

**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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## Abstract

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 non reproductive 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  $99 \pm 13$  to  $163 \pm 17$  nM. These levels are sufficient to exert an estrogenic effect in the testis and liver, as revealed by a twofold increase in luciferase gene expression.  $\beta$ BHC 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  $176 \pm 33$  nM, upregulating the luciferase gene in the liver and inhibiting its expression in the testis.

When genistein was given in combination with  $\beta$ -benzene-hexachloride ( $\beta$ BHC) at doses sufficient to induce accumulation of both in body depots, the genistein mobilized by fasting reversed the action of the mobilized  $\beta$ BHC in the testis.

Acute administration of nutritional doses of genistein inhibited the action of estradiol and reversed the anti-estrogenic action of o,p'-DDT [1,1,1,-trichloro-2(p-chlorophenyl)-2-(o-chlorophenyl)ethane] in the liver and the anti-estrogenic action of  $\beta$ BHC 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 ER-interacting pollutants as well as the dietary modulation of estradiol action.

**Keywords:** phytoestrogens, estrogen receptors, estrogen responsive elements, reporter mice, endocrine disruptors.

## Introduction

There are several chemicals in the environment that are able to bind to estrogen receptors 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 per 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 per day. Dietary intake of soy, produces average blood levels of isoflavones of over 1 $\mu$ M 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; Uehar *et al.* 2000; Nettleton *et al.*, 2004; Vadrine *et al.*, 2006). However, blood levels of 40 nM 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 estrogen receptors (ER $\alpha$  and ER $\beta$ ) 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 ER $\alpha$  and ER $\beta$ , 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 present as food contaminants. The most abundant are organochlorines such as DDT,  $\beta$ BHC, and others (Simonich and Hites, 1995), plasticizers (Toda *et al.*, 2004), phthalates (Inoue *et al.*, 2003), 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 of a mouse model in which the pharmacodynamics of an estrogen receptor 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 non reproductive target organs and to counteract or cooperate with the estrogenic or anti-estrogenic 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, D.C.); 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; Villa *et al.*, 2004; Penza *et al.*, 2004; Mussi *et al.*, 2005), genistein (Brena *et al.*, 2005; Penza *et al.*, 2006) and estradiol (Ciana *et al.*, 2001; Ciana *et al.*, 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 months old heterozygous transgenic male mice of an average weight of 25 grams were screened by PCR analysis for the presence of the transgenic cluster. Before all treatments, the mice were put on an estrogen free diet (Piccioni, Milan) for one week. For the acute treatments, heterozygous male mice (2 months old) were injected i.p. with 100 µl of 17β-estradiol or βBHC or orally treated with genistein at the needed concentration or with 100µl of vehicle (vegetal oil) as control. For loading of compounds the animals were treated i.p. 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 grams (+/- 2 grams) were then put into groups that were either fasted for 48 hours 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  $\mu$ l of 100 mM K<sub>2</sub>PO<sub>4</sub> 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 (Bradford, 1976).

### **Chemicals**

We purchased 17 $\beta$ -estradiol (17 $\beta$ -E<sub>2</sub>) and genistein from Sigma (Pomezia, Italy).  $\beta$ BHC ( $\beta$ -Benzene-Hexachloride), 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 and Gould *et al.* 1988), in tissue extracts at a protein concentration of 1 mg/ml. The light intensity was measured with a luminometer (Digene Diagnostics) over 10 seconds and expressed as relative light units/mg proteins.

### **Genistein assay**

#### *Chow samples preparation*

4 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 x 50 ml of petroleum ether. The defatted extracts were evaporated to dryness under vacuum

at room temperature, and finally redissolved in EtOH/H<sub>2</sub>O (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. (Manach *et al.*, 1998), appropriately modified. To limit the isoflavones losses, a simple treatment procedure was used: 900 µl plasma were acidified with 100 µl of 0.58 M acetic acid solution. The acidified plasma samples were mixed with the enzymes, 5 µl β-glucuronidase and 80 µl arilsulfatase, and treated for 30 minutes at 37°C. For the isoflavones extraction, the samples were mixed with 2.75 ml of acetone, shaken for 1 hour and subsequently centrifuged at 2,400 x g for 45 minutes at 4°C. The supernatant of each samples 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 HPLC system, the samples were centrifuged by microfuge for 2 minutes and 50 µl of clear samples were tested by HPLC.

#### *HPLC-DAD Analysis*

The analyses were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector (Agilent Technologies, California, USA), and a dual pump 515 model liquid chromatographic system, supplied with a diode array system Model 996 (Waters, MA, USA). Isoflavonoids were separated using a 150 × 3.9 mm (4 µm) Nova Pak C18 column (Waters Corporation, Massachusetts USA) operating at 30° C. The mobile phase was a four-step linear solvent gradient system, starting from 95% H<sub>2</sub>O (adjusted to pH 3.2 by H<sub>3</sub>PO<sub>4</sub>) up to 100% CH<sub>3</sub>CN during a 27-min period (Romani *et al.*, 2003), the flow rate was 0.8 ml min<sup>-1</sup>.

### *HPLC-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<sup>-1</sup>, 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 (GC-MS) analysis of $\beta$ BHC**

*Sample extraction.* One millilitre of serum was added to 2 ml of methanol and shaken for 30 minutes. The mix was then added with 6 ml of n-hexane/diethyl ether, shaken for 30 minutes and centrifuged for 10 minutes at 2,000 rpm. 4 ml of the solution were 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  $\mu$ l). As internal standard, a solution of 2,4,6 Trichlorobiphenyl (1  $\mu$ g/ml) was used.

*GC-MS analysis.* Two microlitres of the extracts were injected in 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 programming (injector 240°C, detector 320°C). The instrument used was a GC HP 6890 MS HP 5972-A (HP, CA). p,p'-DDT, p,p'-DDE

and  $\beta$ BHC were identified by Single Ion Monitoring methods (SIM), 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  $\mu\text{g/ml}$ .

### **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-,  $\beta$ BHC-,  $17\beta$ -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  $\beta$ BHC, or 50  $\mu$ g/kg/day of  $17\beta$ -estradiol or vehicle for 3 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 non reproductive 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  $\beta$ BHC ( $p < 0.05$ ), 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  $\beta$ BHC-treated groups, compound mobilization at 15 days reached sufficient levels to strongly reverse fasting-induced reporter down-regulation in the liver (2-fold and 3-fold induction, respectively; Fig.1A). After 30 days, the fasting mobilized  $\beta$ BHC 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  $\beta$ BHC inhibited while genistein significantly induced luciferase expression (Fig. 1B). The effect of  $\beta$ BHC 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  $17\beta$ -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  $17\beta$ -E2 activity peaks at 6 hours in tissues of these mice as measured by luciferase activity. Then is rapidly catabolized. At 24 hours 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  $17\beta$ -estradiol, genistein and  $\beta$ BHC 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  $\beta$ BHC-loaded and fasted mice were due to the mobilization of the compound, we made a quantitative evaluation of the circulating levels of genistein and  $\beta$ BHC. 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  $\beta$ BHC.

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

**Genistein inhibits the activity of  $\beta$ BHC on the ERE-dependent reporter in the testis of loaded, fasted mice.**

We have shown that  $\beta$ BHC 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 anti-estrogen. We set up an experiment in which mice were loaded with  $\beta$ BHC (300 mg/kg/3 days), genistein (150 mg/kg/3 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.  $\beta$ BHC 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 anti-estrogenic action of  $\beta$ BHC.

**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 anti-estrogen in the liver, while *p,p'*-DDT acts as an agonist through estrogen receptor-mediated mechanisms (Di Lorenzo *et al.*, 2002). The following experiment demonstrates that, in the liver, genistein is able to counteract the anti-estrogenic action of *o,p'*-DDT, cooperates with that of the ER agonist *p,p'*-DDT, and antagonizes the positive action of estradiol.

50 mg/kg of *o,p'*-DDT inhibited the expression of luciferase down to a level 35% of the control value. Co-administration of genistein in a dose-response experiment reversed the anti-estrogenic effect of *o,p'*-DDT and induced the reporter two-fold at the nutritional dose of 5,000  $\mu$ g/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  $17\beta$ -E2 (5  $\mu$ g/kg) (Fig. 4C).

50 mg/kg of  $\beta$ BHC inhibited luciferase expression in the testis by 65%. Genistein at 5,000  $\mu$ g/kg reversed this effect (Fig. 4D). In this organ, genistein did not significantly change the extent of luciferase induction by p,p'DDT or estradiol (Fig. 4E and 4F). Possible alterations of the luciferase enzyme activity were tested in vitro after adding  $17\beta$ -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 estrogen receptors ER $\alpha$  and ER $\beta$ , 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; Hedlund *et al.*, 2006; Rannikko *et al.*, 2006).

Fasting induces genistein mobilization in loaded mice, in which it reaches a serum level of 163 nM 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 non-nutritional, 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 anti-estrogenic 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 mg 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 ng amounts per day (Simonich and Hites, 1995). Dietary hormones are also present in blood at much higher concentrations, and their affinity toward estrogen receptors, especially for ER $\beta$ , is higher than those of several xenoestrogens (Kuiper *et al.*, 1998). Competition between the different ligands for binding to estrogen receptors 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).

While genistein acted in the liver by antagonizing the anti-estrogenicity of the organochlorines, it itself displayed anti-estrogenic 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 favourable 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  $\beta$ BHC 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 co-treated 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 5,000  $\mu$ g/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 estrogen receptors compared to estradiol and, as recently observed in our laboratory, to the fact that genistein strongly downregulates ER $\alpha$  in the liver, thus antagonizing ER $\alpha$  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  $\beta$ BHC 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  $\beta$ BHC) 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 behaviour of  $\beta$ BHC 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 is 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 analysed 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 towards the estrogen receptor, this analysis may over-estimate 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 two 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 co-administration 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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## Figures

### Figure 1

#### *Effect of fasting on reporter regulation in transgenic mice loaded with $\beta$ BHC, genistein or 17 $\beta$ -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  $\mu$ g/kg of genistein (oral gavage),  $\beta$ BHC (i.p.) or 17 $\beta$ -estradiol (i.p.), respectively, or vehicle (30 mice per group) once a day for 3 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-,  $\beta$ BHC-, 17 $\beta$ -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 three hours (not shown). The experiments were repeated twice. Bars represent the average  $\pm$  SE; \*,  $p < 0.05$ ; \*\*,  $p < 0.001$ , as compared with the controls.

### Figure 2

#### *Measurements of genistein and $\beta$ BHC in serum.*

The serum of genistein- and  $\beta$ BHC-loaded mice was analyzed for changes in levels of genistein or  $\beta$ BHC, before and after fasting. The white bars indicate the concentration of genistein or  $\beta$ BHC 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 nmoles of genistein or  $\beta$ BHC per liter of serum. The determinations were repeated twice on five mice per group. GEN: genistein-loaded mice  $\pm$  48 h fasting;  $\beta$ BHC:  $\beta$ BHC-loaded mice  $\pm$  48 h fasting. Bars represent the average  $\pm$  SE; \*,  $p < 0.05$ , as compared with the controls.

### Figure 3

#### *Effect of fasting on reporter regulation in testes of transgenic mice loaded with $\beta$ BHC or genistein or both.*

Luciferase values in the testes of genistein-,  $\beta$ BHC-, genistein-plus- $\beta$ BHC- 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  $\beta$ BHC (i.p.), 50 mg/kg of genistein (oral gavage) or both compounds in combination once a day, for 3 consecutive days (total  $\beta$ BHC and genistein 300 mg/kg 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  $-80^{\circ}\text{C}$  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  $\pm$  SE; \*,  $p < 0.05$ ; \*\*,  $p < 0.001$ , as compared with the controls.

### Figure 4

#### *Genistein interferes with the action of organochlorines and estradiol.*

A-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 post injection the mice were orally treated with increasing concentrations of genistein (50, 500, or 5,000  $\mu\text{g}/\text{kg}$ ) 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; Villa et al, 2004; Penza et al 2004; Mussi et al 2005). Controls were orally treated with vegetable oil. C) Adult mice were injected with oil or an oil solution of 5  $\mu\text{g}/\text{kg}$  17 $\beta$ -E2, kept for 6 h and orally treated for the same time with increasing concentrations of genistein (50, 500 or 5,000  $\mu\text{g}/\text{kg}$ ). 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-E) Adult mice were injected with oil or an oil solution of 50 mg/kg  $\beta$ BHC (D) or 50 mg/kg p,p'-DDT (E) and kept for 16 h. At 6 h post injection the mice were orally treated with increasing concentrations of genistein (50, 500 or 5,000  $\mu\text{g}/\text{kg}$ ) 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 three hours (not shown).

The experiments were repeated three times with a total of six animals per group. Bars represent the average  $\pm$  SE of two individual experiments, each performed in triplicate. \*,  $p < 0.05$ , \*\*,  $p < 0.001$ , as compared with the control.

## References

- Adlercreutz, H., Markkanen, H., and Watanabe, S. (1993). Plasma concentrations of phyto-oestrogens in Japanese men. *Lancet* **342**, 1209-1210.
- Beaufrand, M.J., Poullain, B., Klein, D., and Derby, G. (1978). Release of organochloride pesticides during acute weight losses. *Toxicol. Eur. Res.* **1**, 39-43.
- Beck, V., Rohr, U., and Jungbauer, A. (2005). Phytoestrogens derived from red clover: an alternative to estrogen replacement therapy? *J. Steroid Biochem. Mol. Biol.* **9**, 499-518.
- Bhakta, D., Higgins, C.D., Sevak, L., Mangtani, P., Adlercreutz, H., McMichael, A.J., and dos Santos, S. I. (2006). Phyto-oestrogen intake and plasma concentrations in South Asian and native British women resident in England. *Br. J. Nutr.* **95**, 1150-1158.
- Bigsby, R.M., Caperell-Grant, A., and Madhukar, B.V. (1997). Xenobiotics released from fat during fasting produce estrogenic effects in ovariectomized mice. *Cancer Res.* **57**, 865-869.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-251.
- Brena, A., Ciana, P., Sparaciari, P., Bonetti, E., Di Lorenzo, D., Maggi. (2005). Estrogenic activities in rodent estrogen-free diet. Estrogenic activities in rodent estrogen-free diets. *Endocrinology.* **146**, 5144-5150.

Chang, H.C., Churchwell, M.I., Delclos, K.B., Newbold, R.R., and Doerge, D.R. (2000). Mass spectrometric determination of Genistein tissue distribution in diet-exposed Sprague-Dawley rats. *J. Nutr.* **130**, 1963-1970.

Ciana, P., Raviscioni, M., Mussi, P., Vegeto, E., Que, I., Parker, M.G., Lowik, C., Maggi A. (2003) In vivo imaging of transcriptionally active estrogen receptors. *Nat. Med.* **9**, 82-86.

Ciana, P., Di Luccio, G., Belcredito, S., Pollio, G., Vegeto, E., Tatangelo, L., Tiveron, C., and Maggi, A. (2001). Engineering of a mouse for the in vivo profiling of estrogen receptor activity. *Mol. Endocrinol.* **15**, 1104-1113.

Coldham, N.G., and Sauer, M.J. (2000). Pharmacokinetics of [(14)C]Genistein in the rat: gender-related differences, potential mechanisms of biological action, and implications for human health. *Toxicol. Appl. Pharmacol.* **164**, 206-215.

Cornwell, T., Cohick, W., and Raskin, I. (2004). Dietary phytoestrogens and health. *Phytochemistry* **65**, 995-1016. (Review).

Dang, Z.C., Audinot, V., Papapoulos, S.E., Boutin, J.A., and Lowik, C.W. (2003). Peroxisome proliferator-activated receptor gamma (PPARgamma) as a molecular target for the soy phytoestrogen genistein. *J. Biol. Chem.* **278**, 962-967.

Dang, Z., and Lowik, C.W. (2004). The balance between concurrent activation of ERs and PPARs determines daidzein-induced osteogenesis and adipogenesis. *J. Bone Miner. Res.* **19**, 853-861.

de Vet, J.R., Wood, K.V., De Luca, M., Helinski, D.R., and Subramani, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7**, 725-737.

Den Hond, E., and Schoeters, G. (2006). Endocrine disrupters and human puberty. *Int. J. Androl.* **29**, 264-271. (Review).

Di Lorenzo, D., Villa, R., Biasiotto, G., Belloli, S., Ruggeri, G., Albertini, A., Apostoli, P., Ravisconi, M., Ciana, P., and Maggi, A. (2002). Isomer-specific activity of dichlorodiphenyltrichloroethane with estrogen receptor in adult and suckling reporter mice. *Endocrinology* **143**, 4544-4551.

Falconer, I.R. (2006). Are endocrine disrupting compounds a health risk in drinking water ? *Int. J. Environ. Res. Public Health.* **3**, 180-184.

Gao, S., Liu, G.Z., and Wang, Z. (2004). Modulation of androgen receptor-dependent transcription by resveratrol and genistein in prostate cancer cells. *Prostate.* **59**, 214-225.

(10) Gould, S.J., Subramani, S. (1988). Firefly luciferase as a tool in molecular and cell biology. *Anal. Biochem.* **175**, 5-13. (Review).

Hedlund, T.E., Maroni, P.D., Ferucci, P.G., Dayton, R., Barnes, S., Jones, K., Moore, R., Ogden, L.G., Wahala, K., Sackett, H.M., and Gray, K. (2005). Long-term dietary habits affect soy isoflavone metabolism and accumulation in prostatic fluid in caucasian men. *J. Nutr.* **135**, 1400-1406.

Hedlund, T.E., Bokhoven, A., Johannes, W.U., Nordeen, S.K., and Ogden, L.G. (2006). Prostatic fluid concentrations of isoflavonoids in soy consumers are sufficient to inhibit growth of benign and malignant prostatic epithelial cells in vitro. *Prostate*. **66**, 557-566.

Henson, M.C., and Chedrese, P.J. (2004). Endocrine disruption by cadmium, a common environmental toxicant with paradoxical effects on reproduction. *Exp. Biol. Med. (Maywood)*. **229**, 383-392. (Review).

Herron, R.E., and Fagan, J.B. (2002). Lipophilic-mediated reduction of toxicants in humans: an evaluation of an ayurvedic detoxification procedure. *Altern. Ther. Health. Med.* **8**, 40-51.

Hirai, Y., Sakai, S., Watanabe, N., and Takatsuki, H. (2004). Congener-specific intake fractions for PCDDs/DFs and Co-PCBs: modeling and validation. *Chemosphere*. **54**, 1383-1400.

Hoyer, P.B. (2001). Reproductive toxicology: current and future directions. *Biochem. Pharmacol.* **62**, 1557-1564.

Huang, X., Chen, S., Xu, L., Liu, Y., Deb, D.K., Plataniias, L.C., and Bergan, R.C. (2005). Genistein inhibits p38 map kinase activation, matrix metalloproteinase type 2, and cell invasion in human prostate epithelial cells. *Cancer Res.* **65**, 3470-3478.

Inoue, K., Okumura, H., Higuchi, T., Oka, H., Yoshimura, Y., and Nakazawa, H. (2002). Characterization of estrogenic compounds in medical polyvinyl chloride tubing by gas

chromatography-mass spectrometry and estrogen receptor binding assay. *Clin. Chim. Acta* **325**, 157-163.

Kim, J., and Kwon, C. (2001). Estimated dietary isoflavone intake of Korean population based on National Nutrition Survey. *Nutr. Res.* **21**, 947-953.

Kimura, M., Arai, Y., Shimoi, K., and Watanabe, S. (1998). Japanese intake of flavonoids and isoflavonoids from foods. *J. Epidemiol.* **8**, 168-175

King, R.A., and Bursill, D.B. (1998). Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. *Am. J. Clin. Nutr.* **67**, 867-872.

Kuiper, G. G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., and Gustafsson, J. A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor  $\beta$ . *Endocrinology* **139**, 4252-4263.

Legler, J., and Brouwer, A. (2003). Are brominated flame retardants endocrine disruptors? *Environ. Int.* **29**, 879-885. (Review).

Lintelmann, J., Katayama, A., Kurihara, N., Shore, L., Wenzel, A. (2003). Endocrine disruptors in the environment (IUPAC Technical Report). *Pure Appl. Chem.* **75**, 631-681.

Lordo, R.A., Dinh, K.T., and Schwemberger, J.G. (1996). Semivolatile organic compounds in adipose tissue: estimated averages for the US population and selected subpopulations. *Am. J. Public Health.* **86**, 1253-1259.

Manach, C., Morand, C., Crespy, V., Demigne, C., Texier, O., Regeat, F., and Remesy, C. (1998). Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties. *FEBS Lett.* **426**, 331-336.

Mussi, P., Ciana, P., Raviscioni, M., Villa, R., Regondi, S., Agradi, E., Maggi, A., Di Lorenzo, D. (2005). Activation of brain estrogen receptors in mice lactating from mothers exposed to DDT. *Brain Res Bull.* **65**, 241-247.

Nettleton, J.A., Greany, K.A., Thomas, W., Wangen, K.E., Adlercreutz, H., Kurzer, M.S. (2004). Plasma phytoestrogens are not altered by probiotic consumption in postmenopausal women with and without a history of breast cancer. *J. Nutr.* **134**, 1998-2003.

Penza, M., Bonetti, E., Villa, R., Ganzerla, S., Bergonzi, R., Biasiotto, G., Caimi, L., Apostoli, P., Ciana, P., Maggi, A., and Di Lorenzo, D. (2004). Whole body action of xenoestrogens with different chemical structures in estrogen reporter male mice. *Toxicology* **205**, 65-73.

Penza, M., Montani, C., Romani, A., Vignolini, P., Pampaloni, B., Tanini, A., Brandi, ML., Alonso-Magdalena, P., Nadal, A., Ottobrini, L., Parolini, O., Bignotti, E., Calza, S., Maggi, A., Grigolato, P.G., and Di Lorenzo, D. ( 2006 ). Genistein affects adipose tissue deposition in a dose-dependent and gender-specific manner. *Endocrinology* **147**, 5740-5751.

- Rannikko, A., Petas, A., Rannikko, S., Adlercreutz, H. (2006). Plasma and prostate phytoestrogen concentrations in prostate cancer patients after oral phytoestrogen supplementation. *Prostate* **66**, 82-7.
- Romani, A., Vignolini, P., Galardi, C., Aroldi, C., Vazzana, C., and Heimler, D. (2003). Polyphenolic Content in Different Plant Parts of Soy Cultivars Grown Under Natural Conditions. *J. Agric. Food. Chem.* **51**, 5301-5306.
- Safe, S. (1992). Toxicology, structure-function relationship and human and environmental health impacts of polychlorinated biphenyls: progress and problems. *Environ. Health Perspect.* **100**, 258-268.
- Schultz, J.R., Petz, L.N., and Nardulli, A.M. (2005). Cell- and ligand-specific regulation of promoters containing activator protein-1 and Sp1 sites by estrogen receptors alpha and beta. *J. Biol. Chem.* **280**, 347-354.
- Simonich, S.L., and Hites, R.A. (1995). Global distribution of persistent organochlorine compounds. *Science* **269**, 1851-1854.
- Slikker, W., Jr, Scallet, A.C., Doerge, D.R., and Ferguson, S.A. (2001). Gender-based differences in rats after chronic dietary exposure to genistein. *Int. J. Toxicol.* **20**, 175-179.

Toda, C., Okamoto, Y., Ueda, K., Hashizume, K., Itoh, K., and Kojima, N. (2004). Unequivocal estrogen receptor-binding affinity of phthalate esters featured with ring hydroxylation and proper alkyl chain size. *Arch. Biochem. Biophys.* **431**, 16-21.

Uehar, M., Arai, Y., Watanabe, S., and Adlercreutz, H. (2000). Comparison of plasma and urinary phytoestrogens in Japanese and Finnish women by time-resolved fluoroimmunoassay. *Biofactors* **12**, 217-225.

Vedrine, N., Mathey, J., Morand, C., Brandolini, M., Davicco, M.J., Guy, L., Remesy, C., Coxam, V., Manach, C. (2006). One-month exposure to soy isoflavones did not induce the ability to produce equol in postmenopausal women. *Eur. J. Clin. Nutr.* **60**, 1039-1045

Villa, R., Bonetti, E., Penza, M.L., Iacobello, C., Bugari, G., Bailo, M., Parolini, O., Apostoli, P., Caimi, L., Ciana, P., Maggi, A., and Di Lorenzo, D. (2004). Target-specific action of organochlorine compounds in reproductive and nonreproductive tissues of estrogen-reporter male mice. *Toxicol. Appl. Pharmacol.* **201**, 137-148.

Wang, X.J., Bartolucci-Page, E., Fenton, S.E., and You, L. (2006). Altered Mammary Gland Development in Male Rats Exposed to Genistein and Methoxychlor. *Toxicol. Sci.* **91**, 93-103.

Watanabe, S., Yamaguchi, M., Sobue, T., Takahashi, T., Miura, T., Arai, Y., Mazur, W., Wahala, K., and Adlercreutz, H. (1998). Pharmacokinetics of soybean isoflavones in plasma, urine and feces of men after ingestion of 60 g baked soybean powder (kinako). *J. Nutr.* **128**, 1710-1715.

Yamamoto, S., Sobue, T., Sasaki, S., Kobayashi, M., Arai, Y., Uehara, M., Adlercreutz, H., Watanabe, S., Takahashi, T., Itoi, Y., Iwase, Y., Akabane, M., and Tsugane, S. (2001). Validity and reproducibility of a self-administered food-frequency questionnaire to assess isoflavone intake in a Japanese population in comparison with dietary records and blood and urine isoflavones. *J. Nutr.* **131**, 2741-2747.

You, L., Casanova, M., Bartolucci, E.J., Fryczynski, M.W., Dorman, D.C., Everitt, J.I., Gaido, K.W., Ross, S.M., and Heck, H. (2002). Combined effects of dietary phytoestrogen and synthetic endocrine-active compound on reproductive development in Sprague-Dawley rats: genistein and methoxychlor. *Toxicol. Sci.* **66**, 91-104.

Yu, Z., Li, W., and Liu, F. (2004). Inhibition of proliferation and induction of apoptosis by genistein in colon cancer HT-29 cells. *Cancer Lett.* **215**, 159-166.

