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Genistein Accumulates in Body Depots and Is Mobilized during Fasting, Reaching Estrogenic Levels in Serum that Counter the Hormonal Actions of Estradiol and Organochlorines

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Isoflavones are important dietary compounds that are consumed with the daily diet and elicit important biological actions. Here we report on the ability of genistein to partially accumulate in body depots of male mice, be released following fasting, and modulate the actions of estradiol and environmental estrogens in reproductive and nonreproductive target organs of estrogen-reporter mice (ERE-tK-luciferase). After the consumption of 50 mg/kg/day for 3 days, genistein accumulates in body compartments where it remains at functionally active levels for at least 15 days. Following 48 h of fasting, its concentration increased in serum from 99 ± 13 to 163 ± 17nM. These levels are sufficient to exert an estrogenic effect in the testis and liver, as revealed by a twofold increase in luciferase gene expression. β-Benzenehexachloride (βBHC) given at the concentration of 100 mg/kg/day for 3 days also accumulates in the body and is released by fasting, reaching serum levels of 176 ± 33nM, upregulating the luciferase gene in the liver and inhibiting its expression in the testis. When genistein was given in combination with βBHC at doses sufficient to induce accumulation of both in body depots, the genistein mobilized by fasting reversed the action of the mobilized βBHC in the testis. Acute administration of nutritional doses of genistein inhibited the action of estradiol and reversed the antiestrogenic action of *o,p′*-DDT [1,1,1,-trichloro-2(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane] in the liver and the antiestrogenic action of βBHC in the testis. Genistein had an additive effect with the ER agonist *p,p′*-DDT [1,1,1,-trichloro-2,2-bis (*p*-chlorophenyl)ethane] in the liver. The observed effects may be relevant to a protective action of phytoestrogens against estrogen receptor–interacting pollutants as well as the dietary modulation of estradiol action.

Key Words: **phytoestrogens; estrogen receptors; estrogen responsive elements; reporter mice; endocrine disruptors.**

There are several chemicals in the environment that are able to bind to estrogen receptors (ERs) and modulate the transcription of target genes (Kuiper *et al.*, 1998). They include man-made by-products of industrial processes that are found as contaminants in food and water (xenoestrogens) (Lintelmann *et al.*, 2003) and natural compounds produced by plants consumed as food or food supplements (phytoestrogens) (Cornwell *et al.*, 2004).

People are exposed to nutritional estrogens (mainly isoflavones and lignans) (Aldecreutz *et al.*, 1993; Bhakta *et al.*, 2006) depending on their daily intake of certain vegetables. Those most exposed are populations eating soy in their daily diet. Asian populations may eat up to 50 mg/day of the isoflavone genistein (Kim and Kwon, 2001; Kimira *et al.*, 1998; Yamamoto *et al.*, 2001), while for some Western populations (i.e., the Finnish), the daily intake may not exceed 1 mg/day. Dietary intake of soy produces average blood levels of isoflavones of over 1μM in Asian populations and in people consuming isoflavones supplemented products, and levels 10–100 times lower in populations with a diet of variable vegetable content (Adlercreutz *et al.*, 1993; Nettleton *et al.*, 2004; Uehar *et al.*, 2000; Vedrine *et al.*, 2006). However, blood levels of 40nM or higher have been shown to produce estrogenic effects in experiments on animals (Penza *et al.*, 2006). Thus, it is possible that also concentrations of genistein lower than those found in Asian diets are estrogenically active in humans.

Nutritional isoflavones can bind ERs (ERα and ERβ) and exert both ER agonist and ER antagonist action in a tissue-specific and promoter-specific manner (Schultz *et al.*, 2005). Genistein is known to exert estrogenic effects at low doses, through ERα and ERβ, although at higher doses it also productively interacts with other nuclear receptors such as PPARs (Dang and Lowik, 2004) and affects protein kinases, apoptosis, and cell proliferation (Huang *et al.*, 2005; Yu *et al.*, 2004).

In the human diet, there are also several estrogenic compounds that do not play a nutritional role, but that are

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present as food contaminants. The most abundant are organochlorines such as DDT, β-benzene-hexachloride (βBHC), and others (Simonich and Hites, 1995); plasticizers (Toda *et al.*, 2004); phthalates (Inoue *et al.*, 2002); industrial chemicals such as PCBs and dioxins (Hirai *et al.*, 2004; Safe, 1992); detergents (Falconer, 2006); flame retardants (Legler and Brouwer, 2003); and heavy metals (Henson and Chedrese, 2004). Several of these substances have chemical structures that favor their accumulation and persistence in fat tissue from which they can be mobilized under energetic imbalance (e.g., during pregnancy, lactation, disease, and weight loss–inducing diets) and exert estrogenic effects on the whole physiology (Villa *et al.*, 2004). Phytoestrogens are not considered able to accumulate in the organism. Following ingestion, their elimination is fast, with 90% being eliminated after 36 h (King and Bursill, 1998). However, because they are consumed very frequently, a diet containing a normal amount of soy may be sufficient to maintain a certain estrogenic load in the body.

Using a mouse model in which the pharmacodynamics of an ER ligand can be studied by the modulation of a convenient estrogen-dependent enzyme (luciferase), we provide evidence for accumulation of genistein in the organism that may persist for at least 2 weeks. When, following fasting, it is mobilized from body stores, it reaches blood levels that are sufficient to induce estrogenic effects in reproductive and nonreproductive target organs and to counteract or cooperate with the estrogenic or antiestrogenic action of industrial xenoestrogens. Interaction between these two types of estrogens may affect the endocrine system, depending on their relative concentrations, their affinities for the ERs, and their synergistic or mutually competing actions. The actions of phytoestrogens may protect organisms from the actions of ER-interacting pollutants and interfere with the actions of physiological estrogens.

MATERIALS AND METHODS

Experimental Animals

The procedures involving the animals and their care were conducted in accord with 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, DC]; National Academy Press, National Research Council Guide, http://www.nap.edu/readingroom/books/labrats). The ERE-tK-luciferase mouse model (ERE-tK-LUC) has been extensively studied in our laboratories for testing the mode of action of the chemicals used in these experiments: organochlorines (Di Lorenzo *et al.*, 2002; Mussi *et al.*, 2005; Penza *et al.*, 2004; Villa *et al.*, 2004), genistein (Brena *et al.*, 2005; Penza *et al.*, 2006), and estradiol (Ciana *et al.*, 2001, 2003), with respect to their dose response, time course, specificity, sensitivity, and agonism/antagonism of ER ligands. ERE-tK-LUC transgenic mice were kept in animal rooms maintained at a temperature of 23°C, with natural light/dark cycles. For the experiments, we used heterozygous littermates obtained by mating our founders with C57BL/6J wild-type mice. Two-month-old heterozygous transgenic male mice of an average weight of 25 g were screened by polymerase chain reaction analysis for the presence of the transgenic cluster. Before all treatments, the mice were put on an estrogen-free diet (Piccioni, Milan) for 1 week. For the acute treatments, heterozygous male mice (2 months old) were injected ip with 100 µl of 17β-estradiol or βBHC or orally treated with genistein at the needed concentration or with 100 µl of vehicle (vegetable oil) as control. For loading of compounds, the animals were treated ip for three consecutive days with 100 mg/kg of βBHC or 50 µg/kg of 17β-estradiol and orally with 50 mg/kg of genistein. The mice with an average body weight of 25 g (± 2 g) were then put into groups that were either fasted for 48 h or fed *ad libitum*. The animals were sacrificed by cervical dislocation and the tissues dissected and immediately frozen on dry ice. Tissue extracts were prepared by homogenization in 500 µl of 100mM K_2PO_4 lysis buffer (pH 7.8) containing 1mM dithiothreitol, 4mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 4mM ethylenediaminetetraacetic acid, and 0.7mM 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 (Bradford, 1976).

Chemicals

We purchased 17β-estradiol (17β-E2) and genistein from Sigma (Pomezia, Italy). βBHC, *p,p′*-DDT [1,1,1,-trichloro-2,2-bis(*p*-chlorophenyl)ethane], and *o,p′*-DDT [1,1,1,-trichloro-2(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane] were purchased from Superchrom (Milan, Italy).

Enzymatic Assay

Luciferase enzymatic activity was measured, as reported by de Vet *et al.* (de Vet *et al.*, 1987; Gould and Subramani, 1988), in tissue extracts at a protein concentration of 1 mg/ml. The light intensity was measured with a luminometer (Digene Diagnostics) over 10 s and expressed as relative light units/mg proteins.

Genistein Assay

Chow samples preparation. Four grams of each chow sample was ground with a mill and extracted with 100 ml of 70% ethanol, adjusted to pH 3.2 with formic acid for one night at room temperature. The extracts were defatted with 3 × 50 ml of petroleum ether. The defatted extracts were evaporated to dryness under vacuum at room temperature, and finally redissolved in EtOH/H_2O (70:30 pH 3.2 with formic acid), to a final volume of 7 ml.

Plasma samples preparation. Sample preparation for plasma isoflavones analysis was performed according to the methods described by Manach *et al.* (1998), appropriately modified. To limit the isoflavones losses, a simple treatment procedure was used: 900 µl plasma was acidified with 100 µl of 0.58M acetic acid solution. The acidified plasma samples were mixed with the enzymes, 5 µl β-glucuronidase and 80 µl 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 × g for 45 min at 4°C. The supernatant of each sample was dried by speed vacuum at room temperature. The organic phase was than redissolved in 300 µl of methanol and 200 µl of water. Before being injected in the high-performance liquid chromatography (HPLC) system, the samples were centrifuged by microfuge for 2 min, and 50 µl of clear samples were tested by HPLC.

HPLC-DAD analysis. The analyses were carried out using an HP 1100L liquid chromatograph equipped with a DAD detector (Agilent Technologies, CA), and a dual pump 515 model liquid chromatographic system, supplied with a diode array system Model 996 (Waters Corporation, MA). Isoflavonoids were separated using a 150 × 3.9 mm (4 µm) Nova Pak C18 column (Waters Corporation) operating at 30°C. The mobile phase was a four-step linear solvent gradient system, starting from 95% H_2O (adjusted to pH 3.2 by H_3PO_4) up to 100% CH_3CN during a 27-min period (Romani *et al.*, 2003), the flow rate was 0.8 ml/min.

High-performance liquid chromatography–mass spectrometry analysis. High-performance liquid chromatography–mass spectrometry (HPLC-MS) analyses were performed using an HP 1100 MSD API, ESI interface, coupled with an 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, nebulizer pressure 30 psi, 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 0–12 ng on the basis of authentic standard, and determination was directly performed by HPLC-DAD.

Gas Chromatography–Mass Spectrometry Analysis of βBHC

Sample extraction. One milliliter of serum was added to 2 ml of methanol and shaken for 30 min. The mix was than added with 6 ml of n-hexane/diethyl ether, shaken for 30 min, and centrifuged for 10 min at 2000 rpm. Four milliliters of the solution was concentrated to 1 ml in a vacuum evaporator and eluted on a Florisil cartridge (6 ml, 1 g; Supelco, Bellefonte, PA) and a silica cartridge (6 ml, 1 g; Supelco) sequentially. The extracts were brought to dryness, and the derivatives were dissolved in n-hexane (100 µl). As internal standard, a solution of 2,4,6-trichlorobiphenyl (1 µg/ml) was used.

Gas chromatography–mass spectrometry analysis. Two microliters of the extracts was injected in gas chromatography–mass pectrometry (GC-MS) and separated by chromatography on a PONA fused silica capillary column (HP, CA) with helium as carrier gas (flow rate, 1 ml/min constant flow) and by temperature programing (injector 240°C, detector 320°C). The instrument used was a GC HP 6890 MS HP 5972-A (HP). *p,p′*-DDT, *p,p′*-DDE, and βBHC were identified by single-ion monitoring methods by the following ions: 217/219, 235/237, 246/248. They were quantified by the standard addition method (10 and 100 ng/ml solutions). Concentrations are expressed as micrograms per milliliter.

Statistical Analysis

Statistical analysis was performed by two-way ANOVA test followed by *post hoc* Bonferroni analysis.

RESULTS

Genistein Released from Body Depots Reaches Estrogenic Levels in the Organism

We have assessed ERE-dependent luciferase expression in estrogen-reporter mice (Ciana *et al.*, 2001) before and after 48 h of fasting in genistein-, βBHC-, 17β-estradiol–, and vehicle-loaded mice.

Thirty ERE-tK-LUC mice per group were loaded with 50 mg/kg/day of genistein, or 100 mg/kg/day of βBHC, or 50 µg/kg/day of 17β-estradiol, or vehicle for three consecutive days (accumulation phase). Compound-loaded and control mice were left untreated for 15, 30, or 90 days on an estrogen-free diet and assessed for the presence of residual estrogenic activity in a reproductive and a nonreproductive tissue (testis and liver; Fig. 1, white bars). Luciferase activity significantly above control levels was detectable at 15 days in the livers of mice loaded with βBHC ($p < 0.05$), indicating the persistence of this compound in the body. No activity on reporter expression was detected in genistein- and estradiol-loaded mice.

We then verified if fasting could induce an increase in compound bioavailability sufficient to affect ERE-dependent gene expression. Fifteen mice from each group were fasted for 48 h. Fasting itself caused a dramatic reduction in reporter activity in the liver, to 20% of the control value ($p < 0.001$), and significantly activated the ERs in the testis (1.7-fold induction; $p < 0.001$).

In the genistein- and βBHC-treated groups, compound mobilization at 15 days reached sufficient levels to strongly reverse fasting-induced reporter downregulation in the liver (twofold and threefold induction, respectively; Fig. 1A). After 30 days, the fasting mobilized βBHC to levels sufficient to upregulate the reporter (1.9-fold induction), while at 90 days luciferase expression was not higher than in fed mice, although it was not significantly lower as in the unloaded group. In the genistein-loaded group, after 30 days, reporter expression was not repressed by fasting as it was in control mice, indicating some residual activity of the phytoestrogen that was not visible at 90 days.

In the testis, although fasting itself slightly activated the ERs, at 15 days βBHC inhibited, while genistein significantly induced luciferase expression (Fig. 1B). The effect of βBHC at 30 days was less evident. At this time, the action of genistein was not significantly different from the control.

No differences in ER activity were detected in 17β-E2–loaded mice before or after 48 h of fasting, compared to vehicle-treated mice, indicating clearance of the estrogen shortly after loading. We have shown in recent works that 17β-E2 activity peaks at 6 h in tissues of these mice as measured by luciferase activity, than is rapidly catabolized. At 24 h, luciferase is back to basal level (Ciana *et al.*, 2001, Villa *et al.*, 2004). Possible alterations of the luciferase enzyme activity were tested *in vitro* after adding 17β-estradiol, genistein, and βBHC to the enzyme reaction. No changes were observed, indicating that the function of the enzyme is not directly affected by the chemicals (not shown).

Serum Levels of Genistein Mobilized during Starvation

To verify if the observed differences in reporter activation in the genistein- and βBHC-loaded and fasted mice were due to the mobilization of the compound, we made a quantitative evaluation of the circulating levels of genistein and βBHC. HPLC-DAD and HPLC-MS analysis were performed on the sera of untreated and genistein-loaded mice before and after the 48-h fasting period. GC-MS was used to assay βBHC.

As shown in Figure 2, fasting was accompanied by an increase in genistein levels from 99 ± 13 to 163 ± 17nM ($p < 0.05$). βBHC increased from 74 ± 16 to 176 ± 33nM ($p < 0.05$).

Genistein Inhibits the Activity of βBHC on the ERE-Dependent Reporter in the Testis of Loaded, Fasted Mice

We have shown that βBHC inhibits ERE-dependent luciferase expression in the testes of 2-month-old male mice loaded with the chemical and fasted (48 h) after 15 days, thus acting as an antiestrogen. We set up an experiment in which mice were loaded with βBHC (300 mg/kg/3 days), genistein (150 mg/kg/3

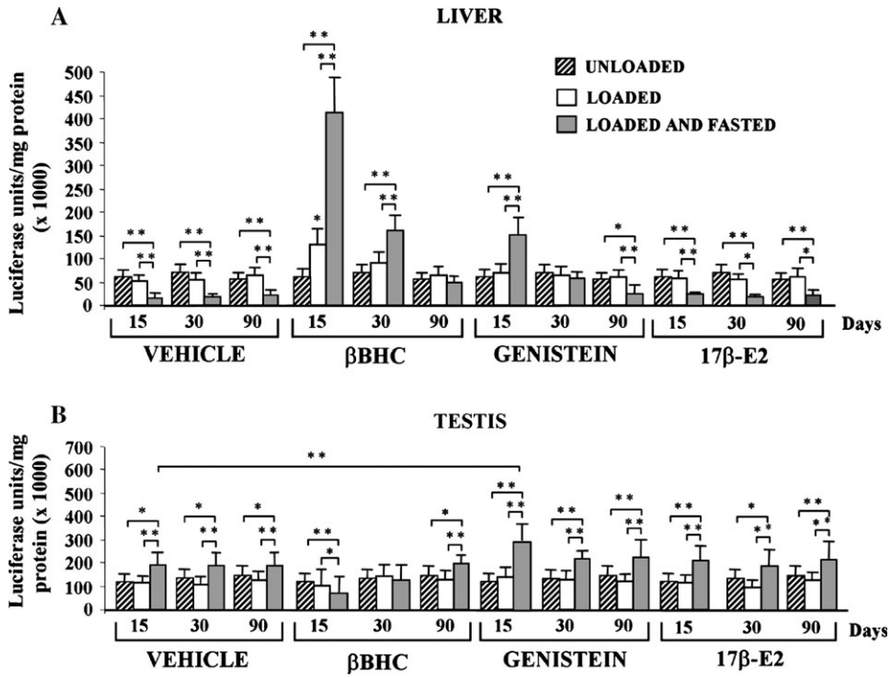

FIG. 1. Effect of fasting on reporter regulation in transgenic mice loaded with βBHC, genistein, or 17β-estradiol. Mice were put on an estrogen-free diet for 1 week before any treatment and for the entire course of the experiments. Mice were loaded with 50 mg/kg, 100 mg/kg, or 50 μg/kg of genistein (oral gavage), βBHC (ip), or 17β-estradiol (ip), respectively, or vehicle (30 mice per group) once a day for three consecutive days and left without any other treatment for the following 15, 30, or 90 days. Luciferase activity in the liver (A) and testes (B) of genistein-, βBHC-, 17β-estradiol– or vehicle-loaded mice after 15, 30, or 90 days on an estrogen-free diet, with or without 48 h of fasting. Fifteen mice from each group were then put on complete food deprivation for 48 h, with water *ad libitum.* At the end of the fasting period, all the mice were sacrificed and the tissues collected and stored at − 80°C until assayed. Luciferase activity is expressed as relative light units normalized to protein concentration. The half-life of the luciferase enzyme used to generate these mice is 3 h (not shown). The experiments were repeated twice. Bars represent the average ± SE; $*p < 0.05$; $**p < 0.001$, as compared with the controls.

days), or both compounds in combination. After 15 days of resting, mice were fasted for 48 h, and luciferase activity in the testis was recorded. βBHC and genistein were mobilized and targeted the testis with opposite actions on the ERE-dependent reporter (Fig. 3). In mice loaded with both compounds simultaneously, genistein was able to revert the antiestrogenic action of βBHC.

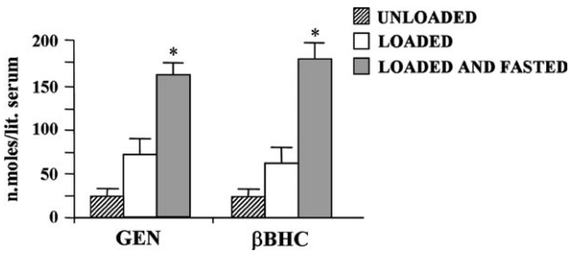

FIG. 2. Measurements of genistein and βBHC in serum. The serum of genistein- and βBHC-loaded mice was analyzed for changes in levels of genistein or βBHC, before and after fasting. The white bars indicate the concentration of genistein or βBHC in serum of compound-loaded mice before fasting (day 15 from loading) and the gray bars the concentrations after 48 h of fasting (day 17 from loading). Values are expressed as nanomoles of genistein or βBHC per liter of serum. The determinations were repeated twice on five mice per group. GEN, genistein-loaded mice ± 48 h fasting; βBHC, βBHC-loaded mice ± 48 h fasting. Bars represent the average ± SE; $*p < 0.05$, as compared with the controls.

Genistein Antagonizes or Cooperates with the Action of Organochlorines and Estradiol in Testis and Liver of Acutely Treated Estrogen-Reporter Mice

We have previously reported that the DDT isomer *o,p′*-DDT is an efficient antiestrogen in the liver, while *p,p′*-DDT acts as an agonist through ER-mediated mechanisms (Di Lorenzo *et al.*, 2002). The following experiment demonstrates that, in the liver, genistein is able to counteract the antiestrogenic action of *o,p′*-DDT, cooperates with that of the ER agonist *p,p′*-DDT, and antagonizes the positive action of estradiol.

o,p′-DDT (50 mg/kg) inhibited the expression of luciferase down to a level 35% of the control value. Coadministration of genistein in a dose-response experiment reversed the antiestrogenic effect of *o,p′*-DDT and induced the reporter twofold at the nutritional dose of 5000 μg/kg (Fig. 4A). Genistein's action was additive with that of *p,p′*-DDT (Fig. 4B). At the same doses, genistein also inhibited the estrogenic effect of physiological concentrations of 17β-E2 (5 μg/kg) (Fig. 4C).

βBHC (50 mg/kg) inhibited luciferase expression in the testis by 65%. Genistein at 5000 μg/kg reversed this effect

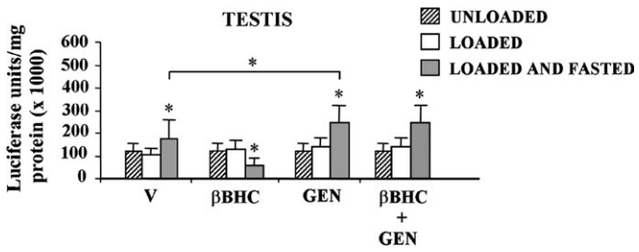

FIG. 3. Effect of fasting on reporter regulation in testes of transgenic mice loaded with βBHC or genistein or both. Luciferase values in the testes of genistein-, βBHC-, genistein-plus-βBHC– and vehicle-loaded mice, after 15 days of resting on an estrogen-free diet and after 48 h of fasting. Mice were put on an estrogen-free diet for 1 week before any treatment and for the entire course of the experiments. Mice were loaded with 100 mg/kg of βBHC (ip), 50 mg/kg of genistein (oral gavage), or both compounds in combination once a day, for three consecutive days (total βBHC and genistein 300 and 150 mg/kg, respectively) and left without any other treatment for the following 15 days (20 mice per group). Ten mice from each group were then put on complete food deprivation, with water *ad libitum*. At the end of the 48 h of fasting, all the mice were sacrificed and the tissues collected and stored at − 80°C until assayed. Luciferase activity is expressed as relative light units normalized to protein concentration. The experiments were repeated twice. V, vehicle; GEN, genistein. Bars represent the average ± SE; $*p < 0.05$; $**p < 0.001$, as compared with the controls.

(Fig. 4D). In this organ, genistein did not significantly change the extent of luciferase induction by *p*,*p*′-DDT or estradiol (Figs. 4E and 4F). Possible alterations of the luciferase enzyme activity were tested *in vitro* after adding 17β-estradiol, genistein, *o*,*p*′-DDT, and *p*,*p*′-DDT to the enzyme reaction. No changes were observed, indicating that the function of the enzyme is not directly affected by the chemicals (not shown).

These results indicate that nutritional doses of genistein inhibit the hormonal effects of xenoestrogens in tissues of male mice and may interfere with the physiological action of estradiol

DISCUSSION

Genistein is a nutritional isoflavone that possesses hormonal activity through its binding to various nuclear receptors (Dang *et al.*, 2003; Gao *et al.*, 2004; Kuiper *et al.*, 1998). The best characterized is its action through the ERs, ERα and ERβ, by which it induces several biological responses that may be beneficial for human health. For this reason, genistein has been proposed as a potential therapeutic compound that should be integrated into our diets (Beck *et al.*, 2005). Its effects, as observed in mouse models, are dependent on dose and gender (Penza *et al.*, 2006; Slikker *et al.*, 2001), so the ''healthy'' safe dose should be defined and the biology of this molecule better characterized before it is put to pharmacological use. Pharmacokinetics and pharmacodynamics studies on genistein have been conducted in animal models (Chang *et al.*, 2000) and humans (King and Bursill, 1998; Watanabe *et al.*, 1998), in which it appears that it is quickly eliminated from plasma, dropping to residual levels 12–24 h from administration (King and Bursill, 1998) with gender-specific pharmacokinetics (Chang *et al.*, 2000; Watanabe *et al.*, 1998). Our results establish that part of the consumed genistein may transiently accumulate in the body, persisting for weeks at preferential sites of accumulation from which it is mobilized during energy imbalance. In rats, the largest amount of the absorbed compound is not found in fat (Coldham and Sauer, 2000). Maximum concentrations were measured in the small intestine and cecum at 2 and 7 h after administration. The liver, plasma, and reproductive tissues, including uterus, ovary, vagina, testis, prostate, and prostatic fluid, also accumulate higher concentrations of genistein than other peripheral organs (Chang *et al.*, 2000; Hedlund *et al.*, 2005, 2006; Rannikko *et al.*, 2006).

Fasting induces genistein mobilization in loaded mice, in which it reaches a serum level of 163nM after 48 h of fasting. This concentration is below the K_i for tyrosine kinase inhibition, supporting a receptor-mediated action in target cells.

There are also nonnutritional, synthetic estrogenic compounds that contaminate food and are consumed daily with the diet. Several organochlorines, for example, have been characterized as estrogenic (Di Lorenzo *et al.*, 2002; Kuiper *et al.*, 1998); they are environmentally ubiquitous and can enter the food chain and accumulate in the adipose tissues of animals and humans, where they can remain for years (Safe, 1992), accumulating to higher levels with advancing age (Lordo *et al.*, 1996). Periodically, mainly in physiological situations associated with dietary restrictions (Beaufrand *et al.*, 1978; Bigsby *et al.*, 1997; Herron and Fagan, 2002), they are mobilized, increase their blood levels, and may evoke an abnormal estrogenic or antiestrogenic response.

Concern over the activity of environmental estrogens is in regard to their action as ER modulators in the whole body and its suspected link to chronic and degenerative diseases (Den Hond and Schoeters, 2006; Hoyer, 2001).

In this work, we have characterized the total estrogenic action deriving from simultaneous exposure to estrogens from different classes, including physiological, nutritional, and environmental estrogens. We found that the estrogenic action of a toxic environmental compound may be influenced by the presence of nutritional estrogens such as genistein. These natural compounds are generally consumed with the diet at higher concentrations than contaminating chemicals. Phytoestrogens are consumed in milligram amounts per day (Aldercreutz *et al.*, 1993; Bhakta *et al.*, 2006 Kimira *et al.*, 1998), while environmental chemicals such as organochlorines and other persistent pesticide residues are consumed in nanogram amounts per day (Simonich and Hites, 1995). Dietary hormones are also present in blood at much higher concentrations, and their affinity toward ERs, especially for ERβ, is higher than those of several xenoestrogens (Kuiper *et al.*, 1998). Competition between the different ligands for binding to ERs may result in overlapping or contrasting actions on the regulation of target genes. The outcome of this competition may be dependent on each compound's relative affinity for the receptors (Kuiper *et al.*, 1998).

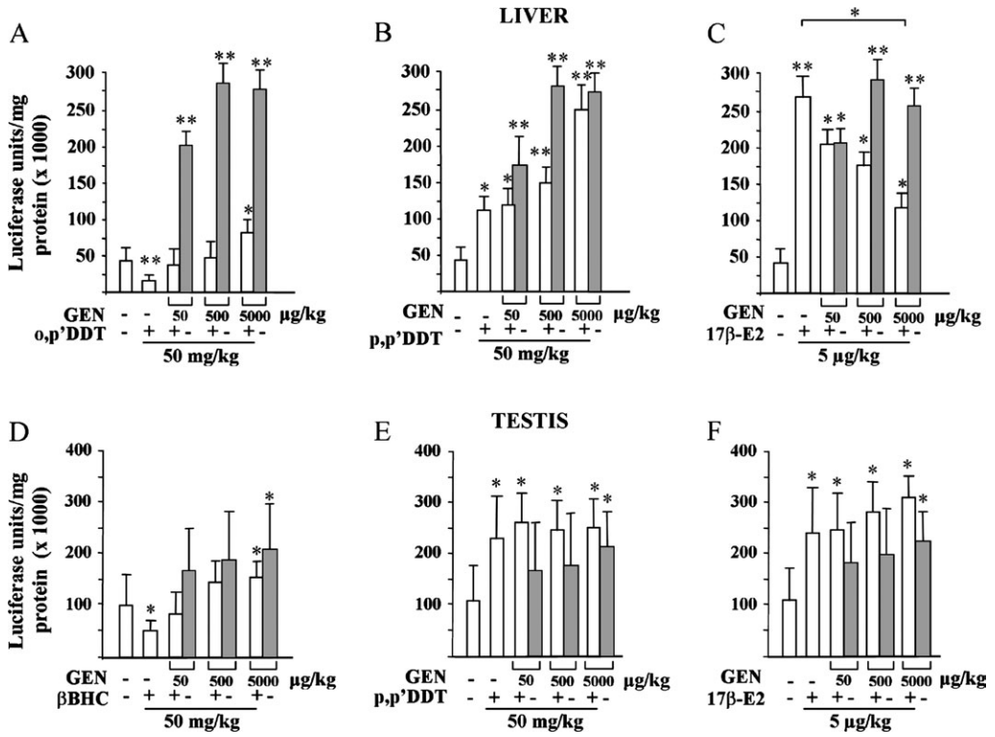

FIG. 4. Genistein interferes with the action of organochlorines and estradiol. (A and B) Adult mice were injected with oil or an oil solution of 50 mg/kg *o,p′*-DDT (A) or *p,p′*-DDT (B) and kept for 16 h. At 6 h after injection, the mice were orally treated with increasing concentrations of genistein (50, 500, or 5000 µg/kg) and kept for the remaining 10 h. The single dose of the administered organochlorines was chosen from previous experiments already published (Di Lorenzo *et al.*, 2002; Mussi *et al.*, 2005; Penza *et al.*, 2004; Villa *et al.*, 2004). Controls were orally treated with vegetable oil. (C) Adult mice were injected with oil or an oil solution of 5 µg/kg 17β-E2, kept for 6 h, and orally treated for the same time with increasing concentrations of genistein (50, 500, or 5000 µg/kg). Luciferase activity was measured in livers and testis (F) of mice killed at defined time points after treatment. The experiments were repeated three times with a total of six animals per group. (D and E) Adult mice were injected with oil or an oil solution of 50 mg/kg βBHC (D) or 50 mg/kg *p,p′*-DDT (E) and kept for 16 h. At 6 h after injection, the mice were orally treated with increasing concentrations of genistein (50, 500, or 5000 µg/kg) and kept for the remaining 10 h. Controls were orally treated with vegetable oil. Doses of genistein are plotted on a logarithmic scale. (F) Mice were treated as in (C). The half-life of the luciferase enzyme used to generate these mice is 3 h (not shown). The experiments were repeated three times with a total of six animals per group. Bars represent the average ± SE of two individual experiments, each performed in triplicate. $*p < 0.05$, $**p < 0.001$, as compared with the control.

While genistein acted in the liver by antagonizing the antiestrogenicity of the organochlorines, it itself displayed antiestrogenic characteristics in the presence of estradiol. The molecular interaction among different agents at the receptor level has particular importance because nutritional phytoestrogens can modify responses to physiological estrogens and toxic compounds. Competition with toxic compounds should have favorable effects on human health and might represent a new rationale for dietary advice. Interference with estradiol has unknown outcomes and should be studied in more detail during development, at different ages, during pathological states, and in a gender-specific manner.

Genistein, when given as a single oral bolus and when mobilized from body stores weeks after administration, competed with the action of *o,p′*-DDT and βBHC in liver and testis, respectively, and interfered with estradiol action in the liver. Less striking was the interaction of genistein with the ER agonist *p,p′*-DDT in mice cotreated with both ER ligands, in which case we observed an additive effect on the reporter in the liver, which was not visible in the testis.

A tissue-specific pharmacodynamic of genistein and estradiol may be responsible for the different combined interactions observed in the organs analyzed.

These data show that genistein is an efficient estrogen in the tissues analyzed. However, at the nutritional dose of 5000 µg/kg body weight, while being an agonist in both the liver and testis when given alone, it appears to behave as a partial antagonist when given in conjunction with physiological doses of estradiol. This is probably due to the 100-fold lower potency of genistein on ERs compared to estradiol and, as recently observed in our laboratory, to the fact that genistein strongly downregulates ERα in the liver, thus antagonizing ERα upregulation by estradiol (not shown).

The simultaneous presence of different ER ligands affects the response of the compounds given singly. Genistein at dietary doses counteracted the inhibitory action of βBHC on

the ERE reporter in the mouse testis when the compounds were mobilized from body stores. Accumulation followed by fasting was used to minimize the differences in the toxicokinetics of the two compounds (they are much faster for genistein than for βBHC) when they were given acutely (Penza *et al.*, 2004; Villa *et al.*, 2004). The fact that the levels of genistein released during fasting are capable of altering the toxicological behavior of βBHC and *o*,*p*′-DDT allows us to hypothesize that it is also effective in changing the effect of other toxicants active through the ERs. Interactions of genistein with other endocrine-disrupting agents have recently been reported (Wang *et al.*, 2006; You *et al.*, 2004), although it was not clear that the interaction between the compounds analyzed, genistein and methoxychlor, was occurring through the ERs. Data on the interaction between two or more chemicals are important in interpreting toxicological experiments in animals exposed to single chemicals and for the epidemiological evaluation of the effects of pollutants on human health in countries with different traditional diets.

In this work, we have analyzed the acute and delayed action of genistein following accumulation/fasting release, through the ERs; however, other mechanisms could be involved. For instance, while the results here strongly indicate a dose-dependent effect following liberation of genistein from body stores, it must be kept in consideration that the analytical methods used measured total serum genistein after cleaving glucuronide and sulfate conjugates. Since conjugated forms of genistein are not active toward the ER, this analysis may overestimate the actual activity of genistein in the serum, since over 95% of circulating genistein is usually conjugated. Therefore, while the experiments strongly indicate that this ''unloading'' is the cause of the changes observed in luciferase reporter activity, one cannot rule out a pharmacodynamic effect. There are different possible mechanisms that may participate to modulate the end point used in our model (luciferase). For instance, the 3-day ''loading'' of genistein may cause changes in steroidogenesis or steroid metabolism that can persist for 2 weeks, perhaps magnified by the effects of fasting which is known to alter enzyme activities. We are currently studying the serum concentrations of free (unconjugated) genistein to understand which of these two possible mechanisms is responsible for the observed effects.

Another effect of the chemicals used in our study that might add a further level of complexity to the performed analysis is the regulation of peripheral estrogen synthesis. However, although organochlorines and genistein may regulate aromatase, this was not an effect that changed our data. In fact, the state of ER activation in these animals was monitored with or without coadministration of the aromatase inhibitor Arimidex (0.5 mg/kg). Although this treatment was able to block the female estrous cycle in a parallel experiment, we did not observe changes in luciferase activity that reached significance (not shown).

Environmental estrogens have been found to vary in their capacity to mobilize from sites of accumulation and, consequently, to have different biological actions on the uterus (Bigsby *et al.*, 1997). We found that the isoflavone genistein can also accumulate in the body and be mobilized during periods of energy imbalance. Although it is much less persistent than the organochlorines we tested and other industrial chemicals, its serum concentration increased during fasting and reached estrogenic levels in mice previously loaded with this compound. These results seem to indicate that some dietary regimens may favor genistein accumulation and its bioavailability, which in turn may result in fluctuating estrogenic actions that may antagonize or provide an additive effect to other environmental or endogenous estrogens.

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