Salidroside induces apoptosis and autophagy in human colorectal cancer cells through inhibition of PI3K/Akt/mTOR pathway

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Abstract. The role of salidroside in colon cancer remains unknown. Here we show that salidroside, a phenylpropanoid glycoside extracted from Rhodiola rosea, exhibited potent anti-proliferative properties in human colorectal cancer cells via inducing apoptosis and autophagy. We ascertained that salidroside exerts an inhibitory effect on the proliferation of human colorectal cancer cells in a dose-dependent manner. In addition, salidroside induced cell apoptosis, accompanied by an increase of chromatin condensation and nuclear fragmentation, and a decrease of Bcl-2/Bax protein expression ratio. We also found that salidroside induced autophagy, evidenced by increased LC3+ autophagic vacuoles, positive acridine orange-stained cells, enhanced conversion of LC3-I to LC3-II, and elevation of Beclin-1. Treatment with autophagy-specific inhibitors [3-methyladenine (3-MA) and bafilomycin A1 (BA)] enhanced salidroside-induced apoptosis, indicating that salidroside-mediated autophagy may protect HT29 cells from undergoing apoptotic cell death. Additionally, salidroside decreased the phosphorylation of PI3K, Akt and mTOR. Treatment with PI3K inhibitor LY294002 augmented the effects of salidroside on the expression of Akt and mTOR. These findings indicate that salidroside could suppress the PI3K/Akt/mTOR signaling pathways. This study may provide a rationale for future clinical application using salidroside as a chemotherapeutic agent for human colorectal cancer.

Introduction

Colorectal cancer (CRC) is the most common digestive malignant and devastating primary tumor. Based on global estimates, it is the third most commonly diagnosed cancer in males and the second in females (1). Despite early diagnosis and treatment such as surgery and chemotherapy, colon cancer can reappear at a later time, even if the cancer was entirely removed during the initial treatment. Therefore, the current challenge is to identify new effective less toxic chemotherapeutic agents that are need in treatment of colon cancer.

Targeting programmed cell death (PCD) has become a promising approach in the fight against cancer, which mainly includes modulation of apoptosis and autophagy (2,3). Type I PCD, apoptosis, is a biological process with a crucial role in normal development and tissue homeostasis (4). Type II PCD, autophagic cell death, is a highly conserved cellular degradation process characterized by the presence of abundant intracellular autophagic vacuoles termed autophagosomes. Autophagosomes participate in the recycling of cellular components by sequestering damaged organelles and misfolded proteins, targeting them for lysosomal degradation (5-7). Apoptosis and autophagy are two distinct processes, coordinately regulating cell survival and cell death, and occur simultaneously in cancers (8,9). Accumulated evidence has shown that apoptosis and autophagy is a response to various anticancer therapies in many kinds of cancer cells (10-12). In addition, the apoptosis and autophagy mechanisms are involved in CRC and play an important role in the multifactorial etiology of CRC (13). So the modulation of apoptosis and autophagy might be applied in a potential cancer therapy for the treatment of colon cancer cells.

Rhodiola rosea L, also known as ‘golden root’, is a perennial herbaceous plant of the Crassulaceae family, widely distributed at high-altitudes regions (14). It has long been used as adaptogen traditional Chinese medicine (15). Reports on the anticancer effect of Rhodiola extracts have been published (16,17). Salidroside, a major component of Rhodiola rosea, has been reported to have significant antitumor effects, such as inhibiting cell proliferation, arresting cell cycle, and promoting apoptosis of the human bladder, breast, lung or liver cancer cells (16,18-21). The existing evidence indicates that salidroside plays antitumor role by inhibiting tumor metastasis, reducing new angiogenesis and changing the tumor microenvironment (22-24). In addition, it is also reported that it can inhibit proliferation, decrease the migration and invasion of colon carcinoma SW1116 cells in JAK2/STAT3-dependent pathway (25). However, the relative molecular mechanisms still need to be studied.

Studies have found that salidroside could decrease the growth of bladder cancer cell lines via inhibition of the mTOR pathway and induction of autophagy (16). In addition,
mTOR has emerged as an effective target for colorectal cancer therapy (26). Increasing evidence demonstrates that PI3K/Akt/mTOR signaling plays a key role in regulation of apoptosis and autophagy, and targeting PI3K/Akt/mTOR signaling has been proposed to be a promising strategy for cancer treatment (27-29). Thus, this study aimed to investigate whether salidroside modulates apoptosis and autophagy in HT29 human colon cancer cells and to further elucidate the role of the PI3K/Akt/mTOR signaling in regulation of cell death.

Materials and methods

Materials. Salidroside (purity >99%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). RPMI-1640 was purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Sijiqing (Hangzhou, China). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, trypsin, Acridine orange (AO), LY294002, 3-methyladenine, Bafilomycin A1, Hoechst 33342, antibodies for the detection of LC3 (#L7543) and Beclin-1 (#B6186) was purchased from Sigma (St. Louis, MO, USA). Bcl-2 (#15071), Bax (#2772), PI3K (#4292), p-PI3K at Tyr458 (#4228), Akt (#9272), p-Akt at Ser473 (#9271), mTOR (#2972), p-mTOR at Ser2448 (#2971) and GAPDH (#5174) were purchased from Cell Signaling Technology (Beverly, MA, USA). HRP-labeled goat anti-rat IgG(H+L) (#A0562), FITC-labeled goat anti-rabbit IgG (H+L) (#A0192), HRP-labeled goat anti-rabbit IgG(H+L) (#A0208), FITC-labeled goat anti-rabbit IgG (H+L) (#A0562) were purchased from Beyotime Institution of Biotechnology (Haimen, China).

Cell culture. The human colon cancer HT-29 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). HT-29 cells were cultured in RPMI-1640 containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and were kept at 37°C in a humidified atmosphere composed of 5% CO2 and 95% air. Salidroside was diluted in cell culture medium and regulated to final concentrations of 0.5, 1 and 2 mM, and cultured for the indicated time periods. To investigate the mechanisms for salidroside-induced apoptosis and autophagy, cells were pretreated with the 10 μM LY294002 (a PI3K inhibitor), 10 nM BA (an autophagy-lysosomal inhibitor) or 10 mM of 3-MA (an autophagy inhibitor) for 30 min, then co-treated with 2 mM salidroside for further 48 h. Cells were treated with fresh medium as vehicle control.

Cell proliferation assay. Cell proliferation was assayed by MTT. Briefly, the cells were seeded in 96-well plates at a density of 1x10^4 cells/well. After treatment, 0.5 mg/ml MTT was added to each well and the plates were incubated for another 4 h at 37°C. The formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance was determined at 550 nm on an EIX-800 MicroElisa reader (Bio-Tek Inc., Winooski, VT, USA). The cell viability were expressed as a percentage of the controls.

Hoechst 33342 staining. To quantify and assess nuclear morphology, HT29 cells were cultured on 24-well culture plates. After treatment, cells were fixed for 20 min with 4% paraformaldehyde in PBS at room temperature. After staining for 10 min with 10 μg/ml Hoechst 33342, the cells were visualized and photographed under a DMR fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with fluorescence excitation at 340 nm and emission at 510 nm. The apoptotic index was calculated as: [apoptotic cells number / total cells number] x 100 (%). At least four different fields from each well were selected to count ≥500 cells to calculate the rate of apoptosis.

Immunofluorescence analysis of LC3 distribution. Cells (1x10^5 cells/cm^2 in 24-well plates) were fixed in 4% paraformaldehyde for 30 min at room temperature. Subsequently, the cells were permeabilized with 0.5% Triton X-100 and blocked with 1% bovine serum albumin in PBS for 1 h, followed by incubation in anti-LC3 antibody (1:100) overnight at 4°C, washed and incubated with FITC-labeled goat anti-rabbit IgG (H+L) (1:500) for 2 h at 37°C, rinsed with PBS, and counter-stained with Hoechst 33342 for 10 min. Images were obtained using a fluorescence microscope (488-nm filter; Olympus BX51, Japan).

Acridine orange staining. Acridine orange staining was used to detect autophagy induction. After seeding, HT-29 cells were washed with phosphate-buffered saline (PBS), stained with 1 μg/ml acridine orange for 15 min at 37°C. Photographs were obtained with a fluorescence microscope (Axioscop, Carl Zeiss, Thomswood, NY, USA) equipped with a mercury 100-W lamp, 490-nm band-pass blue excitation filters, a 500-nm dichroic mirror and a 515-nm long-pass barrier filter. Autophagic lysosomes appeared as orange/red fluorescent cytoplasmic vesicles according to their acidity, while the nuclei were stained green.

Western blot assay. Proteins (35 μg/sample) were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% non-fat milk for 1 h and incubated with the following antibodies: LC3, Beclin-1, Bcl-2, Bax, PI3K, p-PI3K at Tyr458, Akt, p-Akt at Ser473, mTOR, p-mTOR at Ser2448 and GAPDH at 1:1,000 overnight at 4°C. The membranes were washed with TBS/T (TBS with 0.05% Tween-20) and then incubated with HRP-labeled goat anti-rabbit IgG(H+L) (1:3,000) or HRP-labeled goat anti-rabbit IgG(H+L) (1:1,000) at room temperature for 1 h. The reaction was visualized using ECL and detected using a Luminescent Image Analyzer LAS-4000 mini. The images were quantified with Multi Gauge. For each sample, band intensities were normalized to GAPDH.

Statistical analysis. Statistical differences were evaluated by GraphPad Prism 5.0 (San Diego, CA, USA). Statistical significance was determined by one-way analysis of variance (ANOVA) and subsequent Tukey’s test. Differences were considered significant at p<0.05. The data are expressed as mean ± SD of three independent experiments.

Results

Salidroside inhibits growth and induces apoptosis in HT-29 colon cancer cells. HT-29 colon cancer cells were treated in indicated time periods with various concentrations of
salidroside to investigate the cytotoxic activity of salidroside against these cells. Cell viability was then assessed using MTT assay. MTT assay revealed a dose-dependent and time-dependent cytotoxicity of salidroside on these cells (Fig. 1A). Compared with control group, treatment with salidroside (0.5, 1 and 2 mM) for 48 h significantly inhibited cell viability to 86.17±6.28, 71.38±6.48 and 67.65±6.39%, respectively, and 0.5, 1 and 2 mM salidroside treatment for 72 h inhibited cell viability to 59.81±5.94, 52.23±4.86 and 44.12±3.71%, respectively. However, treatment with salidroside for 24 h had

Figure 1. The ability of salidroside to induce apoptosis in HT29 colon cancer cells. (A) The HT29 cells were treated with different concentration of salidroside and incubated for different times. Cell viabilities were determined by MTT assay. (B) Hoechst 33342 staining was used to detect the morphological features of apoptosis in HT29 cells (condensed chromatin and fragmented nuclei). Arrowheads indicate apoptotic cells. (C) The apoptosis index was calculated. (D) The expression levels of Bcl-2 and Bax were analyzed by western blotting. Anti-GAPDH antibody was used for normalization. (E) The intensity of bands was quantified by densitometric analysis. All data are expressed as mean ± SD from three independent experiments. **p<0.01 and ***p<0.001 vs. control group.
Figure 2. Salidroside induces the formation of autophagic vacuoles. Treating the cells with salidroside for 48 h prominently enhanced the formation of autophagic vacuoles as determined by immunofluorescent staining for LC3.

Figure 3. Salidroside induces the accumulation of acidic vesicular organelles. Cells were treated with 1 and 2 mM salidroside for 48 h and stained with acridine orange. Green and red fluorescence in acridine orange-stained cells were observed under a fluorescence microscope.
no inhibitory effect. In subsequent experiments, 1 and 2 mM salidroside treatment for 48 h was used to observe the effects of salidroside on HT-29 colon cancer cells.

The nuclear Hoechst 33342 staining assay was used to detect apoptosis of HT29 cells. Pretreatment with 1 and 2 mM salidroside for 48 h displayed typical morphological features of apoptosis including chromatin condensation, nuclear shrinkage, and the formation of a few apoptotic bodies, and the percentage of nuclear condensation increased to 37.9±3.7 and 50.4±4.2% from 7.1±3.4%, compared with the control group (Fig. 1B and C).

Western blot analysis was used to evaluate the expression of Bcl-2 and Bax. A decreased ratio of Bcl-2/Bax was found after salidroside treatment (Fig. 1D and E). These results revealed that salidroside could induce apoptosis in HT-29 colon cancer cells.

**Salidroside induces autophagy in HT-29 colon cancer cells.** To determine whether salidroside induces autophagy in colon cancer cells, we used immunofluorescence to examine the intracellular distribution of LC3, an autophagy marker (30). Results showed that control HT29 colon cancer cells exhibited weak and diffuse cytoplasmic staining with LC3-associated green fluorescence, whereas those treated with salidroside exhibited an increase in LC3 staining intensity, which is a typical feature of LC3 distribution within autophagosomes (LC3-II) (Fig. 2).

To further determine the effect of salidroside on autophagy, we analyzed the accumulation of acidic vesicular organelles. Vital staining of HT-29 colon cancer cells with acridine orange revealed the appearance of acidic vesicular organelles with bright red fluorescence after salidroside treatment (Fig. 3). Conversely, the majority of control cells exhibited only minimal red fluorescence.

**Salidroside inhibits the activation of PI3K/Akt/mTOR signaling pathway.** Since we have observed that salidroside could induce apoptosis and autophagy in HT29 colon cancer cells, we further investigated the possible mechanisms. The PI3K/Akt/mTOR signaling pathway is a key pathway related to cell survival/death (9,32), then we investigated if this pathway plays a central role in salidroside-mediated cell death. As shown in Fig. 6A and B, salidroside treatment causes significant decrease in the phosphorylation levels of PI3K, Akt, and mTOR, and there was no change observed in the total PI3K, Akt, and mTOR protein level. There was a 16.9, 33.2

![Figure 4. Effects of salidroside on the protein levels of LC3 and Beclin-1 in HT29 cells. (A) The expression levels of LC3 and Beclin-1 were analyzed by western blotting. Anti-GAPDH antibody was used for normalization. (B) The intensity of bands was quantified by densitometric analysis. All values represent mean ± SD of three independent experiments. *p<0.05, **p<0.01 vs. control.](image-url)
and 36.2% decline in the ratio of p-PI3K over PI3K, 19.6, 21.3 and 31.2% decrease in the ratio of p-Akt over Akt, and 30.5, 34.7 and 46.3% reduction in the ratio of p-mTOR over mTOR in HT29 colon cancer cells when treated with salidroside at 0.5, 1 and 2 mM, respectively. In addition, treatment of HT29 cells with 10 µM LY294002 plus 2 mM salidroside decreased
the ratio of p-Akt/Akt and p-mTOR/mTOR more than treatment with salidroside alone (Fig. 6C and D). Thus, these data suggested that the salidroside inhibited the activation of PI3K/Akt/mTOR signaling pathway.

**Discussion**

In our research, we found that salidroside inhibited the growth of HT-29 human colorectal cancer cells concentration- and time-dependently, which is consistent with the results that salidroside inhibited proliferation, decreased the migration and invasion of SW1116 cells (25). However, whether the anticancer effect of salidroside is related with apoptosis and autophagy have not been elucidated.

Apoptosis is an active process in which apoptotic cells undergo chromatin condensation and fragmentation followed by the formation of apoptotic bodies (33). Several genes have been shown to regulate apoptosis. The proteins of the Bcl-2 family represent a critical checkpoint in major apoptotic signal transduction cascades (34). In addition, apoptotic cell death is typically determined by the ratio of Bcl-2/Bax (35-37). In this study, we found that salidroside induced cell apoptosis, accompanied by an increase of chromatin condensation and nuclear fragmentation, and a decrease of Bcl-2/Bax expression ratio. We thus examined whether salidroside induced autophagy.

Autophagy in cancer has begun to be investigated and has been suggested as a novel potential target for improved anticancer therapies (38-40). Autophagy is characterized by engulfment of cytoplasm and organelles into double-membrane bound structures, autophagosomes, and delivery to and subsequent degradation in lysosomes (41). Autophagy allows degradation of the cytoplasmic contents under certain stress conditions such as oxidative stress, nutrient starvation, misfolded protein accumulation, and irradiation is a temporary survival mechanism. Recent studies shown that autophagy is needed for cancer survival and tumorigenesis (42,43). Autophagy can serve as a mechanism of self-defense by recycling essential molecules, and by contributing to therapy resistance (44-46). Conversely, autophagy can inhibit tumor progression (45). Extensive autophagy can also result in destruction of vital cell constituents, committing the cell to death (47). LC3 and ATG12, two ubiquitin-like protein
systems, play an important role for the autophagosomal membrane formation and expansion (48). LC3 consists of two forms, LC3-I and its cleavage form, LC3-II. The conversion of soluble LC3-I to the membrane bound LC3 II is considered as one of the makers of autophagy induction in the cells. Detecting LC3-II by immunoblotting or immunofluorescence systems, play an important role for the autophagosomes formation during autophagy (50). Autophagy is characterized morphologically by the formation of LC3+ autophagic vacuoles and accumulation acidic vesicular organelles (30,51). Our data showed that salidroside treatment increased the formation of LC3+ autophagic vacuoles and the accumulation of acidic vesicular organelles. Western blot analysis found that salidroside remarkably increased the ratio of LC3-II/LC3-I and Beclin-1 protein expression in a dose-dependent manner. It is obviously that salidroside induced autophagy in HT29 colon cancer cells.

3-MA is a popular inhibitor of the autophagic agent. It has been reported to inhibit the activity of PI3-kinase (a kinase that is essential for vesicle nucleation, the first phase of autophagosome formation) and blocks the formation of preautophagosome, autophagosome, and autophagic vacuoles (52). BA, a known inhibitor of the late phase of autophagy, prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes (53). Addition of 3-MA or BA attenuated the formation of LC3+ autophagic vacuoles and inhibited the increase of LC3-II/I ratio and Beclin-1 protein expression induced by salidroside in HT29 colon cancer cells. Moreover, pre-treatment with 3-MA or BA augmented the inhibitory effects of salidroside on the expression of Bcl-2/Bax ratio and the cell viability. Collectively, these results indicated that inhibition of autophagy decreased cell viability and increased apoptosis, which revealed that autophagy provided a protective mechanism against salidroside-induced apoptosis.

Increasing evidence suggests that cross-talk between apoptosis and autophagy is made especially complicated by the fact that they share many common regulatory molecules, such as Bcl-2 and the PI3K/Akt/mTOR signaling pathway (33,54). PI3K activates the downstream serine/threonine kinase Akt, which in turn, through a cascade of regulators, triggers the phosphorylation and activation of the serine/threonine kinase mTOR (55). PI3K/Akt/mTOR, a major intracellular signaling pathway, has received much attention in recent years given its role in cell proliferation, growth, protein synthesis, motility, and other functions of pro-tumorigenic impact (57). Inhibition of PI3K/Akt/mTOR signaling pathway causes cell death associated with apoptosis and/or autophagy (58,59). As shown by our western blot analysis, salidroside significantly decreased the activation of PI3K, Akt, and mTOR in HT-29 human colorectal cancer cells. In addition, PI3K inhibitor LY294002 could further inhibit the activation of Akt and mTOR induced by salidroside treatment. Thus, the inhibition of the PI3K/Akt/mTOR signaling pathway contributes, at least in part, to the apoptosis-inducing and autophagy-inducing effect of salidroside in HT-29 human colorectal cancer cells.

In this study, we demonstrated the cell growth inhibitory effect of salidroside on HT-29 human colorectal cancer cells. We elucidated the underlying mechanism that involves cross-talk between apoptosis and autophagy via inhibition of PI3K/Akt/mTOR signaling pathways. In conclusion, we have provided a basis for molecular mechanism of salidroside in colon cancer treatment. The potential application of salidroside in inhibiting colon cancer cell proliferation makes it an attractive agent for colorectal cancer research, and possibly treatment.

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