Saikosaponin-d suppresses the expression of cyclooxygenase-2 through the phospho-signal transducer and activator of transcription 3/hypoxia-inducible factor-1α pathway in hepatocellular carcinoma cells

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Abstract. Hepatocellular carcinoma (HCC) is one of the most common malignancies and accounts for ~6% of all types of human cancer worldwide, particularly in Asia. The incidence and mortality rates in the USA have also rapidly increased. Saikosaponin-d (SSD), a saponin derivative extracted from several species of *Bupleurum* (*Umbelliferae*), possesses unique biological activities, including anti-inflammatory, antihepatitic and immunomodulatory effects. Our previous studies have demonstrated that SSD inhibits the proliferation and induces the apoptosis of HCC SMMC-7721 cells by downregulating the expression of cyclooxygenase (COX)-2 and decreasing the production of prostaglandin E2. However, the specific mechanism underlying how SSD controls the expression of COX-2 remains to be elucidated. In the present study, it was demonstrated that hypoxia inducible factor-1α (HIF-1α) was responsible for the expression of COX-2 under hypoxic conditions in HCC cells, and the activation of signal transducer and activator of transcription 3 (STAT3) was required for the expression of HIF-1α. SSD treatment inhibited STAT3 activation [phosphorylation of STAT3 (p-STAT3)], reduced the protein level of HIF-1α and decreased the expression of COX-2. These results suggested that SSD may target HCC cells by suppressing the expression of COX-2 through the p-STAT3/HIF-1α pathway.

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Introduction

Hepatocellular carcinoma (HCC), accounting for 70-85% of the total liver cancer burden, is one of the most common malignancies and is the third leading cause of cancer mortality worldwide, with an estimated >500,000 new cases per year (1,2). At present, curative therapies, including resection, liver transplantation and ablation, provide effective treatment for only a small number of patients presenting with early stage HCC in the clinic. The majority of patients with intermediate-advanced HCC are only eligible for the mainstream palliative treatments, including transarterial chemoembolization and systemic therapy with molecular targeted drugs (3). However, therapies against liver cancer to date have not been completely effective. In this context, the development of new, effective therapeutic approaches for liver cancer remains one of the most challenging goals in cancer research.

Numerous traditional Chinese plants have been identified to possess biological activities with potential therapeutic applications. Saikosaponin-d (SSD), a saponin derivative extracted from several species of *Bupleurum* (*Umbelliferae*), has been traditionally used in the treatment of infectious diseases due to its anti-inflammatory, antipyretic and analgesic effects (4,5). Previous studies have demonstrated that SSD also has hepatoprotective, antifibrotic (6,7) and immunomodulatory (8,9) activities. Furthermore, traditional use and scientific studies have suggested that SSD is a potential candidate as an anticancer agent (10,11), which has been demonstrated to have anti-proliferative and apoptotic effects on various cancer cells, including human leukemia cancer, non-small cell lung cancer (12) and hepatic cancer (13). Our previous study demonstrated that SSD inhibits the proliferation and induces the apoptosis of HCC SMMC-7721 cells by downregulating cyclooxygenase (COX)-2 at the mRNA and protein level and inhibiting the production of prostaglandin E2 (PGE2) (14). However, the specific mechanism underlying how SSD controls COX-2 expression remains to be elucidated.

COX-2, a key inducible enzyme in prostanoid biosynthesis, is overexpressed in solid malignancies, including colon, prostate, breast and HCC (15). A significant negative correlation between the overexpression of COX-2 and the survival rates of patients in various types of cancer has been reported in retrospective studies (16-18). Inhibiting the activity or expression of COX-2 has shown promise for tumor therapy in animal models and cancer patients (19,20). In HCC patients, the protein expression of COX-2 correlates well with the differentiation grades, suggesting that abnormal COX-2 expression has an important effect in hepatocarcinogenesis (21). It is well established that non-steroidal anti-inflammatory drugs (NSAIDs) have anti-tumor effects by acting on COX-2 (22). SSD has a similar pharmacological activity to NSAIDs, and it has been documented that SSD inhibits HCC cell proliferation by modulating COX-2 expression (14). However, how SSD regulates the expression of COX-2 remains to be elucidated.

Materials and methods

Cell culture and reagents. The liver cancer cell lines SMMC-7721 and HepG2, obtained from the Transform Medical Center of Xi'an Jiaotong University (Xi'an, China), were cultured in RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin/streptomycin, and incubated at 37˚C in a humidified 5% CO_2 atmosphere. The Janus kinase 2 (JAK2) selective inhibitor AG-490, hypoxia simulator cobalt chloride ($CoCl_2$), mammalian target of rapamycin (mTOR) and SSD were obtained from Sigma (Poole, UK). AG-490, rapamycin and SSD were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA), and interleukin-6 (IL-6) was dissolved in acetic acid (Sigma-Aldrich). For all experiments, final concentrations of the tested regents were prepared on the day of assessment by diluting the stock with RPMI-1640 medium and the final concentration of DMSO was <0.1%, which was not considered to be harmful to the cells.

Cell proliferation assay. The effect of SSD on cell proliferation was examined using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. SMMC-7721 cells were plated in 96-well plates at a density of $5x10^3$ cells per well and were allowed to grow to 70% confluence. After 24 h, the cells were randomly separated into four groups and were treated with SSD at 2.5, 5.0, 10 and 15 μg/ml, respectively. After 0, 24, 48 and 72 h, 20 μl of MTT test solution, which was freshly prepared, was added to each well. After 4 h incubation, the supernatant was discarded and 150 μl DMSO was added to dissolve the crystal. All analyses were performed in triplicate. The absorbance was measured on an ELISA reader (Thermo Fisher Scientific, Waltham, MA, USA) at a test wavelength of 490 nm. Proliferation inhibition rate (%) = (control well A490 - experiment well A490) / control well A490 x 100%.

Western blot analysis. Tumor cells were plated in 100 mm cell culture dishes (Nunc A/S, Roskilde, Denmark) with ~$300x10^4$ cells per dish. When cells grew to 60-70% confluence, they were randomly separated into different groups to be treated with either $CoCl_2$, $CoCl_2$ + rapamycin, $CoCl_2$ + AG490 or $CoCl_2$ + SSD. After 24 h, whole cell protein extracts were prepared by lysing cells with radioimmunoprecipitation assay lysis buffer supplemented with a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and phosphate inhibitor PhosStop (Roche Diagnostics). Protein concentration was quantified using the Bradford method. For western blotting, total cell lysates (~100 μg per lane) were subjected to SDS-PAGE. The protein was then transferred onto polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA, USA) using semi-dry transfer instruments (Bio-Rad Laboratories, Hercules, CA, USA) at 15 V for 30 min. The membranes were incubated with blocking buffer (0.05% Tween 20 with 5% nonfat milk) for 1 h at room temperature followed by anti-COX-2, anti-HIF-1α or anti-phospho-STAT3 primary rabbit anti-human monoclonal antibody (Cell Signaling Technology, Inc., Danvers, MA, USA; 1:1,000) dilution buffer overnight at 4˚C. Following washing three times with washing buffer (blocking buffer without 5% nonfat milk) for 10 min each time, the membranes were incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (polyclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:5,000) for 1 h at 37˚C. The membranes were washed again and detection was performed using an enhanced chemiluminescence western blotting detection system (Pierce Biotechnology, Inc., Rockford, IL, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). Total cellular RNA was isolated from each of the experimental groups using TRIzol solution (Invitrogen Life Technologies). RT was performed on RNA samples, followed by PCR amplification. For RT, 1.0 μg of the RNA sample was added to 20 μl of RT reaction mixture (Fermentas, Waltham, MA, USA). The reaction was performed by treating the samples at 65˚C for 5 min, at 42˚C for 60 min and at 70˚C for 5 min. PCR was conducted using the following primers specific for each of the target genes: HIF-1α, sense 5'-CATTAGAAAGCAGTTCCGCAAGC-3' and antisense 5'-CAGTGGTAGTGGTGGCATTAGC-3'; COX-2, sense 5'-AGTATCACAGGCTTCCATTGACCAG-3' and antisense 5'-CCACAGCATCGATGTCACCATAG-3'; β-actin, sense 5'-ATCGTGCGTGACATTAAGGAGAAG-3' and antisense 5'-AGGAAGGAAGGCTGGAAGAGTG-3'. The PCR was initiated in a thermal cycle programmed at 94˚C for 5 min, 94˚C for 30 sec, 58˚C for 30 sec, 72˚C for 60 sec, and amplified for 30 cycles with HIF-1α and β-actin, and 35 cycles with COX-2. The amplified products were visualized on 1.5% agarose gels.

Immunocytochemical staining. Immunocytochemical staining was performed on the coverslips obtained from the experimental groups. The antibodies against HIF-1α and COX-2 were purchased from Beijing Biosynthesis Biotechnology Co., Ltd (Beijing, China) and used according to the manufacturer's instructions. Briefly, the coverslips were incubated for 20 min in 3% H_2O_2. Following washing with phosphate-buffer saline, the coverslips were incubated with the appropriately diluted first antibody (1:400) at 4˚C overnight in a humid chamber, followed by treatment with biotinylated immunoglobulin for 12 min after washing, and then with streptavidin/horseradish peroxidase for 12 min at

37˚C. The color reaction was developed using diaminobenzidine working solution (Tiangen Biotech Co., Ltd., Beijing, China) for 3-5 min and counterstained with hematoxylin.

Statistical analysis. The results were analyzed for statistical significance using Student's t-test between the incubation conditions of normoxia and hypoxia under multiple exposure conditions using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

SSD inhibits SMMC-7721 cell proliferation and alters its cell morphology. MTT assay was used to detect the effect of SSD on SMMC-7721 cell proliferation. SMMC-7721 cells were treated with SSD at various concentrations (2.5, 5, 10 and 15 μg/ml) for 0, 24, 48 and 72 h. The results demonstrated that the growth inhibitory effect of SSD on SMMC-7721 cells was in a time- and dose-dependent manner (Fig. 1). Following treatment with 10 μg/ml SSD, SMMC-7721 cell proliferation activity was significantly reduced. Morphologically, the cells detached from the bottle and became round. In addition, a transparent vacuolar structure and pyknosis of the nucleus was observed (Fig. 2). This phenomenon was most clear at 72 h.

HIF-1α is necessary for COX-2 expression in HCC cells. Hypoxia commonly occurs in solid tumors and HIF-1α is critical in the hypoxia adaptation process. Several studies have investigated the importance of COX-2 in tumorigenesis and analysis has identified COX-2 as a direct target for HIF-1α in colorectal tumor cells (12,13). Furthermore, COX-2 upregulation represents a pivotal cellular adaptive response to hypoxia with implications for colorectal tumor cell survival and angiogenesis. Therefore, the present study aimed to determine whether COX-2 expression was controlled by HIF-1α in HCC. $CoCl_2$ was able to inhibit the degradation of HIF-1α under normoxic conditions and is used to simulate hypoxia in experiments (23). SMMC-7721 cells were subjected to $CoCl_2$-stimulated hypoxia and protein extracts were prepared over time at several concentrations of $CoCl_2$. Western blotting revealed that HIF-1α and COX-2 protein levels were rapidly induced by $CoCl_2$ in a time- and dose-dependent manner (Fig. 3A). The gene expression of HIF-1α and COX-2 was also detected. Although HIF-1α mRNA levels did not alter with different concentrations of $CoCl_2$, COX-2 mRNA levels increased under $CoCl_2$-stimulated hypoxic conditions in a dose-dependent manner in RT-PCR analysis (Fig. 3B).

To further examine the effect of HIF-1α on COX-2 induction, SMMC-7721 cells were treated with rapamycin, a reagent which could inhibit the synthesis of HIF-1α. Western blotting and RT-PCR analysis demonstrated that rapamycin eliminated COX-2 upregulation at the protein and mRNA levels under $CoCl_2$-stimulated hypoxia conditions (Fig. 3C and D). This suggested that HIF-1α was an upstream regulator of COX-2 expression in HCC.

Inhibition of STAT3 phosphorylation reduces the expression of HIF-1α and COX-2. The activated form of STAT3 (p-STAT3) is highly expressed in several malignancies, which has been demonstrated to induce HIF-1α protein synthesis in human breast tumor MCF-7 cells (24). However, the association between COX-2, HIF-1α and p-STAT3 in HCC cells remains to be elucidated. In order to determine the association between p-STAT3 and HIF-1α/COX-2, SMMC-7721 cells were treated with AG-490 for 30 min prior to the addition of $CoCl_2$. AG-490 is the selective inhibitor of JAK2, which can inhibit the activation of STAT3 (25). The results demonstrated that AG-490 resulted in the downregulation of HIF-1α and COX-2 at the protein level under the hypoxic conditions simulated by $CoCl_2$ (Fig. 4A). In order to confirm this effect, this was repeated on HepG2 cells and the result was the same as that observed in SMMC-7721 cells (Fig. 4B).

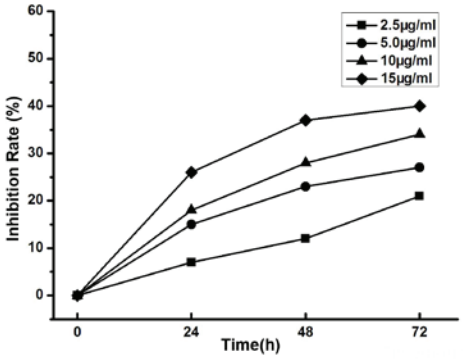

Figure 1. Effects of proliferation on SMMC-7721 cells treated with SSD. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay demonstrated that SSD inhibited the growth of SMMC-7721 cells in a dose- and time-dependent manner. A higher inhibition rate corresponded to higher drug doses and longer drug treatment time periods. Values are expressed as the mean ± standard deviation of three independent experiments. *$P<0.05$, compared with the untreated control. SSD, Saikosaponin-d.

Effects of SSD on the protein expression of p-STAT3, HIF-1α and COX-2. In order to determine the mechanism of SSD targeting in HCC cells, the expression of COX-2, HIF-1α and p-STAT3 was determined by immunocytochemistry following SSD treatment. The results suggested that p-STAT3 staining in SMMC-7721 tumor cells demonstrated nuclear localization, with HIF-1α located in the cytoplasm and particularly in the nucleus, and COX-2 expressed in the cytoplasm and nuclear membrane. SSD not only significantly reduced the expression of HIF-1α and COX-2 induced by $CoCl_2$, but also deceased the expression of p-STAT3 and COX-2 induced by IL-6 (Fig. 5). These results were partially verified by western blotting, which indicated that SSD inhibited the protein expression of COX-2, HIF-1α and p-STAT3 (Fig. 6).

Discussion

HCC is one of the most common types of malignancy worldwide, the incidence and mortality rate of HCC are extremely high in Asia and have also increased rapidly in the United States (1,2). Due to the occult onset of HCC, the majority of HCC patients are at an advanced stage when diagnosed and to date there remains no completely effective therapy

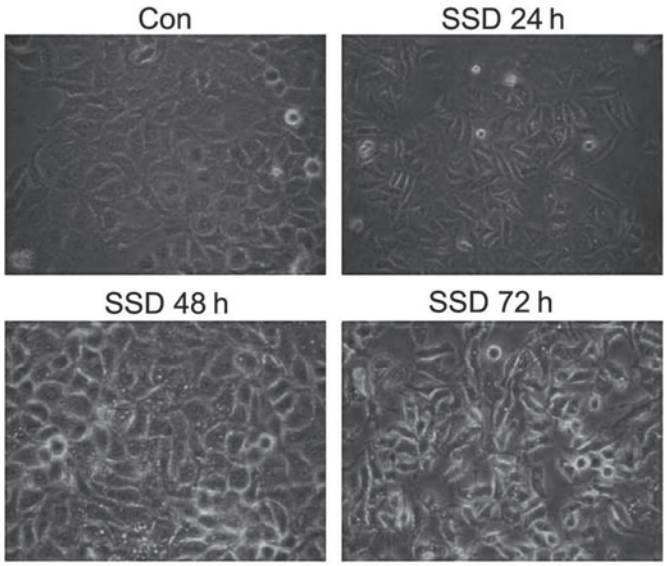

Figure 2. SSD suppresses SMMC-7721 cell growth in a time-dependent manner. Cell growth was inhibited, the number of cells markedly decreased with time and the shape of SMMC-7721 cells became round and detached from the bottle. Con, control; SSD, Saikosaponin-d.

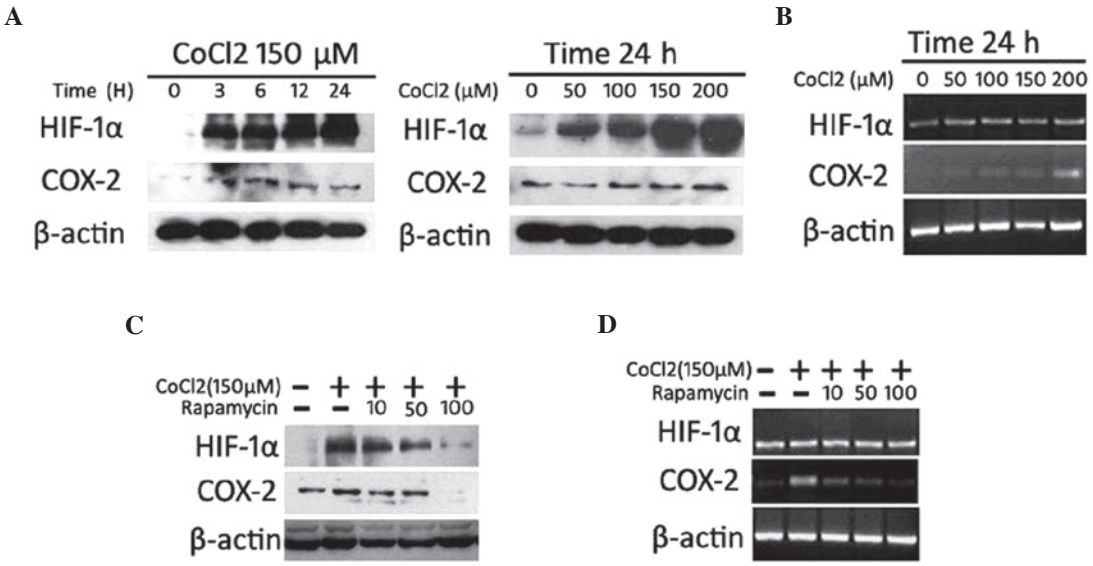

Figure 3. HIF-1α is necessary for COX-2 expression. (A) Western blot analysis of HIF-1α and COX-2 in SMMC-7721 cells simulated by hypoxia induced by $CoCl_2$. (B) RT-PCR analysis of HIF-1α and COX-2 in SMMC-7721 cells simulated by hypoxia induced by $CoCl_2$. (C) Western blot analysis of HIF-1α and COX-2 in SMMC-7721 cells exposed to $CoCl_2$ in the presence of increasing concentrations of rapamycin for 2 h. (D) RT-PCR analysis of HIF-1α and COX-2 in SMMC-7721 cells exposed to $CoCl_2$ in the presence of increasing concentrations of rapamycin for 24 h. $CoCl_2$, cobalt chloride; HIF-1α, hypoxia-inducible factor 1α; COX-2, cyclooxygenase-2; RT-PCR, reverse transcription-polymerase chain reaction.

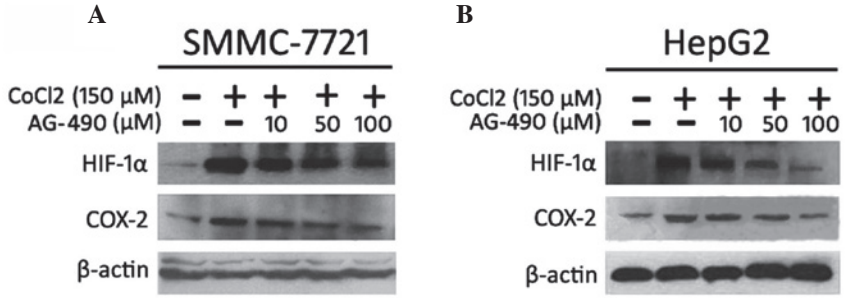

Figure 4. AG490 inhibits HIF-1α and COX-2 protein expression. AG490 inhibited HIF-1α and COX-2 protein expression in (A) SMMC-7721 cells and (B) HepG2 cells in a dose-dependent manner. $CoCl_2$ (150 μmol/l) treatment was used to simulate hypoxic conditions. $CoCl_2$, cobalt chloride; COX-2, cyclo-oxygenase-2; HIF-1α, hypoxia-inducible factor 1α.

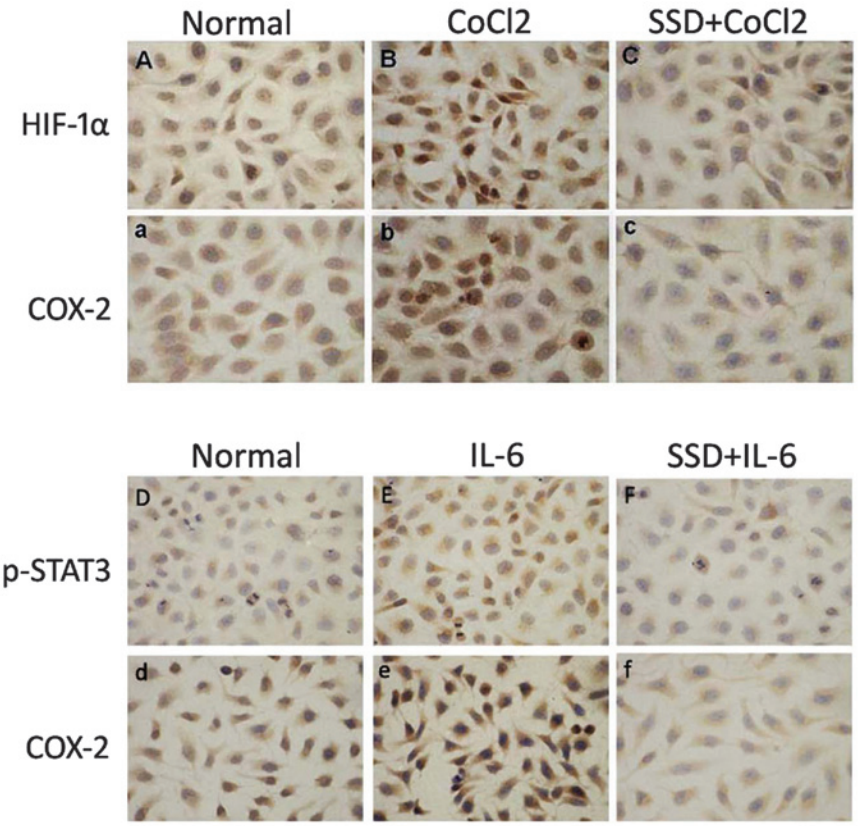

Figure 5. Immunocytochemical staining of p-STAT3, HIF-1α and COX-2 protein in SMMC-7721 cells. (A and a) Respective expression of HIF-1α and COX-2 in normally controlled SMMC-7721 cells; (B and b) Respective expression of HIF-1α and COX-2 in SMMC-7721 cells exposed to $CoCl_2$ (150 μmol/l) for 24 h; (C and c) Respective expression of HIF-1α and COX-2 in SMMC-7721 cells treated with SSD (10 μg/ml) for 30 min prior to the addition of $CoCl_2$ (150 μmol/l). (D and d) Respective expression of p-STAT3 and COX-2 in normally controlled SMMC-7721 cells; (E and e) Respective expression of p-STAT3 and COX-2 in SMMC-7721 cells exposed to IL-6 (25 ng/ml) for 24 h; (F and f) Respective expression of p-STAT3 and COX-2 in SMMC-7721 cells treated with SSD (10 μg/ml) for 30 min prior to the addition of IL-6 (25 ng/ml). HIF-1α, hypoxia-inducible factor 1α; COX-2, cyclooxygenase-2; SSD, Saikosaponin-d; p-STAT3, phosphorylated signal transducer and activator of transcription 3; IL-6, interleukin 6.

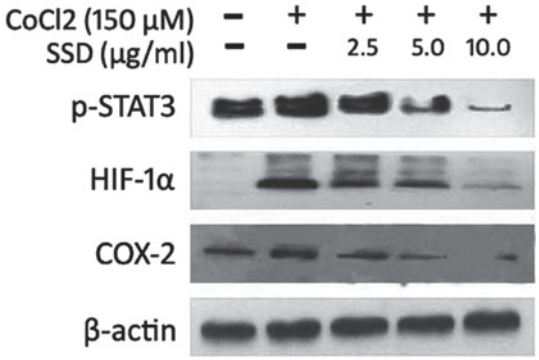

Figure 6. SSD inhibits p-STAT3, HIF-1α and COX-2 protein expression. $CoCl_2$ was used to simulate hypoxic conditions. SSD significantly reduced the protein expression of p-STAT3, HIF-1α and COX-2 induced by $CoCl_2$, in a dose-dependent manner. SSD, Saikosaponin-d; HIF-1α, hypoxia-inducible factor 1-α; COX-2, cyclooxygenase-2; pSTAT3, phosphorylated signal transducer and activator of transcription 3.

for HCC (3). Numerous active compounds extracted from traditional plants, including SSD, have been demonstrated to have anti-tumor activities (10,11). SSD could not only inhibit growth and differentiation of human leukemia cells (26) and glioma cells (27), but was also be able to increase the radiosensitivity of HCC SMMC-7721 cells by adjusting the G_0/G_1 and G_2/M checkpoints of the cell cycle (28). The present study demonstrated that SSD inhibited COX-2 expression through the STAT3/HIF-1α signaling pathway, which may be the specific antitumor mechanism of SSD.

COX, the key enzyme for prostanoid biosynthesis, has two isoforms: COX-1 and COX-2. COX-1 is constitutively expressed in several tissues and cell types, whereas COX-2 is an inducible enzyme expressed only in response to certain stimuli. COX-2 is overexpressed in a subset of malignant tumors and accumulating evidence suggests that COX-2 may be important in tumorigenesis through multiple mechanisms (15). Previous studies have confirmed that COX-2 is not only overexpressed in HCC, but also correlates well with the differentiation grades of HCC (21). Our previous study (14) found that SSD inhibited COX-2 expression in HCC SMMC-7721 cells, which confirmed the hypothesis that SSD, with similar pharmacological activities as NASIDs, could inhibit SMMC-7721 proliferation through the COX-2 pathway.

The association between tumors and microenvironments has attracted more attention (29). The hypoxic microenvironment, one of the basic features in solid tumors, characterized by deficiency in oxygen and nutrients, leads to epigenetic and genetic adaptation of clones and an increase in invasiveness and metastasis (30). These hypoxic adaptations, including

increasing vascularization, activation of proto-oncogenes, increasing glucose transportation and inducing glycolytic enzymes and various apoptotic-related genes make the tumors more difficult to treat and confers increased resistance to chemotherapy and radiotherapy (31). It is believed that the HIF-1 complex, composed of a heterodimer pair of HIF-1α and HIF-1β is important in mediating these adaptations. At present, HIF-1α has emerged as an important transcription factor in cancer biology and is expressed in the early stages of several types of human malignant tumor, including HCC (32). Csiki *et al* (33) demonstrated that COX-2 is upregulated in hypoxic lung cancer cells in an HIF-1-dependent manner. Another study provided the first evidence, to the best of our knowledge, demonstrating that HIF-1 directly binds a specific hormone response element located at the COX-2 promoter (34). Dai *et al* (35) demonstrated a positive correlation between HIF-1α and COX-2 in HCC. The present study found that hypoxia, imitated by $CoCl_2$, could induce the expression of HIF-1α accompanied by the protein level of COX-2 in SMMC-7721 cells. Rapamycin, a selective inhibitor of mTOR, could inhibit the expression of HIF-1α and the COX-2 protein, and SSD had a similar effect. The results of the present study were consistent with previous studies, demonstrating that HIF-1α was obligatory for COX-2 expression in HCC cells and is possibly an important upstream factor for COX-2.

The level of HIF-1α can be regulated not only by hypoxia through a ubiquitin-proteasome pathway, but can also be modulated by several other pathways. The JAK/STAT3 pathway appears to be important in modulating HIF-1α expression. Activated STAT3 can increase HIF-1α protein levels by inhibiting HIF-1α degradation and accelerating its *de novo* synthesis in ischemic rat kidneys and hypoxic human renal carcinoma cells (36). STAT3 knockout eliminates estrogen receptor-α-induced HIF-1α and subsequent vascular endothelial growth factor (VEGF) production (37). In human breast cancer MCF-7 cells, STAT3 regulates HIF-1α, and targeting STAT3 with siRNA knockdown inhibits $CoCl_2$-mediated HIF-1α nuclear accumulation and recruitment on the VEGF promoter (38). The present study demonstrated that activated STAT3 was involved in the expression of HIF-1α in HCC cells, and p-STAT3 was able to increase HIF-1α protein levels but not mRNA levels. This suggested that activation of STAT3 modulated HIF-1α expression through transcriptional or posttranscriptional mechanisms in HCC. The results of the present study suggested that p-STAT3 may be the upstream regulator of HIF-1α and COX-2 and that there was a p-STAT3/HIF-1α/COX-2 signal transduction pathway in HCC SMMC-7721 cells. In the present study, SSD inhibited SMMC-7721 growth accompanied by a reduction in the expression of p-STAT3, HIF-1α and COX-2. This indicated that SSD may suppress HCC SMMC-7721 proliferation by inhibiting the expression of COX-2 through the p-STAT3/HIF-1α signaling pathway.

In conclusion, the present study provided primary evidence that HIF-1α promoted COX-2 expression under hypoxic conditions in HCC cells and HIF-1α was induced by activated STAT3. SSD may suppress HCC SMMC-7721 cell proliferation by inhibiting the expression of COX-2 through the p-STAT3/HIF-1α signaling pathway.

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