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Suppression of TNF-α-induced dysregulation of adipocytokine and insulin signaling in 3T3-L1 adipocytes by a diarylheptanoid from *Curcuma comosa*

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ABSTRACT: Adipocyte and adipose tissue dysfunction are primary defects in obesity and link obesity to metabolic diseases such as insulin resistance and type 2 diabetes mellitus (type 2 DM). Tumor necrosis factor (TNF-α) released from adipose tissue inhibits insulin signaling, thereby, inducing insulin resistance in adipocytes. Here, we investigated the preventive effect of 1,7-diphenyl-(4E,6E)-4,6-heptadien-3-ol (DPHD), a diarylheptanoid from *Curcuma comosa*, on TNF-α-induced adipocyte dysfunction using 3T3-L1 mature adipocytes. Exposure to TNF-α (10 ng/ml) for 24 h induced lipolysis and decreased triglyceride accumulation in adipocytes, indicating insulin resistance. Pretreatment with DPHD at concentrations of 0.1, 1, and 10 µM inhibited the lipolytic effect of TNF-α in a dose-dependent manner. TNF-α also increased the expression of genes encoded for proinflammatory cytokines, including interleukin 6 (*Il6*) and resistin (*Retn*), while it decreased the expression of genes involved in lipolysis and fatty acid oxidation, including perilipin (*Plin1*) and adiponectin (*adipoq*). Consistent with increased lipolysis, TNF-α decreased the expression levels of key proteins in the insulin signaling pathway, including insulin receptor substrate-1 (IRS-1), the phosphorylated phosphoinositide 3-kinase (p-PI3K), phosphorylated AKT (p-AKT), and glucose transporter 4 (GLUT4) in adipocytes. However, pretreatment with DPHD attenuated the changes in gene and protein expressions induced by TNF-α. The preventive effect of DPHD was found to be mediated through the downregulation of the nuclear factor NF-kB p65, a key transcriptional regulator of genes encoding pro-inflammatory cytokines. These results suggested the potential role of DPHD in effectively preventing the dysfunction and insulin resistance of adipocytes associated with TNF-α.

KEYWORDS: diarylheptanoid, insulin resistance, inflammation, 3T3-L1 adipocyte, TNF-α

INTRODUCTION

Obesity is associated with a higher risk of developing metabolic chronic diseases, including insulin resistance, type 2 DM, hypertension, and cardiovascular disease (CVD) [1]. The prevalence of obesity is increasing worldwide, and the global prevalence is expected to reach 18% in males and 25% in females in 2025 [2]. In obesity, excessive accumulation of lipids in adipocytes leads to adipocyte hypertrophy and adipose tissue dysfunction, which is a key determinant of metabolic diseases in individuals with obesity [3]. Inhibition of lipid accumulation in adipocytes might potentially be used in the prevention of adipocyte hypertrophy [4]. Adipocyte hypertrophy is associated with several changes in adipocyte function and the production of anti- and pro-inflammatory cytokines. Moreover, free fatty acids are released into the circulation, where they contribute to insulin resistance [5].

Dysfunctional adipocytes and macrophages in adipose tissue secrete proinflammatory cytokines, such as interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α), into the circulation. The resulting persistent high circulating concentrations of these cytokines contribute to chronic, low-grade inflammation [6]. The amount of TNF-α released positively correlates with the level of adiposity, and this cytokine has deleterious effects on adipocyte function through various mechanisms. It directly inhibits the activation of insulin signaling in adipocytes by inhibiting the tyrosine kinase activity of the insulin receptor (IR) [7] and induces lipolysis in adipocytes, thereby increasing mobilization of free fatty acids (FFAs), which exacerbate insulin resistance [8]. The effect of TNF-α in adipocytes has been reported to involve greater translocation of nuclear factor kB (NF-kB) into the nucleus [9], which further aggravates inflammation. Obese mice lacking TNF-α display lower circulating FFA concentrations and are protected against the obesity-induced reduction in insulin receptor signaling in adipose tissue, which significantly preserves insulin sensitivity [10]. Therefore, agents that disrupt the action of TNF-α on adipocytes and suppress local inflammation might potentially prevent obesity-related metabolic diseases.

Curcuma comosa Roxb (*C. comosa*) is a member of the Zingiberaceae family that has been demon-

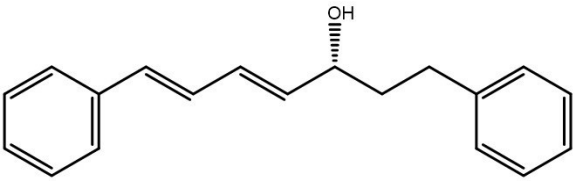

Fig. 1 Chemical structure of a non-phenolic diarylheptanoid (3R)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol (DPHD) obtained from *C. comosa*.

strated to possess an estrogen-like activity [11]. Increasing evidence demonstrates that *C. comosa* extract has a variety of pharmacological effects, including an anti-hypercholesteremic effect in rabbits and hamsters [12], and anti-oxidant and anti-inflammatory effects [13]. In addition, *C. comosa* extract reduces the expression of several pro-inflammatory cytokines, such as IL-1, MCP-1, and TNF-α, in the aorta of rabbits fed a high-cholesterol diet [14]. A non-phenolic (3R)-1,7-diphenyl-(4E,6E)-4,6-heptadien-3-ol (DPHD) is the most abundant biologically active compound of *C. comosa*. An extract containing DPHD was previously demonstrated to reduce the expression of pro-inflammatory cytokine TNF-α in thioacetamide-induced hepatotoxicity mice [15]. The extract also alleviated insulin resistance in the skeletal muscle of estrogen-deprived rats, which are characterized by obesity and dyslipidemia [16]. Furthermore, DPHD reduced the accumulation of visceral adipose tissue and the expression of TNF-α and resistin in the adipose tissue of ovariectomized (OVX) rats, where OVX caused hyperlipidemia and larger adipocyte size in visceral adipose tissue [17]. DPHD restored the lipid metabolites associated with metabolic disturbance and inflammation in OVX rats, including lysophosphatidylcholines and arachidonic acid [18]. However, the anti-inflammatory effect and improvement of insulin resistance of DPHD in adipose tissue have not been characterized. Therefore, in the present study, we investigated the preventive effect of DPHD on the TNFα-induced dysregulation of adipocytokines and insulin signaling in 3T3-L1 adipocytes to understand further the underlying molecular mechanisms.

MATERIALS AND METHODS

Preparation of DPHD from *C. comosa* extract

C. comosa is commonly known as *Wan chak motluk* in Thai. Rhizomes of *C. comosa* were collected from Kampangsaen District, Nakhon Pathom Province, Thailand, and subjected to taxonomic identification by Soontornchainaksaeng and Jenjittikul in 2010 [19]. The voucher herbarium specimen SCMU-300 was deposited at the Department of Plant Science, Faculty of Science, Mahidol University, Thailand. The collected rhizomes were sliced into small pieces, dried, ground, and then extracted three times with three volumes of boiling ethanol (95%). A dark brown viscous liquid of *C. comosa* extract was finally obtained by vacuum-removal of the pooled ethanol solvent. The composition of the extract was evaluated by high-performance liquid chromatography (HPLC) with ultraviolet (302 nm) detection. The HPLC traces for the major diarylheptanoid components of the extract samples were compared with data kept in our records. A portion of the ethanol extract was subjected to column chromatography, using silica gel as adsorbent, *n*-hexane-dichloromethane followed by dichloromethane-methanol as a gradient elution solvent system, and *n*-hexane-dichloromethane (1:4 v/v) as elution solvent; and diarylheptanoid DPHD (Fig. 1) was obtained. The structure of the DPHD obtained was confirmed by nuclear magnetic resonance and mass spectroscopy to be the same as that of the previously isolated DPHD [20], and its purity was estimated to be >98 by HPLC. The *C. comosa* extract contained 21.0 mg DPHD per gram.

Culture, differentiation, and treatment of adipocytes

Mouse 3T3-L1 preadipocytes were purchased from the American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's-modified Eagle's medium (DMEM) containing 4.5 g/l glucose (Invitrogen, USA), 10% bovine calf serum (BCS) (Hyclone, USA), 10 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO_2. To differentiate 3T3-L1 preadipocytes into mature adipocytes, cells were seeded onto 6-well plates of DMEM at a density of 8×10^4 cells per well and cultured, with the culture medium changed every 2 days, until confluent. Then, the cells were kept remaining in the medium for another 2 days to be set as Day 0. On Day 0, cells were cultured in DMEM containing 10% fetal bovine serum (FBS) (Hyclone), 10 µg/ml bovine insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 1 µM dexamethasone (Sigma Aldrich, USA) for 2 days to initiate the differentiation. The cells were then incubated in DMEM supplemented with 10% FBS and 10 µg/ml bovine insulin for another 2 days, and thereafter in DMEM containing 10% FBS until Day 8, with the medium being refreshed every 2 days. On Day 8, more than 90% of the cells displayed adipocyte morphology, as evidenced by the accumulation of lipid droplets. The fully differentiated 3T3-L1 adipocytes were then used in experiments. To investigate the protective effect of DPHD against TNF-α-induced adipocyte dysfunction, these 3T3-L1 adipocytes were pretreated with or without various concentrations of DPHD for 6 h before the addition of 10 ng/ml recombinant mouse TNF-α (Sigma Aldrich), followed by further incubation for 24 h. Dimethyl sulfoxide (DMSO) (Sigma Aldrich) was

used as the solvent at a final concentration of $< 0.1\%$ in all the experiments.

Cell viability assay

The percentage of viable cells was assessed using a 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the DPHD-treated cells were incubated with 100 µl MTT solution (5.0 mg/ml) (Sigma Aldrich) dissolved in serum-free DMEM for 4 h at 37 °C, during which a violet precipitate, formazan, accumulated. All the liquid in each well was then removed, and 100 µl of DMSO was added. The absorbance of the recovered DMSO at 570 nm was determined using a Multiskan™ microplate spectrophotometer (Thermo Fisher Scientific, USA).

Lipolysis assay

A lipolysis assay was performed by following lipolysis in 3T3-L1 cells and determined by Colorimetric Assay Kit according to the manufacturer's instructions (Sigma Aldrich). In brief, differentiated 3T3-L1 adipocytes were washed twice with 100 µl of Lipolysis Wash Buffer and, then, incubated with 100 nM isoproterenol for 1 h to initiate lipolysis. Aliquots (20 µl) of samples were quantified using a glycerol assay. The samples were incubated with glycerol buffer and reagents for 30 min in the dark, and their absorbances at 540 nm were measured using a Multiskan™ microplate reader. The glycerol content of the samples was calculated against a glycerol standard.

Oil red O staining

The accumulation of intracellular lipids was assessed using Oil red O staining. After treatment, the medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS) and fixed in 10% formaldehyde in PBS for 1 h at room temperature (25 °C). The fixed cells were washed with 60% isopropanol and dried at room temperature. Oil red O working solution (Sigma Aldrich) was added, and the cells were incubated for 20 min at room temperature. After three washes with distilled water to remove excess stains, the stained lipid droplets were examined under a light microscope at 10× magnification, and images were captured. The amount of intracellular lipid was quantified by incubating the stained cells with 100% isopropanol for 20 min to extract the dye. The absorbance of the obtained solution was measured at 520 nm using a microplate reader.

RNA preparation and real-time quantitative PCR

Adipocyte RNA was extracted using a Trizol reagent (Invitrogen), following the manufacturer's protocol. cDNA was synthesized from the RNA using an iScript™ cDNA Synthesis Kit (BioRad, USA). Real-time PCR analysis was performed using SYBR Green I dye (Invitrogen) and an ABI PRISM7500 Sequence Detection System and analysis software (Applied Biosystems, USA). The sequence of primers used is provided in Table S1.

Western blot analysis

Cells were lysed using modified RIPA lysis buffer (Thermo Fisher Scientific) containing a protease inhibitor cocktail (Sigma Aldrich) for 20 min on ice, then centrifuged at $16\,000 \times g$ for 20 min at 4 °C. The supernatants were collected, and their protein concentrations were measured using a Bicinchoninic Acid Assay Kit (Pierce Biotechnology Inc., USA). Lysate samples containing equal amounts of protein were mixed with 2× Laemmli buffer and heated for 5 min at 95 °C. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred to nitrocellulose membranes by electro-blotting. The membranes were then incubated overnight at 4 °C with primary antibodies. The following primary antibodies were used: anti-IRS-1 (#2382), anti-total PI3K (#4292), anti-phosphorylated PI3K p85 (#4228), anti-total AKT (#9272), anti-phosphorylated AKT Ser473 (#9271), and anti-PPARγ (#2435) antibodies from Cell Signaling Technology, Inc., USA; anti-GLUT4 (ab654) and anti-β-actin antibodies from Abcam, UK; anti-GAPDH antibody from Thermo Fisher Scientific; and anti-p50 and anti-p65 NF-kB antibodies from Santa Cruz Biotechnology, Inc, USA. After washing, the membranes were incubated with species-matched secondary antibodies conjugated with horseradish peroxidase. Specific protein bands were visualized using the Enhanced Super Signal West Pico Chemiluminescent System (Thermo Fisher Scientific). The intensities of the target protein bands were measured using ImageJ software (National Institutes of Health, USA), and relatively expressed to the β-actin or GAPDH bands.

Immunofluorescent staining

Cells were fixed in 4% formaldehyde at room temperature for 10 min and then permeabilized with PBS-Triton X-100 for 20 min. The cells were washed three times with PBS containing 0.1% Triton X-100 (PBST) and blocked in PBST containing 5% BSA for 1 h at 37 °C. The cells were washed and incubated for 1 h at 37 °C with anti-GLUT4 or anti-beta actin antibodies (Abcam) diluted in a blocking solution containing 2% Triton X-100 (1:200). After washing with PBST, the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgM monoclonal antibody or Alexa Fluor 594-conjugated anti-rabbit IgG polyclonal antibody in the dark for 1 h at 37 °C. The cells were washed in PBST and stained with 4′6-diamidino-2-phenylindole (Sigma Aldrich) for 5 min to label nuclei. After washing with PBST, the cells were examined using an Olympus FV1200 laser scanning confocal microscope (Tokyo, Japan), and images were captured.

The intensity of the GLUT4 staining was quantified.

Statistical analysis

Data were expressed as mean and standard error of the mean (SEM). Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by the Tukey-Kramer *post-hoc* test, in Prism version 7 (GraphPad Software, USA). Statistical significance was accepted when $p < 0.05$.

RESULTS

Effects of DPHD on cell viability of adipocytes

The effect of DPHD on the viability of mature adipocytes was first determined using an MTT assay. Incubation of the cells with 0.1–25 µM DPHD for 24 or 48 h did not affect their viability. However, after 72 h of incubation with DPHD at 50 µM or 100 µM, the number of viable cells had significantly reduced (Fig. S1). Therefore, DPHD was used at concentrations <25 µM in further experiments.

Effects of DPHD on lipolysis induced by TNF-α in adipocytes

The present study used TNF-α at 10 ng/ml to induce adipocyte dysfunction. Treatment of TNF-α at 10 ng/ml either alone or in combination with DPHD at concentrations of 0.1, 1, and 10 µM for 24 h did not affect cell viability of 3T3-L1 adipocytes (Fig. 2A). However, as shown in Fig. 2B, TNF-α significantly increased lipolysis in adipocytes, with approximately 2.25-fold more of released glycerol compared with the control; and pre-treatment of the adipocytes with DPHD attenuated the lipolytic effect of TNF-α in a dose-dependent manner. Consistent with the lipolytic effect, TNF-α reduced triglyceride accumulation in the adipocytes, and DPHD also decreased the accumulation of triglyceride in a dose-dependent manner (Fig. 2C,D).

Effects of DPHD on TNF-α-induced dysregulation of adipocytokines

One of the most important molecular effects underpinning TNF-α-mediated lipolysis is the downregulation of the *Plin1* gene that encodes for perilipin. Therefore, we measured the expression of *Plin1* and the adipokines and cytokines involved in insulin resistance, including IL-6, resistin, and adiponectin. TNF-α decreased the expression of the *Plin1* and *Adipoq* gene, which encodes adiponectin, while increasing the expression of IL-6 and Retn, which encodes resistin. However, pretreatment with DPHD prevented these changes in a dose-dependent manner (Fig. 3A–D). These results suggested that DPHD might prevent TNF-α-induced lipolysis by increasing the expression of perilipin and, hence, maintain normal levels of adipocytokines in adipocytes.

Effects of DPHD on TNF-α-induced impairments in the insulin signaling pathway

TNF-α decreased the levels of key proteins in the insulin signaling pathway, including insulin receptor substrate-1 (IRS-1), the phosphorylated form of phosphoinositide 3-kinase (p-PI3K), and the phosphorylated form of AKT (p-AKT) in the adipocytes. Pretreatment with DPHD abrogated these effects in a dose-dependent manner. However, the effects were statistically significant only at DPHD concentrations of 1 and 10 µM (Fig. 4). The negligible effect at 0.1 µM DPHD could be due to the variability of protein levels in the control group. TNF-α also reduced the expression of peroxisome proliferator-activated transcription factor γ (PPARγ), a transcription factor involving in the regulation of insulin sensitivity and the expression of insulin-dependent glucose transporter, GLUT4. However, DPHD prevented the reduction in PPARγ and GLUT4 protein expressions (Fig. 5A). The effects of DPHD on the localization and expression of GLUT4 in the adipocytes were confirmed by immunofluorescence staining (Fig. 5B). These results suggested that DPHD restored adipocyte insulin sensitivity through the activation of PPARγ.

Effects of DPHD on protein levels of NF- κB p50 and NF- κB p65 in adipocytes treated with TNF-α

PPARγ has been shown to have an anti-inflammatory effect by inhibiting nuclear factor-κB (NF-κB). In this study, the levels of NF-κB p50 and NF-κB p65, key components of the canonical NF-κB signaling pathway, were measured. The level of NF-kB p65 protein was significantly higher in adipocytes treated with TNF-α, and this effect was suppressed by DPHD (Fig. 6). Of note, DPHD at 10 µM caused a marked reduction in NF-kB p65 protein expression compared with the control, and the level of NF-kB p50 protein was not at all affected by the DPHD treatments ($p > 0.05$). These results could imply that the inhibitory effect of DPHD on the TNF-α-induced dysregulation of adipokines and insulin signaling was achieved through a reduction in NF-kB p65 expression, thereby limiting the transcriptional effects of NF-kB p65 in the nucleus. The reduction of NF-kB p65 might be a secondary effect resulting from the activation of PPARγ.

DISCUSSION

In this study, we demonstrated for the first time that DPHD isolated from *C. comosa* protected against TNF-α-induced lipolysis, dysregulation of adipocytokines, and impaired insulin signaling in 3T3-L1 adipocytes, elucidating the molecular mechanisms underlying the preventive effects of DPHD. We induced insulin resistance in 3T3-L1 adipocytes using recombinant TNF-α protein to mimic the dysfunctional adipose tissue typically observed in obese individuals. The treated adipocytes exhibited increased lipolysis, triglyceride

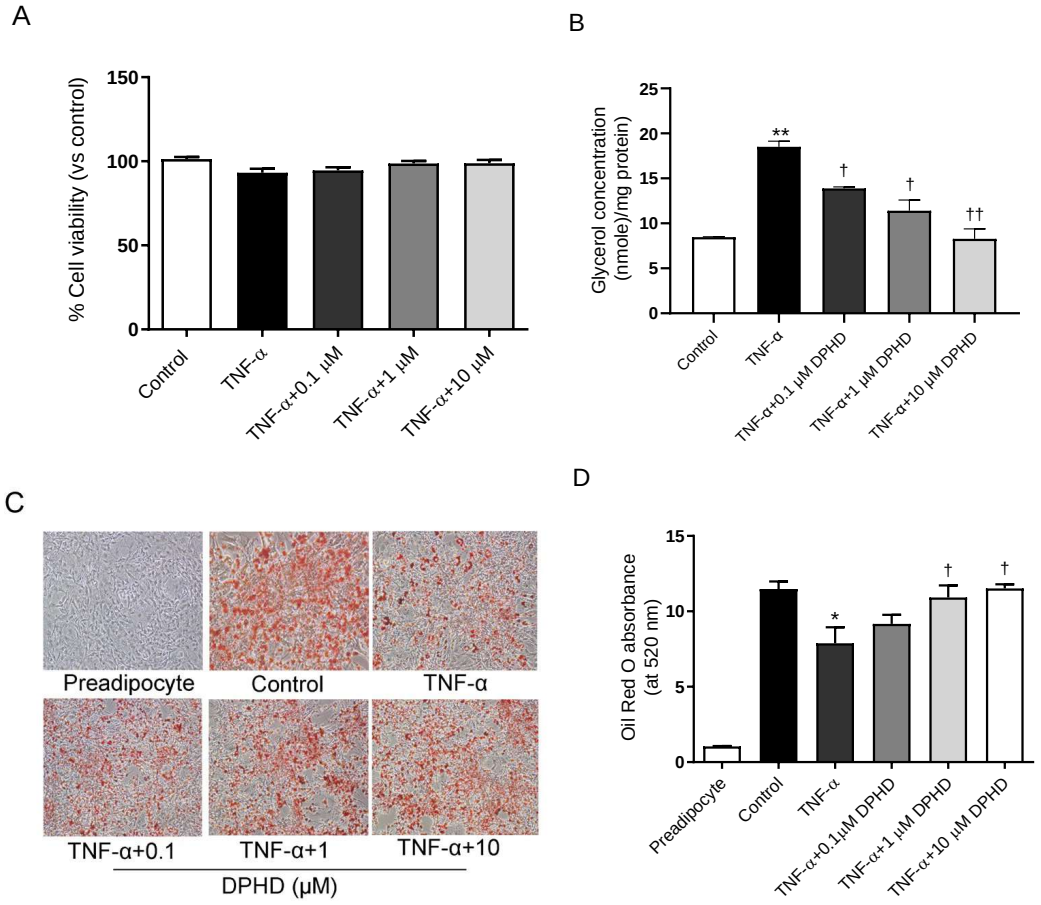

Fig. 2 Effects of DPHD pretreatment on viability, lipolysis, and triglyceride accumulation of TNF-α treated adipocytes: (A), cell viability (MTT assay); (B), lipolysis (lipolysis (3T3-L1) kit); (C), intracellular lipid content (Oil red O staining); and (D), amount of stained lipid (Oil red O absorbance at 520 nm). Prior to each assay, 3T3-L1 adipocytes were pre-incubated with DPHD at various concentrations for 6 h and then treated with TNF-α at 10 ng/ml for 24 h. Data are presented as mean ± SEM of three independent experiments. ** $p < 0.01$ and * $p < 0.05$ vs. control. †† $p < 0.01$ and † $p < 0.05$ vs. TNF-α.

accumulation, and altered gene expression levels, including *Plin1*, *Adipoq*, *IL-6*, and *Retn*, alongside reduced expression of insulin signaling proteins such as IRS-1, p-PI3K, p-AKT, and GLUT4 induced by TNF-α. Remarkably, all these TNF-α-mediated effects were inhibited by pretreatment with DPHD. Additionally, DPHD prevented the reduction in PPARγ expression, a critical transcription factor that regulates insulin sensitivity in adipocytes. The detrimental effects of TNF-α were associated with the upregulation of NF-κB, a transcription factor responsible for controlling pro-inflammatory cytokine production, which DPHD successfully inhibited.

Adipose tissue dysfunction plays a crucial role in the development of insulin resistance and metabolic diseases in individuals with obesity. Dysfunctional adipose tissue releases higher levels of pro-inflammatory cytokines and adipokines, such as IL-6, TNF-α, and resistin, while producing lower amounts of adiponectin [21]. TNF-α contributes to insulin resistance by directly interfering with insulin signaling through its receptor and consequently blocking biological actions of insulin [22]. Preventing inflammatory disorders by blocking TNF-α and TNF-α signaling could be an effective strategy for treating insulin resistance and T2DM.

In the present study, TNF-α increased the expression of *IL-6* and *Retn* (encoding resistin) genes, while decreasing the expression of *Adipoq* (encoding adiponectin). Both IL-6 and resistin are secreted by dysfunctional adipocytes, and their circulating concentrations are high in individuals with obesity and insulin resistance. Conversely, adiponectin, an adipokine with insulin-sensitizing and anti-inflammatory activities, is present in a lower concentration in individuals with obesity than normal persons. Pretreatment with DPHD

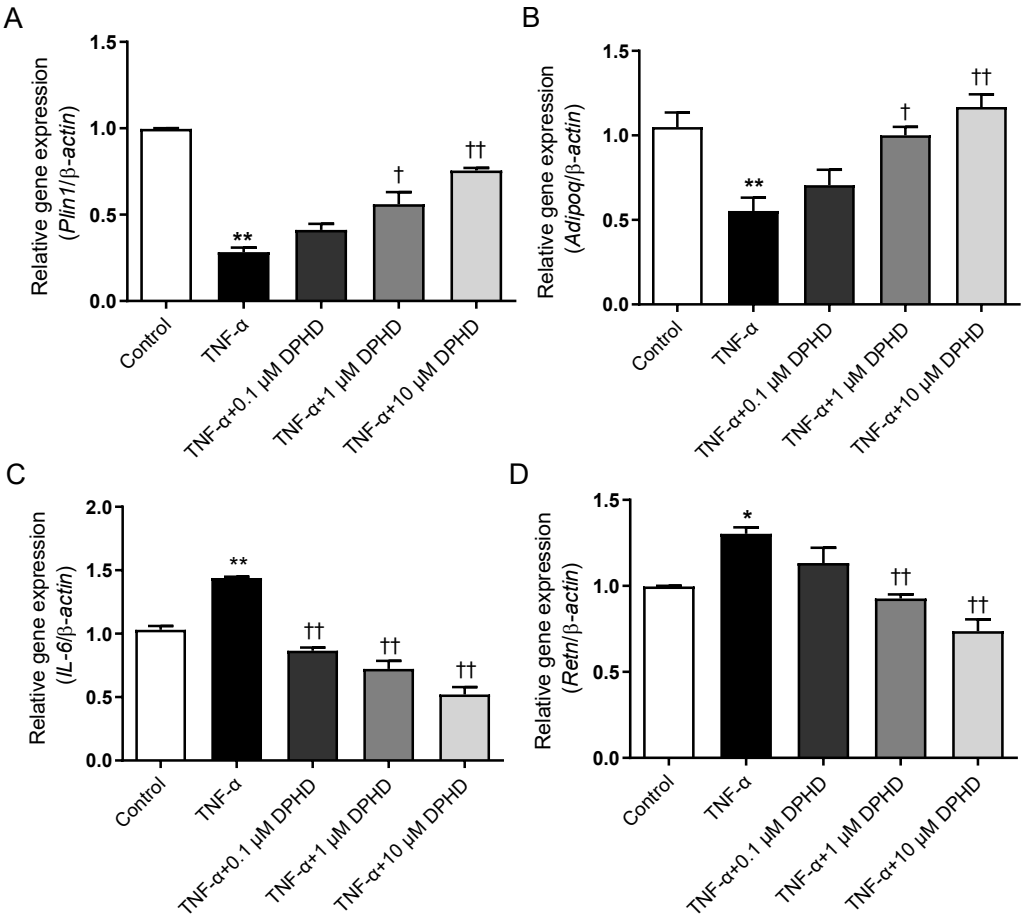

Fig. 3 Effects of DPHD on the expression of the adipocytokine-encoding genes: (A), *Plin1*; (B), *Adipoq*; (C), *IL-6*; and (D), *Retn*. 3T3-L1 adipocytes were pre-incubated with DPHD for 6 h and then treated with TNF-α. β-actin was used as the reference gene. Data are presented as mean ± SEM of three independent experiments. ** $p < 0.01$ and * $p < 0.05$ vs. control. †† $p < 0.01$ and † $p < 0.05$ vs. TNF-α.

prevented the increases in *IL-6* and *Retn* gene expression induced by TNF-α and increased the expression of *Adipoq*. These effects might underpin the anti-inflammatory properties of the compound. Previously, DPHD was demonstrated to have anti-inflammatory effects in both *in vitro* and *in vivo* studies by suppressing the release of proinflammatory cytokines. Moreover, DPHD was shown to reduce TNF-α and resistin expression in the visceral adipose tissue of rats with longstanding ovariectomy [23] and to suppress the increase in TNF-α expression in the aortic ring of OVX rats [24].

In addition to their deleterious effects on adipokines, TNF-α was reported to directly inhibit insulin signaling by interfering with the transduction of signal between insulin receptor and insulin receptor substrate (IRS) [22], leading to the development of insulin resistance. Additionally, TNF-α was shown to reduce the tyrosine phosphorylation of IRS-1 while increasing its serine phosphorylation [25], blocking signal transduction. TNF-α could also induce insulin resistance in 3T3-L1 adipocytes by decreasing the expression of IRS-1 and GLUT4 without a loss of insulin receptor-mediated signal transduction [26]. In the present study, we could not detect changes in the phosphorylation of serine residues in IRS-1. However, we found a low IRS-1 expression level and low phosphorylation of other key signaling proteins (phosphorylated AKT and phosphorylated PI3K) and GLUT4 in TNF-α-treated adipocytes, indicating the dysregulation of insulin signaling. In line with one previous report, the elevated phosphorylation of signaling proteins enhanced insulin signaling transduction [27].

The activation of insulin signaling increases lipid storage in adipocytes by stimulating triacylglycerol synthesis and inhibiting its breakdown. In addition, insulin signaling protects perilipin, a protein that coats

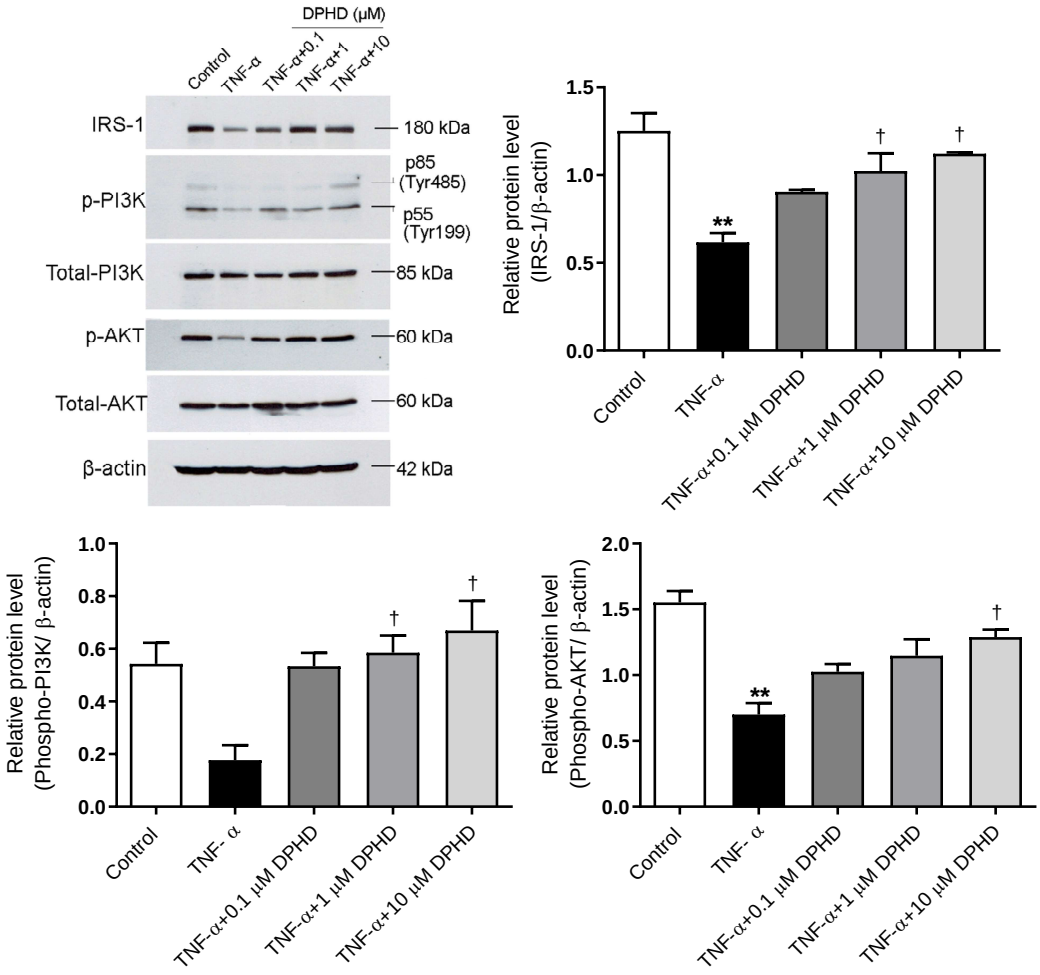

Fig. 4 Effects of DPHD on protein levels of the insulin signalling-pathway key components. The expression of IRS-1, p-PI3K, and p-AKT were measured. Sample blots and intensities of the protein bands normalized to the loading control were shown. The band intensities for phosphorylated proteins were normalized to those of the corresponding total proteins and the loading control, β-actin. Data are presented as mean ± SEM of three independent experiments. ** $p < 0.01$ and * $p < 0.05$ vs. control. †† $p < 0.01$ and † $p < 0.05$ vs. TNF-α.

lipid droplets in adipocytes, from lipases. TNF-α interferes with insulin signaling and reduces perilipin expression, resulting in greater adipocyte lipolysis. The FFAs that are released as a consequence can cause peripheral (muscle) insulin resistance [28], and TNF-α-induced lipolysis causing an increase in circulating FFA concentrations in individuals with obesity. However, the inhibition of TNF-α action by chemical or genetic means has been shown to restore insulin sensitivity [29]. The present study found that TNF-α promoted lipolysis and increased triglyceride mobilization in adipocytes, leading to less triglyceride accumulation in adipocytes. Consistent with this, perilipin expression was downregulated in adipocytes treated with TNF-α. DPHD could sustain the basal level of insulin signaling intermediates and GLUT4, indicating functional insulin signaling. Regular insulin signaling preserves the perilipin expression, thereby preventing adipocyte lipolysis. The modulating effects of DPHD might be related to its anti-inflammatory properties.

To understand the molecular mechanism of DPHD in preventing the deleterious effects of TNF-α on induction of inflammation and insulin resistance in adipocytes, we focused on PPARγ, a transcription factors that play a vital role in regulating insulin sensitivity and inflammation [30]. The level of PPARγ was previously shown to be negatively regulated by TNF-α [31]. In line with the previous report, TNF-α markedly decreased the level of PPARγ in adipocytes. DPHD effectively maintained high levels of PPARγ

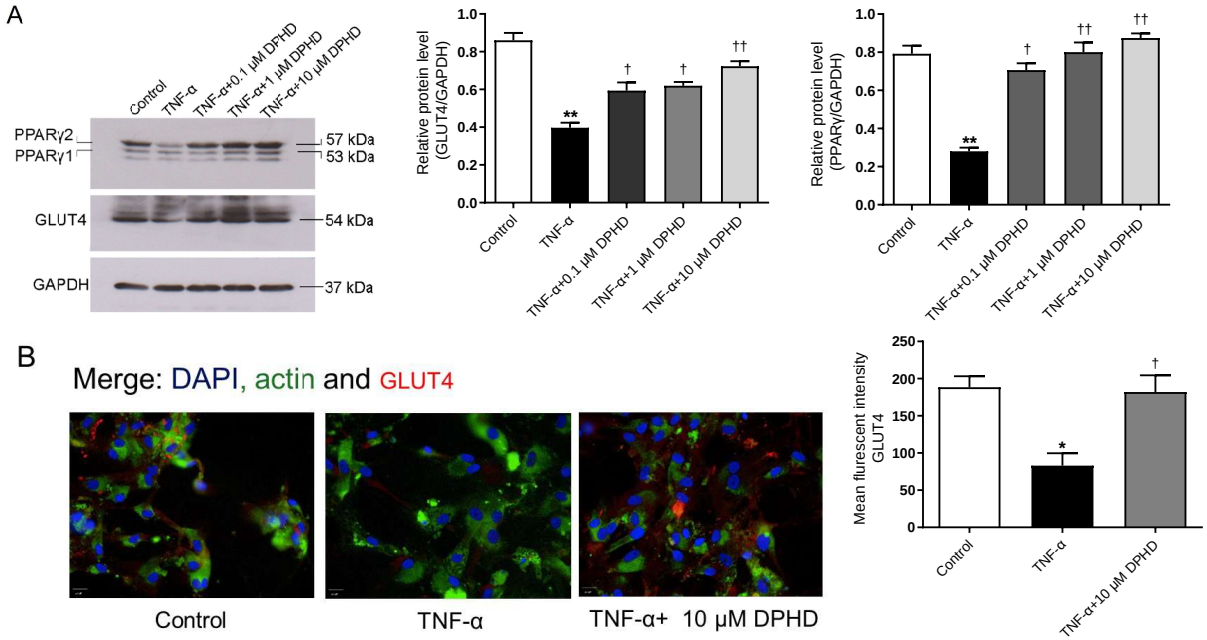

Fig. 5 Effects of DPHD on protein levels of PPARγ and GLUT4. (A), band intensities of the target proteins normalized to those of the loading control GAPDH; (B), immunofluorescence staining for GLUT4 along with the mean fluorescence intensity. Data are presented as mean ± SEM of three independent experiments. ** $p < 0.01$ and * $p < 0.05$ vs. control. †† $p < 0.01$ and † $p < 0.05$ vs. TNF-α.

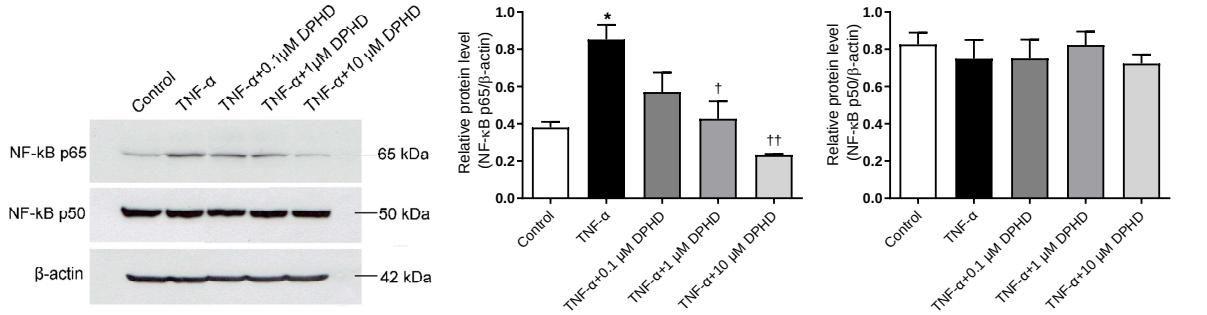

Fig. 6 Effects of DPHD on the protein levels of NF-kB p65 and NF-kB p50 in adipocytes treated with TNF-α for 24 h. Sample blots and protein band intensities were shown, normalized to that of β-actin. Data are presented as mean ± SEM of three independent experiments. ** $p < 0.01$ and * $p < 0.05$ vs. control. †† $p < 0.01$ and † $p < 0.05$ vs. TNF-α.

and their target genes, including adiponectin, resistin, and *Glut4*. Treatment with PPARγ agonists effectively repressed the expression of resistin gene in 3T3-L1 adipocytes [32] while increasing the expression of adiponectin [33]. Apart from the modulating effect on adipokines, which directly affect insulin sensitivity, PPARγ also exerts an anti-inflammatory effect in adipose tissue [34]. The anti-inflammatory effect of DPHD against TNF-α in adipocytes might be associated with the activation of PPARγ.

PPARγ has been reported to exert its anti-inflammatory effects by inhibiting NF-κB-mediated transcription. PPARγ inhibits NF-κB transcriptional activity by interfering with DNA binding or activating IκB kinase, inhibiting the expression of most proinflammatory cytokines [35]. Additionally, TNF-α has been shown to inhibit PPARγ by promoting its association with NF-κB, which reduces PPARγ's DNA binding and transcriptional activity [36]. The activation of NF-κB is associated with the expression of various pro-inflammatory mediators, including IL-6 and TNF-α [37]. This study found TNF-α to specifically upregulate the NF-kB p65, while the NF-kB p50 remained unaffected. The NF-kB p65 possesses several phosphorylation sites that enhance its activity, leading to various cellular responses. In contrast, NF-kB p50 lacks

the equivalent level of phosphorylation-dependent activation [38]. A variety of stimuli can solely activate the NF-kB p65, which is sufficient to upregulate the transactivation of target genes involved in inflammatory responses [39]. Interestingly, DPHD appeared to attenuate the increase in NF-kB p65 levels, suggesting that the reduced expression of IL-6 could result from decreased NF-κB expression. This finding aligned with previous research indicating that DPHD reduced TNF-α expression in mouse liver [15]. Furthermore, DPHD suppressed the expression of IkappaB kinase and the activation of NF-κB in PMA-stimulated cells [40]. These results suggested that DPHD ameliorated TNF-α-induced adipokine dysregulation and impairment of insulin signaling in the adipocytes through the activation of PPARγ, subsequently decreasing NF-κB and IL-6 expression. However, the direct effect of DPHD on the activation of PPARγ needs to be confirmed using genetic knockdown and pharmacologic inhibition.

In conclusion, this study highlighted the potential of DPHD to prevent the dysregulation of adipokines and the impairment of insulin signaling in adipocytes caused by TNF-α. Subsequently, the study results suggested that DPHD could serve as a promising treatment for obesity-related metabolic diseases. However, the positive effects of DPHD observed in this study were based on experiments conducted with a single cell line, where DPHD was directly administered to the cells. To validate these effects and understand the mechanisms of action of DPHD, further studies using adipocytes from various sources or in obese animal models would be needed. The results of such studies would accurately reflect the conditions in obese individuals and, hence, provide valuable insights into the pharmacokinetics of the compound.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2025.040.

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Appendix A. Supplementary data

Table S1 Primer sequences.

Gene	Accession number	Forward primer	Reverse primer
Plin1	NM_001113471.1	5′-GACAAGGAGTCAGCCCCTTC-3′	5′-CTCACAAGGCTTGGTTTGGC-3′
Adipoq	NM_009605	5′-GGAGATGCAGGTCTTCTTGG-3′	5′-CTTCTCCAGGCTCTCCTTTC-3′
IL-6	NM_031168.1	5′-ACGGACTACAACCAGTTCGC-3′	5′-GGGACAGCTCCTTGGTTCTT-3′
Retn	NM_022984.4	5′-ACTCCCTGTTTCCAAATG-3′	5′-GCTCAAGACTGCTGTGCC-3′
β-*actin*	NM_007393	5′-CTGGTCGTCGACAACGGCTC-3′	5′-CATCATCACACCCTGGTGCC-3′

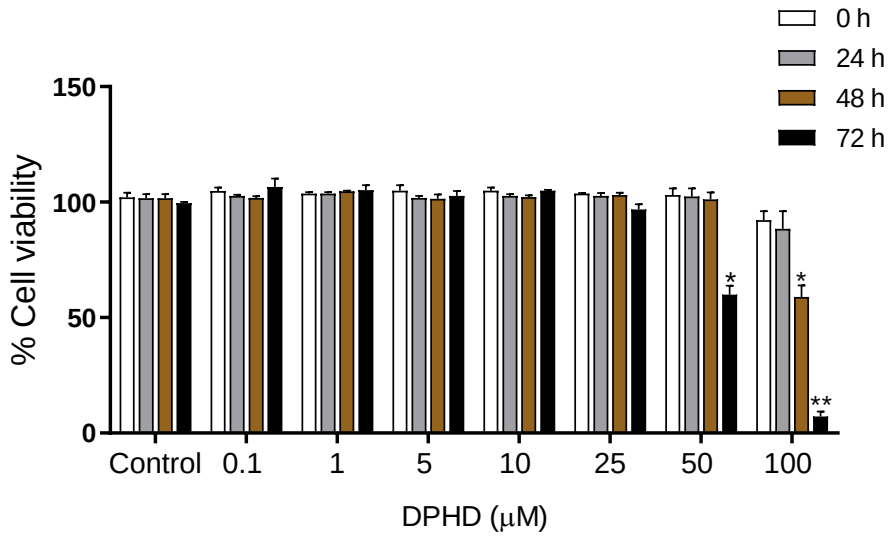

Fig. S1 Effects of DPHD on viability of 3T3-L1 adipocytes. Cells were incubated with DPHD at concentrations of 0.1–100 μM for 24, 48, or 72; then, cell viability was determined using an MTT assay. Data are presented as mean ± SEM of three independent experiments. ** $p < 0.01$ and * $p < 0.05$ vs. control.