2,3,7,8-Tetrachlorodibenzo-p-dioxin-mediated induction of amphiregulin in the developing mouse kidney

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Introduction

Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) results in a multitude of adverse effects in experimental animals and humans[1]. One of the most sensitive teratogenic responses to TCDD is hydronephrosis of the murine kidney as a result of gestational and early postnatal exposure[2-4]. TCDD-induced hydronephrosis is characterized by hyperplasia of the developing ureteric luminal epithelium resulting in occlusion and restriction of urine flow[5].

It has been suggested that the epidermal growth factor receptor (EGFR) and its cognate ligands play a role in the pathogenesis of AHR-mediated hydronephrosis[6-9]. Indeed, TCDD-induced alteration of EGFR/EGFR-ligand expression and EGFR signaling has been demonstrated in several systems implicating the crosstalk between the AHR and EGFR signaling pathways[10-15].

Although, EGFR and its cognate ligands appear to be targets of the AHR, their role in AHR-mediated hydronephrosis is not well defined. Analyses using genetically-modified mice suggest that neither EGF or TGF-alpha are required for the development of TCDD-induced hydronephrosis, but that growth factor availability substantially influences the incidence and severity of hydronephrosis[8,9].

Since the EGFR signaling pathway has been implicated in AHR-mediated hydronephrosis we undertook a candidate gene approach and examined expression of the EGFR and its cognate ligands in response to a maternal TCDD dose.

Materials & Methods

Animals

Transgenic C57BL/6 mice were housed in clear plastic cages with litter and bedding under temperature and light (22 °C and 12 h light/dark cycle) controlled conditions and given mouse chow and water *ad libitum*. Care and treatment of the mice were in compliance with the guidelines of the Canadian Council on Animal Care and the protocol was approved by the Hospital for Sick Children Animal Care Committee. Female mice were housed overnight with male mice and females checked for vaginal plugs the next morning, which was designated as gestational day 0 (GD 0). On GD 13 or 16, dams were injected *i.p.* with a single dose of 30 microgram/kg of TCDD or vehicle (corn oil) alone. Dams were sacrificed on GD 17 by cervical dislocation and fetuses removed and decapitated. Maternal and fetal tissues were dissected and stored in RNA*later* (Ambion, Austin, Tx) at -80 °C until processed.

RNA Isolation from Animal Tissues

Total RNA was isolated from maternal liver, kidney, and ureter and fetal skin, lung, heart, liver, bladder, kidney, and ureter using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocolfollowed by treatment with DNase I (Amersham Biosciences, Piscataway, NJ) to remove any contaminating genomic DNA. In order to obtain sufficient material for these studies, tissues were pooled from one or more litters (~9 fetuses/litter) within the same treatment group to make up one biological replicate.

Cell Culture, Treatments, and RNA Isolation

The mouse hepatoma cell line, Hepa-1c1c7 (Hepa-1) was kindly provided by Dr. David S. Riddick of the University of Toronto and the TAO BP^rc1 cell line was purchased from the American Type Culture Collection (Manassas, VA). Cells were grown as a monolayer in alpha-minimal essential medium (alpha-MEM) (Wisent, Saint-Jean-Baptiste de Rouville, Quebec) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Oakville, ON) and maintained at 37 °C in a 5% CO_2 incubator. Cells were treated at ~50% confluency with the test chemicals in alpha-MEM in the absence of serum. Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Mississauga, ON) which included DNA digestion using the RNase-Free DNase Set (Qiagen, Mississauga, ON).

RT-PCR

One microgram of total RNA was reverse-transcribed to cDNA and target genes amplified by RT-PCR. PCR products from animal experiments were resolved by electrophoresis on 1.5% agarose gels stained, visualized with ethidium bromide and digitally recorded using a Gel Documentation System (Diamed, Mississauga, ON). PCR products from cell culture experiments were radio-labeled during the PCR reaction by incorporation of ^{32}P-labeled dCTP (2 microCi/reaction) and quantified as previously described[16].

Results

Effects of TCDD on Candidate Genes in Developing Murine Kidney and Ureter

At the transcriptional level, amphiregulin, a ligand for the EGFR, was markedly induced in fetal ureters within 24 h of *in utero* TCDD exposure and remained elevated 4 d after TCDD treatment as compared to vehicle (corn oil) exposed fetuses. The level of amphiregulin mRNA was also increased in fetal kidneys and to a much lesser extent in fetal lungs after a 4 d TCDD exposure. Similarly, a marginal increase in amphiregulin mRNA was detected in skin from TCDD-exposed fetuses at 4 d but not at 24 h. Thus, the early robust response of amphiregulin to TCDD exposure appears to be specific to the developing ureter.

It has recently been reported that amphiregulin is transcriptionally regulated by Wilms tumor suppressor (WT1), a critical factor in mouse kidney development[17]. To determine whether TCDD-activated AHR was signaling through WT1 to induce amphiregulin gene expression, WT1 transcript levels were assessed in the developing kidney and ureter. We found no change in WT1 mRNA levels in the TCDD-treated group compared to the vehicle-treated group, demonstrating that WT1 gene expression is not transcriptionally regulated by the AHR in this mouse model.

Epiregulin, another ligand for the EGFR, was also induced at the mRNA level in ureters of TCDD-exposed fetuses compared to vehicle-exposed fetuses. However, the expression of other candidate genes such as those encoding the EGFR itself and other EGFR ligands was not induced by TCDD in the developing kidney and ureter.

Effects of TCDD on Amphiregulin Gene Expression in the Mature Murine Ureter

To determine whether the amphiregulin response to TCDD in mouse fetal ureters was specific to the developing fetus, we investigated the effects of TCDD on amphiregulin mRNA levels in adult mice. Amphiregulin mRNA was not expressed at detectable levels in kidneys of control or TCDD-treated mice. However, basal amphiregulin expression was detectable in ureters from adult control animals, the level of which was slightly elevated in ureters of TCDD-treated adult mice. Our results suggest that the early response of amphiregulin to TCDD exposure seen in fetal ureters occurs to a much lesser extent in mature ureters.

AHR Dependency of TCDD-Mediated Amphiregulin Induction in Hepa-1 cells

Preliminary experiments revealed that amphiregulin mRNA was induced by TCDD in Hepa-1 cells, the prototypical AHR-responsive mouse cell line. Treatment of Hepa-1 cells for 24 h with 10^{-10}–10^{-7} M TCDD resulted in a significant increase in amphiregulin expression as compared with cells treated with vehicle alone. A TCDD dose-response relationship was observed with a maximal response occurring at a concentration of 10^{-9} M.

The expression of amphiregulin mRNA was significantly increased in Hepa-1 cells 3 h after TCDD treatment

compared to cells treated with vehicle alone and maximally increased by 6 h, the levels of which remained elevated at 24 h after treatment.

To determine the degree of dependence of TCDD-mediated induction of amphiregulin on the AHR, we employed the Hepa-1 variant cell line TAO BP^rc1, which has extremely low expression of AHR as compared to Hepa-1 wild type cells. Consistent with our *in vivo* results, amphiregulin mRNA was significantly induced in wild type cells after treatment with 1 nM TCDD. However, no change in amphiregulin gene expression was observed in TAO BP^rc1 cells after TCDD treatment indicating that TCDD-mediated induction of amphiregulin is via the AHR.

Discussion

We have demonstrated that of the genes encoding the EGFR ligands only amphiregulin and epiregulin were altered in their expression in the developing ureter in response to TCDD. Amphiregulin and epiregulin transcripts were induced substantially in fetal ureters within 24 h of a maternal dose of TCDD with the induction of amphiregulin being sustained for at least 4 d after treatment. Amphiregulin mRNA was also induced in fetal kidneys after a 4 d TCDD exposure compared to controls. Amphiregulin is a regulator of cell proliferation and migration whose principal physiological role appears to be in branching morphogenesis of developing organs. In mice, amphiregulin is an inducer of ureteric bud branching[17]. Amphiregulin is a developmentally important factor, the dysregulation of which may be detrimental to the developing embryo. This would be consistent with our hypothesis that TCDD dysregulates the expression of developmentally important genes involved in the regulation of cell proliferation in the mouse kidney and/or ureter, resulting in a hyperplastic response within the ureter and eventually in hydronephrosis.

The findings from the present study do provide a molecular basis for which the EGFR pathway may be involved in AHR-mediated hydronephrosis and other dioxin-mediated toxicities.

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