Research article

Barley sprout extract containing policosanols and polyphenols regulate AMPK, SREBP2 and ACAT2 activity and cholesterol and glucose metabolism in vitro and in vivo

Ji Hae Lee a,b, So Young Lee a,b, Bobae Kim a,b, Woo Duck Seo c, Yaoyao Jia a,b, Chunyan Wu a,b, Hee-jin Jun a,b, Sung-Joon Lee a,b,c,⁎

a Department of Food Bioscience and Technology, College of Life Sciences and Biotechnology, Korea University, Seoul, 136-713, Republic of Korea
b Graduate School of Life Sciences and Biotechnology, Korea University, BK21-PLUS, Seoul, 136-713, Republic of Korea
c Department of Functional Crop, National Institute of Crop Science (NICS), Rural Development Administration (RDA), Miryang, 627-803, Republic of Korea

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ABSTRACT

Mechanism of barley sprout extract (BS), which contains policosanols and polyphenols, on cholesterol and glucose metabolism was investigated. BS reduced the intracellular cholesterol concentrations in the HepG2 cells and the plasma cholesterol concentrations in the mice by the activation of AMPK and the subsequent phosphorylation inhibition of HMGCR. BS suppressed the nuclear translocation of SREBP2, reducing the transcription of HMGCR. AMPK activation with BS reduced the fasting glucose and hepatic triglyceride concentrations in mice by repressing the hepatic gluconeogenic genes, including fructose-1, 6-bisphosphatase and pyruvate carboxylase and the plasma levels of the proinflammatory cytokines tumor necrosis factor-α and interleukin-6. The activation of hepatic autophagy by BS was confirmed by induced protein expressions of LC3-II and LAMP. In conclusion, BS activates AMPK and hepatic autophagy and inhibits SREBP2, resulting in hypcholesterolemia and hypoglycemic activities and improvements in the symptoms of hepatic steatosis.

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1. Introduction

Chronically elevated plasma cholesterol concentrations increase the risk of atherosclerosis and coronary heart disease and increase the major risk of mortality worldwide. Hypercholesterolemia is correlated with the prevalence of obesity, metabolic syndrome, and the occurrence of fatty livers, which has a 20 to 30% prevalence in general population (Carroll, Kit, Lacher, & Yoon, 2013; Targher et al., 2005). Lowering the cholesterol is essential for maintaining health status, especially among the middle-aged population.

Statins, the 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) inhibitors, are widely prescribed as cholesterol-lowering therapy (Hebert, Gaziano, Chan, & Hennekens, 1997), but long-term and high dose of administration of these drugs could cause side effects, including rhabdomyolysis, proteinuria, and nephropathy, and some patients do not respond to statin treatment (Golomb & Evans, 2008). Alternatively, nutritional dose of phytochemicals have moderate metabolic effects and could avoid safety issues (Liu, 2004).

One potential target for dietary interventions for metabolic disorders is AMPK, which is a cellular sensor of energy metabolism and a regulator for cholesterol and glucose metabolism and hepatic lipid metabolism (Carling, Thornton, Woods, & Sanders, 2012; Luo, Saha, Xiang, & Ruderman, 2005; Steinberg & Kemp, 2009). The activation of AMPK by phosphorylation of Thr 172 by AMPK activation inhibits the mammalian target of rapamycin (mTOR), cholesterol biosynthesis phosphorylation of HMGCR at Ser 727, inhibiting the enzyme activity and blocking hepatic cholesterol biosynthesis (Steinberg & Kemp, 2009). AMPK activation is known to improve insulin resistance, primarily to control the hepatic glucose output (Ruderman, Carling, Prentki, & Cacicedo, 2013).

AMPK activation inhibits the mammalian target of rapamycin (mTOR) pathway to improve insulin sensitivity by upregulating insulin receptor substrate-1, alleviating insulin resistance and hyperglycemia.
The inhibition of AMPK on the mTOR pathway activates the autophagy process, which degrades lipid droplets and ameliorates hepatic lipid accumulation and liver steatosis (Kim, Kundu, Viollet, & Guan, 2011; Mihaylova & Shaw, 2011). AMPK directly phosphorylates sterol regulatory element binding protein-2 (SREBP2), which inhibits the nuclear translocation of SREBP2, downregulating HMGCR transcription (Li et al., 2011).

Barley sprout extract (BS) contains policosanols with substantial levels of polyphenols. Policosanols mainly exist in plant cuticle wax and are a natural mixture of long chain alcohols that have between 24 and 32 carbon atoms and can reduce plasma cholesterol concentrations by the AMPK dependent phosphorylation inhibition of HMGCR (Singh, Li, & Porter, 2006). Previous studies focused on policosanols from sugar cane, which are major source of policosanols and mainly consists of octacosanol; the policosanols in BS have a different proportion, with hexacosanol (>60%) as the major policosanols type (Irmak, Dunford, & Milligan, 2006).

Metformin and 5-aminomidazole-4-carboxamide-1-β-D-ribofuranoside are potent synthetic AMPK activators, and flavonoids have been identified as AMPK activators from natural phytochemicals. The AMPK activation by flavonoids suppresses fatty acid synthesis in hepatocytes and adipogenesis in adipocytes (Liu et al., 2011; Ono & Fujimori, 2011). A large scale cohort study revealed that the intake of prevalent polyphenol flavonoids reduced the relative risk of coronary heart disease (Mink et al., 2007). Flavonoid-rich foods have been reported to reduce plasma LDL and glucose concentrations, as has been shown in human meta-analysis data (Hooper et al., 2008). These metabolic benefits could be caused by AMPK activation. The prevalent polyphenol flavonoid in BS is saponarin, and the biological activity of saponarin is largely unknown. In this study, we investigate metabolic effect and mechanisms of action of BS on cholesterol and glucose metabolism and hepatic steatosis in human HepG2 cells and high fat diet (HFD) fed mice.

2. Material and methods

2.1. Sample preparation and composition analysis

The BS (Hordeum vulgare L.) were harvested as described previously in the Department of Functional Crop, National Institute of Crop Science (NICS), Rural Development Administration (Miryang, Republic of Korea) and provided to Korea University for further experiments (Seo et al., 2013). Briefly, barley seeds (Daejin cultivar) were imbibed in water for 1 day prior to germination and kept in the dark for 2 days at 22–25 °C. After germination, the seeds were transferred to a soil bed and grown under 60% relative humidity at 22–24 °C. The BS was harvested 10 days after sprouting, when the extract showed the highest AMPK activation activity (Seo et al., 2013). The BS was then dried and the plasma was collected by cardiac puncture and the plasma was collected by cardiac puncture. The plasma was freeze-dried and the plasma was collected by cardiac puncture. Tissue samples, including liver and adipose tissues, were collected and snap frozen in liquid nitrogen or fixed in 4% formaldehyde for the histological analysis.

2.2. Cell culture and treatment

HepG2 cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and grown in DMEM (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37 °C in 5% CO2. The cells were seeded into six-well culture plates (2.5 × 105 cells/mL), treated with metformin (1 mM), a statin (2 μM) or BS (100, 200, and 400 μg/mL) for 24 h, and then subjected to a 3-h phosphor–protein extraction procedure. Cells were homogenized in RIPA buffer made fresh and containing a cocktail of protease and phosphatase inhibitors (Abcam, Cambridge, MA, USA) (Beg & Brewer, 1982).

2.3. Cellular lipid staining and quantification

The lipophilic compounds in the HepG2 cells were stained with the 1,10-diododecyl-3,3′,3′-tetramethyldiindocarbocyanine perchlorate (DiI) fluorescence dye (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Briefly, the cells were washed with cold PBS and incubated with the DiI dye in serum free DMEM (5 μM, 1 mL per well in 6-well plate) for 20 min. The cells were washed for another 30 min, and images were acquired using a confocal laser scanning microscope (LSM 5 Exciter, Carl-Zeiss, Oberkochen, Germany).

For cellular lipid measurements, HepG2 cells were washed twice with 1 mL PBS, treated with 1 mL hexane/isopropanol (2:1) for 30 min at room temperature, and then the lipid extracted solvent was transferred to test tubes. The wells were again washed with 1 mL hexane/isopropanol, and the washing solutions were transferred to the corresponding test tubes. The organic solvent was removed using a vacuum centrifuge, and the lipids were resuspended in 95% ethanol. The intracellular cholesterol was quantified with a cholesterol/cholesteryl ester kit (Abcam) according to the manufacturer’s instructions. After lipid extraction, cells were lysed with RIPA buffer and centrifuged at 13,000 g for 10 min to collect the supernatant to measure cellular protein concentrations. Lipid levels were normalized to the total cellular protein concentration as determined using the BCA protein assay (Pierce Biotechnology, Rockford, USA).

2.4. Animals and diets

The animal care and handling procedures were performed according to protocols approved by the Animal Experimentation and Ethics Committee of Korea University (Protocol No. KUIACUC-2013-139). C57BL/6J male mice (8 weeks old, n = 6 per group) were purchased from Samtako (Seoul, Korea) and maintained in a temperature-controlled (25 °C) specific-pathogen-free facility with 12 h light/dark cycles. The mice were fed an AIN-76A-based high fat diet (45% of calories from fat, Table S1) for 4 weeks to develop obesity. The mice were randomly divided into two groups, and the tested materials, including BS, were orally administered for 12 weeks (4.8 mg/kg/day). BS feeding concentration was determined after dose–response experiment in vivo (data not shown). The body weight was measured twice per week. Food and water were provided ad libitum. After 12 weeks of feeding, the mice were sacrificed and the plasma was collected by cardiac puncture. Tissue samples, including liver and adipose tissues, were collected and snap frozen in liquid nitrogen or fixed in 4% formaldehyde for the histological analysis.

2.5. Plasma cholesterol, glucose, and cytokine analysis

The plasma samples were collected retro-orally after 12 h of fasting, and the total cholesterol, HDL, and LDL were determined enzymatically with an automatic analyzer (Cobas C111, Roche, Basel, Switzerland). The plasma glucose concentrations were measured in the blood after overnight fasting. The glucose concentrations were quantified with an enzymatic method as described previously (Lee et al., 2011). For oral glucose tolerance test (OGTT), mice fed BS for 2 weeks were orally administered with glucose (1.5 g/kg body weight in PBS) after overnight fasting. Blood glucose concentrations were measured at different time points using a portable glucose meter (Accu-Check Go, Roche). Insulin sensitivity index and homeostatic model assessment-insulin resistance (HOMA-IR) were assessed by the following equations: Insulin sensitivity index = 1/log([fasting insulin (μU/mL)] + log([fasting glucose (mg/dL)])), HOMA-IR = [Fasting insulin (μU/mL) × Fasting glucose (mg/dL)]/405 (Katz et al., 2000; Matthews et al., 1985). The plasma tumor necrosis factor-α (TNFα),
interleukin-6 (IL-6), and insulin levels were quantified using ELISA kit according to the manufacturer’s instructions. The ELISA kits were purchased from Novus Biologicals (Littleton, CO, USA), Thermo scientific (Rockford, IL, USA), and Millipore (Bedford, MA, USA) respectively.

2.6. Hepatic lipid quantification

Hepatic lipids were extracted from mouse liver by adding 1 mL acetone to 40 mg liver tissue. After 12 h of incubation at room temperature, supernatants were collected and evaporated using vacuum centrifuge (Speedvac, N-Biotek, Bucheon, Korea). Concentrates were dissolved in 100 μL 80% (v/v) ethanol, and hepatic cholesterol and triglyceride concentrations were determined enzymatically using the Cobas C111 (Roche, Forrenstrasse, Switzerland) with enzymatic quantification methods (Kim et al., 2013).

2.7. Histological analysis of liver and adipose tissue

The liver and epididymal adipose tissue samples were fixed in 4% formaldehyde and stained with hematoxylin and eisin. The adipocyte sizes were determined using an upright microscope and software (Axio Imager M1, Carl-Zeiss, Oberkochen, Germany).

2.8. Total RNA extraction and quantitative real-time PCR (qPCR)

The total RNA was extracted from HepG2 or liver tissue using a RNAiso Plus Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. qPCR was performed with the Bio-Rad iQ SYBR Green Supermix reagent and a Bio-Rad iQ5 cycler system (Richmond, CA, USA). Amplification was performed using an initial denaturation step at 95 °C for 30 s, followed by 60 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and extension at 68 °C for 20 s. The fluorescent signal was detected automatically at the end of each PCR cycle. Primer sequences used to amplify the genes of interest are presented in Table S2. The expression levels were calculated with the threshold cycle (Ct) method according to the manufacturer’s guidelines and normalized by the cyclophilin-β expression level (Hoang et al., 2012).

2.9. Protein extraction and immunoblotting analysis

HepG2 cells or liver tissue samples were lysed at 4 °C in lysis 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% protease inhibitor cocktail. The protein concentration was determined using the Bradford reagent, and bovine serum albumin was used as the standard. Protein samples (40 μg) in Laemmli sample buffer (4:1 v/v) were heated for 5 min and resolved by 8–10% SDS–PAGE. The separated proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) at 100 V for 1 h. Nonspecific binding was blocked with 5% skim milk in TBS-T buffer for 1 h at room temperature. The membranes were incubated with the primary antibody overnight at 4 °C. Upon incubation, the membranes were washed with TBS-T for 40 min and incubated with the secondary antibody for 1 h at room temperature. Antibodies for SREBP2, ACAT2, AMPK, p-AMPK, HMGCR, α-tubulin, INSIG1, and LAMP1 were purchased from Santa Cruz Biotechnology (CA, USA). p-HMGCR was obtained from Biorbyt (Cambridge, UK). ATG5, ATG9A, ATG16L1, and LC3 were purchased from Novus Biologicals (CO, USA). Secondary anti-mouse, goat, and rabbit were produced in Immunogen (USA). The immunoreactive protein bands were detected with an enhanced chemiluminescence system (Animal Genetics, Seoul, Korea), visualized using a ChemiDoc XRS + System (Bio-Rad), and quantified with Gel-Pro Analyzer software (Jia et al., 2012). The protein expression levels were normalized with the expression of α-tubulin.

2.10. Statistical analysis

The in vitro and in vivo data are expressed as the means ± SEM. The student’s t-test was performed for comparisons between the two groups. The statistical significance level was set at P < 0.05.

3. Results

3.1. BS contains policosanols and polyphenols

The results of the compound analysis of BS were previously published (See et al., 2013). Policosanols are known as AMPK activators, and the policosanols in BS were quantified after ethanol (80%, v/v) extraction. The concentrations of policosanols and major flavonoids in BS were quantified with gas chromatography–mass spectrometry as described previously (See et al., 2013). The total policosanols concentration was 5.74 mg/g BS that 61% hexacosanol and 5 other different policosanols types (Table 1). The BS contained 19.65 mg/g BS of total polyphenol concentration. The major polyphenols in BS are flavonoids, including saponarin (isovitexin–7–O-glucoside) as a major compound as described (Ferreres, Andrade, Valentao, & Gil-Izquierdo, 2008) along with significant quantities of orientin (luteolin–8–glucoside) and vitexin (apigenin–8–glucoside) (See et al., 2013). Saponarin concentration was 5.34 mg/g in the tested batch of BS.

3.2. BS reduces cellular cholesterol accumulation by activating AMPK and suppressing SREBP2

The hypocholesterolemic effects of policosanols and polyphenols containing BS were investigated in HepG2 cells. BS stimulation significantly decreased the intracellular cholesterol in lipid-loaded HepG2 cells (Fig. 1). Lipid staining with a fluorescent dye, DiI, showed a reduction in the cellular lipid accumulation. The intracellular total and free cholesterol values were reduced by 24% and 18%, respectively (P<0.05 vs. control in both comparisons). The cholesterol ester concentration was significantly lowered by 45% (P<0.05). The ratio of free-cholesterol ester was increased with borderline significance in the BS group (P=0.05) compared with the control, suggesting a reduction in the accumulation of intracellular cholesterol.

Previous in vitro studies by other groups have demonstrated that policosanols at 5–50 μg/mL have significant hypocholesterolemic effects (Oliaro-Bosso et al., 2009; Singh et al., 2006). Considering the policosanols content of the BS used in this study (5.74 mg/g BS), 871–8710 μg/mL BS provides 5–50 μg/mL of policosanols. Our data revealed that the 400 μg/mL concentration has significant cellular cholesterol lowering activity by regulating gene expression and protein phosphorylation. This effect of BS may be accounted for in part by the additional hypocholesterolemic compounds, such as polyphenols (1.96%), used in the formulation.

Policosanols are known to inhibit HMGCR through AMPK activation, and AMPK and HMGCR phosphorylation were examined. The results suggested that BS tended to induce AMPK phosphorylation at Thr 172 (+68% compared with the control) and HMGCR phosphorylation at Ser 871 (Fig. 2A and F), albeit not significantly. SREBP2 is a key transcription factor in the regulation of cellular cholesterol homeostasis and induces the transcription of HMGCR (Miserez et al., 2002). SREBP2 is anchored in the endoplasmic reticulum (ER) membranes (precursor SREBP2, pSREBP2) and transferred to the nucleus (mature SREBP2, mSREBP2) before regulating the transcription of its target gene (Rawson, 2003). We assessed the gene expression of SREBP2 by qPCR and the expression of the pSREBP2 and mSREBP2 proteins by immunoblotting assays (Fig. 2B, C, and F). The transcription level of SREBP2 was reduced by BS treatment. These results are in line with a study that showed that the AMPK activator, metformin, downregulated SREBP2 expression (Yang, Craddock, Hong, & Liu, 2009). The ratio of mSREBP2 to pSREBP2 was altered marginally. As a result of the
reduction in mSREBP2, the transcription of HMGCR was reduced dose dependently (−66% at 400 μg/mL of BS treatment vs. control, Fig. 2D and F). BS reduced acetyl-CoA acetyltransferase-2 (ACAT2) transcription (−68% at 400 μg/mL of BS treatment vs. control, P < 0.01 vs. control, Fig. 2E), which lowered the intracellular cholesteryl ester concentrations and downregulated by AMPK, indirectly (Hong, Varanasi, Yang, & Leff, 2003; Pramfalk et al., 2009). These results suggested that BS inhibits cholesterol biosynthesis and accumulation in cultured hepatocytes.

3.3. BS feeding reduces plasma cholesterol concentrations by SREBP2 suppression and AMPK activation in HFD fed mice

*In vivo* experiments were performed with HFD fed mice. C57BL/6J mice were fed a HFD for 4 weeks and then fed BS for an additional 12 weeks. Previously, we performed a dose–response experiment for orally administered BS in C57BL6/J mice for 12 weeks. The dose (4.8 mg/kg) selected for the mouse experiment in this study was the minimum effective dose identified in our preceding dose–response study. The body weight tended to decrease in the BS group compared with the vehicle-fed controls (Fig. 3A). The food intakes in control and BS group were similar, 2.30 ± 0.05 and 2.21 ± 0.01 g/day, respectively. The adipocyte size was not altered in the BS group (4285 vs. 4075 μm² in control and BS, Fig. 3B). The infiltration of inflammatory cells was predominant in the adipose tissue of the control mice group, while the BS group showed markedly reduced inflammatory cell infiltration. The plasma concentration of the inflammatory cytokines IL-6 was reduced with borderline significance (P = 0.052) in the BS fed mice (Fig. 3C). The TNFα concentrations were unaltered. The total and LDL cholesterol concentrations were significantly decreased by 12% (P < 0.05) and 33% (P < 0.01), respectively, after 12 weeks of feeding (Fig. 4A). Plasma lipid-protein profiling assessed with FPLC analysis showed similar trends (Fig. 4B). The HDL-to-LDL cholesterol ratio was significantly higher in the BS group compared with the controls at 12 weeks of feeding (Fig. 4A and B). The hepatic cholesterol concentration was similar between the groups, but the free cholesterol concentrations and free-to-cholesterol ester ratio (+34% vs. control, P < 0.05) were increased in the BS group (Fig. 4C). This ratio represents the ratio of effluxed cholesterol to stored cholesterol in the cells. The liver weight and intracellular triglyceride concentrations were unaltered (Fig. 4C).

AMPK and its target gene/protein expressions in liver were evaluated to investigate the impact of BS on cholesterol metabolism (Fig. 5). AMPK phosphorylation was induced significantly by 34% in the BS liver, increasing HMGCR phosphorylation with borderline significance (P = 0.087), which inhibits enzyme activity (Fig. 5A and F). The expression ratio of mSREBP2-pSREBP2 and induction of insulin-induced protein-1 (INSIG1) were unaltered compared with the controls (Fig. 5B, C, and F), but mSREBP2 in the BS group was significantly decreased (−37% vs. control, P < 0.01). The protein expression level of the HMGCR was reduced accordingly (Fig. 5D and F). ACAT2 protein expression was reduced by BS feeding (−46%, P < 0.05, Fig. 5E and F). This could increase the free cholesterol concentration compared with the cholesteryl ester concentration. These results demonstrate that BS activates AMPK and impairs SREBP2 translocation to nucleus in mice; these results are in accordance with the *in vitro* results. These effects inhibit HMGCR activity and inhibit the expression of HMGCR. The combined effects of HMGCR and ACAT2 repression reduce the plasma LDL, total cholesterol concentrations and increase hepatic free cholesterol- to-cholesterol ester ratio.

**Table 1**

Composition of the BS extract.

<table>
<thead>
<tr>
<th>Policosanols (mg/g of BS extract)</th>
<th>Polyphenols (mg/g of BS extract)</th>
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<tbody>
<tr>
<td>Tricosanol (C23H48O)</td>
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<tr>
<td>0.02 ± 0.00</td>
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<td>0.48 ± 0.00</td>
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<td>1.03 ± 0.01</td>
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<td>0.10 ± 0.00</td>
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<tr>
<td>19.65 ± 0.36</td>
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</table>
3.4. AMPK activation with BS improves glucose control by suppressing gluconeogenesis gene expression

AMPK activation leads to the reduction of hepatic gluconeogenesis. Plasma fasting glucose and insulin concentration in BS fed mice were quantified. The results suggested that the fasting glucose and insulin levels in the BS group were reduced with borderline significance compared with the controls at 12 weeks (−24 and −75%; P = 0.09 and 0.079, respectively, for glucose and insulin at 12 weeks, Fig. 6A and B). Accordingly, glucose tolerance was improved in BS fed mice compared...
with controls assessed in OGTT (AUC, −20% vs. control, \( P < 0.05 \), Fig. 6C). Additionally, insulin sensitivity index and HOMA-IR were significantly improved in BS group compared with controls indicating increased insulin sensitivity by BS feeding (Fig. 6B). The expression of gluconeogenesis genes, including fructose-1,6-bisphosphatase, pyruvate carboxylase, phosphoenolpyruvate carboxyl kinase, and glucose-6 phosphatase, was not altered significantly, suggesting that alternative mechanisms regulate glucose metabolism and insulin sensitivity in mice by BS feeding (data not shown). Inflammatory cytokines are negatively correlated with glucose homeostasis. As described in Fig. 3, BS feeding showed a reduction in the plasma IL-6 concentrations, and this proinflammatory cytokine by 46% (Fig. 3C). Obesity is a state of chronic inflammation with high concentrations of plasma cytokines. IL-6 affects the development of insulin resistance and glucose metabolism disorders by inhibiting insulin receptor and insulin receptor substrates through serine phosphorylation (Glund & Krook, 2008; Popa, Netea, van Riel, van der Meer, & Stalenhoef, 2007). These results suggest that BS reduces the fasting glucose concentrations, and improves insulin sensitivity by suppressing plasma IL-6 concentrations of pro-inflammatory cytokine.

3.5. BS activates hepatic autophagy

Autophagy is directly promoted by AMPK activation and degrades subcellular organelles, including lipid droplets (Kim et al., 2011). The activation of autophagy could be a therapeutic target for liver steatosis by maintaining hepatic lipid homeostasis. Because of the BS fed mice had reduced hepatic lipid accumulation, as shown in Fig. 4B, we analyzed if autophagy activation was involved. BS stimulation in the HepG2 cells induced the autophagy-related gene-16L1 (Fig. 7A) compared with the controls. The markers for the autophagosome (LC3II) were up-regulated with borderline significance in the HepG2 cells with BS treatment (\( P = 0.08 \) for LC3II). The in vivo study revealed similar results to ATG9 and LC3II (Fig. 7B). These results demonstrate that the AMPK activator BS induced hepatic autophagy, contributing to the reduction in hepatic lipid accumulation.

4. Discussion

The pharmacological activities of natural compounds have been proven scientifically, and their applications for preventing chronic diseases, including inflammation and cardiovascular disease, have been suggested (Harvey, 2008). In the present study, BS was shown to contain a high concentration of policosanols and polyphenols; BS displayed hypcholesterolemic and hypoglycemic effects and ameliorated hepatic steatosis.

Policosanols are mixtures of long chain fatty alcohols and have been reported to have hypcholesterolemic effects, especially the policosanols from sugar cane. Sugar cane policosanols have been used as functional foods and food supplements in several countries for their cholesterol lowering activities. Some human clinical trials showed that the policosanols from sugar cane are hypercholesterolemic and hypertensive (Varady, Wang, & Jones, 2003), and other studies did not confirm significant cholesterol reductions with policosanols from sugar...
cane. Accordingly, the efficacy of the policosanols from sugar cane has been somewhat controversial. Since results from human trials have not been consistent. However, BS contains polyphenols and policosanols and may have additional multiple roles in the regulation of cellular and plasma cholesterol metabolism. The results from this study demonstrated that BS significantly reduces the cholesterol concentration in vivo and in vitro by multiple mechanisms (Fig. 8). First, BS suppresses HMGCR activity by direct phosphorylation. HMGCR, a rate-limiting enzyme in cholesterol biosynthesis, catalyzes mevalonate production from HMG-CoA. The phosphorylation of AMPK on Thr 172 was induced by BS treatment, and the active AMPK subsequently phosphorylated HMGCR on Ser 872 in HepG2 cells and mouse livers, indicating that BS reduced the HMGCR enzyme activity in vitro and in vivo. Second, BS represses SREBP2-dependent HMGCR transcription. In the posttranslational modification of SREBP2, it has been reported that AMPK-dependent phosphorylation of SREBP2 hinders the ER-to-Golgi transport of pSREBP2 (Li et al., 2011). The activation of AMPK inhibits HMGCR gene expression via potentially by the phosphorylation inhibition of SREBP2 processing, which repress HMGCR transcription. Third, BS reduced ACAT2 expression. Cholesteryl esters are stored in lipid droplets, and free cholesterols are effluxed from cells. ACAT2 synthesizes cholesteryl ester from free cholesterol and fatty acids, and reduced ACAT2 expression indicates decreased synthesis of cholesteryl ester and a subsequent increase in cholesterol efflux. ACAT2 could be regulated by several mechanisms, including the downregulation of ACAT2 expression by the AMPK-dependent inhibition of HNF4α, a transcription factor of ACAT2 expression (Hong et al., 2003; Pramfalk et al., 2009). HNF4α phosphorylation at Ser 304 by AMPK reduces DNA binding stability, suppressing target gene expression, including ACAT2 (Chavalit, Rojvirat, Muangsawat, & Jitrapakdee, 2013). Collectively, the results from our study demonstrated that BS reduced the plasma and intracellular cholesterol concentrations via the reduction of HMGCR activity, gene expression, and ACAT2 expression.

Liver steatosis is a critical public health issue because of its increasing prevalence and the progression to liver cirrhosis. To provide protection from fatty liver disease, the reduction of lipid synthesis, the induction of lipid oxidation, and lipid degradation are suggested as potential therapeutic and preventive strategies (Anderson & Borlak, 2008). As shown in our results, AMPK activation leads to a reduced level of cholesterol biosynthesis by multiple mechanisms and leads to elevated fatty acid oxidation.

Intracellular lipid molecules are maintained in lipid droplets and metabolized by cytoplasmic neutral hydrolases to provide lipids for cells. Recently, an alternative pathway of lipid metabolism through the lysosomal degradative pathway of autophagy was demonstrated. In this type of lipid metabolism, the TG molecules in lipid droplets and cholesterol are taken up by autophagosomes and delivered to the lysosomes for degradation by acidic hydrolases. Free fatty acids generated by autophagy from the degradation of TG facilitate β-oxidation rates. Autophagy plays a role in regulating the intracellular lipid metabolism.
stores, the cellular levels of free lipids, such as fatty acids, and energy metabolism.

Our results demonstrated that the expression of autophagy markers was upregulated with BS stimulation, including ATG16L1 (autophagosome formation) in HepG2 cells and LC3 (autophagy maturation) in mouse livers. These autophagic pathways are initiated and promoted by the AMPK-dependent inhibition of the mTOR signaling pathway (Inoki, Kim, & Guan, 2012). These findings demonstrate that BS ameliorated hepatic lipid accumulation by activating the autophagic pathway in addition to its regulation of cholesterol metabolic pathways.

Excessive hepatic glucose production is a risk factor for hyperglycemia in humans. The de novo synthesis of glucose from pyruvate is carried out by a series of gluconeogenic enzymes, including fructose-1,6-bisphosphatase, pyruvate carboxylase, phosphoenolpyruvate carboxylase, and glucose-6 phosphatase (data not shown). However, the expression of these gluconeogenic genes was not altered significantly with BS, suggesting that alternative mechanisms are involved in the regulation of glucose metabolism and insulin sensitivity in mice fed BS.

In addition to policosanols, the polyphenol flavonoids in BS might play a key role in the regulation of AMPK, SREBP2, and ACAT2. AMPK, a serine/threonine protein kinase, is activated by several natural compounds, including resveratrol, epigallocatechin gallate, berberine, and quercetin. AMPK activation could induce ATP generation through pathways such as glycolysis and fatty acid beta-oxidation. ATP-consuming pathways, including fatty acid and cholesterol synthesis and gluconeogenesis, are suppressed by AMPK activation. BS contains several unique flavonoid polyphenols, including saponarin, orientin and vitexin, and the biological activity of the polyphenols in the purified forms would confirm the potent activation of AMPK with BS containing with policosanols and polyphenols.

BS (400 μg/mL, the concentration used in the in vitro experiment) contains 5.8 μM policosanols (2.3 μg/mL) and 3 μM saponarin, along with other flavonoids. Policosanols at 5 μg/mL have been demonstrated to reduce cellular acetate uptake and AMPK phosphorylation in HepG2 cells (Singh et al., 2006), suggesting that the concentration of policosanols in BS is sufficient to activate AMPK and trigger a subsequent reduction in cellular cholesterol synthesis.

In a cell-free kinase assay, several BS flavonoids, including luteolin 7-glucoside, honoorientin, and vitexin, directly induced AMPK activity at low μM concentrations (Fig. S1). Saponarin, however, did not directly activate AMPK activity but did induce AMPK phosphorylation in cultured cell experiments (Fig. S1). Preliminary results revealed that the effect of 0.1 mM saponarin on AMPK phosphorylation is comparable to that of 1 mM metformin, a positive control. Interestingly, saponarin induces an increase in intracellular calcium concentrations, indicating that activation of CAMKK-β (unpublished data), the kinase upstream of AMPK, might be involved in saponarin-mediated AMPK phosphorylation. Further experiments are required; however, these results collectively indicate that activation of AMPK by BS is achieved by multiple compounds via multiple mechanisms.

5. Conclusion

We demonstrate that BS regulates AMPK, SREBP2, and ACAT2 by multiple biological mechanisms, which results in reduced plasma, LDL cholesterol levels, and fasting glucose concentrations, ameliorating hepatic steatosis. BS induces hepatic lipid accumulation, at least in part, by activating the autophagic pathway. The intake of BS might provide the metabolic benefits of cholesterol and glucose control. Further human studies may confirm this effect.
Fig. 7. BS ameliorates hepatic steatosis through the activation of the autophagy pathway. (A) The protein levels of ATG5, ATG9A, ATG16 L1, LAMP1, LC3-I, and LC3-II in HepG2. (B) The protein levels of ATG5, ATG9A, ATG16 L1, LAMP1, Beclin1, LC3-I, and LC3-II in C57BL/6J mice. ⁎ P < 0.05, ⁎⁎ P < 0.01, vs. control. The data are represented as the means ± SEM.

Fig. 8. Effect of BS on cholesterol and glucose metabolism. Various phytochemicals in BS have multiple benefits, including hypercholesterolemia, hypoglycemia, and the amelioration of liver steatosis by AMPK mediated pathways.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2015.03.041.

References


