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The constitutively active Ah receptor (CA-AhR) mouse as a model for dioxin exposure – Effects in reproductive organs

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ABSTRACT

The dioxin/aryl hydrocarbon receptor (AhR) mediates most toxic effects of dioxins. *In utero*/lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) impairs fetal/neonatal development and the developing male reproductive tract are among the most sensitive tissues. TCDD causes antiestrogenic responses in rodent mammary gland and uterus and in human breast cancer cell lines in the presence of estrogen. Also, more recently an estrogen-like effect of TCDD/AhR has been suggested in the absence of estrogen. A transgenic mouse expressing a constitutively active AhR (CA-AhR) was developed as a model mimicking a situation of constant exposure to AhR agonists. Male and female reproductive tissues of CA-AhR mice were characterized for some of the effects commonly seen after dioxin exposure. Sexually mature CA-AhR female mice showed decreased uterus weight, while an uterotrophic assay in immature CA-AhR mice resulted in increased uterus weight. In immature mice, both TCDD-exposure and CA-AhR increased the expression of the estrogen receptor target gene Cathepsin D. When co-treated with 17β-estradiol no increase in Cathepsin D levels occurred in either TCDD-exposed or CA-AhR mice. In sexually mature male CA-AhR mice the weights of testis and ventral prostate were decreased and the epididymal sperm reserve was reduced. The results of the present study are in accordance with previous studies on dioxin-exposed rodents in that an activated AhR (here CA-AhR) leads to antiestrogenic effects in the presence of estrogen, but to estrogenic effects in the absence of estrogen. These results suggest the CA-AhR mouse model as a useful tool for studies of continuous low activity of the AhR from early development, resembling the human exposure situation.

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1. Introduction

The polyhalogenated aromatic hydrocarbons are a family of toxicologically important environmental contaminants that includes polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic congener and has been used as a prototype compound to investigate the toxic potential of PCDDs, PCDFs and coplanar PCBs. Exposure during early life-stages appears to be the most critical and the male reproductive organs are among the most susceptible and sensitive target tissues for *in utero* and lactational TCDD exposure (EC-SCF, 2000, 2001; WHO-ECEH/IPCS, 2000). Several studies in rats show reduced epididymal sperm count, decrease of both ventral prostate size and anogenital distance as well as a feminized sexual behavior (Mably et al., 1992; Bjerke et al., 1994; Gray et al., 1995; Sommer et al., 1996; Faqi et al., 1998; Ohsako et al., 2001).

The dioxin/aryl hydrocarbon receptor (AhR) mediates most, if not all, effects of dioxins. TCDD, the most well characterized ligand of AhR, has for a long time been regarded as having antiestrogenic properties such as inhibition of 17β-estradiol (E2)-induced uterine weight increase and decreased levels of estrogen and progesterone receptors in uterus of rats and mice (Safe, 1995). In Ah-responsive MCF-7 human breast cancer cells, TCDD inhibits 17β-estradiol-induced cell proliferation and decreases the mRNA levels of Cathepsin D (Gallo et al., 1986; Romkes et al., 1987; Gierthy et al., 1988). One of the suggested mechanisms of inhibitory AhR-estrogen receptor (ER) crosstalk involves direct interaction of the AhR complex with functional DREs within the regulatory part of estrogen responsive genes, such as Cathepsin D (Safe, 1995). Interestingly, ligand activated AhR was more recently shown to be a member of a ubiquitin ligase complex promoting proteosomal degradation of ERα (Ohtake et al., 2007). On the contrary, estrogenic effects of TCDD have also been reported (Abdelrahim et al., 2003; Watanabe et al., 2004;

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Boverhof et al., 2006). Hence, these data indicate a more complex interaction between the AhR and the estrogen signalling pathways. When activating AhR by 3-methylcholanthrene (3-MC), it can form a complex with unliganded ERα, that is recruited to estrogen responsive genes resulting in transcriptional activation both *in vitro* and *in vivo* (Ohtake et al., 2003). In addition, in transient transfection assays the AhR partner factor Arnt has been shown to co-activate ER-dependent gene expression (Brunnberg et al., 2003). Moreover, estrogen signalling seems to influence the AhR pathway since it has been shown that ERα is recruited with ligand-bound AhR to AhR regulated genes such as CYP1A1 and thereby modifying AhR transcriptional activity (Matthews et al., 2005).

We have developed transgenic mice expressing a constitutively active dioxin receptor (CA-AhR) (McGuire et al., 2001). The mutant Ah receptor is expressed and functionally active in all organs studied (Andersson et al., 2002, 2003; Brunnberg et al., 2006; Wejheden et al., 2010). The CA-AhR mice show several signs that are typical for dioxin toxicity such as thymic atrophy and liver enlargement (Andersson et al., 2002). These mice (CA-AhR mice) develop invasive tumors of the glandular stomach that correlate with increased mortality (Andersson et al., 2002). Other findings in these mice include a reduced population of B1 lymphocyte in the peritoneal cavity (Andersson et al., 2003) and enlarged heart and kidneys (Brunnberg et al., 2006). Additionally, adult female CA-AhR mice show a general bone loss in both trabecular and cortical bone tissue while male CA-AhR mice seem to be unaffected (Wejheden et al., 2010).

The aim of the present study was to further characterize the CA-AhR mouse model with regard to effects on male and female reproductive organs and the agonistic and/or antagonistic interactions with the estrogen pathway.

2. Materials and methods

2.1. Animals

In the study with 3 month old CA-AhR mice, the animals were backcrossed with the C57BL/6-strain (Charles River, Germany) for at least 10 generations before the experimental study (Wejheden et al., 2010). In the uterotrophic study transgenic CA-AhR and wild-type control animals with background described in (Andersson et al., 2002) were used. Animals were held in ventilated filter-top cages and received conventional rodent feed and tap water *ad libitum*, and were exposed to a 12 h light/dark cycle. All animal procedures were approved by the local ethical committee. Wild-type and CA-AhR mice were sacrificed by CO_2 asphyxiation followed by cervical dislocation. Body and organ weights were measured. The organs were frozen in −80 °C for further analysis.

2.2. Uterotrophic assay with immature mice

Female wild-type mice 20 d old, received on day one an intraperitoneal (i.p.) injection of TCDD 3 μg kg^{-1} bw or corn oil together with 20 ng of 17β-estradiol (E2) corresponding to approximately 2 μg kg^{-1} bw dissolved in ethanol, or vehicle only. Transgenic CA-AhR females of the same age received E2 or vehicle as described for wild type females. The E2 or vehicle exposure was administered once a day for three consecutive days and 24 h later the mice were sacrificed and treated as described above.

2.3. RNA-isolation, RT-PCR and qRT-PCR

Total RNA was extracted in TRIzol reagent (Invitrogen, UK). After DNase treatment (DNase I, Invitrogen), 1 μg of total RNA was reversely transcribed using 200 units of Superscript Reverse Transcriptase (Invitrogen, UK) and random hexamer primers (Amersham Biosciences). cDNA was amplified using 1 unit of Platinum Taq polymerase (Invitrogen, UK). Reverse transcriptase polymerase chain reaction (RT-PCR) primers and programs used for CA-AhR, CYP1A1 and β-actin amplification have previously been reported (Chatelain et al., 1995; Vogel et al., 1997; Andersson et al., 2003) The PCR products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide and visualized and photographed under UV light.

Quantitative RT-PCR (qRT-PCR) was used to quantify the expression of CYP1A1 in the testis. The diluted (1:10) cDNA was placed in a 20 μL reaction mixture containing 10 μL of POWER SYBR Green Mix (Applied Biosystems) and 300 nM of each primer. Primer sequence was obtained from PrimerBank with the PrimerBank ID 6753564a3 (Wang and Seed, 2003), forward primer CTACAGGACATTTGAGAAGGGC and reverse primer AGGTCCAAAACAATCGTGATGAC resulting in a PCR product of 135 bp. Primers were purchased from Thermo Scientific. All amplification reactions were performed in triplicates using 96-well optical reaction plates (MicroAmp, Applied Biosystems) on an ABI Prism® 7300 Sequence Detection System using standard conditions. Relative mRNA expression levels of each sample were calculated after normalization to an endogenous reference gene (β-Actin) and relative to the mean of all wild type samples, based on the cycle threshold (C_T) (Livak and Schmittgen, 2001). The relative copy number was calculated by the expression $2^{-(\Delta\Delta CT)}$.

2.4. Northern blot analysis

RNA blotted filters were hybridized with ^{32}P-labeled cDNA fragments of Cathepsin D in formamide-containing buffer (Sambrook et al., 1989) or Ultrahyb solution (Ambion). The filters were stripped and reprobed with labeled cDNA fragments of 18S rRNA. Band intensity was quantified by Phosphoimager analysis (Fuji Film), where differences in loading input was corrected for by normalization against the band for 18S rRNA. A specific probe was generated by amplifying cDNA templates from uterus tissue using the following primers, forward CAAGTCCAGCACCTATGTGAAGAA and reverse GGACACCTTCTCACAAGGAATCATATA. The cDNA was sequenced using DYEnamic™ ET terminator cycle sequencing premix kit (Amersham) in order to ascertain that the correct fragment had been amplified.

2.5. Epididymal sperm count

The sperm reserve of epididymis was counted using a modified version of a previously described method (Hamm et al., 2003). Briefly, the whole epididymis was thawed on ice, weighed, and minced in a weight boat. Icecold 0.9% saline with 0.3% Triton X-100 (1 mL) was added, mixed with tissue and the mixture was transferred to a tube. An additional 1 mL of saline was used to rinse the weigh boat and the entire mixture was homogenized by pipetting 10 times with a Pasteur pipette. The mixture was allowed to stand for 1 h and sperm numbers were determined by diluting the samples and manually counting intact sperm (head attached to tail) using a hemocytometer and light microscope. Sperms from each epididymis were counted in triplicates. The number of sperms was expressed as per gram epididymis.

2.6. Statistical method

Two-tailed Student's *t*-test was used to compare CA-AhR, TCDD and wild-type control mice. Due to data of uterine weight not being normally distributed a Mann-Whitney *U* test was applied. A *p*-value less than 0.05 was considered to indicate statistical significance.

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