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Title: Kahweol Inhibits Lipid Accumulation and Induces Glucose-Uptake through Activation of AMP-Activated Protein Kinase (AMPK)

Article Type: Article

Keywords: coffee beans; kahweol; adipogenesis; AMPK; glucose uptake

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Running Title: Kahweol Activates AMPK

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ABSTRACT

Weight loss ≥5% is sufficient to significantly reduce health risks for people with obesity; therefore, development of novel weight loss compounds with reduced toxicity is urgently required. After screening of natural compounds with anti-adipogenesis properties in 3T3-L1 cells, we determined that kahweol, a coffee-specific diterpene, inhibited adipogenesis. Kahweol reduced lipid accumulation and expression levels of adipogenesis and lipid accumulation-related factors. Levels of Phosphorylated AKT and phosphorylated JAK2, which induce lipid accumulation, decreased in kahweol-treated cells. Notably, kahweol treatment significantly increased AMP-activated protein kinase (AMPK) activation. We showed that depletion of AMPK alleviated the reduction in lipid accumulation resulting from kahweol treatment, suggesting that inhibition of lipid accumulation by kahweol is dependent on AMPK activation. Moreover, we detected a more rapid reduction in blood glucose levels in mice administrated kahweol than in control mice. Therefore, we suggest that kahweol has anti-obesity effects and should be studied further for possible therapeutic application.

INTRODUCTION

More than one-third of adults in the United States (US) have obesity, a disease characterized by a body mass index (over 30 kg/m^2. Obesity increases the risk of mortality and morbidity, because it is accompanied by type 2 diabetes, heart disease, stroke, hypertension, respiratory problems, and several types of cancer [1]. Therefore, pharmacotherapeutic drugs have been developed and used for treating obesity. Initially, the thyroid hormone was selected as a therapeutic target, and Amphetamine became popular for weight-loss in the late 1930s given its ability to suppress appetite [2]. Sibutramine was approved for long-term use in November 1997, and Orlistat, a gastric and pancreatic lipase inhibitor that reduces dietary fat absorption, was approved by the FDA for long-term use in 1999. According to the results of several meta-analyses from the 2000s, anti-obesity drugs demonstrated effectiveness in facilitating moderate weight reduction. The latest approvals were for the naltrexone-bupropion combination and liraglutide in 2014 [2].

However, safety concerns for anti-obesity drugs have emerged, and some drugs have been withdrawn from the market. For example, fenfluramine and dexfenfluramine were found to be associated with heart valve disease, and sibutramine also provoked severe cardiovascular events [3]. Moreover, even with current drugs on the market, such as orlistat, lorcaserin, naltrexone-bupropion, phentermine-topiramate, and liraglutide, treatment decisions are driven by coexisting medical conditions [4]. For example, liraglutide may be a more appropriate agent in people with diabetes because of its glucose-lowering effects. Conversely, the use of naltrexone-bupropion in patients with chronic opiate or alcohol dependence is associated with neuropsychiatric complications [5]. Finally, most weight-loss drugs are classified as controlled substances by the US Drug Enforcement Administration, and many states have passed stringent regulations on the prescription and utilization of scheduled drugs. The Endocrine Society also suggests the use of approved weight loss medications for long-term weight maintenance to ameliorate comorbidities and to enhance adherence to behavioral changes [6].

Nevertheless, a weight loss of 5% or more is sufficient to significantly reduce health risks in individuals with impaired glucose tolerance, hypertension, or nonalcoholic fatty liver disease [7]. The

goal of medicating for obesity is to help more patients achieve increased weight loss. A barrier to drug approval has been the concern that weight loss medications might be used by individuals with little or no health risks, thereby mandating a low side-effect profile for approval of any drug. This limits the available options for patients who have obesity-related health problems that could improve with weight loss [1, 8, 9].

Therefore, the primary objective of our study was to identify anti-obesity drugs by screening natural products, without significant side effects. We used a library using in clinic with other purpose to cure diseases, such as antibiotics and anti-cancer agents. We also established a screening method to determine lipid accumulation using 3T3-L1 cells. After screening with natural products, we identified kahweol, which is a diterpene found in the beans of *Coffea arabica* and structurally related to cafestol (Fig. 1A and B). Recent research suggests that kahweol may have beneficial effects on bones by inhibiting osteoclast differentiation [10]. Another recent study showed that kahweol has anti-inflammatory and anti-angiogenic effects [11], offering a possible mechanism for epidemiological studies showing an association between unfiltered coffee consumption and decreased risk of cancer.

In this study, we examined kahweol's effect on adipocyte differentiation and lipid accumulation. Because AMP-Activated Protein Kinase (AMPK) is a serine/threonine kinase regulating lipid metabolism and glucose transport, AMPK is known as therapeutic target of anti-obesity and diabetic agent [12]. Interestingly, we determined that kahweol has a potent effect on the activation of AMPK, thereby increased glucose uptake in blood of sugar taken mice. Therefore, we suggest that kahweol could be an ideal agent for obesity attenuation and suggest clinical trials further examine its potential application.

RESULTS

Kahweol reduces lipid accumulation in 3T3-L1 cells

Excessive increase of adipocyte number (hyperplasia) and adipocyte size (hypertrophy) contributes to obesity. In adults, obesity is caused by an increase in adipocyte size in white adipose tissue resulting from excessive storage of triglyceride. Thus, inhibition of triglyceride accumulation in adipocytes is a promising strategy for prevention and treatment of obesity. To not effect on mitotic clonal expansion, a period where cells proliferate during adipocyte differentiation, 3T3-L1 cells were treated with different concentrations of kahweol 2 days after MDI-induction. Lipid accumulation in 3T3-L1 cells was measured using Oil Red O staining on day 6 (Fig. 1C). Kahweol (25 μg/ml) significantly reduced lipid accumulation, whereas cafestol did not reduce lipid accumulation (Figs. 1C and D). To confirm that the inhibitory effect of kahweol on lipid accumulation is not a result of cell cytotoxicity, a cell viability assay was performed. We confirmed that there was no difference in cell viability (Fig. 1E). We tested whether kahweol treatment affects protein expression of PPARγ, C/EBPα, FABP4, and FASN, which regulate adipocyte differentiation and lipid metabolism. Kahweol significantly reduced expression of these proteins in a dose-dependent manner (Fig. 1F).

Kahweol suppresses the expression of adipogenesis and lipid accumulation-related genes in 3T3-L1 cells

We identified that 25 μg/ml kahweol had an inhibitory effect on lipid accumulation through dose-dependent treatment (Fig. 2A). To examine how kahweol treatment affects adipogenesis and lipid accumulation-related genes during adipocyte differentiation, cell lysates were prepared on day 0, 2, 4, and 6. We treated cells with kahweol 2 days after adipogenic stimuli, focusing on the effect of kahweol on lipid accumulation. Kahweol significantly repressed mRNA (Fig. 2B) and protein expression (Fig. 2C) of PPARγ, C/EBPα, FABP4, and FASN after 2 days of treatment, suggesting that kahweol affects both adipogenesis and lipid accumulation.

Kahweol reduces MDI-induced adipocyte differentiation and lipid droplet size

To demonstrate the effect of kahweol on MDI-induced adipogenesis, 3T3-L1 cells were treated with MDI and kahweol at day 0. Kahweol treatment reduced adipocyte differentiation and lipid accumulation (Fig. 3A). We also examined the effect of kahweol on late adipocyte differentiation. 3T3-L1 cells were incubated with kahweol from days 6 to 10. Kahweol treatment slightly reduced lipid accumulation, and we showed that lipid droplet size in kahweol-treated 3T3-L1 cells was smaller than that of control cells (Fig. 3B).

To identify kahweol's effect on MDI-induced mitotic clonal expansion, we measured cell number on day 0 and 2. On day 2, vehicle-treated 3T3-L1 cell number increased about 1.8-fold compared with that in day 0. Kahweol-treated 3T3-L1 cell number also increased, compared with that at day 0, but increased significantly less than vehicle-treated samples (Fig. 3C). These data indicate that kahweol represses adipocyte differentiation through inhibition of mitotic clonal expansion and reduces lipid droplet size.

Kahweol inhibits lipid accumulation and increase glucose uptake through activation of AMPK

Many studies have reported that kahweol suppresses activation of AKT and ERK1/2 [10, 13]. AKT and ERK1/2 pathways were also reported to regulate adipocyte maturation [14]. Therefore, we examined changes in activation of these signaling pathways, which are involved in adipocyte differentiation and lipid metabolism (Fig. 4A). Phosphorylation of AKT and JAK2 decreased after kahweol treatment; in contrast, phosphorylation of ERK1/2 was not affected by kahweol treatment. Interestingly, we found that kahweol treatment significantly increased the phosphorylation of AMPK and its downstream target Acetyl-CoA carboxylase (ACC). We also confirmed that kahweol treatment increased phosphorylated AMPK and ACC in a time-dependent manner (Fig. 4B). Many studies reported that AMPK is activated by phosphorylation and that it inhibits adipocyte differentiation and increases glucose uptake and fatty acid oxidation [12].

Furthermore, we investigated whether kahweol could inhibit lipid accumulation through AMPK activation. The inhibitory effect of kahweol on lipid accumulation was alleviated by knockdown of AMPK using siRNAs (Fig. 4C). Knockdown of AMPK also reduced the effect of

kahweol on expression of adipogenesis and lipid metabolism-related genes (Fig. 4D). Together, these results suggest that kahweol suppresses lipid accumulation by upregulation of AMPK activation. Activation of AMPK promotes cellular glucose uptake through glucose transporter. Metformin known as AMPK activator has anti-diabetic effect [15]. Since kahweol also had the effect of AMPK activation as metformin, we hypothesized that kahweol would show anti-diabetic effect. In 3T3-L1 cells, kahweol treatment elevated glucose uptake in a dose-dependent manner (Fig. 4E). In addition, we tested whether kahweol could improve glucose homeostasis in vivo. 8 week-old mice were administered kahweol every 2 days by oral gavage. After 2 weeks, we performed glucose tolerance test (GTT). Clearance of blood glucose was faster in kahweol-treated mice than in control mice (Fig. 4F).

DISCUSSION

Coffee is one of the most widely consumed beverages in the world. Moreover, it contains a wide array of components that have potential health implications. Several epidemiological studies associate coffee consumption with a reduced incidence of various chronic diseases such as diabetes, cardiovascular diseases, and neurodegenerative diseases [16, 17]. Experimental studies show that coffee consumption reduces fat accumulation and collagen deposition in the liver and promotes antioxidant capacity through an increase in glutathione as well as modulation of gene and protein expression of several inflammatory mediators. The diterpenes cafestol and kahweol have been implicated as the components in boiled coffee that are responsible for its hypercholesterolaemic effects [18]. It was also shown that cafestol and kahweol may reduce the activity of hepatic LDL receptors and thereby cause extracellular accumulation of LDL [19]. However, the mechanisms of action by which it exerts its beneficial effects are not fully understood.

In this study, we determined the molecular mechanism by which kahweol inhibits lipid accumulation in 3T3-L1 cells. First, we established a screening method using adipogenesis of 3T3-L1 cells. We treated cells with selected compounds from natural compound libraries 2 days after initiation of adipogenesis and then quantified lipid in adipogenic 3T3-L1 cells by Oil Red staining. Among these compounds, we found that kahweol, a coffee-specific diterpene, has a strong anti-lipogenic effect compared to its structural analog cafestol. Kahweol inhibited lipid accumulation and adipogenesis in a dose and time-dependent manner, but cafestol had no effect on these in 3T3-L1 cells. Many studies on kahweol have defined its inhibitory effects on cancer, angiogenesis, and inflammation. For example, kahweol suppresses cell proliferation by inducing cyclin D1 proteasomal degradation via ERK1/2, JNK, and GKS3β-dependent threonine-286 phosphorylation [20] and heat Shock Protein 70 Expression [21] in human colorectal cancer cells. Kahweol blocks STAT3 phosphorylation and induces apoptosis in human lung adenocarcinoma A549 cells [22]. The inhibitory effect of kahweol on endothelial cells was demonstrated by targeting MMP-2 and uPA [11] and inhibition of VEGFR2 expression and its downstream effector AKT, but not ERK, was also observed [23]. Here, we detected that kahweol treatment reduced the expression of adipogenic factors, such as

PPARγ and C/EBPα, which induce adipogenic differentiation, lipid synthesis related factor, FASN, and the lipid accumulation promoting factor FABP4.

Interestingly, we determined that kahweol induces activation of AMPK. AMPK is an evolutionarily conserved serine/threonine kinase that was originally identified as the key player in maintaining cellular energy homeostasis [12]. The well-defined mechanisms of AMPK activation involve phosphorylation at T172 of the α-subunit and by binding of AMP and/or adenosine diphosphate (ADP) to the γ-subunit. Adenosine triphosphate (ATP) competitively inhibits the binding of both AMP and ADP to the γ-subunit, suggesting that AMPK is a sensor for AMP/ATP or ADP/ATP ratios. The first known function for AMPK was the regulation of lipid metabolism. AMPK inhibits de novo synthesis of fatty acids (FAs), cholesterol, and triglycerides (TGs), and activates FA uptake and β-oxidation (FAO). After glucose is transported into cells, it is phosphorylated by hexokinases to generate glucose-6-phosphate. Glucose-6-phosphate is then consumed in several metabolic pathways, including glycolysis, glycogen synthesis, and the pentose phosphate pathway. Among these pathways, glycolysis and glycogen synthesis are regulated by AMPK. AMPK increases glucose uptake by blocking endocytosis and by promoting GLUT1 and GLUT4 expression [24]. Because AMPK regulates diverse metabolic and physiological processes and is dysregulated in major chronic diseases, such as obesity, inflammation, diabetes, and cancer, AMPK activation is a promising strategy for alleviation of metabolic dysfunction. Many research showed that AMPK activators inhibit adipocyte differentiation *in vitro* and ameliorate adiposity in high fat diet fed obese mice [25-27]. Metformin, a typical AMPK activator was well-known as an anti-diabetic drug for the treatment of type 2 diabetes [15].

In this study, kahweol induces phosphorylation of AMPK. The effect of kahweol treatment on reducing lipid accumulation was decreased by depletion of AMPK, suggesting that the inhibitory effect of kahweol on lipid accumulation was due to phosphorylation of AMPK. Moreover, mice were fasted for 15 h and then fed glucose with or without kahweol. Administration of kahweol further decreased the blood glucose level compared with glucose treatment alone. However, we do not yet know how kahweol increases phosphorylation of AMPK. Phosphorylation at T172 of the AMPK α-

subunit is regulated by at least three kinases and three phosphatases: liver kinase B1 (LKB1), calcium/calmodulin-dependent kinase kinase 2 (CaMKK2) [28], and TGFβ-activated kinase 1 (TAK1) [29]. When energy is depleted, high levels of AMP and ADP bind to CBS3 of the AMPK γ-subunit. This prevents phosphatases from accessing T172 of the AMPK α-subunit, thereby increasing its phosphorylation. In addition, binding of AMP and (to a lesser extent) ADP to CBS3 stimulates LKB1-mediated phosphorylation. Finally, the binding of AMP, but not ADP, to CBS1 increases its intrinsic AMPK activity by inducing its allosteric activation. Currently, we are studying how kahweol regulates phosphorylation of AMPK by examining AMPK-related signaling pathways and/or the kinases that are regulated by kahweol.

Taken together, we suggest that kahweol has anti-obesity effects and should be studied further for possible therapeutic application.

MATERIALS AND METHODS

Materials and methods are available in supplementary information.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

FIGURE LEGENDS

Fig. 1. Kahweol inhibits lipid accumulation in 3T3-L1 cells, whereas cafestol has no effect on lipid accumulation. (A) Structure of kahweol. (B) Structure of cafestol. (C, D) Oil Red O staining of kahweol- and cafestol-treated 3T3-L1 cells. After MDI induction, 3T3-L1 cells were treated with kahweol or cafestol on days 2-6. Oil Red O staining was performed on day 6. Measurement of lipid accumulation. Stained ORO was eluted with 100% isopropanol and measured using the OD_{500}. $^{***}P$ <0.001, DMSO vs. kahweol, DMSO vs. cafestol. (E) Cell viability assays. Confluent 3T3-L1 cells were treated with kahweol for 48 h. (F) Protein expression of PPARγ, C/EBPα, FABP4, and FASN was detected by western blotting. Protein expression was normalized to β-actin.

Fig. 2. Kahweol attenuates expression of adipogenic factors. (A) Oil Red O staining of kahweol-treated 3T3-L1 cells. After MDI induction, 3T3-L1 cells were treated with kahweol on days 2-6. Oil Red O staining was performed on day 6. (B) mRNA expression of PPARγ, C/EBPα, FABP4, and FASN was detected by real-time PCR. RNA samples were prepared on day 0, 2, 4, and 6. 3T3-L1 cells were treated with kahweol on days 2-6. mRNA expression was normalized to β-actin. $^{***}P$ <0.001, DMSO vs. kahweol. (C) Protein expression of PPARγ, C/EBPα, FABP4, and FASN was detected by western blotting. Protein samples were prepared on days 0, 2, 4, and 6. 3T3-L1 cells were treated with kahweol on days 2-6. Protein expression was normalized to β-actin.

Fig. 3. Kahweol inhibits mitotic clonal expansion and reduces lipid droplet size. (A) Oil Red O staining and lipid accumulation of kahweol-treated 3T3-L1 cells. 3T3-L1 cells were treated with MDI and kahweol for day 2. Oil Red O staining was performed on day 6. (B) Oil Red O staining and lipid accumulation of kahweol-treated 3T3-L1 cells. 3T3-L1 cells were treated with kahweol on days 6-10. Oil Red O staining was performed on day 10. (C) Increase of cell numbers during mitotic clonal expansion. 3T3-L1 cells were treated with DMI and kahweol until day 2, and cell numbers were measured on day 0 and 2. $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001, untreated control vs. kahweol,

Fig. 4. Kahweol reduces lipid accumulation and increase glucose uptake through activation of AMPK. (A) Western blot analysis of signal transduction-related proteins. 3T3-L1 cells were treated with kahweol on day 2 and incubated for 24 h. (B) Activity of signal transduction-related proteins, such as AMPK and ACC was detected by western blotting. 3T3-L1 cells were treated with kahweol on day 2. (C) Knockdown of AMPKa1 using small interfering RNA (siRNA). After transfection of AMPKa1 siRNA, 3T3-L1 cells were incubated with MDI. Then, 3T3-L1 cells were treated with kahweol on day 2. $^{***}P < 0.001$, scRNA vs. siAMPK. (D) Protein expression of PPARγ and C/EBPα was detected by western blotting. Protein samples were prepared on day 4. (E) Glucose uptake analysis. 3T3-L1 cells were incubated with kahweol or metformin for 48 h. The remaining level of glucose in media was measured using a glucose assay kit. Metformin was used as a positive control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, untreated control vs. kahweol, untreated control vs. metformin (F) Glucose tolerance test (n=5 for each group). 8 week-old C57BL/6 mice were treated with the vehicle (DMSO) or kahweol (100 mg/kg) every 2 days via oral administration. After 2 weeks, the glucose tolerance test was performed at 0, 15, 30, 60, 90, and 120 min. $^{*}P < 0.05$, vehicle (n=5) vs. kahweol (n=5).

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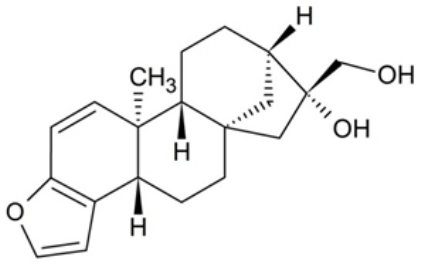
A



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B

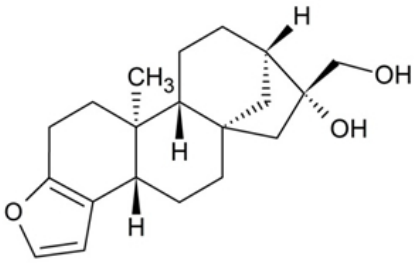

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Fig. 1

Figure 1

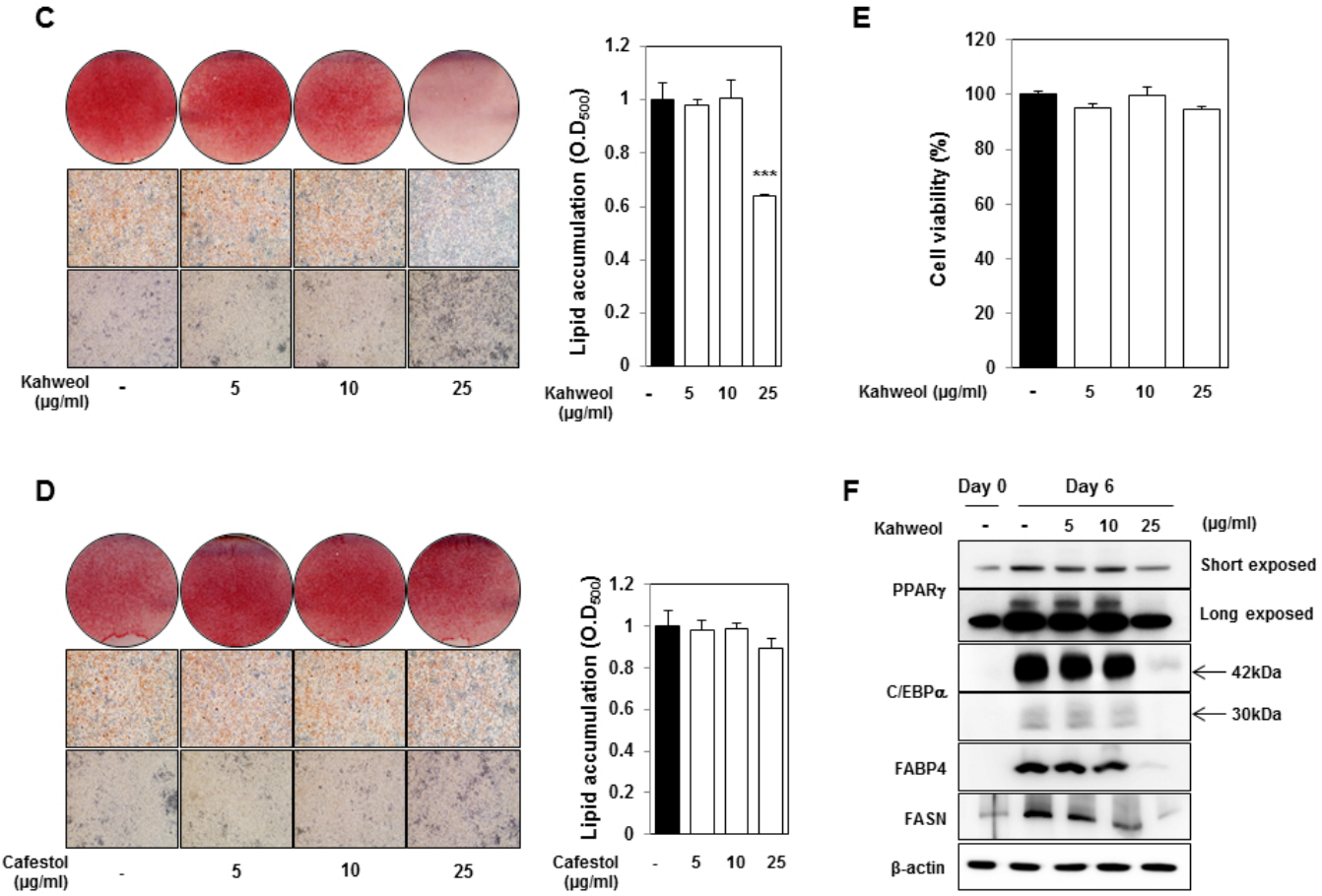

Fig. 2

Figure 2

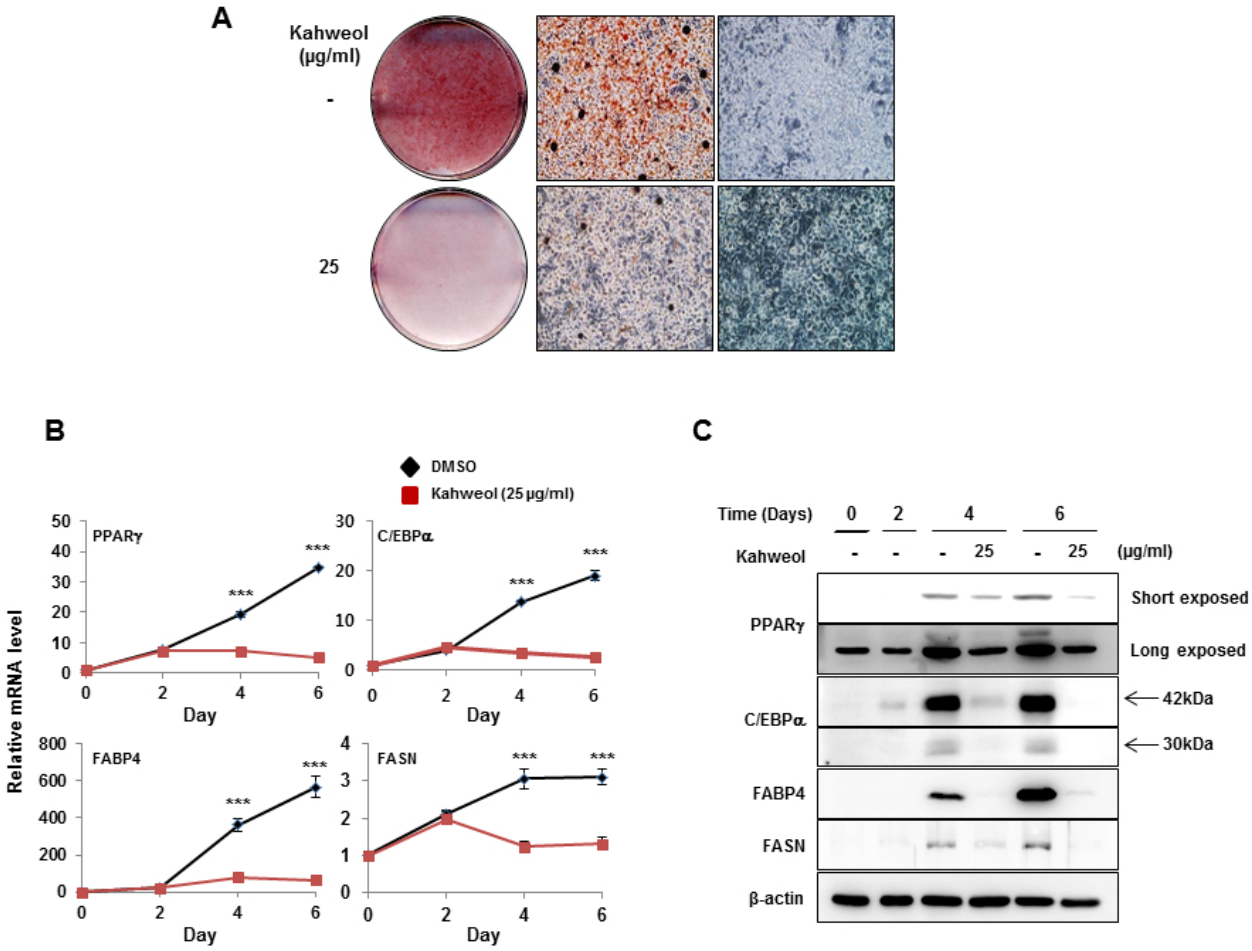

Fig. 3

Figure 3

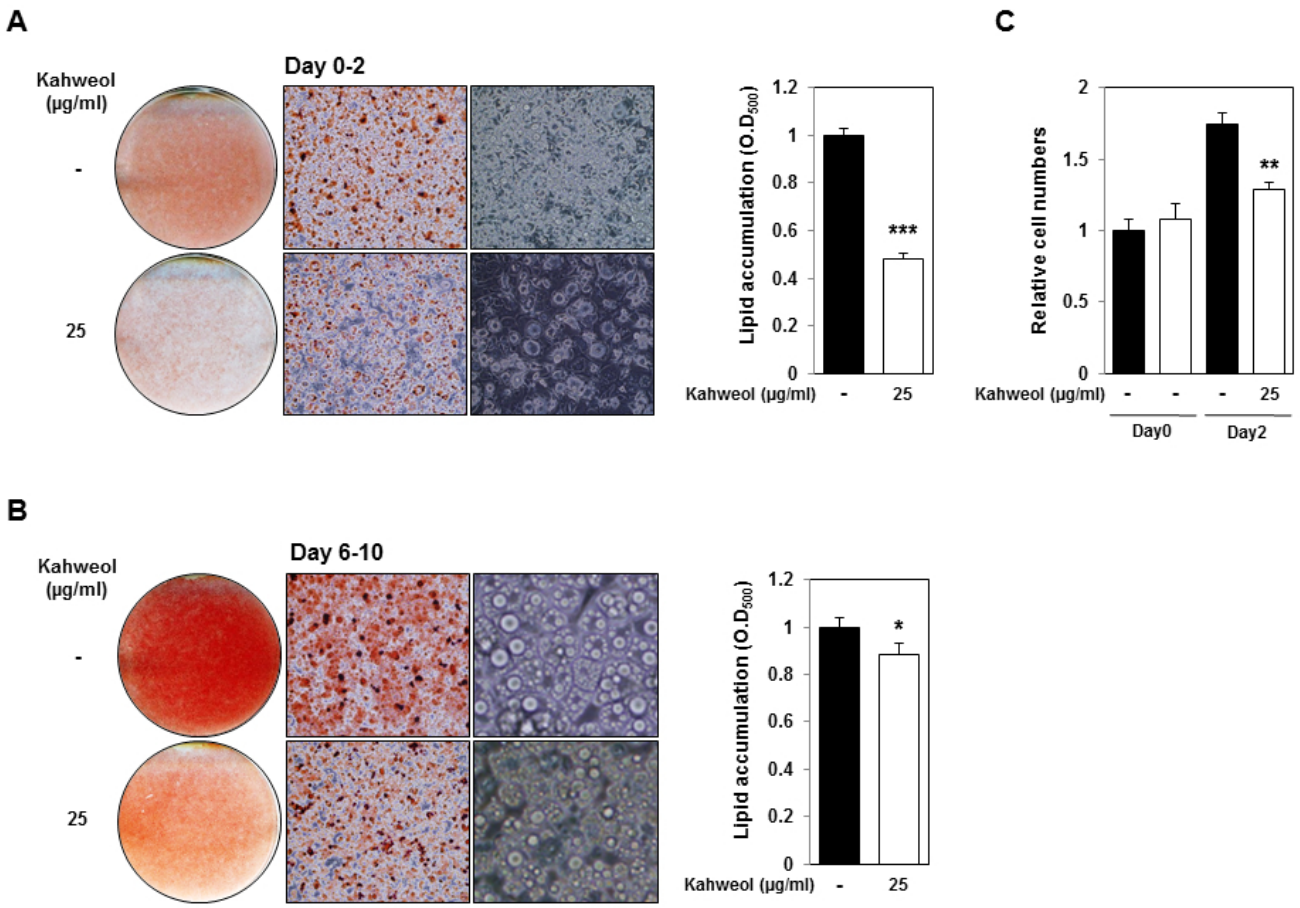

Fig. 4

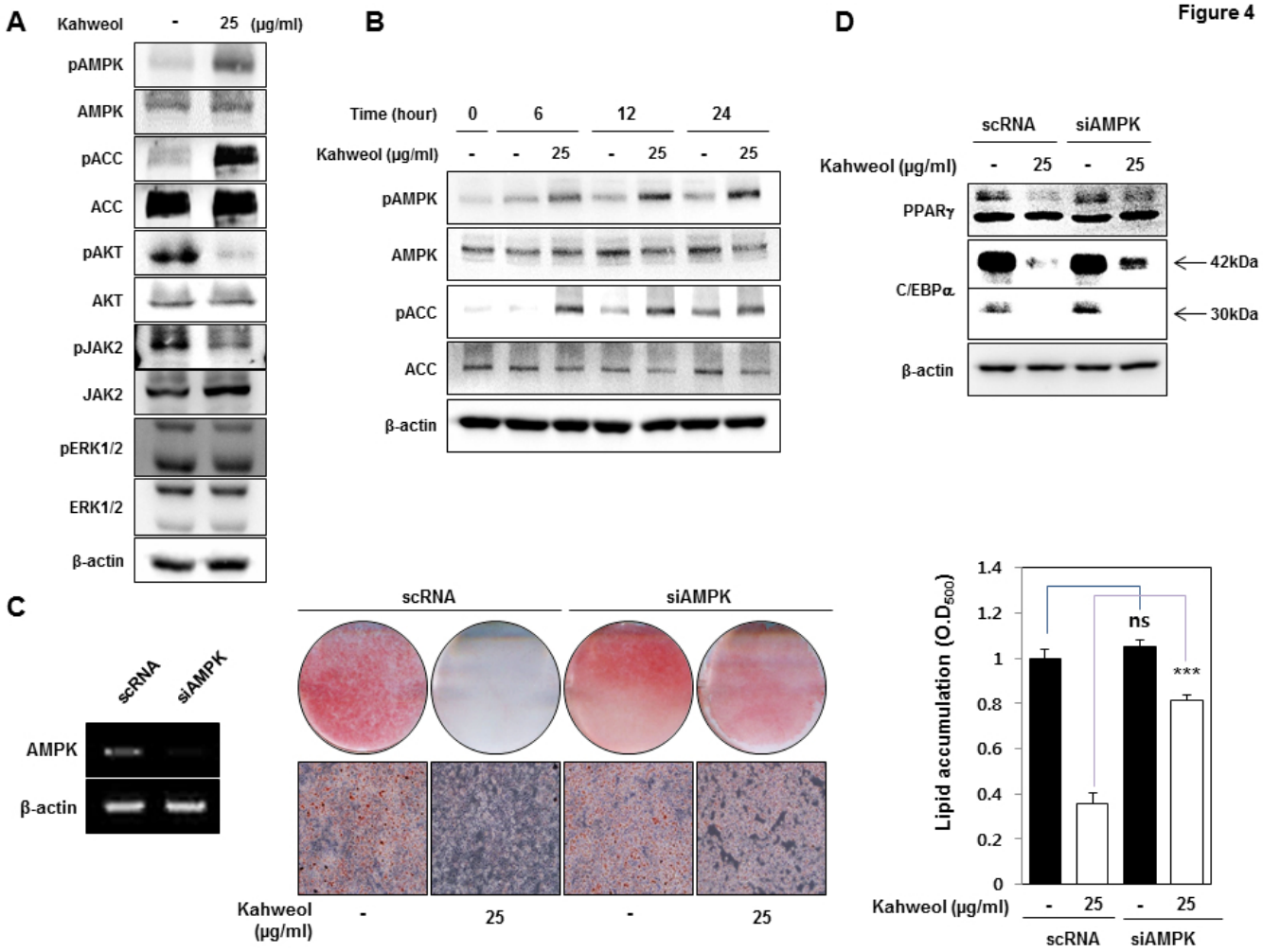

Figure 4

Fig. 5

Figure 4

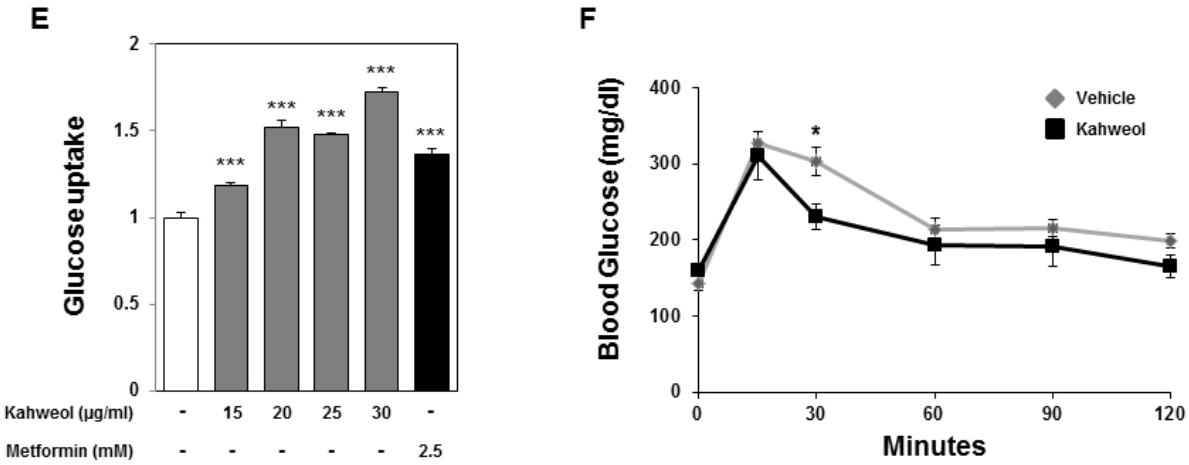

Fig. 6

Supplementary Information

MATERIALS AND METHODS

Cell culture and adipocyte differentiation assay

3T3-L1 cells were kindly provided by professor Jae-woo Kim (Yonsei University). 3T3-L1 cells were maintained and differentiated as previously described (1). Briefly, 3T3-L1 cells were grown to post-confluence with DMEM containing 10% bovine serum. On day 0 post-confluence, 3T3-L1 cells were incubated with DMEM containing 10% fetal bovine serum (FBS), insulin (1μg/ml), isobutylmethylxanthine (520 μM), and dexamethasone (1 μM). After 2 days, media was replaced with DMEM containing 10% FBS and insulin (1 μg/ml). On day 4, media was replaced with DMEM, supplemented with only 10% FBS.

Cell viability assay

3T3-L1 cells were plated in 12-well plates and incubated until confluence. Then, 3T3-L1 cells were treated with kahweol for 48 h. Cell viability was measured using Ez-Cytox (Daeil Lab), according to the manufacturer's protocol (2, 3). This experiment was performed in triplicate.

Oil Red O staining

Differentiated 3T3-L1 cells were incubated with 10% formalin for 10 min and washed with distilled water. Then, cells were stained with Oil Red O (ORO) in 60% isopropanol. Stained ORO was eluted with 100% isopropanol and measured at OD_{500}.

Western blot analysis

Cell lysate extractions were prepared with RIPA buffer (1% Triton X-100; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate; 150 mM NaCl; 50 mM Tris-HCl, pH 7.5; and 2 mM EDTA, pH 8.0) as previously described (1). Primary antibodies (C/EBPα, PPARγ, FABP4, FASN, p-AMPK, AMPK, p-

AKT, AKT, p-JAK2 and JAK2 from Santa Cruz Biotechnology; p-ACC, ACC, p-ERK1/2 and ERK1/2 from Cell Signaling) were used for detection using a FUSION SOLO S (Vilber) detector according to the manufacturer's directions (4-6). The normalization control was anti-β-actin (Santa Cruz Biotechnology).

RNA isolation and real-time PCR analysis

RNA was prepared using the RNA-lysis reagent (5 PRIME) as previously described (7). cDNA (1 μg) was synthesized from RNA using qPCR RT master mix (TOYOBO). The following primers were used: PPARγ: F_5′-agggcgatcttgacaggaaa-3′ and R_5′-cgaaactggcacccttgaaa-3′; C/EBPα: F_5′-gacatcagcgcctacatcga-3′ and R_5′-tcggctgtgctggaagag-3′; FABP4: F_5′-catcagcgtaaatggggatt-3′ and R_5′-tcgactttccatcccacttc-3′; FASN: F_5′-tgggttctagccagcagagt-3′ and R_5′-accaccagagaccgttatgc-3′; β-actin: F_5′-ggctgtattcccctccatcg-3′ and R_5′-ccagttggtaacaatgccatgt-3′. Real-time PCR was performed using SYBR Green Master Mix (TOYOBO) with an instrument from Applied Biosystems (8-10).

Transfection of small interfering RNA

3T3-L1 cells were plated in a 6-well plate and incubated for 24 h. 3T3-L1 cells were transfected with mouse AMPKa1 siRNA at 50 nM (GenePharma) using the Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's protocol (11, 12). After 24 h, media was replaced with DMEM containing 10% bovine serum. On the next day, differentiation of 3T3-L1 cells was induced in DMEM containing 10% FBS, insulin (1 μg/ml), isobutylmethylxanthine (520 μM), and dexamethasone (1 μM).

Glucose uptake assay

3T3-L1 cells were plated in a 12-well plate and incubated for 4 day. 3T3-L1 cells were incubated with DMEM containing insulin, isobutylmethylxanthine and dexamethasone. After 2 days, media were replaced with DMEM containing kahweol or metformin. After 48 h, glucose uptake was quantitated

using a glucose assay kit (Eton Bioscience), according to the manufacturer's protocol. This experiment was performed in triplicate.

Glucose tolerance test (GTT)

For glucose tolerance tests, wild-type male C57BL/6 mice were fasted overnight. After 15 h, fasting blood glucose levels were measured using a glucometer. Then, glucose solution (1 g/kg) was administered by intraperitoneal injection. Blood glucose levels were measured at 15, 30, 60, 90, and 120 minutes. Animal study was approved by the Yonsei University Health System Institutional Animal Care and Use Committee.

Statistical analysis

We employed unpaired *t*-tests to analyze comparisons between two groups. Statistical analysis was performed using Prism 5. P values < 0.05 were considered to be significant.

Reference

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