Dehydrotrametenolic Acid Induces Preadipocyte Differentiation and Sensitizes Animal Models of Noninsulin-Dependent Diabetes Mellitus to Insulin

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We recently discovered that the triterpene acid compound dehydrotrametenolic acid promotes adipocyte differentiation in vitro and acts as an insulin sensitizer in vivo. This natural product has been isolated from dried sclerotia of Poria cocos WOLF (Polyporaceae), a well-known traditional Chinese medicinal plant. We examined the effects of dehydrotrametenolic acid on plasma glucose concentration in obese hyperglycemic db/db mice. Dehydrotrametenolic acid can reduce hyperglycemia in mouse models of noninsulin-dependent diabetes mellitus (NIDDM) and act as an insulin sensitizer as indicated by the results of the glucose tolerance test. These terpenoids and thiazolidine type of antidiabetic agents such as Ciglitazone, although structurally unrelated, share many biological activities: both induce adipose conversion, activate peroxisome proliferator-activated receptor γ (PPAR γ) in vitro, and reduce hyperglycemia in animal models of NIDDM. Dehydrotrametenolic acid is a promising candidate for a new type of insulin-sensitizing drug. This finding is very important for the development of insulin sensitizers that are not of the thiazolidine type.

Key words adipocyte differentiation; noninsulin-dependent diabetes mellitus; triterpene; dehydrotrametenolic acid; pachymic acid; peroxisome proliferator-activated receptor γ (PPAR γ)

Poria cocos WOLF is a parasite commonly found on roots of pine trees, it grows naturally in China, Korea, Japan, and North America. Dried sclerotia of P. cocos WOLF are used in the Chinese drug Hoelen and are used in combination with other drugs in more than 52 traditional Chinese prescriptions as a diuretic, sedative, and tonic medicine. Poria cocos WOLF consists of 90% β-glucan and 10% various terpenes by dry weight. The predominant triterpenes in P. cocos WOLF are pachymic acid, tumulosic acid and eburicoic acid, which are the lanostane type of triterpene acids. Pachymic acid also has been isolated from the European fungus Fomitopsis pini-cola. Pachymic acid and dehydrotumulosic acid isolated from P. cocos WOLF actively inhibited phospholipase A2 from snake venom. The sclerotia of P. cocos WOLF are usually prescribed as an antipalpitation drug. Kaminaga et al. demonstrated that triterpenes of P. cocos WOLF have a potent inhibitory effect on tumor promotion in mice skin two-stage carcinogenesis and on 12-0-tetradecanoyl phobol-13-acetate-induced inflammation in mice. Tai et al. reported that triterpenes extracted from P. cocos WOLF with an exomethylene group at C-24, such as pachymic acid, exhibited antiemetic activity in frogs. Until this study there has been no report concerning the antidiabetic effect of P. cocos WOLF.

In a previous study, we established preadipose cell line ST 13 from adult ddN mice and discovered the physiological modulator or pharmacological agent that controls terminal differentiation of preadipocytes. We also demonstrated that Ciglitazone (ADD4743) prostaglandins D2, F2α, A2, E1, and E2 induce ST 13 adipose conversion. Of these adipose inducers, the most potent adipose promoter is Ciglitazone (ADD4743), which was originally synthesized as an oral antidiabetic drug by Takeda Chemical Industries Ltd. (Japan). Retinoids and 1,25-dihydroxyvitamin D3, the active form of vitamin D3, inhibited ST 13 and 3T3 L1 preadipocyte differentiation at physiological concentrations. Furthermore, we demonstrated the presence of nuclear vitamin D3 receptors in both preadipose cell lines. Peroxisome proliferator-activated receptor γ (PPAR γ), which belongs to the nuclear receptor superfamily, is expressed at high levels in the adipose tissue and functions as a master regulator of adipocyte differentiation. Recent studies show that the ability of thiazolidinediones to bind and activate PPAR γ correlate with their ability to reduce hyperglycemia in animal models of noninsulin-dependent diabetes mellitus (NIDDM) and obesity. We found that a methanol extractive fraction of P. cocos WOLF is a potent inducer of adipocyte differentiation. We attempted to identify the active component from P. cocos WOLF and tested whether this compound activates PPAR γ or reduces plasma glucose in animal models of NIDDM and obesity.

There are two possible mechanisms of action in insulin-sensitizing drug. One mechanism is the activation of PPAR as induced by thiazolidinediones, and another mechanism is the activation of retinoid X Receptor (RXR) as induced by the rexinoid LG100268. To recognize the direct repeating core sequence, retinoic acid receptor, vitamin D receptor, and PPAR γ form a heterodimer with the RXR. The rexinoid LGD 1069, which is presently undergoing phase II human clinical trials for cancer therapy, decreases fasting glucose levels to 55% of vehicle-treated control levels. Antidiabetic thiazolidinedione drugs such as Pioglitazone, BRL 49653, Ciglitazone, Troglitazone, form a high-affinity ligand with PPAR γ whose structure contains a thiazolidine frame.

Here we report that extracted lanostane-type triterpenes pachymic acid, dihydrotumulosic acid, and several triterpenes from P. cocos WOLF induce adipose conversion, activate PPAR γ, and act as an insulin sensitizer in an animal model of NIDDM.

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MATERIALS AND METHODS

Chemicals  Pachymic acid; dehydrotrametenolic acid; hydroxyheptacyclic acid; and poricoic acid A, B, and C were isolated from selerotia or surface layers of *P. cocos* WOLF.\(^{17,18}\) Polypropenolic acid and 3-α-acetyl-16α-hydroxy-dehydratemen- 
ic acid were hydrolyzed from their phthalimidomethyl esters during the isolation process. Ciglitazone (ADD4743), 5-[4-(1-methylcyclohexylmethoxy)benzyl] 2,4-thiazolididine- 
dione was kindly provided by Takeda Chemical Industries Ltd. TNT-coupled reticulocyte lysate systems were purchased from Promega, (Madison, WI, U.S.A.). ³⁵S methionine was obtained from Amersham (Arlington Heights, IL, U.S.A.).

Plasmid and Transfection/Transactivation Assay  The pcMX-PPAR γ1 expression vector was kindly provided by Dr. Ronald M. Evans (Salk Institute for Biological Studies). Adipocyte-specific fatty acid binding protein P2 (apoP2) reporter plasmid (pGL3aP2-5.4-luciferase) was constructed by ligating the promoter fragment (−5.4 kb) into the pGL3 basic vector (Promega). NIH 3T3 cells were transiently transfected with various expression plasmids using TransFast (Promega). After transfection, cells were incubated for 48 h in a culture medium with or without increasing the concentration of test compounds. Cell lysates were produced using a reporter lysis buffer (Promega) according to the manufacturer’s instructions. Luciferase activity was determined by the Dual-luciferase reporter assay system (Promega).

Protease Digestion Assay  The protease digestion assay was performed by the method of Allan et al.\(^{19}\) The plasmid pSG5-mouse PPAR γ1 and pSG5 RXR α were used to synthesize ³⁵S-radiolabeled PPAR γ1 and RXR α in a coupled transcription/translation system according to the manufacturer’s instructions (Promega). The transcription/translation re- actions were subsequently incubated for 20 min at 25 °C with 10⁻⁵ M pachymic acid or 0.1% vehicle dimethylsulfoxide (DMSO) and digested with trypsin at 20 °C for 20 min. The trypsin digestion was terminated by the addition of denaturing gel loading buffer. The products of the digestion were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Cell Culture and Induction of Adipose Conversion  Stocks of ST 13 cells were maintained in Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (1:1) supplemented with 10% calf serum (growth medium). To induce adipose conversion, 5 × 10⁵ cells were seeded onto 24-well plastic culture dishes with the growth medium and were refed with medium supplemented with 10% fetal bovine serum and test compounds on the following day (day 1). The medium was changed every 3 d up to day 14. When adipose conversion was evaluated. Each compound was dissolved in DMSO. The concentration of DMSO in the culture medium did not exceed 0.1% and vehicle-treated control cells were received 0.1% or 0.01% DMSO.

Estimation of Triacylglycerol and Protein Content  The cell monolayer was rinsed twice with phosphate buffered saline (PBS)\(^{-}\) and dried briefly before triacylglycerol extraction with isopropanol. The phospholipid, cholesterol, and free fatty acids in the extracts were removed by adsorption to silicic acid. Triacylglycerol was assayed colorimetrically. The residual cell sheet was solubilized with 1 M NaOH and used for the estimation of cell-layer protein content according to the method of Lowry et al.

In Vivo Studies  Obese male db/db C57BLKS/J mice (genotypes +/+ ) and their lean counterparts db/+ mice were obtained from Japan Clea. All institutional guidelines for animal care and use were applied in this study. Obese and lean mice (7 weeks old at the commence- ment of the study) were orally administered vehicle (0.5% carboxymethyl cellulose) or dehydrotrametenolic acid (110 mg/kg/d) by gavage once daily for 14 d. The gavage volume was 10 ml per 1 kg body weight. Plasma glucose concentrations were determined from blood obtained by tail bleeds. The compound was suspended in 0.5% carboxymethyl cellulose and administered orally to mice by a zonde. In the glu- cose tolerance test, mice received a 10% glucose solution (1.0 g/kg) after a 3-h fast. Serum insulin was determined using commercially available ELIZA kits, with mouse insulin used as the standard (Morinaga Institute of Biological Science, Yokohama, Japan).

RESULTS

Effect of Triterpene Compounds Isolated from *Poria cocos* on ST 13 Adipose Conversion  The structures of triterpenes extracted from *P. cocos* WOLF and ciglitazone are presented in Fig. 1. When ST 13 preadipose cells were treated with 10⁻⁶ M extracted triterpene compounds, they rapidly and uniformly differentiated into lipid-accumulating adipose cells within 7–8 d after cell seeding (Fig. 2A). Each compound was added from day 1 until day 14. Cells were harvested 14 d after cell seeding and were evaluated for triacylglycerol and protein contents. The most active lanostane- 
type triterpenoid, pachymic acid (10⁻⁵ M), and the antidiabetic agent Ciglitazone (3×10⁻⁶ M) were similar in their degrees of adipose conversion and lipid accumulation (Fig. 2B).

None of the triterpene acids affected the growth rate of ST 13 cells at concentrations below 10⁻⁵ M. A slight retardation of growth was observed in cells treated with 10⁻⁵ M of Cigli- 
tazone. In the triterpenes isolated from *P. cocos* WOLF, lanostane-type triterpene acids, such as pachymic acid, dehydro- 
trametenolic acid, polypropenolic acid C, dehydrotrametenolic acid, and 3-O-acetyl-16α-hydroxy-dehydratemenolic acid, have the ability to induce differentiation of preadipocytes. Other extracted triterpene acids, such as 3,4-secolanosta- 
7,9(11)-diene-type triterpene acids and poricoic acids A, B, D, are weak inducers of adipose conversion (Fig. 2B).

Protease Protection of PPAR γ1 with Pachymic Acid  Proteolytic analysis has previously demonstrated that ligands of the steroid, thyroid, LXR and PPAR γ receptors can, upon binding, specifically alter the conformation of the receptors.\(^{19–21}\) PPAR γ1 and RXR α were synthesized in vitro by a TNT-coupled transcription system. It was sub- 
sequently preincubated with 1% DMSO (vehicle-treated control) or 10⁻⁵ M pachymic acid and then incubated with H₂O or with increasing concentrations of trypsin. Digestion prod- 
ucts were analyzed by SDS-PAGE followed by autoradiogra- 
phy. As shown in Fig. 3A, pachymic acid produced a par- 
tially protease-resistant 27-kDa fragment of PPAR γ1 possibly reflecting a conformational change in PPAR γ1. However, pachymic acid fail to produce such a protease-resistant frag- 
ment of RXR α (Fig. 3B). These data support the notion that
the antidiabetic actions of pachymic acid are mediated through its direct binding to PPAR γ and the subsequent change to the active conformation of the receptor.

**Triterpene Compounds Activate a PPAR γ** PPAR γ appears to be involved in differentiation processes because the activation of PPAR γ triggers adipocyte differentiation and stimulates expression of several genes critical to adipogenesis. Lanostane-type triterpenes, such as pachymic acid and dehydrotrametenolic acid, promote adipocyte conversion of ST 13 in vitro (Figs. 2A, B). We tested whether these terpene derivatives activate PPAR γ. Like other members of the ligand-activated nuclear receptor superfamily, PPAR γ contains a central DNA-binding domain that binds to cis-acting elements in the promoter of its target genes. Adipocyte P2 (aP2) gene, which encodes an intracellular lipid-binding protein specifically expressed in adipocytes, is one of the target genes of PPAR γ. The proximal promoter region (5.4 kb) of the aP2 gene has two binding sites (PPAR-response element) for transcription factor PPAR γ. To determine whether these terpene compounds have the ability to activate PPAR γ, transactivation experiments were performed using transfected NIH 3T3 cells that express PPAR γ and PPAR-response element containing a reporter construct (aP2-5.4-luciferase). These terpene compounds activated the reporter construct in a dose-dependent manner, which correlates with their ability to induce adipocyte conversion in vitro (Fig. 4).

**Lanostane-Type Triterpene Dehydrotrametenolic Acid Lowers Plasma Glucose Concentrations and Acts as an Insulin Sensitizer in an Animal Model of NIDDM**

The predominant constituent of *P. cocos* Wolf is known to be pachymic acid. This triterpene is the most active inducer of adipose conversion among the extracted triterpenes. Pachymic acid is slightly unstable in basic solvent, however as compared with dehydrotrametenolic acid. Therefore, we investigated the effectiveness of dehydrotrametenolic acid as an orally administered drug. NIDDM model db/db mice lack a functional leptin receptor. These mice are highly insulin-resistant hyperglycemic, hypertriglyceridemic, and hyperinsulinemic. The diabetic status of these mice progress with time. Severe atrophy and degeneration of the islet of Langelhans leads to decreased plasma insulin levels in db/db mice after 10 weeks. Thus, we used 7-week-old db/db mice for 14 d oral treatment of dehydrotrametenolic acid. The average fasting plasma glucose levels of these mice were 250 mg/dl and 500 mg/dl at 7 weeks and 9 weeks old, respectively. Daily oral treatment of male db/db mice with dehydrotrametenolic acid prevented the time-dependent increase in plasma glucose concentrations and lowered plasma insulin levels (Figs. 5A, B). When administered orally to obese db/db mice at 110 mg/kg/d for 14 d, dehydrotrametenolic acid had no significant effect on the body weights of these mice (Fig. 5C).

The results of glucose tolerance test are shown in Fig. 6. Dehydrotrametenolic acid treatment normalized the abnormal response of plasma glucose to an oral glucose load. A highly significant difference (p<0.0005) was observed between mice treated and not treated with dehydrotrametenolic acid. These results indicate that orally administered dehydrotrametenolic acid is active on NIDDM.

**DISCUSSION**

In hope of discovering a new antidiabetic insulin sensitizer, extracts from several medicinal plants mixed with hot water or DMSO were screened. The objective was to identify compounds with the ability to promote differentiation of ST 13 preadipocytes to adipocytes. Previous studies indicate that...
at least one of the ligands for PPAR γ (such as thiazolidine derivatives) and ligands for RXR are insulin sensitizers. Ligands of both PPAR γ and RXR induce adipocyte differentiation in vitro. We identified a new structural type of insulin sensitizer from the medicinal plant P. cocos WOLF using this screening method; the results presented here demonstrate that dehydrotrametenolic acid is an active insulin sensitizer in obese hyperglycemic db/db mice. Two possible mechanisms for the induction of adipose conversion are suggested by these results. One is the activation of PPAR γ and another is the activation of RXR, both of which act as insulin sensitizers.

Nine triterpenoids isolated from P. cocos WOLF induced differentiation of ST 13 and 10T1/2 from preadipocyte to adipocyte. Dehydrotrametenolic acid and pachymic acid activated PPAR γ, but did not activate RXR based on the reporter assay (in preparation). These results indicate that dehydrotrametenolic acid acts as an insulin sensitizer by activating PPAR γ. The results of the protease protection assay suggest that dehydrotrametenolic acid binds PPAR γ, however, whether the dehydrotrametenolic acid binds PPAR γ directly remains to be determined.

The crude extract from whole P. cocos WOLF at concentration of 10 mg/ml can induce adipose conversion, and we did not find a potent inhibitory component from P. cocos WOLF affecting adipose conversion. Therefore, crude extract of P. cocos WOLF may be effective as oral antidiabetic agents. In Japan, the surface layer of P. cocos WOLF is not usually used as the raw material for the medicine, although the extract from the surface layer of P. cocos WOLF prepared with methanol can induce ST 13 preadipocyte differentiation. Tai et al. identified in the extract the following compounds; tumulosic acid, dehydrotumulosic acid, phthalimidomethyl eburicoate, phthalimidomethyl dehydroeburicoate, phthalimidomethyl trametenolate, phthalimidomethyl 3β-hydroxylanosta 7,9(11),24-trien-21-oate dehydroeburicoic acid, 3-epi-dehydrotrametenolic acid, methyl 3-epi-25-hydroxydehydrotrumulosate, and 9-secolanostanetriterpenes. We have to clarify the active triterpene in these components. Synthesis of these triterpene acids seems to be difficult because of the nu-

Fig. 2A. Pachymic Acid and Ciglitazone Induce Differentiation of ST 13 Preadipose Cells to Adipose Cells

ST 13 cells were cultured with 10−7 m insulin-containing medium for 14 d in the presence of (A) vehicle (DMSO 0.1%), (B) 1×10−5 m pachymic acid or (C) 1×10−5 m antidiabetic agent, Ciglitazone. Cells were fixed and stained with oil-red O on day 14.

Fig. 2B. Effect of Lanostane-Type Triterpene Acids on ST 13 Adipocyte Differentiation

Approximately 5×103 cells were seeded onto 24-well plastic tissue culture dishes. Drugs were introduced to culture on the following day with or without insulin (10−7 m) until day 14, when cultures were harvested to estimate triacylglycerol and protein content for triplicate wells. Data are expressed as mean±S.D. (n=3).
merous asymmetrical carbon molecules in their structure. Bohlmann et al. reported on the genes of mono- or diterpene synthases of plants and fungus. In the future if cloning of the gene encoding the enzyme for triterpene synthesis is successful, large amounts of triterpene can be produced easily.

Prostaglandin J-2 derivatives, oxidized low-density lipoprotein (oxLDL), 9-hydroxyoctadecanoic acid (9-HODE), and hydroxyoctadecanoic acid (HODE) have been identified as endogenous activators of the PPAR γ subtype. Many reports suggest that these natural ligands of PPAR γ may play

Fig. 3. Tryptic Partial Proteolysis of Pachymic Acid and (A) 35S-Labeled PPAR γ Protein or (B) 35S-Labeled RXR α Protein Were Incubated with 10^{-5} M Pachymic Acid or Vehicle (DMSO 0.1%) and Digested with Increasing Amounts of Trypsin at 20 °C for 20 min. Assay were then analyzed by SDS-PAGE and autoradiography. An arrow indicates the 27 kDa protease-resistant fragment of PPAR γ.

Fig. 4. Effects of Triterpene Acids on Transactivation of aP2 Promoter by PPAR γ. NIH 3T3 cells were transfected with an aP2 promoter luciferase construct (pGL3-aP2-5.4-luciferase), pCMX-PPAR γ expression vector, and pRL-TK. After treatment with test compounds for 2 d, cells were harvested and the Dual-luciferase assay was performed as described in Materials and Methods. Data are expressed as mean±S.D. (n=3).

Fig. 5. Dehydrotrametenolic Acid Decrease Hyperglycemia and Hyperinsulinaemia in db/db Mice

(A) Plasma glucose; (B) serum insulin ; (C) body weights were measured in obese db/db and lean db/+ mice administered vehicle or dehydrotrametenolic acid (110 mg/kg/d). Mean±S.D. (n=5). *Significantly different (p<0.01) from the vehicle treated control group as determined by Student’s unpaired t-test.
an important role in the regulation of lipid metabolism in vivo. Synthesis ligand of PPAR γ (such as thiazoldinedions), nonsteroidal anti-inflammatory agents like indomethacin, flufenamic acid and the triterpenes isolated from P. cocos may also play act as regulators of lipid metabolism in vivo. B. M. Spiegelman suggested the application of the ligands of PPAR γ to animal models of inflammation and atherosclerosis. Further investigations concerning therapeutic activity of dehydrotrametenolic acid or pachymic acid on NIDDM, inflammatory disease, or atherosclerosis are warranted.

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