Coplanar Polychlorinated Biphenyls Activate the Aryl Hydrocarbon Receptor in Developing Tissues of Two TCDD-Responsive lacZ Mouse Lines

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In utero exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) can have an immediate impact on developmental processes that then lead to long-term deficits in function. To define the specific tissues affected by TCDD during development, we developed a lacZ-reporter gene mouse model driven by activation of the aryl hydrocarbon receptor (AhR). Exposure to TCDD on gestational day (GD) 14 results in strong activation of the lacZ transgene in numerous tissues including fore and hind paws, ear, and genital tubercle. Experiments were conducted to examine the ability of alternative AhR ligands to activate our model system. The coplanar polychlorinated biphenyl congeners 3,4,5,3′,4′-pentachlorobiphenyl (PCB126) and 3,4,3′,4′-tetrachlorobiphenyl (PCB77) both induced staining in fetal tissues identical to that observed following TCDD exposure. Exposure of fetuses to the PCB mixture Aroclor 1254 and the non-coplanar congener 2,3,6,2′,5′-pentachlorobiphenyl (PCB95) did not result in any activation of the lacZ transgene. In addition to the testing of alternative ligands, another line of reporter mice was generated to determine the potential influence of the site of insertion of the lacZ transgene on the reported observations. Both TCDD and the coplanar PCBs induced a similar pattern of staining in the new line as compared to that observed in the original lacZ reporter mouse line. The ability of AhR ligands, other than TCDD, to activate the AhR-mediated transgene, in combination with the insertion-site independence of the response, strengthens the data previously derived from this model and increases the utility of this system for investigations examining AhR-mediated events during development.

Key Words: aryl hydrocarbon receptor; polychlorinated biphenyls; dioxin; lacZ.

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is an important cellular target for environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). TCDD and other related halogenated aromatic hydrocarbons (HAHs) bind to the AhR and initiate a process involving translocation of the receptor to the nucleus, association of AhR with other proteins, binding of the complex to dioxin responsive elements (DREs) on DNA, and finally transcription of selected genes. The AhR signaling pathway and its resultant gene products are known to influence both developmental and cell-signaling processes, as well as metabolism of endogenous and exogenous compounds (Hankinson, 1995).

A great deal of our understanding of AhR function comes from investigating the effects of exposure to TCDD and other HAHs. The responses to AhR ligands varies with dose and time of exposure, but they include effects such as immunotoxicity, neurotoxicity, and alterations in developmental processes (Birnbaum and Tuomisto, 2000). Targeted deletion of AhR results in abnormalities in growth (Gonzalez and Fernandez-Salguero, 1998; Lahvis and Bradfield, 1998), supporting a role of the AhR in development. Experiments designed to investigate the interaction of AhR with other signaling pathways (Hayashida et al., 2004; Puga et al., 2000) and the identification of potential endogenous ligands (Song et al., 2002) are beginning to shed light on additional aspects of AhR function. Based on these studies, there is growing evidence that the AhR plays a critical role in the regulation of growth and differentiation.

As a means of examining AhR activation in specific cells and tissues following in vivo exposure to AhR ligands, our laboratory developed a transgenic mouse model containing a bacterial β-galactosidase reporter gene under the exclusive control of several DREs (Wille et al., 1998). AhR-mediated transcription of the β-gal enzyme, and subsequent treatment of the tissues to reveal β-galactosidase-mediated X-gal staining, can identify tissues where the AhR pathway is activated by either endogenous or exogenous agents. In our initial characterization of this model, 24 h exposure to TCDD, beginning at gestational day (GD) 13, resulted in X-gal staining in numerous regions including craniofacial structures, ear, palate, paws, and genital tubercle. Control animals exposed to vehicle revealed only faint, but detectable X-gal staining in genital tubercle, strongly suggesting activation of the AhR by an endogenous compound.
or process (Willey et al., 1998). Thus, the DRE-lacZ reporter mouse represents a model that can examine the activation of AhR via potential endogenous ligands, as well as target the sites of action of exogenous AhR ligands.

In addition to TCDD, other compounds can activate AhR-mediated transcription. Polychlorinated biphenyls (PCBs) are a family of ubiquitous environmental contaminants that possess widely varying toxic potential dependent primarily on chlorination pattern and density (Tilson and Kodavanti, 1998). This group of toxicants can be separated into two groups designated by the chlorine substitution of the ortho positions of the biphenyl ring. Polychlorinated biphenyl congeners without chlorines in the ortho positions are called “coplanar” because the two phenyl rings can assume a planar state. Chlorination of the ortho positions does not favor alignment of the phenyl rings; therefore the other class of PCB congeners is termed “ortho-substituted” or “non-coplanar.”

Non-ortho chlorine-substituted, or coplanar PCBs, have been implicated as developmental and immune system toxicants and are suspected carcinogens (Tilson and Kodavanti, 1998). By virtue of their ability to assume a planar state, which structurally resembles TCDD, coplanar PCBs are believed to elicit many of their effects through the AhR. Given these observations, we hypothesized that the DRE-lacZ reporter mouse would respond to coplanar PCBs in a manner similar to TCDD and should have little or no reactivity to ortho-substituted congeners.

In addition to testing the efficacy of coplanar PCBs in the original DRE-lacZ line, we tested another line of DRE-lacZ reporter mice generated in our laboratory. A potential limitation of the observations made using the original DRE-lacZ mouse line is that the responses we observed after TCDD exposure could be dependent on factors particular to the site of insertion of the lacZ transgene into the mouse genome. Thus, it is possible that the lacZ induction observed in particular tissues was not a direct response of AhR signal transduction, but rather a consequence of positional effects caused by endogenous regulators, mutations or recombinations, or concatemer-mediated effects (Pinkert, 2002). It is also possible that lacZ induction was not observed in certain tissues because that particular site of insertion was obstructed by tissue-specific factors that restricted AhR access and transactivation. A new line was created to confirm that the AhR ligand-mediated effects on the lacZ reporter gene are reproducible after random insertion, and thus the activity of the transgene is not as likely to be dependent on the site of insertion into the mouse genome. In addition to the well-characterized TCDD response, the new line (referred to as the W-line) was also tested with PCBs to determine its response to other AhR agonists.

MATERIALS AND METHODS

Selection and preparation of toxicants. 3,4,5,3',4'-Pentachlorobiphenyl (PCB126), 3,4,3',4'-tetrachlorobiphenyl (PCB77), 2,3,6,2',5'-pentachlorobiphenyl (PCB95), and Aroclor 1254 (A1254; Lot # 124–191, >99% purity) were obtained from Accustandard (New Haven, CT). PCB126 and PCB77 were selected for their respective potencies in activating the AhR (Hestermann et al., 2000) and their use in numerous in vivo toxicity studies (Crofton et al., 2000; Geller et al., 2000). PCB95 was chosen to maintain the same number of chlorines as PCB126, in addition to the significant activity of PCB95 in several model systems (Gafni et al., 2004; Schantz et al., 1997). Finally, A1254 is one of the more well-studied technical PCB mixtures that was used in industry and subsequently released into the environment (Ericksion, 1997). Thus, when compared to single PCB congeners, A1254 represents a more environmentally relevant toxicant mixture to study in terms of its potential impact on human health. We selected the 1 mg/kg concentration as the highest dose of any of the PCBs used in this study based on the study of Hesterman et al. (2000), which reports that PCB77 has about 1000× less affinity for the AhR and about 500× less relative potency for the receptor as compared to TCDD. The Aroclor mixture and PCB95 were also used at this concentration to provide a measure of dose equivalency. It is possible that considerably higher doses of the Aroclor 1254 mixture would activate our model, but because of potential general toxic effects in the mother and relevancy to other studies, such high concentrations were not examined.

TCDD serves as our positive control for activation of the lacZ transgene. Exposure of mice to 5 μg/kg TCDD po will reliably produce β-galactosidase activity in the previously mentioned tissues, and staining has been observed after exposure to concentrations as low as 1 μg/kg po (personal observation). However, we selected the dose of 30 μg/kg because this concentration had been used in previous experiments, and it has been well demonstrated that this dose maximally elicits lacZ reporter gene activity in this murine model. Individual congeners were dissolved in dioxane to create concentrated stock solutions. Aliquots of the stock solutions were taken to dryness under a gentle nitrogen stream. The toxicants were then dissolved in olive oil via thorough mixing for a minimum of 12 h. A1254 was dissolved directly in olive oil in an identical manner.

Animal handling and dosing. Timed-pregnant DRE-lacZ dams were generated by overnight pairing, followed by removal of the male and examination for a sperm plug. Successful matings were further verified by steady weight gain and gentle palpation for fetuses. For the purposes of gestational dating, the first day after pairing was termed gestational day (GD) 0. Dams were dosed at GD 14 via oral gavage of olive oil control or olive oil containing TCDD or PCBs. A minimum of three dams, with at least three pups per dam, were used for each exposure condition.

Twenty-four hours after dosing, the dams were sacrificed by CO2 overdose, and the entire uterus was removed to cold PBS. The condition and position of each fetus in the uterus was recorded, then each fetus was removed and placed in PBS. The fetuses were fixed in 10% neutral buffered formalin for 20 min at room temperature, followed by extensive rinsing in PBS.

X-gal staining. Based on a modification of Cheng et al. (1993), β-galactosidase activity was visualized by incubation of the intact fetus in a solution containing 5 mM K2Fe(CN)6, 5 mM K3Fe(CN)6, 2 mM MgCl2, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, 1 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal) in PBS (pH 7.9–8.0). Fetal tissues were fixed in this solution for 5 h at 37°C. and then rinsed thoroughly in PBS. Presence of the blue dibromo-dichloro indigo X-gal precipitate formed via the cleavage of the X-gal substrate by β-galactosidase was recorded with an Olympus SZX12 stereomicroscope (Melville, NY) and Image Pro Plus software (Media Cybernetics, Silver Spring, MD). After X-gal staining, individual fetuses were tested via polymerase chain reaction (PCR) analysis to verify the presence of the lacZ reporter gene (Willey et al., 1998).

Comparative assessment of X-gal staining in fetuses. Activation of AhR in the DRE-lacZ mouse model results in a recognizable pattern of staining throughout the fetus (Willey et al., 1998). The X-gal staining pattern in the whole fetuses reported here was most strongly visualized in the ears, fore and hind paws, and genital tubercle (GT). However, these tissues do not represent the entire staining profile observed in the fetus after TCDD exposure (see Willey et al., 1998). For the purposes of this study, these sites were selected...
because of their intense, reproducible staining, ease of identification, and ability to serve as a means to rapidly screen and compare AhR activation across treatment conditions. All results are based on observations made from at least three dams per treatment condition, with a range of 3–10 fetuses per dam.

In an attempt to characterize the staining observed in the fetuses, we developed a scale of staining intensity for the respective tissues examined in this study. Olive oil–treated controls provided us with the background level of staining; thus, no staining was observed in either ear or paw, and minimal staining was seen in genital tubercle only (data not shown; however, the background staining pattern of the controls matched that of PCB95-treated fetuses, Fig. 3). Wild-type (WT) controls exposed to vehicle or TCDD showed no staining in any regions examined. Using tissues from fetuses exposed to 30 μg/kg TCDD as our maximal response (for example, see Fig. 7), we visually graded the fetuses based on the amount and intensity of the X-gal precipitate in the selected tissues. Any tissue showing either no staining or background staining was scored – or “background,” respectively; any visible staining, +; moderate staining, ++; and maximal staining, ++++. Our effort to apply a semi-quantitative approach to characterizing the staining observed in the fetuses is based purely on extensive observation of our model and serves as a means for comparisons within these experiments only.

Histological examination of X-gal staining. To more readily compare the X-gal staining profile of mice exposed to PCBs with those administered TCDD, an examination of the tissue-specific distribution of β-galactosidase activity was performed. Whole fetuses were processed according to the above X-gal method and then embedded in Tissue-Tek O.C.T. (Sakura Finetek USA, Torrance, CA) and cut into 12-μm sections using a Microm HM 500 OM cryostat (Richard-Allen Scientific, Kalamazoo, MI). The sections were thawed onto slides, counter-stained with eosin, and mounted with GVA mounting medium (Zymed, San Francisco, CA). Images were captured using an Olympus BH2 microscope and Image Pro Plus software.

RESULTS

Coplanar PCBs Induce AhR-Mediated Transcription of β-Galactosidase

Microscopic examination of whole fetuses harvested from timed-pregnant dams dosed with 50–500 μg/kg PCB126 po revealed staining of ears, paws, and GT (Fig. 1). Application of the grading scale of intensity and character of the staining suggests a dose-dependent relationship between the concentration of PCB126 administered and the activation of AhR-induced transcription (Table 1). Exposure of fetuses to PCB77 also induced staining in the ear, paw, and GT (Fig. 2), albeit at doses much higher (1000 μg/kg, po) than those required to observe staining after exposure to PCB126.

Neither PCB95 (Fig. 3) nor the A1254 mixture (data not shown) elicited any response of the AhR-mediated lacZ reporter gene. Exposure of the fetuses to concentrations as high as 1000 μg/kg failed to result in any staining of the selected tissues above background levels. The concentrations of PCBs used here did not appear to adversely affect the dam, and no weight loss was observed following the 24 h exposure period (data not shown).

Histological examination of X-gal staining in tissue sections of fetuses treated with PCB126, as compared to fetuses exposed to TCDD further substantiated the similarities between the staining profiles of these two AhR ligands. X-gal staining in the GT was generally localized to the ventral regions of the structure and was most predominant in the glans and urethral seam, ending around the proximal urethral opening (Fig. 1A and Fig. 4). In the paw, X-gal staining was closely associated with the footpads of both the fore and hind limbs. Staining was localized to the metatarsal and tarsal pads of the hind limb (Fig. 1B) and their analogous pads on the forelimb. Curiously, the tarsal pads showed a more heavily focused staining pattern (Fig. 5 A–D), whereas the metatarsal pads displayed a more ring-like staining profile (Fig. 1B and Fig. 7B). No staining was observed in the digital pads of any paws. In addition to the staining localized to the pads, the junctions between the digits showed strong reporter gene signaling. The X-gal staining in the ear was localized to a region of the outer ear termed the “external acoustic meatus” (Fig. 6). These
regions of PCB126-induced AhR activity correspond well to those associated with TCDD exposure.

**New DRE-lacZ Line Responds to Both TCDD and PCBs**

Exposure of fetuses derived from the W-line to 30 \( \mu \text{g/kg} \) TCDD produced considerable staining in the ear, the fore and hind paws, and GT (Fig. 7). This line was similarly responsive to PCB126 and PCB77 at the same doses as employed with the established DRE-lacZ mouse line (data not shown). As observed in the original line of DRE-lacZ mice, the W-line fetuses did not respond to administration of A1254 or C95 (data not shown).

**DISCUSSION**

The goal of this study was to examine the response of an established DRE-lacZ mouse line to known AhR ligands other than TCDD. In addition, we sought to determine the potential influence of the site of integration of the transgene on the activity of the reporter gene by testing the response of another mouse line, generated using similar techniques, to known AhR ligands.

Exposure of pregnant dams to PCB126 and PCB77 resulted in activation of the AhR/DRE-driven pathway, leading to transcription of the lacZ transgene in specific tissues of the developing fetus. Figures 1 and 2 demonstrate that the tissue-specific profile of PCB-induced staining of the fetus is comparable to that observed after exposure to TCDD (Fig. 7). Additionally, cryotome sectioning of stained fetuses treated with PCB126 or TCDD revealed a similar pattern of \( \beta \)-galactosidase–positive cells at the cellular level (Figs. 4–6).

Although in certain cases the staining patterns didn’t exactly overlap, or the intensity of staining varied, the general pattern and characteristics of the staining observed between the TCDD- and PCB126-treated fetuses was quite similar. Some of these differences may be attributed to slightly different orientation of the tissue during cryotome slicing, as well as to inter-individual variations in staining and the differences in activity of the ligands at the AhR.

Identification of the exact cellular localization and specific cell type(s) with which the X-gal signal is associated in the various tissues was not attempted in this study. Our intention was to compare the activity of AhR ligands other than TCDD, and to confirm the pattern of AhR activation. Further studies can now be initiated, in specific tissues, using co-localization of the reporter gene signal with other cellular markers to positively identify cell types affected by TCDD or PCB exposure during development.
The presence of AhR and its accessory proteins in mouse embryos has been reported as early as GD 13.5 (Jain et al., 1998) in tissues such as palate, lung, liver, urogenital sinus, and GT. Thus our reported activation of the lacZ reporter gene corresponds to several previously identified sites of expression of AhR. This observation, in addition to the similarity in staining profile between PCB126 and TCDD, suggests that in utero exposure to PCB126 initiates activation of pathways potentially involved in AhR-mediated alterations in neonatal development.

PCB126 has previously been employed to examine the effects of developmental exposure to a coplanar PCB congener that is a known AhR agonist. Rice and colleagues employed a dosing regime of 0.25 or 1 µg/kg/day for 5 days a week, beginning 35 days prior to mating and continuing through gestation and lactation (Crofton and Rice, 1999; Rice and Hayward, 1999). In terms of cumulative dose, the concentrations of PCB126 used in the present studies are well within those used in the Rice studies and, in fact, are in the lower range of concentrations. However, the effects of acute versus chronic dosing may not necessarily be similar. With respect to the concentrations of coplanar congeners employed here, our data demonstrate the transplacental potency of these compounds for the AhR and the sensitivity of the DRE-lacZ mouse model to AhR ligands.

FIG. 3. Gestational day 15 fetus exposed to 1000 µg/kg 2,3,6,2',5'-pentachlorobiphenyl (PCB95) for 24 h via maternal gavage. A. Anterior fetus showing lack of X-gal staining in tissues of the external ear. B. Posterior fetus showing lack of X-gal staining in hind paw and background levels of X-gal in GT.

FIG. 4. 12-µm sagittal sections through the genital tubercle (GT) of a gestational day 15 fetus exposed for 24 h to 30 µg/kg tetrachlorodibenzo-p-dioxin (A,C) or 150 µg/kg 3,4,5,3',4'-pentachlorobiphenyl (PCB126) (B,D) via maternal gavage. Sections A & B are from more lateral regions of the GT, whereas C & D are closer to the midline.

FIG. 5. 12-µm sections through the hind paw of GD 15 fetus exposed for 24 h to 30 µg/kg tetrachlorodibenzo-p-dioxin (A,C) or 150 µg/kg 3,4,5,3',4'-pentachlorobiphenyl (PCB126) (B,D). Sections A and B are 4× images of varying orientations of the paw, and C and D are the corresponding 20× images indicated by the boxes. Locations of digits and footpads are indicated to provide the orientation of the sections.
The observation that higher doses of PCB77 are required to elicit a similar intensity of β-galactosidase staining as that seen following PCB126 (1000 μg/kg versus 50 μg/kg) exposure maintains the structure–activity relationships (SARs) established for the effects of PCBs on the AhR. Hesterman et al. (2000) demonstrated a considerably higher EC50 value for PCB77 than for PCB126 in EROD induction (0.73 versus 0.029 nM, respectively) and a 1000-fold increase in relative potency of induction of Cytochrome P-450 1A (CYP1A) protein in Poeciliopsis lucida hepatocellular carcinoma (PLHC-1) cells when comparing PCB126 to PCB77. In the whole animal, some tenfold higher concentrations of PCB77 were required to elicit similar alterations in sex-based behavior after exposure to PCB126 (Amin et al., 2000). The fact that our data support established PCB SARs further strengthens our confidence that the DRE-lacZ mouse models in vivo AhR function.

The intense staining observed in GT after TCDD exposure suggests significant activation of AhR-mediated events in this developing tissue. Willey et al. (1998) reviewed the effects of TCDD on phallic development and reproduction, supporting the observation that early disruption of differentiation processes by TCDD exposure leads to long-term functional deficits. More recent experiments describe the effects of TCDD on the development of the closely associated urogenital sinus (UGS) and its impact on prostate gland formation. TCDD exposure at GD 13 acts specifically on the UGS to reduce budding, and this reduction eventually leads to alterations in duct formation of the prostate (Ko et al., 2004a; Lin et al., 2004). This effect is mediated by TCDD-induced activation of the AhR in ventral UGS mesenchymal cells (Ko et al., 2004a), and it does not involve AhR-mediated disruption of normal androgen signaling (Ko et al., 2004b). It is interesting to note that the region of staining in the genital tubercle in our model exposed to TCDD or PCBs was also predominantly in the ventral mesenchymal tissues. The observations from Peterson’s group, combined with ours, illustrate the influence that TCDD may have on the developing reproductive system. Our present data predict that other exogenous AhR ligands, such as the coplanar PCBs used here, would induce similar effects during UGS differentiation.

The creation of a new line of DRE-lacZ mice was prompted by the potential influence of intrinsic regulatory regions surrounding the reporter gene transcript. Weis et al. (1991) demonstrated that regulatory regions, intrinsic to the site of insertion of the transgene, influenced the activity of a lacZ reporter gene, driven by regulatory elements from a major histocompatibility complex. By creating another mouse line, the W-line, under the assumption that the chances of insertion into the same site in the genome as in the original DRE-lacZ...
line are miniscule, we tested whether the lacZ response was integration-site dependent. After exposure to both TCDD and coplanar PCBs, the distribution of staining in fetuses derived from the W-line was identical to that seen in the original DRE-lacZ mouse model. While this observation cannot entirely eliminate integration-site dependence, it does serve to significantly minimize the chances that any positional effects are driving the response of the transgene, and it confirms the utility of this mouse model in investigations of AhR-mediated toxicity and the potential function of the AhR in normal development.

Our use of the DRE-lacZ mouse model has extended beyond experiments that examine the general reporter gene activity in investigations of tissue specific responses to TCDD. Williamson et al. (2005) used the reported transcriptional activation of AhR in immature cerebellar granule cells after acute TCDD exposure in early postnatal mice. We have also collaborated with Dr. Richard Peterson’s group at the University of Wisconsin to examine localization of TCDD-mediated AhR activity in the developing prostate (Kelman et al., 2005). The increasing application of this mouse model in investigations examining TCDD-mediated toxicity necessitates studies such as those described here.

In summary, these data confirm that the activation of β-galactosidase observed is dependent on signaling through the AhR and is not some phenomenon derived from TCDD exposure alone. Additionally, generation of a new line of DRE-lacZ mice indicates that the effects of both TCDD and PCBs on the DRE-driven lacZ reporter gene are most likely insertion-site independent. Both of these investigations serve to validate the DRE-lacZ mouse as a suitable model for TCDD-induced toxicity and strengthen the observations we have previously reported from this mouse model.

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