Dietary Phytoestrogens Activate AMP-Activated Protein Kinase With Improvement in Lipid and Glucose Metabolism

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OBJECTIVE—Emerging evidence suggests that dietary phytoestrogens can have beneficial effects on obesity and diabetes, although their mode of action is not known. Here, we investigate the mechanisms mediating the action of dietary phytoestrogens on lipid and glucose metabolism in rodents.

RESEARCH DESIGN AND METHODS—Male CD-1 mice were fed from conception to adulthood with either a high soy–containing diet or a soy-free diet. Serum levels of circulating isoflavones, ghrelin, leptin, free fatty acids, triglycerides, and cholesterol were quantified. Tissue samples were analyzed by quantitative RT-PCR and Western blotting to investigate changes of gene expression and phosphorylation state of key metabolic proteins. Glucose and insulin tolerance tests and euglycemic-hyperinsulinemic clamp were used to assess changes in insulin sensitivity and glucose uptake. In addition, insulin secretion was determined by in situ pancreas perfusion.

RESULTS—In peripheral tissues of soy-fed mice, especially in white adipose tissue, phosphorylation of AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase was increased, and expression of genes implicated in peroxisomal fatty acid oxidation and mitochondrial biogenesis was upregulated. Soy-fed mice also showed reduced serum insulin levels and pancreatic insulin content and improved insulin sensitivity due to increased glucose uptake into skeletal muscle. Thus, mice fed with a soy-rich diet have improved adipose and glucose metabolism.

CONCLUSIONS—Dietary soy could prove useful to prevent obesity and associated disorders. Activation of the AMPK pathway by dietary soy is likely involved and may mediate the beneficial effects of dietary soy in peripheral tissues. ***Diabetes*** **57:1176–1185, 2008**

A better understanding of the factors regulating lipid and glucose metabolism are of eminent interest due to the pandemic development of obesity and its related metabolic disorders. Preventive strategies to control excess body weight and insulin resistance have mainly focused on physical exercise and total caloric intake. Over the past years, however, an increasing body of evidence indicates that estrogens are important regulators of glucose and adipose metabolism. They are known to modulate the distribution of body fat and adipose tissue metabolism either through the direct regulation of adipogenesis and lypolysis or indirectly via modulation of the energy balance (rev. in 1). Similarly, estrogens play an important role in glucose homeostasis and are known to modulate insulin sensitivity (2). Postmenopausal women develop visceral obesity and insulin resistance and are at an increased risk for diabetes; estrogen replacement therapy normalizes these abnormalities (3,4). In rodent models of type 2 diabetes, female mice are protected against hyperglycemia unless they are ovariectomized, whereas in male animals, estrogen perfusion reverses diabetes (5). Finally, estrogen receptor (ER)α or aromatase-knockout mice display several phenotypes associated with the metabolic syndrome, such as increased adiposity, glucose intolerance, and insulin resistance (6,7), demonstrating the fundamental implication of estrogens in these metabolic processes.

Phytoestrogens are nonsteroidal compounds of natural origin that can bind to both ERα and ERβ and behave as estrogen mimics (8). The isoflavones genistein and daidzein, predominantly found in soybean and soybean-derived products, are a major source of phytoestrogens in human diets. Multiple studies in humans and animals suggest that, similar to estrogens, dietary phytoestrogens also play a beneficial role in reducing obesity and diabetes and improving glucose control and insulin resistance. In humans, isoflavone consumption is correlated with a decrease in BMI and serum insulin levels (9), and dietary phytoestrogens improve insulin resistance in ovariectomized cynomolgus monkeys (10). In mice, exposure to

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ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; AUC, area under curve; DEXA, dual-energy X-ray absorptiometry; ER, estrogen receptor; ERRα, estrogen receptor–related receptor α; FFA, free fatty acid; GLP-1, glucagon-like peptide 1; GTT, glucose tolerance test; HPLC, high-performance liquid chromatography; IRβ, insulin receptor β; IRS, insulin receptor substrate; ITT, insulin tolerance test; mAb, monoclonal antibody; mTOR, mammalian target of rapamycin; PGC, peroxisome proliferator–activated receptor γ co-activator; PPAR, peroxisome proliferator–activated receptor; ROS, reactive oxygen species; TG, triglyceride; WAT, white adipose tissue.

pure dietary genistein at doses of 500-1500 ppm has antilipogenic effects and decreases adipose deposition (11). Similarly, rodents fed with a soy-rich diet have significantly decreased adiposity compared with animals fed with a phytoestrogen-free diet (12,13). Recently, we showed that mice exposed to dietary phytoestrogens maintain a lean phenotype associated with increased energy expenditure and locomotor activity coupled with a marked shift toward the use of lipids (14). However, the molecular and physiological mechanisms underlying the metabolic action of phytoestrogens on glucose metabolism and fat deposition have not yet been determined. Elucidating these mechanisms could represent a major contribution to the rational use of such natural compounds for prevention and management of metabolic diseases.

RESEARCH DESIGN AND METHODS

Animal care and diets. Outbred CD1 male and female mice were fed with a high soy-containing diet (high phytoestrogen) (Harlan Teklad 8604; Harlan Teklad, Madison, WI) or a soy-free diet (low phytoestrogen) (Phytoestrogen Reduced Rodent Diet I; Ziegler Brothers, Gardner, PA) 3 weeks before mating so that the offspring of the pairing would be exposed solely to high- or low-phytoestrogen diets. The isoflavone content of these two closed-formula diets was previously reported to be ~198 ppm daidzein and 286 ppm genistein equivalents in the high-phytoestrogen diet and nondetectable in the low-phytoestrogen diet (15). Both diets were equivalent in terms of carbohydrate, protein, fat, amino acids, vitamins, mineral content, gross energy content, metabolizable energy, and digestable energy (14,15). In the low-phytoestrogen diet formulation, soy was omitted and replaced by lactic casein and dried skim milk.

Animal protocols used in these studies were approved by the Geneva Veterinarian Office. Animals had free access to food and water. Temperature was maintained between 19 and 21°C, and lights were on at 7:00 A.M. and off by 7:00 P.M.

Serum phytoestrogen levels. The concentrations of total genistein, daidzein, and equol were determined in individual serum samples collected at 8 A.M. from adult mice (23–26 weeks old) exposed to high-phytoestrogen (n = 11) or low-phytoestrogen (n = 11) diets as previously described (14,16).

Measurements of body composition and fat depot. Peripheral dual-energy X-ray absorptiometry (DEXA; PIXImus; GE-Lunar, Madison, WI) was used to measure in vivo percent fat mass of mice. Adult male mice were killed between 9:00 A.M. and 11:00 A.M. after a 2-h fasting. Adipose tissues were dissected out, weighted, and expressed as a percentage of the total weight of the animal.

Blood and tissue chemistry. Blood was collected in the morning by cardiac puncture from mice that had fasted for 2 h. Sera and relevant peripheral tissues were stored at −20°C and used subsequently to assess the levels of metabolic hormones. Leptin was assessed using kits from Linco Research (Lausanne, Switzerland). Serum insulin was assayed using a kit from Dia Sorin (Saluggia, Italy), whereas free fatty acids (FFAs) and triglycerides (TGs) were measured by colorimetric assays. Serum and tissue cholesterol concentrations were determined as described elsewhere (17). AMP-to-ATP ratio in skeletal muscle was assessed by quantifying AMP, ADP, and ATP using a high-performance liquid chromatography (HPLC) system as previously described (18). Briefly, tissues were frozen between precooled metal plates and directly extracted in ice-cold 5% perchloric acid. After centrifugation, supernatant was stored at −80°C before HPLC analysis.

Glucose and insulin analyses. For glucose tolerance tests (GTTs), animals fasted overnight (11 h) were injected intraperitoneally with 1.5 g glucose/kg body wt. Plasma glucose levels were measured at 0, 15, 30, 60, 90, and 120 min with Glucometer DEX (Bayer). For determination of plasma insulin concentrations during GTTs, blood was collected from the tail vein, and measurements were performed by ELISA (Kit Mercodia Ultrasensitive Mouse Insulin ELISA). For insulin tolerance tests (ITTs), mice fasted for 3 h were injected intraperitoneally with 0.75 units insulin/kg body wt (Novo Nordisk Pharma, Küsnacht, Switzerland). Glucose levels were measured at 0, 20, 40, 60, and 120 min as described above.

For measurement of insulin content, whole pancreata of 6-month-old high- and low-phytoestrogen–fed mice were extracted in acid-ethanol (74% ethanol and 1.4% HCl). Samples were sonicated and centrifuged before radioimmunoassay (19).

For pancreas perfusion, whole pancreata of high- and low-phytoestrogen–fed animals were perfused in situ with 1.5 ml/min Krebs-Ringer HEPES buffer. The perfusate contained the glucose and glucagon-like peptide 1 (GLP-1) concentrations indicated in the text, each applied for 20 min. During the first 20-min equilibration period, the medium contained 1.4 mmol/l glucose, and no effluent was sampled. Thereafter, aliquots were collected every minute for measurement of insulin (19). Differences in insulin secretion between animals and groups were assessed by the median test, which compared the areas under the secretion curve.

Euglycemic-hyperinsulinemic clamps. Twenty-five– to 28-week-old high- and low-phytoestrogen–fed male mice were fasted for 3 h, anesthetized with sodium pentobarbital (55 mg/kg i.p.), and subjected to a euglycemic-hyperinsulinemic clamp as previously described (20). At the end of these euglycemic-hyperinsulinemic clamps, a bolus of 9.25 mBq 2-deoxy-D-[1-^{3}H]glucose (Amersham Biosciences, Dübendorf, Switzerland) was injected intravenously to determine the in vivo insulin-stimulated glucose uptake of various tissues (21).

Western blot. Frozen tissues were mechanically homogenized (Polytron; Kinematica, Lucerne, Switzerland) in ice-cold radioimmunoprecipitation assay buffer (50 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 1% Nonidet P-40, 0.5% Na deoxycholate, and 0.1% SDS), including 10 mmol/l NaF, 5 mmol/l orthovanadate, and a cocktail of protease inhibitors (Roche Diagnostics, Mannheim, Germany). After clearing the lysates by centrifugation at 10,000 rpm for 10 min, total protein content was measured using a BCA protein assay kit (Pierce, Lausanne, Switzerland). Samples containing 10 μg total proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane (Amersham Biosciences, Dübendorf, Switzerland), and relevant proteins were revealed using enhanced chemiluminescence detection (ECL Advance, Amersham Biosciences). Protein levels were quantified using the ChemiDoc XRS from Bio-Rad and the Quantity One software. Antibodies against insulin receptor β (IRβ), insulin receptor substrate 1 (IRS1), phospho-IRS1 (Y632), phospho-IRS1 (S636/639), IRS2 were from Santa Cruz Biotechnology; phospho-IRβ (Y1131), Akt, phospho-Akt (T308 and S473), AMP-activated protein kinase (AMPK) α (pan α1 and α2, monoclonal antibody [mAb] 2532), phospho-AMPKα (pan α1 and α2 T172, mAb 2535), mammalian target of rapamycin (mTOR), phospho-mTOR (S2448), p70 S6 kinase, and phospho-p70 S6 kinase (T389) were from Cell Signaling Technology (Danvers, MA). Phospho–acetyl-CoA carboxylase (phospho-ACC) (S79) antibody was from Phoenix Pharmaceutical, and anti-actin was from Chemicon.

Real-time RT-PCR. Total RNAs from adipose tissue (epididymal), muscle (quadriceps), and liver were extracted using RNeasy mini kit from Qiagen (Hombrechtikon, Switzerland) according to the manufacturer's protocol. One microgram total RNAs was reverse transcribed with the Superscript II Reverse Transcriptase from Invitrogen (Life Technologies, Gaithersburg, MD) according to manufacturer's instructions, and one-twentieth of cDNA template was used as template. Quantitative RT-PCRs were performed as previously described (22). The relevant primers used for quantitative RT-PCR are listed in Supplemental Table 1, which is detailed in the online appendix (available at http://dx.doi.org/10.2337/db07-0630).

Statistical analysis. Results are expressed as means ± SE of n experiments. The nonparametric unpaired t test was used for statistical analysis. Differences were considered statistically significant if P was <0.05.

RESULTS

Circulating isoflavone levels and decreased adiposity in mice fed with dietary phytoestrogens. Male CD1 mice were fed from conception to adulthood with either a high soy-containing diet (high phytoestrogen) or a soy-free diet (low phytoestrogen). The isoflavone content of these two closed-formula diets was previously reported to be ~600 and 10–15 ppm, respectively (15). The levels of isoflavones in plasma were directly correlated; mice fed on high-phytoestrogen diet displayed significantly higher levels of genistein, daidzein, and equol compared with animals fed with the low-phytoestrogen diet (Fig. 1*A*). In mice consuming the high-phytoestrogen diet, the average steady-state serum concentrations of genistein (181 ng/ml) and daidzein (128 ng/ml) were well within the range observed in humans consuming soy foods and nutritional supplements (23,24), whereas the daidzein metabolite, equol, was present at higher concentrations (1,715 ng/ml).

As we previously reported (15), exposure to the soy-based diet significantly decreased weight, overall fat, and adipose tissue weight of adult male mice (Fig. 1*B* and *C*).

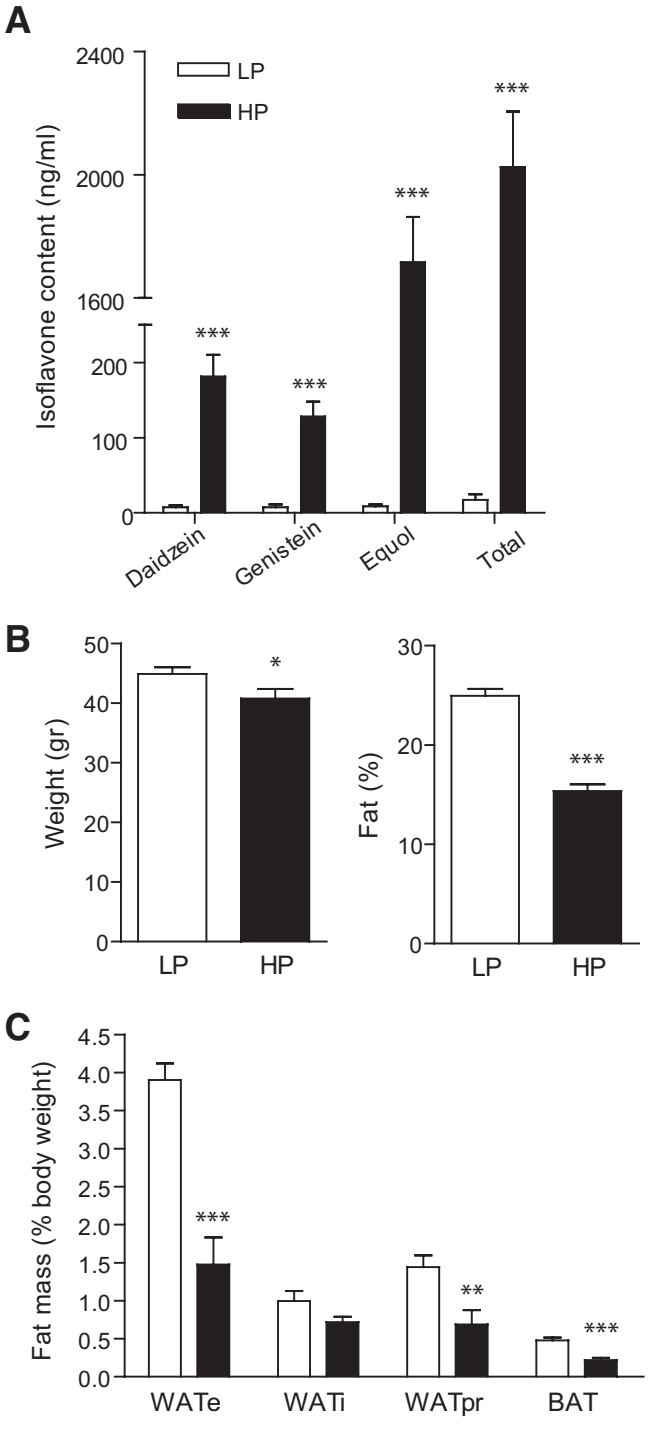

FIG. 1. Leanness in mice exposed to a soy-rich diet. *A*: Circulating serum isoflavone levels of adult male mice fed either with high-phytoestrogen (HP) or low-phytoestrogen (LP) diet. For each phytoestrogen measured, high-phytoestrogen–fed animals displayed significantly higher daidzein, genistein, equol, or total isoflavone levels compared with low-phytoestrogen–fed mice. *B*: Total weight and fat content as measured by DEXA analysis was significantly reduced in adult high-phytoestrogen–fed animals (P120, postnatal day 120). *C*: Intra-abdominal fat was drastically reduced with significant reduction in epididymal and perirenal WATs of high-phytoestrogen–fed animals. ■, high-phytoestrogen–fed animals; □, low-phytoestrogen–fed animals. BAT, brown adipose tissue; WATe, epididymal WAT; WATi, inguinal WAT; WATpr, perirenal WAT. Results are means ± SE (n = 7–11/group), *P < 0.05, **P < 0.01, ***P < 0.001 vs. low phytoestrogen.

Epididymal, inguinal, and perirenal white adipose tissue (WAT) fat depositions of high-phytoestrogen–fed mice were ~50% lower in comparison with low-phytoestrogen–fed mice. This fat mass reduction in soy-fed mice has been attributed exclusively to reduced fat storage (14).

Improved hormonal profile in high-phytoestrogen–fed animals. Because estrogenic treatment and adipose mass have a substantial influence on hormonal and metabolic parameters, we compared the serum profiles of high- and low-phytoestrogen–fed adult mice (Fig. 2*A*). Ghrelin levels were unchanged, whereas leptin levels were significantly lower in high-phytoestrogen–fed mice, presumably reflecting the decrease in adipose tissue mass. Cholesterol levels were also reduced in the serum of high-phytoestrogen–fed mice, but TG and FFA levels did not differ (Fig. 2*A*, *bottom panel*). Because of their leanness, we hypothesized that levels of cholesterol and TG would be lower in peripheral tissues of high-phytoestrogen–fed mice. We found important reductions in TGs and cholesterol in WAT and, to a lesser extent, in muscle (Fig. 2*B*). In liver, TGs were reduced while cholesterol levels remained unchanged.

Increased phosphorylation and gene expression of proteins essential for fatty acid oxidation in peripheral tissues of high-phytoestrogen–fed mice. Our previous study suggested that mice fed with dietary phytoestrogens are leaner because of increased locomotor activity and preferential use of lipids as fuel source (14). Exercise is known to activate the fuel-sensing enzyme AMPK, which in turn improves glucose uptake and increases fatty acid oxidation in peripheral tissues (25,26). In particular, phosphorylation by AMPK inhibits ACC activity, thus decreasing the production of malonyl-CoA, an inhibitor of mitochondrial fatty acid import and oxidation. We assessed the phosphorylation of AMPK and ACC in skeletal muscle, liver, and WAT. In comparison with low-phytoestrogen–fed mice, high-phytoestrogen–fed mice displayed higher phosphorylation of AMPK (T172) and similar increases in the phosphorylation of its downstream target ACC (S79) (Fig. 3*A*–*C*). Consistent with a direct effect of phytoestrogens on adipose and muscle tissues, phosphorylation of AMPK and ACC tends also to increase in C2C12 myotubes and 3T3-L1 adipocytes after a 30-min exposure to 0.1–10 μmol/l to the isoflavone genistein (data not shown). AMPK can also be activated by increased AMP-to-ATP ratio (27), which could result from the increased locomotor activity of high-phytoestrogen–fed mice. We therefore determined the adenine nucleotide content of skeletal muscles by HPLC. Surprisingly, the AMP-to-ATP ratio was unchanged between high- and low-phytoestrogen skeletal muscles (low phytoestrogen, 0.0253 ± 0.0018; high phytoestrogen, 0.0244 ± 0.0017; P = 0.736), suggesting that the activation of AMPK mediated by dietary soy does not involve global changes in AMP, ADP, or ATP.

Enhanced expression of genes essential for peroxisomal fatty acid oxidation and mitochondrial metabolism in high-phytoestrogen–fed mice. To test the hypothesis that mitochondrial metabolism is altered in high-phytoestrogen–fed mice, we used quantitative RT-PCR and found that the expression of genes essential for mitochondrial biogenesis and peroxisomal fatty acid oxidation, respectively peroxisome proliferator–activated receptor (PPAR)γ co-activator (PGC-1α) and PPARα, were upregulated in WAT, skeletal muscle, and liver of high-phytoestrogen–fed animals (Fig. 3*D* and *E*). PPARα is known to mediate the expression of genes promoting fatty acid β-oxidation in particular when coactivated with PGC-1α (28) and also to regulate peroxisomal β-oxidation

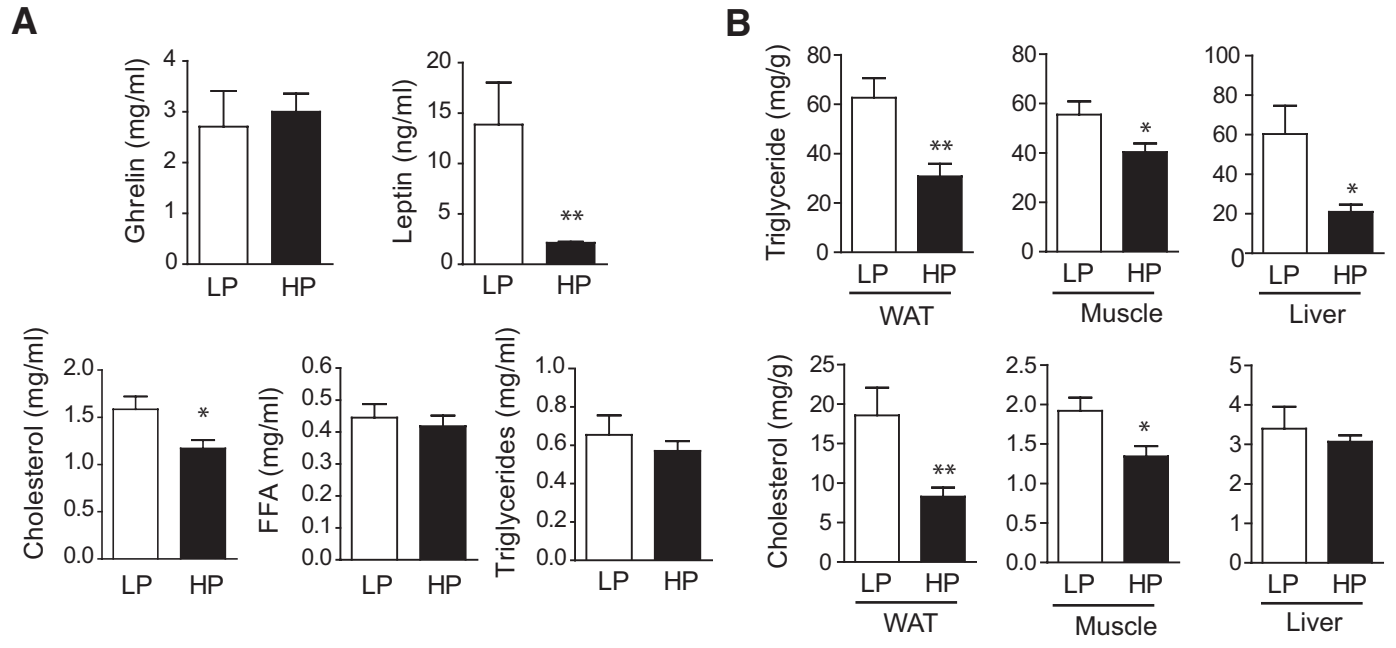

FIG. 2. Improved hormonal profile and decreased TG and cholesterol in peripheral tissues of high-phytoestrogen–fed (HP) animals. *A*: Comparison of ghrelin, leptin, cholesterol, TG, and FFA levels in the serum of HP and low-phytoestrogen–fed (LP) mice. *B*: Analysis of TG and cholesterol in WAT, muscle, and liver from HP and LP mice. Results are means ± SE (n = 10–12/group). *P < 0.05, **P < 0.01, ***P < 0.001 vs. control.

(rev. in 29). We also found that Acyl-CoA oxidase, a downstream target of PPARα, was upregulated in WAT of high-phytoestrogen–fed mice (Table 1).

Activation of AMPK is known to increase energy levels in skeletal muscle by enhancing oxidative metabolism through promoting the expression and by direct phosphorylation of PGC-1α (30–32). PGC-1α induces the expression of genes involved in heme biosynthesis, ion transport, mitochondrial translation, and protein import and also stimulates mitochondrial biogenesis and respiratory functions (33,34). Consistent with the induction profile of PGC-1α in peripheral tissue (Fig. 3*D*), the expression of genes involved in mitochondrial metabolism was significantly increased in the epididymal WAT of high-phytoestrogen–fed mice compared with muscle and liver (Table 1). *Tfam*, a target of PGC-1α essential for the

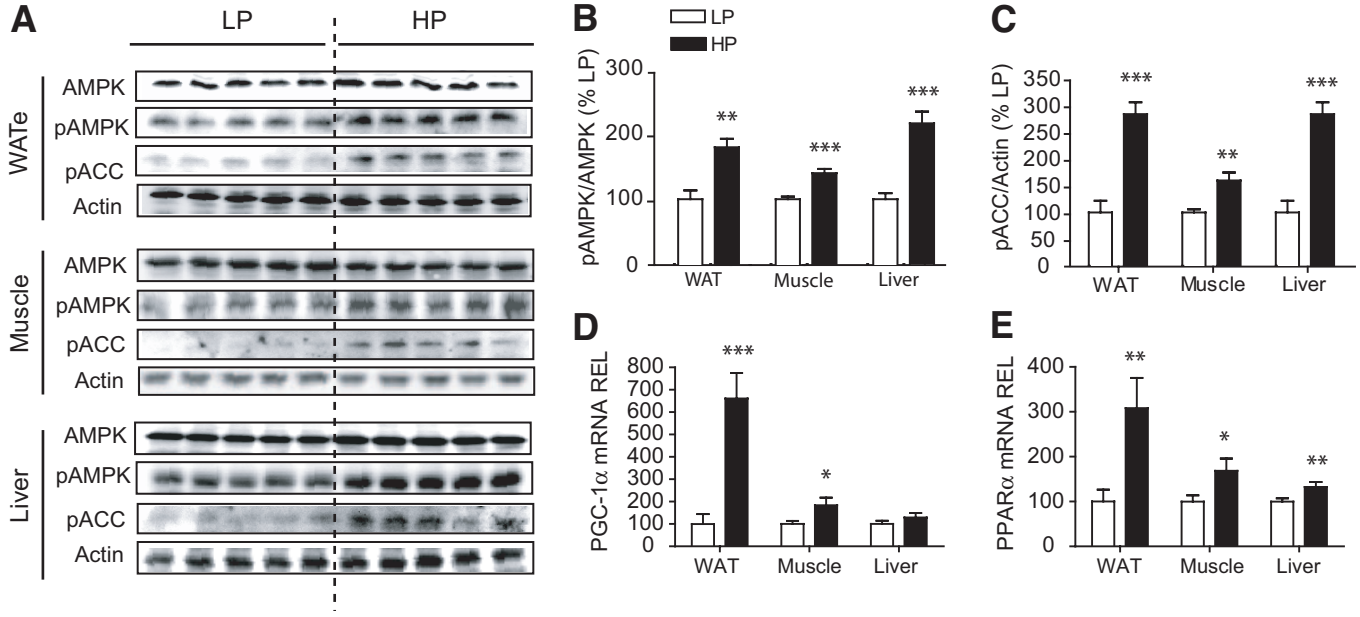

FIG. 3. Increased phosphorylation and gene expression of several proteins essential for fatty acid oxidation in peripheral tissues of high-phytoestrogen–fed (HP) mice. Representative Western blot analysis of total AMPK, phospho-AMPK, and phospho-ACC (*A*) and quantification of the phospho-AMPK–to–AMPK protein ratio (*B*) and of phospho-ACC–to–actin ratio (*C*) in skeletal muscle, liver, and epididymal WAT of HP and low-phytoestrogen–fed (LP) mice. Animals exposed to phytoestrogens exhibit higher levels of PGC-1α (*D*) and PPARα (*E*) gene expression in epididymal WAT, skeletal muscle, and liver as determined by real-time quantitative RT-PCR. Results are means ± SE (n = 5/group [*A–C*] or 10/group [*D* and *E*]) and are shown as percentage of expression in HP mice relative to LP animals (REL). *P < 0.05, **P < 0.01, ***P < 0.001 vs. control.

TABLE 1
Higher mitochondrial biogenesis in WAT of animals exposed to a soy-rich diet

	Epididymal WAT		Muscle		Liver	
	Low phytoestrogen	High phytoestrogen	Low phytoestrogen	High phytoestrogen	Low phytoestrogen	High phytoestrogen
Mitochondrial related regulation of transcription						
Tfam	100 ± 28.5	371.9 ± 36.3*	100 ± 12.6	144.1 ± 10.7†	100 ± 6.8	105.7 ± 7.8
ERRα	100 ± 19.8	132.2 ± 14.2	100 ± 3.6	134.4 ± 12.1†	100 ± 9.3	110.0 ± 8.3
PGC1b	100 ± 23.0	426.2 ± 72.9*	100 ± 9.5	155.6 ± 16.5‡	100 ± 17.9	174.1 ± 25.1*
ROS detoxifying enzymes						
Catalase	100 ± 20.0	219.3 ± 45.1†	100 ± 11.1	85.0 ± 8.7	100 ± 7.4	103.2 ± 7.1
SOD1	100 ± 19.8	204.2 ± 25.8‡	100 ± 6.2	122.3 ± 4.8†	100 ± 7.1	103.0 ± 6.8
SOD2	100 ± 18.0	178.0 ± 21.7†	100 ± 7.3	115.7 ± 9.2	100 ± 4.3	91.8 ± 6.3
Gpx1	100 ± 26.7	180.2 ± 28.6	100 ± 9.0	101.1 ± 6.6	100 ± 9.0	88.9 ± 7.2
Mitochondrial respiratory chain						
ANT	100 ± 18.9	329.3 ± 33.6*	100 ± 7.3	115.6 ± 7.2	100 ± 6.2	116.7 ± 12.4
ATP 5j	100 ± 18.7	316.8 ± 33.4*	100 ± 9.9	126.1 ± 8.8	100 ± 8.7	107.1 ± 7.6
ATP synb	100 ± 20.6	187.0 ± 23.9†	100 ± 4.1	100.8 ± 8.1	100 ± 5.2	118.5 ± 7.4
COX II	100 ± 21.6	377.0 ± 36.8*	100 ± 16.8	166.9 ± 18.0†	100 ± 7.0	122.0 ± 8.0
COX III	100 ± 30.0	254.5 ± 27.8‡	100 ± 9.0	124.6 ± 11.2	100 ± 8.1	110.4 ± 8.1
COX IV	100 ± 20.8	135.9 ± 15.5	100 ± 4.8	114.2 ± 10.3	100 ± 5.7	107.8 ± 7.3
COX IV1	100 ± 22.2	124.7 ± 14.4	100 ± 4.5	124.5 ± 10.8†	100 ± 8.5	117.1 ± 8.7
COX 5b	100 ± 19.3	189.2 ± 22.5‡	100 ± 7.1	116.5 ± 9.2	100 ± 6.7	109.4 ± 6.6
COX 6a	100 ± 20.5	202.2 ± 23.9‡	100 ± 5.4	105.6 ± 6.5	100 ± 6.0	105.6 ± 6.5
COX 7a1	100 ± 24.0	90.5 ± 10.2	100 ± 6.4	111.7 ± 10.6	100 ± 12.0	118.6 ± 13.5
Cytc	100 ± 17.2	246.4 ± 30.5*	100 ± 10.5	110.3 ± 7.6	100 ± 4.9	105.3 ± 6.8
Ndnfb5	100 ± 21.7	120.2 ± 16.3	100 ± 5.6	123.5 ± 14.0	100 ± 7.8	84.6 ± 6.5
Uncoupling						
UCP2	100 ± 10.7	89.9 ± 11.0	100 ± 9.7	73.6 ± 9.2	nd	nd
UCP3	100 ± 21.5	163.5 ± 21.7†	100 ± 13.6	107.3 ± 21.60	nd	nd
Peroxisomal fatty acid oxidation						
ACoAx	100 ± 17.7	239.6 ± 26.3*	100 ± 9.9	75.3 ± 11.6	100 ± 18.8	100.4 ± 14.2

Data are means ± SE, are summarized per group (n = 8–10), and are expressed as percentage relative to low-phytoestrogen tissues (100%). Quantitative real-time PCR of low- and high-phytoestrogen WAT, muscle, and liver. Normalization was performed using four housekeeping genes (Cyclophilin B, Actin-β, Tubulin-α, and Tubulin-β). t test, *$P < 0.001$, †$P < 0.05$, ‡$P < 0.01$ vs. control; nd, not determined.

replication and transcription of mitochondrial DNA (34,35), was upregulated by 3.7-fold in the WAT of high-phytoestrogen–fed mice, whereas the expression of cofactors of PGC-1α, such as the estrogen receptor–related receptor α (ERRα), was unchanged. The expression of PGC-1β, also implicated in the regulation of the mitochondrial metabolic gene programming, increased by 4.2-fold. Genes implicated in reactive oxygen species (ROS) metabolic oxidative phosphorylation, ATP synthesis, and uncoupling were also upregulated in the WAT of high-phytoestrogen–fed mice (Table 1). Taken together, these data suggest that dietary soy promotes fatty acid oxidation and leanness by activating AMPK and improving PGC-1α–related mitochondrial metabolism preferentially in WAT.

High-phytoestrogen–fed mice feature reduced pancreatic insulin content. Although glycemia was comparable (Fig. 4*A*), basal insulin levels were significantly lower in high-phytoestrogen–fed mice (Fig. 4*B*), suggesting that dietary soy could affect total pancreatic insulin content and/or insulin secretion. Total pancreatic insulin content was significantly ($P < 0.01$) reduced in high-phytoestrogen–fed mice (Fig. 4*C*), despite a comparable size of the pancreas (Fig. 4*D*). In addition, pancreas perfusion showed that the release of insulin in response to a basal glucose concentration (1.4 mmol/l) was lower ($P < 0.05$, as assessed by the median test) in high-phytoestrogen than in low-phytoestrogen–fed mice (Fig. 4*E* and *F*). A comparable difference was observed in the presence of postprandial levels of glucose (4.2–8.0 mmol/l), although significance was not reached. Strikingly, the insulin release of high-phytoestrogen–fed mice was not stimulated by these glucose levels over basal value. In contrast, the pancreas of high- and low-phytoestrogen–fed mice secreted similar levels of insulin in response to a supra-physiological stimulation by glucose (16 mmol/l) and in the presence of 1 μmol/l GLP-1 (Fig. 4*E* and *F*). These data indicate that dietary soy exposure reduces both insulin storage and the sensitivity of β-cells to physiological glucose stimulation.

Increased glucose uptake and insulin sensitivity in high-phytoestrogen–fed animals. In comparison with low-phytoestrogen–fed mice, high-phytoestrogen–fed mice already displayed a number of metabolic improvements; we therefore also investigated the ability of high-phytoestrogen–fed mice to respond to a glucose challenge. In GTTs, glucose and insulin levels remained significantly lower in high-phytoestrogen–fed mice at all time points (Fig. 5*A* and *B*). ITTs showed that the insulin-mediated decrease in glycemia was more pronounced in high-phytoestrogen–fed than in low-phytoestrogen–fed mice (Fig. 5*C*). The insulin resistance index, calculated from the area under curve (AUC) in GTTs and ITTs, was 1.63-fold lower for high-phytoestrogen–fed mice than for low-phytoestrogen–fed animals (Table 2). These results were confirmed in euglycemic-hyperinsulinemic clamp studies performed in high- and low-phytoestrogen–fed mice: The glucose infusion rate required to maintain normoglycemia was significantly higher in high-phytoestrogen–fed mice (Fig. 5*D*). No significant differences in FFA concentrations during the clamp between high- and low-

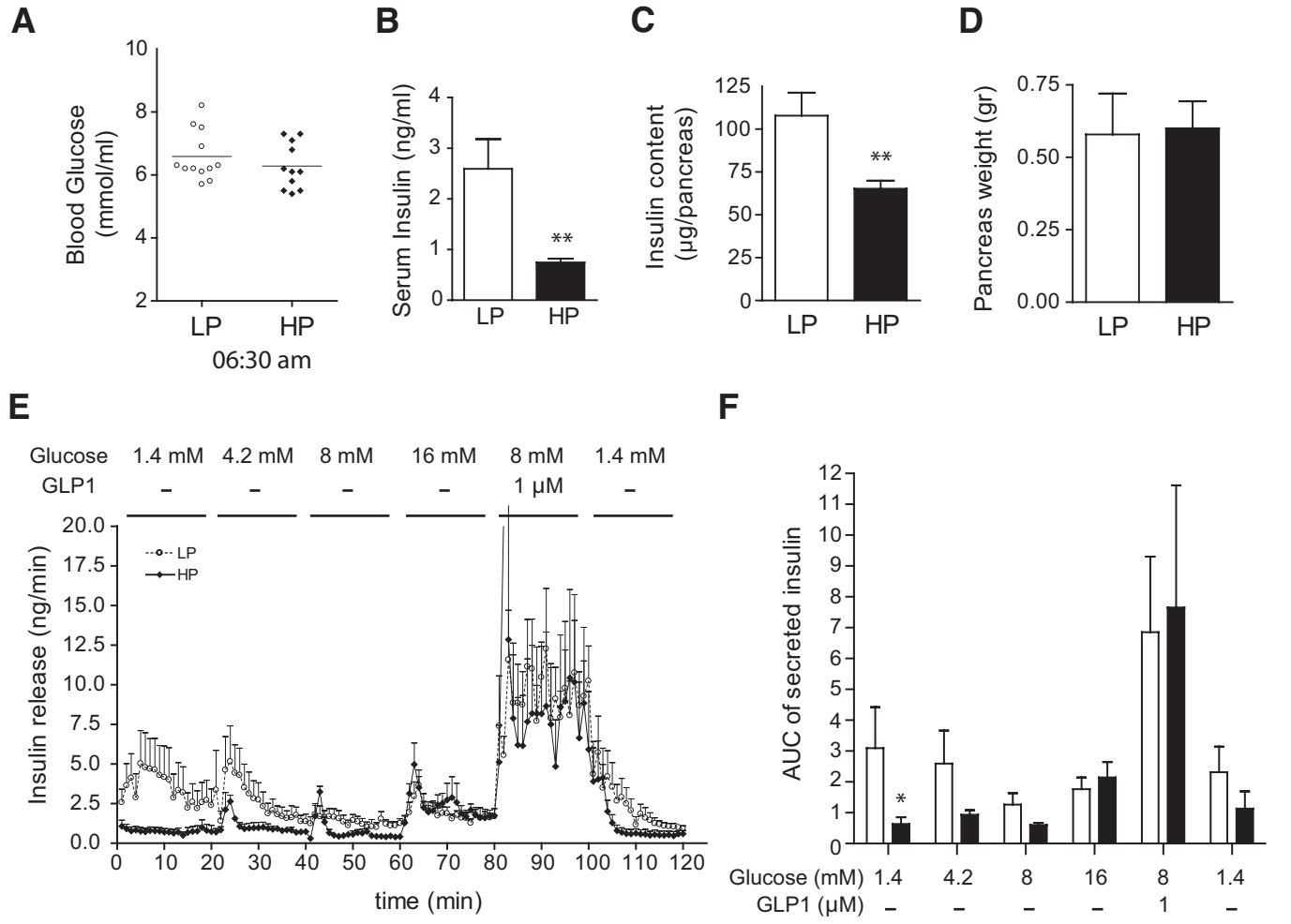

FIG. 4. Reduced pancreatic insulin content and circulating insulin on high-phytoestrogen–fed (HP) mice. Despite similar blood glucose levels (*A*), serum insulin levels (*B*) are reduced in HP mice compared with low-phytoestrogen–fed (LP) mice. Total insulin content is reduced in HP mice (*C*), although pancreas mass is similar (*D*). *E*: Insulin secretion from in situ perfused pancreas was evaluated during six 20-min periods that consecutively tested glucose concentrations from 1.4–16 mmol/l glucose. LP mice (○) displayed a variable insulin output at basal levels, whereas that of HP mice (♦) was consistently very low. Strikingly, HP mice did not increase their insulin output over basal level in response to 4.2–8.0 mmol/l glucose but released insulin as LP mice when stimulated by either 16 mmol/l glucose or GLP-1. After GLP-1 stimulation, return to basal level appeared faster in HP mice. *F*: AUC of insulin release from perfused pancreas in the presence of different glucose and GLP-1 concentrations. ■, HP animals; □, LP animals. Data are means ± SE of the indicated number of animals. *$P < 0.05$, **$P < 0.01$ vs. control.

phytoestrogen–fed animals were observed (data not shown). Tissue-specific glucose uptake measured at the end of euglycemic-hyperinsulinemic clamps (Fig. 5*E* and *F*) indicated that the insulin-stimulated glucose utilization index was significantly higher in high-phytoestrogen–fed mice for all skeletal muscles tested; this index was also higher for the WAT and BAT tissues of high-phytoestrogen–fed animals, although statistical significance was reached only by the inguinal WAT. There was no difference in glucose uptake in brain and heart. Because skeletal muscles account for ~42% of the total body mass in male mice, our data suggest that the increased whole-body insulin sensitivity of high-phytoestrogen–fed mice is, to a large extent, a consequence of increased insulin sensitivity in skeletal muscles.

Improved insulin sensitivity via AMPK-dependent reduction of the mTOR/S6K1 pathway. Glut4-dependent glucose transport across the sarcolema of skeletal muscle is stimulated either by the classical insulin signaling pathway and/or in response to exercise where involvement of AMPK has been demonstrated (36). Recently, both pathways have been proposed to converge at the level of mTOR to modulate insulin sensitivity (37). To gain insight into the mechanism by which glucose uptake is increased in high-phytoestrogen–fed mice, we characterized both the mTOR/S6K1 and the insulin-induced Akt signaling cascades in low- and high-phytoestrogen skeletal muscles in basal and insulin-stimulated conditions (clamp) (Fig. 6*A* and *B*). Interestingly, phosphorylation of IRS1 (Y632) in high-phytoestrogen–fed mice was lower than in low-phytoestrogen–fed mice in basal conditions, probably reflecting the lower serum insulin levels in high-phytoestrogen–fed mice. Akt phosphorylation (T308/S473) was also reduced, albeit not reaching statistical significance. Upon insulin stimulation, phosphorylation of IRS1 (Y632) was similar in low- and high-phytoestrogen–fed mice, but IRS1 (S636/S639), mTOR (S2448), and S6K1 (T389) levels were significantly lower in high-phytoestrogen–fed mice. In contrast, phosphorylation of Akt (T308/S473) was significantly higher in high-phytoestrogen–fed mice in the insulin-stimulated state. Recent evidence indicates that, in response to insulin, S6K1 phosphorylates IRS1 on two inhibitory serine residues (S636/S639), thus preventing further activation of the phosphatidylinositol 3-kinase/Akt signaling pathway (38,39). Moreover, activation of AMPK in vitro leads to the inhibition of the mTOR pathway and reduces IRS1 phosphorylation on S636/S639

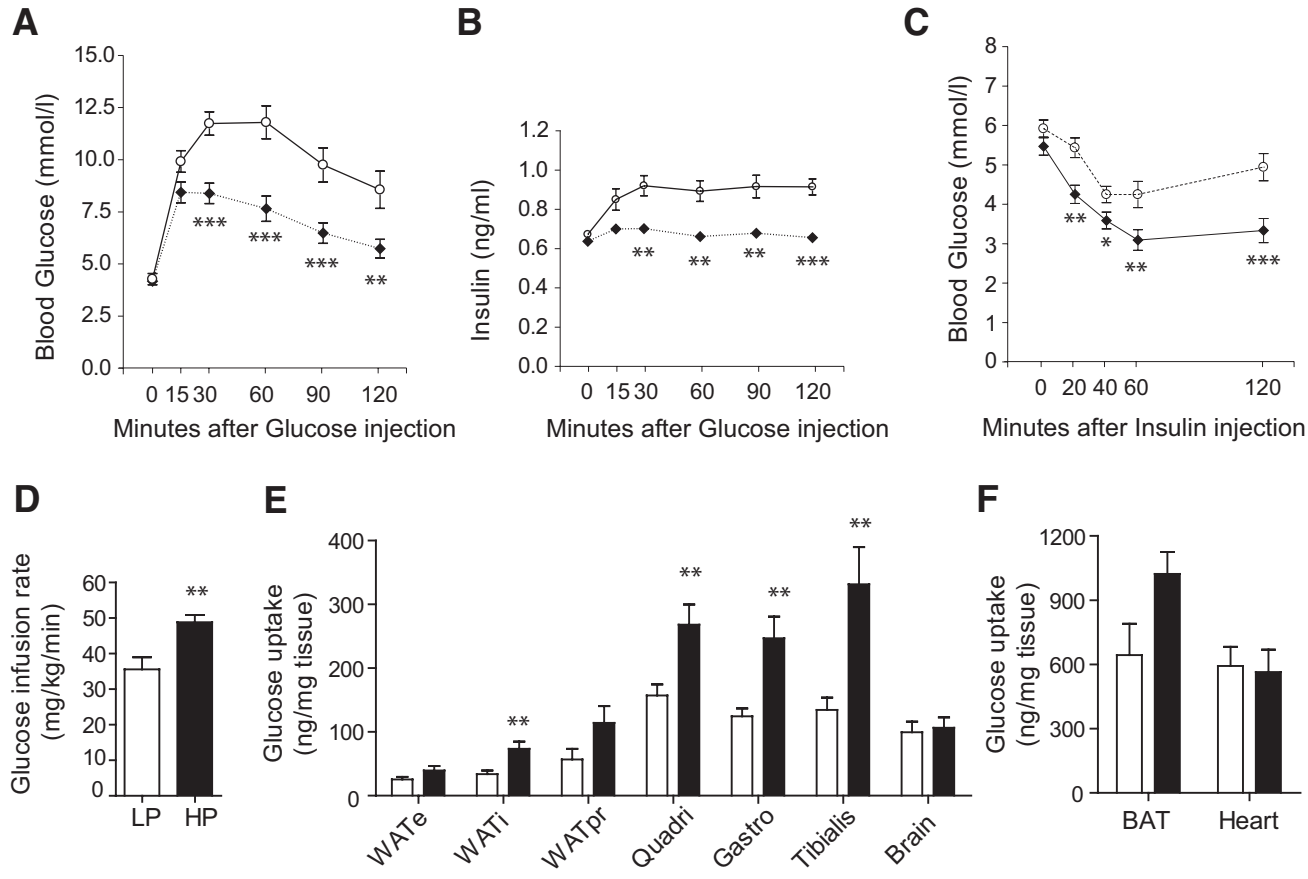

FIG. 5. Improved insulin sensitivity and glucose uptake in high-phytoestrogen–fed (HP) mice. Blood glucose (*A*) and insulin (*B*) levels were measured at the indicated points before and after intraperitoneal glucose administration (1.5 mg/g body wt) in overnight-fasted HP and low-phytoestrogen–fed (LP) animals (n = 14–19/group in *A* and n = 6/group in *B*). *C*: Blood glucose was measured before and after intraperitoneal insulin administration (0.75 unit/kg, n = 14–19/group). ○, LP animals; ♦, HP animals. *D*: Glucose infusion rate in mice fed high- and low-phytoestrogen diets for 6 months measured during euglycemic-hyperinsulinemic clamp studies (n = 9–10 per group). *E* and *F*: Tissue glucose uptake during clamp studies (n = 6–7 per group). Results are means ± SE and are shown as percent of expression in HP mice relative to LP animals (REL) unless specified. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control. Quadri, quadriceps; Gastro, gastrocnemius; WATe, epididymal WAT; WATi, inguinal WAT; WATpr, perirenal WAT.

(37). Thus, the increased AMPK activation in high-phytoestrogen–fed mice (Fig. 3*A* and *B*) may correlate with the significant reduction of mTOR and S6K-1 phosphorylation and with a decreased phosphorylation of the IRS1 inhibitory serines (S636/S639) after insulin stimulation. Overall, our results suggest that dietary soy improves insulin sensitivity in skeletal muscles, which may in part be due to reduced repression of the insulin signaling exercised by AMPK-dependent inhibition of the mTOR/S6K1 axis.

TABLE 2
Insulin resistance indexes of low- and high-phytoestrogen–fed mice

Mice	AUC by GTT	AUC by ITT	Insulin resistance index
Low phytoestrogen	2,916 ± 187	964 ± 53	2,849 ± 297
High phytoestrogen	2,083 ± 136*	799 ± 53†	1,747 ± 210‡

Data are means ± SE. Insulin resistance indexes for animals from each group are shown, calculated as the product of AUC of the GTT and the ITT. *P < 0.001, †P < 0.05, ‡P < 0.01 compared with values from low-phytoestrogen–fed mice.

DISCUSSION

The metabolic syndrome is characterized by obesity, insulin resistance, and a predisposition to hypertension, dyslipidemia, and type 2 diabetes. A common feature linking these metabolic abnormalities is the dysregulation of AMPK, a major sensor of systemic energy status (26). Phosphorylation of AMPK and its downstream target ACC inhibits malonyl CoA production, thus enhancing carnitine palmitoyl-transferase 1 activity and import of fatty acids into mitochondria for oxidation and energy production (40). In the present study, we provide evidence that dietary phytoestrogens reduce body weight and lipid levels and improve insulin action. Most importantly, we show that dietary soy and, by inference, its component phytoestrogens activate AMPK activity in both adipose tissue and skeletal muscle and induce a variety of metabolic effects consistent with AMPK activation. This includes reduced lipid content in adipocytes, increased phosphorylation of AMPK and ACC, increased expression of PGC-1α and genes involved in mitochondrial biogenesis and ROS-detoxifying enzymes, increased insulin sensitivity, and reduction of the mTOR/S6K1 pathway in skeletal muscle. We further document that phytoestrogens also reduce the pancre-

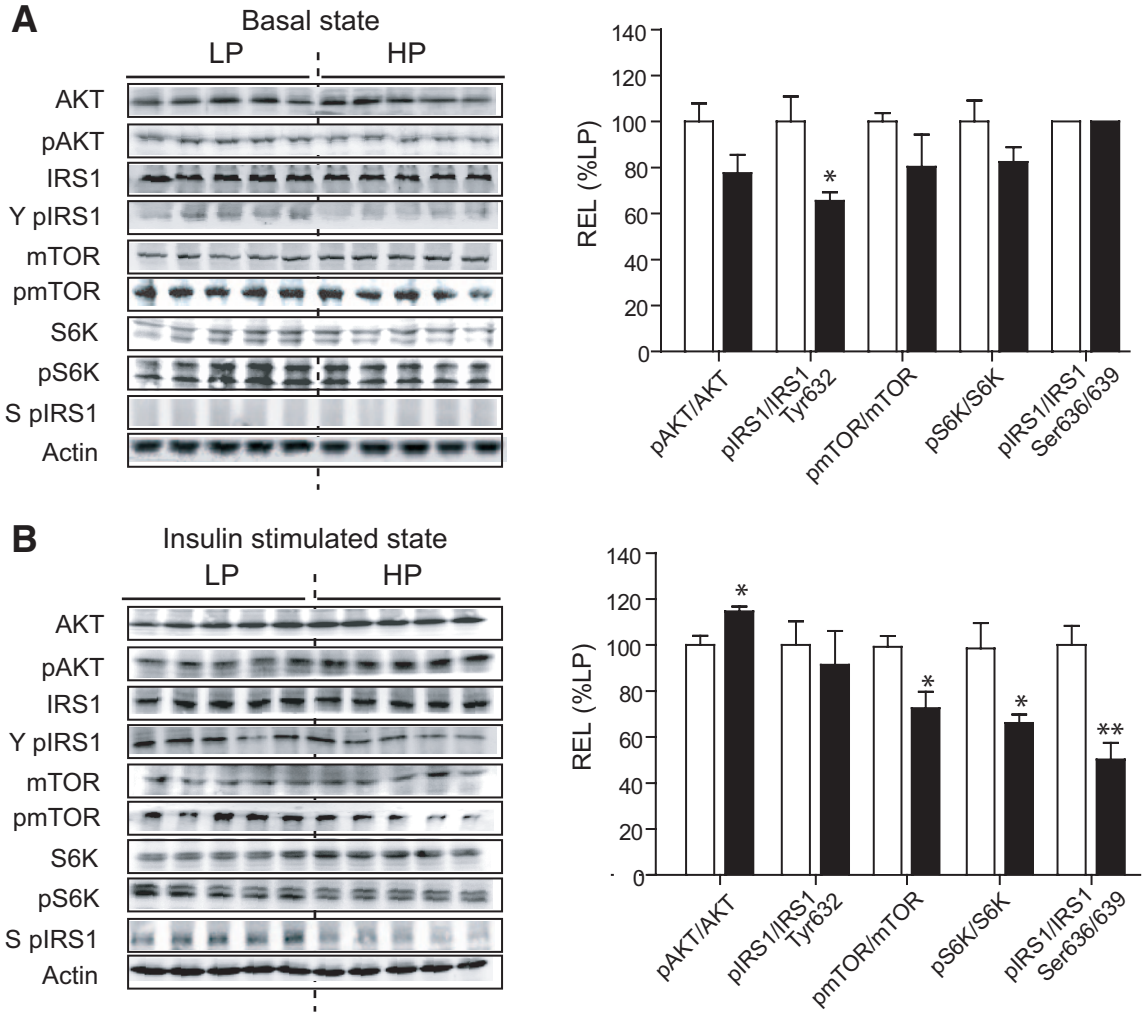

FIG. 6. Improvement of insulin signaling in high-phytoestrogen–fed (HP) mice. Representative Western blot analysis of intracellular insulin effectors in skeletal muscle of HP and low-phytoestrogen–fed (LP) mice under basal (*A*) or insulin-stimulated (*B*) conditions from the clamp experiment. ■, HP animals; □, LP animals. Quantification of phospho-protein–to–protein ratios indicate that phosphorylation of mTOR and its downstream effectors S6K and IRS1 (Ser636/S639) are significantly reduced, whereas Akt phosphorylation is improved under insulin-stimulated conditions in HP mice. Results are means ± SE (n = 5/group) and are shown as percentage of expression in HP mice relative to LP animals (REL) unless specified. *P < 0.05, **P < 0.01 vs. control.

atic storage of insulin and the glucose threshold for stimulation of pancreatic β-cells.

AMPK activation and improvement in various metabolic parameters, such as glucose tolerance and insulin sensitivity observed in high-phytoestrogen–fed animals could either be the result of a direct effect of dietary soy or a secondary consequence due to changes in body weight and leanness. We favor the first hypothesis because in vitro exposure of cultured 3T3-L1 adipocytes to genistein has been reported to significantly activate AMPK and ACC (41). This suggests that phytoestrogens could act directly on adipocytes, a cell type known to express both ERα and ERβ (8,42). It has also been shown that chronic estrogen exposure upregulates AMPK activity in skeletal muscle of ovariectomized mice (43) and that in C2C12 myocytes, estrogens rapidly activate AMPK phosphorylation through nongenomic effects involving membrane-bound ERs (43). Consistent with these studies, we also found that short-term exposure of terminally differentiated C2C12 myotubes and 3T3-L1 adipocytes to genistein at doses <10 μmol/l activate the AMPK, although this was highly dependent on culture conditions and nutrient availability (data not shown).

In an attempt to determine in vivo whether AMPK activation is the cause or an effect of leanness in mice fed with dietary soy, we designed an experiment in which a group of animals was fed the soy-free diet, but feed was restricted to a level that would give them the same body weight as mice fed with the soy-rich diet (Supplemental Fig. 1). Despite the fact that these feed-restricted low-phytoestrogen–fed mice had a similar body weight to high-phytoestrogen–fed mice, fat mass was not reduced as much, indicating that dietary soy affects body composition. Unfortunately, these differences in body composition render comparison between the different groups hazardous and speculative. Nevertheless, feed-restricted low-phytoestrogen–fed mice exhibited GTT and ITT profiles similar to low-phytoestrogen–fed mice, whereas at equal body weight, high-phytoestrogen–fed mice with unrestricted access to food and feed-restricted low-phytoestrogen–fed mice showed significantly different profiles (Supplemental Fig. 1*D* and *E*). In adipose tissues, AMPK activation in feed-restricted low-phytoestrogen–fed mice was at an intermediate level, falling between that of high- and low-phytoestrogen–fed mice. These data, although difficult to interpret, suggest that improvement of insulin

sensitivity and to some extent AMPK activation is not due to changes in body weight.

These beneficial effects of a soy-rich diet are probably related, at least in part, to the increased locomotor activity of high-phytoestrogen–fed mice because exercise induces AMPK phosphorylation in skeletal muscle, adipose tissue, and liver (44,45). Our previous study showed reduced levels in *AgRP* mRNA levels in the hypothalamus of high-phytoestrogen–fed mice consistent with increased metabolic rate and voluntary exercise (14). In addition, *AgRP* mutant mice display an age-related lean phenotype correlated with increased metabolic rate, locomotor activity, and preferential use of lipids (46). A recent publication reported that specific silencing of ERα in the hypothalamus leads to a phenotype resembling the metabolic syndrome (47), demonstrating the importance of the central regulation by estrogens in the control of energy balance. Whether phytoestrogens are able to modulate hypothalamic signals to regulate the energy balance remains to be determined.

Another factor potentially modulating AMPK activation is the adipokine leptin, which was found to selectively stimulate phosphorylation and activation of α2 AMPK in skeletal muscle through an early activation directly on muscle and a later activation through hypothalamic neurons and stimulation of the α-adrenergic pathway (48). However, we found that the levels of leptin were reduced in high-phytoestrogen–fed mice, presumably reflecting smaller adipocytes, thus likely not contributing to the AMPK activation in soy-fed mice. Alternatively, dietary soy and possibly phytoestrogens could bypass the central action of leptin potentially through the melanocortin system and activation of the hypothalamic-sympathetic nervous system axis.

In conclusion, the present in vivo study shows that consumption of a soy-rich diet reduces whole-body adiposity and improves insulin sensitivity, at least in part, by activating AMPK in multiple tissues, including WAT and skeletal muscles. Collectively, these effects induce changes in biochemical processes and gene expression that lead to catabolism of fuel stores and improved glucose uptake. Whether these effects are due exclusively to isoflavones or also associated with soy proteins or other micronutrients remain unclear. The ubiquity of these compounds in human diet emphasizes the importance of further research in this field and warrant further studies for exploiting the preventive or curative potential of soybean compounds for metabolic diseases.

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