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Methyl Cinnamate Inhibits Adipocyte Differentiation via Activation of the CaMKK2−AMPK Pathway in 3T3-L1 Preadipocytes

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ABSTRACT: Methyl cinnamate, an active component of *Zanthoxylum armatum*, is a widely used natural flavor compound with antimicrobial and tyrosinase inhibitor activities. However, the underlying bioactivity and molecular mechanisms of methyl cinnamate on adipocyte function and metabolism remain unclear. The aim of this study was to investigate the inhibitory effect of methyl cinnamate on adipogenesis in 3T3-L1 preadipocytes. Methyl cinnamate markedly suppressed triglyceride accumulation associated with down-regulation of adipogenic transcription factor expression, including sterol regulatory element binding protein-1 (SREBP-1), peroxisome proliferator-activated receptor γ (PPARγ), and CCAAT/enhancer-binding protein α (C/EBPα). Additionally, methyl cinnamate-inhibited PPARγ activity and adipocyte differentiation were partially reversed by the PPARγ agonist troglitazone. Furthermore, methyl cinnamate stimulated Ca^{2+}/calmodulin-dependent protein kinase kinase 2 (CaMKK2) and phospho-AMP-activated protein kinase (AMPK) expression during adipogenesis. This study first revealed methyl cinnamate has antiadipogenic activity through mechanisms mediated, in part, by the CaMKK2−AMPK signaling pathway in 3T3-L1 cells.

KEYWORDS: *methyl cinnamate, adipogenesis, PPARγ, C/EBPα, AMPK, CaMKK2*

INTRODUCTION

The incidence of obesity is dramatically rising worldwide and is a major contributor to the global burden of metabolic disorders. Excessive accumulation of adipose tissue mass contributes to the development of obesity and increased risk for various diseases, such as coronary heart disease and type 2 diabetes.[1] Adipose tissue mass expansion is caused by both hyperplasia and hypertrophy of adipocytes. The development of fat cells from preadipocytes is known as adipogenesis.[2] For these reasons, the control adipogenesis may be a target to prevent and treat obesity and metabolic disorders. The 3T3-L1 preadipoctye cell line is a well-established in vitro model for searching for antiobesity agents in numerous studies. The current focus is on finding natural materials with antiobesity activity due to the serious side effects of antiobesity drugs on the market.[3] Several studies have revealed that dietary phytochemicals possess high potential for obesity treatment and prevention through the inhibition of 3T3-L1 adipocyte differentiation; these compounds include β-asarone,[4] genistein,[5] capsaicin,[5] and curcumin.[6]

Adipogenesis is the process of adipocyte formation from precursor cells and is regulated by a cascade of transcription factors. Two adipogenic factors, peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPs), are considered to be key regulators of adipogenesis, inducing downstream adipocyte specific gene activation and maintaining the phenotype of adipocytes. Furthermore, the expression of the transcription factor sterol regulatory element binding protein (SREBP-1) also increases PPARγ activity during adipocyte differentiation.[2,7] AMP-activated protein kinase (AMPK) is a key factor that controls cellular energy homeostasis and metabolism. Ca^{2+}/calmodulin-dependent protein kinase kinase 2 (CaMKK2) has been identified to act as an upstream kinsase of AMPK and regulates AMPK activity in mammalian cells including adipocytes.[8−10] Recently, one study reported activation of the CaMKK2−AMPK signaling regulates the early phase of adipogenesis.[11] In addition, AMPK also attenuates SREBP-1, PPARγ, and C/EBPα expression to inhibit fat accumulation during adipogenesis.[12,13]

Zanthoxylum armatum DC (Rutaceae) is a wild shrub distributed mainly in southeast Asia, the Himalayas, and China. It has a long history of use as a spice and as a folk medicine as a carminative, analgesic, stomachic, and anthelmintic in Ayurvedic and Chinese medicine. Recent studies have demonstrated the extract of *Z. armatum* possesses anti-inflammatory, antinociceptive, and hepatoprotective activities.[14−16] *Z. armatum* essential oil exhibited good antimicrobial and anthelmintic activities.[17] Methyl cinnamate (Figure 1A) is one of the components of *Z. armatum* and is widely used in the flavor industry.[18,19] According to our preliminary finding, *Z. armatum* has antiadipogenic activity, and methyl cinnamate is the active component, producing potent activity. Previous

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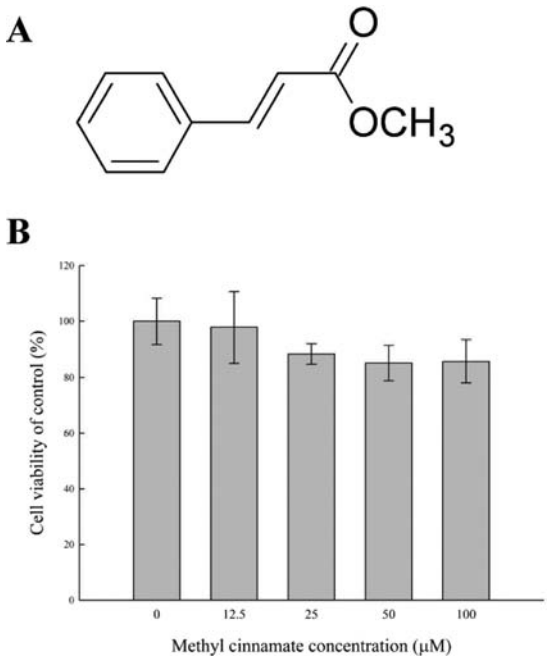

Figure 1. Cytotoxic effect of methyl cinnamate on 3T3-L1 cells: (A) structure of methyl cinnamate; (B) results of MTT assay to analyze cell viability after increasing concentrations (12.5, 25, 50, and 100 μM) of methyl cinnamate were used to treat 3T3-L1 cells for 9 days. The results are presented as the mean ± SD of at least three independent experiments. (∗) $p < 0.05$ compared to the untreated control.

research has indicated that methyl cinnamate possesses antimicrobial activity and is a tyrosinase inhibitor that can prevent food browning.[20] However, there are no studies investigating the antiobesity effect of methyl cinnamate. In this study, we used the 3T3-L1 cell model and found that methyl cinnamate inhibited adipocyte differentiation by attenuating expression of the adipogenic transcription factors SREBP-1, PPARγ, and C/EBPα and the transcriptional activity of PPARγ. In addition, methyl cinnamate activated the CaMKK2−AMPK signaling cascade involved in the regulation of adipogenesis.

MATERIALS AND METHODS

Materials. 3T3-L1 preadipocytes were purchased from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). Fetal bovine serum (FBS) was obtained from Biological Industries (Taipei, Taiwan). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Grand Island, NY). Insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, troglitazone, and compounds with structures similar to that of methyl cinnamate were obtained from Sigma Chemical (St. Louis, MO). Anti-mouse PPARγ, C/EBPα, resistin, β-actin, and α-tubulin monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-AMPK and anti-phospho-AMPK antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-phospho-ACC antibody was purchased from Millipore. Anti-CaMKK2 antibody was purchased from GeneTex (Irvine, CA).

Cell Culture and Adipocyte Differentiation. 3T3-L1 cells were cultured in 12-well plates (6×10^4/well) in high-glucose DMEM containing 10% FBS, 10000 U/mL penicillin, and 10 mg/mL streptomycin until confluent. Two days after confluence (day 0), the cells were stimulated to differentiate the hormone mixture MDI (0.5 mM IBMX, 0.5 μM dexamethasone, and 10 μg/mL insulin) in 10% FBS/DMEM. After 3 days of induction (day 3), the cells were maintained in 10% FBS/DMEM containing 10 μg/mL insulin, and the medium was replaced every 3 days. The cells were incubated at 37 °C in humidified 10% CO_2. Methyl cinnamate was added in medium at various concentrations (12.5, 25, 50, and 100 μM) throughout the differentiation process.

Triglyceride Assay. Methyl cinnamate treated 3T3-L1 cells and control cells were collected on day 9. Cells were washed with PBS and scraped on ice in 100 μL of lysis buffer (pH 7.5, 50 mM Tris-HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, and 0.1% SDS). The cell lysates were used for cellular triglyceride content determination using a GPO-PAP test kit (Merck, Darmstadt, Germany). Total cellular protein was measured using a DC protein assay (Bio-Rad, Hercules, CA). The triglyceride content was normalized to the total protein concentration, and the results were expressed as the relative lipid content compared to the MDI-treated positive control cells.

Cell Viability Assay. The cytotoxicity of methyl cinnamate on 3T3-L1 cells was examined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.[21] Cells (1×10^4/well) were cultured in 96-well plates. After treatment with methyl cinnamate, cells were washed twice with PBS, and then MTT reagent was added to each well. Cells were incubated at 37 °C for 1 h, and

Table 1. Primer Sets for the Real-Time RT-PCR

gene name	primer sequence[a]	size (bp)	accession no.
aP2	F: GTGAAAACTTCGATGATTACATGAA R: GCCTGCCACTTTCCTTGTG	66	NM_024406
C/EBPα	F: CGCAAGAGCCGAGATAAAGC R: CACGGCTCAGCTGTTCCA	112	NM_007678
GAPDH	F: GTATGACTCCACTCACGGCAAA R: GGTCTCGCTCCTGGAAGATG	101	NM_008084
PPARγ2	F: TGCTGTTATGGGTGAAACTCTG R: GAAATCAACTGTGGTAAAGGGC	217	NM_011146
SREBP-1	F: TGTTGGCATCCTGCTATCTG R: AGGGAAAGCTTTGGGGTCTA	190	NM_011480
adiponectin	F: TCCTGGAGAGAAGGGAGAGAAAG R: TCAGCTCCTGTCATTCCAACAT	84	NM_009605

[a]F, forward primer; R, reverse primer.

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dimethyl sulfoxide was added to dissolve the formazan crystals. The absorbance was then measured at 570 nm with a spectrophotometer.

Oil Red O Staining. Cells were washed with PBS and then fixed with 3.7% formaldehyde for 30 min. The fixed cells were washed three times with distilled water, and Oil Red O solution (5 mg Oil Red O/mL isopropanol) was added to each well. The cells were then incubated at room temperature for 2 h. Plates were rinsed three times with distilled water and allowed to air-dry for examination under a light microscope (Olympus CK 40). Images were captured with a digital camera (Olympus C-5050).

Glycerol-3-phosphate Dehydrogenase (GPDH) Activity Assay. 3T3-L1 cells were incubated with methyl cinnamate (12.5, 25, 50, and 100 μM) in MDI throughout the differentiation process. On day 9, the cells were washed twice with cold PBS and harvested in enzyme extraction buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, and protease inhibitor) on ice. Then, the cells were disrupted by sonication and centrifuged at 12000*g* for 20 min at 4 °C. The supernatant was collected and incubated with the reaction mixture (100 mM triethanolamine−HCl, 0.12 mM NADH reduced form, 2.5 mM EDTA, 0.1 mM DTT, and 0.2 mM dihydroxyacetone phosphate). GPDH activity was determined by measuring the decrease in the absorbance of NADH at 340 nm. The results were normalized to the total protein content, and 1 unit of enzyme was defined as the amount of enzyme required to convert 1.0 μmoL of dihydroxyacetone phosphate to α-glycerophosphate per minute at pH 7.4 and 25 °C .

RNA Extraction and Real-time RT-PCR. Total RNA was extracted from 3T3-L1 cell pellets using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was synthesized with the oligo(dT) primers using SuperScript II reverse transcriptase reagents (Life Technologies, Carlsbad, CA). Quantitative PCR was conducted with SYBR green reagent (Finnzymes Oy, Espoo, Finland) using a LightCycler real-time PCR system (Roche Diagnostics, Manheim, Germany). Primer sets are summarized in Table 1. The housekeeping gene glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used as an internal control.

Western Blot Analysis. Cultures were washed three times with ice-cold PBS and lysed with protein lysis buffer containing protease and phosphatase inhibitors. Equal amounts of protein were loaded onto SDS-PAGE gels, and the proteins were electrophoresed and then transferred onto membranes. The membranes were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween-20. Then the membranes were incubated overnight at 4 °C with primary antibodies. The membranes were incubated with horseradish peroxide labeled secondary antibody for 1 h after being washed with PBS containing 0.1% Tween-20, and the blots were developed using an enhanced chemiluminescence system (ECL, Amersham). The immune complex was detected using a luminescence imaging system (LAS-4000, Fujifilm, Japan).

PPARγ Activity Assay. 3T3-L1 cells treated with methyl cinnamate or troglitazone were collected, and the nuclear fraction was prepared as described by Andrews and Faller[22] on day 8. PPARγ activity was measured by using PPARγ transcription factor assay kit (Cayman Chemical, Ann Arbor, MI). Briefly, the nuclear fraction was added to a 96-well plate coated with a specific double-stranded DNA (dsDNA) sequence containing peroxisome proliferator response element (PPRE). Then PPARγ contained in the nucleus extract binds to immobilized PPRE and is detected with PPARγ specific primary antibody. The secondary antibody conjugated with horseradish peroxidase (HRP) was added, and the absorbance was measured at 450 nm.

Statistical Analysis. All results were expressed as the mean ± standard deviation (SD). The statistical significance was analyzed by one-way ANOVA and Tukey's multiple-comparison test. A p value of <0.05 was considered to be statistically significant.

RESULTS

Methyl Cinnamate Inhibits Adipocyte Differentiation. To determine the cytotoxicity of methyl cinnamate, 3T3-L1 cells were treated with methyl cinnamate at various concentrations (12.5, 25, 50, and 100 μM), and the cell viability was measured using the MTT assay. No significant adipocyte toxicity was observed when the cells were treated with methyl cinnamate up to 100 μM; the cell viability was >80% relative to the control (Figure 1B). During adipogenesis, the morphology of the 3T3-L1 preadipocytes becomes more rounded, and there is an increase in lipid droplet accumulation in the cells. Next, to examine the effect of methyl cinnamate on lipid accumulation during adipogenesis, various doses of methyl cinnamate were used to treat 2 day postconfluent preadipocytes during MDI-induced differentiation. On day 9, the cells were stained with Oil Red O. Methyl cinnamate decreased cellular lipid accumulation in a dose-dependent manner as shown in Figure 2A. Treatment with 100 μM methyl cinnamate reduced the lipid content by up to 75% relative to MDI-treated positive control cells (Figure 2B). These results suggested that methyl cinnamate inhibited cellular lipid formation and adipocyte differentiation not due to cytotoxicity.

Methyl Cinnamate Suppresses GPDH Activity during Adipogenesis. GPDH is an enzyme involved in triglyceride synthesis in adipocytes. As shown in Figure 2C, methyl cinnamate at 25, 50, and 100 μM significantly reduced GPDH activity during adipocyte differentiation. Consistent with the triglyceride assay results, the GPDH activity assay revealed that methyl cinnamate exhibited a suppressive effect on lipid accumulation during adipogenesis.

Methyl Cinnamate Decreases Adipocyte-Specific Marker Expression. PPARγ and C/EBPα are key transcription factors that regulate adipocyte differentiation. To determine whether methyl cinnamate influences the expression levels of adipocyte-related transcription factors, quantitative real-time RT-PCR was performed to analyze the expression of PPARγ and C/EBPα. The mRNA expression levels of PPARγ and C/EBPα were significantly decreased by 0.51- and 0.22-fold, respectively, with respect to MDI-treated positive control cells at a concentration of 100 μM (Figure 3A,B). We also demonstrated that methyl cinnamate attenuated the expression of SREBP-1 (Figure 3C), and the downstream targets of PPARγ, aP2 and adiponectin (Figure 3D,E). Consistent with the mRNA results, Western blot analysis revealed that methyl cinnamate markedly reduced the protein expression levels of PPARγ and C/EBPα (Figure 4A). Additionally, methyl cinnamate decreased the protein level of resistin, which is an adipocyte-secreted hormone, in mature adipocytes (Figure 4B).

Methyl Cinnamate Activates CaMKK2−AMPK Signaling. Several studies have shown that AMPK is involved in adipogenesis through regulation of adipogenic transcription factors. We questioned whether methyl cinnamate inhibits adipogenesis through AMPK signaling pathway. The cells were treated with methyl cinnamate (12.5, 25, 50, and 100 μM), and the protein levels were assayed on day 2. The immunoblotting results show that methyl cinnamate increased the expression of phospho-AMPK in a dose-dependent manner but did not alter the expression of total AMPK. In addition, the expression of the downstream target protein of AMPK, phospho-ACC, was increased with increasing concentrations of methyl cinnamate compared to the MDI-treated positive control cells (Figure 4C). To address whether CaMKK2, the upstream kinase of AMPK, was involved, we analyzed the protein expression of CaMKK2. As Figure 4C shows, methyl cinnamate markedly increased the expression of CaMKK2 on day 2. Furthermore, methyl cinnamate stimulated both CaMKK2 and AMPK in differentiated cells on days 1−3, indicating that methyl

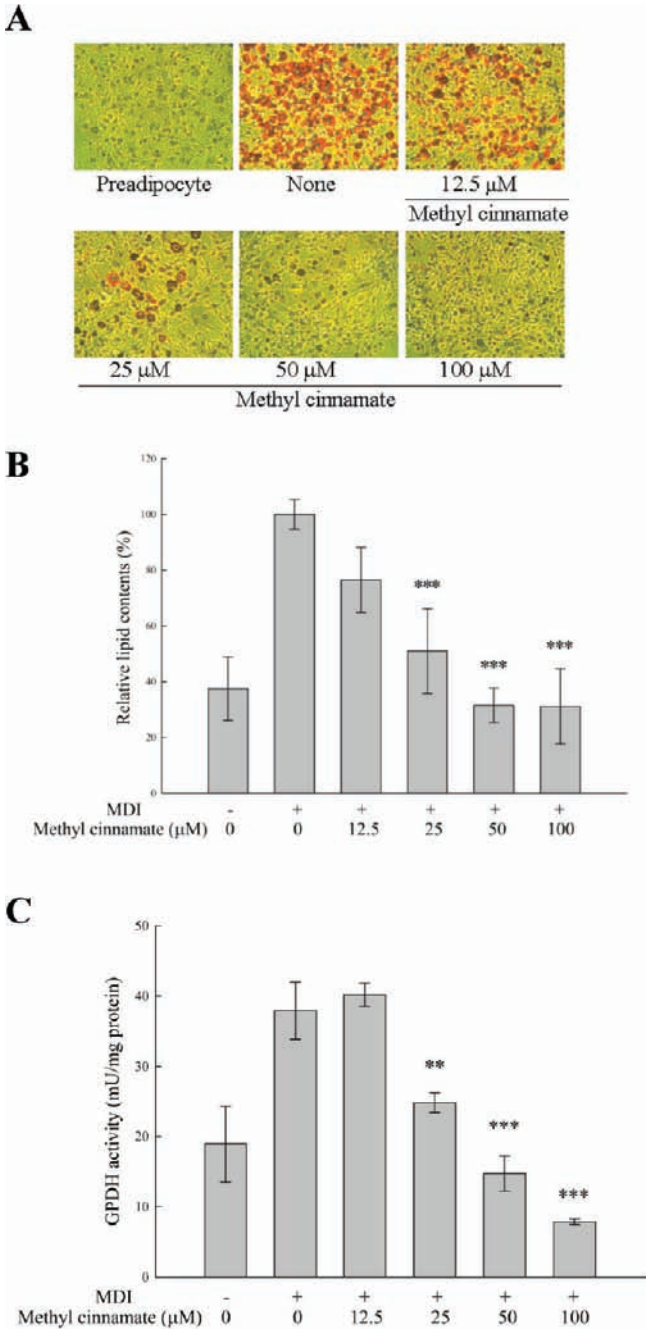

Figure 2. Methyl cinnamate decreased triglyceride accumulation and GPDH activity during adipogenesisL (A) Oil Red O staining on day 9 after 3T3-L1 cells were induced to differentiate with MDI in the presence or absence of methyl cinnamate (12.5, 25, 50, and 100 μM); (B) cellular lipid content of 3T3-L1 preadipocytes treated with the indicated concentration of methyl cinnamate measured on day 9; (C) GPDH activity assay performed after 3T3-L1 cells were treated with the indicated concentration of methyl cinnamate. The results are presented as the mean ± SD of at least three independent experiments. (**) $p < 0.01$ and (***) $p < 0.001$ compared to MDI-treated positive control cells.

cinnamate activated CaMKK2−AMPK signaling in the early stage of differentiation (Figure 4D).

Methyl Cinnamate Suppresses PPARγ Transcriptional Activity. PPARγ is the master control of adipogenesis, and as shown in Figure 3D,E methyl cinnamate decreases expression of the PPARγ target gene, aP2, and adiponectin. We tested whether methyl cinnamate affects PPARγ activity in the nuclear fraction. 3T3-L1 cells treated with MDI and/or troglitazone, a PPARγ ligand, highly activated PPARγ activity, whereas treating cells with methyl cinnamate suppressed the MDI-induced PPARγ activity. However, this inhibitory effect of methyl cinnamate was reversed by the addition of troglitazone (Figure 5A). Inhibition of triglyceride accumulation by methyl cinnamate was also rescued by troglitazone treatment in Oil Red O staining results (Figure 5B). These results suggested methyl cinnamate inhibits adipogenesis through an antagonistic effect on PPARγ transcriptional activity.

Structure−Activity Relationship for Antiadipogenesis Effect of Methyl Cinnamate and Flavor Compounds. To investigate the relationship between structure and function, we examined the antiadipogenic effect of several phenylpropane derivatives, including ethyl cinnamate, cinnamic acid, methyl L-3-phenyllacetate, methyl phenylacetate, and 3-phenylpropionic acid (Figure 6A), all of which have structures similar to that of methyl cinnamate. We treated 3T3-L1 cells with 100 μM of each test compound during MDI-induced differentiation. Cellular lipid content was measured on day 9. As shown in Figure 6B, methyl cinnamate significantly reduced the lipid levels to 40%, ethyl cinnamate reduced the lipid levels to 63% compared with the MDI-treated positive control, and the other compounds had no significant inhibitory effect on lipid accumulation in adipocytes.

DISCUSSION

Accumulating evidence indicates that inhibition of adipogenesis could be primarily achieved through regulating the expression and activity of multiple molecular targets. In the present study, we found that methyl cinnamate suppressed adipocyte differentiation via activating CaMKK2−AMPK signaling and down-regulating adipogenic transcription factors, SREBP-1, PPARγ and C/EBPα, as well as inhibiting the activity of PPARγ and GPDH. Oil Red O staining and TG analysis demonstrated that methyl cinnamate at a concentration of 25 μM was sufficient to significantly reduce intracellular lipid accumulation of 3T3-L1 cells, and the cells assume a morphology similar to that of preadipocytes. These results support the notion that the antiadipogenic activity of methyl cinnamate is mediated through negatively regulating lipid formation.

Adipogenesis is induced by a cascade of transcription factors. In our study, methyl cinnamate suppressed the MDI inducing mRNA and protein expression of PPARγ and C/EBPα on day 5 of 3T3-L1 cells. During the early stage of adipogenesis, SREBP-1, the upstream factor of PPARγ, enhances the expression of PPARγ and C/EBPα. In turn, PPARγ induces its downstream target genes to stimulate lipid synthesis and promote adipocyte differentiation.[7,23] Various natural products with antiobesity effects such as resveratrol,[24] epigallocatechin gallate (EGCG),[25] and curcumin[26] were reported to inhibit adipogenesis by blocking these factors. Figure 3 shows methyl cinnamate down-regulated the expression of SREBP1, PPARγ, and C/EBPα. The downstream target genes of PPARγ, aP2, and adiponectin expression were also suppressed by methyl cinnamate in differentiated cells. aP2 plays an important role in fatty acid metabolism, and decreased expression of aP2 is related to decreased utilization of fatty acid.[27] Adiponectin is exclusively released in mature adipocytes,[1] and suppression of adipogenesis reduces its expression. Thus, methyl cinnamate effectively inhibited lipid formation and adipogenesis by attenuating adipogenic transcription factors.

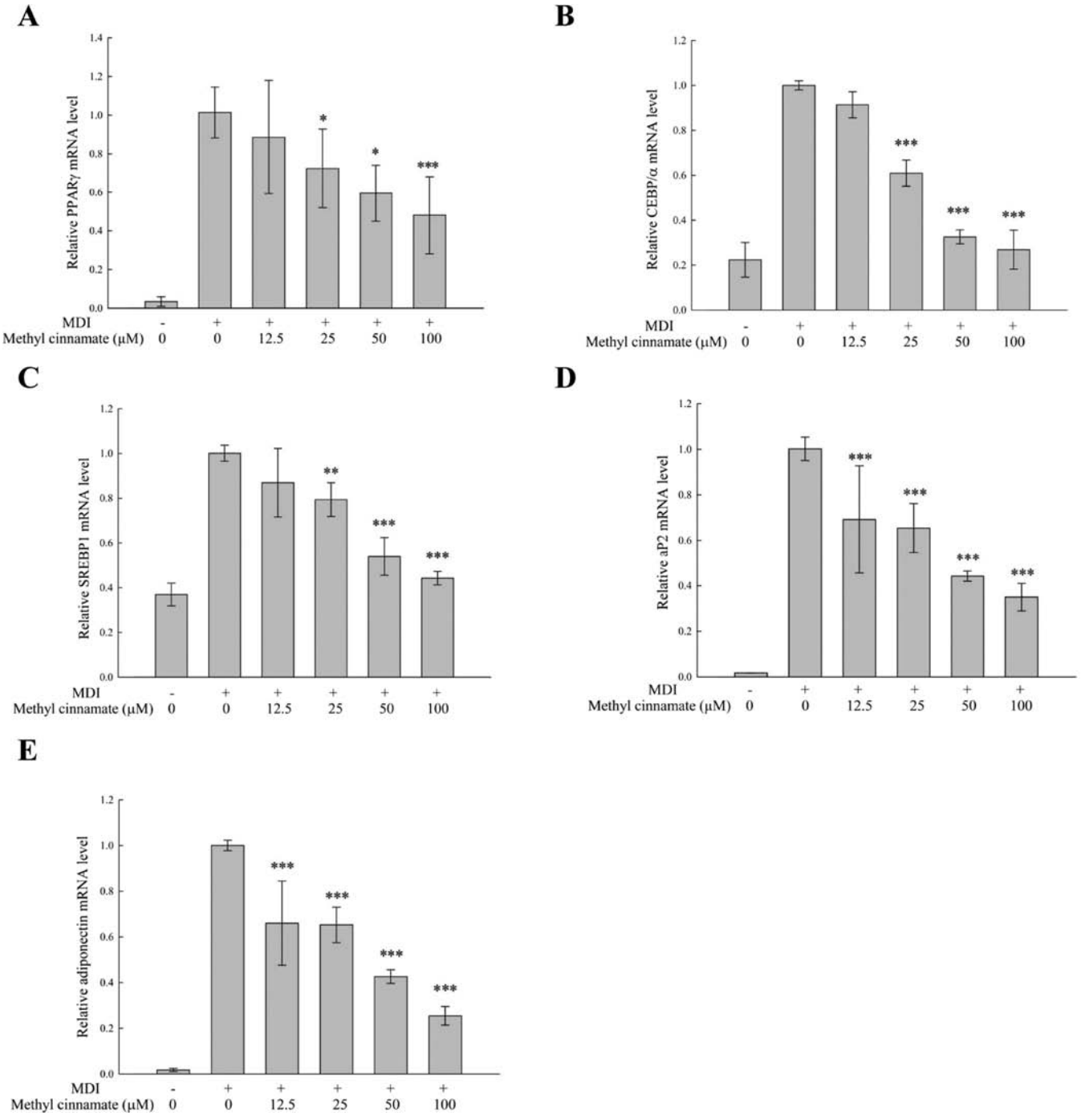

Figure 3. Methyl cinnamate attenuated the adipogenic-specific marker gene expression during adipogenesis: relative mRNA expression of the adipogenic transcription factors (A) PPARγ, (B) C/EBPα, and (C) SREBP-1 and the PPARγ downstream target genes (D) aP2 and (E) adiponectin. 3T3-L1 cells were induced to differentiate in the presence of methyl cinnamate (12.5, 25, 50, and 100 μM), and the cells were collected on day 5. Total RNA was isolated for quantitative RT-PCR to analyze the gene expression of adipocytes. Results were compared to those of MDI-treated positive control cells after normalization to an internal control, GAPDH. The results are presented as the mean ± SD of at least three independent experiments. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ compared to MDI-treated positive control cells.

In our results, we found methyl cinnamate activated the CaMKK2−AMPK pathway, which is involved in negative regulation of adiposity. Huang et al. have concluded that activated AMPK signaling is beneficial for treating obesity, diabetes, and cancer.[12] Some studies have shown amino-imidazole carboxamide ribonucleotide (AICAR), an activator of AMPK, inhibits 3T3-L1 adipocyte differentiation by blocking the expression of SREBP-1, PPARγ, and C/EBPα.[28,29] These findings support our results that methyl cinnamate stimulated AMPK activation and resulted in suppression of SREBP-1, PPARγ, and C/EBPα expression during differentiation. In addition, inactivation of ACC through AMPK phosphorylation leads to decreased lipid synthesis and prevents fat accumulation.[12,13] As shown in Figure 4C, methyl cinnamate up-regulated AMPK and phospho-ACC in a dose-dependent manner. Thus, methyl cinnamate inhibited lipid formation in

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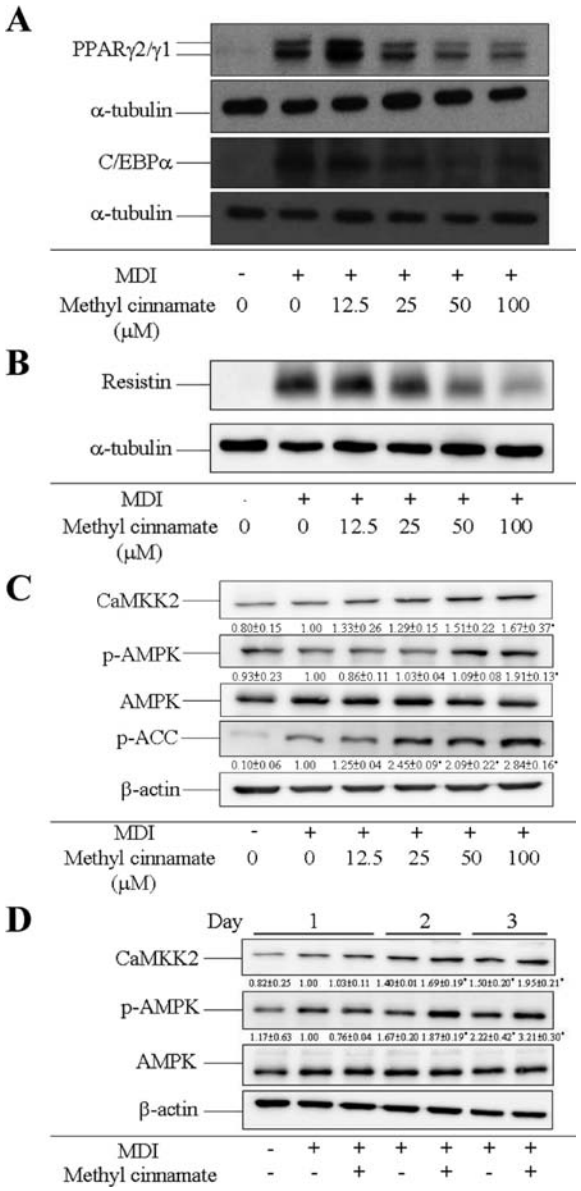

Figure 4. Methyl cinnamate inhibited adipocyte transcription factor expression and activated CaMKK2−AMPK signaling during adipogenesis. 3T3-L1 cells were induced to differentiate in the presence of methyl cinnamate (12.5, 25, 50, and 100 μM). The cells were collected on day 5 for Western blot analysis of (A) C/EBPα and PPARγ and on day 9 for (B) resistin. (C) Western blot analysis was used to determine (C) the CaMKK2, phospho-AMPK, and phospho-ACC protein levels on day 2 and (D) the protein level of CaMKK2 and phospho-AMPK on the indicated days. α-Tubulin and β-actin were used as internal controls. Densitometry analyses are presented as relative ratios of CaMKK2/β-actin, phospho-AMPK/AMPK, or phospho-ACC/β-actin, and each value shows the mean ± SD (n = 3). (*) $p < 0.05$ compared to MDI-treated positive control cells.

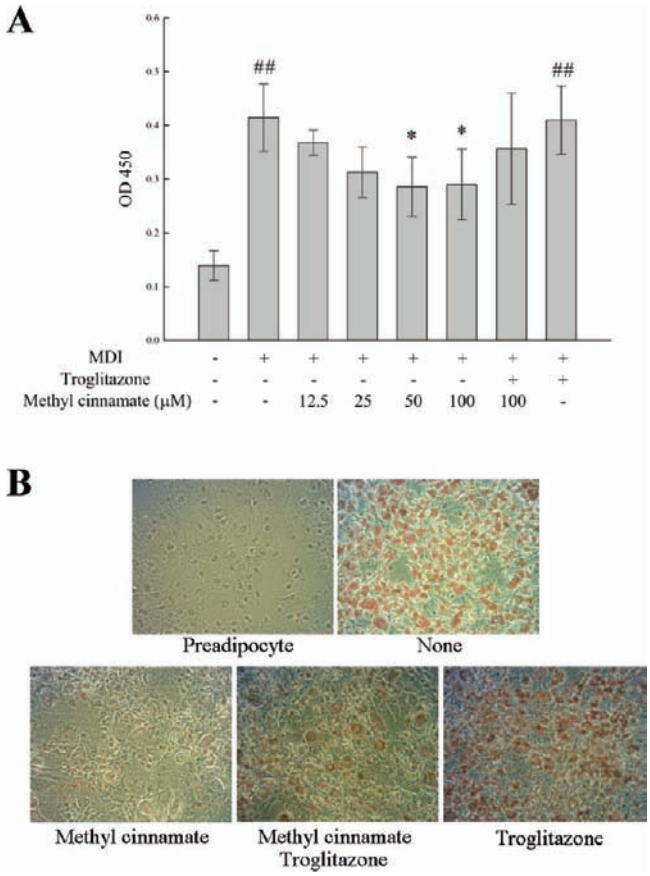

Figure 5. Methyl cinnamate suppressed PPARγ transcriptional activity. (A) 3T3-L1 cells were treated with indicated concentrations of methyl cinnamate or 10 μM troglitazone. On day 8, PPARγ transcriptional activity was detected by ELISA-based colorimetric method. The results are presented as the mean OD_{450} ± SD of at least three independent experiments. (*) $p < 0.05$ compared to MDI-treated positive control cells; (##) $p < 0.01$ compared to MDI-untreated preadipocytes. (B) Oil Red O staining demonstrated cellular lipid accumulation of 3T3-L1 cells treated with 100 μM methyl cinnamate or 10 μM troglitazone on day 9.

3T3-L1 preadipocytes through activation of AMPK. The present finding that methyl cinnamate increased the abundance of CaMKK2 and phospho-AMPK at the early phase of adipogenic differentiation (Figure 4C,D) suggests that CaMKK2−AMPK signaling may be responsible for the antiadipogenic effect of methyl cinnamate. CaMKK2 phosphorylates and activates AMPK in response to elevated intracellular Ca^{2+}.[8,9] A recent paper indicates that Ca^{2+} /CaMKK2 signaling is involved in the activation of AMPK in adipocytes.[10] Results from Lin et al. that CaMKK2−AMPK signaling participates in the inhibition of adipogenesis further implicate CaMKK2 as a novel target in preventing obesity.[11] It has been shown that elevation of intracellular Ca^{2+} activates CaMKK2/AMPK[8,9] and that high levels of Ca^{2+} inhibit the differentiation of 3T3-L1 preadipocytes.[30,31] Accordingly, our finding that methyl cinnamate increased CaMKK2 expression may suggest that the effect is mediated by increasing cellular Ca^{2+} levels in 3T3-L1 cells, albeit the underlying mechanisms remain to be elucidated.

PPARγ is not only critical for adipogenesis but also plays a pivotal role in lipid biosynthesis, inflammation, and glucose metabolism.[32] We showed methyl cinnamate antagonizes the MDI-mediated activation of PPARγ transcriptional activity in differentiated cells, and this effect was diminished in the presence of troglitazone, a strong agonist of PPARγ. A similar effect was also observed in lipid accumulation analysis (Figure 5A,B). These results together suggest that methyl cinnamate exerts its inhibitory effect on adipogenesis as an antagonist of PPARγ. On the other hand, activation of PPARγ is a therapeutic target for type 2 diabetes to increase insulin sensitivity. The thiazolidinediones (TZDs), antidiabetic drugs,

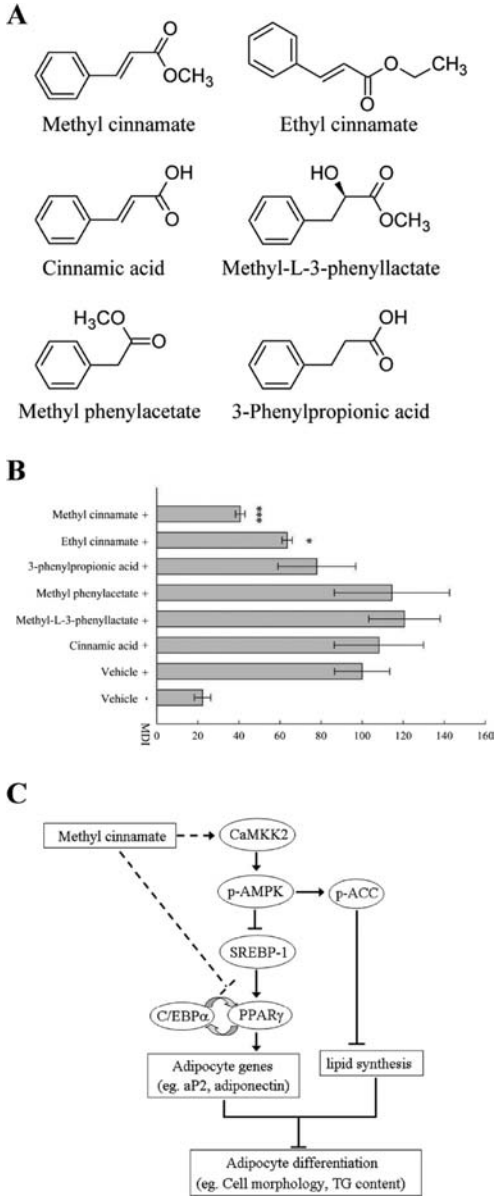

Figure 6. Effect of compounds structurally similar to methyl cinnamate on adipogenesis: (A) structures of methyl cinnamate, ethyl cinnamate, cinnamic acid, methyl L-3-phenyllacetate, methyl phenylacetate, and 3-phenylpropionic acid. (B) 3T3-L1 cells were treated with 100 μM test compounds and MDI, and the cells were collected to determine the cellular lipid contents on day 9. The vehicle was 0.1% DMSO. The results are presented as the mean ± SD of at least three independent experiments. (*) $p < 0.05$; (***) $p < 0.001$ compared to MDI-treated positive control cells. (C) Proposed mechanism of methyl cinnamate-mediated inhibition of adipogenesis. Methyl cinnamate inhibits adipocyte differentiation by activating CaMKK2−AMPK signaling that leads to down-regulation of the expression of SREBP-1, PPARγ, and C/EBPα and, in turn, the adipogenic specific genes aP2 and adiponectin. Besides, methyl cinnamate enhances the activation of AMPK and increases phospho-ACC levels to impair lipid synthesis in 3T3-L1 adipocytes. In another way, methyl cinnamate might negatively regulate PPARγ activity. These actions contribute the inhibition effect of methyl cinnamate on adipocyte differentiation. (→, activation; —|, inhibition).

are strong PPARγ agonists and potent insulin-sensitizing agents. However, PPARγ activation is a risk factor of obesity and osteoporosis because of increased adipogenesis and bone marrow adiposity.[32] Several lines of evidence indicate that TZD increases fracture risk in postmenopausal women and diabetes patients.[33,34] PPARγ antagonists have been reported to effectively inhibit adipogenesis and improve insulin sensitivity in vitro and in vivo.[35] Many natural compounds, including EGCG, berberine, and curcumin, have been reported to suppress PPARγ expression and act as AMPK activators, which are used in the prevention of many metabolic diseases.[5,6,12] Methyl cinnamate, with the bioactivity of modulating PPARγ expression and AMPK activation, might be beneficial for treating obesity and metabolic disorders.

Research has revealed that methyl cinnamate has antimicrobial activity against *Escherichia coli*, *Candida albicans*, and *Bacillus subtilis*, is a tyrosinase inhibitor that can prevent food browning, and repels mycophages in *Tricholoma matsutake*.[20,36] In this study, we found that methyl cinnamate had an inhibitory effect on adipogenesis, and we examined the antiadipogenic effect of some compounds with structures similar to that of methyl cinnamate. The results showed that methyl cinnamate and ethyl cinnamate significantly reduced triglyceride accumulation in 3T3-L1 cells. The ester form of phenylpropane seems to be more effective with respect to inducing antiadipogenic activity than the acid form, and the absence of the double bond in the propyl chain of the phenylpropane ester abolished the inhibitory effect. Methyl cinnamate exhibited a better inhibitory effect than ethyl cinnamate. Therefore, our results suggest that the double bond in the propyl chain of phenylpropane ester is important for the antiadipogenic activity and that there is a preference for the methyl group.

Methyl cinnamate has been approved as a flavor ingredient by the U.S. Food and Drug Administration (FDA) and as a substance that may be used in foodstuffs by the Council of Europe.[37] In addition, many studies have confirmed the safety of methyl cinnamate by evaluating acute toxicity, skin irritation, and genotoxicity.[37] In vivo studies have demonstrated that methyl cinnamate can be metabolized to form hippuric acid (67% in rats) and glucosiduronic acid (3% in rats) and is excreted in the urine.[38] The therapeutic potentials of volatile components have been discussed. A variety of natural aroma and flavor constituents of plants have antibacterial, antiviral, antioxidant, anticancer, and antidiabetic activities. Therefore, investigating the function of flavor molecules might be a way to identify new drugs from nature, and flavor constituents may be useful in the prevention and treatment of disease.[39] In summary, this is the first report that methyl cinnamate has an inhibitory effect on adipogenesis in vitro. We propose methyl cinnamate inhibits adipogenesis by activating CaMKK2−AMPK signaling and suppressing expression of adipogenic transcription factors and, in turn, adipocyte specific genes and lipid synthesis (Figure 6C). Methyl cinnamate is considered to be safe and is currently used as a fragrance agent and a food additive. Therefore, methyl cinnamate might have the potential to be developed as an antiobesity agent and as a treatment for obesity-related metabolic syndrome.

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■ ABBREVIATIONS USED

ACC, acetyl-CoA carboxylase; AICAR, aminoimidazole carboxamide ribonucleotide; AMPK, AMP-activated protein kinase; aP2, adipocyte fatty acid binding protein; C/EBP, CCAAT/enhancer-binding protein; EGCG, epigallocatechin gallate; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; GPDH, Glycerol-3-phosphate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PPARγ, peroxisome proliferator-activated receptor γ; SPREB-1, sterol regulatory element binding protein-1; TZD, thiazolidinedione.

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