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ORIGINAL CONTRIBUTION

Platyconic acid, a saponin from Platycodi radix, improves glucose homeostasis by enhancing insulin sensitivity in vitro and in vivo

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Abstract
Background Previous research demonstrated that the crude saponins of Platycodi radix improve glucose metabolism by enhancing insulin sensitivity in type 2 diabetic animals; however, which individual saponins are the most potent insulin sensitizers is unknown.
Objectives This study investigated which saponin(s) have anti-diabetic action in vitro and in vivo.
Methods The insulin-stimulated glucose uptake and PPAR-γ agonistic actions of six saponins from Platycodi radix were investigated in 3T3-L1 adipocytes, and glucose-stimulated insulin secretion was determined in Min6 cells. Four individual saponins (20 mg/kg body weight) were orally administered to low-dose streptozotocin-injected diabetic mice fed a high-fat diet for 8 weeks to evaluate glucose tolerance by oral glucose tolerance testing (OGTT), insulin sensitivity by insulin tolerance testing, and insulin signaling in the liver and adipose tissues.
Results Platyconic acid (PA) most effectively increased insulin-stimulated glucose uptake in 3T3-L1 adipocytes, possibly in part by working as a peroxisome proliferator-activated receptors (PPAR)-γ activator; however, none of the saponins improved glucose-stimulated insulin secretion in insulinoma cells. PA-treated diabetic mice exhibited the lowest peak serum glucose levels and highest serum insulin levels during the first part of OGTT. PA also improved insulin sensitivity: PA increased glycogen accumulation and decreased triacylglycerol storage in liver, which was associated with enhanced hepatic insulin signaling, while PA potentiated the expression of adiponectin and PPAR-γ in adipose tissue, and improved insulin signaling and increased GLUT4 translocation into the membranes.
Conclusions PA improves glucose homeostasis in type 2 diabetic mice, partly by enhancing hepatic and adipocyte insulin sensitivity, possibly by activating PPAR-γ.

Keywords Platyconic acid · Platycodine D · Platycodi radix · PPAR-γ · Liver

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Introduction

Type 2 diabetes is a heterogeneous metabolic disorder that is characterized by peripheral insulin resistance combined with inadequate compensatory insulin secretion [1, 2]. Asians experience a subtype of type 2 diabetes that occurs on a background of naturally low insulin secretory capacity, and insulin resistant Asians typically progress to type 2 diabetes more rapidly than their Western counterparts [3, 4]. Thus, type 2 anti-diabetic compounds for Asians should have the ability to reduce insulin resistance and especially to improve glucose-stimulated insulin secretion. Some of the most commonly used Asian botanicals

exhibiting such anti-diabetic properties, including ginseng and bitter melon (*M. charantia*), include saponins among their bioactive compounds [5].

Triterpenoidal saponins are the major bioactive components of Platycodi radix, the root of *P. grandiflorum* (Balloon Flower), and have efficacy as interventions for metabolic disorders such as obesity, hyperlipidemia, and inflammation in rats and mice [6, 7]. It was previously demonstrated that the water extracts of Platycodi radix and its crude mixture of triterpenoidal saponins exert anti-diabetic effects by increasing insulin sensitivity and glucose-stimulated insulin secretion in the 90% pancreatectomized rat type 2 diabetes animal model [8]. The individual triterpenoidal saponins in Platycodi radix include deapioplatycoside E (DPE, 7%), platycoside E (PE, 12%), deapioplatycodin D3 (3.5%), platycodin D3 (PD3, 10%), polygalacin D2 (7.5%), platycodin D2 (PD2, 8.6%), platycodin D (PD, 17%), and platyconic acid (PA, 15%) [9]. However, few studies have investigated the functions of the individual triterpenoidal saponins or the mechanisms responsible for their anti-obesity and anti-diabetic properties. Nevertheless, some studies have demonstrated their anti-inflammatory effects [10–13], suggesting that saponins from Platycodi radix may protect against diabetes through anti-inflammatory actions, which could protect pancreatic β-cells. However, a previous study [8] in diabetic rats found that crude saponins of Platycodi radix improved glucose homeostasis mainly by enhancing hepatic insulin sensitivity, possibly as a consequence of decreased fat storage and stimulated insulin signaling in the liver of diabetic rats. Thus, it is interesting and important to investigate which triterpenoidal saponins have the greatest anti-diabetic action and their mechanisms of action. The major saponins of Platycodi radix, PA, PD, PD2, PD3, PE, and DPE, were tested for anti-diabetic actions on the main targets for anti-diabetic agents, peroxisome proliferator-activated receptors (PPAR)-γ agonistic and insulinotropic activity, in cell-based experiments and those showing promise were tested further for in vivo anti-diabetic efficacy in a type 2 diabetes animal model.

Materials and methods

Preparation of individual saponins

Dried roots of 20-year-old *P. grandiflorum* plants were purchased from Seoul, Korea in 2005, verified and a voucher specimen stored in the herbarium (No. 2005-03) by Dr. Kim YS (Korea Research Institute of Chemical Technology, Taejeon, Korea). Saponins were extracted from dried roots and separated and purified using sequential fractionation and HPLC to obtain the major saponins: PA, PD, PD2, PD3, PE, and DPE as previously described [13].

Insulin-stimulated glucose uptake in 3T3-L1 adipocytes

3T3-L1 fibroblasts were grown and differentiated into adipocytes as described by Shimaya et al. and Park et al. [14, 15]. Briefly, the adipocytes were differentiated for 9–12 days in 24-well plates at 4×10^4 cells/well in high-glucose DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) [15]. The medium was switched to low-glucose DMEM containing 0.3% bovine serum albumin (BSA) and DMSO, PA, PD, PD2, PD3, PE, or DPE (2 and 20 μM) and incubated at 37 °C for 16 h. The medium was switched to a Krebs–Ringer–Hepes buffer (KRB) containing 20 mM glucose and either 1 ng/mL insulin with vehicles (DMSO) or with 2 or 20 μM respective saponins. Each well was further incubated at 37 °C for 30 min. Cells treated with 10 nM insulin were used as a positive control. After 20 min of incubation, 0.1 μCi 2-deoxy-D-[^{3}H] glucose was added into the KRB buffer for 10 min. At the end of the incubation, the buffer was removed and the cells were washed three times with ice-cold phosphate-buffered saline (PBS). The cells were solubilized in 0.5 M NaOH and 0.1% SDS and ^{3}H counted using a Wallac Liquid Scintillation Counter (Waltham, MA, USA) to determine glucose uptake. Non-specific glucose uptake was measured in cells treated with vehicles or single compounds without insulin.

Triacylglycerol accumulation in 3T3-L1 adipocytes

Triacylglycerol accumulation was determined by adding the vehicle (DMSO) or 20 μM PA, PD, PD2, PD3, PE, or DPE to media with 1 mg/mL insulin (Sigma Co.), 50 μM dexamethasone (Sigma Co.), and 0.8 mM isobutylmethyl xanthine (Sigma Co.), for 4 days during the differentiation of 3T3-L1 fibroblasts, and then the cells were treated with vehicle or extracts without differentiation inducers for 6 or more days [15]. Fresh DMSO and respective saponins were added whenever the media was switched. After 6 days, the cells were lysed with a lysis buffer (20 mM Tris buffer, pH 7.4, containing 1 mM EDTA, 150 mM NaCl, and 1% NP40) and the triacylglycerol contents in the cell lysates measured using a Trinder kit (Young Dong Pharmaceutical Co., Seoul). The results for each treatment were expressed as the percent difference in triacylglycerol accumulation from control (vehicle treatment).

PPAR-γ activation

PPAR-γ activity was measured in human embryonic kidney (HEK) 293 cells since they are more satisfactory for transfecting vectors and have less endogenous PPAR-γ activity than 3T3-L1 adipocytes. HEK 293 cells were seeded into 96-well plates at 1×10^4 cells/well 24 h before

transfection. The cells were transiently transfected with a PPRE-luciferase construct (firefly pGL3-DR-1-luciferase; 0.12 μg DNA/well), pSV-SPORT-PPAR-γ expression vector (0.12 μg DNA/well), and pSV-SPORT-retinoid X receptor (RXR)-α vector (0.08 μg DNA/well) with a Lipofectamine PLUS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. These vectors were generously provided by Dr. Bruce Spiegelman (Department of Cell Biology, Harvard Medical School). For the assessment of transfection efficiency, renilla phRL-TK vector (10 ng DNA/well) was also transfected (Promega, Madison, WI). After 2 h of transfection, 2 or 20 μM of PA, PD, PD2, PD3, PE, DPE, or rosiglitazone (RGZ; GlaxoSmithKline, Brentford, Middlesex, England), or vehicles (DMSO) were added into media for 40 h after which the media was changed to serum-free DMEM containing 0.1% bovine serum albumin (BSA), which also contained the respective extracts, and incubated for 12 h. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) in an Aureon PhL luminometer (Aureon Biosystems, Vienna, Austria). Results were expressed as ratios of firefly luciferase activity to renilla luciferase activity.

Glucose-stimulated insulin secretion of Min6 cells

Min6 cells grown as previously described [15] in a 24-well plate at 6×10^4 cells/well were incubated with high-glucose DMEM containing 0.3% BSA and 20 μM of PA, PD, PD2, PD3, PE, DPE, or DMSO as a vehicle for 16 h. Exendin-4 (2.5 nM; Sigma Co., St. Louis, MO)-treated cells were used as a positive control. After washing the cells with PBS, the Min6 cells were treated with the designated respective saponins in low- (2 mM) or high-glucose (20 mM) Krebs–Ringer buffers (KRB) containing 20 mM Hepes pH 7.4 and 5 mg/mL BSA for 30 min. Insulin concentrations in supernatants from all wells were measured using a radioimmunoassay kit (Linco Research, St. Charles, MO) and a Packard Cobra gamma-counter (Packard Instrument Co., Inc., Meriden, CT).

Experimental design of animal study

C57BL/6J mice are susceptible to high-fat diet-induced obesity and insulin resistance resulting in hyperinsulinemia and accompanied by mild to moderate hyperglycemia, making them a model for early-stage and pre-type 2 diabetes. High-fat fed C57BL/6J mice can be made to produce a more consistent and advanced type 2 diabetes model that mimics Asian type 2 diabetes, without hyperinsulinemia, by treating them with low-dose streptozotocin (STZ) to impair, but not destroy, insulin secretory capacity [16–18]. Accordingly, male C57BL/6J mice (8–10 weeks old) were purchased from Dae Han Biolink Co. (Seoul, Korea). After a 2 week quarantine, the mice were intraperitoneally injected with 20 mg STZ/kg bw (Sigma Co.) for 2 consecutive days to induce the symptoms of type 2 diabetes. STZ-induced diabetic mice exhibiting fasting hyperglycemia (about 12 mM) after 7 days were randomly divided into six groups. Non-diabetic mice were injected with citrate buffer. A high-fat diet was used to induce peripheral insulin resistance and exacerbation of β-cell failure [17, 18]. The high-fat diet consisted of a modified AIN-94 diet with 40 energy percent (En%) carbohydrates, 20 En% protein, and 40 En% fat [19]. The major carbohydrate, protein, and fat sources were starch plus sugar, casein, and lard (CJ Co, Seoul). Three saponins with potent in vitro anti-diabetic activity (PA, PD, and PE) were selected for in vivo testing and one saponin with low activity (DPE) was used to confirm the consistency between in vivo and in vitro results. The diabetic mice were randomly divided into six different groups of 15, provided free access to a high-fat diet and were orally administered with 20 mg/kg body weight of PA, PD, PE, DPE, RGZ, or cellulose (a non-nutritive diabetic control) for 8 weeks. Saponins, RGZ, and cellulose were dissolved in 10% DMSO and administered by oral gavage each day. Fifteen non-diabetic normal control mice were also given cellulose (20 mg/kg body weight) each day. All animals were housed individually in a light (12 h on/12 h off) and temperature-controlled room with free access to diets and water. All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA) and were approved by the Institutional Animal Care and Use Committee of Hoseo University.

Glucose homeostasis

Fasting serum glucose and insulin levels, food intakes, and body weights were measured every week. At the end of the experimental period, an oral glucose tolerance test (OGTT) was performed in overnight-fasted animals by orally administering 2 g glucose/kg body weight. Blood glucose levels were measured by tail bleeding at 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 120 min after glucose loading with a portable glucometer, ACCU-CHEK (Roche Diagnostics, Indianapolis, IN) and checked by determining serum glucose levels using a Beckman Analyzer II (Beckman, Brea, CA). Serum insulin levels were measured at 0, 20, 40, 80, and 120 min during OGTT using a radioimmunoassay kit (Linco Research, Billerica, MA). Insulin tolerance was determined in fed mice intraperitoneally injected with diluted insulin (regular human insulin from Eli Lilly and Co.; 1 U/kg body weight). Blood glucose concentrations were measured at 0, 15, 30, and 60 min after insulin injection. After the insulin tolerance test (ITT), mice were

freely provided diets and water for 2 days after which they were deprived of food for 14 h. Blood was collected to measure non-esterified fatty acid (NEFA; Waco Diagnostics, Richmond, VA) and adiponectin (Linco Research). Mice from each group were then anesthetized with a mixture of ketamine and xylazine and 5 U insulin/kg body weight was injected through the inferior vena cava for 10 min and then they were killed, tissues collected, frozen with liquid nitrogen, and stored at −70 °C for immunoblotting analyses.

Liver glycogen and triacylglycerol content analysis

Glycogen content was assayed as described previously [20]. The liver was homogenized with 1.5N perchloric acid, and the lysate was treated with α-amyloglucosidase to hydrolyze glycogen. The hydrolysate was neutralized with NaOH to pH 7.4 and centrifuged at 3,000 rpm for 10 min and the glucose concentration measured using a glucose oxidase kit (Young Dong Pharm., Seoul, Korea); liver glycogen was calculated from the glucose concentrations. Triacylglycerol was extracted with chloroform–methanol (2:1, vol/vol) from the liver and resuspended in pure chloroform [21]. After evaporating chloroform, the residue was suspended with PBS with 0.1% triton X-100 and the suspension was sonicated and boiled for 5 min. The triacylglycerol contents of the suspensions were assayed using a Trinder kit (Young Dong Pharm., Seoul, Korea).

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

The white adipose tissues from four rats from each group were collected at the end of treatment. Total RNA was isolated from the adipose tissues using a monophasic solution of phenol and guanidine isothiocyanate (Trizol reagent, Gibco-BRL, Rockville, MD), followed by extraction and precipitation with isopropyl alcohol [22]. The cDNA was synthesized from equal amounts of total RNA with superscript III reverse transcriptase, and polymerase chain reaction (PCR) was performed with high-fidelity Taq DNA polymerase. Equal amounts of cDNA were mixed with sybergreen mix and they were analyzed using a realtime PCR machine (BioRad Laboratories, Hercules, CA). The expression level of the gene of interest was corrected for that of the housekeeping gene, glyceraldehydes 3-phosphate dehydrogenase (GAPDH). The primers used to detect mouse PPAR-γ were forward 5′-GAGCATGGTGCCTTCGCTGAT-3′, reverse 5′-CAAC CATTGGGTCAGCTCTTG-3′; GAPDH forward 5′-AA CGGGAAGCTCACTGGCAT-3′; and reverse 5′-GCTT CACCACCTTCTTGATG-3′. The primers were designed to sandwich at least one intron in order to distinguish between the products derived from mRNA to genomic DNA.

Immunoblot analysis

Liver samples were lysed with 20 mM Tris buffer (pH 7.4) containing 2 mM EGTA, 137 mM NaCl, 1% NP40, 10% glycerol, and 12 mM α-glycerol phosphate with complete protease inhibitor (Roche). White adipose tissues were homogenized in buffer (10 mM Tris HCl, 1 mM EDTA, 250 mM sucrose, pH 7.4) and fractionated as previously described [22]. Briefly, the homogenate was centrifuged at $1{,}000\times g$ for 15 min, fat cake was discarded, and the fat-free extract was used for lysates to measure insulin signaling in the white adipose tissue. Total membranes of white adipose tissues were separated for GLUT4 detection by centrifuging the lysate at $146{,}000\times g$ at 4 °C for 75 min and resuspending the pellets in the same lysis buffer. Protein contents in the whole lysate and total membrane fraction were assayed using a Bio Rad protein assay kit (Bio-Rad, Hercules, CA); lysates and total membrane with equivalent amounts of protein (30–50 μg) were resolved by SDS–PAGE. Lysates of the liver and adipose tissues were immunoblotted with phospho-Akt^{ser478}, phospho-AMP-Kinase^{thr172} (AMPK), phospho-acetyl CoA carboxylase^{ser72} (ACC), adiponectin, β-actin (Cell Signaling Technology, Danvers, MA), and phosphoenolpyruvate carboxykinase (PEPCK), generously provided by Dr. Daryl K. Granner of Vanderbilt University. Tyrosine phosphorylation of insulin receptor substrate-2 (IRS2) was measured by immunoblotting with anti-phosphotyrosine antibody PY20 after immunepreciptation with IRS2 protein (Millipore Co., Billerica, MA). The expression of glucose transporter-4 (GLUT4; Chemicon, Temecula, CA) was measured in total membrane fraction by immunoblotting. The intensity of protein expression was determined using Imagequant TL (Amersham Biosciences, Piscataway, NJ). Each experiment was repeated 3 times for each group.

Statistical analysis

Statistical analysis was performed using SAS software, and all results are expressed as a mean ± standard deviation. The anti-diabetic effects of individual saponins were evaluated by a one-way analysis of variance. Significant differences in the main effects among groups were identified by Tukey tests at $P < 0.05$. Comparisons between the control group of diabetic mice and the non-diabetic group were evaluated by two-sample t tests at $P < 0.05$.

Results

Insulin-stimulated glucose uptake in 3T3-L1 adipocytes

Treatment with saponins and low-dose (0.2 nM) insulin were compared with higher dose (10 nM) insulin alone to evaluate the sensitizing action of each saponin (Table 1). A saponin would be considered a stronger insulin sensitizer candidate if 0.2 nM insulin-stimulated glucose uptake increased to levels approaching that of 10 nM insulin. PA, PD, PD3, and PE increased insulin-stimulated glucose uptake; however, PA was most effective but less effective than RGZ, a commercial insulin sensitizer (Table 1). Non-specific glucose uptake was not altered by saponin treatments (data not shown).

Triacylglycerol accumulation in 3T3-L1 adipocytes

PPAR-γ activators are insulin sensitizers that typically increase insulin-stimulated glucose uptake and facilitate triacylglycerol accumulation in adipocytes [23]. PA (and PD, PD3 and PE by lesser amounts) increased triacylycerol accumulation by 1.7-fold in 3T3-L1 adipocytes, but by much less than RGZ (Table 2), suggesting that they might work as PPAR-γ activators, as does RGZ.

PPAR-γ activation

HEK 293 cells were transiently transfected with PPER-luciferase, PPAR-γ, and RXR-α vector to measure PPAR-γ activity following saponin treatment. PA dose-dependently increased luciferase activity more than the other saponins, but less than RGZ (Table 2), suggesting PA is a mild PPAR-γ activator.

Glucose-stimulated insulin secretion in Min6 cells

Saponins had no significant effect on insulin secretion in Min6 cells although only high-dosage treatment of PA appeared to slightly elevate insulin secretion ($P = 0.09$, data not shown).

Body weight and serum glucose levels of diabetic mice

Diabetic mice had higher food intakes, lower body and epididymal fat pad weights, higher fasting and post-prandial serum glucose and NEFA levels, and lower serum insulin levels than non-diabetic (Table 3). Diabetic mice also had increased insulin resistance. Therefore, STZ-injected mice displayed hyperglycemia due to both insufficient insulin secretion and response. In addition, serum adiponectin levels, a modulator of glucose and fatty acid metabolism, were lower in the STZ-injected mice. These results indicated that STZ-injected mice showed type 2 diabetic symptoms. Neither saponins nor RGZ affected food intake or body weight in the diabetic mice. PA administration for 8 weeks resulted in decreased fasting glucose but not insulin levels compared to the cellulose-administered control group (Table 3). RGZ treatment lowered fasting serum glucose levels more than PA treatment but not to the levels of non-diabetic mice. Post-prandial serum glucose levels were lower and insulin levels

Table 1 Insulin-stimulated glucose uptake in 3T3-L1 adipocytes ($n = 5$)

	Low-dosage treatment with insulin[1] (dpm/μg protein)	High-dosage treatment with insulin[2] (dpm/μg protein)	High-dosage treatment without insulin[3] (dpm/μg protein)
DMSO	11.4 ± 2.5^{c}	12.2 ± 2.4^{c}	4.1 ± 0.8
Platyconic acid	24.5 ± 4.9^{a}	37.6 ± 6.8^{a}	4.3 ± 0.8
Platycodine D	$20.6 \pm 5.2^{a,b}$	26.5 ± 4.8^{b}	4.0 ± 0.8
Platycodine D2	11.2 ± 2.5^{c}	12.7 ± 2.8^{c}	4.3 ± 0.9
Platycodine D3	18.7 ± 4.1^{b}	22.8 ± 4.8^{b}	4.2 ± 0.8
Platycoside E	17.5 ± 3.9^{b}	20.6 ± 4.5^{b}	4.5 ± 0.9
Deapioplaycoside E	11.3 ± 2.5^{c}	12.5 ± 2.9^{c}	4.3 ± 0.7
Rosiglitazone	$38.7 \pm 7.3^{\dagger\dagger\dagger}$	$57.3 \pm 9.7^{\dagger\dagger\dagger}$	4.4 ± 0.9
DMSO + 10 nM insulin	61.2 ± 8.4		

[1] Two micrometer of each saponin plus 0.2 nM insulin or 0.2 μM of rosiglitazone plus 0.2 nM insulin

[2] Two micrometer of each saponin plus 0.2 nM insulin or 2 μM of rosiglitazone plus 0.2 nM insulin

[3] Twenty micrometer of each saponin or 2 μM of rosiglitazone. Values are Mean ± SD

[a,b,c] Means on the same column were significantly different at $P < 0.05$

[†††] Significant difference between the control and rosiglitazone at $P < 0.001$

Table 2 Triacylglycerol accumulation during differentiation from 3T3-L1 fibroblasts to adipocytes and PPAR-γ activity in HEK293 cells ($n = 5$)

	Triglyceride accumulation in 3T3-L1 adipocytes[1] (% changes of the basal)	Relative luciferase activity in HEK 293 cells[2]	
		Low-dosage treatment[3]	High-dosage treatment[3]
DMSO	100 ± 11^{c}	1.12 ± 0.15^{b}	1.09 ± 0.18^{c}
Platyconic acid	179 ± 20^{a}	1.83 ± 0.22^{a}	2.62 ± 0.31^{a}
Platycodine D	141 ± 23^{b}	1.32 ± 0.15^{b}	1.81 ± 0.24^{b}
Platycodine D2	109 ± 16^{c}	1.11 ± 0.14^{b}	1.15 ± 0.13^{c}
Platycodine D3	$136 \pm 19^{b,c}$	1.26 ± 0.16^{b}	$1.47 \pm 0.19^{b,c}$
Platycoside E	$140 \pm 22^{b,c}$	1.37 ± 0.15^{b}	$1.57 \pm 0.17^{b,c}$
Deapioplaycoside E	106 ± 16^{c}	1.15 ± 0.14^{b}	1.17 ± 0.15^{c}
Rosiglitazone	$239 \pm 31^{\dagger\dagger\dagger}$	$2.72 \pm 0.30^{\dagger\dagger\dagger}$	$3.90 \pm 0.44^{\dagger\dagger\dagger}$

[1] Twenty micrometer of each saponin and 2 μM of rosiglitazone were treated

[2] HEK 293 cells were transfected with PPRE-luciferase construct, a pSV-SPORT-PPAR-γ expression vector, a pSV-SPORT-retinoid X receptor (RXR)-α vector renilla phRL-TK vector to measure PPAR-γ activity without endogenous PPAR-γ interference

[3] Low- and high-dosage treatments were 2 and 20 μM of each saponin and 0.2 and 2 μM of rosiglitazone. Relative luciferase activity was calculated by the ratios of firefly luciferase activity to renilla luciferase activity. Values are Mean ± SD

[a,b,c] Means on the same column were significantly different at $P < 0.05$

[†††] Significant difference between the control and rosiglitazone at $P < 0.001$

higher in diabetic mice administered with PA than in controls (Table 3). Unlike fasting serum glucose levels, RGZ did not reduce post-prandial serum glucose levels more than PA, probably due to its inability to increase serum insulin levels in a post-prandial state. Serum NEFA levels were decreased, and serum adiponectin levels were increased equally by PA and RGZ. Therefore, PA protected against the exacerbation of insulin resistance in type 2 diabetic mice, although the protection was somewhat less than by RGZ.

Oral glucose tolerance test

As expected, STZ-induced diabetic mice exhibited greater glucose intolerance during the OGTT than non-diabetic mice (Fig. 1a) due to impaired early phase and late phase serum insulin release. Peak serum glucose levels of PA-treated mice were lower and quickly decreased during the 60–120 min period compared to the control group (Fig. 1a). RGZ-treated mice showed exactly the same pattern as PA-treated mice in the first phase of the OGTT. PD exhibited a similar pattern in the OGTT curve as PA, but was less effective than PA. The AUC of serum glucose during early-phase OGTT was lowest in the mice treated with PA and RGZ, which were the only groups significantly lower than the control group, and the PD group was not significantly different from PA or control groups (Fig. 1b). In the late phase of the OGTT, the AUC of the PA group was lower than in controls or the other saponin groups, but not as low as in the RGZ group. The AUC for insulin during the early phase of OGTT was higher in the PA group than the control group but the AUC for insulin during the late phase was not significantly different among diabetic mice regardless of their treatment (Fig. 1b).

Insulin tolerance test

ITT revealed insulin resistance in all the STZ-treated animals. Intraperitoneal injections of insulin decreased the serum glucose levels of all mice but the glucose levels of non-diabetic mice decreased much faster than those of diabetic mice. Serum glucose levels decreased most rapidly in the RGZ, PA, and PD groups among the treated groups, but not as rapidly as in the non-diabetic mice (Fig. 2), demonstrating that PA attenuated insulin resistance similarly to RGZ in diabetic mice.

Insulin action and signaling in the liver

STZ-induced diabetic mice had decreased hepatic glycogen storage and increased triacylglycerol accumulation compared to non-diabetic mice; both of which were significantly attenuated by PA and by PD to a lesser extent (Fig. 3a). PA had a similar effect as RGZ on glycogen and triacylglycerol storage in the liver. PA and RGZ potentiated the phosphorylation of IRS2 and Akt and decreased the expression of PEPCK, major regulators of gluconeogenesis, although the effect of RGZ on Akt was greater

Table 3 Body weight and food intake and serum glucose, insulin, and non-esterified fatty acids

	Diabetic control ($n = 15$)	PA ($n = 15$)	PD ($n = 15$)	PE ($n = 15$)	DPE ($n = 15$)	RGZ ($n = 15$)	Normal control ($n = 15$)
Initial body weight (g)[1]	30.3 ± 3.3	29.8 ± 3.1	30.5 ± 3.4	29.9 ± 3.1	30.3 ± 3.2	29.9 ± 3.5	31.1 ± 2.7
Final body weight (g)[2]	29.1 ± 3.9	29.4 ± 3.4	28.9 ± 3.2	29.2 ± 3.1	28.9 ± 3.1	30.7 ± 3.3	37.2 ± 3.6††
Final epididymal fat pads (g)	0.35 ± 0.07	0.38 ± 0.06	0.36 ± 0.07	0.37 ± 0.05	0.34 ± 0.06	0.39 ± 0.07	0.46 ± 0.09†
Average food intake (g)	3.7 ± 0.5	3.8 ± 0.4	3.6 ± 0.5	3.9 ± 0.5	3.7 ± 0.5	4.0 ± 0.5	2.9 ± 0.4††
Initial fasting serum glucose (mM)	11.7 ± 1.7	12.2 ± 1.8	11.8 ± 1.6	12.2 ± 1.9	12.3 ± 1.8	12.1 ± 1.7	5.1 ± 0.6†††
Final fasting serum glucose (mM)	14.3 ± 1.7^{a}	11.2 ± 1.3^{b}	12.8 ± 1.5^{a}	13.8 ± 1.6^{a}	13.7 ± 1.6^{a}	9.8 ± 1.4^{c}	5.7 ± 0.7†††
Final post-prandial serum glucose levels (mM)[3]	19.9 ± 2.3^{a}	16.7 ± 2.0^{b}	18.2 ± 1.9^{a,b}	19.1 ± 2.1^{a}	19.4 ± 2.0^{a}	15.9 ± 1.9^{b}	7.7 ± 1.1†††
Initial fasting serum insulin (ng/mL)	0.45 ± 0.07	0.45 ± 0.06	0.46 ± 0.07	0.44 ± 0.08	0.43 ± 0.05	0.45 ± 0.06	0.66 ± 0.09††
Final fasting serum insulin (ng/mL)	0.50 ± 0.08	0.54 ± 0.08	0.51 ± 0.07	0.52 ± 0.07	0.53 ± 0.08	0.52 ± 0.07	0.94 ± 0.11††
Final post-prandial serum insulin state (ng/mL)	1.72 ± 0.34^{b}	2.25 ± 0.36^{a}	1.94 ± 0.38^{a,b}	1.83 ± 0.29^{b}	1.79 ± 0.27^{b}	1.79 ± 0.27^{b}	3.94 ± 0.53†††
Final serum adiponectin (μg/mL)	2.5 ± 0.4^{c}	3.6 ± 0.5^{a}	3.1 ± 0.5^{b}	2.6 ± 0.5^{c}	2.4 ± 0.4^{c}	3.9 ± 0.6^{a}	4.2 ± 0.4†††
Serum non-esterified fatty acids (mg/dL)	145.5 ± 18.6^{a}	93.6 ± 12.5^{c}	118.4 ± 14.7^{b}	137.8 ± 19.4^{a}	140.3 ± 18.9^{a}	98.9 ± 13.8^{c}	85.6 ± 10.8†††

Values are Mean ± SD

[1] The measurement of 12–14 h fasted mice the day after dividing STZ-treated and citrate buffer-treated mice into the assigned groups

[2] The measurement of 12–14 h fasted mice after 8 weeks of assigned treatments

[3] After 2 h later providing food to rats

[a,b,c] Means on the same row with different superscripts were significantly different at $P < 0.05$ by Tukey's test

[†] Significantly different from the control of diabetic mice at $P < 0.05$

[††] $P < 0.01$

[†††] $P < 0.001$

than PA. PA supplementation also increased AMPK and ACC phosporylations, which were associated with decreased triacylglycerol accumulation in the liver (Fig. 3b).

PPAR-γ expression and insulin signaling in white adipose tissues

PA increased PPAR-γ expression in the white adipose tissues, and PD did not increase PPAR-γ expression although it was a little greater than the control (Fig. 4a). The increment of PPAR-γ expression by PD was less than RGZ. The phosphorylation of Akt in adipose tissues increased in the ascending order of the control, PE, DPE < PD < PA and RGZ as did GLUT4 expression in the total membrane of the adipose tissues; adiponectin expressions were modified in the same manner as Akt phosphorylation (Fig. 4b), indicating that PA enhanced insulin sensitivity in adipose tissues by increasing the translocation of GLUT4 into the membrane and by increasing adiponectin expression by as much as RGZ.

Discussion

A previous study in 90% pancreatectomized diabetic rats fed a high-fat diet found that crude saponins of Korean Platycodi radix decreased body weight and visceral fat, improved insulin sensitivity, enhanced tyrosine phosphorylation of anti-insulin receptor substrate-2 and serine^{473} phosphporylation of Akt, and decreased phosphoenolpyruvate carboxykinase expression [8]. However, no study has determined which saponin(s) from Platycodi radix improves glucose homeostasis with respect to insulin sensitivity and insulin secretion. If Platycodi radix is to be developed as an intervention for the treatment of diabetes, it is important to be able to produce standardized extracts with consistent concentrations of the active ingredients.

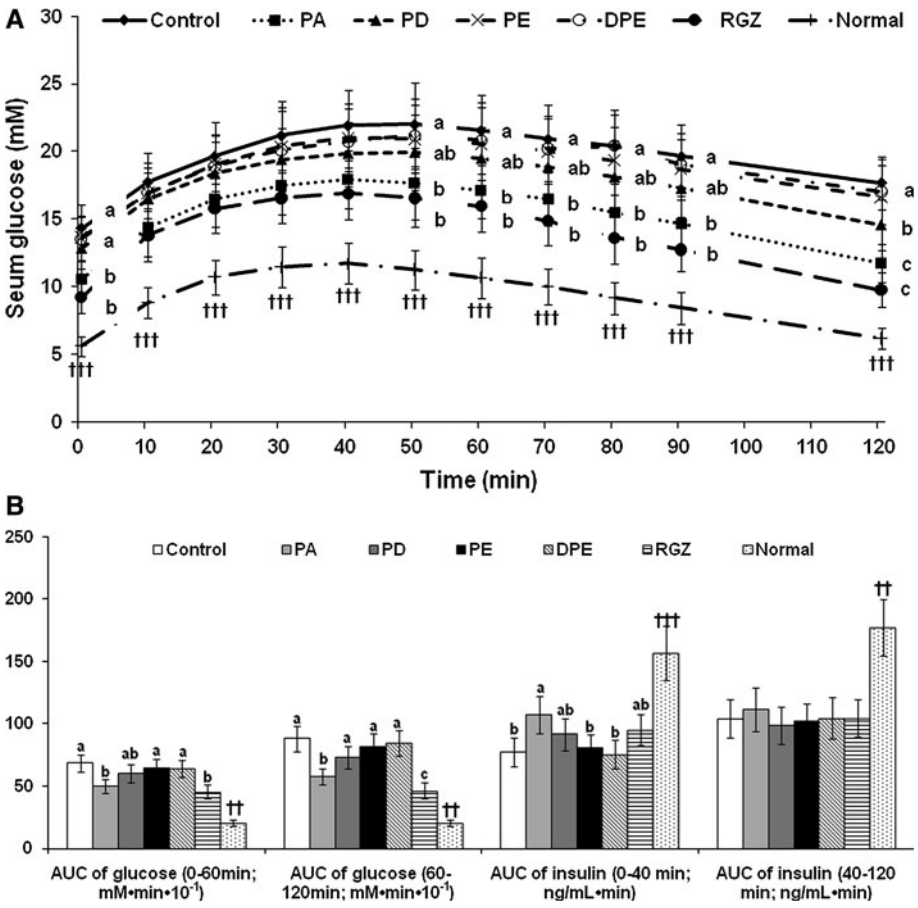

Fig. 1 Changes in serum glucose and insulin levels during oral glucose tolerance test. After an 8-week treatment period with cellulose, platyconic acid (PA), platycodine D (PD), platycoside E (PE), deapioplaycoside E, or rosiglitazone (RGZ), an oral glucose tolerance test was performed in overnight-fasted mice by orally administering 2 g/kg bw of glucose. **a** serum glucose levels during OGTT. **b** The average of the area under the curve (AUC) of glucose and insulin. The sample size in each group was the same as in Table 1. Values are means ± SD. [a,b,c]Lines and bars with different superscripts were significantly different at $P < 0.05$. [††]Means of the non-diabetic group were significantly different from the control group of the diabetic mice at $P < 0.01$; [†††]at $P < 0.001$

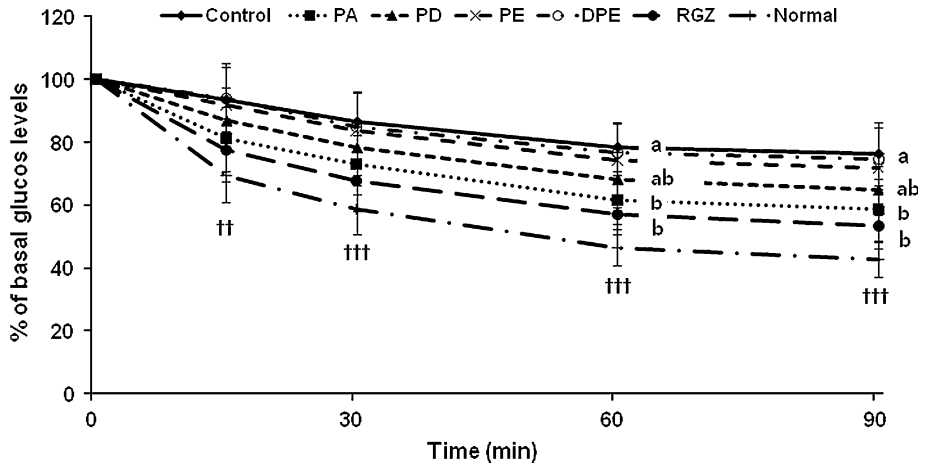

Fig. 2 Changes in serum insulin levels during the insulin tolerance test. After an 8 week administration of cellulose, platyconic acid (PA), platycodine D (PD), platycoside E (PE), deapioplaycoside E, or rosiglitazone (RGZ), an insulin tolerance test was performed in fed mice by intraperitoneal injection of insulin (1 U/kg bw). The sample size in each group was the same as in Table 1. [a,b]Lines with different superscripts were significantly different at $P < 0.05$. [††]Means of the non-diabetic group were significantly different from the control group of the diabetic mice at $P < 0.01$; [†††]at $P < 0.001$

Also, the traditional medicine, Platycodi radix, uses the roots from very mature plants that are expensive, but young plants are comparatively inexpensive. Identifying the anti-diabetic bioactive compounds would facilitate the optimization of growth duration for obtaining a cost effective product. The present study first screened the 6 major saponins identified in Platycodi radix for in vitro anti-diabetic potential; three were found worthy of further in vivo investigation, and a fourth was used to confirm that the lack of potent activity would also be realized in vivo. The in

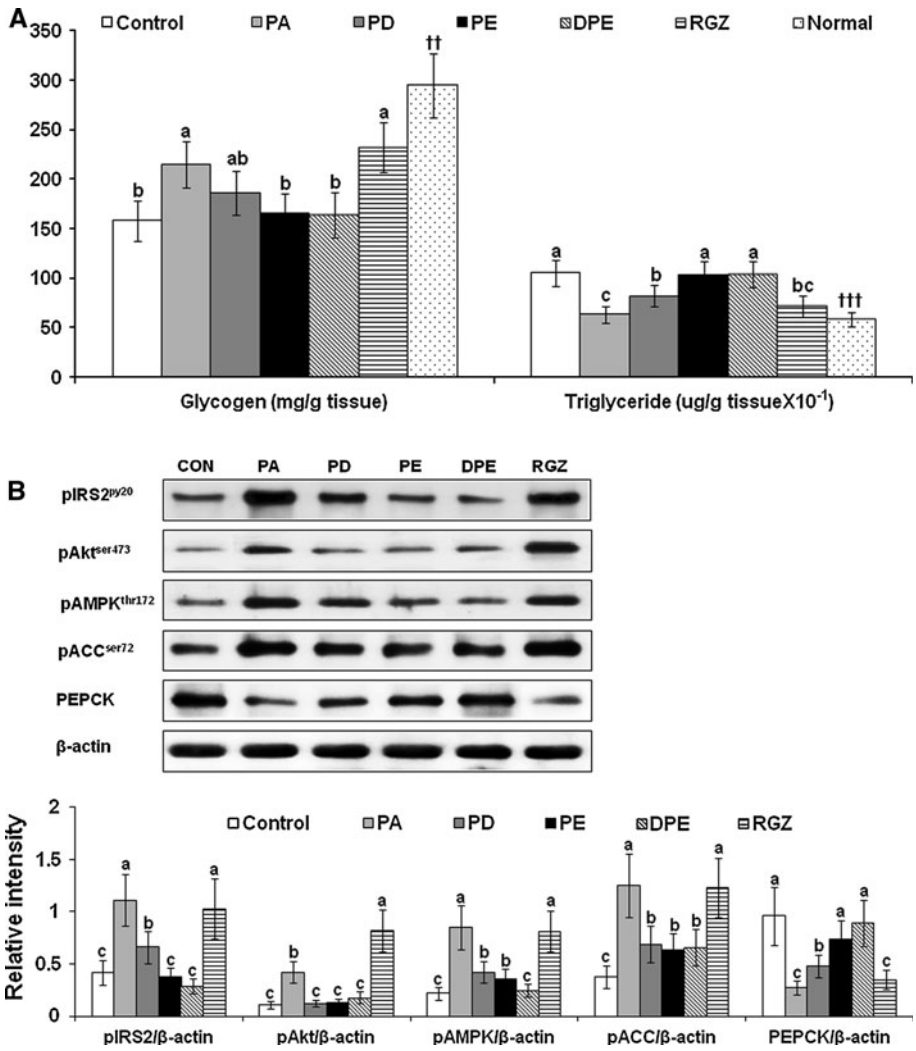

Fig. 3 Hepatic glycogen and triacylglycerol contents and insulin signaling in liver. After 10 min of insulin (5 U/kg body weight) stimulation through the inferior vena cava at the end of each experimental period, the livers were collected from the mice administered with cellulose, platyconic acid (PA), platycodine D (PD), platycoside E (PE), deapioplaycoside E, or rosiglitazone (RGZ) for 8 weeks, and immediately lysed with a lysis buffer. **a** Glycogen and triacylglycerol contents. **b** The phosphorylation levels of the Akt, AMPK, and ACC and expression of PEPCK, were determined by immunoblotting with specific antibodies. The intensity of protein expression was determined using an Imagequant TL. The experiments were repeated 4 times and the values are mean ± SD. [a,b,c]Means on the bars with different superscripts were significantly different at $P < 0.05$. [††]Means of the non-diabetic group were significantly different from the control group of the diabetic mice at $P < 0.01$; [†††]at $P < 0.001$

vitro study revealed that PA (and PD to a lesser extent) improved insulin-stimulated glucose uptake and enhanced PPAR-γ activity in 3T3-L1 adipocytes, which was consistent with previous studies using crude saponin extracts from Platycodi radix by Kwon et al. and Kim et al. [8, 24].

Prior to the in vivo study, we established that the dose and duration of intravenous injection of STZ (20 mg/kg body weight for 2 consecutive days) resulted in fasting serum glucose levels of 12.0 ± 1.7 mM, and that high-fat diet facilitated the development of insulin resistance, resulting in mice that closely mimic human type 2 diabetes. Other investigators have also used similar STZ and dietary treatments for simulating human type 2 diabetes [25–27]. Therefore, the model used in the present study is suitable for testing anti-diabetic agents for the treatment of type 2 diabetes.

The crude saponins of Platycodi radix, especially PD, reportedly decrease body weight and adipose tissues by inhibiting pancreatic lipase, thereby inhibiting fat absorption in the small intestine [28]. In addition, Lee et al. [29] recently reported that PD inhibited intracellular triglyceride accumulation in 3T3-L1 adipocytes by suppressing the expression genes involved in lipid metabolism including PPAR-γ. Our previous study [8] also revealed that the crude saponins decreased body weight and fat without fat malabsorption in mild type 2 diabetic rats. However, in the present study intracellular triglyceride accumulation was increased in PD-treated 3T3-L1 adipocytes and PPAR-γ activity was induced. In addition, exogenous fat was not excreted into the feces of mice fed high-fat diet containing individual saponins (data not shown) nor did saponins affect body weight. This weight loss discrepancy might be associated with the severity of diabetes or differences in species. Similar to PA, berberine lowered serum glucose levels but also failed to decrease body weight in STZ-induced diabetic rats fed a high-fat diet, despite decreased food intakes [15]. Therefore, in this study neither loss of body weight nor decreased energy intake was responsible for the glucose lowering effects.

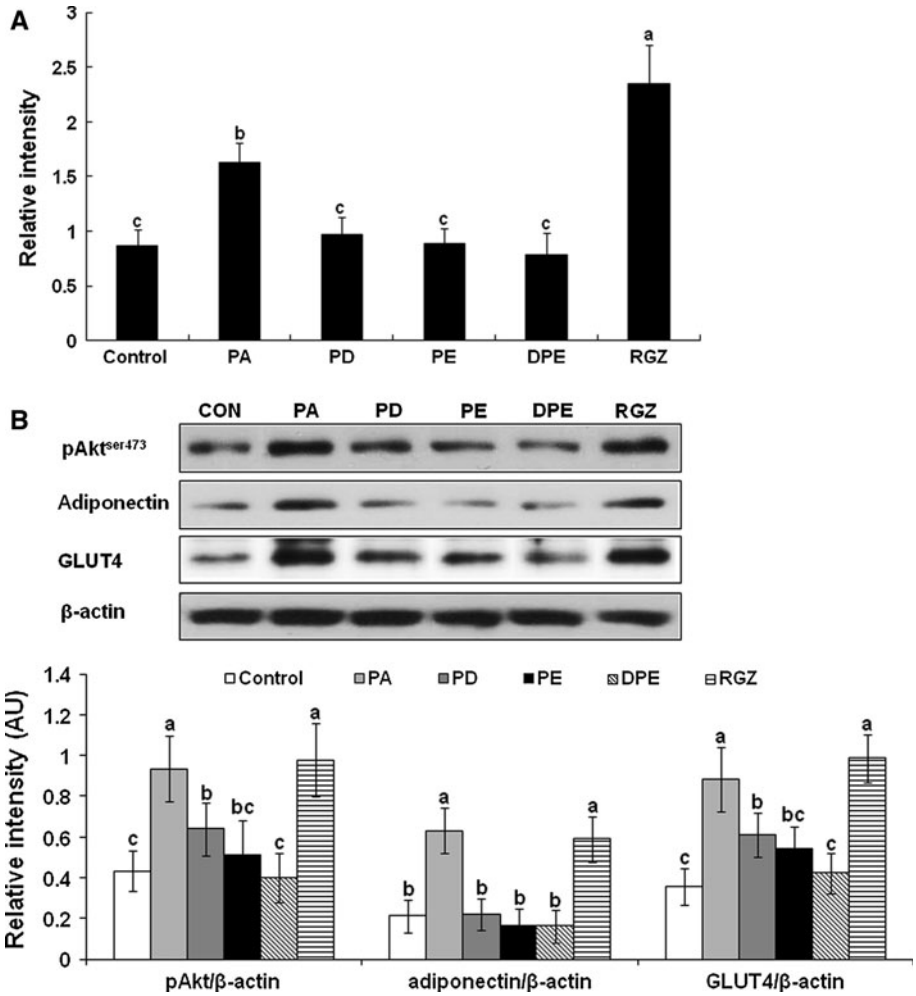

Fig. 4 PPAR-γ expression and insulin signaling of the white adipose tissues. After 10 min of insulin (5 U/kg body weight) stimulation through the inferior vena cava, the white adipose tissues collected from the mice administered with cellulose, platyconic acid (PA), platycodine D (PD), platycoside E (PE), deapioplaycoside E, or rosiglitazone (RGZ) for 8 weeks, were immediately lysed with trizol reagent for measuring mRNA and a lysis buffer for determining insulin signaling. **a** The mRNA of PPAR-γ was measured in cDNA generated from the adipose tissues. **b** The phosphorylation levels of Akt and expression of adiponectin and GLUT4 were determined by immunoblotting with specific antibodies. The GLUT4 expression was measured from the cell membrane of the adipose tissues. The intensity of protein expression was determined using an Imagequant TL. These experiments were repeated four times for the adipose tissues, and the values are mean ± SD. [a,b,c]Means of the bars with different superscripts were significantly different at $P < 0.05$

PA improved glucose homeostasis primarily by improving insulin sensitivity, as evidenced by OGTT and insulin tolerance test, but did result in higher early phase insulin AUC in the OGTT, suggesting that PA might potentiate both insulin sensitivity and release. However, Kwon et al. [8] showed that the crude saponins of Platycodi radix improved glucose homeostasis by improving insulin sensitivity but not by modulating glucose-stimulated insulin secretion in 90% pancreatectomized rats. Kim et al. [24] also revealed that a saponin-rich fraction from Platycodi radix reduced postprandial glucose levels and the insulin resistance index in mice fed a high-fat diet, and decreased the fatty acid biosynthesis to oxidation ratio.

The liver is central to nutrient homeostasis, storing postprandial glucose as glycogen or converting it to fatty acids; during the fasting state it produces glucose. The integration of insulin signals with those generated by counter-regulatory hormones and neuronal inputs establishes a dynamic network that coordinates systemic nutrient homeostasis [1, 30]. Dysregulation of hepatic insulin signaling is associated with metabolic syndrome and diabetes. A previous study revealed that hepatic insulin signaling is substantially attenuated in diabetic rats [31]. Hepatic insulin signaling in the fasting state is mainly regulated by phosphorylation of IRS2 to suppress gluconeogenesis [32, 33]. Activated IRS2 contributes to the serine^{473} phosphorylation of Akt, which is essential for controlling hepatic gluconeogenesis, as suggested by the complete loss of Akt phosphorylation in IRS2 liver specific knockout mice with a background of IRS1 [33]. In these mice, changes in hepatic gene expression strongly favor hepatic glucose production, including increased glucose 6-phosphatase and PEPCK, and decreased glucokinase [33]. The present study showed that PA potentiated IRS2 → Akt signaling in the liver, which resulted in decreased PEPCK expression to suppress gluconeogenesis in a hyperinsulinemic state. This may be associated with improved glucose tolerance in PA-treated diabetic mice.

Thiazolidinediones (TZDs) are a class of synthetic PPAR-γ ligands that improve insulin resistance in target tissues such as adipose tissues and are used to treat type 2

diabetes [34]. Since some herbs contain PPAR-γ agonists which improve insulin resistance [35], the present study determined whether the individual saponins activate PPAR-γ and if so, by what mechanism. PA, and PD to a lesser degree, somewhat increased PPAR-γ expression and exhibited moderate in vitro and in vivo PPAR-γ activating behavior: PA and PD increased GLUT4 translocation into the membrane and adiponectin expression in the adipose tissues and also potentiated serine phosphorylation of Akt. GLUT4 and adiponectin are known to be PPAR-γ target genes [36]. These results were consistent with those of other studies of PPAR-γ agonists [36, 37]. Previous studies have revealed that the major mechanism of increased glucose uptake by PPAR-γ agonists is the rapid translocation of GLUT4 proteins from the cytosol to the plasma membrane through insulin-dependent phosphoinositide 3-kinase/Akt signaling [36, 37]. In addition, some studies have shown that rosiglitazone improves steatosis and transaminase levels, an effect related to an improvement in insulin sensitivity. However, there were no improvement in other parameters of liver injury [38, 39]. In contrast, Yoon et al. [29] reported that PD down-regulated PPAR-γ by upregulating Kruppel-like factor in 3T3-L1 adipocytes. Thus, the effect of PD on PPAR-γ activity is still controversial, but PA may be a potential PPAR-γ activator and possible agonist, and reduces triglyceride accumulation in the liver by enhancing hepatic insulin sensitivity.

In summary, PA protected against the development of type 2 diabetes by increasing insulin-stimulated glucose uptake though apparent PPAR-γ activation; PD displayed a similar but lesser activity as PA. Eight weeks of PA supplementation (20 mg/kg bw) decreased peak serum glucose levels during OGTT and made serum glucose levels quickly drop after the peak in type 2 diabetic mice. Serum insulin levels during the first part of the OGTT were increased by PA but were not different in the later phases among the groups. PA also improved insulin tolerance with increased hepatic glycogen accumulation and decreased triacylglycerol storage due to potentiated hepatic insulin signaling pathways. PA substantially improved glucose homeostasis with a similar action mechanism as RGZ, although RGZ was a somewhat better anti-diabetic agent than PA. In conclusion, this study provides evidence that dietary supplementation with PA may improve glucose homeostasis by improving insulin sensitivity in the liver and adipose tissues by potentiating insulin signaling pathways, and by activating PPAR-γ in type 2 diabetic mice.

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Conflict of interest All authors have no conflict of interest.

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