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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. H_2O_2 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.

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

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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.

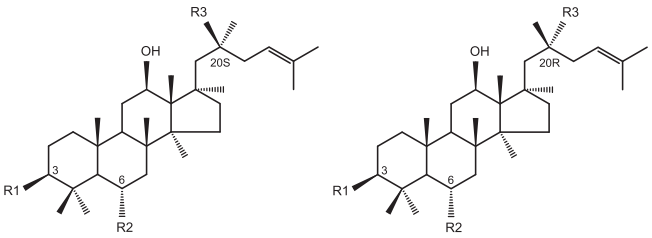
Ginseng, the root of *Panax ginseng* Meyer, has been used as adaptogenic agent for centuries in Korea, and modern science has shown that ginseng saponins (ginsenosides) are major active ingredients of ginseng. The ginsenosides are classified as dammarane-type, ocotillol-type, or oleanane-type oligoglycosides, and the most abundant dammarane-type ginsenosides can be further classified as protopanaxadiol (PPD) or protopanxatriol (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, 20*S*-Rg3, 20*R*-Rg3, Re, Rg1) and aglycosylated metabolites [compound K (Comp. K), 20*S*-PPT, 20*R*-PPD, and 20*S*-PPD] were purchased from Ambo Institute (Daejeon, Korea) (Fig. 1). Compound C (an inhibitor of AMPK) and STO-609 (an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase kinase β, CAMKKβ) 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-glial 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



Ginsenoside	R1	R2	R3
Rb1	-O-Glc^{2}- Glc	-H	-O-Glc^{6}- Glc
Rb2	-O-Glc^{2}- Glc	-H	-O-Glc^{6}- Ara(p)
Rc	-O-Glc^{2}- Glc	-H	-O-Glc^{6}- Ara(f)
20S-Rg3	-O-Glc^{2}- Glc	-H	-H
20R-Rg3	-O-Glc^{2}- Glc	-H	-H
Comp. K	-OH	-H	-O-Glc
20S-PPD	-OH	-H	-OH
20R-PPD	-OH	-H	-OH
Re	-OH	-O-Glc^{2}- Rha	-O-Glc
Rg1	-OH	-O-Glc	-O-Glc
20S-PPT	-OH	-OH	-OH

Fig. 1. Chemical structures of ginsenosides and aglycosylated metabolites. Numerical superscripts indicate the carbons at glycosidic bonds. Ara(f), arabinofuranose; Ara(p), arabinopyranose; Comp. K, compound K; Glc, glucose; PPD, protopanaxadiol; PPT, protopanaxatriol; Rha, rhamnose.

(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% CO_2. 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 fluorescent microscope (Eclipse Ti-U; Nikon, Kanagawa, Japan), and phenotypic changes during HSC activation were verified by GFAP and α-SMA immunoblotting, as previously described [13,28]. 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

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