RESEARCH ARTICLE

Hyperin Extracted from Manchurian Rhododendron Leaf Induces Apoptosis in Human Endometrial Cancer Cells Through a Mitochondrial Pathway

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Abstract

Background: A number of effective prevention measures have been introduced in attempts to substantially reduce both the incidence and mortality due to many kinds of cancer. The search for new anti-cancer compounds in foods or in plant medicines is one realistic and promising approach to prevention. Chinese medicines provide a rich pool of novel and efficacious agents for cancer prevention and treatment. Previously it was demonstratrated that hyperin extracted from the Manchurian rhododendron leaf reduces the proliferation of many cancer cells. The present study was carried out to evaluate its effects on human endometrial cancer cell viability and apoptosis and to investigate its mechanisms of action in RL952 cells. Methods: Cell viability was measured using the MTT assay. Intracellular calcium ions were detected using laser-scanning confocal microscopy. The effects of hyperin on apoptosis related proteins in RL952 cells were examined using Western blot analysis. Results: The growth of RL952 cells was inhibited by treatment with hyperin. OD values of caspase-3 and caspase-9 were increased and expression of bcl-2 was increased and bax was decreased in protein levels in RL952 cells after 24 h of hyperin treatment, Moreover, intracellular calcium accumulation occurred in hyperin-treated cells. Conclusions: These results suggest that hyperin may play an important role in tumor growth suppression by inducing apoptosis in human endometrial cells via a Ca^{2+}-related mitochondrion apoptotic pathway in RL952 cells.

Keywords: Hyperin - endometrial cancer cells - apoptosis - Ca^{2+}

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Introduction

Cancer is the second leading cause of death in industrialized countries, and as common gynecologic malignant tumors, endometrial cancer is the second most deadly cancer among women in the world. Cyclophosphamide, methotrexate, and 5-fluorouracile (termed CMF regimen) as well as anthracyclines and paclitaxel belong to the chemotherapeutical drugs for cancer. However, the development of drug resistance and severe side effects of standard anticancer drugs necessitates the search for novel treatment options for this disease. The discovery of new natural and synthetic products for cancer treatment is of great urgency to improve prospects of affected women for cure from their disease (Narendra et al., 2010).

A wide variety of biological activities from medicinal plants have recently been reported, in addition to their traditional medicinal effects. Herbal medicines have attracted considerable interest as alternative cancer remedies because of their low toxicity and costs. Endometrial cancer rates are continuously increasing in Asia due to persistent high incidences of obesity, diabetes and hypertension, which are the three co-existed in patients with endometrial cancer, correlated to high fat diet. In China, As flavonols glycoside compounds, hyperin has been extensively used for clinical treatment of anti-oxidation and analgesia, but it is not clear whether it have anti- tumor effect as other flavonols. Our previously study demonstrated in our laboratory that flavonols from seeds of Apium graveolens showed antiproliferative activities in the human carcinoma cell lines BGC-823 (Gao et al., 2011). Therefore, the present study was carried out to evaluate the effects of hyperin on human endometrial cancer cell line RL952 cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and to investigate the effects of hyperin on cell proliferation, intracellular calcium and apoptosis regulatory proteins (Figure 1).

Materials and Methods

Chemicals and reagents

RPMI-1640 medium, HEPES (4-hydroxyethyl piperazine ethanesulfonic acid), fetal calf serum and trypsogen were purchased from GIBCO (Canada). 3-(4,

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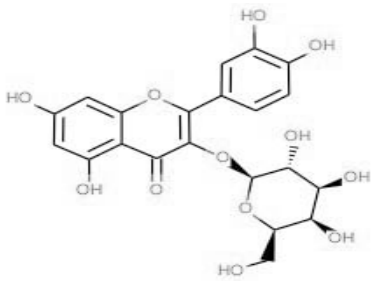

Figure 1. Structure of Hyperin

5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT), penicillin, streptomycin, bovine serum albumin and trypsin were purchased from Amresco Chemical Co. Ltd. (USA). MTT and Fluo 3/AM were purchased from Sigma Chemical Co. (St. Louis, MO) and all chemicals were of analytical grade and were obtained from Tianjin Chemical Reagents Co. Ltd. (Tianjin, China).

Preparation of hyperin

Hyperin was made and purified from Manchurian rhododendron leaf according to the established methods (Li and Chen, 2005) with slight modifications. Hyperin with 99% or higher purity was used in all experiments. Hyperin was dissolved in dimethyl sulfoxide (DMSO) as a stock solution, stored at -20 °C, and diluted with medium before experiments. The final DMSO concentration did not exceed 0.1% throughout the study. The control groups were treated with 0.1% DMSO in the corresponding experiments.

Cell culture

RL952 cells were obtained from the Chinese Type Culture Collection (Shanghai Institute of Cell Biology, Chinese Academy of Science, Shanghai, China), cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, the medium was changed every other day. When the cultures were 80 to 90% confluent, the cells were washed with phosphate-buffered saline (PBS, pH 7.4), detached with 0.25% trypsin, centrifuged and re-plated onto 96- or 24-well plates at an appropriate density according to each experimental scale.

Cell viability and cytotoxicity

The cultured cells at the exponential growth phase were harvested from the culture flasks by trypsin and then resuspended in fresh medium. The cell suspensions were dispensed into a 96-well microplate at 100 μl/well and incubated in an incubator with 5% CO_2 at 37 °C. After 24 h, 200 μl of various concentrations (0 to 500 μM) of hyperin were added and incubated for 24, 48, and 72 h to evaluate their anti-proliferation effects on RL952. The cell proliferation in the microplate was determined using the MTT (3-(4, 5-dimethylthiazol-2-yle) 2, 5-diphenyl-tetraloziumbromide) assay after incubation (Chang et al., 2008). Twenty microliters of PBS solution containing 5 mg/ml MTT was added to each well. After incubation for 4 h, the cells from each well were solubilized with 100 μl DMSO for optical density determination at 570 nm. Cell proliferation activity was expressed as the percentage of MTT counts of treated cells relative to those of the control (% of control). The percentage of cell growth inhibition was calculated as follows:

Inhibition (%)=[A570(control)–A570(drug)]/A570 (control)×100%

Measurement of intracellular calcium

After confluence, RL952 cells on a coverslip were loaded by the $[Ca^{2+}]_i$ indicator Fluo-3/AM in HEPES solution at 37 °C in the dark for 30 min. HEPES solution contains (concentration in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, HEPES 5, and glucose 10. The pH was brought to 7.4 with NaOH. The final concentration of Fluo-3/AM was 5 μM. After loading with Fluo-3/AM, a fluorescence image of $[Ca^{2+}]_i$ was taken using a laser-scanning confocal microscope (Bio-Rad Radiance2100, U.S.A) at 600×, and qualitative changes of $[Ca^{2+}]$i were inferred from the fluorescence intensity using SimplePCI Imaging Systems (SimplePCI, Compix Inc., USA).

Measurement of caspase-3 and caspase-9 activities

The activation of caspase-3 and caspase-9 were determined with the colorimetric kit (Nanjing kaiji Bio-Tek Corporation, China). RL952 cells (1×10^6 cells/mL) were harvested and washed once with PBS. After the RL952 cells were lysed, reaction buffer was added to the RL952 cells followed by the additional 5μL of caspase-3 or caspase-9 colorimetric substrate (DEVD-pNA) and incubated in a 96-well plate for 4 h at 37 °C in a CO_2 incubator. The plate was then read with a microplate reader at 405 nm. Activities of caspase-3 and caspase-9 were expressed relative to theoretical density value (OD).

Western blot analysis

The 20 μg of protein in each 20-μl sample was electrophoresed through 10% SDS-PAGE gels as previously described (Rasmussen et al., 2008). Separated proteins were incubated with primary antibodies overnight at 4 °C, transferred to nitrocellulose membranes, and blocked with a 5% skim milk solution. They were incubated with secondary antibodies for 1 h at 37 °C. Each antigen-antibody complex was visualized by enhanced chemiluminescense (ECL) western blotting detection kits (Amersham Pharmacia Biotech, Piscataway, NJ), and band densities were determined using Chemi Doc Software (BioRad); β-Actin was used as a loading for normalization.

Statistical analysis

All experiments were repeated three times. The results of multiple experiments are given as the mean ± SE. Statistical analysis was performed using the statistical software package SPSS 13.0 (SPSS). A p-value of 0.05 (two-sided) was considered statistically significant.

Results

Cytotoxicity assays

The anti-proliferative activity of hyperin on RL952

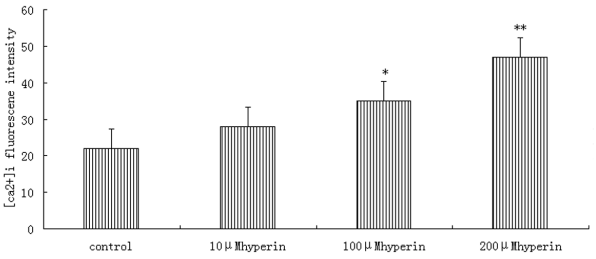

Figure 2. Effects of Hyperin on Intracellular [Ca^{2+}] in RL952 Cells. Qualitative changes of $[Ca^{2+}]_i$ were inferred from the fluorescence intensity after hyperin treatment for 24 h, using SimplePCI Imaging Systems. Data are presented as mean ± SD (error bar). * P<0.05 vs. control and **P<0.01 vs. control

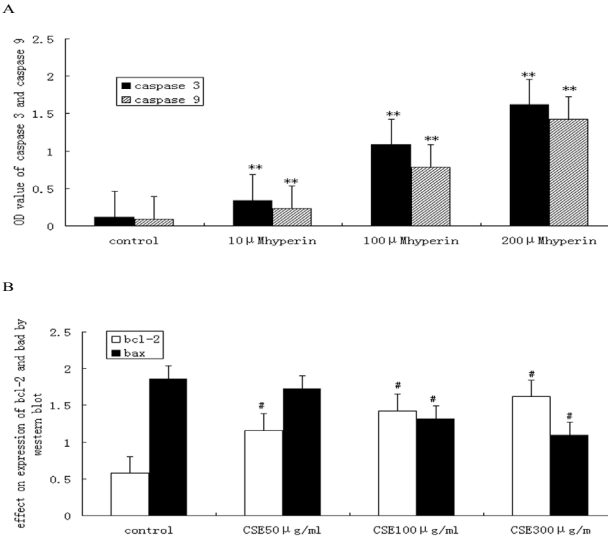

Figure 3. Hyperin Induced Apoptosis in RL952 Cells After 24h Following the Treatment. (A) OD value of caspase 3 and caspase 9 with the colorimetric kit. a: vehicle treated; b: 10 μM; c: 100 μM; d: 200 μM. (B) Western blot analysis of the expressions of the level of bcl-2 and bax protein. #P<0.01 vs. control

cells was determined by using the MTT reduction assay. The effects of 0-500μM hyperin on viability of RL952 cell lines after exposure for 24, 48 and 72 h. The IC50 value was 38.67 μM after 72 h treatment, 47.82 μM after 48 h, and 69.14 μM after 24 h. Hyperin inhibited the growth of RL952 cells in a time- and dose-dependent manner.

Effects of hyperin on $[Ca^{2+}]_i$ in RL952 cells

To explore whether hyperin-induced apoptosis involved $[Ca^{2+}]_i$, we used the $[Ca^{2+}]_i$ indicator Fluo-3/AM to detect $[Ca^{2+}]_i$ changes after hyperin treatment with various densities. As shown in Figure 2, $[Ca^{2+}]_i$ fluorescence intensity in the group treated with 2000 μM hyperin was brighter than were the vehicle treated and lower concentration groups (P<0.01), and hyperin treatment with 10, 100 and 200 μM induced an increase by 38±2.63%, 54±4.27%, and 72±7.51% vs. the vehicle treated (22.33±2.04%) (P < 0.01, n=4) in Fluo-3/AM fluorescence intensity after 24 h of treatment, respectively. These results suggest that hyperin can induce a dose-dependent $[Ca^{2+}]_i$ influx and might induce apoptosis or necrosis that follows via calcium ion overload.

Effect of hyperin on apoptosis in RL952 cells

Caspases are important regulators of apoptosis. Therefore, we investigated the involvement of caspase-3 and caspase-9 in hyperin -induced apoptosis. In the untreated RL952 cells, OD value of caspase-3 was 0.025 ± 0.004, and the OD value of caspase-9 was 0.016 ± 0.004 (Figure 3A). After treatment with hyperin (10–100 μM) for 48 h, a dose-dependent increase of caspase-3 and caspase-9 activities was observed. The highest activities of caspase-3 and caspase-9 were found upon exposure to 200 μM hyperin. The OD values were 0.214± 0.011 and 0.167 ± 0.034, respectively, and were significantly higher than those in the control group.

To determine whether apoptosis induced by hyperin was due to a caspase pathway, we next investigated the levels of bcl-2 and bax, which was the core protein in the caspase cascade of RL952 cells after hyperin treatment for 24 h. Figure 3B shows the expression of bax was increased and bcl-2 was decreased after hyperin treatment for 24 h compared to the vehicle treated, which indicated that hyperin indeced the apoptosis process in RL952 cells.

Discussion

There have been reports of flavonoids inducing apoptosis in cancer cells (Wang et al., 1999; Park et al., 2008). The results of the present study clearly demonstrate that hyperin suppressed RL952 cell viability through inducing apoptosis.

Many flavonoids possess antitumor activity towards various human cancer cell lines and xenograft systems of human tumors, suggesting that they may be promising candidates for novel anticancer agents (Middleton, et al., 2000; Nijveldt et al., 2001). In addition, it was shown that the levels of $[Ca^{2+}]_i$ in RL952 cells increased significantly when the cells were cultured with hyperin at 100-200 μM (Figure 2).

Ca^{2+} overload has even been suggested to be the final common pathway for all types of cell death. Over the last few years, several studies have shown that increases of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) occur, both at early and late stages of the apoptotic pathway (Martikainen et al. , 1991; Kruman et al., 1998)

More specifically, it has been suggested that both Ca^{2+} release from the endoplasmic reticulum (ER) and capacitive Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+} channels are apoptogenic (Zirpel et al., 1998; Tombal et al., 1999). There are also data suggesting that very high intracellular Ca^{2+} levels can promote cell death through necrosis, whereas lower intracellular Ca^{2+} increases induced by milder insults promote cell death through apoptosis (Choi, 1995; Sara Leo et al., 2005). In this study, the $[Ca^{2+}]_i$ fluorescence intensity of cells loaded with Fluo-3/AM under a fluorescence microscope in the group treated with 200 μM hyperin was obviously brighter than were the control and lower concentration groups.

Commonly known, apoptosis is a highly regulated death process by which cells undergo inducible non-necrotic cellular suicide. It plays an important role in anti-carcinogenesis (Kaufmann, 2001). Data obtained has been reported to be one of the earliest intracellular events of apoptosis (Desagher and Martinou, 2001; Han et al., 2006). These results suggested that hyperin -induced intracellular

calcium ion plays an important role in eliciting early signals for triggering apoptosis. Decreased mitochondrial membrane potential regulates mitochondrial permeability transition pore (MPT) opening (Korge, 2001), and it is associated with cytochrome c release (Bustamante, 2004). High Bax/Bcl-2 ratio also resulted in cytochrome c release and apoptosis (Yang, 1997). In this study, we found that hyperin increased Bax/Bcl-2 ratio, which could explain hyperin -induce intracellular calcium ion release, and resulting in the activation of caspase-9. Caspase-9 activates the effector pro-caspases, including pro-caspase-3, an effector caspase of apoptosis. We next investigated the activity of caspase-3, which is considered to play a central role in many types of stimulus-induced apoptosis (Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997). Western blot showed that expression level of Bcl-2 was decreased,while that of Bax was increased and significantly decrease the Bax/Bcl-2 ratio. These results suggest that FAC could activate caspase-8 via the mitochondria-dependent pathway, and caspase-8 could then activate the downstream effector caspase-3, which in turn cleaves cytoskeletal and nuclear proteins, finally inducing apoptosis.

We characterized the mechanisms by which hyperin exerts its inhibitory effects on RL952 cells by Ca^{2+}-apoptosis. Our results show that hyperin demonstrates significant cytotoxicity in RL952 cells. The cytotoxic mechanisms of hyperin relate to its effects on apoptosis and Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+}channels. Hyperin could induce apoptosis through mitochondria-dependent and death receptor-dependent apoptotic pathways. These results suggest that hyperin deserves further study as a potential anti-cancer drug.

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