Ginsenoside 20(S)-Protopanaxatriol (PPT) Activates Peroxisome Proliferator-Activated Receptor γ (PPARγ) in 3T3-L1 Adipocytes

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Peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor of ligand-activated transcription factors, regulates the expression of key genes involved in lipid and glucose metabolism or adipocyte differentiation. Ligands for this receptor have emerged as potent insulin sensitizers used in the treatment of Type2 diabetes. Ginseng saponins or ginsenosides are reported to provide anti-diabetic activity as well as to modulate glucose metabolism, although the mechanism remains unclear. In this study, we examined the effect of ginsenosides on activation of PPARγ and adipogenesis in 3T3-L1. Using a GAL-4/PPARγ transactivation assay, 20(S)-protopanaxatriol (PPT), one of the ginsenoside metabolites, was found to increase PPARγ-transactivation activity dose-dependently with similar activity as troglitazone, a well-known PPARγ agonist. PPT enhanced adipogenesis by increasing the expression of PPARγ target genes such as aP2, LPL and PEPCK. Furthermore, PPT significantly increased expression of glucose transporter 4 (GLUT4). These results indicate that PPT can be developed as a PPARγ agonist for the improvement of insulin resistance associated with diabetes.

Key words PPARγ; PPT; GAL-4/PPARγ transactivation assay; 3T3-L1 pre-adipocyte

Peroxisome proliferator-activated receptor γ (PPARγ), one of the subtypes of PPARs, is a ligand-dependent transcription factor of the nuclear hormone receptor superfamily. 1 PPARγ is expressed not only in the adipose tissue but also in the immune system organ, adrenals and small intestine.2 It is a target for therapeutic intervention in cardiovascular diseases, various cancers and diabetes.3 PPARγ is an essential transcription factor in adipogenesis4 and the overexpression of PPARγ enhances adipogenesis in vitro.5 PPARγ is a regulator of the insulin resistance in adipose tissue and it is the predominant molecular target for the insulin-sensitizing TZD drugs such as troglitazone, pioglitazone and rosiglitazone.6 TZDs, which are anti-diabetic drugs, improve insulin resistance, promote adipocyte differentiation and induce apoptosis in large adipocytes via PPARγ activation in adipocytes. 7

Ginseng has a long history of medicinal usage traditionally in the oriental region as a general tonic to promote health. There are extensive reports that ginseng has many pharmacological effects on immune, cardiovascular, central nervous systems and endocrine.8,9 The molecular components responsible for ginseng actions are ginsenosides, which are also known as ginseng saponins. The basic structure of ginsenosides consists of a gomane steroid nucleus with 17 carbon atoms arranged in four rings. The characteristic biological response for each ginsenoside is attributed to the difference in the type, position and number of sugar moieties attached by a glycosidic bond at C-3 and C-6.10

The two major group of ginsenosides are the protopanaxadiol and protopanaxatriol groups. The protopanaxadiol group includes Rb1, Rb2, Rc, Rd and Rh2, while the protopanaxtriol group includes Re, Rf, Rg1, Rg2, Rg3, and Rh1. 20(S)-protopanaxatriol (PPT) is an aglycone of ginsenosides Re, Rf, Rg1, Rg2, and Rh1, while 20(S)-protopanaxadiol (PPD) is that of ginsenosides Rb1, Rb2, Rc, Rd and Rh2.

The blood glucose-lowering effect of ginseng root has been investigated frequently.11,12 Recently, the extract of Panax ginseng berry and ginsenoside Re were reported to have anti-diabetic effect in an ob/ob mouse model.13 However, there is only limited data on the anti-diabetic biological mechanism of ginsenosides. Therefore, to discover anti-diabetic mechanism of ginsenosides, we investigated PPARγ ligand binding activity of ginsenosides. We carried out transactivation assays for PPARγ ligands using 19 kinds of ginsenosides, and found that PPT (Fig. 1), aglycone of ginsenoside Rg2, had the highest PPARγ ligand-binding activity than other ginsenosides tested. In this study, we hypothesized that PPT exerts a beneficial antidiabetic effect by activation of PPARγ.

MATERIALS AND METHODS

Chemical Reagents Ginsenoside 20(S)-protopanaxatriol or PPT was provided by Ambo Institute (Seoul, Korea). Troglitazone, a specific PPARγ agonist, was purchased from Sigma (St. Louis, MO, U.S.A.). These compounds were diluted with dimethyl sulfoxide (DMSO) to prepare the stock solution (10—100 mM).

Cells Monkey COS-7 kidney cells and mouse 3T3-L1 pre-adipocytes were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, U.S.A.). Cells were cultured in DMEM (Dulbecco’s Modified Eagle’s Medium) supplemented with antibiotics (100 U/ml of penicillin A and 100 U/ml of streptomycin) and 10% heat-inactivated fetal bovine serum (Gibco, Rockville, MD, U.S.A.) and maintained at 37°C in a humidified incubator containing 5% CO2.

Fig. 1. Chemical Structure of 20(S)-Protopanaxatriol (PPT)
Cell viability was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. 14)

Transactivation Assay PPARγ ligand-binding activity was measured using a GAL-4/PPARγ transactivation assay. 15) COS-7 monkey kidney cells were inoculated into a 96-well culture plate at 1.5×10^4 cells/well, and incubated in 5% CO₂/air at 37°C for 24 h. DMEM (Gibco, Grand Island, NY, U.S.A.) containing 10% fetal bovine serum (FBS) and 10 ml/1 penicillin-streptomycin (5000 IU/ml and 5000 mg/ml) (Gibco, Grand Island, NY, U.S.A.) was used as the medium. Cells were washed with Dulbecco’s phosphate-buffered saline (DPBS) (Sigma, St. Louis, MO, U.S.A.) and transfected with pFA-hPPARγ, pFR-Gal4 (UAS-Gal4-luciferase) and pFR-β-galactosidase (Stratagene, La Jolla, CA, U.S.A.) using Genejuice (Novagen, Madison, WI, U.S.A.). In an internal control, pFA and pFR-Gal4 (UAS-Gal4-luciferase) were transfected into COS-7 cells. 24 h after transfection, the medium was changed with DMEM containing 10% FBS and each sample, and the cells were further cultured for 24 h. Then, the cells were washed with DPBS, to which luciferase assay substrate (Promega, Madison, WI, U.S.A.) was added. The intensity of emitted luminescence was determined using a CytoFluor Series 4000 multwell luminescence plate reader (PerSeptive Biosystems Inc., Framingham, MA, U.S.A.). PPARγ ligand-binding activity of the test sample was expressed as the relative luminescence intensity to that of control.

Adipogenesis and Quantification of Lipid Accumulation To induce adipogenesis, 3T3-L1 cells (1×10⁵ cells/well) were plated into culture 96-well plate and maintained for 2 d after reaching confluence. Then, medium were exchanged with differentiation medium (DMEM containing 10% FBS, 0.5 mM isobutylmethylxanthine (IBMX), 1 μM dexamethasone and 10 μg/ml insulin) and cells were incubated for 2 d. Finally, differentiation medium was replaced with adipocyte growth medium (DMEM supplemented with 10% FBS and 10 μg/ml insulin), which was refreshed every 2 d. 16) At day 1, 3, 5, 7 after differentiation medium was added, cells were retrieved and neutral lipid accumulation was measured using a previously published method. 17) Briefly, cells were washed with PBS, fixed with 10% buffered formalin and stained with Oil Red O solution (0.5 g in 100 ml isopropanol) for 10 min. After removing the staining solution, the dye retained in the cells was eluted into isopropanol and OD₅₉₅ was determined.

RNA Preparations and Real-Time RT-PCR Total RNA was isolated with RNeasy Mini Kit (Qiagen, Valencia, CA, U.S.A.) at adipogenesis after day 3. Using M-MLV Reverse Transcriptase (Promega, Madison, WI, U.S.A.), the total RNA was reverse-transcribed according to the manufacturer’s instructions with a thermal cycler (Perkin-Elmer PCR Thermal Cycler, Perkin-Elmer, Wellesley, MA, U.S.A.). To quantify mRNA expressions, PCR was performed using a fluorescence temperature cycler (Chromo4, real-time PCR System, BioRad, Hercules, CA, U.S.A.). The oligonucleotide primers of PPARγ target genes were designed using a PCR primer selection program at the website of the Virtual Genomic Center from the GenBank database as follows: lipoprotein lipase (LPL, sense: 5′-ATCCATGATGGAGCTTAACCAG-3′ and antisense: 5′-CTTGCATCCCAATACTTCC-GACCA-3′), adipocyte fatty acid-binding protein (aP2, sense: 5′-AAAGACACGCTCCTCTCAAGTT-3′ and antisense: 5′-TGACCAATCCCATTTACGC-3′), phosphoenol pyruvate carboxykinase (PEPCK, sense: 5′-TACATAC-TACCGAAGGCAAAG-3′ and antisense: 5′-CTTTGATG- CACCCCTGGAAT-3′) and β-actin (sense: 5′-TGGAACTC- CTGTGGCATCCATGAAC-3′ and antisense: 5′-TAAACGGCAGCTCAGTACGTCGG-3′). Briefly, the reaction solution (20 μl final volume) contained 3 μl MgCl₂, 2.0 μl of DyNaMo HS SYBR Green qPCR kit mater mix (Finnzymes, Espoo, Finand) and 100 pm of each primer. The standard amplification program included 30 cycles of three steps each, which involved heating the product to 95°C with a 20 s hold, annealing to 60°C with a 20 s hold, and extending to 72°C with a 10 s hold.

Western Blot Analysis Differentiated 3T3-L1 cells were homogenized in the buffer (1% Triton X-100, sodium pyrophosphate 100 mM, HEPES 50 mM, pH 7.4, PMSF 2 mM). The homogenates were centrifuged at 12000 rpm for 20 min and the supernatants were collected for GLUT4 analysis. Protein extracts were separated using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose membranes. The nitrocellulose membranes were incubated in phosphate buffered saline containing 5% skim milk and 0.1% Tween-20 at 4°C overnight to reduce non-specific binding and blotted with GLUT4 antibody (1:1000, Santa Cruz Biotechnology, CA, U.S.A.) according to the manufacturer’s instructions. After incubation with peroxidase-conjugated secondary antibodies (1:2000, Santa Cruz Biotechnology, CA, U.S.A.), proteins were visualized using enhanced chemiluminescence. Band intensities were quantified by densitometry.

Statistical Analysis The data were presented as means± S.E.M. and statistically analyzed using Student’s t-test. Differences were considered significant when p<0.05.

RESULTS

PPARγ Ligand-Binding Activity To functionally examine whether PPT had any effect on the transactivating activity of PPARγ, COS-7 cells were transfected with GAL4/PPARγ expression vector, together with a luciferase report construct including GAL4 binding element in minimal promoter and luciferase activity was determined. As shown in Fig. 2A, PPT increased GAL4/PPARγ transactivating activity in a dose-dependent manner. PPT at a concentration of 10 μM increased the activation by 5.5 fold compared to that of the 0.01% DMSO control, which was almost equivalent to troglitazone, a well-known PPARγ agonist. The concentrations of PPT used in these assays had no influence on cell viability (data not shown). However, BADGE (bisphenol A diglycidyl ether), a PPARγ antagonist, 18) suppressed PPT-induced transactivation of PPARγ in a dose-dependent manner (Fig. 2B), supporting that PPT can play a role in PPARγ-mediated transactivation as a PPARγ ligand.

Effect of PPT on Adipogenesis PPARγ is essential for adipocyte differentiation and is a key transcription factor for induction of adipogenic marker genes. To investigate whether PPT affects adipocyte differentiation, we treated the 3T3-L1 cell with 10 μM of PPT for 7 d and measured adipogenesis by oil red staining. As shown in Fig. 3, incubation of 3T3-L1
cells with PPT resulted in increase of lipid droplets compared with 0.01% DMSO control (Fig. 3), suggesting that PPT can induce adipogenesis via PPARγ activation in 3T3-L1 cells.

**PPARγ Target Gene Expression** To examine the effect of PPT on expression of PPARγ target genes involved in adipocyte differentiation, we measured the mRNA expression of adipogenic genes in differentiated 3T3-L1. PPT at 10 μM increased expression of adipocyte fatty acid-binding protein (aP2), LPL, and PEPCK by 7.7, 8.9, 3.9 fold, respectively (Fig. 4). These results demonstrate that PPT can induce adipocyte differentiation through increasing the expression of aP2, LPL, and PEPCK via PPARγ activation.

**GLUT4 Protein Expression** Since PPARγ agonist also regulates glucose metabolism, we investigated the effect of PPT on glucose metabolism in adipocyte. As shown in Fig. 5, when 3T3-L1 cells were treated with 10 or 25 μM of PPT, GLUT4 protein was significantly increased compared with the 0.01% DMSO control. These results suggest that PPT can play a role as an insulin-sensitizer through upregulation of GLUT4.

**DISCUSSION**

PPARγ is a master regulator of adipocyte differentiation and PPARγ agonists suppress insulin resistance in adipose tissue in addition to skeletal muscle and liver as an insulin...
sensitizer.\(^{19}\) Activation of PPAR\(^\gamma\) in adipose tissue improves its ability to store lipids, thereby reducing lipotoxicity in muscle and liver.\(^{20}\) The activation of PPAR\(^\gamma\) involves activation of genes encoding molecules that promote a combination of lipid storage and lipogenesis, such as aP2, LPL, and SCD-1. This activation causes body-wide lipid repartitioning by increasing the triglyceride content of adipose tissue and lowering free fatty acids and triglycerides in circulation, liver, and muscle, thereby improving insulin sensitivity.\(^{21}\) Therefore, PPAR\(^\gamma\) agonists ameliorate hyperglycemia by reversing lipotoxicity-induced insulin resistance. PPAR\(^\gamma\) ligands also regulate the expression of several other genes that enhance glucose metabolism in adipocyte, including insulin-responsive glucose transporter GLUT4 and c-Cbl association protein (CAP).\(^{22}\) Increased glucose uptake into adipocytes directly increases whole body glucose disposal. Therefore, potent PPAR\(^\gamma\) agonists have been the focus of intense academic and pharmaceutical research for treatment of diabetes. It has been reported that natural compounds from several medical plants have potential as PPAR\(^\gamma\) agonists. Saururrufuran A from *Saururus chinensis* (Saururaceae),\(^{23}\) flavonoids such as chrysin, apigenin and kaempferol\(^{24}\) and phenolic compounds from *Glycyrrhiza uralensis* (Fabaceae)\(^{25}\) have been recently identified as PPAR\(^\gamma\) agonists.

The present study showed that PPT potentially activated PPAR\(^\gamma\) as revealed by the GAL4/PPAR\(^\gamma\) transactivation assay. This activity was further confirmed by BADGE (an antagonist of PPAR\(^\gamma\)), which binds to the PPAR\(^\gamma\) ligand binding domain and strongly inhibits PPAR\(^\gamma\) ligand-mediated transactivation. BADGE blocked PPT-induced PPAR\(^\gamma\) transactivation, indicating that PPT can activate PPAR\(^\gamma\) as an agonist. Furthermore, we observed that PPT stimulated adipogenesis in 3T3-L1 adipocytes with similar potential to trogilitazone, a well known PPAR\(^\gamma\) agonist, through increasing the expression of PPAR\(^\gamma\) target genes involved in adipogenesis such as aP2, LPL and PEPCK. Moreover, PPT significantly increased expression of GLUT4 in 3T3-L1 adipocytes (Fig. 5), which may lead to enhancement of insulin sensitivity. All together, these results suggest that PPT, as a novel compound of naturally occurring PPAR\(^\gamma\) agonists, contributes to insulin-sensitizing effects of PPAR\(^\gamma\) through regulating lipid and glucose metabolism in 3T3-L1 adipocyte.

In conclusion, PPT promotes differentiation of adipocyte via PPAR\(^\gamma\) activation and increases expression of GLUT4 associated with insulin sensitivity in 3T3-L1 adipocyte. Therefore, PPT might improve insulin resistance by reducing lipotoxicity in muscle and liver through increasing ability to store lipid in adipocyte and enhancing insulin sensitivity through increase of GLUT4 expression in adipocyte and then provide antidiabetic effects.

**REFERENCES**