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Schizandrin B mitigates rifampicin-induced liver injury by inhibiting endoplasmic reticulum stress

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ABSTRACT

*Schisandra chinensis* is widely used and effective in protecting liver. There are many mechanisms of drug-induced hepatocyte injury, among which endoplasmic reticulum (ER) stress-induced cell injury plays an important role. However, little is known about whether *schisandra chinensis* can inhibit rifampicin (RFP)-induced hepatocyte injury by affecting ER stress. In our study, firstly, L02 cells were treated with different concentrations of RFP for different time intervals, and the apoptosis, survival rate and endoplasmic reticulum stress gene and protein expressions of GRP78, PERK, ATF4, CHOP, ATF6, ARMET, p-IRE1 and XBP-1 were measured. We found that RFP increased apoptosis of L02 cells, decreased cell survival, and increased the gene and protein expression levels of GRP78, PERK, ATF4, CHOP, ATF6, ARMET, p-IRE1 and XBP-1, suggesting that RFP could induce hepatocyte injury, and the degree of injury was positively correlated with the dose and time of RFP. Next, we treated RFP-damaged hepatocytes with Schizandrin B. We found that schizandrin B increased cell survival rate in dose-dependent and time-dependent manner, decreased cell apoptosis rate, and reduced protein and gene expression levels of GRP78, PERK, ATF4, CHOP, ATF6, ARMET and XBP-1. These results indicate that schizandrin B alleviates RFP-induced injury in L02 cells by inhibiting ER stress.

**Keywords:** Rifampicin; Schizandrin B; Endoplasmic reticulum stress; L02 cells; Cell injury
INTRODUCTION

The endoplasmic reticulum (ER) is a very important subcellular organelle; it is distributed throughout the cytoplasm of all eukaryotic cells, except mature red blood cells, and maintains cell homeostasis by inducing cell responses to internal and external stimuli, mainly by promoting new intracellular synthesis. Proteins are modified, folded and oligomerized to form the correct conformation and to facilitate transport\(^1\). When cells are damaged by external factors, the body produces an adaptive response, namely the unfolded protein response (UPR), to restore cell homeostasis. Several studies have shown that the UPR may be beneficial to cells\(^2,3,4\), but severe ER stress and defective UPR can cause cell death and inflammation\(^5\). Studies have shown that acetaminophen (APAP) can induce drug toxicity by promoting ER stress\(^6\).

The main causes of drug-induced liver injury (DILI) in mainland China are Chinese herbal medicines, antituberculosis drugs and dietary supplements, and the annual incidence of DILI in China was approximately 23.80 per 100,000, much higher than in other countries\(^7\). Rifampicin (RFP) is a drug widely used in the clinical treatment of tuberculosis in mainland China and has been reported to cause liver damage in many cases\(^8,9,10\). Regarding the mechanism of RFP-induced liver injury, studies have confirmed that RFP can induce ER stress and produce hepatotoxicity, while 4-phenylbutyrate can reduce RFP-induced injury by inhibiting the PERK-ATF4-CHOP pathway during ER stress\(^11\).

Schisandra chinensis (SC) is a commonly used traditional Chinese medicine included in the "Pharmacopeia of the People's Republic of China" and has liver protective functions. Pharmaceutical preparations of the main components of this medicine are widely used in the treatment of liver dysfunction. These pharmaceutical preparations may not only have protective effects on nonalcoholic fatty liver disease through the upregulation of PPAR-\(\alpha/\gamma\) and the downregulation of NF-\(\kappa B\) signaling pathway\(^12\) but also attenuate APAP-induced liver damage by inhibiting oxidative stress\(^13\). Recently, it was discovered that the extract of Schisandra chinensis inhibited alcohol-induced oxidative stress in rats\(^13,14\) and reduced the severity of liver injury\(^15\). SC polysaccharide has also been reported to inhibit liver lipid accumulation in mice with nonalcoholic fatty liver disease by downregulating the expression
of sterol regulatory element-binding proteins\textsuperscript{16}). The main bioactive substance involved in the hepatoprotective effect of SC are lignans, including deoxyschizandrin, gomisin A and gomisin N. Among SC lignans, schisandrol B and schisantherin A have the strongest liver protection effects against APAP-induced hepatotoxicity\textsuperscript{17, 18}). Studies have found that gomisin N can inhibit ER stress, prevent the accumulation of triglyceride in HepG2 cells, and treat hepatic steatosis\textsuperscript{19}). schisandrol B protected mice from APAP-induced liver injury by activating the NRF2/ARE signaling pathway\textsuperscript{20}). However, whether SC can protect the drug-induced liver injury caused by RFP by inhibiting the ER stress pathway has not been reported. Therefore, our study aimed to explore whether and how the ER stress signaling pathway promotes the protective effect of schizandrin B (Sch B) (Fig. 1)on RFP-induced toxicity.

**MATERIALS AND METHODS**

**Drugs**

Schizandrin B (110765) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products. RFP (Prod #R3501) was purchased from Sigma-Aldrich (St. Louis, MO, USA). schizandrin B was dissolved in a DMSO (Sigma-Aldrich) solution to a final concentration of 0.1 mol/L. The final concentration of RFP was 100 mg/mL in DMSO.

**Cell culture**

L02 cells (normal human liver cells) were purchased from the Fudan Cell Bank. The cells were cultured in RPMI-1640 medium, which was purchased from HyClone Corporation (Logan, UT, USA). The RPMI-1640 medium was mixed with penicillin (100 U/mL), fetal bovine serum (10%, heat-inactivated), and streptomycin (100 µg/mL) according to the user instructions. The cells were kept in an environment with constant humidity at 5% CO\textsubscript{2} at 37°C. After the fourth generation and when 60-70% confluency was reached, the L02 cells were used for experiments.

**MTT assay**

Cell viability was determined using the MTT assay. Briefly, L02 cells in the culture dish were evenly distributed and initially plated at a density of $5 \times 10^3$ cells/well (using a 96-well
plate). After successful plating, the cells were incubated for 24 h with antibiotic-free and serum-free medium. After 24 h, fresh RPMI-1640 medium was used instead of the culture supernatant. Different concentrations of schizandrin B and RFP were added to the cells for 48 or 72 h. The L02 cells were washed twice in phosphate-buffered saline (PBS) buffer after the medium was removed. MTT (Sigma-Aldrich) was dissolved in PBS to a concentration of 5 mg/mL. Then, a mixed solution, consisting of MTT solution (10 μL) and RPMI-1640 medium (90 μL), was added to each well. Next, the cells were incubated in the mixed solution at 37 °C for 4 h, after which the solution was removed from each well and replaced with 150 μL of DMSO. Finally, each 96-well plate was shaken on a shaker at room temperature for 15 min, and the absorbance of each well was measured at 570 nm using a microplate reader (SPECTRA MAX 190, MD, USA). The control cell viability was set to 100%, and cell viability was determined by comparing the number of viable cells with control cells.

**Western blot analysis**

After treating with schizandrin B and RFP, all L02 cells were first collected and then lysed with RIPA lysis buffer. The RIPA lysis buffer consisted of 1% Triton X-100, 50 mM Tris-HCl (pH 8.0), 100 mM methylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 10% SDS, 10% sodium deoxycholate and 10% NP-40. After cell lysis, proteins were extracted. Then, an equal amount of protein from each sample and a known molecular weight marker were applied to a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel for electrophoresis. The gels were divided into 12% and 8%. After electrophoresis, the proteins were first transferred to polyvinylidene difluoride (PVDF) membranes for 150 min, and then the membranes were blocked with 5% skim milk for 1 hour at room temperature. After blocking, the membranes were washed three times with PBST for 10 min each time. PBST was prepared by mixing 0.1% Tween-20 and 0.01 M PBS. Then, the membranes were probed with primary antibodies against the following proteins at the indicated dilutions by incubating overnight at 4 °C in blocking buffer: ATF4 (1:1,000), 78 kDa glucose-regulator protein (GRP78) (1:1,000), ATF6 (1:1,000), PERK (1:1000), ARMET (1:1,000), XBP-1 (1:1,000), CHOP (1:1,000), p-IRE1 (1:1,000) and β-actin (1:5,000). The antibodies against
ATF4, GRP78, ATF6, PERK, ARMET, XBP-1, CHOP, and p-IRE1 were obtained from Cell Signaling Technology (Beverly, MA, USA). The β-actin antibody was purchased from Proteintech. After the membranes were washed three additional times, the membranes were incubated with a horseradish peroxidase secondary antibody (mouse or rabbit, 1:4,000) for one hour on a shaker at room temperature. Then, enhanced chemiluminescence (ECL) regents were added, which were purchased from Thermo Fisher Scientific (Waltham, MA, USA), and the membranes were detected by using a Gel Catcher 3400 (Shanghai, China). Finally, the data were analyzed using a gel recording system from Clinx (Shanghai, China).

**Immunofluorescence staining**

Poly-lysine-coated foamed, rinsed and autoclaved coverslips were prepared. Then, the L02 cells were digested and inoculated onto the poly-lysine-coated coverslips in a 24-well plate overnight. One treatment group was given 200 μM RFP, another treatment group was given 200 μM RFP and 100 μM schizandrin B, and the blank group was given the same volume of DMSO. After 48 h of treatment, the cell-covered coverslips were removed, washed 3 times with precool PBS, and fixed with precooled 4% paraformaldehyde at 4 °C for 20 min. Then, the coverslips were washed three times with PBS for 5 min after the fixative was discarded, and subsequently, the coverslips were sealed with 0.1% bovine serum albumin (BSA) solution at room temperature for 30 min. Afterwards, the blocking solution was discarded, and the primary antibody at a dilution of 1:50 was added and incubated at room temperature for 1 hour. The primary antibody was discarded, and the coverslips were washed 3 times with PBS for 10 min each time. Fluorescently labeled secondary antibody at a dilution of 1:200 was added in the dark and incubated for 2 h at room temperature. Then, after the secondary antibody was discarded, the coverslips were washed 3 times with PBS for 10 min each time in the dark. Finally, the coverslips were mounted with 80% glycerol, viewed under a fluorescence microscope (Olympus IX73P2F, SN: 2J44787) and photographed.

**Flow cytometric assay**

L02 cells were treated with drugs. Then, each group of cells was separately collected into a flow tube and centrifuged by a centrifuge (MSD97K49, Beckman, USA) at 1000 rpm.
for 5 min. After centrifugation, the supernatant was decanted out of the flow tubes. Then, each flow tube was washed with 1 mL of PBS and centrifuged at 1000 rpm for 5 min. Afterwards, the PBS was discarded. After adding 50 μL of 1 x binding buffer and 2.5 μL of Annexin-V-FITC (20 μg/mL) to each flow tube, the cells were incubated in the refrigerator at 4 °C for 30 min in the dark or incubated for 15 min at room temperature in the dark. Next, the cells in each flow tube were incubated with 250 μL of 1 x binding buffer and 2.5 μL of PI (50 μg/mL) for 5 min in the dark. The cells were detected by flow cytometer (Flowsight, Merck, Germany) within 1 hour (note: a negative control tube was included). Cells negative for both Annexin V-FITC and PI staining were considered living cells. Cells positive for Annexin V-FITC were considered early apoptotic cells, while cells positive for PI alone were considered necrotic cells. Cells positive for both Annexin V-FITC and PI were considered late apoptotic cells. Apoptosis is the sum of the early apoptotic cells and late apoptotic cells, accounting for the percentage of all cells.

**Quantitative reverse transcription polymerase chain reaction (qRT-PCR)**

All test procedures were performed in accordance with the manufacturer's instructions, and total RNA was separated by using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). The RNA concentration was measured by using a Quawell Q5000 (San Jose, CA, United States), and RNA was reverse-transcribed by using a reverse transcription system (TaKaRa Bio, Japan). The PCR consisted of the following components in a final volume of 10 μL: 0.4 μL of 5' and 3' primers at a concentration of 10 μmol/L; 1 μL of the cDNA samples; 5 μL of SYBR Premix Ex Taq™ II; and 3.2 μL of ddH2O. Amplification was carried out in the following three steps for 40 cycles: denaturation at 95°C for 5 s, annealing at 60°C for 30 s, and extension at 60°C for 30 s. β-actin was used as an internal control. The primer sequences (5' to 3') were as follows: GRP78 forward, tggctggaagccccagcaag and GRP78 reverse, cttcaccagttggggaggg; XBP1 forward, catggcagccccagcag and XBP1 reverse, cttctcctgtgggctcaggt; PERK forward, aggtgactgtggagcct and PERK reverse, aaggcttggcctccactgga; ATF4 forward, cagcagccactaggtaccg and ATF4 reverse, ttctctccctgcctctct; CHOP forward, tcttcctcctctctcctg and CHOP reverse, cactctgacctgcttct; ATF6 forward, getgccagccacccaga and ATF6 reverse,
gagggcagaactccaggtgc; ARMET forward, cgacctgagcacagtggacc and ARMET reverse, ggctgccttgggggcatatt; and β-actin forward, gagctacgagctgcctgacg and β-actin reverse, tgccagggcagtcatctct.

Statistical analysis

All of the data were statistically analyzed using SPSS 13.0 (Chicago, IL, United States) software. Experimental results are presented as the mean ± standard error of the mean (SEM) as determined from more than three separate experiments. One-way analysis of variance was used for comparisons among groups, and then the results were subjected to a least significant difference test. Differences with $P < 0.05$ were considered significant.

RESULTS

Effect of RFP and schizandrin B on the survival rate of L02 cells

Different concentrations of schizandrin B and RFP were applied for 48 h, and the absorbance was measured by MTT to calculate the IC50. The MTT assay was used to detect the inhibitory effect of schizandrin B on normal human hepatocyte activity, and the results showed that the inhibition rate of treated cells reached 50% at 180.1 μM (Fig. 2A). When the concentration was 50 μM, there was no inhibitory effect on cell viability; thus, 50 μM was selected as the protective concentration, and the experiment was carried out. The MTT method confirmed that at an RFP concentration of approximately 200 μM, the cell inhibition rate reached 50% (Fig. 2B); thus, 200 μM was selected as the model concentration. The L02 cells were administered 200 μM RFP or the combination of 50 μM schizandrin B and 200 μM RFP for 72 h. The results showed that the combination of schizandrin B and RFP significantly increased the survival rate of L02 cells, indicating that schizandrin B can reduce the damage of RFP on L02 cells (Fig. 2C).

Effects of RFP on the expression of ER stress pathway proteins and genes

In this study, L02 cells were treated with 200 μM RFP for 12 h, 24 h, or 48 h, as shown in Figure 3A. RFP can activate the ER stress pathway and increase the protein expression of GRP78, PERK, ATF4, CHOP, ATF6, ARMET, p-IRE1 and XBP-1 in time-dependent manner, with the most significant expression occurring at 48 h. At the gene level, L02 cells were treated with 200 μM RFP for 12 h, 24 h, or 48 h. RFP enhanced the gene expression of
GRP78, PERK, ATF4, and CHOP in a time-dependent manner (Fig. 3B). We found that changes in gene expression began to occur 12 h after treatment. At 12 h, RFP induced the mRNA expression of ATF4 (p < 0.05) and CHOP (p < 0.01) and did not significantly affect the mRNA expression of PERK and GRP78. At the 24-hour time point, RFP began to activate the mRNA expression of PERK (p < 0.05) and the mRNA expression of GRP78 (p < 0.01). At 48 h, the gene expression of GRP78, PERK, ATF4, and CHOP was significantly increased (both p < 0.01). Taken together, these results indicated that 200 μM RFP had an effect on the ER stress pathway at the gene and protein levels in a time-dependent manner.

**Schizandrin B inhibits the effect of RFP on the expression of ER stress pathway proteins and genes**

To determine whether schizandrin B could inhibit RFP-induced ER stress, L02 cells were treated with 200 μM RFP or the combination of 25 μM, 50 μM, or 100 μM schizandrin B and 200 μM RFP for 48 h. Our results indicated that schizandrin B can inhibit the RFP-induced protein expression of GRP78, PERK, ATF4, CHOP, ATF6, ARMET and XBP-1 in a concentration-dependent manner (Fig. 4A and 4C). At the gene level, schizandrin B could inhibit the RFP-induced gene expression of GRP78, PERK, ATF4, CHOP, ATF6, ARMET and XBP-1 (both p <0.01) in a concentration-dependent manner (Fig. 4B and 4D). The results showed that schizandrin B could inhibit the RFP-induced expression of proteins and genes in the ER stress pathway and that the effect was concentration-dependent.

**Schizandrin B inhibits the RFP-induced apoptosis of L02 cells**

To determine whether schizandrin B could inhibit RFP-induced apoptosis in L02 cells, L02 cells were treated with 200 μM RFP or the combination of 50 μM schizandrin B and 200 μM RFP for 48 h and then subjected to flow cytometry. The combined administration of schizandrin B and RFP significantly reduced the level of RFP-induced apoptosis (Fig. 5A). We then treated L02 cells with 25 μM, 50 μM, or 100 μM schizandrin B and 200 μM RFP for 48 h and detected apoptotic factors. The results showed that schizandrin B could inhibit the RFP-induced expression of the apoptotic factors PARP, Caspase3 and cleaved caspase3 and inhibited protein expression. The effect was concentration-dependent (Fig. 5B). These two experiments showed that schizandrin B could effectively inhibit the RFP-induced apoptosis.
of L02 cells in a concentration-dependent manner.

**The effect of schizandrin B on the protein localization of GRP78, PERK, ATF6, p-IRE1 and XBP-1 in RFP-treated L02 cells**

Immunofluorescence experiments indicated that the protein expression of GRP78, PERK, ATF6, p-IRE1 and XBP-1 in the control group was limited and that these proteins did not enter the nucleus. The protein expression of GRP78, PERK, ATF6, p-IRE1 and XBP-1 in the group treated with RFP alone was significantly higher than in the control group, and these proteins entered the nucleus. Compared with the RFP-treated group, the combined schizandrin B and RFP treatment decreased the expression of the above proteins and reduced the nuclear import of these proteins (Fig. 6), indicating that schizandrin B could inhibit the expression of RFP-induced ER stress pathway-related proteins in cells.

**DISCUSSION**

SC is a traditional Chinese herbal medicine that has been used for thousands of years. SC has not only antioxidant, anticancer, anti-inflammatory, and heart-protective effects but also a very good liver protective effect21). The ER stress-induced inflammatory response is an important mechanism of liver steatosis 22). Both in vivo and in vitro experiments showed that SC extract (SCE) effectively attenuated ER stress, improved symptoms of nonalcoholic fatty liver and prevented the development of nonalcoholic fatty liver23). Through the Nrf2-mediated signaling pathway, SCE could regulate the effect of drug-metabolizing enzymes and drug transporters to protect the liver24). Schizandrin B is an extract of SC with a strong antioxidant effect25). According to a previous report, schizandrin B effectively enhanced the antioxidant status of mitochondrial glutathione and induced the expression of heat shock protein 25/70, thereby reducing the toxic effect of CCl4 on liver cells; this protective effect was especially obvious under oxidative stress26, 27). Pretreatment with schizandrin B could reduce the degree of damage to I/R injured cardiomyocytes in rats28). In addition, this protection might work through the PI3K/Akt signaling pathway29). Schizandrin B also has an effect on the endoplasmic reticulum stress pathway. By inhibiting its PERK and ATF6 pathway, schizandrin B could attenuate myocardial cell apoptosis and play a protective role in myocardial I/R injury30). Previous studies have shown that schizandrin B has a good
protective effect on the liver, but as far as we know, whether this protective mechanism is related to ER stress has not been previously reported.

Liver injury is the most common adverse reaction caused by antituberculosis drugs in antituberculosis treatment. Patients may stop antituberculosis treatment due to antituberculosis drug-induced liver injury, which may lead to severe liver failure\(^{31}\). In recent years, there have been many studies on ER stress. The activation of the ER stress pathway could induce apoptosis, while xanthatin can induce the apoptosis of liver cancer cells by activating ER stress\(^{32}\). In normal liver cells, RFP also induced liver injury via the activation of ER stress\(^{33}\).

The three signaling pathways in the UPR, namely PERK-ATF4-CHOP, ATF-6 and IRE1-XBP1, maintain ER homeostasis. After ER function is damaged and unfolded proteins accumulate in the ER lumen, the UPR sensor responds by activating these three signaling pathways. When ER stress persists too long or is too severe, apoptosis signals prevail\(^{34}\). The stress level of the ER is characterized by protein misfolding, unfolded protein aggregation in the cavity and disordered calcium ion balance\(^{35}\). GRP78, which is regarded as the main regulator of the UPR, binds unfolded proteins in the absence of ER stress. GRP78 releases three UPR signals when unfolded proteins accumulate in the ER\(^{36}\). Each of three UPR sensory proteins—IRE1, ATF6 and PERK—are activated by ER stress. Once IRE1\(\alpha\) isolates GRP78 and activates RNase activity, immature XBP1 transcripts are processed into mature XBP1 mRNA\(^{37}\). XBP1 is a transcription factor that improves the expression of GRP78\(^{38}\). Therefore, GRP78 is one of the core participants in ER stress response\(^{39}\). By activating PERK, eIF2\(\alpha\), and ATF4 can further activate the transcription of genes involved in cell apoptosis, including CHOP, which plays a vital role in ER stress-mediated apoptosis and has been involved in many diseases, including liver diseases, diabetes, neurodegenerative disease and metabolic diseases\(^{40}\). Within contrast to the IRE1 and ATF6 signaling pathways, the activation of the PERK signaling pathway is largely sustained when ER stress is not alleviated and may drive cell death\(^{41}\). Previous research has shown that the PERK-eIF2\(\alpha\) pathway mediates the induction of human bronchial epithelial cell apoptosis by cigarette smoke extract\(^{42}\). In cultured cells, ATF4 induces the transcription of genes related to the
PERK signaling pathway in the UPR, including genes involved in antioxidant response and amino acid metabolism and the apoptotic transcription factor CHOP (GADD153/DDIT3)\textsuperscript{43}).

CHOP, also known as growth arrest and DNA damage 153, is a 29 kDa protein that is significantly increased when cell homeostasis is disturbed by ER stress\textsuperscript{44}). Thus, CHOP is widely believed to be the main factor that regulates apoptosis during ER stress. Additionally, apoptotic molecules, such as death receptor 5 and ER oxidase, are considered targets of CHOP\textsuperscript{45}). The increased expression of several classical ER stress signals, such as GRP78, IRE1α, PERK and CHOP, and the activation of the ER stress pathway, can inhibit the growth of glioma cells\textsuperscript{46}). In this study, we first observed that increasing RFP concentrations and treatment times gradually decreased the survival rate of L02 hepatocytes, gradually increased the apoptotic rate, and upregulated GRP78, PERK, ATF4, CHOP, ATF6, ARMET, p-IRE1 and XBP-1 expression, suggesting that the ER stress pathway was widely activated. Next, we administered schizandrin B to treat hepatocytes damaged by RFP. We found that the survival rate of damaged hepatocytes was improved and that the apoptotic rate was decreased. Moreover, the expression of GRP78, PERK, ATF4, CHOP, ATF6, ARMET and XBP-1 was expression, suggesting that the ER stress pathway was inhibited. The degree of inhibition was positively correlated with the concentration of schizandrin B.

These results indicate that schizandrin B is a potential alternative to treat RFP-induced liver damage. The mechanism of the hepatoprotective action of schizandrin B may be the inhibition of the ER stress pathway. Because our experiments were performed in vitro using only one cell line, this mechanism should be further validated in animal studies in the future.

\textbf{ACKNOWLEDGEMENTS}

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\textbf{Conflict of interest}

The authors declare no conflict of interest.
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Fig. 1. The chemical structure of Schizandrin B (Sch B).
Fig. 2. Effect of RFP and schizandrin B on the survival rate of L02 cells. (A) Effect of different concentrations of schizandrin B on the cell viability of L02 cells. (B) Effect of different concentrations of RFP on the cell viability of L02 cells. (C) Effect of 200 μM RFP and the combination of 50 μM schizandrin B and 200 μM RFP on the cell viability of L02 cells at different times (0 h, 4 h, 8 h, 12 h, 24 h, 48 h, 72 h). The experiment was repeated three times.
Fig. 3. The effect of RFP on the expression of ER stress pathway proteins and genes. (A) L02 cells were treated with 200 μM RFP for 12 h, 24 h or 48 h. Western blot analysis showed that RFP could upregulate the protein expression of GRP78, PERK, ATF4, CHOP, ATF6, ARMET, p-IRE1 and XBP-1 in a time-dependent manner, with the most significant expression occurring at 48 h. (B) Treatment of L02 cells with 200 μM RFP for 12 h, 24 h, and 48 h. After 12 h, RFP began to significantly increase ATF4 and CHOP gene expression. After 24 h, RFP began to significantly increase GRP78, PERK gene expression in a time-dependent manner. The gene expression of the control group was set to 1.0, and the experimental group data were compared with the control group and expressed as the mean ± SEM. P < 0.05 was considered to be significant (* p < 0.05 and ** p < 0.01). All experiments were repeated three times.
Fig. 4. Effect of RFP on ER stress pathway protein and gene expression. L02 cells were treated with 200 μM RFP or the combination of 25 μM, 50 μM, or 100 μM schizandrin B and 200 μM RFP for 48 h. (A) Western blot analysis showed that 25 μM schizandrin B could inhibit RFP-induced GRP78, PERK, ATF4, and CHOP protein expression, and the inhibitory effect of schizandrin B on protein expression was concentration-dependent. (B) q-PCR analysis showed that 25 μM schizandrin B inhibited the RFP-induced gene expression of GRP78, PERK, ATF4, and CHOP, and the inhibitory effect of schizandrin B on gene expression was concentration-dependent. (C) Western blot analysis showed that 25 μM schizandrin B could inhibit the expression of ATF6, ARMET and XBP-1 induced by RFP, and the inhibitory effect of schizandrin B on protein expression was concentration-dependent. (D) q-PCR analysis showed that 25 μM schizandrin B could inhibit RFP-induced ATF6, ARMET, XBP-1 gene expression, and the inhibitory effect of schizandrin B on gene expression was concentration-dependent. The gene expression of the control group was set to 1.0, and the experimental group data were compared with the control group and expressed as the mean ± SEM. P < 0.05 was considered to be significant (##p<0.01 and **p<0.01). All experiments were repeated three times.
Fig. 5. Schizandrin B inhibits the RFP-induced apoptosis of L02 cells. (A) Treatment of L02 cells with 200 μM RFP or the combination of 50 μM schizandrin B and 200 μM RFP for 48 h. The flow cytometry results of the RFP-treated group were significantly higher than those of the control group; however, in the group administered both RFP and schizandrin B, schizandrin B significantly reduced the level of apoptosis induced by RFP. (B) L02 cells were treated with 25 μM, 50 μM, or 100 μM schizandrin B combined with 200 μM RFP for 48 h, and apoptotic factors were detected. Western blot analysis showed that 25 μM schizandrin B could downregulate the RFP-induced expression of the apoptotic factors PARP, Caspase3 and cleaved caspase3 and that the downregulation of protein expression was concentration-dependent. (Color figure can be accessed in the online version.)
Fig. 6. Immunostaining of GRP78, PERK, ATF6, p-IRE1 and XBP-1 in L02 cells (x200). Cells were treated with 200 μM schizandrin B and 100 μM RFP for 48 h. The nuclei were counterstained with DAPI (blue), and the final merged image is representative of three independent experiments.  （Color figure can be accessed in the online version.)