Involvement of the Mitochondrion-dependent and the Endoplasmic Reticulum Stress-signaling Pathways in Isoliquiritigenin-induced Apoptosis of HeLa Cell

YUAN Xuan1, ZHANG Bo3, GAN Lu4, WANG Zhen Hua4, YU Ba Cui3, LIU Liang Liang3, ZHENG Qiu Sheng2,*, and WANG Zhi Ping1,###

1. Lanzhou University Second Hospital, Lanzhou University, Lanzhou 730000, Gansu, China; 2. Life Science School, Yantai University, Yantai 264000, Shandong, China; 3. Key Laboratory of Xinjiang Endemic Phytomedicine Resources, Ministry of Education, School of Pharmacy, Shihezi University, Shihezi 832002, Xinjiang, China; 4. Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou 730000, Gansu, China

Abstract

Objective Isoliquiritigenin (ISL), a licorice chalconoid, is considered to be a bioactive agent with chemopreventive potential. This study investigates the mechanisms involved in ISL-induced apoptosis in human cervical carcinoma HeLa cells.

Methods Cell viability was evaluated using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. Apoptosis was determined by flow cytometry using an Annexin V-FITC Apoptosis Detection Kit. The intracellular ROS levels were assessed using a 2, 7-dichlorofluorescein probe assay. The mitochondrial membrane potential was measured with the dual-emission potential-sensitive probe 5, 5′, 6, 6′-tetra-chloro-1, 1′, 3, 3′-tetraethyl-imidacarbocyanine iodide (JC-1). The degradation of poly-ADP-ribose polymerase (PARP) protein, the phosphorylation of PKR-like ER kinase (PERK), the phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) expression, and the activation of caspase-12 were analyzed via western blot analysis.

Results ISL significantly inhibited the proliferation, the increase in ROS levels and apoptotic rates of HeLa cells in a concentration-dependent manner. Moreover, ISL induced mitochondrial dysfunction, caspase activation, and PARP cleavage, which displayed features of mitochondria dependent on apoptotic signals. Besides, exposure of HeLa cells to ISL triggered endoplasmic reticulum (ER) stress, as indicated by the increase in p-eIF2α and GRP78 expression, ER stress-dependent apoptosis is caused by the activation of ER-specific caspase-12.

Conclusion The findings from our study suggest that ISL-induced oxidative stress causes HeLa cell apoptosis via the mitochondrion-dependent and the ER stress-triggered signaling pathways.

Key words: ISL; HeLa cells; ROS; Mitochondria; ER stress; Apoptosis


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**Correspondence should be addressed to WANG Zhi Ping, Tel: 86-0931-8912106, Fax: 86-0931-8942821, E-mail: erywzp@lzu.edu.cn; ZHENG Qiu Sheng. Tel: 86-0993-2057003, Fax: 86-0993-2057005, E-mail: zqsyt@sohu.com

Biographical note of the first author: YUAN Xuan, female, born in 1982, Ph. D candidate, majoring in tumor molecular biological.

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INTRODUCTION

Soliquiritigenin (ISL) is existing in licorice and vegetables such as shallots and bean sprouts. ISL has shown a variety of biological activities including antioxidant[3-4], anti-inflammatory[5], estrogenic[6], chemopreventive[7-8], and anti-tumor properties[9]. ISL reportedly inhibits cell growth and induces apoptosis in lung[10], gastric[11], hepatic[12], prostatic[13-14], colon[15-17], and skin cancer cells[18]. These studies strongly support the use of ISL in chemoprevention of human cancer.

The endoplasmic reticulum (ER), a major intracellular organelle, is a quality control system for intracellular protein homeostasis and it controls the synthesis, folding, and delivery of biologically active proteins[19-20]. The accumulation of unfolded or misfolded proteins in the ER lumen, which induces a coordinated adaptive program, are called the unfolded protein response (UPR), causing ER stress[21]. Increasing evidence show that ER stress plays a critical role in the apoptosis regulation caused by oxidative stress[22-23]. On the other hand, the production of reactive oxygen species (ROS) is induced by misfolded ER proteins, which activate the UPR and contribute to the induction of apoptosis[24-25].

ROS are formed as byproducts of the normal cellular metabolism of oxygen[26]. Organisms have developed antioxidant systems that include antioxidant enzymes, such as superoxide dismutase (SOD), and antioxidant molecules, such as glutathione (GSH), to balance these harmful radicals[27]. However, oxidative stress, a condition characterized by a dramatic increase in ROS levels and disruption of antioxidant balance, results in oxidative damage to cellular structures, signal transduction, and cell death[28]. In addition, ROS damages both DNA and proteins. Then, chemopreventive agents induce apoptosis in cancer cells through ROS generation[29-31].

Several lines of evidence indicate that ISL has diverse pharmacological effects such as antioxidant[3-4] anti-inflammatory[5], and anti-tumor[9,32-33]. However, few studies have shown that ISL induces apoptosis via oxidative stress, disrupting cellular functions and eventually causing apoptosis in HeLa. Therefore, this study is aimed to explores the role of ROS and the possible mechanisms of ISL-induced apoptosis in HeLa cells.

MATERIALS AND METHODS

Chemicals

ISL was purchased from Jiangxi Herb Tian gong Technology Co., Ltd. (Jiangxi, China). Dulbecco’s modified Eagle’s medium (DMEM), dimethylsulfoxide (DMSO) and acridine orange/ethidium bromide (AO/EB), N-acetylcyesteine (NAC), a precursor of glutathione, provide important protection against ROS, and Annexin V/PI apoptosis kit, molecular Probes 2’,7’-dichlorodihydrofluorescein diacetate (H2DCFDA) were purchased from Sigma (St. Louis, Missouri, USA). Fetal bovine serum (FBS) was purchased from Tianjin Hao Yang Biological Manufacture Co., Ltd. (Tianjin, China). The antibodies used in this study were purchased from Santa Cruz Biotec (Santa Cruz, CA, USA). Penicillin and streptomycin were obtained from Shandong Sunrise Pharmaceutical Co., Ltd. (Shandong, China). ISL was dissolved in DMSO and diluted with fresh medium to achieve the desired concentration. The final concentration of DMSO did not exceed 0.2% in the fresh medium, and DMSO at this concentration had no significant effect on the cell viability. Unless indicated otherwise, the other reagents were purchased from Sigma.

Cell Culture

HeLa cells were purchased from China Center for Type Culture Collection (Wuhan, China). The cells were maintained in DMEM supplemented with 10% FBS, 100 µ/mL penicillin and 100 µg/mL streptomycin at 37 °C with 5% CO2. The cells were split every 3 days and were diluted every day before each experiment.

Cell Viability Assay

Cell viability was measured by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] assay[34]. In brief, cells were washed with fresh media and cultured in 96-well plates (1×10^5 cells/mL) and then incubated with ISL (0, 2, 5, 10, 30, 40, 60 µg/mL) for 24 h. After incubation, the medium was aspirated and fresh medium containing 10 µL of 5 mg/mL MTT was added. After 4 h, the medium was removed and replaced with blue formazan crystal dissolved in 100 µL dimethyl sulfoxide (DMSO). Absorbance at 570 nm was measured using a fluorescent plate reader (Millipore Corp., Bedford, MA). The data were expressed as percent cell viability compared with control (DMSO).
**Morphological Assay (AO/EB)**

In order to explore whether ISL induces apoptosis in HeLa cells, the cells were plated on a four-well chamber slides at 20,000 cells/slide, and treated with increasing concentrations of ISL for 24 h to examine whether ISL induces apoptosis in HeLa cells. The cells were stained by 4 μL of prepared acridine orange/ethidium bromide (AO/EB) working solution (100 mg/L AO and 100 mg/L EB in phosphate buffered saline) for 30 min in the dark at 37 °C. The cells in the slides were then inspected using fluorescence microscope.

**Detection of Intracellular Reactive Oxygen Species (ROS) Lever**

ROS generated in HeLa cells was assessed using 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA) probe. H₂DCFDA was deacetylated intracellularly by using a nonspecific esterase, which was further oxidized by cellular peroxidases, yielding 2,7-dichlorofluorescein (DCF), a fluorescent compound (λEX/λEM=485 nm/535 nm). Briefly, the cells were incubated with the indicated concentrations of ISL with or without NAC (200 μmol/L) for 2 h. Cells were then washed in phosphate buffered saline (PBS) and incubated with 30 μmol/L H₂DCFDA at 37 °C for 30 min, as indicated in the instructions of the manufacturer. DCF was detected by using a FACStar flow cytometer (Becton Dickinson). Viable cells were negative for both PI and annexin V-FITC; apoptotic cells were positive for annexin V-FITC and negative for PI, whereas late apoptotic dead cells displayed strong annexin V-FITC and PI labeling. Non-viable cells, which underwent necrosis, were positive for PI but negative for annexin V-FITC.

**Measurement of Mitochondrial Membrane Potential**

In order to measure the mitochondrial membrane potential, the dual-emission potential-sensitive probe 5, 5′, 6, 6′-tetra-chloro-1, 1′, 3, 3′-tetaethyl-imidacarbocyanine iodide (JC-1) was used. JC-1 is a green-fluorescent monomer at low membrane potential, with the membrane potential of energized mitochondria promoting the formation of red-fluorescent J-aggregates. The ratio of red to green fluorescence of JC-1 depends only on the membrane potential, with a decrease being indicative of membrane depolarization. HeLa cells treated with ISL were harvested in the absence or presence of NAC (200 μmol/L). Then, the cells were loaded with 2 mg/L of JC-1 at 37 °C for 20 min and analyzed afterwards by using a plate reader (Millipore Corp., Bedford, MA, USA).

**Measurement of Caspase Activities**

The activities of caspase-3 and caspase-9 were assessed by using the caspase-3 and caspase-9 Colorimetric Assay Kits (R&D systems, Inc., Minneapolis, USA), which are based on the spectrophotometric detection of the color reporter molecule p-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA (caspase-3) and LEHD-pNA (caspase-9) as an index respectively. In brief, cells were incubated with the designated concentration of ISL in the absence or presence of NAC (200 μmol/L). The cells were then washed by PBS and suspended in 5 volumes of lysis buffer (20 mmol/L Hepes, pH 7.9, 20% Glycerol, 200 mmol/L KCl, 0.5 mmol/L EDTA, 0.5% NP40, 0.5 mmol/L DTT, 1% protease inhibitor cocktail (from Sigma). The lysates were then collected and stored at -20 °C until.

Apoptosis was determined by staining cells with annexin V fluorescein isothiocyanate (FITC) and propidium iodide (PI) labeling. Briefly, 1.5×10⁵ cells/mL were incubated with ISL with or without NAC (200 μmol/L) for 24 h. Afterwards, the cells were washed twice with ice-cold PBS, and then 5 μL of annexin V-FITC (PharMingen, San Diego, CA) and 5 μL of PI (1 mg/mL) were then applied to stain cells. The status of cell staining was analyzed by using the FACStar flow cytometer (Becton Dickinson). Viable cells were negative for both PI and annexin V-FITC; apoptotic cells were positive for annexin V-FITC and negative for PI, whereas late apoptotic dead cells displayed strong annexin V-FITC and PI labeling. Non-viable cells, which underwent necrosis, were positive for PI but negative for annexin V-FITC.

**Measurement of Malondialdehyde (MDA) Content**

The formation of MDA, a substance produced during lipid peroxidation, was determined by the thiobarbituric acid reactive substances (TBARS) test. Briefly, after exposure to ISL alone or in combination with NAC for 24 h, HeLa cells were harvested and aliquots of 10% supernatant were incubated with 0.8% TBA. The mixture was heated in 95 °C water bath for 1 h. Afterwards, n-butanol and pyridine (15:1, V/V) were added and the mixture was centrifuged. The organic phase was collected to measure fluorescence at excitation and emission wavelengths of 515 and 553 nm respectively. A standard curve was generated by using 1, 2, 3, 3-tetramethoxypropane. MDA content was expressed as nmol/mg protein. Protein content was measured by the method of Bradford (1976).

**Detection of Cell Apoptotic Rates by Flow Cytometry**

Apoptosis was determined by staining cells
use. Protein concentration was determined by the Bradford method. Supernatants, containing 100 µg of total protein, were added to 96-well plates (Nunc, Roskilde, Denmark) with the DEVD-pNA and LEHD-pNA at 37 °C for 1-2 h to determine caspase-3 and caspase-9 activities. The optical density of each well was measured at 405 nm by using the microplate reader (Millipore Corp., Bedford, MA). Each plate contained the multiple wells of a given experimental condition and multiple control wells. The activities of caspase-3 and caspase-9 were expressed in arbitrary absorbance units (absorbance at a wavelength of 405 nm).

**Western Blot Analysis and Statistical Analysis**

The soluble lysates (15 µL per lane) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto the nitrocellulose membranes (Amersham Biosciences, New Jersey, USA) and blocked with 5% nonfat milk in Tris-buffered saline with Tween (TBST) for 2 h at room temperature. Membranes were incubated with anti-glucose-regulated protein, 78/immunoglobulin heavy chain-binding protein (78 kD glucose-regulated protein, GRP78) antibody (1:200), anti-poly-ADP-ribose polymerase (PARP) antibody (1:500), anti-caspase-12 antibody (1:200), anti-phosphorylation of α-subunit of eukaryotic initiation factor 2 (eIF2α) antibody (1:200), anti-phosphorylation of PKR-like ER kinase (PERK) antibody, anti-β-actin antibody (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% milk/TBST at 4 °C overnight. After washing membranes with TBST five times, the membranes were incubated with horseradish peroxidase-conjugated antibody for 1 h at room temperature. Western blots were developed by using enhanced chemiluminescence (ECL, thermo) and were exposed on Kodak radiographic film.

The data were presented as means±S.E. from at least three independent experiments and evaluated through the analysis of variance (ANOVA) followed by student’s t test. The values of P<0.05 were considered statistically significant. The analyses were performed by using the Origin 6.0 software (Origin Lab Corporation, Northampton, MA, USA).

**RESULTS**

**Effects of ISL on Cell Viability and ROS Production in HeLa Cells**

HeLa cells were cultured for 24 h with different ISL concentrations (0, 2, 5, 10, 30, 40, and 60 µg/mL) in order to examine its effects on HeLa cell viability. Cell viability was measured using an MTT assay. A significant concentration-dependent reduction in cell viability was observed, and cell inhibition rates ranged from 25% to 76% after 24 h of ISL treatment (Figure 1). After the HeLa cells were exposure to ISL (10, 30, and 60 µg/mL) for 2 h, the intracellular ROS (using DCF fluorescence as an indicator for ROS formation) and MDA (an index of oxidative damage to membrane lipids) levels were significantly increased in a concentration-dependent manner compared with the control group (Figure 2). The antioxidant N-acetylcysteine (NAC, a precursor of glutathione, 200 µmol/L) effectively prevented ISL-induced ROS formation and MDA levels (Figure 2).

![Figure 1. Effects of ISL on cell viability of HeLa cells.](image)

**ISL-induced Apoptosis Is Mediated by a Mitochondrion-dependent Pathway in HeLa Cells**

Morphologic measurement results obtained by using AO/EB staining (Figure 3A) showed that normal viable HeLa cells were stained green (Ctr), and the apoptotic HeLa cells (10, 30, and 60 µg/mL) appeared as bright green arcs in its earliest stage and with condensed, yellow/orange nuclei in its late stage. Annexin V-FITC-PI double-staining was used to detect phosphatidyl serine (PS) externalization, a hallmark of early apoptosis, to prove whether ISL-induced apoptosis occurs. As shown in Figure 3B,
Figure 2. Effects of ISL on ROS and MDA production in HeLa cells. The cells were treated with ISL (0, 10, 30, and 60 µg/mL) with or without NAC (200 µmol/L). (a) ROS was determined via flow cytometry; (b) oxidative damage to membrane lipids (lipid peroxidation) was measured by using the levels of MDA. Data are presented as the mean±S.E. of the three independent experiments. *P<0.05, **P<0.01 compared with the control group; #P<0.05 compared with the ISL group alone (30 µg/mL).

Figure 3. ISL induced apoptosis in HeLa cells. The cells were treated with or without the indicated amounts of ISL for 24 h with or without NAC (200 µmol/L). (A) Morphologic measurements in HeLa cells were carried out via AO/EB fluorescence staining. (B) Detection of apoptotic rates conducted via flow cytometry. (C) Caspase-3 and caspase-9 activity were examined. The data in C and D were presented as the mean±S.E. of the three independent experiments. *P<0.05, **P<0.01 compared with the control group; #P<0.05, ##P<0.01 compared with the ISL group alone (30 µg/mL).

the apoptotic rates were markedly increased among ISL-treated cells, which indicates that these cells were in early apoptosis. In order to evaluate the apoptotic signaling induced by ISL, caspase-9 and caspase-3 activity were measured. Caspase-9 is involved in the activation of the caspase cascade responsible for apoptosis induction, which then cleaves and activates caspase-3. Caspase-3 activity is
an integral step in most apoptotic events. In this study, treatment with ISL (10, 30, and 60 µg/mL) induced remarkable caspase-9 and caspase-3 activation (Figure 3C). Pretreatment with NAC (200 µmol/L) effectively prevented ISL-induced HeLa cell apoptosis (Figure 3). These results suggest that ISL-induced apoptosis may be associated with ROS production. In order to determine whether ISL-induced apoptosis is mediated through mitochondrial dysfunction, the mitochondrial membrane potential (MMP) is measured by using the mitochondrial-sensitive dye JC-1. As shown in Figures 4A, JC-1 accumulated in the untreated control cells, where it displayed bright red fluorescence, which indicates a high potential. By contrast, JC-1 poorly accumulated in ISL-treated cells, which displayed green fluorescence, thereby indicating disruption of the MMP. This result was further confirmed by calculating the mean red/green fluorescence ratio using a plate reader, which showed a concentration-dependent decrease in MMP after ISL treatment (Figure 4B). Meanwhile, as shown in Figure 4C, the levels of the 89 kD cleaved PARP fragment (the active form) was significantly increased after the HeLa cells were exposed to ISL for 24 h. These ISL-induced responses were alleviated by 200 µmol/L NAC (Figure 4).

**ISL Induces the Endoplasmic Reticulum (ER) Stress Response in HeLa Cells**

The involvement of ER stress signaling in the responses triggered by ISL-induced apoptosis was evaluated based on the phosphorylation patterns of PKR-like ER kinase (PERK) and α-subunit of eukaryotic initiation factor 2 (eIF2α). PERK, an ER-resident transmembrane kinase, is known to auto-phosphorylate its cytoplasmic kinase domain in response to the accumulation of unfolded proteins in the ER lumen. Activated PERK subsequently phosphorylates several cytosolic proteins such as eIF2α. As shown in Figure 5, ISL increased the levels of p-PERK, p-eIF2α, and GRP78, and the cleavage of caspase-12 with in 24 h of exposure. These effects were reversed by 200 µmol/L NAC (Figure 5).

**DISCUSSION**

With the multistep development of human tumors, cancer cells acquire biological capabilities such as sustained proliferative signaling, evasion of growth suppressors, cell death resistance, replicative immortality, induction of angiogenesis, activation of invasion and metastasis, reprogramming of energy metabolism, and evasion of immune destruction, which constitute an organizing principle for rationalizing the
Mitochondria are very sensitive to oxidative stress, demonstrated to play a crucial role in cell apoptosis. Mitochondrial dysfunction has been shown to be involved in ISL-induced cytotoxic responses and ER stress activation, and ER stress in ISL-treated HeLa cells. Moreover, these ISL-induced cytotoxic responses are alleviated by cotreatment with the antioxidant NAC. Therefore, these findings indicate that oxidative stress mediates mitochondrial-dependent and ER stress-activated apoptotic signals involved in ISL-induced HeLa cell apoptosis.

Mitochondrial dysfunction has been demonstrated to play a crucial role in cell apoptosis. Mitochondria are very sensitive to oxidative stress, and excess ROS causes perturbations in mitochondrial function, and subsequently triggers apoptosis. Killing tumor cells via cytotoxic therapy, for example, chemotherapy, γ-irradiation, immunotherapy, or suicide gene therapy, is predominantly mediated by triggering apoptosis in cancer cells. Recent studies have indicated that ISL induces prostate cancer cell apoptosis by disrupting mitochondrial function and activating caspase cascade signals. However, the role of ROS is not considered. The results of this study show that ISL increased intracellular ROS levels, and that ISL is capable of inducing HeLa cell apoptosis by reducing MMP. In addition, the levels of 89 kD fragment (active form) of PARP significantly increases, which is associated with the activation of caspase-3 and caspase-9; all these effects of ISL are antagonized by NAC.

The ER is the primary intracellular organelle responsible for protein folding, maturation, and trafficking. Unfolded or misfolded proteins accumulate in the ER lumen when the ER homeostasis is disturbed, resulting in ER stress. The three ER transmembrane proteins PERK, inositol-requiring protein-1 (IRE1a), and activating transcription factor-6 sense ER stress and induce specialized responses to recover or maintain ER function. Activated PERK phosphorylates the α-subunit of eukaryotic initiation factor-2 (eIF2α) and eventually leads to the general inhibition of translation, thereby reducing the load of newly synthesized proteins that are translocated to the ER. Activated IRE1α splices X-box binding protein 1 (XBP1) mRNA. The XBP1 protein encoded by the spliced XBP1 mRNA enhances the capacity of the ER by upregulating the expression of ER chaperone proteins, such as the 78 kD glucose-regulated protein (GRP78), and reduces unfolded or misfolded proteins in the ER via the ER-associated protein degradation system (ERAD). ERAD regulates the degradation of unfolded or misfolded proteins in the ER through the ubiquitin (Ub)-proteasome system. However, if these protective responses fail and ER stress persists, specialized apoptotic pathways are activated to eliminate the damaged cells. ER stress-dependent apoptotic cell death is caused by the activation of ER-specific caspase-12. In the present study, we used the phosphorylation of PERK and eIF2α as markers for ER stress, the expression of GRP78 as a marker for the protective ER stress response, and the expression of caspase-12 (full length) as a marker for the apoptotic ER stress response, in ISL-triggered HeLa cancer cells apoptosis. As showed in Figure 5, the expression of p-PERK, p-eIF2α, ER-related chaperones GRP78, and caspase-12 degradation were significantly increased.
after exposure to ISL for 24 h. These effects are antagonized by cotreatment with NAC. Also, these results indicate that increased ER stress is involved, at least partially, in the ISL-triggered apoptosis of HeLa cells.

In conclusion, ISL induces oxidative stress in HeLa cells. The evidence demonstrates that ISL triggers oxidative stress to induce HeLa cell apoptosis through mitochondrial dysfunction, leading to the cleavage of PARP and activation of the caspase cascade-mediated signaling pathway. ISL also induces the expression of ER stress-related markers (GRP78), which subsequently trigger caspase-12 cleavage, resulting in apoptosis. Our evidence also demonstrates that ISL-induced apoptosis is implicated in ROS production. Moreover, extensive but fragmentary studies have shown that (i) ISL induces apoptosis in cancer cells; (ii) ISL induces excessive ROS generation in focal cerebral ischemia; and (iii) ISL induces apoptosis by disrupting mitochondrial function and activating caspase cascade signals in several cell types. However, ISL triggers oxidative stress to induce apoptosis through mitochondrial dysfunction in HeLa cells. Besides, ISL induces the expression of ER stress-related markers (GRP78) that subsequently trigger caspase-12 cleavage, resulting in apoptosis. A combination of these results provide more complete information on a better understanding of cancer prevention by using natural products. Evidence based on the aforementioned observations suggests that ISL has an important chemopreventive effect on human cancer.

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