

AMPK and SREBP-1c mediate the anti-adipogenic effect of β -hydroxyisovalerylshikonin

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Abstract. β -hydroxyisovalerylshikonin (β -HIVS), which is a natural naphthoquinone compound, is one of the main chemicals isolated from a therapeutic plant, *Lithospermum erythrorhizon*. In the present study, we demonstrated that β -HIVS inhibited the adipogenesis of 3T3-L1 cells through AMP-activated protein kinase (AMPK)-mediated modulation of sterol regulatory element binding protein (SREBP)-1c. The anti-adipogenic effect of β -HIVS was accompanied by the increased phosphorylation of AMPK and precursor SREBP-1c. In β -HIVS-treated 3T3-L1 cells, AMPK was activated and phosphorylated precursor SREBP-1c, preventing the cleavage of precursor SREBP-1c to mature SREBP-1c. Expression of the fat-forming enzymes, acetyl-CoA carboxylase (ACC)1, fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD)1, which are transcribed by mature SREBP-1c, were downregulated, resulting in reduced intracellular fat accumulation. The anti-adipogenic effect of β -HIVS was significantly attenuated by AMPK knockdown. Knockdown of AMPK using siRNA decreased the phosphorylation of precursor SREBP-1c and increased the levels of mature SREBP. The levels of the fat-forming enzymes, ACC1, FAS and SCD1, as well as intracellular fat accumulation were also significantly increased by AMPK knockdown. These results suggest that β -HIVS activated AMPK, which was followed by the downregulation of mature SREBP-1c and fat-forming enzymes, leading to the inhibition of adipogenesis.

Introduction

Obesity is defined by the excessive formation of adipose tissue, and thus an understanding of the molecular mechanism behind adipose tissue formation, i.e., adipogenesis, is necessary to find ways of preventing and treating obesity and obesity-related diseases including type 2 diabetes, hyperlipidemia and cardiovascular disease (1). Adipogenesis is mediated by the coordinated expression of the genes involved in adipocyte differentiation and intracellular fat accumulation (2-4). The 3T3-L1 cell line has been widely used as an *in vitro* model of adipogenesis, and the treating these cells with 3-isobutyl-1-methylxanthine, dexamethasone and insulin is known to upregulate the major transcription factors of adipogenesis, peroxisome proliferator-activated receptor γ (PPAR γ) and CCAAT/enhancer-binding protein α (C/EBP α), which directly transcribe various adipocyte marker genes, including fatty acid-binding protein (FABP)4 (2-4).

AMP-activated protein kinase (AMPK) is a major regulator of cellular energy homeostasis (5), and it regulates carbohydrate and fat metabolism in order to maintain the cellular energy balance (6-8). AMPK is activated by the increased ratio of AMP/ATP, and it is known to phosphorylate various target proteins involved in cell growth and metabolism (9). Dysregulation of AMPK is involved in various disorders including metabolic diseases, cardiovascular disease, cancer and dementia (10). Recently, Li *et al* reported that sterol regulatory element binding protein (SREBP)1c was one of the target proteins directly phosphorylated by AMPK (11). AMPK was found to phosphorylate the Ser372 residue of SREBP-1c, which inhibited the proteolytic cleavage of the precursor form of SREBP-1c (precursor SREBP-1c) into mature SREBP-1c, resulting in the inhibition of hepatic steatosis in insulin-resistant mice. SREBP-1c has also been identified as one of the transcription factors involved in adipogenesis (12). However, to the best of our knowledge, AMPK-mediated modulation of SREBP-1c has never been suggested to be the anti-adipogenic mechanism of any compound which inhibits adipogenesis.

Shikonin is a major chemical ingredients of *Lithospermum erythrorhizon*, a therapeutic plant used for the treatment of macular eruption, measles, sore-throat, carbuncles and burns (13). *Lithospermum erythrorhizon* contains various shikonin compounds, including shikonin, acetylshikonin, isobutyrylshikonin and β -hydroxyisovalerylshikonin (β -HIVS) (14). In the present study, we demonstrated the anti-adipogenic effect

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Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; C/EBP α , CCAAT/enhancer-binding protein α ; FABP, fatty acid-binding protein; FAS, fatty acid synthase; β -HIVS, β -hydroxyisovalerylshikonin; PPAR γ , peroxisome proliferator-activated receptor γ ; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element binding protein

Key words: AMP-activated protein kinase, sterol regulatory element binding protein-1c, 3T3-L1, β -hydroxyisovalerylshikonin, adipogenesis

of β -HIVS and elucidated its molecular mechanisms. It was found that β -HIVS activated AMPK, resulting in the increased phosphorylation of SREBP-1c, preventing its proteolytic maturation. This was followed by the reduced expression of fat-forming enzymes, including acetyl-CoA carboxylase (ACC)1, fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD)1, which resulted in reduced intracellular fat accumulation.

Materials and methods

Chemicals and reagents. The cell culture reagents, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin, were all obtained from Life Technologies (Grand Island, NY, USA). Anti-PPAR γ (#2430), anti-FABP4 (#2120), anti-p-AMPK (#2535), anti-AMPK (#2603), anti-FABP4 (#2120) and anti-p-SREBP1c (#9847) antibodies, and anti-mouse (#7076S)/anti-rabbit (#7074) secondary antibody were all purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-C/EBP α (sc-61) and anti- β -actin (sc-47778) antibodies were all purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Anti-SREBP1c (557036) was purchased from BD science (Franklin Lakes, NJ, USA). AMPK small interfering (si)RNA and control siRNA were both purchased from Santa Cruz Biotechnology, Inc. Lipofectamine RNAiMAX transfection reagent was purchased from Invitrogen (Carlsbad, CA, USA). Shikonin, acetylshikonin, isobutyrylshikonin and β -HIVS were all purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. The 3T3-L1 preadipocytes were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and subcultured every 2 days. The cells were seeded in 6-well plates at a density of 1.5×10^5 cells/well. Two days after reaching confluence (day 0), the 3T3-L1 cells were differentiated in a differentiation-induction medium containing DMEM, supplemented with 1 μ g/ml insulin, 0.25 μ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 10% FBS, for 2 days. The cells were then maintained in a differentiation-maintenance medium containing DMEM, supplemented with 1 μ g/ml insulin and 10% FBS. The differentiation-maintenance medium was replaced every 2 days until the cells were harvested.

Oil Red O staining of intracellular fat. The 3T3-L1 cells were seeded in 12-well plates at a density of 7.5×10^4 cells/well. After differentiation for 7 days, the cells were washed with phosphate-buffered saline (PBS), and incubated for 4 h in 4% paraformaldehyde (Sigma-Aldrich). The cells were then washed with distilled water and stained with 0.3% Oil Red O (Sigma-Aldrich) solution for 1 h. After washing them 3 times with distilled water, the cells were completely dried and observed under an inverted microscope (Olympus, Tokyo, Japan). Photographic images were captured and then 100% isopropanol (Sigma-Aldrich) was added to each well to extract the stained Oil Red O dye. The contents of the extracted dye were measured at 540 nm using a spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Cell viability assay. Cell viability was determined using a CellCountEZTM Cell Survival assay kit (purchased from

Rockland Immunochemicals, Limerick, PA, USA), as previously described (15); viability was determined based on the ability of viable mammalian cells to convert hydroxyethyl disulfide into mercaptoethanol. The amount of mercaptoethanol produced from hydroxyethyl disulfide can be measured in the extracellular culture media since mercaptoethanol produced inside cells is extruded quickly by cells through an active transport mechanism.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The 3T3-L1 cells were seeded into 12-well plates at a density of 7.5×10^4 cells/well, and, after differentiation, total RNA was extracted using an RNeasy kit (Qiagen, Hilden, Germany). One microgram of total RNA was reverse transcribed at 37°C using the cDNA reverse transcription kit (Applied Biosystems, Inc., Foster City, CA, USA). qPCR was performed using the 7000 Real-Time PCR System (Applied Biosystems) in a final volume of 20 μ l, which included the TaqMan Gene Expression Master Mix, 250 nM of TaqMan probe, an optimized concentration of each primer and 1 μ l of the reverse transcription product containing cDNA. The reaction mixtures were preheated at 95°C for 10 min to activate the enzyme and then subjected to 40 cycles of melting at 95°C for 15 sec and annealing/extension at 60°C for 1 min. The efficiency of RT-qPCR was approximately 100%. The Assays-on-Demand gene expression products (Applied Biosystems) were used to evaluate the mRNA levels of PPAR γ (Mm00440945_m1), C/EBP α (Mm01265914_s1), SREBP-1c (Mm00550338_m1), FABP4 (Mm00445880_m1), FAS (Mm01253292_m1), ACC1 (Mm01304257_m1), SCD1 (Mm00772290_m1) as well as the level of 18S rRNA (Hs99999901_s1). 18S rRNA was used as an internal control, as previously described (16). For each sample, the mRNA level was normalized against the level of 18S rRNA, and the ratio of normalized mRNA in each sample to that in the control sample was determined using the comparative Ct method (17).

Protein extraction and western blot analysis. Cells were harvested using a cell scraper in ice-cold PBS, and lysed with RIPA buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS and a protease inhibitor cocktail (Sigma-Aldrich) for 30 min at 4°C. The total cell lysates were then obtained after centrifuging at 20,000 \times g for 20 min at 4°C to remove the insoluble materials. The protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Twenty micrograms of protein were separated using 10% polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membranes at 180 mA for 1 h. The membranes were then blocked for 2 h at room temperature with Tris-buffered saline (TBS) containing 5% skim milk. Blocked nitrocellulose membranes were incubated with TBS containing 0.1% Tween-20 and a 1:1,000-dilution of primary antibody overnight at 4°C followed by a 1:1,000-dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Peroxidase activity was visualized using an ECL kit (Pierce).

Transfection with siRNA. Two days after reaching confluence, 3T3-L1 cells were incubated in serum-free medium for 1 h and

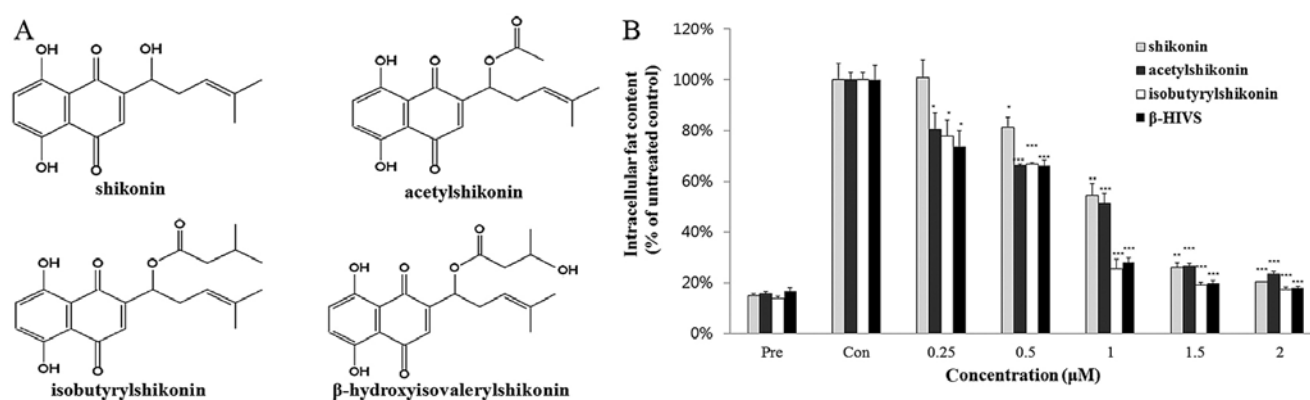


Figure 1. Comparison of the anti-adipogenic activities of shikonin compounds. (A) Chemical structures of shikonin compounds contained in *Lithospermum erythrorhizon*. (B) The inhibitory effect of shikonin compounds on intracellular fat accumulation. Intracellular fat droplets were stained with Oil Red O and extracted for measurement by spectrophotometric analysis at 540 nm on day 7 of 3T3-L1 adipocyte differentiation. *P<0.05, **P<0.01, ***P<0.001 compared with untreated adipocytes (Con). Pre denotes undifferentiated preadipocytes.

then transfected with 60 nM of AMPK siRNA or 60 nM of control siRNA using Lipofectamine RNAiMAX transfection reagent. Six hours later, the transfected cells were differentiated by replacing the medium with differentiation-induction medium. Total RNA and protein were extracted for RT-qPCR and western blot analysis, respectively.

Analysis of nuclear SREBP-1c levels. In the present study, the cells were harvested using cell scrapers, and the nuclear extracts were prepared using a nuclear extract kit (Active Motif, Carlsbad, CA, USA). Protein concentrations in the nuclear extracts were determined using a BCA protein assay kit (Pierce). Twenty micrograms of nuclear protein were separated using 10% polyacrylamide gel electrophoresis and analyzed by western blot analysis using an anti-SREBP-1c antibody followed by secondary antibody.

Statistical analyses. All data are represented as the means \pm standard deviation (SD) of at least three replicated experiments. Statistically significant differences between treated and untreated samples were detected using unpaired t-tests. All analyses were performed using SPSS v.14 (SPSS, Inc., Chicago, IL, USA). A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Comparison of the anti-adipogenic effect of various shikonin compounds. It has previously been reported that *Lithospermum erythrorhizon* contains various shikonin compounds, including shikonin, acetylshikonin, isobutyrylshikonin and β -hydroxy-isovalerylshikonin (β -HIVS) (14). These shikonin compounds have the common basic structure of naphthoquinone, while the attached chemical groups are slightly different (Fig. 1A). To determine which compound exerted the greatest anti-adipogenic effect, the 3T3-L1 cells were treated with the four shikonin compounds at various concentrations for 7 days. Analysis of the effects on intracellular fat content revealed that isobutyrylshikonin and β -HIVS exerted a greater anti-adipogenic effect than shikonin and acetylshikonin (Fig. 1B). However, the intracellular fat content

of 3T3-L1 cells treated with isobutyrylshikonin or β -HIVS for 7 days were similar. Thus, in the subsequent experiment, 3T3-L1 cells were treated with isobutyrylshikonin or β -HIVS on days 0-2, 3-4 and 4-7 of adipogenic differentiation (Fig. 2). The anti-adipogenic effect of β -HIVS was found to be slightly greater than that of isobutyrylshikonin (Fig. 2C and D). These data indicate that β -HIVS exerted the greatest anti-adipogenic effect among the shikonin compounds, and further studies were therefore conducted using β -HIVS.

Anti-adipogenic effect of β -HIVS. In order to determine the most effective concentration of β -HIVS required for anti-adipogenic activity, the 3T3-L1 cells were treated with β -HIVS at various concentrations (0.25, 0.5, 1, 1.5 and 2 μ M) for 7 days. The Oil Red O staining revealed that intracellular fat droplet formation was inhibited by β -HIVS in a dose-dependent manner, and that β -HIVS markedly inhibited fat droplet formation at a concentration of 2 μ M, almost to the control level (Fig. 3A and B). Cytotoxicity was not observed when the 3T3-L1 cells were treated with β -HIVS at concentrations of up to 2 μ M for 7 days, and cell viability was maintained at >90% of the untreated control cells (Fig. 3C), indicating that the inhibitory effects of β -HIVS on fat accumulation were not mediated by cytotoxicity. Further experiments were conducted with β -HIVS at a concentration of 2 μ M.

During adipogenesis of the 3T3-L1 cells, the mRNA expression of the fat-forming enzymes, ACC1, FAS and SCD1, was upregulated, and this effect was significantly reduced by β -HIVS treatment (Fig. 4A-C). The major transcription factors of adipogenesis, PPAR γ and C/EBP α , which are markedly upregulated during adipogenesis and are involved in the transcription of adipocyte marker genes including FABP4, were also significantly reduced by β -HIVS treatment (Fig. 4D-F).

It is noteworthy that ACC1, FAS and SCD1 have been reported to be transcribed by a transcription factor, SREBP-1c. It has also been reported that the mRNA expression of SCD1 is induced by the binding of nuclear SREBP-1c to its promoter (18), and SREBP-1c is the transcription factor that binds to a regulatory element in the enhancer of the FAS gene (19). The level of ACC1 mRNA is also controlled by the binding of SREBP-1c to the ACC1 promoter (19,20). It has

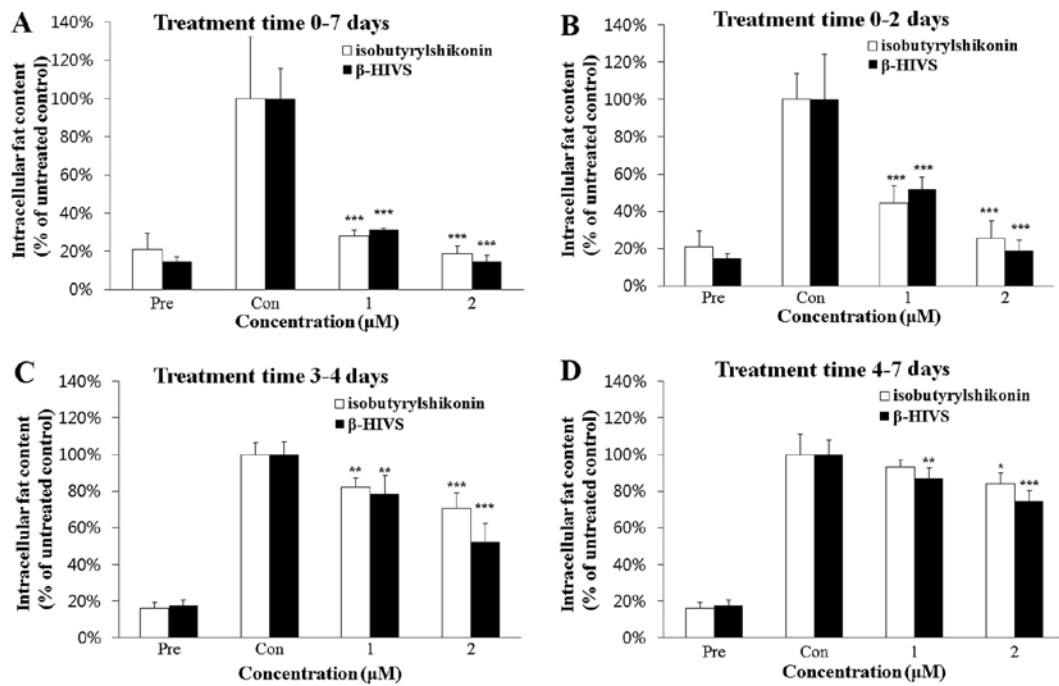


Figure 2. Comparison of the anti-adipogenic activity of isobutyrylshikonin and β -hydroxyisovalerylshikonin (β -HIVS). The 3T3-L1 cells were treated with β -HIVS or isobutyrylshikonin during days (A) 0-7, (B) 0-2, (C) 3-4 and (D) 4-7. Intracellular fat droplets were stained with Oil Red O and extracted for measurement by spectrophotometric analysis at 540 nm on day 7 of 3T3-L1 adipocyte differentiation. * P <0.05, ** P <0.01, *** P <0.001 compared with the untreated adipocytes (Con). Pre denotes undifferentiated preadipocytes.

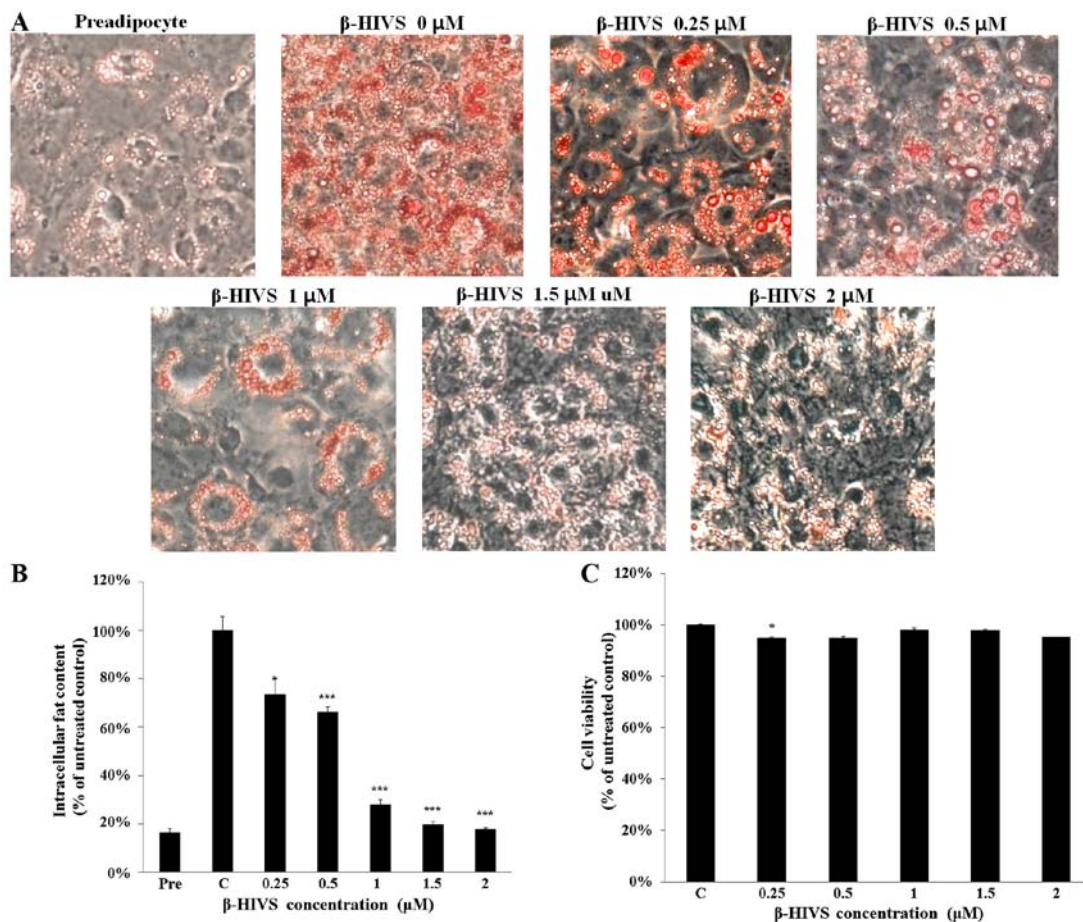


Figure 3. Anti-adipogenic activity and cytotoxicity of β -hydroxyisovalerylshikonin (β -HIVS). (A) Intracellular fat droplets were stained with Oil Red O on day 7 of 3T3-L1 adipocyte differentiation and examined using light microscopy. (B) Stained Oil Red O dye was extracted by isopropyl alcohol and measured by a spectrophotometer at an optical density of 540 nm. (C) Cell viability was measured on day 7 of 3T3-L1 adipocyte differentiation. * P <0.05, *** P <0.001 compared with the untreated adipocytes. Pre denotes undifferentiated preadipocytes.

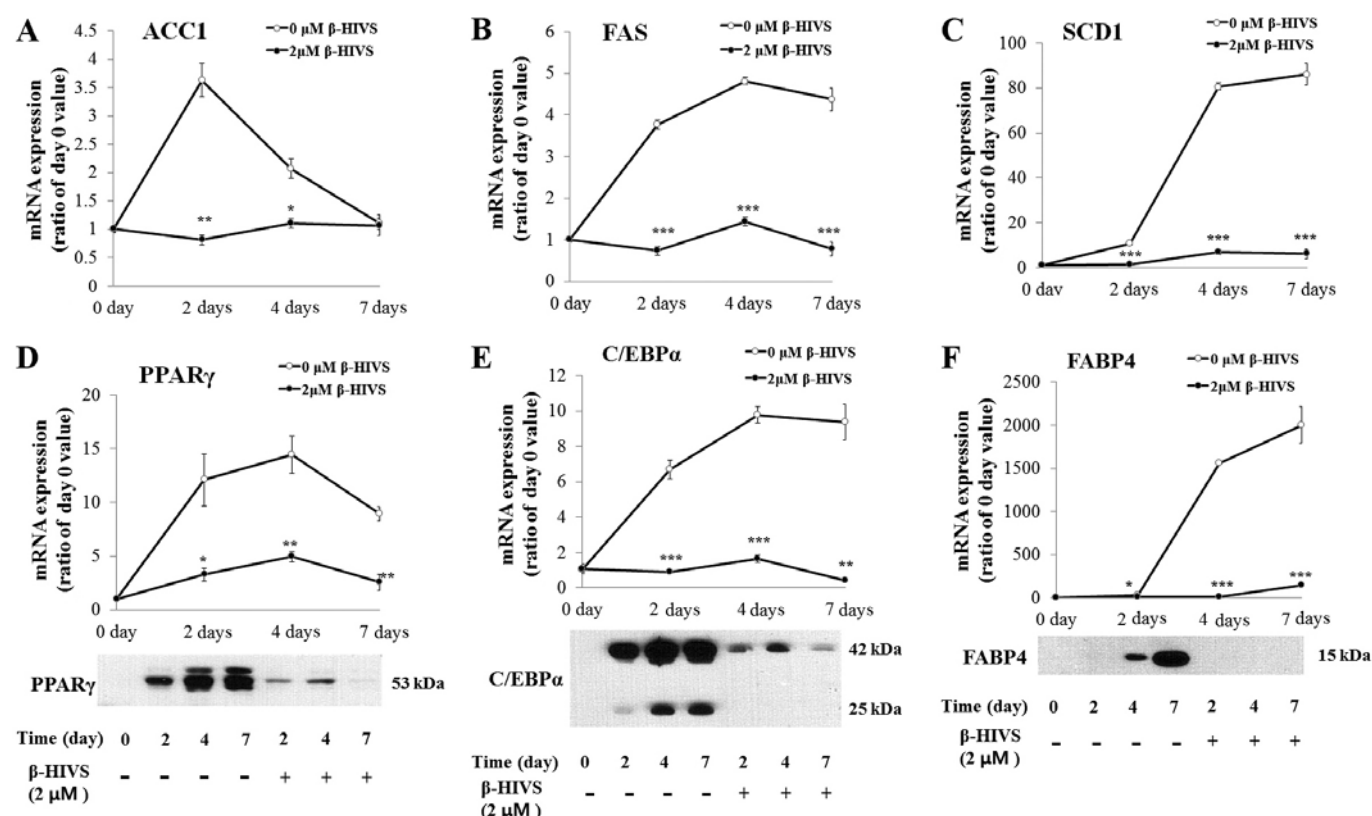


Figure 4. Effects of β -hydroxyisovalerylshikonicin (β -HIVS) on the expression of fat-forming enzymes and adipocyte-specific genes. (A-C) Effects of 2 μ M of β -HIVS on the mRNA expression of fat-forming enzymes, acetyl-CoA carboxylase (ACC)1, fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD)1, in 3T3-L1 cells differentiated for 0, 2, 4 and 7 days. (D-F) Effects of 2 μ M of β -HIVS on the mRNA and protein expression of adipocyte-specific genes such as major adipogenic transcription factors, peroxisome proliferator-activated receptor γ (PPAR γ) and CCAAT/enhancer-binding protein α (C/EBP α), and an adipocyte marker gene, fatty acid-binding protein (FABP)4, in 3T3-L1 cells differentiated for 0, 2, 4 and 7 days. * P <0.05, ** P <0.01, *** P <0.001 compared with the untreated adipocytes on the same differentiation day.

also been reported that SREBP-1c induces the expression of PPAR γ , which collaborates with C/EBP α to transcribe adipocyte marker genes such as FABP4 (21).

Effects of β -HIVS on AMPK activation and SREBP-1c downregulation. AMPK is known to suppress adipogenesis when it is activated by phosphorylation (22). To elucidate the association between β -HIVS and the activation of AMPK, we determined whether β -HIVS induces AMPK phosphorylation during adipogenesis, and it was found that the p-AMPK level was markedly upregulated in the β -HIVS-treated cells compared with the untreated cells. The total AMPK protein level was almost unchanged, demonstrating that β -HIVS induced the phosphorylation of AMPK without affecting its total protein level (Fig. 5A). 5-Aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) is the most well-known activator of AMPK, which activates AMPK by increasing its phosphorylation, and it was thus necessary to compare the AMPK-activating effect of β -HIVS with AICAR (23). The levels of p-AMPK and p-precursor-SREBP-1c, which is one of the products made by p-AMPK, were measured in order to compare the AMPK-activating effect of AICAR and β -HIVS at various concentrations (AICAR: 25, 150, 250 and 500 mM vs. β -HIVS: 0.1, 0.5, 1 and 2 μ M). The results revealed that the levels of p-AMPK and p-precursor-SREBP-1c were increased by AICAR and β -HIVS (Fig. 5B). However, β -HIVS

increased the levels of p-AMPK and p-precursor-SREBP-1c at a much lower concentration, <1/100,000, compared with AICAR, demonstrating that β -HIVS is a much more effective AMPK activator compared with AICAR.

Li *et al* previously reported that phosphorylated/activated AMPK directly phosphorylates precursor SREBP-1c at the Ser372 residue, which prevents the proteolytic maturation of precursor SREBP-1c into mature SREBP-1c and the translocation of mature SREBP-1c into the nucleus (11). When the 3T3-L1 cells were treated with β -HIVS for 6, 12, 24 and 48 h, the level of p-precursor-SREBP-1c was increased similarly to the p-AMPK level. As a result, the level of precursor SREBP-1c (unphosphorylated) and the proteolytic cleavage of precursor SREBP-1c (unphosphorylated) to mature-SREBP-1c was decreased by β -HIVS treatment. Furthermore, the level of nuclear mature SREBP-1c was also decreased, as the phosphorylation of Ser372 prevented nuclear translocation of SREBP-1c (Fig. 5C). When the 3T3-L1 cells were treated with β -HIVS for longer periods of time, of 2, 4 and 7 days, similar patterns of change were observed in the phosphorylation, proteolytic maturation and nuclear translocation of SREBP-1c. The level of precursor SREBP-1c (unphosphorylated) and its proteolytic cleavage into mature SREBP-1c, followed by its nuclear translocation, were reduced by the increase of p-precursor SREBP-1c in the β -HIVS-treated cells compared with the untreated cells (Fig. 5D).

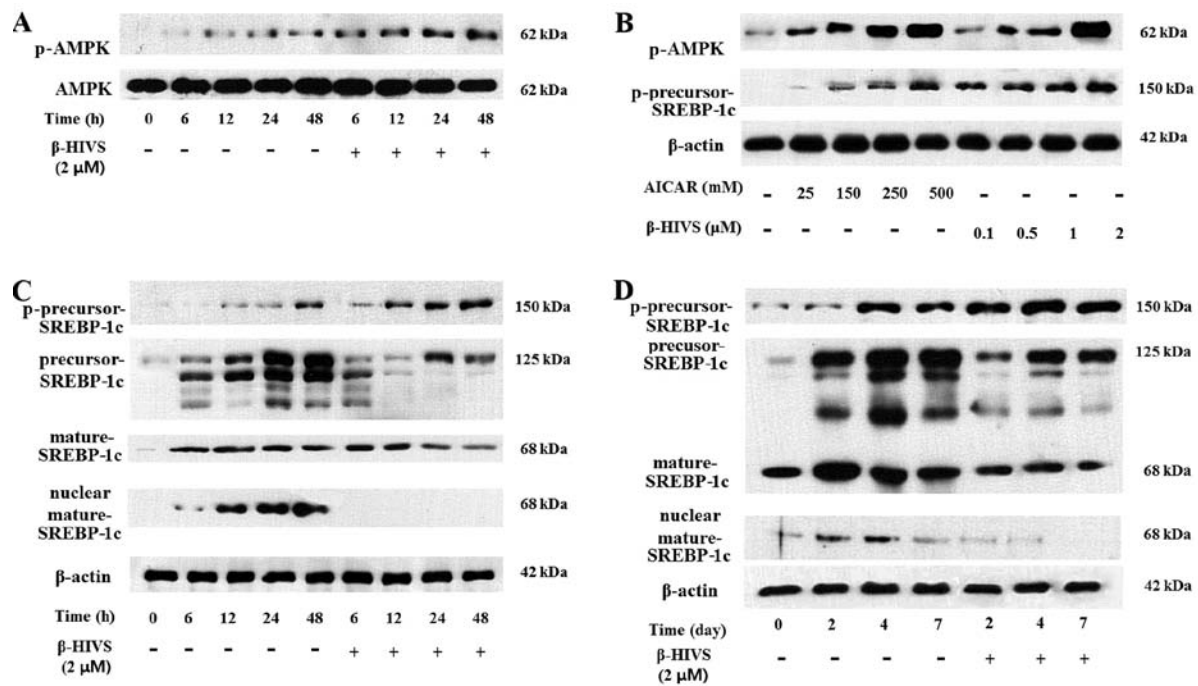


Figure 5. Effects of β -hydroxyisovalerylshikonic acid (β -HIVS) on AMP-activated protein kinase (AMPK) and sterol regulatory element binding protein (SREBP)1c during adipogenesis. (A) Levels of phosphorylated (p-)AMPK and total AMPK in 3T3-L1 cells differentiated for 6, 12, 24 and 48 h in the presence or absence of β -HIVS. (B) Levels of p-AMPK and p-precursor SREBP-1c in 3T3-L1 cells differentiated for 24 h in the presence of various concentrations of 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) and β -HIVS. (C and D) Levels of p-precursor SREBP-1c, unphosphorylated precursor/mature SREBP-1c and nuclear mature SREBP-1c in 3T3-L1 cells differentiated for 6, 12, 24 and 48 h (C) and 2, 4, 7 days (D) in the presence or absence of β -HIVS.

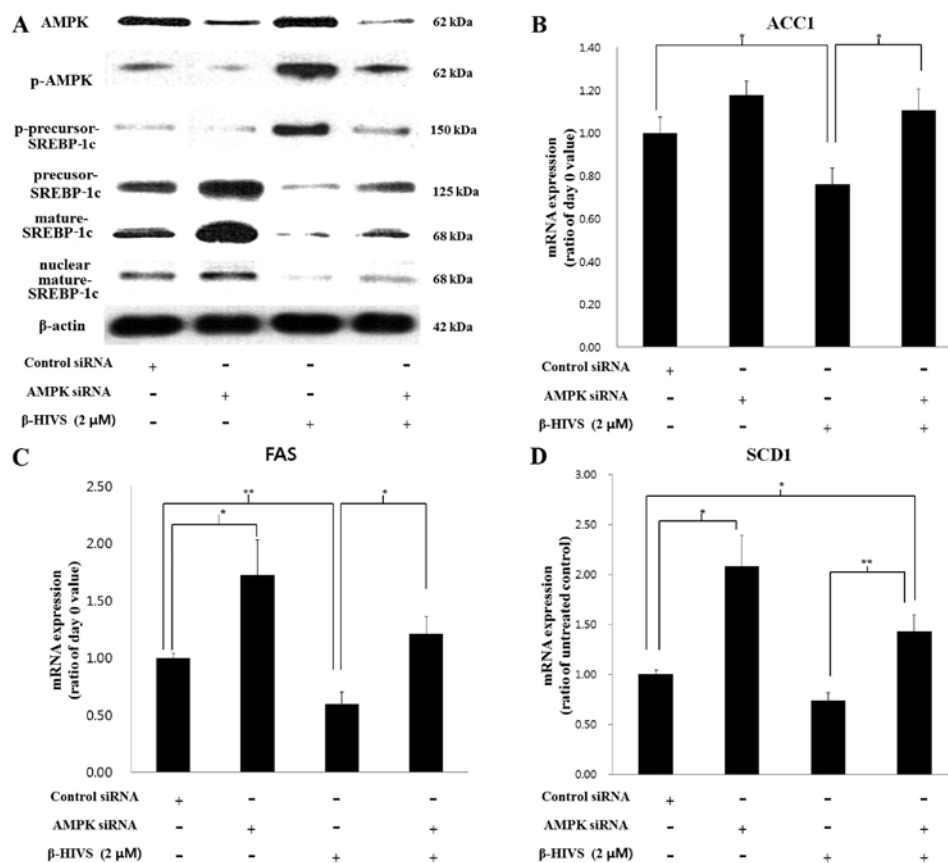


Figure 6. Effects of AMP-activated protein kinase (AMPK) knockdown on sterol regulatory element binding protein (SREBP)1c and the fat-forming enzymes. (A) Effects of AMPK siRNA transfection on the protein levels of AMPK, phosphorylated (p-)AMPK, p-precursor SREBP-1c, unphosphorylated precursor/mature SREBP-1c and nuclear mature SREBP-1c. (B-D) Effects of AMPK siRNA transfection on the mRNA levels of the fat-forming enzymes, acetyl-CoA carboxylase (ACC)1 (B), fatty acid synthase (FAS) (C) and stearoyl-CoA desaturase (SCD)1 (D). Following transfection with the control siRNA or the AMPK siRNA, the 3T3-L1 cells were differentiated for 7 days in the presence or absence of 2 μ M of β -hydroxyisovalerylshikonic acid (β -HIVS). * P <0.05, ** P <0.01.

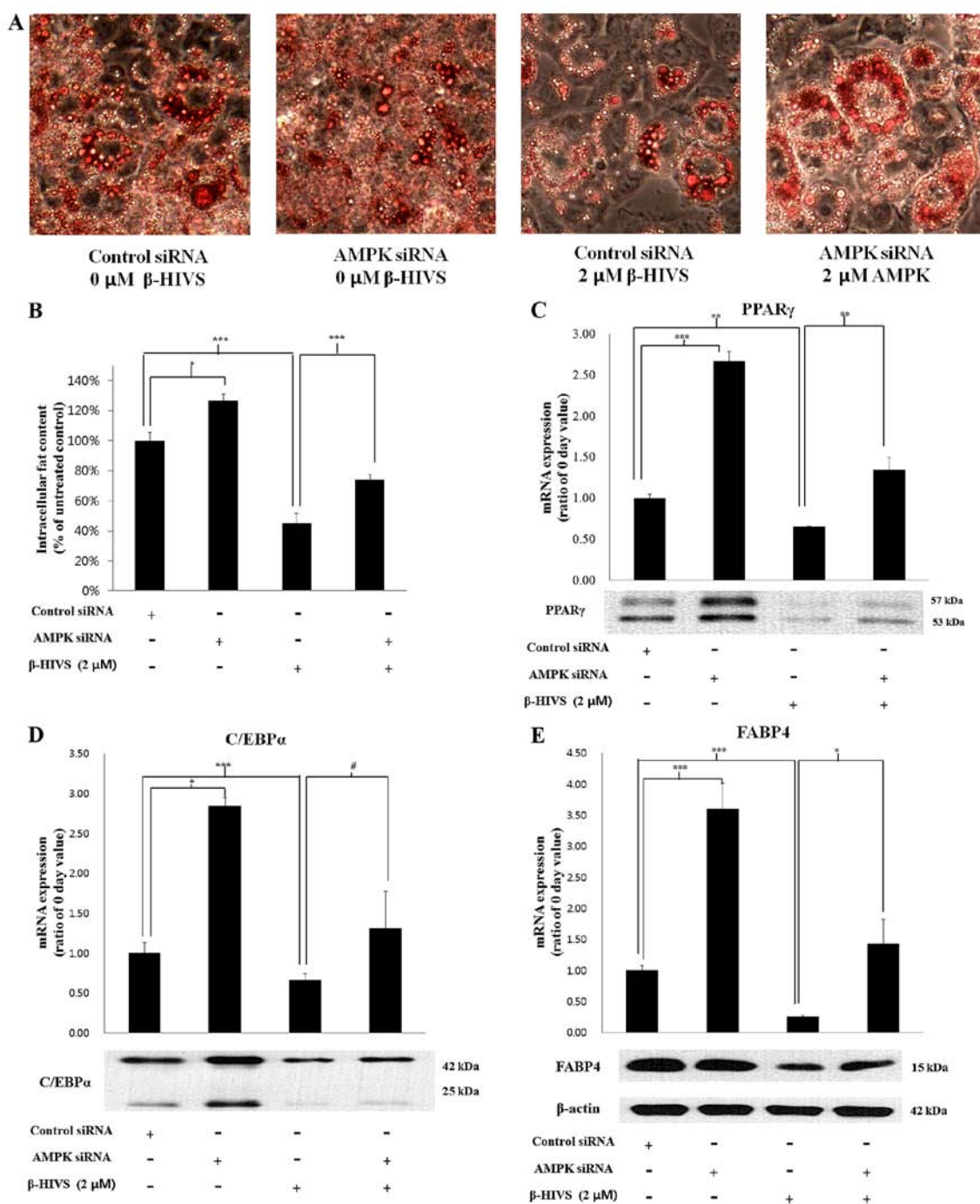


Figure 7. Effects of AMP-activated protein kinase (AMPK) knockdown on intracellular fat accumulation and adipocyte-specific gene expression. (A and B) Effects of AMPK siRNA transfection on intracellular fat accumulation. Intracellular fat droplets were stained with Oil Red O dye and examined by light microscopy (A). Oil Red O dye in the cells was extracted using isopropyl alcohol and measured by spectrophotometry at 540 nm (B). (C-E) Effects of AMPK siRNA transfection on the mRNA and protein levels of adipocyte-specific genes, (C) peroxisome proliferator-activated receptor γ (PPAR γ), (D) CAAT/enhancer-binding protein α (C/EBP α) and (E) fatty acid-binding protein (FABP4). Following transfection with control siRNA or AMPK siRNA, the 3T3-L1 cells were differentiated for 7 days in the presence or absence of β -hydroxyisovalerylshikonin (β -HIVS). * P <0.1, * P <0.05, ** P <0.01, *** P <0.001.

Effects of AMPK knockdown on the anti-adipogenic effect of β -HIVS. To confirm the essential role which AMPK plays in the anti-adipogenic mechanism of β -HIVS, we performed siRNA-mediated knockdown of AMPK in 3T3-L1 cells in the presence or absence of β -HIVS. The siRNA-mediated knockdown of AMPK reduced the levels of AMPK and p-AMPK. We noted that p-precursor-SREBP-1c, which is a product made by p-AMPK, was also reduced by AMPK knockdown. As a result, levels of precursor SREBP-1c (unphosphorylated),

mature SREBP-1c and nuclear mature SREBP-1c were increased by AMPK knockdown (Fig. 6A). Mature SREBP-1c is a transcription factor required for the expression of the fat-forming enzymes ACC1, FAS and SCD1, the levels of which were all significantly increased by AMPK siRNA compared to transfection with control siRNA (Fig. 6B-D). SREBP-1c is also known to induce self mRNA transcription by binding to its own promoter (24,25). In the present study, it was also found that the SREBP-1c mRNA level was increased by

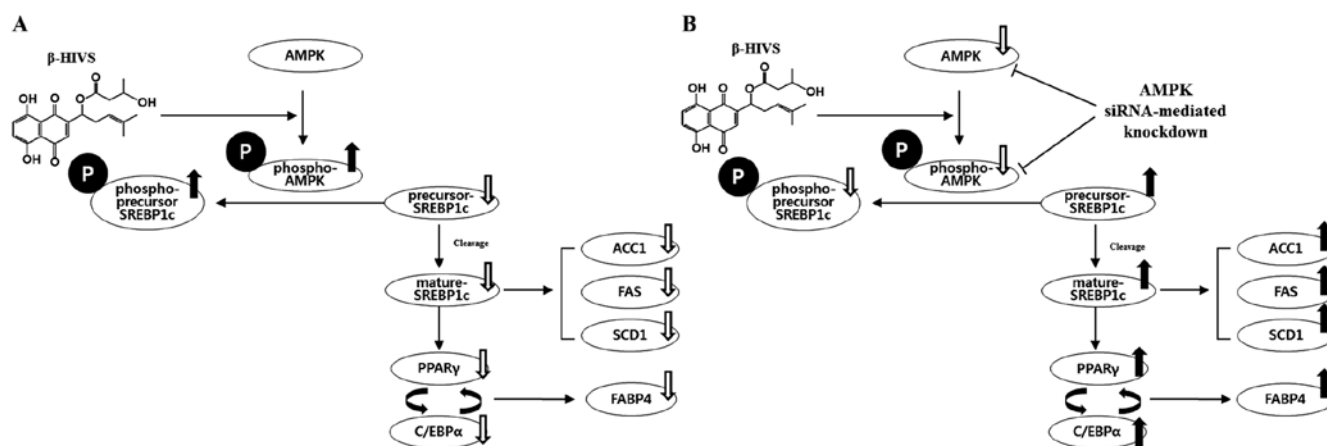


Figure 8. Molecular mechanisms for the anti-adipogenic effect of β -hydroxyisovalerylshikonin (β -HIVS) and effect of AMPK knockdown. (A) β -HIVS activates AMPK by increasing its phosphorylation. Phosphorylated (p-)AMPK directly phosphorylates precursor-sterol regulatory element binding protein (SREBP)-1c into p-precursor SREBP-1c, which cannot be cleaved into mature SREBP-1c, and the level of mature SREBP-1c level decreases. A decreased level of mature SREBP-1c, which is a transcription factor required for the expression of acetyl-CoA carboxylase (ACC)1, fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD)1 and peroxisome proliferator-activated receptor γ (PPAR γ), leads to reduced intracellular fat accumulation. (B) AMPK siRNA reduces the levels of AMPK and p-AMPK, which leads to the reduced phosphorylation of precursor SREBP-1c and an increased level of unphosphorylated precursor SREBP-1c. Unphosphorylated precursor SREBP-1c is cleaved into mature SREBP-1c, which is a transcription factor required for the expression of ACC1, FAS, SCD1 and PPAR γ , leads to increased intracellular fat accumulation.

AMPK knockdown in β -HIVS-treated cells, possibly through the reduction in SREBP-1c phosphorylation and increase in mature SREBP-1c level (data not shown).

We noted that as a result of the effects of AMPK knockdown on the expression of the fat-forming enzymes, intracellular fat accumulation, which had been suppressed by the anti-adipogenic activity of β -HIVS, was significantly recovered by AMPK siRNA compared to transfection with the control siRNA (Fig. 7A and B). AMPK knockdown also reduced the effect of β -HIVS on the expression level of the major adipogenic transcription factors, PPAR γ and C/EBP α , and an adipocyte marker gene, FABP4, transcribed by them. The mRNA and protein levels of PPAR γ , C/EBP α and FABP4, which had been suppressed by β -HIVS, were significantly increased by AMPK knockdown (Fig. 7C-E).

The results of the present study demonstrated that AMPK is involved in the anti-adipogenic mechanism of β -HIVS through the modulation of SREBP-1c phosphorylation, maturation and nuclear translocation as well as the transcription of downstream fat-forming enzymes and adipogenic transcription factors. A possible molecular mechanism for the anti-adipogenic activity of β -HIVS and the effects of AMPK knockdown are described in Fig. 8.

Discussion

A number of natural compounds have the potential to exert anti-obesity effects through the inhibition of adipogenesis (26). As previously noted, β -HIVS is one of the natural shikonin compounds contained in a therapeutic plant, *Lithospermum erythrorhizon* (14). In the present study, modulations of AMPK and SREBP-1c were analyzed in relation to the anti-adipogenic mechanisms of β -HIVS, resulting in novel findings. Firstly, we noted that β -HIVS exerted the greatest anti-adipogenic effect of the four shikonin compounds. Secondly, β -HIVS effectively activated AMPK by increasing its phosphorylation at a much

lower concentration, $<1/100,000$, compared with AICAR, a well-known AMPK activator. Thirdly, β -HIVS increased the phosphorylation of precursor SREBP-1c through the activation of AMPK, which prevented the cleavage of precursor SREBP-1c into mature SREBP-1c and its nuclear translocation. Accordingly, the expression of genes transcribed by mature SREBP-1c, which included fat-forming enzymes and an adipogenic transcription factor, were decreased, resulting in reduced intracellular fat accumulation. Finally, knockdown of AMPK attenuated the anti-adipogenic activity of β -HIVS, including the expression of fat-forming enzymes and an adipogenic transcription factor as well as intracellular fat accumulation, through the modulation of SREBP-1c.

AMPK is a serine/threonine kinase expressed in various tissues such as the skeletal muscle, liver and adipose tissue (27), and it regulates metabolic processes by upregulating catabolism and downregulating anabolism of lipid and carbohydrate (6-8). SREBPs are a family of transcription factors that regulate lipid homeostasis by controlling the expression of enzymes required for the synthesis of endogenous cholesterol, fatty acids, triacylglycerols and phospholipids. Accordingly, SREBPs have been identified as a novel therapeutic target for metabolic diseases such as insulin resistance, type 2 diabetes, fatty liver and atherosclerosis (28). The three SREBP isoforms, SREBP1a, SREBP-1c and SREBP2, play different roles in lipid metabolism. SREBP-1c is involved in fatty acid synthesis and lipogenesis, whereas SREBP2 and SREBP1a are mainly involved in cholesterol synthesis. SREBPs are synthesized as inactive precursors, and upon activation, the precursors undergo proteolytic cleavage to release the mature SREBPs into the nucleus (29). A previous study demonstrated AMPK-SREBP-1c modulation by revealing that activated AMPK directly phosphorylates precursor SREBP-1c at the Ser372 residue, which suppresses the proteolytic cleavage of precursor SREBP-1c into mature SREBP-1c and inhibits its nuclear translocation (11).

Many phytochemicals, including resveratrol, epigallocatechin gallate, berberine, quercetin, rutaecarpine analogues and ursolic acid, have previously been reported to inhibit adipogenesis by activating AMPK but the molecular mechanisms downstream of AMPK have not been fully elucidated (30-32). It is noteworthy that until the present study, AMPK-SREBP-1c modulation has never been suggested as the anti-adipogenic mechanism of any compound which inhibits adipogenesis, to the best of our knowledge. In the present study, the detailed molecular mechanism for the anti-adipogenic activity of β -HIVS, which was found to be an efficient AMPK activator, was elucidated. β -HIVS activated AMPK to increase the phosphorylation of precursor SREBP-1c, which inhibited the formation of mature SREBP-1c, necessary for the transcription of fat-forming enzymes as well as a major transcription factor of adipogenesis.

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