Estrogen receptor mediates the effects of pseudoprotodiocins on adipogenesis in 3T3-L1 cells

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1Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing, People’s Republic of China; 2Life Science Division, Graduate School at Shenzhen, Tsinghua University, Shenzhen, People’s Republic of China; 3Department of Natural Products Chemistry, Shenyang Pharmaceutical University, Shenyang, People’s Republic of China; and 4Department of Cell Biology, School of Basic Medicine, Peking Union Medical College, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, People’s Republic of China

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Xiao J, Wang NL, Sun B, Cai GP. Estrogen receptor mediates the effects of pseudoprotodiocins on adipogenesis in 3T3-L1 cells. Am J Physiol Cell Physiol 299: C128–C138, 2010. First published April 28, 2010; doi:10.1152/ajpcell.00538.2009.—Estrogen receptors (ERs) play a pivotal role in adipogenesis; therefore, compounds targeting ERs may also affect fat formation. Recent studies have shown that the Dioscorea plant (commonly called yam) exhibits an antiobesity effect on rodents. However, the active compounds and underlying mechanisms responsible for this effect are not yet fully understood. We evaluated the effects of pseudoprotodiocin (PPD), a steroid saponin from Dioscorea nipponica Makino (a type of Dioscorea), on adipogenesis and the mechanisms underlying this effect. Treatment with PPD at the onset of adipogenic differentiation resulted in significantly decreased adipogenesis in both in vitro and in vivo experimental systems. An increased amount of ERs mRNA, protein, and the accumulation of ERs in the nucleus were also observed. However, the expression pattern of ERβ was not altered. Furthermore, the antiadipogenic effect of PPD was found to be ER dependent. It was also accompanied by the decreased expression of several genes involved in adipogenesis, including lipoprotein lipase (LPL), leptin, CCAAT/enhancer-binding-protein-α (C/EBPα), and peroxisome proliferator-activated receptor-γ (PPARγ), as well as the increased expression of some negative factors of adipogenesis, including preadipocyte factor 1 (Pre-1), GATA-binding protein 2 (GATA-2), GC-induced leucine-zipper protein (GILZ), and C/EBP homologous protein (CHOP-10). In addition to its estrogenic action, PPD also abolished the p38 mitogen-activated protein kinase (p38 MAPK) activation. Our results suggest that PPD inhibits adipogenesis in an ER-dependent manner and induces the expression of ERα. These findings may provide a lead toward a novel agent that can be used to treat obesity.

obesity; Dioscorea nipponica Makino; steroid saponin

Obesity has been closely correlated with sex hormone balance (28). Postmenopausal women exhibit characteristic abdominal weight gain, and ovariectomized female rodents demonstrate an increased body weight and fat stores. These effects can be prevented using hormone replacement therapies (HRT; reviewed in Ref. 47). Evidence from both humans and laboratory animals has demonstrated a pivotal role of estrogens and estrogen receptors (ERs) in fat formation and metabolism. There are two isoforms of the ER: ERα and ERβ. Several studies have shown that both ER isoforms function in fat formation and metabolism. Both male and female ERα-deficient mice, as well as aromatase knockout mice, possess 50–100% more adipose tissue than wild-type controls. This appears to be due to increases in both adipocyte number and size, suggesting a role for ERα in adipocyte growth and proliferation (10). Estradiol has also been found to inhibit fat accumulation in adipocytes expressing ERα (20). Moreover, multiple studies in humans and animals suggest that phytosterogens that are similar in structure to estrogens can play a beneficial role in reducing obesity and improving glucose control (for review see Ref. 3). HRT also appears to reduce the degree of central obesity (16); however, in postmenopausal women, HRT may lead to breast and uterine tumor progression (23, 34), which limits its applications. Consequently, targeting ERs with compounds that lack mitogenic activity in tumor cells may be a suitable approach to treat related endocrine diseases (31) and may also be applicable as an antiobesity therapy.

Dioscorea plants, otherwise known as yams, are important agricultural crops in tropical regions and are grown for their large tubers in parts of Africa, Asia, and Oceania (38). In China, saponins extracted from Dioscorea nipponica Makino have been used in folk medicine as a cure for coronary heart disease, asthma, rheumatoid arthritis, and hyperlipidemia, as well as a source of hormonal sterol synthesis precursors (8, 50). Steroid saponins isolated from Dioscorea have been shown to exert estrogenic effects (7), and Dioscorea plants have been reported to promote the health of postmenopausal women (32). Recently, studies have also revealed that the Dioscorea plant possesses antiobesity effects. Kwon and colleagues (24) reported that Sprague-Dawley rats fed a high-fat diet containing 5% Dioscorea nipponica Makino and 40% beef tallow gained significantly less body weight and adipose tissue than control animals fed a high-fat diet alone during an 8-wk experimental period (24). Another Dioscorea plant, Rhizoma Dioscoreae Tokoron, also affects obesity, and its activity is partially in response to the suppression of the sterol regulatory element-binding protein 1-dependent lipogenic pathway (46). However, the underlying mechanisms of the antiobesity activity of Dioscorea and its active compounds are not fully understood.

Pseudoprotodiocins (PPD) is a steroidal saponin compound found in plant species of the Dioscorea and Tribulus terrestris families (11, 27) (Fig. 1). PPD has been reported to have an inhibitory effect on some cancer cell lines (12, 21). To date, the biological and pharmacological activities of PPD are widely unknown. The purpose of the present study was to characterize the effect of PPD, isolated from Dioscorea nipponica Makino (a type of Dioscorea), on fat formation. The aglycone part of PPD is similar to estrogen; therefore, we hypothesized that PPD may function through ERs. In this report, we demonstrate...
that in 3T3-L1 cells, PPD inhibited the postconfluent expansion of preadipocytes and their subsequent differentiation into adipocytes. The modulation of fat formation is associated with a selective induction in ERα expression and inhibition in the p38 mitogen-activated protein kinase (p38 MAPK) activation.

**MATERIALS AND METHODS**

**Materials**

Pseudoprotodiocisin (CAS registry no. 102115-79-7) was kindly provided by Dr. Nai-Li Wang (Department of Natural Products Chemistry, Shenyang Pharmaceutical University). All tissue culture materials were from Gibco (Grand Island, NY). Insulin, dexamethasone, and 3-isobutyl-1-methylxanthine were from Sigma (St. Louis, MO). Rabbit polyclonal anti-ERα, rabbit polyclonal anti-ERβ, mouse monoclonal anti phosphorylated p38, mouse monoclonal anti-phosphorylated ERK1/2, mouse monoclonal anti-peroxisome proliferator-activated receptor (PPAR)-γ, mouse monoclonal anti-GAPDH, FITC-labeled anti-rabbit, horseradish peroxidase-conjugated secondary antibodies and protein A/G Plus agrose beads were from Santa Cruz Biotechnology (Santa Cruz, CA). The Enhanced Chemiluminescence (ECL) Detection Kit was from Amersham Pharmacia Biotech (Piscataway, NJ).

**Cell Culture**

3T3-L1 preadipocytes (obtained from City University of Hong Kong) were cultured in DMEM supplemented with 10% (vol/vol) calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 1 mM pyruvate at 37°C in 5% air-5% CO2. To induce differentiation, 2-day postconfluent 3T3-L1 preadipocytes were stimulated for 72 h with 0.5 mM 3-isobutyl-1-methylxanthine, 1 mM dexamethasone, and 5 μg/ml insulin (MDI), which were added to DMEM-10% FBS culture medium. On day 3, the MDI medium was replaced with DMEM-10% FBS with 5 μg/ml insulin. On day 6, the DMEM-10% FBS was replaced with DMEM-10% FBS, which was changed every 2 days until analysis on days 8–9.

**Concentration of PPD**

In preliminary experiments, we evaluated the cytotoxicity of PPD using a colorimetric cytotoxicity assay (Cytotoxicity Assay; Promega). PPD with concentrations above 10 μM resulted in significant cytotoxicity in 3T3-L1 cells. Therefore, we set the concentration of PPD at 0, 1 μM, 2 μM, and 4 μM. PPD was reconstituted as a 10 mM stock solution in DMSO, filter sterilized, and stored at −20°C. For each experiment, cells received PPD premixed with culture medium.

**Proliferation Assay**

Preconfluent 3T3-L1 preadipocytes were seeded in 96-well plates at a density of 10,000 cells in 100 μl of medium per well. PPD, in doses ranging from 0 to 4 μM, was added to the culture medium. At the end of experiment, a colorimetric proliferation assay [Cell Proliferation Kit (MTT); Roche Diagnostics, Mannheim, Germany] was performed as directed by the manufacturer. To calculate the absorbance values at each dose and time point, the mean absorbance of two blank wells (containing PPD in the culture medium but no cells) was subtracted from the mean absorbance of six wells containing cells.

**Cytotoxicity Assay**

Preconfluent 3T3-L1 preadipocytes were seeded in 24-well plates at a density of 50,000 cells in 1 ml of medium per well or in six-well plates at a density of 130,000 cells in 2 ml of medium per well. PPD, in doses ranging from 0 to 4 μM, was added to phenol red-free culture medium with or without cells. At the end of experiment, the medium was removed from the wells and stored at 4°C. Each remaining cell monolayer was washed three times with 37°C PBS, and the cells were scraped on ice in 1 ml lysis medium (0.05% Triton X-100 in PBS) per well. The lysate was stored at 4°C until a colorimetric cytotoxicity assay (Cytotoxicity Assay) was performed as directed by the manufacturer. The percent lactate dehydrogenase (LDH) release was calculated as follows: % LDH release = [LDH in culture supernatant/LDH in culture supernatant + LDH in cell lysates] × 100.

What should be emphasized is that although LDH release per well increased with cell expansion, the total LDH content (LDH in culture supernatant + LDH in cell lysates) per well also increased in proportion to the cell number. Thus, the percent LDH release shown in Figs. 2B, 3B, and 4B is not related to cell number, although it can directly reflect the percent LDH release per cell.

**Oil Red O Staining**

3T3-L1 adipocytes were washed with PBS, fixed with 10% formaldehyde in PBS (pH 7.4), and stained with 0.5% Oil Red O.

**Triglyceride Assay**

On days 8–9 after differentiation, 3T3-L1 cells were washed with PBS, scraped on ice in 100 ml of saline solution (2 M NaCl, 2 mM EDTA, and 50 mM sodium phosphate, pH 7.4), sonicated to homogenize the cell suspension, and assayed for total triglycerides (GPO-Trinder; Sigma) as previously described (45). The results are expressed as total triglycerides per cellular protein (DC Protein Assay; Bio-Rad, Hercules, CA).

**Real-Time RT-PCR**

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). A total of 2 μg total RNA was reverse transcribed using the Super-Script First-Strand Synthesis System (Invitrogen). cDNA was synthesized from the isolated RNA, and cycle time (Ct) values were obtained using real-time PCR with the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), an ABI PRISM 7300 Sequence Detection System and analysis software (Applied Biosystems), as previously described (4). Primers were designed using Applied Biosystems Primer Express Software (version 2.0), and the primer sequences are shown in Table 1.

**Immunoblot Analysis**

Cells were washed twice in ice-cold PBS with 1 mM orthovanadate and placed immediately in sample buffer [1% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1% NaN3, 10 mg/ml aprotinin, 1 mM pepstatin, 16.4 mg/ml leupeptin, 1 mM phenylmethylsulfonfluoride (PMSF), 0.1 mM Na3VO4, 2% SDS, and 10% glycerol] without dithiothreitol (DTT). The lysates were heated, and the protein concentration was determined before the addition of 100 mM DTT. The protein concentration was determined using the Bio-Rad DC Protein Determination Kit, with bovine serum albumin as the standard.
The 3T3-L1 cells were seeded in a six-well plate. Two-day confluent cells were treated in the presence or absence of 2 μM PPD for 2 days and the cells were then trypsinized and suspended in DMEM with 10% FBS. After centrifugation, cell pellets were resuspended in PBS, after which 10^7 cells (500 μl) were subcutaneously injected on the backs of 6-wk-old athymic BALB/c nude mice. We repeatedly injected PPD-treated and PPD-untreated cells every week for a period of 6 wk. The cells grew and developed into mature lipid-laden fat pads over the next 3–4 wk. At 6 wk after implantation, mice were anesthetized via inhaled metofane, and the fat pads were excised. The adjacent skin and subcutaneous muscle tissues were removed. The plugs were then fixed overnight, and the transplants were weighed and analyzed histologically as previously described (44). Two groups of mice, PPD-treated mice and PPD-untreated nude mice, were investigated.

**Histology**

The specimens were fixed overnight in 10% buffered formalin, dehydrated, and embedded in paraffin wax. Sections (5 mm) were stained with hematoxylin and eosin (H&E) and examined. Photographs were taken under a light microscope (Olympus BH-2).

**Statistical Analysis**

The data are expressed as means ± SD. The significance of the differences between the means was assessed using Student's t-test, with P < 0.05 considered significant. A one-way ANOVA with Bonferroni corrections was used to determine significance for multi-

<table>
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*ER*, estrogen receptor; *PPARγ*, peroxisome proliferator-activated receptor-γ; *C/EBP*, CCAAT/enhancer-binding-protein; *PAI-1*, plasminogen activator inhibitor-1; *TNF-α*, tumor necrosis factor-α; *VEGFA*, vascular endothelial growth factor A; *Pre-1*, preadipocyte factor 1; *GATA-2*, GATA-binding protein 2; *GILZ*, GC-induced leucine-zipper protein; *CHOP-10*, C/EBP homologous protein; *LPL*, lipoprotein lipase; *F*, forward; *R*, reverse.

albumin as the standard. The samples were heated for 5 min at 95°C, separated using 10% SDS-PAGE, and analyzed using immunoblot, as previously described (25). The immunoblots were developed with the ECL kit.

**Indirect Immunofluorescence Assay**

3T3-L1 cells cultured on Lab-Tek chamber slides (Nunc, Naperville, IL) were washed with PBS and fixed with 10% formaldehyde in PBS (pH 7.4). The cells were treated with 0.3% Triton X-100 in PBS for 15 min at room temperature to increase permeability. After blocking with 10% normal goat serum in PBS at room temperature for 1 h, the cell monolayers were screened using a standard indirect immunofluorescence staining procedure that uses polyclonal antibodies to ERα or ERβ (1:200) and a FITC-labeled goat anti-rabbit antibody (1:200) as a secondary antibody (Santa Cruz Biotechnology). The quantitative analyses of the intercellular fluorescence were performed using Image-Pro Plus software (version 6.0). The ratio of nuclear to cytoplasmic fluorescence was calculated and is expressed as the mean ± SE.

**De Novo Adipose Formation Model**

All mice used in this study were handled in compliance with the guidelines for the care and use of laboratory animals established by the Chinese Council on Animal Care, and all animal protocols were approved by the Tsinghua University Animal Care and Use Committee. The 3T3-L1 cells were seeded in a six-well plate. Two-day
ple comparisons. The calculations were performed using SPSS (version 11.0) statistical software.

RESULTS

Effect of PPD on Proliferation of 3T3-L1 Cells

PPD has no effect on the proliferation of preconfluent preadipocytes. For terminal differentiation to adipocytes depends on proliferation of both pre- and postconfluent preadipocytes (29). We first determined whether PPD affects the proliferation of preconfluent preadipocytes. PPD, in doses ranging from 0 to 4 μM, was added at the time of plating, and cell proliferation at several points during the course of growth was measured. As shown in Fig. 2A, PPD had no effect on the proliferation of 3T3-L1 preadipocytes. By introducing ICI 182,780 (an ER antagonist), we also demonstrated that the lack of effect of PPD at this early stage of differentiation didn’t rely on the lack of ER. As shown in Fig. 2B, although all cells released LDH over time, roughly 90% more LDH was released at 72 h than at 24 h; PPD did not significantly induce more LDH release per cell at any time point in the experiment compared with the DMSO (0.1% DMSO)-treated cells.

PPD inhibits mitotic clonal expansion in an ER-dependent manner. After stimulation with MDI differentiation medium, growth-arrested postconfluent 3T3-L1 cells undergo two rounds of mitotic clonal expansion before exiting the cell cycle and expressing adipocyte-specific genes (18). We next determined whether PPD affects the mitotic clonal expansion of postconfluent preadipocytes. Two-day postconfluent 3T3-L1 cells were cultured in MDI with various doses of PPD, and proliferation was measured 72 h later. As shown in Fig. 3A, PPD inhibited postconfluent mitotic clonal expansion of 3T3-L1 preadipocytes in a dose-dependent manner. This inhibitory effect of PPD can be reversed by ICI 182,780. However, PPD did not significantly trigger more LDH release per cell during this process (Fig. 3B).

PPD Inhibits Adipogenesis in Vitro and in Vivo

PPD exerts a time-restricted inhibitory effect on adipogenesis in 3T3-L1 cells. We next examined the dependency of adipogenesis on the administration time of the PPD. PPD, ranging from 0 to 4 μM, was added at several time points (day −3, day 0, day 3, and day 6) for 72 h. Two-day postconfluent 3T3-L1 cells were differentiated using the MDI protocol, and quantitative analyses were performed by measuring the optical density at 510 nm of extracts of Oil Red O-stained cells. As shown in Fig. 4A, PPD inhibited adipogenic differentiation only when it was administered on day 0, and the inhibitory effect was dose dependent. As shown in Fig. 4B, PPD did not trigger more LDH release per cell in this process. Additionally, mRNA levels of PPAR and CCAAT/enhancer-binding-protein-α (C/EBPα) were also inhibited when PPD was administered at day 0, which may further confirm the time-restricted effect of PPD (Fig. 4, C and D).

Fig. 3. Proliferative and cytotoxic effects of PPD on 3T3-L1 cells during postconfluent mitotic clonal expansion. Two-day postconfluent 3T3-L1 cells were cultured in MDI medium cotreated with 2 μM PPD in the presence or absence of ICI. Ind, cells that were differentiated using the MDI protocol; DMSO, medium without the inducers of differentiation; vehicle, differentiation medium with DMSO. The degree of cell proliferation (A, expressed as the percentage of control) and the cytotoxicity of PPD (B, expressed as the percent LDH release) were examined at day 3 (the onset of differentiation was designated day 0). The results are presented as means ± SE (n = 8 per group). Within each experiment, the different letters (a, b, c, d) indicate statistically significant differences between the means (P ≤ 0.05).
Inhibition of de novo adipose tissue formation in nude mice via PPD. Although the role of PPD in adipocyte differentiation has been examined in cultured cells, its role in in vivo adipogenesis has not yet been explored. When injected into mice, 3T3-L1 preadipocytes differentiate and form fat pads that are indistinguishable from normal adipose tissue (36). Thus, the 3T3-L1 cells in vivo transplantation model was used to explore the role of PPD in in vivo adipogenesis. To address this issue, 2-day postconfluent 3T3-L1 cells were treated with 2 μM PPD for 48 h. These cells were repeatedly implanted subcutaneously into athymic mice once a week for 6 wk. As shown in Fig. 5A, cells without PPD treatment formed substantial adipose tissue developed 6 wk after implantation. The weight of the fat pads revealed a significant reduction of fat formation in the mice injected with cells pretreated with PPD. The number and size of adipocytes decreased at the 6-wk time point (Fig. 5B; supplemental data for this article can be found online at the American Journal of Physiology-Cell Physiology web site). No obvious macrophage infiltration in both groups was observed (Fig. S1). Furthermore, the mRNA levels of several genes involved in adipogenesis, such as PPARγ, C/EBPα, lipoprotein lipase (LPL), and leptin, were also downregulated in PPD-treated transplants at both the 1-wk and 6-wk time points (Fig. 5D).

Angiogenesis is required for adipose tissue expansion in obesity (4). Although no decreased neovascularure in PPD-treated transplants was detected by H&E staining (Fig. 5C), mRNA levels of the gene expression of plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor A (VEGFA)—also known as the three angiogenic factors expressed in adipose tissue—were downregulated in PPD-treated transplants at both the 1-wk and 6-wk time points (Fig. 5D).

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Fig. 4. Time-restricted effect of PPD. 3T3-L1 cells were differentiated according to the MDI protocol. DMSO, medium without the inducers of differentiation; vehicle, differentiation medium with DMSO. PPD was administrated at several time points (day −3, day 0, day 3, or day 6) and maintained for 48 h. On days 8–9, quantitative analyses were performed by measuring the optical density (OD) at 510 nm of extracts generated from Oil Red O-stained cells (A), cytotoxicity of PPD (β, expressed as percent LDH release), and mRNA level of two adipogenic master genes, peroxisome proliferator-activated receptor-γ (PPARγ) (C) and CCAAT/enhancer-binding-protein-α (C/EBPα) (D). Relative mRNA levels were normalized to that of GAPDH. The value from DMSO (0.1% DMSO)-treated cells for each gene was defined as 1. The results are presented as means ± SE (n = 8 per group). Within each experiment, the different letters (a, b, c, d) indicate statistically significant differences between the means (P ≤ 0.05).
PPD inhibited adipogenesis in an ER-dependent manner. Two-day postconfluent 3T3-L1 cells were differentiated using the MDI protocol, and the cells were cotreated with DMSO (0.1% DMSO) or PPD, in doses ranging from 0 to 4 μM, at the onset of differentiation (day 0) and maintained for 48 h. As shown in Fig. 6A, we observed a dose-dependent inhibitory effect of PPD. Furthermore, the PPD-treated cells accumulated approximately 30–80% of the intracellular triglyceride content of the controls, as shown by both Oil Red O staining (Fig. 6B) and triglyceride accumulation (Fig. 6C). Furthermore, the antiadipogenic effect of PPD can be reversed byICI 182,780, an ER antagonist. And ICI 182,780 alone is not sufficient to promote differentiation.

PPD-induced ERα expression and nuclear ERα accumulation. Studies examining the predominant biological effects of estrogen have revealed that two distinct intracellular receptors, ERα and ERβ, appear to have overlapping but different roles (39). Therefore, experiments were conducted to determine whether PPD can alter the expression pattern of the ERs. Two-day postconfluent 3T3-L1 cells were differentiated using the MDI protocol, and the cells were cotreated with DMSO (0.1% DMSO) or 2 μM PPD at the onset of differentiation (day 0) and maintained for 48 h. Both forms of the ER were detected in 3T3-L1 cells using real-time RT-PCR and immunoblot, and the amount of ERα protein increased significantly in response to PPD (Fig. 7, A and B). This PPD-induced increase in the amount of ERα mRNA and protein was paralleled by an increase in the accumulation of ERα in the nucleus, which was monitored using an indirect immunofluorescence assay and quantitative analysis of the ratio of nuclear to cytoplasmic fluorescence (Fig. 7, C and D). In contrast, neither the amount of ERβ mRNA and protein nor the subcellular localization in 3T3-L cells changed with PPD treatment.

ER-dependent regulation of genes involved in adipogenesis by PPD. We examined the effect of PPD on the mRNA level of genes involved in adipogenesis. Two-day postconfluent 3T3-L1 cells were differentiated using the MDI protocol, and the cells were cotreated with DMSO (0.1% DMSO) or 2 μM PPD at the onset of differentiation (day 0) and maintained for 48 h. As shown in Fig. 8A, the mRNA level of LPL, leptin, C/EBPβ, and PPARγ at day 8 was significantly downregulated by PPD, whereas treatment with ICI 182,780, abolished this effect. The level of these genes in control cells was consistent with previous studies that demonstrated that the expression level of these genes increases as the cells differentiate towards the terminal adipocyte state (18, 20, 37).

Previous studies have shown that C/EBPβ and -δ are the first transcriptional factors induced after MDI induction which can mediate PPARγ and C/EBPα transcription (29). Here, we evaluated the mRNA levels of C/EBPβ and -δ at days 2 and 5. As shown in Fig. 8B, PPD resulted in reduced mRNA levels of C/EBPβ and -δ at day 2 which can be abolished by ICI 182,780, whereas PPD exerted no significant effect at day 5. As shown in Fig. 8C, PPD also caused an induction in some negative regulators of adipogenesis, such as Pre-1, GATA-binding protein 2, GC-induced leucine-zipper protein, and C/EBP homologous protein (CHOP-10), which can also be reversed by ICI 182,780.

ER-dependent inhibition of the MAPK signaling pathway by PPD. The MAPK signaling pathway is involved in adipogenesis, and the phosphorylation of ERK1/2 and p38 are key...
events in adipocyte differentiation (2, 26). To examine the effects of PPD on ERK1/2 and p38, 2-day postconfluent 3T3-L1 cells were differentiated using the MDI protocol, and the cells were cotreated with DMSO (0.1% DMSO) or 2 μM PPD at the onset of differentiation (day 0) and maintained for 48 h. The cell lysates were analyzed using an immunoblot with either an anti-phospho-ERK1/2 antibody or an anti-phospho-p38 antibody, which detect phosphorylated ERK1/2 and p38, respectively. As shown in Fig. 8D, PPD induced the dephosphorylation of p38 in 3T3-L1 cells. The treatment of 3T3-L1 cells with ICI 182,780 inhibited the PPD-induced dephosphorylation of p38. In contrast, PPD cotreatment did not alter the phosphorylation of ERK1/2 during adipogenesis significantly (Fig. 8E).

**DISCUSSION**

In the present study, we characterized the effect of PPD, a naturally occurring active compound from *Dioscorea nipponica* Makino, on adipogenesis using both in vitro and in vivo experimental systems. The in vitro adipogenesis model using the 3T3-L1 cell line showed that treatment with PPD caused a reduction in fat formation, elevated the total amount of ERα expression, and increased the accumulation of ERα in the nucleus. Furthermore, in vivo transplantation of 3T3-L1 cells treated with PPD also impeded fat pad formation in nude mice. Our data demonstrate that PPD is an active compound in *Dioscorea nipponica* Makino that contributes to the antiobesity activity of the plant. To the best of our knowledge, this research is the first to shed light on the effects of PPD on fat formation.

Steroid saponins are externally distributed in plants, with some possessing a structure similar to that of sex steroid hormones. Therefore, they can potentially also affect sex ste-
roid hormone receptors, specifically estrogen receptors, which they can affect directly (30). In the present study, the inhibitory effect of PPD on postconfluent preadipocyte expansion and the subsequent differentiation were attenuated by ICI 182,780. As a potent steroidal antiestrogen, ICI 182,780 can bind to both ER subtypes (48). However, studies of ER expression in both testis and efferent ductules also showed that ICI 182,780 decreased the expression of ERα, but not ERβ. The downregulation of ERα protein by ICI 182,780 is thought to be caused by a change in the ERα conformation, which leads to a rapid loss of the receptor (49). In our present study, ICI 182,780 could knock down PPD-induced expression in both ERα and ERβ, whereas only ERα was sensitive to PPD treatment (Fig. 7). Although ERα and ERβ have overlapping tissue distribution, a number of molecular mechanisms may explain the different roles of ERα and ERβ, including their differences in ligand affinity and transactivation, distinct cofactor interactions, and putative heterodimerization (5). In this study, PPD triggered an increase in ERα mRNA and protein expression, as well as an accumulation of ERα in the nucleus, while it had no effect on ERβ expression or localization (Fig. 7). Although both ERα and ERβ are expressed in adipocytes, ERα is the predominantly expressed isoform (22). Mature, male ERα-deficient mice and double (ERα/ERβ) knockout mice have increased body weight and adipose tissue concomitant with higher levels of cholesterol and leptin, a sensor with a high caloric supply (37). In addition, estradiol-induced ERα-mediated transcriptional suppression of LPL, an enzyme that catabolizes plasma triglycerides (20). Our results are consistent with previous work stating that PPD suppresses the elevated expression of LPL and leptin mRNA. Furthermore, adipogenic transcriptional factors (Fig. 8, A and B) and several negative regulators of adipogenesis (Fig. 8C) were also changed by PPD. In contrast, ERβ-deficient mice have normal body weight and adipose tissue compared with wild-type mice (37). These studies clearly indicate that ERα, but not ERβ, is a regulator of white adipose tissue mass. This finding may partially explain why, in the results, PPD had no effect on the ERβ isoform (Fig. 7). However, previous studies have also shown that adipose tissue and body weight decrease in ERα-deficient animals that have had an ovariectomy, suggesting the possible involvement of ERβ in adipogenesis (15). The reason why the expression pattern of ERβ was not altered by PPD treatment is to be determined. Potentially, PPD may be ERα selective because its conformational structure may be more prone to bind to ERα. This selective induction of ER isoforms may also provide a clue to identify other ER-selective compounds in plants.

It is well established that hormones regulate adipogenesis via the MAPK pathway, especially the ERK1/2 and p38 pathway (2, 26). Transient activation of the MEK/ERK signaling pathway during the early stages of adipogenesis is likely to be required to promote the differentiation process (41). p38 MAPK is also required for adipogenesis, and the activation of p38 promotes the spontaneous differentiation of preadipocytes (13). In the current study, we observed that the phosphorylation of both ERK1/2 and p38 is elevated after MDI induction. However, only phospho-p38 was downregulated by PPD, which can be reversed by ICI 182,780, suggesting that p38 MAPK may also be involved in the ER-dependent anti-adipogenic effect of PPD.

**Fig. 7.** Effect of PPD on the ER isoforms. DMSO, medium without the inducers of differentiation; vehicle, differentiation medium with DMSO. Real-time RT-PCR (A), immunoblot (B), indirect immunofluorescence (C; bar = 10 μm), and quantitative analyses of the ratio of nuclear to cytoplasmic fluorescence (D) of ER isoforms revealed the selective elevated expression and nuclear accumulation of ERα by PPD. In A, relative mRNA levels were normalized to that of GAPDH, and the value from DMSO (0.1% DMSO)-treated cells for each gene was defined as 1. The results are presented as means ± SE (n = 8 per group). Within each experiment, PPD was added in the presence or absence of ICI and the different letters (a, b, c) indicate statistically significant differences between the means (P ≤ 0.05).
MAPKs could also regulate C/EBPβ:H9252 or -H9254 dephosphorylation of p38 may directly cause reduced C/EBPβ:H9252 activity (1). Thus, PPD-induced depressed expression in ER:H9251 clonal expansion of postconfluent preadipocytes is a prerequisite for their further differentiation into adipocytes mediated by conformational changes in the chromatosome (18). This facilitates the binding of adipogenic transcriptional factors to their templates during the early stages of adipogenesis (18). In addition, cotreatment with pioglitazone, a PPARγ agonist, does not suppress the antiadipogenic effect of PPD, suggesting that PPARγ is not a direct target of PPD. So PPD may affect adipogenesis before the activation of PPARγ. The time-restricted effect of PPD may also partially explain why PPD exerts an effect when treated in a very short window; that is, PPD acts on two early-stage transcriptional factors, C/EBPβ:H9252 and -H9254. It also inhibits mitotic clonal expansion of preadipocytes.

Because PPD only plays a role when administered at the onset of adipogenesis (Fig. 4A), we propose that PPD may exert an influence on some events occurring in the first 72 h of adipogenesis. Previous studies have shown C/EBPβ:H9253 and C/EBPβ as the first transcription factors induced after exposure of the preadipocytes to the differentiation cocktail (29). In addition, the activities of C/EBPβ and β mediate the expression of PPARγ and C/EBPα, both of which increased after MDI stimulation (29). As shown in Fig. 8B, PPD resulted in reduced mRNA levels of C/EBPβ and -β at day 2. It exerted no effect on the expression of C/EBPβ and -β at day 5 although their levels declined. Thus, the antiadipogenic effect of PPD may be mediated through C/EBPβ and -β, resulting in a reduction in PPARγ and C/EBPα (Fig. 8A). Studies have also shown that ERα and C/EBPβ, or -β can interact and form a complex that can inhibit their transcription (6, 42). As previously reported, p38 MAPks could also regulate C/EBPβ transcriptional activity (1). Thus, PPD-induced depressed expression in ERα and dephosphorylation of p38 may directly cause reduced C/EBPβ or -β transcriptional activity. This will trigger a cascade of downregulated PPARγ, C/EBPα, and other genes involved in adipogenesis. Furthermore, the time-restricted effect of PPD may also be due to its inhibitory effect on postconfluence expansion of preadipocytes. During adipogenesis, the mitotic clonal expansion of postconfluent preadipocytes is a prerequisite for their further differentiation into adipocytes mediated by conformational changes in the chromatosome (18). This facilitates the binding of adipogenic transcriptional factors to their templates during the early stages of adipogenesis (18). In addition, cotreatment with pioglitazone, a PPARγ agonist, does not suppress the antiadipogenic effect of PPD, suggesting that PPARγ is not a direct target of PPD. So PPD may affect adipogenesis before the activation of PPARγ. The time-restricted effect of PPD may also partially explain why PPD exerts an effect when treated in a very short window; that is, PPD acts on two early-stage transcriptional factors, C/EBPβ and -β. It also inhibits mitotic clonal expansion of preadipocytes.

In accordance with what was observed in vitro, PPD also inhibited adipogenesis in vivo by triggering a cascade of downregulated master adipogenic transcriptional factors (PPARγ and C/EBPα), as well as other genes involved in adipogenesis such as LPL and leptin in the transplanted 3T3-L1 cells (Fig. 5D). Alternatively, PPD accumulation within the transplanted cells may also act as a “paracrine,” leading to reduced fat pad formation. Recently, subcutaneously implanted adipose cells were reported to have interacted with the surrounding cells and tissues; thus, the functions of adipose cells might be modified by stimuli from their surroundings (35). Apart from the observed effect that PPD treatment re-
sulted in a depressed expression of genes involving in adipogenesis (Fig. 8D), the antiadipogenic effect of PPD may also relate to the dynamic interactions between differentiating adipocytes, stromal cells, and angiogenesis in living obese adipose tissue. In the present study, mRNA levels of three angiogenic factors known to be expressed in adipose tissue, PAI-1, TNF-α, and VEGFA, were also downregulated in PPD-treated transplants at the one wk time point (Fig. 5D). These data demonstrated that PPD inhibited early-stage angiogenesis/vessel development. It has been reported that macrophage development also plays a role in adipogenesis (19). However, there is no obvious macrophage infiltration in both groups, suggesting that monocyte/macrophage development may not play a role in the antiadipogenic effect of PPD in vivo. Furthermore, when fat pads form, newly injected PPD-pre-treated cells can also affect surrounding preexisting preadipocytes in various stages of development.

Although PPD caused a significant inhibition of adipogenesis, we cannot deduce its effect on humans directly from our present studies. As we know, adipogenesis is regulated in two stages: hormonally and neurally. Apart from affecting the ERs in adipose tissue directly (9), PPD may also affect ERα in hypothalamic neurons and play a role in controlling energy balance, the maintenance of normal body weight (14, 33), and the modulation appetite or energy expenditure (9). Another reason is that all the data in the present study are based on mouse cells. Studies have shown that data obtained by studying the differentiation of mouse adipocytes cannot always be extrapolated to human adipocytes (17, 43). Thus, the effects of PPD on human adipocyte differentiation are yet to be determined in future studies.

In conclusion, PPD causes an inhibition of postclonal expansion of 3T3-L1 preadipocytes and their subsequent differentiation into adipocytes by partly altering the expression pattern of the ERα isofrom and may also be involved in blocking the p38 MAPK pathway. Moreover, PPD appears to mainly act during the early stages of adipogenesis.

Another issue that should be addressed is that the use of PPD, a nonapproved herbal medicine, in extensive traditional applications may result in a possible risk of both acute and chronic toxicities, which is a major biosafety concern (40). In our study, PPD was effective at micromolar concentrations and had no obvious cytotoxicity in 3T3-L1 preadipocytes (Figs. 2 and 3). Previous studies have also reported that PPD can inhibit the proliferation of some cancer cell lines (12, 21). So, unlike HRT, PPD may reduce weight gain in menopausal women without the detrimental side effects, including ovarian and breast cancer. Although rigorous examination of the adverse effects of PPD should be carried out, this compound is a promising agent for the treatment of obesity and could potentially be used to treat other hormone-related diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


