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Isoimperatorin enhances 3T3-L1 preadipocyte differentiation by regulating PPARγ and C/EBPα through the Akt signaling pathway

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Abstract. Lipodystrophic patients have an adipose tissue triglyceride storage defect that causes ectopic lipid accumulation, leading to severe insulin resistance. The present study investigated the potential role of isoimperatorin on 3T3-L1 adipocyte differentiation. mRNA and protein levels of differentiation- and lipid accumulation-associated genes, as well as the adipogenesis-related signaling pathway were analyzed in control and isoimperatorin-treated differentiated 3T3-L1 adipocytes using reverse transcription-quantitative PCR and western blot analysis. Results determined that isoimperatorin promoted 3T3-L1 fibroblast adipogenesis in a dose-dependent manner compared with standard differentiation inducers. Isoimperatorin significantly increased mRNA and protein expression of the crucial adipogenic transcription factors peroxisome proliferator activated receptor-γ (PPARγ) and CCAAT enhancer binding protein-α (C/EBPα). mRNA expression of the downstream adipogenesis-related genes sterol regulatory element-binding transcription factor 1c, adipocyte protein 2, fatty acid synthase, adiponectin and diacylglycerol *O*-acyltransferase 2 were also significantly increased following isoimperatorin treatment. The underlying mechanism likely involved activation of the Akt signaling pathway. Taken together, the present findings indicated that isoimperatorin may alter PPARγ and C/EBPα expression via the Akt signaling pathway, resulting in promotion of adipogenesis. The results highlighted the potential use of isoimperatorin as a therapeutic agent for preventing diabetes.

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Introduction

Type 2 diabetes mellitus (T2D), commonly recognized as a collection of prolonged metabolic disorders, is a public health issue with increasing prevalence worldwide (1). Ectopic lipid deposition in skeletal muscle and the liver may lead to insulin resistance and diabetes (2,3). Adipose tissue is the largest organ used in humans for lipid storage and mobilization based on energy requirements: It contains several types of cells, including mature adipocytes. As an endocrine organ, adipose tissue contributes to the complex regulatory homeostasis of energy intake, and to the metabolism of glucose and lipids (4). An increasing number of studies have identified that adipose tissue dysfunction may cause metabolic syndrome, such as T2D, atherosclerosis, cardiovascular disease and even cancer (5,6). Consequently, adipocytes have emerged as a possible pharmacological target in T2D (7).

Adipocyte differentiation is complex with the process orchestrated by a cascade of transcription factors and other regulatory proteins. Nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer binding protein-α (C/EBPα) are activated during adipocyte differentiation. PPARγ acts together with retinoid X receptor to induce differentiation by hormonal stimulation (8,9). In the early stages of adipocyte differentiation, C/EBPβ and C/EBPδ are both expressed earlier than C/EBPα. C/EBPβ and C/EBPδ induce C/EBPα and PPARγ expression, and then together with PPARγ and C/EBPα stimulate the expression of the downstream adipose-specific genes involved in glucose uptake, adipose phenotype, and lipid accumulation (10,11).

Signaling pathways such as PI3K/Akt, Wnt/β-catenin and mitogen-activated protein kinase signaling mediate the adipogenic transcriptional cascade involved in adipogenesis (12). PI3K/Akt signaling is important in PPARγ and C/EBPα upregulation and adipogenesis (13). Fibroblasts lacking Akt activation, or when Akt activation is inhibited, cannot differentiate from preadipocytes into adipocytes (14). By contrast, Akt activation can promote embryonic fibroblast differentiation into mature adipocytes even if other adipogenic factors are absent (15).

Recently, dietary phytochemicals that have beneficial effects on obesity and T2D by regulating adipocyte

differentiation have become a highly attractive topic, as natural products have lower risks of adverse effects compared to synthetic drugs (16). Isoimperatorin (ISOIM; Fig. 1) is included in the 6,7-furanocoumarin family of compounds and is the main effective element in the Umbelliferae family (17). *Heracleum*, *Angelica dahurica*, Chinese angelica, coastal glehnia root and *Peucedanum ostruthium* are members of the Umbelliferae family, which are all widely used as traditional medicines in many countries (18). ISOIM displays anti-inflammatory (19), anti-hypertension (20), analgesic (19), anti-cancer (21), and hepatoprotective properties (22). Additionally, dietary furocoumarin imperatorin, an ISOIM isomer, increases glucagon-like peptide secretion, reducing blood sugar in rodents by activating G protein-coupled bile acid receptor 1 (23). In addition, lipodystrophic patients have an adipose tissue triglyceride storage defect that causes ectopic lipid accumulation, leading to the development of severe insulin resistance (2,3). Furthermore, increased fat capacity storage in adipose with low fat mobilization leads to the expansion of fat mass, and may also be considered the best means of storing lipids in a harmless compartment (3).

The present study investigated the underlying molecular mechanism by which ISOIM regulates the differentiation of 3T3-L1 adipocytes and the accumulation of lipids. These finding may contribute to the development of novel drugs that ameliorate diabetes.

Materials and methods

Materials. ISOIM (≥98% purity; $C_{16}H_{14}O_4$) was obtained from Wuhan Jonk Biological Technology Co., Ltd. and maintained in 100 mM stock solutions in dimethyl sulfoxide then stored at -20˚C.

Cell culture and differentiation. 3T3-L1 fibroblasts were purchased from the Stem Cell Bank, Chinese Academy of Sciences and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Zeta Life, Inc.) and 1% streptomycin/penicillin under a humidified 5% CO_2 incubator at 37˚C. Two days after reaching confluence, the culture medium was switched to differentiation medium: DMEM containing 10% FBS and 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone and 10 μg/ml insulin (all Sigma-Aldrich; Merck KGaA) for 2 days at 37˚C. Then, cells were maintained in differentiation medium II: DMEM containing 10% FBS and 10 μg/ml insulin. The medium was replaced every 2 days. To observe the impact of ISOIM on adipogenesis, the cells were treated with 20 μM ISOIM, which was included in each medium used, from day 0 to 6 during differentiation.

Cell Counting Kit-8 (CCK-8). Cell viability was determined using CCK-8 kit (Vazyme) according to the manufacturer's protocol. Cells ($1x10^4$ per well) were seeded in a 96-well plate and incubated overnight at 37˚C, before being treated with 0-60 μM ISOIM for 48 h at 37˚C. Then, 10 μl CCK-8 solution was added to each well and the plate was incubated at 37˚C for 2 h. A microplate reader (Thermo Fisher Scientific, Inc.) was used to measure the absorbance at 450 nm.

Oil red O staining. Following the six-day induction of 3T3-L1 fibroblast differentiation, the accumulation of lipids was visualized using lipid-specific oil red O staining (Sigma-Aldrich; Merck KGaA). The cells were washed twice with PBS then were fixed for 30 min at room temperature in 4% formalin. The cells were washed with 60% isopropanol and incubated with oil red O working solution (0.5 g oil red O in 100 ml isopropanol diluted with water in a 3:2 ratio then filtered) at room temperature for 30 min. Samples were washed twice in water then imaged with and inverted microscope (Primovert; Carl Zeiss Microscopy, LLC) at a magnification of x200. Oil red O stain was extracted from the cells using 60% isopropanol and the absorbance measured at 490 nm using a microplate reader (Multiskan FC; Thermo Fisher Scientific, Inc.).

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using RNAiso Plus Reagent (Takara Bio, Inc.). A total of 500 ng total RNA was reverse transcribed to complementary DNA using a PrimeScript RT Reagent Kit (Takara Bio, Inc.) with the following thermocycling parameters: 37˚C for 15 min and 85˚C for 5 sec. A SYBR Green kit (Takara Bio, Inc.) was used for qPCR, with measurement using an iQ5 real-time qPCR detection system (Bio-Rad Laboratories, Inc.). The thermocycling conditions were as follows: Initial denaturation at 94˚C for 5 min; 40 cycles of 94˚C for 10 sec, 60˚C for 30 sec, 72˚C for 30 sec; and a final elongation at 72˚C for 10 min. The internal control was β-actin. mRNA levels were quantified using the comparative threshold cycle $2^{-\Delta\Delta Cq}$ method (24). The primer sequences were as follows: C/EBPβ forward, 5'-AAGCTGAGCGACGAGTACAAGA-3' and reverse, 5'-GTCAGCTCCAGCACCTTGTG-3'; PPARγ forward, 5'-CCAAGAATACCAAAGTGCGATCA-3' and reverse, 5'-CCCACAGACTCGGCACTCAAT-3'; C/EBPα forward, 5'-CTGATTCTTGCCAAACTGAG-3' and reverse, 5'-GAGGAAGCTAAGACCCACTAC-3'; sterol regulatory element-binding transcription factor 1c (SREBP1c) forward, 5'-GGAGCCATGGATTGCACATT-3' and reverse, 5'-GGCCCGGGAAGTCACTGT-3'; adipocyte protein 2 (aP2) forward, 5'-AAGAAGTGGGAGTGGGCTTTG-3' and reverse, 5'-CTCTTCACCTTCCTGTCGTCTG-3'; fatty acid synthase (FAS) forward, 5'-ATCAAGGAGGCCCATTTTGC-3' and reverse, 5'-TGTTTCCACTTCTAAACCATGCT-3'; diacylglycerol *O*-acyltransferase 2 (DGAT2) forward, 5'-CCTTCCTGGTGCTAGGAGTG-3' and reverse, 5'-CCAGTCAAATGCCAGCCA-3'; adiponectin forward, 5'-AAAGGGCTCAGGATGCTACTG-3' and reverse, 5'-TGGGCAGGATTAAGAGGAACA-3'; adipose triglyceride lipase (ATGL) forward, 5'-TTCGCAATCTCTACCGCCTC-3' and reverse, 5'-AAAGGGTTGGGTTGGTTCAG-3'; hormone-sensitive lipase (HSL) forward, 5'-GCTGGGCTGTCAAGCACTGT-3' and reverse, 5'-GTAACTGGGTAGGCTGCCAT-3'; and β-actin forward, 5'-GTCCCTGACCCTCCCAAAAG-3' and reverse, 5'-GCTGCCTCAACACCTCAACCC-3'.

Western blot analysis. Cells were lysed in radioimmunoprecipitation lysis buffer (Appligen) containing protease inhibitor cocktail (Beijing ComWin Biotech Co., Ltd.). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Beijing ComWin Biotech Co., Ltd.). Proteins (30 μg/lane)

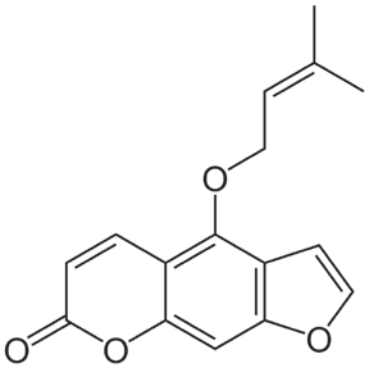

Figure 1. Chemical structure of isoimperatorin.

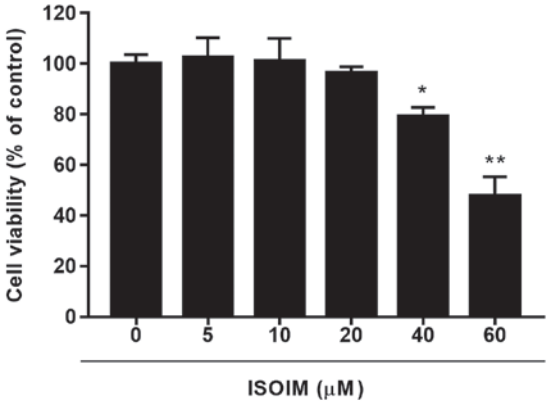

Figure 2. Effects of ISOIM on 3T3-L1 preadipocyte viability. 3T3-L1 cells were cultured with various concentrations of ISOIM (0, 5, 10, 20, 40 and 60 μM) for 48 h, and then cell viability was detected. Data are presented as the mean ± standard deviation from five independent experiments. *P<0.05 and **P<0.01 vs. control. ISOIM, isoimperatorin.

were separated via SDS-PAGE on a 10% gel, and transferred to polyvinylidene difluoride membranes (EMD Millipore; Merck KGaA). Membranes were blocked for 1.5 h at room temperature using 5% non-fat milk in Tris-buffered saline containing 0.1% Tween. Membranes were then incubated with primary antibodies against PPARγ (cat. no. 2430), ATGL (cat. no. 2138), HSL (cat. no. 4107), Akt (cat. no. 9272), phosphorylated Akt-pS473 (cat. no. 9271) and β-actin (cat. no. 4970; all Cell Signaling Technology, Inc.) and C/EBPα (cat. no. ab40764; all 1:1,000; Abcam) at 4˚C overnight. Following washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibodies (cat. no. 7074; 1:3,000; Cell Signaling Technology, Inc.) for 1.5 h at room temperature. An enhanced chemiluminescent peroxidase substrate (EMD Millipore; Merck KGaA) was used to visualize the protein bands. ImageJ software (1.8.0_112 version; National Institutes of Health) was used to quantify band density. The expression level of the protein was calculated using the ratio of the target protein intensity to β-actin.

Statistical analysis. Each experiment was performed three times independently unless stated otherwise. Statistical analysis was performed using SPSS v18.0 (SPSS, Inc.) and GraphPad Prism 6 (GraphPad Software, Inc.). Quantitative data were analyzed using Student's t-test and one-way analysis of variance followed by Tukey's post hoc test for multiple group comparisons. Data were presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxic effects of ISOIM on 3T3-L1 preadipocytes. To select a subtoxic ISOIM dose for later adipogenesis study, ISOIM cytotoxicity to 3T3-L1 fibroblasts was evaluated. ISOIM inhibited cell viability in a dose-dependent manner. At 40 and 60 μM, ISOIM significantly decreased cell viability to 79.2±3.50 and 47.7±7.56% of the control, respectively (Fig. 2). To avoid obvious ISOIM cytotoxicity, <40 μM ISOIM was selected for subsequent experiments to detect the impact on lipid accumulation.

ISOIM enhances 3T3-L1 cell lipid accumulation. To investigate the possible effect of ISOIM on adipogenesis, 3T3-L1 fibroblasts were treated with 0, 5, 10 or 20 μM ISOIM for 6 days in the differentiation mediums. ISOIM promoted 3T3-L1 adipocyte differentiation (Fig. 3A). The cells were treated with isopropanol to release the oil red O stain into the solution, which represented the cytoplasmic accumulation of lipid droplets, with 10 and 20 μM ISOIM absorbance significantly increased to 24.0 and 34.3% relative to the control. However, the 5 μM group and the control were not significantly different (Fig. 3B).

ISOIM increases adipogenesis-related mRNA and protein expression in differentiated adipocytes. To examine ISOIM adipogenic efficacy during differentiation, mRNA and protein expression of the genes related to adipogenesis were measured in 3T3-L1 fibroblasts treated with or without 20 μM ISOIM for 6 days. Compared with the control, ISOIM-treated differentiated adipocytes had higher PPARγ, C/EBPα and SREBP1c mRNA levels, but not C/EBPβ mRNA levels (Fig. 4A). In accordance with the aforementioned findings, western blot analysis demonstrated that ISOIM increased PPARγ (2.7 fold) and C/EBPα (2.6 fold) protein expression, two important adipogenesis transcriptional factors, in comparison to the control (Fig. 4B and C). Moreover, mRNA expression of PPARγ target genes and differentiation markers of late-stage adipocytes including aP2 (2.8 fold), FAS (1.9 fold), adiponectin (2.4 fold) and DGAT2 (1.7 fold) increased relative to the control (Fig. 4D). Results suggested that ISOIM promoted 3T3-L1 cell adipogenesis by upregulating PPARγ and C/EBPα.

ISOIM does not significantly affect lipolysis-related mRNA and protein expression in differentiated adipocytes. The study assessed whether ISOIM accelerated adipogenesis by downregulating lipolysis-related genes. mRNA expression of the genes encoding the lipolytic enzymes ATGL and HSL increased slightly but not significantly compared to the control (Fig. 5A). Similarly, there was no marked difference in the ATGL and HSL protein levels between the ISOIM and control groups (Fig. 5B and C). These findings demonstrated that ISOIM did not affect lipolysis.

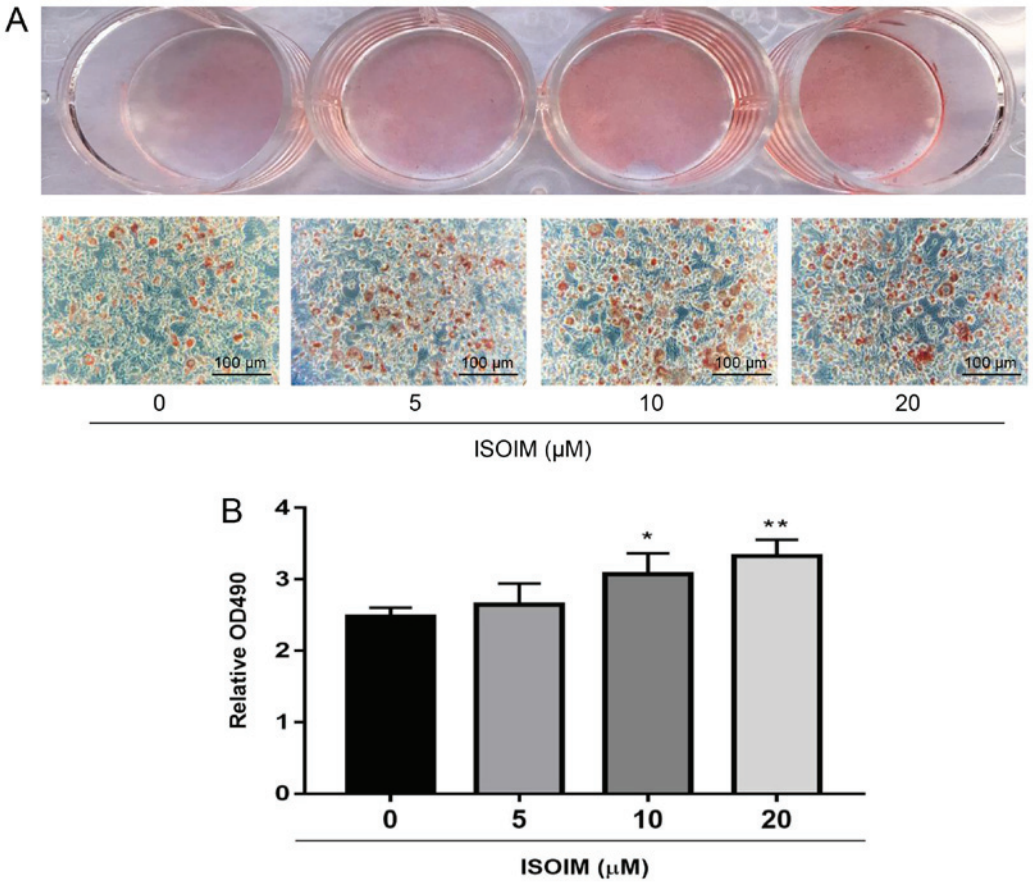

Figure 3. ISOIM promotes 3T3-L1 cell adipogenic differentiation. (A) Representative day 6 images of oil red O-stained differentiated and treated cells following treatment with various concentrations of ISOIM (0, 5, 10 and 20 μM; magnification, x200). (B) Measurement of oil red O extracted with isopropanol at OD490. Data are presented as mean ± standard deviation from three independent experiments. *P<0.05 and **P<0.01 vs. control. ISOIM, isoimperatorin; OD, optical density.

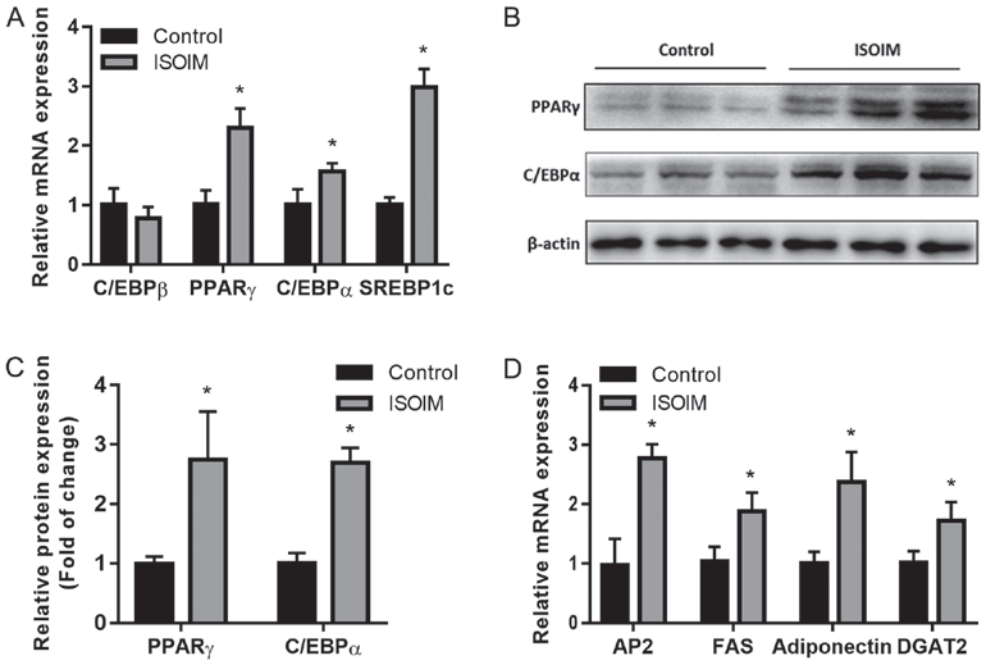

Figure 4. ISOIM (20 μM) induces mRNA and protein expression of adipogenesis-related genes. (A) RT-qPCR measurement of transcription factor mRNA expression. (B) Representative western blots and (C) quantification of PPARγ and C/EBPα protein expression. (D) RT-qPCR measurement of adipogenesis-related downstream genes. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05 vs. control. ISOIM, isoimperatorin; RT-qPCR, reverse transcription-quantitative PCR; C/EBPβ, CCAAT/enhancer binding protein β; PPARγ, peroxisome proliferator-activated receptor γ; C/EBPα, CCAAT/enhancer binding protein α; SREBP1c, sterol regulatory element-binding transcription factor 1c; aP2, adipocyte protein 2; FAS, fatty acid synthase; DGAT2, diacylglycerol *O*-acyltransferase 2.

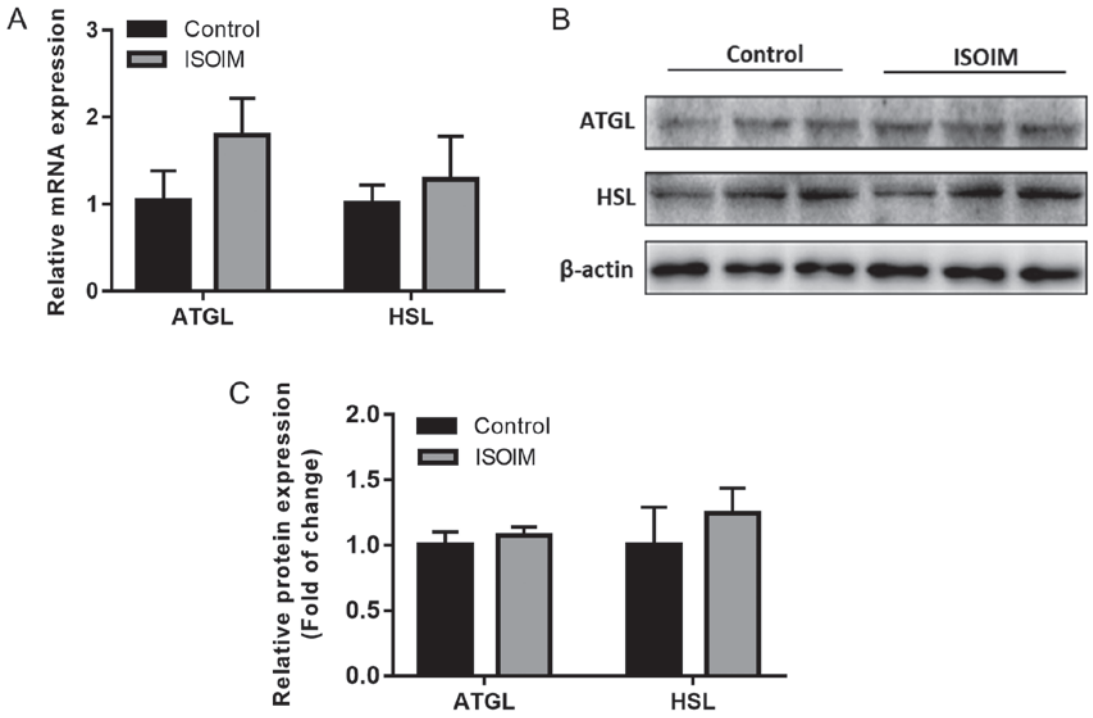

Figure 5. Effects of ISOIM (20 μM) on mRNA and protein expression of lipolytic genes. (A) Reverse transcription-quantitative PCR measurement of ATGL and HSL mRNA expression. (B) Representative western blots and (C) quantification of ATGL and HSL protein expression. Data are presented as the mean ± standard deviation from three independent experiments. ISOIM, isoimperatorin; ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase.

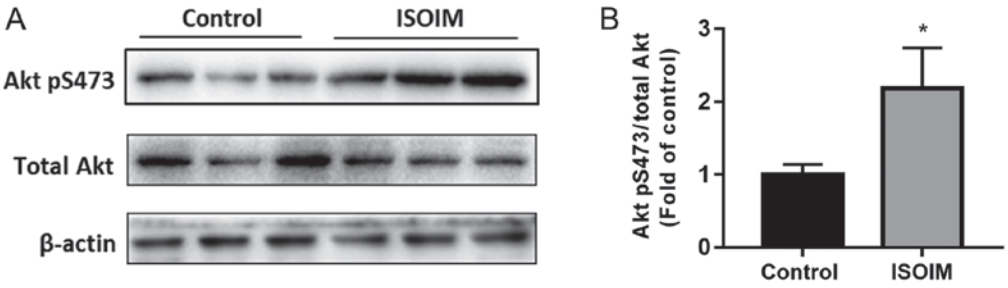

Figure 6. Effects of ISOIM on Akt phosphorylation during adipocyte differentiation. Differentiation medium was used to differentiate 3T3-L1 fibroblasts into adipocytes, which were treated with ISOIM for 6 days. (A) Representative western blots and (B) quantification of Akt and p-Akt protein expression. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05 vs. control. ISOIM, isoimperatorin; p, phosphorylated.

Effect of ISOIM on the Akt signaling pathway. Akt has an important role in adipocyte differentiation, which affects lipid metabolism through the insulin pathway (13). To define the underlying molecular mechanism of ISOIM-induced adipocyte differentiation, differentiated 3T3-L1 adipocytes were treated with ISOIM and Akt protein phosphorylation was detected. In addition to inducing the differentiation of 3T3-L1 cells with insulin, 3T3-L1 cells were treated with 20 μM ISOIM for 6 days. ISOIM increased Akt phosphorylation significantly (2.2 fold) compared with the control (Fig. 6A and B). These results suggest that ISOIM increased Akt phosphorylation during adipocyte differentiation.

Discussion

ISOIM has anti-inflammatory (19), anti-hypertension (20), analgesic (19), anti-cancer (21) and hepatoprotective properties (22). In the present study, ISOIM promoted adipogenesis in 3T3-L1 cells in a dose-dependent manner, and significantly increased lipid accumulation in the cells. Mechanistically, ISOIM stimulated activation of the insulin signaling pathway by phosphorylating Akt, critical for PPARγ and C/EBPα expression and transcriptional activity. This may have led to enhanced expression of PPARγ, C/EBPα, aP2, FAS, adiponectin and DGAT2 genes, which are involved in the differentiation of 3T3-L1 adipocytes.

Adipogenesis is complex and regulated by a cascade of transcription factors and other regulatory proteins (25). Previous studies have indicated that C/EBPβ is pivotal in regulating adipogenesis, and its activation is essential for initiating mitotic clonal expansion in adipocyte differentiation (26-28). Following a 16-20 h delay, C/EBPβ activates PPARγ and C/EBPα expression coordinately through regulatory elements of C/EBP in their respective genes' proximal promoters (29,30). However, the present study determined that ISOIM did not regulate C/EBPβ mRNA expression in

mature adipocytes. PPARγ is the adipocyte differentiation master regulator, during which it modulates the expression of several genes, whereas C/EBPα adipogenic activity relies on PPARγ being present. The terminally differentiated state is maintained by this transcription cascade via the induction of high expression levels of PPARγ and C/EBPα in a positive feedback loop (31-33). The present study determined that ISOIM increased PPARγ and C/EBPα mRNA and protein expression significantly, indicating that ISOIM can enhance adipocyte differentiation effectively by upregulating PPARγ and C/EBPα, rather than C/EBPβ.

For the downstream gene products of adipogenesis, SREBP1c binds to the FAS promoter to promote lipogenesis whilst also increasing aP2 expression levels in adipocytes (34). aP2 expression is found primarily in mature adipose tissue, which regulates lipid and glucose metabolism (35). Long-chain fatty acids are catalyzed and synthesized from acetyl-coenzyme A (CoA) by FAS, a multifunctional lipogenesis enzyme (36). Adipocytes secrete adiponectin, which modulates glucose regulation and fatty acid oxidation (37,38). DGAT2 catalyzes triglyceride formation from diacylglycerol and acyl-CoA, considered the terminal and sole committed step in the synthesis of triglycerides, and is essential for adipogenesis (39). The present study determined that ISOIM significantly increased SREBP1c, aP2, FAS, adiponectin and DGAT2 expression, which would likely mean enhanced fatty acid transport and synthesis of lipids.

Lipolysis affects lipid accumulation in adipocyte differentiation. A lipolytic enzyme cascade catalyzes triglyceride hydrolysis in adipocytes, where ATGL and HSL form the first and second steps (40). Therefore, the effect of ISOIM on lipolysis in 3T3-L1 adipocytes was investigated. Results determined that there was a trend for increased ATGL and HSL mRNA levels that was not statistically significant. Similarly, ATGL and HSL protein levels did not significantly change, which indicated that ISOIM does not regulate triglyceride hydrolysis during 3T3-L1 adipocyte differentiation; thus ISOIM does not affect lipid accumulation through triglyceride hydrolysis.

Finally, the signaling pathway involved in 3T3-L1 adipocyte differentiation was investigated. Akt has an essential function in adipocyte differentiation into lipids (41). Akt deletion impairs the ability of the 3T3-L1 cells to differentiate into adipocytes (15). In addition, PPARγ and C/EBPα transcript upregulation activates Akt, thereby increasing 3T3-L1 cell glucose uptake and differentiation into adipocytes (13). Akt phosphorylation regulates several biological processes (42), and is essential in PPARγ expression induction (14). Numerous studies have reported that Akt regulates PPARγ and adipocyte differentiation. Balakrishnan *et al* (43) reported that hyperglycemia in 3T3-L1 adipocytes is ameliorated by *Moringa concanensis* Nimmo upregulation of PPARγ and C/EBPα via the Akt signaling pathway. Conversely, Choe *et al* (44) reported that 3T3-L1 cell adipogenesis is attenuated by water-extracted plum (*Prunus salicina* L. cv. Soldam) via the PI3K/Akt signaling pathway. The present study determined that compared with the control, ISOIM increased Akt phosphorylation levels during the differentiation of 3T3-L1 preadipocytes. Therefore, the present results indicated that ISOIM may enhance adipocyte differentiation and lipid accumulation by enhancing PPARγ and C/EBPα expression via the Akt pathway.

To summarize, the present study demonstrated that ISOIM increased the differentiation and accumulation of lipids of 3T3-L1 cells. Moreover, these results demonstrate that ISOIM may have potential as a natural agent for the prevention and improvement of diabetes.

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Availability of data and materials

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

Authors' contributions

TJ and SG conceived and designed the experiments. TJ, XS and ZY performed the experiments. TJ and XW analyzed the data. TJ and SG drafted the manuscript. The final manuscript was read and approved by all authors.

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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