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(2*S*)-7,4′-dihydroxy-8-prenylflavan stimulates adipogenesis and glucose uptake through p38MAPK pathway in 3T3-L1 cells

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ABSTRACT

Adipose tissue plays a key role in the development of obesity and diabetes. Natural products are one of the main sources for discovering new lead compounds. In the present study, (2*S*)-7,4′-dihydroxy-8-prenylflavan (DHPF), a natural prenylated flavan isolated from *Morus yunnanensis*, was found to significantly promote adipogenesis and increase glucose uptake in 3T3-L1 cells. Real-time PCR results showed that DHPF increased the expression of glucose and lipid metabolism-related genes (C/EBPα, PPARγ, aP2, GLUT4 and adiponectin) and decreased the expression of inflammatory cytokine TNF-α. Western blotting further revealed that DHPF activated p38 MAPK at the initial stage of 3T3-L1 preadipocyte differentiation. DHPF-induced activation of p38, adipogenesis and glucose uptake were effectively blocked by SB203580, a specific p38 inhibitor. These results indicate that DHPF could stimulate adipogenesis and increase glucose uptake through the p38 MAPK pathway, and DHPF may be useful for the prevention and treatment of obesity-associated disorders such as type 2 diabetes (T2D).

1. Introduction

Adipose tissue plays an important role in the regulation of glucose and lipid metabolism, insulin action, energy balance and inflammation [1,2]. Adipose tissue dysfunction in obesity and lipodystrophy is closely related to incidence of T2D, atherosclerotic vascular diseases and inflammation [3]. In mature adipocytes, aP2 (aFABP, adipocyte-specific fatty acid-binding protein) is highly expressed and serves to bind and transport free fatty acids (FFAs). Glucose transporter type 4 (GLUT4) is also highly expressed and responds to insulin to lower blood glucose. In addition, as an endocrine organ, adipose tissue can express and secrete adipokines to regulate lipid and glucose metabolism. For example, adiponectin exerts an insulin-sensitizing effect, while tumor necrosis factor-α (TNF-α) impairs insulin sensitivity. Thiazolidinediones (TZDs), a class of peroxisome proliferator-activated receptor gamma (PPARγ) agonists for treating T2D, stimulate adipogenesis and increase insulin sensitivity [4,5]. However, the clinical use of TZDs has induced weight gain, fluid retention, congestive heart failure, and bone fractures [6,7]. Therefore, development of new potent drugs to regulate adipocyte function is urgently needed to protect obese patients from insulin resistance and other obesity-related health disparities.

Plants are one of the main sources of natural compounds. The genus *Morus* (Moraceae) has important economic and medicinal values. The leaves of some *Morus* plants are indispensable food for silk-worms. In particular, the root bark and leaves of some *Morus* species have been used in traditional Chinese medicines for the treatment of diabetes and hypertension [8]. (2*S*)-7,4′-dihydroxy-8-prenylflavan (DHPF) is a prenylated flavan isolated from the leaves of *Morus yunnanensis* Koidz [9]. It has been documented that prenylated flavans have multiple functions such as antibacterial and cytotoxic effects [10,11], and suppression of nuclear factor-κB [12], aromatase [13] and tyrosinase activities [9]. However, the effect of DHPF on adipocytes has not been studied.

Abbreviations: aP2, aFABP, adipocyte fatty acid-binding protein; FFA, free fatty acid; GLUT4, glucose transporter type 4; IL, interleukin; TG, triglyceride; TZDs, thiazolidinedione.

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The aim of this study was to evaluate the possible effects of DHPF on adipogenesis and glucose uptake in murine 3T3-L1 preadipocytes, and the possible mechanism is also being investigated.

2. Materials and methods

2.1. Reagents

Insulin, dexamethasone, 3-isobutyl-1-methylxanthine, and SB203580 were purchased from Sigma. Dulbecco's modified Eagle's medium (DMEM) and newborn calf serum (NCS) were obtained from Invitrogen. Fetal bovine serum (FBS) was obtained from Hyclone. 2-deoxy-D-[2−^{3}H] glucose was obtained from Perkin–Elmer.

DHPF was isolated from the leaves of *M. yunnanensis* Koidz. and the identification was detailed in our previous study [9]. The purity of DHPF was assessed as >98% by analytical HPLC [Agilent 1200; YMC C_{18} column (250 × 4.6 mm, 5 μm, YMC Co., Ltd., Japan); MeOH–H_2O (70: 30); UV detection, 210 nm; flow rate, 1.0 ml/min].

2.2. Cell culture and treatment

3T3-L1 preadipocytes were cultured in DMEM with 10% NCS (Gibco) and maintained in a 5% CO_2 humidified atmosphere at 37 °C. Two days after confluence, the cells were stimulated to differentiate by a mixture, which contains 0.125 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone and 2 μg/ml insulin in DMEM with 10% FBS (HyClone), in the presence or absence of DHPF or rosiglitazone. Two days later, the medium was changed by the DMEM containing 10% FBS plus 2 μg/ml insulin with or without DHPF or rosiglitazone every 2 days for another six days.

2.3. Triglyceride measurement

As previously described [14], the fully differentiated 3T3-L1 adipocytes were lysed by repeated freezing and thawing (three times). The amount of triglyceride (TG) was determined by a commercial enzyme assay kit (Rongsheng, Shanghai, China).

2.4. Oil Red O staining

Oil Red O staining was performed as described in our previous work [14]. Briefly, the fully differentiated 3T3L-1 cells were washed twice with PBS (pH 7.4) and fixed by 10% formalin for 15 min at 37 °C. Then the fixative was aspired, and the cells were washed twice with PBS and stained with filtered Oil Red O (0.6% in 60% isopropanol; Sakura Finetek USA Inc.) for 30 min. Then the cells were washed twice with distilled water and photographed.

2.5. Western blotting

The expression levels of GLUT4, aP2, PPARγ, C/EBPα, phospho-p38, p38, and β-actin protein were detected using western blot analysis as described previously [14].

2.6. Real-time PCR

The gene expression levels were examined by RT-PCR as previously described [14]. The primers for amplification were as follows: GLUT4 (forward, 5′-TCCTTCTATTTGCCGTCCTC-3′, and reverse, 5′-TGTTTTGCCCCTCAGTCATT-3′), PPARγ (forward, 5′-TTTTCAAGGGT GCCAGTTTC-3′, and reverse, 5′-AATCCTTGGCCCTCTGAGAT-3′), C/EBPα (forward, 5′-ACTCGCTCCTTTTCCTACCG-3′, and reverse, 5′-CCCCAACACCTAAGTCCCTC-3′), adiponectin (forward, 5′-GCACTGGC AAGTTCTACTGCAA-3′, and reverse, 5′-GTAGGTGAAGAGAACGGC CTTGT-3′), TNF-α (forward, 5′-CGTGGAACTGGCAGAAGAGG-3′, and reverse, 5′-CTGCCACAAGCAGGAATGAG-3′), and β-actin (forward, 5′-CACGATGGAGGGGCCGGACTCATC-3′, and reverse, 5′-TAAA-GACCTCTATGCCAACACAGT-3′). The levels of transcripts were normalized to *β-actin* and presented as mean ± SD.

2.7. Glucose uptake

The fully differentiated 3T3L-1 cells were starved for 4 h. Then the cells were rinsed twice with Krebs-Ringer-Hepes (KRH) buffer, and incubated in KRH buffer with or without 20 ng/ml insulin for 15 min. After incubation with 0.5 Ci/ml (final concentration) 2-deoxy-D-[2−^{3}H] glucose in KRH buffer at 37 °C for 10 min, the cells was quickly washed twice with ice-cold KRH buffer to terminate reaction. Then the cells were solubilized with 0.2% Triton X-100 in KRH buffer overnight and the radioactivity was detected with a scintillation counter (Beckman Instruments).

2.8. Statistical analysis

Quantitative data were expressed as the mean ± SD of experiments in triplicate. Statistical analysis was performed by one-way ANOVA followed by post hoc test (LSD). The results were considered statistically significant at $P < 0.05$.

3. Results and discussion

DHPF is a prenylated flavan isolated from *Morus yunanensis* (Fig. 1). In this study, we first investigated the effect of DHPF on adipogenesis in 3T3-L1 cells, the most commonly used *in vitro* model for studying adipogenesis. Adipogenesis was assessed by Oil Red O staining and quantification of triglyceride content. As shown in Fig. 2A and B, 8 days of DHPF treatment induced observable increase in lipid accumulation (Oil Red O staining) and a dramatic increase in triglyceride content in a concentration-dependent manner. Moreover, Fig. 2D showed that DHPF significantly increased the protein levels of aP2 and GLUT4, two well-known adipogenic markers. GLUT4 is the primary transporter for glucose uptake in adipose tissue [15]. Decreased expression of GLUT4 and impaired responsiveness of GLUT4 to insulin were observed in adipocytes from patients with obesity and diabetes [16,17]. In accordance with the up-regulation of GLUT4 expression, DHPF significantly enhanced both basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes (Fig. 2C). As the positive control, the PPARγ agonist rosiglitazone markedly induced adipogenesis and increased glucose uptake (Fig. 2A–D). Therefore, these data suggest that DHPF was capable of promoting both adipogenesis and glucose uptake in 3T3-L1 cells.

Adipose tissue is not only a reservoir of energy in the body, but also an endocrine organ secreting adipokines such as adiponectin and TNFα [18–20]. In obese individuals, the down-regulation of adiponectin expression and the up-regulation of TNFα expression

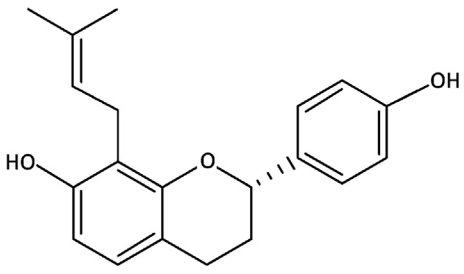

Fig. 1. Chemical structure of DHPF.

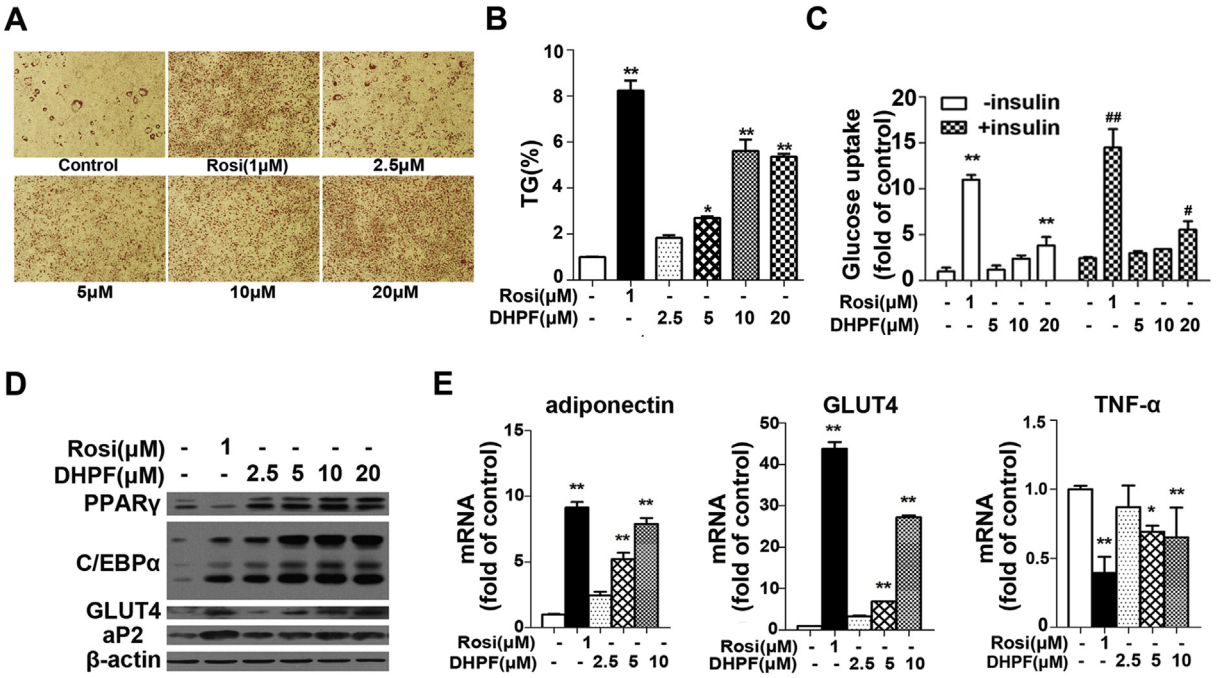

Fig. 2. The effect of DHPF on adipogenesis. 3T3-L1 cells were treated with differentiation cocktail and various concentrations of DHPF for 8 days. (A and B) The accumulation of lipid was detected by Oil Red O staining and triglyceride measurement (n = 3). (C) Basal and insulin-stimulated glucose uptake were determined (n = 3). (D) the protein levels of PPARγ, C/EBPα, GLUT4 and aP2 were detected by western blotting. (E) The mRNA levels of adiponectin, GLUT4 and TNF-α were detected by quantitative RT-PCR (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$ versus the untreated control; $\#p < 0.05$, $\#\#p < 0.01$ versus insulin-treated only group. Rosi, Rosiglitazone.

are observed in adipose tissue, both of which contribute to insulin resistance associated with obesity [21,22]. Fig. 2E showed that DHPF treatment resulted in a significant increase of adiponectin expression and a substantial decrease of TNFα expression. Combined with the up-regulation of glucose uptake, it is conceivable that DHPF may be a potential lead compound to combat T2D.

The transcription factors PPARγ and CCAAT/enhancer binding protein α (C/EBPα) are the master regulators of adipogenesis [23]. PPARγ and C/EBPα cooperate to activate the expression of genes involved in adipocyte function and terminal differentiation, such as aP2, GLUT4 and adiponectin [24,25]. Since Fig. 2D showed that 8 days of DHPF treatment significantly increased the protein levels of PPARγ and C/EBPα, we next further examined the temporal expression patterns of these two key transcription factors in 3T3-L1 cells. DHPF gradually up-regulated the mRNA levels of PPARγ and C/EBPα from Day 2–8 (Fig. 3A and B). Immunoblot analysis showed

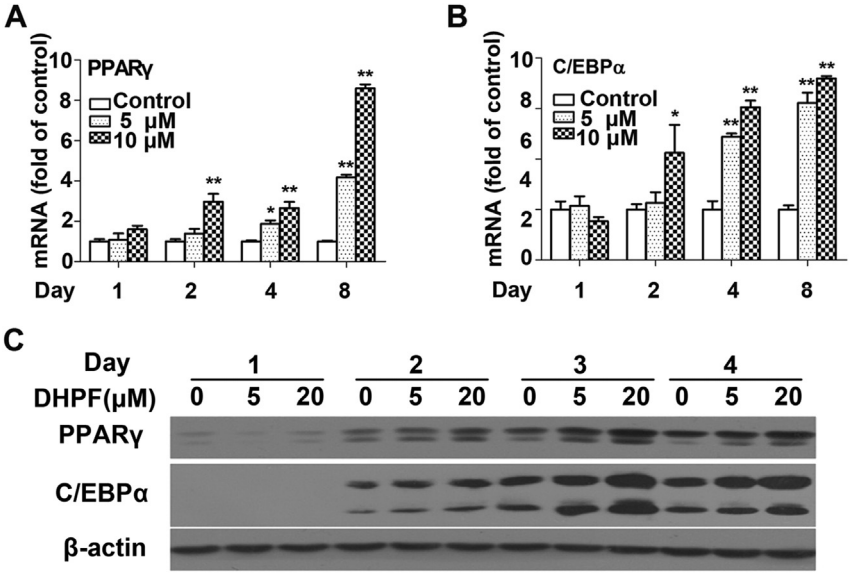

Fig. 3. The effect of DHPF on the kinetic expression of PPARγ and C/EBPα in 3T3-L1 cells. (A and B) Cells were treated with differentiation cocktail and DHPF for varying time as shown, and then the mRNA levels of PPARγ and C/EBPα were detected by quantitative RT-PCR (n = 3). (C) After 1–4 days of treatment with DHPF, the protein levels of PPARγ and C/EBPα were detected by western blotting. $^{*}p < 0.05$, $^{**}p < 0.01$ versus the untreated control.

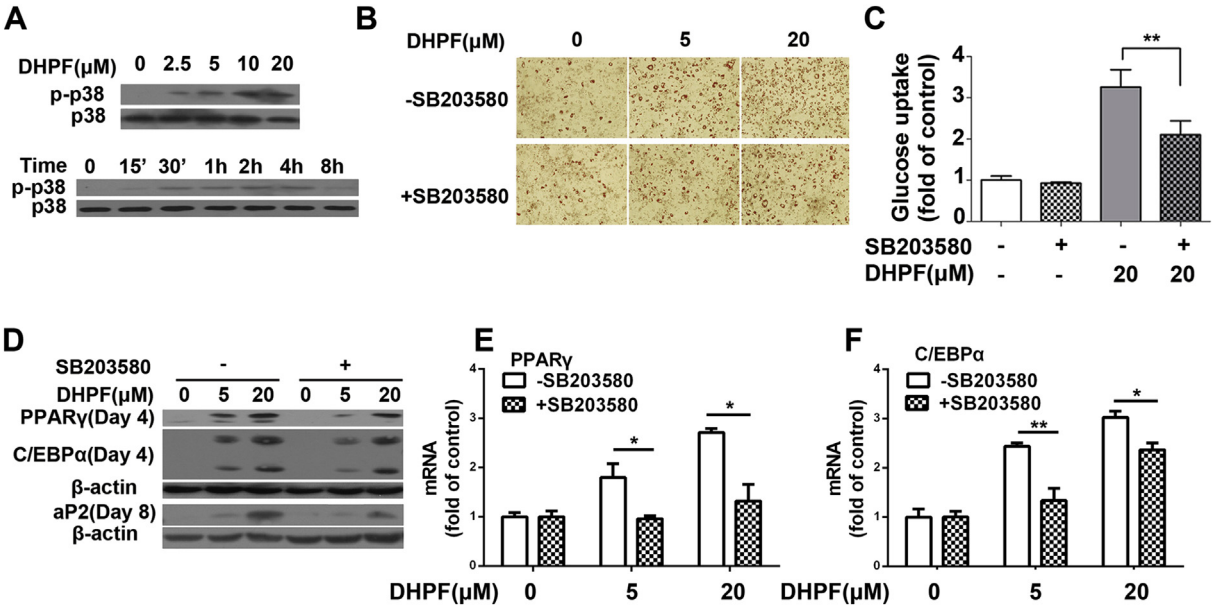

Fig. 4. DHPF promotes 3T3-L1 preadipocyte differentiation through p38MAPK activation. (A) After confluence, 3T3-L1 cells were starved for 4 h in serum-free DMEM with 0.2% BSA and then treated with different concentrations of DHPF for 4 h or 20 μM DHPF for different time. The level of phosphorylated p38MAPK was detected by western blotting. (B–D) Cells were treated with differentiation cocktail and DHPF in the presence or absence of SB203580 for 8 h, and then only treated with DHPF and differentiation cocktail afterward for 8 days. (B) Accumulation of lipid was detected by Oil Red O staining. (C) Basal glucose uptake was measured (n = 3). (D) The protein levels of PPARγ, C/EBPαand aP2 were analyzed by western blotting. (E and F) Cells were treated for 4 days and the mRNA levels of PPARγ and C/EBPα were detected by quantitative RT-PCR (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, compared to DHPF-treated only group. Rosi, Rosiglitazone.

a similar result (Fig. 3C). These data suggest that DHPF promotes 3T3-L1 preadipocyte differentiation via up-regulation of PPARγ and C/EBPα expression.

However, we found that DHPF did not affect the PPARγ activity in a luciferase reporter assay, whereas rosiglitazone increased it apparently (data not shown), indicating that DHPF promotes adipogenesis through a mechanism distinct from rosiglitazone. It has been reported that p38 mitogen-activated protein kinase (MAPK) plays an important role in adipocyte differentiation, with its activity peaking early during adipocyte conversion. As a key factor for adipocyte differentiation, C/EBPβ is induced by p38 activation during the early stage of adipogenesis [26], and then promotes the expression of PPARγ and C/EBPα [23]. To further investigate the mechanism underling DHPF–induced adipogenesis, the level of p38 phosphorylation was examined. We found that p38 phosphorylaiton was up-regulated at 15 min after addition of 20 μM DHPF and markedly increased after 30 min of treatment (Fig. 4A). In addition, DHPF dose-dependently enhanced the phosphorylation of p38 after 4 h of treatment (Fig. 4A). To investigate whether the p38 MAPK pathway participates in DHPF-stimulated adipocyte differentiation, 3T3-L1 cells were treated with DHPF in the presence or absence of SB203580 (a specific p38 inhibitor). SB203580 significantly inhibited DHPF-induced lipid accumulation and glucose uptake after 8 days of treatment (Fig. 4B and C). In addition, SB203580 attenuated DHPF-stimulated up-regulation of PPARγ and C/EBPα expression (Day 4) as well as aP2 expression (Day 8) (Fig. 4D–F). Taking together, our data suggest that the effect of DHPF on adipogenesis is attributed to the activation of p38 MAPK signaling at least partly.

In conclusion, DHPF, a prenylated flavan isolated from *M. yunnanensis*, was found to promote adipogenesis 3T3-L1 cells by enhancing the expression of PPARγ and C/EBPα through the p38 MAPK pathway. Moreover, DHPF can decrease the expression of TNFα, and increase the expression of adiponectin and GLUT4, resulting in a significant up-regulation of glucose uptake in 3T3-L1 adipocytes. Therefore, DHPF might be a potential lead compound for prevention and treatment of T2D.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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