Ginsenoside Rg1 exerts anti-apoptotic effects on non-alcoholic fatty liver cells by downregulating the expression of SGPL1

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Abstract. Non-alcoholic fatty liver disease (NAFLD) has a high incidence, and can lead to liver cirrhosis and even hepatocellular carcinoma in severe cases. To the best of our knowledge, there is currently no safe and effective treatment for the management of this disease. Ginsenoside Rg1 (Rg1) is an active monomer derived from ginseng and notoginseng. In the present study, HHL-5 hepatocytes were used to establish an *in vitro* cell model of NAFLD by medium- and long-chain fat emulsion treatment, and the effects of Rg1 on adipose accumulation, apoptosis and the expression levels of apoptosis-related proteins in HHL-5 hepatocytes were examined. The results demonstrated that Rg1 inhibited the accumulation of fat in HHL-5 cells, while inhibiting apoptosis, and Rg1 downregulated the expression levels of the pro-apoptotic protein Bax and upregulated the expression levels of the anti-apoptotic protein Bcl-2, indicating that Rg1 could promote the stability or integrity of mitochondria and exert an anti-apoptotic effect by regulating Bcl-2 family proteins. In addition, Rg1 markedly downregulated the expression levels of sphingosine-1-phosphate lyase 1 (SGPL1), a key enzyme in the sphingosine signaling pathway, in HHL-5 cells with steatosis, and increased the expression levels of the downstream pro-survival signals phosphorylated (p-)Akt and p-Erk1/2. Furthermore, overexpression of SGPL1 abolished the anti-apoptotic effect of Rg1 on SGPL1-overexpressing HHL-5 cells with steatosis, and downregulated the expression levels of pro-survival proteins, such as Bcl-2, p-Akt and p-Erk1/2, whereas the expression levels of pro-apoptotic Bax were markedly increased. In conclusion, although there are some reports regarding the protective effect of Rg1 on fatty liver cells, to the best of our knowledge, the present study is the first to report that Rg1 may exert an anti-apoptotic effect on fatty liver cells by regulating SGPL1 in the sphingosine signaling pathway. Rg1 is the main component of the prescription drug Xuesaitong in China; therefore, the findings of the present study may provide a theoretical molecular basis for the use of Rg1 or Xuesaitong in the treatment of patients with NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to a large category of liver diseases, the incidence of which is increasing yearly; studies have reported prevalence rates of ~31% in South America (1), 25-30% in Japan (2), 28% in Italy (3) and ~20% in China (4). In general, 15-20% of patients with NAFLD tend to progress to non-alcoholic steatohepatitis, which, if not addressed, can progress to liver failure or hepatocellular carcinoma (5). A number of pathogenetic factors, such as fat deposition, oxidative stress and endoplasmic reticulum stress, can induce apoptosis, which serves an important role in the development of NAFLD (6). The search for effective NAFLD therapeutic targets and the development of related drugs have recently attracted attention.

Ginsenoside Rg1 (Rg1) is an active monomer component isolated from ginseng and *Panax notoginseng*. Due to its extensive pharmacological effects and the minor risk of side effects, it has garnered attention with a number of researchers (7-12). Monomeric Rg1 has been reported to exert numerous biological effects, including anti-inflammation and anti-oxidation (7,8), inhibition of cardiac hypertrophy (9), anti-aging (10), neuroprotection and memory improvement (11,12). In addition, Rg1 may reduce the content of intracellular triglycerides by activating the AMP-activated protein kinase/NF-κB signaling pathway (13), and could alleviate the fatty degeneration of HepG2 cells induced by palmitic acid. Peng *et al* (14) reported

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that, compared with ursodeoxycholic acid, Rg1 better regulated fat metabolism to reduce liver damage in NAFLD rats. Xiao *et al* (15) revealed that the therapeutic effect of Rg1 on NAFLD rats was mediated through upregulation of Bcl-2 and pro-caspase-3 expression, and downregulation of Bax expression, which has an anti-apoptotic effect on liver cells. However, the specific molecular mechanism underlying the anti-apoptotic effects of Rg1 in NAFLD needs to be explored further.

In the present study, the effects of Rg1 on the apoptosis of steatotic HHL-5 liver cells and the underlying mechanism were investigated. Furthermore, the role of a key lyase, sphingosine-1-phosphate lyase 1 (SGPL1), in the sphingosine kinase signaling pathway and its involvement in the anti-apoptotic effects of Rg1 on steatotic HHL-5 cells was examined.

Materials and methods

Cell culture. The HHL-5 human hepatocyte cell line was purchased from Procell Life Science & Technology Co., Ltd. HHL-5 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific) containing 10% FBS (Gibco; Thermo Fisher Scientific) at 37˚C in a humidified incubator supplied with 5% CO_2. The 293T cell line was obtained from Kunming Cell Bank of Chinese Academy Sciences and cultured in DMEM supplemented with 10% heat-inactivated FBS at 37˚C in a humidified incubator supplied with 5% CO_2.

Cell viability assay. HHL-5 cell viability was measured using an MTS assay (CellTiter 96 AQueous MTS Reagent; Promega Corporation) according to the manufacturer's protocol. HHL-5 cells were seeded into 96-well plates at a density of $5x10^4$ cells/ml (100 μl/well) and cultured for 24 h. The medium was then discarded and replaced with 1% medium- and long-chain fat emulsion (MCE; Baxter Qiaoguang Healthcare) for 24 h at 37˚C. Subsequently, 0.2, 0.4 and 0.6 mM Rg1 (Chengdu Push Bio-technology Co., Ltd.) was added to the HHL-5 cells, followed by incubation for a further 20 h at 37˚C (treatment groups). The model (Mod) group were treated with MCE only; in the recovery (Rec) group, following 1% MCE treatment for 24 h, MCE was removed without Rg1 treatment. The structure of Rg1 is shown in Fig. 1A. For cell viability analysis, 20 μl MTS/phenazine methosulfate (20:1 in volume) was added to the medium, followed by incubation for 4 h at 37˚C. Cell viability was finally determined using a microplate reader (Nanjing DeTie Laboratory Equipment Co., Ltd.) at 490 nm.

Oil-red O staining. HHL-5 cells were seeded at a density of $2x10^4$ cells/ml into 24-well plates, treated with 1% MCE (Mod group), and then cultured with 0.2, 0.4 and 0.6 mM Rg1 for a further 24 h (treatment groups). For Rec group, following 1% MCE treatment for 24 h, MCE was removed without Rg1 treatment. The cells were washed once with PBS and fixed with 95% ethanol for 20 min at room temperature. Subsequently, the cells were stained with freshly prepared Oil Red O solution (Wuhan Servicebio Technology Co., Ltd.; Oil Red:deionized water, 3:2) for 10 min at room temperature. After staining, the cells were washed with double-distilled water to remove the unbound staining solution and observed under an inverted light

phase-contrast microscope (magnification, x200; PH-XDS5; PhenixOptics). The adipose accumulation was semi-quantified using ImageJ v2.1.4.7 (National Institutes of Health), and the result was presented as average optical density (AOD).

Assessment of cell apoptosis. HHL-5 cells ($2x10^5$ cells/ml) in 6-well plates were treated with various concentrations of Rg1 (0.2, 0.4 and 0.6 mM, treatment groups) at 37˚C for 24 h after 24-h 1% MCE treatment. Similarly, 12- or 24-h 1% MCE-treated HHL-5-SGPL1 cells were treated with 0.6 mM Rg1 for 24 h. The Mod group were treated with MCE; in the Rec group, MCE was removed without Rg1 treatment. After collection by precipitation, cells were washed with PBS and stained with Annexin V-FITC/PI at room temperature for 15 min in the dark (Annexin V-FITC Apoptosis Detection kit; MilliporeSigma) according to the manufacturer's instructions. The numbers of viable, necrotic and apoptotic cells were assessed using a flow cytometer (CyFlow Space; Sysmex Partec) and FloMax Software Version 2 (Sysmex Partec GmbH).

Reverse transcription-quantitative PCR (RT-qPCR). Following treatment with Rg1 (0.2, 0.4 and 0.6 mM for 24 h after 24-h 1% MCE treatment, treatment groups), total cellular RNA was extracted from HHL-5 cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Similarly, total cellular RNA from HHL-5 cells transfected with empty lentiviral vector pLVX-IRES-Neo for 48 h was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.). RNA was reverse transcribed into cDNA using MMLV reverse transcriptase (cat. no. M1701; Promega Corporation) according to the manufacturer's instructions. Subsequently, mRNA expression levels were measured using GoTaq qPCR Master Mix (cat. no. A6001; Promega Corporation) and an ABI7000 cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences used were as follows: Sphingosine kinase 1 (SPHK1) forward, 5'-CAGTGGATGGGGAATTGATGG-3' and reverse, 5'-AAG GGCTCTTCTGGCGGTG-3'; SGPL1 forward, 5'-AGTATG GCTATGCCCCAAAAGG-3' and reverse, 5'-CCACCCTGC CAATCTGTATCG-3'; Bax forward, 5'-GTCACTGAAGCG ACTGATGTCCC-3' and reverse, 5'-CAAAGATGGTCACGG TCTGCC-3'; Bcl-2 forward, 5'-GTGGATGACTGAGTACCT GAACCG-3' and reverse, 5'-GACAGCCAGGAGAAATCA AACAGAG-3'; and GAPDH forward, 5'-GGTGAAGCAGGC GTCGGAG-3' and reverse, 5'-GGAGTGGGTGTCGCTGTT GAA-3'. The thermocycling conditions were as follows: Initial denaturation at 94˚C for 5 min, followed by 40 cycles at 94˚C for 15 sec and 60˚C for 20 sec. Relative changes in mRNA expression levels were calculated and analyzed using the relative quantification $2^{-\Delta\Delta Cq}$ method (16). GAPDH was used as an internal control to normalize the variability in expression levels.

Western blotting. After HHL-5 cells were treated with 1% MCE for 24 h at 37˚C, the cells were then treated with different concentrations (0.2, 0.4 and 0.6 mM) of Rg1 for 24 h (treatment group) at 37˚C; for HHL-5-SGPL1 cells, 1% MCE was first treated for 12 h at 37˚C, and then treated with 0.6 mM Rg1 (treatment group) at 37˚C. The model (Mod) group were
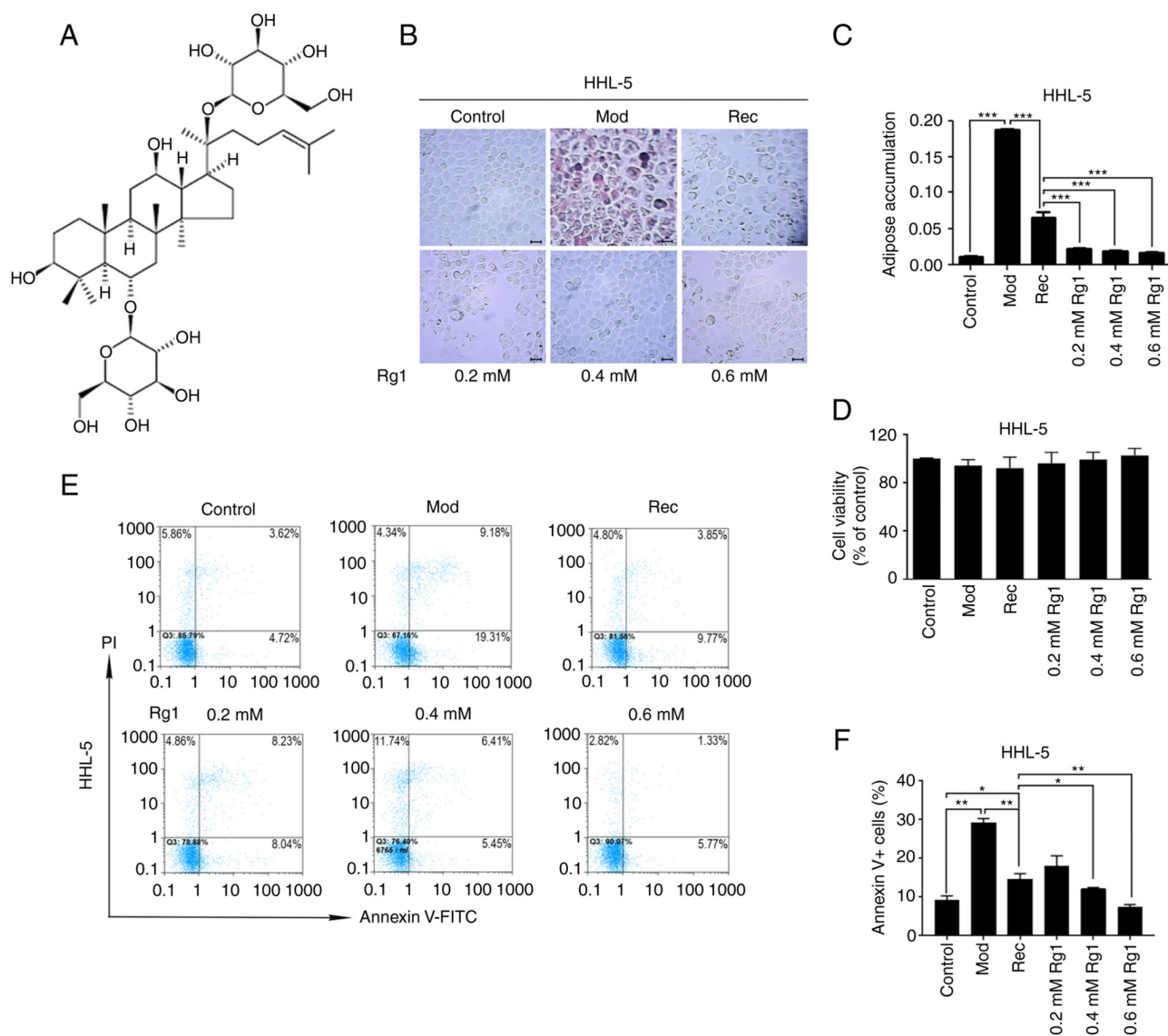
Figure 1. Effects of Rg1 on steatosis, proliferation and apoptosis of HHL-5 hepatocytes. (A) Structure of Rg1. HHL-5 cells were exposed to MCE (1%) for 24 h and treated with Rg1 (0.2, 0.4 or 0.6 mmol/l) for 24 h. (B) Oil red O staining. Magnification, x200; scale bar, 20 μM. (C) Adipose accumulation (AOD) was semi-quantified using ImageJ software. (D) HHL-5 cell viability. (E) HHL-5 cells were stained with PI and Annexin V-FITC and apoptosis was assessed by flow cytometry. (F) Apoptotic rate (Annexin V⁺) of HHL-5 cells was estimated. *P<0.05, **P<0.01 and ***P<0.001. MCE, medium- and long-chain fat emulsion; Mod, model; Rec, recovery; Rg1, ginsenoside Rg1; AOD, average optical density.

treated with MCE only at 37°C; in the recovery (Rec) group, following 1% MCE treatment for 12 h or 24 h at 37°C, MCE was removed without Rg1 treatment. The treated cells were then washed with PBS and lysed with radio immunoprecipitation assay lysis buffer (MilliporeSigma) on ice for 30 min. Subsequently, the cells were sonicated on ice with 10 1-sec bursts with a 3 sec interval by an ultrasonic crusher at medium power. The cell lysates were centrifuged at 14,000 x g for 15 min at 4°C, and the supernatants were then collected. The protein concentration was determined using a BCA assay (BCA Protein Assay Kit; Beyotime Institute of Biotechnology). Equal amounts of the lysed proteins (20 μg/lane) were separated by 12% SDS-PAGE. The proteins were then transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk in PBS with 0.05% Tween-20 (PBST) for 1 h at room temperature. Subsequently, the membranes were incubated with the primary antibodies at 4°C overnight. After

washing with PBST three times, the secondary antibodies (1:5,000; cat. nos. 31430 and 31460; Thermo Fisher Scientific, Inc.) were added and the membranes were incubated at room temperature for 1 h, followed by washing twice with PBST and once with PBS. Enhanced chemiluminescence reagent (Immobilon Western Chemiluminescent HRP Substrate; cat. no. WBKLS0500; MilliporeSigma) was then added and the X-ray film was exposed to the blots. Primary antibodies (dilution 1:500 for p-Erk1/2 and p-Akt antibodies or 1:1,000 for the rest of antibodies) against phosphorylated (p)-Erk1/2 (cat. no. 4370), Erk1/2 (cat. no. 4695), p-Akt (cat. no. 4060), Akt (cat. no. 4691), Bcl-2 (cat. no. 3498S) and Bax (cat. no. 2772S) were obtained from Cell Signaling Technology, Inc. A polyclonal antibody against SGPL1 (cat. no. abx103371) was purchased from Abbexa Ltd. The antibody against SPHK1 (AF5536) was obtained from R&D Systems. β-actin antibody (1:8,000; cat. no. A1978; MilliporeSigma) was used as a loading

control. Protein bands were scanned and semi-quantified using ImageJ software (v2.1.4.7; National Institutes of Health).

Lentiviral packaging and cell transduction. The full-length coding sequence of SGPL1 (NM_003901.4) was synthesized by Sangon Biotech Co., Ltd. Subsequently, the SGPL1 fragment was inserted into linearized pLVX-IRES-Neo plasmid (Clontech Laboratories, Inc.), and the recombinant plasmid was named pLVX-IRES-Neo-SGPL1. Lentiviruses carrying SGPL1 were packaged by transfection of three plasmids, namely pLVX-IRES-Neo-SGPL1, pCMV-VSV-G and pCMV-Δ8.9 (1.5:0.5:1.1 μg in 35-mm dish), into 293T cells after the cells reached 50% confluence using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C. Plasmid extraction and transfection were performed according to the manufacturer's instructions. After transfection for 72 h, viruses were collected by filtering supernatant using a 45-μm filter and added to pre-seeded HHL-5 cells. Specifically, 200 μl packaged virus and 300 μl medium were added to pre-seeded HHL-5 cells in 24-well plates (4×10^4 cells/ml, 500 μl). After lentiviral infection for 24 h, the G418 (0.8 mg/ml) was added to screen for 6 days, during the screening the dead cells were removed and new culture media with G418 was added. Viruses were collected by filtering supernatant using a 45-μm filter, and multiplicity of infection was 1.34. The positive SGPL1-overexpressing HHL-5 cells (HHL-5-SGPL1 cells) were then selected and the virus titer was calculated at 72 h post-infection. The calculated titer of the virus was 1.34×10^5 IFU/ml. With the virus titer, the number of HHL-5 cells infected with the viruses can be estimated, and the multiplicity of infection was 1.34. The HHL-5-SGPL1 cells were then subjected to gene expression detection and apoptosis analysis after 1% MCE (12 h) and/or Rg1 treatment (0.6 mM for 24 h). HHL-5-SGPL1 cells were maintained at a concentration of 0.8 mg/ml G418.

Statistical analysis. The experiments were performed at least three times and all data analysis was performed using GraphPad Prism (version 7.0; GraphPad Software, Inc.). All data are presented as the mean ± SD. Paired Student's t-test was used for comparisons between two groups, and one-way ANOVA with post-hoc intergroup comparisons using Tukey test was used for comparisons among multiple groups. For comparisons between two independent variables, two-way ANOVA with Bonferroni post hoc test was applied. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Rg1 reduces adipose accumulation of HHL-5 liver cells. During the occurrence of NAFLD, one of the most significant pathological changes is adipose accumulation. In order to clarify the anti-steatotic effect of Rg1 on liver cells, HHL-5 hepatocytes were treated with 1% MCE to induce cell steatosis (Mod group) and were then treated with Rg1. Subsequently, it was evaluated whether Rg1 reduced the accumulation of fat in HHL-5 cells by Oil Red O staining. Compared with in the Mod group, the Rec group exhibited a reduction in the number of fat particles, but still more than in the Rg1 treatment groups (Fig. 1B and C). Furthermore, the cell state of the

Rg1 treatment group gradually returned to normal with the increase in Rg1 treatment concentration (0.2, 0.4 and 0.6 mM) (Fig. 1B and C). Additionally, a cell viability assay was used to detect the effect of Rg1 on the viability of steatotic HHL-5 cells. The data demonstrated that Rg1 slightly promoted the viability of steatotic HHL-5 cells; however, the results were not statistically significant ($P > 0.05$; Fig. 1D).

Rg1 reduces the apoptosis of steatotic HHL-5 cells. In the present study, the anti-apoptotic effect of Rg1 on steatotic HHL-1 cells was examined using flow cytometry. The results showed that, compared with that of the control group, the apoptotic rate (percentage of Annexin V$^+$ cells) in the Mod and Rec groups was markedly increased, whereas in the treatment groups, the apoptotic rate gradually decreased with increasing Rg1 concentrations (0.4 and 0.6 mM) compared with that of the Rec group (Fig. 1E and F).

Effect of Rg1 on the expression levels of Bcl-2 family proteins. The effect of Rg1 on the expression levels of Bax and Bcl-2 in steatotic HHL-5 hepatocytes was examined by western blotting. The data demonstrated that the expression levels of Bax were significantly increased in the Mod group compared with in the control group, whereas the expression levels of Bax were decreased in the treatment group in a Rg1 concentration-dependent manner (0.2, 0.4 and 0.6 mM) compared with in the Rec group (Fig. 2A and B). Conversely, the protein expression levels of Bcl-2 were not significantly altered in the Mod group, but Bcl-2 was significantly upregulated in the treatment groups compared with in the Rec group (Fig. 2A and B). Additionally, the mRNA expression levels of Bax and Bcl-2 were detected using RT-qPCR. The results revealed that the transcription levels of Bax were upregulated in the Mod and Rec groups compared with in the control group, whereas these were gradually downregulated in the Rg1 treatment group in a concentration-dependent manner (Fig. 2C). By contrast, the transcription levels of Bcl-2 were downregulated in the Mod and Rec groups compared with in the control group, whereas they were gradually increased in the Rg1 treatment group (Fig. 2C).

Rg1 blocks SGPL1 expression. In order to elucidate whether the relevant molecules in the sphingosine lipid signaling pathway mediated the anti-apoptotic effect of Rg1 in the process of hepatocyte steatosis, the present study first detected the transcription levels of SPHK1 and SGPL1 in steatotic HHL-5 cells following Rg1 treatment. SPHK1 and SGPL1 are the two key enzymes of sphingosine-1-phosphate (S1P) metabolism (17). The results revealed that, compared with in the Rec group, the transcription levels of SPHK1 were significantly increased in the high-concentration Rg1 treatment group (0.6 mM), whereas the transcription levels of SGPL1 were significantly decreased in steatotic HHL-5 cells after Rg1 treatment (0.6 mM; Fig. 3B). The present study further examined the protein expression levels of SPHK1 and SGPL1 (Fig. 3A and C). Notably, the protein expression levels of SPHK1 were not significantly altered in steatotic HHL-5 hepatocytes following Rg1 treatment. By contrast, the protein expression levels of SGPL1 were increased in the Mod and Rec groups compared with in the control group, but were significantly decreased following
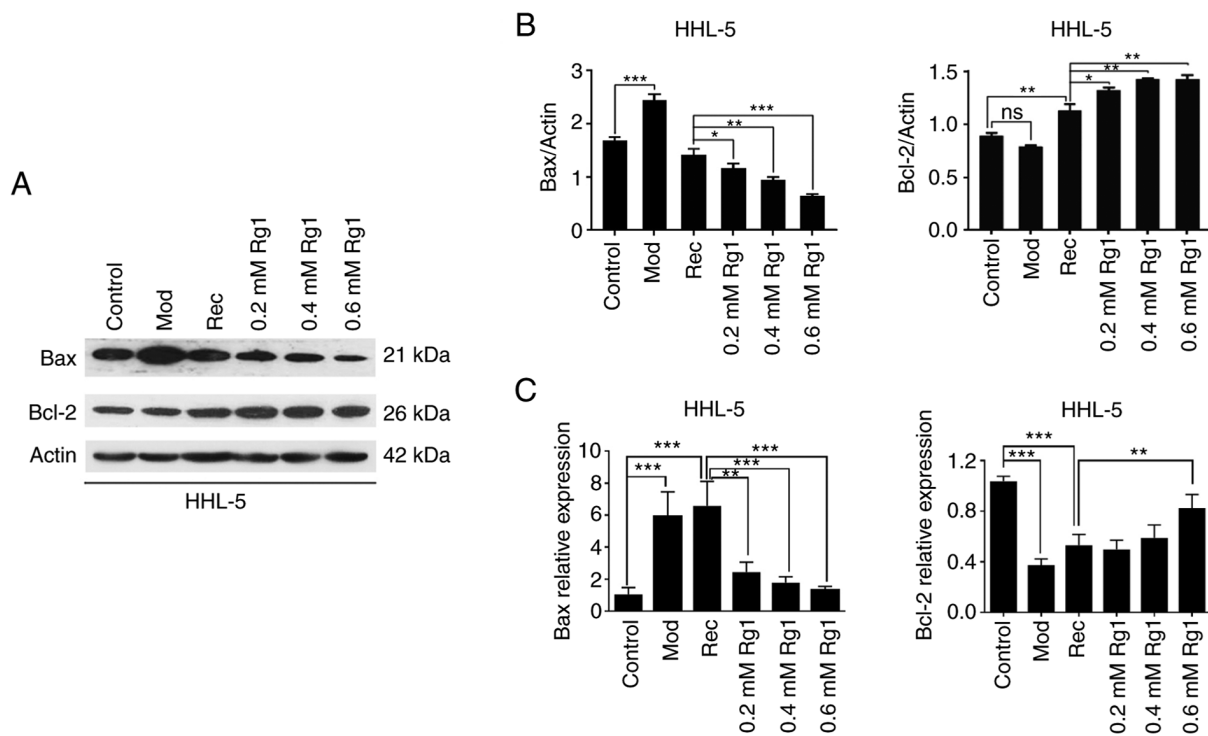
Figure 2. Anti-apoptotic effects of Rg1 are mediated by Bcl-2 proteins. (A) Bax and Bcl-2 protein expression levels were examined in HHL-5 cells treated with Rg1 (0.2, 0.4 and 0.6 mM) after exposure to MCE (1%) by western blotting. (B) Bax and Bcl-2 protein expression levels were normalized to actin expression using ImageJ software. (C) Bax and Bcl-2 mRNA expression levels were detected in HHL-5 cells using reverse transcription-quantitative PCR. *P<0.05, **P<0.05 and ***P<0.001. MCE, medium- and long-chain fat emulsion; Mod, model; Rec, recovery; Rg1, ginsenoside Rg1.

Rg1 treatment (0.4 and 0.6 mM). Additionally, the present study further determined the activities of the anti-apoptotic signaling molecules Akt and Erk1/2 downstream of the sphingosine signaling pathway (17,18). The results demonstrated that the protein expression levels of p-Akt and p-Erk1/2 were downregulated in the Mod and Rec groups compared with in the control group; however, the expression levels of p-Akt and p-Erk1/2 were increased following Rg1 treatment in a concentration-dependent manner (Fig. 3A and C).

Overexpression of SGPL1 abrogates the anti-apoptotic effects of Rg1. In order to further clarify if the anti-apoptotic effect of Rg1 on steatotic hepatocytes was mediated by downregulation of SGPL1, SGPL1 was overexpressed in HHL-5 hepatocytes; a hepatocyte line stably overexpressing SGPL1 was established and named HHL-5-SGPL1. During the establishment of HHL-5-SGPL1 cells, the lentivirus carrying the overexpressed SGPL1 gene was packaged. The results revealed that the expression levels of SGPL1 in HHL-5-SGPL1 cells were increased 18.93-fold compared with SGPL1 expression in normal HHL-5 cells (Fig. 4A), indicating that SGPL1 was stably expressed in HHL-5-SGPL1 cells. Furthermore, flow cytometry was used to detect the anti-apoptotic effect of Rg1 on steatotic HHL-5-SGPL1 cells and wild-type HHL-5 cells. The results revealed that there was no significant difference in the apoptotic rates between the Rg1 high-concentration (0.6 mM) treatment group and the Rec group in steatotic HHL-5-SGPL1 cells (Fig. 4B and C). However, the apoptotic rate in the Rg1 high-concentration (0.6 mM) treatment group was decreased compared with the Rec group in wild-type HHL-5 cells (Fig. 4B and C). Additionally, western blot analysis

demonstrated that high-concentration (0.6 mM) Rg1 treatment only slightly downregulated SGPL1 expression in steatotic HHL-5-SGPL1 cells compared with in wild-type HHL-5 cells, in which SGPL1 expression was substantially decreased (Fig. 4D and E). Furthermore, the pro-apoptotic protein Bax (control group) was markedly upregulated in HHL-5-SGPL1 cells compared with in normal HHL-5 cells, and it was not significantly downregulated following Rg1 treatment in HHL-5-SGPL1 cells compared with cells in the Rec group (Fig. 4D and E). By contrast, compared with those in normal HHL-5 cells, the expression levels of Bcl-2 (control group) in HHL-5-SGPL1 cells were significantly downregulated, and Bcl-2 expression was not significantly increased after Rg1 treatment in HHL-5-SGPL1 cells compared with in the Rec group (Fig. 4D and E). Additionally, the expression levels of p-AKT and p-Erk1/2 (control group) in HHL-5-SGPL1 cells were significantly decreased compared with those in normal HHL-5 cells, although Rg1 treatment increased their expression levels in HHL-5-SGPL1 cells compared with cells in the Rec group (Fig. 4D and E), indicating Rg1 may have exerted a pro-proliferative role in HHL-5-SGPL1 cells. As a control, there was no significant difference in the expression levels of SGPL1, and apoptosis-related genes Bax and Bcl-2, between HHL-5 cells and HHL-5 cells infected with the empty lentiviral vector pLVX-IRES-Neo (Fig. 4F).

Discussion

NAFLD is the most prevalent liver disease in humans with the number of patients approaching two billion worldwide (19). In ~25% of patients, NAFLD subsequently leads to steatohepatitis,
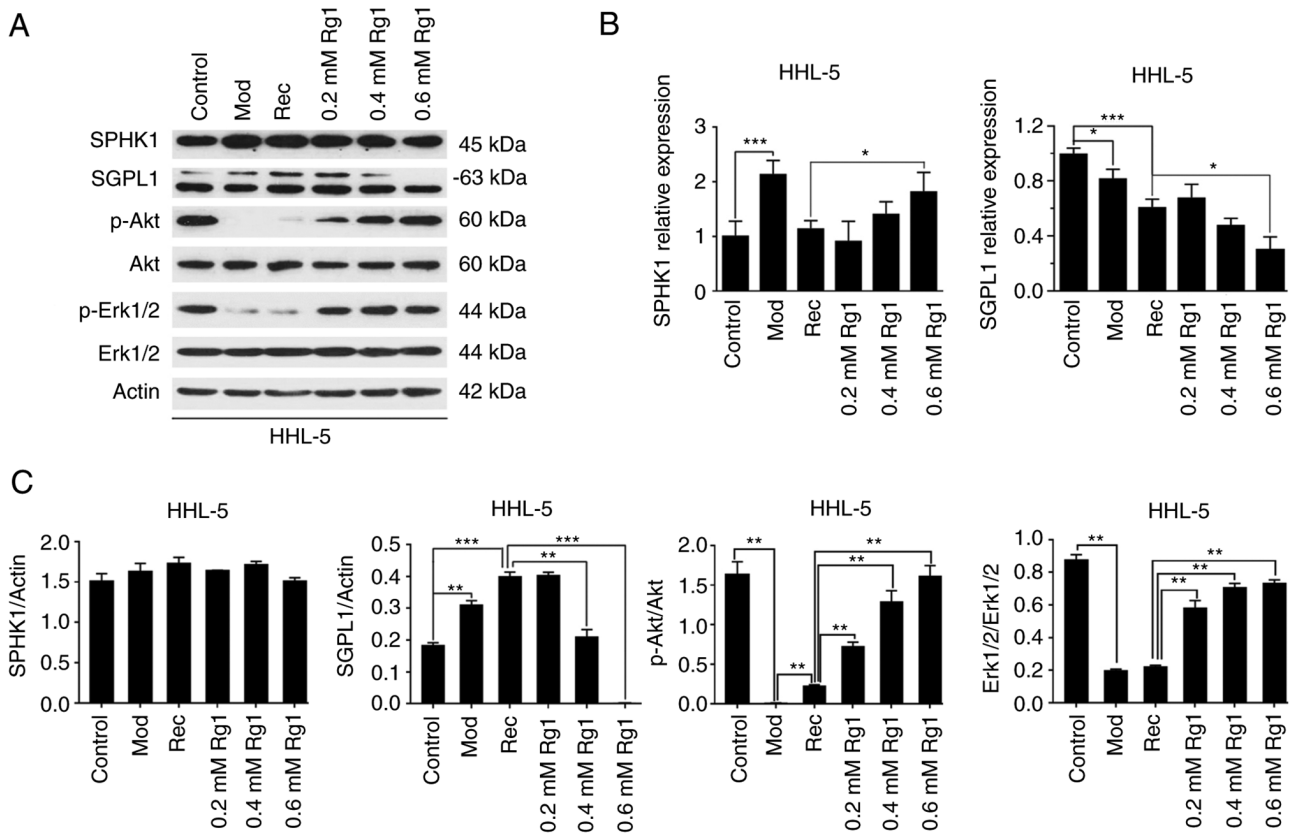
Figure 3. Effect of Rg1 on sphingolipid pathway proteins. (A) SPHK1, SGPL1, p-Akt, p-Erk1/2, Akt and Erk1/2 expression levels were detected in HHL-5 cells treated with Rg1 (0.2, 0.4 and 0.6 mM) after exposure to MCE (1%) by western blotting. (B) SPHK1 and SGPL1 mRNA expression levels were detected in HHL-5 cells using reverse transcription-quantitative PCR. (C) SPHK1 and SGPL1 protein expression levels were normalized to actin expression, and p-Akt and p-Erk1/2 protein expression levels were normalized to Akt or Erk1/2 using ImageJ software. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$; MCE, medium- and long-chain fat emulsion; Mod, model; p-, phosphorylated; Rec, recovery; SGPL1, sphingosine-1-phosphate lyase 1; SPHK1, sphingosine kinase 1; Rg1, ginsenoside Rg1.

liver cirrhosis and hepatocellular carcinoma (20). The search for effective therapeutic targets for NAFLD and the development of related drugs have attracted increasing attention. Rg1 is an active monomer isolated from *Panax ginseng* and *Panax notoginseng*, which has been widely studied due to its extensive pharmacological action and few side effects (7-12).

In the present study, HHL-5 hepatocytes were used to establish a NAFLD cell model and it was revealed that Rg1 had the ability to decrease adipose granule aggregation in HHL-5 cells. Furthermore, Rg1 inhibited the apoptosis of steatotic HHL-5 cells by regulating the expression levels of Bcl-2 family proteins, Bax and Bcl-2. Bcl-2 family molecules include two major categories: Pro-apoptotic protein molecules, such as Bax, and anti-apoptotic protein molecules, such as Bcl-2 (21). The balance between pro-apoptotic and anti-apoptotic Bcl-2 family proteins regulates the apoptotic fate of cells by regulating the stability and integrity of the mitochondrial membrane (22). In the present study, Rg1 downregulated the expression levels of Bax and upregulated the expression levels of Bcl-2, which may enhance the stability of mitochondria, thereby making HHL-5 hepatocytes resistant to the pro-apoptotic effects induced by fat accumulation. The results revealed the regulation effects of Rg1 on Bax and Bcl-2 expression, which was consistent with the anti-apoptotic effects of Rg1 on hepatocyte apoptosis, as detected by flow cytometry. Notably, it is well known that the mechanism underlying the protective effects of Rg1 against NAFLD is multifaceted (14,15,23,24).

For example, it has been reported to involve regulation of the expression of lipid metabolism enzyme genes, such as peroxisome proliferator-activated receptor α, and reductions in blood lipids, blood sugar and inflammatory factor levels, thereby reducing liver fat accumulation and exerting hepatoprotective effects (25).

The damage caused by hepatocyte steatosis and apoptosis serves an important role in the pathogenesis of NAFLD (7). The mechanism underlying apoptosis in NAFLD has been suggested to be multifaceted (26), and may involve inflammatory factors (27), lipid peroxidation damage (28) and endoplasmic reticulum stress (29). The sphingosine signaling pathway serves an important role in the physiological processes of lipid metabolism, cell survival and apoptosis (30). The present study revealed that Rg1 downregulated SGPL1 expression in steatotic HHL-5 hepatocytes. SPHK1 and SGPL1 are two key enzymes in S1P metabolism. SPHK1 catalyzes sphingosine to produce S1P, whereas SGPL1 is responsible for the irreversible cleavage of S1P into hexadecenal and ethanolamine phosphate (17). S1P is transported out of the cell by the ATP-binding cassette transporter (31), binds to its receptor (including sphingosine-1-phosphate receptor 1-5), and activates downstream signaling pathways (17,18), such as the Akt and Erk1/2 signaling pathways. In the present study, although Rg1 treatment could upregulate the transcription levels of SPHK1 in HHL-5 cells, Rg1 had no effect on SPHK1 protein expression. The possible reason is that the regulation
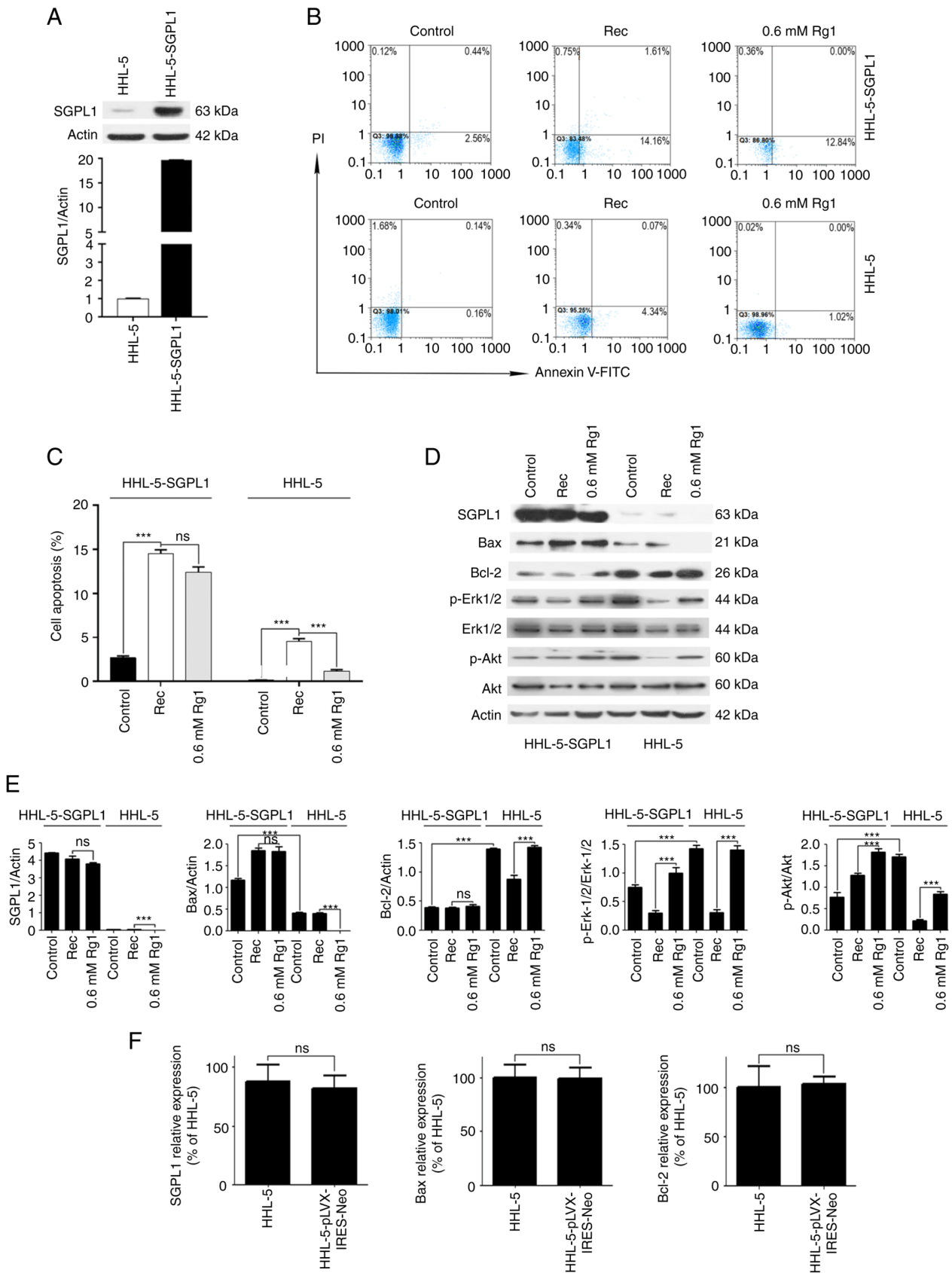
Figure 4. Overexpression of SGPL1 inhibits the anti-apoptotic effect of Rg1. (A) SGPL1 expression was analyzed by western blotting in normal HHL-5 and HHL-5-SGPL1 cells established by infecting HHL-5 cells with a lentivirus packaged with SGPL1 overexpression plasmids. (B) HHL-5-SGPL1 and HHL-5 cells were treated with 1% MCE for 12 h (Rec) and with Rg1 (0.6 mM) for 24 h, and stained with PI and Annexin V-FITC. Cell apoptosis was assessed by flow cytometry, and (C) analyzed statistically. (D) SGPL1, Bax, Bcl-2, p-Akt, Akt, p-Erk1/2 and Erk1/2 expression levels in HHL-5-SGPL1 and HHL-5 cells were examined by western blotting. (E) SGPL1, Bax and Bcl-2 protein expression levels were normalized to actin expression, and p-Erk1/2 and p-Akt protein levels were normalized to Akt or Erk1/2 using ImageJ software. (F) SGPL1, Bax and Bcl-2 gene relative expression was detected in HHL-5 cells and HHL-5 cells transfected with the empty lentiviral vector pLVX-IRES-Neo. ns, no significance. ***$P<0.001$; HHL-5-SGPL1 cells, SGPL1-overexpressing HHL-5 cells; p-, phosphorylated; Rec, recovery; SGPL1, sphingosine-1-phosphate lyase 1; Rg1, ginsenoside Rg1.

of SPHK1 expression by Rg1 is multifaceted, and that it is not only limited to effects on mRNA transcription levels, but also involves protein translation and protein stability. Since Rg1 downregulated SGPL1 expression in HHL-5 steatotic liver cells, it was hypothesized that Rg1 may have the effect of upregulating S1P levels, thus exerting anti-apoptotic effects through its downstream signaling pathways. It can be seen from the present results that the overexpression of SGPL1 abolished the anti-apoptotic effect of Rg1 on HHL-5-SGPL1 cells, and markedly downregulated the expression levels of pro-survival molecules, such as Bcl-2, p-Akt and p-Erk1/2. Conversely, following overexpression of SGPL1, the expression levels of the pro-apoptotic molecule Bax in HHL-5-SGPL1 cells were markedly increased. It may be concluded that the anti-apoptotic effect of Rg1 in steatotic HHL-5 hepatocytes was blocked by overexpression of SGPL1.

Notably, when HHL-5 cells overexpressing SGPL1 were treated with 1% MCE and flow cytometry was performed, it was observed that the treatment time of MCE needed to be reduced from 24 to 12 h, since when used for 24 h, it markedly increased the apoptotic rate (data not shown). Due to too much apoptosis, not enough cells could be collected for flow cytometry analysis. This indicated that cells were too sensitive to adipogenesis when SGPL1 was highly expressed, so the treatment time of MCE was shortened. Too high an apoptotic rate makes the experiment impossible, indicating that the overexpression of SGPL1 in HHL-5 cells enhanced the sensitivity of cells to steatosis. The aforementioned findings revealed that overexpression of SGPL1 promoted apoptosis during hepatocyte steatosis, whereas Rg1 served an anti-apoptotic role and protected liver cells by downregulating the expression levels of SGPL1.

Several studies (32-34) have reported that SGPL1 serves a role in the inhibition of cell survival and tumor chemotherapeutic drug sensitivity. The inactivation of SGPL1 has been reported to induce resistance of tumor cells to chemotherapy drugs and promote cell survival (32). Matula *et al* (32) reported that resistance to the chemotherapy drugs oxaliplatin, cisplatin and docetaxel was associated with decreased expression levels of SGPL1 in gastroesophageal cancer. Degagné *et al* (33) also reported that the loss of SGPL1 promoted colon carcinogenesis, and similar findings were also reported in prostate cancer (34). Therefore, the downregulation of SGPL1 and the inhibition of apoptosis in HHL-5 cells mediated by Rg1 treatment in the present study were similar to the lack of SGPL1 activity in tumor cells leading to drug resistance and promoting cell survival, since abolition of SGPL1 promoted cell survival. Overexpression of SGPL1 promoted HHL-5 hepatocyte apoptosis, which revealed that Rg1 served an anti-apoptotic effect by downregulating SGPL1. Furthermore, Rg1 is one of the main components of the current prescription drug Xuesaitong in China (35). Xuesaitong is mainly used to treat cardiovascular and cerebrovascular diseases, such as cerebral infarction (36,37) and coronary heart disease (38). In the present study, Rg1 reduced liver cell steatosis and inhibited cell apoptosis. Therefore, it may be hypothesized that Xuesaitong or Rg1 could have potential clinical applications in patients with NAFLD.

In conclusion, although the mechanism by which Rg1 inhibits cell apoptosis has been widely reported (39-41), to the best of the authors' knowledge, the present study was the first
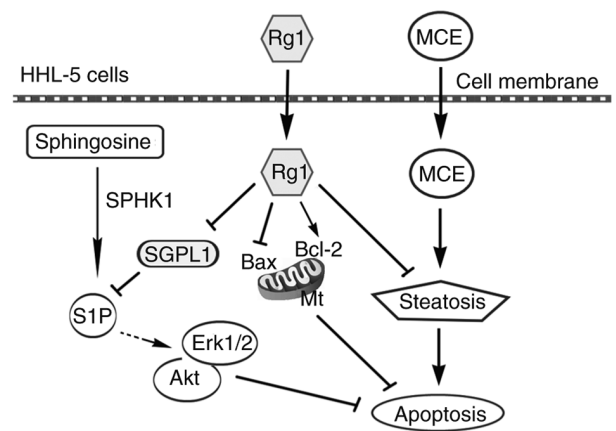

Figure 5. Molecular mechanism underlying the inhibitory effects of Rg1 on apoptosis in steatotic HHL-5 hepatocytes. MCE, medium- and long-chain fat emulsion; SPHK1, sphingosine kinase 1; SGPL1, sphingosine-1-phosphate lyase 1; S1P, sphingosine-1-phosphate; Mt, mitochondria; Rg1, ginsenoside Rg1.

to report that Rg1 exerted an anti-steatotic effect on hepatocyte apoptosis by downregulating a key enzyme, SGPL1, in the sphingosine signaling pathway (Fig. 5). On the one hand, Rg1 inhibited MCE-induced fat aggregation in HHL-5 cells; on the other hand, Rg1 upregulated Bcl-2 and downregulated Bax expression levels to enhance mitochondrial stability. In addition, Rg1 downregulated SGPL1, and subsequently upregulated pro-survival p-Erk1/2 and p-Akt proteins via the sphingosine phosphate signaling pathway. These aforementioned findings may indicate the mechanism underlying the anti-apoptotic effects of Rg1 on steatotic HHL-5 hepatocytes. However, the present study is limited to the anti-apoptotic effects of Rg1 on a steatotic hepatocyte model *in vitro*, and whether Rg1 can also serve an anti-apoptotic effect on steatotic hepatocytes by downregulating SGPL1 and sphingosine kinase pathways *in vivo* requires further investigation. In addition, the specific molecular mechanism by which Rg1 downregulates SGPL1 needs to be further elucidated.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GL and HX performed all the experiments. GL, YL and LC conceived and designed the study. GL and HX wrote the main manuscript. GL, HX, XC and CM analyzed the data. XC and CM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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