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Research report

Phytoestrogens decrease brain calcium-binding proteins but do not alter hypothalamic androgen metabolizing enzymes in adult male rats

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Abstract

Phytoestrogen [plant estrogenic-like molecule(s)] research has grown rapidly in recent years due to their potential health benefits. However, little is known about phytoestrogen's effects on the CNS. Androgen metabolizing enzymes are known to regulate neuroendocrine functions and reproductive behaviors, while calcium-binding proteins are associated with protecting against neurodegenerative diseases. Therefore, we examined aromatase and 5α-reductase enzyme activities in the medial basal hypothalamic and preoptic area (mbh–poa) and characterized mbh–poa and amygdala (amy) calbindin and calretinin levels (via Western analysis) from animals fed a phytoestrogen-free (P-free) vs. a phytoestrogen-containing diet [(P-600); that had 600 μg/g of phytoestrogens]. After approximately 5 weeks on the diets, the male rats were killed at 105 days. P-600 plasma phytoestrogen levels were 78-fold higher than the P-free values and the mbh–poa phytoestrogen content was 8-fold higher than the P-free group, demonstrating the passage of phytoestrogens into brain. In general, brain aromatase or 5α-reductase activity levels were not significantly altered by the experimental diets. However, independent of brain site (i.e., mbh–poa or amy) the abundance of calbindin from male P-600 rats was significantly lower than P-free animals. Conversely, for calretinin there were no significant alterations in the mbh–poa tissue site, while in the amy a similar pattern of expression was seen to that of the calbindin results. These data suggest that consumption of phytoestrogens via a soy diet for a relatively short interval can significantly: (1) elevate plasma and brain phytoestrogens levels and (2) decrease brain calcium-binding proteins without altering brain androgen metabolizing enzymes. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Soy; Phytoestrogens; Hypothalamus; Calbindin; Calretinin; Aromatase; 5α-Reductase; Rat; Western

1. Introduction

Phytoestrogens are estrogen-like molecules found in many plants (that have a diphenolic, nonsteroidal structure), such as, fruits, vegetables, legumes, whole-grain and especially soy products [1,6,34,39,41,42]. These estrogen mimics have the ability to selectively bind estrogen receptors ($\beta > \alpha$) [18], and recently have received a great deal of investigative attention due to the potential protective effects against age-related diseases (e.g., cardiovascular disease and osteoporosis) and hormone-dependent cancers (i.e., breast and prostate cancer) [1,3,17,33,43]. However, little is known about the effects phytoestrogens have on the central nervous system (CNS).

We recently reported that a soy diet containing moderate levels of phytoestrogens can alter the brain calcium-binding protein, calbindin, during prenatal development [49] and exert slight but significant effects on the androgen-metabolizing enzyme, 5α-reductase, in the hypothalamus of adult male rats [52]. Both biological parameters represent important elements in the structure/function relationship of the development of the rat CNS perinatally and the operation/survival of neurons postnatally [4,5,13,20,21,35]. Calbindin (CALB) and calretinin (CALRET) are calcium-binding proteins that serve potentially important roles in the development and function of the central nervous system (CNS) [4,13]. However, the most abundant calcium-binding protein in neurons is calbindin that apparently sequesters/regulates intracellular calcium during genesis of the CNS. Also, it is thought to act as a potential neuroprotective factor against programmed cell death

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(apoptosis) that is associated with neurodegenerative diseases, such as Parkinson's and Huntington's disease [13]. On the other hand, the aromatase and 5α-reductase enzymes are major pathways of androgen metabolism in brain [5,20,21,35]. Both enzymes activities in peripheral tissue sites have been shown to be inhibited by phytoestrogens (in vitro) [2,7,14,16,50]. The aromatase and 5α-reductase pathways represent important mechanisms of steroid hormonal action in the CNS, especially in the hypothalamic region during perinatal/postnatal development and both play regulatory roles in neuroendocrine functions, reproductive endocrine physiology and sexual behaviors [5,20,21,35]. To determine whether a soy diet containing relatively high levels of phytoestrogens influence brain androgen metabolizing enzymes and calcium-binding proteins levels in adult male rats, we examined aromatase and 5α-reductase enzyme activities in the medial basal hypothalamic and preoptic area (mbh–poa) and characterized mbh–poa and amygdala (amy) CALB and CALRET levels (via Western analysis) from animals fed a phytoestrogen-free vs. a phytoestrogen-containing diet. [Due to the fact that brain calcium-binding proteins have not been characterized in female rats (during the estrous cycle) only male rats were examined in this study.]

2. Materials and methods

2.1. Animals and phytoestrogen diets

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

At 70 days of age, 36 rats were randomly assigned to two treatment groups: (1) Phytoestrogen sterol-free diet (referred to hereafter as the P-free diet) or, (2) the Phytoestrogen-containing diet (referred to hereafter as the P-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 tap water and the diets. The P-free rat diet was obtained from Ziegler Bros. (Gardner, PA, USA), balanced and matched for equivalent percentage content of protein, carbohydrate, and fat to that of the commercially available P-600 diet (Harlan-Teklad). 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 under gradient elution conditions, with internal controls, as described elsewhere [6,44]. The P-600 diet contained a total of 600 μg/g of phytoestrogens while the phytoestrogen content in the P-free diet was below the limits of HPLC detection. [The P-600 diet contained the following isoflavones (mean expressed in μg/g): daidzin = 224.1; genistin = 319.0; glycitin = 39.5; and the aglycones detected were, daidzein = 8.9 and genistein = 8.6.]

After 35 days on the treatment diets (starting at 70 days old), the male rats were killed at 105 days of age. At this time, blood (for phytoestrogen plasma content analysis) and the brain tissue sites were collected using landmark boundaries (for phytoestrogen content analysis, aromatase/5α-reductase activity determination and the abundance of the calcium-binding proteins, CALB and CALRET) as outlined elsewhere [15,22,25,49,52]. The animals and methods of this study were approved by the Institute of Animal Care and Use Committee (IACUC) at Brigham Young University.

2.2. Plasma phytoestrogen levels

The concentration and type(s) of phytoestrogens were analyzed from pooled (by treatment) plasma ($n = 18$) samples by gas chromatography/mass spectrometry. This was performed by liquid–solid extraction and liquid–gel chromatographic techniques to isolate the phytoestrogen fractions using standard methods with internal controls to valid the assay [45]. The assay precision for the individual isoflavones was 6% to 10%, coefficient of variation. The obtained values were expressed in nanograms per milliliter.

2.3. Brain phytoestrogen levels

The levels of the phytoestrogens, genistein and daidzein were determined by time-resolved fluoroimmunoassay (TR-FIA) in lyophilized brain tissue (i.e., the mbh–poa) samples [in a subset of animals, (pooled by treatment, $n = 6$ per group)] using standard methods with internal controls.

2.4. Brain aromatase and 5α-reductase activities

Aromatase activity was determined in the mbh–poa brain tissue region. In animals by treatment group (representing two independent experiments, total $n = 20$ per treatment), the isolated brain tissue samples were incubated in 200 μl of DMEM with a saturating concentration of [1β-^{3}H]testosterone (2.5 μM; DuPont/New England Nuclear, Boston, MA, USA) for 1 h. Using standard assay procedures [24,25], the rates of aromatase activity in each tissue sample were determined by the 'tritiated water' release assay where aromatization of the substrate was isolated from the aqueous phase of the reaction mixture. Subsequently, an aliquot of the isolated and purified aqueous phase was quantified by scintillation counting for aromatase activity. The protein content of each tissue fragment was determined by the method of Lowry et al. [30]. Aromatase activity rates were expressed in femtomoles per hour (of incubation) per milligram protein.

Table 1

A. Phytoestrogen concentrations in plasma samples from adult male rats on phytoestrogen diets

	P-600 diet	P-free diet
Daidzein (ng/ml)	1000.5 + 226.8	16.9 + 0.9
Genistein (ng/ml)	433.5 + 98.5	10.0 + 1.1
Equol (ng/ml)	1031.0 + 26.3	4.8 + 0.0
Total (ng/ml)	2465.0[a]	31.7

Pooled plasma samples ($n = 18$ per treatment group). Each value represents the mean + S.E.M.; the pooled samples were analyzed twice. Phytoestrogen concentrations were measured by gas chromatography/mass spectrometry [45].

B. Phyotestrogen levels in the medial basal hypothalamic (mbh) and preoptic area (poa) from adult male rats on phytoestrogen diets

	P-600 diet	P-free diet
Daidzein (ng/g)	41.9	4.9
Genistein (ng/g)	27.2	3.9
Total (ng/g)	69.1[b]	8.8

Pooled brain samples ($n = 6$ per treatment group). Each value represents the mean from duplicate assayed samples. Phytoestrogen concentrations were determined by time-resolved fluoroimmunoassay.

[a] Significantly different vs. P-free diet.

[b] Significantly higher vs. the P-free group. There were no significant differences in food intake during the treatment interval.

To determine 5α-reductase activity in the brain tissue samples, an aliquot of the chloroform phase (100 μl) (from the extracted reaction mixture) was evaporated to dryness, redissolved in 30 μl chloroform containing 10 μg each of five nonradioactive steroids [5α-androstane-3β,17-dione, androstenedione, 5α-dihydrotestosterone (5α-DHT), testosterone and 5α-androstane-3α,17β-diol (3α-diol)]. Each prepared sample was applied to precoated 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 v/v/v), 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 [23]. Using these conditions, the predominate enzyme activity measured was 5α-reductase type 1 which apparently is the major 5α-reductase type expressed in adult rats of this brain tissue site [38]. The 5α-reductase activities were expressed as specific activity rate(s) in picomoles per hour (of incubation) per milligram protein.

2.5. Brain calbindin and calretinin Western analysis

Calbindin (CALB) and calretinin (CALRET) levels in the mbh–poa and amy tissue samples were determined by Western blot analysis, as previously reported by our laboratory [22,26,49,51]. In brief, the brain tissue samples (30 μg; by treatment and brain site) were resolved on 14% Tris-glycine gels, transferred onto Millipore-Immobilon-P membranes (Millipore, Bedford, MA, USA) and then incubated with a rat CALB antibody (1:50,000; kindly provided by A.M. Iacopino at Baylor College of Dentistry, Dallas, TX) or a rat CALRET antibody (1:10,000) purchased from Swant Antibodies (Bellinzona, Switerland). (Adult rat cerebellum served as positive controls in these experiments and the specificity of the CALB antibody has been previously reported by our laboratory [49]). The bound antibody-complex for each sample on the immunoblots was detected by enhanced chemiluminescence (ECL) Western blotting system (Amersham, Arlington

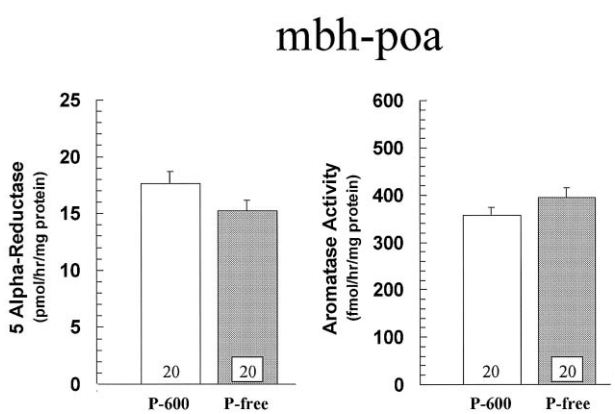

Fig. 1. Effect of phytoestrogen diets on 5α-reductase (left panel) and aromatase (right panel) activities in the mbh–poa at the end of the treatment interval. The number of animals per group is shown at the base of each bar, representing combined data from two separate experiments. The animals on the phytoestrogen-containing diet, P-600, are shown by the open bars and the animals on the phytoestrogen-free diet, P-free, are shown by the gray bars. The bars represent the mean + S.E.M. There were no significant differences between the treatments for mbh–poa 5α-reductase or aromatase activity levels.

Height, IL, USA). The intensities of the immunoreactive bands were captured using an imaging analysis system (Fotodyne, Harland, WI, USA). The optical densities of the bands were quantified using the NIH Imaging program (version 1.61). The presented results are data derived from three independent immunoblots for CALB or CALRET. In each immunoblot, the lowest intensity band (amy P-600) was assigned an arbitrary value of one (for the CALB and CALRET results). All other bands were then expressed as a fraction of this value and analyzed for significantly differences in band intensities, as previously performed by our laboratory [22,26,27,29,49,51].

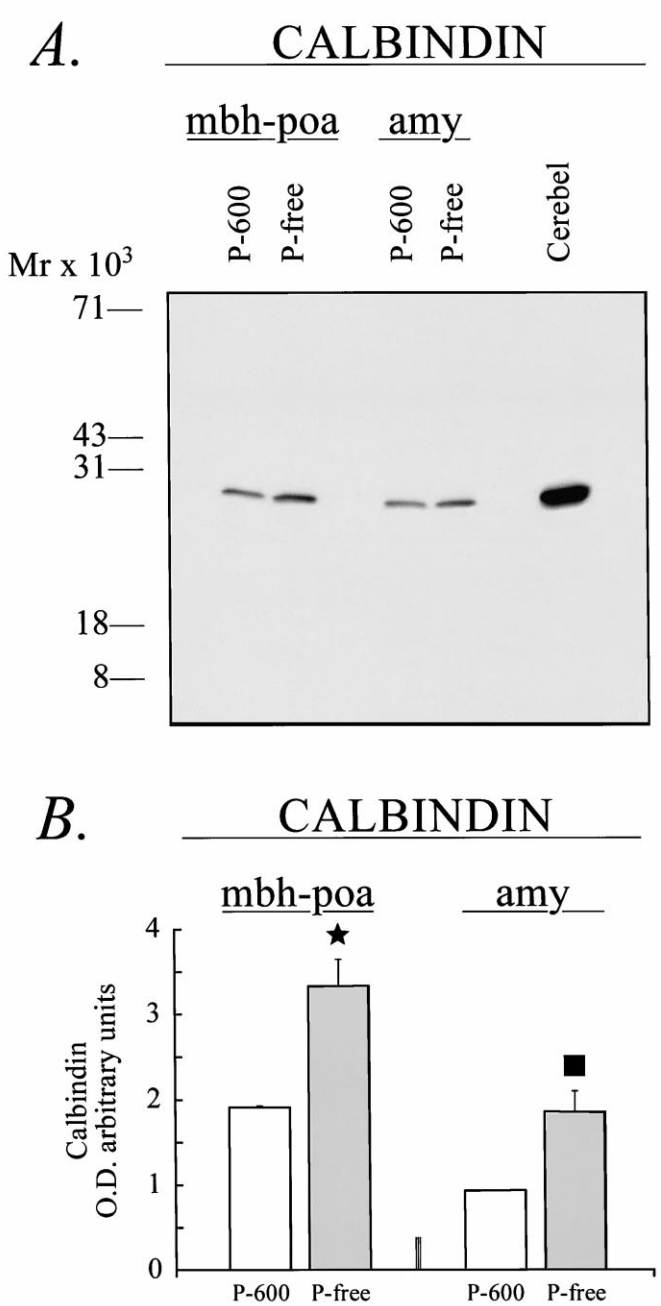

Fig. 2. (A) Western analysis of mbh–poa and amy calbindin abundance in adult male rats fed diets containing phytoestrogens, P-600, or lacking phytoestrogens, P-free. Prestained molecular weight standards ($Mr \times 10^3$) are shown by the horizontal bars on the left in the figure above. Rat cerebellum (Cerebel) served as positive controls in each Tris-glycine gel. (B) Densitometric analysis of the mbh–poa and amy calbindin Western autoradiograms (shown in A). For each immunoblot, the lowest band intensity (amy P-600) was assigned an arbitrary unit of 1 and all other band intensities were expressed as a fraction of this value. The histogram above represents data derived from three independent immumoblots expressed as the mean + S.E.M. (★) Significantly greater mbh–poa P-free levels compared to mbh–poa P-600 values. (■) Significantly greater amy P-free levels compared to amy P-600 values.

2.6. Statistical analysis

The data derived from the adult male rats were tested by analysis of variance (ANOVA), followed by pairwise comparisons (via Neuman–Kuels analysis) to detect significant differences between the treatment groups ($\alpha = p < 0.05$).

3. Results

3.1. Plasma phytoestrogens

The levels and types of phytoestrogens were determine in pooled plasma samples by treatment. The animals in the P-600 group displayed significantly higher levels of phytoestrogens in their plasma (total isoflavones = 2465

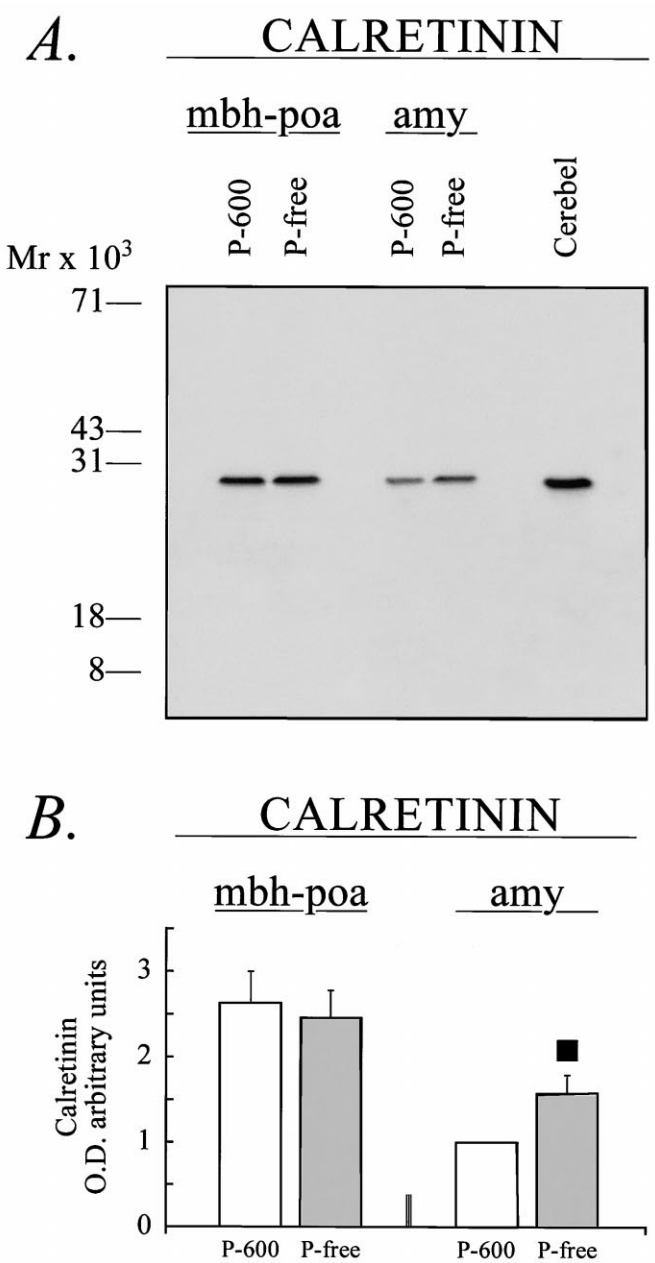

Fig. 3. (A) Western analysis of mbh–poa and amy calretinin abundance in adult male rats fed diets containing phytoestrogens, P-600, or lacking phytoestrogens, P-free. Prestained molecular weight standards ($Mr \times 10^{-3}$) are shown by the horizontal bars on the left in the figure above. Rat cerebellum (Cerebel) served as positive controls in each Tris-glycine gel. (B) Densitometric analysis of the mbh–poa and amy calbindin Western autoradiograms (shown in A). For each immunoblot, the lowest band intensity (amy P-600) was assigned an arbitrary unit of 1 and all other band intensities were expressed as a fraction of this value. The histogram above represents data derived from three independent immumoblots expressed as the mean + S.E.M. (■) Significantly greater amy P-free levels compared to amy P-600 values.

ng/ml) compared to the P-free treatment group (total isoflavones = 31.7 ng/ml) (Table 1A). Indeed, the total circulating concentration of plasma phytoestrogens was 78 times higher in the P-600 versus the P-free animals. In the plasma of P-600 diet-fed animals, daidzein and equol were the major components while genistein made up the remaining fraction of the circulating level of plasma phytoestrogens. In the P-free-diet-fed group the phytoestrogens, daidzein, genistein and equol were present but at very low circulating levels in plasma.

3.2. Brain (mbh–poa) phytoestrogen content

In evaluating the influence of the phytoestrogen diets on brain androgen metabolizing enzymes, the content of phytoestrogens was determined in mbh–poa samples by time-resolved fluoroimmunoassay (Table 1B). In the P-600 group there was a significant 8-fold higher level of the phytoestrogens, daidzein and genistein compared to the P-free values, indicating that phytoestrogens pass the blood–brain barrier in a similar manner to that of steroid molecules.

3.3. Mbh–poa aromatase and 5α-reductase enzyme activities

Since the phytoestrogen content in the brain samples paralleled that of the P-600 and the P-free circulating plasma values, we ascertained whether brain aromatase or 5α-reductase enzyme activities were altered by the diet treatments (Fig. 1). In either case, mbh–poa 5α-reductase activity levels (Fig. 1, left panel) or mbh–poa aromatase activity rates (Fig. 1, right panel) were not significantly altered by consumption of the P-600 vs. the P-free diets.

3.4. Calbindin and calretinin mbh–poa and amy Western analysis

Western analysis of the brain immunoblots revealed a protein band by the CALB specific antibody at approximately 28,000 Da (Fig. 2A), whereas, the CALRET antibody recognized a protein band with an apparent molecular weight of 29,000 Da (Fig. 3A). After the immunoblots were scanned and quantified the following results were obtained. The CALB results are displayed in Fig. 2B. Independent of brain site (i.e., mbh–poa or amy), the abundance of CALB from male P-600 rats was significantly lower than animals on the P-free diet. Conversely, for the CALRET results (Fig. 3B) there were no significant alterations in the mbh–poa tissue site, while in the amy a similar pattern of expression was seen to that of the CALB results. In the latter instance, amy CALRET abundance was significantly lower in the P-600 vs. the P-free-fed male rats. These data suggest that high levels of dietary phytoestrogens can significantly decrease calcium-binding proteins in rat brain.

4. Discussion

4.1. Phytoestrogens, hormone-dependent diseases and CNS effects

Substantial research attention has been devoted to phytoestrogens in recent years producing several avenues of investigation into their putative roles in preventing a number of cancers (e.g., breast and prostate) as well as age-related disorders such as, cardiovascular disease and osteoporosis [1,3,17,33,42,43]. In contrast to the linkage of phytoestrogens protecting against hormone-dependent and age-related disorders, there is a clear lack of data on the effects of phytoestrogens in the CNS. The present study addressed this issue, in part, by quantifying androgen metabolizing enzymes in the mbh–poa of the brain (regions that are known to regulate important neuroendocrine functions, reproductive endocrine physiology and sexual behaviors) from adult male rats fed phytoestrogen-containing vs. phytoestrogen-free diets. The impetus to conduct these studies originates from in vitro data where both the aromatase and 5α-reductase enzyme pathways of androgen metabolism are inhibited by phytoestrogens in reproductive tissue sites that are associated with the prevention of hormone-dependent cancers like breast and prostate [1–3,14,16,50]. We have therefore quantified the major enzymes involved in androgen metabolism in the brain and additionally, have determined the influence of dietary isoflavones on the calcium-binding proteins, CALB and CALRET because these play important roles in regulating intraneuronal cellular calcium [4,13,31]. CALB is present in relatively high abundance in neurons and protects against neurodegenerative diseases where it apparently defends against programmed cell death. This neuroprotective mechanism appears to be important in neurological disorders such as Parkinson's and Huntington's disease [13].

4.2. Diet, plasma and brain phytoestrogen levels

To validate the phytoestrogen composition of the diets utilized in this study, HPLC analysis revealed that the high phytoestrogen diet contained 600 μg/g of total phytoestrogens, whereas, the diet lacking phytoestrogens was below the limits of HPLC detection for phytoestrogens. After the adult male rats were exposed to these diets for approximately 35 days (from 70 to 105 days of age) the plasma phytoestrogen levels in the P-600 group (@ 2465 ng/ml) were similar to those seen in adult humans on an Asian soy-based diet (of 20 to 40 g of soy intake per day) [1,3]. On the other hand, the very low plasma phytoestrogen levels in the rats fed a P-free diet was similar to that seen in adults on a Western diet where soy foods are rarely consumed [1,3]. Given, the physiochemical similarity between phytoestrogens and estradiol it was not surprising to find high levels of daidzein and genistein in the mbh–poa brain sites of P-600 fed rats. These data provide evidence

that phytoestrogens enter the lipophilic environment of the brain, confirming an earlier report which showed that after intraperitoneal injection of genistein and daidzein brain levels increased within 20 min [10,42]. Equol could not be measured in the brain tissue by TR-FIA, but it is presumed to also be present and therefore it is reasonable to hypothesize that notably higher levels of equol occur in the P-600 brain samples.

4.3. Phytoestrogens and brain aromatase and 5α-reductase enzyme activity levels

Although there were significantly higher levels of phytoestrogens in the plasma and brain sites of the P-600 vs. the P-free animals, there were no significant alterations in the brain androgen metabolizing enzymes, aromatase, or 5α-reductase between the animals on the two diets. These data are in agreement with previous findings where brain aromatase activity levels were unchanged in adult male rats receiving 200 μg/g of dietary phytoestrogens vs. a diet lacking phytoestrogens [52]. Conversely, the slight but significant changes in brain 5α-reductase of adult animals on a moderate phytoestrogen (200 μg/g) vs. a diet free of phytoestrogens we reported previously [52] were not observed in the present study when a high phytoestrogen diet (600 μg/g) was examined. One explanation for the difference in these results may be the relative variability of brain 5α-reductase enzyme activity rates we have seen in animals on phytoestrogen-free diets (unpublished data) vs. stable brain 5α-reductase levels in animals on phytoestrogen-containing diets [52]. While phytoestrogens inhibit aromatase and 5α-reductase (examining in vitro systems) [2,14,16,50], it is known that the factors regulating brain aromatase are quite different [21,35], while the regulation of 5α-reduction in brain is unknown [5,20]. There is evidence showing pharmacological doses of genistein injected into rats prenatally, increase sexually dimorphic nuclear volumes in female offspring to male-like patterns [8,9], however, it is not known if dietary phytoestrogens have any effects on the genesis of the CNS.

4.4. Dietary phytoestrogens and brain calbindin and calretinin levels

It has been hypothesized that calcium-binding proteins perform a critical role in the process of programmed neuronal cell death because of their ability to regulate intracellular calcium [4,13,31]. It has been postulated that the primary function of calcium-binding proteins is to buffer intracellular calcium levels, thus functioning as a neuroprotective agent against premature apoptosis [4,13,31]. When neurons are exposed to toxic levels of calcium, it is thought that cells expressing sufficient levels of CALB experience less neurodegeneration than cells in areas where CALB is less abundant [13]. Thus, the concept that calcium plays an important role as sculptor and destroyer of neural circuitry has been clearly established [31].

Previously, experimental evidence was presented showing that CALB (and CALRET), specifically in the mbh–poa, is expressed in the rat in a dimorphic fashion [22,29]. Males displayed significantly greater levels than females and that these differences in calcium-binding protein levels may be involved in the establishment of sexually dimorphic brain structures [22]. Moreover, the expression of CALB during prenatal development is regulated by testosterone in a positive manner [51] and CALB is colocalized with the aromatase enzyme within sexually dimorphic hypothalamic structures during perinatal development [28]. Furthermore, we have shown that CALB mRNA levels are quite abundant in the mbh–poa region of rats compared to other brain regions such as the hippocampus or cerebellum [27,48]. Therefore, due to the functional significance of the hypothalamic region and the importance of calcium-binding proteins in neuronal protection/survival, we examined the effects of phytoestrogen diets on mbh–poa and amy CALB and CALRET levels by Western analysis.

In this study, adult male rats fed a phytoestrogen diet (of 600 μg/g; essentially equivalent to a typical Asian diet, based on the obtained plasma phytoestrogen levels) displayed a significant reduction in mbh–poa CALB levels compared to animals fed a phytoestrogen-free diet. This inhibition by high dietary phytoestrogens was also seen in the amy for both CALB and CALRET, indicating that dietary phytoestrogens may reduce calcium-binding proteins in different brain sites. However, significant alterations in mbh–poa CALRET were not observed by Western analysis and the reason for the lack of a treatment effect in this brain site is unknown. Notably, the inhibition of mbh–poa CALB by dietary phytoestrogens in the present study was somewhat expected since we recently reported similar findings in prenatal female (but not male) animals fed a moderate phytoestrogen diet (@ 200 μg/g) vs. a phytoestrogen-free diet [49].

It is not known exactly how CALB/CALRET expression is inhibited by phytoestrogens. Nevertheless, it is intriguing to speculate that the relative high affinity phytoestrogens have for ERβs may: (a) influence gene expression or (b) block endogenous estrogen signals [18], that may result in decreasing CALB expression. Regulation of CALB gene expression by estrogen via a 5′ flanking region mechanism is known [11]. Although, the complex nature of ERβ variants expressed in various brain sites in modulating the hormonal action of phytoestrogens is unknown and will require labor-intensive research efforts to unravel the pathways of signal transduction via the different forms of ERβ receptors [19,36,37]. Another possible explanation for reduced brain CALB expression may be due to decreased circulating plasma testosterone levels in the P-600 vs. the P-free animals (unpublished data). A positive correlation between testosterone and CALB levels has been established during prenatal development [51].

In summary, these data suggest that dietary soy phytoestrogens, or the lack thereof, for a relatively short interval

can significantly influence brain calcium-binding proteins in adult male rats. While dietary soy phytoestrogens have been linked to protecting against hormone-dependent cancers and age-related disease [1,3,17,33,42,43], the present findings have important implications on the neuroprotective effects of calcium regulating molecules in the brain. This may be of importance during pre- and postnatal development and especially in aging. Whether these observations in rats have relevance to humans is not known, but are important to establish. There is some interest now in the possible role of phytoestrogens on cognition, memory and dementia, given the potentially beneficial effects of estrogen therapy in this area [12,32,46,47]. Likewise, there is some evidence suggesting a link between high cerebrovascular mortality rates in the Japanese population being associated with high consumption of plant products [40]. Clearly, the scarcity of data regarding the effects of phytoestrogens on CNS development/function justifies further research given the recognized importance of estrogen in the brain [12,32,46,47].

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References

[1] H. Adlercreutz, Evolution, nutrition, intestinal microflora, and prevention of cancer: a hypothesis, Proc. Soc. Exp. Biol. Med. 217 (1997) 241–246.
[2] H. Adlercreutz, C. Bannwart, K. Wahala, T. Makela, G. Brunow, T. Hase, P.J. Arosemena, J.T. Kellis Jr., L.E. Vickery, Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens, J. Steroid Biochem. Mol. Biol. 44 (1994) 147–153.
[3] H. Adlercreutz, W. Mazur, Phyto-estrogens and Western diets, Ann. Med. 29 (1997) 95–120.
[4] K.G. Baimbridge, M.R. Celio, J.H. Roger, Calcium-binding proteins in the nervous system, Trends Neurosci. 15 (1992) 303–308.
[5] F. Celotti, C. Melcangi, L. Martini, The 5α-reductase in the brain: molecular aspects and relation to brain function, Front. Neuroendocrinol. 13 (1992) 163–215.
[6] L. Coward, N.C. Barnes, K.D.R. Setchell, S. Barnes, Genistein, daidzein, and their β-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets, J. Agric. Food Chem. 41 (1993) 1961–1967.
[7] B.A. Evans, K. Griffiths, M.S. Morton, Inhibition of 5α-reductase in genital skin fibroblasts and prostate tissue by dietary lignans and isoflavonoids, J. Endocrinol. 147 (1995) 295–302.
[8] K.A. Faber, C.L. Hughes Jr., The effect of neonatal exposure to diethylstilbestrol, genistein, and zearalenone on pituitary responsiveness and sexually dimorphic nucleus volume in the castrated adult rat, Biol. Reprod. 45 (1991) 649–653.
[9] K.A. Faber, C.L. Hughes Jr., Dose–response characteristics of neonatal exposure to genistein on pituitary responsiveness to gonadotropin releasing hormone and volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in postpubertal castrated female rats, Reprod. Toxicol. 7 (1993) 35–39.
[10] P.H. Gamache, T.J. Maher, K.D.R. Setchell, T.-H. Wu, I.N. Acworth, The transfer of non-steroidal dietary estrogens into brain, Soc. Neurosci. Abstr. 22 (1996) 1967.
[11] R.K. Gill, S. Christakos, Regulation by estrogen through the 5′-flanking region of the mouse calbindin-28K gene, Mol. Endocrinol. 9 (1995) 319–326.
[12] V.W. Henderson, Estrogen, cognition, and a woman's risk of Alzheimer's disease, Am. J. Med. 103 (1997) 11S–18S.
[13] A.M. Iacopino, E.M. Quintero, E.K. Miller, Calbindin-D_{28K} a potential neuroprotective protein, Neurodegeneration 3 (1994) 1–20.
[14] A.R. Ibrahim, Y.J. Abul-Hajj, Aromatase inhibition by flavonoids, J. Steroid Biochem. Mol. Biol. 37 (1990) 257–260.
[15] N.A. Jacobson, D.R. Ladle, E.D. Lephart, Aromatase cytochrome P450 and 5α-reductase in the amygdala and cortex of perinatal rats, NeuroReport 8 (1997) 2529–2533.
[16] J.T. Kellis Jr., L.E. Vickery, Inhibition of human estrogen synthetase (aromatase) by flavones, Science 225 (1984) 1032–1034.
[17] D.C. Knight, J.A. Eden, A review of the clinical effects of phytoestrogens, Obstet. Gynecol. 87 (1996) 897–904.
[18] G.G.J.M. Kuiper, B. Carlsson, K. Grandien, E. Enmark, J. Haggbald, S. Nilsson, J. Gustafsson, Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β, Endocrinology 138 (1997) 863–870.
[19] G.G. Kuiper, P.J. Shughrue, I. Merchenthaler, J.A. Gustafsson, The estrogen receptor beta subtype: a novel mediator of estrogen action in neuroendocrine systems, Front. Neuroendocrinol. 19 (1998) 253–286.
[20] E.D. Lephart, A review of brain 5α-reductase: cellular, enzymatic, and molecular perspectives and implications for biological function, Mol. Cell. Neurosci. 4 (1993) 473–484.
[21] E.D. Lephart, A review of brain aromatase cytochrome P450, Brain Res. Rev. 22 (1996) 1–26.
[22] E.D. Lephart, Dimorphic expression of calbindin-D_{28K} in the medial basal hypothalamus from perinatal male and female rats, Dev. Brain Res. 96 (1996) 281–284.
[23] E.D. Lephart, S. Andersson, E.R. Simpson, Expression of neural 5α-reductase messenger ribonucleic acid: comparison to 5α-reductase activity during prenatal development in the rat, Endocrinol. 127 (1990) 1121–1128.
[24] E.D. Lephart, E.R. Simpson, Techniques for the assay of aromatase activity, in: M.R. Waterman, E.F. Johnson (Eds.), Methods of Enzymology, Vol. 206, Academic Press, Orlando, 1991, pp. 477–483.
[25] E.D. Lephart, E.R. Simpson, M.J. McPhaul, M.W. Kilgore, J.D. Wilson, S.R. Ojeda, Brain aromatase cytochrome P-450 messenger RNA levels and enzyme activity during prenatal and perinatal development in the rat, Mol. Brain Res. 16 (1992) 187–192.
[26] E.D. Lephart, H. Talyor, N.A. Jacobson, M.A. Watson, Calretinin and calbindin-D_{28K} in male rats during postnatal development, Neurobiol. Aging 19 (1998) 253–257.
[27] E.D. Lephart, M.A. Watson, Effects of early postnatal maternal separation on hypothalamic–preoptic area and hippocampal calbindin-D_{28K} in male and female infantile rats, Neurosci. Lett. 267 (1999) 41–44.
[28] E.D. Lephart, M.A. Watson, L. Mathias, R.W. Rhees, S. Diano, T.L. Horvath, Co-localization of aromatase cytochrome P450 and calbindin-D28K and androgen regulation of calbindin-28K during perinatal development, Soc. Neurosci. Abstr. 23 (1997) 342.
[29] E.D. Lephart, M.A. Watson, R.W. Rhees, D.R. Ladle, N.A. Jacobson, Development expression of calretinin in the medial basal hypothalamus and amygdala from male and female fats, Neurosci. Res. 28 (1997) 269–273.

[30] O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randall, Protein determination with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[31] M.P. Mattson, Calcium as sculptor and destroyer of neural circuitry, Exp. Gerontol. 27 (1992) 29–49.
[32] B.S. McEwen, Clinical review 108: the molecular and neuroanatomical basis for estrogen effects in the central nervous system, J. Clin. Endocrinol. Metab. 84 (1999) 1790–1797.
[33] A.L. Murkies, G. Wilcox, S.R. Davis, Phytoestrogens — review, J. Clin. Endocrinol. Metab. 83 (1998) 297–303.
[34] P.A. Murphy, Phytoestrogen content of processed soybean products, Food Technol. 34 (1982) 60–64.
[35] F. Naftolin, Brain aromatization of androgens, J. Reprod. Med. 39 (1994) 257–261.
[36] H.B. Patisaul, P.L. Whitten, L.J. Young, Regulation of estrogen receptor beta MRNA in the brain: opposite effects of 17beta-estradiol and the phytoestrogen, coumestrol, Mol. Brain Res. 67 (1999) 165–171.
[37] D.N. Petersen, G.T. Tkalcevic, P.H. Koza-Taylor, T.G. Turi, T.A. Brown, Identification of estrogen receptor beta2, a functional variant of estrogen receptor beta expressed in normal rat tissues, Endocrinology 139 (1998) 1082–1092.
[38] A. Poletti, P. Negri-Cesi, M. Rabuffetti, A. Colciago, F. Celotti, L. Martini, Transient expression of the 5α-reductase type 2 isozyme in the rat brain in late fetal and early postnatal life, Endocrinology 139 (1998) 2171–2178.
[39] K.R. Price, G.R. Fenwick, Naturally occurring oestrogens in foods — a review, Food Addit. Contam. 2 (1995) 73–106.
[40] S. Seely, Diet and cerebrovascular disease: search for linkages, Med. Hypotheses 9 (1982) 509–515.
[41] K.D.R. Setchell, Naturally occurring non-steroidal estrogens of dietary origin, in: J. McLachlan (Ed.), Estrogens in the Environment: Influence on Development, Elsevier, New York, 1985, pp. 69–85.
[42] K.D.R. Setchell, Phytoestrogens: biochemistry, physiology and implications for human health of soy isoflavones, Am. J. Clin. Nutr. 68 (1998) 1333S–1346S.
[43] K.D.R. Setchell, A. Cassidy, Dietary isoflavones — biological effects and relevance to human health, J. Nutr. 129 (1999) 758S–767S.
[44] K.D.R. Setchell, M.B. Welsh, C.K. Lim, High-performance liquid chromatographic analysis of phytoestrogens in soy protein preparations with ultraviolet, electrochemical and thermospray mass spectrometric detection, J. Chromatogr. 386 (1987) 315–323.
[45] K.D.R. Setchell, L. Zimmer-Nechemias, J. Cai, J.E. Heubi, Exposure of infants to phyto-oestrogens from soy-based infant formula, Lancet 350 (1997) 23–27.
[46] B.B. Sherwin, Estrogen and cognitive function in women, Proc. Soc. Exp. Biol. Med. 217 (1998) 17–22.
[47] J.W. Simpkins, P.S. Green, K.E. Gridley, M. Singh, N.C. de Fiebre, G. Rajakumar, Role of estrogen replacement therapy in memory enhancement and the prevention of neuronal loss associated with Alzheimer's disease, Am. J. Med. 103 (1997) 19S–25S.
[48] E. Stuart, E.D. Lephart, Dimorphic expression of MBH–POA calbindin mRNA levels during perinatal development and adult brain tissue distribution of calbindin mRNA, Mol. Brain. Res. 73 (1999) 60–67.
[49] H. Taylor, E.M. Quintero, A.M. Iacopino, E.D. Lephart, Phytoestrogens alter hypothalamic calbindin-D_{28K} levels during prenatal development, Dev. Brain Res. 114 (1999) 277–281.
[50] C. Wang, T. Makela, T. Hase, H. Adlercreutz, M.S. Kurzer, Lignans and flavonoids inhibit aromatase enzyme in human preadipocytes, J. Steroid Biochem. Mol. Biol. 50 (1994) 205–212.
[51] M.A. Watson, H. Taylor, E.D. Lephart, Androgen-dependent modulation of calbindin-D_{28K} in hypothalamic tissue during prenatal development, Neurosci. Res. 32 (1998) 97–101.
[52] K.S. Weber, N.A. Jacobson, K.D.R. Setchell, E.D. Lephart, Brain aromatase and 5α-reductase, regulatory behaviors and testosterone levels in adult rats on phytoestrogen diets, Proc. Soc. Exp. Biol. Med. 22 (1999) 131–135.