Aryl Hydrocarbon Receptor 2 Mediates 2,3,7,8-Tetrachlorodibenzo-p-dioxin Developmental Toxicity in Zebrafish

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The aryl hydrocarbon receptor (AHR) is a member of the basic helix-loop-helix PAS family of proteins that includes the aryl hydrocarbon receptor nuclear translocators (ARNT, ARNT2, and ARNT3), hypoxia inducible factor-1α (HIF1-α), endothelial-specific PAS protein-1 (EPAS-1/HIF2-α), single minded (SIM), and others. These proteins are involved in the sensation of and adaptation to changing environmental conditions (Gu et al., 2000). The components of the AHR signaling pathway have been well characterized in mammals because of their importance in mediating responses to environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The unliganded AHR resides in the cytoplasm in a complex with at least three chaperone proteins, two molecules of HSP90 (Perdew et al., 1988) and one molecule of the aryl hydrocarbon interacting protein (AIP, ARA9, XAP2) (Carver et al., 1997; Ma and Whitlock, 1997; Meyer et al., 1998). These chaperone proteins keep the AHR in the cytoplasm in the appropriate confirmation to bind ligand. Once bound by ligand, the AHR translocates to the nucleus, dissociates from the chaperone proteins, and dimerizes with ARNT. The AHR/ARNT heterodimer associates with specific DNA sequences termed dioxin–response elements (DREs) in the promoters of target genes, such as cytochrome P4501A1, leading to alterations in gene expression (Schmidt and Bradfield, 1996).

Embryos of various fish species including zebrafish are responsive to TCDD, and all display similar endpoints of developmental toxicity including cardiovascular dysfunction, edema, hemorrhages, craniofacial malformations, growth arrest, and mortality (Henry et al., 1997; Tanguay et al., 2003; Walker and Peterson, 1994). However, zebrafish have distinct advantages compared to other fish species for use in laboratory studies because they develop rapidly and externally and large numbers of embryos can be obtained regularly. Also, abundant information on zebrafish development is available, and sequence of the full zebrafish genome is near completion. Many molecular and genetic techniques have been developed for use in zebrafish that allow perturbations in gene expression to be rapidly studied.
The cloning and characterization of components of the AHR signaling pathway in zebrafish and other fish species revealed an important difference from that in mammals: fish possess at least two genes for the Ahr, whereas mammals have only one. The two classes of fish Ahr are denoted as Ahr1 and Ahr2 (Hahn et al., 1997). Both forms have been identified in zebrafish (Andreasen et al., 2002a; Tanguay et al., 1999) and Fundulus heteroclitus (Karchner et al., 1999), and Ahr2s have been described in Atlantic tomcod (Roy and Wirgin, 1997) and rainbow trout (Abnet et al., 1999).

Full-length cDNAs for zebrafish AHR1 (zfAHR1), AHR2 (zfAHR2), and ARNT2 (zfARNT2) have been cloned and their translation products functionally characterized (Andreasen et al., 2002a; Tanguay et al., 1999, 2000). zfAHR2 and zfARNT2b form a functional heterodimer in vitro that specifically recognizes DREs in gel shift experiments and induces DRE-driven transcription in COS-7 cells treated with TCDD (Tanguay et al., 1999, 2000). In contrast, zfAHR1 and zfARNT2b form only a weak interaction with DREs in gel shift experiments and produce minimal DRE-driven transcription in TCDD-treated COS-7 cells (Andreasen et al., 2002a). Consistent with these results, radioligand binding assays demonstrate that zfAHR2 but not zfAHR1 binds TCDD (Andreasen et al., 2002a). Tissue-specific expression patterns of mRNA in the zebrafish embryo demonstrate that zfAHR2 and zfARNT2a,b,c mRNAs colocalize in those embryonic tissues where zfCYP1A induction is observed after TCDD exposure (Andreasen et al., 2002b). Also, zfAHR2 mRNA has a wide tissue distribution in adult zebrafish compared to zfAHR1, which is expressed in only a few tissues including liver and to a far lesser extent in heart, kidney, and swimbladder. These results suggest that, despite the fact that zfAHR1 shares more sequence similarity with mammalian forms of the receptor than zfAHR2 (Andreasen et al., 1999, 2000), zfAHR2 mRNA has a wide tissue distribution in adult zebrafish compared to zfAHR1, which is expressed in only a few tissues including liver and to a far lesser extent in heart, kidney, and swimbladder. These results suggest that, despite the fact that zfAHR1 shares more sequence similarity with mammalian forms of the receptor than zfAHR2 (Andreasen et al., 2002a), zfAHR2 is more likely to be the isoform that mediates responses to TCDD in zebrafish.

Development of Ahr−/− null mice lines played a fundamental role in understanding TCDD signaling in mammals and clearly demonstrated the role of the AHR in mediating responses to TCDD (Fernandez-Salgueiro et al., 1996; Mimura et al., 1997). Although it is currently not possible to generate zfAhr2−/− null zebrafish, morpholino oligonucleotides provide an effective method to specifically and transiently knock down protein expression in the zebrafish embryo (Nasevicius and Ekker, 2000). Morpholinos are chemically modified oligonucleotides designed to target a specific mRNA sequence, such as the region surrounding the AUG start site on mRNA, in order to block ribosome access and inhibit initiation of protein translation. Morpholinos microinjected into 1–2 cell stage zebrafish embryos generate a zebrafish that is “morphant” for a particular protein. In the present study we use a morpholino to specifically knock down levels of zfAHR2 in zebrafish embryos to test the hypothesis that zfAHR2 mediates TCDD developmental toxicity.

MATERIALS AND METHODS

Zebrafish aryl hydrocarbon receptor 2 morpholino (zfahr2-MO), obtained from Gene Tools (Corvallis, OR), was designed with sequence complementary to zfahr2 cDNA (GenBankTM accession # AF063446). Zfahr2-MO overlapped the translation start site of zfahr2 mRNA in order to block initiation of translation by starting 4 bp upstream of the AUG start codon and continuing to 18 bp downstream. Sequence of the zfahr2-MO was: 5′ GTACCGATACCCCTCCATACGGT3′, and it was fluorescent tagged at the 3′ end to monitor injection success. The standard control morpholino sold by Gene Tools (5′CTCTACCTCTAGTTAACATTATA 3′) was used as the control morpholino (control-MO). Prior to embryo injection, morpholinos were diluted to 0.1 mM in 1x Danieau’s solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM HEPES, pH 7.6) as described by Nasevicius and Ekker (2000).

In vitro transcription and translation (TNT) of zfAHR. In vitro transcription and translation of zfahr1 and zfahr2 cDNA was performed using the TNT-coupled rabbit reticulocyte lysate system (Promega, Madison, WI). Re-combinant proteins were produced from pBK-CMV expression constructs (Andreasen et al., 2002a; Tanguay et al., 1999) with T3 RNA polymerase. Reactions were performed according to manufacturer’s protocol, except that to a 12.5 μl reaction was added 75 ng of template DNA, 0.75 μl of 35S-methionine, and either no morpholino or 500 nM final concentration of control-MO or zfahr2-MO. After 90 min incubation at 30°C radioactive translation products were resolved by 8% polyacrylamide gel electrophoresis, dried, and phosphorimaged.

Zebrafish embryos and microinjection of morpholinos. Fertilized eggs were obtained from adult AB strain zebrafish bred in our laboratory as described by Westerfield (1995). Embryos were raised at a water temperature of 27°C, water was changed daily, and embryos kept beyond 144 hpf were fed paramecia.

For microinjection newly fertilized eggs, collected at 20-min intervals, were injected with either zfahr2-MO or control-MO. Embryos were injected at the 1–2 cell stages with approximately 15 nl of the appropriate morpholino solution, resulting in about 13 ng of morpholino delivered to each embryo. Embryos were allowed to develop for approximately 2 h, after which unfertilized eggs or embryos damaged by injection were discarded. Viable embryos injected with zfahr2-MO were observed for fluorescence as an index of injection success, which demonstrated uniform distribution of the morpholino. Only zfahr2-MO injected embryos exhibiting fluorescence at 2 h post fertilization (hpf) were used.

Waterborne exposure of embryos to TCDD. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) of > 99% purity was obtained from Chemsys, Lenexa, KS and dissolved in 0.1% DMSO. From approximately 3–4 hpf, uninjected embryos, control-MO injected embryos, and zfahr2-MO injected embryos, respectively, were maintained in egg water (60 mg/L Instant Ocean Salts) containing either vehicle (0.1% DMSO) or TCDD (0.4 ng/ml, a concentration that produces toxicity in greater than 95% of the embryos). Embryos were statically exposed to either vehicle or TCDD for 1 h with gentle rocking in 5- or 20-ml glass scintillation vials, with no more than 6 embryos/ml of solution. Thereafter embryos were rinsed with egg water that was both vehicle and TCDD-free and transferred to either 1 well of a 24-well plate or to a 100-mm petri dish containing egg water.

Experimental design. Six treatment groups were used: vehicle uninjected, vehicle + Control-MO, vehicle + zfahr2-MO, TCDD uninjected, TCDD + Control-MO, and TCDD + zfahr2-MO. For all experiments n was defined as a set of embryos exposed to vehicle or TCDD in the same vial. These sets ranged from 1–50 embryos, depending on the experiment. For the assessment of edema, red blood cell (RBC) perfusion rate, RBC morphology, evaluation of lower jaw morphology, and measurement of body length embryos were exposed to TCDD or vehicle individually, and n = 1 is defined as a single embryo. For analysis of zfCYP1A protein expression, embryos were exposed in groups of 4, and n = 1 is defined as a group of 4 embryos. For mRNA time
course experiments, embryos were exposed in groups of 50 per vial and sets of 10 embryos were used for each time point analyzed. Each set of 10 embryos was used as a pool for RNA preparation. In this case, n = 1 refers to the pool of RNA from a single group of 10 embryos. Finally, to analyze swimbladder inflation, embryos were exposed to TCDD or vehicle in groups of 10, and n = 1 is defined as a single group of 10 embryos. Experiments were repeated with total n values ranging from 4–12, as indicated in the figure legends.

**zfCYP1A mRNA abundance.** RNA was isolated from pools of vehicle and TCDD-exposed embryos using a Qiagen RNasea Mini kit according to manufacturer’s instructions. cDNA was produced from 1 μg of each RNA sample using Superscript II (Invitrogen) and oligo (dT) primer in 20 μl, and the Light Cycler (Roche Applied Science, Indianapolis, IN) was used for quantitative real-time PCR. One μl of each cDNA sample was used for each PCR in the presence of SYBR Green according to manufacturers instructions. To confirm specific product formation, gel electrophoresis and thermal denaturation (melt curve analysis) were used. Primers used to amplify zfCYP1A and β-actin have been described previously (Andreasen et al., 2002a).

**Whole mount immunolocalization of zfCYP1A.** Tissue-specific expression of zfCYP1A in zebrafish embryos was determined using monoclonal antibody Mab1-12-3 (Park et al., 1986). This antibody was used previously to specifically detect zfCYP1A protein in the zebrafish embryo (Andreasen et al., 2002b) and in several other fish species (Guiney et al., 1997; Iwata and Stegeman, 2000; Schleizenger and Stegeman, 2000; Smolowitz et al., 1992; Stegeman et al., 1989, 1991; Van Veld et al., 1997). Immunohistochemistry was performed as described earlier (Andreasen et al., 2002b). Embryos were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS). After fixation embryos were washed 3x in PBS, dehydrated in a methanol series, and stored at –20°C overnight. For staining, embryos were rehydrated gradually into PBS and permeated by digestion in collagenase (1mg/ml) for 30–40 min. Permeabilized larvae were blocked in 10% normal calf serum in PBS with 0.1% Tween-20 (PBST) for 1 h before addition of Mab 1-12-3 (0.3 μg/ml). After overnight incubation with the antibody embryos were washed several times in PBST and incubated with a secondary antibody (Alexa-488 conjugated goat anti-mouse, Molecular Probes, Eugene, OR) for 5 h at room temperature. Embryos were washed 3x for 10 min in PBST and visualized by epifluorescence microscopy.

**Pericardial sac and yolk sac areas.** Embryos were mounted in 3% methylocellulose, observed using a Nikon Eclipse TE300 inverted microscope, and photographed using Universal Imaging Corporation Metamorph imaging software and a Princeton Instruments Micromax charge-coupled device (CCD) camera. To measure pericardial and yolk sac areas, lateral view images of each embryo were taken at the same magnification, outline of the pericardial sac and yolk sac, respectively, was traced, and the area within each tracing was determined by Scion Image for Windows available from Scion Corporation at http://www.scioncorp.com.

**Red blood cell (RBC) perfusion rate.** As an index of regional blood flow, RBC perfusion rate was measured in two readily standardized vessels: an intersegmental vein located in the posterior quarter of the trunk and the posterior cerebral vein of the head (Teraoka et al., 2002). Briefly, the number of RBCs passing through each vein in 10 s was determined by time-lapse recording using the same imaging system described for determination of pericardial and yolk sac area. To capture images fast enough, data was streamed directly to the RAM using a 1-ms exposure time, which allowed 10 frames/s to be captured. Perfusion of each vein with RBCs was measured for 10 s (100 frames). The stack of images was then converted to a movie using Metamorph imaging software. Each frame was played for 2–3 s so the number of RBCs flowing past a designated point in the vein could be counted. Nomenclature for veins was that of Isogai et al. (2001).

**Erythrocyte morphology.** Blood collection and RBC shape determination was performed as described previously (Belair et al., 2001). RBCs were collected by cardiac puncture of anesthetized zebrafish (4 mg/ml tricaine containing 1% bovine serum albumin in calcium and magnesium-free PBS, pH 7.4). Blood was collected directly on a glass slide, and RBCs were observed by DIC microscopy and photographed. The percentage of RBCs that were elliptical and round, respectively, was determined by two observers independently. At least 40 RBCs were counted per determination.

**Lower jaw morphology and body length.** Cartilage in the lower jaw was stained with alizarin blue using the protocol of Kelly and Bryden (1983) as modified by Neuhausser et al. (1996). Embryos were fixed overnight in 4% paraformaldehyde in PBS and then washed twice for 40 min in PBST. Pigmentation was reduced by bleaching for 2 h in 30% H2O2, after which embryos were washed for 40 min in PBST and transferred to a solution of 0.1% alizarin blue, 1% concentrated HCl, and 70% ethanol to stain overnight. Embryos were cleared in acidic ethanol (5% concentrated HCl, 70% ethanol) for 4 h, dehydrated in an ethanol series, and stored in glycerol. The ventral side of the cartilage-stained head of each embryo was photographed using a Nikon CoolPix 5000 digital camera attached to a Leica WILD M8 microscope. Photographs were then analyzed using the Scion image software to determine lower jaw length and width.

**Swimbladder inflation and other endpoints at late stages of development.** To assess swimbladder inflation, embryos were observed every 24 h beginning at 144 hpf and ending at 192 hpf using a Bausch and Lomb stereomicroscope (x20–x30). Each embryo was scored as having an inflated or uninflated swimbladder. Cumulative percentage of embryos with inflated swimbladders was determined for each treatment group. These same embryos were also observed to determine effects of the morpholino on endpoints of toxicity just prior to mortality. That is, when mortality was first detected in each TCDD treatment group, respectively, all remaining embryos in that group were observed for various endpoints of TCDD toxicity. Representative embryos exhibiting these endpoints were photographed, and all remaining embryos were euthanized with MS-222.

**Statistical analysis.** For all endpoints, except swimbladder inflation, significance was determined using a two-way ANOVA followed by the Fisher LSD test. Levene’s test for homogeneity of variances was performed before the ANOVA. Data sets which did not pass Levene’s test were transformed by log10 transformation (zfCYP1A mRNA abundance, pericardial edema, yolk sac edema) or square root transformation (RBC perfusion rate), and the transformed data was analyzed by two-way ANOVA. Swimbladder inflation data, assumed not to be normally distributed, was evaluated using the Kruskal-Wallis k-sample test followed by the Wilcoxon-Mann-Whitney test to analyze for significant differences among treatments. All statistical analyses were performed using Statistica 6.0 software package. Results are presented as mean ± SE; level of significance was p ≤ 0.05.

**RESULTS**

Antisense morpholino oligos are specific inhibitors of translation, which function by binding to complementary sequences on mRNA to block access by ribosomes. To confirm that the zfahr2-MO blocks translation of zfAHR2, an *in vitro* transcription and translation reaction was performed using zfahr2 cDNA (Fig. 1, lanes 1–3) or zfahr1 cDNA (lanes 4–6) as a template. When zfahr2 cDNA was used in the absence of morpholino, a 123 kDa zfahr2 protein was produced (Fig. 1, lane 1). Addition of control-MO to the reaction had no effect on translation of zfahr2 (Fig. 1, lane 2). However, addition of zfahr2-MO to the reaction caused a complete block in translation of zfahr2 protein, demonstrating effectiveness of the morpholino (Fig. 1, lane 3).

Although the zfahr1 and zfahr2 proteins share 58% amino acid identity in the N-terminal half, they share little sequence similarity in the region surrounding the AUG start site. Therefore it would be expected that zfahr2-MO would
zfahr2-MO specifically decreases levels of zfAHR2 without affecting those of zfAHR1. To ensure that zfahr2-MO specifically decreases levels of zfAHR2, zfAHR2 cDNA was transcribed and translated in vitro in the presence of [35S]-methionine. The reaction was performed either in the absence of morpholino (lane 1) or in the presence of control-MO (lane 2) or zfahr2-MO (lane 3). To determine specificity of the zfahr2-MO, zfAHR1 cDNA was transcribed and translated in the absence of morpholino (lane 4) or in the presence of control-MO (lane 5) or zfahr2-MO (lane 6). Both morpholinos were used at a final concentration of 500 nM. [35S]-labeled proteins were resolved on an 8% SDS polyacrylamide gel, and the dried gel was phosphorimagged.

FIG. 1. Effect of zfahr2-MO on in vitro translation of zfAHR2 and zfAHR1. To determine effectiveness of zfahr2-MO at blocking translation of zfAHR2, zfAHR2 cDNA was transcribed and translated in vitro in the presence of [35S]-methionine. The reaction was performed either in the absence of morpholino (lane 1) or in the presence of control-MO (lane 2) or zfahr2-MO (lane 3). To determine specificity of the zfahr2-MO, zfAHR1 cDNA was transcribed and translated in the absence of morpholino (lane 4) or in the presence of control-MO (lane 5) or zfahr2-MO (lane 6). Both morpholinos were used at a final concentration of 500 nM. [35S]-labeled proteins were resolved on an 8% SDS polyacrylamide gel, and the dried gel was phosphorimagged.

have no effect on translation of zfAHR1. To ensure that zfahr2-MO specifically decreases levels of zfAHR2 without affecting those of zfAHR1, the zfahr2-MO was added to an in vitro transcription and translation reaction containing zfAHR1 cDNA. In the absence of morpholino, the 97.5 kDa zfAHR1 protein was produced (Fig. 1, lane 4) and addition of control-MO to the reaction had no effect (Fig. 1, lane 5). Addition of the zfahr2-MO to the zfAHR1 translation reaction also had no effect on the production of zfAHR1 (Fig. 1, lane 6), demonstrating that zfahr2-MO is able to block translation of zfAHR2 specifically without affecting that of zfAHR1.

To determine if induction of zfCYP1A mRNA by TCDD in zebrafish embryos is mediated by zfAHR2, a time course study was conducted (Fig. 2). TCDD and vehicle-exposed embryos that were either uninjected or injected with a control-MO or zfahr2-MO were used. Quantitative real-time PCR for zfCYP1A mRNA was performed and its relative abundance determined in the whole embryo. At 24 hpf, embryos treated with TCDD showed about a 100-fold induction of zfCYP1A mRNA compared to vehicle-treated embryos. Injection of control-MO had no effect on induction in TCDD-treated embryos. However, embryos injected with zfahr2-MO and treated with TCDD failed to induce zfCYP1A mRNA to levels above those seen in vehicle-treated embryos, indicating that zfAHR2 mediates zfCYP1A induction in zebrafish.

Because morpholino oligos are effective only transiently, zfCYP1A mRNA abundance was determined at later times in development to determine how long zfahr2-MO would be effective at decreasing zfAHR2 signaling (Fig. 2). At 48, 72, and 96 hpf, TCDD caused a significant induction of zfCYP1A mRNA compared to vehicle control, with the largest induction observed at 72 hpf. Injection of control-MO had no effect on induction of zfCYP1A mRNA at any time. In contrast to what was observed at 24 hpf, induction of zfCYP1A mRNA did occur at 48, 72, and 96 hpf in TCDD-exposed embryos injected with zfahr2-MO. However, the magnitude of this induction was significantly reduced when the TCDD group was compared to the TCDD + zfahr2-MO group. At 72 hpf, a 400-fold induction was observed in the TCDD group compared to a 136-fold induction in the TCDD + zfahr2-MO group. We interpret these findings to mean that, at 24 hpf, zfahr2-MO was able to completely block expression of zfAHR2, because no induction of zfCYP1A was observed. However, by 48 hpf some zfAHR2 is present but at a reduced level, which remains decreased until 96 hpf.

Low constitutive expression of zfCYP1A mRNA (relative abundance of approximately 0.001) was detected in vehicle treated embryos at all times (Fig. 2). zfahr2-MO had no effect on constitutive zfCYP1A expression at any time, suggesting that zfAHR2 may not be involved in regulating constitutive levels of zfCYP1A message.

To determine if zfCYP1A induction throughout the entire embryo is mediated by zfAHR2, whole mount immunolocalization of zfCYP1A was performed using the Mab1-12-3 antibody. In Figure 3, results are shown for a representative embryo at 72 hpf in the vehicle group (top panels), TCDD group (middle panels), and TCDD + zfahr2-MO group (bottom panels). Panels on the left show zfCYP1A immunostaining in the trunk and on the right in the head. In vehicle-exposed embryos, very little staining was observed (Fig. 3, top panels).
Weak levels of zfCYP1A immunofluorescence were seen in the intersegmental vessels and caudal artery and vein in the trunk. No staining was observed in the head. In contrast, TCDD-exposed embryos show significant immunostaining at this time (Fig. 3). Area of the pericardial sac was used as a measure of pericardial edema. Significant increases in pericardial sac area are seen in TCDD-treated embryos at 96 hpf (Fig. 4A). Control-MO had no effect on pericardial edema formation after TCDD exposure. However, injection of zfahr2-MO prevented the accumulation of edema fluid in the pericardial sac, so pericardial sac area was similar to that seen in vehicle-exposed embryos.

Yolk sac area was measured at 120 hpf as an index of yolk sac edema, and at this time TCDD caused a significant increase in yolk sac area (Fig. 4B). Yolk sac area increases after TCDD exposure because of edema fluid accumulation in the yolk sac and also because TCDD decreases the absorption of yolk sac constituents. Injection of the control-MO had no effect on the TCDD-induced increase in yolk sac area. However, injection of the zfahr2-MO before TCDD exposure prevented yolk sac edema fluid accumulation and the decrease in yolk absorption, such that zfahr2 morphants treated with TCDD were indistinguishable from vehicle controls.

Photographs illustrating the protection that the zfahr2-MO affords against TCDD-induced edema can be seen in representative embryos at 120 hpf (Fig. 4C). The edema that occurs after TCDD exposure is illustrated for the pericardial sac (ps) and yolk sac (ys) of a TCDD embryo, but edema is not present in either the vehicle or TCDD + zfahr2-MO embryo. In fact, embryos injected with zfahr2-MO and treated with TCDD were observed through 240 hpf, and edema fluid never accumulated in the pericardial and yolk sacs. Also, injection of the zfahr2-MO in vehicle-treated embryos had no effect on pericardial or yolk sac areas.

One of the earliest endpoints of TCDD developmental toxicity observed in the zebrafish embryo is a reduction in blood flow. This is observed earliest in vessels of the trunk and later in the head. To determine if this reduction in blood flow is mediated by zfAHR2, RBC perfusion rate was determined in an intersegmental vein (isv) in the most posterior quarter of the trunk (Fig. 5A) and the posterior cerebral vein (PeCV) in the head (Fig. 5B). RBC perfusion rates in both vessels were evaluated at 72, 96, and 120 hpf, with results shown for 120 hpf (Fig. 5).
Blood flow was markedly reduced in the isv of TCDD-treated embryos at 72 and 96 hpf, with flow almost completely ceasing by 120 hpf (Fig. 5A). RBC perfusion of the PeCV was not significantly reduced in TCDD-treated embryos until 96 hpf and, as with the isv, had almost completely ceased by 120 hpf (Fig. 5B). Injection of the control-MO had no effect on the reduction in RBC perfusion rates caused by TCDD in either vessel. However, injection of the zfahr2-MO completely prevented the striking reduction in blood flow observed in TCDD-exposed embryos in both vessels at all times analyzed and caused perfusion rates to return to those seen in vehicle-treated embryos. Injection of the zfahr2-MO had no effect on RBC perfusion rates in vehicle-treated embryos.

We examined the lower jaw in zfahr2 morphants treated with TCDD to determine if the mechanism by which lower jaw growth is reduced is dependent on zfAHR2 signaling (Fig. 6). Embryos from each treatment group were evaluated at 96 hpf for total body length (Fig. 6A) and relative lower jaw length (Fig. 6B) and width (Fig. 6C). TCDD caused a small but significant decrease in body length of the no-morpholino and control-MO injected embryos (Fig. 6A). However, embryos injected withzfahr2-MO and treated with TCDD had no reduction in body length.

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FIG. 6. Effect of the zfahr2-MO on TCDD-induced reduction in body length and relative lower jaw growth in the zebrafish embryo. To obtain measurements of lower jaw length and width, embryos from each treatment group were fixed at 96 hpf, and alcian blue staining was performed. Body length (A), relative lower jaw length (B), and relative lower jaw width (C) were determined at 96 hpf. Also shown are representative photographs of the alcian blue staining patterns for cartilage observed at 96 hpf on the ventral side of the head after exposure to vehicle, TCDD, or TCDD + zfahr2-MO (D). The way in which lower jaw measurements were made is illustrated (vehicle inset). To determine jaw length, a straight line was drawn connecting the bottom edges of the hyosymplectic cartilage (dotted line, D); then a second line was drawn at a 90° angle from the dotted line to the most anterior edge of Meckel’s cartilage (solid single arrow, D). The distance of the second line is a measure of lower jaw length. To determine lower jaw width, a straight line was drawn connecting the posterior ends of the Meckel’s cartilage (double-sided arrow, D). Body length was determined as the distance from the tip of the snout to the junction point of the posterior trunk and caudal fin. Values are mean ± SE of n = 12. Cartilage abbreviations: mk, Meckel’s; ch, ceratohyal; hs, hyosymplectic cartilage. Bar = 100 μm. Other conditions as in Fig. 4 legend.

hpf, and this was also observed in control-MO injected embryos treated with TCDD (Fig. 6B). Injection of zfahr2-MO protected against the TCDD-induced reduction in relative lower jaw length such that jaw length in TCDD-treated zfahr2 morphant embryos was not significantly different from vehicle controls.

TCDD also caused a significant decrease in relative lower jaw width in no-morpholino and control-MO injected embryos treated with TCDD (Fig. 6C). Unlike what was observed for lower jaw length, the lower jaw width in zfahr2 morphants treated with TCDD was only slightly greater than in no-morpholino or control-MO injected embryos treated with TCDD and was still significantly decreased when compared to vehicle-treated embryos.

The protection afforded by the zfahr2-MO against TCDD-induced lower jaw malformations can be seen in representative embryos stained with alcian blue at 96 hpf (Fig. 6D). In the representative TCDD embryo, both the length and width of the Meckel’s cartilage is reduced, and orientation of the ceratohyal cartilages is changed so that a more obtuse angle is formed where the two sides of the cartilage meet. Injection of the zfahr2-MO provided some protection against these effects of TCDD. The two sides of the ceratohyal cartilage form a more acute angle at their juncture in TCDD-exposed zfahr2 morphants, and the length and width of Meckel’s cartilage is also greater when compared to the representative TCDD embryo. However, the width of Meckel’s cartilage in TCDD-exposed zfahr2 morphants was still decreased compared to vehicle-exposed embryos.

One of the later-occurring effects of TCDD in the zebrafish
embryo is anemia, which may be secondary to a block in the switch from primitive to definitive hematopoesis (Belair et al., 2001). These two phases of erythropoiesis can be distinguished because the primitive RBCs have a flattened circular morphology, and the definitive RBCs have an elliptical shape (Fig. 7C). Definitive RBCs never form in TCDD-treated embryos, resulting in anemia at 120 hpf (Belair et al., 2001). In the present study, morphology of the RBCs in zebrafish embryos was examined at 144 hpf, a time at which the switch from primitive to definitive erythropoiesis should have occurred and almost all RBCs should be elliptical (Fig. 7). As expected, approximately 90% of RBCs examined in vehicle-treated embryos from all groups were elliptical (Fig. 7B). However, no-morpholino and control-MO injected embryos treated with TCDD had approximately 90% round RBCs (Fig. 7A) and only 10% elliptical cells (Fig. 7B). In contrast, zfahr2 morphants treated with TCDD produced 90% RBCs with elliptical morphology (Fig. 7B) similar to vehicle-exposed embryos. Lastly, injection of the zfahr2-MO had no effect on the pattern of RBC morphology observed in vehicle-treated embryos.

Failure of the swimbladder to inflate after TCDD exposure is an effect of dioxin that is commonly observed in early life stages of several fish species. Embryos from each treatment group were examined for successful swimbladder inflation to determine if zfahr2-MO could protect against this late-occurring endpoint (Fig. 8A). Swimbladder inflation in vehicle-treated embryos began at approximately 120 hpf and continued until 192 hpf, at which time the percentage of embryos in each group with successful swimbladder inflation was determined. On average, 70–80% of vehicle-exposed embryos inflated their swimbladders. However, when embryos were treated with TCDD, 0% were able to inflate their swimbladders. All embryos injected with the control-MO and treated with TCDD also failed to inflate their swimbladders. Furthermore, unlike what had been observed with many other endpoints of TCDD developmental toxicity, injection of the zfahr2-MO did not protect against this TCDD effect.

Zebrafish embryos from all treatment groups were observed to determine the onset of mortality. No mortality was observed in any of the vehicle-exposed groups until 240 hpf, at which time a small increase in mortality (5%) occurred. Mortality in no-morpholino and control-MO TCDD-exposed embryos began at 192 hpf. In contrast, onset of mortality in zfahr2-MO TCDD-exposed embryos was delayed until 240 hpf. Photographs of a representative TCDD embryo at 192 hpf and a TCDD + zfahr2-MO embryo at 240 hpf demonstrate that the two types of embryos display very different endpoints of TCDD toxicity (Fig. 8B). The TCDD embryo exhibits extensive pericardial sac (a), yolk sac (b), and meningeal edema (c), has a severely shortened lower jaw (d), and lacks swimbladder inflation (e). The embryo also had a complete cessation of blood flow, and many embryos had hemorrhages. In striking contrast, the embryo injected with the zfahr2-MO had a normal swimbladder inflation, normal pericardial sac, and normal yolk sac.
contrast, the TCDD + zfahr2-MO embryo has only a shortened lower jaw and a lack of swimbladder inflation. Unlike what was observed in the TCDD embryo, no edema ever formed, no hemorrhages were observed, and only a reduction, not a complete cessation, of blood flow was seen.

**DISCUSSION**

**zfahr2 Morpholino**

We used an antisense morpholino approach to knock down expression of zfAHR2 to investigate its role in mediating TCDD developmental toxicity. Morpholino technology is the only viable, sequence-specific gene inactivation method available for use in zebrafish embryos. Morpholinos injected at the single-cell stage persist for at least the first 50 h of development; however, they progressively lose effectiveness due to inactivation and dilution (Ekker and Larson, 2001). The control-MO used in this study was the standard control morpholino sold by Gene Tools. While this provided a control for the injection and for the presence of a morpholino sequence in the embryo, it did not match the base composition of the zfahr2-MO used. Therefore it was not a control for specificity of the zfahr2-MO. In two different studies, however, we used a 4-base mismatch of the zfahr2-MO as a more stringent control, and similar results were found as with the standard control-MO used here (Dong et al., submitted; Teraoka et al., 2003).

In vehicle-exposed embryos, the zfahr2-MO used at this concentration did not cause toxicity or a mistargeting phenotype. Since zfAHR2 mRNA expression in zebrafish embryos is very low (Tanguay et al., 1999), the zfahr2-MO was expected to block translation of zfAHR2 on most mRNA targets. However, since there is no antibody available for immunolocalization of zfAHR2 in zebrafish, the extent to which zfahr2-MO decreased zfAHR2 abundance in embryos was not able to be determined. Instead, we monitored the effect of the zfahr2-MO on zfAHR2 signaling by assessing expression of zfCYP1A mRNA. At 24 hpf, a complete block in induction of zfCYP1A mRNA by TCDD was found in zfahr2 morphants, demonstrating that zfAHR2 mediates TCDD induction of zfCYP1A.

However, between 48 and 96 hpf, there was some zfCYP1A induction in zfahr2 morphants treated with TCDD. We interpret this to mean that effectiveness of zfahr2-MO in blocking zfAHR2 translation was declining and there was now sufficient zfAHR2 to mediate zfCYP1A induction. An alternative possibility is that zfAHR1 mediates zfCYP1A induction at later time points. We think this is unlikely in view of our in vitro results with zfAHR1 and the fact that there is no dramatic increase in zfAHR1 mRNA expression during this time in either vehicle- or TCDD-exposed embryos (Andreasen et al., 2002a). Therefore, later TCDD induction of zfCYP1A is not likely to be due to increased zfAHR1 expression. Taken together, the zfCYP1A results demonstrate that the zfahr2-MO decreased zfAHR2 signaling until 96 hpf. This is a critical developmental period for the manifestation of TCDD toxicity.
Developmental Cardiovascular Toxicity

The cardiovascular system is a primary target of TCDD developmental toxicity. In zebrafish embryos, zfAHR2 and zfARNT2b mRNAs colocalize in the heart and developing vasculature (Andreasen et al., 2002b), and in zebrafish and lake trout larvae the vasculature is one of the first sites of CYP1A induction after TCDD exposure (Andreasen et al., 2002b; Guiney et al., 1997). These results suggest that activation of zfAHR2 by TCDD in the cardiovascular system may lead to embryo toxicity, and results of the present study support this hypothesis. Immunohistochemical staining for zfCYP1A protein in zfahr2 morphants demonstrated that zfahr2-MO decreased zfCYP1A induction by TCDD in both the heart and vasculature.

A decrease in RBC perfusion rate is one of the earliest effects seen in TCDD-exposed zebrafish embryos. Decreases in blood flow can be observed at approximately 72 hpf in the trunk and later in vessels of the head and gills (Belair et al., 2001; Dong et al., 2001, 2002; Henry et al., 1997; Teraoka et al., 2002). By 120 hpf, heart rate is reduced, and blood flow appears to cease throughout the embryo. A major finding was that RBC perfusion rates in TCDD-treated zfahr2 morphants were the same as vehicle-treated embryos. This indicates that the inhibitory effect of TCDD on blood flow is mediated by zfAHR2.

At later stages of development, loss of RBCs due to hemorrhage and anemia also contributes to the decrease in RBC perfusion rates caused by TCDD (Belair et al., 2001; Henry et al., 1997). Belair et al. (2001) suggested that anemia may be caused by TCDD blocking the switch from primitive to definitive hematopoiesis, which normally occurs from 48 to 120 hpf, with primitive round RBCs declining in numbers and being replaced by elliptical definitive-phase adult cells (Amatruda and Zon, 1999). Zebrafish embryos exposed to TCDD are unable to replace the primitive round RBCs with definitive elliptical RBCs and therefore become anemic. At 144 hpf, zfahr2 morphants treated with TCDD were not anemic and had approximately 90% elliptical RBCs, indicating that they can undergo the switch from primitive to definitive hematopoiesis. Thus, zfAHR2 signaling is required for hematopoiesis to be disrupted by TCDD.

Edema

Edema is a hallmark endpoint of TCDD developmental toxicity in fish, birds, and mammals (Peterson et al., 1993). In TCDD-exposed zebrafish and lake trout embryos high levels of CYP1A are expressed in the vascular endothelium preceding the onset of edema (Andreasen et al., 2002b; Guiney et al., 2000), and in lake trout similar TCDD dose response curves are observed for both CYP1A protein induction in the vascular endothelium and larval mortality (Guiney et al., 1997). It has also been shown in lake trout that the edema fluid is an ultrafiltrate of blood (Guiney et al., 2000). These results suggest that activation of the AHR pathway in endothelial cells may increase vascular permeability leading to edema. Alternatively, TCDD may initially decrease cardiac output, leading to a reduction in renal blood flow. This could impair osmoregulatory function of the kidneys, with the decrease in glomerular filtration leading to an increase in blood volume and edema formation. A final hypothesis is that TCDD may disrupt development of other osmoregulatory organs such as the gills or skin, or the edema could be secondary to impaired development of the heart. zfAHR2 and zfARNT2b mRNAs colocalize in all of these organs (Andreasen et al., 2002b), but there is as yet no evidence that kidney, gill, or skin osmoregulatory function is disrupted by TCDD. Whatever the mechanism of TCDD-induced edema, it is clear that zfAHR2 is required, because zfahr2 morphants are completely protected against this effect of dioxin.

Critical Window for Anemia and Edema

Knocking down zfAHR2 expression allowed us to determine what endpoints of TCDD toxicity are zfAHR2-dependent and which ones have a critical window of exposure between 0 and 96 hpf, the period of morpholino effectiveness. Since treatment with the zfahr2-MO decreases zfAHR2 levels only transiently and TCDD persists in the embryo throughout development, the zfAHR2 pathway will be activated by TCDD once the morpholino has been eliminated. Therefore, any endpoint of TCDD developmental toxicity that is permanently blocked by the zfahr2-MO must involve a developmental process that is completed before zfAHR2 signaling returns to the embryo. Belair et al. (2001) suggest that TCDD produces ischemia, edema, and anemia by disrupting critical developmental processes that are completed before 96 hpf. Results of the present study support this interpretation. TCDD-exposed zfahr2 morphants evaluated at 168–240 hpf do not exhibit edema and have only a slight reduction in blood flow. Therefore, it is likely that a transient developmental event, occurring during the window ofmorpholino effectiveness, is being disrupted by TCDD, leading to edema and the profound reduction in blood flow.

Jaw Malformation

Effects of TCDD on jaw development have been documented in rainbow trout (Hornung et al., 1999), zebrafish (Henry et al., 1997; Teraoka et al., 2002), and several other fish species (Tanguay et al., 2003; Walker and Peterson, 1994). In zebrafish the primary effect appears to be inhibition of chondrogenesis. Cartilage components of the lower jaw are shortened, and their orientation is altered by TCDD (Henry et al., 1997; Teraoka et al., 2002). TCDD does not affect generation of the components but inhibits their growth. This effect has been dissociated from a reduction in blood flow to the lower jaw (Teraoka et al., 2002). zfAHR2 and zfARNT2b mRNAs colocalize in the lower jaw, and high levels of zfCYP1A induction are observed in this tissue after TCDD exposure.
Suggested roles for the AHR in a number of normal development. However, only a few roles appear to have any developmental defects. It is possible that zfAHR2 may play in normal development. Since TCDD appears to inhibit cartilage growth and not formation, a critical period of TCDD exposure in order to cause the jaw malformation is not apparent. Beginning TCDD exposure at any time from 0 to 84 hpf decreased jaw length at 96 hpf (Teraoka et al., 2002). We also observed that, while protection against the TCDD-induced decrease in lower jaw growth by the zfahr2-MO was observed at 96 hpf, it had subsided by 240 hpf. These results support the hypothesis that TCDD impairs lower jaw growth by activation of zfAHR2. Because there is continuous growth of the lower jaw, activation of the AHR2 pathway at any time can disrupt this growth, leading to a shortened lower jaw.

Impaired Swimbladder Inflation and Mortality

In TCDD-exposed zebrafish embryos the swimbladder forms but never inflates with air (Henry et al., 1997). zfahr2 morphants were not able to inflate their swimbladders after TCDD exposure. Although it is possible that zfAHR2 is not mediating this effect of TCDD, it seems more likely, because this endpoint occurs later in development, that the morpholino was simply not effective in decreasing zfAHR2 expression at this time.

While zfahr2 morphants were not protected against mortality caused by TCDD, they were completely protected against pericardial and yolk sac edema. Since they did not exhibit edema or profound circulatory failure, their cause of death is unclear. Because they fail to inflate their swimbladder and have craniofacial malformations, it is possible that they have difficulty feeding, which may lead to mortality.

Role of zfAHR2 in Normal Development

Comparison of Ahr−/− null and wild-type mouse lines have suggested roles for the AHR in a number of normal developmental processes such as hepatic growth and development (Fernandez-Salgueiro et al., 1996; Schmidt et al., 1996), peripheral immune system function (Fernandez-Salgueiro et al., 1996), cardiac development (Fernandez-Salgueiro et al., 1996; Thackaberry et al., 2002), and vascular remodeling (Lahvis et al., 2000). The present study does not provide any insight into the physiological role that zfAHR2 may play in normal zebrafish development, because zfahr2 morphants did not appear to have any developmental defects. It is possible that zfAHR2 is not essential for normal development. However, only a transient knockdown of zfAHR2 was evaluated, and although zfAHR2 levels were decreased in zfahr2 morphants, there may still have been enough present to carry out its physiological functions. A second possibility is that there is functional redundancy between zfAHR2 and zfAHR1. By decreasing levels of only zfAHR2, zfAHR1 may be able to compensate and carry out the normal function. It has been hypothesized that the multiple functions of the mammalian AHR may have been partitioned between the two forms of AHR in fish (Hahn, 2002). One of the fish AHRs may have maintained the ability to bind exogenous ligands and upregulate xenobiotic metabolizing enzymes while the other form retained the ability to carry out the physiological functions of AHR. The results of this study clearly demonstrate that zfAHR2 has retained the ability to mediate responses to exogenous ligands, although the mechanism by which activation of this pathway by TCDD causes toxicity is still unclear. Whether or not zfAHR1 has retained other functions involved in normal development remains to be determined.

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