Regulation of the Aromatic Hydrocarbon Receptor (AHR) by *In-Utero* and Lactational Exposure to 2, 3, 7, 8-Tetrachlorodibenzo-*p*-Dioxin (TCDD)

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Abstract. The aromatic hydrocarbon receptor (AHR) mediates the toxicity of several halogenated aromatic hydrocarbons, including 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD). In order to better understand the systemic endocrine disruption induced by AHR agonists, we examined the regulation of AHR by exposure to TCDD during gestation and lactation. Pregnant rats were given an oral dose of 1 µg TCDD/kg body weight or vehicle control on day 15 of gestation, and female pups sacrificed on post-natal day 21. Total RNA from ovaries, uteri, and hypothalami was immobilized on a nylon membrane and probed with a murine AHR cDNA. The gel mobility-shift assay was used to assess changes in the DNA-binding activity of AHR *in vitro*. Ovarian AHR mRNA and DNA-binding capability were decreased 2.1- and 1.7-fold, respectively; while in the uterus, there was a 1.3- and 1.5-fold increase in message and DNA binding. Hypothalamic mRNA for AHR showed no change, while there was a 1.7-fold increase in DNA-binding activity. Our results show that *in-utero* and lactational exposure to TCDD alters the expression of AHR in a tissue-specific fashion, and that this altered regulation may either be a part or a cause of the general endocrine disruption observed in these animals.

Key words: Aromatic hydrocarbon receptor, TCDD, Ovary, Hypothalamus, Uterus.

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The halogenated aromatic hydrocarbon 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a potent disruptor of vertebrate endocrine systems, with exposure in rats *in utero* and through lactation more potent than equivalent doses given to adults [reviewed in 1]. The actions of TCDD are mediated by a soluble, ligand-inducible transcription factor termed the aromatic hydrocarbon receptor (AHR) that binds to an upstream enhancer element (dioxin response element, DRE) to alter target gene expression [2, 3].

The transformation of AHR into an active transcription complex by TCDD results in the direct regulation of a number of endocrine genes; however, regulation of AHR itself remains poorly understood. The promoter of the murine AHR gene is GC-rich and does not contain TATAA or CCAAT boxes, although Sp1, AP-1, E-box (c-Myc), and CREB-binding sites are present [4]. TCDD has been shown to down-regulate AHR in the placentas of mice exposed *in utero* [5] and in cells in culture [6, 7]. Pollenz [8] has recently demonstrated in Hepa-1 and NIH-3T3 cells that AHR, but not the essential co-factor AHR-nuclear translocator factor, is depleted following TCDD exposure. These data clearly demonstrate that AHR is auto-

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regulated by agonists both *in vivo* and *in vitro*.

Exposure to TCDD during development results in abnormal embryonic and fetal development; for example, hydronephrosis, cleft palate, thymic hypoplasia, and in some cases, pre- and post-implantation embryonic loss [1, 9]. Blankenship *et al.* [10] have shown that exposure of cultured mouse blastocysts to TCDD results in accelerated differentiation, although culturing embryos in media containing an AHR anti-sense oligonucleotide reduced the incidence of blastocyst formation and mean embryo cell number [11]. These data suggest that AHR has a function in embryonic cell proliferation.

Because AHR appears to play a role in early embryo growth and later fetal and neonatal development, the disruption of AHR expression by xenobiotic agonists such as TCDD may play a significant role in the induction of developmental abnormalities by *in-utero* and lactational exposure to these compounds: in female rats, these include decreased ovarian, brain, and body mass in adulthood, abnormalities in vaginal morphology, inflammatory cervical lesions, and cystic hyperplasia of the endometrium [12]. Female rats born to mothers exposed to 1 $\mu\text{g/kg}$ on gestation day 15 exhibited reduced serum concentrations of 17 β -estradiol and systemic alterations in estrogen receptor, as well as in reduced fertility and fecundity [12, 13]. In the present study, we report changes in the expression of AHR mRNA and DNA-binding activity in 21-day-old female Holtzman rats exposed to TCDD *in utero* and during lactation. Ovarian, uterine, and hypothalamic tissue were examined as part of our efforts to better understand the disruption of the female reproductive axis by perinatal exposure to TCDD.

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 ($22 \pm 1^\circ\text{C}$), and humidity ($55 \pm 5\%$); and a 14 h L: 10 h D 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 $\mu\text{g/kg}$) and nine pregnant rats received an equivalent volume of vehicle control (corn oil/acetone, 19/1, v/v, 2 ml/kg) by feeding. TCDD (98% purity) was purchased from Cambridge Isotope Laboratories (Woburn, MA).

On the day of birth, litters were adjusted to five males and five females to allow for similar lactational exposure to TCDD. Litter weights were obtained at birth and post-natal days 7, 14, and 21. On post-natal day 21, female pups were sacrificed by decapitation under CO_2 anesthesia. Post-natal day 21 was chosen in order to follow the pups' exposure through lactation. The ovaries, uteri, and hypothalami were removed, pooled into groups of six without regard for litter, and frozen on dry ice.

RNA Isolation

Total RNA was isolated using the acid-guanidinium isothiocyanate method [14]. The quantity of RNA was determined using absorption at 260 nm on a spectrophotometer. To confirm the quality of the RNA visually and verify the accuracy of the spectrophotometric reading, 1 μg of RNA was electrophoresed through a 1% agarose gel stained with ethidium bromide and visualized using a UV light source.

Slot-blot assay

Twenty-five μg of total RNA were immobilized on nylon membrane using a slot-blot manifold and hybridized to a randomly primed murine AHR cDNA (courtesy of Dr. CA Bradfield, Northwestern School of Medicine [15]) at 42°C overnight. Blots were washed and used for autoradiography.

Protein Isolation

Whole-cell protein extracts were prepared following the method of Bettini *et al.* [16]. In brief, pooled tissues were homogenized with a Dounce homogenizer in 5 ml of HDK buffer (25 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) pH 7.5, 1 mM dithiothreitol (DTT), 400 mM KCl, 2 $\mu\text{g/ml}$ pepstatin A, 2 $\mu\text{g/ml}$ leupeptin, 6 $\mu\text{g/ml}$ of N- α -p-tosyl-L-lysine chloromethylketone and 6 $\mu\text{g/ml}$ L-1-tosylamido-2-phenylethyl chloromethylketone). The homogenate was incubated on ice for 1 h and then centrifuged at $500 \times g$ for 10 min at 4°C . Supernatant was diluted to 10% with respect to glycerol and centrifuged at $120,000 \times g$ for 1 h at 4°C . The

resultant supernatant was collected, aliquoted, and snap frozen in ethanol/dry ice. Protein concentrations were determined using Bio-Rad protein assay dye (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin (fraction V) standards. Aliquots were stored at -80°C until use and were not re-used after being thawed.

Gel mobility shift assay

Twenty μg of protein were incubated with 80 fmol of [^{32}P] end-labeled, double-stranded dioxin response element (DRE) from the murine cytochrome P4501A1 (*cyp1A1*) gene (5'-GGGCCAGA GCTCGCGTGTCTGGCGGGAC). Binding reactions occurred in the presence of 1 μg poly dI-dC, and with or without 200-fold molar excess of non-radioactive competing DRE (specific competitor) or 12,000-fold molar excess of poly dI-dC (non-specific competitor). The binding buffer was 12% glycerol, 12 mM HEPES pH 7.9, 48 mM KCl, 4 mM Tris pH 7.9, 1 mM DTT, 1 mM EDTA. Binding reactions were carried out for 30 min at 24°C and subsequently resolved in a non-denaturing, 12% acrylamide gel at 10°C . Gels were dried and used for autoradiography.

Data analysis

All bands generated by autoradiography were analyzed by using densitometry (Optimas images, Madison, WI). Each assay was replicated a minimum of three times with equivalent relative results.

Results

Administration of 1 μg TCDD/kg maternal body mass did not result in overt signs of maternal toxicity, and pups were born on the expected day. Neonatal mortality for control- and TCDD-exposed pups was 0 and 10%, respectively. Pup mass was reduced at birth and remained so to the time of sacrifice.

Exposure to TCDD *in utero* and during lactation resulted in tissue-specific changes in AHR, both in terms of mRNA and DNA-binding activity. In whole ovary, AHR mRNA and DNA-binding was decreased 2.1- and 1.7-fold, respectively. In uterine extracts, AHR mRNA was increased by 1.3-fold and DNA-binding by 1.5-fold. Messenger RNA encoding for AHR in the hypothalamus was not changed, although there was a 1.7-fold decrease in

DNA-binding activity. These results are summarized in Figs. 1 and 2.

In ovarian and uterine protein extracts, but not in hypothalamic extracts, multiple shifted bands were observed (Fig. 2). While both bands are believed to possess authentic AHR based on specific and non-specific competition experiments, the composition of the DNA-binding complexes is not currently known. Binding of AHR to DRE was competitively displaced by the addition of 200-fold excess of unlabeled DRE, as depicted by elimination of ALL bands to non-specific background (data not shown).

Discussion

AH receptor mRNA and DNA-binding activity were detected in ovary, uterus, and hypothalamus in peripubertal rats. This represents the first time that this molecule has been localized to these tissues, and provides the first data showing agonist regulation of AHR in the reproductive tract.

The regulation AHR mRNA and DNA-binding

Fig. 1. AH receptor mRNA from 21-day old rats exposed *in utero* and via lactation to TCDD. Results are representative of three replicates. Graphical representation of slot-blot expressed as optical density (O.D.) units. C, control-exposed; T, TCDD-exposed; OV, ovary; UT, uterus; HYP, hypothalamus.

Fig. 2. AH receptor DNA-binding activity from 21-day old rats exposed *in-utero* and via lactation to TCDD. Results are representative of three experiments. (A) Results from gel mobility shift assay. Arrows indicate AHR/DRE complexes formed *in vitro*, "probe" refers to radiolabelled DRE that remained uncomplexed with AHR. Note the presence of two discreet shifted complexes in ovarian and uterine extracts. Specific and non-specific competition experiments are not shown. (B) Graphical representation expressed as optical density (O.D.) units. C, control-exposed; T, TCDD-exposed; OV, ovary; UT, uterus; HYP, hypothalamus.

activity by *in-utero* and lactational exposure to TCDD was tissue specific in the 21-day-old female rat. While the endocrinologic consequences of this altered regulation are unclear, it is possible that changes in the expression of AHR were due to alterations in intracellular levels of cAMP, as the murine AHR promoter possess two CREB response elements [4]. This is supported by the fact that Enan *et al.* [17] have shown that TCDD alters cAMP in human luteinized granulosa cells *in vitro*. In addition, we have recently discovered that *in-utero* and lactational exposure to TCDD results in increased FSH concentrations (Chaffin *et al.*, submitted), potentially acting to increase intracellular cAMP in granulosa cells. The rise in cAMP may have therefore acted as a regulator of AHR, although multiple factors (e.g., steroids) likely played a role in the regulation of AHR.

Contrary to the ovary, where AHR was decreased, uterine AHR was increased by *in-utero* and lactational exposure to TCDD. This direction in change was unexpected as TCDD has been shown to down-regulate AHR in several other systems [5–7]. While both the mechanisms for the increase

and the consequences are unknown, it is clear that the *in-vivo* response of AHR to chronic TCDD exposure is not a consistent down-regulation as would be predicted based on classical endocrine-type regulation.

In hypothalamic extracts, AHR DNA-binding was decreased by 1.7-fold, while no change was observed in AHR mRNA. This was potentially due to the low sensitivity of the slot-blot assay versus the gel-shift assay, although it is possible that the decrease in DNA-binding was a result of translational or post-translational changes rather than altered gene expression [3]. An alternative possibility is that an essential cofactor for DNA binding, the AHR nuclear translocator factor (ARNT), was decreased in hypothalami from exposed individuals.

In both ovarian and uterine protein extracts, dioxin-response element DNA was able to produce two bands in the gel shift assay (Fig. 2). Both bands are believed to represent authentic AHR based upon competition assays in which specific, but not non-specific, competitor was able to reduce binding to background levels. The composition of these complexes is unknown, but may represent another level of AHR regulation in that differential changes in DNA binding activity were observed between the two bands. We hypothesize that the lower molecular weight bands in the multiple-shifted complexes may be due to single-stranded binding proteins, but as yet have no confirming evidence. We believe that the reason we do not see these faster-migrating complexes in hypothalamic extracts is that there may be reduced concentrations of single-stranded binding proteins in this tissue, since less DNA replication occurs in neuronal tissues.

The presence of AHR in the ovary, uterus, and hypothalamus suggests that these tissues may be direct targets of TCDD, and may in part help to explain the systemic disruption of the female reproductive endocrine system observed by Gray & Ostby [12] and Chaffin *et al.* [13]. In addition, the localization to these tissues suggests that AHR may play an endogenous role in reproduction.

Previous research has suggested significant cross-talk between AHR and the estrogen receptor as a mechanism of TCDDs antiestrogenicity [18]. The interaction between TCDD and the estrogen signal is exceedingly complex. We have observed, for example, that while TCDD regulates ER, a 12-h

exposure to 17 β -estradiol did not alter hepatic AHR mRNA in immature female rats (Chaffin *et al.*, submitted). Thus, the interaction between these two receptor pathways may not be bi-directional, implying that the reduction in serum estradiol was not the sole factor in the regulation of AHR.

In conclusion, the data presented herein indicate that the Ah receptor is regulated *in vivo* by *in-utero* and lactational exposure to the agonist TCDD. While the physiologic consequences of this are unknown, it is clear that the regulation of AHR is complex and controlled by multiple cell- and tissue-specific factors.

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