Coumestrol Antagonizes Neuroendocrine Actions of Estrogen via the Estrogen Receptor α

DENA A. JACOB,[2,*] JENNIFER L. TEMPLE,* HEATHER B. PATISAUL,† LARRY J. YOUNG,† AND EMILIE F. RISSMAN[1,*]

Department of Biology and Neuroscience Program, University of Virginia, Charlottesville, Virginia 22904–4328; †Center for Behavioral Neuroscience, Emory University, Atlanta, Georgia 30329

The phytoestrogen coumestrol has estrogenic actions on peripheral reproductive tissues. Yet in the brain this compound has both estrogenic and anti-estrogenic effects. We used estrogen receptor α knockout mice (ERαKO) to determine whether coumestrol has estrogenic actions in mice and also if these effects are mediated by the classic ERα. Female wild-type (WT) and ERαKO mice were ovariectomized and treated with estradiol (E_2), dietary coumestrol, both, or neither compound. Ten days later the animals were sacrificed, blood was collected, and brain tissues were perfused. Fixed brains were sectioned and immunocytochemistry was employed to quantify progesterone receptors (PR) in the medial preoptic (POA) and ventromedial nucleus of the hypothalamus (VMN). Plasma was assayed for luteinizing hormone (LH). Estrogen treatment induced PR immunoreactivity in both regions in brains of WT females. In ERαKO mice, lower levels of PR were induced. The stimulatory effects of E_2 on PR were attenuated in the POA by cotreatment with coumestrol, and the same trend was noted in the VMN. WT ovariectomized females treated with E_2 had low levels of LH, while LH was high in untreated females and even higher in ovariectomized females treated with coumestrol. ERαKO females in all treatment groups had high levels of LH. Taken together, the results show that coumestrol has anti-estrogenic actions in the brain and pituitary and that ERα mediates these effects. [Exp Biol Med Vol. 226(4):301–306, 2001]

Key words: phytoestrogen; progesterone receptor; luteinizing hormone; nutrition; estrogen receptor β

Phytoestrogens are nonsteroidal, estrogen-like compounds produced by plants, particularly the legumes and soy-based foods. Coumestrol is a coumestan phytoestrogen found at high concentrations in clover and alfalfa sprouts, but also at lower concentrations in sunflower seeds, lima bean seeds, pinto bean seeds, and round split peas (1). When consumed, these plant estrogens have various effects on reproductive physiology including uterine proliferation in immature female rats (2, 3) and infertility in sheep (4). Diets rich in soy, particularly vegetarian and traditional Asian diets, have been associated with a number of beneficial effects such as the reduced risk of breast cancer, prostate cancer, heart disease, and a decrease in menopausal symptoms (5, 6). In addition, the regression of mammary tumors in rats adhering to a soybean diet demonstrates possible chemopreventative effects of phytoestrogens (7, 8). Although it is not fully understood how phytoestrogens exert their effects, numerous studies have demonstrated their potential for nutritional and pharmaceutical use (9–11).

Phytoestrogens bind to both the estrogen receptor alpha (ERα) and the newly cloned estrogen receptor beta (ERβ) (12–14). Use of ERαKO mice has demonstrated ERα's major role in various reproductive functions such as the induction of PR in the brain (15, 16), E_2 negative feedback on LH (17), and mating behavior (18, 19). However, ERαKO females treated with E_2 do show an attenuated induction of PR in several brain regions (16), and ERβKO females are subfertile (20), thus ERβ is involved in female reproduction. Estrogen receptor β has been detected in the hypothalamus, cerebellum, and olfactory lobe of the brain, as well as in the prostate, uterus, ovary, and lung (21). Although many differences in the distribution of ERα and ERβ in the brain exist, the expression of the two receptors overlaps considerably in the preoptic area (POA), bed nucleus of the stria terminalis (BnST), and throughout the lower brain stem (22).

Binding studies using the purified receptor proteins have shown that coumestrol actively competes for both re-

This work was supported by NIH awards R01 MH57759 and K02 MH01349 (to E. F. R.) and by NICHD/NIH through cooperative agreement (U54 HD28934) as part of the Specialized Cooperative Centers Program in Reproduction Research. J. L. T. was supported by a NSF pre-doctoral fellowship.

[1] To whom requests for reprints should be addressed at P.O. Box 40–0328, Department of Biology, Gilmer Hall, University of Virginia, Charlottesville, VA 22904–4328. E-mail: Rissman@virginia.edu

[2] Current address: Neuroscience Program, University of Massachusetts, Amherst, MA 01003–7710.

Received August 3, 2000.
Accepted November 10, 2000.

0037-9727/01/2264-0301$15.00

ceptor subtypes, but has a 2-fold higher binding affinity for ERβ than ERα (12, 13). This is likely to have functional significance given the differential distributions of the two receptor subtypes. These same studies confirm that coumestrol not only binds to estrogen receptors, but also initiates gene transcription through both subtypes.

Although these assays are helpful in determining whether or not coumestrol and other phytoestrogens have estrogenic properties, they cannot accurately predict the activity of these compounds *in vivo* because of the obvious absence of cellular binding proteins and the exclusion of normal metabolism. This necessitates the use of *in vivo* studies. In the few that have already been undertaken, coumestrol has been shown to have both estrogenic and antiestrogenic effects on reproductive physiology, gene activation (12, 33), and behavior (3). These studies indicate that coumestrol can cross the blood-brain barrier and affect estrogen-dependent gene expression, but do not conclusively demonstrate through which estrogen receptor subtype it produces these effects.

To clarify which ER subtype coumestrol acts upon, this study was conducted in knockout mice that lack functional ERα (ERαKO). Based on a study by Patisaul and colleagues (23), we hypothesized that coumestrol works through ERβ and thus, ERαKO and their wild-type (WT) littermates would display similar effects after coumestrol treatment.

Materials and Methods

Subjects. Subjects used in the following studies were female mice of a mixed 129/J and C57BL/6J background. Mice were generated by pairing heterozygous (HET) animals (one functional copy of the ERα gene) to produce a 1:2:1 ratio of WT:HET:knock out offspring that were genotyped by PCR analysis of tail DNA (24). Each animal was housed individually at weaning (21 days of age) and had *ad libitum* access to water and food (Purina mouse chow No. 5001). Twenty-four WT (+/+) and 24 ERα knockout (ERαKO; -/-; homozygous ERα-deficient) littermates were used. All animals were kept on a 12:12 light:dark cycle (lights off at 1300 hr).

Between 45 and 50 days of age, all mice were given powered mouse chow in place of pellets. Two days later, females were ovariectomized under ketamine/xylazine anesthesia (100 mg/kg ketamine/10 mg/kg xylazine). Half of the animals of each genotype (12 WT, 12 ERαKO) received a Silastic implant containing E_2 (5 mm, 1:1 17β-E_2:cholesterol, o.d. 2.125 mm, i.d. 1 mm). The other animals received a blank implant (same size Silastic tubing without any hormone). This E_2 implant yields a plasma concentration equivalent to that experienced during mouse proestrous (about 80 pg/ml) (15). The capsules were implanted subcutaneously in the mid-scapular region. After ovariectomy, half of the females in each genotype and hormone condition were given 0.02% coumestrol mixed in their chow and the other half were fed powdered mouse chow only. In this manner, eight groups were formed: WT/OVX/no coumestrol, WT/OVX/coumestrol, WT/OVX + E_2/no coumestrol, WT/OVX + E_2/coumestrol, ERαKO/OVX/no coumestrol, ERαKO/OVX/coumestrol, ERαKO/OVX + E_2/no coumestrol, and ERαKO/OVX + E_2/coumestrol. There were six females in each group.

Ten days after surgery, animals in each group were deeply anesthetized with sodium pentobarbital (0.1 mg/kg) prior to perfusion through the aorta. Blood was collected via cardiac puncture. The mice were then perfused briefly with heparinized saline, followed by 4% paraformaldehyde. Brains were removed, cryoprotected overnight in 30% sucrose, and frozen in cold *N*-methyl butane. Frozen tissue was stored at −70°C until sectioned. Blood samples were spun in a centrifuge and plasma was extracted, quickly frozen, and stored at −70°C until assayed for LH.

Immunocytochemistry Procedures. Brains were sectioned coronally at 30 μm on a cryostat and divided into a series of four vials containing antifreeze. Alternate sections from each brain were processed for progesterone receptor immunoreactivity (PR-ir). Sections were rinsed in TBS and treated with sodium borohydride. Tissue was then incubated for 48 hr at 4°C in progesterone receptor primary antibody (DAKO, Sigma Chemical Company, St. Louis, MO). This antibody was raised in rabbit against the human progesterone receptor and used at a concentration 0.325 μg/ml. After 48 hr, the tissue was rinsed thoroughly in TBS and incubated in biotinylated goat anti-rabbit IgG (1:500, Vector Labs, Burlingame, CA) for 1 hr. Tissue was rinsed and incubated in avidin-biotin-peroxidase complex (ABC, Vector Elite Kit, Vector Labs) for 1 hr. Immunoreactivity was visualized using nickel-intensified diaminobenzidine (DAB) with hydrogen peroxide as the chromogen for 25 min.

LH Assay. The assay for LH was conducted using radioimmunoassay (RIA) by the University of Virginia Core Ligand and Assay Laboratory (National Institutes of Health U54 HD-36199). All samples were run in duplicate in a single assay to eliminate interassay variability. Plasma LH was measured by a supersensitive two-site sandwich immunoassay using monoclonal antibodies MAB1 (no. 58187) and TMA (no. 5303, Medix Kaunianinen, Finland) against mouse and human, respectively. This assay has a sensitivity of 7 pg/tube and the intraassay coefficient of variability of the controls ranged from 1.7% to 10.1%. This assay has been previously validated for use in the mouse (25).

Immunocytochemical Data Analysis. An observer blind to the treatment of the animals counted the numbers of PR-ir cells. Labeled neurons were counted in the medial preoptic area (POA) and the ventral medial nucleus of the hypothalamus (VMH). We employed an Olympus BX60 light microscope and an Olympus U-CMAD-2 camera to project images onto a computer screen. We used Scion Image (a downloadable program offered to the public for free at http://scrc.dcrt.nih.gov/imaging/index.html#image) in order to automate counting of cells in these

areas. The images were captured and saved for each animal from matched sections of the brain using well-defined landmarks and a mouse brain atlas (26). For analysis of data, a threshold was placed on the section that was two standard deviations higher (darker) than the background. A standard-size box was then placed around the area of interest and the program calculated the size of the labeled area within the box. We then divided this total labeled area by the average cell size for individual cells in each area (determined by measuring cell sizes from each area from six randomly chosen animals and taking the average). This gave us an estimate of the number of cells in the area of interest. In order to verify this, we compared numbers produced by the computer program and number of cells counted by hand and saw no differences.

Data Analysis. The number of PR-ir cells in each area and the plasma LH levels were analyzed using a series of two-way analyses of variance (ANOVA) comparing hormone and food treatment within each genotype. In addition, one-way ANOVAs were performed to test for overall affect of hormone, food, and genotype. *Post hoc* comparisons were made using a Student-Newman-Keuls test. The data were considered significant if $P < 0.05$.

Results

Coumestrol Reduces the Effects of E_2 on PR-ir in Female Brain. Estradiol treatment augmented PR-ir cell numbers in both the POA and VMN ($F[1, 48] = 24.8$ and 21.5, respectively; $P < 0.0001$; Figs. 1, 2, and 3). These effects were more robust in WT than in ERαKO females ($F[1, 21] = 27.74$ [WT] versus 14.88 [ERαKO]).

As pictured in Figure 2, WT mice treated with E_2 and coumestrol had significantly fewer PR-ir cells in the POA than E_2-treated WT mice on regular chow ($F[1, 21] = 3.58$; $P < 0.05$; Figs. 1A and 2). In ERαKO females coumestrol consumption had no impact on E_2 up-regulation of PR ($P > 0.05$). Ovariectomized animals that did not receive E_2 had low numbers of PR-ir cells, which were not influenced by coumestrol ($P > 0.05$; Fig. 1B).

In the VMN of WT females, E_2 was equally effective in induction of PR-ir cells in females that did and did not receive coumestrol supplement ($F[1, 49] = 16.29$ and 18.64, respectively; $P < 0.001$). Estradiol had a reduced effect on PR-ir in ERαKO VMN. The OVX + estradiol-treated ERαKO mice were only significantly different from OVX ERαKOs when they did not have coumestrol ($t[11] = 3.57$; $P < 0.05$). Ovariectomized females had few PR-ir cells and these numbers were not affected by coumestrol treatment ($F[1, 49] = 41.99$ and 37.19, respectively; $P < 0.000001$; Fig. 3).

Coumestrol Elevates LH Levels in OVX WT Females. WT OVX animals had significantly higher plasma LH levels when they consumed coumestrol as compared with regular food ($F[1, 49] = 6.31$; $P < 0.05$; Fig. 4A). WT females treated with E_2 had low plasma levels of LH regardless of coumestrol treatment. The ERαKO females, regardless of hormone or coumestrol treatment, had high plasma concentrations of LH ($P > 0.05$; Fig. 4B).

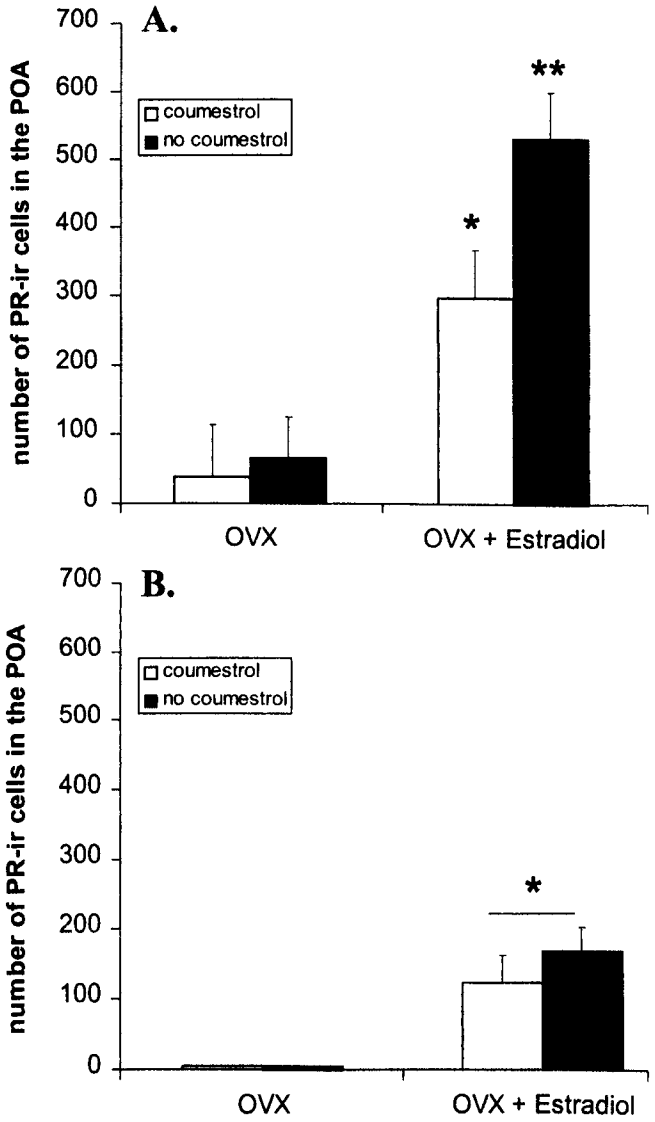

Figure 1. The mean number (± SEM) of PR-ir cells in the POA of ovariectomized (OVX) WT (A) or ERαKO (B) females untreated or given estradiol and fed mouse chow with or without coumestrol supplementation. *, Significantly different from OVX females. **, Significantly different from OVX and from OVX + estradiol + coumestrol females. $P < 0.05$. $n = 6$ mice per group.

Discussion

The present data show that in the mouse, coumestrol has anti-estrogenic actions on two classic neuroendocrine responses. In the POA, and to a lesser degree in the VMN, coumestrol antagonized the effects of E_2 on PR-ir cell induction. In addition, coumestrol amplified LH concentrations, which result from a release from negative feedback after ovariectomy. Since these effects were not noted in ERαKO mice, it suggests that in both of these cases coumestrol acts on the ERα.

Other studies conducted with coumestrol suggest that this phytoestrogen has estrogenic actions on female rat peripheral reproductive tissues (2, 27). Yet in the female rat

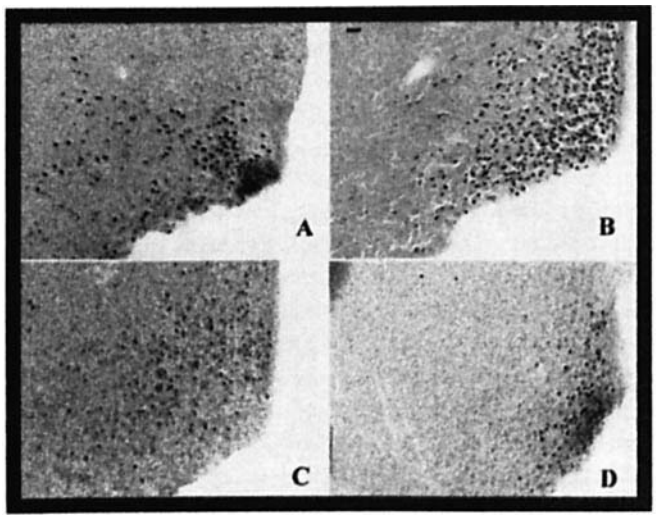

Figure 2. Photomicrograph of PR-ir cells in the POA of WT females treated with E_2 + coumestrol (A), WT females treated with E_2 without coumestrol (B), ERαKO females treated with E_2 + coumestrol (C), and ERαKO females treated with E_2 without coumestrol (D). Scale-bar (shown in B) = 20 μm.

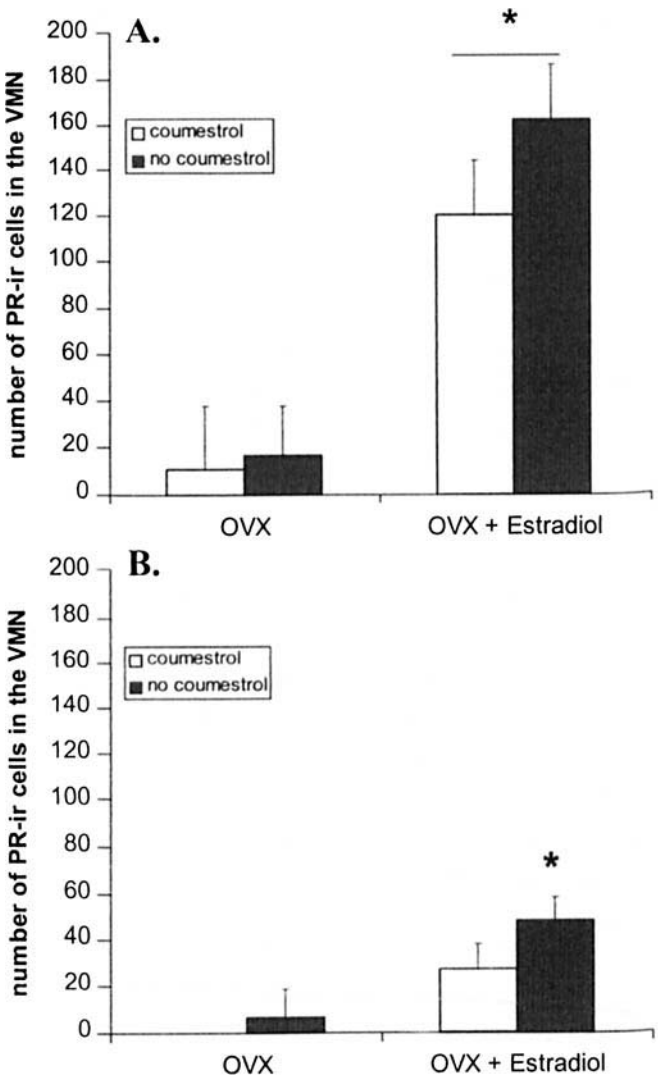

Figure 3. The mean number (± SEM) of PR-ir cells in the VMN of ovariectomized (OVX) WT (A) or ERαKO (B) females untreated or given estradiol and fed mouse chow with or without coumestrol supplementation. *, Significantly different from OVX females. $P < 0.05$. $n = 6$ mice per group.

brain, coumestrol has an antiestrogenic action on ERβ mRNA regulation in the paraventricular nucleus (PVN). In fact, while E_2 decreased expression of ERβ mRNA, coumestrol treatment increased ERβ in this region (23). Although our data were collected in a different species and different brain regions, and we examined a different dependent variable, we reached the same conclusion. In both cases coumestrol acts in an opposite direction as compared with E_2. However, in our present study coumestrol treatment in the absence of E_2 had no effect on PR. Perhaps this is because so few cells contain PR-ir when E_2 is not present that it is not possible to assess any further reduction.

An estrogenic effect of coumestrol has also been reported in immature female rat hypothalamic/POA tissues. Cytosolic PR binding was examined after rats consumed a 0.01% coumestrol diet for 4 days. Progesterone receptor binding was increased by 34% in the hypothalamic/POA area as compared with animals fed regular chow. However, this same treatment had a much more pronounced effect in uterus and pituitary. Moreover, no data on estrogen responses were presented, so it is difficult to determine if this is a weak or strong effect (2). In another report by this same group, a stronger effect was noted in the hypothalamus (28), but again, control data are lacking. Nevertheless, our results are contradictory to these rats studies. Aside from the obvious species difference, it should be noted that we fed our mice twice as much coumestrol for a longer time period and this alone could contribute to these differences.

Our LH data yield the same general conclusion, which is that coumestrol opposes the action of E_2. Several studies have examined the effects of coumestrol on LH. High concentrations of coumestrol in diets of ewes lead to a decrease in LH pulse amplitude during the breeding season (29). This may suggest that coumestrol acts as an anti-estrogen in breeding sheep experiencing E_2-LH positive feedback. In other studies coumestrol has been administered during the neonatal period and adult hypothalamic-pituitary functions were examined. In female rats, neonatal coumestrol treatment interfered with the ability of estradiol to elicit an LH surge in adults (30). Thus, coumestrol may have an estrogenic effect in neonates and may masculinize the LH surge system. Neonatal males treated with coumestrol had no discernable differences in plasma LH levels as adults when compared with the controls (31). Yet neonatal treatment with coumestrol did affect male rat sexual behavior in a manner consistent with coumestrol acting as an anti-estrogen (32). However, the sexually dimorphic nucleus of the rat POA is not affected by neonatal treatments with coumestrol (33).

The extent to which ERα and ERβ have dependent or separate roles on reproduction is not yet known. The differential distribution of the two ER subtypes suggests that they may have independent actions. For example, in the rat

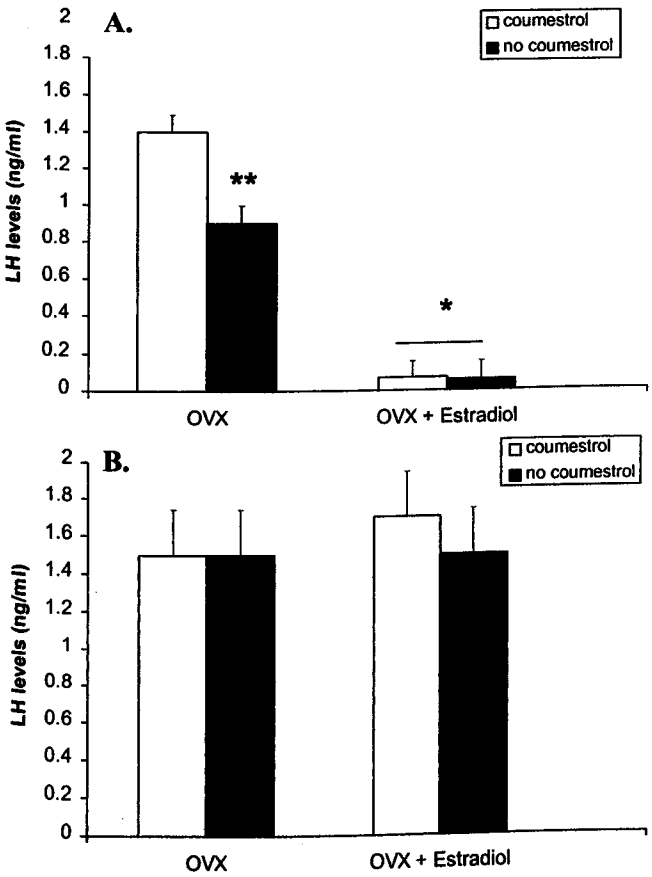

Figure 4. Mean (± SEM) plasma LH levels in ovariectomized (OVX) WT (A) or ERαKO (B) females untreated or given estradiol and fed mouse chow with or without coumestrol supplementation. *, Significantly different from OVX females. **, Significantly different from OVX + estradiol + coumestrol. $P < 0.05$. $n = 6$ mice per group.

brain both ERα and ERβ are abundant in the BnST, the POA, and the amygdala, but the PVN and the supraoptic nucleus (SON) contain only ERβ, while the VMN contains primarily ERα (34–36). In the mouse, ERβ mRNA is present in many of the same regions as ERα, and both genes are found in the POA, BnST, and PVN (37). The distribution of ERβ seen in the rat brain is similar in rhesus monkeys and sheep (38, 39), suggesting that it is somewhat conserved across species. Yet sex differences, during development, and differences in the concentrations of ERα and β in specific brain regions may reveal that ERα and β have different functional roles in different species. Despite speculation that ERα is essential for reproductive function (40), we have found that lack of a functional ERβ in mice has an impact on several estrogen-induced neuroendocrine responses. In addition, residual PR induction is present in estrogen-treated ERαKO mice (16) and it is likely that ERβ causes this response (41).

This is the first study to examine the effects of a phytoestrogen in ERαKO mice and the first to examine the effects of coumestrol on LH production in an adult mouse. These results are particularly compelling given that phytoestrogens are increasingly being touted as a healthful, natural estrogen replacement therapy for postmenopausal women, and are sold in caplet and powder forms in health food and grocery stores. Although a multitude of studies have tried to elucidate if and how these compounds are chemoprotective (6, 10, 11), the results are contradictory and inconclusive. Part of the confusion comes from the use of assays that do not take the potential role of both ER subtypes into consideration. By identifying which ER subtypes respond to coumestrol, our study helps to elucidate the mechanism by which this phytoestrogen produces its effects, and may in turn help determine whether or not coumestrol is truly as healthful as advertised.

We thank Dr. Dennis Lubahn for providing us with ERαKO heterozygotes to set up our breeding colony. We are indebted to Xia Li, Emily Linde, and Aileen Wills for technical assistance.

1. Franke AA, Custer LJ, Cerna CM, Narala K. Rapid HPLC analysis of dietary phytoestrogens from legumes and from human urine. PSEBM **208:**18–26, 1995.
2. Whitten PL, Russell E, Naftolin F. Effects of a normal, human-concentration, phytoestrogen diet on rat uterine growth. Steroids **57:**98–106, 1992.
3. Whitten PL, Lewis C, Russel E, Naftolin F. Potential adverse effects of phytoestrogens. J Nutr **125:**771S–776S, 1995.
4. Adams NR. Detection of the effects of phytoestrogens on sheep and cattle. J Anim Sci **73:**1509–1515, 1995.
5. Murkies A, Lombard C, Strauss B, Wilcox G, Burger H, Morton M. Dietary flour supplementation decreases post-menopausal hot flushes: Effect of soy and wheat. Maturitas **21:**189–195, 1995.
6. Patisaul HB, Whitten PL. Dietary phytoestrogens. In: Naz RK, Ed. Endocrine Disruptors: Effects on Male and Female Reproductive Systems. Boca Raton: CRC Press, pp389–423, 1999.
7. Troll W, Wiesner R, Shellabarger CJ, Holtzmann S, Stone JP. Soybean diet lowers breast cancer incidence in irradiated rats. Carcinogenesis **1:**469–472, 1980.
8. Barnes S, Grubbs C, Setchell KDR, Carlson J. Soybeans inhibit mammary tumors in models of breast cancer. In: Pariza M, Ed. Proceedings Mutagens and Carcinogens in the Diet. New York: Wiley-Liss, pp239–253, 1989.
9. Barnes S, Peterson GT, Coward L. Rational for the use of genistein-containing soy matrices in the chemoprevention trails for breast and prostate cancer. J Cell Biochem **22:**181–187, 1995.
10. Adlercreutz H. Phytoestrogens: Epidemiology and a possible role in cancer protection. Environ Health Perspect **130:**103–112, 1995.
11. Strauss L, Santti R, Saarinen N, Streng T, Joshi S, Makela S. Dietary phytoestrogens and their role in hormonally dependent disease. Toxicol Lett **102–103:**349–354, 1998.
12. Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe S H, Van Der Saag PT, Van Der Berg B, Gustafsson J-Å. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology **139:**4252–4263, 1998.
13. Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Häggblad J, Hilsson S, Gustafsson J-Å. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology **138:**863–870, 1997.
14. Makela S, Savolainen H, Aavik E, Myllarniemi M, Strauss L, Taskinen E, Gustafsson J-Å, Hayry P. Differentiation between vasculoprotective and uterotrophic effects of ligands with different binding affinities to estrogen receptors alpha and beta. Proc Natl Acad Sci U S A **96:**7077–7082, 1999.
15. Shughrue PJ, Lubahn DB, Negro-Vilar A, Korack KS, Merchenthaler

I. Responses in the brain of estrogen receptor α-disrupted mice. Proc Natl Acad Sci U S A **94:**11008–11012, 1997.
16. Moffatt CA, Rissman EF, Shupnik MA, Blaustein JD. Induction of neural progestin receptors by estradiol in estrogen receptor-α gene disrupted mice. J Neurosci **18:**9556–9563, 1998.
17. Wersinger SR, Haisenleder DJ, Lubahn DB, Rissman EF. Steroid feedback on gonadotropin release and pituitary gonadotropin subunit mRNA in mice lacking a functional estrogen receptor α. Endocrine **11:**137–143, 1999.
18. Wersinger SR, Sannen K, Villalba C, Lubahn DB, Rissman EF, De Vries GJ. Masculine sexual behavior is disrupted in male and female mice lacking a functional estrogen receptor α gene. Hormones Behavior **32:**176–183, 1997.
19. Rissman EF, Wersinger SR, Fugger HN, Foster TC. Sex with knockout models: Behavioral studies of estrogen receptor α. Brain Res **835:**80–90, 1999.
20. Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M., Korach KS, Gustafsson J-Å, Smithies O. Generation and reproductive phenotypes of mice lacking estrogen receptor β. Proc Natl Acad Sci U S A **95:**15677–15682, 1998.
21. Couse JF, Lindzey J, Grandien K, Gustafsson J-Å, Korach KS. Tissue distribution and quantitative analysis of estrogen receptor-α and estrogen receptor-β mRNA in the wild-type and ERα knock-out mouse. Endocrinology **138:**4613–4621, 1997.
22. Kuiper GGJM, Shughrue PJ, Merchenthaler I, Gustafsson J-Å. The estrogen receptor β subtype: A novel mediator of estrogen action in neuroendocrine systems. Frontiers Neuroendocrinol **19:**253–286, 1998.
23. Patisaul H, Whitten P, Young L. Regulation of estrogen receptor beta mRNA in the brain: Opposite effects of 17β-estradiol and the phytoestrogen, coumestrol. Molec Brain Res **67:**165–171, 1999.
24. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci U S A **90:**11162–11166, 1993.
25. Fallest PC, Trader GL, Darrow JM, Shupnik MA. Regulation of rat luteinizing hormone beta gene expression in transgenic mice by steroids and a gonadotropin-releasing hormone antagonist. Biol Reprod **53:**103–109, 1995.
26. Franklin KBJ, Paxinos G. The Mouse Brain in Stereotaxic Coordinates. San Diego: Academic Press, 1997.
27. Ashby J, Tinwell H, Soames A, Foster J. Induction of hyperplasia and increased DNA content in the uterus of immature rats exposed to coumestrol. Environ Health Perspec **107:**819–822, 1999.
28. Whitten PL, Russell E, Naftolin F. Effects of a normal, human-concentration, phytoestrogen diet on rat uterine growth. Steroids **57:**98–106, 1992.
29. Montgomery GW, Martin GB, Le Bars J, Pelletier J. Gonadotropin release in ovariectomized ewes fed different amounts of coumestrol. J Reprod Fertil **73:**457–463, 1985.
30. Whitten PL, Lewis C, Naftolin F. A phytoestrogen diet induces the premature anovulatory syndrome in lactationally exposed female rats. Biol Repod **49:**1117–1121, 1993.
31. Awoniyi CA, Roberts D, Chandrashekar V, Veeramachaneni DN, Hurst BS, Tucker KE, Schlaff WD. Neonatal exposure to coumestrol, a phytoestrogen, does not alter spermatogenic potential in rats. Endocrine **7:**337–341, 1997.
32. Whitten PL, Lewis C, Russel E, Naftolin F. Phytoestrogen influences on the development of behavior and gonadotropin function. PSEBM **208:**82–86, 1995.
33. Register B, Bethel MA, Thompson N, Walmer D, Blohm P, Ayyash L, Hughes C Jr. The effect of neonatal exposure to diethylstilbestrol, coumestrol, and beta-sitosterol on pituitary responsiveness and sexually dimorphic nucleus volume in the castrated adult rat. PSEBM **208:**72–77, 1995.
34. Li X, Schwartz PE, Rissman EF. Distribution of estrogen receptor-ββlike immunoreactivity in rat forebrain. Neuroendocrinology **66:**63–67, 1997.
35. Shughrue PL, Lane MV, Merchenthaler I. Comparative distribution of estrogen receptor α and β mRNA in the rat central nervous system. J Comp Neurol **388:**507–525, 1997.
36. Österlund M, Kuiper GGJM, Gustafsson J-Å, Hurd YL. Differential distribution and regulation of estrogen receptor-α and -β mRNA within the female rat brain. Molec Brain Res **54:**175–180, 1998.
37. Shughrue PL, Scrimo P, Lane MV, Merchenthaler I. The distribution of estrogen receptor-β mRNA in forebrain regions of the estrogen receptor-α knockout mouse. Endocrinology **138:**5649–5652, 1997.
38. Scott CJ, Tilbrook AJ, Simmons DM, Rawson JA, Chu S, Fuller PJ, Ing NH, Clarke IJ. The distribution of cells containing estrogen receptor-α (ERα) and ERβ messenger ribonucleic acid in the preoptic area and hypothalamus of the sheep: Comparison of males and females. Endocrinology **141:**2951–2962, 2000.
39. Gundlah C, Kohama S, Mirkes S, Garyfallou V, Urbanski H, Bethea C. Distribution of estrogen receptor beta mRNA in hypothalamus, midbrain, and temporal lobe of spayed macaque: Continued expression with hormone replacement. Brain Res Molec Brain Res **76:**191–204, 2000.
40. Couse JF, Korach KS. Exploring the role of sex steroids through studies of receptor deficient mice. J Mol Med **76:**497–511, 1998.
41. Temple JL, Fugger HN, Li X, Shetly SJ, Gustafsson J-Å, Rissman EF. Estrogen receptor β regulates sexually dimorphic neural responses to estradiol. Endocrinology **142:**510–513, 2001.