Biochemical and Molecular Actions of Nutrients

Soy Isoflavones Exert Antidiabetic and Hypolipidemic Effects through the PPAR Pathways in Obese Zucker Rats and Murine RAW 264.7 Cells[1]

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ABSTRACT The hypocholesterolemic and anti-atherosclerotic mechanism by which soy may exert a beneficial effect remains unclear. Peroxisome-proliferator activated receptors (PPAR) are promiscuous nuclear receptors that regulate the transcription of genes involved in lipid and glucose homeostasis and lipid metabolism within the cell. We hypothesize that the isoflavones improve lipid and glucose metabolism by acting as an antidiabetic PPAR agonist. Male and female obese Zucker rats (OZR) were used as a model of Type 2 diabetes, and OZR fed a high isoflavone soy protein diet displayed improvements in lipid metabolism consistent with results in humans treated with antidiabetic PPAR agonists such as the fibrates or glitazones. Liver triglyceride and cholesterol concentrations were lower in all OZR fed high-isoflavone soy protein diets than in rats fed low-isoflavone and casein diets ($P < 0.05$). Concurrently, PPAR-directed gene expression was evaluated in a cell culture model. An isoflavone-containing soy extract doubled PPAR-directed gene expression ($P < 0.05$) in RAW 264.7 cells containing either a PPARα or PPARγ expression plasmid. A similar induction was observed when the soy isoflavones genistein or daidzein were used to treat cells. Both isoflavones doubled PPARα-directed gene expression ($P < 0.05$), whereas they increased PPARγ-directed gene expression 200–400% ($P < 0.05$). This study suggests that soy isoflavones improve lipid metabolism, produce an antidiabetic effect, and activate PPAR receptors. J. Nutr. 133: 1238–1243, 2003.

KEY WORDS: • *PPAR* • *cholesterol* • *diabetes* • *isoflavones* • *soy*

Soy intake has been linked to improved blood lipid levels in humans and animals and decreased arterial fatty streaks in animals, thereby reducing the risk of developing atherosclerosis (1–6). However, the physiological mechanism by which soy may improve blood lipid profiles has been the subject of speculation and investigated but is still not known with any certainty. It is unclear which soy components may contribute to the lipid-lowering property of soy, and numerous studies have been conducted to determine which components of soy exert bioactive effects (3,7). Soy components include protein, lipids, fiber and phytochemicals including isoflavones. Some researchers have focused on isoflavones as an important bioactive component of soy. The three main isoflavones found in soybeans are genistein, daidzein and glycitein (3).

Considerable research effort has focused on isoflavones as the main hypolipidemic agent in soy because of their antioxidative and mild estrogenic activity (4,8,9). In fact, some studies have shown that removal of the isoflavone-containing fraction of soy protein results in the loss of soy's beneficial effect on blood lipids (10,11). However, other recent studies reported minimal effects of soy isoflavones on blood lipid levels alone (12–15). Because of these conflicting results, it is possible that isoflavones exert some of their beneficial effects mainly when they are part of an intact soy source (7,16).

Recent studies have also provided evidence that soy consumption alleviates some of the symptoms associated with Type 2 diabetes such as insulin resistance and glycemic control (17,18). Some of these effects may be the end result of the improved blood lipid profile caused by soy consumption. However, it remains a possibility that soy has a positive and direct effect on the management of diabetes by some yet-unrecognized mechanism.

One such mechanism may be by peroxisome-proliferator activated receptors (PPAR),[3] nuclear receptors that participate in cellular lipid homeostasis and insulin action (19–23). Upon ligand binding, PPAR are activated and bind to peroxisome-proliferator response element (PPRE) sequences located within the promoters of PPAR-regulated genes (20,24). PPAR are "promiscuous" receptors, so named because they can be activated by many different ligands. Ligands for PPARγ include some unsaturated fatty acids and their derivatives as well as glitazones, insulin-sensitizing drugs used to

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[3] Abbreviations used: C, casein; FER, feed-efficiency ratio; GTT, glucose tolerance test; HIS, high isoflavone-containing soy protein; LIS, low isoflavone-containing soy protein; OZR, obese Zucker rat; PPAR, peroxisome-proliferator activated receptor; PPRE, peroxisome-proliferator response element.

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treat Type 2 diabetes (20,25). Ligands for PPARα include some saturated and unsaturated fatty acids as well as the group of drugs termed fibrates, which are hypolipidemic agents used to manage elevated blood lipid levels and Type 2 diabetes (26). Generally, PPARα controls the transcription of many genes involved in lipid catabolism, whereas PPARγ controls the expression of genes involved in adipocyte differentiation and insulin sensitization (20). Together, activation of PPARα and PPARγ increases β-oxidation and insulin sensitization, whereas blood and liver lipid concentrations are typically reduced. The increase in adipocyte differentiation attributed to PPARγ activation often results in weight gain because of increased body fat, resulting in the paradoxical effect of PPARγ agonist treatment: increased fat mass alongside improvements in other metabolic lipid variables (27).

Considering the promiscuous ligand binding properties of PPAR and the effects of PPAR on lipid metabolism, we hypothesized that soy isoflavones exert a beneficial hypolipidemic and antidiabetic effect by activation of the PPAR receptors.

MATERIALS AND METHODS

Rat diets. Experimental diets were prepared and feed consumption was measured as described previously (28,29). Only the protein component of the diet was modified; all other nutrients were held constant and based on AIN-93 guidelines (30). The protein sources used were high-isoflavone soy protein (HIS), low-isoflavone soy protein (LIS) and casein (C) (**Table 1**).

Rat study. The Southern Illinois University Animal Care and Use Committee approved all rat studies. Nine-wk-old male and female obese Zucker rats (OZR; Harlan, Indianapolis, IN) were acclimated to the animal facility for 2 wk and all were then randomly assigned to C, LIS or HIS diets (Table 1). Rats consumed food and water ad libitum, and food intake was recorded daily. Body weight was recorded weekly. Male OZR consumed the diets for 8 wk and the female OZR consumed the diets for 11 wk. Blood was collected by cardiac puncture; a bilateral thoracotomy followed to assure death. Blood from fasting rats was collected by cardiac puncture, heparinized and centrifuged. Plasma aliquots were then stored at −70°C. The feed-efficiency ratio (FER) was determined. Rats and organs were examined for any overt pathological conditions.

TABLE 1

Composition of experimental diets

Ingredient[1]	Control (C)	Low-isoflavone soy protein (LIS)	High-isoflavone soy protein (HIS)
	g/kg diet		
Casein	200	—	—
Soy protein	—	200	200
Sucrose	300	300	300
Cornstarch	350	350	350
Corn oil	50	50	50
Cellulose	50	50	50
Mineral mix	35	35	35
Vitamin mix	10	10	10
Choline bitartrate	2	2	2
DL-Methionine	3	3	3
DL-α-Tocopherol[2]	1.2	1.2	1.2
Total isoflavones	0	<0.009	1.16
Genistein equivalents	0	<0.004	0.696
Aglycone isoflavones	0	<0.005	0.754

1 Protein was either low- or high-isoflavone soy protein, SUPRO® SOY brand isolated soy protein (a Solae protein, a gift from DuPont Protein Technologies International, St. Louis, MO), or casein (ICN Biomedicals, Irvine, CA). All other ingredients were from ICN Biomedicals.

Liver and plasma lipid concentrations. Liver and plasma lipids were analyzed as previously described (28). Briefly, total liver lipids were extracted; total and unesterified cholesterol concentrations were determined colorimetrically by an enzymatic procedure, and cholesteryl esters were calculated by differences (31). Liver triglycerides were measured by a procedure using the Hantzch reaction. Plasma triglycerides were measured by the use of a commercial kit (#320-A; Sigma, St. Louis, MO).

Glucose tolerance test (GTT). During wk 9 and 10, female OZR were deprived of food overnight, anesthetized with ether and bled through the orbital sinus immediately before administration of an intraperitoneal (i.p.) glucose load (2 g glucose/kg body), and at 15, 30, 60, 90, 120, 150, 180, 210 and 240 min later. At the end of the GTT the females were killed. Plasma glucose was measured by use of a commercial glucose kit (#635; Sigma).

Cell culture and reagents. The effect of various soy phytochemicals on PPAR-mediated gene expression was tested in murine macrophage-like RAW 264.7 cells (ATCC, Rockville, MD). G-2535, a soy extract containing unconjugated soy isoflavones, was from Protein Technologies International (St. Louis, MO), and Prevastein HC, a soy extract containing conjugated soy isoflavones was from Cognis (LaGrange, IN). Genistein, daidzein, glycitein and clofibrate were purchased from Sigma. Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA). RAW 264.7 cells were grown in high glucose Dulbecco's modified Eagle's medium with pyruvate (GIBCO, Gaithersburg, MD) containing 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were maintained at 37°C, 5% CO_2 and 100% relative humidity.

Plasmid constructs. To evaluate PPAR-directed gene expression, we used the pTK.PPRE3x luciferase expression plasmid, which is regulated by the PPRE-containing acyl CoA oxidase promoter (32). To study PPARα- and PPARγ-regulated gene expression, a pCMX.PPARα or a pCMX.PPARγ expression plasmid was transiently cotransfected into cells (32). The β-galactosidase plasmid (Promega, Madison, WI) was used as an internal control to monitor transfection efficiency.

Transfections. Near-confluent RAW 264.7 cells were removed from culture plates by use of phosphate-buffered saline containing 10 mmol/L EDTA and replated onto six-well plates at 8.4×10^5 cells/well. Transient cotransfections were performed 18 h later by use of 20 μL of lipofectamine and 9 μL lipofectamine plus reagents per well (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Each well was transfected with 1.5 μg of the pTK.PPRE3x reporter construct, 1.5 μg of the pCMX.PPARα or the pCMX.PPARγ expression plasmids, and 1.5 μg of the β-galactosidase plasmid. After 6 h of transfection, cell media was replaced with media containing 0.5% fetal bovine serum and 1% penicillin-streptomycin.

Cell treatments. Transiently cotransfected cells were kept in the 0.5% serum media for 12 h and treated with one of the following: G-2535, Prevastein HC, genistein, daidzein, glycitein, or vehicle serving as a control (consisting of DMSO at less than 0.1% v/v and/or ethanol at less than 0.9% v/v). Positive controls were 20 μmol/L of pioglitazone (for PPARγ) or 100 μmol/L of clofibrate (for PPARα). Cells were harvested after 6, 9, 12, or 24 h by use of Reporter Lysis Buffer (Promega); lysates were centrifuged at 12,000 × *g* for 2 min at 4°C and the supernatants stored at −70°C. Luciferase activity was determined with a TR717 Microplate Luminometer (Applied Biosystems, Foster City, CA) by use of the luciferase assay reagent according to the manufacturer's protocol (Promega). The β-galactosidase activity was determined by use of *o*-nitrophenyl-β-D-galactopyranoside as a substrate, according to the manufacturer's protocol (Promega). β-Galactosidase activity was assayed at 420 nm (Bio-Tek Instruments, Winooski, VT). Every transfection experiment also included samples not transfected with plasmids to measure baseline levels for the luciferase and β-galactosidase assays. Luciferase assay activity was normalized to β-galactosidase activity for each well in each experiment to control for transfection efficiency. All luciferase values are expressed relative to the mean of the vehicle control.

Statistical analysis. All rat experiments used a block-randomized design. Data were analyzed by one-way ANOVA and post hoc

TABLE 2

Feed efficiency ratio, body weight, visceral fat weight and fasting plasma glucose and insulin in male and female obese Zucker rats fed control (C), LIS or HIS diets for 8 or 11 wk, respectively[1]

Diet group[2]	*n*	FER	Change in body wt	Visceral fat wt[3]	Plasma glucose	Plasma insulin
		g gain/g feed	*g*		*mmol/L*	*nmol/L*
Males						
C	8	0.103	170 ± 19	46.6 ± 1.9	14.0 ± 1.5	0.16 ± 0.02
LIS	8	0.116	174 ± 20	49.2 ± 2.8	10.0 ± 0.8	0.66 ± 0.24[a]
HIS	8	0.148[a]	222 ± 27	55.7 ± 2.9	12.8 ± 1.5	0.28 ± 0.05[b]
Females						
C	4	0.084	176 ± 13	51.2 ± 5.3	10.9 ± 1.8	1.44 ± 0.20
LIS	4	0.090	180 ± 7	54.8 ± 6.5	11.4 ± 1.2	1.87 ± 0.04
HIS	4	0.118[a,b]	245 ± 28[a,b]	57.0 ± 2.1	12.5 ± 0.6	1.59 ± 0.21

[1] Values are means ± SEM.
[2] Diet groups: Control (casein), C; low-isoflavone (< 0.1 mg isoflavone/g protein) soy protein, LIS; or high-isoflavone (5.8 mg isoflavone/g protein) soy protein, HIS.
[3] Corrected for body weight in the statistical analysis. [a] Different from C, $P < 0.05$; [b] Different from LIS, $P < 0.05$.

comparisons (LSD) were made (significance at $P \leq 0.05$) when appropriate. Data are presented as means ± SEM. For the cell experiments, each time-course experiment was initially analyzed by a two-way ANOVA, and when there were significant differences, individual time points were further analyzed post hoc by use of Kruskal–Wallis ANOVA on ranks. All other cell experiments were analyzed by Kruskal–Wallis ANOVA on ranks unless otherwise stated.

RESULTS

Rat studies. Female OZR fed the HIS diet gained more body weight than both the LIS- and C-fed rats ($P < 0.05$) (**Table 2**). This weight change was accompanied by a concomitant increase in FER ($P < 0.05$). The male OZR fed the HIS diet demonstrated similar nonsignificant trends in weight change and FER ($P > 0.1$). There was no increase in visceral fat weight in either the male or female OZR fed the HIS diet (Table 2).

Total liver weight, liver cholesterol and liver triglyceride concentrations were lower in male and female OZR fed the HIS diet compared to both C- and LIS-fed rats ($P < 0.05$). The plasma cholesterol levels were lower in all HIS-fed OZR as well as male LIS-fed OZR compared to C-fed rats ($P < 0.05$) (**Table 3**). Plasma triglycerides were greater in female, but not male HIS-fed OZR ($P < 0.05$) compared to the C- and LIS-fed groups.

Female OZR consuming the HIS diet had improved ($P < 0.05$) glucose tolerance relative to OZR consuming the LIS and C diets (**Fig. 1**). However, there was no (apparent) glucose-sensitizing effect of the HIS diet upon examination of blood glucose concentrations in fasting female or male OZR (Table 2). Fasting plasma insulin concentrations in fasting rats were greater in LIS- than in HIS-fed male OZR, and female OZR had greater insulin concentrations than did male OZR.

Cell studies. G-2535 induced PPAR-regulated luciferase activity in RAW 264.7 cells expressing a PPRE-containing reporter and PPARα expression plasmids (**Fig. 2**A) ($P < 0.001$). When individual time points were analyzed, G-2535 treatment-induced luciferase activity was greater than in cells treated with the vehicle (control) or clofibrate ($P < 0.05$).

G-2535 treatments also promoted luciferase activity to a greater extent than that in the vehicle in cells transiently cotransfected with the PPRE-reporter and PPARγ expression plasmids (Fig. 2*B*) ($P < 0.05$). However, luciferase activity in the G-2535–treated cells was lower than that in cells treated

TABLE 3

Liver weight, liver cholesterol and liver triglyceride concentrations in male and female obese Zucker rats on fed control (C), LIS or HIS diets for 8 or 11 wk, respectively[1]

Diet group[2]	*n*	Liver wt[3]	Liver cholesterol	Liver TG[4]	Plasma cholesterol	Plasma triglyceride
		g	*μmol/g liver*		*mmol/L*	
C (OZR male)	8	33.1 ± 2.2	13.61 ± 1.11	246 ± 17.1	7.41 ± 0.45	3.34 ± 0.56
LIS (OZR male)	8	29.2 ± 2.2	13.35 ± 0.87	233 ± 17.0	5.96 ± 0.46[a]	2.88 ± 0.24
HIS (OZR male)	8	24.5 ± 1.7[a,b]	8.27 ± 0.25[a,b]	131 ± 21.6[a,b]	5.86 ± 0.46[a]	3.13 ± 0.29
C (OZR female)	4	36.9 ± 2.7	17.21 ± 0.60	367 ± 27.7	9.51 ± 0.72	3.26 ± 0.29
LIS (OZR female)	4	36.5 ± 4.3	12.49 ± 0.69[a]	387 ± 19.7	7.91 ± 0.51	2.29 ± 0.14
HIS (OZR female)	4	26.7 ± 3.8[a,b]	8.63 ± 1.11[a,b]	229 ± 50.4[a,b]	5.61 ± 0.77[a,b]	7.95 ± 1.02[a,b]

[1] Values are means ± SEM.
[2] Diet groups: Control (casein), C; low-isoflavone (< 0.1 mg isoflavone/g protein) soy protein, LIS; or high-isoflavone (5.8 mg isoflavone/g protein) soy protein, HIS.
[3] Corrected for body weight in the statistical analysis. [a] Different from C, $P < 0.05$; [b] Different from LIS, $P < 0.05$.
[4] Total triglycerides (TG) as triolein (MW 885.4).

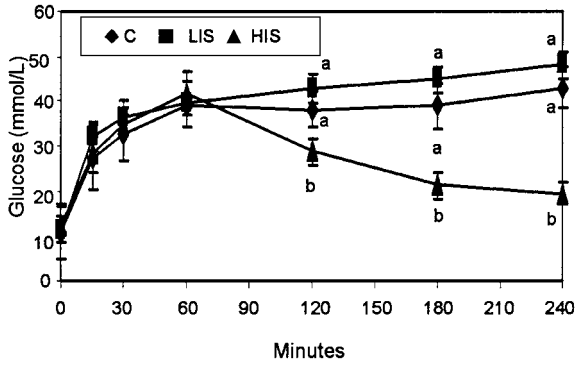

FIGURE 1 Glucose tolerance test (GTT) in female obese Zucker rats (OZR) fed casein (C), low-isoflavone soy protein (LIS) or high-isoflavone soy protein (HIS) diets for 11 wk. Values are means ± SEM, $n = 4$. Means at a time without a common letter differ, $P < 0.05$.

with pioglitazone. When RAW 264.7 cells were transfected with only the PPRE-reporter plasmid and β-galactosidase plasmid, but not an expression plasmid for either the PPARα or PPARγ receptor, there was no induction of luciferase activity in clofibrate, pioglitazone or G-2535 (data not shown) compared to vehicle-treated cells ($P < 0.05$).

To determine the effects of unconjugated compared to conjugated isoflavones and individual isoflavones, cells were treated with vehicle, a positive control (clofibrate or pioglitazone), G-2535, Prevatein HC, genistein, daidzein or glycitein for 24 h. Cells containing the PPRE-reporter and PPARα expression plasmids significantly increased luciferase activity when treated with G-2535 soy extract (220% greater), genistein (140% greater) and daidzein (160% greater) compared to vehicle-treated cells (all $P < 0.05$) (**Fig. 3***A*). PPRE-directed gene expression was not increased by treatment with Prevastein HC or glycitein.

Transiently transfected cells containing the PPRE-reporter and PPARγ expression plasmids also had significantly greater luciferase activity when treated with G-2535 (280% greater), genistein (380% greater) and daidzein (200% greater) compared to vehicle-treated cells (all $P < 0.05$) (Fig. 3*B*). Again, neither Prevastein HC nor glycitein increased PPRE-directed gene expression.

DISCUSSION

We have demonstrated that consumption of an HIS diet improves lipid metabolism and glucose tolerance in male and female OZR and glucose tolerance in female OZR, especially when directly compared to OZR fed the LIS diet. Further, we showed that isoflavone-containing soy extracts and individual soy isoflavones activated PPAR-mediated gene expression. These data suggest that, compared to LIS, the increased level of isoflavones present in the HIS diet improved lipid metabolism and glucose tolerance by PPAR activation. We chose the OZR for the in vivo study because it is an established animal model, especially with respect to diabetes and the metabolic syndrome (33). When fed HIS diets, OZR had an increased FER, and in all OZR fed HIS compared to OZR fed

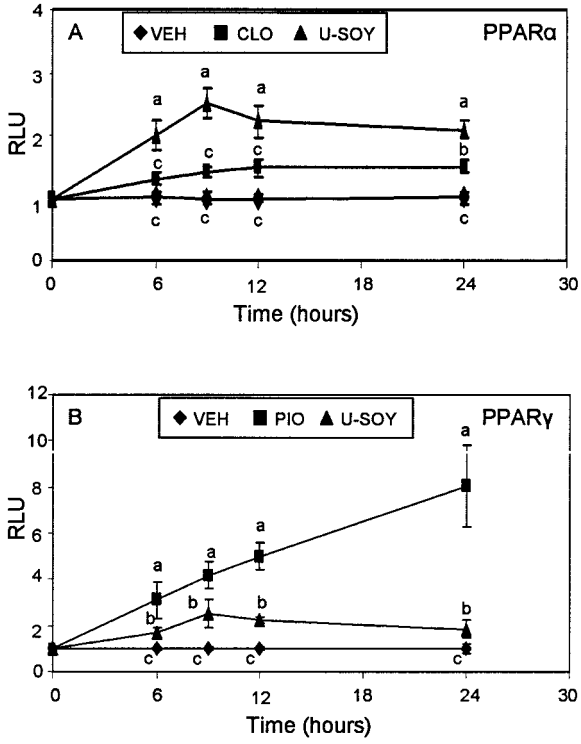

FIGURE 2 The effect of an unconjugated isoflavone-containing soy extract on PPAR activation. Luciferase activity was regulated by the PPRE-containing reporter plasma and was cotransfected into RAW 264.7 cells using the PPARα (*A*) or PPARγ (*B*) expression plasmids. Cells were incubated with a vehicle (VEH), 2.5 mg/L G-2535 (U-SOY), clofibrate (CLO, *A*) or pioglitazone (PIO, *B*) after transfection. Values are means ± SEM, $n = 7$–11. Means at a time without a common letter differ, $P < 0.05$.

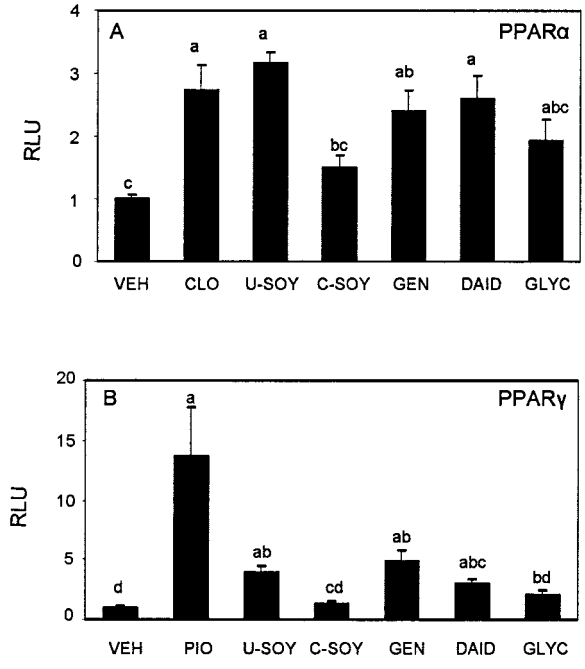

FIGURE 3 The effect of isoflavone-containing soy extracts and soy isoflavones on PPAR activation. Luciferase activity was regulated by the PPRE-containing reporter plasma and was cotransfected into RAW 264.7 cells using the PPARα (*A*) or PPARγ (*B*) expression plasmids. Cells were incubated with either a vehicle (VEH), clofibrate (CLO), pioglitazone (PIO), 2.5 mg/L G-2535 (U-SOY) or Prevastein HC (C-SOY), 9.3 μmol/L genistein (GEN), 9.8 μmol/L daidzein (DAID) or 8.8 μmol/L glycitein (GLYC). Values are means ± SEM, $n = 7$–8. Means without a common letter differ ($P < 0.05$).

LIS ($n = 12$ each) diets, weight gain was greater ($P < 0.05$). In contrast to increased body weight, liver weight, liver cholesterol and liver triglyceride concentrations were all reduced in HIS-fed compared to LIS-fed OZR. Additionally, HIS-fed female OZR had significantly improved glucose tolerance compared to LIS- or C-fed OZR. These data are consistent with PPAR agonist actions and previous research published in OZR (27,28,34). At this time, we are unable to explain the increase in plasma triglycerides in female OZR fed a HIS diet compared to both the C- and LIS-fed rats. This same increase was described previously when Zucker rats were fed a soy diet (35).

In a comparison of HIS- and LIS-fed OZR, plasma cholesterol levels were significantly lower only in the females. Interestingly, a recent report describes results in Golden Syrian F_1B hybrid hamsters, in which males fed a HIS diet had reduced plasma LDL-cholesterol, whereas the females' cholesterol concentrations did not change (36). Species and sex differences may contribute to the differences in animal studies investigating the plasma cholesterol–lowering property of soy. Future research should further investigate gender-based effects of soy isoflavones. Furthermore, the OZR is not the ideal model for studying atherosclerosis and plasma cholesterol concentrations (33).

Our animal studies demonstrated metabolic changes suggestive of a PPAR agonist-like effect of soy isoflavones. Thus, we performed cell culture studies to determine whether isoflavones could influence PPAR action. Cell treatments were designed to resemble concentrations similar to the plasma concentrations of isoflavones measured in vivo (37,38). Exposing cells to a soy extract containing conjugated isoflavones did not affect activation (Fig. 2). However, when cells were exposed to an unconjugated isoflavone-containing soy extract, PPAR-mediated gene expression was increased. Tested individually, genistein and daidzein activated PPARα and PPARγ, whereas glycitein had no effect. Our results are consistent with a recent study reporting genistein-induced activation of PPARγ in KS483 cells (39). Unlike the highly specific estrogen receptors, PPAR bind a wide number of ligands and directly affect lipid metabolism by enhancing transcription of PPAR-regulated genes. This "promiscuous" binding activity suggests that phytochemicals including soy isoflavones bind and activate PPARα and PPARγ (40).

In conclusion, an isoflavone-containing soy diet improved the diabetic phenotype of male and female OZR. Moreover, cell studies provided direct evidence that soy isoflavones affect both PPARα- and PPARγ-directed gene expression. This study suggests that soy isoflavones exert a beneficial effect on lipid and glucose metabolism through activation of the PPAR receptors.

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