Brain Aromatase and 5α-Reductase, Regulatory Behaviors and Testosterone Levels in Adult Rats on Phytoestrogen Diets (44395)

K. Scott Weber,* Nathan A. Jacobson,† Kenneth D. Setchell,‡ and Edwin D. Lephart*‡

Department of Zoology,* Cellular Biology Division, Brigham Young University, Provo, Utah 84602; and Clinical Mass Spectrometry Center,† Children’s Hospital Center, Cincinnati, Ohio 45229

Abstract. The purpose of this study was to examine the short-term effects of phytoestrogens in the diet on regulatory behaviors (food/water intake and locomotor activity), prostate weight, testosterone levels, and brain androgen metabolizing enzyme activity levels in adult male rats. Sprague-Dawley rats were fed phytoestrogen-containing versus phytoestrogen-free diets for 29 days. Standard methods were used to measure open field behavior, reproductive, hormonal parameters, and enzymatic activity levels. The phytoestrogen diet contained ~200 µg/g of isoflavones whereas in the phytoestrogen-free diet, no phytoestrogens were detected by HPLC analysis. There were no significant differences in any of the regulatory behaviors (food/water intake or locomotor activity), prostate weight, or testosterone levels between the treatment groups. Furthermore, there was no significant influence of phytoestrogens on brain aromatase activity levels, in either the medial basal hypothalamic-preoptic area (MBH-POA) or amygdala brain tissue sites examined. However, significant alterations in MBH-POA and amygdala 5α-reductase activities were detected in animals receiving the phytoestrogen-containing versus the phytoestrogen-free diets.

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phytoestrogens (nonsteroidal, estrogen-like substances found in many plants) have received a great deal of scientific investigative attention in recent years (1–3). Various studies have described their potential protective effects in the prevention of: 1) hormone-dependent cancers (e.g., breast and prostate); 2) cardiovascular disease; and 3) osteoporosis (3–8). These estrogen mimics have the ability to bind estrogen receptors (ERα and ERβ) and exert agonistic or antagonistic effects (3, 9, 10).

In mammals, the synthesis of endogenous estrogens is catalyzed by the aromatase cytochrome P450 enzyme. The aromatase enzyme converts androgen substrates to estrogens, primarily in gonadal/reproductive tissues (e.g., ovarian and placental tissue), but this enzyme is in adipocytes as well (11–13). Since phytoestrogens are estrogen-like substances, their potential to inhibit aromatase enzyme activity has been studied. In both human placental tissue and adipocytes, phytoestrogens (lignans and flavonoids) inhibit aromatase enzyme activity in a competitive fashion (14–17). While the protective effects of phytoestrogens against cancer and disease appear to be beneficial in peripheral tissue sites, it is not known how phytoestrogens may affect central nervous system function. This is an important issue since androgen metabolism (via the aromatase and 5α-reductase pathways) within the hypothalamus and limbic brain regions (11, 18, 19), is involved in the establishment of sexually dimorphic structures, regulation of neuroendocrine function, reproductive mechanisms and locomotor/sexual behaviors (8, 18–23).

Although brain plasticity (structural change) is relatively low in adult compared to perinatal animals, we de-
terminated whether phytoestrogens have the ability to alter brain function (by examining the enzymes responsible for androgen metabolism) and behavior in adult male Sprague-Dawley rats. This was accomplished by quantitating medial basal hypothalamic-preoptic area (MBH-POA) and amygdala (AMY) aromatase and 5α-reductase enzyme activity levels in the treated animals. Finally, the regulatory behaviors, feeding (food and water intake) and locomotion (open field activity), were examined along with the measurement of prostate weight and plasma testosterone levels in the male rats tested in this study.

Materials and Methods

**Animals.** Sprague-Dawley (45-day-old) male rats were obtained from Simonsen Lab (Gilroy, CA) and housed in individual plastic cages on a bedding of pine shavings in a controlled environment on a reverse light-dark cycle (lights on 1600–0600 hr; red light illumination during the dark cycle from 0600 to 1600 hr). The animals were given free access to tap water and a standard rat chow (Teklad Rat Diet, Madison, WI) until the animals were 74 days old.

**Phytoestrogen Diets.** Custom rat diets were obtained from Ziegler Bros. (Gardner, PA). The phytoestrogen-containing (NIH-07; referred to hereafter as Phyto-200) and phytoestrogen sterol-free (referred to hereafter as Phyto-free) diets were balanced and matched for equivalent percentage content of protein, carbohydrate, and fat. 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 x 0.46 cm Aquapore (C8; particle size 7 μm) column under gradient elution conditions, with internal controls, as described elsewhere (24, 25). The obtained values were expressed in μg per g. The Phyto-200 diet contained the following isoflavones (mean ± SEM; in μg per g): Daidzin = 81.7 ± 3.2; Genistin = 95.0 ± 0.7; and Glycitin = 16.5 ± 5.3 [The aglycones, daidzein and genistein were not detected (ND)]. In the Phyto-free diet using HPLC analysis, no phytoestrogens were detected.

At 74 days, the male rats were placed on the phytoestrogen-containing (Phyto 200) or phytoestrogen-free (Phyto-free) diets as assigned by treatment group. Animals continued to have free access to water. Food intake and body weight were recorded (in grams) and water intake (in mls) during the pretreatment and treatment intervals. The animals and methods of this study were approved by the IACUC (Institute of Animal Care and Use Committee) at Brigham Young University.

**Aromatase and 5α-Reductase Assays.** The medial basal hypothalamic and preoptic (MBH-POA) and amygdala (AMY) brain tissue sites were collected, as previously described (26–28), from 103-day-old animals. Aromatase and 5α-reductase activities were determined simultaneously in each MBH-POA and AMY tissue sample. For aromatase activity, the “tritiated water release” assay was performed, whereas the 5α-reductase rates were determined by thin layer chromatography and scintillation counting of the isolated 5α-metabolites, described in detail and validated elsewhere (26–28).

In brief, the isolated brain tissue samples were incubated in 200 μl of DMEM (pH 7.0; Sigma Chemical Co., St. Louis, MO) with a saturating concentration of [1β-3H]testosterone (2.6 μM; New England Nuclear Corp., Boston, MA) for 1 hr. Using these assay conditions, the predominant enzyme activity measured was 5α-reductase type 1 (29, 30), which apparently is the major 5α-reductase type expressed in brain in adult rats (29, 30). The protein content of each tissue fragment was determined by the method of Lowry et al. (31). Aromatase activity rates were expressed in fmol/ hr (of incubation)/mg protein, and the 5α-reductase activities were expressed in pmol/ hr (of incubation)/mg protein.

**Locomotor Activity.** During the pretreatment interval, at 83 days of age (or 9 days on the solid Phyto-200 or Phyto-free diets), and at 97 days of age (or 23 days on the treatments), the locomotor activity of the rats from each treatment group was measured by open field tests [conducted at 1400 hr (during the dark cycle when rats are most active) under red light conditions] (32). One rat (per open field test by treatment) was placed on a round table (4 ft in diameter, 3 ft off of the ground, with 4 x 4-inch boxed grids), and the ambulatory activity was videotaped. Two observers later counted the number of squares that each rat entered in 3 min. The locomotor activity levels were averaged (for each open-field test), and the correspondence (analyzed by a Pearson Product Moment Correlation) between the two observers in recording open-field behavior was r = 0.96 for the entire testing interval.

**Radioimmunoassay of Plasma Testosterone.** At the same time that enzyme activities were determined (in animals 103 days old), trunk blood was collected by decapitation and plasma was prepared and stored at −20°C until assayed. Plasma testosterone levels were determined by radioimmunoassay using a kit from Diagnostic Systems Laboratories (Webster, TX). All samples were run in a single assay (in duplicate), and the intra-assay coefficient of variance was less than 4%.

**Plasma Concentrations of Phytoestrogens.** The concentration and type(s) of phytoestrogens were analyzed from pooled (by treatment) plasma (n = 18) samples by gas-chromatography/mass spectrometry, with liquid-solid extraction and liquid-gel chromatographic techniques to isolate the estrogenic fractions using standard assay methods with internal controls (24). The obtained values were expressed in ng/ml.

**Statistical Analysis.** The data derived from the adult male rats (for each measured parameter) were tested by analysis of variance (ANOVA), followed by pairwise comparisons (via Tukey’s analysis) to detect significant differences between the treatment groups (α = P < 0.05).
Results

To determine the efficacy of the diets used in this study, the concentration of phytoestrogens was determined in each diet (see Materials and Methods section) and in pooled plasma samples. The animals receiving the phytoestrogen-containing diet (Phyto-200) displayed high levels of phytoestrogens in plasma compared to animals that received the phytoestrogen-free (Phyto-free) diet (Table I). In fact, the total concentration of total isoflavones was 35-fold higher in the Phyto-200 versus the Phyto-free animals.

When food/water intake, body and ventral prostate weights, and testosterone levels were analyzed, there were no significant differences among these parameters across the Phyto-200 versus the Phyto-free diet treatment groups. [Testosterone levels in the Phyto-200 = (3.5 ng/ml)] versus the Phyto-free group = (3.2 ng/ml), n = 18 per group].

Since phytoestrogens have similar physiochemical and physiological characteristics to endogenous estrogens, we determined the influence of the phytoestrogen diets on locomotor behavior by open-field tests in all of the animals (by treatment group) during the pretreatment and treatment intervals. During the pretreatment interval or at 9 days and 23 days on the treatments, there were no significant alterations in open-field activity across the Phyto-200 versus the Phyto-free groups.

In a subset of the treatment animals (10 out of 18 rats), aromatase cytochrome P450 and 5α-reductase enzyme activities were determined by standard assays in the amygdala (AMY) and the medial basal hypothalamic-preoptic area (MBH-POA) brain tissue sites. There were no significant alterations in brain aromatase levels regardless of the brain site examined (i.e., amygdala or MBH-POA) across the Phyto-200 versus the Phyto-free treatment groups. [Expressed in pmol/ hr/mg protein ± SEM: Amygdala; Phyto-200 = 547 ± 44 vs. Phyto-free = 484 ± 24; MBH-POA; Phyto-200 = 259 ± 11 vs. Phyto-free = 300 ± 21; n = 10 per group].

For brain 5α-reductase enzyme activity levels, unexpected results were obtained. Surprisingly, in the AMY, a slight but significant increase in 5α-reductase activity was recorded in the Phyto-200 vs. the Phyto-free treated-group (Fig. 1). Whereas, in the MBH-POA brain tissue site, a slight but significant decrease in 5α-reductase levels was observed in the Phyto-200 compared to the Phyto-free treated-male adult animals (Fig. 1).

Discussion

To our knowledge, this is the first study to examine phytoestrogens measured in plasma along with the determination of brain steroid metabolizing enzyme activity levels (of androgen metabolism) from animals on different phytoestrogen diets. In this study, the phytoestrogen diet (Phyto-200) contained approximated 200 µg per gram of phytoestrogens whereas in the phytoestrogen-free diet, phytoestrogens were below the detection limits of the HPLC method. When the concentration of isoflavones was determined in pooled plasma samples (by GS/MS), an expected relatively high concentration was found in samples from animals on the phytoestrogen-containing compared to the phytoestrogen-free diet. The beneficial effects of long-term or life-long consumption of diets containing phytoestrogens (via soy plant products) have been reported and reviewed, especially in the last few years (3–8). Circumstantial evidence suggests that phytoestrogens may have a protective role for a number of cancers (e.g., breast and prostate) as well as beneficial effects on other hormone-dependent diseases, such as cardiovascular disease and osteoporosis (3, 33, 34). On the other hand, potential health hazard issues have been raised in reference to human consumption of

Table I. Phytoestrogen Concentrations in Plasma Samples from Adult Male Rats on Phytoestrogen Diets

<table>
<thead>
<tr>
<th>Phyto-200 diet</th>
<th>Phyto-free diet</th>
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<tbody>
<tr>
<td>Daidzein</td>
<td>202.8 ng/ml</td>
</tr>
<tr>
<td>Genistein</td>
<td>121.0 ng/ml</td>
</tr>
<tr>
<td>Equol</td>
<td>371.7 ng/ml</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>694.7 ng/ml</strong></td>
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</tbody>
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*Note*. Pooled plasma samples (n = 18 per treatment group). Phytoestrogen concentrations were measured by gas chromatography/mass spectrometry (25, 26).
phytoestrogens during neonatal and infantile development (35), particularly the consumption of soy infant formulas that have reflectively high levels of isoflavones (24, 36).

Estrogenic hormones are known to significantly decrease feeding behavior, alter body-weight composition, and significantly increase behavioral activity levels, such as running wheel and open-field behaviors (37, 38); however, there were no significant differences in open-field behavior activity levels between animals fed a diet with or without phytoestrogens. Furthermore, no significant differences were detected in ventral prostate weights or circulating testosterone levels across the treatment groups in the present study. In previous longer-term studies, ventral prostate parameters and other reproductive measurements were influenced by phytoestrogens (8, 39–42). However, phytoestrogen levels were not measured in these previous studies and therefore, such differences are difficult to reconcile. It is possible that the previous observed effects are time- and/or dose-dependent since these longer-term studies administered phytoestrogens (either in the diet or by injection) during early development.

The importance of the aromatase cytochrome P450 enzyme in reproductive organs for normal hormone-dependent development and function is clearly established (43). Phytoestrogens, on the other hand, have the ability to inhibit aromatase activity in placental and adipocyte tissue (14–17). The link between modest phytoestrogen inhibition of aromatase and the apparent protection phytoestrogens have against cancer (breast and prostate) is an intriguing connection, since estrogens are mitogenic in some tissues. Conversely, the aromatase enzyme plays a pivotal role in the local formation of estrogen(s), particularly in specific regions of the brain areas such as the hypothalamus and limbic regions (11, 18–22). Most importantly, androgen metabolism in these brain regions is involved in: 1) the establishment of sexually dimorphic structures during development; 2) the regulation of neuroendocrine functions; and 3) the regulation of reproductive behavior (11, 18, 19, 21–23, 26). In contrast to the moderate in vitro inhibition of aromatase in peripheral tissues (14–17), we did not detect any alterations in AMY or MBH-POA aromatase levels in animals on the two diets. The regulatory factors for the aromatase enzyme in brain are, however, quite different from those in peripheral tissue sites and thus may explain these results (11).

When brain 5α-reductase enzyme activity was examined, surprisingly, slight but significant alterations were detected in brain 5α-reductase activity levels. This was unexpected since all previous reports examining brain 5α-reductase enzyme activity indicated that the levels were relatively stable regardless of brain sites assayed, even when hormonal treatments or castration was employed (23). Nevertheless, slight but significantly higher rates of AMY 5α-reductase were observed in animals on the Phyto-200 diet compared to animals administered the Phyto-free diets. On the other hand, in the MBH-POA tissue site animals on the phytoestrogen-containing diet (Phyto-200) displayed a moderate but significant decrease in 5α-reductase levels compared to the treatment group on the Phyto-free diet. The regulatory factor(s) for brain 5α-reductase are unknown (23), but this latter finding is in agreement with recent data where phytoestrogens in skin and prostate tissue inhibit 5α-reductase enzyme activity (44). This is an important finding since this is the first report to show that phytoestrogen diets can significantly alter 5α-reductase enzyme activity in neural tissue. These insights are intriguing in light of the potential positive actions of phytoestrogens in health and disease.