Isoliquiritigenin inhibits the proliferation of human renal carcinoma Caki cells through the ROS-mediated regulation of the Jak2/STAT3 pathway

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Abstract. Isoliquiritigenin (ISL) is a flavonoid with chalcone structure that has been noted in licorice and shallot, which are generally used in traditional Chinese medicine. ISL has demonstrated various pharmacological effects including antioxidant, anti-inflammatory and antitumor activity. However, the molecular mechanisms underlying the anticancer effects of ISL remain poorly understood. The present study revealed that ISL significantly decreased viability and induced apoptosis in human renal carcinoma Caki cells. The ISL-induced apoptosis was associated with the cleavage of caspase-9, -7 and -3, and that of PARP. Moreover, ISL increased the expression of pro-apoptotic protein Bax and diminished the expression of anti-apoptotic protein Bcl-2, and Bcl-xl, thereby increasing cytochrome c release. Treatment of cells with ISL also induced the expression of p53 through downregulation of murine double minute 2 (Mdm2). Furthermore, ISL generated reactive oxygen species (ROS), and pretreatment with ROS scavenger N-acetyl cysteine (NAC) and NADPH oxidase inhibitor diphenyleneiodonium abrogated the ISL-induced apoptosis. One of the key oncogenic signaling pathways is mediated through signal transducer and activator of transcription 3 (STAT3), which promotes abnormal cell proliferation. Incubation of cells with ISL markedly diminished phosphorylation and DNA binding activity of STAT3, and reduced expression of STAT3 responsive gene products, such as cyclin D1 and D2. ISL also attenuated constitutive phosphorylation of upstream kinase, Janus-activated kinase 2 (Jak2). Pretreatment with NAC abrogated the inhibitory effect of ISL on activation of STAT3 and blocked the cleavage of caspase-9, -7 and -3, and that of PARP in Caki cells. Taken together, the present study provides the first report that ISL induces apoptosis in Caki cells via generation of ROS, which causes induction of p53 and inhibition of the STAT3 signaling pathway.

Introduction

Renal cell carcinoma (RCC) is the most common type of malignant neoplasm arising from the kidney (1). RCC has the highest mortality rate of all genitourinary cancer and its incidence has steadily risen (2). Since RCC is resistant to conventional chemotherapy, it commonly recurs after treatment; thus, patients with metastatic RCC have poor prognosis (3). Typically, the drugs used to treat metastatic RCC are targeted to vascular endothelial growth factor (VEGF) receptors and mammalian target of rapamycin (mTOR) signaling. However, while most patients initially respond to these drugs, its resistance results in subsequent progression of RCC. Thus, to address the unmet need to identify additional targets for RCC, newly developed therapeutic agents are required for effective treatment.

Signal transducer and activator of transcription 3 (STAT3) is a transcriptional factor that mediates the signaling pathway of various cytokines and growth factors (4). Constitutive activation of STAT3 has been observed in human cancer including breast, ovarian and prostate cancer (5-7). It has been recently reported that a high frequency of STAT3 activation is observed in RCC, particularly in metastatic disease (8). STAT3 protein has been shown to play an important role in supporting cell survival and proliferation (9). In cancer cells, STAT3 becomes constitutively active through the phosphorylation of its serine 727 (S727) and tyrosine 705 (Y705) residues by upstream Janus-activated kinases (Jaks) or the Src family kinases (9). Phosphorylated STAT3 then undergoes dimerization and is translocated to the nucleus where it binds to specific DNA response elements in the promoters of target genes (10). STAT3 transactivates the genes encoding various cell survival proteins, such as cyclins, survivin, Bcl-2 and

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Bcl-xl (11). This evidence supports the rationale for targeting STAT3 for suppression of tumor growth in several cancers including RCC (12).

Apoptosis is triggered by extrinsic and/or intrinsic pathways and is regulated by various ligands (13). The extrinsic pathway originates from interaction between death receptors and its ligands such as Fas and TNFα. The intrinsic pathway is closely related to the permeabilization of the mitochondrial membrane, commanded by the Bcl-2 superfamily (14). Reactive oxygen species (ROS) are mainly generated by the mitochondria in most mammalian cells. ROS can cause cellular apoptosis via both the extrinsic cell death receptor and the intrinsic mitochondrial cell death pathways (15). It has been reported that elevated ROS formation and the subsequent apoptosis induction is a novel approach to treat cancer (16).

Since evasion of apoptosis is one of the hallmarks of cancer, it has long been attempted to develop anticancer drugs that can selectively induce apoptosis in cancer cells (17). Various phytochemicals such as polyphenols modulate differentiation of cells and induce apoptosis in cancer cells (18). Isoliquiritigenin [2',4',4-trihydroxychalcone (ISL)] (Fig. 1A) is a flavonoid with chalcone structure that has been noted in licorice and shallot, which are generally used in traditional Chinese medicine (19). ISL shows pharmacological effects including antioxidant, anti-inflammatory and antitumor activity (20-22). In addition, ISL has been proved to induce apoptosis and inhibit cell proliferation in breast, liver, colon and cervical cancer cells (23-27). Moreover, ISL was found to inhibit tumor growth and angiogenesis in lung and breast cancer mouse models (22,28). Previously, Yamazaki et al reported that ISL suppressed the viability of murine RCC with concomitant inhibition of nitric oxide production by lipopolysaccharide-stimulated macrophages, resulting in decreased pulmonary metastasis (29). However, thus far, there have been no studies regarding the effects of ISL in human RCC. Therefore, we aimed to investigate molecular mechanisms underlying the anticancer effects of ISL on human renal carcinoma Caki cells. The present study revealed that ISL induced antiproliferative and apoptotic effects in Caki cells through mitochondrial-dependent caspase activation and interference with the STAT3 signaling pathway via ROS generation.

Materials and methods

Materials. ISL, N-acetyl cysteine (NAC), diphenyleneiodonum (DPI) and β-actin antibody were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies against cleaved caspase-9, -7, -3 and PARP as well as Bcl-2, Bcl-xl, Bax, STAT3, p-STAT3 (Y705), p-STAT3 (S727), Jak2, p-Jak2, cyclin D1 and cyclin D2 were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Primary antibodies against each of p53 and murine double minute-2 (Mdm2), and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 2',7'-Dichlorofluorescein diacetate (DCF-DA) was obtained from Invitrogen (Carlsbad, CA, USA). Hank's balanced salt solution (HBSS) was purchased from Mediatech (Herndon, VA, USA).

**Cell culture and treatment.** Caki cells were provided by Dr. T.K. Kwon (Keimyung University, Daegu, Korea) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin G and 100 µg/ml streptomycin) at 37°C in a humidified incubator containing 5% CO₂ and 95% air. The cells were plated at an appropriate density according to each experimental scale.

**Cell viability assay.** Cell viability was measured by the MTT assay. Cells (2x10⁵) were incubated in triplicate in a 96-well plate in the presence of ISL in a final volume of 100 µl for different time intervals at 37°C. Thereafter, 10 µl of MTT solution (5 mg/ml) was added to each well and incubated for 4 h. Medium was removed, formazan was dissolved in dimethyl sulfoxide (DMSO) and absorbance at 570 nm was measured using a microplate reader (Tecan Trading AG, Männedorf, Switzerland). Cell viability was expressed as the relative percentage of the control.

**Annexin V staining.** Annexin V staining was performed using fluorescein isothiocyanate (FITC)-Annexin V staining kit (BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions. Briefly, ISL-treated cells were washed with HBSS solution and resuspended in binding buffer containing Annexin V and propidium iodide (PI). Fluorescence intensity was measured using flow cytometry (BD Biosciences, La Jolla, CA, USA).

**Western blot analysis.** Cells were lysed with RIPA buffer, and collected protein samples were quantified using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The protein samples were electrophoresed using 8-15% sodium dodecyl sulfate-polyacrylamide gel and immunoblot analysis was carried out according to the protocol previously described (30). Immunoblot membranes were incubated with SuperSignal Pico Chemiluminescent or Dura Luminol substrates (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instruction and visualized with ImageQuant LAS 4000 (Fujifilm Life Science, Tokyo, Japan).

**Measurement of ROS accumulation.** Cells were treated with ISL in the presence or absence of NAC and DPI for 24 h and then loaded with 25 µM of DCF-DA. After incubation for 30 min at 37°C, the cells were washed twice with HBSS solution, suspended in complete media and examined under a fluorescence microscope to detect the intracellular accumulation of ROS. Total cellular ROS were quantified using a flow cytometry (BD Biosciences).

**Electrophoretic mobility gel shift assay (EMSA).** The nuclear extract was prepared from cells incubated with or without ISL. The STAT3 oligonucleotide probe 5'-AGC TTC ATT TCC CGT AAA TCC CTA-3' (Biomedic, Korea) was labeled with [γ-32P]-ATP using T4 polynucleotide kinase. The EMSA was performed according to a previously described protocol (31).

**Statistical analysis.** When necessary, data are expressed as mean ± SD of the results of at least three independent
experiments, and statistical analysis for single comparison was performed using the Student's t-test and a p-value <0.05 was considered to indicate a statistically significant result.

**Results**

**ISL induces apoptosis in Caki cells.** We initially examined the effect of ISL on the viability of Caki cells. Incubation of cells with ISL (5, 10, 20 or 50 µM) significantly reduced the cell viability (Fig. 1B). In addition, to elucidate whether ISL-mediated cytotoxicity resulted from the induction of apoptosis, FACS analysis was performed. Caki cells treated with ISL (5, 20 or 50 µM) for 48 h were analyzed by flow cytometry using double staining with Annexin V and PI to quantify the population of cells undergoing apoptosis. As shown in Fig. 1C, incubation of Caki cells with ISL for 48 h resulted in apoptotic cell death. Quantification of apoptotic cells and statistical analysis of ISL-induced apoptosis are presented in Fig. 1C (right panel).

**ISL induces apoptosis through the mitochondrial pathway in Caki cells.** Mitochondria are a central regulator of the intrinsic pathway in apoptosis triggered by various stimuli (32). The loss of mitochondrial membrane potential leads to increased mitochondrial membrane permeability, and then released cytochrome c, which induces activation of the caspase cascade (32). The activation of caspases is a critical event in the proteolytic cascade elicited by apoptotic stimuli. We investigated the involvement of caspase activation in the ISL-induced apoptosis of Caki cells. Treatment of Caki cells with ISL induced the cleavage of caspase-9, -7 and -3, and PARP (Fig. 2A). Incubation of cells with ISL increased the expression of p53 and diminished the expression of its cytosolic repressor protein Mdm2 in a concentration-dependent manner (Fig. 2B). To elucidate whether the apoptosis may be mediated by mitochondrial alterations, we evaluated the expression of anti-apoptotic or pro-apoptotic proteins in the ISL-treated Caki cells. Anti-apoptotic proteins such as Bcl-2 and Bcl-x1 reside in the outer mitochondrial membrane and
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block the release of cytochrome c, whereas pro-apoptotic protein Bax stimulates its release. Since Bcl-2 family proteins regulate the mitochondrial membrane integrity, the effect of ISL on the expression of Bcl-2 family proteins was then examined. As shown in Fig. 2C, incubation with ISL reduced the expression of Bcl-2 and Bcl-xl, while ISL increased expression of Bax in Caki cells. In addition, the level of cytochrome c expression was increased by treatment of ISL in the Caki cells with a concomitant decrease in Bcl-2 expression (Fig. 2C).

ISL induces generation of ROS in Caki cells. Since it has been postulated that ROS production contributes to apoptosis triggering in various cancer cell lines (33), the effect of ISL on ROS generation was examined. Treatment of cells with ISL (5, 20 or 50 µM) for 24 h led to ROS generation as revealed by FACS analysis after DCF-DA staining (Fig. 3A). As a positive control, Caki cells were incubated with hydrogen peroxide (500 µM) under the same experimental condition. To verify that the increase in ROS level was involved in the ISL-induced apoptosis, we utilized the ROS scavenger NAC and specific NADPH oxidase inhibitor DPI. It has been known that NADPH oxidases catalyze the production of superoxide, a type of ROS. Treatment of Caki cells with NAC and DPI abrogated the ISL-induced ROS generation (Fig. 3B and C), following cell death (Fig. 3D and E). Quantification of apoptotic cell death under each experimental condition as well as statistical analysis are presented in the right panel of Fig. 3D and E.

Role of ROS in ISL-induced inhibition of STAT3 signaling and induction of apoptosis in Caki cells. We next examined the role of ISL-induced ROS generation in the blocking of STAT3 signaling and induction of apoptosis in Caki cells. Incubation of Caki cells with ISL in the absence or presence of NAC revealed that inhibition of ROS generation abrogated the inhibitory effect of ISL on the phosphorylation of Jak2 following STAT3 activation (Fig. 5A) as well as its DNA binding activity (Fig. 5B). Moreover, pretreatment of NAC averted the ISL-induced cleavage of caspase-9 -7 and -3 and PARP (Fig. 5C). These findings suggest that the ISL-induced ROS generation plays a pivotal role in apoptosis and inactivation of STAT3 signaling in the ISL-treated Caki cells.

ISL-induced apoptosis in Caki cells is mediated through inactivation of STAT3 signaling. STAT3 protein is constitutively activated in various human cancer cells and aberrant STAT3 signaling is implicated as playing a pivotal role in the initiation and progression of cancer (34). It has been known that STAT3 transcriptional activity is stimulated by its simultaneous tyrosine and serine phosphorylation via activation of Jak kinases (35). Incubation with ISL inhibited the STAT3 DNA binding activity following inhibition of constitutive phosphorylation of STAT3 at both Y705 and S727 residues (Fig. 4A and B). To elucidate the mechanism of ISL-induced inactivation of STAT3, we examined the effect of ISL on the phosphorylation of upstream kinase, Jak2, which is known to phosphorylate STAT3. As shown in Fig. 4A, ISL attenuated the constitutive phosphorylation of Jak2 in Caki cells. In addition, ISL attenuated the expression of STAT3 target gene products such as cyclin D1 and cyclin D2 (Fig. 4C).
Figure 3. ISL induces ROS generation in Caki cells. (A) Cells were treated with ISL (5, 20 or 50 µM) for 24 h, and then examined for the intracellular accumulation of ROS. DCF-DA fluorescence staining was measured by flow cytometry. Hydrogen peroxide (H$_2$O$_2$) (500 µM) was used as a positive control. (B and C) Effect of antioxidants on ISL-generated ROS in Caki cells. Cells were treated with NAC (5 mM) and DPI (50 nM) for 1 h before treatment with ISL (50 µM) for 24 h. ROS levels were measured using flow cytometry. This graph shows quantification and statistical analysis of ROS generation by ISL treatment in the presence or absence of (B) NAC and (C) DPI. The experiment was carried out in triplicate and the data are presented as mean ± SD. (D and E) Roles of ROS in ISL-induced apoptosis in Caki cells. Cells were treated with NAC (5 mM) and DPI (50 nM) for 1 h before treatment with ISL (50 µM) for 48 h. The apoptotic index (%) was determined using flow cytometry. This graph shows statistical analysis of apoptosis by ISL treatment in the presence or absence of (D) NAC and (E) DPI. Data are representative of three independent experiments; *p<0.001, compared to control.
Discussion

Approaches to RCC therapy have many limitations and novel strategies are urgently required. It has been reported that dysregulated cell growth is a hallmark of cancer development, and naturally occurring substances derived from plant-based diet have been suggested for used in the therapy of various types of cancers (36). In addition, Jak/STAT3 signaling controls the ability of pre-neoplastic lesions to transform into a malignant tumor, which suggests that intervention of the Jak/STAT3 pathway provides the opportunities for new approaches in cancer therapy (37). Cumulative evidence supports activation of STAT3 as an oncogenic pathway in many cancers including RCC (5-8). To address the unmet need to identify therapeutic
targets for RCC, researchers have recently identified the Jak/STAT3 pathway. Our group and other investigators have suggested that kinase inhibitors of this pathway and phytochemicals suppressing STAT3 cause marked tumor growth inhibition in RCC in vitro and in vivo models (38,39).

Chalcone possesses a basic structure of two benzene rings connected by an unsaturated carbon chain and are the intermediate precursors for all flavonoid compounds. ISL is a chalcone found in licorice root and other plants, and shows various pharmacological properties including antitumor, antioxidant, and anti-allergic activities in vitro (40-43). ISL also contains an α,β-unsaturated carbonyl group as a Michael acceptor site, suggesting that ISL-induced ROS is mediated through the depletion of intracellular GSH. The electrophilic center such as the α,β-unsaturated carbonyl group is prone to undergo Michael addition reactions with nucleophiles, such as the free sulfhydryl group of cysteine residues located in reduced glutathione or many cellular proteins (44). In the present study, we provide the novel finding that ISL attenuated the constitutive phosphorylation, nuclear localization and DNA binding activity of STAT3 in Caki cells. Thus, we propose that ISL may directly bind to GSH and/or cysteine residues of STAT3. Phosphorylation of STAT3 initiates their nuclear translocation, followed by activated STAT3 dimers, and binds to specific promoter regions in DNA (45). In addition, it was reported that statin, a small molecular inhibitor of STAT3, prevented STAT3 dimerization and phosphorylation, and this was accompanied by alkylation of four cysteine residues (Cys251, 259, 367 and 426) in unphosphorylated STAT3 (46). Moreover, it has been shown that specific cysteine residues of STAT3 undergo covalent modifications and/or oxidation, and then changes in the cellular redox status affect the transcriptional activity of STAT3 (47). It is likely that ISL-induced ROS may cause oxidative modification of cysteine residues of Jak2 or STAT3 proteins, thereby blunting the survival of Caki cells. Our findings suggest that pretreatment with NAC abrogated the inhibitory effects of ISL on the activation of Jak2/STAT3 signaling implying that ISL can induce oxidative modification of these kinases. Similar to this finding, natural products such as withaferin A and carnosol were found to induce apoptosis through inhibition of Jak2/STAT3, accompanied by generation of ROS (48,49).

The role of ROS in the apoptosis of cancer cells is quite controversial. Although some investigators suspect that ROS

![Figure 6. A schematic diagram showing the molecular mechanisms of apoptosis induction by ISL in Caki cells.](image-url)
play a role in pro-tumorigenic signaling, other investigators have reported that ROS induce apoptosis (50). It seems likely that many antitumor agents cause oxidative stress such as ROS, which also contributes to their anticancer effects (51). Li et al revealed that ROS induce apoptosis through suppression of Bcl-2 expression and an increase in the Bax protein level in squamous cell carcinoma cells (52). The present study, also demonstrated that upregulation of the Bax/Bcl-2 ratio may contribute to the apoptosis in ISL-stimulated Caki cells. In addition, NAC treatment rescued cells from ISL-induced apoptosis by blocking the cleavage of caspases and PARP. Schumacker explained that cancer cells may be more vulnerable to oxidative stress, since they function with a higher level of ROS-mediated growth signaling than normal cells (53). Therefore, ISL has important value in the treatment of RCC, based on induction of oxidative stress-mediated apoptotic signals.

Recent studies have confirmed that the Jak2/STAT3 pathways are implied in the regulation of apoptosis and survival of tumor cells. STAT3 can also enhance its DNA binding activity by activating growth factors such as IL-6 and IL-17 to regulate the transcription of the corresponding target genes. It continuously transduces the survival signal to tumor cells to play a role in the promotion of tumor cell survival and inhibition of apoptosis (54). Therefore, in the present study, the effects of ISL on activation of STAT3 were estimated, and inhibition of the Jak2/STAT3 pathway resulted in apoptosis in ISL-treated Caki cells. In addition, ISL downregulated the expression of STAT3-related anti-apoptotic and survival genes such as Bcl-2, Bcl-xl, cyclin D1 and D2 in the Caki cells. Intracellular kinases, such as Jak2, have been shown to phosphorylate STAT3. The inhibitory effect of ISL on the phosphorylation of Jak2 through ROS generation interfered with STAT3 signaling pathway in the Caki cells. STAT3 becomes constitutively active through the phosphorylation by Src kinase as well as Jak. Moreover, a synergistic effect on tumor inhibition by co-treatment of Src and STAT3 inhibitors in RCC was noted (38). In accordance with these studies, we found that incubation of Caki cells with ISL inhibited Src activation and pretreatment with NAC abrogated the inhibitory effect of ISL on the phosphorylation of Src activation (data not shown). These data imply the role of Src in STAT3 activation in ISL-treated Caki cells. In summary, the present study demonstrated for the first time that ISL induces apoptosis in Caki cells through generation of ROS, which also contributes to their anticancer effects (51).

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References


