MDA-MB-231 and MCF-7 cells after 48 h. All cells were stained with FITC-conjugated annexin V in a buffer containing propidium iodide and analyzed by flow cytometry. For each group of cells, the percentage of surviving cells is shown in the lower left quadrant. The lower right quadrant indicates the percentage of early apoptotic cells (Annexin V-positive cells). The percentage of late apoptotic cells is shown in the upper right quadrant (Annexin V- and PI-positive cells). The percentage of cells in each quadrant is shown. The results are representative of three independent experiments. (B) Cells were treated by salidroside (0–20 μM) for 24 h. Apoptosis was measured by an ELISA kit (Roche) that detects DNA histone fragmentation using the protocol provided by the manufacturer. Signal was assayed for absorption at 405 nm in the Ultra plate reader and expressed as a fold increase over untreated control cells. The percentages of each phase are represented as mean ± standard deviation from three independent experiments performed in duplicate. $^{*}p < 0.05$ compared to control using the Student–Newman–Keuls test.

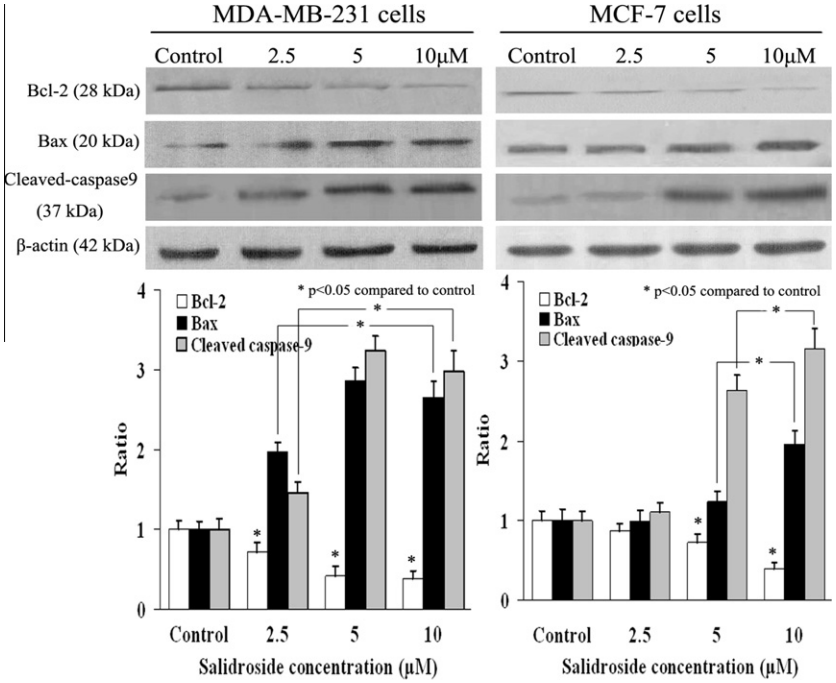

Fig. 4. Effect of salidroside on protein levels of Bcl-2, Bax and cleaved caspase 9 in MDA-MB-231 and MCF-7 cells. Cells were untreated (control) or treated with 2.5-, 5- and 10-μM salidroside for 24 h. The expression of Bcl-2, Bax and cleaved caspase 9 was investigated by Western blot. β-Actin was used to correct for protein loading. Figures show the representative blots from one of three experiments that gave similar results. Bars represent the mean ± standard deviation from three independent experiments. $^{*}p < 0.05$ compared to control using the Student–Newman–Keuls test.

the untreated controls, and the percentage of apoptotic cells was more prominent in the MDA-MB-231 cells than that in the MCF-7 cells. For example, after salidroside treatment of increasing concentrations (0–20 μM), the apoptotic percentages were 174.1%, 324.5%, 539.7% and 676.2% for MDA-MB-231 cells, and 108.3%, 119.4%, 285.2% and 487.5% for MCF-7 cells, compared with the baseline 100% of the control (Fig. 3B). These results suggested that salidroside increased the total percentage of apoptotic cells in both cell lines in a concentration-dependent manner ($p < 0.05$).

It is known that suppression of anti-apoptotic members or activation of pro-apoptotic members of the Bcl-2 family usually leads to an altered mitochondrial membrane permeability, which allows the release of cytochrome c into the cytosol and the subsequent activation of caspase 9 [27]. We further evaluated the expression of apoptosis-related proteins to analyze the underlying mechanisms of the above findings. As shown in Fig. 4, salidroside downregulated the expression of Bcl-2 and upregulated the expression of both Bax and cleaved caspase 9 in a concentration-dependent manner ($p < 0.05$). In MDA-MB-231 cells, Bax expression was significantly upregulated at a salidroside concentration of 2.5 μM, indicating that Bax may be an early response protein. These results suggested that salidroside induced apoptosis of MCF-7 and MDA-MB-231 cells by suppressing anti-apoptotic proteins. Together, these findings suggested that salidroside induced the apoptosis of MCF-7 and MDA-MB-231 cells by suppressing anti-apoptotic proteins and may play a role in the mitochondrial pathway.

4. Conclusions

In this study, we are the first to provide evidence that salidroside is highly effective in inhibiting cell proliferation and inducing apoptotic cell death in ER-negative and ER-positive breast cancer cells, and salidroside may be a promising candidate for breast cancer treatment. Therefore, it is very important to understand the molecular effects of salidroside and evaluate its efficacy and side effects in vivo.

Acknowledgments

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