Blocking Expression of AHR2 and ARNT1 in Zebrafish Larvae Protects Against Cardiac Toxicity of 2,3,7,8-Tetrachlorodibenzo-p-dioxin

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The zebrafish (Danio rerio) has become an attractive vertebrate model for studying developmental processes, and is emerging as a model system for studying the mechanisms by which xenobiotic compounds perturb normal development. Embryos treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) shortly after fertilization exhibit a range of adverse effects on the heart: an early reduction in cardiac myocyte number, followed by a change in heart looping and morphology, with an apparent compaction of the ventricle and overall decrease in heart size. These changes are accompanied by impaired cardiac function including a decrease in cardiac output and eventually irreversible ventricular standstill. The mechanisms involved in mediating effects of TCDD on the heart remain unknown. However, it is widely accepted that aryl hydrocarbon receptor (AHR) activation mediates endpoints of TCDD toxicity in vertebrates. In zebrafish, there are multiple forms of AHR and AHR nuclear translocator protein (ARNT) raising the question about whether different endpoints of TCDD toxicity are mediated by different components of the AHR/ARNT pathway. To address this question we used morpholino oligonucleotide technology to specifically block the expression of zfAHR2, zfARNT1, zfARNT2, and zFCYP1A, and assessed the previously described effects of TCDD on heart morphology, size, and function in the developing morphants. We report that blocking zfAHR2 and zfARNT1 expression provided protection against the TCDD-mediated alteration in heart morphology, reduced cardiac myocyte number, decreased cardiac output and ventricular standstill in zebrafish larvae, while the zfARNT2 and zFCYP1A morpholinos did not block the TCDD-induced cardiac toxicity.

Key Words: 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCDD; zebrafish; embryo; heart; cardiovascular; Ah receptor; AHR2; ARNT1; CYP1A; antisense morpholino.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a persistent, bioaccumulative environmental contaminant, as well as a potent developmental toxicant and human carcinogen (Cole et al., 2003). In piscine, avian, and mammalian species cardiovascular system is sensitive to TCDD toxicity. For example, in monkeys TCDD treatment results in ventricular dilation, cardiac enlargement, edema, hemorrhage, and an increase in extracellular matrix proteins in heart tissue (Riecke et al., 2002). In mice, in utero exposure to TCDD reduces heart-to-body weight ratio and cardiocyte proliferation during gestation, followed by postnatal cardiac hypertrophy (Thackaberry et al., 2005). Similarly, in chick embryos in ovo TCDD exposure results in ventricular hypertrophy and decreased cardiomyocyte proliferation, along with increased free intracellular calcium in cardiac myocytes and reduced contractility (Canga et al., 1993; Heid et al., 2001; Walker and Catron, 2000). TCDD also decreased heart size and caused cardiac dysfunction in early life stage rainbow trout and zebrafish (Antkiewicz et al., 2005; Hornung et al., 1999). An early effect of dioxin in zebrafish embryos treated shortly post fertilization is a reduction in cardiomyocyte number at 48 h post fertilization (hpf). This is followed by decreased heart size, altered cardiac looping with a lengthened tubular appearance, as well as altered vascular remodeling, pericardial edema, and decreased ventricular contraction culminating in ventricular standstill (Antkiewicz et al., 2005; Belair et al., 2001; Bello et al., 2004).

The exact mechanisms by which TCDD exerts its adverse effects remain largely unidentified. However, TCDD is a known agonist for aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor that translocates from the cytoplasm to the nucleus upon ligand binding, where it dimerizes with the AHR nuclear translocator protein (ARNT). The AHR/ARNT heterodimer then binds to a characteristic DNA sequence termed the dioxin response element (DRE) to regulate transcription of downstream genes (Schmidt and Bradfield, 1996; Tanguay et al., 2003). Among the known AHR/ARNT target genes, cytochrome P450 1A (CYP1A) is the best characterized, and induction of cyp1a expression has been used as a marker of AHR pathway activation (Nebert et al., 1990; Tanguay et al., 1999). However, studies in zebrafish larvae showed that CYP1A knockdown with a zfCYP1A morpholino afforded no protection from TCDD toxicity (Carney et al., 2004; Prasch et al., 2004). Regardless of the nature of its downstream target genes, it is widely accepted that the AHR/ARNT pathway mediates...
TCDD toxicity. This is mostly due to protection from TCDD toxicity provided by knocking out AHR and ARNT proteins. This has been observed in experiments with Ahr<sup>−/−</sup> null mouse lines (reviewed in Mimura and Fujii-Kuriyama, 2003), as well as in zebrafish in which AHR or ARNT levels have been knocked down by morpholino oligonucleotide (MO) injection (Prasch et al., 2003, 2006).

While mammals have only one form of AHR, zebrafish possess at least three forms: zfAHR1A, zfAHR1B, and zfAHR2 (Andreasen et al., 2002; Karchner et al., 2005; Tanguay et al., 1999). ZfAHR2 and zfAHR1B bind TCDD with high affinity and induce DRE-driven transcription, but zfAHR1A does not (Karchner et al., 2005; Tanguay et al., 1999, 2000). Similarly, zebrafish have multiple ARNT isoforms, including splice variants of zfARNT1 and zfARNT2 (Prasch et al., 2006; Tanguay et al., 2000).

Studies in zebrafish embryos indicate that zfAHR2 and zfARNT1 are important isoforms for producing TCDD effects; morpholino knockdown of either zfAHR2 or zfARNT1 blocks overt endpoints of dioxin toxicity including reduced blood flow, craniofacial malformation, and edema (Prasch et al., 2003, 2006). ARNT1 is involved in mediating TCDD toxicity in mammals as well. Transgenic mice expressing decreased levels of ARNT1 exhibit protection against several hallmark endpoints of dioxin toxicity (Wallisser et al., 2004). While zfARNT2 had previously been thought to be the important dimerization partner for zfAHR2 (Tanguay et al., 2000), TCDD toxicity was unaffected in experiments using zfAHR2 morpholinos or zfARNT2<sub>−/−</sub> mutants (Prasch et al., 2004).

While it is clear that blockade of zfAHR2 and zfARNT1 are sufficient to rescue zebrafish embryos from many endpoints of TCDD toxicity, it is not known whether blockade of these isoforms is sufficient to rescue all of the effects of TCDD. This is especially true for cardiotoxicity, where TCDD produces a range of defects including changes in morphology, cell number, and function, which were not examined in the initial experiments using the zfahr<sub>2</sub>- and zfarnt<sub>1</sub>-MOs. Furthermore, zfARNT2 appears to be functional as an AHR-binding partner in vitro, making it possible that this ARNT isoform might play a role in TCDD toxicity that has not been noticed. Due to the importance of the cardiovascular system as a target of TCDD toxicity in embryonic zebrafish, we tested the hypothesis that zfAHR2 and zfARNT1 are essential for TCDD-induced cardiac toxicity in zebrafish larvae. Morpholino experiments show that knockdown of zfAHR2 or zfARNT1 offers protection from dioxin-induced cardiotoxicity but knockdown of zfARNT2 or zfCYP1A does not.

**MATERIALS AND METHODS**

**Zebrafish lines and embryos.** A cmhc2:dsRed2-nuc line of transgenic zebrafish that expresses red fluorescent protein (RFP) specifically in the cardiomyocyte nuclei (a generous gift from Dr Geoffrey Burns) was used to determine the number of cardiac myocytes. Albino embryos (AB background) were used to obtain an unobstructed ventral view of heart morphology. The wild type AB strain of zebrafish was used for all other experiments. Pooled embryos from group matings were used in all experiments to control for variability between different clutches of embryos. All fish were bred and embryos were raised according to the procedures described by Westerfield (1995). When applicable, fish were anesthetized with 1.67 mg/ml tricaine (MS222, Sigma).

**Morpholinos and microinjection.** All morpholinos were obtained from Gene Tools (Philomath, OR) and used as previously reported (Carney et al., 2004; Prasch et al., 2003, 2004, 2006). The morpholinos were designed to block the translation of all protein isoforms of the corresponding gene. The morpholino sequences used were as follows:

- zfahr<sub>2</sub>-MO—5’ GTACCAGATCCCTCTCATGTT 3’
- zfahr<sub>1</sub>-MO—5’ GGATCTGCTATGTCATGCGCCA 3’
- zfart<sub>2</sub>-MO—5’ AGCGGCTTGGTGTGCCATATGCGCT 3’
- zfahr<sub>2</sub>-MO—5’ TGGGATACCCAGTTCAGGCT 3’

All morpholinos were fluorescent tagged at the 3’ end to assess injection success. The Gene Tools standard control morpholino (5’ CTCTTCACCT-CAGTTACAATTTA 3’) was used as a control (control-MO [CMO]). The microinjection (Narishige IM300 Microinjector; Tokyo, Japan) of newly fertilized eggs from AB strain zebrafish was performed at the 1–2 cell stages with approximately 2 nl of the appropriate morpholino solution (0.15mM CMO, 0.1mM zfahr<sub>2</sub>-MO, 0.12mM zfahr<sub>1</sub>-MO, 0.15mM zfart<sub>2</sub>-MO, 0.15mM zfcyp1a-MO). 1X Danio’s solution (58mM NaCl, 0.7mM KCl, 0.4mM MgSO<sub>4</sub>, 0.6mM Ca(NO<sub>3</sub>)<sub>2</sub>, 5mM N-(2-hydroxyethyl)-piperazine-N’-2-ethanesulfonic acid, pH 7.6) was used to dilute all morpholino stocks to the concentration appropriate for injection. The morpholino solution was injected approximately at the border between the cell and yolk, with a needle positioned such that it was piercing the cell. Embryos were screened under fluorescent light to determine injection success and even distribution of morpholino within the embryo at approximately 2 h postinjection.

**TCDD exposure.** TCDD of >99% purity from Chemsyn (Lenexa, KS) was dissolved in dimethyl sulfoxide (DMSO) for preparation of dosing solutions. All embryos were treated shortly following MO injection (approximately 4–6 hpf) by 1-h static waterborne exposure to either 0.1% DMSO (vehicle) or 0.4 ng/ml (ppb) TCDD in glass scintillation vials with rocking. The number of embryos per 1 ml of dosing solution never exceeded 10. After the 1-h exposure, embryos were transferred to TCDD-free, fresh fish water (60 mg/l Instant Ocean Salts; Aquarium Systems, OH).

**Imaging.** Live embryos were mounted in 3% methyl cellulose. Ventral view images were obtained with an Optronics MicroFire camera mounted on a Leica MZ16 stereomicroscope. For all other imaging we used a Nikon TE300 inverted microscope with a Princeton Instruments Micromax charge-coupled device camera or a high-speed Motion Scope camera (Redlake, CA).

**SV-BA distance.** Changes in heart morphology caused by TCDD exposure were quantified at 72 and 96 hpf by measuring the distance between the sinus venosus (SV) and bulbus arteriosus (BA) regions of the heart in lateral view images, as described previously (Antkiewicz et al., 2005). The MetaMorph Imaging System (Universal Imaging Corporation) was used to determine the length of a straight line connecting the two structures on acquired images.

**Cardiac myocyte count.** Cardiac myocytes were counted at 72 hpf in TCDD- and vehicle-treated cmhc2:dsRed2-nuc transgenic zebrafish (Mably et al., 2003) using the method described by Antkiewicz et al. (2005). Briefly, embryos were anesthetized and flat-mounted on a glass microscope slide under a coverslip in Lebovitz’s L15 cell culture media (Invitrogen, CA, formerly Gibco). Epifluorescence images were captured and nuclei were counted to yield the cell number.

**Incidence of ventricular standstill.** Zebrafish larvae were assessed for incidence of ventricular standstill at 96 hpf as described previously (Antkiewicz et al., 2005). All larvae demonstrating a persistent lack of visible ventricular contraction at 96 hpf were scored as exhibiting the ventricular standstill. At least three separate experiments were performed (n ≥ 6 larve per each MO).
injection per experiment), and the average percentage of larvae exhibiting ventricular standstill for each injection and treatment group was calculated.

**Cardiac function.** End-diastolic volume (EDV) and end-systolic volume (ESV), as well as stroke volume and cardiac output were determined at 60 hpf as described previously, from time-lapse recordings (250 frames/s) of a lateral view of the beating larval zebrafish heart (Carney et al., 2006). Frames representative of the ventricle in end-systole and end-diastole were used to calculate volume of the ventricle for both states according to the method of discs (Coucelo and Joaquim, 2000; Schiller et al., 1989). This method assumes that ventricular volume can be represented as the sum of the volumes of individual discs into which the ventricle can be sliced. The total ventricular volume can therefore be represented by the following equation:

\[ V = \sum_{i=1}^{n} A_i \times T, \]

where \( A \) is the planimetered area and \( T \) the disc thickness. The two-dimensional image of the ventricle at end-diastole and end-systole was divided into slices of 10 \( \mu \)m thickness as previously described (Carney et al., 2006). Time-lapse recordings of the heart were also used to calculate heart rate (HR), which was determined from the number of frames in which three consecutive heart contractions occurred. Stroke volume (SV) was calculated from the approximated EDV and ESV:

\[ SV = \frac{EDV}{C255} - ESV \]

Cardiac output (CO) was calculated from SV and HR:

\[ CO = SV \times HR \]

**Statistics.** One-way or factorial analysis of variance followed by the Fisher least significant difference test were used to determine statistical significance. The assumption of unequal variances for all data sets was checked using Levene’s test. Results are presented as mean ± standard error of the mean, and asterisks indicate a significant difference between TCDD and the representative control (DMSO), unless indicated otherwise. Level of significance was \( p \leq 0.05 \) for all comparisons. All analyses were performed using the Statistica 7.0 software package. In all cases, the observer was blinded to the treatment group until measurements or until scoring was completed.

## RESULTS

**Blocking zfAHR2 and zfARNT1 Expression Protects Zebrafish Larvae against TCDD-Induced Morphology Defects**

We have used an experimental system designed to mimic the route of exposure to TCDD and other lipophilic contaminants observed in the environment, where the biggest source of exposure for fish is maternal transfer via the egg yolk (Cook et al., 1991; Monteverdi and Di Giulio, 2000). In our experiments, newly fertilized eggs were exposed to TCDD for 1 h, rinsed, and left to develop in fresh water. Since the developing zebrafish have no way of clearing the lipophilic TCDD, this produces a continuous exposure over several days (Heiden et al., 2005). The dioxin-treated embryos develop normally for approximately 48 h, undergoing normal pattern formation and organogenesis, with normal heart development and looping, as well as normal blood circulation. However, cardiac morphology becomes altered between 48 and 72 hpf. The heart becomes elongated and tube like, and the previously looped heart assumes new shape such that the atrium and ventricle are no longer in a side-by-side orientation (Antkiewicz et al., 2005). This response to dioxin is even more evident at 96 hpf as shown in Figure 1. In the vehicle control larvae (panels a, c, e, g, and i), the ventricle and atrium lie side by side when viewed from the ventral perspective. The atrium is rounded and looped close to the ventricle. This normal appearance was observed for

![FIG. 1. Morpholino knockdown of AHR pathway components protects zebrafish larvae from TCDD-induced alterations in heart morphology. Albino zebrafish embryos at 1 cell stage were injected with the indicated MOs, and subsequently exposed to TCDD as described in the Methods. Representative photographs of the embryo heart at 96 hpf are shown in ventral view, with the heart chambers outlined in red. Larvae injected with control MO are shown in panels a and b, zfahr2-MO in c and d, zfarn1-MO in e and f, zfarn2-MO in g and h, zfcp1a-MO in i and j (DMSO vehicle control and TCDD, respectively). Abbreviations: (A) Atrium, (V) Ventricle. Scale bar = 100 \( \mu \)m.](attachment:zebrafish_morphology.png)
all MO-injected embryos exposed to DMSO vehicle. In contrast, exposure to TCDD produced an elongated heart morphology in which the atrium appeared stretched out, and was no longer lying looped beside the ventricle. This previously characterized response was plainly evident in the embryos receiving the CMO (Fig. 1b) and in uninjected embryos (not shown). Injection of the zfahr2- or zfarnt1-MOs offered protection from TCDD cardiac toxicity as seen in panels d and f of Figure 1. In contrast, the zfarnt2 or zfcyp1a MOs (panels h and j) were no more effective than the CMO in protecting the embryos from heart malformation.

In previous experiments, the efficacy of the zfarnt1-MO has been shown to diminish more rapidly over time than that of the zfahr2-MO (Carney et al., 2004; Prasch et al., 2003, 2006). Consistent with this, we observed weaker protection with the zfarnt1-MO than with the zfahr2-MO at time points after 72 hpf. While there was an obvious difference between the protection afforded by the zfahr2- and zfarnt1-MOs and the lack of protection produced by the other MOs, there is some evidence that by 96 hpf, neither the zfahr2-MO nor the zfarnt1-MOs completely blocked changes in morphology produced by TCDD (see Fig. 1, panel d).

TCDD also induces a morphological change that resembles stretching of the heart. A method for quantifying this effect has previously been reported in which the distance between the inflow (SV) and outflow tracts (BA) of the heart is measured from lateral view images (Antkiewicz et al., 2005). We used this to test the ability of the MOs to protect against this effect of TCDD. At 72 hpf, both the zfahr2-MO and the zfarnt1-MO provided complete protection from the TCDD-induced increase in BA-SV distance, but by 96 hpf the protection afforded by the zfarnt1-MO was reduced (Fig. 2). This is consistent with a shorter duration of efficacy for the zfarnt1-MO. Nonetheless, both morpholinos produced a significantly less severe response to TCDD compared to the uninjected or CMO controls (Fig. 2B). No protection against this endpoint of TCDD cardiac toxicity was provided by either zfcyp1a-MO or zfarnt2-MO at both 72 and 96 hpf (Fig. 2).

Blocking zfAHR2 and zfARNT1 Protects Zebrafish Larvae from TCDD-Induced Reduction in Cardiac Myocyte Number

One of the earliest signs of TCDD toxicity in developing zebrafish is a reduction in cardiomyocyte number (Antkiewicz et al., 2005; Carney et al., 2006). This can be assessed using a cmlc2::dsRed2-nuc line of transgenic zebrafish, which expresses Discosoma RFP specifically in the nuclei of cardiac myocytes, allowing these to be counted (Antkiewicz et al., 2005; Mably et al., 2003). The zfahr2- and zfarnt1-MOs prevented the TCDD-mediated decrease in cardiac myocyte number at 72 hpf. In contrast, un.injected, CMO, zfcyp1a-MO, and zfarnt2-MO–injected embryos were not protected against this toxic effect of TCDD (Fig. 3).

FIG. 2. Zfahr2-MO and zfarnt1-MO protect zebrafish larvae from a TCDD-induced increase in SV-BA distance. Morpholino-injected embryos treated with TCDD or vehicle as described for Figure 1 were photographed, and the distance between the SV and BA was measured using MetaMorph software as described in the Methods. (A) SV-BA distance in zebrafish larvae hearts at 72 hpf. (B) SV-BA distance at 96 hpf. Values are mean ± standard error for n ≥ 8. The asterisk indicates a significant difference between TCDD and DMSO treatment. A filled diamond indicates a significant difference in response to TCDD between the test MO and the CMO. The level of significance for all comparisons was p < 0.05.

Blocking zfAHR2 and zfARNT1 Protects Zebrafish Larvae from TCDD-Induced Changes in Heart Function

One sign of impaired cardiac performance in TCDD treated zebrafish larvae is ventricular standstill (Antkiewicz et al., 2005). This is manifested as a heart with a beating atrium and complete absence of visible ventricular contraction. It is not clear whether this represents atrioventricular conduction block, the inability of the ventricular muscle to contract, or the inability to relax. The incidence of TCDD-induced ventricular standstill is approximately 0% at 72 hpf, 50% at 96 hpf, and 75% at 120 hpf. Thus, the time frame for observing this form of TCDD cardiotoxicity is at the limit of MO knockdown effectiveness, as these MOs have been determined to be effective for 72–96 hpf (Carney et al., 2004; Prasch et al., 2003, 2004, 2006), while the ventricular standstill is best observed at 120 hpf. However, we found that the approximately 50% incidence of ventricular standstill induced by TCDD at 96 hpf was sufficiently reliable to reveal an effect of the zfahr2- and zfarnt1-MOs (Fig. 4). Injection of the zfahr2-MO and zfarnt1-MO resulted in
significant protection against ventricular standstill, while the CMO, \textit{zfarn2}-MO, and \textit{zfcyp1a}-MO had no effect on the frequency of TCDD-induced ventricular standstill at 96 hpf. Moreover, this effect of TCDD was never observed in any of the DMSO-treated larvae. Therefore, the DMSO vehicle control values have not been plotted in Figure 4.

Carney et al. (2006) used high-speed video microscopy to show that TCDD reduces cardiac output. Stroke volume was estimated by assessing ventricular EDV and ESV along with heart rate from two-dimensional lateral view imaging of the beating heart. We used this method to calculate stroke volume and cardiac output in vehicle control and TCDD-exposed embryos injected with CMO, \textit{zfahr2}-MO, \textit{zfarn1}-MO, \textit{zfarn2}-MO, \textit{zfcyp1a}-MO, as well as in un.injected embryos (Fig. 5). In order to optimize use of the limited effective life of the morpholinos, we used the earliest time point at which we could see a reproducible effect of TCDD. We found that TCDD treatment resulted in significantly decreased stroke volume and

![FIG. 3. \textit{zfahr2}-MO and \textit{zfarn1}-MO protect zebrafish larvae from a TCDD-induced decrease in cardiac myocyte number. Transgenic \textit{cmlc2::dsRed2-nuc} zebrafish embryos expressing RFP specifically in cardiomyocyte nuclei were injected with the indicated MOs and subsequently treated with TCDD (0.4 ppb) or vehicle (0.1% DMSO) shortly after fertilization as described in the legend to Figure 1. Cardiac myocytes were counted at 72 hpf as described in the Methods. Epifluorescent images were captured and cells were counted in at least four hearts per treatment group. Values represent the mean ± standard error (n ≥ 4). An asterisk indicates a significant effect of TCDD compared to vehicle control.](image1)

![FIG. 4. \textit{zfahr2}-MO and \textit{zfarn1}-MO provide protection against a TCDD-induced ventricular standstill. Zebrafish embryos were injected with MOs as indicated, and treated with TCDD (0.4 ppb) or vehicle (0.1% DMSO) as described for Figure 1. Cardiac myocytes were assessed as described in the Methods. Data are shown for TCDD treated larvae only; ventricular standstill was not observed in DMSO control larvae. Values are mean ± standard error for three separate experiments (n = 5 larvae per experiment). An asterisk indicates a significant difference between a test morpholino group and the control morpholino (p < 0.05).](image2)

![FIG. 5. \textit{zfahr2}-MO and \textit{zfarn1}-MO protect zebrafish larvae from a TCDD-induced decrease in stroke volume and cardiac output. Zebrafish embryos were injected with MOs as indicated, and treated with TCDD (0.4 ppb) or vehicle (0.1% DMSO) as described for Figure 1. Time-lapse recordings were used to follow ventricular movement from a lateral view in embryos at 60 hpf. (A) Stroke volume was calculated as described in the Methods by estimating ESV and EDV. (B) Heart rate was calculated from the number of frames captured during three consecutive, complete ventricular contractions in the time-lapse recordings. (C) Cardiac output was calculated as the product of stroke volume and heart rate. Values are mean ± standard error with n/C21 5. Significant effect of TCDD compared to vehicle control is indicated by an asterisk. A filled diamond indicates a significant difference in response to TCDD between the test MO and the CMO. The level of significance for all comparisons was p < 0.05.](image3)
cardiac output by 60 hpf, while no significant effect on heart rate was observed. It should be noted that ventricular standstill is not observed at this time; the heart rate measured reflects the coupled beating for the atrium and the ventricle. The TCDD-induced decrease in stroke volume and cardiac output was not observed in embryos injected with the zfahr2-MO or the zfarnt1-MO. However, the effect of TCDD was not blocked by the zfarnt2- or zfcypla-MOs.

DISCUSSION

Many of the known signs of TCDD toxicity in zebrafish larvae have been shown to occur via activation of the AHR signaling pathway. MO knockdown of either zfAHR2 or zfARNT1 protects zebrafish larvae from TCDD-induced pericardial edema, peripheral ischemia, and jaw malformation, indicating that these responses require zfAHR2 and zfARNT1 (Carney et al., 2004; Dong et al., 2004; Prasch et al., 2003, 2006). Recently, zfARNT1 and zfAHR2 have also been shown to mediate the TCDD-induced inhibition of regenerative growth in zebrafish (Mathew et al., 2006). However, because these studies did not closely examine the adverse effects of TCDD exposure on the developing heart, and the zebrafish arnt2+/− mutant was found to develop signs of cardiac malfunction (Hill et al., 2005), it remained possible that components such as zfARNT2 might act as an AHR dimerization partner to produce cardiotoxicity.

We found that morpholino blockade of either zfAHR2 or zfARNT1 was sufficient to protect embryos from the tube-like alteration in heart morphology, disrupted looping, decreased cardiomyocyte number, ventricle silencing, as well as decreased stroke volume and cardiac output. Moreover, injection of the zfarnt2-MO or zfcypla-MO had no effect on these endpoints of TCDD-induced cardiac toxicity. Thus, all TCDD responses in zebrafish larvae so far examined appear to be mediated by the AHR2/ARNT1 heterodimer. We have not observed any effect with a zfAHR1 morpholino, and since the efficacy of this morpholino remains difficult to prove these inconclusive experiments were not reported. Nonetheless, we can conclude that AHR1 isofoms are not likely to efficiently substitute for AHR2 in producing the TCDD-induced cardiotoxicity, since blocking zfAHR2 alone was sufficient to prevent all examined endpoints of cardiac toxicity. Also, while zfAHR1 has been indicated in toxicity of some components of weathered crude oil, it has only been reported so for non-dioxin-like compounds (Incardona et al., 2005). In addition, we observed no protection against the TCDD cardiac toxicity from MOs directed against zfCYP1A, and zfARNT2, though there is some evidence that cytochrome P450 may be involved in some forms of dioxin toxicity (Smith et al., 2001; Uno et al., 2004).

The level of protection afforded by the morpholinos differed between the zfahr2-MO and the zfarnt1-MO, especially at time points later than 72 hpf. This is not surprising given the transient nature of MOs administered by only a single injection immediately after fertilization. With the growth of the developing embryo, morpholinos are constantly diluted by steadily increasing cell numbers contributing to a decrease in efficacy (reviewed in Sumanas and Larson, 2002). This has been well documented in earlier morpholino work, especially with regards to the ability of individual morpholinos to block CYP1A induction (Carney et al., 2004; Prasch et al., 2003, 2004, 2006). Thus, the differences between the zfahr2- and zfarnt1-MOs seen in the heart are entirely consistent with previous work.

The effects of TCDD on heart morphology seem to suggest that TCDD acts by disrupting early processes of pattern formation and organogenesis. If these early processes are the important targets for TCDD disruption, then the heart should become insensitive to TCDD after they have been completed. The MOs should protect the developing heart against TCDD during the critical processes of heart field specification, tube formation, and looping, because these processes have all been completed by 48 hpf, well within the range of MO action. With these processes completed, the MO-protected heart should be resistant to TCDD even after the MOs have ceased to function. However attractive this model may be, it is not consistent with several published reports indicating that TCDD does not prevent initial organ formation. An embryo carrying a lethal dose of TCDD forms a functional, normal looking, looped heart as it approaches 48 hpf, only to have it become malformed and nonfunctional over the next 3 days (Antkiewicz et al., 2005; Henry et al., 1997). Similarly, TCDD can cause heart deformation and loss of circulation when introduced at 72 hpf, a time point at which looping is completed (Belair et al., 2001; Carney et al., 2006). Administration of TCDD at 72 or 96 hpf produces almost exactly the same morphological changes as seen in the hearts in embryos exposed at fertilization. In our experiments, the developing zebrafish have no way of clearing the lipophilic TCDD administered shortly after fertilization (Heiden et al., 2005). This produces an exposure that continues into the time frame where the MOs fail at around 72–96 h after injection. This residual TCDD is then free to alter heart morphology and function.

In addition to establishing the involvement of zfAHR2 and zfARNT1 dimerization partners in TCDD-induced cardiac toxicity, our study reports that cardiac function is affected by TCDD as early as 60 hpf. It is not clear whether the observed decrease in cardiac output is a direct effect of dioxin or rather a consequence of the TCDD-induced effect on heart morphology and size. Nevertheless, it is clearly one of the earliest signs of TCDD toxicity in the zebrafish embryo. Decreased cardiomyocyte numbers have been observed at 48 hpf (Antkiewicz et al., 2005), and decreased red blood cell perfusion rate has been reported in trunk vessels as early as 72 hpf (Carney et al., 2004; Prasch et al., 2003, 2006; Teraoka et al., 2002), as well as in the mesencephalic vein of the brain at 50 hpf (Dong et al., 2002). At higher TCDD exposure concentrations (6 ng/ml)
blood flow in caudal trunk vessels was also decreased as early as 60 hpf (Belair et al., 2001). Thus, our results place a loss of cardiac output in a similar time frame as the previously reported effects on circulation and heart cell number.

It should be noted that the method we employed to assess the stroke volume and cardiac output assumes spherical or cylindrical shape of the heart chambers. More specifically, it presumes that ventricular volume can be represented as the sum of the volumes of individual discs into which the ventricle can be sliced. Therefore, the change in heart morphology caused by TCDD could potentially have an effect on these measurements. However, our data suggest that this would be a minimal effect, as serial sections and stereology work indicate that the heart chambers are essentially circular in cross-section in both TCDD-exposed and control hearts at all inspected time points; at the same time the shape of the ventricle remains largely unaltered by TCDD, save for an overall compaction (Antkiewicz et al., 2005; Carney et al., 2006).

We set out to determine whether MOs previously shown to block gross endpoints of toxicity were also capable of blocking more subtle signs of toxicity in the developing heart. We were especially interested in testing the effects of the zfarma2-MO, since zfARNT2 is an effective binding partner for zfAHR2 in vitro. CYP1A was included due to its historical interest as a target for AHR/ARNT signaling. However, a plentitude of new target genes for AHR is emerging now through variety of approaches (Marchand et al., 2005; Niermann et al., 2003; Thomsen et al., 2004). These, along with the set of TCDD-induced transcripts in the developing zebrafish heart recently identified by Carney et al. (2006), provide a much more substantial set of AHR/ARNT target genes for future study. The complexity of the AHR signaling pathway makes teasing apart the mechanisms of TCDD toxicity difficult, and is in part responsible for our current lack of understanding of TCDD toxicity. While much has yet to be learned regarding the specific molecular mechanisms that underlie TCDD-induced cardiac toxicity, our work presented here confirms hypotheses that such toxicities are AHR2/ARNT1 driven in zebrafish.

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