Research article

20S-Protopanaxadiol, an aglycosylated ginsenoside metabolite, induces hepatic stellate cell apoptosis through liver kinase B1—AMP-activated protein kinase activation

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Abstract
Background: Previously, we reported that Korean Red Ginseng inhibited liver fibrosis in mice and reduced the expressions of fibrogenic genes in hepatic stellate cells (HSCs). The present study was undertaken to identify the major ginsenoside responsible for reducing the numbers of HSCs and the underlying mechanism involved.

Methods: Using LX-2 cells (a human immortalized HSC line) and primary activated HSCs, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assays were conducted to examine the cytotoxic effects of ginsenosides. H2O2 productions, glutathione contents, lactate dehydrogenase activities, mitochondrial membrane permeabilities, apoptotic cell subpopulations, caspase-3/-7 activities, transferase dUTP nick end labeling (TUNEL) staining, and immunoblot analysis were performed to elucidate the molecular mechanism responsible for ginsenoside-mediated cytotoxicity. Involvement of the AMP-activated protein kinase (AMPK)-related signaling pathway was examined using a chemical inhibitor and small interfering RNA (siRNA) transfection.

Results and conclusion: Of the 11 ginsenosides tested, 20S-protopanaxadiol (PPD) showed the most potent cytotoxic activity in both LX-2 cells and primary activated HSCs. Oxidative stress-mediated apoptosis induced by 20S-PPD was blocked by N-acetyl-L-cysteine pretreatment. In addition, 20S-PPD concentration-dependently increased the phosphorylation of AMPK, and compound C prevented 20S-PPD-induced cytotoxicity and mitochondrial dysfunction. Moreover, 20S-PPD increased the phosphorylation of liver kinase B1 (LKB1), an upstream kinase of AMPK. Likewise, transfection of LX-2 cells with LKB1 siRNA reduced the cytotoxic effect of 20S-PPD. Thus, 20S-PPD appears to induce HSC apoptosis by activating LKB1—AMPK and to be a therapeutic candidate for the prevention or treatment of liver fibrosis.

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1. Introduction

Liver fibrosis is the result of an excessively exuberant wound healing response to chronic hepatic damage caused by imbalance between extracellular matrix (ECM) production and degradation. ECM overproduction caused by chronic damage leads to progressive loss of hepatic parenchyma and a progressive advance to cirrhosis [1,2]. Activated hepatic stellate cells (HSCs) are one of the major executors of ECM accumulation in the liver. In their quiescent state, HSCs reside in the perisinusoidal space of Disse and store vitamin A. Repeated liver injury induces the transdifferentiation of quiescent HSCs into proliferative, contractile, and fibrogenic myofibroblast-like cells [2]. Evidence indicates that liver fibrosis is a dynamic, reversible process, and thus, reducing numbers of activated HSCs is regarded as a critical strategy for reversing liver fibrogenesis [2,3]. Although no drug has been approved for the treatment of liver fibrosis, it has been reported that several natural products, including berberine and guggulsterone, can cause the
apoptosis of activated HSCs and reduce liver fibrosis in experimental models [4,5].

AMP-activated protein kinase (AMPK), an evolutionarily conserved heterotrimeric Ser/Thr kinase, is considered a master regulator of nutritional status and energy homeostasis and is activated by several upstream kinases, including liver kinase B1 (LKB1) [6,7]. AMPK activation in liver reduces lipid synthesis by inducing the phosphorylation of acetyl-CoA carboxylase (ACC), decreases protein metabolism by inhibiting mammalian target of rapamycin complex 1 (mTORC1), induces autophagy by activating unc-51-like protein kinase 1, and protects cells and mitochondria from oxidative stress [7–9]. Thus, AMPK activation in liver relieves metabolic imbalances caused by alcohol intake or high calorie diet and protects tissues from toxic stimuli. Interestingly, activated AMPK attenuates liver fibrosis by inhibiting the proliferation of activated HSCs and ECM accumulation [10–12]. Although it has been reported that AMPK activation leads to HSC apoptosis [4,13], the relationship between AMPK activation and HSC apoptosis remains to be further established.

Ginseng, the root of Panax ginseng Meyer, has been used as an adaptogenic agent for centuries in Korea, and modern science has shown that ginseng saponins (ginsenosides) are major active ingredients in ginseng [14]. The ginsenosides are classified as dammarane-type, oleanane-type, or protopanaxadiol; protopanaxatriol; and the most abundant dammarane-type ginsenosides can be further classified as protopanaxadiol (PPD) or protopanaxatriol (PPT) types [14]. Although ginsenosides have been credited with the diverse pharmacological activities of ginseng [15], they are poorly absorbed in the gastrointestinal tract, and thus, the compounds responsible for the effects of orally administered ginseng are believed to be metabolites produced in the gastrointestinal tract [16–19].

Studies have shown that ginseng and ginsenosides ameliorate diverse liver diseases by inducing the activation of AMPK [20–23]. In addition, certain ginsenosides have been reported to inhibit liver fibrosis [24,25] and to induce HSC apoptosis [26]. In a previous study, we found Korean Red Ginseng inhibited liver fibrosis induced by carbon tetrachloride in mice and decreased the expressions of transforming growth factor-β (TGF-β)-dependent fibrogenic genes in HSCs [27]. Although ginseng may regress fibrosis in liver, the major ginsenosides that contribute to reductions in activated HSC numbers have yet to be identified. Thus, in the present study, we sought to identify the ginsenosides responsible for reducing the numbers of HSC and the underlying molecular mechanisms involved.

2. Materials and methods

2.1. Reagents

Korean Red Ginseng extract (RGE) was kindly provided by KT&G Central Research Institute (Daejeon, Korea), as described previously [27]. Ginsenosides (Rb1, Rb2, Rc, 20S-Rg3, 20R-Rg3, Re, Rg1) and aglycosylated metabolites [compound K (Comp. K), 20S-PPD, 20R-PPD, and 20S-PPD] were purchased from Ambo Institute (Daejeon, Korea) (Fig. 1). Compound C (an inhibitor of AMPK) and STO-609 (an inhibitor of Ca2+/-PPD) were supplied by Calbiochem (San Diego, CA, USA). Anti-poly(ADP-ribose)polymerase (PARP), anti-procaspase-3, anti-phosphorylated AMPK (Thr172), anti-phosphorylated ACC (Ser79), anti-ACC, anti-phosphorylated LKB1 (Ser428), anti-LKB1, anti-Bcl-2, anti-gial fibrillary acidic protein (GFAP), and horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-Bax and anti-AMPK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-acetyl-L-cysteine (NAC), rhodamine123, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), anti-α-smooth muscle actin (α-SMA) antibody, anti-β-actin antibody, and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture, isolation of murine primary HSCs, and treatment

LX-2 cells (a human immortalized semi-activated HSCs cell line) were kindly provided by Dr S.L. Friedman (Mount Sinai School of Medicine, New York, NY, USA). Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO2. HSC isolation was conducted according to national regulations regarding the use and welfare of laboratory animals, and was approved by the Institutional Animal Care and Use Committee at Daegu Haany University (Approval No. DHU2016-061). Male ICR mice were perfused using pronase/collagenase, and primary HSCs were isolated by gradient centrifugation, as previously described [28]. Isolated cells were cultured on six-well plate in Dulbecco’s modified Eagle’s medium containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin for 6 d to acquire activated HSCs. The purity of isolated HSCs was confirmed by UV positivity using a fluorescence microscope (Eclipse Ti-U; Nikon, Kanagawa, Japan), and phenotypic changes during HSC activation were verified by GFAP and α-SMA immunoblotting, as previously described [14,28]. RGE and NAC were dissolved in water. Ginsenosides, DCFH-DA, rhodamine123, compound C, and STO-609 were dissolved in dimethyl sulfoxide. For all experiments, cells were grown until 80–90% confluent, incubated in medium without FBS for 12 h, and then

![Fig. 1. Chemical structures of ginsenosides and aglycosylated metabolites. Numerical superscripts indicate the carbons at glycosidic bonds. Ara(f), arabinofuranose; Ara(p), arabinopyranoside; Comp. K, compound K; Glc, glucose; PPD, protopanaxadiol; PPT, protopanaxatriol; Rha, rhamnose.](image-url)
exposed to RGE or ginsenoside for the indicated times. Untreated cells were used as controls.

2.3. Cell viability assay

To examine the cytotoxicities of ginsenosides, cells were plated at 5 × 10^4 cells/well in 24-well plates, serum-starved for 12 h, and then treated with 0.3–10 mg/mL of RGE or 1–10 μM of ginsenosides for the indicated times. After treatments, viable cells were stained with MTT (0.5 mg/mL, 4 h), and viabilities were assessed as previously described [8].

2.4. Preparation of whole cell lysates and immunoblot analysis

Cells were lysed in radioimmunoprecipitation buffer containing Xpert protease inhibitor cocktail (GenDEPOT, Barker, TX, USA), sodium fluoride (1 mM), β-glycerophosphate (1 mM), sodium orthovanadate (1 mM), and sodium pyrophosphate (2.5 mM). After incubation for 30 min on ice, cell lysates were collected by centrifugation at 15,000 g for 30 min. Protein concentrations were determined using a bicinchoninic acid assay kit (Thermo, Rockford, IL, USA). Equal amounts of protein were resolved by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK). Immunoreactive proteins of interest were visualized using an enhanced chemiluminescence detection kit (PerkinElmer, Boston, MA, USA). Immunoblot intensities were quantified by densitometric analysis (Image J, rsb.info.nih.gov/ij).

2.5. Measurement of intracellular H$_2$O$_2$ production

The levels of intracellular H$_2$O$_2$ production were determined by measuring increases in dichlorofluorescin fluorescence. After treating LX-2 cells with 20S-PPD for 24 h, cells were stained with 20 μM of DCFH-DA for 1 h. Dichlorofluorescin fluorescence was measured using an automated microplate reader (Tecan Infinite 200 PRO, Männedorf, Switzerland) at excitation/emission wavelengths of 485 nm/530 nm.

2.6. Measurement of reduced glutathione

LX-2 cells were treated with 20S-PPD for 24 h, and levels of reduced glutathione (GSH) were determined using a GSH BIOXYTECH GSH-400 kit (Oxis International Inc., Portland, OR, USA). Absorbance at 405 nm was monitored using a microplate reader (Tecan).

2.7. Measurement of lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activities in media were measured using an LDH assay kit (Cayman, Ann Arbor, MI, USA). Briefly, 20S-PPD-treated media were incubated for 30 min at room temperature with an assay buffer containing LDH diaphorase, lactic acid, NAD$, and tetrazolium salt. Absorbance at 490 nm was then measured using a microplate reader (Tecan).

2.8. Flow cytometric analyses

Mitochondrial membrane permeability (MMP) was assessed using rhodamine123, a membrane permeable cationic fluorescent dye [81]. Cells were treated with 10 μM of 20S-PPD for 24 h, stained with 0.05 μg/mL rhodamine123 for 1 h, and harvested by trypsinization. For some experiments, cells were pretreated either with 10 mM of NAC or 3 μM of compound C for 1 h, and then exposed to 10 μM of 20S-PPD for 24 h. A dead cell apoptosis kit (Invitrogen, Carlsbad, CA, USA) was used to quantify subpopulations of apoptotic cells. Changes in MMP and dead cell subpopulations were determined using a flow cytometer (Partec, Münster, Germany). A total of 10,000 events were recorded during each analysis.

2.9. Caspase-3/-7 activities assay

Caspase-3/-7 activities in cell lysates were determined using a Caspase-Glo 3/7 assay kit (Promega, Madison, WI, USA). 20S-PPD-treated LX-2 cells were harvested and resuspended in phosphate-buffered saline (PBS), and Caspase-Glo 3/7 reagent was added. Cell lysates were then incubated at room temperature for 2 h, and luminescence intensities were measured using a GloMax 20/20 luminometer (Promega). Measured luminescence intensities were normalized with respect to the protein contents of lysates.

2.10. Terminal deoxynucleotidyl transferase dUTP nick end labeling staining

After treatment with 3 μM or 10 μM of 20S-PPD for 18 h, LX-2 cells were stained using an In Situ Apoptosis Detection Kit (Abcam, Cambridge, MA, USA). Briefly, cells were washed with PBS, fixed in PBS containing 4% paraformaldehyde, rehydrated in Tris-buffered saline, and permeabilized by adding 20 mg/mL of protease K. After inactivating endogenous peroxidase, cells were labeled with terminal deoxynucleotidyl transferase, incubated with streptovadin–horseradish peroxidase conjugate, and then developed using diaminobenzidine. The stained cells were observed under a light microscope (Eclipse Ti-U; Nikon, Kanagawa, Japan).

2.11. Small interfering RNA transfection

Scrambled siRNA (si-Con) and small interfering RNA (siRNA) directed against LKB1 (si-LKB1) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were transfected with siRNA (100 pmol each) for 24 h by using Fugene HD transfection reagent (Invitrogen), and then treated with 20S-PPD for the indicated times.

2.12. Statistical analysis

One-way analysis of variance was used to determine the significance level of differences between multiple group means, and unpaired Student t test was used to determine the significance level of differences between the means of two groups. A p value < 0.05 was considered significant.

3. Results

3.1. 20S-PPD decreased HSC viability

To explore the mechanistic basis of the antifibrotic effect of RGE, we treated LX-2 cells with 0.3–10 mg/mL of RGE, and then measured cell viability using an MTT assay. Exposure to RGE (1–10 mg/mL) for 24 h significantly and concentration-dependently decreased cell viability as compared with untreated control cells (Fig. 2A, left). When LX-2 cells were treated with 10 mg/mL of RGE for 6–48 h, cell viabilities were time-dependently decreased by RGE treatment (Fig. 2A, right). Because treatment with RGE for 24 h is sufficient incubation time to show differences in LX-2 cell viabilities, cells were treated with major ginsenosides and aglycosylated metabolites for 24 h to identify the ginsenoside responsible for RGE-mediated HSC cytotoxicity. Treatment with PPD-type
ginsenosides (Rb1, Rb2, Rc, and 20R-Rg3) or PPT-type ginsenosides (Re and Rg1) at concentrations up to 10μM for 24 h did not affect LX-2 cell viability (Figs. 2B and 2C). However, treatment with the PPD-type ginsenoside 20S-Rg3 (10μM) significantly decreased cell viability. In addition, treatments with aglycosylated metabolites (Comp. K, 20R-PPD, and 20S-PPD) for 24 h also concentration-dependently reduced LX-2 cell viability, whereas treatment with 20S-PPT did not (Fig. 2D). The IC50 values of Comp. K, 20R-PPD, and 20S-PPD were 3.39 ± 0.87μM, 8.52 ± 0.59μM, and 2.05 ± 0.59μM, respectively.

Ginsenoside Rb1 has an O-linked di-glucose residue at C3 and C20 on its PPD scaffold. By contrast, the ginsenoside metabolite Comp. K has a hydroxyl group at C3 and an O-linked glucose at C20, and 20S-PPD has only hydrogen at C3 and C20 (Fig. 1). To examine the effect of ginsenoside aglycosylation on LX-2 cell viability, we treated cells with Rb1, Comp. K, or 20S-PPD (3μM each). As was expected, treatment time with Rb1 (3μM) for up to 24 h did not decrease cell viability. When LX-2 cells were incubated with 3μM of 20S-PDD, cell viability was reduced significantly at 6 h, and this then gradually decreased. However, cell viability started to decrease significantly at 12 h after LX-2 cells were treated with 3μM of Comp. K (Fig. 3A). The viabilities of 20S-PDD- and Comp. K-treated cells at 24 h were 14.75 ± 1.00% and 55.40 ± 5.17% of that of controls, respectively. Next, we isolated primary HSCs from ICR mice and cultivated them for 6 d to activate the cells. Cell purity and identity were confirmed by UV positivity, GFAP (a marker of quiescent HSCs), and α-SMA (a marker of activated HSCs) immunoblotting (Fig. 3B, upper). When primary activated HSCs were treated with Rb1, Comp. K, or 20S-PDD (3μM each) for 24 h, 20S-PDD was found to reduce α-SMA protein levels (Fig. 3B, lower). In addition, 20S-PDD (3μM, 24 h) had the most potent cytotoxic effect against primary activated HSCs (Fig. 3C).

3.2. 20S-PDD promoted oxidative stress

To determine whether oxidative stress was involved in 20S-PDD-mediated HSC death, LX-2 cells were pretreated with NAC (10mM; a representative antioxidant) and then incubated with 10μM of 20S-PDD for 24 h. We found that 20S-PDD-mediated cytotoxicity was significantly inhibited by NAC pretreatment (Fig. 4A). Next, we monitored intracellular H2O2 production using DCFH-DA. Treatment with 1–10μM of 20S-PDD for 24 h increased intracellular H2O2 production in a concentration-dependent manner. However, NAC pretreatment completely abolished the H2O2 accumulation induced by 20S-PDD (Fig. 4B). We also measured the level of reduced GSH (a major endogenous antioxidant that regulates redox homeostasis by scavenging reactive oxygen species). Intracellular concentrations of reduced GSH were
significantly diminished after treatment of LX-2 cells with 3 μM or 10μM of 20S-PPD for 24 h (Fig. 4C). In addition, we further investigated whether reactive oxygen species generation by 20S-PPD was mediated by mitochondrial membrane dysfunction. We observed that 20S-PPD (10μM) significantly increased LX-2 cell populations with low rhodamine123 fluorescence intensity as compared with controls, and that this 20S-PPD-induced change was prevented by NAC pretreatment (10mM) (Fig. 4D). Furthermore, LX-2 cells treated with 20S-PPD released LDH to medium in a concentration-dependent manner (Fig. 4E).

3.3. 20S-PPD induced HSC apoptosis

To determine the type of cell death induced by 20S-PPD, LX-2 cells were treated with 3μM or 10μM of 20S-PPD for 24 h and double stained with fluorescein isothiocyanate-conjugated annexin V and propidium iodide (PI). Cell subpopulations were then investigated by flow cytometry (Fig. 5A). The results showed that 20S-PPD concentration-dependently increased the proportion of early apoptotic cells (annexin V-positive and PI-negative stained cells). Proportions of early apoptosis were 25.12 ± 12.47% and 63.03 ± 12.70% in cells treated with 3μM or 10μM of 20S-PPD, respectively. Next, we monitored the expression levels of apoptosis-related proteins to confirm apoptosis induction by 20S-PPD. When cells were treated with Comp. K or 20S-PPD (both at 3μM) for 24 h, 20S-PPD was found to cause more PARP cleavage than Comp. K. Although Comp. K treatment tended to increase PARP cleavage as compared with controls, no statistical difference was observed (Fig. 5B). Furthermore, 20S-PPD increased Bax expression, reduced the expressions of procaspase-3 and Bcl-2 in a concentration-dependent manner (Fig. 5C), and significantly increased caspase-3/-7 activities and transferase dUTP nick end labeling (TUNEL) staining intensities of LX-2 cells (Fig. 5D).

3.4. AMPK activation was involved in 20S-PPD-mediated apoptosis

To determine whether 20S-PPD activates AMPK in HSCs, we monitored the phosphorylations of AMPK and ACC (a downstream substrate of AMPK) in LX-2 cells. The phosphorylations of AMPK and ACC were both increased by 20S-PPD at concentrations of 1–10μM, whereas 20S-PPD did not affect the expression levels of AMPK and ACC (Fig. 6A, upper). Maximum AMPK phosphorylation was observed after treatment with 1μM of 20S-PPD for 6 h, and this phosphorylation was sustained for 24 h (Fig. 6A, lower). Consistently, treatment with 20S-PPD increased the phosphorylations of AMPK and ACC more than the same concentrations of Rb1 or Comp.
K in LX-2 cells and primary activated HSCs (Fig. 6B). To investigate the role of AMPK activation in \(^{20}S\)-PPD-induced HSCs apoptosis, AMPK activation was blocked by pretreating LX-2 cells with compound C, and \(^{20}S\)-PPD-mediated cytotoxicity was then monitored. Cell viability results showed that pretreatment with compound C (1 \( \mu \)M) significantly inhibited the \(^{20}S\)-PPD-induced apoptosis of LX-2 cells (Fig. 6C, lower). Inhibition of AMPK activity by compound C was confirmed by immunoblotting (Fig. 6C, upper). Treatment with compound C alone did not alter cell viability (data not shown). Furthermore, mitochondrial impairment induced by \(^{20}S\)-PPD was also significantly attenuated by compound C pretreatment (Fig. 6D).

### 3.5. \(^{20}S\)-PPD phosphorylated AMPK by activating LKB1

To identify the upstream kinase involved in \(^{20}S\)-PPD-mediated AMPK activation, we investigated whether LKB1 (an upstream kinase of AMPK) was phosphorylated by \(^{20}S\)-PPD. LKB1 phosphorylation started to increase at 6 h after treating LX-2 cells with 1 \( \mu \)M of \(^{20}S\)-PPD and was sustained for up to 24 h (Fig. 7A), which paralleled \(^{20}S\)-PPD-mediated AMPK phosphorylation (Fig. 6A). Transfection with LKB1 siRNA decreased \(^{20}S\)-PPD-mediated AMPK phosphorylation (Fig. 6A), and inhibited \(^{20}S\)-PPD-mediated cytotoxicity (Fig. 7C). However, chemical inhibition of CAMKK\(b\) by STO-609 had no effect on \(^{20}S\)-PPD-mediated AMPK phosphorylation or cytotoxicity (Fig. 7D).

### 4. Discussion

It is generally accepted that the diverse pharmacological activities of \(P\). ginseng are derived from a group of steroidal saponins, the ginsenosides [15]. When \(P\). ginseng is orally administered, ginsenosides cannot be easily absorbed into tissues because of their hydrophilicities. Thus, ginsenosides are converted into metabolites...
in the gastrointestinal tract by acid hydrolysis or intestinal microflora. It has been reported that the fully aglycosylated metabolite, PPD, can be produced from ginseng extract, Rb1, Rg3, or Comp. K by intestinal microflora [16,18,19,29], and that aglycosylated metabolites are more potent than their parent compounds [26,29,30]. Studies have shown that 20S-PPD inhibits the growth of Helicobacter pylori [29], attenuates the progression and growth of castration-resistant prostate cancer [31], enhances the sensitivity of chemotherapy against colorectal cancer [16], and protects neurons from permanent focal cerebral ischemic damage [32]. Although Park et al [26] reported that Comp. K triggers apoptosis in T-HSC/CI-6 cells (a rat-derived HSC line), the effects of other ginsenosides on HSCs remain to be established.

Among the 11 ginsenosides and aglycosylated metabolites tested, we found that 20S-PPD exhibited the greatest cytotoxic activity in HSCs (Figs. 2, 3). Our results indicate that 20S-PPD increased H₂O₂ production, depleted GSH levels, impaired MMP, and enhanced LDH leakage. Furthermore, pretreatment with NAC attenuated 20S-PPD-mediated H₂O₂ production, mitochondrial dysfunction, and cytotoxicity (Fig. 4), indicating that oxidative stress is involved in HSC death. Oxidative stress changes the Bcl-2/Bax expression ratio and opens the mitochondrial permeability.

![Fig. 5](image)

**Fig. 5.** Induction of apoptosis by 20S-PPD. (A) Flow cytometry analyses of cells double stained with fluorescein isothiocyanate-conjugated annexin V and PI. LX-2 cells were treated with 3μM or 10μM of 20S-PPD for 24 h. Percentages of healthy (low annexin V and low PI stained cells), early apoptotic (high annexin V and low PI stained cells), late apoptotic (high annexin V and high PI stained cells), and necrotic (low annexin V and high PI stained cells) cells were shown in the bar graph. (B) PARP cleavage. LX-2 cells were treated with 20S-PPD or Comp. K (3μM each) for 24 h and cell lysates were immunoblotted for PARP. (C) Expressions of apoptosis-related proteins. LX-2 cells were treated with 20S-PPD or Comp. K (3μM each) for 24 h and cell lysates were immuno-blotted for PARP. (D) Caspase-3/-7 activities. (E) TUNEL staining. LX-2 cells were treated with 3μM or 10μM 20S-PPD for 18 h and then stained by TUNEL. The relative expression levels of proteins were determined by scanning densitometry (B and C). Data represent the means ± SDs of three separate experiments. Significant versus untreated controls, * p < 0.05, ** p < 0.01; significant as compared between 20S-PPD and Comp. K treated cells, ## p < 0.01. Comp. K, compound K; PARP, poly(ADP-ribose)polymerase; PI, propidium iodide; PPD, protopanaxadiol; SD, standard deviation; TUNEL, transferase dUTP nick end labeling.
transition pore, which promotes caspase-dependent apoptosis [33,34]. Caspase-3 and caspase-7 are executor caspases and are responsible for the cleavage of more than 100 different proteins including that of PARP [35]. PARP cleavage causes defects in the DNA repair system and accelerates DNA fragmentation [36]. In accord with previous reports, the present study shows that 20S-PPD concentration-dependently reduced the expression of Bcl-2 (an antiapoptotic protein), increased the expression of Bax (a pro-apoptotic protein), and enhanced caspase-3/-7 activities. In addition, 20S-PPD concentration-dependently increased PARP cleavage, TUNEL staining intensity, and the proportions of early apoptotic cells (Fig. 5). Moreover, 20S-PPD induced the cleavage of PARP more than Comp. K at same concentration (Fig. 5B), which suggests that the apoptotic activities of ginsenosides are potentiated by the loss of sugar moieties.

Accumulated evidence shows AMPK is a double-edged sword in terms of determining the fate of cells against a variety of stresses. Previously, we reported that AMPK activation by several natural products protects hepatocytes from oxidative stress [8,37]. In addition, other groups have reported that AMPK activation attenuates hepatocytes apoptosis in different experimental models, such as tumor necrosis factor α-induced hepatitis and hepatic ischemia-reperfusion [38,39]. However, AMPK activation in hepatocellular carcinoma cells causes cell cycle arrest and apoptosis [40,41]. AMPK

**Fig. 6.** Activation of AMPK by 20S-PPD. (A) AMPK activation. LX-2 cells were treated with 1–10μM of 20S-PPD for 6 h (upper) or 1μM of 20S-PPD for 1–24 h (lower). Levels of phosphorylated AMPK and ACC in cell lysates were assessed by immunoblotting. Equal protein loadings was confirmed by immunoblotting for β-actin. Results were confirmed by repeated experiments. (B) Effect of aglycosylated ginsenosides on AMPK activation. LX-2 cells (upper) or primary activated HSCs (lower) were treated with Rb1, Comp. K or 20S-PPD (1μM each) for 6 h. (C) The effects of compound C on 20S-PPD-mediated cytotoxicity. LX-2 cells were pretreated with 1μM or 3μM of compound C for 1 h, and subsequently exposed to 1–10μM of 20S-PPD for 24 h. (D) Effect of compound C on 20S-PPD-mediated mitochondrial dysfunction. LX-2 cells were pretreated with 3μM of compound C for 1 h and then incubated with 10μM of 20S-PPD for 24 h. Data represent the means ± SDs of three separate experiments. Significant versus untreated controls or treatment with compound C alone, **p < 0.01; significant as compared between 20S-PPD alone and 20S-PPD plus compound C treated cells, #p < 0.05, ##p < 0.01. ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; Comp. K, compound K; HSCs, hepatic stellate cells; PPD, protopanaxadiol; SD, standard deviation.
suppresses proliferation, migration, chemokine secretion, and profibrogenic genes expressions in HSCs [10–12,42,43], which are the major phenotype changes observed during HSC activation [3]. Blockage of AMPK has been reported to attenuate the ability of adiponectin to inhibit HSC proliferation [11,12], and AMPK activation in HSCs degrades p300 coactivator, dissociates protein interactions between Smad3 and p300, and thereby inhibits the TGF-β-mediated expressions of ECM proteins [10]. Furthermore, AMPK activation by curcumin in HSCs upregulates antioxidant and adipogenic genes by inducing peroxisome proliferator-activated receptor-γ coactivator-1α, and downregulates the expressions of profibrogenic genes by inhibiting aerobic glycolysis [42,43].

Although several studies have suggested that AMPK activation leads to HSC apoptosis [4,13], the relationship between AMPK activation and HSC apoptosis remains to be further established. The present results indicate that 20S-PPD was the most cytotoxic and that it also triggered AMPK phosphorylation in HSCs (Figs. 3, 6B). Moreover, 20S-PPD-mediated mitochondrial dysfunction and apoptosis were blocked in the presence of compound C (an AMPK inhibitor) (Figs. 6C and 6D). Therefore, our results imply that AMPK activation is involved in the 20S-PPD-mediated apoptosis of HSCs. mTORC1 is another regulator of cell growth in response to energy status or environmental stress [44]. mTORC1 is a protein complex that contains mTOR, regulatory associated protein of mTOR (raptor), GβL, and PRAS40 [44,45]. AMPK activation inhibits mTORC1 activity and that of its downstream kinase, p70 ribosomal S6 kinase, via the phosphorylation of raptor [46]. Our supplementary results indicate that 20S-PPD increased the phosphorylation of raptor, decreased the GβL expression, and reduced levels of phosphorylated mTOR and S6 (a downstream substrate of p70 ribosomal S6 kinase) in LX-2 cells (Fig. S1). Although identities of the downstream signaling molecules that regulate HSC apoptosis

![Fig. 7. Role of LKB1 on 20S-PPD-mediated AMPK activation.](A) LKB1 phosphorylation. LX-2 cells were treated with 20S-PPD (1 μM) for the indicated times. Levels of phosphorylated LKB1 in cell lysates were measured by immunoblotting. Equal protein loadings were confirmed by immunoblotting for LKB1. (B) Effect of LKB1 knockdown on 20S-PPD-mediated AMPK activation. LX-2 cells were transfected with scrambled siRNA (si-Con) or LKB1 siRNA (si-LKB1) and then treated with 20S-PPD (10 μM) for 6 h. (C) Effect of LKB1 knockdown on 20S-PPD-mediated cytotoxicity. si-LKB1 transfected cells were treated with 10 μM of 20S-PPD for 24 h and cell viabilities were measured. (D) Effect of STO-609 on 20S-PPD-mediated cytotoxicity. LX-2 cells were pretreated with 1 μg/mL STO-609 and then exposed to 10 μM of 20S-PPD for 6 h (upper) or to 1–10 μM of 20S-PPD for 24 h (lower). Data represent the means ± SDs of three separate experiments. Significant versus untreated controls, *p < 0.05, **p < 0.01; significant as compared between 20S-PPD-treated cells, #p < 0.05, ##p < 0.01. ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; LKB1, liver kinase B1; PPD, protopanaxadiol; N.S., not significant; SD, standard deviation.
remain to be further identified, our findings suggest the involvement of mTORC1 inhibition in AMPK-mediated apoptosis induced by 20S-PPD.

Changes in AMP/ATP ratio and the activations of LKB1, CaMKK, protein kinase A, and TGF-β-activated kinase have been reported to regulate AMPK activity [6,7,47]. Ginsenosides and aglycosylated metabolites activate AMPK via multiple upstream activators [22]. It has been established that LKB1 activation by Rg1 or Rg2 inhibits hepatic glucose production [23,48]. Comp. K decreases matrix stress through inhibition of AMPK-mediated GSK3β-mediated apoptosis of colon cancer cells and increase nitric oxide production [30]. However, the activations of CaMKK by Comp. K or Rg3 induce the apoptotic effect of ginsenosides examined in the present study, 20S-PPD mediates AMPK phosphorylation in Cx-43 dependent manner.

In conclusion, our results demonstrate that aglycosylation potentiates the cytotoxicities of ginsenosides against HSCs. Of the ginsenosides examined in the present study, 20S-PPD most potently induced the apoptosis of HSCs via LKB1-dependent AMPK activation. Taken together, these findings suggest that 20S-PPD is a potential, therapeutic candidate for the prevention or treatment of liver fibrosis. Conflicts of interests

The authors declare that they have no conflicts of interests.

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Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgr.2017.01.012.

References

[1] Moreira RK. Hepatic stellate cells and liver fibrosis. Arch Pathol Lab Med 2007;131:1728–34.
[2] Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005;115:209–18.
[3] Lee YA, Wallace MC, Friedman SL. Pathobiology of liver fibrosis: a translational success story. Gut 2015;64:830–41.
[4] Wang N, Xu Q, Tan HY, Hong M, Li S, Yuen MF, Feng Y. Berberine inhibition of PPAR-γ mediates AMPK-mediated hepatoprotection against alcohol-induced liver injury in mice through suppression of inflammation. Phytomedicine 2016;23:583–8.
[5] Han JY, Lee S, Yang JH, Kim J, Sim J, Kim MG, Jeong TC, Ku SK, Cho IJ, Ki SH. Korean Red Ginseng attenuates ethanol-induced steatosis and oxidative stress via AMPK-dependent activation. J Ginseng Res 2015;39:105—15.
[6] Jeong KJ, Kim CW, Chung SH. AMP-activated protein kinase: an emerging target for ginseng. J Ginseng Res 2014;38:83—8.
[7] Yuan HD, Kim DY, Quan HY, Kim SJ, Jung MS, Chung SH. Ginsenoside Rg2 inhibits NF-κB and nuclear factor κB receptor-associated protein expression and inactivates NF-κB–induced apoptosis. J Nutr Biochem 2008;19:641—4.
[8] Cho SJ, Kim YW, Han CY, Kim EH, Anderson RA, Lee YS, Lee CH, Hwang SJ, Kim SC. E-cadherin antagonizes transforming growth factor (TGF-β) gene induction in hepatic stellate cells by inhibiting RhoA-dependent Smad3 phosphor- ylation. Hepatology 2010;52:2033—43.
[9] Bae EA, Han MJ, Cho MK, Park SY, Kim DH. Metabolism of 20S- and 20R-ginsenoside Rg1 by human intestinal bacteria. Biol Pharm Bull 2000;23:1481—5.
[10] Geng J, Peng W, Huang Y, Fan H, Li S. Ginsenoside-Rg1 from Panax notoginseng prevents hepatic fibrosis induced by thioacetamide in rats. Eur J Pharmacol 2006;543:162—9.
[11] Park EJ, Zhao YZ, Kim J, Sohn DH. A ginsenoside metabolite, 20-O-beta-d-glucopyranosyl-20(S)-protopanaxadiol, triggers apoptosis in activated rat hepatic stellate cells via caspase-3 activation. Planta Med 2006;72:1250—3.
[12] Ku SH, Yang JH, Ku SK, Kim SC, Kim YW, Cho IJ. Red ginseng extract prevents against carbon tetrachloride-induced liver fibrosis. J Ginseng Res 2013;37:35—3.
[13] Cho SJ, Kim YW, Han CY, Kim EH, Anderson RA, Lee YS, Lee CH, Hwang SJ, Kim SC. E-cadherin antagonizes transforming growth factor (TGF-β) gene induction in hepatic stellate cells by inhibiting RhoA-dependent Smad3 phosphor- ylation. Hepatology 2010;52:2033—43.
[14] Bae EA, Han MJ, Cho MK, Park SY, Kim DH. Metabolism of 20S- and 20R-ginsenoside Rg1 by human intestinal bacteria and its relation to in vitro biological activities. Bioll Pharm Bull 2002;25:58—63.
[15] Shin DJ, Kim JE, Lim TG, Jeong EH, Park G, Kang MJ, Park JS, Yeom MH, Oh DK, Bode AM, et al. 20-O-β-D-Glucopyranosyl-20(S)-protopanaxadiol suppresses UV-induced MMP-1 expression through AMPK-mediated mTOR inhibition as a downstream of the PKA-LKB1 pathway. J Cell Biochem 2014;115:1702—11.
[16] Cao B, Qi Y, Yang Y, Liu X, Xu D, Gao W, Zhan Y, Xiong Z, Zhang A, Wang AR, et al. (20S)-Protopanaxadiol inhibition of progression and growth of castration-resistant prostate cancer. PLoS One 2014;9:e111201.
[17] Xu H, Xu X, Qu S, Chen Y, Wang Z, Sai D. Protective effect of Panax quinquefolium 20(S)-protopanaxadiol saponins, isolated from Panax quinquefolium, on permanent focal cerebral ischemic injury in rats. Exp Ther Med 2014;7:165—70.
[18] Xu LM, Xue C, Lin CY, Hung MF, Shen JH, Hwang TL. Intracellular glutathione depletion by oridonin leads to apoptosis in hepatic stellate cells. Molecules 2014;19.3237—47.
[19] Wu J, Huang RW, Lin DJ, Peng J, Wu XY, Lin Q, Pan XL, Song YQ, Zhang MH, Hou M, et al. Expression of survivin and bax/bcl-2 in peroxisome proliferator activated receptor-γ gene induction in peroxisome proliferator activated receptor-γ ligands induces apoptosis on human myeloid leukemia cells in vitro. Annals Oncol 2005;16:455—60.
[20] Yu H, Cullen SP, Sheridan C, Litthi CH, Geng J, Martin SJ. Executive caspase-3 and caspase-7 are functionally distinct proteases. Proc Natl Acad Sci U S A 2005;108:12815—9.
[21] Herceg C, Wang ZQ. Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. Mutat Res 2001;477:97—110.
[37] Dong GZ, Jang EJ, Kang SH, Cho IJ, Park SD, Kim SC, Kim YW. Red ginseng abrogates oxidative stress via mitochondria protection mediated by LKB1–AMPK pathway. BMC Complement Altern Med 2013;13:64.

[38] Cai L, Hu K, Lin L, Ai Q, Ge P, Liu Y, Dai J, Ye B, Zhang L. AMPK dependent protective effects of metformin on tumor necrosis factor-induced apoptotic liver injury. Biochem Biophys Res Commun 2015;465:381–6.

[39] Zhang C, Luo Y, Li Q, Chen M, Zhao Q, Deng R, Wu C, Yang A, Guo Z, Wang D, et al. Recombinant adiponectin ameliorates liver ischemia reperfusion injury via activating the AMPK/eNOS pathway. PLoS One 2013;8:e66382.

[40] Cai X, Hu X, Cai B, Wang Q, Li Y, Tan X, Hu H, Chen X, Huang J, Cheng J, et al. Metformin suppresses hepatocellular carcinoma cell growth through induction of cell cycle G1/G0 phase arrest and p21cip and p27kip expression and downregulation of cyclin D1 in vitro and in vivo. Oncol Rep 2013;30:2449–57.

[41] Lee CW, Wong LL, Tse EY, Liu HF, Leong YY, Lee JM, Hardie DG, Ng IO, Chung YP. AMPK promotes p53 acetylation via phosphorylation and inactivation of SIRT1 in liver cancer cells. Cancer Res 2012;72:4394–404.

[42] Lian N, Jin H, Zhang F, Wu L, Shao J, Lu Y, Zheng S. Curcumin inhibits aerobic glycosylation in hepatic stellate cells associated with activation of adenosine monophosphate-activated protein kinase. J Biochem Mol Biol 2016;49:189–96.

[43] Zhai X, Qiao H, Guan W, Li Z, Cheng Y, Jia X, Zhou Y. Curcumin regulates peroxisome proliferator-activated receptor-γ coactivator-1α expression by AMPK pathway in hepatic stellate cells in vitro. Eur J Pharmacol 2015;746:56–62.

[44] Wulfischleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell 2006;124:471–84.

[45] Shaw RJ. LKB1 and AMP-activated protein kinase control of mTOR signalling and growth. Acta Physiol 2009;196:65–80.

[46] Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell 2008;30:214–26.

[47] Djouder N, Tuerk RD, Suter M, Salvioni P, Thali RF, Scholz R, Vahtomeri K, Auchi Y, Rechsteiner H, Brunisholz RA, et al. PKA phosphorylates and inactivates AMPKα1 to promote efficient lipolysis. EMBO J 2010;29:469–81.

[48] Kim SJ, Yuan HD, Chung SH. Ginsenoside Rg1 suppresses hepatic glucose production via AMP-activated protein kinase in HepG2 cells. Biol Pharm Bull 2010;33:325–8.

[49] Hien TT, Kim ND, Pokharel YR, Oh SJ, Lee MY, Kang KW. Ginsenoside Rg3 increases nitric oxide production via increases in phosphorylation and expression of endothelial nitric oxide synthase: essential roles of estrogen receptor-dependent PI3-kinase and AMP-activated protein kinase. Toxicol Appl Pharmacol 2010;246:171–83.

[50] Kim DY, Park MW, Yuan HD, Lee HJ, Kim SH, Chung SH. Compound K induces apoptosis via CAMK-IV/AMPK pathways in HT-29 colon cancer cells. J Agric Food Chem 2009;57:10573–8.