An Active Part of *Artemisia sacrorum* Ledeb. Attenuates Hepatic Lipid Accumulation through Activating AMP-Activated Protein Kinase in Human HepG2 Cells

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**Artemisia sacrorum** Ledeb. (Compositae) (ASL) is a traditional Chinese medicine used to treat different hepatic diseases. However, a hypolipidemic effect of ASL on fatty liver disease has not been reported. Therefore, we investigated whether 95% ethanol eluate (EE), an active part of ASL, would attenuate hepatic lipid accumulation in human HepG2 cells by activating AMP-activated protein kinase (AMPK). Significant decreases in triglyceride levels and increases in AMPK and acetyl-CoA carboxylase (ACC) phosphorylation were observed when the cells were treated with 95% EE. EE down-regulated the lipogenesis gene expression of sterol regulatory element-binding protein 1c (SREBP1c) and its target genes, such as fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1), in a time- and dose-dependent manner. In contrast, the lipolytic gene expression of peroxisome proliferator-activated receptor-α (PPAR-α) and CD36 increased in a time- and dose-dependent manner. These effects were abolished by pretreatment with compound C, an AMPK inhibitor. However, there were no differences in the gene expression of SREBP2, low density lipoprotein receptor (LDLR), hydroxymethyl glutaryl CoA reductase (HMG-CoA), or glucose transporter 2 (GLUT2). At the same time, 95% EE significantly increased the gene expression of acyl CoA oxidase (ACOX) in a time- and dose-dependent manner. Thus, AMPK mediated 95% EE induced suppression of SREBP1c and activation of PPAR-α respectively. These finding indicate that 95% EE attenuates hepatic lipid accumulation through AMPK activation and may be active in the prevention of serious diseases such as fatty liver, obesity, and type-2 diabetic mellitus.

**Key words:** *Artemisia sacrorum* Ledeb.; AMP-activated protein kinase; HepG2 cells

Liver is a major organ of energy storage involved in whole-body glucose, lipid, and energy metabolism. Fatty liver disease (FLD) is one of the most common chronic liver diseases and is characterized by the accumulation of lipids in the hepatocytes. In the various stages of FLD, hepatic lipid accumulation (mainly triglyceride) has become a significant public health concern because it can lead to more harmful hepatitis and cirrhosis. Moreover, fatty liver and hepatic triglyceride (TG) accumulation plays important roles in the development of metabolic syndrome, including the obesity, hyperlipidemia, and diabetes. AMPK is known to play a major role in energy homeostasis and is considered a key master switch regulating glucose and lipid metabolism. Because AMPK is considered a cellular energy sensor, it is important to gain an understanding of the mechanism by which hepatic AMPK coordinates hepatic energy metabolism. In the liver, activated AMPK inhibits energy-consuming biosynthetic pathways, including the fatty acid and cholesterol biosynthesis, yet switches on catabolic pathways that generate ATP, such as fatty acid oxidation, glucose uptake, and glycolysis. In other words, AMPK activation in the liver increases synthesis of fatty acid, TG, and cholesterol and fatty acid oxidation. Thus AMPK represents an attractive target for therapeutic intervention in the treatment of hepatic disorders.

*Artemisia sacrorum* Ledeb. (Compositae) (ASL) is an oriental folk medicine that has been used traditionally for thousands years to prevent and treat chronic and acute hepatitis. The application of *Artemisia sacrorum* Ledeb. has a long history, and its notable hepatoprotective effects have been widely recognized in folk memory. But the hypolipidemic effect of ASL has not been reported. In the present study, we...
demonstrated that 95% ethanol elute (EE), an active part from ASL, was inhibited hepatic TG accumulation through activation of AMPK in human hepatoma HepG2 cells.

Materials and Methods

Chemicals and antibodies. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). MTS reagent was obtained from Promega (Madison, WI). Antibodies for AMPK, phosphor-AMPK, ACC, and phosphor-ACC antibody were from Cell Signaling Technology (Beverly, MA), and actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Reverse transcriptase was from Promega (Mannheim, Germany), and compound C (an AMPK inhibitor) was from Calbiochem (Darmstadt, Germany). Protein extraction kit, an EASY-BLUE total RNA extraction kit, and an ECL-reagent kit were from Intron Biotechnology (Beverly, MA). Infinitiy™ triacylglycerol reagent was from Asan Pharmaceutical. (Hwaseong, Korea).

Preparation of an active part of Artemisia sacrorum Ledeb. (ASL). The aerial part of Artemisia sacrorum Ledeb. was extracted twice with boiling water for 2 h and 1 h respectively and the aqueous extract was combined and concentrated to 0.5 g (crude drug)/ml and loaded on a D-101 macroporous resin column, and the column was eluted with water, 50% ethanol and 95% ethanol, and thereby water eluate (WE), 50% ethanol eluate (50% EE), and 95% ethanol eluate (95% EE) were obtained. After evaporation, the solutions were freeze-dried under a 50% ethanol eluate (50% EE), and 95% ethanol eluate (95% EE) were combined and concentrated to 0.5 g (crude drug)/ml and loaded on a D-101 macroporous resin column, and the column was eluted with

Cell culture. The HepG2 cell line was purchased from the American Type Culture Collection (Gaithersburg, MD). The cells were cultured in DMEM containing 10% FBS, 100 unit/ml of penicillin, and 100 μg/ml of streptomycin, and kept at 37°C in a 6-well plate and treated with various concentrations (0, 25, 50, and 100 μg/ml) of 95% EE for 24 h. The cells were fixed with 4% formaldehyde for 1 h, and then they were washed twice with cold phosphate-buffered saline (PBS) and harvested in a protein extraction kit. Then the insoluble protein was removed by centrifugation at 13,000 rpm for 20 min. The supernatant was collected from the lysates and protein concentrations were determined using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. Western blot analysis. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested in a protein extraction kit. Then the insoluble protein was removed by centrifugation at 13,000 rpm for 20 min. The supernatant was collected from the lysates and protein concentrations were determined using a Bio-Rad protein assay reagent (Bio-Rad Laboratories) following the manufacturer’s instructions. Equal amounts of proteins (40 μg) were resolved by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membrane was further incubated with the indicated primary antibody, followed by secondary antibody conjugated with horseradish peroxidase. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Uppsala, Sweden) and then exposed to X-ray film.

Results

At the beginning, to examine cellular toxicity, various concentrations of WE, 50% EE, and 95% EE (0, 25, 50, and 100 μg/ml) were treated HepG2 cells for 24 h. Three parts from ASL did not show any cellular toxicity up to 100 μg/ml. Hence, we screened for AMPK activation with 50% EE, 95% EE, and WE. The results showed that 95% EE activated AMPK in a dose-dependent manner (50 and 100 μg/ml) were treated HepG2 cells for 24 h. Three parts from ASL did not show any cellular toxicity up to 100 μg/ml. Hence, we screened for AMPK activation with 50% EE, 95% EE, and WE. The results showed that 95% EE activated AMPK in a dose-dependent manner (50 and 100 μg/ml). Because it has been reported repeatedly that AMPK activation is associated with lipid accumulation and fatty acid β-oxidation in the liver, triglycerides were measured with 95% EE, and the triglyceride concentrations showed in a dose-dependent manner. Hence we decided to carry on further research on the expression of AMPK target genes.

Effects of 95% EE on cell proliferation

To examine the cellular toxicity of 95% EE, various concentrations of 95% EE (0, 25, 50, and 100 μg/ml) were treated HepG2 cells for 24 h. As shown in Fig. 1A, 95% EE did not show any cellular toxicity up to 100 μg/ml.
Effects of 95% EE on AMPK phosphorylation

To screen out the AMPK active part, we first examined AMPK activation by western blot analysis. The 95% EE part significantly phosphorylated AMPK and ACC in a dose-dependent manner in the HepG2 cells (Fig. 2). Hence, we used a part of 95% EE. At the present time, separation and identification of compounds from part of 95% EE are being performed and active compounds will be confirmed after in vitro and in vivo experiments. To investigate the effects of 95% EE on AMPK phosphorylation, HepG2 cells were treated with 100 µg/ml of 95% EE for the indicated times. After each time period, cells were harvested and total cell lysates were extracted. The activation states of the α subunit of AMPK (AMPKα) and ACC were determined by immunoblotting using phosphorylated antibodies. 95% EE activated AMPKα-Th172 in a time-dependent manner in the HepG2 cells (Fig. 3A). Consistently with the increase in AMPK activity, the phosphorylation of ACC-Ser79, the best-characterized phosphorylation site of AMPK, also increased in a time-dependent manner (Fig. 3A). Next, HepG2 cells were exposed to the indicated concentrations of 95% EE for 24 h. As shown in Fig. 3B, AMPK and ACC were both phosphorylated by 95% EE in a dose-dependent manner. Hence, to verify the effects of 95% EE's AMPK activation, we attempted to inhibit AMPK and ACC activity by a pharmacological approach. While pretreatment of HepG2 cells with compound C (10 µM), an AMPK inhibitor, 95% EE-induced phosphorylation of AMPK and ACC was significantly attenuated (Fig. 3C, D). These results indicate that 95% EE has a metabolic role in HepG2 cells through AMPK activation.

Effects of 95% EE on lipogenesis, lipolysis, and cholesterol synthesis gene expression

It has been reported that activation of AMPK in the liver leads to increased fatty acid oxidation and simultaneously to inhibition of lipid synthesis and cholesterol synthesis. Therefore, to investigate the effect of 95% EE on the expression of the genes involved in lipid metabolism, the expression of target genes responsible for lipogenesis, lipolysis, cholesterol
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synthesis, and glucose uptake was examined by RT-PCR. As shown in Figs. 4 and 5, 95% EE significantly inhibited the expression of genes such as SREBP1c, FAS, and SCD1 in a time- and dose-dependent manner (Fig. 4A, B). All these are associated with fat synthesis. In contrast, 95% EE significantly increased the expression of lipolytic genes such as PPAR-α and CD36 in a time- and dose-dependent manner (Fig. 4C, D). Hence, to determine whether the effects of 95% EE on lipid metabolism are mediated by...
AMPK activation, we used AMPK inhibitor compound C. 95% EE-induced decreases in SREBP1c, FAS, and SCD1 gene expression were abolished by pretreatment with compound C (Fig. 5A). In contrast, the 95% EE-induced increases in PPAR-α and CD36 gene expression were significantly decreased by pretreatment with compound C (Fig. 5B). All the above results strongly indicate that 95% EE plays important roles in lipid metabolism in HepG2 cells by AMPK activation. However, there were no changes in the gene expression of SREBP2, HMG-CoA, LDLR, or GLUT2 (Fig. 6A, B). These data indicate that 95% EE attenuates lipid accumulation through regulation of expression of the genes involved in lipogenesis and lipolysis, but has no effect on cholesterol synthesis and glucose uptake. On the other hand, 95% EE significantly increased the gene expression of acyl CoA oxidase (ACOX) in a time- and dose-dependent manner (Fig. 6A, B).

**Discussion**

Use of plant medicine in the treatment of liver diseases has a long history, especially in Eastern medicine. Artemisia sacrorum Ledeb. is a traditional Chinese medicine that is widely used for prevent and treat different chronic and acute hepatitis. However, there are few, or no, scientific studies on the protective effects of this plant medicine on liver injury. Recently, we reported that another active part of ASL protected against acetaminophen-induced liver injury through inhibition of lipid peroxidation and down-regulation of TNF-α mediated apoptosis. In the meantime, we are attempting to determine whether ASL activates AMPK and affects lipid metabolism in HepG2 cells.

AMPK is a serine/threonine kinase activated following a rise in the intracellular AMP:ATP ratio. Numerous studies have indicated that AMPK plays a key role in the regulation of lipid metabolism.
Activation of AMPK leads to the phosphorylation and regulation of a number of downstream targets involved in lipid metabolism in order to maintain energy status.\(^1,2\) Based on this, AMPK cascades have emerged as novel targets in the treatment of fatty liver. Among the large number of AMPK downstream targets, HMG-CoA reductase and ACC are well identified.\(^2\) ACC is an important rate-controlling enzyme in the synthesis of malonyl-CoA, which inhibits carnitine palmitoyltransferase 1 (CPT-1), the rate-limiting step in mitochondrial and fatty acid oxidation. In other words, inhibition of ACC by AMPK through phosphorylation leads to a fall in malonyl-CoA content and a subsequent decrease in fatty acid synthesis and an increase in mitochondrial fatty acid oxidation through the regulation of CPT-1, which catalyzes the entry of long-chain fatty acyl-CoA into the mitochondria.\(^1,10\) In the present study, 95% EE time- and dose-dependently stimulated increases in AMPK and ACC phosphorylation in HepG2 cells (Fig. 3A, B). These phosphorylations were attenuated in the presence of compound C, an AMPK inhibitor (Fig. 3C, D). This suggests that AMPK activation is required for the phosphorylation (at Ser79) and inhibition of ACC. Thus the activation of AMPK by 95% EE inhibits ACC, decreases malonyl-CoA levels, and leads to stimulation of fatty-acid oxidation.

Numerous studies have indicated that AMPK activation directly inhibits ACC and HMG-CoA activity and inhibits ACC and HMG-CoA expression indirectly via suppression of sterol regulatory element binding protein 1s (SREBP1).\(^5,21,22\) SREBPs are a family of transcription factors that consist of SREBP1a, SREBP1c, and SREBP2.\(^2\) SREBP1c is a critical transcription factor that stimulates several lipogenic enzymes involved in fatty-acid synthesis, FAS, ACC, and SCD1, in the liver,\(^1,25\) whereas SREBP2 is relatively specific to regulation of the genes involved in cholesterol synthesis and uptake, LDLR and HMG-CoA. GLUT2 is one of the main glucose transporters in the liver. Regulation of GLUT2 in the liver is controlled by the promoter region and has a relation with various metabolic states.\(^26\) Hence, we investigated the effects of 95% EE on the expression of the target genes involved in TG, cholesterol synthesis, and glucose uptake. As shown in Fig. 3, 95% EE significantly inhibited the expression of genes including SREBP1c, FAS, and SCD1, all associated with TG biosynthesis, in time- and dose-dependent manner (Fig. 4A, B). In contrast, 95% EE significantly increased the expression of lipolytic genes including PPAR-\(\alpha\) and CD36 in time- and dose-dependent manner (Fig. 4C, D). ACOX is another rate-limiting marker enzyme of free fatty acid metabolism in peroxisome. Gloerich et al. reported that PPAR-\(\alpha\) activation increases ACOX, leading to peroxisomal \(\beta\)-oxidation.\(^27\) In our study, 95% EE significantly increased the gene expression of ACOX in time- and dose-dependent manner (Fig. 6A, B). However, there were no changes in the gene expression of SREBP2, LDLR, HMG-CoA, or GLUT2, proteins responsible for cholesterol synthesis and glucose uptake (Fig. 7A, B). In addition, 95% EE-induced decreases in SREBP1c, FAS, and SCD1 gene expression and 95% EE-induced increases in PPAR-\(\alpha\) and CD36 gene expression were repaired by treatment with compound C (Fig. 5A, B). Moreover, it significantly decreased TG accumulation, in a dose-dependent manner (Fig. 1B, C). From these results, we may conclude that 95% EE stimulated fatty acid \(\beta\)-oxidation through AMPK activation and ACC inactivation, and thereby inhibited the gene expression of SREBP1c, FAS, and SCD1, and further inhibited TG synthesis. Through AMPK activation and ACC inactivation, it also increased the gene expression of lipolysis, including CD36, PPAR-\(\alpha\), and thereby stimulated fatty-acid oxidation and decreased TG content in HepG2 cells.

In summary, our results suggest that 95% EE inhibited hepatic TG accumulation through the activation of AMPK in human hepatoma HepG2 cells. These findings might prove to be of benefit in treatment strategies for fatty liver and obesity-related disorders.

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References

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