

der (16). *In vitro* studies show that, at low doses, genistein efficiently binds both estrogen receptors, although ER β is bound with higher affinity (10). At high doses, genistein was reported to act as a tyrosine kinase inhibitor (17, 18), an antioxidant (19), and a steroid-metabolizing enzyme modulator (20). In addition, at high concentration, genistein may inhibit the action of estrogen receptors by acting through nuclear receptors such as the peroxisome proliferator-activated receptors (PPARs) (21). These data suggest that genistein may activate or inhibit estrogen-dependent pathways depending on the extent of intake. Furthermore, recent studies on adipose tissue in women (22) and female mice (7) indicate that genistein inhibits adipose deposition and decreases adipose mass, and that this activity occurs through regulation of the expression of specific genes.

The aim of this study was to understand the effect of genistein in male mice and its mechanisms of action at low and high doses. Our results indicate that genistein is adipogenic in young immature mice at levels similar to those present in Western and Eastern diets, in soy milk, or in food supplements containing soy. This effect of genistein, not observed in 4-wk-old female mice, may be relevant to the gender-specific regulation of adipose development, deposition, function, and metabolism during growth and adulthood.

Materials and Methods

Chemicals

We purchased 17 β -estradiol (E2) and genistein from Sigma-Aldrich (Pomezia, Italy); β -glucuronidase type HP2S and arilsulfatase type H2 were purchased from Sigma. Authentic standards of genistein were purchased from Extrasynthèse S.A. (Lyon, Nord-Genay, France). All the solvents used were of HPLC-grade purity (BDH Laboratory Supplies, Poole, UK).

Experimental animals

The procedures involving animals and their care were conducted in accord to institutional guidelines, which comply with national and international laws and policies [National Institutes of Health, Guide for the Care and Use of Laboratory Animals, 1996 (7th edition) (Washington, D.C.); National Academy Press, National Research Council Guide, www.nap.edu/readingroom/books/labrats]. Four-week-old mice were purchased from Harlan (Udine, Italy) and kept in animal facilities at a temperature of 23 C, with natural 12-h light/12-h dark cycles. Before treatments, mice were always put for 7 d on a soy-free, estrogen-free diet (Piccioni, Milan, Italy; www.totofood.it/presentazione.htm). The average caloric intake was 8.83 calories per mouse per day without or with genistein up to the dose of 50,000 μ g/kg·d and 8.05 calories per mouse per day in mice fed the 200,000 μ g/kg·d genistein dose.

For acute experiments, wild-type or transgenic ERE-tK-LUC mice (23–25) were treated sc with E2 or by gavage with genistein in 100 μ l of vegetable oil or with 100 μ l of vehicle as control. For the 15-d treatments wild-type mice were counterbalanced for body weight and food intake and put into groups of 25 mice each: group 1, the following oral doses of genistein were administered daily by gavage at: 0, 50, 500, 5000, 50,000, or 200,000 μ g/kg; group 2, subgroup A, dietary genistein, 800 ppm of food pellets; and subgroup E2, E2 (5 μ g/kg·d). Body weight and food intake were measured daily. The animals were killed by cervical dislocation and the tissues were dissected and immediately frozen on dry ice until assayed. For the experiments with β ERKO mice, six mice per group were used. β ERKO mice were kindly provided by P. Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire and Institut Clinique de la Souris, Collège de France, Illkirch, France) (26).

mRNA quantification by real-time RT-PCR

Total RNA from epididymal fat was extracted from 30 mg of tissue using the RNeasy Lipid Tissue Kit (Qiagen, Milan, Italy). RNA for each sample was reversed transcribed using high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using Assay on Demand kits based on TaqMan chemistry (Applied Biosystems). RT-PCR were performed on an ABI PRISM 7000 Sequence Detection System instrument, and data analysis was done with the ABI PRISM 7000 SDS software (Applied Biosystems). 18S RNA was used as the reference housekeeping gene. Specific oligonucleotide pairs were designed by the Applied Biosystems service. Calculations were done as described for the Comparative Method in the User Bulletin 2 of ABI PRISM sequence detection system.

Protein isolation and Western analysis of ER α and ER β in epididymal fat

Tissues were excised and homogenized in lysis buffer [1 M HEPES (pH 7.9), 2 M MgCl₂, 10% (vol/vol) glycerol, 5 M NaCl, 0.5 M EDTA, 0.1% Triton X-100, 14.2 M mercaptoethanol, 100 mM PMSF] supplemented with a protease inhibitor cocktail. Homogenized tissues were centrifuged for 25 min at 13,000 rpm in a cold room. Protein concentration was determined by the Bradford's assay (27). Equal amount of proteins from each treatment group were boiled in 1 \times Laemmli buffer (50 mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 175 mM β -mercaptoethanol). Proteins were separated by SDS-PAGE and electrophoresed to polyvinylidene difluoride membrane. Membranes were blocked in Blotto [5% milk, Tris-buffered saline (10 mM Tris-HCl, pH 8.0; 150 mM NaCl), and 0.05% Tween 20] and probed with primary antibodies ER α (R21) (1:1000) and ER β (CO1531) (antibodies were a gift from G. Greene, The Ben May Institute for Cancer Research, Chicago, IL). After incubation with peroxidase-conjugated secondary antibody, immunoglobulins were visualized using the ECL detection system (Pierce, Milan, Italy). Densitometric quantitation of ER α levels relative to actin levels was performed using the Matrix software program (Quantavision, Montreal, Canada).

Adipocyte size and number

Fresh epididymal adipose tissue was fixed in formaldehyde, embedded in paraffin, and sliced into 3- μ m sections. Sections were stained with hematoxylin, counterstained with eosin, then coverslipped with DPX mountant for microscopy (BDH Laboratory Supplies). Images acquisition was obtained by a digital camera (Nikon Digital Camera DMX 1200; Nikon, Melville, NY). Adipocyte area and diameter were calculated at \times 20 magnification with a dedicated software (Image Pro Plus; Imaging and Computer, Milan, Italy). In all samples, at least four different fields of three different tissue sections have been evaluated. Adipocyte number was calculated by counting the nuclei in a fixed field area. Adipocyte volume was calculated following the formula: $4/3r^3\pi$.

Glucose and insulin tolerance tests

For glucose tolerance tests, animals were fasted overnight for 12 h and blood samples were obtained from the tail vein. Animals were then injected ip with 2 g/kg body weight of glucose, and blood samples were taken at the indicated intervals.

For insulin tolerance tests, fed animals were used. Animals were injected ip with 0.75 IU/kg body weight of soluble insulin (Humulina Regular; Lilly, Madrid, Spain).

Blood glucose was measured in each sample using an Accu-check compact glucometer from Roche Diagnostic GmbH (Mannheim, Germany).

Genistein assay

Sample preparation. Sample preparation for plasma isoflavones analysis was performed according to the methods described by Manach *et al.* (28), appropriately modified. To limit the isoflavone losses, a simple treatment procedure was used: 900 μ l plasma were acidified with 100 μ l of 0.58 M acetic acid solution. The acidified plasma samples were mixed with the enzymes, 5 μ l β -glucuronidase, and 80 μ l arilsulfatase, and treated for 30 min at 37 C. For the isoflavones extraction, the samples

were mixed with 2.75 ml of acetone, shaken for 1 h, and subsequently centrifuged at $2400 \times g$ for 45 min at 4°C. The supernatant of each sample was dried by Speed Vacuum at room temperature. The organic phase was then redissolved in 300 μ l methanol and 200 μ l water. Before being injected in the HPLC system, the samples were centrifuged by microfuge for 2 min, and 50 μ l of clear samples were tested by HPLC.

HPLC-diode array detection (DAD) analysis. The analyses were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector (Agilent Technologies, Palo Alto, CA), and a dual pump 515 model liquid chromatographic system, supplied with a diode array system Model 996 (Waters Corp., Milford, MA). Isoflavonoids were separated using a 150×3.9 mm (4 μ m) Nova Pak C18 column (Waters Corp.) operating at 30°C. The mobile phase was a four-step linear solvent gradient system, starting from 95% H₂O (adjusted to pH 3.2 by H₃PO₄) up to 100% CH₃CN during a 27-min period (29); the flow rate was 0.8 ml min⁻¹.

HPLC-mass spectrometry (MS) analysis. HPLC-MS analyses were performed using a HP 1100 MSD API, ESI interface, coupled with a HP 1100L liquid chromatography equipped with a DAD detector (Agilent Technologies). The HPLC-MS analysis was performed using the same HPLC-DAD condition with water adjusted to pH 3.2 by HCOOH. Mass spectrometer operating conditions were: nitrogen gas temperature 350°C at a flow rate of 12 L min⁻¹, nebulizer pressure 30 ψ , quadrupole temperature 30°C, and capillary voltage 3500 V. The mass spectrometer operated in positive and negative mode at 80–180 eV fragmentor values.

Identification of genistein was carried out using its retention time and both spectroscopic and spectrometric data. Genistein was quantified by a five-point regression curve (r^2 , 0.9994) operating in the range of 0–12 ng on the basis of authentic standard, and determination was directly performed by HPLC-DAD.

Plasma lipids

Total plasma cholesterol and triglycerides were measured using colorimetric methods with commercially available kits (Roche Molecular Biochemicals).

Luciferase enzymatic assay

Tissue extracts were prepared by homogenization in 500 μ l of 100 mM K₂PO₄ lysis buffer (pH 7.8) containing 1 mM dithiothreitol, 4 mM EGTA, 4 mM EDTA, and 0.7 mM phenylmethylsulfonylfluoride, with three cycles of freezing-thawing and 30 min of microfuge centrifugation at maximum speed. Supernatants containing luciferase were collected, and protein concentration was determined by Bradford's assay (27).

Luciferase enzymatic activity was measured, as reported by de Vet *et al.* (30), in tissue or cell extracts at protein concentration of 1 mg/ml. The intensity of the light was measured with a luminometer (Digene Diagnostics, Gaithersburg, MD) over 10 sec and expressed as relative light units per milligram of proteins.

Total RNA extraction and Genechip hybridization

Total RNA was obtained from 100–300 mg of tissue using the RNeasy Lipid Tissue kit (Qiagen) and following the manufacturer's instruction. The quality of all tissue samples was controlled strictly to verify the RNA integrity before use in microarray experiments. RNA quantity was evaluated spectrophotometrically, and the quality was assessed with the Agilent 2100 bioanalyzer (Agilent Technologies). Only samples with good RNA yield and no RNA degradation (28S:18S >1.5 and RNA integrity >8.5) were retained for further experiments. Information about RNA sample integrity from a bioanalyzer electrophoretic trace was obtained by the use of a new software algorithm that has been developed (www.agilent.com/chem/labonachip). Labeling of samples and hybridization to the Affymetrix (Santa Clara, CA) Gene Chip Mouse Expression Arrays 430A containing about 22,000 probe sets, were performed following the manufacturer's protocols, as described (31).

Bioinformatics

Background correction, normalization, and gene expression values were computed using the Affymetrix MAS5 algorithm (32). A second normalization step was conducted using the loess method (33). To select

genes of more interest, a nonspecific filtering was first applied. Genes were retained if at least 75% of samples in the overexpressed group were called present according to the Affymetrix presence call algorithm (32). Differential expression among experimental groups were evaluated by means of ANOVA models fitted at single gene level (34). Correction for multiple comparisons (four samples for each treatment) was conducted on the F statistics *P* values controlling for the false discovery rate using the q-value algorithm. Genes with a q-value smaller than 20% were retained. Comparisons of interest (genistein 5,000 *vs.* control; genistein 200,000 *vs.* control; genistein 5,000 *vs.* genistein 200,000; E2 *vs.* control; E2 *vs.* genistein 5,000; and E2 *vs.* genistein 200,000) were evaluated at the single gene level and a *P* value lesser than 5% was considered as significant. A hierarchical clustering using Pearson correlation as distance matrix were performed to graphically show the results of the analysis (not shown in this article).

All the analyses were performed using the R and Bioconductor software and the limma package [Refs. 34 and 35 and the 2006 R Development Core Team: A Language and Environment for Statistical Computing; Foundation for Statistical Computing, Vienna, Austria (<http://www.R-project.org>)]. The average fold change of the genes reported in Fig. 5 was 2 or more.

Statistical analysis

Data are expressed as mean \pm SE. Pair-wise comparisons were made using Student's *t* test. A probability level of *P* < 0.05 was considered statistically significant.

Results

Adipose tissue deposition in male mice chronically exposed to genistein

In 4-wk-old male mice treated orally with genistein or E2, epididymal and renal fat pad weights were significantly higher than controls at genistein doses less than 50,000 μ g/kg·d (Fig. 1, A and B); in addition, they were significantly lower in mice given the 200,000 μ g/kg·d dose. Fat pad changes were associated with a significantly lower level of triglycerides and cholesterol in plasma in the groups treated with estrogen or with 5,000 μ g/kg genistein (Table 1). The effects of genistein were always highest at the concentration of 5,000 μ g/kg·d, followed by a decrease at higher doses. Genistein increased renal fat weight at a dose of 50 μ g/kg·d.

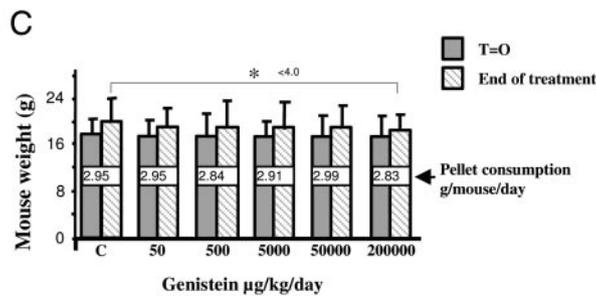
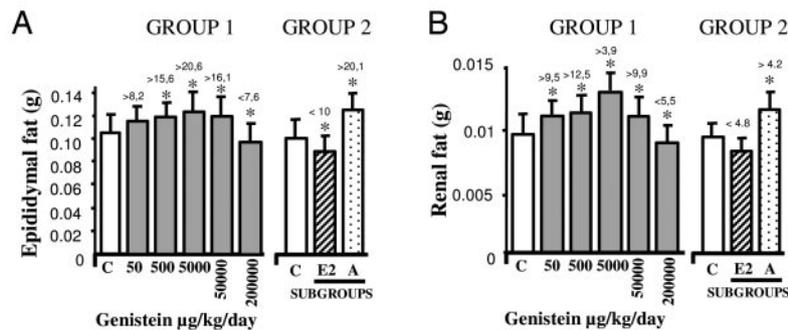
The levels of serum genistein of both groups at 12 h after administration are reported in Table 2. These results indicate that circulating genistein concentrations must reach levels higher than 35 nM and up to 100 nM, to elicit adipogenic action, but must reach levels higher than 100 nM for antiadipogenic action.

To assess whether the observed effects were gender specific, 4-wk-old healthy females were put on the same SFDS diet and treated with the same doses of genistein. Most interestingly, in these animals, genistein consumption did not change fat deposition, with the exception of the highest dose, which induced a significant decrease (Fig. 1, D and E). Body weights changed significantly in both sexes only at the dose of 200,000 μ g/kg·d (4.0% decrease in males and 4.3% in females). In these groups, food intake decreased by 4.7% in males and by 5.2% in females (Fig. 1, C and F). Thus, fat increase did not reflect a general increase in body weight or in food consumption.

Adipocytes size and number

To better understand the effects of genistein in male mice, our study continued with a more in-depth analysis of fat

MALES



FEMALES

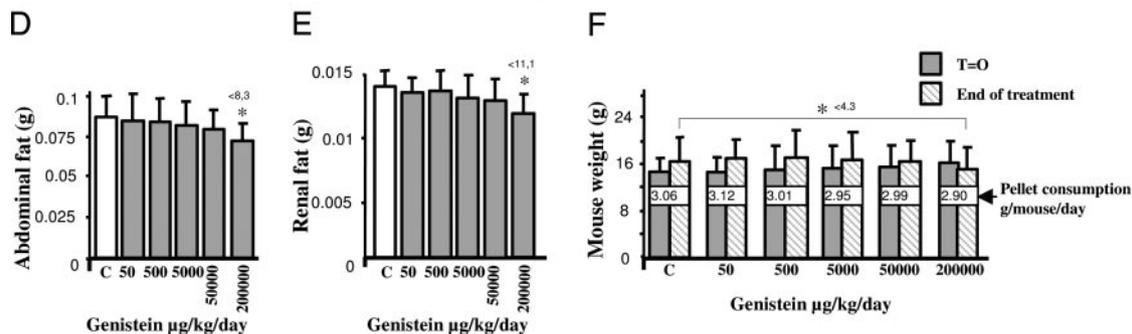


FIG. 1. Effect of genistein on epididymal and renal fat weight. A and B, Four-week-old male mice were put for 1 wk on a soy-free standard diet. Group 1 was divided into six subgroups of 20 males each, which received genistein by oral gavage at doses of 0, 50, 500, 5,000, 50,000, or 200,000 $\mu\text{g}/\text{kg}/\text{d}$, respectively. Epididymal and renal fat pad weights were measured (y-axis; grams). Group 2, subgroup A (20 mice) received dietary genistein (800 ppm) mixed in the soy-free diet. Subgroup E2 received 5 $\mu\text{g}/\text{kg}/\text{d}$ of sc administered E2. Control group = vegetable oil. Treatments were given once a day for 15 d. C, Body weights (grams) and food intake (grams per mouse per day) of mice in group 1. Measurements were made daily at 1700 h. Bars indicate the average weight for each group of mice at the beginning (T=0) and before animals were killed (end of treatment). D and E, Twenty-four-week-old female mice were treated in the same way as the male mice with increasing doses of genistein (0–200,000 $\mu\text{g}/\text{kg}/\text{d}$). Abdominal and renal fat pads were measured. F, The body weights and food intake of female mice are shown. Data are mean \pm SE. *, $P < 0.05$ vs. controls. Numbers above bars in A, B, D, and E indicate the average weight change (percent) compared with controls.

tissue. Stereological examination of the adipocytes revealed that the increase in fat weight primarily resulted from an increase in adipocyte volume. It is worth noting that adipocyte size was increased only in animals treated with doses of genistein less than 200,000 $\mu\text{g}/\text{kg}$. On the contrary, in this high-dose group, adipocyte size became very heterogeneous (size range, 504–1148 $\mu\text{m}^2/\text{cell}$). E2 treatment also caused very heterogeneous changes in cell volume (size range, 626–1754 $\mu\text{m}^2/\text{cell}$) (Fig. 2, A and B).

We hypothesized that the aforementioned effects on adipose tissue deposition and adipocyte size might regulate peripheral insulin resistance. Therefore, we performed an insulin tolerance test in animals treated during 15 d with vehicle, E2 5 $\mu\text{g}/\text{kg}/\text{d}$ or genistein 5,000 $\mu\text{g}/\text{kg}/\text{d}$ or 200,000

$\mu\text{g}/\text{kg}/\text{d}$ (Fig. 2C). In Fig. 2C, an improvement of insulin sensitivity is manifested in E2-treated mice compared with vehicle-treated animals after 60 min of insulin injection. This situation is similar to that induced by pharmacological doses of genistein (200,000 $\mu\text{g}/\text{kg}/\text{d}$) (compare open circles with open squares). Interestingly, a significant difference in insulin tolerance was manifested in a comparison of the effects of low and high doses of genistein. Treatment with the lower dose of genistein (5,000 $\mu\text{g}/\text{kg}/\text{d}$) produced a mild insulin resistance compared with the result with the high genistein dose (200,000 $\mu\text{g}/\text{kg}/\text{d}$). This insulin-resistance state was also manifested in a comparison of genistein-treated (5,000 $\mu\text{g}/\text{kg}/\text{d}$) and E2-treated (5 $\mu\text{g}/\text{kg}/\text{d}$) animals (compare open circles with filled squares). When glucose tolerance tests

TABLE 1. Effect of genistein and E2 on plasma lipids

Group	n	Plasma triglycerides and cholesterol	
		Triglycerides (mg/dl)	Cholesterol (mg/dl)
Control	5	130 ± 22	90 ± 8
Genistein (5,000 μg/kg·d)	5	66 ± 9 ^a	78 ± 7 ^a
Genistein (200,000 μg/kg·d)	5	111 ± 19	84 ± 8
E2 (5 μg/kg·d)	5	77 ± 13 ^a	73 ± 8 ^a

Mice were treated for 15 d with 5,000 or 200,000 μg/kg genistein per day or 5 μg/kg E2 on the estrogen-free soy-free diet. Values are reported as means ± SE.

^a $P < 0.05$, significant difference from compound-treated *vs.* vehicle-treated mice.

were performed, no significant differences were manifested among the different conditions (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>). This finding indicates that insulin release from β-cells was adapted to compensate for the mild insulin intolerance generated by low doses of genistein.

Acute estrogenicity of genistein in the adipose tissue

To assess whether genistein was acting through estrogen receptor pathways, we evaluated the response of the estrogen-dependent reporter luciferase in ERE-tk-Luciferase mice (Fig. 3, A and B). Genistein could not modulate the estrogen-responsive element (ERE)-dependent promoter in fat, contrary to its effects in the lung (Fig. 3C) and in other tissues such as the liver (Penza, M., C. Montani, A. Romani, P. Vignolini, P. Ciana, A. Maggi, B. Pampaloni, L. Caimi, and D. DiLorenzo, manuscript in preparation; and Ref. 25). E2, at a concentration of 5 μg/kg, repressed luciferase expression as much as 50% at 6 h; thus, the effect of genistein did not overlap with that of E2 on target promoters in this tissue. To gain further insights into the mechanism of action of genistein, we studied the regulation of ERα and ERβ. ERα and ERβ proteins were strongly down-regulated at doses of 50 and 500 μg/kg·d (Fig. 3D). A weaker modulation was observed at doses higher than 5,000 μg/kg. For the time-course analysis of acute treatments reported in Fig. 3, D and E, we used the dose of 500 μg/kg because this is the dose at which we begin to see a significant effect on the growth of the epididymal fat (Fig. 1A). ERα protein down-regulation (Fig. 3D) was similar to that observed with E2, whereas ERβ was less affected by the steroid. ERα and ERβ protein down-regulation was paralleled by a decrease in receptor mRNA levels (Fig. 3E); in this case, genistein action was more dramatic and persistent with ERβ.

We also evaluated the regulation of the endogenous progesterone receptor (PR) gene by real-time RT-PCR. PR

TABLE 2. Levels of genistein in serum in mice

Treatment	Group 1 (μg/kg)						Group 2
	0	50	500	5,000	50,000	200,000	Subgroup A
Genistein	28 ± 4	35 ± 6	66 ± 7	74 ± 7	98 ± 11	223 ± 43	90 ± 8 (males) 369 ± 133 (females) ^a

Data for genistein in serum are given as nm mean ± SE of three measurements. Genistein was assayed 12 h after the last gavage in group 1 and 12 h after transfer of mice on a soy-free diet in group 2, subgroup A.

^a Genistein levels found in female mice fed the same dietary regimen of males in subgroup A.

mRNA levels were strongly decreased by both genistein and E2 (Fig. 3E).

Modulation of lipogenic and adipocyte differentiation factors in epididymal fat

Next we evaluated the ability of genistein (15-d treatments) to modulate adipocyte differentiation factors [PPARγ, liver X receptor α (LXRα), retinoid X receptor α (RXRα), sterol regulatory element-binding protein 1 (SREBP1)], the adipogenic enzymes lipoprotein lipase (LPL) and leptin, and factors important for fatty acid use, uncoupling protein 1 (UCP1) and long-chain acyl-CoA dehydrogenase (LCAD) (Fig. 4A).

At the adipogenic dose of 5,000 μg/kg·d, genistein augmented levels of PPARγ, LXRα, RXRα, and UCP1 mRNA. RXRα mRNA was significantly increased at a genistein concentration as low as 500 μg/kg·d. A significant induction was also observed when 800 ppm genistein was given with the pellets (subgroup A) (Fig. 4B). The 200,000 μg/kg dose showed no effect. E2 treatment significantly decreased LPL, leptin, PPARγ, and LXRα. Modulations of these factors by genistein and E2 directly correlated with changes in epididymal fat weight. UCP1, a β-oxidation factor not directly correlated with adipose mass, was induced by both of these hormones.

When ER mRNAs were analyzed in these chronically treated mice, ERβ was strongly down-regulated in all treatment groups (from 82% in group 1 to 76% and 33% in subgroups A and E2 of group 2, respectively). In contrast to the results of the acute treatments, ERα was slightly but significantly down-regulated only by E2 (Fig. 4, C and D).

Genome expression profiles in the epididymal fat of genistein-treated mice

Because of the lack of regulation of the ERE consensus promoter at all genistein doses and of LPL, leptin, PPARγ, LXRα, and RXRα genes at the highest dose of genistein, we hypothesized that genistein may regulate a completely different set of adipose-associated genes depending on the dose. Thus, we searched the genome for genes differentially regulated by genistein, to gain further insights into the molecular basis of its adipogenic action at nutritional doses *vs.* its opposite effect at pharmacological doses (Fig. 5A and supplemental Table 1A). Microarray DNA analysis revealed changes in 42 adipose-associated genes known to play a role in fat metabolism (Fig. 5B and supplemental Table 1B). Among the genes differentially expressed under the two treatments, we selected the two genes that showed opposing regulation: phospholipase A2 group 7 (PLA2g7) and phos-

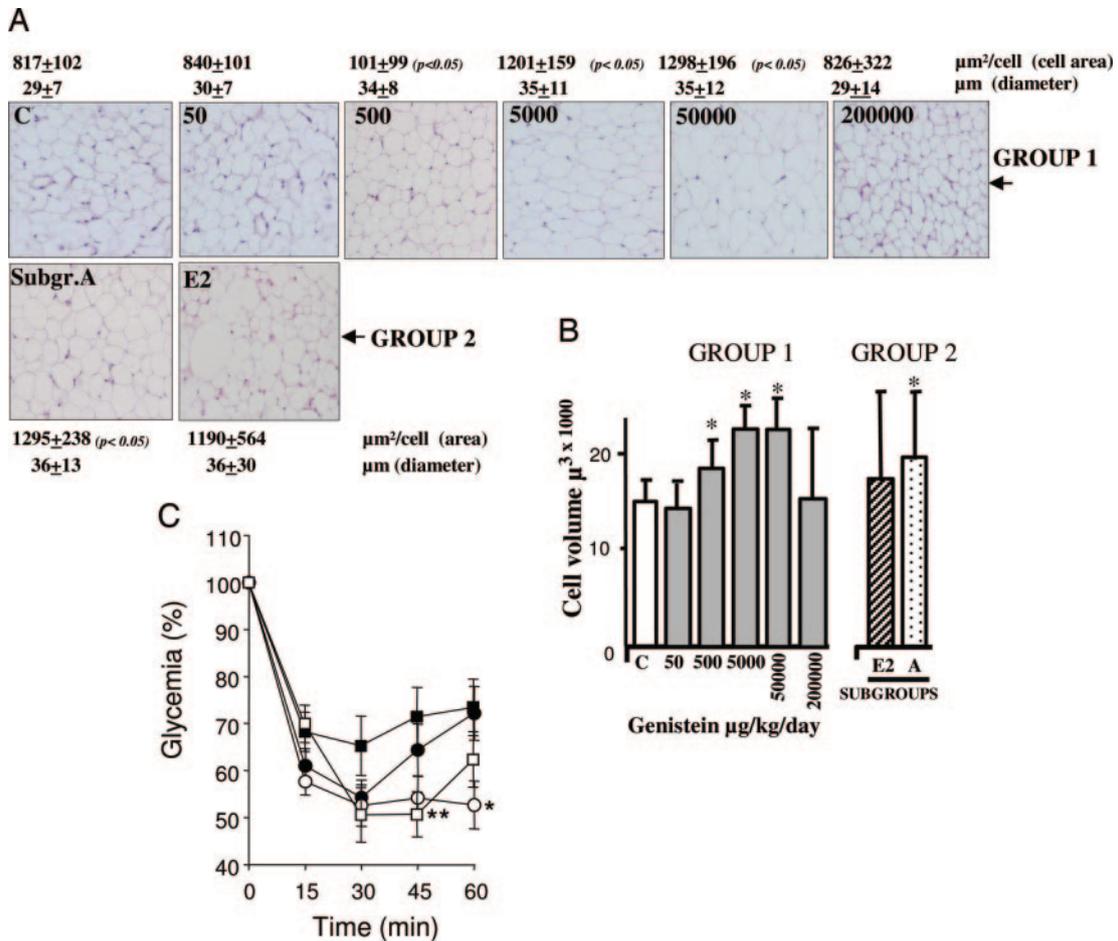


FIG. 2. Histological examination of adipocyte size and insulin tolerance test. Epididymal adipose tissue was fixed in formaldehyde and paraffin embedded. Sections (3 µm) were stained with hematoxylin and eosin. A, Cells were photographed at ×20 magnification with a digital camera (Nikon Digital Camera DMX 1200). B, Adipocyte volume was measured using a dedicated software (Image Pro Plus; Imaging and Computer). In all samples, at least four different fields of three different tissue sections were evaluated for each sample. Data represent the average result of the analysis of the epididymal fat of five different mice. Data shown as mean ± SE. C, Insulin tolerance test. Awake, fed mice treated with: vehicle (●), E2 (○), 200,000 µg/kg·d genistein (□), or 5,000 µg/kg·d genistein (■), were injected ip with 0.75 IU of soluble insulin. n = 10; * P < 0.05; **, P < 0.025.

pholipid transfer protein (PLT_p). These changes were confirmed by real-time RT-PCR and show up-regulation with a genistein dose of 5,000 µg/kg and down-regulation with a dose of 200,000 µg/kg (Fig. 6).

We then compared the expression profiles obtained with genistein and E2 treatments on the whole genome and on a subset of genes of fat metabolism (Fig. 5, A and B, and supplemental Table 1, C and D). Comparison revealed that genistein at both doses regulates a very different set of genes compared with E2, although slightly more overlap is evident between the antiadipogenic genistein dose and E2.

ERβ is required for the response of the epididymal and renal fat tissues to genistein

Because all treatments of genistein down-regulated ERβ much more strongly than ERα, we then assessed the ER-subtype specificity of genistein in modulating epididymal and renal fat by using the two newly isolated genes PLA2g7 and PLT_p. In βERKO mice, the genistein dose of 5,000 µg/kg showed a nonsignificant trend to increasing the weight of the adipose tissue; this increase was significant in wild-type mice.

Most important, the 200,000 µg/kg dose did not cause a significant decrease in the fat pads weights and was not able to down-regulate the PLA2g7 and PLT_p genes in contrast to what was observed in wild-type mice (Fig. 7, A, B, and D), indicating a role of ERβ especially evident at the pharmacological dose of genistein. Genistein did not caused a significant decrease in total body weight or food consumption in βERKO mice (Fig. 7C); a decrease was observed in wild-type mice at the 200,000 µg/kg·d genistein.

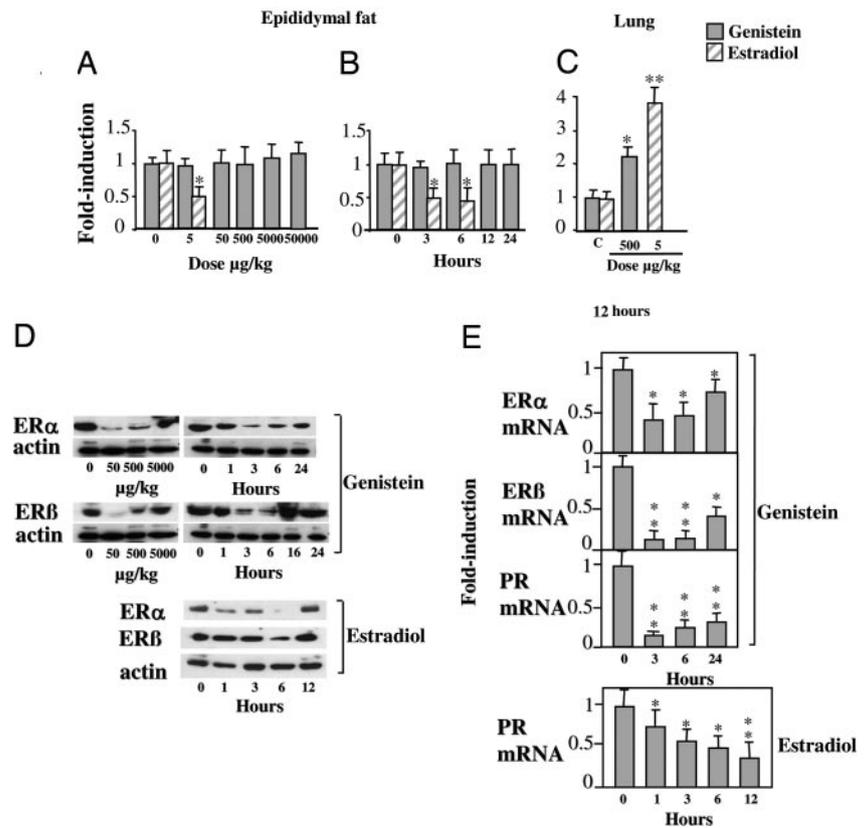
Interestingly ERβ ablation partially increased the basal expression of leptin, LPL, and PLA2g7 (2-, 1.5-, and 1.5-fold) inhibited the effect of E2 on epididymal fat mass growth and diminished the E2-induced regulation of the lipogenic factors leptin, PLA2g7, and LXRA by 50, 40, and 20%, respectively. These results demonstrate a role of ERβ for the regulation of fat metabolism by estrogens.

Discussion

These results show that genistein has important effects on the growth of adipose tissue in young mice, which is dependent on the dose of the compound and gender. Nutri-

ERE-tK-LUC mice

FIG. 3. Estrogenic effects of genistein in epididymal fat. **A** and **B**, ERE regulation in epididymal adipose tissue. Effect of genistein and E2 on reporter regulation in adipose tissue of transgenic mice. Male mice, reporters of estrogen action (ERE-tK-Luciferase), were put on the estrogen-free diet for 1 wk and treated as follows: genistein by oral gavage; dose-response analysis (0, 5, 50, 500, 5,000, or 50,000 $\mu\text{g}/\text{kg}$) at 12 h (■) and 5 $\mu\text{g}/\text{kg}$ E2 for 6 h (▨); and time-course analysis (0, 3, 6, 12, or 24 h) with 500 $\mu\text{g}/\text{kg}$ of genistein by oral gavage (■) and with 5 $\mu\text{g}/\text{kg}$ E2 (0, 3, 6 h) (▨). Mice (six mice per point) were then killed and tissues were collected and stored at -80°C until assayed. **C**, ERE regulation in the lungs of genistein- and E2-treated mice at 12 h. Luciferase activity, measured in tissue extracts (light units), was normalized for protein concentration. Values are expressed as fold induction. Bars represent the average \pm SE. *, $P < 0.05$; **, $P < 0.001$, compared with the relative controls. **D**, Dose-response (0–5,000 $\mu\text{g}/\text{kg}$) and time-course (0–24 h) regulation of ER α and ER β proteins in epididymal fat of mice treated with genistein or E2 as indicated. Only specific bands are shown. **E**, Real-time RT-PCR of ER α and ER β mRNAs and the target PR gene mRNA in epididymal fat of mice treated with genistein or E2 as indicated. Each value is the mean \pm SE of the results from tissues of three different mice. *, $P < 0.05$; **, $P < 0.001$ vs. controls.



tional doses of genistein ($<50,000 \mu\text{g}/\text{kg}\cdot\text{d}$) induce adipose tissue deposition in 4-wk-old male mice, but not in healthy females of the same age. These doses correspond to typical daily intakes of genistein present in soy-containing diets (36). A significant decrease in fat pads was detected only in mice fed the pharmacological dose of $200,000 \mu\text{g}/\text{kg}$, a decrease similar to that produced by $5 \mu\text{g}/\text{kg}$ E2. The increase in adipose tissue deposition at nutritional doses of genistein correlated with mild peripheral insulin resistance, especially when compared with animals treated with E2 ($5 \mu\text{g}/\text{kg}\cdot\text{d}$) or pharmacological doses of genistein. Although this finding suggests that the low dose of genistein may not be beneficial against peripheral insulin resistance, the effect appears to be compensated by insulin release from β -cells.

Body weight changes in mice given dietary genistein, were previously reported in obese or ovariectomized mice (7, 37). Our results show that genistein does not significantly affect food consumption and body weight in young, healthy, male mice or females, except when they are fed the dose of $200,000 \mu\text{g}/\text{kg}\cdot\text{d}$. Genistein did not change skeletal muscle or developing testes weight at any concentrations; however, an effect was observed in the testicles with E2 exposure (8.3% decrease) (data not shown), indicating that the adipogenic action of genistein does not reflect a general increase in organ weight. In addition, in βERKO mice, genistein did not significantly affect total body weight and food intake although treated animals ate about 25% more and weighed slightly more than wild-type mice (6, 38).

Adipogenic actions were produced by serum levels of

genistein in the nanomolar range ($<100 \text{ nM}$), which is below the K_i for tyrosine kinase inhibition. In studying adiposity in mice, it has to be considered that a concentration of 200 ppm of genistein is generally present in normal soy-containing rodent diets, giving a concentration of genistein in serum of $70 \pm 9 \text{ nM}$, which falls between the concentrations found in our mice fed the 500 and the $5,000 \mu\text{g}/\text{kg}\cdot\text{d}$ dose. At these doses, genistein caused a significant increase in the epididymal and renal fat pads.

Higher serum levels were found in our female mice and in other studies conducted with females (mice, rats and humans) in which genistein inhibited adipose deposition (7, 39, 40). The levels found in our male mice 12 h after the treatments might result from the fact that the pharmacokinetics of genistein are faster in males compared with females (41). This, together with the observation that the $5,000 \mu\text{g}/\text{kg}\cdot\text{d}$ dose affects neither the abdominal nor renal adipose mass of intact females, suggests gender-specific metabolism of the phytoestrogen that further contributes to the different results observed in the two sexes.

To understand the molecular basis of the adipogenic action of genistein, we have studied different nuclear adipogenic factors that have coordinated actions on fat growth and development (42). PPAR γ is a major factor involved in *de novo* fatty acid synthesis, adipocyte differentiation, lipid accumulation, and adipocyte survival/maintenance (43, 44). Genistein influenced PPAR γ transcripts in our mice, and these increases positively correlated with adipose mass changes. RXR α is a hetero-dimeric partner of PPAR γ and

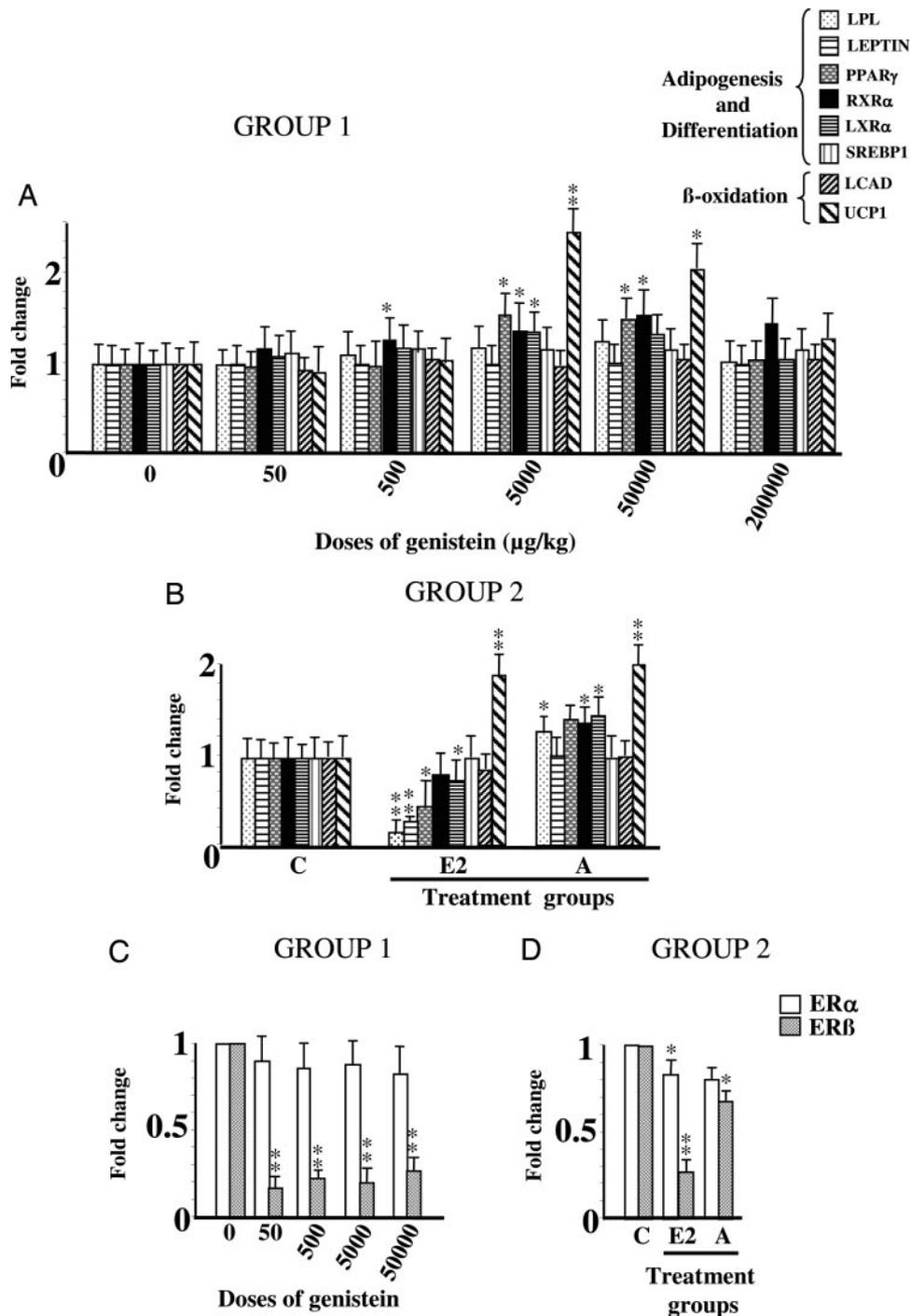


FIG. 4. Effect of genistein on markers of lipogenesis and adipose differentiation. Epididymal fat tissue of mice treated with genistein (groups 1 and 2) was evaluated for changes in expression of adipogenic and adipocyte differentiating factors. Total RNA was extracted and reverse transcribed into cDNA. ER α , ER β , LPL, leptin, PPAR γ , RXR α , LXR α , SREBP1, LCAD, and UCP1 mRNA levels were measured by real-time RT-PCR on six mice per treatment. A and B, LPL, leptin, PPAR γ , RXR α , LXR α , SREBP1, LCAD, and UCP1 mRNA levels in group 1 (0, 50, 500, 5,000, and 50,000 $\mu\text{g}/\text{kg}$) and group 2. Expression is shown relative to the 18S gene. C and D, ER α and ER β mRNA levels in group 1 (0, 50, 500, 5,000, and 50,000 $\mu\text{g}/\text{kg}\cdot\text{d}$) and group 2, subgroup A: dietary genistein added to estrogen-free pellets (800 ppm); subgroup E2: E2 (5 $\mu\text{g}/\text{kg}$). C, controls. Data shown as mean \pm SE. *, $P < 0.05$; **, $P < 0.001$ vs. controls.

plays key roles in adipocyte differentiation, hypertrophy, survival, and lipolysis, and regulates key genes for glucose homeostasis, as shown in mice in which RXR α and PPAR γ are ablated selectively (45, 46). Although RXR α can heterodimerize with many nuclear receptors, the PPAR γ /RXR α -heterodimer may represent an important factor for adipose function because impairments of its signaling, lead to adipose tissue disorders (45).

LXR α is another member of the nuclear receptor superfamily whose expression was shown to be important in lipid metabolism and adipocyte differentiation (47). Like PPAR γ ,

LXR α is recognized as an estrogen-inhibited factor in fat. Heterodimers between PPAR γ and LXR α , although in competition for their common heterodimeric partner RXR (48), target promoters of genes negatively regulated by estrogens (49). All these factors and other factors known to play roles in fat metabolism are positively modulated by adipogenic doses of genistein.

LPL, which is reported to be inhibited by genistein in females after adipose mass decrease (7), and leptin (50) were not inhibited in our male mice exposed to the high genistein dose, although they were strongly inhibited by E2. These

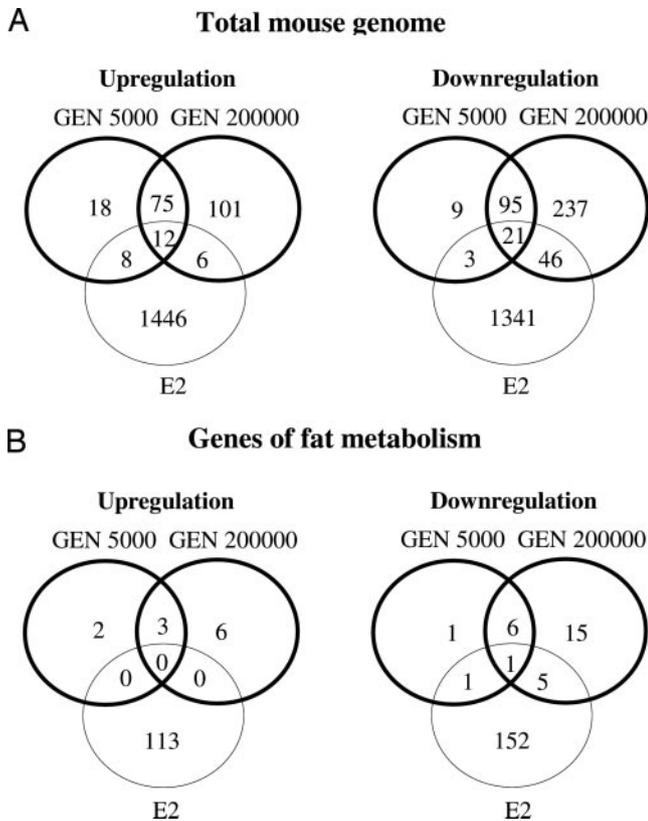


FIG. 5. Comparison between the genes that changed expression after treatment with genistein and E2. Changes in the expression of total genes (A) or a subset of genes (B) involved in fat metabolism (59). Classification of genes differentially expressed after treatment with: genistein (5,000 or 200,000 $\mu\text{g}/\text{kg}\cdot\text{d}$) (dark circles) or E2 (5 $\mu\text{g}/\text{kg}\cdot\text{d}$) (light circles) relative to the control group, was based on Venn diagrams. The up- or down-regulation for the genes in epididymal fat of treated mice relative to the control group is shown. The genes that changed their expression are reported in supplemental Table 1. Genes involved in fat metabolism were selected from the literature and according to Ref. 60. Up- or down-regulation was detecting using *t* tests with a significance criterion of 5% at the gene level.

results show that nutritional genistein exerts an effect opposite to that of E2 with regard to the adipose growth and deposition and to the regulation of adipose regulatory genes. In addition, the high antiadipogenic dose did not induce the same changes induced by E2 on these adipogenic genes.

The only factor important in fat metabolism that showed a similar response to both these hormones is the uncoupling protein 1 (UCP1), which is strongly induced by the adipogenic genistein concentrations and by E2. This finding may suggest that these hormones also regulate adipose function through the regulation of the mobilization and/or use (β -oxidation) of energy substrates independently from tissue mass changes (51, 52). This idea is also supported by the correlation between UCP1 induction and the decrease in plasma lipids observed in the 5,000 $\mu\text{g}/\text{kg}$ genistein and E2 groups.

The statistically significant effect of genistein on epididymal fat deposition began at 500 $\mu\text{g}/\text{kg}\cdot\text{d}$ and peaked at 5,000 $\mu\text{g}/\text{kg}\cdot\text{d}$. These doses are equivalent to the consumption of genistein occurring in populations eating moderate to high amounts of soy or soy-derived products (53, 54). At doses

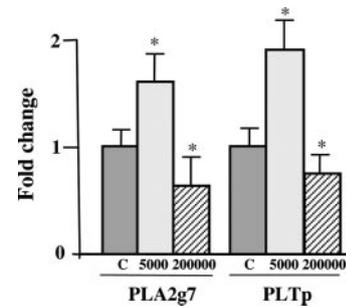


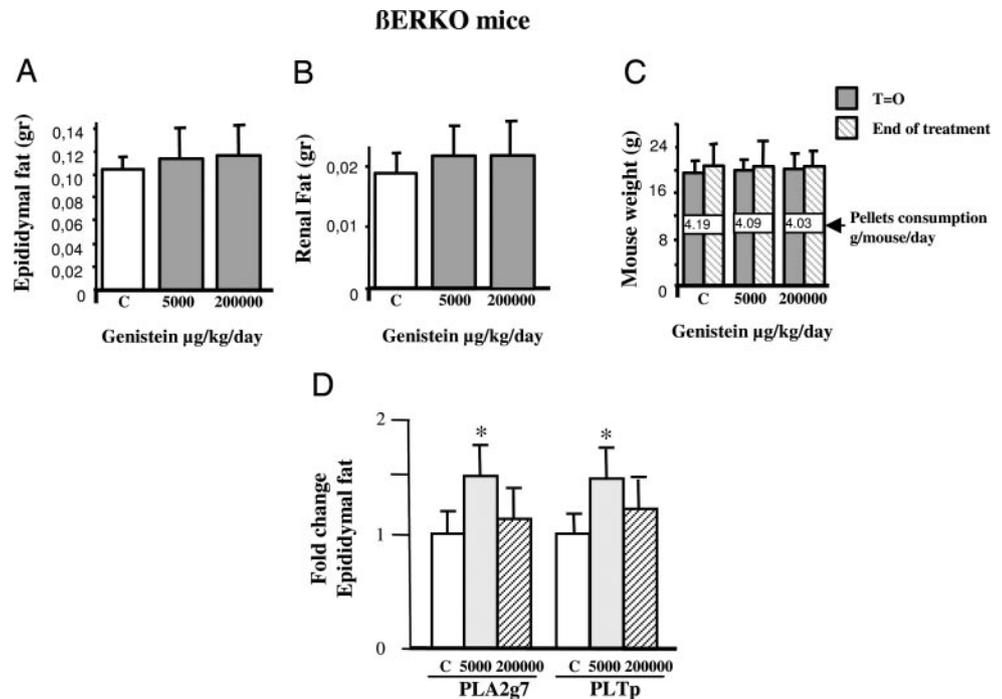
FIG. 6. Effect of genistein on the expression of PLA2g7, PLTp genes. Epididymal fat tissue of mice treated with genistein (5,000 or 200,000 $\mu\text{g}/\text{kg}$) was evaluated for changes in expression of the PLA2g7 and PLTp genes. Total RNA was extracted and reverse transcribed into cDNA. mRNA levels were measured by real-time RT-PCR on six mice per treatment. Expression is shown relative to the 18S gene. Controls (dark grey bars), 5,000 $\mu\text{g}/\text{kg}$ (light gray bars), 200,000 $\mu\text{g}/\text{kg}$ (dashed bars). Data shown as mean \pm SE. *, $P < 0.05$ vs. controls.

higher than 50,000 $\mu\text{g}/\text{kg}\cdot\text{d}$, adipose growth begins to decrease. Recent results (55) suggest that, at pharmaceutical doses, genistein may also, as a ligand, engage other nuclear receptors beyond the ERs, contributing to the production of the bell-shaped response (56) of the adipose tissue to this compound and possibly leading to the significant decrease in tissue weight at the 200,000 $\mu\text{g}/\text{kg}$ dose. Genistein was shown to be a ligand of PPAR γ and to activate PPAR-responsive elements in cell culture systems at doses much higher than those activating the ERs. PPAR γ activation is also accompanied by a decrease in ER action. A balance in the recruitment of these two receptor systems has been shown to modulate the response of the adipose cells to genistein *in vitro* (21) and should be investigated if the same outcome occurs *in vivo*.

Estrogen receptor recruitment by genistein at the doses used in this study, is evidenced by the fact that the compound caused a strong down-regulation of ER α and ER β protein and mRNAs in acutely treated mice and of ER β mRNA in chronically treated mice. A lack of ER α down-regulation has also been observed in our laboratory in the livers of mice chronically exposed (4 wk) to the liver-specific agonist tamoxifen (data not shown). This observation points to a mechanism of compensation in receptor expression, that can probably differ for the two receptor subtypes and that appears to depend on the time of exposure to a specific ER ligand. After acute administration of genistein, this compensation appears to be faster for ER α ; ER α mRNA levels returned to 80% of that of the controls at 24 h. At this time point, ER β mRNA was still 65% down-regulated. An estrogen receptor turnover dependent on the time of exposure to a specific ligand, has been observed also in other studies (57) where the authors reported that short-term treatment of endothelial cells with E2 causes only ER α down-regulation, in contrast to the up-regulation of ER α and down-regulation of ER β after long-term E2, and that regulation required the binding of E2 to ER α and ongoing gene transcription. A ligand- and time-dependent modulation of ERs levels is an important aspect of the pharmacology of estrogens and of all the compounds that are able to bind and activate the estrogen receptors (58).

A lack of correlation between mRNA and protein levels is also apparent from our data. This may occur when different

FIG. 7. Dose-dependent effects of genistein in β ERKO male mice. Dose-dependent action of genistein in epididymal (A) and renal (B) fat of β ERKO male mice. C, Body weights and food intake are shown. Mice were treated orally for 2 wk with vehicle (\square) or 5,000 or 200,000 $\mu\text{g}/\text{kg}$ of genistein (\blacksquare). Epididymal and renal fat pads were excised and weighed. The expression of the PLA2g7 and PLTp genes was determined in the epididymal fat of five mice per treatment by real-time PCR. The fold-changes with respect to untreated control β ERKO mice are reported in D. Expression is shown relative to the 18S gene. Control (\square), 5,000 $\mu\text{g}/\text{kg}$ (\blacksquare); 200,000 $\mu\text{g}/\text{kg}$ (\boxtimes). Data shown as mean \pm SE. *, $P < 0.05$ vs. control.



regulatory mechanisms converge on the same factor. A mechanism controlled by genistein on estrogen receptors, beyond the control of transcription or stabilization of mRNA, may consist of altered modulation of protein stability/degradation, through changes of their ubiquitination. This kind of control has been shown to occur for estrogen receptors and coregulators, in a ligand-dependent manner (59).

It has been reported that estrogens play a major role in adipose tissue through ER α and that this receptor subtype is expressed at notably higher levels in adipose tissue (60). Our results show that ER β , which is expressed mainly in fully differentiated and mature adipocytes (61, 62), also plays a role in the antiadipogenic effect of high doses of genistein. This pathway was initially revealed in mice in which estrogen receptor β was ablated (β ERKO) (26). Our results may suggest a control of ER β vs. genistein-activated ER α and may indicate that ER β is required for the antiadipogenic action of genistein.

Assuming a role of estrogen receptors for genistein action, the estrogenicity of genistein has also been studied in the epididymal fat of estrogen reporter mice (23). Interestingly, although genistein modulated several endogenous genes, it did not change the expression of two consensus EREs in tandem, present in the construct used to generate the estrogen-reporter mouse line ERE-tK-LUC. We speculate that this may result from the lack of flanking regulatory sequences (which are present on endogenous and more complex promoters) that may cooperate with the minimal EREs for the receptor action and are recognized by adipose-specific factors; it also possibly is attributable to the lack of adipose-associated coregulators required by genistein-activated ERs. The fact that the lack of regulation of the ERE-tK promoter is adipose specific is proved by showing that genistein regulates this promoter in the lung and in several other tissues studied (Ref. 25; and; Penza, M., C. Montani, A. Romani, P.

Vignolini, P. Ciana, A. Maggi, B. Pampaloni, L. Caimi, and D. DiLorenzo, manuscript in preparation). Physiological doses of E2 (5 $\mu\text{g}/\text{kg}$) significantly down-regulated the same promoter in the adipose tissues but not in other tissues such as the lung and the liver (24, 25) where it induced the ERE-tK-LUC reporter. These results may support the fact that down-regulation of estrogen-dependent genes are an important step in the inhibition of adipose growth. In addition, they emphasize the fact that the recognition of genistein-responsive promoters is partially different from that elicited by E2-activated ERs in adipocytes and likely involves also non-ER-mediated pathways.

These results provided the rationale for searching the mouse genome for genes that were differentially expressed during adipose mass changes in mice exposed to the 5,000 and 200,000 $\mu\text{g}/\text{kg}\cdot\text{d}$ genistein doses and to E2. Using gene expression profiles obtained from epididymal fat, we isolated a few differentially expressed genes known from the literature to be involved in fat metabolism (adipogenesis or lipogenesis and lipolysis) (49, 63). PLA2g7 and PLTp were two genes induced in the 5,000 $\mu\text{g}/\text{kg}$ group and repressed in the 200,000 $\mu\text{g}/\text{kg}$ group that may be directly involved in the dose-dependent actions of genistein. PLTp is a protein closely related to adipose mass, which is elevated together with HDL cholesterol by PPAR α and LXR α activation in mice (64, 65). It is also an important factor for converting the atherogenic lipoprotein profile of obese subjects into a weight-loss-associated profile that is less atherogenic (66). Moreover, PLTp haplotypes are associated with obesity-related phenotypes (67). PLA2g7 is the gene encoding platelet activating factor-acetylhydrolase, an adipose and circulating enzyme likely to play a role in obesity-related inflammation (68). In addition to having an anti-inflammatory activity by degrading PAF, platelet activating factor-acetylhydrolase may also exert proinflammatory activity by massively hy-

drolizing phospholipids to generate lysophosphatidylcholine and free oxidized fatty acids, both proinflammatory mediators responsible for the proatherogenic activity of oxidized LDL (69). Up-regulation of these genes in adipocytes may also result in changes in the extracellular matrix (tissue remodeling) to accommodate adipose tissue expansion (70).

Interestingly, computer analysis of the 5'-flanking sequences of the PLA2g7 and PLTP genes revealed the presence of possible EREs centered at position -318 with respect to the start site of transcription in the PLA2g7 gene (aGT-CAGacTGAGct) and at -3752 in the PLTP gene (CGgCAGc-cTGAaCa). These elements may be responsible for the regulation of these genes by genistein-activated ERs suggesting the regulation of pathways in common with E2. Functional data confirming this hypothesis may generate additional interest in the role of these hormones in obesity-related atherogenesis.

In our DNA microarray study, a total of 648 genes changed expression. There was poor overlap between genes whose expression was altered in the genistein groups and those changed in the E2-treated group; a slightly better match was evident between the 200,000 $\mu\text{g}/\text{kg}$ dose of genistein and E2.

These results provide evidence that the physiological effects of genistein, although mediated by estrogen receptors and, in particular, ER β , are produced through the regulation of pathways that differ from those regulated by E2, even at the dose that produced adipose mass changes similar to those induced by E2.

Most available studies on the action of isoflavones on adipose and body weight, have been performed in female animals. However, the results of our work suggests that, in light of the possible use of these compounds in weight-loss diets and for the prevention of diseases related to intraabdominal fat deposition (71, 72), the effects of genistein have to be analyzed in more detail in humans.

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