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In Utero and Lactational Exposure of Female Holtzman Rats to 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin: Modulation of the Estrogen Signal[1]

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ABSTRACT

The environmental pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin) induces severe reproductive defects in male rats when exposure occurs in utero and during lactation. Yet there is currently a paucity of information regarding the effects of this exposure paradigm in females. In the current study, we examine the effects of TCDD during fetal and perinatal development on the estrogen-signaling system in peripubertal female rats. Pregnant Holtzman rats were given 1 μg/kg TCDD or vehicle control by gavage on gestational Day 15. Body weights were reduced, though not significantly, on postnatal Day 21. While ovarian and uterine wet weights were not increased by TCDD exposure, the percentage of body weight attributed to the ovary was increased significantly. Through use of ribonuclease protection and gel-shift assays, exposed females were compared with nonexposed counterparts for estrogen receptor (ER) mRNA and DNA-binding activity in the following tissues: hypothalamus, pituitary (mRNA only), uterus, and ovary. ER mRNA levels increased in the hypothalamus, uterus, and ovary, and decreased in the pituitary. The results of the DNA-binding assays paralleled the mRNA results in the uterus, while DNA-binding activity was decreased in the hypothalamus and was unchanged in ovarian protein extracts. Circulating concentrations of estrogen were significantly lower in TCDD-exposed rats than in controls. These data suggest that the decrease in serum estrogen may be a cause of the alterations in ER mRNA; the changes in ER DNA-binding activity may indicate alterations in either translation or posttranslational receptor processing. Overall, this study shows that TCDD may act systemically in this model, and these effects should not necessarily be characterized as antiestrogenic.

INTRODUCTION

The environmental pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin), formed during incineration procedures [1], is a potent disrupter of vertebrate endocrine systems. TCDD induces a wide variety of untoward effects, including reproductive, metabolic, and neoplastic effects [2]. While the health consequences to both feral and laboratory-housed animals are apparent, the risk to humans remains controversial and unsubstantiated.

TCDD administered acutely results in a wide range of endocrine responses in laboratory animals, including alterations in thyroxine, prolactin, corticosteroids, androgens, and estrogens [3–7]; there are several other identified targets of TCDD action, notably the cytochromes P450 [8]. Recently, Li et al. [9] have observed persistent estrus, a reduction in eCG-stimulated ovarian weight, enhancement by TCDD of eCG-stimulated estrogen production, and decreased peak concentrations of FSH and LH. These data suggest that TCDD may act both directly and via centrally mediated mechanisms to disrupt ovarian function in the adult rat.

The actions of TCDD are mediated by a soluble, ligand-activated transcription factor, termed the aromatic hydrocarbon receptor, that binds TCDD saturably and with high affinity [2, 10]. The active transcription complex binds a well-characterized DNA motif termed the dioxin-response element [11], resulting in the modulation of target gene expression [12–14] and the potential progression to a neoplastic state.

TCDD is a potent tumor promoter in laboratory animals [2]; however, long-term treatment appears to reduce the incidence of uterine, mammary, and pituitary neoplasias [15]. Tumors in these tissues are often estrogen-dependent [16], and so TCDD has been suggested to act in an antiestrogenic fashion [17]. In female rhesus macaques fed a diet consisting of 500 ppt TCDD for 9 mo, for example, serum estrogen and progesterone concentrations were decreased [18]. It has been suggested that TCDD may act through the induction of cytochrome P450IA1 to hydroxylate and metabolize estrogen [19–21]. In MCF-7 human breast cancer cells, TCDD acts to increase hydroxylation at the C-2, -4, -15, -16, and -17 positions [19, 21, 22]. In other models, TCDD apparently acts to decrease expression of the estrogen receptor (ER) in immature and adult rats [23–27] and in MCF-7 cells [28]. DeVito et al. [27], using CD1 mice, showed that while acute exposure to TCDD resulted in a dose-dependent decrease in hepatic and uterine ER, serum estrogen concentrations were unaffected. There are no further data to suggest that these two potential mechanisms of TCDD action are mutually exclusive of one another, although the level of interaction between these mechanisms may be specific to the model.

The potency of TCDD is dependent upon species, strain, developmental stage, gender, and tissue type. In one study, Sprague-Dawley rats and guinea pigs were found to be sensitive to TCDD-induced ER down-regulation while hamsters were not [29]. Using weanling female Sprague-Dawley rats, White et al. [30] showed that TCDD was incapable of reversing the estrogen-induced decrease in uterine ER protein, verifying the specificity of TCDD in this model. Contrary to the situation with exposure during adulthood, when certain species are relatively resistant (e.g., hamster [31]), TCDD appears to have similar potencies among species when exposure occurs in utero and through lactation [32].

Peterson and coworkers [33–37] and Gray et al. [38] have demonstrated that exposure to TCDD during critical

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periods of development results in a range of endocrine abnormalities in male Holtzman rats born to dams given a single oral dose of 1 μg TCDD/kg maternal body weight on gestational Day 15.

In female rats, Gray and Ostby [39] examined the effects of TCDD administered on gestational Day 8 or 15 in Long-Evans and Holtzman rats. In both strains, ovarian, brain, and body weights were decreased in adulthood. Exposure at gestational Day 15 resulted in vaginal abnormalities in both strains, such as clefting of the phallus and a thread of tissue extending perpendicularly across the vagina, and the onset of vaginal opening was delayed. In all TCDD-exposed animals, inflammatory cervical lesions and cystic hyperplasia of the endometrium were present. In breeding trials, stud males had a longer latency to ejaculation, and in exposed females, vaginal bleeding was present during intromission. Most importantly, only 17% of exposed females were able to produce a fifth litter, vs. 61% of vehicle-exposed animals. Of animals exposed on gestational Day 8, only 64% produced a first litter (vs. 88% of nonexposed animals), and only 51% produced a second litter while 78% of nonexposed animals were able to do so.

The present study was designed to determine the effects of in utero/lactational exposure to TCDD on ER mRNA levels and DNA-binding activity throughout the female reproductive system. Data were obtained from ovarian, uterine, pituitary, and hypothalamic tissues, and circulating concentrations of estrogen were determined.

MATERIALS AND METHODS

Animals and Treatments

Pregnant female Holtzman rats were obtained from Harlan Sprague Dawley Inc. (Madison, WI) on gestational Day 12. Upon arrival, animals were housed individually in an environment of constant temperature (22 ± 1°C) and humidity (55 ± 5%) and on a 12L:12D lighting schedule. Animals were fed commercial food (Rat Chow 5012; Purina Mills, St. Louis, MO) and water ad libitum. On the morning of gestational Day 15, nine pregnant rats were administered a single oral dose of TCDD (1.0 μg/kg), and nine pregnant rats received an equivalent volume of vehicle control (corn oil:acetone; 19:1 [v:v], 2 ml/kg) by gavage ([32] and references therein). TCDD (98% purity) was purchased from Cambridge Isotope Laboratories (Woburn, MA).

On the day of birth, nine litters per treatment were adjusted to five males and five females to allow for similar lactational exposure to TCDD. Litter weights were obtained on postnatal Days 7, 14, and 21. On postnatal Day 21, female pups were killed by decapitation under CO_2 anesthesia. Postnatal Day 21 was chosen in order to follow the pups' exposure through lactation. The ovaries, uteri, hypothalami, and pituitaries were removed, weighed, randomly pooled into groups of six without regard to litter, and frozen on dry ice. The hypothalamus was delineated anteriorly by the optic chiasm, posteriorly by the mammillary bodies, laterally by the sulci formed with the temporal lobes, and superiorly by a plane 3 mm dorsal to the ventral surface of the median eminence. Serum samples were prepared from trunk blood collected into serum separator tubes. Three animals were killed independent of litter on postnatal Day 60, and the ovaries were removed, pooled, and frozen on dry ice for RNA analysis.

RNA Isolation

Total RNA was isolated by the acid-guanidinium isothiocyanate method [40]. The quantity of RNA was determined spectrophotometrically with absorption set at 260 nm. To further evaluate the integrity of the RNA, 1 μg was electrophoresed through a 1% agarose gel stained with ethidium bromide and visualized by means of a UV light source.

Ribonuclease Protection Assay

An ER cDNA probe was linearized and transcribed in vitro through use of SP6 RNA polymerase and labeled by the incorporation of [^{32}P]UTP (DuPont NEN, Boston, MA). ER cDNA was kindly provided by Dr. Kelly Mayo, Northwestern University, Evanston, IL. After transcription, we removed template DNA by using RQ1 RNase-free DNase (Promega Biotech, Madison, WI), and the transcripts were extracted with phenol:chloroform (1:1). Five micrograms of total RNA was hybridized overnight at 45°C with 2.5 × 10^5 cpm of ER cRNA in 80% formamide/0.4 M NaCl/40 mM PIPES. Radiolabeled transcripts that remained unhybridized were removed by digestion with 10 U RNase One (Promega Biotech) in 10 mM Tris-Cl/300 mM NaCl/5 mM EDTA, and the protected transcripts were resolved on an 8% denaturing acrylamide gel. To verify that hybridization was to endogenous mRNA rather than contaminating template DNA, and to ensure the efficacy of the RNase digestion, the ER cRNA probe was treated as above with the omission of RNA as well as with or without the addition of RNase.

Protein Isolation

Whole cell protein extracts were prepared according to the method of Bettini et al. [41]. In brief, pooled tissues were homogenized with a Dounce homogenizer in 5 ml of HDK buffer (25 mM HEPES [pH 7.5], 1 mM dithiothreitol [DTT], 400 mM KCl, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 6 μg/ml *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, and 6 μg/ml 1-L-tosylamido-2-phenylethyl chloromethyl ketone). The homogenate was incubated on ice for 1 h and then centrifuged at 500 × *g* for 10 min at 4°C. Supernatant was made 10% with respect to glycerol and centrifuged at 120 000 × *g* for 1 h at 4°C. The resultant supernatant was collected, aliquoted, and snap-frozen in ethanol/dry ice. Protein concentrations were determined with use of Bio-Rad protein assay dye (Bio-Rad Laboratories, Hercules, CA) and BSA standards. Aliquots were stored at −80°C until use and were not reused after being thawed. Protein was not isolated from pituitaries, as insufficient material was available.

Gel-Shift Assay

Protein (20 μg) was incubated with 80 fmol of [^{32}P]-end-labeled, double-stranded estrogen response element (ERE; 5′-CAAAGTCA*GGTCA*CAG*TGACC*TGATCAAA-3′ [41]) in the presence of 1 μg poly dI-dC, and with or without 200-fold molar excess of nonradioactive competing ERE or 12 000-fold molar excess of poly dI-dC. The binding buffer was 12% glycerol, 12 mM HEPES (pH 7.9), 48 mM KCl, 4 mM Tris (pH 7.9), 1 mM DTT, and 1 mM EDTA. Binding reactions were carried out for 30 min at 24°C and subsequently resolved in a nondenaturing 12% acrylamide gel at 10°C. The authenticity of the binding reaction was verified by the addition of the ER monoclonal

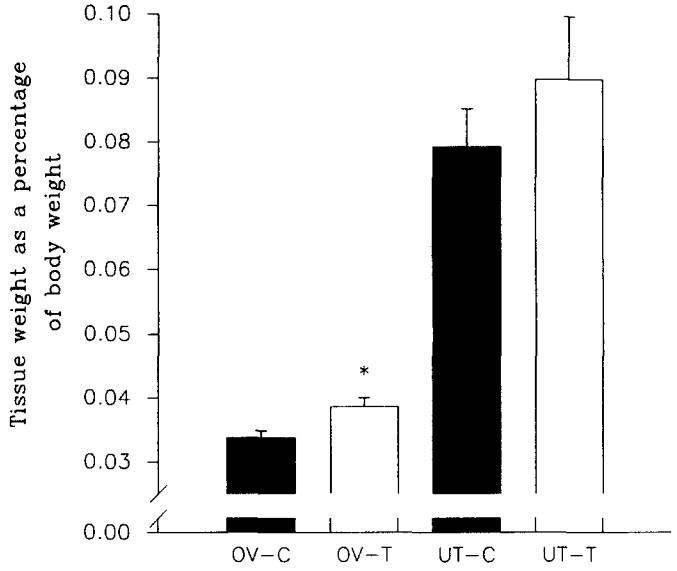

FIG. 1. Tissue weight expressed as a percentage of whole body weight in postnatal Day 21 female rats exposed to TCDD in utero and through lactation. OV-C, ovary nonexposed (n = 28); OV-T, ovary exposed (n = 17); UT-C, uterus nonexposed (n = 9); UT-T, uterus exposed (n = 7). *Significantly different from values in nonexposed females ($p < 0.05$).

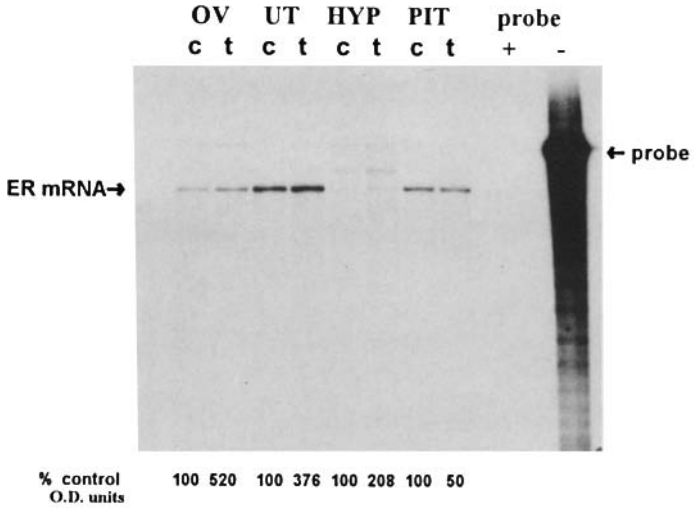

FIG. 2. Ribonuclease protection assay of ER expression using 5 μg of total RNA isolated from pooled ovaries (OV), uteri (UT), hypothalami (HYP), or pituitaries (PIT). The percentage of control optical density units is given below each lane and represents the results of a single assay. The right two lanes represent the radiolabeled ER cRNA in the absence of RNA with (+) or without (−) the addition of RNase One, demonstrating the specific hybridization and the efficacy of the RNase treatment. c, nonexposed; t, exposed.

antibody H222 (courtesy Dr. G. Greene, Ben May Inst., Chicago, IL). One microliter of H222 was incubated with protein extracts for 20 min on ice before the addition of ERE.

Estrogen RIA

Circulating concentrations of estrogen were determined (n = 19 individuals per group) with use of 200 μl of serum and the estradiol Coat-A-Count kit, validated for rat (Diagnostic Products Corp., Los Angeles, CA). All samples were run in duplicate with estrogen standards. The within-assay coefficient of variation was 3.2%.

Statistical Analysis

All bands generated by autoradiography were quantified with a densitometer (Optimas Bioscan, Madison, WI) and represent the results of a single assay. Body weights were compared by a two-way ANOVA with repeated measures. Ovarian and uterine wet weights were compared within each treatment to ensure that there was no litter effect on tissue mass (ovary pooled mean ± SEM = 10.6 ± 0.8; ovary litter mean = 10.6 ± 0.5; uterine pooled mean = 49.3 ± 3.2; uterine litter mean = 50.7 ± 3.8). Tissues were compared by use of *t*-test both as raw weights and as percentages of body mass. The gel-shift and RNA assays were replicated with a minimum of three different RNA or protein pools. Relative optical density units were generated as a percentage of controls. With use of these values, the interassay coefficient of variation was calculated to be 16.5%. For the estrogen RIA, data were spline-transformed and analyzed using a *t*-test and the results expressed as mean ± SEM. Significance was set at $p < 0.05$. The data are representative of two separate experiments.

RESULTS

Pregnant female dams given 1 μg TCDD/kg body weight on gestational Day 15 did not exhibit signs of overt toxicity, and pups were born on the expected day. Offspring mortality of vehicle- and TCDD-exposed pups as measured by visual inspection of dead pups at birth was 0% and 10%, respectively. Weaning weights of female pups were decreased, although not significantly, by exposure to TCDD. Ovarian weights of pups exposed in utero and through lactation were not significantly different from those of controls when expressed as ovarian mass (nonexposed [C] = 3.15 ± 0.12 mg; exposed [T] = 3.88 ± 0.13). When these weights were converted to a percentage of body weight, the increase was significant (C = 0.034 ± 0.001%; T = 0.039 ± 0.001; Fig. 1). Uterine mass was not significantly changed in exposed animals (C = 54.5 ± 5.05 mg; T = 50.0 ± 4.50), nor was it changed when expressed as a percentage of body weight (C = 0.079 ± 0.006; T = 0.090 ± 0.01; Fig. 1).

The relative abundance of ER (protein and mRNA) in control tissues based on optical density values was as predicted (uterus > pituitary > ovary > hypothalamus), while in utero and lactational exposure to TCDD had varied effects on ER expression based on the particular tissue type and assay used. With use of RNase protection assays, it was observed that TCDD exposure increased hypothalamic ER mRNA to 2.1 times that of controls, while in pituitaries from TCDD-exposed animals the ER mRNA was 50% lower than in nonexposed counterparts (Fig. 2).

At the level of the uterus, TCDD-exposed animals had 3.8 times the abundance of ER mRNA seen in controls, while a 5.2 times increase was observed in ovarian ER mRNA vs. that in controls (Fig. 2). Total ovarian RNA from 60-day-old females exposed in utero and through lactation to TCDD or to vehicle control was slot-blotted and probed with an ER cDNA. The relative amount of ER mRNA remained elevated at this age (data not presented).

In order to associate changes in ER mRNA with potential alterations in ER function, we performed gel-shift assays using protein extracts from these same treatment groups. The specificity of the binding reaction was verified by the reduction of signal to background levels with use of a 200 times molar excess of nonradioactive ERE DNA.

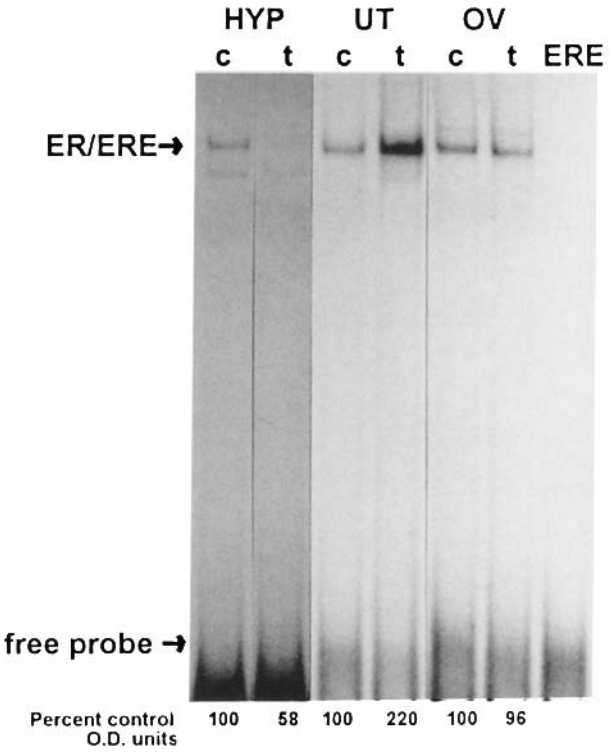

FIG. 3. Gel-shift assay using whole cell protein extracts from pooled ovaries (OV), uteri (UT), or hypothalami (HYP) probed with a [^{32}P]-ERE. The lane labeled ERE included 200-fold molar excess of nonradioactive ERE in the binding reaction. The percentage of control optical density units is given below each lane and represents the results of a single assay. c, nonexposed; t, exposed.

Signal intensity was not reduced by the addition of 12 000 times molar excess of poly dI-dC to the binding reaction. Each tissue tested for DNA-binding activity was supershifted with monoclonal antibody H222 in order to verify the presence of ER (data not presented).

In whole hypothalamic protein extracts, ER DNA-binding activity was reduced by up to 58% that of vehicle-exposed animals (Fig. 3). ER DNA-binding activity in control-exposed uterine protein extracts was increased 2.2 times in exposed vs. nonexposed animals, while ovarian ER DNA-binding activity was not changed with TCDD exposure (Fig. 3; Table 1).

Serum estrogen concentrations were 145.7 pg/ml ± 17.0 (n = 19) in nonexposed females and 63.8 pg/ml ± 9.2 (n = 17) in TCDD-exposed animals ($p < 0.05$; Fig. 4).

DISCUSSION

Gray and Ostby [39] have recently shown that in utero and lactational exposure to TCDD on gestational Day 15 resulted in a number of reproductive abnormalities in adulthood including, for example, reduced ovarian and brain weight, vaginal deformities, delayed puberty, cervical lesions, and a reduction in the percentage of females producing a fifth litter. We have extended these studies to demonstrate that exposure to TCDD on gestational Day 15 resulted in changes to ovarian and uterine mass, ER mRNA and DNA-binding activity, and serum estrogen concentrations in the weanling Holtzman rat.

Exposure of female Holtzman rats to TCDD in utero and through lactation resulted in a significant increase in ovarian mass when expressed as a percentage of body weight

TABLE 1. Summary of the variations in ER mRNA and DNA-binding activity.*

Tissue	ER mRNA	ER DNA-binding
HYP	↑	↓
PIT	↓	—
UT	↑↑	↑
OV	↑↑↑	nc

* Arrows indicate changes with respect to 21-day-old vehicle-exposed females; nc: indicates no change; —: not performed due to insufficient material. HYP, hypothalamus; PIT, pituitary; UT, uterus; OV, ovary.

on postnatal Day 21, and no significant changes in the percentage of body weight were attributed to the uterus. While Gray and Ostby [39] showed that in utero and lactational exposure to TCDD resulted in a decrease in ovarian mass in adulthood, we attribute these differences to the age of the animals and support the notion that the effects of TCDD on the female reproductive system are specific to the particular model [30]. The fact that ovarian weight was not significantly increased with TCDD exposure, while comprising a significantly greater percentage of body weight, may be the result of an interaction between the small increase in raw ovarian weight and the slight decrease in body weight. The relationship of ovarian and uterine mass to the observed changes in serum estrogen and ER is unclear.

In the rat, as in other species, exogenous estrogen stimulates follicular development through mediation by the ER (e.g., [42, 43]). However, endogenous estrogen plays multiple roles in the control of follicle development (reviewed in [44, 45]). At hypothalamic and anterior pituitary cells, the effects may be mediated by catechol estrogens, formed in part by the TCDD-inducible cytochromes P4501A1 and 1A2. Catechol estrogens compete for both estrogen- and dopamine-binding sites, perhaps altering LH/FSH secretion [46, 47]. From the RIA data alone, it is not possible to determine whether the decrease in estrogen was a result of increased metabolism in the form of hydroxylation [19–22] or a decrease in estrogen biosynthesis.

Estrogen regulates the ER in a tissue-specific manner. In the preoptic area/hypothalamus, it was found that estrogen decreased the amount of immunoreactive ER in the adult

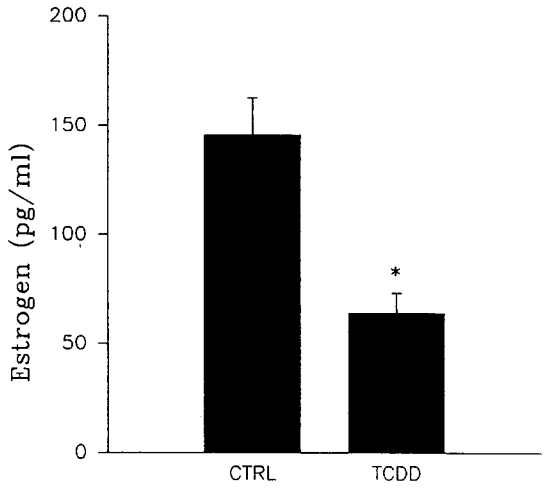

FIG. 4. Effects of in utero/lactational exposure to TCDD on circulating concentrations of estrogen in 21-day-old female rats. Data are presented as the mean (n = 19 for control and 17 for exposed) ± 1 SEM. *Significantly different from values for control-exposed females ($p < 0.05$).

rat [47], while in the pituitary, estrogen increased the amount of ER mRNA [49]. At both the ovary and uterus, estrogen may be a negative regulator of ER [30, 41, 48]. In systems perturbed by agents that alter serum estrogen concentrations, it should be possible to predict the direction of change in ER expression. The results from the present study are in good agreement with predictions of ER alteration based upon serum estrogen.

In TCDD-exposed animals, serum estrogen was decreased; hypothalamic, uterine, and ovarian ER mRNA was increased by TCDD exposure and remained elevated on postnatal Day 60, while pituitary ER mRNA was decreased by TCDD exposure. The gel-shift assays, however, yielded some unexpected results. While the uterine gel shift paralleled changes in ER mRNA, ovarian ER DNA-binding activity did not change, and hypothalamic DNA binding decreased with TCDD exposure. These data may indicate an uncoupling of transcription and translation or changes in receptor function, although these points remain to be tested. While the results from the present study do not directly address the issue of the mechanism of TCDD's antiestrogenicity, they do suggest that in this model, TCDD may act to regulate estrogen; and the decrease in estrogen may be the stimulus for alterations in ER expression.

In conclusion, we have observed in weanling female rats that in utero and lactational exposure to TCDD on gestational Day 15 resulted in decreased serum estrogen and in increased ER mRNA in the ovary, uterus, and hypothalamus while a decrease was seen in pituitary ER mRNA. Exposed females had higher levels of ER DNA-binding activity in the uterus, but there was no change in the ovary, and there was a decrease in hypothalamic protein extracts. Ovaries from exposed pups constituted a greater percentage of total body weight than in vehicle-exposed females. The mechanism for the reduction in female fertility that accompanies in utero and lactational exposure to TCDD remains unknown, although it could be linked to the estrogenic effects observed by Gray and Ostby [39], such as clefting of the phallus and hypospadias. We now believe, therefore, that the actions of TCDD in this model are exceedingly complex and that they should not necessarily be defined as purely anti-estrogen or estrogenic.

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