Barley sprout extracts reduce hepatic lipid accumulation in ethanol-fed mice by activating hepatic AMP-activated protein kinase

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ABSTRACT

Chronic alcohol consumption leads to hepatic lipid accumulation and alcoholic fatty liver disease. Previously, we demonstrated that barley sprout extract, which contains saponarin as an active compound, reduces hepatic steatosis. In this study, we investigated the effect of barley sprout extracts (BSE) on hepatic lipid accumulation in a mouse model of alcoholic fatty liver disease. Seven-week-old C57BL/6 mice were fed an alcohol-containing diet (5% ethanol) and a low or high dose of BSE (100 or 200 mg/kg body weight, respectively) for 10 days. The high dose of BSE significantly decreased hepatic lipid accumulation compared with the ethanol-only control group. In the second animal study, mice were fed an alcohol-containing diet for 10 days, followed by a 45% high-fat diet with oral administration of BSE (100 or 200 mg/day/kg body weight) for 4 weeks. Mice in both BSE-fed groups showed reduced hepatic steatosis. In the livers of mice fed BSE, phosphorylation of AMP-activated protein kinase (AMPK) was increased, and expression of hepatic autophagy markers was elevated. In cultured hepatocytes, BSE (200 μg/mL) increased the rate of fatty acid oxidation and reduced that of fatty acid synthesis. Taken together, these findings suggest that BSE promotes degradation of lipid droplets and subsequent activation of fat oxidation by activating AMPK in the liver, thus protecting against development of hepatic steatosis in alcohol-fed mice. Saponarin, a major flavonoid in BSE and an activator of AMPK, increased the activity of microsomal triglyceride transfer protein, which suggests that the reduction in hepatic triglyceride levels was mediated by this component of BSE. In conclusion, BSE ameliorated hepatic steatosis in a mouse model of ethanol-induced fatty liver by activating AMPK, an effect possibly mediated by the saponarin component.

1. Introduction

Alcoholic fatty liver disease (AFLD) is a major chronic liver disease worldwide (Arteel, Marsano, Mendez, Bentley, & McClain, 2003). Excessive alcohol consumption results in lipid accumulation in the liver, which leads to fatty liver, alcoholic hepatitis, and alcoholic cirrhosis (Diehl, 2002; Livero & Acco, 2016). Hepatic steatosis is the most common early symptom of AFLD and is characterized by the accumulation of lipid droplets within hepatocytes without prominent infiltration of inflammatory macrophages (Yeh & Brunt, 2014). No drug has been approved for AFLD (Gao & Bataller, 2011); therefore, prevention of hepatic steatosis due to alcohol consumption is an important issue (Louvet & Mathurin, 2015). Prevention of hepatic steatosis is particularly important in patients with AFLD because of the possibility of progression to more severe forms of AFLD (You & Crabb, 2004). Hepatic lipid metabolism is highly complex and regulated by multiple pathways, including that centered around AMP-activated protein kinase (AMPK) (Viollet et al., 2006). AMPK is a crucial regulatory protein for cellular energy homeostasis, and its activation stimulates oxidation of fuel molecules, including stored fats, and suppresses anabolic metabolism, such as lipid and protein syntheses (Hardie, 2008; Mihaylova & Shaw, 2011). Thus, activation of AMPK could be an effective strategy for reducing hepatic steatosis.

Several natural substances induce AMPK activation (Ajmo, Liang, Rogers, Pennock, & You, 2008; Liu et al., 2014; Noh et al., 2011). The sprouts from natural grains such as barley have potent antioxidant activity and high levels of flavonoids (Park, Seo, & Kang, 2015). In a previous study, we showed that barley sprouts contain high levels of...
polyphenols and flavonoids; the major flavonoid identified in barley sprouts was saponarin (Lee et al., 2015; Seo, Lee, Jia, Wu, & Lee, 2015). In addition, we reported that barley sprout ethanol (EtOH) extract (80% v/v; BSE) exerted hypocholesterolemic and hypoglycemic effects by stimulating AMPK activity and its signaling pathways in cultured hepatocytes and in high-fat diet (HFD)-fed mice (Lee et al., 2015). Thus, in this study, we investigated the effects of BSE on lipid accumulation and steatosis in the liver during or after ingestion of alcohol using a mouse model of AFLD. The results suggest that BSE can alleviate AFLD by activating the AMPK signaling pathway and inducing expression of autophagy-related proteins. Moreover, saponarin was at least in part responsible for these effects.

2. Materials and methods

2.1. Sample preparation and composition analysis

Barley sprouts (Hordeum vulgare L.) were harvested at the Department of Functional Crops, National Institute of Crop Science, Rural Development Administration (Jeonju, Republic of Korea) as described previously (Seo et al., 2013). Briefly, barley seeds (Kunalbori 1) were imbibed in water for 1 day prior to germination and kept in the dark for 2 days at 22–25 °C. The germinated barley was grown in a growth chamber (DSGC768, Dongseo Science, Anyang, Republic of Korea) under 60% relative humidity. Thirty days after germination, barley leaves were harvested, freeze-dried and extracted using EtOH (80%, v/v) at room temperature for 24 h. The extracts were filtered and evaporated under a vacuum and subsequently freeze-dried to yield dry powders. BSE composition was analyzed as described previously (Seo et al., 2013). Total polyphenols were determined by the Folin–Ciocalteu method, as described previously (Singleton, Orthofer, & Lamuela-Raventos, 1999). To quantify policosanols, the lipophilic fraction was isolated by adding hexane, and gas chromatography mass spectrometry (GC–MS) analysis was performed using the Agilent Technologies 7890A series GC system coupled to the 5975C single quadrupole MS (Agilent Technologies, Palo Alto, CA, USA) with the HP-5MS (5% diphenyl–95% dimethylsiloxane co-polymer) capillary GC column (30 m × 0.25 μm × 0.25 μm film thickness; Agilent Technologies). The oven temperature was programmed to increase from 150 to 325 °C at a rate of 4 °C/min and then maintained at 320 °C for 5 min. Helium was used as the carrier gas at a flow rate of 1.8 mL/min. The sample (1 μL) was injected into the GC by an autosampler (Agilent Technologies). The split ratio was 1:5. For MS detection, the electron impact ion source and transfer line temperatures were set to 200 and 280 °C, respectively, and the ionization energy was set to 70 eV. Data collection and analysis were conducted using the GC–MSD Chemstation (Agilent Technologies) (Seo et al., 2013). The results showed that BSE contained 167.4 mg/g total polyphenols, 58.0 mg/g flavonoids, and 19.4 mg/g saponarin (Supplemental Table 1).

2.2. Animal experiments

Mice were fed EtOH to induce AFLD (Bertola, Mathews, Ki, Wang, & Gao, 2013). C57BL/6 male mice (6 weeks old, 20–23 g) were purchased from Samtako (Kyunggido, Korea). Two animal experiments were performed. In the first experiment, mice were fed a commercial

![Fig. 1. BSE ameliorated hepatic lipid accumulation in EtOH-fed mice. BSE reduced hepatic triglyceride concentrations, lipid accumulation, and macrophage infiltration into liver tissue. After daily oral administration of BSE for 10 days, EtOH-fed mice were euthanized, and organs were collected. (A) Experimental design. (B) Hepatic triglyceride concentrations. (C) H & E staining, Oil red O staining, and F4/80 staining of mouse livers. Images were obtained and analyzed as described in the Materials and methods section (magnification ×200). Data are means ± SEM, and the significance of differences was determined by one-way ANOVA. Different letters indicate significant differences among the groups. BSE, barley sprout extract; C, control; E, EtOH; L, LBSE; H, HBSE.](image)
chow diet (Central Lab. Animal Inc., Seoul, Korea) for 1 week and then randomly assigned into one of the following four groups: the normal diet group, fed a Lieber-DeCarli control liquid diet without EtOH (negative control, n = 5); the EtOH control group, fed a Lieber-DeCarli control liquid diet containing EtOH (5%, v/v, EtOH control, n = 5); and two BSE groups, fed a Lieber-DeCarli control liquid diet containing EtOH (5%, v/v) and orally administered a low or high dose of BSE (100 or 200 mg/day/kg body weight; LBSE and HBSE groups, respectively; n = 4). The negative and EtOH control mice were orally administered vehicle (double-distilled water). The mice were fed the liquid diet for 10 days. On day 11, the mice were fed a bolus of bine EtOH (31.5% EtOH, v/v); 9 h later the mice were deeply anesthetized with isolufore and then euthanized (Fig. 1A). The gavage volume of bing alcohol was calculated as described below (Bertola et al., 2013):

Gavage volume (μL) of 31.5%EtOH per mouse = body weight (g) × 20

In the second experiment, negative control mice were fed a Lieber-DeCarli control liquid diet without EtOH followed by an AIN-76A-based HFD (HFD group, 45% of calories from fat, n = 5) for 4 weeks. The other three groups were fed a Lieber-DeCarli liquid diet with EtOH (5%, v/v) for 10 days to induce AFDL. The experimental mouse model was used to mimic alcoholic humans who consume a HFD, which is a high-risk group for AFDL. Before the start of the test diet, Mice from the control and ethanol groups were randomly selected and sacrificed, and the development of fatty liver disease was confirmed by measurement of hepatic triglyceride levels and H&E staining of liver tissues (Fig. S2). Then, mice were randomly assigned into three groups and fed as follows for another 4 weeks: mice fed a HFD (HFD-EtOH group, n = 5) and mice fed a HFD and orally administered a low or high level of BSE (100 or 200 mg/day/kg body weight; LBSE and HBSE group, respectively, n = 5; Fig. 3A). Mice were maintained on a 12 h light/dark cycle at a controlled temperature of 21–25 °C and humidity of 50–60%. All mice were fasted for overnight before sacrifice. All experiments involving animals were performed according to a protocol approved by the Animal Experiment Committee of Korea University (Protocol No. KUACUC-2015-81).

2.3. Measurement of serum and liver triglyceride levels

Hepatic lipids were extracted from mouse livers by adding 1 mL aceton to 40 mg liver tissue. After 12 h of incubation at room temperature, supernatants were collected and evaporated using a vacuum centrifuge (Speedvac, N-Biotek, Bucheon, Korea). Concentrates were dissolved in 100 μL 80% (v/v) ETOH. Serum triglycerides and cholesterol levels, hepatic triglyceride levels, and alanine aminotransferase and aspartate aminotransferase activities were determined enzymatically using the Cobas C111 system (Roche, Forrenstrasse, Switzerland) and enzymatic quantification methods (Jun et al., 2014).

2.4. Histological analysis of liver tissue

Livers were fixed with 4% paraformaldehyde and then stained with hematoxylin and eosin (H&E) in the Histopathology Department of Anam Korea University Hospital (Seoul, Korea). Hepatic steatosis was determined by staining of 10-μm-thick frozen sections with Oil Red O (Sigma-Aldrich, St Louis, MO, USA). The H&E and Oil Red O-stained sections were viewed under the Axio Imager M1 microscope (Carl-Zeiss, Oberkochen, Germany). Immunohistochemistry was performed using the ImmunoCruz Rat ABC Staining System (Santa Cruz, CA, USA) according to the manufacturer's instructions. Briefly, parafern-embedded liver sections were incubated in deionized water to quench endogenous peroxidase activity, rehydrated in PBS, blocked with PBS containing 1.5% blocking serum for 60 min at room temperature, and then incubated with an F4/80 primary antibody at 4 °C overnight. The following day, the sections were washed three times with PBS. Sections were then incubated with a biotinylated secondary antibody at room temperature for 30 min and washed three times with PBS. Inflammatory cells were stained by incubating the liver sections with peroxidase substrate for 10 min, followed by washing in deionized water. Hematoxylin was used to stain the nuclei, and sections were mounted in Prolong Gold antifade reagent (Invitrogen, Carlsbad, CA, USA). The sections were visualized under the Axio Imager M1 microscope (Carl-Zeiss) (Jia, Wu, Kim, Kim, & Lee, 2016).

2.5. Immunoblot analysis

Mouse liver tissue was homogenized in RIPA buffer (10 mM Tris-HCl, pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 1 mM EDTA) containing 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich) and centrifuged at 17,500 × g for 10 min at 4 °C to collect the supernatant. Protein concentrations were assessed using Bradford reagent (Bio-Rad). Protein samples were mixed with sample buffer (1 M Tris-HCl (pH 6.8), 15% SDS, 25% glycerol, 12.5% 2-mercaptoethanol, and 0.02% bromophenol blue), denatured by boiling for 5 min at 100 °C, run on an SDS-PAGE gel, transferred onto nitrocellulose membranes (Daeilab Service Co. Ltd., Seoul, Korea), and immunoblotted using the appropriate primary and secondary antibodies. Immunoblots were analyzed using the ChemiDoc™ Touch imaging system (Bio-Rad). Antibodies specific for AMPK, phosphorylated AMPKα (Thr172), and β-actin were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). The anti-microtubule-associated protein 1A/1B-light chain 3 (LC3) antibody was obtained from Novus Biologicals (Littleton, CO, USA). Protein levels were normalized to that of β-actin (Wu et al., 2015).

2.6. Transmission electron microscopy (Helman, Temko, Nye, & Fallon)

Liver tissues were fixed in 2.5% glutaraldehyde with 0.1 M sodium cacodylate (pH 7.43) and stored at 4 °C overnight. Tissues were then fixed with 2% osmium tetroxide, serially dehydrated in ethyl alcohol (50–100%), stained with 0.5% uranyl acetate, and embedded in Spurr's resin. Samples were sectioned using an ultramicrotome (Leica, Wetzlar, Germany), and ultrathin sections were stained with 1% uranyl acetate and 2% lead citrate. Grids were visualized by TEM (JEOL1010, JEOL, Tokyo, Japan) at 80 kV. Three non-repeating micrographs of randomly selected fields were observed for each liver sample (n = 3/group). The numbers of autophagy vacuoles and lipid droplets per unit area (100 μm²) in each sample were counted using Image J (NIH).

2.7. Fatty acid oxidation, fatty acid synthesis, and ketone body analysis

HepG2 cells were cultured in 24-well plates at 2.5 × 10^5/well for 24 h. Next, the cells were incubated in the presence of palmitic acid and oleic acid (400 μM each) for 24 h to induce lipid accumulation. On the following day, cells were treated with BSE (100 or 200 μg/mL), 2 mM metformin, or 1 μM GW7647, a peroxisome proliferator-activated receptor (PPAR)-α agonist, for 24 h. The rates of fatty acid oxidation and synthesis were measured by determining [1-14C] palmitate and [1-14C] acetate (45–60 mCi/mmol, Perkin Elmer, Norwalk, CT, USA) levels, respectively, as described previously (Kim et al., 2013). To evaluate fatty acid oxidation, cells were incubated with [1-14C] palmitate for 1 h. Next, 500 μL culture medium were collected and mixed with 500 μL 0.5 mM NaOH and 0.5 mM HCl, followed by incubation for 30 min at room temperature. After addition of counting solution (1 mL), 14C-labeled CO₂ was quantified using a scintillation counter (Hidex 300sl, Hidex, Finland). To assess the rate of fatty acid synthesis, HepG2 cells were incubated with [1-13C] acetate for 1 h, washed three times with 0.14 M KCl, incubated with 0.5 M NaOH (0.75 mL), and harvested for preparation of cell lysates. After addition of 2 mL EtOH and 1 mL distilled water, cell lysates were incubated at 90°C for 90 min, followed by addition of 7 M HCl (0.5 mL) and petroleum ether (7.5 mL), followed by complete drying under N₂ gas. Counting solution (1 mL) was added to
the samples, and radioactivity was quantified using a scintillation counter (Hidex 300sl, Hidex) (Kim et al., 2016). Protein concentrations were assessed by the Bradford method with Bio-Rad Protein Assay Reagent (Bio-Rad), and used to normalize the radioactivity data. Hepatic ketone body levels were measured using a \( \beta \)-hydroxybutyrate assay kit (Cayman, MI, USA).

2.8. Luciferase assay

HEK293-T cells were seeded in 24-well plates at \( 8 \times 10^4 \)/well. On the following day, the cells were co-transfected with a Forkhead box O3 (FOXO3) expression vector (product #60643, BPS Biosciences, San Diego, CA, USA), a FOXO firefly luciferase reporter vector (product #60643, BPS Biosciences), and a Renilla luciferase constitutive expression vector (internal control, Promega, Madison, WI, USA) using Lipofectamine 2000 transfection reagent (Invitrogen). This reporter vector contains the firefly luciferase gene under the control of multimers of the FOXO response element. The FOXO reporter was premixed with Renilla luciferase expression vector. After transfection for 16 h, cells were treated with or without EtOH (100 mM) and with or without saponarin (50 \( \mu \)M) or A-769662 (positive control for AMPK; Cayman Chemical, Ann Arbor, MI, USA) for 48 h. Medium was changed at 12 h intervals. Luciferase activities were measured using a dual luciferase reporter assay system (Promega) according to the manufacturer's protocol (Jia et al., 2011).

2.9. Microsomal transfer triglyceride protein (MTP) activity assay

HepG2 cells were cultured until confluence, then treated with or without EtOH (100 mM) and with or without saponarin (100 \( \mu \)M) for 48 h. Medium was changed at 12 h intervals. Collected cells were homogenized in homogenization buffer (pH 7.4; 100 mM Tris, 150 mM NaCl, 1 mM EDTA, 100 mM phenylmethylsulfonyl fluoride, and 1 mg/mL leupeptin) by ultrasonication (Active Motif, Carlsbad, CA, USA). MTP activity was measured using an MTP activity kit (MAK110, Sigma-Aldrich) according to the manufacturer's instructions. Briefly, 100 \( \mu \)g homogenized HepG2 cell samples were added to a 96-well plate (10 \( \mu \)L), and 190 \( \mu \)L MTP assay mixture were added to each well. The MTP assay mixture contains buffer and donor and acceptor particles. This kit measures MTP activity in terms of the transfer of neutral lipids on the donor particles to acceptor particles by assessing changes in the fluorescence intensity of the samples. The reaction plate was sealed and incubated for 3 h at 37 °C. At 30 min intervals, MTP activity was measured as the increase in fluorescence (\( \lambda_{exc} = 465/\lambda_{em} = 535 \) nm) in the samples using a luminescence meter (Victor2, Perkin Elmer) (Seo et al., 2017).

2.10. Statistical analysis

The in vitro and in vivo data are shown as means ± SEM. Group means were compared by one-way analysis of variance. Different letters indicate significant differences among the groups. Differences with a value of \( P < 0.05 \) were considered to be statistically significant.
3. Results

3.1. BSE decreased hepatic lipid accumulation and inflammatory macrophage infiltration in EtOH-fed mice

To investigate the effect of BSE on the development of AFLD, mice were fed control diets or pair-fed Lieber-DeCarli liquid diets with EtOH (5%, v/v) for 10 days. Mice were orally administered BSE (100 or 200 mg/kg body weight) daily during the feeding period (Fig. 1A). After the feeding period, the body, liver, and white adipose tissue weights of mice were not different among the four groups (Fig. S1). Plasma aspartate aminotransferase and alanine aminotransferase levels were not different between the BSE groups and EtOH control group (data not shown), suggesting that BSE did not exert liver toxicity. EtOH feeding increased liver triglyceride levels 1.8-fold compared with the control groups (21.3 and 11.6 mg/g liver in the EtOH and control groups, respectively). Hepatic triglyceride levels were significantly lower in the HBSE group compared with the EtOH control group (Fig. 1B). Histopathological analysis showed that EtOH feeding induced accumulation of lipid droplets (including microsteatosis) in the liver, whereas administration of BSE reduced the number of lipid droplets in the liver (Fig. 1C). Consistent with this observation, BSE feeding decreased hepatic lipid accumulation, as assessed by Oil Red O staining, compared with the EtOH group. These results suggest that BSE administration reduces hepatic fat accumulation in EtOH-fed mice. Hepatic macrophage infiltration and induction of inflammatory markers are features of AFLD progression; thus, inflammatory macrophage infiltration of the liver was assessed by F4/80 staining. The livers of BSE-fed mice showed reduced numbers of inflammatory macrophages compared with EtOH-fed control mice. These results suggest that BSE administration reduces hepatic steatosis and hepatic macrophage infiltration in mice with acute AFLD.

3.2. BSE promoted AMPK phosphorylation and hepatic autophagy

Next, we investigated the molecular mechanisms underlying the effect of BSE on AFLD. We reported previously that BSE stimulates activation of AMPK (Lee et al., 2015). This effect could ameliorate AFLD, as AMPK plays a key role in regulating cellular lipogenesis, fatty acid synthesis and oxidation, and autophagy. Thus, we examined the effects of BSE on AFLD via activation of AMPK. Phosphorylation of AMPK at Thr172 is critical for its activation, and BSE administration increased phosphorylation of hepatic AMPKThr172 by 161% and 212% in the LBSE and HBSE groups, respectively (Fig. 2A). The AMPK pathway activates autophagy, which results in lipid droplet degradation and reduced hepatic steatosis. We next examined the levels of autophagy-related proteins in the livers of the mice. BSE administration increased the expression of two markers suggestive of induction of hepatic autophagy: LC3-II levels and the LC3-II/LC3-I ratio (Fig. 2B). BSE feeding significantly increased the number of autophagic vacuoles, although the number of lipid droplets was significantly lower than that in the EtOH group (Fig. 2C). Taken together, these results suggest that BSE
induced AMPK phosphorylation and the expression of factors related to autophagy, which reduced hepatic lipid accumulation, in the livers of EtOH-fed mice.

3.3. BSE decreased hepatic lipid accumulation and inflammatory macrophage infiltration in EtOH-HFD-fed mice

We next investigated the effects of BSE on C57BL/6 mice fed a HFD followed by an EtOH-containing liquid diet. This experiment was designed to investigate the effects of BSE on AFLD patients who consume a Western diet. The mice were fed an EtOH-containing diet for 10 days to induce AFLD and then fed a HFD and orally administered BSE or vehicle (double-distilled water) for 4 weeks (Fig. 3A). Initial body weight was similar among all groups (Fig. S3A). At the end of the 4-week feeding period, the body weight of mice in the HFD-LBSE and HFD-HBSE groups was significantly lower than that of mice in the HFD-EtOH group (HFD-EtOH vs. HFD-HBSE, \( P = 0.05 \), Fig. S3). The liver and white adipose tissue weights were slightly, but not significantly, increased in the HFD-EtOH group (Fig. S3B). EtOH-HFD-fed mice showed 1.2-fold increased hepatic triglyceride levels compared with the HFD-control mice (32.5 and 27.8 mg/g liver, respectively). BSE feeding for 4 weeks significantly decreased hepatic triglyceride levels by 28% in the HFD-HBSE group compared with the control group and by 38% compared with the HFD-EtOH group (Fig. 3B). Histological analysis revealed a dramatic reduction in the number of lipid droplets in the BSE groups compared with the HFD-EtOH group (Fig. 3C). These results indicate that BSE administration inhibits hepatic lipid accumulation and thus ameliorates AFLD in mice fed a HFD. Furthermore, the livers of mice administered BSE showed reduced inflammatory macrophage infiltration compared with EtOH-fed mice. These results suggest that BSE reduces hepatic inflammation. Collectively, the results of the above experiments suggest that BSE reduces hepatic fat accumulation in EtOH-fed mice.

3.4. BSE promoted AMPK phosphorylation and induced hepatic autophagy in EtOH-HFD-fed mice

Next, we investigated the effect of BSE on the AMPK-induced autophagic pathway in mice fed a HFD followed by an EtOH-containing liquid diet. The livers of mice in the HFD-LBSE and HFD-HBSE groups exhibited significantly enhanced AMPK phosphorylation, LC3-II expression, and LC3-II/LC3-I ratio compared with the HFD-EtOH group (Fig. 4A–B), suggesting activation of the AMPK-autophagic pathway; these results are in agreement with those of the first experiment (Fig. 2). TEM revealed that the number of autophagic vacuoles was significantly higher in the livers of BSE-treated mice compared with EtOH-treated mice, whereas the number of lipid droplets was significantly lower in the livers of mice in the HFD-HBSE group compared with the HFD-EtOH group. These findings suggest that the BSE mediated increase in phosphorylated AMPK and LC3 levels may reduce the development of fatty liver.
3.5. BSE induced hepatic β-oxidation and reduced fatty acid synthesis

Alcohol consumption induces hepatic fat accumulation by inhibiting fatty acid oxidation and stimulating fatty acid synthesis (Reddy & Rao, 2006). Activation of AMPK reduces intracellular lipid accumulation by regulating the rates of fatty acid synthesis and oxidation and by activating the autophagic pathway. Thus, the effect of BSE (100 or 200 μg/mL) on fatty acid oxidation and synthesis rates was examined in HepG2 cells (Fig. 5A–B). HepG2 cells stimulated with 200 μg/mL BSE showed a significantly increased rate of fatty acid oxidation and decreased rate of fatty acid synthesis. The intracellular concentration of β-hydroxybutyrate was increased (Fig. 5C), while that of intracellular malonyl-CoA was decreased (Fig. 5D); these are markers of ketone body formation and fatty acid synthesis, respectively. Collectively, these results suggest that BSE induces hepatic fatty acid oxidation and ketogenesis and suppresses fatty acid synthesis, thus reducing lipid accumulation in hepatocytes. These metabolic changes may be due to activation of AMPK, a key regulator of cellular energy metabolism.

3.6. Saponarin regulated hepatic triglyceride metabolism

Saponarin, apigenin-6-C-glucosyl-7-O-glucoside, is a major flavonoid in barley (Hordeum vulgare L.) sprouts that activates AMPK (Seo et al., 2015). Thus, we investigated the effect of saponarin on AFLD using HepG2 cells. Saponarin at 100 and 200 μM significantly reduced cellular lipid accumulation in a dose-dependent manner compared with the EtOH control group (Fig. 6A–B).

To identify the mechanism by which saponarin modulates hepatic lipid accumulation, we investigated key target molecule(s) in hepatic triglyceride metabolism. First, we examined whether saponarin regulates FoxO3 transcription. The transcription factor FoxO3, a member of the forkhead box protein family, is directly activated by AMPK phosphorylation. Phosphorylated FoxO3 is translocated to the nucleus where it stimulates the expression of autophagy-related genes, leading to degradation of intracellular lipid droplets and triglyceride hydrolysis. Saponarin did not increase transcription activity of FoxO3 (Fig. 6C). Second, the effect of saponarin on MTP was investigated. EtOH administration exacerbates hepatic steatosis by inhibiting very-low-density lipoprotein (VLDL) secretion from hepatic lipid droplets. MTP mediates the transfer of triglycerides from the cytosol into newly synthesized VLDL; therefore, VLDL secretion contributes to the reduction in hepatic intracellular triglyceride concentration. Saponarin treatment significantly increased MTP activity in EtOH-treated hepatocytes. These results suggest that saponarin increases VLDL secretion, resulting in reduced hepatic lipid accumulation (Fig. 6D). A luciferase assay showed that saponarin did not affect the activity of PPARs and liver X receptor (Data not shown). These results suggest that saponarin is responsible for the BSE-mediated reduction in hepatic lipid accumulation in AFLD. In addition to activation of the AMPK-autophagy pathway, induction of MTP activity and VLDL secretion may be a mechanism of the effect of saponarin.

4. Discussion

AFLD is caused by chronic alcohol abuse and may or may not be superimposed by metabolic risk factors such as obesity, dyslipidemia, and insulin resistance, which is different from the etiology of non-AFLD (Traversy & Chaput, 2015). A considerable portion of AFLD patients...
develop a more severe form of liver disease, such as alcohol-induced liver cirrhosis, which is a major public health problem worldwide. Alcohol-induced liver cirrhosis is reportedly responsible for 1% of all deaths and approximately half of all liver cirrhosis deaths globally (Williams, 2006). Thus, the development of an appropriate AFLD prevention and treatment strategy is a critical global health issue. Abstinence is the first-line treatment for AFLD. Regulation of hepatic lipid metabolism, including suppression of lipid synthesis and activation of lipid oxidation and degradation, is a potential therapeutic and preventive strategy (Anderson & Borlak, 2008). Accordingly, dyslipidemia drugs (such as fibrates and statins) as well as diabetes drugs (such as metformin) have been suggested for AFLD treatment; however, no drug effective against AFLD is available. Phytochemicals and other natural substances have potential in this regard.

In this study, BSE reduced hepatic lipid accumulation in a mouse model of AFLD. Oral administration of BSE reduced hepatic lipid accumulation and steatosis both during and after ingestion of alcohol, which suggests the potential of BSE for prevention and treatment of AFLD. BSE did not show toxicity, as indicated by its lack of effect on plasma AST and ALT levels, which is in agreement with previous studies. BSE ameliorated hepatic lipid accumulation and reduced the levels of markers of inflammation by activating the AMPK signaling pathway and autophagy.

BSE has several biological activities. We reported previously that BSE regulates cholesterol and glucose metabolism by activating AMPK and hepatic autophagy, resulting in improved hepatic health issue (Lee et al., 2015). BSE suppressed posttranslational activation of sterol regulatory element binding protein-2, which reduced the expression of 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. BSE also suppressed the plasma levels of interleukin-6 and C-reactive protein in mice, suggestive of considerable anti-inflammatory activity in vivo. We reported previously that saponarin, the major flavonoid in BSE, exerted effects on cultured human hepatocytes and myocytes (Seo et al., 2015). In both cell types, saponarin stimulated AMPK phosphorylation and increased intracellular calcium levels, which were diminished by co-treatment with STO-609, an inhibitor of CAMKK. These findings suggest that saponarin activated the calcium/CAMKK-β/AMPK signaling axis. Moreover, BSE reportedly attenuates alcoholic fatty liver injury in mice (Lee et al., 2016). In this study, BSE influenced hepatic lipid accumulation and the levels of pro-inflammatory cytokines. These liver enzyme levels were reduced as well in mice fed BSE. These results and those of the present study showed that BSE exerts hypolipidemic effects on AFLD. BSE increased the fatty acid oxidation rate and reduced the fatty acid synthetase rate, likely due to activation of AMPK by saponarin. These findings suggest that the lipid-lowering activity of BSE can also be applied to AFLD. These results indicate that, irrespective of the cause of steatosis, BSE may ameliorate hepatic lipid accumulation.

In this study, we investigated whether saponarin regulates the expression of other factors involved in hepatic triglyceride metabolism. Saponarin did not alter FoxO3 activity but increased MTP activity in vitro. These results suggest that saponarin reduces intracellular triglyceride levels by stimulating VLDL secretion and MTP activation. Thus, saponarin regulates hepatic intracellular triglyceride metabolism by activating AMPK and MTP. The effects of saponarin on AMPK and MTP activity are less potent than those of the synthetic drugs used as positive controls; however, the saponarin-mediated reduction in AMPK and MTP activity could lower hepatic triglyceride accumulation.

AMPK activation decreased intracellular triglyceride levels by several mechanisms and induces fatty acid oxidation. Lipids are stored as cytosolic lipid droplets and degraded by cytoplasmic neutral hydrolases to release lipids for cellular use. In addition, an alternative lipid metabolic pathway involving the autophagy pathway (Ding, Manley, & Ni, 2011). In this type of lipid metabolism, the triglyceride molecules in
lipid droplets and cholesterol are absorbed by autophagosomes and delivered to lysosomes for degradation by acidic hydrolases. Free fatty acids produced from the hydrolysis of triglycerides and used for β-oxidation. Autophagy is critical in regulating the cellular lipid levels, such as fatty acids, and modulating energy metabolism (Singh et al., 2009). Results from this study demonstrated that the expression of autophagy markers, including LC3-II (autophagy maturation) were upregulated by BSE in HepG2 cells. These autophagic pathways are initiated and promoted by AMPK-dependent inhibition of the mTOR signaling pathway. Therefore, BSE ameliorated hepatic lipid accumulation by activating the autophagic pathway in addition to its regulation of cholesterol metabolism.

MTP, a key chaperone in the assembly and secretion of apolipoprotein B-containing lipoproteins, is localized in the endoplasmic reticulum of hepatocytes and enterocytes (Lin, Zhao, Shen, & Xu, 2014). Suppression of MTP activity is suggested to cause hepatic steatosis in AFDL, and insulin resistance and activation of MTP could ameliorate hepatic steatosis (Seo et al., 2017). Activation of MTP or MTP expression by natural substances containing polyphenolic compounds reportedly reduced hepatic steatosis by transferring intracellular triglycerides to secreted VLDL particle. In this study, we showed that saponarin in BSE activates MTP activity, which led to reduced intracellular triglyceride levels.

BSE regulates hepatic steatosis by multiple mechanisms. First, BSE activates AMPK, which directly activates fatty acid oxidation and suppresses fatty acid synthesis by phosphorylation of target proteins involved in cellular energy metabolism. Second, AMPK activation increases autophagic activity, resulting in degradation of intracellular lipid droplets. Third, BSE activates MTP, which reduces intrahepatic lipid accumulation. These effects of BSE contribute to the amelioration of hepatic steatosis in the livers of AFDL patients.

The effects of BSE should be further investigated and confirmed in human subjects. The hypocholesterolemic effects of BSE containing high levels of hexacosanol have not been confirmed in human subjects, due to compliance issues, small sample sizes, and a lack of information on the saponarin content of BSE. Therefore, human trials should take into consideration the issues encountered in previous human trials.

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Reference


