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RESEARCH ARTICLES

Thiacremonone, a sulfur compound isolated from garlic, attenuates lipid accumulation partially mediated via AMPK activation in 3T3-L1 adipocytes☆

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Abstract

Garlic extracts exert anti-cancer and anti-inflammatory effects. However, the anti-adipogenic effect of garlic-derived compounds remains unclear. In this study, we examined the effect of thiacremonone, a sulfur compound isolated from garlic, on adipocyte differentiation using 3T3-L1 cells. We found that thiacremonone significantly inhibited 3T3-L1 differentiation via down-regulation of adipogenesis-related transcription factors and adipogenic markers. The inhibitory effect mainly occurred at the early phase of differentiation in 3T3-L1 cells. There was no cytotoxic effect of thiacremonone in 3T3-L1 cells and treatment of differentiating 3T3-L1 cells with thiacremonone resulted in AMPK activation, which led to an attenuated expression of acetyl CoA carboxylase-1 (ACC-1), an essential enzyme for the synthesis and usage of fatty acids. Moreover, thiacremonone enhanced the mRNA level of carnitine palmitoyltransferase (CPT-1). The modulating effect of thiacremonone on expressions of genes involved in lipolysis was partially abrogated by treatment with compound C, an AMPK inhibitor. Taken together, these results indicated that thiacremonone-induced AMPK activation, inhibition of ACC-1 expression and concomitant recovery of CPT-1 expression resulted in the suppression of intracellular lipid droplet levels, suggesting that thiacremonone may induce reduction of lipid synthesis and increases in fatty acid oxidation partially mediated via AMPK activation. Thiacremonone may be a promising compound for the treatment of obesity.

Keywords: Thiacremonone; Adipocyte; AMPK; Bioflavonoid; PPARγ

1. Introduction

Obesity increases the prevalence of a host of other diseases including type 2 diabetes, hyperlipidemia, hypertension, cardiac injury and cancer [1]. Natural products that modulate the expression of proteins involved in the metabolic disorders have attracted much attention. There are two major proteins, AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor γ (PPARγ), which regulate adipocyte differentiation [2]. AMPK is a metabolic master switch regulating glucose and lipid metabolism [3]. AMPK deactivates acetyl CoA carboxylase 1 (ACC-1), a rate-limiting enzyme required for the synthesis of malonyl CoA, a critical substrate for fatty acid biosynthesis and a potent inhibitor of fatty acid oxidation, thereby inhibiting lipogenesis [4]. AMPK also activates the activity of malonyl CoA decarboxylase, thus inhibiting the supply of substrate for lipogenesis [5]. Furthermore, reduction of malonyl CoA increases lipid oxidation by relief of carnitine palmitoyltransferase 1 (CPT-1) inhibition, an outer mitochondrial membrane enzyme that regulates the entry of saturated fatty acids into mitochondria where they undergo subsequent oxidation [6]. Increased mitochondrial fatty acid oxidation results in decreased lipid accumulation and increased insulin sensitivity [7]. Activation of AMPK by pharmacological agents holds considerable potential for reversing metabolic abnormalities associated with type 2 diabetes [7]. In addition to

Abbreviations: Thia, thiacremonone; PPAR, peroxisome proliferator-activated receptor; AMPK, adenosine 5′-phosphate-activated protein kinase; C/EBP, CCAAT/enhancer binding protein; aP2, adipocyte fatty acid binding protein; FAS, fatty acid synthase; ACC, acetyl CoA carboxylase; CPT, carnitine palmitoyl CoA transferase; TZD, troglitazone; PPRE, peroxisome proliferator response element; Pref-1, pre-adipocyte factor -1.

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AMPK, PPARγ, a well-known regulatory protein involved in both diabetes and obesity, is a key member of an adipogenic transcription factor family which is activated under conditions of adipocyte differentiation [8]. Adipogenesis requires a network of transcription factors and multiple signaling pathways for the development of the adipocyte phenotype. When pre-adipocytes are stimulated by an adipocyte differentiation hormonal cocktail, CCAAT/enhancer binding proteins (C/EBPs), such as C/EBPβ and C/EBPδ, are induced and followed by induction of C/EBPα and PPARγ [9,10]. These adipogenic transcription factors regulate the expression of genes involved in adipogenesis and lipogenesis such as adipocyte fatty acid binding protein (aP2) and fatty acid synthase (FAS) [11]. Therefore, the inhibition of PPARγ expression with specific ligands can induce anti-obesity effects. In this study, we identified a candidate anti-obesity compound in garlic. This plant, traditionally used for improving human health in oriental countries, contains certain active components including bioflavonoids [12]. Recently, 2,4-dihydroxy-2,5-dimethylthiophene-3-one, a sulfur-containing compound (named thiacremonone) isolated from garlic, was found to inhibit colon cancer cell growth through induction of apoptotic cell death by modulation of NF-κB [13]. However, the biochemical mechanisms underlying the anti-obesity effects of thiacremonone are still unclear. Therefore, in this study, we examined whether thiacremonone could suppress adipocyte differentiation and activate AMPK signaling in 3T3-L1 adipocyte cells. Based on our results, we suggest that thiacremonone may be used as an AMPK activator for treating obesity and obesity-related disorder.

2. Material and methods

2.1. Reagents and antibodies

Thiacremonone (2,4-dihydroxy-2,5-dimethylthiophene-3-one, purity >97%, ~1% thiacremonone in garlic) was isolated and characterized as previously described (Fig. 1A) [14–16]. Thiacremonone was dissolved in 0.1% DMSO. Methylisobutylxanthine, dexamethasone, insulin, C75 (a potent derivative of the classic FAS inhibitor, cerulenin), AMPK inhibitor compound C (6-[4-(2-piperidin-1-ylethoxy) phenyl]-3-pyridin-4-ylpyrazolo [1,5-*a*] pyrimidine) and oil red O were purchased from Sigma-Aldrich Sigma (St. Louis, MO, USA). The plasmids expressing PPARα, PPARδ and PPARγ were prepared as previously described [14–16]. Troglitazone (TZD), Wy14,643 (Wy) and GW501516 (GW) were from Calbiochem (La Jolla, CA, USA). The specific antibodies against AMPK-alpha and phosho-AMPK-alpha (Thr 172) were from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cell viability assay

To determine the effects of thiacremonone on 3T3-L1 cell viability, cell viability assay was performed using an MTS assay. Cell lines and thiacremonone (0, 0.3, 0.6, 1.2 mM)-treated cells were treated with MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reagent (Promega, Southampton, England, UK) at 24 h and submitted for absorbency measurement at 490 nm using an enzyme-linked immunosorbent assay plate reader (Berthold Technologies, Bad Wildbad, Germany).

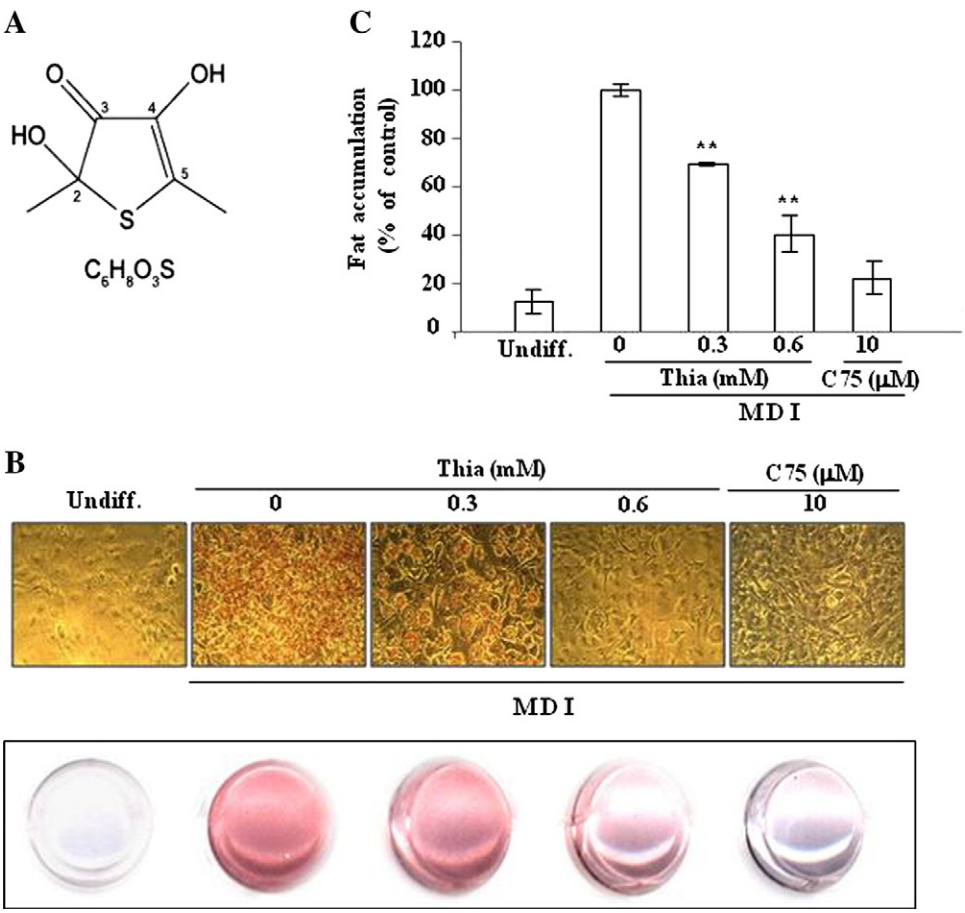

Fig. 1. Effect of thiacremonone on 3T3-L1 adipocyte differentiation. (A) Chemical structure of thiacremonone (2,4-dihydroxy-2,5-dimethylthiophene-3-one). (B) 3T3-L1 pre-adipocytes were differentiated with MDI-differentiation medium in the absence or presence of thiacremonone (Thia) for 8 days. After 8 day, cells and accumulated lipids stained with oil red O were photographed. (C) Accumulated lipids were stained with oil red O, eluted and quantitated by spectrophotometrical analysis at 510 nm. Three independent experiments were performed in triplicate; points represent the mean±S.D. from a representative experiment of three experiments. C75 was used as a FAS inhibitor. $^{**}P<.01$, MDI-treated cultures without Thia (0 mM) vs. the MDI and Thia-treated group.

2.3. Cell culture and adipocyte differentiation

3T3-L1 pre-adipocyte cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS (HyClone Laboratories, Logan, UT, USA) at 37°C in a humidified atmosphere of 5% CO_2. Cells were subcultured every 2–3 days at approximately 80–90% confluency. 3T3-L1 pre-adipocytes were plated in six-well plates with the culture medium. Cells were plated at a density that allowed them to reach confluence in 3 days. The day when cells were initiated for differentiation is designated as Day 0. At this point (Day 0), cells were switched to MDI-differentiation medium (DMEM, 10% FBS, 0.5 mM methylisobutylxanthine, 1 μM dexamethasone and l0 μg/ml insulin) for 2 days. The medium was changed to DMEM containing 1 μg/ml insulin 2 days later. Thereafter, cells were maintained in the culture medium since Day 4, and the process of differentiation proceeded until Day 8, when pre-adipocytes became mature adipocytes. In order to elucidate the effects of thiacremonone on adipogenic differentiation, thiacremonone (0.3, 0.6 mM) was added to 3T3-L1 cells at Day 0. The same concentration of thiacremonone was added at 2-day intervals when the culture medium was replaced. At Day 8, cells were harvested.

2.4. Oil red O staining

Cells on Day 8 were washed twice with PBS, fixed with 0.5% glutaraldehyde for 3 h at room temperature, washed with PBS and dried completely. The fixed cells were then stained with a 0.2% oil red O in working solution that made up isopropanol diluted (6:4) in distilled water for 1 h at room temperature and then washed twice with phosphate buffered saline (PBS). Lipid droplets were stained and observed by light microscopy (Nikon, Tokyo, Japan). Stained oil droplets were dissolved with isopropanol and quantified by spectrophotometrical analysis at 510 nm.

2.5. Transcriptional activity assay

In order to assess the transcriptional activities of PPARs, HEK293 cells (human embryonic kidney cells) were maintained in complete DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were plated in 24-well plates at 1.5×10^5 cells per well. The next day, they were transiently transfected with plasmids expressing PPARα, PPARδ or PPARγ with a (PPRE×3)-thymidine kinase-luciferase reporter construct (1 μg per well), using a Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. After 24 h, the cells were treated with thiacremonone in the absence or presence of specific ligands (0.5, 5 μM): TZD, GW501516 and Wy14,643. After 24 h, cells were harvested and lysed in the recommended lysis buffer for luciferase assay (Promega, Madison, WI, USA). The luciferase activities were evaluated at least in triplicate. The assay results were expressed as an average of three independent experiments±S.D. in relative luciferase activity units.

2.6. Reverse-transcription PCR

In order to conduct reverse-transcription PCR (RT-PCR), total RNA was isolated using an easy-BLUE total RNA extraction kit (iNtRon Biotechnology, South Korea) according to the manufacturer's protocol. Reverse transcription was performed using ProSTAR (Stratagene, La Jolla, CA, USA). After strand cDNA was synthesized by using Oligo (dT), cDNA was used for amplification of specific target genes by PCR. The sequences of the PCR primer pairs for amplification of each gene were as follows: PPARγ (sense, 5′-tgatatcgaccagctgaatcca-3′; antisense, 5′-ccgctcgagctaata-caagtccttgt-3′), C/EBPα (sense, 5′-atggagtcggccgacttctac-3′; antisense, 5′-caggaa ctcgtcgttgaaggc-3′), aP2 (sense, 5′-acatgatcatcagcgtaaatggg-3′; antisense, 5′-tcataa cacattccaccagc-3′), SREBP-1 (sense, 5′- ctcaggtcatgttggaaacc-3′; antisense, 5′-agacag ggagttctcagatg-3′), fatty acid synthase (FAS) (sense, 5′-ccctgaaatcccagcacttc-3′; antisense, 5′-ggcatggctgctgtaggggt-3′), ACC-1 (sense, 5′-gtcagcggatgggcggaatg-3′; antisense, 5′-cgccgg atgccatgctcaac-3′), uncoupling protein-1 (UCP-2) (sense, 5′-gcagctttgaagaacgagaca-3′; antisense, 5′-cttccggagcatggtaag-3′), GAPDH (sense, 5′-accacagtccatgccatcac-3′; antisense, 5′-tccacc accctgttgctgta-3′). GAPDH was used as the RNA loading control. PCR products were separated on 1% agarose gels and stained with ethidium bromide.

2.7. Quantitative real-time PCR

Real-time PCR performed with relative quantification protocol on a Chromo 4 Real-Time PCR system (Bio-Rad, Hercules, CA, USA), using iQ SYBR Green Supermix (Bio-Rad) for amplification detection. Primer pairs producing a unique band and no primer dimerization were selected for the quantitative real-time PCR (qRT-PCR) assays. The sequences of the PCR primer pairs were as follows: Pref-1 (sense, 5′-ctttcggccacagcacctat-3′; antisense, 5′-tgcactgccatggttcctt-3′), C/EBPδ (sense, 5′-gatctgcacggcctgttgta-3′; antisense, 5′-ctccac tgcccacctgtca-3′), C/EBPβ (sense, 5′- gtttcgggacttgatgcaatc-3′; antisense, 5′-aacaaccccgcaggaacat-3′), C/EBPα (sense, 5′-ggaacagctgagccgtgaac-3′; antisense, 5′-gcaacccgaaaccatcct-3′), PPARγ (sense, 5′-gatgcactgcctatgagcactt-3′; antisense, 5′-agaggtccacagagctgattcc-3′), β-actin (sense, 5′-ctg ggacgatatggagaaga-3′; antisense, 5′-agaggcatacagggacaaca-3′). Results were analyzed with the Opticon Monitor 3 software (version 3.1) (Bio-Rad) and using an efficiency calibrated model. All target genes were normalized to the housekeeping gene, β-actin. All values were calculated from three independent trials.

2.8. Western blot analysis

Cells were washed three times with ice-cold PBS, scraped on ice and lysed in RIPA buffer (50 mM NaCl, 10 mM Tris, 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 5 mM EDTA and 0.1 M Na-orthovanadates, pH 7.4). Total cell proteins (100 μg) were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% skimmed milk overnight and incubated with primary antibodies (diluted 1:1000) for 1 h at room temperature. After washing with Tris-buffered saline containing 0.05% Tween-20 (TBST), the membrane was then incubated with horseradish peroxidase conjugated secondary antibodies (diluted 1:3000) for 1 h at room temperature. Antibody binding on the PVDF membrane was detected with an enhanced chemiluminescence (ECL) solution (Amersham Bioscience, Buckinghamshire, UK) and X-ray film.

2.9. Statistical analysis

The experiments were conducted either in duplicate or in triplicate, and all experiments were repeated at least three times with similar results. The data were expressed as the means±S.D. Statistical analysis was done using the Student's *t* test, with the following significance levels: $^*P<.01$, $^{**}P<.001$.

3. Results

3.1. Thiacremonone inhibited 3T3-L1 adipogenesis

To examine whether thiacremonone is cytotoxic, 3T3-L1 cells were exposed to high concentrations of thiacremonone (0, 0.3, 0.6, 1.2 mM) and cytotoxicity was determined by an MTS assay. Thiacremonone did not affect cell viability even at a high concentration of 1.2 mM (200 μg/ml; data not shown). The treatment concentrations of thiacremonone (0.3, 0.6 mM) were determined in the following experiments because 1.2 mM is too high and there was no cytotoxicity in 3T3-L1 cells even at a higher concentration. To examine the effects of thiacremonone on adipogenesis, thiacremonone was added to the medium every 2 days during cell differentiation. To measure adipogenesis, cells were stained with oil red O on Day 8 of differentiation and showed that thiacremonone significantly inhibited lipid accumulation by up to 40% in the cytoplasm of 3T3-L1 adipocytes. C75, a potent FAS inhibitor used for a positive control, efficiently inhibited intracellular fat accumulation in differentiated adipocytes (Fig. 1B and C). These results suggest that thiacremonone efficiently inhibited adipocyte differentiation.

3.2. Thiacremonone inhibited the expression of adipogenesis-related transcription factors and markers during the early stage of differentiation

3T3-L1 adipogenic differentiation requires a network of transcription factors and adipogenic markers [17,18]. When pre-adipocytes are stimulated by an adipocyte differentiation hormonal cocktail, CCAAT/enhancer binding proteins (C/EBPs), such as C/EBPβ and C/EBPδ, are induced and followed by induction of C/EBPα and PPARγ, two transcription factors which are up-regulated and work cooperatively to enhance the expression of several other adipogenic markers such as aP2 and FAS [9,10]. Therefore, the inhibition of PPARγ expression with specific ligands can induce anti-obesity effects. In order to investigate whether thiacremonone inhibits PPARγ, which is involved in adipocyte differentiation, HEK293 cells were transiently transfected with PPARα, PPARδ or PPARγ with (PPRE×3)-thymidine kinase-luciferase expression vectors. Cells were then treated with specific ligands for each of these nuclear receptors in the presence or absence of thiacremonone. While known specific ligands strongly activated reporter genes, thiacremonone itself did not cause any significant change in the basal level of transcriptional activities of PPARα, PPARδ or PPARγ (Fig. 2A–C). Similarly, thiacremonone did not inhibit specific ligand-stimulated

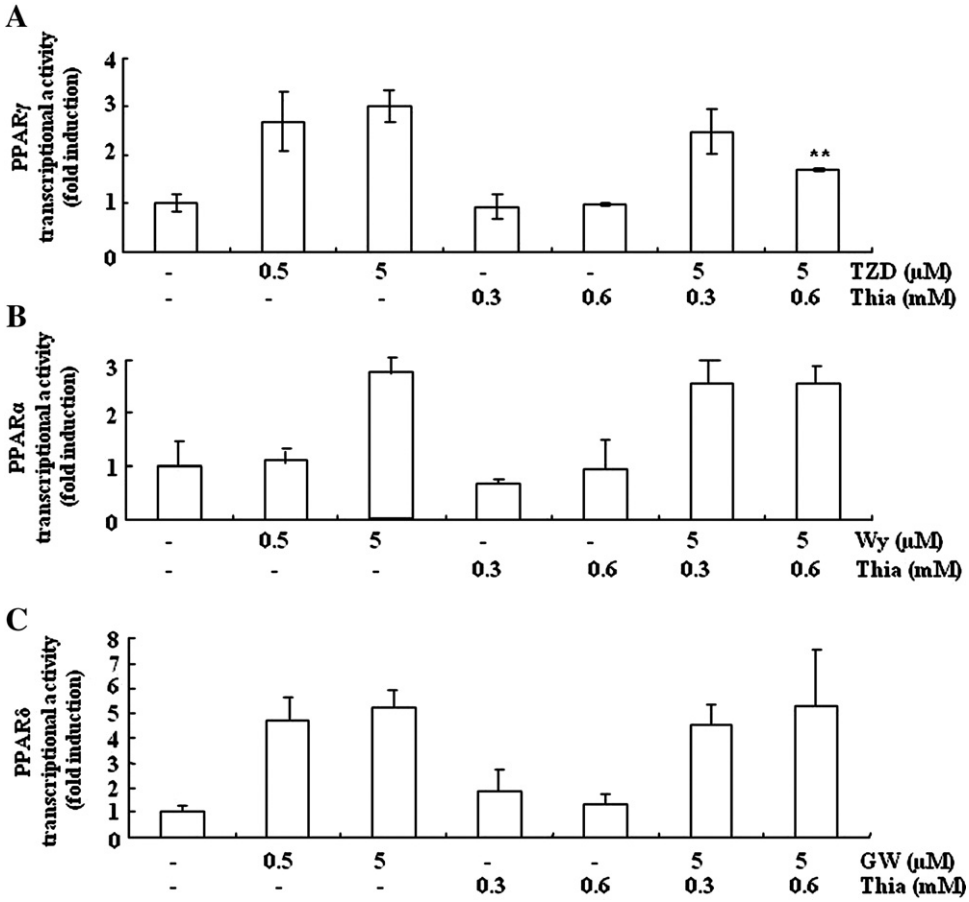

Fig. 2. Effect of thiacremonone on PPAR transactivation. HEK293 cells were co-transfected with the reporter construct (PPRE-thymidine kinase-luciferase reporter vector) and PPARγ (A), PPARα (B), and PPARδ (C) expression vectors. Cells were then treated with thiacremonone in the presence or absence of specific agonists (TZD for PPARγ, Wy14,643 for PPARα and GW501516 for PPARδ) for 24 h. Values are expressed as the mean±S.D. of three different experiments done in triplicate. **P<.01, Thia and the TZD-treated group vs. the TZD-treated group.

transcriptional activities of PPARα (Fig. 2B) or PPARδ (Fig. 2C). However, it inhibited troglitazone (TZD)-induced PPARγ transcriptional activity at a high concentration (0.6 mM) (Fig. 2A). Thus, thiacremonone can be considered to be a weak PPARγ antagonist that is able to inhibit the TZD-induced transcriptional activity of PPARγ. These results indicate that thiacremonone inhibits adipogenesis via inhibition of PPARγ activation. Pre-adipocyte factor-1 (Pref-1) is an early differentiation marker which is highly expressed in 3T3-L1 pre-adipocytes and disappears after their differentiation into mature adipocytes [19]. To further elucidate whether thiacremonone regulates the expression of C/EBPβ and C/EBPδ, two transcription factors upstream of PPARγ and C/EBPα were quantitated by using qRT-PCR analysis. Thiacremonone significantly suppressed the expression levels of C/EBPβ and C/EBPδ on Day 2 of the early differentiation of 3T3-L1 cells (Fig. 3A). In addition, Pref-1 was significantly reduced during differentiation, and this down-regulation was abolished when cells were treated with thiacremonone (Fig. 3A). Taken together, thiacremonone reduced the levels of C/EBPβ and C/EBPδ, and subsequently inhibited the induction of C/EBPα and PPARγ. Thus, thiacremonone can effectively inhibit the early stage of adipocyte differentiation. PPARγ and C/EBPα regulate the expression of their target genes such as aP2, ADD1/SREBP1 and FAS [12]. We next elucidated whether the expressions of PPARγ and C/EBPα target genes were inhibited by thiacremonone during the differentiation. The mRNA expression levels of aP2, ADD1/SREBP1 and FAS were inhibited by thiacremonone (Fig. 3B).

3.3. Enhanced AMPK activation was involved in the inhibitory effects of thiacremonone on 3T3-L1 adipogenesis

To investigate whether AMPK, one of the critical factors for the prevention of obesity [7], is activated by thiacremonone during 3T3-L1 differentiation, the level of phosphorylated AMPKα, which represents the enzymatic activity of AMPK, was analyzed and compared with the total level of AMPKα. As shown in Fig. 4A, AMPK phosphorylation was induced by thiacremonone treatment during the early differentiation of 3T3-L1 cells. To further confirm the above results, adipocytes were pretreated with AMPK inhibitor compound C for 30 min and then exposed to thiacremonone for 6 h. AMPK activation induced by thiacremonone was partially abrogated by pretreatment with AMPK inhibitor compound C (Fig. 4B).

3.4. Thiacremonone modulated the expression of genes involved in fatty acid metabolism and energy expenditure

Long-term AMPK activation regulates the expression of genes involved in fatty acid synthesis, fatty acid oxidation and energy expenditure [3]. Therefore, we examined whether thiacremonone is able to regulate these genes. Indeed, thiacremonone treatment activated AMPK (Fig. 4B) and up-regulated UCP-2 gene, which are involved in energy expenditure [20], while the AMPK inhibitor compound C inhibited AMPK phosphorylation and UCP-2 gene expression induced by thiacremonone as expected (Fig. 4B and C). ACC is an essential enzyme for the synthesis and usage of fatty acids.

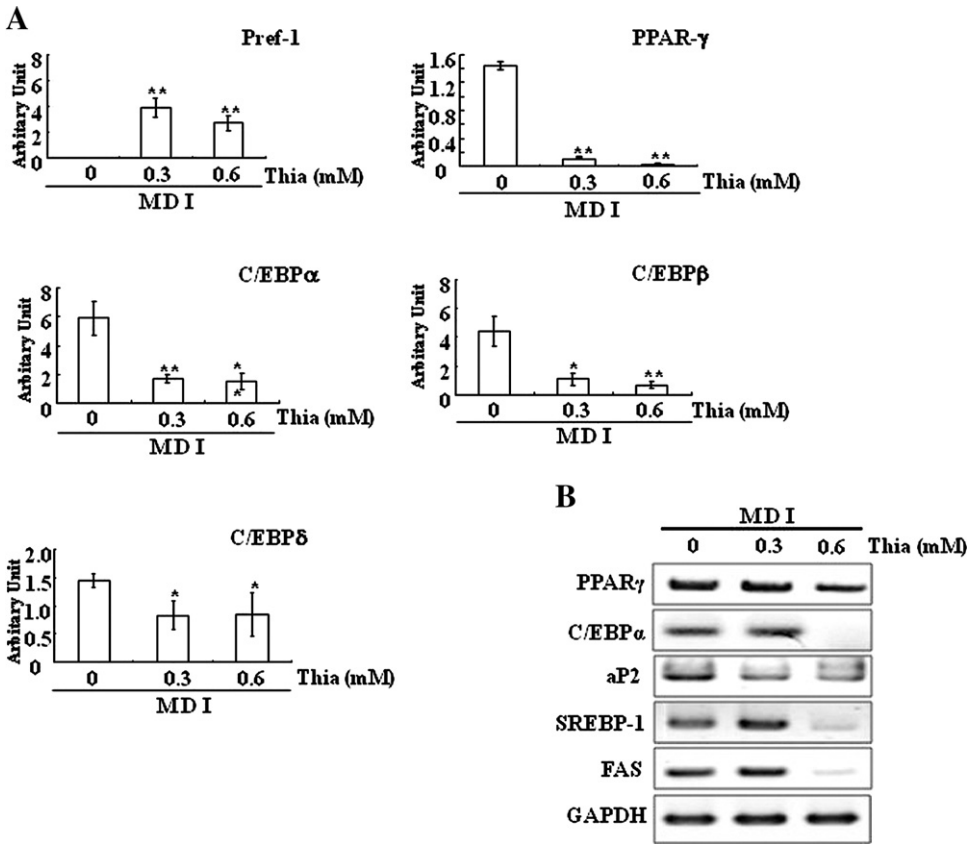

Fig. 3. Effect of thiacremonone on the expression of transcription factors and their target adipogenic genes. (A) 3T3-L1 pre-adipocytes were treated with thiacremonone for 8 days during adipocyte differentiation by MDI-differentiation medium, and mRNA levels of each gene were estimated by quantitative real-time PCR with β-actin as a reference gene. The expression levels of C/EBPβ and C/EBPδ, two transcription factors upstream of PPARγ and C/EBPα, were estimated by quantitative real-time PCR on Day 2 of differentiation. The values of the *y* axes represent the mRNA ratios of the respective genes to β-actin. Values are expressed as the mean±S.D. from a representative of three different experiments. $^{*}P<.05$, $^{**}P<.01$, MDI vs. the Thia-treated group. (B) 3T3-L1 pre-adipocytes were treated with thiacremonone for 8 days during adipocyte differentiation by MDI-differentiation medium. The mRNA levels of each gene were examined by RT-PCR. GAPDH was used as an internal control.

ACC activity is inhibited by p-AMPK [21]. Thiacremonone treatment phosphorylated AMPK and suppressed ACC-1 expression in 3T3-L1 adipocytes and also induced a concomitant increase in the expression of CPT-1 mRNA, an outer mitochondrial membrane enzyme that regulates the entry of saturated fatty acids into the mitochondria where they undergo subsequent oxidation (Fig. 4C). The modulating effects of thiacremonone on these lipolysis genes were partially abrogated by AMPK inhibitor treatment (Fig. 4C). Taken together, these results suggest that thiacremonone may induce a reduction of lipid synthesis and increases in fatty acid oxidation partially mediated via AMPK activation.

4. Discussion

Garlic and garlic-derived compounds are associated with pharmacological effects such as anti-tumor, anti-inflammatory and antioxidant activities [22]. Recently, the sulfur-containing compound thiacremonone was isolated from garlic [23] and was found to induce apoptosis in human colon cancer cell and inhibit cell growth [13]. However, its anti-obesity effects have not been elucidated. Controlling adipocyte differentiation is important for pharmacological intervention and treatment of obesity. AMPK and PPARγ appear to be involved in adipocyte differentiation and maturation and thus can be potential drug targets for treatment of obesity [24]. In the present study, we examined the effect of thiacremonone on adipocyte differentiation of 3T3-L1 cells. Our study was focused on examining whether thiacremonone inhibits 3T3-L1 adipocyte differentiation by regulating adipogenic gene expression by modulating AMPK and PPARγ transcriptional activity. During differentiation, thiacremonone significantly inhibited 3T3-L1 adipogenesis and neutral fat accumulation (Fig. 1B and C). It is notable that the concentration of thiacremonone used in this study was much higher than that usually used. Indeed, 3T3-L1 cells were very tolerable to thiacremonone. PPARγ and C/EBPs play a role in the initiation of adipocyte differentiation and induce the synthesis of various adipogenic genes [25]. Thiacremonone also significantly inhibited the expression levels of C/EBPα and PPARγ, two master regulators of adipogenesis (Fig. 3), indicating that thiacremonone might inhibit 3T3-L1 differentiation via suppressing the expression of adipogenesis-related transcription factors and markers. Down-regulation of late adipogenic makers such as aP2 and FAS by thiacremonone further supported this speculation. Meanwhile, PPARγ transcriptional activity was reduced (Fig. 2), supporting that thiacremonone down-regulated PPARγ expression as well as its transcriptional activity. AMPK phosphorylates the transcriptional coactivator p300 and induces its interaction with PPARγ [26]. It was supposed that thiacremonone might suppress PPARγ transcriptional activity via activating AMPK and phosphorylating the transcriptional coactivators and hence leading to the inhibition of their abilities to interact with PPARγ. In addition, thiacremonone inhibited the mRNA expression of C/EBPβ and C/EBPδ, specific adipogenic markers of the early differentiation stage in 3T3-L1 cells, whereas Pref-1, a specific pre-adipogenic marker, was observed in thiacremonone-treated 3T3-L1 cells (Fig. 3). These data indicate that thiacremonone regulates adipocyte differentiation during the early stage of differentiation by inhibiting C/EBPβ and C/EBPδ expression.

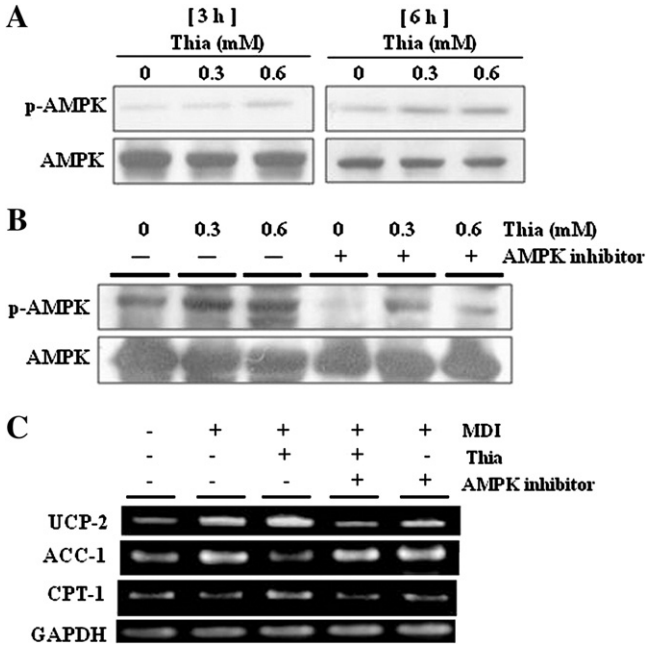

Fig. 4. Effects of thiacremonone on genes involved in fatty acid oxidation and energy expenditure in 3T3-L1 adipocyte cells. (A) AMPK activation during the process of adipocyte differentiation. At Day 2, adipocytes were treated with thiacremonone for 3 and 6 h. AMPK phosphorylation and AMPK levels were determined by Western blot analyses. Equal amounts (50 μg) of cell extracts were loaded into the SDS-PAGE wells. (B) During the process of adipocyte differentiation, adipocytes were pretreated with the AMPK inhibitor compound C (20 μM) at Day 2 and exposed to thiacremonone for 6 h. AMPK phosphorylation and AMPK levels were determined by Western blot analyses. Equal amounts (50 μg) of cell extracts were loaded into the SDS-PAGE wells. Results were confirmed by at least two repeated experiments. (C) Adipocytes were pretreated with the AMPK inhibitor compound C (20 μM) at Day 2 for 30 min and exposed to thiacremonone for 6 h. mRNA levels of each gene involved in fatty acid oxidation and energy expenditure were examined by RT-PCR. GAPDH was used as an internal control.

Several adipogenic markers were also down-regulated in thiacremonone-treated cells. Overall, these results demonstrated that thiacremonone inhibited 3T3-L1 differentiation via exerting an inhibitory effect on the signaling cascade that ultimately culminated in adipogenesis. We speculated that AMPK activation was partially involved in the inhibitory effect of thiacremonone on adipogenesis since AMPK activation has been reported to inhibit adipocyte differentiation [27]. The level of UCP-2 gene expression was increased by an AMPK activator, AICAR, and decreased by compound C, an AMPK inhibitor [28,29]. Thiacremonone induced AMPK activation and up-regulated UCP-2 gene expression, while compound C inhibited AMPK phosphorylation and down-regulated UCP-2 gene expression induced by thiacremonone. ACC is an essential enzyme for the synthesis and usage of fatty acids, and ACC activity is inhibited by phosphorylation of AMPK. Thiacremonone phosphorylated AMPK, and the level of ACC1 gene expression was decreased (Fig. 4C). Also, thiacremonone concomitant recovery of CPT-1 expression in the 3T3-L1 cells (Fig. 4C). These data suggest that increased mitochondrial fatty acid oxidation and interruption of lipogenesis result in decreased lipid accumulation (Fig. 1B and C). AMPK also mediates the suppression of lipogenic gene expression such as ACC and FAS via decreased action of the transcription factor SREBP-1c [30]. As expected, ACC-1 and FAS expressions were also down-regulated by thiacremonone (Figs. 3B and 4C). Although AMPK and PPARγ signaling pathways were involved in the anti-adipogenesis effects, no direct correlation was confirmed between the AMPK and PPARγ signaling pathways modulated by thiacremonone. However, these studies strongly suggest that AMPK and PPARγ are one of the targets of and are modulated by thiacremonone, and the thiacremonone-associated anti-obesity effects may be exerted due to inhibition of adipocyte differentiation.

In conclusion, we have investigated the inhibitory effects of thiacremonone on 3T3-L1 adipogenesis for the first time. Thiacremonone inhibited 3T3-L1 adipogenic differentiation via down-regulating the expression of C/EBPβ, C/EBPδ and downstream adipogenic factors, as well as activating the AMPK signaling pathway. Therefore, thiacremonone may be used to prevent obesity and obesity-related disorders.

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