

An active part of *Artemisia sacrorum* Ledeb. inhibits adipogenesis via the AMPK signaling pathway in 3T3-L1 adipocytes

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Abstract. *Artemisia sacrorum* Ledeb. (Compositae) (ASL) has long been used in Oriental folk medicine to treat diverse hepatic diseases. In this study, we investigated the effect of ASL on adipocyte differentiation in 3T3-L1 cells. ASL significantly suppressed 3T3-L1 differentiation in a concentration-dependent manner. A significant increase of AMP-activated protein kinase (AMPK) was observed when the cells were treated with ASL. Activation of AMPK was also demonstrated by measuring the phosphorylation of acetyl-CoA carboxylase, a substrate of AMPK. These effects were abolished by pre-treatment with the AMPK inhibitor, compound C. In addition, ASL down-regulated the adipogenesis-related gene expression of the sterol regulatory element-binding protein 1c (SREBP1c) and its target genes, such as fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD1) and glycerol-3-phosphate acyltransferase (GPAT) in a concentration-dependent manner. These effects were abolished by pre-treatment with compound C. ASL significantly reduced the gene expression of the peroxisome proliferator-activated receptor γ (PPAR γ) and of the CCAAT/enhancer binding protein- α (C/EBP α), two key transcription factors in adipogenesis. Meanwhile, adipocyte fatty acid binding protein (aP2) gene expression was also reduced in a concentration-dependent manner. These findings indicated that ASL exerts anti-adipogenic activity via AMPK activation and may act to prevent obesity.

Introduction

Obesity is a complex multifactorial chronic disorder that is associated with many health problems, including hyper-

lipidemia, hypertension, type 2 diabetes, coronary heart disease, cancer, respiratory complications, and osteoarthritis (1,2). Obesity is caused by an imbalance between the energy intake and expenditure that may lead to a pathological growth of adipocytes (3). Adipocytes play a central role in regulating adipose mass and obesity. In obese or overweight individuals, the increased adipose mass can affect adipose cell size. In other words, the increased adipose mass in obesity is not only caused by adipose tissue hypertrophy, but also by adipose tissue hyperplasia, which triggers the transformation of pre-adipocytes into adipocytes (4,5). Thus, adipocyte differentiation and the amount of fat accumulation are associated with the development of obesity.

The AMP-activated protein kinase (AMPK) is a heterotrimeric protein consisting of one catalytic subunit (α) and two non-catalytic subunits (β and γ). When AMP binds to the γ -subunit, AMPK activation is promoted by stimulating phosphorylation at the threonine residue within the kinase domain (6). AMPK has been proposed to act as a metabolic master switch in response to alterations in the cellular energy charge. Once activated, AMPK leads to a concomitant inhibition of energy-consuming biosynthetic pathways and activation of ATP-producing catabolic pathways (7,8). Among its identified roles, AMPK has been implicated in the control of adipose glucose and lipid homeostasis by many additional effects both on genes and specific enzymes (9,10).

Anti-obesity drugs such as orlistat, sibutramine, and topiramate have been used to treat obesity (11). However, these drugs have many side effects, including dry mouth, anorexia, insomnia and gastrointestinal distress (12). Due to the adverse side effects associated with many anti-obesity drugs, recent drug trials have focused on herbal medicines. The *Artemisia sacrorum* Ledeb. (ASL) has been used in Oriental folk medicine to prevent and treat chronic and acute hepatitis. The application of ASL has a long history, and its hepatoprotective effects have been widely recognized in some areas of China (13). However, there have been no reports on the effects of ASL on adipocyte differentiation in relation to obesity. In this study, we examined whether ASL inhibits adipocyte differentiation, and whether this effect is associated with the AMPK signaling pathway in 3T3-L1 pre-adipocytes.

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Materials and methods

Materials. The preparation of plant material was described in our previous study (14). In the present study, we used the 50% ethanol eluate part of ASL. 3T3-L1 fibroblast cells were purchased from the American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), bovine calf serum (BCS), and penicillin-streptomycin were purchased from Gibco-BRL Life Technologies (Grand Island, NY). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for AMPK, phosphor-AMPK, ACC and phosphor-ACC antibody were purchased from Cell Signaling Technology (Beverly, MA, USA), and peroxisome proliferator-activated receptor γ (PPAR γ), CCAAT/enhancer binding protein- α (C/EBP α) and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Reverse transcriptase was supplied by Promega (Mannheim, Germany) and compound C (an AMPK inhibitor) was from Calbiochem (Darmstadt, Germany).

Cell culture and adipocyte differentiation. Mouse 3T3-L1 pre-adipocytes were cultured at 37°C under a humidified, 5% CO₂ atmosphere in DMEM supplemented with 10% BCS, 100 U/ml of penicillin, and 100 μ g/ml of streptomycin. 3T3-L1 cells were incubated for 2 days until confluence. At this point (day 0), the cells were exposed to adipogenic differentiation medium (DM; DMEM containing 5% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 mM dexamethasone and 10 μ g/ml insulin) for 4 days. The DM was replaced after 2 days with DMEM containing 5% FBS and 10 μ g/ml insulin (day 6), and then exchanged with DMEM containing 5% FBS every other day. During adipocyte differentiation, 3T3-L1 cells were treated with ASL at a concentration of 0, 25, 50 or 100 μ g/ml between days 0 to 4. The positive control was treated with 10 μ M of pioglitazone (PIO) and the same concentration of adipogenic DM. The cytotoxicity level of ASL in 3T3-L1 cells was evaluated by the MTS assay. ASL did not show any cellular toxicity up to 200 μ g/ml concentration (data not shown) and thus concentrations from 0-100 μ g/ml of ASL were employed in this study.

Measurement of triglyceride content. For triglyceride determination, cells were collected and lysed in lysis buffer (25 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA and 1 mM EGTA). The cellular contents of triglyceride were measured according to the manufacturer's instructions, for Infinity™ triacylglycerol reagents. The protein concentration was determined using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

Oil-Red O staining. After differentiation, cells were gently washed with phosphate-buffered saline (PBS) twice, fixed with 10% formalin for 1 h, and then stained with filtered Oil-Red O solution (60% isopropanol and 40% water) for 2 h at room temperature. The Oil-Red O staining solution was removed and the plates were washed with water and dried. The pictures were taken using an Olympus microscope (Tokyo, Japan). The stained lipid droplets were dissolved in isopropanol and quantified by either ELISA (540 nm) or fluorescence microscopy.

Western blot analysis. The cells were washed twice with ice-cold 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 following the manufacturer's instructions. Equal amounts of proteins (40 μ g) 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 incubation with horseradish peroxidase-conjugated secondary antibodies. Protein bands were detected using an enhanced chemiluminescence Western blot analysis detection kit (Amersham, Uppsala, Sweden) and then exposed to X-ray film.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated using an Easy Blue total RNA extraction kit (Intron Biotechnology Inc., Seoul, Korea) according to the manufacturer's instructions. First-strand cDNA synthesis was performed as described previously (15), using 5 μ g of RNA, oligo (15)-dT primers, and reverse transcriptase in a total volume of 50 μ l. PCR reactions were performed in a total volume of 20 μ l comprised of 2 μ l of cDNA product, 0.2 mM of each dNTP, 20 pmol of each primer and 0.8 units of Taq polymerase. Oligonucleotide primer sequences used in the PCR amplification were as follows; sterol regulatory element-binding protein 1c (SREBP1c), forward 5'-GCGCTACCGGTCTTCTATCA-3' and reverse 5'-TGCTGCCAAAAGACAAGGG-3'; fatty acid synthase (FAS), forward 5'-GATCCTGGAACGAGAACAC-3' and reverse 5'-AGACTGTGGAACACGGTGGT-3'; stearoyl-CoA desaturase 1 (SCD1), forward 5'-CGAGGGT TGTGTGTGATCTG-3' and reverse 5'-ATAGCACTGTT GGCCCTGGA-3'; glycerol-3-phosphate acyltransferase (GPAT), forward 5'-GGTAGTGGATACTCTGTCGTCCA-3' and reverse 5'-CAGCAACATCATTCGGT-3'; PPAR γ , forward 5'-AGGCCGAGAAGGAGAAGCTGTTC-3' and reverse 5'-TG GCCACCTCTTTGCTCTTGCTC-3'; C/EBP α , forward 5'-GG GTGAGTTCATGGAGAATGG-3' and reverse 5'-CAGTTTG GCAAGAATCAGAGCA-3'; adipocyte fatty acid binding protein (aP2), forward 5'-TCTCACCTGGCCTCTTCCTTTG GCTC-3' and reverse 5'-TTCCATCCAGGCCTCTTCCTTTG GCTC-3'; actin, forward 5'-GGACTCCTATGGTGGGTGAC GAGG-3' and reverse 5'-GGGAGAGCATAGCCCTCGTAG AT-3'. PCR was performed at 95°C for 30 sec, followed by 30 sec at 50°C (FAS, C/EBP α), 55°C (PPAR γ), 56°C (SCD1), 58°C (SREBP1c, GPAT, actin) or 60°C (aP2) and 1 min at 72°C. The last cycle was followed by a final extension step at 72°C for 10 min. The RT-PCR products were electrophoresed on a 1% agarose gel under 100 V and stained with 0.5 μ g/ml ethidium bromide. Scanning densitometry was performed with the I-MAX Gel Image analysis system (Core-Bio, Seoul, Korea). Actin was amplified as a control gene.

Statistical analysis. All data were expressed as the mean \pm standard error (SE), and differences between groups were analyzed by the Student's t-test. Mean values were considered significantly different at $p < 0.05$.

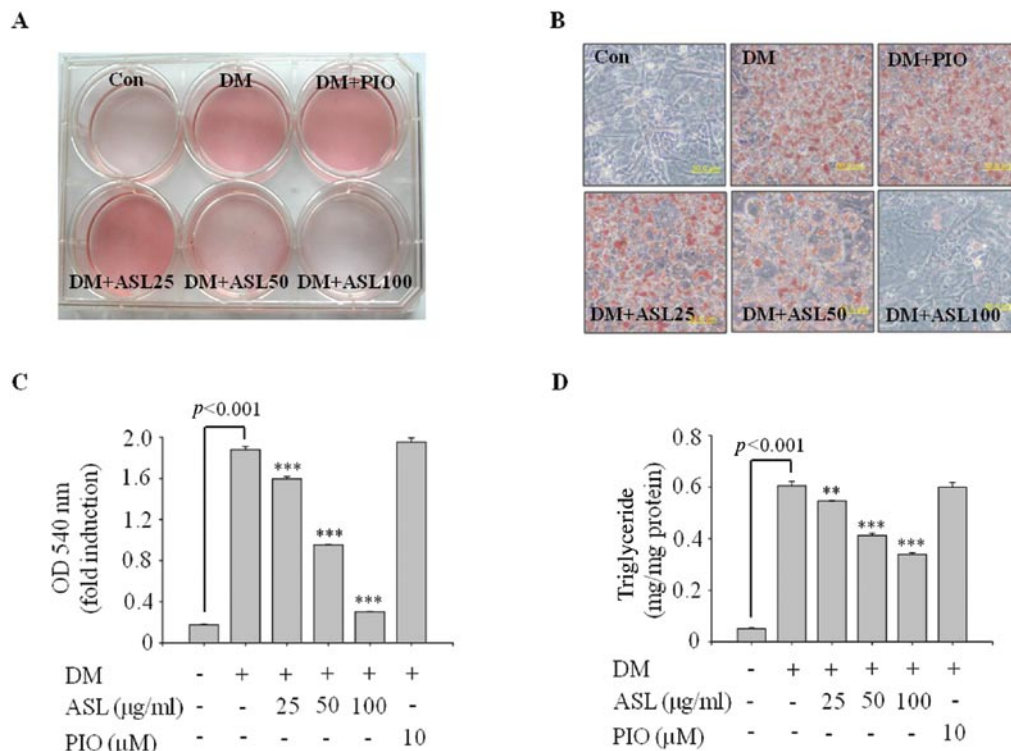


Figure 1. Effects of ASL on lipid accumulation in 3T3-L1 cells. Confluent cells were treated with various concentrations of ASL (0, 25, 50 or 100 $\mu\text{g/ml}$) from day 0 to 4. Cell differentiation was examined at day 8 by Oil-Red O staining (A and B). Stained intercellular oil droplets were eluted with isopropanol and quantified by spectrophotometrical analysis at 540 nm (C). Triglyceride content was measured in 3T3-L1 cells (D). Data are represented as the mean \pm SE of triplicate experiments. ** $p < 0.01$ and *** $p < 0.001$, as compared to differentiated control; DM, differentiation medium; PIO, pioglitazone.

Results

Effects of ASL on adipocyte differentiation. To explore the effects of ASL on adipocyte differentiation, 3T3-L1 cells were treated with various concentrations of ASL (0, 25, 50 or 100 $\mu\text{g/ml}$) from day 0 to 4. We examined cell differentiation on day 8. Lipid accumulation was quantified by Oil-Red O staining and triglyceride measurement. As shown in Fig. 1A and B, ASL markedly inhibited the accumulation of lipid droplets in a concentration-dependent manner. The absorbance values of eluted Oil-Red O solution in adipocytes represent the lipid droplet accumulation in the cytoplasm. As shown by Oil-Red O elution (Fig. 1C), ASL treatment significantly reduced the absorbance value of the eluted dye, when compared to the fully differentiated cells in the absence of ASL ($p < 0.001$). In addition, ASL significantly reduced triglyceride accumulation in a concentration-dependent manner (Fig. 1D). Compared to the DM control level, fat storage was reduced by 9.8% ($p < 0.01$) at 25 $\mu\text{g/ml}$, 31.9% ($p < 0.001$) at 50 $\mu\text{g/ml}$, and 43.9% ($p < 0.001$) at 100 $\mu\text{g/ml}$ of ASL, respectively. These results suggest that ASL efficiently inhibited adipocyte differentiation in 3T3-L1 pre-adipocytes and may have potential anti-obesity effects.

Effects of ASL on AMPK phosphorylation. To investigate whether inhibition of adipocyte differentiation by ASL is mediated by AMPK activation, 3T3-L1 cells were exposed to the indicated concentrations of ASL for 4 days. The activation states of the α subunit of AMPK (AMPK α) and ACC were determined by immunoblotting, using phosphorylated

antibodies. ASL activated AMPK α -Thr¹⁷² in a concentration-dependent manner (Fig. 2A). Consistent with the increase in AMPK activity, the phosphorylation of ACC-Ser⁷⁹, which is the best-characterized phosphorylation site of AMPK, also increased in a concentration-dependent manner (Fig. 2A). Therefore, to verify the effect of ASL's AMPK activation, we attempted to inhibit the AMPK and ACC activity through a pharmacological approach. When the 3T3-L1 cells were pre-treated with compound C (20 μM), an AMPK inhibitor, the ASL-induced phosphorylation of AMPK and ACC were significantly attenuated (Fig. 2B). These results indicate that ASL inhibits adipogenesis through AMPK activation.

Effects of ASL on AMPK target gene expression. To investigate the effects of ASL on the expression of genes involved in lipid metabolism, the expression of AMPK target genes responsible for adipogenesis was examined by RT-PCR. As shown in Fig. 3A, ASL significantly inhibited the expression of genes such as, SREBP1c, FAS, SCD1 and GPAT, which are all associated with adipogenesis, in a concentration-dependent manner. Furthermore, to assess whether the effects of ASL on lipid metabolism are mediated by AMPK activation, we used the AMPK inhibitor, compound C. ASL-induced decreases in SREBP1c, FAS, SCD1 and GPAT gene expression were abolished by pre-treatment with compound C (Fig. 3B). These results strongly indicate that the ASL-induced suppression of adipogenesis in 3T3-L1 cells is mediated via AMPK activation.

Effects of ASL on PPAR γ and C/EBP α expression. PPAR γ and C/EBP α , which are mainly found in adipose tissue, are

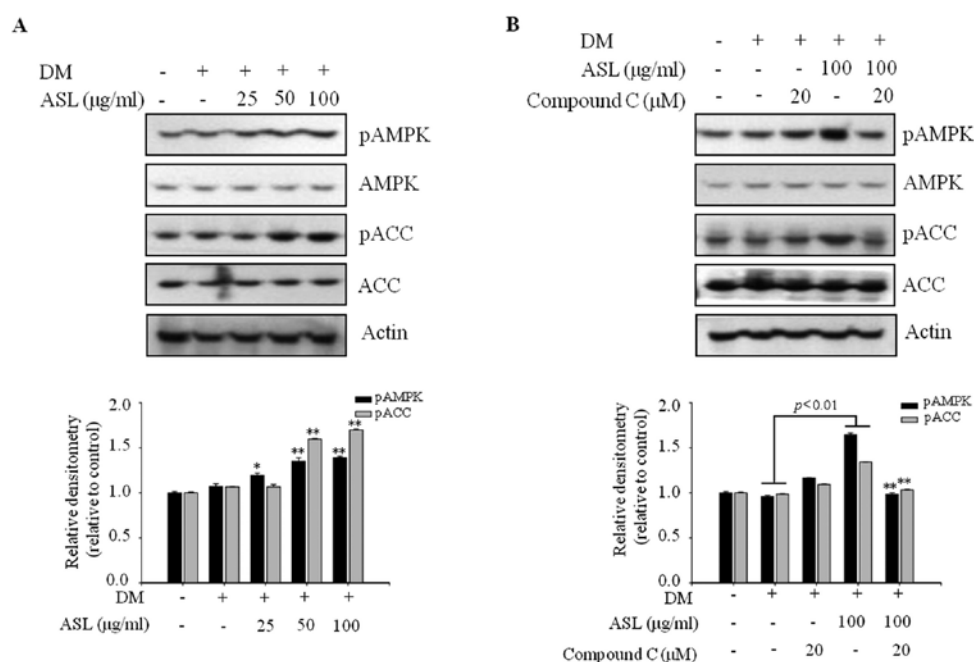


Figure 2. Effects of ASL on AMPK and ACC phosphorylation in 3T3-L1 cells. Confluent cells were treated with various concentrations (0, 25, 50 or 100 $\mu\text{g/ml}$) of ASL from day 0 to 4 (A). Cells were pre-treated with compound C for 1 h, and then treated with 100 $\mu\text{g/ml}$ of ASL from day 0 to 4 (B). On day 8, completely differentiated cells were lysed to extract total protein. Protein extracts were prepared and subjected to Western blot analysis using phosphor-AMPK (pAMPK), AMPK, phosphor-ACC (ACC), ACC and β -actin antibodies. β -actin protein levels were used as an internal control to evaluate relative expression of the protein. Data are represented as the mean \pm SE (n=3). * $p<0.05$ and ** $p<0.01$, as compared to differentiated control; DM, differentiation medium.

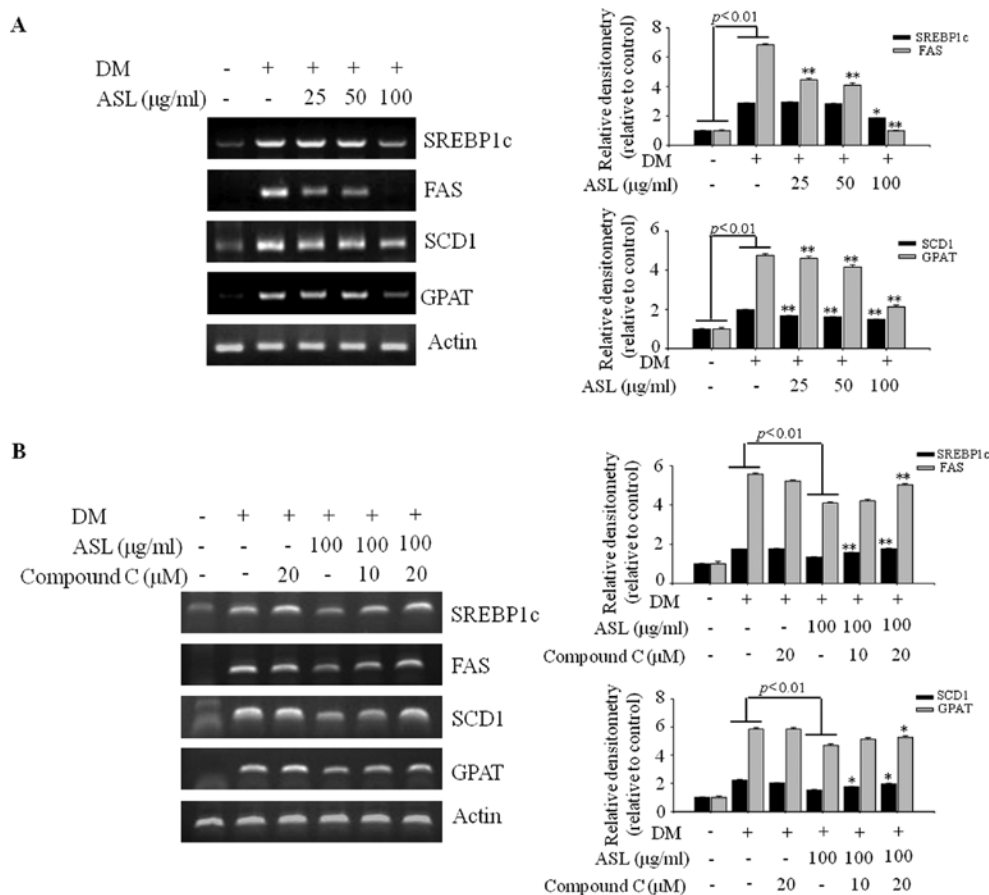


Figure 3. Effects of ASL on SREBP1c, FAS, SCD1 and GPAT gene expression in 3T3-L1 cells. Confluent cells were treated with various concentrations (0, 25, 50 or 100 $\mu\text{g/ml}$) of ASL from day 0 to 4 (A). Cells were pre-treated with compound C for 1 h, and then treated with 100 $\mu\text{g/ml}$ of ASL from day 0 to 4 (B). On day 8, completely differentiated cells were used to extract total mRNA. The expression of adipogenesis-related AMPK target genes, such as SREBP1c, FAS, SCD1 and GPAT, was measured by RT-PCR. Data are represented as the mean \pm SE (n=3). * $p<0.05$ and ** $p<0.01$, as compared to differentiated control; DM, differentiation medium.

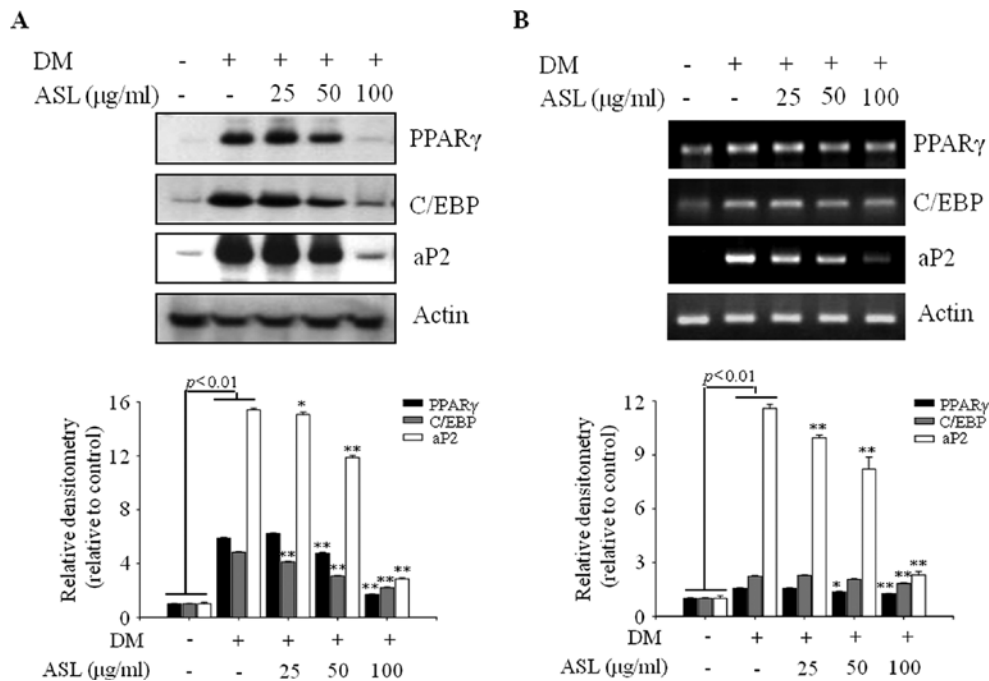


Figure 4. Effects of ASL on PPAR γ , C/EBP α and aP2 expression in 3T3-L1 cells as examined by Western blot analysis (A) and RT-PCR (B). Confluent cells were treated with various concentrations (0, 25, 50 or 100 μ g/ml) of ASL from day 0 to 4. On day 8, cells were completely differentiated into adipocytes. Data are represented as the mean \pm SE (n=3). *p<0.05 and **p<0.01, as compared to differentiated control; DM, differentiation medium.

the key transcription factors in adipogenesis and lipogenesis (16). To further investigate the mechanism of differentiation, of 3T3-L1 cells the protein and mRNA expression levels of PPAR γ and C/EBP α were monitored by Western blot analysis and RT-PCR, respectively. ASL markedly suppressed the protein (Fig. 4A) and mRNA (Fig. 4B) levels of PPAR γ and C/EBP α in a concentration-dependent manner. PPAR γ and C/EBP α promote terminal differentiation by transactivating the expression of downstream adipocyte-specific genes, including aP2 (17). According to our results, the protein (Fig. 4A) and mRNA (Fig. 4B) levels of aP2 were also reduced in a concentration-dependent manner. These results suggest that ASL inhibited 3T3-L1 differentiation by suppressing the expression of PPAR γ and C/EBP α .

Discussion

ASL has been used in Oriental folk medicine to prevent and treat diverse forms of chronic and acute hepatitis (13). The application of ASL has a long history and its hepatoprotective effect has been widely recognized. Even at present, ASL is often used to treat hepatitis in some regions, especially in the Yanbian area of China. We have previously demonstrated that ASL extracts had a protective effect in acetaminophen-induced liver injury in mice (14,18). In addition, ASL was shown to increase bile secretion volume and have an antibacterial effect (19). Nevertheless, the molecular mechanisms of ASL in the differentiation of 3T3-L1 cells have not been defined. The present study demonstrated for the first time that ASL suppressed 3T3-L1 adipocyte differentiation and lipid accumulation via the AMPK signaling pathway.

Numerous studies have demonstrated that adipocyte differentiation and the amount of fat accumulation are

associated with the occurrence and development of obesity. Our results show that ASL significantly inhibited adipocyte differentiation and lipid accumulation in a concentration-dependent manner (Fig. 1A, B and C). Meanwhile, ASL significantly reduced TG accumulation in a concentration-dependent manner (Fig. 1D), which indicates that ASL inhibited adipogenesis during adipocyte differentiation, and may have potential anti-obesity effects.

AMPK is a pivotal enzyme that regulates diverse signals in metabolic pathways. AMPK plays a key role in the regulation of carbohydrate and fat metabolism, is a potential drug target for the treatment of obesity, diabetes, and fatty liver disease, and appears to be intimately involved in adipocyte differentiation and maturation (20). In order to elucidate the molecular mechanism by which ASL inhibits adipogenesis via AMPK signaling, we evaluated the role of AMPK in anti-adipogenesis. Our results show that ASL stimulated the phosphorylation of AMPK and of its substrate, ACC in 3T3-L1 cells (Fig. 2A), and that ASL-mediated AMPK and ACC phosphorylation in these cells were completely inhibited by compound C, an AMPK inhibitor (Fig. 2B). Activation of AMPK is involved in the regulation of downstream target genes that belong to diverse pathways in adipose tissue (21). Thus, AMPK cascades have emerged as important targets in the treatment of obesity (22,23). Numerous studies have indicated that AMPK activation inhibits ACC activity directly through phosphorylation, and inhibits ACC expression indirectly via the suppression of SREBP1c (24-26). In the present study, we investigated the effects of ASL on the expression of AMPK target genes associated with adipogenesis. ASL attenuated the gene expression of SREBP1c, central to the intracellular surveillance of lipid catabolism and *de novo* biogenesis, in a concentration-dependent manner (Fig. 3A). Genes for

FAS, SCD1 and GPAT, well-known target molecules of SREBP1c, were also suppressed in a concentration-dependent manner. In addition, ASL-induced decreases in SREBP1c, FAS, SCD1 and GPAT gene expression were abolished by pre-treatment with compound C in a concentration-dependent manner (Fig. 3B). These results suggest that ASL suppressed adipocyte differentiation through AMPK activation and ACC inactivation, and thereby inhibited the gene expression of SREBP1c, FAS, SCD1 and GPAT, which in turn inhibited lipid accumulation.

Adipocyte differentiation is regulated by the coordinated expression of various transcription factors, such as PPAR γ and C/EBP α (27). PPAR γ and C/EBP α are the most important transcriptional regulators known to play central role in adipogenesis (28). In the present study, ASL markedly down-regulated the protein and mRNA levels of PPAR γ and C/EBP α , as well as those of their downstream target, aP2, in a concentration-dependent manner (Fig. 4). These results suggest that ASL inhibited adipocyte differentiation and adipogenesis by affecting the transcriptional factor cascade upstream of PPAR γ and C/EBP α , possibly resulting in the inhibition of lipid accumulation by inhibiting adipogenesis.

In conclusion, our results suggest that ASL is able to inhibit adipocyte differentiation and adipogenesis through the activation of AMPK in 3T3-L1 cells. These findings might be of great benefit to the development of treatment strategies for obesity and obesity-related disorders in the future.

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