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Estrogenic effects of zearalenone on the expression of progestin receptors and sexual behavior in female rats

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Abstract

Zearalenone is a resorcylic acid lactone compound that is produced by fungal infection of edible grains and is believed to influence reproduction by binding to estrogen receptors. In order to study the potential estrogenic effects of this compound in the brain, we examined the effects of zearalenone on the expression of neuronal progestin receptors and feminine sexual behavior in female rats. Ovariectomized rats were treated with zearalenone (0.2, 1.0, or 2.0 mg), estradiol benzoate, or vehicle daily for 3 days. They were then either perfused, and progestin receptors visualized by immunocytochemistry, or injected with progesterone and tested for sexual receptivity with male rats. Progestin receptor-containing cells were counted in the medial preoptic area and ventromedial hypothalamus. The two highest doses of zearalenone increased the concentration of neuronal progestin receptors, as did 10 μg of estradiol. The highest dose of zearalenone (2 mg) also induced progestin receptor staining density comparable to that of 10 μg of estradiol benzoate. In behavioral tests, ovariectomized animals treated with 2 mg of zearalenone followed by progesterone showed levels of sexual receptivity comparable to females treated daily with estradiol benzoate (2 μg) followed by progesterone. These studies suggest that, although structurally distinct and less potent than estradiol, zearalenone can act as an estrogen agonist in the rat brain.

Keywords: Phytoestrogens; Dietary estrogens; Estrogen receptors; Uterus; Lordosis; Hypothalamus; Preoptic area

Introduction

Animals are exposed to natural and synthetic compounds with estrogen-like actions, which are prevalent in the environment. Natural compounds with estrogenic effects include phytoestrogens of several classes, such as isoflavones, coumestans, lignans and rescorcylic acid lactones. These naturally occurring dietary estrogens bind to both estrogen receptor subtypes and have either agonistic or antagonistic effects (Kuiper et al., 1998; Nikov et al., 2000).

The dietary estrogen, zearalenone is a phenolic rescorcylic acid lactone, produced by *Fusarium*, a mold which infects cereals and grains, such as corn, oats, and hay (Coulombe, 1993). Although not produced by plants, zearalenone may be considered a phytoestrogen in that it is naturally occurring and found associated with grains (Nikov et al., 2000) that are common in animal feed and in human diets (Kuiper-Goodman et al., 1987; Schollenberger et al., 1999). Zearalenone has estrogenic properties in a number of species. In rats, the reproductive consequences of zearalenone exposure include decreased fertility, resorption or deformities of fetuses, and abortion at high dietary concentrations (Kuiper-Goodman et al., 1987). Zearalenone and its analogs (at concentrations that have been found in corn products) induce proliferation of MCF 7 cells, an estrogen-dependent response (Kuiper-Goodman et al., 1987; Makela et al., 1994).

Zearalenone binds to estrogen receptors (ERs) in vitro with similar affinity for both forms of estrogen receptor, ERα and ERβ (Kuiper et al., 1998). Despite its having a lower affinity for estrogen receptors than 17β estradiol (100–1000 times less), zearalenone and its metabolite, α-zearalanol, act

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through ERs (Kiang et al., 1978; Martin et al., 1978; Nikov et al., 2000) to activate transcription of estrogen-responsive genes in vivo (Gray et al., 1985; Katzenellenbogen et al., 1979; Mehmood et al., 2000) and in vitro (Kuiper et al., 1998; Mayr, 1988).

Most of the studies of the estrogenic effects of zearalenone have concentrated on effects on peripheral reproductive organs. However, the brain is also a target for estrogens, and phytoestrogens cross the blood–brain barrier (Chang et al., 2000; Lephart et al., 2000) and alter neuronal gene expression in rats (Lephart et al., 2000; Pan et al., 1999; Patisaul et al., 1999, 2001, 2002; Whitten et al., 2002). We examined the effects of zearalenone in vivo in rats by determining its effects on neural progestin receptor (PR) expression, and progesterone-facilitated sexual behavior, both well-characterized, estrogen-dependent responses. In addition, as an index of peripheral estrogenic effects, uterine weights were determined.

Materials and methods

Animals

Thirty-one female, Sprague–Dawley rats (175–200 g) obtained from Charles River Laboratories (Wilmington, MA) were group-housed (3–4 per cage) in suspended wire-mesh cages. Rats were maintained on a 14:10 h light/dark cycle (lights on at 2000 h and lights off at 1000 h) with food and water freely available. Rats were allowed to acclimate to the laboratory for 1 week prior to surgery. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts, Amherst.

Surgery

Rats were anesthetized with a cocktail of ketamine HCl (26 mg/kg), xylazine HCl (5 mg/kg), and acepromazine (0.9mg/kg, i.p.) and ovariectomized via a mid-ventral incision. Treatment was initiated 1 week after surgery.

Hormone treatment for immunocytochemistry

Zearalenone (Sigma-Aldrich Co., St. Louis, MO) and estradiol benzoate (EB) were dissolved in vehicle (propylene glycol with 10% ethanol) and sonicated until clear (15–20 min). Rats received sc injections of 0.1 ml vehicle, 10 μg of EB, or one of three doses of zearalenone (0. 2 mg, 1 mg, or 2 mg) daily for 3 consecutive days.

Perfusion

On day 4, rats were given a lethal dose of pentobarbital (89 mg/kg solution) and chloral hydrate (425 mg/kg solution). In order to assess the effects of each treatment on a peripheral estrogen-dependent response, the uterus was excised just above the cervix prior to perfusion, carefully dissected free of associated fat and connective tissues, and weighed. Perfusions were carried out via a cardiac cannula with 1 ml of heparin solution (5000 IU in saline) followed by 25 ml of saline and approximately 350 ml of 4% paraformaldehyde. Flow rate was maintained at approximately 25 ml/min. Brains were placed in 0.1 M sodium phosphate buffer (pH = 7.2) containing 20% sucrose for at least 24 h followed by immersion in phosphate buffer containing 30% sucrose at 4°C, until the brains were saturated. Forty micron transverse sections from the rostral preoptic area through the caudal ventromedial hypothalamus were cut on a freezing rotary microtome. Sections were stored in glycerol-based cryoprotectant at −20°C, until they were immunostained.

Immunohistochemistry

A set of every fourth section from each animal was used for immunohistochemistry. Cryoprotectant was removed from free-floating sections with three rinses (5 min) in 0.05 M Tris-buffered saline (TBS; pH 7.6). These initial rinses were followed by incubation in blocking solution for 20–30 min. Blocking solution consisted of TBS containing 20% normal goat serum (NGS), 1% bovine serum albumin and 1% H_2O_2, to reduce non-specific staining and endogenous peroxidase activity. Sections were then incubated in primary incubation buffer in modified TBS containing NGS, 0.02% sodium azide, 0.1% gelatin and 0.2% Triton-X (Gel-TBS) and primary antibody against human progestin receptor (MAB 462, 1:1000, DakoCytomation, Inc., Carpenteria, CA) for 72 h at 4°C.

Following incubation in primary antibody, sections were rinsed three times with Gel-TBS (5 min). Sections were then incubated for 90 min in Gel-TBS containing 1.5% NGS and 2 μg/ml of biotinylated goat-anti-rabbit IgG antiserum. The sections were then rinsed with Gel-TBS (2 × 5 min) and TBS (1 × 5 min), followed by incubation for 90 min in TBS containing 1% biotinylated horseradish peroxidase H complex (Vector ABC Elite Kit, Vector Laboratories, Inc., Burlingame, CA). After three rinses in TBS (5 min), PRs were visualized by reaction in 0.05% diaminobenzidine and 0.05% H_2O_2, and this reaction was halted by rinsing in TBS (3 × 5 min). Sections were then mounted on slides and coverslipped with DePex mounting medium for microscopic examination.

Analysis

Representative sections of rostral, middle, and caudal ventrolateral–ventromedial hypothalamus (VMHvl) were carefully matched based on histological landmarks, as were rostral and caudal medial preoptic area (MPO) (Fig. 1). These areas were chosen based on previous work showing high levels of estrogen-induced PRs in these areas (Blaustein and Erskine, 2002).

Progestin receptor-immunoreactive (PR-ir) cells were counted using a Leitz Dialux 20 microscope, under Kohler illumination, connected to a MTI CCD 72 digital camera, which was connected to a Power MacIntosh G3 computer. Images of representative areas were captured at 100× magnification and analyzed using NIH Image program V1.62 (available at http://www.rsb.info.nih.gov/nih-image/). Prior to capture of images, the camera was calibrated. The black and gain levels were adjusted, so that the blank portion of the slide produced gray levels between 2 and 10, and an opaque black mark produced gray levels of approximately 240. The density threshold option was adjusted to give minimum and maximum gray levels between two and four times the standard deviation of the total mean pixel-density. For each area analyzed, all images were captured in one session to decrease variability. Only those areas which occupied between 10 and 200 pixels at 10× magnification were considered cells. Image-capture and analysis were conducted with the experimenter blind to treatment groups.

Estrous behavior testing

Testing took place during the dark phase of the light/dark cycle under red light illumination in a rectangular clear Plexiglas arena with pine-chip bedding. Females were placed in the arena with a sexually experienced male until 10 mounts were achieved, including ejaculations if they occurred. If the female received an ejaculation, she was placed in an adjacent arena with another male until testing was completed. Lordosis ratings were scored on a 4-point scale (0–3)(Hardy and Debold, 1971). Lordosis quotients were calculated by dividing the number of lordosis responses by the total number of mounts × 100.

Screening

All animals were screened for progesterone-facilitated estrous behavior after sc injections of 5 μg EB 48 h and 500 μg progesterone 4 h before testing. Four rats were eliminated from the study because of failure to meet a criterion lordosis quotient of 70.

Progesterone facilitated estrous behavior

One week after screening, rats were treated once a day for three days with 0.1 ml vehicle, 2 μg EB, 0.2, 1, or 2 mg zearalenone sc. On the fourth day, animals received 500 μg of progesterone sc 4 h before behavior testing during the dark cycle.

Statistical analysis

One-way analysis of variance, followed by a Fisher-Protected Least Significant Difference post hoc test was used to determine significant differences among groups.

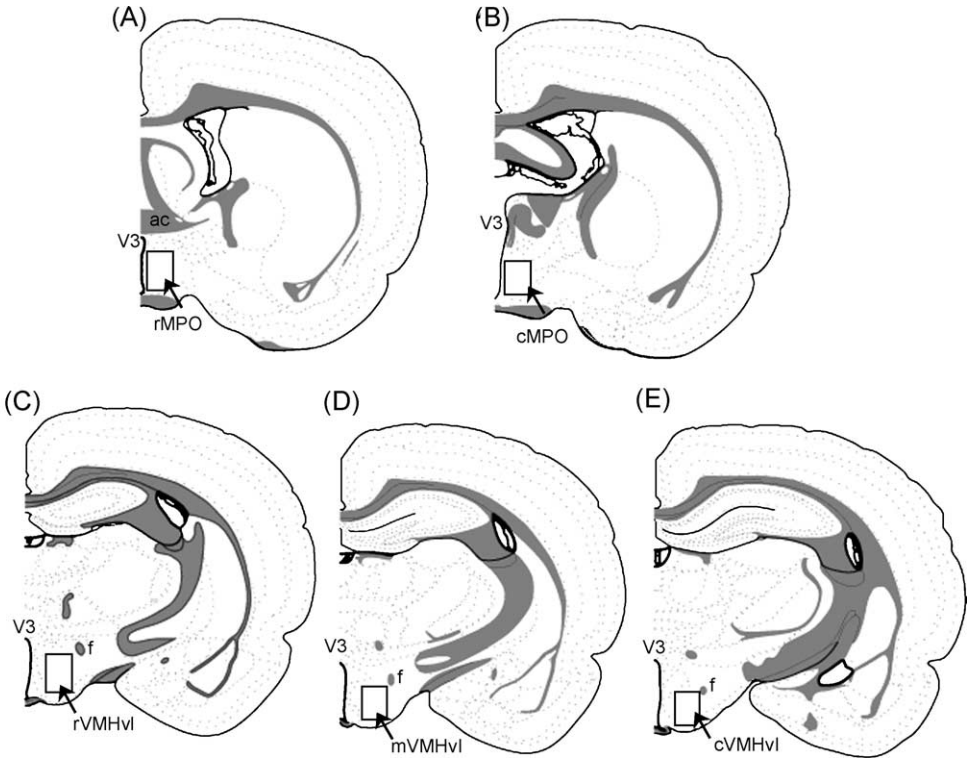

Fig. 1. Drawings of brain regions at two levels of the MPO and three levels through the VMHvl. Areas in which cells were counted are indicated by large squares over the MPO or VMHvl. Anterior commissure, ac; rostral medial preoptic area, rMPO; caudal medial preoptic area, cMPO; rostral ventromedial hypothalamus ventrolateral region, rVMHvl; mid ventromedial hypothalamus ventrolateral region, cVMHvl; caudal ventromedial hypothalamus ventrolateral region, cVMHvl; third ventricle, V3.

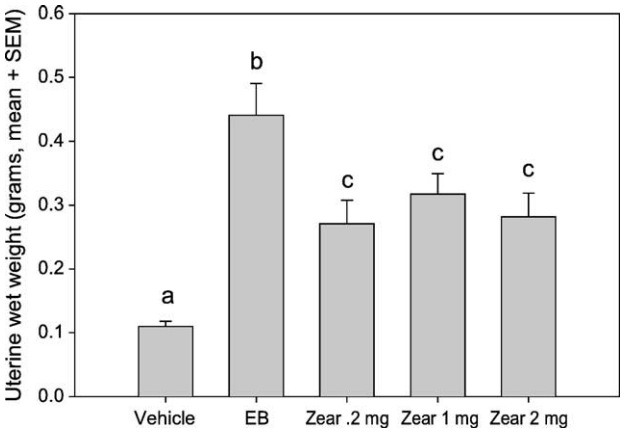

Fig. 2. Uterine weights (mean ± SEM) of animals injected daily for three days with vehicle, 10 μg EB, 0.2, 1.0, or 2.0 mg zearalenone (Zear). Different letters above standard error bars indicate statistically significant differences among experimental groups. n = 5–6 per group.

Contrasts were considered statistically significant at a level of $P < 0.05$. Any positive results discussed in the text were statistically significant at least at this level.

Results

Uterine weight

Uterine wet weight was used as an index of peripheral estrogenic response. All doses of zearalenone (0.2, 1.0, and 2.0 mg) increased uterine weights in contrast to vehicle controls (Fig. 2). However, none of the zearalenone treatments increased uterine weight to the same levels as those obtained from animals treated with EB.

PR induction

PR-ir was detected in all treatment groups at the three levels of the VMHvl and both levels of the MPO. The number of cells expressing PR-ir was low in animals treated with 0.2 mg of zearalenone (Figs. 3 and 4A) and was not statistically different from vehicle controls at any level. However, the number of PR-ir cells was higher than controls

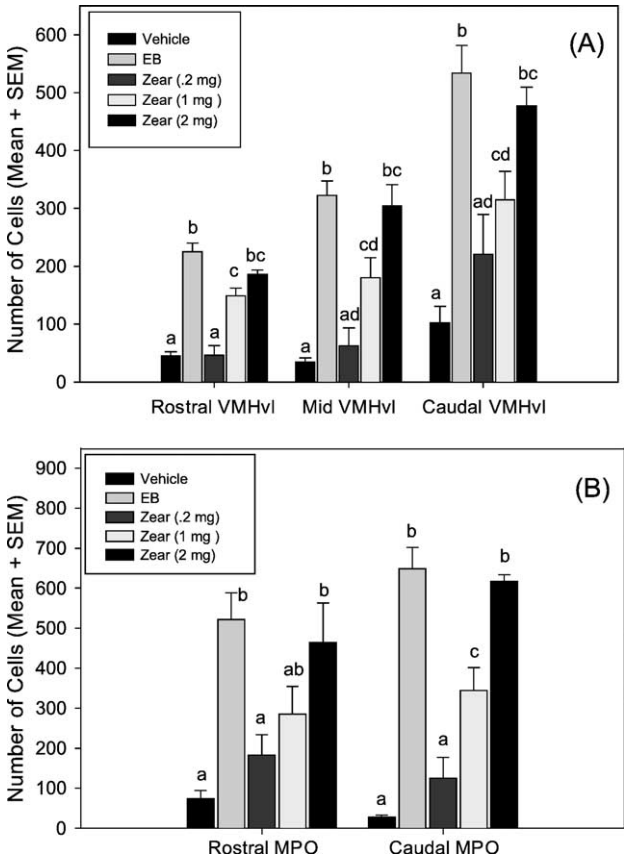

Fig. 4. Numbers of progestin receptor-containing cells (mean ± SEM) in (A) the ventromedial hypothalamus ventrolateral region (VMHvl; n = 4–7 per group) and (B) the medial preoptic area (MPO; n = 4–7 per group with the exception of 2 mg zearalenone group in the rMPO for which n = 3) and after treatment with zearalenone (Zear), EB or vehicle. Different letters above bars indicate statistically significant differences among experimental groups.

in all areas in rats treated with 1.0 or 2.0 mg of zearalenone (Fig. 4). At the highest dose of zearalenone, the numbers of cells expressing PR-ir were not significantly different from the estradiol-treated, positive control animals.

The dose-response pattern in the rostral and caudal MPO was similar to that observed in the VMHvl (Figs. 4A and B). The 1 and 2 mg doses of zearalenone induced higher levels of PR-ir in the rostral and caudal MPO than in vehicle-

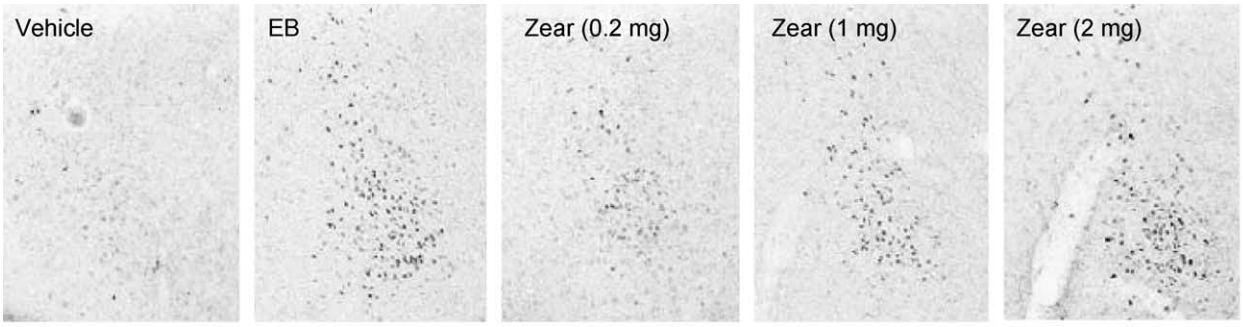

Fig. 3. Progestin receptor immunostaining in the rostral VMHvl of female rats after treatment with vehicle, estradiol benzoate (10 μg) or one of three concentrations of zearalenone (Zear: 0.2, 1.0 or 2.0 mg) daily for 3 days.

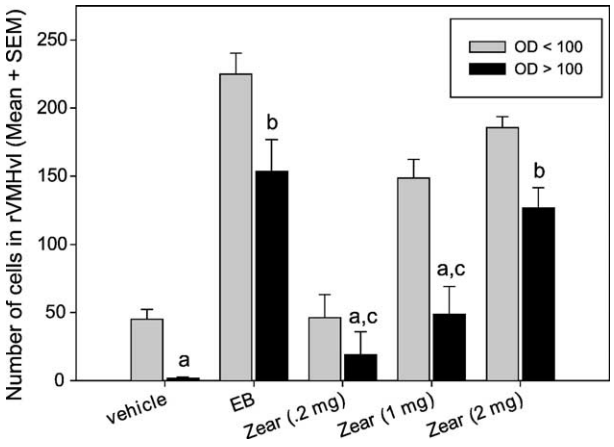

Fig. 5. Numbers of cells (mean ± SEM) that are lightly staining (with pixel density < 100) or darkly staining (with pixel density > 100) for PR immunoreactivity in the ventrolateral region of the ventromedial hypothalamus after zearalenone (Zear) treatment. Different letters above bars indicate statistically significant differences among experimental groups. n = 4–7 per group.

treated animals with significantly higher levels of PR-ir induced by 2.0 mg zearalenone. At the highest dose of zearalenone, the number of cells expressing PR-ir did not differ significantly from the number in EB-treated rats.

In order to determine if there were differences in the apparent density of PRs per cell in the VMHvl, we examined differences in the density of diaminobenzidine reaction. Using a mean pixel density of over 100 as a criterion for "darkly labeled," the average number of darkly stained cells was highest in animals treated with either EB or 2 mg zearalenone (Fig. 5). In the 0.2 and 1 mg zearalenone groups, the numbers of darkly stained cells were less than half of the number of lightly stained PR-ir cells in each group. Therefore, the high dose of zearalenone, like EB, induced apparent high levels of PR-ir in many of the cells.

Progesterone-facilitated estrous behavior

The lordosis quotients and lordosis ratings (not shown; results not different from lordosis quotients) for animals treated with 2 mg zearalenone were significantly higher than those of vehicle-treated animals (Fig. 6). The lordosis quotient of the rats receiving 2 mg zearalenone also did not differ significantly from EB-treated control animals. In contrast, neither 0.2 nor 1 mg zearalenone increased the lordosis quotient above vehicle-treated control animals. Only one female out of six was sexually receptive after treatment with 1 mg of zearalenone.

Discussion

Zearalenone, administered by subcutaneous injection for 3 days, acts like an estrogen receptor agonist in the brain by increasing the expression of progestin receptors and priming rats to express sexual behavior in response to progesterone. This is consistent with previous work showing that zearalenone is estrogenic in some peripheral reproductive responses in farm and laboratory animals (Stob et al., 1962), and that zearalenone increases uterine weight (Christensen et al., 1965) and other indicators of estrogenic activity (Katzenellenbogen et al., 1979; Kiang et al., 1978) in rats. Furthermore, the present data extend these previous results to include estrogenic effects of zearalenone in the brain and on sexual behavior.

The fact that even the lowest dose of zearalenone increased uterine weight suggests that the uterus may be more sensitive than the brain to short-term zearalenone exposure by subcutaneous injection. A dose of 0.2 mg/day of zearalenone for 3 days significantly increased uterine weight, while the same dose did not increase PR-ir in the brain significantly relative to controls. This could reflect differences in the sensitivity of each measure to estradiol, as the uterus is more sensitive to estradiol than the brain (Kelner et al., 1982). Alternatively, it could reflect differences in the availability of zearalenone to each tissue, since the extent of zearalenone's ability to cross the blood–brain barrier has not been established.

Increasing the dose of zearalenone resulted in an increase in the number of PR-ir cells. Although treatment with 0.2 mg of zearalenone for 3 days did not significantly increase the numbers of PR-ir cells, either 1 or 2 mg of zearalenone was sufficient to increase PR-ir cell numbers contrasted with vehicle-injected controls. The highest dose of zearalenone (2 mg for 3 days) induced PR-ir levels comparable to those observed with a 10-μg dose of estradiol benzoate.

Zearalenone only had estrogenic properties on sexual behavior when administered at the highest dose of 2 mg/day. This dose of zearalenone was approximately as effective as three daily injections of 2 μg of EB administered prior to injection of progesterone and behavior testing. The effectiveness of zearalenone to prime proges-

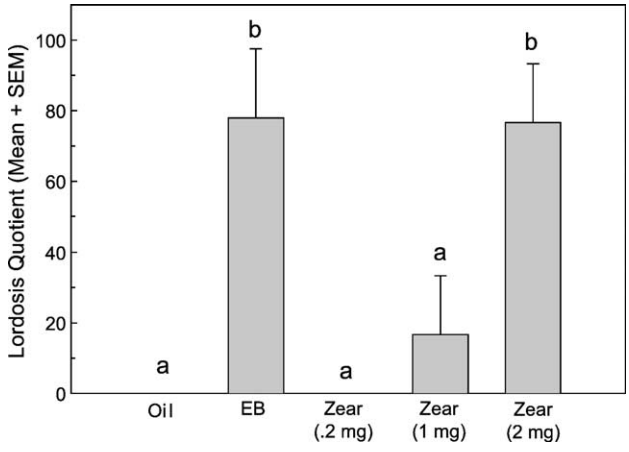

Fig. 6. Induction of progesterone-facilitated sexual behavior by zearalenone (Zear) or EB in female rats. Different letters above bars indicate statistically significant differences among experimental groups. n = 5–6 per group.

terone-facilitated sexual behavior suggests that zearalenone, like estradiol, can act on a variety of estrogen-sensitive genes in addition to the progestin receptor to initiate the cascade of molecular events necessary to lead to changes in estrous behavior.

There was an apparent inconsistency between doses of zearalenone that primed rats for progesterone-facilitated sexual behavior and doses that induced progestin receptors in the brain. At the 1 mg/day dose, zearalenone significantly increased PR-ir in the VMHvl and MPO contrasted with oil-treated controls. However, this dose was not sufficient to reliably prime females to show progesterone-facilitated estrous behavior with only one animal out of six showing lordosis responses. For this reason, an analysis of optical density of PR-ir was done. Perhaps the 1 mg dose of zearalenone induced only low levels of PR-ir in many cells. In fact, a contrast of PR-ir cells in the VMHvl at each dose of zearalenone showed that, although the 1 mg dose increased PR-ir cell number, the vast majority of these were cells with light reaction product in contrast to the animals treated with the high dose of zearalenone or EB. These findings confirm the importance of the apparent density of PRs expressed in each cell, as well as the number of PR-containing cells in initiating the cellular and molecular events leading to estrous behavior. It would be expected that cells with higher levels of progestin receptors would be more sensitive than those with lower levels (Blaustein and Olster, 1989).

After ingestion, zearalenone is metabolized in the gut before entering the bloodstream. Therefore, we administered zearalenone by subcutaneous injection to avoid variability between animals in intestinal microflora that could affect the metabolism and availability of the compound (Kuiper-Goodman et al., 1987). One of the metabolites, α-zearalanol, is more potent than zearalenone itself on some responses (Kuiper-Goodman et al., 1987).

Although some recent work on other phytoestrogens suggests that phytoestrogens are exclusively antiestrogenic in the adult brain (Jacob et al., 2001; Patisaul et al., 1999, 2001, 2002; Whitten et al., 2002), estrogenic effects have been observed as well (Ferguson et al., 2002; Pan et al., 1999). Although we have not excluded the possibility of antiestrogenic effects with the present experimental design, the results of the present study demonstrate that zearalenone can act in an estrogenic manner in the adult female rat brain, as is the case in neonatal rats (Faber and Hughes, 1991). Zearalenone, of course, is not always considered to be a true phytoestrogen; although it is present in grain, it is actually produced by a fungus. Additional work will be necessary to determine if zearalenone can act as an antiestrogen at other doses or treatment schedules. Nevertheless, the present results, in conjunction with those showing antiestrogenic effects in the brain of a variety of dietary estrogens, demonstrates the complexity in determination of whether or not dietary estrogens are likely to have overall positive or negative effects in neuronal function.

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