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In vitro anti-obesity effects of sesamol mediated by adenosine monophosphate-activated protein kinase and mitogen-activated protein kinase signaling in 3T3-L1 cells

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Abstract Sesamol is a phenol derivative of sesame oil and a potent anti-oxidant, anti-inflammatory, anti-hepatotoxic, and anti-aging compound. We investigated the effects of sesamol on the molecular mechanisms of adipogenesis in 3T3-L1 preadipocytes. The intracellular lipid accumulation accompanied by increased extracellular release of free glycerol was decreased during differentiation on treating 3T3-L1 with sesamol. Sesamol treatment on 3T3-L1 inhibited adipogenic differentiation by down-regulating adipogenesis-related factors (C/EBPα, PPARγ, and SREBP-1). Lipid accumulation was repressed by decreasing fatty acid synthase and by up-regulating lipolysis-response genes (HSL and LPL). The molecular mechanisms of sesamol-induced inhibition in adipogenesis were mediated by increased levels of phosphorylated adenosine monophosphate-activated protein kinase and its substrate acetyl-CoA carboxylase. Sesamol treatment, in turn, modulated the different members of the mitogen-activated protein kinase family by suppressing phosphorylation of ERK 1/2 and JNK and by increasing the phosphorylation of p38. In summary, sesamol inhibits adipogenic differentiation by reducing phosphorylation levels of ERK 1/2 and JNK while inducing lipolysis by activating p38 and AMPK. Our results demonstrate that the molecular mechanisms of *in vitro* anti-obesity effects of sesamol are due to the combined effects of preventing both lipid accumulation and adipogenesis.

Keywords: sesamol, adipogenesis, anti-obesity, AMPK, lipolysis, MAPK

Introduction

Adipocytes play an important role in the maintenance of metabolic balance by secreting lipid hormones, adipokines, and various transcription factors; (1) however, the over-expansion of adipocytes leads to obesity, thereby causing a variety of pathological disorders including hypertension, type II diabetes mellitus, heart disease, cancer, and osteoarthritis (2-6). Due to the harmful effects of obesity, therapeutic approaches using natural products have been studied for preventing obesity and the diseases caused by obesity (7). Natural compounds for the treatment of obesity have been discovered with a focus on the mechanisms involved in restoring the balance between energy intake and expenditure, stimulating adipocyte apoptosis, inhibiting preadipocyte differentiation and lipogenesis, and increasing lipolysis (8). Therefore, considerable effort has been made to discover natural products and bioactive compounds that can prevent obesity, with a specific focus on the mechanisms of adipocyte proliferation and differentiation (9). Recent studies showed that several natural bioactive compounds found in ginseng, coptidis, and melon may play roles in the prevention of obesity (10).

Adipocyte differentiation is strongly related to obesity. The 3T3-L1 preadipocytes, which can differentiate into mature adipocytes, are a well-characterized *in vitro* model for studying adipocyte differentiation (11,12). The peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs) are the major regulators during lipid-laden adipocyte differentiation from preadipocytes (13). In order to induce adipogenesis, 3T3-L1 preadipocytes are treated with a hormonal cocktail MDI containing 3-isobuthyl-1-methylxanthine, dexamethasone, and insulin to activate C/EBPβ and C/EBPδ, which, in turn, up-regulate gene expressions of C/EBPα and PPARγ (14). Adenosine monophosphate-activated protein kinase (AMPK) is an αβγ-heterotrimeric enzyme complex, which acts as a sensor of cellular energy status by monitoring the AMP to ATP ratio (15). The mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinases (ERKs), jun aminoterminal kinase (JNK), and p38 mitogen-activated protein kinases (p38), play different

roles in adipogenic differentiation. ERKs promote adipocyte differentiation through up-regulating major transcription factors, including C/EBPs and PPARγ, whereas suppressing ERK activity in 3T3-L1 inhibits adipocyte differentiation (16,17). JNK increases adipogenesis, thereby inducing obesity in the mouse model when activated (18). Unlike ERKs and JNK, p38 may play a negative role in adipocyte differentiation, during which its activity is decreased (19).

Sesame products derived from *Sesamum indicum* are traditionally used as health foods. It has been known that sesame oil inhibits lipogenesis and adipocytes *in vivo* (20). There are two major active constituents of sesame oil, sesamol and sesamolin. Sesamol (5-hyroxy-1,3-benzodioxole) is one of the active metabolites of sesamolin. Previous studies showed that sesamol acts as a metabolic regulator and possesses hepatoprotective, cardioprotective, antiatherogenic, chemopreventive, radical scavenging, anti-aging, and hypolipidemic effects (21,22). It was also demonstrated that sesamol reduces both diabetic states and neuropathic pain in rats by attenuating oxidative–nitrosative stress and inflammation (23). A recent study showed that sesamol can control hyperlipidemia and ischemic heart disease in a mouse model (22). However, a detailed anti-obesity sesamol signaling pathway has not yet been clearly elucidated. In this study, we show that sesamol treatments modulate a major adipogenic induced-signaling pathway, thereby reducing adipogenic differentiation in 3T3-L1 preadipocytes.

Materials and Methods

Materials The 3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Sesamol, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), insulin, Oil Red O, and Compound C were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Welgene (Daegu, Korea), and fetal bovine serum (FBS), penicillin, and streptomycin were purchased from PAA Laboratories (Cölbe, Germany). Antibodies against PPARγ, FAS, SREBP-1, pAMPKs, pACC, ERK, pERK, β-actin, and HRP-conjugated anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against p38 MAPK, p-p38 MAPK, JNK, and pJNK were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture and induction of adipogenic differentiation The 3T3-L1 preadipocytes were grown in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2. To induce adipogenic differentiation, cells were plated at a density to reach 80–90% confluence (~5×10^4 cells/well) in 3 days, during which the medium was switched to MDI (DMEM containing 10% FBS, 0.5 mM IBMX, 1 μM dexamethasone, and 10 μg/mL of insulin). After 3 days, dexamethasone and IBMX were removed and the cells were maintained in the medium containing insulin for an additional 4 days. To determine the effects of sesamol on adipocyte differentiation, various concentrations (0, 50, 100, and 150 μM) of sesamol were added for adipocyte induction. After 7 days, when the differentiation of control cells was completed, the cells were subjected to further analysis or harvested to prepare cell-free extracts.

Measurement of cell viability To evaluate the general cytotoxicity of sesamol on 3T3-L1 cells, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] assay was conducted. The 3T3-L1 preadipocytes and adipocytes cultured with DMEM medium in 96-well plates were treated with sesamol (0, 50, 100, and 150 μM) for 24 h and then MTT reagent (Sigma-Aldrich) was added. Absorbance measurement at 570 nm was conducted using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Oil Red O staining Cells were gently washed twice with ice-cold phosphate buffered saline (PBS), fixed with 10% formalin (Sigma-Aldrich) for 30 min at room temperature, and then stained with 60% Oil Red O (Sigma-Aldrich) solution in isopropanol for 10 min. After the staining solution was removed, the cells were rinsed with 70% isopropanol (Sigma-Aldrich) and PBS buffer. Images of stained lipid droplets were captured by a phase-contrast microscope (Leica, Wetzlar, Germany). Quantification was performed by dissolving the dye in 100% isopropanol and the absorbance was measured at 490 nm using a microplate reader.

Measurement of intracellular triglyceride content and free glycerol release Adipocytes differentiated for 7 days under the treatment with sesamol at concentrations of 0, 1, 50, 100, and 150 μM were harvested and homogenized in a 5% NP-40 (Sigma-Aldrich) buffer. Cell homogenates were then incubated at 85°C for 5 min and cooled to room temperature. Cellular triglyceride content was measured using the Triglyceride Quantification Kit (Biovision, Palo Alto, CA, USA) according to the manufacturer's protocols. To measure free glycerol released from the cells, the culture medium was collected and subjected to measure free glycerol using the Free Glycerol Assay Kit (Biovision) following the manufacturer's instructions. In both the assays, spectroscopic quantification was performed by measuring the absorbance at 570 nm with a microplate reader.

Western blot analysis Total cell lysates were prepared in RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.25% sodium deoxycholate) (Biosolution, Seoul, Korea) containing a protease inhibitor cocktail and phosphatase inhibitor cocktail I (Sigma-Aldrich). Total cell proteins (30 μg) were separated by 10% SDS-polyacrylamide (Bio-Rad Laboratories, Hercules, CA, USA) gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA), and then hybridized with their respective primary antibodies. The membrane was incubated with secondary antibodies and the immunoreactive proteins bound to antibodies were detected with ECL Plus western blotting detection

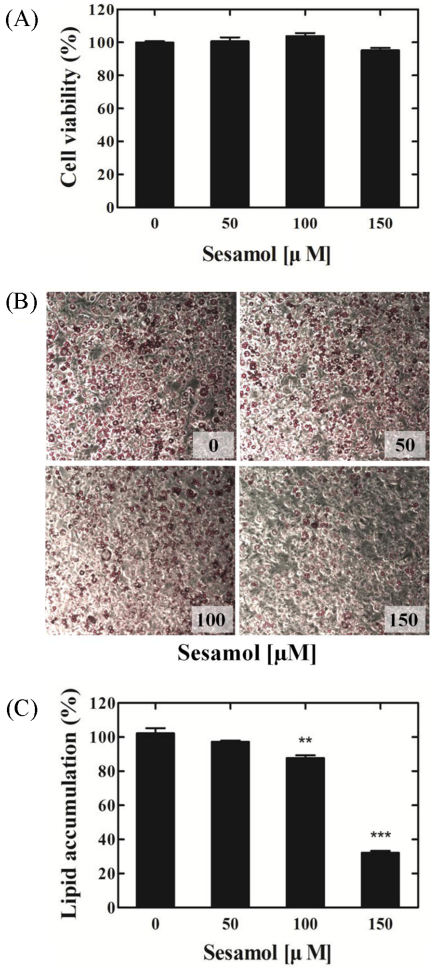

Fig. 1. Effects of sesamol on adipocyte differentiation. (A) Cell viability of 3T3-L1 preadipocyte. 3T3-L1 cells were treated with sesamol at various concentrations. (B) MDI differentiation medium was treated with various doses of sesamol. Cells were stained with Oil Red O after 7 days. (C) Stained oil droplets were dissolved in 100% isopropanol and the absorbance was measured at 490 nm. All values are significantly different (p<0.05) compared with control by Tukey's multiple comparison test.

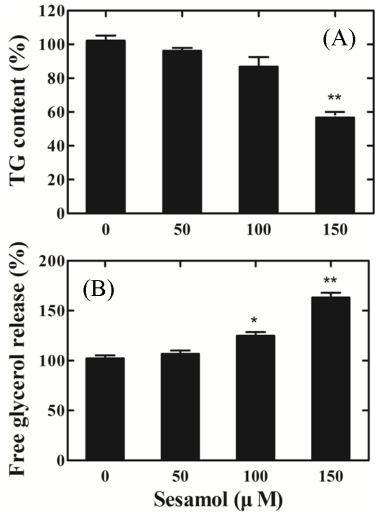

Fig. 2. Effects of sesamol on lipid accumulation and lipolysis activity in 3T3-L1 adipocytes. Cells were incubated at MDI differentiation medium with various doses of sesamol. (A) Lipid accumulation was measured by triglyceride assay. (B) The lipolysis activity of sesamol was determined using a free glycerol assay. Absorbance was measured by a plate reader at 570 nm. All values are significantly different (*p<0.05, **p<0.005) compared with control by Tukey's multiple comparison test.

reagents (Amersham Bioscience, Buckinghamshire, UK). Images were acquired using Bio-Rad ChemiDoc XRS (Bio-Rad Laboratories) and quantified by Quantity One Image Software (Bio-Rad Laboratories).

Quantitative real-time PCR analysis Total RNA was extracted from the cells using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA using the Promega Reverse Transcription System kit (Promega, Madison, WI, USA) following the manufacturer's protocol. The amplification of specific target genes from cDNA was performed in a PCR assay reaction with 2.0 mM $MgCl_2$, 0.5 μM of each forward and reverse primer, and 1×SYBR Green PCR Master Mix (Kapa Biosystems, Woburn, MA, USA). The sequences of PCR primers were as follows: HSL (forward: 5-tgagattgaggtgctgtc-3; reverse: 5-gaggtgagatggtaactgt-3), LPL (forward: 5-agaatcgctgtaacaatctg-3; reverse: 5-tgaatcttgacttggtaatgg-3), and β-actin (forward: 5-cgttgaca tccgtaaagac-3; reverse: 5-gagccagagcagtaatct-3) (Cosmogenetech, Busan, Korea). The following cycling conditions were employed: denaturing at 95°C for 3 min, followed by 40 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. β-Actin was amplified in parallel as an internal control. The relative RNA levels were analyzed by the changes in fluorescence using the CFX Connect™ real-time PCR detection system (Bio-Rad Laboratories).

Statistical analysis Experiments were conducted in triplicate and repeated at least three times. All values are expressed as means± SEM. One-way ANOVA using Tukey's multiple comparison analysis was performed to determine the significant differences among groups. Statistical significance was considered as p<0.05.

Results and Discussion

In this study, we investigated the *in vitro* anti-obesity effects of sesamol by examining the lipid accumulation and the activation levels of key adipogenic regulators during adipogenic differentiation in 3T3-L1 cells. 3T3-L1 cells were induced differentiation with various concentrations of sesamol and their differentiation was evaluated by Oil Red O staining. The results showed that sesamol is not toxic up to 150 μM (Fig. 1A), whereas the differentiation of preadipocytes to mature lipid-storing adipocytes was suppressed by sesamol in a dose-dependent manner (Fig. 1B and 1C). Triglyceride (TG) formation in the cells is another marker for mature adipocytes (24). Therefore,

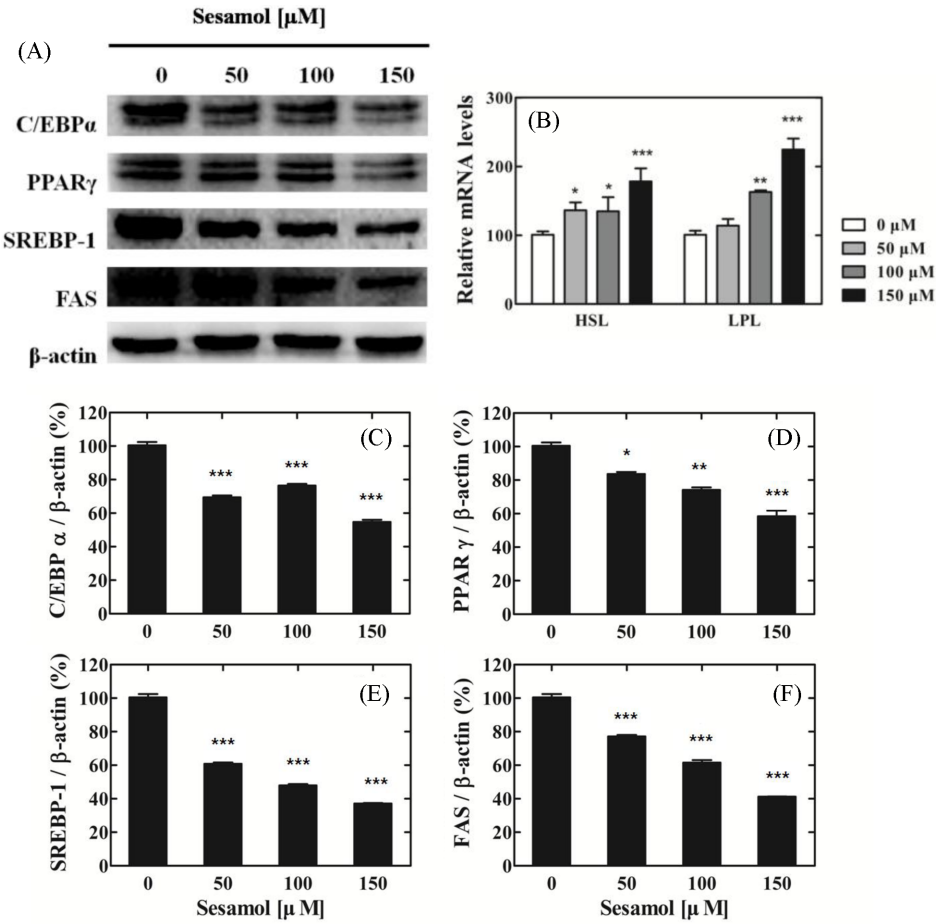

Fig. 3. Effects of sesamol on the expression of adipocyte specific factors and lipolysis related genes. Adipocytes were incubated for 7 days with sesamol. (A) Cell lysates were prepared on day 7 and subjected to Western blot analysis to detect the expression of adipogenic transcriptional factors and enzyme. (B) mRNA levels involved in lipolysis were examined by qRT-PCR analysis. All values are significantly different (*p<0.05, **p<0.005, ***p<0.001) compared with control by Tukey's multiple comparison test. (C-D) Protein expression level in (A) was quantitatively analyzed.

we examined whether the inhibitory effects of sesamol on lipid accumulation is associated with TG metabolic processes. Sesamol treatment during differentiation resulted in the reduction of intracellular contents of TG in a dose-dependent manner (Fig. 2A). In order to determine whether the reduced amount of TG is the result of lipolysis that releases free glycerol into the medium, extracellular levels of free glycerol were measured. The data revealed that sesamol treatment increased the amount of free glycerol release into the medium, indicating that sesamol activates lipolysis (Fig. 2B). These results suggest that treatment of 3T3-L1 preadipocytes with sesamol significantly retarded adipocyte differentiation and lipid accumulation in parallel with an increase in extracellular free glycerol release (Fig. 1 and 2). Several phytochemicals are known to inhibit adipogenesis by inducing apoptotic pathways (25). However, it is unlikely that sesamol mediates apoptosis of adipocytes because sesamol treatment was not cytotoxic to 3T3-L1 preadipocytes and adipocytes at the concentration we used in this experiment (Fig. 1 and data not shown). Thus, attenuation of lipid accumulation by sesamol implies that sesamol may inhibit adipogenic differentiation.

The promoters of numerous adipose tissue-specific genes are transactivated by C/EBPα through the association with PPARγ at shared binding sites that leads to synergistic activation of adipogenesis (26). We found that both the transcription factors were significantly down-regulated in the cells treated with 150 μM sesamol (C/EBPα 1.85-fold and PPARγ 1.75-fold) compared with untreated controls (Fig. 3A). Sesamol treatment also reduced the protein level of SREBP-1, which is a stimulator for lipogenic gene expression, in a dose-dependent manner (Fig. 3A). Accordingly, the expression of fatty acid synthase (FAS), one of the major SREBP-1 regulating enzymes, was significantly inhibited by sesamol (Fig. 3A). Sesamol down-regulates such major adipogenic transcription factors, leading to the suppression of adipocyte differentiation.

Adipogenesis was well known to be regulated by the various signaling pathways, including Wnt, PI3K, and MAPK (16,27,28), whereas the activation of MAPK has been implicated in the major mechanism of adipocyte differentiation (29-31). Previous studies demonstrated that the ERK signaling pathway participates in various cellular processes, including cell cycle progression, cell survival,

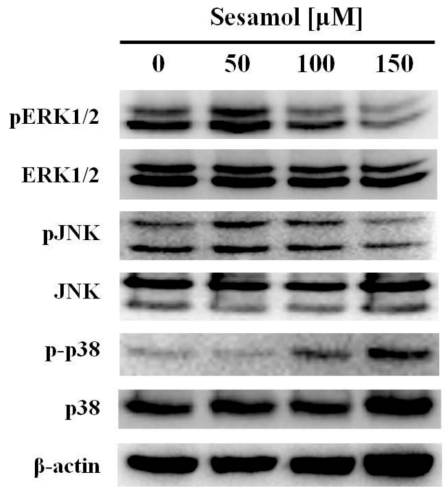

Fig. 4. Effects of sesamol on MAPK pathway. The 3T3-L1 preadipocytes were differentiated in MDI induction media with various doses of sesamol (0, 50, 100, and 150 μM). Cell lysates were prepared on day 7 and subjected to Western blot analysis to detect the expression of p-ERK, ERK, p-JNK, JNK, p-p38, and p38.

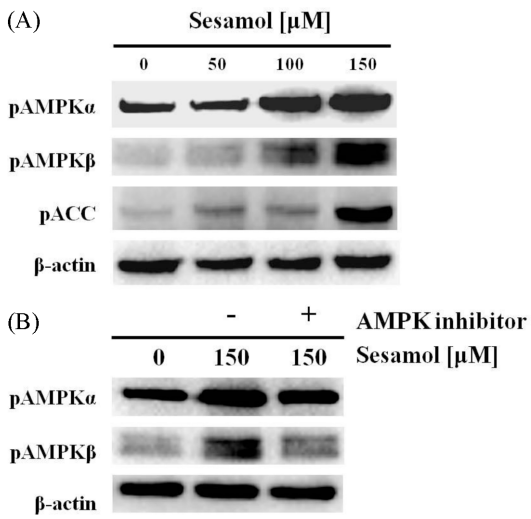

Fig. 5. Effects of sesamol on AMPK pathway. The 3T3-L1 preadipocytes were differentiated in MDI induction media with various doses of sesamol (0, 50, 100, and 150 μM). (A) Cell lysates were prepared on day 7 and subjected to Western blot analysis to detect the expression of pAMPKα, pAMPKβ, and pACC. (B) Cells were pretreated with 20 μM of AMPK inhibitor compound C for 30 min and exposed to sesamol.

differentiation, metabolism, and proliferation (32,33). In addition, ERK signaling pathway is one of the upstream signaling pathways of PPARγ, which plays an important role in adipogenic differentiation (16,32,33). JNK, a member of the MAPK family, regulates the transcriptional activity of PPARγ and both ligand-dependent and ligand-independent transactivation throughout adipocyte differentiation (27). In the case of p38, another member of the MAPK family, inhibition of its activity increase adipogenesis in a previous study (34). We therefore examined the role of MAPK pathway in sesamol-mediated suppression of adipocyte differentiation. Adipogenic induction was conducted in 3T3-L1 preadipocytes with sesamol in an MDI differentiation medium, and the activation levels of ERK1/2, JNK, and p38 were determined by Western blot analysis (Fig. 4). When compared with untreated controls, the levels of phosphorylation in ERK1/2 versus total ERK1/2 protein were significantly decreased by sesamol treatment. Moreover, the activation of JNK was inhibited by sesamol, whereas p38 phosphorylation was prominently increased by sesamol treatment. As expected, sesamol significantly down-regulated the activation of ERK and JNK in 3T3-L1 cells. These results suggest that sesamol regulates protein expression of the major adipogenic transcription factors, PPARγ and cEBPα, through modulating the activation of ERK and JNK MAPK pathways. Moreover, the expression levels of another member of the MAPK family (p38), which can inhibit adipocyte differentiation (35), increased with sesamol treatment. The data suggest that sesamol efficiently modulates adipogenic differentiation through the MAPK signaling pathway.

Protein expression levels of FAS and SREBP-1, which are implicated in fatty acid synthesis, were suppressed by sesamol treatment (Fig. 3A). These decreases may be due to the activation of AMPK pathway (Fig. 5), which has an inhibitory effect on TG synthesis and a stimulating effect on fatty acid oxidation (36). The activation of AMPK pathway inhibits the transcription of SREBP-1, leading to a decrease in the expression of triglyceride synthesis-related genes such as FAS. In adipocytes, phosphorylated AMPK inhibits the activation of acetyl CoA carboxylase (ACC), which converts acetyl CoA into malonyl CoA. When the level of malonyl CoA is decreased, long-chain fatty acyl-CoA can readily enter into the mitochondria and increase the level of fatty acid oxidation (37). Phosphorylated AMPK also inhibits adipocyte differentiation in 3T3-L1 via down-regulating the expression of PPARγ and C/EBPβ (38). Therefore, the activation of AMPKs mediates the reduction of the risk of obesity and insulin resistance via the stimulation of fatty acid oxidation in the mitochondria by increasing the import of fatty acid (39). Our data confirmed that the levels of phosphorylated AMPK and ACC were increased, which, in turn, suggest a decrease in fatty acid levels (Fig. 5). Moreover, the levels of SREBP-1 and FAS were down-regulated by sesamol treatment in a dose-dependent manner (Fig. 3A), suggesting that sesamol may stimulate lipolysis in 3T3-L1 adipocytes via AMPK signaling pathways. Lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL) metabolize TG into two free fatty acids and glycerol in the adipose cells (40). In order to examine whether the sesamol-mediated lipolysis is associated with HSL and LPL, the gene expression levels were investigated by quantitative real time RT-PCR (Fig. 3B). We showed that the sesamol treatment up-regulated the HSL and LPL expression in a dose-dependent manner (Fig. 3B). These results revealed that sesamol increased lipolysis not only by activating the AMPK pathway, which is associated with the lipolysis metabolic pathway, but also by regulating the expression RNA levels of lipolysis-related enzymes such as LPL and HSL.

In summary, our data suggest that sesamol suppresses adipogenesis through modulating MAPK pathway and stimulates lipolysis in mature adipocytes via up-regulating the AMPK signaling pathway. Moreover, the down-regulation of the major transcription factors involved in TG synthesis decreased TG accumulation. These findings provide an insight into the role of sesamol in molecular mechanisms underlying fat cell differentiation and lipolysis and suggest that sesamol may be used as a food ingredient to help control obesity. Most of these studies await further evaluation with validated clinical treatment of obesity with sesamol and identification of the key factors involved in the anti-adipogenesis.

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Disclosure The authors declare no conflict of interest.

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