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2,4,5-TMBA, a Natural Inhibitor of Cyclooxygenase-2, Suppresses Adipogenesis and Promotes Lipolysis in 3T3-L1 Adipocytes

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ABSTRACT: Obesity is a global health problem. Because of the high costs and side effects of obesity-treatment drugs, the potential of natural products as alternatives for treating obesity is under exploration. 2,4,5-Trimethoxybenzaldehyde (2,4,5-TMBA) present in plant roots, seeds, and leaves was reported to be a significant inhibitor of cyclooxygenase-2 (COX-2) activity at the concentration of 100 μg/mL. Because COX-2 is associated with differentiation of preadipocytes, the murine 3T3-L1 cells were cultured with 100 μg/mL of 2,4,5-TMBA during differentiation and after the cells were fully differentiated to study the effect of 2,4,5-TMBA on adipogenesis and lipolysis. Oil Red O staining and triglyceride assay revealed that 2,4,5-TMBA inhibited the formation of lipid droplets during differentiation; moreover, 2,4,5-TMBA down-regulated the protein levels of adipogenic signaling molecules and transcription factors MAP kinase kinase (MEK), extracellular signal-regulated kinase (ERK), CCAAT/enhancer binding protein (C/EBP)α, β, and δ, peroxisome proliferator-activated receptor (PPAR)γ, adipocyte determination and differentiation-dependent factor 1 (ADD1), and the rate-limiting enzyme for lipid synthesis acetyl-CoA carboxylase (ACC). In fully differentiated adipocytes, treatment with 2,4,5-TMBA for 72 h significantly decreased lipid accumulation by increasing the hydrolysis of triglyceride through suppression of perilipin A (lipid droplet coating protein) and up-regulation of hormone-sensitive lipase (HSL). The results of this in vitro study will pioneer future in vivo studies on antiobesity effects of 2,4,5-TMBA and selective COX-2 inhibitors.

KEYWORDS: *2,4,5-TMBA, 3T3-L1 adipocyte, adipogenesis, lipolysis*

INTRODUCTION

Obesity, characterized by excessive lipid accumulation in adipose tissue, is significantly associated with the development of many chronic diseases. Adipose tissue undergoes dynamic remodeling throughout adulthood. The growth of adipose tissue involves the formation of new adipocytes from preadipocytes as well as the increase in the size of adipocytes. Pluripotent cells that reside in the vascular stroma of adipose tissue may commit to preadipocytes under stimulation. The preadipocytes will undergo mitotic clonal expansion when treated with differentiation inducers. Following clonal expansion, preadipocytes then undergo terminal differentiation into adipocytes.[1] Transcription factors CCAAT/enhancer binding protein (C/EBP)β and δ are first induced in response to the adipogenic factors, then in turn activate peroxisome proliferator-activated receptor (PPAR)γ and C/EBPα,[2] which are essential for the expression of a large group of genes that produce the adipocyte phenotype.[3] In the presence of adipogenic stimulation, several signaling pathways have been demonstrated to be involved in the activation of C/EBPs and PPARγ. Among them, activation of the MEK/MAPK (MAP kinase kinase/mitogen-activated protein kinase) signaling pathway in the early stage of differentiation is likely to be required to promote adipogenesis by enhancing PPARγ and C/EBPα expression.[4]

Treatments that inhibit the differentiation of preadipocytes or promote the lipolysis of adipocytes provide effective approaches for treating obesity. Because of the high costs and side effects of obesity-treatment drugs, the potential of natural compounds as alternatives for treating obesity is under exploration. 2,4,5-Trimethoxybenzaldehyde (TMBA) (Figure 1A) is a bitter principle present in plant roots, seeds, and leaves. 2,4,5-TMBA isolated from carrot (*Daucus carota* L.) seeds significantly inhibits cyclooxygenase II (COX-2) activity at the concentration of 100 μg/mL compared to three commercial nonsteroidal anti-inflammatory drugs Aspinin, Ibuprofen, and Naproxen at their IC_{50} values 180, 2.52, and 2.06 μg/mL, respectively.[5] COX-2 plays a crucial role in inflammatory reactions; moreover, it was found to be involved in the modulation of adipogenesis in 3T3-L1 cells.[6] 15-deoxy-$\Delta^{12,14}$-postaglandin (PG)J_2 (15d-PGJ_2), a COX-2 derived prostaglandin, has been known to activate PPARγ and promote efficient differentiation of fibroblasts to adipocytes.[7] Fajas et al. reported that COX-2 inhibition by selective COX-2 inhibitors limits cell cycle reentry required before terminal adipocyte differentiation.[8] The animals deficient in COX-2 had attenuated adipose tissue differentiation and showed significant reduction in total body weight and percent body fat.[6] On the basis of these reports, we were interested in exploring the antiobesity potential of 2,4,5-TMBA, a natural COX-2 inhibitor.

In this study, 2,4,5-TMBA at the concentration of 100 μg/mL as used in Momin's study[5] was cultured with preadipocytes during differentiation (coculture study) or with fully differentiated adipocytes (postculture study) to investigate the effects of 2,4,5-TMBA on the differentiation of preadipocytes and

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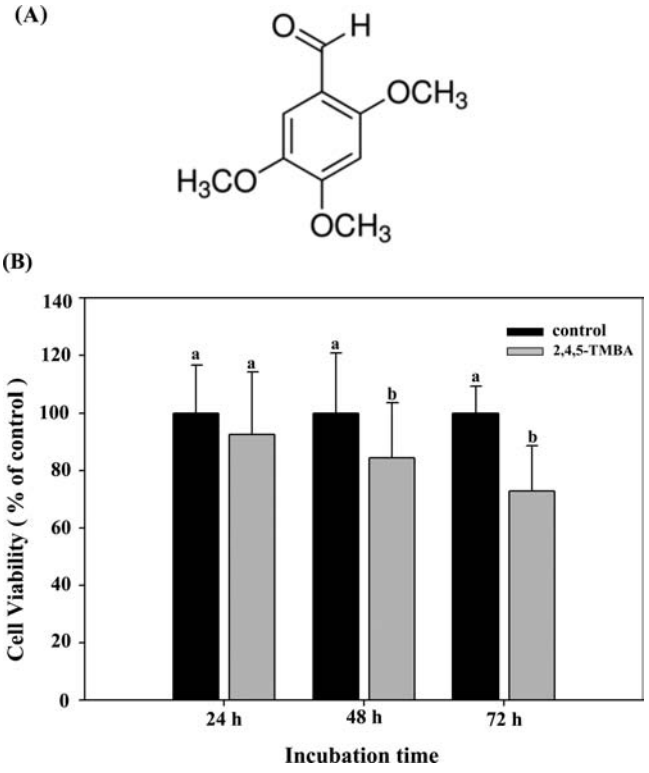

Figure 1. (A) Structure of 2,4,5-trimethoxybenzaldehyde (TMBA). (B) Effect of 2,4,5-TMBA on cell viability of 3T3-L1 adipocytes. Fully differentiated mature adipocytes were treated with 2,4,5-TMBA (100 μg/mL) for 24−72 h. Values indicate the means ± SD. Groups with the same letter are not significantly different (α = 0.05).

lipolysis of mature adipocytes. 3T3-L1 mouse embryonic fibroblast, adipose like cell line was used in the study because it is a widely used cell model for adipocyte research.[9] Differentiation of preadipocytes was induced by treatment with a cocktail of inducers, including insulin, dexamethasone, and methylisobutylxanthine. In addition to lipid accumulation, expression of several transcription factors and proteins associated with adipogenesis and lipolysis were examined. The results of this in vitro study will pioneer future animal studies on antiobesity effects of 2,4,5-TMBA and selective COX-2 inhibitors.

MATERIALS AND METHODS

Chemicals. 2,4,5-Trimethoxybenzaldehyde, Dulbecco's modified Eagle's medium (DMEM), 3-isobutyl-1-methylxanthine (IBMX), insulin, and dexamethasone (DEX) were obtained from Sigma (St. Louis, MO). Bovine calf serum (BCS), fetal bovine serum (FBS), and penicillin−streptomycin were obtained from Gibco (Grand Island, NY). Anti-PPARγ and anti-hormone sensitive lipase antibodies were purchased from Cayman (Ann Arbor, MI). Anti-C/EBPα, β, δ, anti-aP2, anti-MEK, anti-JNK, anti-p38MAPK, and anti-pyruvate antibodies were purchased from Epitomics (Burlingame, CA). Anti-β-actin and anti-COX-2 antibodies were obtained from Novus (Littleton, CO). Antileptin and antiadiponectin antibodies were obtained from Enzo (Farmingdale, NY). Anti-ERK, anti-acetylcarboxylase, and anti-pyruvate dehydrogenase antibodies were obtained from Cell Signaling (Danvers, MA). Anti-ADD1/SREBP1 antibody was obtained from Biovision (Milpitas, CA). Anti-citrate synthetase antibody was purchased from Abcam (Cambridge, UK). Goat anti-rabbit peroxidase-conjugated antibody was purchased from Jackson ImmunoResearch (West Grove, PA). Adiponectin and leptin ELISA kits were obtained from Assaypro (San Diego, CA) and Peprotech (Pucky Hill, NJ), respectively.

Cell Culture. 3T3-L1 preadipocytes (BCRC 60159) purchased from Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan) were seeded into 6-well plates at a concentration of 10^5/well and cultured in DMEM supplemented with 10% bovine calf serum at 37 °C in a humidified atmosphere containing 5% CO_2. Two days after confluence, cells were cultured in FBS-containing DMEM (10%, v/v) with the addition of adipogenic factors (0.5 mM IBMX, 1 μM DEX, 5 μg/mL insulin) to induce differentiation (Day 0). Two days later (Day 2), the medium was changed to DMEM supplemented with 10% FBS and 5 μg/mL insulin for another two days. Afterward (Day 4), the medium was changed to DMEM supplemented with 10% FBS only. For the coculture study, 2,4,5-TMBA (0.1 g dissolved in 2 mL of DMSO) was added to the medium from Day 0 to Day 8 (final concentration 100 μg/mL). Control samples were prepared by adding isovolumetric DMSO to the culture medium. For the postculture study, 2,4,5-TMBA was added to the medium on Day 8 (when the cells were fully differentiated) at a final concentration of 100 μg/mL, followed by another 72-h culture.

MTT Assay. 3T3-L1 cells were seeded in 96-well plates at a concentration of 10^4/well. Twenty-four hours after seeding, the cells were treated with 100 μg/mL of 2,4,5-TMBA for 24 h or for the whole 8-day differentiation period. Fully differentiated adipocytes were also treated with 100 μg/mL of 2,4,5-TMBA for 24−72 h to test the cytotoxicity. At the end of treatment, cells were cultured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a final concentration of 0.5 mg/mL for another 4 h. The purple MTT formazan was dissolved by DMSO and the absorbance at 570 nm was taken with a spectrophotometer. The absorbance is proportional to the viability of adipocytes.

Oil Red O Staining of Adipocytes and Quantification of Triglyceride Accumulation. Formation of lipid droplets in adipocytes was observed by Oil Red O staining. Cells were washed twice with phosphate-buffered saline (PBS) and fixed with 10% formalin for 1 h, followed by the washes with water twice. Cells were stained with Oil Red O (three parts of 0.35% Oil Red O dye in isopropanol and two parts of water) for 15 min at room temperature and then washed twice with water before photographing under a microscope. To assay the triglyceride accumulated in adipocytes, the lipid droplets in water-washed stained cells were dissolved in isopropanol and then quantified by measuring the absorbance at 520 nm. The results were expressed as the lipid content relative to that of the control group.[10]

Assay of Adipokines and Glycerol Release. At the end of treatment, the culture medium was collected for the analysis of leptin, adiponectin, and glycerol. The production and release of leptin and adiponectin were analyzed by the Peprotech ELISA kit (Pucky Hill, NJ) and Assaypro ELISA kit (San Diego, CA), respectively. Glycerol is the product of triglyceride hydrolysis. The release of glycerol into the medium was measured by Adipolysis Assay Kit (Cayman, Ann Arbor, MI).

Western Blotting. At the end of treatment, the cells were washed twice with cold PBS, harvested, and then resuspended in lysis buffer containing 1% Nonidet P 40, 150 mM sodium chloride, and 50 mM Tris-HCl, pH 7.5. The cell suspension was centrifuged at 10000*g* for 30 min, and the supernatant was collected and subjected to further centrifugation at 105000*g* for 60 min. The supernatant was collected for immunoblot analysis. The protein content in supernatant samples was determined by Dc Protein Assay Kit (Bio-Rad, Hercules, CA). Equal amount of proteins were denatured and separated by gel electrophoresis before being transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Pittsburgh, PA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing Tween (TBST), 20 mM Tris-HCl, pH 8.3, 137 mM NaCl, and 0.1% Tween-20 for 1 h and then incubated sequentially with primary antibody for 3 h and horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive proteins were detected using an enhanced chemiluminescence kit

(ECL, PerkinElmer, Waltham, MA), and the film was analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis. Statistical analysis was performed using the Statistical Analysis System (SAS Institute, Cary, NC). Analysis of variance (ANOVA) and Student−Newmann−Keuls multiple range test were used to determine the significant difference among means (α = 0.05).

RESULTS

Cytotoxic Effect of 2,4,5-TMBA on 3T3-L1 Cells. To investigate the cytotoxicity of 2,4,5-TMBA at the concentration of 100 μg/mL, the viability of mature adipocytes and preadipocytes was estimated. When treated with 100 μg/mL of 2,4,5-TMBA for 24, 48, or 72 h, the viability of fully differentiated 3T3-L1 adipocytes was decreased by 8.35, 15.54, and 27.26%, respectively (Figure 1B). When the preadiocytes were treated with 100 μg/mL of 2,4,5-TMBA for 24 h before differentiation medium was supplemented, the cell viability was decreased by 26.46%. When the preadipocytes were cocultured with 100 μg/mL of 2,4,5-TMBA during differentiation, the cell viability was decreased by 25.82% at the end of the 8-day differentiation period (data not shown). On the basis of the results, 100 μg/mL is a reasonable concentration to be applied to 3T3-L1 cells.

Effect of 2,4,5-TMBA on the Expression of MEK and MAPKs during Differentiation. Phosphorylation of the MEK/MAPK (ERK, JNK, and p38MAPK) signaling pathway is required for the differentiation of preadipocytes. Coculturing with 2,4,5-TMBA during differentiation significantly ($p < 0.05$) suppressed the expression of phosphorylated MEK and ERK1 (ERK1, but not ERK2, is essential for the differentiation of 3T3-L1). The expression of JNK and p38MAPK was also inhibited by 2,4,5-TMBA, but the effect was not statistically significant (Figure 2A).

Effect of 2,4,5-TMBA on the Expression of C/EBPs, PPARγ, and ADD1 during the Differentiation of Adipocytes. C/EBPβ, C/EBPδ, C/EBPα, PPARγ, and ADD1 are key transcription factors involved in adipogenesis. Activation of C/EBPβ and C/EBPδ leads to the expression of PPARγ and C/EBPα. In the coculture study, C/EBPβ, C/EBPδ, and C/EBPα were down-regulated by 2,4,5-TMBA (Figure 2B). Expression of PPARγ1 and PPARγ2 was both suppressed by 2,4,5-TMBA, but the effect on PPARγ2 was not statistically significant ($p > 0.05$). The expression of adipocyte determination and differentiation-dependent factor 1 (ADD1) was also significantly down-regulated by the treatment of 2,4,5-TMBA during differentiation.

Effect of 2,4,5-TMBA on Lipid Accumulation during Differentiation of Adipocytes. When 2,4,5-TMBA was added to the differentiation medium at the final concentration of 100 μg/mL, the triglyceride formed in the adipocytes was significantly inhibited by 27.64% (Figure 3A). Treatment with 2,4,5-TMBA during differentiation significantly inhibited the expression of COX-2, which has been reported to be positively associated with adipogenesis (Figure 3B).[6] Perilipin A, the lipid droplet coating protein which is crucial for the intact structure of a lipid droplet, was slightly down-regulated by 2,4,5-TMBA ($p > 0.05$). Newton et al. demonstrated a significant change in expression of pyruvate dehydrogenase (PD), pyruvate carboxylase (PC), and citrate synthase (CS) during differentiation.[11] In this study, expression of PD was slightly ($p > 0.05$) inhibited by 100 μg/mL of 2,4,5-TMBA but PC and CS were not down-regulated. Acetyl-CoA carboxylase (ACC) is the rate-limiting

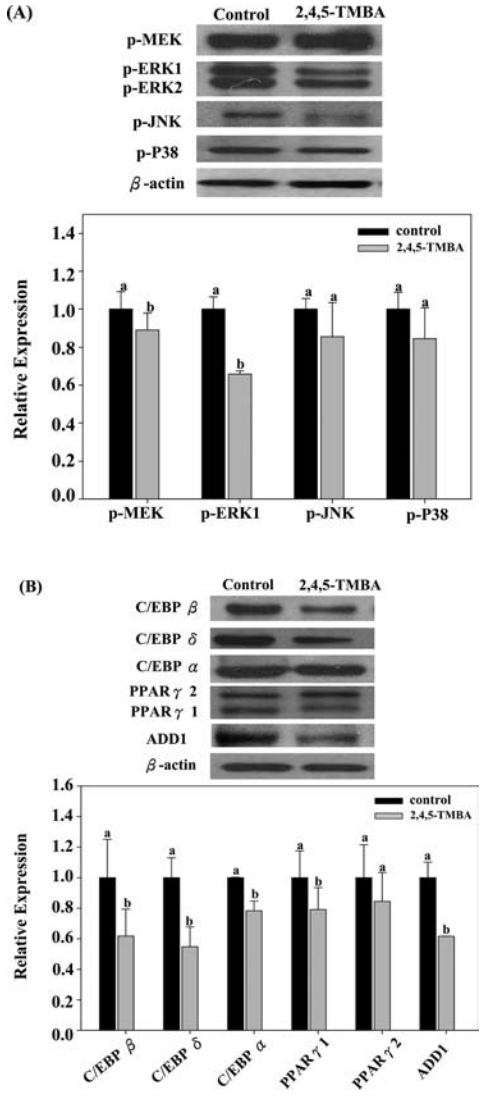

Figure 2. Effects of 2,4,5-TMBA on the protein expression of (A) phosphorylated MEK, ERK1/2. JNK, and p38MAPK; (B) C/EBPα, C/EBPβ, C/EBPδ, PPARγ1/2, and ADD1 during differentiation. 3T3-L1 preadipocytes were cultured with 2,4,5-TMBA (100 μg/mL) during differentiation for 8 days. The relative expression of proteins was calculated according to the reference band of β-actin. Values are the means of three replicated cultures (α = 0.05).

enzyme in fatty acid synthesis. Figure 3B shows that the expression of ACC was significantly suppressed by 2,4,5-TMBA during differentiation ($p < 0.05$).

Effect of 2,4,5-TMBA on Lipolysis in Fully differentiated Adipocytes. To investigate the effect of 2,4,5-TMBA on triglyceride hydrolysis, fully differentiated adipocytes were treated with 100 μg/mL of 2,4,5-TMBA for 72 h. The results of Oil Red O staining and triglyceride content show that 2,4,5-TMBA decreased the amount of lipid accumulated in adipocytes by 26.47% (Figure 4A). Because glycerol is the product of triglyceride hydrolysis, the amount of glycerol released into medium was also measured (Figure 4A). After 72 h of incubation with 100 μg/mL of 2,4,5-TMBA, the amount of glycerol released was increased by 22.91%. The results of triglyceride accumulation and glycerol release indicate an increase of lipolysis by the treatment of 2,4,5-TMBA.

Effect of 2,4,5-TMBA on the Expression of HSL and Perilipin A in Fully differentiated Adipocytes. Hormone

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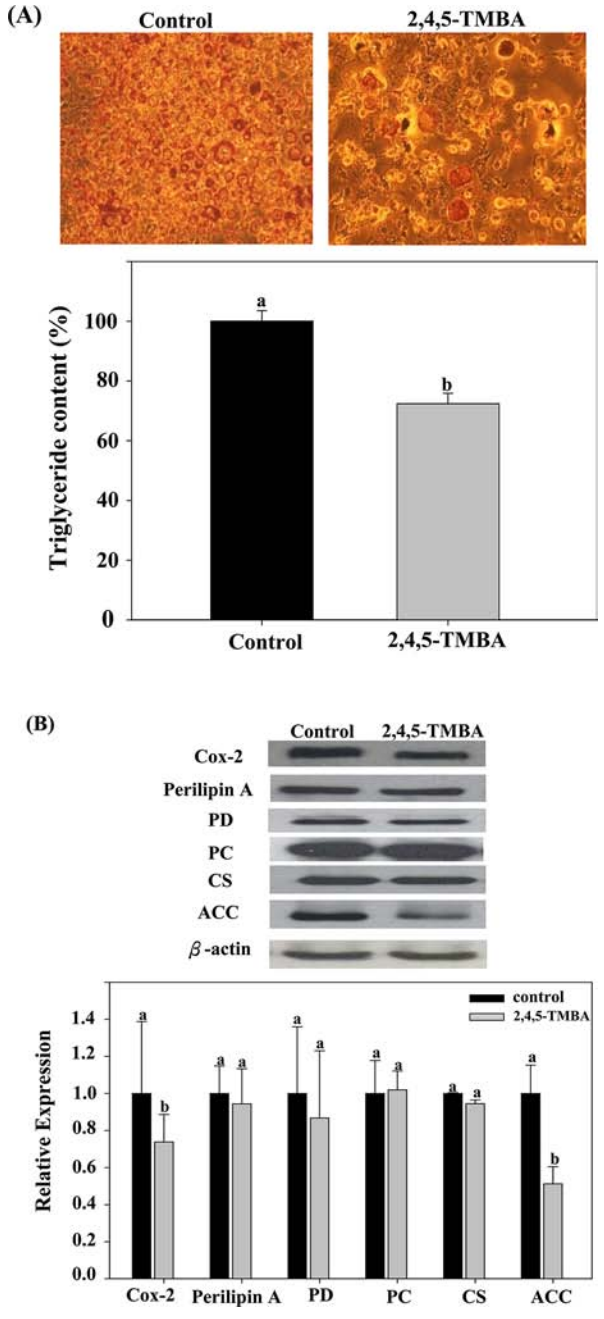

Figure 3. Effects of 2,4,5-TMBA on (A) Oil Red O staining and triglyceride accumulation; (B) protein expressions of COX-2, perilipin A, pyruvate dehydrogenase (PD), pyruvate carboxylase (PC), citrate synthase (CS), and acetyl-CoA carboxylase (ACC) during differentiation. 3T3-L1 preadipocytes were cultured with 2,4,5-TMBA (100 μg/mL) during differentiation for 8 days. The relative expression of proteins was calculated according to the reference band of β-actin. Values are the means of three replicated cultures (α = 0.05).

sensitive lipase (HSL) plays a crucial role in the hydrolysis of triglyceride. However, for triglycerides in lipid droplets to be accessed by HSL, perilipin A covered on the surface of lipid droplets needs to be removed. Treatment with 100 μg/mL 2,4,5-TMBA for 72 h significantly ($p < 0.05$) suppressed the expression of perilipin A and up-regulated HSL in mature adipocytes (Figure 4B).

Effect of 2,4,5-TMBA on the Secretion of Adipokines in Fully differentiated Adipocytes. Adipose tissue is not only the storage site of energy but also an endocrine organ. The hormones derived from adipocytes are called adipokines and have been shown to be a quantitative marker of adiposity. Leptin and adiponectin are two adipokines produced by adipose tissue. Treatment with 100 μg/mL of 2,4,5-TMBA for 72 h decreased the secretion of leptin by 27.11%. Secretion of adiponectin was also inhibited by 2,4,5-TMBA, but the effect was not statistically significant ($p > 0.05$) (Figure 4C).

DISCUSSION

This study was conducted to provide in vitro evidence for antiadipogenic effect of 2,4,5-TMBA, a COX-2 inhibitor found in plants. COX-2 not only is an inflammatory mediator but also is involved in the modulation of adipogenesis. Inhibitors of COX-2 repress the clonal expansion phase of adipogenesis and limit cell cycle reentry required before terminal differentiation.[8] Ghoshal et al. found that deficiency of COX-2 attenuated the differentiation of adipose tissue in mice.[6] In our study, coculture with 2,4,5-TMBA during differentiation inhibited the expression of COX-2 and adipogenesis, which coinsides with previous findings.

Although several signaling pathways are involved in the modulation of adipogenesis, transient activation of the MEK/MAPK signaling pathway is required for the differentiation of preadipocytes.[12] Adipogenic stimuli from the membrane activate MEK, which leads to the phosphorylation of a group of mitogen-activated protein kinase (MAPK): extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38 MAPK. Phosphorylated ERK1, but not ERK2, is essential for the differentiation of 3T3-L1 fibroblasts to adipocytes. By using ERK1$^{-/-}$ mice, Bost et al. demonstrated that animals lacking ERK1 have decreased adiposity and fewer adipocytes than wild-type animals; moreover, cultures of preadiocytes isolated from ERK1$^{-/-}$ mice exhibit impaired adipogenesis.[13] JNK is a crucial mediator of obesity. In obese animals, JNK activity is abnormally elevated; in contrast, adiposity is significantly decreased in JNK knockout mice.[14] Activity of p38MAPK is required for adipogenesis. Pharmacological inhibition of p38MAPK during adipocyte differentiation markedly reduced triglyceride accumulation and adipocyte markers expression.[15] In this study, the coculture of 3T3-L1 preadiocytes with 2,4,5-TMBA during differentiation down-regulated the phosphorylation of MEK, ERK1, JNK, and p38MAPK, indicating a suppression of the signaling pathway required for adipogenesis (Figure 5). Several natural compounds also target MEK/MAPK pathways for inhibition of adipogenesis. Lii et al. reported the suppression of adipogenesis through ERK by diallyl trisulfide.[16] Moreover, both EGCG[(−)-epigallocatechin gallate] and evodiamine (a major alkaloidal compound extracted from the fruit of *Evodia fructus*) suppress adipocyte differentiation through MEK/ERK pathways.[17,18]

Studies in cell lines have shown that activation of MEK/MAPKs is rapidly followed by the expression of transcription factors C/EBPβ and C/EBPδ, which further induces the expression of PPARγ and C/EBPα, the two transcription factors that oversee the entire terminal differentiation process and are needed for the life of mature adipocytes.[19] C/EBPβ and C/EBPδ have a synergistic role in terminal adipocyte differentiation. Adipogenesis is reduced in embryonic fibroblasts derived from mice lacking either C/EBPβ or C/EBPδ; moreover, embryonic fibroblasts derived from C/EBPβ and C/EBPδ double knockout mice do not differentiate into mature

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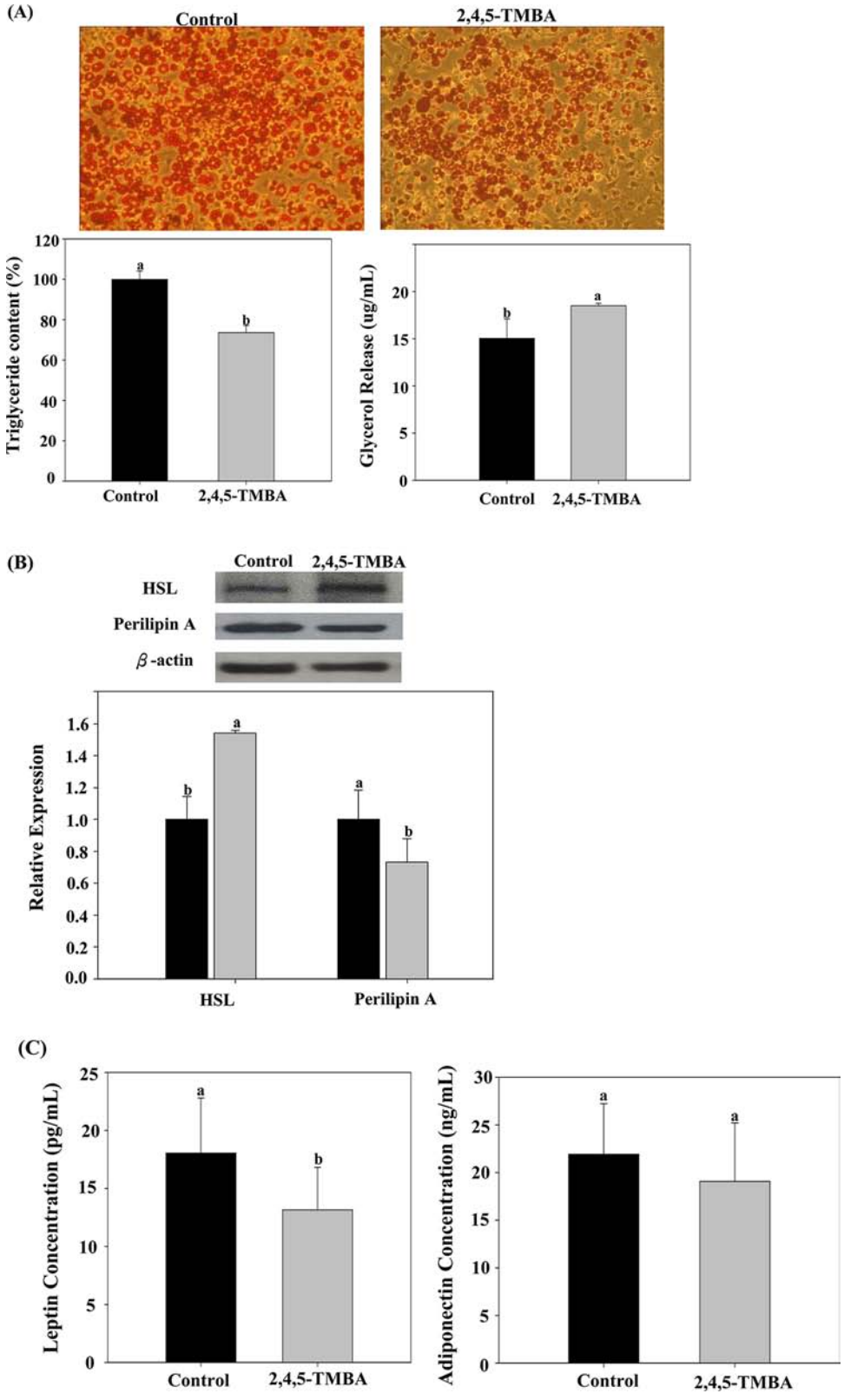

Figure 4. Effects of 2,4,5-TMBA on (A) Oil Red O staining, triglyceride accumulation, and glycerol release; (B) protein expression of hormone sensitive lipase (HSL) and perilipin A; (C) secretion of leptin and adiponectin in fully differentiated adipocytes. Mature adipocytes were cultured with 2,4,5-TMBA (100 μg/mL) for 72 h. The relative expression of proteins was calculated according to the reference band of β-actin. Values are means of three replicated cultures (α = 0.05).

adipocytes.[20] PPARγ is the master regulator of adipogenesis. When activated, PPARγ enhances the expression of C/EBPα, then both factors synergistically induce the genes involved in adipogenesis. PPARγ can promote adipogenesis in C/EBPα-deficient cells, but C/EBPα has no ability to promote adipogenesis in the absence of PPARγ.[21] In addition to

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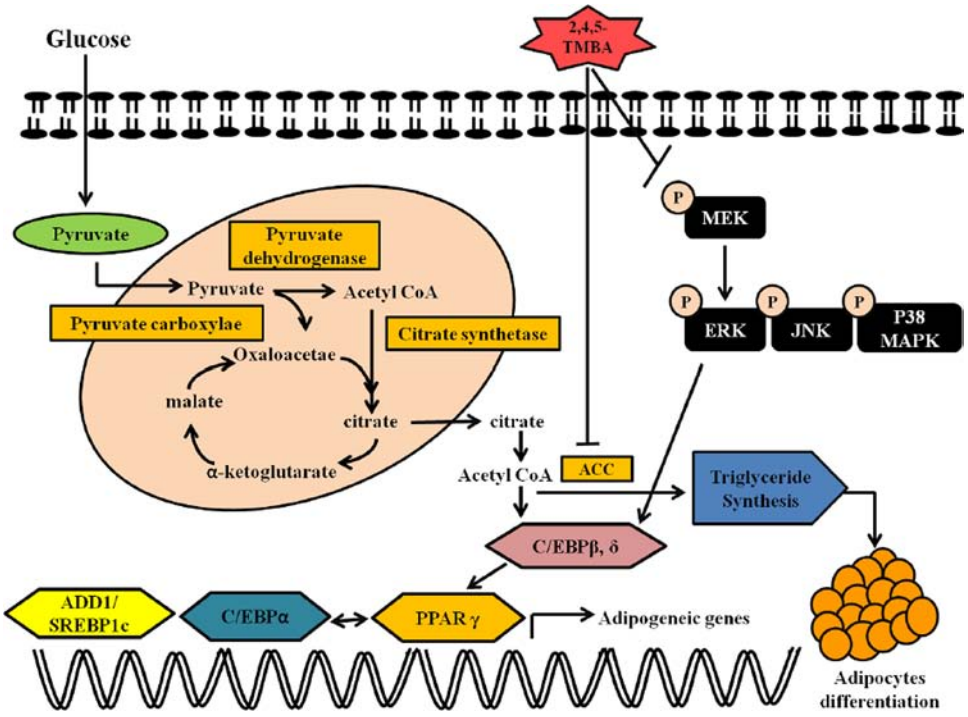

Figure 5. Proposed mechanism for the suppression of adipogenesis by 2,4,5-TMBA.

PPARγ and C/EBPα, ADD1 (adipocyte determination and differentiation-dependent factor 1) is another transcription factor associated with adipogenesis. Ectopic expression of ADD1 was shown to inhibit preadipocyte differentiation, while overexpression of this protein significantly up-regulated the adipogenic activity of PPARγ.[22] In our study, 2,4,5-TMBA not only suppressed MEK/MAPKs but also down-regulated transcription factors C/EBPs, PPARγ, and ADD1, leading to the inhibition of adipogenesis. In previous studies, inhibitory effects of natural compounds on adipogenic transcription factors in adipocytes were investigated. Capsacin, a major pungent ingredient in red pepper, significantly inhibited the expression of PPARγ and C/EBPα.[23] Both resveratrol and quercetin decreased the expression of PPARγ, but only the combination of two compounds was able to down-regulate C/EBPα and significantly inhibit lipid accumulation in adipocytes.[24]

The increase in the size of adipocytes arises in part from progressive lipid accumulation during adipogenesis. Because mitochondria are the sites that govern the extent of triglyceride accumulation, Newton et al. analyzed the mitochondrial proteome in 3T3-L1 adipocytes at different stages of adipogenesis and reported the significant up-regulation of three key enzymes involved in the TCA cycle: pyruvate dehydrogenase (PD), pyruvate carboxylase (PC), and citrate synthase (CS). PD and PC allow pyruvate to enter the TCA cycle as acetyl-CoA and oxaloacetate, respectively.[11] CS combines oxaloacetate and acetyl-CoA into citrate and is considered the overall rate-limiting enzyme for the TCA cycle. In our study, the culture of 3T3-L1 preadipocytes with 2,4,5-TMBA during differentiation did not significantly down-regulate PD, PC, and CS. However, the expression of acetyl-CoA carboxylase (ACC), the rate-limiting enzyme in fatty acid synthesis, was significantly suppressed by 2,4,5-TMBA. According to the role of ACC, down-regulation of this protein may provide a possible mechanism for inhibition of lipid accumulation in adipocytes treated with 2,4,5-TMBA. Ejaz et al. observed an inhibition of ACC activity in the mice which were fed a high-fat diet supplemented with curcumin. These animals showed significantly reduced adiposity and body weight gain.[25]

The hallmark of adipocyte differentiation is the formation and enlargement of intracellular lipid droplets. The composition of lipid droplet coating proteins changes as lipid droplets enlarge and mature. At the late stage of lipid droplet formation and maturation, perilipin replaces the other droplet coating proteins, becoming the only coating protein resides on the large lipid droplets.[26] Perilipin A is the predominant isoform of perilipin proteins found in mature adipocytes. Adipose tissue mass in *perilipin* null mice is only 30% of that in wild type animals;[27] however, expression of perilipin in adipose tissue is elevated in obese subjects.[28] Translocation of perilipin A from the surface of lipid droplets to cytosol is essential for the hydrolysis of lipids by adipocyte lipase.[29] Tansey et al. have reported an elevated lipid hydrolytic activity in isolated adipocytes from *perilipin* null mice.[27] In this study, we observed an inhibition of the expression of perilipin A when mature adipocytes were cultured with 2,4,5-TMBA. Suppression of perilipin A would attenuate the formation of lipid droplets and facilitate lipid hydrolysis by the action of hormone sensitive lipase (HSL), the key enzyme involved in the hydrolysis of triglyceride. HSL is highly expressed in adipocytes and exclusively controls the hydrolysis of triglyceride.[30] Lipolytic stimulation promotes the translocation of perilipin A from the surface of lipid droplets to cytosol and the docking of HSL to the surface of lipid droplets, where HSL gains access to its lipid substrates.[31] In this study, treatment with 2,4,5-TMBA for 72 h inhibited the expression of perilipin A but enhanced the expression of HSL, leading to the significant decrease in lipid accumulation as observed by Oil Red O staining.

Leptin and adiponectin are two of the adipokines secreted by adipose tissues. Both hormones have been shown to be valuable quantitative markers of adiposity in humans.[32] Plasma leptin

concentration positively correlates with body fat content. High leptin level has been related to metabolic syndrome and cardiovascular diseases.[33] Adiponectin is the most abundant protein secreted by adipose tissue. In contrast to most adipokines, adiponectin is decreased in obesity and increased in response to weight reduction.[34] In our study, secretion of leptin and adiponectin was suppressed by treatment of 2,4,5-TMBA. Maeda et al. have shown that PPARγ ligands increase the expression and secretion of adiponectin.[35] Because the expression of COX-2 was suppressed by 2,4,5-TMBA, the production of COX-2 derived ligands for PPARγ was therefore decreased, resulting in less secretion of adiponectin from adipocytes.

In conclusion, our results showed that 2,4,5-TMBA suppressed adipogenesis and promoted lipolysis in 3T3-L1 adipocytes. 2,4,5-TMBA exerted an antiadipogenic effect through down-regulation of MEK/MAPKs, C/EBPs,, ADD1, PPARγ, COX-2, ACC, and perilipin A. Moreover, 2,4,5-TMBA decreased the accumulation of triglycerides in mature adipocytes by increasing the hydrolysis of triglyceride through up-regulating the expression of HSL. On the basis of these results, animals studies will be conducted to explore the bioavailability and physiological functions of 2,4,5-TMBA in vivo. Moreover, the antiadipogenic potential of natural and synthetic COX-2 inhibitors is worth studying.

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Notes

The authors declare no competing financial interest.

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