Inhibition of lipid droplet accumulation by *Solanum nigrum* by suppressing adipogenesis and inducing lipolysis, thermogenesis and autophagy in 3T3-L1 cells

JEONG WON CHOI, HYEOK JIN CHOI, GWANG HYEON RYU, JAE WON LEE and JIN BOO JEONG

Department of Forest Science, Andong National University, Andong, Gyeongsangbuk-do 36729, Republic of Korea

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Abstract. It has been reported that *Solanum nigrum* exhibits anti-obesity effects in animal models induced by a high-fat diet. However, research on how *Solanum nigrum* exerts its anti-obesity effects is currently limited. Thus, the present study focused on identifying the mechanism of action associated with the anti-obesity activity of *Solanum nigrum* aerial part (SNAP), which significantly inhibited the accumulation of lipid droplets in differentiating 3T3-L1 cells. Intracellular lipid accumulation in 3T3-L1 cells was analyzed by Oil-Red O staining and glycerol content was analyzed using an ELISA kit. In addition, changes in protein expression within 3T3-L1 cells were analyzed using western blot analysis. It decreased the expression level of adipogenic proteins such as CCAAT/enhancer-binding protein α, Peroxisome proliferator-activated receptor γ, fatty acid binding protein 4, and adiponectin. In addition, SNAP increased the expression levels of lipolytic proteins, such as adipose triglyceride lipase and hormone-sensitive lipase, while decreasing perilipin-1. The treatment of fully differentiated 3T3-L1 cells increased the free glycerol levels. SNAP treatment resulted in increased AMP-activated protein kinase phosphorylation and the expression levels of thermogenic proteins (peroxisome proliferator-activated receptor-γ coactivator 1-α, PR domain containing 16 and uncoupling protein 1) and an autophagic protein (LC3-II). Overall, these results suggested that SNAP inhibited lipid droplet accumulation by suppressing adipogenesis and promoting lipolysis, thermogenesis and autophagy.

Introduction

Abnormal fat accumulation owing to an imbalance between energy intake and consumption causes obesity (1). The rapid increase in the obese population has emerged as a serious problem worldwide because obesity causes serious metabolic diseases, such as diabetes, arthritis, hyperlipidemia, and high blood pressure (1-3). Thus, many drugs have been developed to treat obesity, including orlistat (a lipase inhibitor) and sibutramine (an appetite suppressant) (4). However, the long-term clinical use of these anti-obesity drugs is limited because they cause various side effects, including digestive disorders, diarrhea, and insomnia (5). Therefore, the search for and development of natural anti-obesity agents with few side effects is urgently required (1).

As a medicinal plant belonging to the Solanaceae family, *Solanum nigrum* has been used for the treatment of tuberculosis, diuresis, neurological disorders, ulcers, liver disorders, seizures, and inflammation (6). Numerous studies have shown that *Solanum nigrum* relieves inflammation, protects the liver, lowers blood pressure, and improves glucose tolerance (7-9). In addition, *Solanum nigrum* reportedly reduced the accumulation of hepatic and body fat in mice fed a high-fat diet (10), indicating that it exerts anti-obesity activity. However, since the mechanism of action related to the anti-obesity activity of *Solanum nigrum* has not been elucidated, in this study, we report that *Solanum nigrum* inhibits adipogenesis and activates lipolysis, browning, and autophagy in adipocytes and 3T3-L1 cells.

Materials and methods

Chemicals and antibodies. Dexamethasone (D4902), 3-isobutyl-1-methylxanthine (IBMX) (I5879), insulin (I6634), and Oil Red O (O0625) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibodies against PPARγ (#2430), CEBPα (#8178), FABP4 (#2120), adiponectin (#2789), ATGL (#2138), HSL (#4107), Perilipin-1 (#9349), p-AMPK (#2535), AMPK (#5831), UCP-1 (#14670), LC3 (#2775), β-actin (#5125), anti-rabbit IgG, HRP-linked antibody (#7074) and anti-mouse IgG, HRP-linked antibody (#7076) were purchased from Cell Signaling (Bervely, MA, USA). Antibodies against PGC-1α (sc-518025) and PRDM16 (ab106410) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and Abcam (Cambridge, United Kingdom), respectively.

Sample preparation. Aerial parts and fruit of *Solanum nigrum* were purchased from a local market in Dongdaemun-gu, Seoul. After grinding, 10 g of the powder was immersed in 200 ml of distilled water and left at 80˚C for 6 h for extraction,

Correspondence to: Professor Jin Boo Jeong, Department of Forest Science, Andong National University, 1375 Gyeongdong-ro, Andong, Gyeongsangbuk-do 36729, Republic of Korea
E-mail: jjb0403@anu.ac.kr

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after which the extracts were centrifuged at 15,000 rpm at 4˚C for 10 min, and the clear supernatant was freeze-dried. The extraction yields of SNAP and SNF were 21.35 and 19.26%, respectively. The freeze-dried extracts from aerial parts (SNAP) or fruits (SNF) of *Solanum nigrum* were dissolved in distilled water for treatment of the cells and stored at -80˚C.

Culture and differentiation of 3T3-L1 cells. Mouse adipocytes, 3T3-L1 cells, were used in this study because they are mainly used to verify *in vitro* anti-obesity activity (11). The 3T3-L1 cells (American Type Culture Collection, Manassas, VA, USA, Cat. NO.: CL-173) were maintained in a DMEM/F-12 medium containing 10% bovine calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C under a humidified atmosphere of 5% CO_2. All experiments performed in this study used 3T3-L1 cells at passage numbers 10 or lower. For the differentiation of 3T3-L1 cells, 2 days (D0) after reaching 100% confluence, the cells were cultured in a DMI medium (DMEM/F-12 medium, 10% fetal bovine serum, 1 μM dexamethasone, 0.5 mM IBMX, and 10 μg/ml insulin) for 2 days (D2). Then, the cells were cultured in an insulin medium (DMEM/F-12 medium, 10% fetal bovine serum, and 10 μg/ml insulin) for 2 days (D4). The culture medium (DMEM/F-12 containing 10% fetal bovine serum) was changed every two days (D6-D8).

Measurement of cell viability. The 3T3-L1 cells were further cultured for 2 days in a 6-well plate at full confluence at 37˚C with 5% CO_2. After 2 days, the 3T3-L1 cells were treated with SNAP without DMI and insulin (undifferentiated) or with DMI and insulin (differentiated) and then cultured from D2 to D8. On D8 after SNAP treatment, cell viability was measured using a NucleoCounter NC-250 (Chemometec, Allerod, Denmark) according to the manufacturer's protocol.

Oil Red O staining. The 3T3-L1 cells were fixed in 10% formalin for 1 h at room temperature. They were then dehydrated with 60% isopropanol for 5 min and stained with Oil Red O solution (isopropanol: water=6:4, v/v) for 20 min. After washing the 3T3-L1 cells with distilled water, the stained lipid droplets of 3T3-L1 cells were photographed using a light microscope (Olympus, Tokyo, Japan) and eluted with 100% isopropanol for quantitative analysis. The absorbance of the eluted solution was measured at 500 nm using a microplate reader (Human Co., Xma-3000PC, Seoul, Korea).

Measurement of glycerol content. The 3T3-L1 cells were differentiated for 6 days without SNAP treatment, after which they were treated with SNAP for 48 h, and the free glycerol content was measured using a cell-based glycerol assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. The cell culture medium was mixed with the reconstituted free glycerol assay reagent in a 1:4 ratio and incubated at room temperature for 15 min. After the reaction, absorbance was measured at 540 nm using a microplate reader (Human Cop., Xma-3000PC, Seoul, Korea).

Western blot analysis. Protein changes in SNAP-treated 3T3-L1 cells were analyzed by Western blotting. The cells were collected using RIPA buffer and incubated at 4˚C for 30 min. After centrifugation at 15,000 rpm for 30 min the protein extract was obtained. The total protein extracted from the 3T3-L1 cells was quantified using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). For SDS-PAGE, an equal amount of protein (30 g/well) was subjected to electrophoresis on 12 or 8% acrylamide gels at 150 V and 400 A for 1 h. Proteins separated on the acrylamide gel were transferred onto a nitrocellulose membrane (Thermo Fisher Scientific) for 2 h at 100 and 300 A. After blocking at room temperature for 1 h, the membranes were incubated with the primary antibodies (1:1,000) at 4˚C overnight. The membranes were then incubated with secondary antibodies (1:1,000) at room temperature for 1 h. After treating the membrane with ECL Western blotting substrate, the protein bands were visualized using an LI-COR C-DiGit Blot Scanner (LI-COR, NE, USA). Quantitative analysis of the visualized protein bands was performed using UN-SCAN-IT gel software version 5.1 (Silk Scientific Inc. Orem, UT, USA).

Statistical analysis. All experiments were repeated at least three times. Statistical analyses were verified using GraphPad Prism version 5.0 (GraphPad Software, Inc.) and data are presented as mean ± standard deviation. Results were considered statistically significant at P-<0.05. Each data point was analyzed using a one-way analysis of variance (ANOVA), and the data were analyzed using the Bonferroni post hoc test. During ANOVA analysis, a normality test (Shapiro-Wilk Test) was performed.

Results

The inhibitory effect of SNAP on lipid droplet accumulation in 3T3-L1 cells is higher than that of SNF. Because obesity is closely related to the accumulation of lipid droplets within adipocytes (12), we investigated whether SNAP and SNF inhibit the accumulation of lipid droplets in 3T3-L1 cells. In previous studies (10), treatment of 3T3-L1 cells with polyphenol extract from aerial parts of *Solanum nigrum* resulted in a rapid decrease in cell viability at concentrations higher than 100 μg/ml. Therefore, in this study, a concentration of 100 μg/ml was designated as a high concentration, and a concentration of 50 μg/ml was designated as a low concentration. SNAP and SNF were administered from the beginning (D0) to the end (D8) of 3T3-L1 cell differentiation, and their inhibitory activity on lipid droplet accumulation was measured using Oil Red O staining (Fig. 1A). As shown in Fig. 1B, SNAP effectively reduced lipid droplet accumulation compared to that in the untreated control group (CON), but SNF did not show such an effect. Because this result demonstrates that only SNAP has anti-obesity activity, we used SNAP in subsequent studies. Furthermore, SNAP inhibited lipid droplet accumulation in a concentration-dependent manner (Fig. 1C). Evaluation of the effect of SNAP on cell viability in undifferentiated or differentiated 3T3-L1 cells showed that SNAP had no effect on cell viability in undifferentiated 3T3-L1 cells, but slightly decreased cell viability in differentiated 3T3-L1 cells (Fig. 1D).

SNAP inhibits adipogenesis in 3T3-L1 cells. To verify whether the inhibition of adipogenesis by SNAP was involved in the reduction in lipid droplet accumulation, we treated cells with SNAP from D0 to D8 (during all phases of the differentiation process) or from D0 to D2 (at the early phase of the differentiation process) and investigated the extent of lipid droplet accumulation using Oil Red O staining (Fig. 2A). As shown in Fig. 2B, SNAP treatment from D0 to D8 or

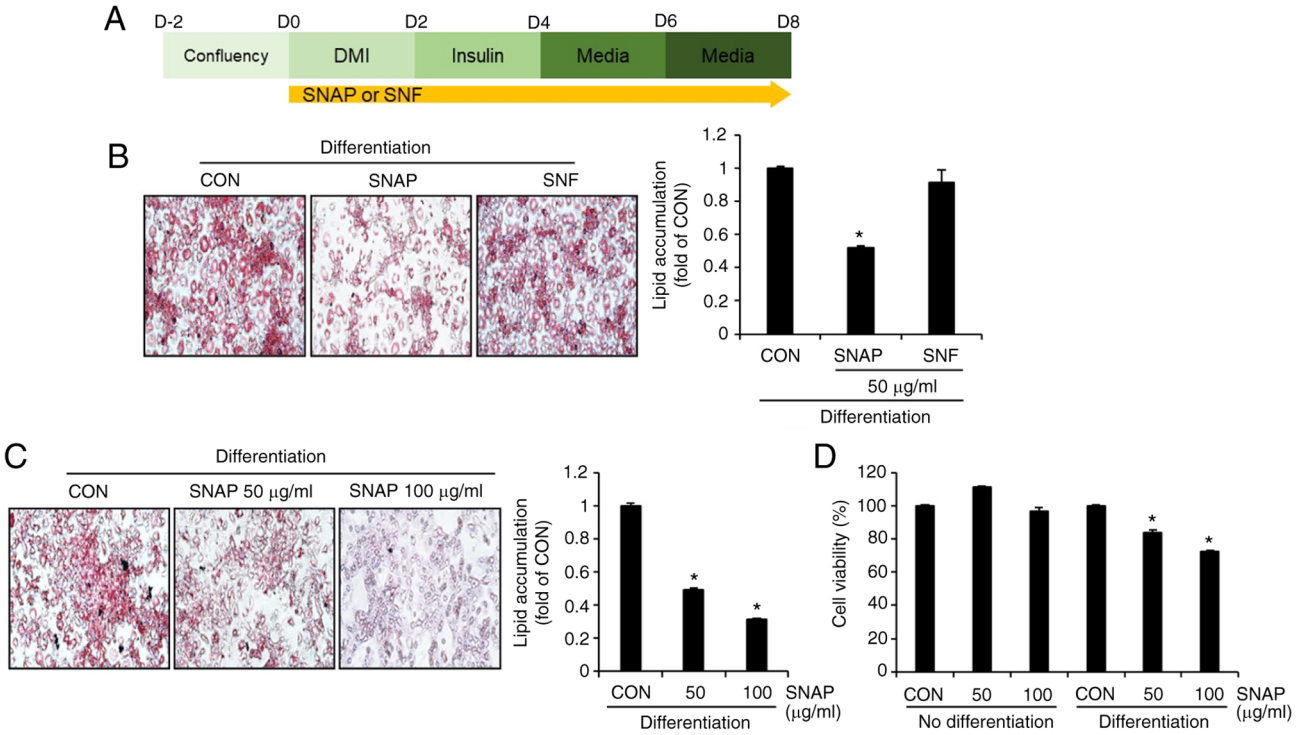

Figure 1. Effect of SNAP and SNF on the accumulation of lipid droplets in 3T3-L1 cells. (A) Experimental design. (B) ORO staining in SNAP and SNF-treated 3T3-L1 cells. (C) ORO staining in SNAP-treated 3T3-L1 cells. (D) Cell viability in SNAP-treated 3T3-L1 cells under the no differentiation or differentiation. *P<0.05 vs. CON group. CON, control group without the sample treatment; DMI, mixture of 0.05 mM IBMX, 1 μM dexamethasone and 10 μg/ml insulin; SNAP, *Solanum nigrum* aerial part extracts; SNF, *Solanum nigrum* fruit extracts.

from D0 to D2 resulted in a significant reduction in lipid droplet accumulation compared to the untreated control group (CON). Thus, we analyzed the expression changes of adipogenesis-related proteins such as PPARγ, CEBPα, FABP4, and adiponectin by SNAP in 3T3-L1 cells treated with SNAP from D0 to D8 using Western blot analysis (Fig. 2C). As shown in Fig. 2D, SNAP dose-dependently downregulated the expression levels of PPARγ, CEBPα, FABP4, and adiponectin. We also investigated the effects of SNAP on the expression of adipogenesis-related proteins during the early stages of differentiation. In 3T3-L1 cells treated with SNAP from D0 to D2 (Fig. 2E), SNAP significantly decreased protein expression levels of PPARγ and CEBPα (Fig. 2F).

SNAP induces lipolysis in 3T3-L1 cells. To investigate whether lipolysis is involved in SNAP-mediated inhibition of lipid droplet accumulation, 3T3-L1 cells undergoing differentiation were treated with SNAP from D0 to D8, and changes in the protein expression of ATGL, HSL, and perilipin-1, which are involved in lipolysis, were measured by Western blot analysis (Fig. 3A). As shown in Fig. 3B, the protein expression of ATGL and HSL increased, whereas that of perilipin-1 decreased in SNAP-treated 3T3-L1 cells compared to untreated cells (CON). To further demonstrate whether SNAP induces lipolysis, 3T3-L1 cells were allowed to accumulate lipid droplets from D0 to D6, followed by treatment with SNAP from D6 to D8 (Fig. 3C). Changes in lipid droplet accumulation, free glycerol content, and protein expression of ATGL, HSL, and perilipin-1 were investigated. SNAP treatment from D6 to D8 led to a decrease in lipid droplet accumulation (Fig. 3D) and an increase in free glycerol content (Fig. 3E) in 3T3-L1 cells compared to the untreated cells (CON). In addition, the protein expression of ATGL and HSL was upregulated, whereas that of perilipin-1 was downregulated in SNAP-treated 3T3-L1 cells compared to untreated cells (CON).

SNAP induces thermogenesis and autophagy in 3T3-L1 cells. To verify whether thermogenesis and autophagy contribute to the SNAP-mediated inhibition of lipid droplet accumulation, 3T3-L1 cells undergoing differentiation were treated with SNAP from D0 to D8 (Fig. 4A), and the changes in the expression of the thermogenesis-related proteins (p-AMPK, AMPK, UCP-1, PGC-1α, and PRDM16) and the autophagy-related protein (LC3-II) were measured by Western blot analysis. As shown in Fig. 4B, treatment with SNAP did not affect the protein expression of AMPK but effectively phosphorylated AMPK and significantly increased the protein expression of UCP-1, PGC-1α, and PRDM16 in 3T3-L1 cells. Furthermore, the protein expression of LC3-II was significantly increased in 3T3-L1 cells treated with SNAP (Fig. 4C).

Discussion

The pursuit of a comfortable lifestyle and unhealthy eating habits leads to obesity, a global health problem (13). Obesity occurs when there is excessive accumulation of fat in adipose tissue (13). White adipose tissue (WAT) is known to increase because of excessive lipid accumulation within adipocytes, which is a major characteristic of obesity (14). Adipogenesis, the process by which preadipocytes differentiate into mature adipocytes, increases the number of mature adipocytes in WAT (15-17). Therefore, agents that inhibit adipogenesis can be developed as anti-obesity agents (14). PPARγ, CEBPα, FABP4, and adiponectin are major factors that promote

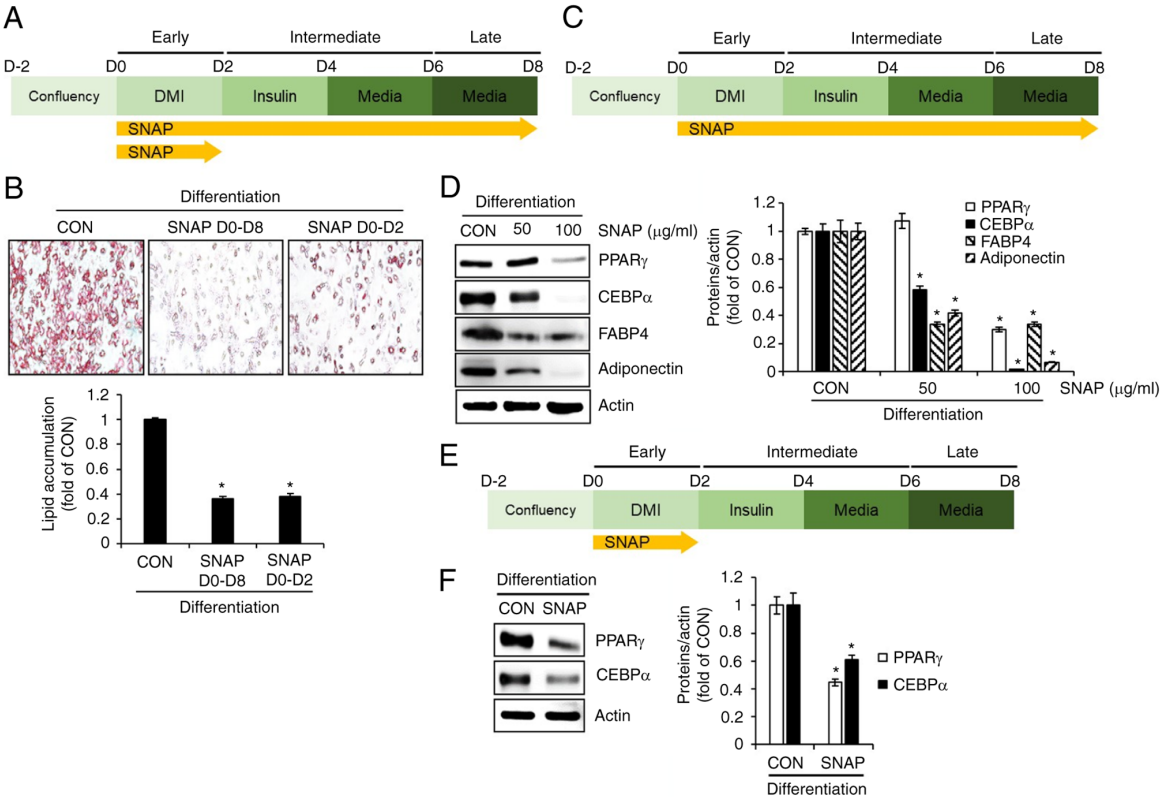

Figure 2. Inhibitory effect of SNAP against the adipogenesis in 3T3-L1 cells. (A) Experimental design. (B) ORO staining in 3T3-L1 cells treated with SNAP (100 μg/ml) from D0 to D8 or from D0 to D2. (C) Experimental design. (D) Western blot analysis in 3T3-L1 cells treated with SNAP from D0 to D8. (E) Experimental design. (F) Western blot analysis in 3T3-L1 cells treated with SNAP from D0 to D2. *P<0.05 vs. CON group. CON, control group without the sample treatment; DMI, mixture of 0.05 mM IBMX, 1 μM dexamethasone and 10 μg/ml insulin; SNAP, *Solanum nigrum* aerial part extracts; CEBPα, CCAAT/enhancer-binding protein alpha; FABP4, fatty acid binding protein 4; PPARγ, peroxisome proliferator-activated receptor gamma.

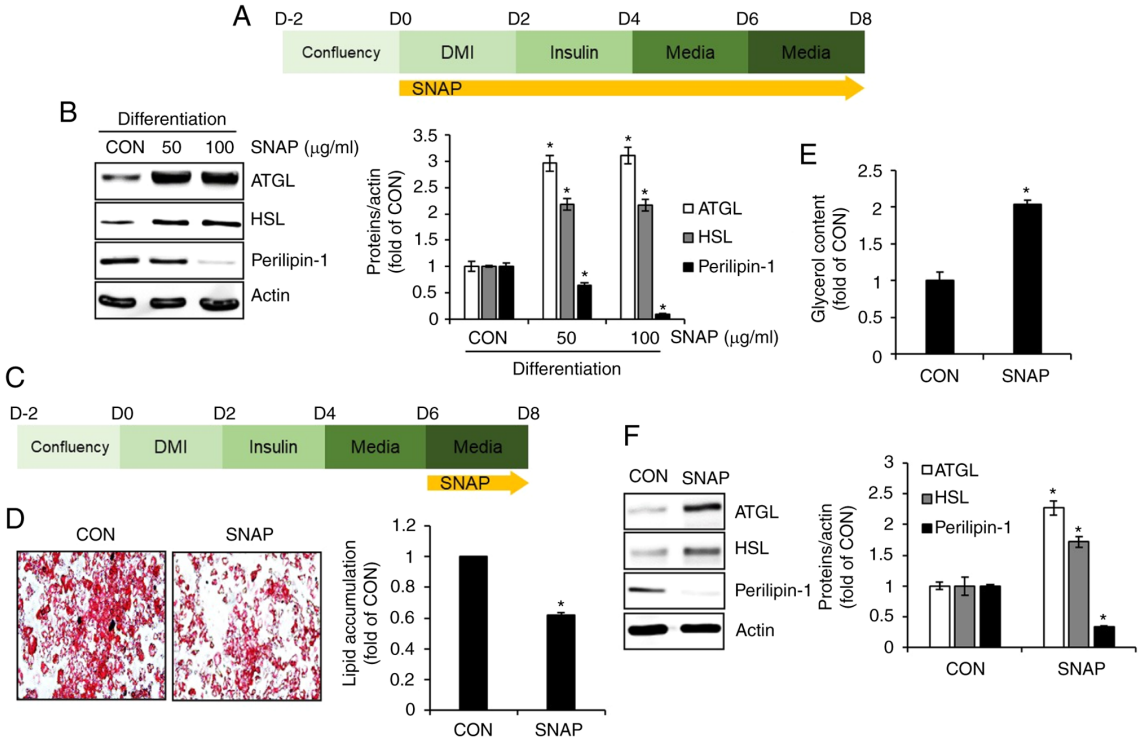

Figure 3. Effect of SNAP on lipolysis in 3T3-L1 cells. (A) Experimental design. (B) Western blot analysis in 3T3-L1 cells treated with SNAP from D0 to D8. (C) Experimental design (D) ORO staining in 3T3-L1 cells treated with SNAP (100 μg/ml) from D6 to D8. (E) Analysis of free glycerol in 3T3-L1 cells treated with SNAP (100 μg/ml) from D6 to D8. (F) Western blot analysis in 3T3-L1 cells treated with SNAP (100 μg/ml) from D6 to D8. *P<0.05 vs. CON group. CON, control group without the sample treatment; DMI, mixture of 0.05 mM IBMX, 1 μM dexamethasone and 10 μg/ml insulin; SNAP, *Solanum nigrum* aerial part extracts; ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase.

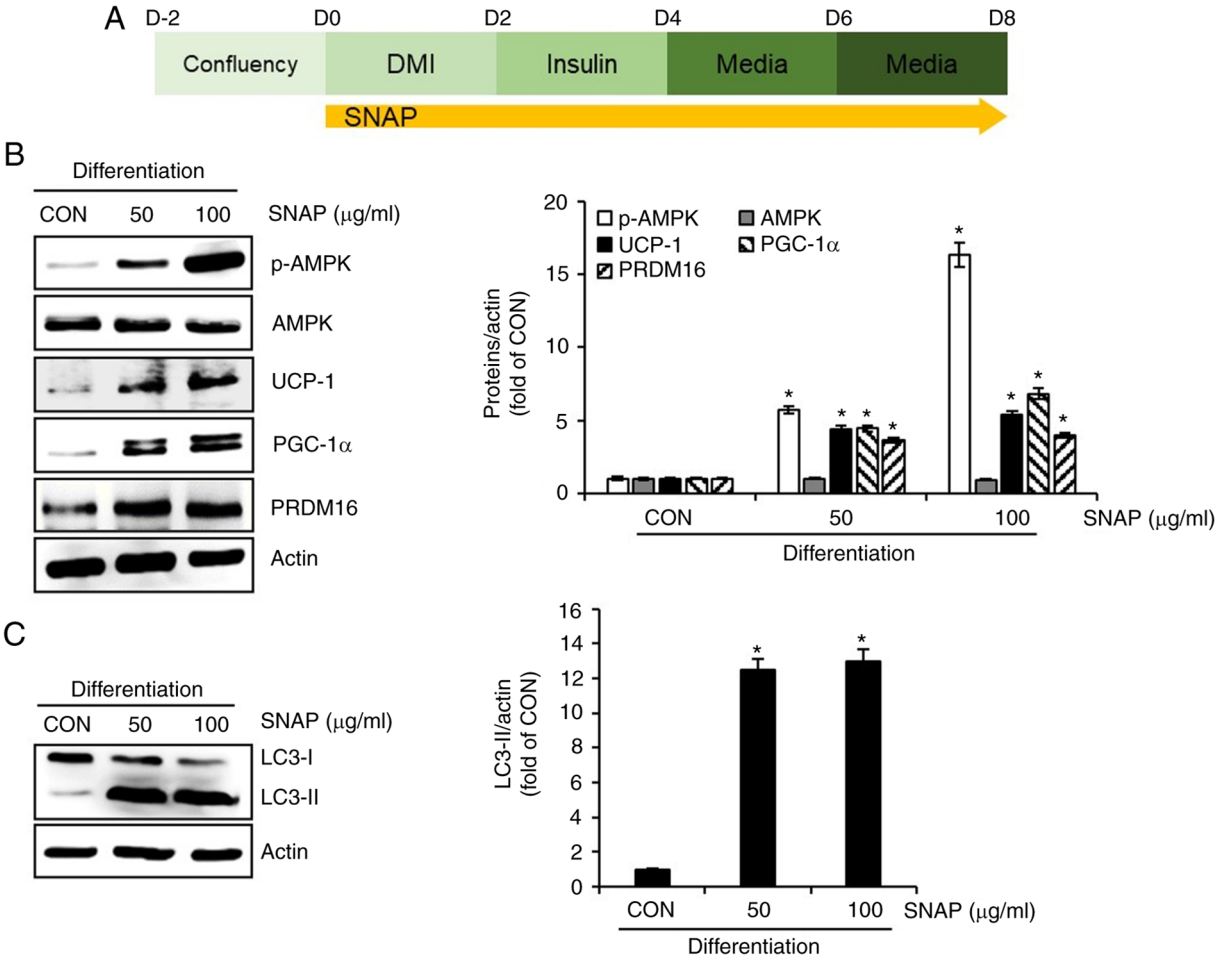

Figure 4. Effect of SNAP on thermogenesis and autophagy in 3T3-L1 cells. (A) Experimental design. (B) Western blot analysis in 3T3-L1 cells treated with SNAP from D0 to D8. (C) Western blot analysis of LC3-I and -II in 3T3-L1 cells treated with SNAP from D0 to D8. *P<0.05 vs. CON group. CON, control group without the sample treatment; DMI, mixture of 0.05 mM IBMX, 1 μM dexamethasone and 10 μg/ml insulin; SNAP, *Solanum nigrum* aerial part extracts; p, phosphorylated; AMPK, AMP-activated protein kinase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; PRDM16, PR domain-containing 16; UCP-1, uncoupling protein 1; LC3, microtubule-associated protein 1A/1B-light chain 3.

adipogenesis in adipocytes (18-22). In this study, we demonstrated that SNAP downregulates the protein expressions of PPARγ, CEBPα, FABP4, and adiponectin in differentiating 3T3-L1 cells. Downregulation of PPARγ and CEBPα expression inhibits weight gain by reducing adipogenesis in an obese mouse model (18,19). FABP4 is overexpressed during adipogenesis and autonomously accounts for 6% of total cytoplasmic proteins in mature adipocytes (20). FABP4 overexpression promotes the progression of obesity, obesity-induced type 2 diabetes, hypertension, and cardiovascular diseases (21). Adiponectin promotes adipogenesis and lipid accumulation in adipocytes (22). While it is a limitation of this study that we did not analyze the expression of other proteins involved in adipogenesis beyond PPARγ, CEBPα, FABP4, and adiponectin, considering the previous reports (18-22) and our results, we believe that SNAP may reduce lipid droplet accumulation by inhibiting adipogenesis.

Lipolysis is the process by which triacylglycerol (TAG) in the adipose tissue sequentially decomposes into diacylglycerol (DAG), monoacylglycerol (MAG), fatty acids (FA), and glycerol (23,24). As a decrease in lipolysis is frequently observed in obesity, reducing stored fat by stimulating hydrolysis through lipolysis is considered a major target for obesity treatment (25). In the present study, we present two pieces of evidence that SNAP induces lipolysis in 3T3-L1 cells. The first piece of evidence suggests that SNAP increases the protein expression of ATGL and HSL and decreases the protein expression of perilipin-1 in 3T3-L1 cells. ATGL and HSL are involved in lipid breakdown during lipolysis. Specifically, ATGL breaks down TAG into DAG and FA, whereas HSL breaks down TAG into DAG and further into MAG (24). Perilipin-1 surrounding lipid droplets in adipocytes inhibits lipolysis by limiting the access of ATGL and HSL to lipid droplets (23). These reports provided the first evidence that SNAP induce lipolysis. Secondly, the amount of free glycerol increased in SNAP-treated 3T3-L1 cells. Because TAG are ultimately hydrolyzed via lipolysis into three molecules of free FA and one molecule of free glycerol (23,24), the increase in free glycerol in SNAP-treated 3T3-L1 cells indicated that lipolysis was induced. These two pieces of evidence demonstrated that SNAP inhibited the accumulation of lipid droplets by inducing lipolysis in adipocytes.

Brown adipose tissue (BAT) generates heat by dissipating excess energy stored in the form of lipids in WAT, a phenomenon referred to as thermogenesis (26). Therefore, as the dissipation of excess energy in the form of heat is effective for weight loss, an increase in BAT is highly effective in

combating obesity (27). In this study, we propose that SNAP induces thermogenesis by presenting evidence that SNAP increases the phosphorylation of AMPK and protein expression of UCP-1, PGC-1α, and PRDM16. AMPK and PRDM16 activate BAT through WAT browning (26,28), and UCP-1 and PGC-1α predominantly expressed in BAT are involved in the dissipation of excess energy as heat (29,30). These previous reports support the evidence for SNAP-mediated thermogenesis presented in this study. Therefore, we propose that thermogenesis may be involved in SNAP-mediated inhibition of lipid droplet accumulation in adipocytes.

Recently, continuous activation of autophagy was reported to inhibit adipocyte maturation via a lipolytic mechanism (31). In adipocytes, autophagy degrades lipid droplets in the cytoplasm and reduces triacylglycerol stored in the lipid droplets (32). In the present study, we observed a significant increase in LC3-II protein expression in SNAP-treated 3T3-L1 cells. Because LC3-II is closely associated with the formation of autophagosomes and autolysosomes, it is commonly used as a key indicator of autophagy induction (33). Furthermore, it has been reported that the increase in ATGL and decrease in perilipin-1 promote autophagic lipolysis (34,35). Based on these previous reports, it has been suggested that SNAP can induce autophagy in adipocytes and that SNAP-induced autophagic lipolysis may contribute to the inhibition of lipid droplet accumulation.

Because this study was conducted *in vitro* to elucidate the anti-obesity mechanisms of SNAP, the lack of *in vivo* studies is a limitation. However, it has recently been reported that *Solanum nigrum* exhibit effective anti-obesity activity in an animal model of HFD-induced obesity (10). Moreover, they reported that *Solanum nigrum* induces lipolysis through the upregulation of PPARα and CPT-1 and inhibits lipogenesis through the downregulation of FAS and HMG-CoR (10). They also confirmed an increase in AMPK phosphorylation in the livers of mice treated with *Solanum nigrum* (10). Although there were differences in the anti-obesity-related proteins analyzed between previous *in vivo* studies and our *in vitro* study, a previous *in vivo* study suggested that SNAP may also exhibit effective anti-obesity activity in an *in vivo* model. Therefore, it is necessary to confirm whether SNAP exhibits anti-obesity activity in an *in vivo* model and whether the changes in anti-obesity-related proteins analyzed in this study are also observed *in vivo*. Furthermore, while previous studies showed that polyphenol extracts from *Solanum nigrum* (SNPE) inhibited lipid accumulation at concentrations of 300-500 μg/ml, this study demonstrated noteworthy inhibition of lipid accumulation at concentrations as low as 50-100 μg/ml of SNAP. Such differences could be attributed to the differences in the extraction solvents, as SNPE is an ethanol extract and SNAP is a water extract. Additionally, differences in growing environments could also be a factor, as SNPE was derived from *Solanum nigrum* grown in Taiwan, whereas SNAP was derived from *Solanum nigrum* grown in Korea.

Although an accurate analysis of the anti-obesity-related components of SNAP is crucial, this study has a limitation in that we could not analyze the specific components of SNAP that inhibit adipogenesis and induce lipolysis, thermogenesis, and autophagy. However, when *Solanum nigrum* was extracted with distilled water, various components such as miquelianin, catechin, rutin, gallic acid, isoquercetin, peltatoside, nuciferine, quercetin, and epigallocatechin gallate were detected (10). Among the various compounds detected in water extracts of *Solanum nigrum*, catechin (36), rutin (37), nuciferine (38), quercetin (39), and epigallocatechin gallate (40) have been reported to exhibit anti-obesity activity. Although the precise ingredients involved in the anti-obesity activity of SNAP have not been identified, based on preliminary reports (36-40), it can be inferred that catechin, rutin, nuciferine, quercetin, and epigallocatechin gallate are involved in the anti-obesity activity of SNAP. However, it is necessary to precisely analyze the anti-obesity ingredients in SNAP using activity-tracing-based ingredient analysis.

In this study, we observed that SNAP exhibited higher inhibitory activity against lipid accumulation in 3T3-L1 cells than SNF at the same concentration. Although this study did not conduct a quantitative analysis of the specific components responsible for inhibiting lipid accumulation in SNAP and SNF, the results suggest that SNAP may contain more components that inhibit lipid accumulation than SNF. Therefore, it is necessary to compare the components that inhibit lipid accumulation in SNAP and SNF in future studies to overcome the limitations of this study.

In this study, we demonstrated that SNAP suppresses the excessive accumulation of lipid droplets by inhibiting adipogenesis and inducing lipolysis, thermogenesis, and autophagy in 3T3-L1 cells. Although the anti-obesity activity of SNAP has already been verified, the novelty of this study lies in its newly identified mechanism of action, which provides new evidence for its anti-obesity activity.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JWC, HJC, GHR and JWL performed the experiments and analyzed the data. JWC and HJC drafted the manuscript. GHR and JWL edited the manuscript. JBJ designed the experiments and wrote and edited the manuscript. JWC, HJC, GHR, JWL and JBJ confirm the authenticity of all the raw data. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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