ELSEVIER

Journal of Chromatography B, 777 (2002) 249–260

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Estrogen receptor expression in the prostate of rats treated with dietary genistein

Abraham Dalu[a], Betty S. Blaydes[a], Corey W. Bryant[a], John R. Latendresse[b], Constance C. Weis[a], K. Barry Delclos[a,*]

[a]*Division of Biochemical Toxicology, National Center for Toxicological Research, Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079, USA*

[b]*Pathology Associates International Inc., Jefferson, AR 72079, USA*

Abstract

Steroid hormones and their receptors play critical roles in the growth, development, and maintenance of the male reproductive tract. Genistein, a naturally occurring isoflavonoid primarily found in soybeans, interacts with estrogen receptors α and β (ERα and β), with preferential affinity for ERβ. This is one mechanism whereby genistein may affect growth and development and potentially alter susceptibility to carcinogenesis. Previous studies have indicated effects of soy and/or genistein in the male rodent reproductive tract under certain exposure conditions. The current study was undertaken to determine if modulation of the expression of ERα and ERβ by dietary genistein may contribute to those effects. Rats in a two-generation study were fed 0, 5, 100, or 500 ppm genistein prior to mating and through pregnancy and lactation. At weaning, male pups were selected in each of the F_1 and F_2 generations and half of the pups continued on the same diet as their dams (G/G, continuous exposure) while their litter mates were placed on control chow (G/C, gestational and lactational exposure) until sacrifice on PND 140. Male reproductive organ weights, serum levels of testosterone and dihydrotestosterone (DHT), and ERα and ERβ protein levels in the ventral and dorsolateral prostate were the endpoints measured. Prostate sections were also evaluated microscopically. Statistically significant elevations in testosterone and DHT were observed in PND 140 animals from the F_1 generation, but they were not accompanied by organ weight changes. Body weight in the continuously dosed 500 ppm F_1 PND 140 animals was depressed relative to control, but organ weights in animals of either generation showed few treatment-related effects. While estrogen receptor levels were quite variable, levels of ERβ in the dorsolateral prostate were significantly depressed in all dose groups in the G/C exposure and the high dose group of the G/G exposure in F_1 rats, but not in F_2 rats. Given the growing body of knowledge on the significance of ERβ in the prostate, the evidence for apparent down regulation of this receptor by genistein may have implications for reproductive toxicity and carcinogenesis that warrant further investigation.
Published by Elsevier Science B.V.

Keywords: Estrogen receptor expression; Prostate; Genistein

1. Introduction

The diet is a major source of human exposure to endocrine active compounds, particularly phytoestrogens [1]. The consumption of soy-containing foods, which contain the isoflavone genistein as the

*Corresponding author. Tel.: +1-870-543-7372; fax: +1-870-543-7136.

E-mail address: bdelclos@nctr.fda.gov (K. Barry Delclos).

1570-0232/02/$ – see front matter Published by Elsevier Science B.V.
PII: S1570-0232(02)00346-X

predominant phytoestrogen, is considerably higher in certain Asian populations than in the Western world [2]. A body of epidemiological, animal, and in vitro work suggests that soy may play a protective role against cancers that are more prevalent in Western countries than in Asia, particularly breast and prostate cancer [3,4]. In fact, soy-derived preparations are being evaluated as preventive and therapeutic agents against prostate cancer in clinical trials [5,6]. Genistein and several other phytoestrogens have been shown to interact with both the classical estrogen receptor, ER-α, and the more recently discovered ER-β, with preferential binding affinity to the latter [7,8]. In addition to this estrogenic activity, genistein has been shown to interact with a variety of other molecular targets, such as tyrosine kinases, through which it might modulate tumor growth [9]. However, the doses required to affect many of these targets are generally much higher than those required for interaction with estrogen receptors [10]. While multiple components of soy are likely to be involved in the inhibition of tumors in various tissues in animal models [11,12], the multiple mechanisms through which genistein has been shown to potentially inhibit tumorigenesis have directed considerable research attention to this isoflavone.

Receptors for both androgens and estrogens are widely expressed in the male reproductive tract and play roles in its development and function [13]. The prominence of prostate cancer and the rising incidence of testicular cancers among young men in some Western populations, as well as the controversial reports of a rise in male reproductive problems, such as the incidence of hypospadias and decreased sperm counts, have led to considerable research into determining whether environmental agents that interact with estrogen and androgen signaling pathways may contribute to adverse effects in human and wildlife populations [13–17]. While the potential role of synthetic environmental chemicals has been a major focus of this work, exposures to naturally occurring phytoestrogens as causative or protective factors also need to be considered. Rates of many of the conditions mentioned above, such as hypospadias [18] and testicular cancer [19], as well as prostate cancer, are lower in Asian populations than in Western populations, indicating that the dietary levels of phytoestrogens to which Asians are exposed do not lead to these problems in Asian males or may even be protective. On the other hand, a recent study conducted in the UK found an increased incidence of hypospadias in males born to vegetarian mothers, which suggested a possible involvement of phytoestrogens [20]. Given the fact that exposure to soy and genistein through the consumption of infant soy formula [21] is greater than that achieved in typical Asian soy-containing diets and that similar high levels of exposure are possible from the intake of soy dietary supplements for cardiovascular benefits and purported relief of diverse adverse menopausal symptoms [22], it seems important to evaluate potential adverse consequences of soy and genistein, and to define the dose levels and exposure conditions under which they might occur.

Developmental exposure of males to potent estrogens has clearly been shown to disrupt the endocrine system and contribute to long term adverse structural and functional changes in the reproductive tract, including cancers [13–17]. Neonatal exposure of rats to high doses of exogenous estrogens has been shown to result in marked developmental abnormalities in the prostate gland and permanent alterations in the growth, secretory function, and activational response to androgen during adulthood [23–25]. Furthermore, estrogen modulates the expression of androgen receptor and ERα and β in the developing and adult rat prostate lobes [25–29]. The testes are also adversely affected by neonatal exposure to high doses of estrogens, and a loss of expression of androgen receptor has been found in association with these changes [13,30]. Several studies have examined the effects of soy or genistein on tissues of the male reproductive tract in rodents. Feeding soy-containing diets to mice and rats from the time of fertilization affected prostate growth [31,32], however, estrogen-like effects were not seen when a soy diet was fed to adult mice [33]. Subcutaneous injections of 2.5 mg genistein/kg per day to adult male mice for 9 days reduced testicular and prostate weights, testicular and serum testosterone concentrations, and pituitary LH content [34]. In the latter study, higher doses were required to produce persistent prostatic changes in neonates. In rats, neonatal administration of 4 mg/kg per day genistein to rats by sc injection produced transient effects on

the epithelial cells of the efferent ducts joining the testis to the initial segment of the epididymis [35] and delayed pubertal spermatogenesis [30]. In the latter study, male rats maintained on a diet containing 15.5% soymeal flour also showed reduced body and testis weights and elevated FSH relative to rats fed a soy-free diet [30]. Casanova et al. [36] saw a small decrease in ventral prostate weight at 1000 ppm. Dalu et al. [37] found that feeding young Lobund–Wistar rats (5–6 weeks old) dietary genistein up to 1000 ppm for 3 weeks did not affect body or prostate weight or serum testosterone, but the 1000 ppm dose did result in down regulation of EGF receptor and the related ErbB2/Neu receptor in the dorsolateral prostate. Fritz et al. [38] found no effect on the time of testicular descent in the male pups exposed to genistein in utero until weaning via dietary administration (25 and 250 ppm) to the dams. Nagao et al. [39] have recently reported reduced body and epididymal weights in rats treated neonatally by gavage with genistein ranging from 12.5 to 100 mg/kg body weight per day as the only significant effects in male pups.

Our laboratory has been investigating the effects of genistein as part of a comprehensive evaluation of several endocrine active compounds sponsored by the National Toxicology Program. Genistein was selected for study because of its estrogenic activity, its presence in soy infant formulas, and the increasingly marketed soy-containing dietary supplements. A series of short term studies with genistein was conducted as a prelude to a multigeneration study, and the results of several of these studies have been reported [40–42]. These studies used dietary administration of genistein to Sprague–Dawley rats from the National Center for Toxicological Research (NCTR) breeding colony from gestation day 7 through postnatal days (PND) 50, 63, or 77, depending on the endpoint examined, in a soy- and alfalfa-free diet at doses ranging from 5 to 1250 ppm. The ingested dose ranged from approximately 0.2 to 200 mg/kg body weight per day in these animals [42]. Blood and tissue levels have also been measured in these animals over this dose range [43], and it is important to note that the serum levels ranged from approximately 10 n*M* to 8 μ*M*, which covers the range of human exposures. It was also demonstrated, consistent with other published data [37,38,44], that reproductive organs contained higher proportions of aglycone, the form of genistein that binds to estrogen receptor, than serum and that the tissue concentrations were within a range compatible with binding to estrogen receptors [45]. In addition, transplacental transfer of genistein was demonstrated under the conditions of the study [46].

In the animals used for the reproductive assessment, which were necropsied at PND 50, several effects in the male reproductive tract were observed that were consistent with weak estrogenic activity [42]. Both the relative and absolute ventral prostate weights showed significant decreasing linear trends and were significantly lower in the 1250 ppm dose group. Dorsolateral prostate weights were not significantly affected. A treatment-related increase of inflammation of the dorsolateral prostate and depletion of secretory fluid in the ventral prostate were noted at 1250 ppm. Effects of genistein on the testis and the epididymis consistent with a possible disruption of or delay in spermatogenesis were noted at 1250 ppm.

In the present study, males treated from conception through PND 21 or PND 140 with dietary genistein were evaluated at PND 140 for alterations in reproductive organ weights and serum testosterone and dihydrotestosterone. Because estrogen receptors play an important role in the growth and development of the male reproductive tract, the expression of ER-α and ER-β proteins was also examined.

2. Experimental

2.1. Chemicals

Genistein (4′,5,7-trihydroxyisoflavone), purity >99%, was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Primary antibodies (mouse anti-ERα and goat anti-ERβ) and secondary antibodies (IgG conjugated to horse radish peroxidase) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). BCA protein assay kits and SuperSignal chemiluminescent substrate were purchased from Pierce (Rockford, IL), and radioimmunoassay kits for the measurement of serum testosterone and dihydrotestosterone (DHT) levels were purchased from Diagnostic Products

Corporation (Los Angeles, CA) and Diagnostic Systems Laboratories (Webster, TX), respectively.

2.2. Animals, diet and treatment

All rats used in the studies described here were generated from a larger multigeneration (PND 140 animals) study designed to assess the toxicity of dietary genistein in a variety of organ systems. Weanling female CD rats were obtained from the NCTR Breeding Colony and placed on an irradiated soy- and alfafa-free diet (5K96, purchased from Purina Mills, St. Louis, MO) 2 weeks prior to mating of 10 to 11-week-old animals with males of similar ages. This diet meets the nutritional specifications of the NIH-31 diet, the autoclavable chow typically used for animal studies at our institution, but casein replaced the protein contributed by soy and alfalfa, soy oil was replaced by corn oil, and the vitamin mix was adjusted for irradiation. The control diet was analyzed for genistein and daidzein after hydrolysis of conjugates, and was found by LC-UV to be below the detection limit of 0.5 ppm [43]. Analysis of 10 different lots of the 5K96 chow by LC-ES–MS, which had a limit of detection of approximately 0.05 ppm, indicated that levels of genistein and daidzein were approximately 0.5 ppm each (Ref. [47] and D. Doerge, personal communication). Stability of genistein in the diet mix was determined for the 5 ppm mix and was found to be within tolerance limits ($\pm$10% of target dose) for up to 6 months after mixing. The rats were maintained under controlled conditions of temperature (23$\pm$3 °C), relative humidity (50$\pm$20%), and illumination (12 h light, 12 h dark). All rats had free access to 5K96 diet and water. All animal husbandry and procedures were conducted according to the Institutional Animal Care and Use Committee guidelines. Both male and female F_0 rats were placed on 0, 5, 100, or 500 ppm of dietary genistein at least 28 days prior to mating. On approximately PND70, the male and female rats were paired for mating to produce F_1 rats. F_1 parental rats continued on the same genistein diet throughout mating, gestation, and lactation. Twelve litters from each dose group in each of the two generations were used in this study. At weaning, half of the F_1 and F_2 male pups from the genistein dosed diet groups were continued on the same dose of dietary genistein (designated G/G) while their littermates were switched to control diet (designated G/C) until PND140. In most cases, a single pair of males was used from each of the litters, giving 12 G/G animals and 12 G/C animals per dose group. In a few cases, two pairs of males were used from some of the 12 litters, resulting in up to 17 animals per dose group. In the latter situation, the statistical model used for analysis took into account the fact that multiple animals from a single litter were used (see below).

At sacrifice, trunk blood was collected after decapitation of non-anesthetized rats for the evaluation of serum testosterone and DHT levels. Ventral prostate, dorsolateral prostate, and testes were removed, weighed, snap frozen (n=5–6) in liquid nitrogen, and stored at −80 °C for the Western blot analysis of ERα, and ERβ. The remainder of the tissues were weighed and then fixed in Bouin's fixative for 24 h for histological evaluation of hematoxylin and eosin (H&E)-stained sections.

2.3. Immunoprecipitation

Immunoprecipitation of the estrogen receptors was carried out prior to Western blot analysis because of weak signals of ER-α and ER-β when tissue lysates were used. Dorsolateral and ventral prostate from each rat were pulverized under liquid nitrogen and lysed in five volumes of lysis buffer according to the procedure provided by Santa Cruz Biotechnologies, (Santa Cruz, CA). Briefly, protein was extracted in lysis buffer containing 1% Triton X-100, 10 m*M* Tris (pH 7.4), 1 m*M* EDTA, 100 m*M* NaCl, 1 m*M* HEPES, 2 m*M* sodium orthovanadate, 1 m*M* PMSF, 1 m*M* leupeptin, and 1 m*M* aprotinin. Protein concentration was determined using Pierce BCA Protein Assay Reagents (Pierce, Rockford, IL). Tissue lysates of the dorsolateral prostate or ventral prostate containing 1 mg of total protein were mixed either with 10 μl mouse anti-estrogen receptor alpha (ERα) monoclonal antibody or with 10 μl goat anti-estrogen receptor beta (ERβ) polyclonal antibody (Santa Cruz Biotechnologies). For immunoprecipitation of ERα or ERβ proteins, the contents were incubated at 4 °C for 30 min on a rotator apparatus, followed by addition of 50 μl of Protein A-Agarose (Oncogene Research Products, Cam-

bridge, MA), and overnight incubation at 4 °C on a rotary apparatus. The samples were washed three times with lysis buffer and centrifuged at 12 000 *g* at 4 °C. The immunoprecipitates were resuspended in Laemmli sample buffer containing 5% β-mercaptoethanol, boiled for 5 min and centrifuged. The supernatant was subjected to 10% SDS–PAGE.

2.4. Western-blot analysis

Immunoprecipitates of ventral and dorsolateral prostate for ERα and ERβ were resolved by 10% SDS–PAGE in reduced sample buffer. The proteins were electrophoretically transferred to a nitrocellulose membrane, blocked with 5% non-fat dry milk in Tris buffered saline containing 0.1% Tween-20 (TBS-T), immunoblotted with anti-ERα, or anti-ERβ primary antibody, followed by incubation with appropriate secondary antibody coupled with horseradish peroxidase (Santa Cruz Biotechnologies), subsequently washed, and the proteins visualized with Pierce SuperSignal chemiluminescent substrate (Pierce, Rockford, IL). The membranes were then exposed to autoradiograph film and developed using a Konica Medical Film Processor (Konica, SRX-101, Wayne, NJ). Densitometric analysis of the protein bands was performed using an I.S.-1000 Digital Imaging System (Alpha Innotech Corporation, San Leandro, CA). Protein levels are reported as percentage of control.

2.5. Determination of serum testosterone and DHT levels

Trunk blood was collected from each rat at the time when they were sacrificed. Serum was prepared and stored at −80 °C until analyzed for the evaluation of testosterone and DHT levels by radioimmunoassay according to the procedures provided by the manufacturer.

2.6. Statistical analysis

Body and organ weights and hormone data were analyzed using the mixed procedure of SAS® v.8. Generation and dose were treated as fixed effects and litter was treated as a random effect. When significant effects were indicated ($P<0.05$), differences in the appropriate least squares means were tested using a *t*-test adjusted for all possible comparisons. Steroid receptor data from densitometric analysis of Western blots were compared to the respective control group by 2-tailed *t*-test analysis ($P\leq0.05$) and a correction for multiple comparisons was applied [48]. All data are summarized as mean values±SEM.

3. Results

3.1. Effects of dietary genistein on body and reproductive organ weights and prostate histology

Body and reproductive organ weights of the F_1 and F_2 PND140 rats treated with dietary genistein are shown in Table 1. There was a significant decreasing trend in the body weights of F_1 continuously dosed (G/G) animals and a significant depression in body weight in the 500 ppm dose group relative to control. There were no significant differences in body weights of F_2 animals of any dose group relative to controls. Similarly, there were no significant treatment-related differences in the weights of dorsolateral prostate, ventral prostate, seminal vesicles, epididymides, and testes of the F_1 and F_2 rats maintained on genistein diet relative to controls whether these analyses were conducted on absolute weights, ratio to body weight, or with body weight as a covariant. When comparisons were made within dose groups (G/G vs. G/C)), the body weights of continuously dosed 500 ppm animals (G/G) were less than those of animals exposed to 500 ppm genistein during gestation and lactation only (G/C). The only other within dose group differences observed were a lower body weight in the F_2 5 ppm G/C group relative to the G/G group and a lower absolute seminal vesicle weight in the F_1 5 ppm G/C group relative to the corresponding G/G group. These differences seem likely to be chance occurrences and not treatment-related effects.

Tissue sections from six to 10 animals from each dose group in the F_1 and F_2 generations were stained with H&E and evaluated. Mild inflammation (both dorsolateral and ventral lobes) and secretory depletion (ventral lobe only) were observed with equal incidence and severity in both control and treated

Table 1
Effects of dietary genistein on the mean body weight and reproductive tract organ weights of PND 140 male Sprague–Dawley rats[a]

Dietary genistein (ppm)[b]	Body weight (g)	Dorsolateral prostate (mg)	Ventral prostate (mg)	Seminal vesicles (g)	Epididimydes (g)	Testes (g)
F_1						
0	490±9	335±0.02	462±0.04	1.46±0.06	1.53±0.08	3.04±0.22
5 (G/G)	497±1	370±0.02	488±0.04	1.62±0.26	1.57±0.1	3.42±0.7
5 (G/C)	480±5	324±0.02	526±0.03	1.52±0.08#	1.69±0.3	3.51±0.06
100 (G/G)	461±38	309±0.02	536±0.05	1.33±0.07	1.81±0.1	3.14±0.07
100 (G/C)	492±14	335±0.01	488±0.03	1.44±0.07	1.46±0.07	3.31±0.19
500 (G/G)	454±9*	336±0.02	508±0.01	1.34±0.05	1.62±0.12	3.29±0.07
500 (G/C)	483±11#	330±0.02	468±0.03	1.29±0.04	1.72±0.19	3.34±0.07
F_2						
0	478±10	311±0.02	512±0.02	1.12±0.06	1.28±0.03	3.28±0.11
5 (G/G)	498±12	325±0.02	543±0.03	1.05±0.06	1.36±0.05	3.32±0.07
5 (G/C)	465±12#	309±0.01	498±0.03	1.05±0.06	1.31±0.04	3.30±0.07
100 (G/G)	489±10	364±0.02	524±0.03	1.08±0.07	1.26±0.06	3.18±0.19
100 (G/C)	478±12	327±0.02	524±0.02	1.05±0.04	1.25±0.09	3.17±0.21
500 (G/G)	454±13	341±0.02	542±0.03	1.01±0.04	1.29±0.04	3.27±0.05
500 (G/C)	494±38#	346±0.03	556±0.05	1.12±0.09	1.33±0.11	3.29±0.27

[a] F_0 male and female rats were exposed to 0, 5, 100 or 500 ppm of dietary genistein for 28 days before mating and were maintained on the same diet during mating. The F_1 and F_2 male pups were exposed to the same concentration of dietary genistein in utero from gestation day 1. At weaning, half of the pups from each dietary genistein group continued on the same concentration of genistein exposed to in utero (designated G/G), whereas the second half of the pups from each dietary genistein group were switched to a control diet at weaning (designated G/C). All rats were necropsied on PND 140. Total body, dorsolateral prostate, ventral prostate, seminal vesicles, epididymis, and testes were weighed at necropsy. Presented in the table are the mean values (±SEM) for each treatment group (N=12–17). Organ weights relative to body weights were also calculated and analyzed, but no significant effects were observed (data not shown). *Denotes significant difference from the respective control group. #Denotes significant differences within dose (G/G vs. G/C) of the same generation ($P \leq 0.05$).

groups; there appeared to be no treatment-related lesions observed in this set of animals at PND 140.

3.2. Serum testosterone and DHT levels

Table 2 shows serum testosterone and DHT levels in F_1 and F_2 PND 140 rats. In F_1 rats, serum testosterone and DHT levels exhibited a linear increasing trend only in rats receiving dietary genistein throughout the experiment (G/G). A significant increase in testosterone levels relative to controls was noted at 500 ppm in both continuously (G/G) and gestationally/lactationally exposed (G/C) animals, while DHT was increased in the G/G and G/C 100 ppm groups and the G/G 500 ppm group. In the F_1 500 ppm G/C group, both testosterone and DHT were significantly lower than in the corresponding G/G group. In the F_2 animals, no significant differences from control were seen in any group for testosterone or DHT.

3.3. Expression of estrogen receptors in ventral and dorsolateral prostate

Estrogen receptor levels in the prostate lobes of PND 140 animals exposed to genistein from conception through PND 140 (G/G) or through PND 21 (G/C) are shown in Table 3. In F_1 animals, ER-β was decreased by approximately 32–42% relative to control in the dorsolateral prostate in all three G/C dose groups and in the 500 ppm G/G dose group. While the mean ER-β levels in these same dose groups were slightly depressed in the F_2 generation, these values were not significantly different after a correction for multiple comparisons to the control was made. Other statistically significant depressions in ER-β levels were seen in the ventral prostate in

Table 2
Effects of two-generation dietary genistein exposure on the serum testosterone (T) and dihydrotestosterone (DHT) levels in male Sprague–Dawley rats[a]

Dietary genistein (ppm)[b]	F_1		F_2	
	T (ng/ml)	DHT (pg/ml)	T (ng/ml)	DHT (pg/ml)
0	1.58±0.02	55±7	1.04±0.14	57±7
5 (G/G)	2.01±0.04	71±11	1.21±0.18	41±4
5 (G/C)	1.39±0.03#	50±6	0.75±0.11#	28±2
100 (G/G)	2.21±0.03	99±21*	1.21±0.22	37±6
100 (G/C)	2.13±0.03	91±22*	0.99±0.11	44±5
500 (G/G)	3.08±0.04*	120±35*	0.84±0.20	40±11
500 (G/C)	2.03±0.03*#	60±10#	0.75±0.11	32±4

[a] F_0 male and female rats were exposed to 0, 5, 100, or 500 ppm of dietary genistein for 28 days before mating and were maintained on the same diet during mating. The F_1 and F_2 pups were exposed to the same concentration of dietary genistein in utero throughout gestation. At weaning, half of the pups from each dietary genistein group continued on the same concentration of genistein exposed to in utero (designated G/G), whereas the second half of the pups from each dietary genistein group were switched to a control diet at weaning (designated G/C). All rats were necropsied on PND 140. Trunk blood was collected for the measurement of serum T and DHT levels by radioimmunoassay. Presented in the table are the mean values of the hormones (±SEM) for each treatment group (N=9–16). *Denotes significant difference from the respective control group. #Denotes significant differences within dose (G/G vs. G/C) of the same generation ($P \leq 0.05$).

the 100 ppm G/C group of both generations. ER-α was significantly depressed in the dorsolateral prostate in the 100 ppm continuously dosed group (G/G) and in the ventral prostate in the 500 ppm G/G group.

4. Discussion

In this study, dietary genistein did not alter body or organ weights, but it did cause increased T and DHT serum levels. Further, ER-β levels in the dorsolateral prostate were depressed. The results of the present studies are consistent with those of our earlier report of the effects of dietary genistein up to 1250 ppm administered through gestation to puberty [42] in that there was little evidence of toxicity to the prostate from dietary genistein in the dose range tested, although chronic effects remain to be evaluated.

Studies from other laboratories have shown effects of isoflavones and/or soy on prostate that vary, possibly due to differences in the dose, method or timing of administration, test animal, the presence of components of soy in addition to isoflavones in the test agent, or other experimental factors. Sharma et al. [49] found that a high incidence of inflammation in the lateral prostate of Sprague–Dawley rats consuming a soy-free diet was not present in animals fed a diet containing 17% soy for 11 weeks as adults. On the other hand, Kwon et al. [50] reported that Sprague–Dawley rats treated by gavage with a soy extract (200 mg/kg genistein, 66 mg/kg daidzein) twice daily for 9 weeks starting at 8 weeks of age had an increased incidence of inflammation in the dorsolateral prostate. No changes in serum testosterone or prostate weight were seen in the latter study. Weber et al. [51] reported a decreased prostate weight, both absolute and relative to body weight, as well as a decreased serum testosterone in adult male Sprague–Dawley rats fed a diet containing approximately 600 ppm soy isoflavones as compared to a soy-free diet. Strauss et al. [34] found that adult NMRI mice treated by s.c. injection with 2.5 mg/kg genistein for 9 days had reduced serum testosterone and LH as well as a decreased ventral prostate weight. Mice treated only on PND 1–3 with 50 or 500 mg/kg per day had a decreased ventral prostate weight as adults, but adverse histological effects, similar to those induced by neonatal exposure to DES, were seen only in the animals treated with the high dose. Nagao et al. [39] dosed Sprague–Dawley rats by gavage on PND 1-5 with doses of genistein ranging from 12.5 to 100 mg/kg body weight and found no effects on serum testosterone, ventral prostate weight, or prostate histology. Mäkelä [31]

Table 3
Effect of dietary genistein on estrogen receptor levels in the dorsolateral and ventral prostates of PND 140 SD rats treated from conception through PND 140 (G/G) or conception through PND 21(G/C)[a]

Dietary genistein (ppm)	Receptor level relative to 0 ppm control[b]			
	F_1		F_2	
	ER-α	ER-β	ER-α	ER-β
Dorsolateral prostate				
0	100±16.9	100±12.5	100±20.8	100±15.5
5 (G/G)	103.3±21	78.8±14.9	79.1±12.6	82.5±9.7
5 (G/C)	150.6±24.4	67.8±1.9[c]	68.5±10.7	83.0±5.9
100 (G/G)	59.3±2.3[c]	89.6±15.6	94.9±8.1	90.4±7.3
100 (G/C)	113.0±14.2	59.1±7.9[c]	81.3±7.0	78.6±7.4
500 (G/G)	92.9±3.9	62.1±7.0[c]	131.6±22.9	97.7±12.9
500 (G/C)	87.7±4.0	57.2±4.2[c]	159.1±16.0	84.1±5.8
Ventral prostate				
0	100±8.6	100±5.5	100±5.1	100±2.6
5 (G/G)	92.7±9.2	128.7±10.7	91.8±4.5	96.3±9.4
5 (G/C)	124.6±2.1	97.6±10.8	86.1±5.0	104.6±5.1
100 (G/G)	113.8±15.9	105.3±10.6	94.4±5.2	83.1±6.2
100 (G/C)	131.2±12.8	48.3±6.4[c]	100.6±5.7	79.8±3.8[c]
500 (G/G)	74.5±6.0[c]	59.2±10.6	87.7±10.2	83.2±5.6
500 (G/C)	71.4±14.3	131.08±16.4	83.7±6.6	62.3±12.2

[a] F_0 male and female rats were exposed to 0, 5, 100 or 500 ppm of dietary genistein for 28 days before mating and were maintained on the same diet during mating. The F_1 and F_2 pups were exposed to the same concentration of dietary genistein in utero throughout gestation. At weaning, half of the pups from each dietary genistein group continued on the same concentration of genistein exposed to in utero (designated G/G), whereas the second half of the pups from each dietary genistein group were switched to a control diet at weaning (designated G/C). All rats were necropsied on PND 140. Immunoprecipitates prepared from tissue lysates were prepared as described in the Experimental section from frozen tissues. Electrophoresis was carried out on a 10% SDS–polyacrylamide gel with immunoprecipitate from 1 mg of total protein. The receptor bands were detected as bands of 62 kDa (ER-β) and 66 kDa (ER-α), as described in the Experimental section.

[b] Mean±SEM.

[c] Significantly different from control, $P<0.05$.

found that Sprague–Dawley rats fed a soy-free diet through pregnancy and lactation and then switched to a diet containing 13% soy meal until 10 weeks of age had increased ventral prostate weight relative to animals fed the soy diet throughout their lives. Similar results, although not statistically significant, were obtained by the same group in NMRI mice at 2 months; that is, mice exposed to a soy-free diet early in life tended to have a higher prostate weight at 2 months than animals exposed to a soy-containing diet throughout life [32]. On the other hand, mice exposed to soy throughout life had higher ventral and dorsal prostate weights at 12 months than animals exposed to a soy-free diet [32]. In neonatally estrogenized mice castrated as adults, exposure to s.c. injections of genistein at 2.5 mg/kg for 10 days induced squamous epithelial metaplasia in the periurethral collecting ducts [34], but feeding a diet containing 7% soy meal, approximately 2–5 mg genistein/kg body weight per day, did not induce metaplasia [33]. In adult Lobund–Wistar rats, s.c. injections of 50 mg/kg genistein twice a day for 31 days induced the male accessory sex glands [52], but feeding concentrations ranging from 25 to 1000 ppm genistein for 3 weeks did not significantly effect the weight of the dorsolateral prostate or serum testosterone [37]. Together, all of these studies indicate that soy and genistein can affect the rodent prostate, but conditions under which these exposures could lead to long term adverse or beneficial consequences are not clear.

As indicated above, previous studies that have

looked at testosterone concentrations in rodents treated with soy or genistein by various routes and at various ages have generally found no effect or a reduction in the serum hormone in males. In our present study, there was a significant increasing trend in both serum testosterone and DHT concentrations in the PND 140 F_1 animals that had been continuously dosed with genistein, and significant increases relative to control levels in both the G/G and G/C 500 ppm groups for testosterone and the G/G and G/C mid dose group (100 ppm) and G/G 500 ppm group for DHT. While high concentrations of genistein have been reported to inhibit several steroidogenic enzymes [10], it is possible that genistein could alter levels of steroid synthetic or metabolizing enzymes to bring increases in testosterone and DHT. For example, Weber et al. [53] have found that treatment of adult rats with a phytoestrogen-containing diet can induce 5α-reductase in the amygdala while at the same time reducing the same enzyme in the medial basal hypothalamic preoptic area. Further, Santti et al. [54] showed altered androgen metabolism in different lobes of the prostate following developmental exposure to DES. Recently, Fritz et al. [55] have also reported significantly increased testosterone concentrations in male rats exposed to 25 and 250 ppm dietary genistein from conception to PND 70. DHT and male reproductive organ weights were not significantly altered in those animals. In the present study, the fact that no effects were observed on organ weights related to the observed increase in testosterone and DHT in the F_1 generation and the fact that no effects were seen in the F_2 generation suggests that the biological relevance, if any, of the observed elevation of serum testosterone remains to be determined in chronic studies.

Detection of estrogen receptor proteins, particularly ER-β, by Western blot at normal tissue levels has been problematic with commercially available antibodies, including the ER-β antibody used in the present study (Santa Cruz Biotechnology, Y-19) [56]. Semi-quantitative measurements of both ER-α and ER-β were able to be made by Western blot after immunoprecipitation. In a preliminary experiment in which animals were exposed to dietary genistein at 0, 25, 250, and 1250 ppm from GD7 through PND 63, there was a statistically significant depression of ER-β in the dorsolateral prostate at all doses that ranged from 32 to 50% of control levels (data not shown). Protein bands of the molecular masses reported for rat ER-α (~66 kDa) and ER-β (~62 kDa) [57] were detected. A reduction in ER-β seen in the dorsolateral prostate in the F_1 animals resulting from genistein treatment (Table 3) deserves further study. Attempts to confirm the reduction of ER-β in the dorsolateral prostate by immunohistochemical staining were made, but the Bouin's fixed tissue available from the study proved unsuitable for this purpose. A recent report by Fritz et al. [55] was published that indicated that dietary genistein at doses ranging from 25 to 1000 ppm reduced expression of mRNA for ER-α, ER-β, and androgen receptor in the rat dorsolateral prostate. In that study, receptor protein levels for ER-β were not evaluated, and ER-α protein levels were reported to be reduced only at the 1000 ppm dose [55]. The same laboratory reported genistein-induced down-regulation of ER-α and androgen receptor, but not ER-β, in transgenic mice prone to postate cancer [58]. If genistein treatment alters the normal balance of ER-α, ER-β, and AR in the prostate, this may be an important factor in the subsequent development of prostatic disease.

Since the initial discovery of ER-β [59], considerable effort has been expended to determine the role of this receptor in the activities of estrogenic compounds. The wide distribution of ER-β in the male reproductive tract suggested a significant role for this receptor in the male [7]. While ER-α had been shown to occur in stromal cells of the prostate, ER-β was found to be highly expressed in prostatic epithelial cells [29,59,60]. Furthermore, ER-β knockout mice showed prostatic hyperplasia with age that was not seen in receptor-positive mice [61,62], and many prostatic tumors have been shown to lack ER-β [65,66]. Although the entire story is not yet clear, since other ER-β knockout mice do not show prostatic hyperplasia [65] and some advanced prostatic tumors do express ER-β [63,64], the data obtained thus far are generally consistent with a loss of ER-β leading to increased proliferation of prostatic tissue. The discovery of a second ER-β that differs from the original ER-β in terms of interaction with ligands [66] and regulation [57] may also complicate the simplistic view that loss of ER-β leads to proliferation. Loss of ER-β has been linked

to an increase of AR in the ventral prostate as one possible mechanism of growth stimulation [62]. Several reports of estrogenic stimulation of prostate growth in mice have linked the stimulation to an up-regulation of AR, although estrogen receptors were not examined in these studies [67–69]. Prins et al. [29] demonstrated that ER-β in prostatic epithelial cells steadily increased between days 1 and 90 and that neonatal exposure of rats to high dose estrogen resulted in a marked decrease in the levels of ER-β in the ventral prostate at day 90, but not in the dorsolateral prostate. This same group had earlier shown that neonatal estrogenization led to a permanent decrease in AR in smooth muscle and epithelial cells in the prostate [26,27]. In the present study, ER-β was significantly depressed in the dorsolateral prostate, but not the ventral prostate. Differences in dosing regimen and the fact that genistein rather than estradiol benzoate was used may explain the discrepancies betweeen this observation and that reported by Prins et al. [29]. The larger multigeneration studies with genistein from which the PND 140 animals in the present study were taken include comprehensive reproductive and, importantly, chronic toxicity assessments that should provide information as to whether the modest changes observed here, which differ significantly from the extreme conditions generated by receptor knockouts or high dose estrogen treatment, may contribute to adverse effects.

The results of the present study together with the results of Fritz et al. [55] suggest that genistein alters the levels of steroid hormone receptors in the prostate. Growth and development of the prostate, including neoplastic growth, are dependent not only on steroid hormones, but on complex interactions among steroids and peptide growth factors [70–72]. Dalu et al. have demonstrated that dietary genistein reduces protein tyrosine phosphorylation at the epidermal growth factor receptor (EGF-R), primarily through down-regulation of EGF-R expression [37]. Genistein-induced alterations of steroid hormone and growth factor receptors in the female reproductive tract and mammary gland [73,74] and the mouse prostate [58] have also been reported. These studies indicate that an understanding of the mechanisms by which genistein causes its effects will require further examination.

Acknowledgements

This study was funded in part through Interagency Agreement 224-93-0001 between the National Institute for Environmental Health Sciences and the United States Food and Drug Administration. A. Dalu was supported by an appointment to the Postgraduate Research Program at the National Center for Toxicological Research, Jefferson, AR, administered by the Oak Ridge Institute for Science and Education through an Interagency Agreement between the US Department of Energy and the US Food and Drug Administration.

References

[1] H. Adlercreutz, W. Mazur, in: G.E. Dunaif, S.S. Olin, J. Scimeca, J.A. Thomas (Eds.), Human Diet and Endocrine Modulation: Estrogenic and Androgenic Effects, ILSI Press, Washington, DC, 1998, p. 135.
[2] M.J. Messina, V. Persky, K.D.R. Setchell, S. Barnes, Nutr. Cancer 21 (1994) 113.
[3] H. Adlercreutz, W. Mazur, Ann. Med. 29 (1995) 95.
[4] S. Barnes, T.G. Peterson, L. Coward, J. Cell. Biochem. 22 (Suppl.) (1995) 181.
[5] M.C. Bosland, I. Kato, J. Melamed, S. Taneja, H. Lepor, P. Torre, P. Walden, A. Zeleniuch-Jacquotte, L.H. Lumey, Urology 57 (Suppl. A) (2001) 202.
[6] D. Urban, W. Irwin, M. Kirk, M.A. Markiewicz, R. Myers, M. Smith, H. Weiss, W.E. Grizzle, S. Barnes, J. Urol. 165 (2001) 294.
[7] G.G.J.M. Kuiper, B. Carlsson, K. Grandien, E. Enmark, J. Haggblad, S. Nilsson, J.-Å. Gustafsson, Endocrinology 138 (1997) 863.
[8] G.G.J.M. Kuiper, J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. Van Der Saag, B. Van Der Burg, J.-Å. Gustafsson, Endocrinology 139 (1998) 4252.
[9] H. Kim, T.G. Peterson, S. Barnes, Am. J. Clin. Nutr. 68 (1998) 1418S.
[10] P.L. Whitten, F. Naftolin, Bailliere's Clin. Endocrinol. Metab. 12 (1998) 667.
[11] M. Messina, S. Barnes, J. Natl. Cancer Inst. 83 (1991) 541.
[12] C.H.T. Adlercreutz, B.R. Goldin, S.L. Gorbach, K.A.V. Höckerstedt, S. Watanabe, E.K. Hämäläinen, M.H. Markkanen, T.H. Mäkelä, K.T. Wähälä, T.A. Hase, T.J. Fotsis, Nutrition 125 (1995) 757S.
[13] K. Williams, C. McKinnell, P.T.K. Saunders, M. Walker, J.S. Fisher, K.J. Turner, N. Atanassova, R.M. Sharpe, Human Reprod. Update 7 (2001) 236.
[14] R.R. Newbold, J.A. McLachlan, in: T. Mori and H. Nagasawa (Eds.), Toxicity of Hormones in Perinatal Life, CRC Press, Boca Raton, FL, 1988, p. 89.

[15] J.A. McLachlan, R.R. Newbold, M.E. Burow, S.F. Li, APMIS 109 (2001) 263.
[16] N.E. Skakkebæk, E. Rajpert-De Meyts, K.M. Main, Human Reprod. 16 (2001) 972.
[17] R.M. Sharpe, Toxicol. Lett. 120 (2001) 221.
[18] L.J. Paulozzi, Environ. Health Perspect. 107 (1999) 297.
[19] F. Levi, C. La Vecchia, P. Boyle, F. Lucchini, E. Negri, Lancet 357 (2001) 1853.
[20] K. North, J. Golding, Br. J. Urol. Int. 85 (2000) 107.
[21] K.D.R. Setchell, L. Zimmer-Nechemias, J. Cai, J.E. Heubi, Lancet 350 (1997) 23.
[22] M.G. Glazier, M.A. Bowman, Arch. Int. Med. 161 (2001) 1161.
[23] J. Rajfer, D.S. Coffey, Invest. Urol. 16 (1978) 186.
[24] F. Gaytan, C. Bellido, R. Aguilar, M.C. Lucena, Biol. Reprod. 35 (1986) 219.
[25] G.S. Prins, C. Woodham, M. Lepinske, L. Birch, Endocrinology 132 (1993) 2387.
[26] G.S. Prins, Endocrinology 130 (1992) 3703.
[27] G.S. Prins, L. Birch, Endocrinology 136 (1995) 1303.
[28] G.S. Prins, L. Birch, Endocrinology 138 (1997) 1801.
[29] G.S. Prins, M. Marmer, C. Woodham, W. Chang, G. Kuiper, J.-Å. Gustafsson, L. Birch, Endocrinology 139 (1998) 874.
[30] N. Atanassova, C. McKinnell, K.J. Turner, M. Walker, J.S. Fisher, M. Morley, M.R. Millar, N.P. Groome, R.M. Sharpe, Endocrinology 141 (2000) 3898.
[31] S. Mäkelä, R. Santii, P. Martikainen, W. Nienstedt, J. Paranko, J. Steroid Biochem. 35 (1990) 249.
[32] S. Mäkelä, L.H. Pylkkänen, R.S.S. Santti, H. Adlercreutz, J. Nutr. 125 (1995) 437.
[33] S. Mäkelä, R. Santti, L. Salo, J.A. McLachlan, Environ. Health Perspect. 103 (Suppl. 7) (1995) 123.
[34] L. Strauss, S. Mäkelä, S. Joshi, I. Huhtaniemi, R. Santti, Mol. Cell. Endocrinol. 144 (1998) 83.
[35] J.S. Fisher, K.J. Turner, D. Brown, R.M. Sharpe, Environ. Health Perspect. 107 (1999) 397.
[36] M. Casanova, L. You, K. Gaido, S. Archibeque-Engle, D.B. Janszen, Hd.'A. Heck, Toxicol. Sci. 51 (1999) 236.
[37] A. Dalu, J.F. Haskell, L. Coward, C.A. Lamartiniere, The Prostate 37 (1998) 36.
[38] W.A. Fritz, L. Coward, J. Wang, C.A. Lamartiniere, Carcinogenesis 19 (1998) 2151.
[39] T. Nagao, S. Yoshimura, Y. Saito, M. Nakagomi, K. Usumi, H. Ono, Reprod. Toxicol. 15 (2001) 399.
[40] National Toxicology Program. Final Report, Protocol E2122.14: Immunotoxicity of Genistein in Male and Female Sprague–Dawley Rats. Research Triangle Park, North Carolina: National Institute of Environmental Health Sciences; 1998.
[41] K.M. Flynn, S.A. Ferguson, K.B. Delclos, R.R. Newbold, Toxicol. Sci. 55 (2000) 311.
[42] K.B. Delclos, T.J. Bucci, L.G. Lomax, J.R. Latendresse, A. Warbritton, C.C. Weis, R.R. Newbold, Reprod. Toxicol. 15 (2001) 647.
[43] C.L. Holder, M.I. Churchwell, D.R. Doerge, J. Agric. Food Chem. 47 (1999) 3764.
[44] N.G. Coldham, M.J. Sauer, Toxicol. Appl. Pharmacol. 164 (2000) 206.
[45] H.C. Chang, M.I. Churchwell, K.B. Delclos, R.R. Newbold, D.R. Doerge, J. Nutr. 130 (2000) 1963.
[46] D.R. Doerge, M.I. Churchwell, H.C. Chang, R.R. Newbold, K.B. Delclos, Reprod. Toxicol. 15 (2001) 105.
[47] D.R. Doerge, M.I. Churchwell, K.B. Delclos, Rapid Commun. Mass Spectrom.. 14 (2000) 673.
[48] Y. Hochberg, Biometrika 75 (1988) 800.
[49] O.P. Sharma, H. Adlercreutz, J.D. Strandberg, B.R. Zirkin, D.S. Coffey, L.L. Ewing, J. Steroid Biochem. Mol. Biol. 43 (1992) 557.
[50] S.-M. Kwon, S.-I. Kim, D.-C. Chun, N.-M. Cho, B.-C. Chung, B.-W. Park, S.-J. Hong, Yonsei Med. J. 42 (2001) 395.
[51] K.S. Weber, K.D.R. Setchell, D.M. Stocco, E.D. Lephart, J. Endocrinol. 170 (2001) 591.
[52] R.L. Schleicher, C.A. Lamartiniere, M. Zheng, M. Zhang, Cancer Lett. 136 (1999) 195.
[53] K.S. Weber, N.A. Jacobson, K.D.R. Setchell, E.D. Lephart, Proc. Soc. Exp. Biol. Med. 221 (1999) 131.
[54] R. Santti, R.R. Newbold, J.A. McLachlan, Reprod. Toxicol. 5 (1991) 149.
[55] W.A. Fritz, J. Wang, I.-E. Eltoum, C.A. Lamartiniere, Mol. Cell. Endocrinol. 186 (2002) 89.
[56] M. Pavao, A.M. Traish, Steroids 66 (2001) 1.
[57] S. Saji, H. Sakaguchi, S. Andersson, M. Warner, J.-Å. Gustafsson, Endocrinology 142 (2001) 3177.
[58] C.A. Lamartiniere, M.S. Cotroneo, W.A. Fritz, J. Wang, R. Mentor-Marcel, A. Elgavish, J. Nutr. 132 (2002) 552S.
[59] G.G.J.M. Kuiper, E. Enmark, M. Pelto-Huikko, S. Nilsson, J.-Å. Gustafsson, Proc. Natl. Acad. Sci. 93 (1996) 5925.
[60] K.M. Lau, I. Leav, S.M. Ho, Endocrinology 139 (1998) 424.
[61] J.H. Krege, J.B. Hodgin, J.F. Couse, E. Enmark, M. Warner, J.F. Mahler, M. Sar, K.S. Korach, J.-Å. Gustafsson, O. Smithies, Proc. Natl. Acad. Sci. USA 95 (1998) 15677.
[62] Z. Weihua, S. Makela, L.C. Andersson, S. Salmi, S. Saji, J.I. Webster, E.V. Jensen, S. Nilsson, M. Warner, J.-A. Gustafsson, Proc. Natl. Acad. Sci. USA 98 (2001) 6330.
[63] I. Leav, K.-M. Lau, J.Y. Adams, J.E. McNeal, M.-E. Taplin, J. Wang, J. Singh, S.-M. Ho, Am. J. Pathol. 159 (2001) 79.
[64] L.G. Horvath, S.M. Henshall, C.-S. Lee, D.R. Head, D.I. Quinn, S. Makela, W. Delprado, D. Golovsky, P.C. Brenner, G. O'Neill, R. Kooner, P.D. Stricker, J.J. Grygiel, J.-A. Gustafsson, R.L. Sutherland, Cancer Res. 61 (2001) 5331.
[65] S. Dupont, A. Krust, A. Gansmuller, A. Dierich, P. Chambon, M. Mark, Development 127 (2000) 4277.
[66] D.N. Petersen, G.T. Tkalcevic, P.H. Koza-Taylor, T.G. Turi, T.A. Brown, Endocrinology 139 (1998) 1082.
[67] F.S. vom Saal, B.G. Timms, M.M. Montano, P. Palanza, K.A. Thayer, S.C. Nagel, M.D. Dhar, V.K. Ganjam, S. Parmigiani, W.V. Welshons, Proc. Natl. Acad. Sci. USA 94 (1997) 2056.
[68] C. Gupta, Proc. Soc. Exp. Biol. Med. 224 (2000) 61.
[69] K.A. Thayer, R.L. Ruhlen, K.L. Howdeshell, D.L. Buchanan, P.S. Cooke, D. Preziosi, W.V. Welshons, J. Haseman, F.S. vom Saal, Human Reprod. 16 (2001) 988.
[70] K.G. Nelson, T. Takahashi, N.L. Bossert, D.K. Walmer, J.A. McLachlan, Proc. Natl. Acad. Sci. 88 (1991) 21.

[71] D.M. Ignar-Trowbridge, M. Pimentel, C.T. Teng, K.S. Korach, J.A. McLachlan, Environ. Health Perspect. 103 (1995) 35.
[72] P. Reinikainen, J.J. Palvimo, O.A. Jänne, Endocrinology 137 (1996) 4351.
[73] N.M. Brown, J. Wang, M.S. Cotroneo, Y.-X. Zhao, C.A. Lamartiniere, Mol. Cell. Endocrinol. 144 (1998) 149.
[74] M.S. Cotroneo, J. Wang, I.-E.A. Eltoum, C.A. Lamartiniere, Mol. Cell. Endocrinol. 173 (2001) 135.