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2,6-Dimethoxy-1,4-benzoquinone Inhibits 3T3-L1 Adipocyte Differentiation via Regulation of AMPK and mTORC1

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ABSTRACT

2,6-Dimethoxy-1,4-benzoquinone is a natural phytochemical present in fermented wheat germ. It has been reported to exhibit anti-inflammatory, antitumor, and antibacterial activities. However, the anti-adipogenic effects of 2,6-dimethoxy-1,4-benzoquinone and the mechanisms responsible have not previously been elucidated. Such findings may have ramifications for the treatment of obesity. 2,6-Dimethoxy-1,4-benzoquinone (5 and 7.5 μM) significantly reduced the expression of various adipogenic transcription factors, including peroxisome proliferator-activated receptor-γ and CCAAT/enhancer binding protein α as well as adipocyte protein 2 and fatty acid synthase. 2,6-Dimethoxy-1,4-benzoquinone upregulated AMP-dependent protein kinase phosphorylation and inhibited the mature form of sterol regulatory element-binding protein 1c. Notably, 2,6-dimethoxy-1,4-benzoquinone attenuated mammalian target of rapamycin complex 1 activity in 3T3-L1 and mouse embryonic fibroblast cells. These findings highlight a potential role for 2,6-dimethoxy-1,4-benzoquinone in the suppression of adipogenesis. Further studies to determine the anti-obesity effects of 2,6-dimethoxy-1,4-benzoquinone in animal models appear warranted.

Introduction

Benzoquinones have been reported to play important roles in bioenergetic transport, oxidative phosphorylation, electron transport processes, and the inhibition of adipogenesis [1–4]. 2,6-Dimethoxy-1,4-benzoquinone (DMBQ) is a natural phytochemical present in fermented wheat germ [5], and has been reported to exhibit various biological properties including anti-inflammatory, antitumor, and antibacterial activities [6–8]. Recent research has shown that wheat germ extract elicits anti-adipogenic and antioxidant effects in 3T3-L1 adipocytes [9]. However, the effect of DMBQ on adipocyte differentiation has not been previously investigated.

Obesity is a common metabolic disorder in developed countries and is associated with metabolic syndrome, which broadly includes insulin resistance, type 2 diabetes, hyperglycemia, dyslipidemia, and various types of cancer [10–13]. An imbalance of energy intake and expenditure results in the enlargement of adipocytes and their proliferation by adipogenesis, the process by which a preadipocyte transforms into a mature adipocyte via the alteration of cellular, morphological, and biochemical properties within adipose tissue [14–18]. Numerous transcription factors, including CCAAT/enhancer binding protein α (C/EBP-α) and peroxisome proliferator-activated receptor-γ (PPAR-γ), play a pivotal role in the formation of mature adipocytes [19]. Lipid synthesis proteins such as fatty acid synthase (FAS) and sterol regulatory ele-

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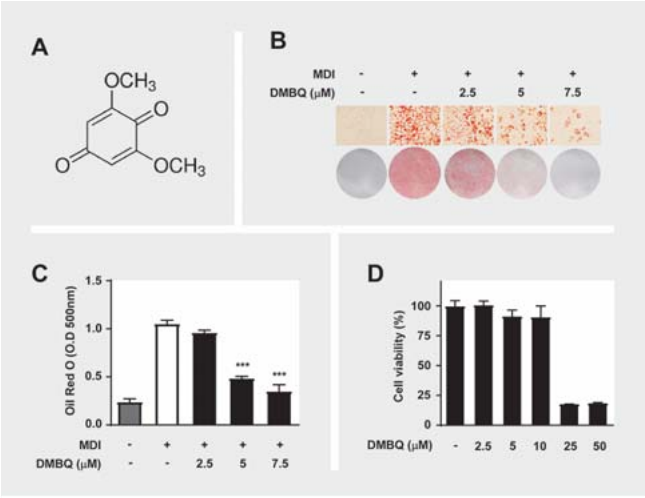
ment-binding protein (SREBP) are involved in fatty acid and cholesterol synthesis, and contribute toward lipid homeostasis.

AMP-dependent protein kinase (AMPK) is activated via elevation of the AMP/ATP ratio, and is a key regulator and energy sensor that integrates nutrients, hormones, and stress signals to maintain whole-body energy balance [20, 21]. AMPK inhibits the ATP consumption pathway, which includes fatty acid and cholesterol synthesis, by interfering with transcription factors and metabolic enzymes such as SREBP-1, acetyl-CoA carboxylase 1 (ACC1), and HMG-CoA reductase [22]. AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide) is an activator of AMPK and suppresses lipid synthesis during adipocyte differentiation by regulating β-oxidation-related proteins [23, 24]. For this reason, AMPKα-knockout mice exhibit increased body weight and fat mass due to increased lipid accumulation by adipocytes when fed a high-fat diet [25].

The mammalian target of rapamycin complex 1 (mTORC1) is another major nutrient sensor that regulates cell metabolism, growth, and protein synthesis [26]. Several studies have demonstrated that the mTORC1 pathway is also involved in the regulation of lipid accumulation during the differentiation of 3T3-L1 preadipocytes [27–29]. mTORC1 enhances SREBP-1 activity, resulting in increased fatty acid and cholesterol synthesis [30]. mTORC1 activity is under the control of several upstream regulators. The PI3K-Akt pathway regulates mTORC1 activity via TSC, and mTORC1 activity is downregulated by AMPK activation in response to energy status [31].

The primary objective of this study was to explore the role of DMBQ in the regulation of adipocyte generation and to investigate the molecular mechanisms of action responsible.



▶ **Fig. 1** Effect of DMBQ on lipid accumulation in 3T3-L1 adipocytes. **A** Structure of DMBQ. **B** DMBQ suppresses lipid accumulation in 3T3-L1 adipocytes. Intracellular lipid content was stained with Oil Red O. 3T3-L1 preadipocytes were differentiated into adipocytes with MDI treatment in the presence or absence of DMBQ for 8 days. **C** Quantification of extracted Oil Red O at 500 nm. **D** DMBQ cytotoxicity in 3T3-L1 cells. Cells were incubated with various concentrations of DMBQ (2.5–50 μM) for 48 h and cytotoxicity was determined by measuring cell viability. Data are mean values ± SD (n = 3); *** $p < 0.001$ versus the group treated with MDI alone.

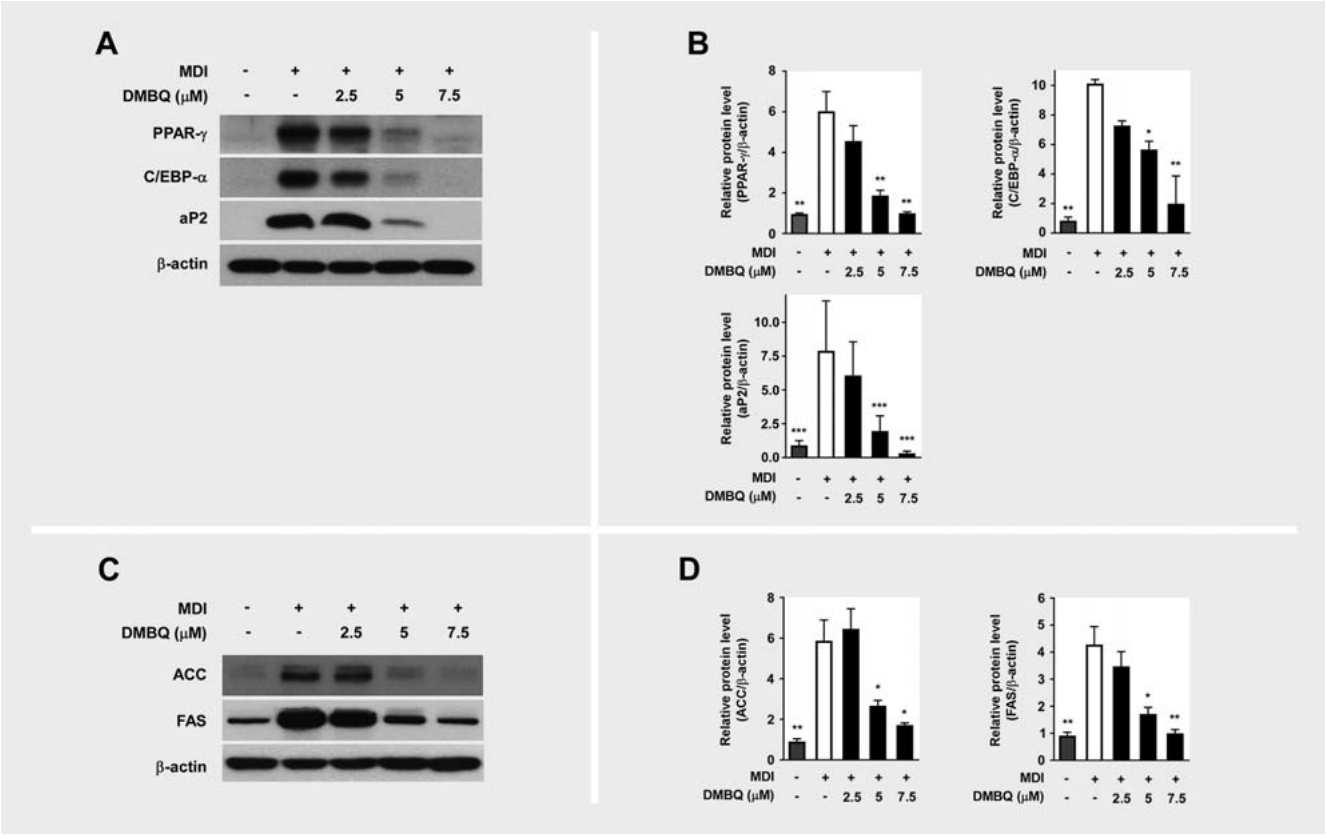
Results

We first examined the effect of DMBQ on MDI-induced (MDI: mixture of isobutylmethylxanthine, dexamethasone, and insulin) adipocyte differentiation in 3T3-L1 cells. The structure of DMBQ is shown in ▶ **Fig. 1 A**. 3T3-L1 cells were maintained in medium containing MDI for 8 days in the presence or absence of DMBQ. On day 8, fully differentiated 3T3-L1 cells exhibited lipid droplets that were strongly stained by Oil Red O, whereas DMBQ (at 2.5, 5, and 7.5 μM) treatment inhibited adipocyte differentiation in a dose-dependent manner (▶ **Fig. 1 B, C**). In comparison to the control cells, treatment with DMBQ significantly downregulated lipid accumulation by 8.7, 54.1, and 66.9 %, respectively. These results indicate that DMBQ prevents MDI-induced lipid accumulation in 3T3-L1 adipocytes. DMBQ did not elicit cytotoxicity up to concentrations as high as 10 μM (▶ **Fig. 1 D**).

PPAR-γ and C/EBP-α are critical transcription factors for adipogenic differentiation [19]. We next evaluated the effect of DMBQ on the protein expression of PPAR-γ, C/EBP-α, and its downstream target adipocyte protein 2 (aP2) by immunoblotting. The MDI-dependent increases in PPAR-γ, C/EBP-α, and aP2 were significantly attenuated at the protein level by DMBQ treatment (▶ **Fig. 2 A, B**). In addition, marked decreases in protein expression of lipogenic enzymes such as ACC (acetyl-CoA carboxylase) and fatty acid synthase (FAS) were observed in DMBQ-treated adipocytes (▶ **Fig. 2 C, D**). These findings suggest that DMBQ suppresses adipocyte differentiation by downregulating adipocyte-specific transcription factors and downstream target genes involved in adipogenesis and lipogenesis.

To clarify whether the activation of AMPK is involved in the inhibition of adipocyte differentiation in the presence of DMBQ, we first examined the effect of DMBQ on AMPK phosphorylation at the protein expression level. DMBQ treatment substantially increased the phosphorylation of AMPK in preadipocytes (▶ **Fig. 3 A**). Activated AMPK is known to inhibit PPAR-γ and SREBP-1 activity [32–34]. Notably, AMPK phosphorylation was significantly increased by DMBQ on day 2 after differentiation, and this effect was maintained until day 8 (▶ **Fig. 3 B**). Compound C (dorsomorphin), a chemical inhibitor of AMPK, was used to confirm the role of DMBQ on AMPK activation. As expected, the upregulation of AMPK phosphorylation by DMBQ was inhibited in the presence of compound C (▶ **Fig. 3 C**). The precursor SREBP-1 is cleaved and forms mature SREBP-1, which translocates to the nucleus and activates the transcription of target genes [35]. We observed that mature SREBP-1 was reduced by DMBQ in a dose-dependent manner (▶ **Fig. 3 D**). Both nuclear and cytosolic SREBP-1 were downregulated by DMBQ treatment (▶ **Fig. 3 E**).

To determine whether AMPK could be involved in the regulation of SREBP1 by DMBQ, 3T3-L1 cells were transfected with anti-AMPK siRNA in the presence or absence of DMBQ. Experiments were conducted to optimize siRNA concentrations for maximal efficacy and specificity for knockdown of AMPK. As shown in ▶ **Fig. 3 F**, a significant reduction in AMPKα expression was only observed in cells treated with siRNA. Although AMPK was down-



▶ **Fig. 2** Effect of DMBQ on adipogenic and lipogenic marker protein expression in 3T3-L1 adipocytes. **A–D** DMBQ inhibits increases in PPAR-γ, C/EBP-α, and aP2 protein expression. **B** Quantification of PPAR-γ, C/EBP-α, and aP2 protein content. **C** DMBQ suppresses ACC and FAS protein expression in 3T3-L1 adipocytes. 3T3-L1 preadipocytes were differentiated into adipocytes in the presence of DMBQ (2.5, 5, 7.5 μM) and cell lysates were recovered after 8 days. Protein expression levels were analyzed by Western blot. Values are means ± SD (n = 3); *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ versus the group treated with MDI alone.

regulated by siRNA knockdown, DMBQ suppressed SREBP1 expression (▶ **Fig. 3F**). These observations suggest that DMBQ regulates SREBP1 through alternative pathways as well as AMPK.

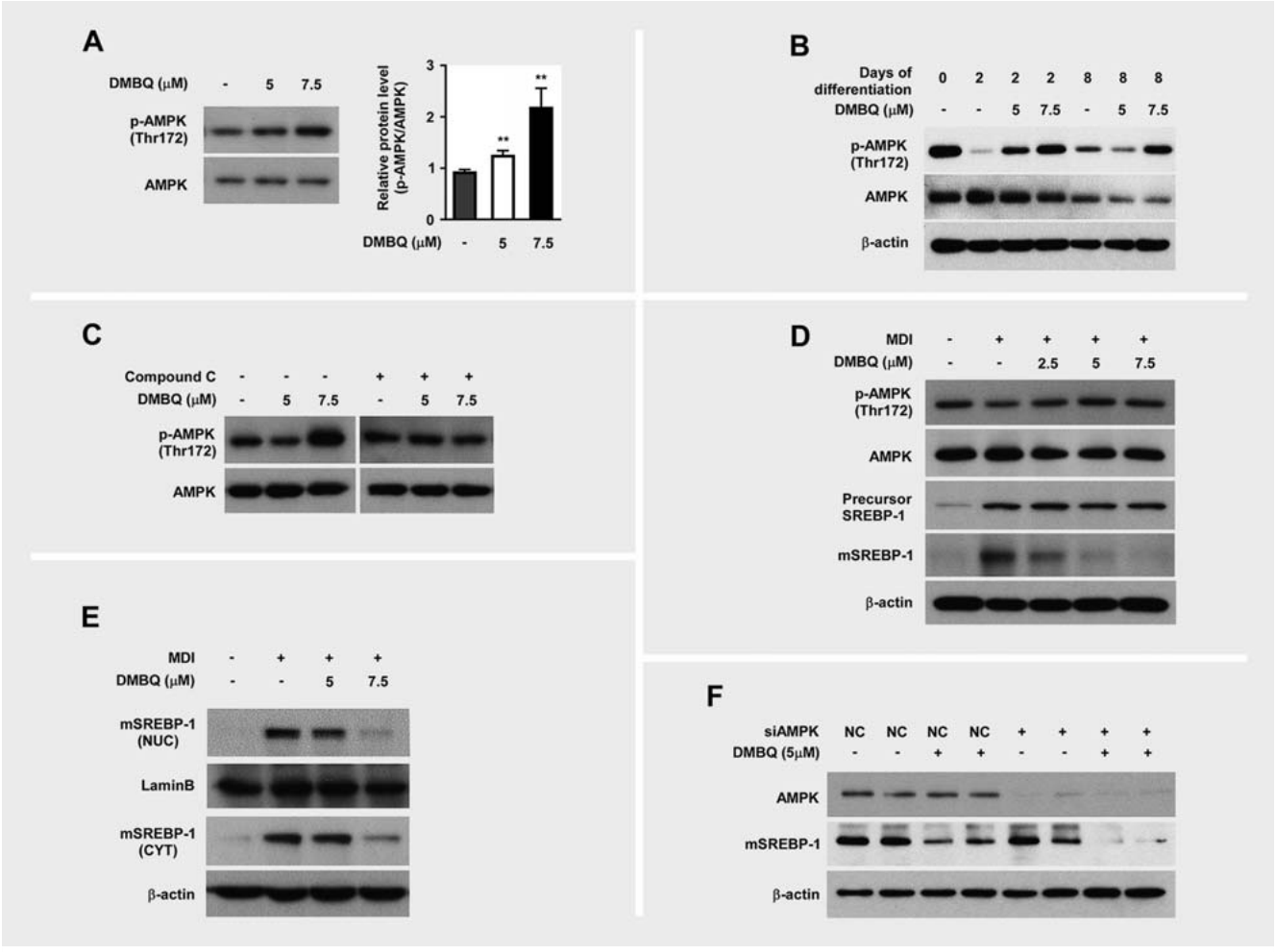
Previous studies have demonstrated that SREBP-1 activation and lipogenesis require the mTOR pathway [30, 36]. We further examined the effect of DMBQ on mTOR signaling in 3T3-L1 and mouse embryonic fibroblast (MEF) cells. The phosphorylation levels of mTOR and 4E-BP1 were reduced in DMBQ-treated cells as compared to the MDI-differentiated cells (▶ **Fig. 4A**). To investigate whether DMBQ regulates insulin-mediated mTOR signaling, MEF cells were stimulated with insulin for 1 h in the presence or absence of DMBQ. DMBQ attenuated insulin-induced phosphorylation of mTOR and 4E-BP1 (▶ **Fig. 4B**). However, phosphorylation of Akt was not decreased by DMBQ (▶ **Fig. 4B**). DMBQ also attenuated phosphorylation of 4E-BP1 in both wild-type (WT) and TSC2-/- MEFs (▶ **Fig. 4C**).

Discussion

Studies have shown that various phytochemicals present in vegetables and plants can regulate the process of adipogenic differentiation [37]. Maslinic acid, a natural triterpene from *Olea europaea L.* (Oleaceae), attenuates 3T3-L1 adipocyte differentiation [38], while Shikonin, a major component of *Lithospermum erythrorhizon Siebold & Zucc.* (Boraginaceae), suppresses early stages of adipocyte differentiation in 3T3-L1 cells [3]. Plant-derived baicalein was also reported to inhibit adipogenesis in 3T3-L1 cells [39]. The present study has identified an anti-adipogenic effect of DMBQ, and explored the molecular mechanisms responsible.

The modulation of AMPK can inhibit the progression of adipogenic differentiation. Fucosterol derived from brown algae suppresses adipogenesis via AMPK activation [40], while Arctigenin found in Arctii Fructus suppresses adipogenic differentiation via the activation of AMPK and reduces obesity in high-fat diet-induced obese mice [41]. We observed that DMBQ increases phosphorylation of AMPK while downregulating mature SREBP-1 and PPARγ expression in 3T3-L1 adipocytes. AMPK inhibits SREBP activation either by inhibiting mTOR or by directly phosphorylating SREBP, resulting in the inhibition of cleavage and translocation of SREBP into the nucleus [34, 42]. AMPK also regulates adipogenesis by inhibiting eIF2α-dependent translation in adipocytes [43]. It has also been reported that activated AMPK inhibits PPARγ transcriptional activity [32].

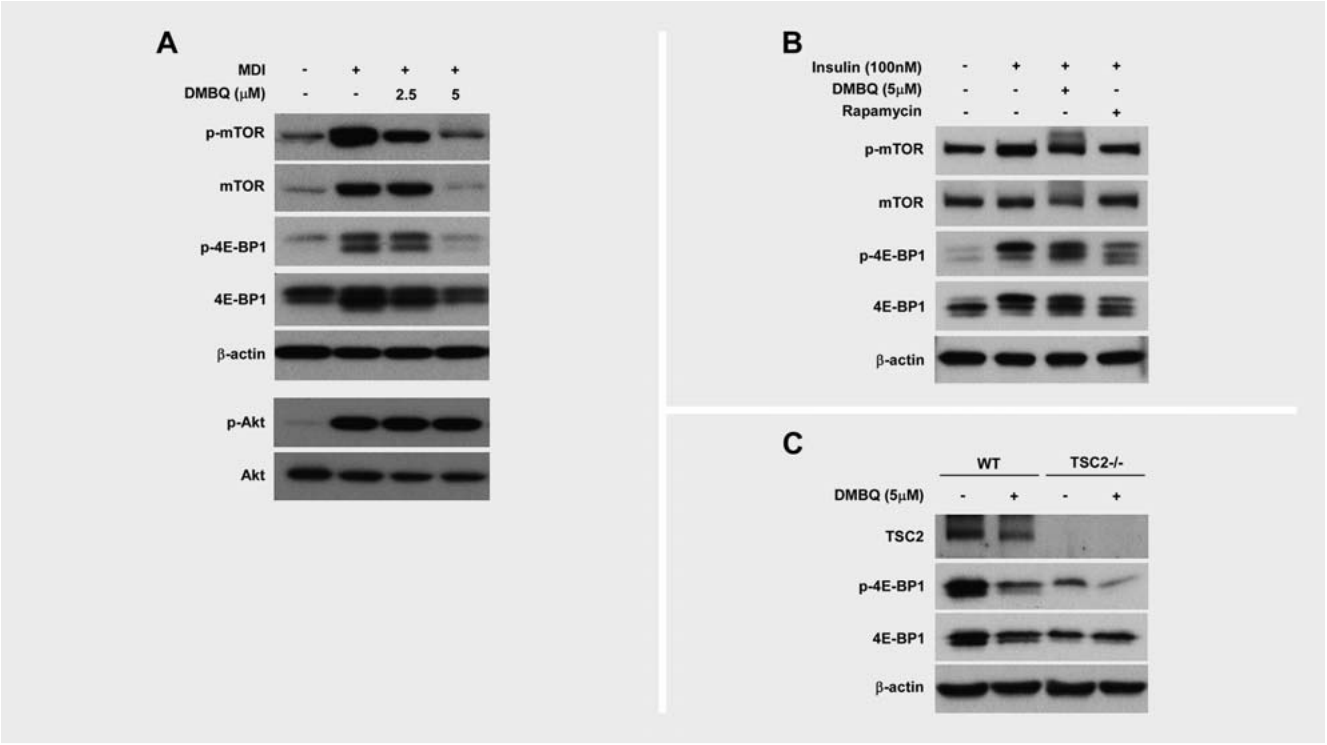
However, our results have shown that AMPK knockdown does not impact the inhibitory efficacy of DMBQ on SREBP-1, suggesting that other signaling factors are involved in DMBQ-dependent



▶ **Fig. 3** Effect of DMBQ on phosphorylation of AMPK and regulation of SREBP in 3T3-L1 adipocytes. **A** DMBQ increases AMPK phosphorylation in a dose-dependent manner in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were treated with DMBQ for 24 h. **B** DMBQ stimulates phosphorylation of AMPK in 3T3-L1 adipocytes. **C** Compound C inhibits DMBQ-dependent increases in AMPK phosphorylation. 3T3-L1 adipocytes were incubated with the AMPK inhibitor compound C (50 μM) for 3 h prior to treatment with DMBQ, and cell lysates were recovered after 24 h. **D** DMBQ inhibits SREBP-1 expression. Differentiating 3T3-L1 cells were treated with varying concentrations of DMBQ (2.5, 5, and 7.5 μM) and cell lysates were recovered after 8 days. **E** Both nuclear (NUC) and cytosolic (CYT) levels of SREBP-1 were attenuated by DMBQ. **F** AMPK knockdown does not affect DMBQ-dependent inhibition of SREBP-1 expression. 3T3-L1 preadipocytes were transfected with siAMPK (20 nM) or scrambled siRNA (negative control, NC, 20 nM) and differentiated with MDI for 8 days. Protein expression was analyzed by Western blot. ** $P < 0.01$ versus control. mSREBP-1, mature SREBP-1, 68 kDa; precursor SREBP-1, 125 kDa.

SREBP-1 regulation. mTORC1 is a major regulator of SREBP1 activity and modulates the transcription, cleavage, and stability of SREBP-1 [44]. It has been demonstrated that mTORC1 regulates SREBP-1 by controlling the nuclear entry of lipin 1 [30]. In the present study, we found that DMBQ inhibited the phosphorylation of mTOR and 4E-BP1 in 3T3-L1 adipocytes without affecting S6K1 phosphorylation. Some studies have shown that mTORC1 regulates the transcription of SREBPs through a mechanism independent of S6K1 [45]. Moreover, other studies have shown that S6K1 could be regulated independent of mTORC1 by Akt-TSC2 [46]. mTORC1 also promotes the translation of PPAR-γ via the 4E-BP-eIF4E axis, and activates PPAR-γ through SREBP-1, which enhances the production of endogenous PPAR-γ ligands [45]. These findings suggest that DMBQ inhibits PPAR-γ by regulating mTORC1-4E-BP1 and mTORC1-SREBP1, which finally contributes to the suppression of adipogenesis.

To better understand how DMBQ regulates mTORC1 activity, we investigated whether DMBQ regulates insulin signaling. DMBQ did not appear to affect insulin-induced Akt phosphorylation, although DMBQ inhibited the phosphorylation of mTOR and 4E-BP1 (data not shown). DMBQ also suppressed the phosphorylation of 4E-BP1 in a TSC2-independent manner (▶ **Fig. 4C**). These results mean that DMBQ regulates mTORC1 activity independent of the Akt-TSC2 pathway. Moreover, mTOR protein content was downregulated by DMBQ (▶ **Fig. 4A, B**). These results suggest that DMBQ influences the mTOR pathway by regulating the stability of the mTOR protein. Several molecules were identified to regulate the stability of mTORC1. For example, the Tel2/Tti1-Tti2 (TTT) complex and Ruvbl1/2 regulate the assembly and stability of



▶ **Fig. 4** Effect of DMBQ on the mTOR pathway in 3T3-L1 adipocytes and MEF cells. **A** DMBQ inhibits phosphorylation of mTOR and 4E-BP1 in 3T3-L1 adipocytes. 3T3-L1 cells were differentiated in the presence or absence of DMBQ for 8 days. For Akt, 3T3-L1 cells were differentiated in the presence or absence of DMBQ for 2 days. **B** DMBQ attenuates insulin-stimulated phosphorylation of mTOR and 4E-BP1 in MEF cells. MEF cells were stimulated with insulin for 1 h in the presence or absence of DMBQ or rapamycin. **C** DMBQ suppresses phospho-4E-BP1 in TSC2-/- MEF cells. WT MEF and TSC2-/- MEFs were treated with DMBQ for 24 h. Protein expression was analyzed by Western blot.

mTORC1 [47–49]. Further study is needed as to how DMBQ regulates mTOR protein expression. It should also be noted that signaling molecules regulating mTOR protein stability could be a target for adipogenesis and obesity.

In summary, our findings reveal that DMBQ enhances AMPK phosphorylation and negatively regulates mTORC1 activity. This results in the downregulation of SREBP-1 and PPAR-γ, and a subsequent decrease in aP2 and FAS expression. These events eventually lead the inhibition of adipocyte differentiation by DMBQ. Further studies are needed to clarify whether DMBQ has the potential to prevent obesity in animal models. Such findings would support its development as a natural agent to prevent obesity and obesity-associated metabolic disorders.

Materials and Methods

Materials

DMEM, FBS, bovine calf serum (CS), and penicillin-streptomycin were obtained from Gibco BRL. Antibodies against pAMPK, AMPK, p-mTOR, mTOR, p4E-BP1, 4E-BP1, TSC2, pAkt, Akt, PPAR-γ, ACC, FAS and C/EBP-α were purchased from Cell Signaling. Anti-β-actin, SREBP-1, aP2, and secondary antibodies were purchased from Santa Cruz Biotechnology. Anti-Lamin B was obtained from Abcam. Isobutylmethylxanthine (IBMX), dexamethasone, insulin, Oil Red O, and DMBQ (purity 97 %) were purchased from Sigma-Aldrich. Compound C (dorsomorphin, purity ≥ 98 %) was obtained from Cayman Chemical and rapamycin (purity ≥ 98 %) was purchased from Tocris Bioscience.

Cell culture

3T3-L1 mouse preadipocytes (ATCC) were cultured in DMEM with 10 % CS and 1 % penicillin-streptomycin-L-glutamine at 37 °C in an atmosphere of 5 % CO_2. $TSC2^{-/-}$ MEFs and littermate control wild-type MEFs were kindly provided by John Blenis's laboratory (Weill Cornell Medicine) and cultured in DMEM supplemented with 10 % FBS and 1 % penicillin-streptomycin at 37 °C in 5 % CO_2.

Cell viability assay

The 3T3-L1 preadipocytes were seeded in 96-well plates at a density of 4×10^3 cells/well. After the cells were preconditioned for 24 h, they were treated with various concentrations of DMBQ for 24 or 48 h. Subsequently, 10 μL of MTT solution in PBS were added to each well, followed by further incubation at 37 °C for 3 h. Cell viability was determined by measuring the absorbance at 450 nm using a microplate reader (Tecan, Infinite M200).

Cell differentiation of 3T3-L1 preadipocytes and Oil Red O staining

3T3-L1 preadipocytes were seeded in 6-well plates at a density of 4×10^5 cells/well. Two days after the cells had reached confluence, they were treated with various concentrations of DMBQ and me-

dium containing 0.5 mM of 3-IBMX, 1 µM of dexamethasone, and 1 µg/mL of insulin (MDI) in DMEM with 10% FBS for 2 days. The medium was then changed to DMEM containing 10% FBS, 1 µg/mL of insulin, and DMBQ for 2 days, and this was changed to fresh DMEM and 10% FBS with DMBQ every 2 days. Following the induction of differentiation, cells were fixed in 10% formaldehyde in PBS for 1 h and stained with a solution of 0.5% Oil Red O in 60% isopropanol for 30 min at room temperature, washed four times with water, and dried. Differentiation was monitored under a microscope and quantified by elution with DMSO before the absorbance was measured at 490 nm.

Cytoplasmic-nuclear fractionation

For nuclear SREBP fractionation studies, protease pellets were added to NE-PER buffer (Thermo Fisher Scientific), and nuclear extracts were prepared according to the manufacturer's protocol. Whole cell lysates were prepared by mixing matched cytoplasmic fractions and nuclear fractions.

siRNA transient transfection

Gene expression was silenced with specific AMPK siRNA (Dharmacon). 3T3-L1 preadipocytes were transfected when preadipocytes reached 50–60% confluence using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. All treatments were administered after 48 h of transfection. Knockdown efficiencies at mRNA and protein levels were tested 8 days after differentiation. The efficiency of transfection was confirmed by Western blot.

Protein extraction and immunoblotting

The cells were lysed in lysis buffer containing 40 mM HEPES (pH 7.4), 120 mM sodium chloride (NaCl), 1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM sodium fluoride (NaF), 1.5 mM sodium orthovanadate (Na_3VO_4), 10 mM β-glycerophosphate, and 1% Triton X-100 supplemented with EDTA-free phosphatase and protease inhibitor cocktail (Thermo Fisher Scientific Inc.). After centrifugation at 13 000 × *g* for 10 min at 4 °C, the supernatants were boiled in SDS loading buffer. Protein extracts (30 or 50 µg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked and immunoblotted with specific primary antibodies.

Statistical analysis

The results are presented as mean values ± standard deviation. The differences between groups were evaluated using one-way analysis of variance (ANOVA) and Bonferroni's post hoc test with GraphPad Prism 5 software.

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Conflict of Interest

The authors declare no conflict of interest.

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