Differentiation In Vitro1–3


Differential Effects of Isoflavones, from *Astragalus Membranaceus* and *Pueraria Thomsonii*, on the Activation of PPARα, PPARγ, and Adipocyte Differentiation In Vitro1–3

P. Shen,* M. H. Liu,* T. Y. Ng,* Y. H. Chan, ‡ and E. L. Yong‡

*Department of Obstetrics and Gynecology, and ‡Department of Biostatistics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 119074

ABSTRACT Compounds that target the peroxisome proliferator-activated receptors PPARα and PPARγ are used to correct dyslipidemia and to restore glycemic balance, respectively. Because the majority of diabetic patients suffer from atherogenic lipid abnormalities, in addition to insulin resistance, ligands are required that can activate both PPARα and PPARγ. In this study, we used chimeric PPARα/γ reporter-gene bioassays to screen herbal extracts with purported antidiabetic properties. Extracts of *Astragalus membranaceus* and *Pueraria thomsonii* significantly activated PPARα and PPARγ. Bioassay-guided fractionation resulted in the isolation of the isoflavones, formononetin, and calycosin from *Astragalus membranaceus*, and daidzein from *Pueraria thomsonii* as the PPAR-activating compounds. We investigated the effects of these and 2 common isoflavones, genistein and biochanin A, using chimeric and full-length PPAR constructs in vitro. Biochanin A and formononetin were potent activators of both PPAR receptors (EC50 = 1–4 μmol/L) with PPARα/PPARγ activity ratios of 1:3 in the chimeric and almost 1:1 in the full-length assay, comparable to those observed for synthetic dual PPAR-activating compounds under pharmaceutical development. There was a subtle hierarchy of PPARα/γ activities, indicating that biochanin A, formononetin, and genistein were more potent than calycosin and daidzein in chimeric as well as full-length receptor assays. At low doses, only biochanin A and formononetin, but not genistein, calycosin, or daidzein, activated PPARγ-driven reporter-gene activity and induced differentiation of 3T3-L1 preadipocytes. Our data suggest the potential value of isoflavones, especially biochanin A and their parent botanicals, as antidiabetic agents and for use in regulating lipid metabolism. J. Nutr. 136: 899–905, 2006.

KEY WORDS: • PPARα • PPARγ • isoflavones • adipocyte differentiation

The metabolic syndrome, wherein patients have both diabetes mellitus and dyslipidemia, is reaching epidemic proportions due to dietary factors and a sedentary lifestyle (1). A major cause of mortality in these patients is atherosclerotic macrovascular disease that results, in large part, from dyslipidemia associated with insulin-resistant diabetes. Peroxisome proliferator-activated receptors (PPAR), 5 a subfamily of the 48-member steroid and nuclear-receptor superfamily, are ligand-dependent transcription factors that control energy homeostasis by regulating carbohydrate and lipid metabolism (2). Like other nuclear receptors, the 3 known subtypes (PPARα, PPARβ/δ, and PPARγ) have N-terminal transactivation domains, central highly conserved DNA-binding domains, and C-terminal ligand-binding domains (LBD). Natural ligands, such as fatty acids and their derivatives, enter a pocket in the LBD (3) activating the receptor, causing it to heterodimerize with its obligate partner, the retinoid receptor. The heterodimer binds to peroxisome proliferator–responsive elements in promoter regions and recruits coregulatory molecules to modulate transcriptional activity of target genes involved in glucose metabolism and lipid homeostasis (4).

PPARα is mainly expressed in tissues such as liver, kidney, heart, and muscles where lipoprotein metabolism is important. Specific PPARα agonists, such as WY14643 (pirinixic acid), regulate genes involved in uptake of fatty acid binding proteins, β-oxidation (acyl-CoA oxidase), and ω-oxidation (e.g., cytochrome P450[CYP]4A6) (5). PPARγ is the predominant therapeutic target of the fibrates, drugs that are widely used to lower serum triglycerides and increase HDL cholesterol in patients with dyslipidaemia, atherosclerosis, coronary heart disease, and obesity. PPARγ, the target for ligands such as 15-deoxy-prostaglandin J2 and thiazolidinediones, is highly
expressed in adipose tissue, where it controls insulin sensitivity, adipocyte differentiation, and lipid storage. Currently, available thiazolidinediones, such as pioglitazone and rosiglitazone, are efficacious in the treatment of type II diabetes mellitus by maintaining plasma glucose and delaying the onset of long-term complications (6).

Unfortunately, pioglitazone and rosiglitazone show modest or even negative effects on blood lipid variables in patients with diabetes (7). PPARα-selective fibrates, such as fenofibrate and gemfibrozil, although efficacious in lowering triglycerides and LDL cholesterol and in raising HDL cholesterol levels in dyslipidemic patients (8), do not have sufficient activity in humans to serve as effective antidiabetic agents. Dual PPARα/γ agonists that target both PPARα and PPARγ may provide optimum therapeutic value for the management of the metabolic syndrome. Compounds with such properties are the focus of intense pharmaceutical research (9–11). Traditional Chinese herbal decoctions and formulations are extremely popular nutritional products consumed for their antidiabetic properties (12), largely without any understanding of their mechanisms of action. We hypothesize that small phenolic molecules present in some “antidiabetic” botanical foods may activate the PPAR-signaling system. Discovery and characterization of such putative PPAR-activating compounds would be an important first step toward their possible application in the management of the metabolic syndrome.

In this study, we used chimeric PPARα/γ reporter-gene biosassays to screen for specific PPAR ligands from herbal extracts with purported antidiabetic properties. The goal was to isolate and characterize PPARα/γ-activating compounds from these botanicals and to compare them with currently available reference compounds.

MATERIALS AND METHODS

Materials. Pioglitazone was a gift from Takeda Chemical Industries. WY14643 was purchased from Cayman Chemicals, and gemfibrozil, although efficacious in lowering triglycerides and LDL cholesterol and in raising HDL cholesterol levels in dyslipidemic patients (8), do not have sufficient activity in humans to serve as effective antidiabetic agents. Dual PPARα/γ agonists that target both PPARα and PPARγ may provide optimum therapeutic value for the management of the metabolic syndrome. Compounds with such properties are the focus of intense pharmaceutical research (9–11). Traditional Chinese herbal decoctions and formulations are extremely popular nutritional products consumed for their antidiabetic properties (12), largely without any understanding of their mechanisms of action. We hypothesize that small phenolic molecules present in some “antidiabetic” botanical foods may activate the PPAR-signaling system. Discovery and characterization of such putative PPAR-activating compounds would be an important first step toward their possible application in the management of the metabolic syndrome.

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Isolation and structural characterization of bioactive compounds. Ethanolic extracts of AM were loaded onto a medium-pressure liquid chromatography column (Merck, 600 g). The column was successively eluted using mixtures of hexane and acetone of increasing polarity (hexane:acetone, from 100:1 to 1:100). Ethanolic extracts of PT were also passed through a silica gel medium-pressure liquid chromatography column (Merck, 600 g). The column was successively eluted using mixtures of hexane and acetone of increasing polarity (hexane:acetone, from 100:1 to 1:100).

Cell culture and reporter-gene bioassays. Cell culture and Treatments: Cells were obtained from American Type Culture Collection. Cerebral carcinoma (HepG2) and rat liver cells from Kunming Institute of Pharmacology. The hepatocellular carcinoma cell line HepG2 and prediabetic 3T3-L1 cells were cultured in DMEM. All media were supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, and 1 mmol/L sodium pyruvate. Plasmids pSG5PL-PPARα, pSG5PL-PPARγ2, and CYP4A6-peroxisome proliferator-responsive element (PPRE)-Luc were gifts of Dr. W. Wahli, University of Lausanne, Switzerland. The pMGal4-PPARα/LBD expression plasmid was constructed by excising pSG5PL-PPARα with BstUI and BamHI and ligating proximally to the DNA binding domain of Gal4p from Saccharomyces cerevisiae. The plasmid pMGal4-PPARγ-LBD was constructed by excising pSG5PL-PPARγ2 with RsaI and blunt-end ligating to Hind III site of pMGal4 expression plasmid (Clontech). The upstream activating sequence (UASg)-luciferase (Luc) reporter gene was constructed by cloning 5 copies of the upstream activating sequence of Gal4p in tandem to a luciferase gene in a pG-Basic vector (14). Chimeric Gal-PPAR, by virtue of its Gal-DNA-binding domain element binds strongly to the heterologous UASg promoter of any co-transfected UASg-Luc reporter.

Reporter-gene biosassays were performed as previously described (15). Briefly, cells were seeded at 40,000 cells/well in 24-well microtiter plates and incubated for 24 h before transfection. 25 ng of the respective Gal-PPAR or full-length PPAR expression plasmids, and/or 250 ng of reporter-gene plasmid (UASg-Luc or CYP4A6-PPRE-Luc) were cotransfected into HeLa, HepG2 cells or differentiated 3T3-L1 preadipocytes with GenePORTER 2 (GTI). Transfected cells were exposed to test samples in charcoal-treated medium for 48 h. Luciferase induction responses for each treatment group were expressed as folds of vehicle-treated cells or percentages of reference drugs.

PPARγ competitor binding assays. A PPARγ competitor assay (PolarScreen; Invitrogen) was applied to evaluate the binding affinity of individual isoflavones to PPARγ-LBD. The assay was performed according to the manufacturer’s instructions. Briefly, recombinant PPARγ-LBD was added to a fluorescent PPARγ ligand (fluoromone PPAR-Green) to form a PPAR-LBD/fluoromone complex with a high polarization index. This complex was added to individual test samples in 96-well plates, incubated at room temperature for 2 h and polarization values measured with TECAN Ultra 384 fluorescence polarization plate reader. PPARγ-specific ligands in test samples displaced the fluorescent fluoromone from the PPAR-LBD fluorescence complex, resulting in lower polarization values. Assays were conducted in triplicate and data presented as means ± SEM. Curve fitting was performed using Prism from GraphPad Software.

Adipocyte differentiation assays. Murine fibroblast or preadipocyte 3T3-L1 cells were seeded at a density of 4 × 10³ cells/well in 24-well plates and cultured to confluence for 2 d in DMEM with 10% heat inactivated fetal calf serum. Postconfluent preadipocytes were exposed to induction medium containing 10% charcoal dextran-stripped serum, insulin (5 mg/L), dexamethasone (1 μmol/L), and 3-isobutyl-1-methylxanthine (0.5 mmol/L). Induction medium was removed after 2 d; cells were washed 3 times, and exposed to increasing doses of test compounds for 8 d. Medium was replenished with appropriate ligands every 2 d. After treatment, cells were washed with PBS, fixed with 4% paraformaldehyde, and lipid droplets were stained with 0.5% oil-red O in 60% isopropanol. Stained cells were examined under phase-contrast photomicrography, and a minimum of 3 experiments were performed in duplicate.

Western blotting. Immunoblotting of total cell lysates were performed according to standard western blotting protocol (15), using anti-PPARγ monoclonal antibody (Santa Cruz Biotechnology).

Statistical analyses. All experiments were performed at least 3 times on separate occasions. Statistical analyses were conducted using SPSS 13.0 (SPSS Inc.). One-way ANOVA was used to determine dose-dependent activities of PPAR ligands, active herbal extracts, and differential effects of isoflavones on PPARα/γ. For multiple comparisons with the vehicle group, Dunnett’s post-hoc test was used (Fig. 2, Fig. 4D, and Fig. 5). For multiple pair-wise group comparisons, Bonferroni adjustments were applied (Fig. 1, Fig. 2B, Fig. 2C, Fig. 4B, Fig. 4C, and Supplementary Fig. 1). Repeated measurement analysis over concentration levels between AM and PT was also performed with Bonferroni correction (Fig. 2B, C). Values presented are means ± SEM and differences were considered significant at P < 0.05.

RESULTS

Chimeric Gal-PPAR reporter-gene biosassays. To differentiate the ligands of PPAR from that of its heterodimeric partner retinoid X receptor (RXR), we used chimeric Gal-PPAR-LBD receptors to screen for PPAR activity. The PPARα-specific ligand, WY14643, dose-dependently activated the chimeric
Gal-PPARα construct, with a 50% effective concentration (EC₅₀) of 5 μmol/L and a maximal activity 14-fold of the vehicle (Fig. 1A). Similarly, the PPARγ-specific ligand, pioglitazone, increased the activity of the Gal-PPARγ system at a maximum of 40-fold of the vehicle with an EC₅₀ of 3 μmol/L (Fig. 1B). There was minimal cross-reaction between pioglitazone and Wy14643 with PPARα and PPARγ, respectively. In addition, ligands for other steroid receptors, such as estradiol and dihydrotestosterone, did not activate chimeric receptors in these bioassays (data not shown).

**Screening of “anti diabetic” herbs for PPAR activity.** Ethanolic extracts of 8 traditional Chinese herbs with purported anti diabetic properties (12) were screened for PPAR activity, using the chimeric Gal-PPAR γ reporter gene bioassay (Fig. 2A). Extracts of AM and PT significantly stimulated both PPARα and PPARγ. In comparison, other herbs did not exhibit any PPAR activity. At a dose of 250 mg/L, AM increased PPARα and PPARγ activity up to 60% and 120% over the vehicle, respectively (Fig. 2A). Dual PPARα and PPARγ activity was dose-dependent, with AM displaying higher activity than PT (Fig. 2B, C). To understand the molecular basis of the PPAR activity of AM and PT, we performed bioassay-guided fractionation of the crude extracts to isolate the PPAR-active components.

**Isolation and structural characterization of PPAR-active compounds from Astragalus membranaceus and Pueraria thomsonii.** Fractionation of AM resulted in 39 fractions, of which 19 and 29 displayed strong PPARα (Fig. 3A) and PPARγ (Fig. 3B) activities. Compounds in fractions 19 and 29 were present in sufficient quantities to be isolated at >95% purity. Structural characterization with mass spectrometry and NMR indicated that they contained calycosin and formononetin (Fig. 3C). Fractionation of PT with medium-pressure, liquid-chromatography silica gel resulted in 54 fractions (data not shown). PPARα-active fraction 50 was crystallized and identified as daidzein using NMR and LC-MS analysis. All 3 PPAR-active compounds characterized (calycosin, formononetin, and daidzein) were isoflavones (Fig. 4).

**Comparative PPAR activity of common isoflavones using chimeric Gal-PPAR assay.** Because genistein and its precursor biochanin A are commonly studied isoflavones, we compared their PPAR actions with those isolated from AM and PT. With the chimeric Gal-PPAR γ assay, biochanin A, formononetin, and genistein were the most potent activators of PPARα with EC₅₀ of 1.3 μmol/L, <1.0 μmol/L, and 16 μmol/L, respectively, comparing favorably with Wy14643 (EC₅₀ of 5 μmol/L) (Fig. 5A, Table 1). In terms of maximal PPARα activity, biochanin A and genistein were the most efficacious, exhibiting up to 65–71% of that observed with Wy14643. However, peak activity of genistein for PPARα was observed only at high doses ≥30 μmol/L, unlike biochanin A, whose peak was 10-fold lower at 3 μmol/L (Fig. 5A). For PPARγ, biochanin A, formononetin, and genistein were the most potent activators (EC₅₀ of 3.7 μmol/L, 2.6 μmol/L, and 23 μmol/L vs. pioglitazone ~3 μmol/L) (Fig. 5B, Table 1). The 5 isoflavones did not differ in their maximal PPARγ activities, which ranged from 20–35% of pioglitazone (Fig. 5B).
**PPARγ-ligand competitor assays.** To understand whether differences in transactivation activity were caused by differential binding affinities, we measured the ability of isoflavones to displace PPARγ-LBD fluorescent-ligand complexes in a polarization competitor assay. As expected, doses of pioglitazone (at least 1 μmol/L) significantly displaced the fluoromeone, whereas estradiol did not (Fig. 5C). All 5 isoflavones significantly displaced fluoromeone at doses of at least 1 μmol/L, indicating that they bind PPARγ at its LBD pocket. In this assay, calycosin and formononetin exhibited poor solubility and precipitated at doses ≥30 μmol/L. Excluding formononetin and calycosin, the 50% inhibitory concentration (IC50) of the other 3 compounds were in the order biochanin A < genistein < daidzein, corresponding to trends in EC50 observed with the Gal-PPARγ transactivation assay (comparing Fig 5B with 5C).

**Bioactivity of isoflavones using full-length PPAR assay.** To determine whether dual PPAR bioactivity of these
TABLE 1
Comparative PPAR activity of commonly consumed isoflavones

<table>
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<tr>
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<th>PPAR in Hela</th>
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<th>PPAR in HepG2</th>
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<td></td>
<td>α</td>
<td>γ</td>
<td>α</td>
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<td></td>
<td>EC50</td>
<td>Cmax¹</td>
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<td></td>
<td>μmol/L</td>
<td>μmol/L</td>
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<td>12</td>
<td>45</td>
</tr>
<tr>
<td>Formononetin³</td>
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<td>2.6</td>
</tr>
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<td>Daidzein</td>
<td>NSA⁴</td>
<td>73</td>
<td>25</td>
</tr>
<tr>
<td>Genistein</td>
<td>NSA⁵</td>
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<td>35</td>
</tr>
<tr>
<td>Biochanin A³</td>
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<td>100</td>
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</tr>
<tr>
<td>Pioglitazone</td>
<td>NSA</td>
<td>NSA</td>
<td>3.6</td>
</tr>
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¹ Maximal activity expressed as percentage of a saturating dose (30 μmol/L) of the PPARα selective ligand, WY14643.
² Maximal activity expressed as percentage of a saturating dose (30 μmol/L) of the PPARγ selective ligand, pioglitazone.
³ Most potent PPARα and PPARγ activators.
⁴ NSA, no significant activity.
⁵ No significant activity up to dose of 30 μmol/L.

Biochanin A was an order of magnitude more potent than calycosin and daidzein and comparable to values observed for WY14643 and pioglitazone for both PPARα and PPARγ. Maximal activities for biochanin A reached 188 and 102% of reference drugs for PPARα and PPARγ, respectively.

**DISCUSSION**

Our study documents that the "antidiabetic" herbs AM and PT are dual PPARα/γ activators due to their isoflavone constituents. Furthermore, closely related isoflavones exhibited significant differences in transcriptional potencies and abilities to regulate adipocyte differentiation. In the chimeric PPARα assay, formononetin and biochanin A had potencies (EC50 of <1.0 μmol/L and 1.3 μmol/L, respectively) that were comparable to those reported for recently synthesized PPARα/γ agonists, such as ragaglitazar (3 μmol/L) (16), tesaglitazar.

**FIGURE 6** Effect of isoflavones on endogenous PPARγ function in adipocytes. (A) Differentiated 3T3-L1 cells were transfected with CYP4A6-PPRE-Luc reporter only, and then exposed to genistein (Gen), formononetin (For), biochanin A (Bio), calycosin (Cal), and daidzein (Dai). Positive control (Pos) was pioglitazone (30 μmol/L). Data are fold-increases in luciferase activity compared with vehicle. *P < 0.05; **P < 0.01, different from vehicle. (B) Immunoblot showing PPARγ protein of differentiated adipocytes. 3T3-L1 cells were exposed to isoflavones (3 μmol/L) and PPARγ protein measured with an anti-PPARγ mouse monoclonal antibody.
(3 μmol/L) (17), and muraglitazar (0.3 μmol/L) (18). With respect to PPARγ, formononetin, and biochanin A (EC50 of 2.6 μmol/L and 3.7 μmol/L, respectively) were less potent than ralaglitazar (0.093 μmol/L) (16), tesaglitazar (0.149 μmol/L) (17), and muraglitazar (0.11 μmol/L) (18). Nevertheless, these isoflavones exhibited balanced PPARα/PPARγ activity ratios of 1.3 in chimeric and almost 1:1 in full-length PPARα/γ assays (Table 1). This balanced-activity PPARα/PPARγ profile may enhance the attractiveness of biochanin and formononetin, and foods containing them, in the management of the metabolic syndrome (1). Synthetic dual PPARα/γ drugs in current development cause considerable side effects, including edema and carcinogenicity in rodent toxicity models (16–20). Isoflavones are very common botanical compounds, and epidemiological evidence suggests that their consumption may aid cancer chemoprevention (21). Our discovery of herbs and isoflavones with PPAR activity might lead to PPAR agonists with improved risk-benefit profiles. Furthermore, these isoflavones and their parent foods are available immediately for clinical evaluation.

Our data add to the increasing evidence (22–24) that isoflavones are dual PPARα/γ activators. Besides being phytoestrogens, isoflavones exhibit antioxidant effects and perturb the action of DNA topoisomerase II (22). Our study indicates that closely related isoflavones have significantly different bioactivities. Biochanin A differs from genistein by only an additional methyl moiety in the phenyl B ring (Fig. 4A), but the former is several-fold more potent than the latter (Table 1). Similarly, formononetin, with an additional methyl moiety, was at least an order of magnitude more potent than its metabolite daidzein. The differences in transactivation potencies were consistently observed across several cell lines and on PPAR-regulated adipocyte differentiation, suggesting that they reflect bona fide functional differences. Differences in transactivation may be partly due to differences in binding affinity, because clear differences in the ability of isoflavone to displace bound PPARα fluoromone were observed. Biochanin A and genistein displayed the strongest binding affinity, corresponding to their strong PPARα transactivity. Further molecular and structural studies are necessary to understand the mechanistic basis for these differences, whether they are related to different abilities to recruit coactivators or corepressors (25), and/or cross-activation of other steroid receptors such as estrogen receptor or RXR (26).

Isoflavones from soy (27,28) and licorice (29) exert antidiabetic and hypolipidemic effects in animal models. There is evidence that soy extracts have antilipidemic properties in humans (30), and evidence is emerging that they play a beneficial role in obesity and diabetes (31). The U.S. FDA recommends the consumption of at least 25 g of soy protein daily for cardiovascular health. Soy-based diets can result in beneficial changes to measures of glycemic control in type II diabetics (31,32). Nonetheless, isoflavones are relatively poorly absorbed, and serum concentrations seldom exceed 10 μmol/L). Nonetheless, these studies are necessary to understand the mechanistic basis for these differences, whether they are related to different abilities to recruit coactivators or corepressors (25), and/or cross-activation of other steroid receptors such as estrogen receptor or RXR (26).

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LITERATURE CITED


