Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate 5α-reductase or testicular steroidogenic acute regulatory peptide levels in adult male Sprague–Dawley rats

K S Weber, K D R Setchell[1], D M Stocco[2] and E D Lephart

Neuroscience Center, Brigham Young University, Provo, Utah 86402, USA

[1]Clinical Mass Spectrometry Center, Children's Hospital Medical Center, Cincinnati, Ohio 45229, USA

[2]Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, Texas 79430, USA

(Requests for offprints should be addressed to E D Lephart, Neuroscience Center, 633 WIDB, Brigham Young University, Provo, Utah 84602, USA; Email: Edwin_Lephart@byu.edu)

Abstract

Nutritional factors, especially phytoestrogens, have been extensively studied for their potential beneficial effects against hormone-dependent and age-related diseases. The present study describes the short-term effects of dietary phytoestrogens on regulatory behaviors (food/water intake, locomotor activity and body weight), prostate weight, prostate 5α-reductase enzyme activity, reproductive hormone levels, and testicular steroidogenic acute regulatory peptide (StAR) levels in adult Sprague–Dawley rats.

Animals were fed either a phytoestrogen-rich diet containing ≈600 μg/g isoflavones (as determined by HPLC) or a phytoestrogen-free diet. After 5 weeks of consuming these diets, plasma phytoestrogen levels were 35 times higher in animals fed the phytoestrogen-rich vs phytoestrogen-free diets. Body and prostate weights were significantly decreased in animals fed the phytoestrogen-rich diet vs the phytoestrogen-free fed animals; however, no significant change in prostate 5α-reductase enzyme activity was observed between the treatment groups. Locomotor activity levels were higher in the phytoestrogen-rich vs the phytoestrogen-free animals during the course of the treatment interval. Plasma testosterone and androstenedione levels were significantly lower in the animals fed the phytoestrogen-rich diet compared with animals fed the phytoestrogen-free diet. However, there were no significant differences in plasma LH or estradiol levels between the diet groups. Testicular StAR levels were not significantly different between the phytoestrogen-rich vs the phytoestrogen-free fed animals.

These results indicated that consumption of dietary phytoestrogens resulting in very high plasma isoflavone levels over a relatively short period can significantly alter body and prostate weight and plasma androgen hormone levels without affecting gonadotropin or testicular StAR levels.

The findings of this study identify the biological actions of phytoestrogens on male reproductive endocrinology and provide insights into the protective effects these estrogen mimics exert in male reproductive disorders such as benign prostatic hyperplasia and prostate cancer.

Journal of Endocrinology (2001) **170,** 591–599

Introduction

Phytoestrogens (a group of natural selective estrogen receptor modulators) are non-steroidal, diphenolic structures found in many plants (e.g. fruits, vegetables, legumes, whole grain and especially soy products) that have the capacity to bind estrogen receptors (α and β) (Bradbury & White 1954, Price & Fenwick 1985, Setchell & Adlercreutz 1988, Knight & Eden 1996, Adlercreutz 1997, Kuiper *et al.* 1997, Setchell & Cassidy 1999). These estrogen mimics have been shown in animal models and in limited clinical investigations to be protective in the prevention of: (1) hormone-dependent cancers (e.g. breast and prostate), (2) cardiovascular disease and (3) osteoporosis (Price & Fenwick 1985, Setchell & Adlercreutz 1988, Knight & Eden 1996, Adlercreutz 1997, Adlercreutz & Mazur 1997, Setchell 1998, Kumar & Besterman-Dahan 1999, Setchell & Cassidy 1999, Adlercreutz *et al.* 2000). The anti-cancer effects of phytoestrogens appear to be associated with several possible mechanisms, including their ability to inhibit tyrosine kinase(s), growth factors, DNA isotopoisomerase, steroidogenic enzymes and act as anti-oxidant and anti-angiogenic agents (Price & Fenwick 1985, Setchell & Adlercreutz 1988, Knight & Eden 1996, Adlercreutz 1997, Adlercreutz & Mazur 1997, Griffiths *et al.* 1998, Setchell 1998, Setchell & Cassidy 1999).

0022–0795/01/0170–591

Online version via http://www.endocrinology.org

Epidemiological data have shown clear relationships between the incidence rates of prostate cancer (PCa) and soy food consumption. In Japan, the incidence of PCa is relatively low compared with that in the USA. Yet, despite the Japanese developing benign prostatic hyperplasia (BPH), rarely does it progress to a malignant cancer condition. This observation has led to the belief that phytoestrogen-rich diets may explain these observations (Adlercreutz 1997, Adlercreutz & Mazur 1997, Griffiths *et al.* 1998, Kumar & Besterman-Dahan 1999, Setchell & Cassidy 1999, Adlercreutz *et al.* 2000). Support for this hypothesis comes from rodent studies where soy diets have been found to be protective against PCa cell growth (Makela *et al.* 1995*a*, Zhang *et al.* 1997, Dalu *et al.* 1998, Landstrom *et al.* 1998, Bylund *et al.* 2000, Choi *et al.* 2000). Several human studies suggest that phytoestrogens inhibit BPH and PCa growth *in vitro* and *in vivo* (Stephens 1997, Griffiths *et al.* 1998, Stephens 1999, Choi *et al.* 2000). In the USA (and in other developed countries), PCa is the second most common cause of cancer death and BPH is a pre-malignant condition representing a major health concern in men. If a dietary approach to preventing either condition proves successful, this will have global implications. For this reason, we have attempted to determine whether high dietary phytoestrogen intake influences reproductive endocrine physiology in adult male rats in a manner that may be beneficial with regard to prevention of these diseases.

In this study, adult male Sprague–Dawley rats were fed either a high phytoestrogen diet or a phytoestrogen-free diet for approximately 5 weeks. Regulatory behaviors (food and water intake) and locomotor (open field) activity were measured along with recording body and prostate weight, prostate 5α-reductase enzyme activity, plasma testosterone, androstenedione, estradiol, luteinizing hormone (LH) and testicular steroidogenic acute regulatory protein (StAR) levels at the end of the study.

Materials and Methods

Animals

Adult male Sprague–Dawley (50-day-old) rats were obtained from Simonsen Laboratories (Gilroy, CA, USA) and were housed in a controlled environment on a reverse light–dark cycle (lights on 1600 to 0600 h; red light illumination during the dark cycle from 0600 to 1600 h). The animals were given free access to water and standard rat chow for approximately 20 days (days 50–70). The standard rat chow diet contains approximately 300 μg/g phytoestrogens.

Phytoestrogen diets

At 70 days of age, 114 rats were randomly assigned to two treatment groups: (1) a sterol-free diet (referred to hereafter as the Phyto-free diet) or (2) the phytoestrogen-rich containing diet (referred to hereafter as the Phyto-600 diet). There were no significant differences in body weight before the animals were assigned to the treatment groups and the animals continued to have free access to water and the diets. The Phyto-free rat diet was obtained from Zeigler Brothers (Gardner, PA, USA), balanced and matched for equivalent percentage content of protein, carbohydrate and fat to that of the commercially available Phyto-600 diet (Harlan-Teklad, Madison, WI, USA). The ingredients for each diet are compared in Table 1. The concentration and type(s) of phytoestrogens in the two diets were analyzed in duplicate by reverse-phase high pressure liquid chromatography (HPLC) using a 25 × 0·46 cm Aquapore (C8; particle size 7 μm) column (Perkin Elmer, Bodman Industries, Aston, PA, USA) under gradient elution conditions, with internal controls, as described elsewhere (Coward *et al.* 1993, Setchell *et al.* 1997). The Phyto-600 diet contained approximately 600 μg/g phytoestrogens while the phytoestrogen content in the Phyto-free diet was below the limits of HPLC detection (see Table 2).

Food (g ± 0·1 g), water (m ± 0·5 ml) and body weight (g ± 0·1 g) measurements were recorded periodically throughout the study. Food intake was recorded by weighing each animal's food tray every 24 h during the treatment interval. Water intake was measured in ml from calibrated water bottles. After 35 days on the treatment diets (starting at 70 days of age), the male rats were killed at 105 days of age. At the time of death, trunk blood was collected, body weight was measured and ventral prostate weight determined (mg ± 0·5 mg). Plasma was prepared from the trunk blood and stored at −20 °C until assayed. The animals and methods used in this study were approved by the Institutional Animal Care and Use Committee at Brigham Young University.

Plasma phytoestrogen levels

The concentration and type(s) of phytoestrogens were analyzed from two pooled (by treatment) plasma (in a subset of animals, total $n=31$ per treatment) samples by gas-chromatography/mass spectrometry. This was preformed by liquid–solid extraction and liquid–gel chromatographic techniques to isolate the phytoestrogen fractions using standard methods with stable isotopic labeled internal standards and control samples to validate the assay (Setchell *et al.* 1997). Concentrations are expressed in ng/ml.

Locomotor activity

In a subset of animals ($n=18$ per group), before the diets were initiated, at 70 days of age and after the diets were administered, at 82 days of age (or 12 days on the diets) and at 99 days of age (or 29 days on the diets) the locomotor

Table 1 Treatment diets

	Unit	Harlan-Teklad 8604	Zeigler Bros, sterol free
Nutrient composition			
Protein	%	24·48	23·14
Fat	%	4·40	5·69
Fiber	%	3·69	2·30
Ash	%	7·84	6·46
Linoleic acid	%	1·87	2·19
Amino acids			
Arginine	%	1·53	1·13
Methionine	%	0·42	0·59
Cystine	%	0·37	0·27
Histidine	%	0·58	0·59
Isoleucine	%	1·24	1·19
Leucine	%	2·04	2·17
Lysine	%	1·46	1·42
Phenylalanine+tyrosine	%	1·84	2·08
Threonine	%	0·94	0·95
Tryptophan	%	0·29	0·26
Valine	%	1·26	1·36
Minerals			
Calcium	%	1·36	1·20
Phosphorus	%	1·01	0·96
Sodium	%	0·29	0·30
Chlorine	%	0·49	0·43
Potassium	%	1·04	0·55
Magnesium	%	0·28	0·16
Sulfur	%	NA	0·20
Iron	mg/kg	352·14	245·49
Manganese	mg/kg	105·39	96·21
Zinc	mg/kg	82·87	59·81
Copper	mg/kg	24·42	13·06
Iodine	mg/kg	2·46	1·84
Cobalt	mg/kg	0·71	0·52
Selenium	mg/kg	0·33	0·37
Vitamins			
Vitamin A	IU/g	12·90	6·59
Vitamin D3	IU/g	2·40	5·08
Vitamin E	IU/kg	90·18	48·84
Choline	mg/g	2·53	1·65
Niacin	mg/kg	63·42	76·56
Pantothenic acid	mg/kg	21·03	31·74
Pyridoxine (vitamin B6)	mg/kg	12·95	9·89
Riboflavin (vitamin B2)	mg/kg	7·85	6·92
Thiamine (vitamin B1)	mg/kg	27·95	17·35
Menadione (vitamin K3)	mg/kg	4·11	3·14
Folic acid	mg/kg	2·72	3·01
Biotin	mg/kg	0·39	0·37
Vitamin B12	mg/kg	51·20	47·88
Vitamin C	mg/kg	0·00	0·00

The ingredients list (first four) for the Harlan-Teklad 8604 diet=soybean meal, corn and wheat flakes, ground corn and wheat middlings; for the Zeigler Brothers, sterol-free diet=corn, wheat, fish meal and wheat middlings (reproduced by permission of the suppliers).
NA, not assayed.

Table 2 HPLC analysis of phytoestrogen content (μg/g) of the Phyto-600 and Phyto-free diets. Duplicate samples of each diet (Harlan-Teklad 8604 (Phyto-600) and Zeigler Brothers, sterol-free (Phyto-free)) were analyzed by reverse phase HPLC (Coward *et al.* 1993, Setchell *et al.* 1997)

	Phyto-600		Phyto-free	
Daidzin	198·2	199·0	ND	ND
Glycitin	47·8	45·8	ND	ND
Genistin	286·1	276·9	ND	ND
Acetylglucoside daidzein	32·4	35·6	NA	NA
Acetylglucoside glycitin	18·6	23·4	NA	NA
Daidzein	10·3	9·6	ND	ND
Glycitein	3·3	4·7	NA	NA
Genistein	9·5	8·6	ND	ND
Total (μg/g)	606·6	603·6	ND	ND

ND=none detected (below the limits of HPLC detection, less than 0·5 μg/g); NA=not assayed.

activity of the rats from each diet group was measured by open field tests (conducted at 1000–1200 h (during the dark cycle when rats are most active) under red light conditions, as previously described; Lephart *et al.* 1996, Weber *et al.* 1999). One rat (per open field test by treatment) was placed on a round table (1·22 m in diameter, 0·91 m off of the ground with 10 × 10 cm boxed grids), and the ambulatory activity was videotaped. Later, three different observers counted the number of squares that each rat entered in three minutes. The locomotor activity levels were averaged (for each animal by treatment per open field test), and the correspondence (analyzed by correlational analysis; Pearson Product Moment Correlation) among the three observers in recording open field behavior was $r=0·98$ for the entire testing interval.

Radioimmunoassay – determination of plasma testosterone, androstenedione, estradiol and LH levels

In a subset of the treated animals ($n=18$ per group), plasma testosterone, androstenedione and estradiol levels were determined by radioimmunoassay (RIA) using kits from Diagnostic Systems Laboratories (Webster, TX, USA). The estradiol values were obtained using an ultra-sensitive kit (RIA). All samples were run in a single assay (in duplicate by hormone tested) and the intra-assay coefficients of variation were 3% for testosterone, 4% for androstenedione and 7% for estradiol. LH levels were measured using reagents obtained from the National Hormone and Pituitary Program. The results obtained are expressed in terms of the LH-RP3 reference standards and the sensitivity of this assay was 0·07 ng/ml (Niswender *et al.* 1968). The samples were run in a single assay in duplicate with an intra-assay coefficient of variation <7%.

Prostate 5α-reductase activity

To determine 5α-reductase activity in ventral prostate tissue (in a subset of animals, $n=13$ per group), the isolated tissue samples were incubated in 200 μl Dulbecco's

minimum essential medium (DMEM) at pH 7·0 (Sigma Chemical Co., St Louis, MO, USA) with a saturating concentration of [1β-^{3}H]testosterone as the substrate (3·0 μM; DuPont/New England Nuclear Corp., Boston, MA, USA) for 1 h. Using standard assay procedures (Lephart *et al.* 1990), the rates of 5α-reductase activity in each tissue sample were determined in the reaction mixture. In brief, at the end of the incubation period, the reaction was stopped and steroids in the reaction mixture were extracted with 5 vol. chloroform with subsequent vortexing. An aliquot of the chloroform phase (100 μl) was evaporated to dryness, re-dissolved in 30 μl chloroform containing 10 μg each of five non-radioactive steroids (5α-androstane-3β,17-dione, androstenedione, 5α-dihydrotestosterone, testosterone and 5α-androstane-3α,17β-diol). Each prepared sample was applied to pre-coated silica gel plastic thin layer chromatography (TLC) plates (20 × 20 cm). The TLC plates were developed with one ascent of the solvent system (dichloromethane, ethyl acetate, methanol; 85:15:3, by vol.), which resolves the major 5α-reduced metabolites from 5β-androgen metabolites, estradiol and estrone. The tritium corresponding to the cold 5α-reduced steroids was quantified by scintillation counting to calculate the 5α-reductase activities as corrected by blanks (reaction tubes containing no tissue) as standards (Lephart *et al.* 1990). Using these conditions, the predominant enzyme activity measured was 5α-reductase type 1 (Normington & Russell 1992) which apparently is the major 5α-reductase type expressed in adult rat prostate tissue (Normington & Russell 1992). The protein content of each tissue fragment assayed was determined by the method of Lowry *et al.* (1951). The 5α-reductase activities were expressed as specific activity rate(s) in pmol/h (of incubation)/mg protein.

Testicular StAR protein Western analysis

The right testis of each Phyto-600 or Phyto-free animal (n=13 per diet group) was dissected and then stored at –85 °C until assayed. The following procedure was performed in a cold room (4 °C). The testicular tissues (by treatments) were thawed, cut into small sections and placed into 30 ml ice-cold TBS buffer, pH 7·0. The testicular tissues were dispersed by passing the tissue/buffer mixture through an 18 gauge needle, then a 20 gauge needle five times each. The dispersed testicular tissue was then placed into a 50 ml conical tube for 5 min to allow the larger cell particles consisting of pieces of seminiferous tubules to settle to the bottom of the tube. Subsequently, 1 ml aliquots of the dispersed testicular tissue were collected for the upper portion of the conical tube, the protein content of each aliquot was determined by a Lowry protein assay (Lowry *et al.* 1951) and finally the samples were lyophilized. Lyophilized Leydig cell samples were analysed for expression of StAR by Western analysis as described previously (Clark *et al.* 1994). The samples were solubilized in sample buffer (25 mM Tris/HCl, pH 6·8, 1% SDS, 5% β-mercaptoethanol, 1 mM EDTA, 4% glycerol and 0·01% bromophenol blue), boiled for 5 min and loaded onto a 12% SDS-PAGE mini-gel (Mini-Protean II System; Bio-Rad, Richmond, CA, USA). Electrophoresis was performed at 200 V for 45 min using a standard SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0·1% SDS, pH 8·3). The proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) at 100 V for 2 h at 4 °C using a transfer buffer containing 20 mM Tris, 150 mM glycine, 20% methanol, pH 8·3. The membrane was incubated in blocking buffer (PBS buffer containing 4% Carnation non-fat dry milk and 0·2% Tween 20), at room temperature for 1 h followed by incubation with a primary antibody against StAR for 30 min. Anti-StAR antisera against amino acids 88–98 of mouse StAR protein were produced in rabbits by Research Genetics (Huntsville, AL, USA). The membrane was washed with PBS containing 0·2% Tween 20 three times for 10 min each time. After incubation with the second antibody, donkey anti-rabbit IgG conjugated with horseradish peroxidase (Amersham, Arlington Heights, IL, USA), the membrane was washed five times for 10 min each time. Specific protein bands were detected by chemiluminescence using the Renaissance Kit (Dupont New England Nuclear, Wilmington, DE, USA), and quantitated using the BioImage Visage 2000 (Townson *et al.* 1996).

Statistical analysis

The data derived from the adult male rats were tested by ANOVA, followed by pairwise comparisons (via Tukey's analysis) to detect significant differences between the treatment groups ($\alpha = P < 0{\cdot}05$). For the open field data, repeated measures were used to detect significant differences between the treatment groups, followed by a post-hoc pairwise comparison via a Neuman–Kuels test ($\alpha = P < 0{\cdot}05$).

Results

The total and individual isoflavones were determined in pooled plasma samples from animals in each diet group. The animals in the Phyto-600 group had significantly higher levels of phytoestrogens in their plasma compared with the Phyto-free treatment group (Fig. 1). The total circulating concentration of plasma isoflavones was approximately 35 times higher in the Phyto-600 (2224 ng/ml) vs the Phyto-free (63 ng/ml) animals. Major components of the plasma phytoestrogen levels in the Phyto-600 animals were equol (≈1000 ng/ml) and daidzein (≈800 ng/ml) while genistein (≈400 ng/ml) made up the remaining fraction of the circulating

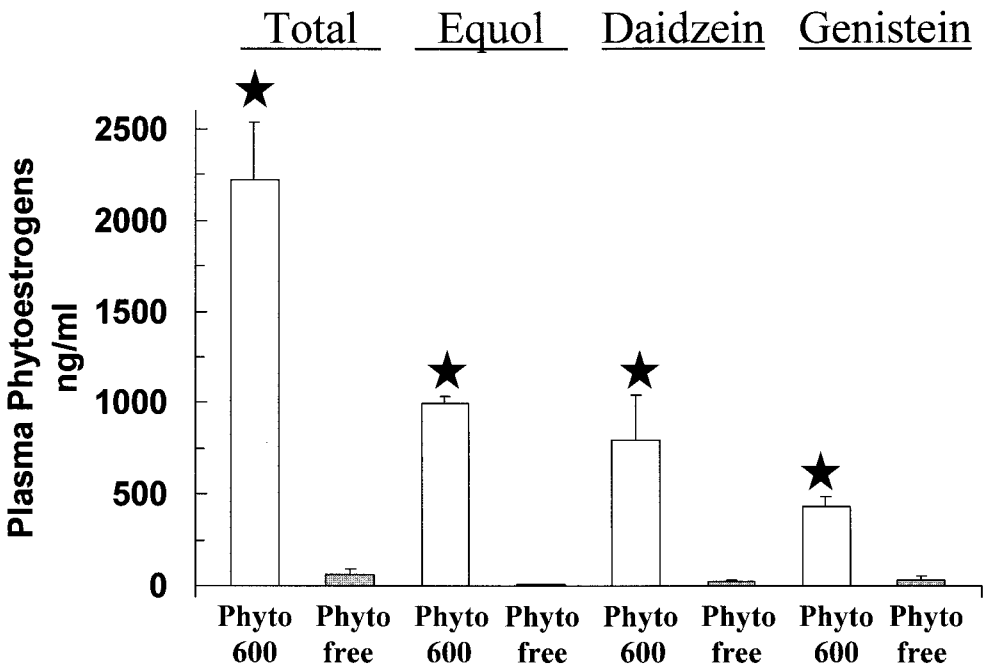

Figure 1 Plasma phytoestrogen concentrations from adult male rats fed the Phyto-600 or the Phyto-free diets for 5 weeks, expressed in ng/ml (means ± S.E.M.). Phytoestrogen concentrations were measured by gas chromatography/mass spectrometry (Setchell *et al.* 1997). $n=31$, this represents the total of two independently pooled plasma samples assayed for each treatment. Total=sum of the three main phytoestrogen metabolites (equol, daidzein and genistein). Significantly higher plasma phytoestrogen levels in the Phyto-600 vs the Phyto-free animals is represented by the solid star.

isoflavones. In the Phyto-free group, the phytoestrogens (equol, daidzein and genistein) were present in plasma but at very low concentrations compared with the Phyto-600 values (Fig. 1).

For food intake, there were no significant differences between the Phyto-600 vs the Phyto-free fed animals near the end of the treatment interval (see Table 3). However, when water intake was examined, Phyto-600 fed animals displayed slight but significantly higher levels than Phyto-free fed animals (Table 3). Also, when body weight was measured at the end of the treatment period (compared to pre-treatment values), we observed a slight but significant decrease in body weight in the Phyto-600 (372·8 + 4·0 g) compared with the Phyto-free fed (391·6 + 3·4 g) animals (Table 3).

Table 3 Food and water intake and body and prostate weights of male rats fed the Phyto-600 vs the Phyto-free diets. Values are means ± S.E.M. ($n=57$)

	Phyto-600	Phyto-free
Food intake (g)	22·5 ± 0·4	21·7 ± 0·4
Water intake (ml)	28·0 ± 0·5#	26·0 ± 0·5
Body weight (g)	372·8 ± 4·0	391·6 ± 3·4*
Prostate weight (mg)	561·5 ± 13·0	605·8 ± 14·1*
Prostate weight/body weight (ratio)	145·6 ± 4·2	157·6 ± 3·7*

Food and water intake represent the last 3 days of the diet treatment interval (averaged before the animals were killed at 120 days of age). Body and prostate weights were determined at the time of death.
There were no significant differences in body weight by diet treatment group (at 70 days of age) before the treatment diets were administered (i.e. Phyto-600 group=304·3 ± 2·7 g vs Phyto-free group=304·7 ± 2·9 g).
#Significantly greater water intake compared with Phyto-free values;
*significantly greater values compared with Phyto-600 values.

Since there were no significant differences in food intake between the Phyto-600 vs the Phyto-free animals, open field tests were conducted to determine whether locomotor activity levels were altered by the diets (Fig. 2). There were no significant differences in open field activity before the animals were fed the Phyto-600 vs the Phyto-free diets. However, after 12 days and at 29 days of feeding on the diets, the Phyto-600 group displayed higher open field locomotor levels compared with the Phyto-free group although the difference did not quite approach statistical significance ($P<0·06$) (Fig. 2), suggesting a potential influence of dietary phytoestrogens on locomotor activity.

For animals fed the Phyto-600 diet, the prostate weight (alone or standardized by body weight) was significantly lower compared with the Phyto-free group (see Table 3). In order to gain an understanding of this result, prostate 5α-reductase activity was measured, since androgen hormonal action (via 5α-dihydrotestosterone) is known to act

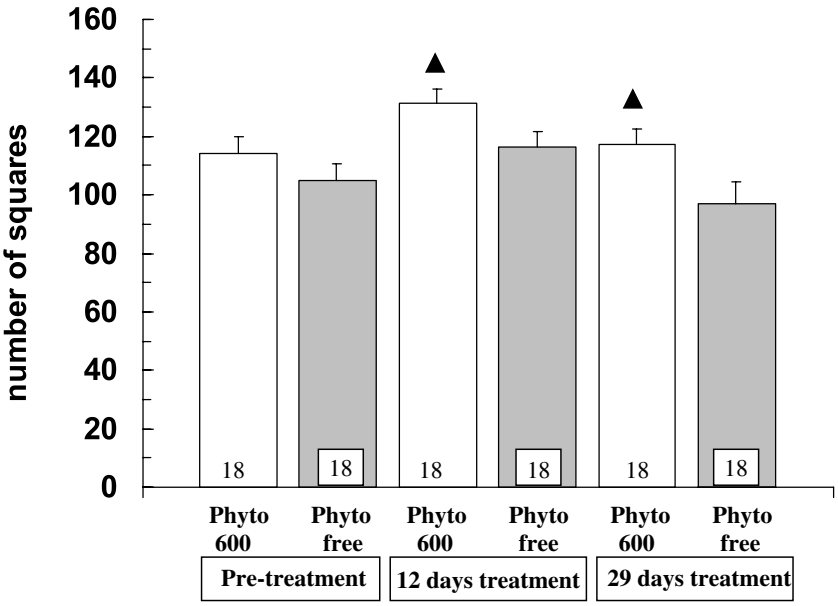

Figure 2 Open field activity of Phyto-600 vs Phyto-free fed male rats. The columns indicate the mean number of squares the rats, by treatment group, entered in a 3-min time-period (Lephart *et al.* 1996, Weber *et al.* 1999). Each bar represents the mean ± S.E.M. for open field activity and the number at the base of each bar indicates the total animals tested per treatment group. ▲ The open field activity levels for the Phyto-600 group at 12 and 29 days of treatment approached significance where the Phyto-600 group displayed higher locomotor activity levels than the Phyto-free group values ($P<0{\cdot}060$, 12 days treatment; and $P<0{\cdot}065$, 29 days treatment).

as a trophic signal in this organ. There were no significant differences between the diet groups for prostate 5α-reductase activity (Phyto-600 = 113·0 ± 3·0 pmol/h of incubation/mg protein vs Phyto-free = 117·5 ± 4·5 pmol/h of incubation/mg protein, $n=13$ per treatment group; data not shown graphically). Therefore, other hormonal signals were analyzed to potentially identify how a significant decrease in ventral prostate weight in the Phyto-600 group occurred.

There was no significant difference in plasma LH values between the animals fed the two phytoestrogen diets (Table 4); however, a significant decrease in plasma testosterone concentrations was observed for animals fed the Phyto-600 vs the Phyto-free diets (Table 4). In fact, plasma testosterone levels were approximately 50% lower in animals fed the Phyto-600 compared with animals fed the Phyto-free diet.

Table 4 Testosterone, LH, androstenedione and estradiol levels in male rats fed the Phyto-600 vs the Phyto-free diets. Values are means ± S.E.M. ($n=18$)

	Phyto-600	Phyto-free
Testosterone (ng/ml)	1·3 ± 0·2	2·6 ± 0·3*
LH (ng/ml)	0·40 ± 0·05	0·37 ± 0·08
Androstenedione (ng/ml)	0·015 ± 0·001	0·097 ± 0·014*
Estradiol (pg/ml)	4·2 ± 0·42	4·5 ± 0·80

*Significantly greater values compared with Phyto-600 values.

To further determine the pattern of steroidogenesis in the Phyto-600 and Phyto-free animals, plasma androstenedione and estradiol levels were also measured by RIA (Table 4). A significant decrease in plasma androstenedione levels was observed in the animals fed the Phyto-600 diet compared with the Phyto-free fed animals (Table 4). The plasma androstenedione concentrations showed the same pattern as that of the plasma testosterone levels, being significantly lower in animals fed the Phyto-600 diet (Table 4). However, in the case of androstenedione, there was a much greater reduction in the Phyto-600 animals vs the Phyto-free fed animals when compared with the testosterone results. Conversely, when plasma estradiol levels were determined, no significant differences were observed between the two groups of animals (Table 4).

To examine whether the significant decrease in plasma testosterone observed in the Phyto-600 diet fed animals was related to changes in testicular StAR protein, levels of this protein were measured by Western analysis. There were no significant differences in testicular StAR protein levels between the two groups (data not shown).

Finally, when testicular characteristics were examined, there were no significant differences in testes weight, Sertoli or Leydig cell number or morphology between the Phyto-600 vs the Phyto-free fed animals (data not shown).

Discussion

Interest in the health effects of phytoestrogens has increased dramatically during the past years where age-related and hormone-dependent diseases appear to be influenced by the consumption of these plant estrogen-like molecules (Setchell & Adlercreutz 1988, Knight & Eden 1996, Adlercreutz 1997, Adlercreutz & Mazur 1997, Murkies *et al.* 1998, Setchell 1998, Kumar & Besterman-Dahan 1999, Setchell & Cassidy 1999, Adlercreutz *et al.* 2000). The present study attempts to understand the possible mechanisms of action of phytoestrogens as they relate to PCa prevention by examining hormonal, reproductive and behavioral responses in an animal model fed a phytoestrogen-rich diet.

The content of the phytoestrogen diet utilized in this study was similar to that previously used. The total isoflavone concentration was approximately 600 μg/g phytoestrogens (designated as the Phyto-600 diet), while the Phyto-free diet had no detectable isoflavones by HPLC with UV detection (Lephart *et al.* 2000). The circulating plasma phytoestrogen concentration of animals fed the Phyto-600 diet (≈ 2200 ng/ml) is similar to that in people eating a typical Asian diet (of approximately 1 μM). Animals fed the Phyto-free diet had very low plasma phyotestrogens levels (≈ 70 ng/ml) reflecting what is observed in humans consuming a typical Western diet. By this approach, we are effectively establishing a dietary model for comparing Asian with Western diets for the consumption of phytoestrogens.

Estrogens are known to alter feeding behavior, body-weight composition and significantly increase locomotor behavior in rats (Gray *et al.* 1979, Mooradian *et al.* 1987). In this study, animals fed the Phyto-600 diet displayed a slight but significant decrease in body weight after 5 weeks on this diet compared with animals fed the Phyto-free diet and this is consistent with the estrogenic hormonal action of these molecules (Mooradian *et al.* 1987, Anderson *et al.* 1988). Furthermore, since phytoestrogens have been shown to transfer into brain tissue (Lephart *et al.* 2000) and have similar physiochemical and physiological characteristics to endogenous estrogens (Setchell & Adlercreutz 1988, Knight & Eden 1996, Adlercreutz 1997, Murkies *et al.* 1998, Setchell 1998, Setchell & Cassidy 1999, Adlercreutz *et al.* 2000), locomotor behavior tests revealed that Phyto-600 fed animals showed slight (but non-significant; $P<0{\cdot}06$) increases in open field behavior compared with the animals fed a Phyto-free diet. This significant decrease in body weight observed in these animals fed the Phyto-600 diet suggests that the Phyto-600 diet may increase locomotor activity. (In another study, we have data to support the notion that Phyto-600 fed animals display significantly increased locomotor activity; however, we have also obtained preliminary data where significant differences in absorption/metabolism may account for the differences in body weight where alterations in leptin and adipose tissue deposition are seen between Phyto-600 and Phyto-free fed animals.)

Long-term dietary studies where animals have been exposed to phytoestrogens show influences on prostate and other markers of reproductive development (Sharma *et al.* 1992, Makela *et al.* 1995*a*, Zhang *et al.* 1997). When reproductive organs were examined, Phyto-600 fed animals displayed a significant reduction in ventral prostate weight compared with Phyto-free fed animals. The mechanism for this change is unknown but may relate to changes in steroid hormone status within the prostate itself by effects on the levels of enzymes regulating steroid hormone production. It is known that phytoestrogens can inhibit prostate 5α-reductase enzyme activity *in vitro* (Evans *et al.* 1995), but our studies did not find significant alterations in prostate 5α-reductase enzyme activity after feeding the Phyto-600 diet that could account for the changes in prostate weight.

However, the Phyto-600 fed animals had significantly lower plasma androgen levels when compared with the Phyto-free fed animals. This reduction in testosterone could account for the reduced prostate weight in these animals, because the ventral prostate is androgen sensitive. In previous (longer term) studies, changes in androgen levels were not noted (Sharma *et al.* 1992, Makela *et al.* 1995*a*, Zhang *et al.* 1997); however, circulating phytoestrogen levels were not measured in these studies and, therefore, such differences are difficult to reconcile. Additionally, we have previously observed a similar pattern of circulating testosterone reduction in animals on the Phyto-600 diet (at approximately 40–50% of Phyto-free values) in three independent studies under the same experimental conditions, representing an n of 39 rats per treatment group. Another possible explanation for this finding is that phytoestrogens have the ability to inhibit the aromatase enzyme in peripheral tissues (Kellis & Vickery 1984, Ibrahim & Abul-Hajj 1990, Adlercreutz *et al.* 1994, Wang *et al.* 1994) and the apparent protection phytoestrogens have against BPH and PCa may be via the reduction in local estrogen formation since estrogenic agents are known to be mitogenic (vom Saal *et al.* 1997, Griffiths *et al.* 1998, Farnsworth 1999, Shibata *et al.* 2000, Yaono *et al.* 2000).

On the other hand, the inter-conversion of testosterone to androstenedione is regulated by the activity of 17β-hydroxysteroid dehydrogenase, an enzyme that has been shown to be influenced by phytoestrogens (Makela *et al.* 1995*b*). Changes in plasma androstenedione levels with

the Phyto-600 diet were much lower compared with those observed for testosterone by dietary treatments, suggesting that the substrate supply or enzyme regulation for testosterone synthesis may be affected by the phytoestrogen-rich diet. No changes in plasma LH or estradiol levels were observed between the Phyto-600 animals and the Phyto-free fed animals that could account for the changes in the observed testosterone levels. Finally, in an attempt to determine whether phytoestrogens may alter cholesterol delivery into the steroidogenic pathway, we measured StAR activity but found no significant differences in testicular StAR levels between the Phyto-600 and the Phyto-free groups. However, it is possible that the 30 kDa StAR protein analyzed in these experiments may not represent newly synthesized StAR and thus the protein measured would not be active in cholesterol transfer (Townson *et al.* 1996). It is also possible that the effects of phytoestrogens may be manifested at the level of protein kinase activity in the prostate. It is known that genistein decreases tyrosine phosphorylation in endocrine tissue (Fioravanti *et al.* 1998). Finally, the decrease in testosterone levels in the Phyto-600 animals remains to be explained but represents a consistent observation (Sharma *et al.* 1992, Landstrom *et al.* 1998). It is possible that phytoestrogens affect the biosynthetic pathways for androgen production by an unknown mechanism.

The reduction in prostate weight in the Phyto-600 fed animals is in agreement with findings by other investigators. Soy-containing diets were shown to inhibit prostatitis and prostate adenocarcinoma in rodents and to reduce prostate size in humans with BPH (Sharma *et al.* 1992, Stephens 1997, 1999, Griffiths *et al.* 1998, Landstrom *et al.* 1998, Adlercreutz *et al.* 2000, Bylund *et al.* 2000). However, in the above rodent studies, prostatic parameters were reduced without changes in testosterone levels among the treatment groups (Sharma *et al.* 1992, Landstrom *et al.* 1998). Thus, the mechanisms across species and presumably among rat strains may represent diverse and complex processes by which phytoestrogens protect against certain types of hormone-dependent cancers and BPH (vom Saal *et al.* 1997, Griffiths *et al.* 1998, Farnsworth 1999, Negri-Cesi *et al.* 1999, Shibata *et al.* 2000, Yaono *et al.* 2000).

In summary, the findings of this study highlight the biological actions of phytoestrogens on male reproductive endocrinology and may explain, in part, the protective effect of these estrogen mimics in male reproductive disorders such as BPH and PCa.

Acknowledgements

This work was supported, in part, by grants from the National Science Foundation, IBN-9507972 (to E D L), and the National Institutes of Health, HD 17481 (to D M S). The authors thank Dr Les Dees at Texas A&M for performing the LH assays.

References

Adlercreutz H 1997 Evolution, nutrition, intestinal microflora, and prevention of cancer: a hypothesis. *Proceedings of the Society for Experimental Biology and Medicine* **217** 241–246.

Adlercreutz H & Mazur W 1997 Phyto-estrogens and Western diets. *Annals of Medicine* **29** 95–120.

Adlercreutz H, Bannwart C, Wahala K, Makela T, Brunow G, Hase T, Arosemena PJ, Kellis JT Jr & Vickery LE 1994 Inhibition of human aromatase by mammalian lignans and sioflavonoid phytoestrogens. *Journal of Steroid Biochemistry and Molecular Biology* **44** 147–153.

Adlercreutz H, Mazur W, Bartels P, Elomaa V, Watanabe S, Wahala K, Landstrom M, Lundin E, Bergh A, Damber JE, Aman P, Widmark A, Johansson A, Zhang JX & Hallmans G 2000 Phytoestrogens and prostate disease. *Journal of Nutrition* **130** 658S–659S.

Anderson WR, Simpkins JW, Brewster ME & Bodor N 1988 Effects of a brain-enhanced chemical delivery system for estradiol on body weight and serum hormones in middle-aged male rats. *Endocrine Research* **14** 131–148.

Bradbury RB & White DE 1954 Estrogens and related substances in plants. *Vitamins and Hormones* **12** 207–233.

Bylund A, Zhang JX, Bergh A, Damber JE, Widmark A, Johansson A, Adlercreutz H, Aman P, Shepherd MJ & Hallmans G 2000 Rye bran and soy protein delay growth and increase apoptosis of human LNCap prostate adenocarcinoma in nude mice. *Prostate* **42** 304–314.

Choi YH, Lee WH, Park KY & Zhang I 2000 p53-Independent induction of p21 (WAF1/CIPI), reduction of cyclin B1 and G2/M arrest by the isoflavone genistein in human prostate carcinoma cells. *Japanese Journal of Cancer Research* **91** 164–173.

Clark BJ, Wells J, King SR & Stocco DM 1994 The purification, cloning and expression of a novel LH-induced mitochondrial protein in MA-10 mouse Leydig tumor cells: characterization of the steroidogenic acute regulatory protein (StAR). *Journal of Biological Chemistry* **269** 28314–28322.

Coward L, Barnes NC, Setchell KDR & Barnes S 1993 Genistein, daidzein, and their β-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets. *Journal of Agriculture and Food Chemistry* **41** 1961–1967.

Dalu A, Haskel JF, Coward L & Lamartiniere CA 1998 Genistein, a component of soy, inhibits the expression of the EGF and ErbB2/Neu receptors in the rat dorsolateral prostate. *Prostate* **37** 36–43.

Evans BA, Griffiths K & Morton MS 1995 Inhibition of 5α-reductase in genital skin fibroblasts and prostate tissue by dietary lignans and isoflavonoids. *Journal of Endocrinology* **147** 295–302.

Farnsworth WE 1999 Estrogen in the etiopathogenesis of BPH. *Prostate* **41** 263–274.

Fioravanti L, Cappelletti V, Miodini P, Ronchi E, Brivio M & DiFronzo G 1998 Genistein in the control of breast cancer cell growth: insights into the mechanism of action *in vitro*. *Cancer Letters* **30** 143–152.

Gray JM, Nunez AA, Siegel LI & Wade GN 1979 Effects of testosterone on body weight and adipose tissue: role of aromatization. *Physiology and Behavior* **23** 465–469.

Griffiths K, Denis L, Turkes A & Morton MS 1998 Phytoestrogens and diseases of the prostate gland. *Baillieres Clinical Endocrinology and Metabolism* **12** 625–647.

Ibrahim AR & Abul-Hajj YJ 1990 Aromatase inhibition by flavonoids. *Journal of Steroid Biochemistry and Molecular Biology* **37** 257–260.

Kellis JT Jr & Vickery LE 1984 Inhibition of human estrogen synthetase (aromatase) by flavones. *Science* **225** 1032–1034.

Knight DC & Eden JA 1996 A review of the clinical effects of phytoestrogens. *Obstetrics and Gynecology* **87** 897–904.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggbald J, Nilsson S & Gustafsson J 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* **138** 863–870.

Kumar NB & Besterman-Dahan K 1999 Nutrients in the chemoprevention of prostate cancer: current and future prospects. *Cancer Control* **6** 580–586.

Landstrom M, Zhang JX, Hallmans G, Aman P, Bergh A, Damber JE, Mazur W, Wahala K & Adlercreutz H 1998 Inhibitory effects of soy and rye diets on the development of Dunning R3327 prostate adenocarcinoma in rats. *Prostate* **36** 151–61.

Lephart ED, Andersson S & Simpson ER 1990 Expression of neural 5α-reductase messenger ribonucleic acid: comparison to 5α-reductase activity during prenatal development in the rat. *Endocrinology* **127** 1121–1128.

Lephart ED, Ladle DR, Jacobson NA & Rhees RW 1996 Inhibition of brain 5α-reductase in pregnant rats: effects on enzymatic and behavioral activity. *Brain Research* **739** 356–360.

Lephart ED, Thompson JM, Setchell KDR, Adlercreutz H & Weber KS 2000 Phytoestrogens decrease brain calcium-binding proteins but do not alter hypothalamic androgen metabolizing enzymes in adult male rats. *Brain Research* **859** 123–131.

Lowry OH, Rosebrough NJ, Farr AL & Randall RJ 1951 Protein determination with the Folin phenol reagent. *Journal of Biological Chemistry* **193** 265–275.

Makela SI, Pylkkanen LH, Santti RS & Adlercreutz H 1995*a* Dietary soybean may be antiestrogenic in male mice. *Journal of Nutrition* **125** 437–445.

Makela S, Poutanen M, Lehtimaki J, Kostian M-L, Santti R & Vihko R 1995*b* Estrogen-specific 17β-hydroxysteroid oxidoreductase type 1 (E.C.1·1·1·62) as a possible target for the action of phytoestrogens. *Proceedings of the Society for Experimental Biology and Medicine* **208** 51–59.

Mooradian AD, Morley JE & Korenman SG 1987 Biological actions of androgens. *Endocrine Reviews* **8** 1–28.

Murkies AL, Wilcox G & Davis SR 1998 Phytoestrogens – review. *Journal of Clinical Endocrinology and Metabolism* **83** 297–303.

Negri-Cesi P, Colciago A, Poletti A & Motta M 1999 5 alpha-reductase isozymes and aromatase are differentially expressed and active in the androgen-independent human prostate cancer cell lines DU145 and PC3. *Prostate* **41** 224–232.

Niswender GD, Midgley AR Jr, Monroe SE & Reichert LE Jr 1968 Radioimmunoassay for rat luteinizing hormone and antiovine LH serum and ovine LH-131-I. *Proceedings of the Society for Experimental Biology and Medicine* **128** 807–811.

Normington K & Russell DW 1992 Tissue distribution and kinetic characteristics of rat 5α-reductase isozymes. *Journal of Biological Chemistry* **27** 19548–19554.

Price KR & Fenwick GR 1985 Naturally occurring oestrogens in foods – a review. *Food Additives and Contaminants* **2** 73–106.

vom Saal FS, Timms BG, Montano MM, Palanza P, Thayer KA, Nagel SC, Dhar MD, Ganjam VK, Parmigiani S & Welshons WV 1997 Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. *PNAS* **94** 2056–2061.

Setchell KDR 1998 Phytoestrogens: the biochemistry, physiology and implications for human health of soy isoflavones. *American Journal of Clinical Nutrition* **68** 1333S–1346S.

Setchell KDR & Adlercreutz H 1988 Mammalian lignans and phtyo-oestrogens – recent studies on their formation, metabolism and biological role in health and disease. In *Role of the Gut Flora in Toxicity and Cancer*, pp 315–345. Ed. IR Rowland. New York: Academic Press.

Setchell KDR & Cassidy A 1999 Dietary isoflavones – biological effects and relevance to human health. *Journal of Nutrition* **129** 758S–767S.

Setchell KD, Zimmer-Nechemias L, Cai J & Heubi JE 1997 Exposure of infants to phyto-oestrogens from soy-based infant formula. *Lancet* **350** 23–27.

Sharma OP, Adlercreutz H, Strandberg JD, Zirkin BR, Coffey DS & Ewing LL 1992 Soy of dietary source plays a preventing role against the pathogenesis of prostatitis in rats. *Journal of Steroid Biochemistry and Molecular Biology* **43** 4557–4564.

Shibata Y, Ito K, Suzuki K, Nakano K, Fukabori Y, Suzuki R, Kawabe Y, Honoma S & Yamanaka H 2000 Changes in the endocrine environment of the human prostate transition zone with aging: simultaneous quantitative analysis of prostatic sex steroids and comparison with human prostatic histological composition. *Prostate* **42** 45–55.

Stephens FO 1997 Phytoestrogens and prostate cancer: possible preventive role. *Medical Journal of Australia* **167** 138–140.

Stephens FO 1999 The rising incidence of breast cancer in women and prostate cancer in men. Dietary influences: a possible preventative role for nature's sex hormone modifiers: the phytoestrogens. *Oncology Reports* **6** 865–870.

Townson DH, Wang XJ, Keyes PL, Kostyo JL & Stocco DM 1996 Expression of the steroidogenic acute regulatory protein (StAR) in the corpus luteum of the rabbit: dependence upon the luteotrophic hormone, 17β-estradiol. *Biology of Reproduction* **55** 868–874.

Wang C, Makela T, Hase T, Adlercreutz H & Kurzer MS 1994 Lignans and flavonoids inhibit aromatase enzyme in human preadipocytes. *Journal of Steroid Biochemistry and Molecular Biology* **50** 205–212.

Weber KS, Jacobson NA, Setchell KDR & Lephart ED 1999 Brain aromatase and 5α-reductase, regulatory behaviors and testosterone levels in adult rats on phytoestrogen diets. *Proceedings of the Society for Experimental Biology and Medicine* **22** 131–135.

Yaono M, Tamano S, Mori T, Kato K, Imaida K, Asamoto M & Shirai T 2000 Lobe specific effects of testosterone and estrogen on 3,2′-dimethyl-4-aminobiphenyl-induced rat prostate carcinogenesis. *Cancer Letters* **150** 33–40.

Zhang JX, Hallmans G, Landstrom M, Bergh A, Damber JE, Aman P & Adlercreutz H 1997 Soy and rye diets inhibit the development of Dunning R3327 prostatic adenocarcinoma in rats. *Cancer Letters* **114** 313–314.

Received 19 February 2001
Accepted 14 June 2001