Heart Malformation Is an Early Response to TCDD in Embryonic Zebrafish

Dagmara S. Antkiewicz,* C. Geoffrey Burns,†‡ Sara A. Carney,* Richard E. Peterson,*§ and Warren Heideman*§†

*Molecular and Environmental Toxicology Center, University of Wisconsin, Madison, Wisconsin 53705; †Developmental Biology Laboratory, Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, Massachusetts 02129; §Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115; and ¶School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53705

Received October 29, 2004; accepted December 22, 2004

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 toxic compounds perturb normal development. When exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) shortly after fertilization, zebrafish embryos exhibit pericardial edema and reduced blood flow by 72 h post fertilization (hpf). To better understand the progression of dioxin toxicity in zebrafish, we have examined the effects of TCDD on heart development. At 72 hpf, TCDD-treated embryos exhibited altered looping, with the atria positioned distinctly posterior to the ventricles, contrary to the looping of control hearts, where the two chambers lied side by side. Moreover, the ventricles in dioxin-exposed hearts became more compact, and the atria elongated in comparison to controls. These defects are not secondary to pericardial edema because they were observed when edema formation was suppressed with osmotic support. In addition to morphological changes, TCDD produced functional deficits in the developing hearts, including blood regurgitation and a striking ventricular standstill that became prevalent by 120 hpf. We also assessed the effect of TCDD on the heart size using stereological measurements, which demonstrated significant reduction in heart tissue volume at 72 hpf. Perhaps our most significant finding was a decrease in the total number of cardiomyocytes in TCDD-exposed embryos by 48 hpf, one day prior to observable effects on peripheral blood flow. We conclude that the developing heart is an important target for TCDD in zebrafish.

Key Words: 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCDD toxicity; zebrafish; heart; development.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a highly lipophilic and persistent environmental pollutant that undergoes bioaccumulation in the food chain. Reproductive and developmental toxicity, immunotoxicity, cardiotoxicity, neurotoxicity, hepatotoxicity, wasting syndrome, and lethality are among the toxic effects of TCDD in wildlife. Fish are the most sensitive vertebrates to the effects of dioxin, especially during embryonic development (Peterson et al., 1993). In a recent analysis, the near extinction of lake trout in Lake Ontario was associated with exposure to TCDD-like chemicals (Cook et al., 2003).

Zebrafish (Danio rerio) have become a model vertebrate for investigating TCDD toxicity (Belair et al., 2001; Bello et al., 2004; Carney et al., 2004; Dong et al., 2002; Henry et al., 1997; Hill et al., 2004; Prasch et al., 2003). The main advantages of this organism as a model are its transparency, fecundity, rapid development, and the availability of many genetic and molecular research tools. Signs of dioxin toxicity in embryonic zebrafish are essentially the same as seen in other fish species. Embryos exhibit in order of appearance: reduction in blood flow, pericardial edema, craniofacial malformations, growth retardation, yolk sac edema and reduced heart rate, anemia, and impaired swim bladder inflation, followed by mortality (Belair et al., 2001; Dong et al., 2002; Henry et al., 1997; Teraoka et al., 2002).

Most of the toxic endpoints seen in TCDD-exposed vertebrates are thought to be mediated by the aryl hydrocarbon receptor (AHR), a ligand activated transcription factor. Inactive AHR resides in the cytoplasm and translocates 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. Induction of cyp1a expression has been used as a marker of AHR pathway activation (Nebert et al., 1990; Tanguay et al., 1999).

TCDD has been shown to have a profound effect on the cardiovascular system in a range of vertebrate species. Acute toxicity study done in rhesus monkeys reported bilateral ventricular dilation, cardiac enlargement, edema, and hemorrhage (Allen et al., 1977). Riecke et al. reported an increase in collagen, fibronectin, laminin, and transforming growth factor β1 expression in myocardium of male marmosets following
a low dose treatment (Riecke et al., 2002). Rats treated with acute toxic doses of TCDD develop decreased blood pressure and decreased heart rate (Hermansky et al., 1988). Mice exposed in utero to TCDD develop cardiac hypertrophy, manifested by increased heart weight (Lin et al., 2001). Interestingly, Ahr null mice also develop hypertrophic hearts, characterized by ventricular wall thickening, and expression of cardiac hypertrophy markers, suggesting that TCDD may be causing toxicity by taking AHR away from its normal function (Thackaberry et al., 2002). The cardiac proliferative index was increased in these animals, suggesting that the cardiac hypertrophy is hyperplastic in nature (Thackaberry et al., 2003). However, an increase in the cardiac myocyte size in the Ahr null mice has also been indicated as a likely cause for the increased heart weight (Vasquez et al., 2003). Taken together, these reports suggest potential involvement of the myocardium in AHR agonist toxicity in mice.

Chicken embryos exposed to dioxin exhibit ventricular hypertrophy in the absence of wall thickening, along with abnormal intracellular calcium modulation in cardiac myocytes and poor contractility (Canga et al., 1993; Heid et al., 2001; Walker and Catron, 2000). Ivnitski-Steele et al. found reduced cardiomyocyte proliferation in chick embryos exposed to TCDD, and suggested that this reduction in cardiomyocyte number could cause thinning of the myocardial wall (Ivnitski-Steele et al., 2001). TCDD reduces the level of vascular endothelial growth factor-A (VEGF-A) as well as the number and size of coronary arteries, and also induces cardiomyocyte growth arrest (Ivnitski-Steele et al., 2004).

The ability of TCDD to produce cardiac defects in fish has not been extensively studied. However, the AHR pathway can be activated by TCDD in the zebrafish heart. TCDD induces CYP1A expression in several tissues in embryonic zebrafish, including endocardium and vascular endothelium, as well as ventricular myocardium in the adult zebrafish (Andreassen et al., 2002; Stegeman et al., 1989; Zodrow et al., 2004). TCDD decreases heart size and cardiac output in rainbow trout larvae (Hornung et al., 1999). Moreover, it has been recently reported that mixtures of polycyclic aromatic hydrocarbons (PAHs), which may activate the AHR, produce cardiac dysfunction in zebrafish embryos (Incardona et al., 2004).

We hypothesized that the heart is a key target for TCDD in developing fish. To test this hypothesis, we examined the effects of TCDD exposure on the hearts of developing zebrafish embryos. Zebrafish are particularly useful for this type of study because severe defects in heart formation do not lead to immediate lethality as in many vertebrate models, and developing zebrafish can survive the first week of life without functional circulation (Sehnert et al., 2002). This allows progressive development of cardiac abnormalities to be readily observed. We used microscopy, histological sections, computer-aided morphometry, and immunohistochemistry to assess the effects of TCDD on cardiac development. We report that TCDD exposure leads to structural malformation, altered looping, and decreased size in the zebrafish heart. We also demonstrate that TCDD has a profound effect on heart function leading to retrograde blood flow and ventricular standstill. The timing of cardiac toxicity relative to other toxic responses indicates that cardiotoxicity is among the very earliest endpoints of toxicity to occur, preceding impairment of the peripheral circulation. This suggests that the heart is a direct target of TCDD toxicity.

**MATERIALS AND METHODS**

**Zebrafish lines and embryos.** For the cardiac myocyte count we used the cmlc2:dsRed2-nuc line of transgenic zebrafish (Mably et al., 2003). In all other experiments we used wild type AB line zebrafish. All fish were bred and embryos were raised in our laboratory according to the procedures described by Westerfield (1995). Fish were anesthetized with 1.67 mg/ml tricine (MS-222, Sigma) as indicated.

**TCDD exposure.** 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) of >99% purity from Chemsyn (Lenexa, KS) was dissolved in dimethyl sulfoxide (DMSO) for preparation of dosing solutions. All embryos were treated shortly after fertilization by 1 h static waterborne exposure to either 0.1% DMSO (vehicle) or 1 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 fresh egg water (60 mg/l Instant Ocean salts).

**Histology and imaging.** Zebrafish larvae were anesthetized, fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4°C, dehydrated in a methanol series, and embedded in paraffin. Embedded embryos were sectioned (5 μm transverse sections) and stained with Giemsa stain (Sigma, St. Louis, MO). Images were obtained with a Nikon Eclipse TE300 inverted microscope, Universal Imaging Corporation Metamorph Imaging software, and a Princeton Instruments Micromax charge-coupled device camera with a CRI microcolor filter. All whole-mount embryo images were captured using a 10× objective, and the tissue sections were photographed using a 20× objective lens.

**Myosin heavy chain immunolocalization.** Embryos were fixed in 4% PFA in PBS, dehydrated in methanol series, hydrated back into the PBS, and permeabilized by digestion with 0.1% Type 1A collagenase (Sigma, St. Louis, MO) for 30 min. After washing with PBST, the embryos were blocked for 1 h at room temperature in blocking solution (5% sheep serum, 2% bovine serum albumin, 1% DMSO, 0.1% Tween20 in PBS), and then incubated overnight at 4°C in a 1:1 dilution of MF-20 primary antibody (Developmental Studies Hybridoma Bank). This was followed with six washes in PBST and overnight incubation with secondary antibody (IgG2b-TRITC, Southern Biotechnology Associated, Inc., Birmingham, AL) at 1:100 dilution. Staining was visualized by epifluorescence microscopy.

**Assessment of TCDD-Induced Cardiac Toxicity.**

**SV-BA distance.** Changes in heart morphology caused by TCDD exposure were measured by positioning embryos in 3% methylcellulose to allow capture of lateral view images and measurement of the distance between the sinus venosus (SV) and bulbus arteriosus (BA) regions of the heart. The Metamorph Imaging software was then used to assess the length of a straight line connecting the two structures on acquired images.

**Tissue volume.** The heart tissue volume was measured from a single transverse sections of paraffin-embedded embryos using stereological software (Stereo Investigator; MicroBrightField, Inc.). We used the Cavalieri method to estimate the volume based on the sum of the surface area of every other heart section, with Gundersen’s coefficient of error ≤ 0.1 for all measurements.
Heart rate. Zebrafish embryos were positioned in 3% methylcellulose and kept on a temperature-controlled microscope stage (27°C) for at least 15 min prior to the heart rate assessment. This time was sufficient for the embryos to equilibrate to the stage temperature. Ventricular and atrial beats were counted in 20 s periods. At least three measurements were taken and their average was used in statistical analysis. In these experiments the rates for TCDD-treated hearts are expressed relative to matched control embryos measured in the same observation session under identical conditions. In other words, the rates in TCDD-exposed embryos are expressed relative to rates in the vehicle-treated embryos to control for temperature sensitivity.

Counting cardiac myocytes. Cardiac myocytes were counted in TCDD- and vehicle-treated cmic2::dsRed2-nuc transgenic zebrafish as previously described (Mably et al., 2003). Briefly, the embryos were anesthetized and flat-mounted on a glass microscope slide under a coverslip in Lebovitz’s L15 cell culture media (Gibco). Epifluorescence images were captured and nuclei were counted to yield the cell number.

Statistics. One-way or factorial ANOVA followed by the Fisher LSD test was used to determine statistical significance. The assumption of unequal variances for all data sets was checked using Levene’s test. The heart rate data could not be transformed to pass Levene’s test due to the binomial character of the distribution. Therefore this data was analyzed using a pair wise t-test with the assumption of unequal variances. Data are presented as mean ± SE, and asterisks indicate a significant difference between TCDD and the representative control (DMSO), unless indicated otherwise. The level of significance was p ≤ 0.05 for all comparisons. All analyses were performed using the Statistica 6.0 software package. In all cases, the observer was blinded to the treatment group until measurements or scoring was completed. This has the caveat that TCDD exposure leads to fairly obvious changes in morphology.

RESULTS

Effects of TCDD on Heart Morphology

Zebrafish embryos exposed to TCDD develop pericardial edema and a significant decrease in blood flow by 72 hpf (Belair et al., 2001; Bello et al., 2004; Carney et al., 2004; Dong et al., 2002; Henry et al., 1997; Prasch et al., 2003). We examined the hearts of developing zebrafish embryos that had been exposed to TCDD immediately after fertilization. By 72 hpf we observed a clearly evident alteration in heart morphology (Fig. 1). This change became progressively more pronounced and was quite obvious by 96 hpf. Instead of the looped, S-shaped hearts seen in the vehicle-treated fish, hearts from TCDD-exposed embryos were elongated and string-like. In control embryos, the normal looping process places the ventricle and atrium side by side, so that the two chambers largely overlap each other in lateral view. In contrast, the hearts were stretched out in the TCDD-treated embryos such that the ventricle was positioned anterior to the atrium. Thus in the TCDD-exposed embryos the chambers can be easily distinguished with little if any overlap. Moreover, in the treated animals the atria were thin and elongated and the ventricles appeared smaller and more compact than normal.

In order to quantify the effect of TCDD on zebrafish hearts, the distance between the junction of the heart with the inflow tract at the sinus venosus (SV) and the junction with the outflow tract at the region of the bulbus arteriosus (BA) was determined (Fig. 2). This “SV-BA distance” was measured in pixels from digital images of lateral view whole mount embryos as indicated by the arrows in Figure 2A. The resulting numbers provide an index of the change in heart morphology due to the TCDD treatment, and reflect the change in cardiac looping. TCDD produced significant increases in the BA-SV distance, compared to controls, at all time points examined: 72, 96, and 120 hpf (Fig. 2B). More specifically, while the vehicle-treated hearts underwent the process of looping and compaction, this was not observed in the TCDD-exposed hearts.

It should be noted that the defects caused by TCDD did not appear to be due to a failure to initially form a functional heart. We found that control and TCDD-exposed hearts appeared indistinguishable from each other at 48 hpf (not shown), consistent with the previously reported normal blood flow at 48 hpf (Belair et al., 2001; Dong et al., 2002). Thereafter, however, the TCDD-exposed hearts progressively deteriorated in morphology and function compared to the control hearts.

Osmotic Support Blocks Edema, but Not Heart Malformation

It was possible that the increase in pericardial edema caused by TCDD could lengthen the distance between the heart...
attachment points at the inflow and outflow tracts, indirectly leading to a mechanical stretching of the hearts treated with TCDD. In order to rule out this possibility, we grew zebrafish embryos with osmotic support (175 mM mannitol) to suppress the pericardial edema. As previously described, such a solution effectively prevented the development of edema in TCDD-treated fish (Hill et al., 2004). However, blocking the edema with mannitol did not prevent the TCDD effects on heart morphology. As can be seen in Figure 3, while mannitol prevented pericardial sac edema in the TCDD-exposed fish, the hearts still became elongated with an alteration in looping. This indicates that the changes in heart morphology caused by TCDD are not a secondary consequence of edema.

TCDD and Specific Heart Structures

We stained TCDD-exposed and control embryos with the MF-20 antibody (Fig. 4). This antibody binds to myosin, and because myosin is more prevalent in the ventricle than in the atrium the MF-20 antibody has been used to distinguish the ventricle from the atrium. This distinction between the ventricle and atrium was not disrupted by TCDD, and the staining of the ventricle with MF-20 remained more pronounced than the atrial staining in the TCDD-exposed hearts. To further investigate the effects of TCDD on heart morphology, we examined serial transverse sections of control and TCDD-treated zebrafish. Figure 5 shows a set of sections representative for both groups of embryos at 96 hpf. These sections show in more detail the change in looping, compaction of the ventricle, elongation of the atrium, and shrinking of luminal area caused by TCDD. The images shown are representative of the different regions of the hearts starting at the anterior end of the heart at the bulbus arteriosus and extending towards the posterior end towards the sinus venosus.

A complete set of sections from which these images were taken is shown as a figure in the supplementary material. The apparent elongation of the TCDD-treated heart was evident...
in that more sections were needed for the representation of the TCDD-exposed heart. Many of these additional sections contain the atrium, consistent with the stretched morphology of this chamber. The cross sectional area of both heart chambers and their lumens was dramatically reduced by TCDD. The degree of looping is reflected by the number of cross sections in which both the atrium and the ventricle can be seen side by side. In the control hearts, several sections on either side of the atrioventricular (AV) junction contained tissue from both chambers. In contrast, in the TCDD-treated hearts the only sections containing both the atrium and ventricle are at the AV boundary. Finally, the ventricle in the TCDD-treated heart was found in fewer sections than in the control, consistent with the seemingly reduced, compacted ventricle seen in the whole mount images of TCDD-treated hearts in Figure 1.

**TCDD Affects Heart Size**

Based on the microscopic and histological examination of the TCDD-exposed embryos, the TCDD-treated hearts consistently appeared smaller in comparison to controls. We hypothesized that TCDD decreases the size of the developing hearts by reducing the heart tissue. To test this hypothesis we used serial transverse sections of paraffin-embedded zebrafish at 72 and 96 hpf, and stereological software (Stereo Investigator; MicroBrightField, Inc.) to produce a quantitative measure of the heart tissue volume. As shown in Figure 6, hearts exposed to TCDD had notably reduced tissue volume compared to control hearts at both 72 and 96 hpf.

One plausible mechanism to explain the TCDD-induced heart size reduction is a decrease in heart cell number. We tested this hypothesis by counting individual cardiac myocytes in the *cmic2::dsRed2-nuc* line of transgenic zebrafish. This line...
of fish expresses *Discosoma* red fluorescent protein (RFP) from the promoter of a cardiomyocyte-specific gene, *cmlc2*. The RFP expressed in these fish contains a nuclear localization sequence fused in frame that restricts the RFP to the nuclei of cardiomyocytes, allowing one to distinguish between individual cells (Fig. 7A) and specifically count cardiac myocytes (Mably et al., 2003).

The TCDD-exposed embryos exhibited a significant decrease in cardiac myocyte number at 48, 72, and 96 hpf (Fig. 7B). This reduction was observed as early as 48 hpf, appearing ahead of all other signs of TCDD toxicity reported in the zebrafish larva. It is especially notable that this precedes the emergence of measurable changes in trunk blood flow by approximately one day.

**Effects on Heart Rate**

The effects of TCDD exposure on the developing zebrafish heart were not limited to the alterations in heart morphology. In addition, we found heart function to be profoundly affected. Initially both chambers of the TCDD-treated hearts exhibited normal contraction, with the ventricle contracting immediately after the atrium. However, by 96 hpf ventricular rate was significantly decreased in the TCDD-exposed embryos, and by 120 hpf the ventricles in the TCDD-exposed group had ceased beating almost entirely (Fig. 8A). It must be noted that this measurement of rate is somewhat misleading because the decrease in average rate is primarily due to a steady increase in the incidence of complete absence of ventricular contraction (ventricular standstill) (Fig. 8B). Thus the average ventricular rate is decreased at 96 hpf not because the ventricles tended to uniformly beat at a slower rate, but instead because a significant fraction of the embryos had no ventricular beat at all, lowering the average.

Blocking edema formation with mannitol as an osmotic support could not reduce the ventricular standstill. Despite a complete lack of edema, we observed ventricular standstill in 100% of TCDD-exposed embryos (N = 6) in mannitol at 120 hpf. Embryos not exposed to TCDD showed no ventricular standstill.

It is important to note that we very rarely observed ventricular arrhythmias that resembled partial AV nodal block. Instead, the vast majority of treated embryos displayed either a normal ventricular rate, or complete ventricular standstill. Indeed, at 96 hpf over 50% of embryos exhibited complete ventricular standstill, and by 120 hpf the incidence of complete ventricular standstill averaged over 75%.
Although the atrial rate was not changed in the TCDD-treated fish compared to controls, the character of atrial contraction differed between the two groups at 96 and 120 hpf. In the control embryos the entire atrium contracted evenly and simultaneously, while the atria in the TCDD treatment group exhibited a slower peristaltic-like wave of contraction (see Movies 3 and 4 in the Supplemental Data).

In addition to the change in ventricular rate and the qualitative changes in atrial conduction, we consistently observed blood cell regurgitation at the outflow tract and AV junction, suggesting a defect in valve function (Movie 2 in Supplemental Data). This points to the possibility of altered valve structure or altered valve contraction.

DISCUSSION

The effects of TCDD on the morphology of the developing zebrafish heart are evident by 72 hpf. These effects include altered looping and a string-like appearance, with the ventricle located distinctly anterior to the atrium. Moreover, the ventricle appears smaller than normal, the atrium is elongated, and both chambers have a smaller lumen. The total amount of heart tissue is reduced in TCDD-exposed embryos, due at least in part to a reduction in myocardocyte number beginning at 48 hpf. Valvular regurgitation of blood and a striking ventricular standstill are also observed in the heart of TCDD-exposed embryos. These defects become progressively more apparent as time passes. Taken together, the steady decrease in heart size coupled with the severe defects in ventricular function, including ventricular standstill and retrograde blood flow would be expected to have a substantial impact on the ability of zebrafish to circulate blood.

Disruption of heart development in zebrafish embryos exposed to TCDD in the ppb range is of particular importance in light of the growing body of evidence demonstrating that the cardiovascular system is also a key target of TCDD toxicity in humans (Kim et al., 2003; Pesatori et al., 1998; Vena et al., 1998). A recent study of the effects of Agent Orange exposure on Korean and Vietnam war veterans showed that higher levels of exposure were associated with increased frequency of ischemic and valvular heart disease (Kim et al., 2003). Our results indicate that zebrafish are useful as a model system for studying the effects of AHR agonists on the vertebrate heart.

Timing of TCDD Effects

In zebrafish embryos exposed to TCDD immediately after fertilization the overall circulation appears normal at 48 hpf (Belair et al., 2001; Dong et al., 2002). The blood flow rate then declines over the next two days until it halts at around 96 hpf (Belair et al., 2001; Carney et al., 2004; Henry et al., 1997). This is consistent with the effects of TCDD on the heart. At 48 hpf we could see no morphological difference between the hearts of TCDD-exposed and control embryos (not shown). However, by 72 hpf morphological and functional defects are readily apparent. Thus, zebrafish embryos can develop a heart with a functional circulation despite the presence of TCDD, but after formation, the heart becomes increasingly affected by TCDD until cardiac function is completely abolished.

Other major signs of dioxin toxicity appearing at around 72 hpf include pericardial edema, decreased peripheral blood flow, and altered vascular remodeling (Belair et al., 2001; Dong et al., 2002; Prasch et al., 2003; Teraoka et al., 2002). Since blood flow, vessel formation, heart development, and edema are all intertwined, this coincidence of TCDD effects makes it difficult to determine which responses are the direct result of TCDD exposure and which are indirect, secondary to the primary effect.
The elongation of the heart by TCDD might be due to the failure of the attachment point between the heart and the common cardinal vein (CCV) to migrate dorsally, thus mechanically stretching the heart. From approximately 72 to 96 hpf, the heart’s attachment point to the inflow tract migrates dorsally, in a process that appears to contribute to the heart’s looping and compaction within the pericardium. This migration is inhibited by TCDD exposure (Bello et al., 2004). However, in TCDD-exposed embryos, the defect in heart morphology is readily apparent by 72 hpf. Thus, the alteration of heart morphology is present by the beginning of the process of dorsal migration, and therefore cannot be secondary to the disruption of CCV regression. We cannot, however, rule out the possibility that blockade of CCV dorsal migration might contribute to the elongated heart morphology at later time points.

Similarly, the formation of pericardial edema could move the inflow and outflow attachment points apart, causing the elongated heart morphology. However, we were able to suppress the edema formation with mannitol without rescuing the cardiac morphological defects caused by TCDD. This indicates that the change in heart morphology is not secondary to edema.

Even more difficult to separate are the effects on blood flow and heart function. Defects in the heart might well be expected to produce a concurrent reduction in circulation. On the other hand, circulatory failure is known to have effects on heart development (Hove et al., 2003). However, the fact that TCDD produces a reduction in cardiomyocyte number as early as 48 hpf indicates that TCDD produces effects on the heart that precede changes in circulation. This suggests that the effects on the heart are not secondary to failing circulation and that the heart is a direct target for TCDD. This idea is strengthened by microarray experiments demonstrating large-scale changes in gene expression in heart cells within 2 h of TCDD exposure, well before any measurable change in circulation (manuscript in preparation). However, small changes in the circulation or vasculature that we cannot observe could conceivably produce substantial changes in gene expression in the heart.

**Early Effects on Myocardium**

The stereology and cell count results suggest that TCDD produces smaller hearts by inhibiting growth or increasing death of heart cells. In normally developing embryos, after initial heart tube formation myocardial cells begin proliferation to increase wall thickness. By 120 hpf, the ventricular myocardium has become multilayered and extensive trabeculation has formed (Hu et al., 2000). The decreased myocardial cell number produced by TCDD would be expected to inhibit this maturation process and produce the smaller hearts found in TCDD-treated zebrafish larvae.

Our counts of cardiomyocyte number consistently were lower than those reported by Mably et al. (2003). While it is possible that the difference is related to staging and growth rate differences between the two studies, the discrepancy seems likely due to differences in instrumentation or technique. Since our number is the lower one, we may be consistently not scoring some cells that would have been scored by Mably et al. With only our report and that of Mably et al., it is difficult to know how far our results are from the true number of cardiomyocytes. It should be noted, however, that in these embryos the cardiomyocyte nuclei are quite distinct, and few in number. This makes the cell counting method relatively robust.

Reports describing mouse and chick models indicate that the myocardium is a target of dioxin toxicity (Ivnitski et al., 2001; Thackaberry et al., 2003; Vasquez et al., 2003). While we saw a reduction in myocyte number, myocardium development can be influenced by signals from endothelial cells (Trinh le and Stainier, 2004). Therefore, endocardial cells could potentially be the main targets for TCDD in disrupting heart formation. This idea is supported by the fact that the AHR/ARNT pathway is very responsive to TCDD in vascular endothelium (Andreasen et al., 2002; Carney et al., 2004; Guiney et al., 1997; Prasch et al., 2003).

**Effects on Heart Function**

Our experiments show that TCDD has a striking effect on the ventricular beat, a condition that we describe as ventricular standstill. Our data does not distinguish between a block of impulse propagation at the AV node, and inability of the ventricle to contract in response to an action potential. We have therefore refrained from referring to the observed phenomenon as an AV conduction block. We did not observe progressive arrhythmias commonly associated with AV block. Arrhythmias in which the ventricle would beat asynchronously with the atrium, skipping a contraction sporadically, were observed only rarely. Instead, we observed the ventricles either beating in synchrony with the contracting atrium, or not contracting at all (see digital movies in Supplement).

One possible mechanism to explain the ventricular standstill is a failure of the action potential to travel beyond the AV junction. An alternative hypothesis is that the action potential travels across the ventricle, but contraction is blocked. Our results do not distinguish between these two mechanisms. An additional possible mechanism might be that increasing pressure in the pericardium could be inhibiting the heartbeat by compressing the heart and preventing filling in a process analogous to cardiac tamponade. We consider this mechanism unlikely because such a mechanism should affect the atrium at least as much as the ventricle, yet the atrial beat is unaffected. Additionally, we have conducted experiments in which pericardial edema was blocked by the addition of osmotic support with mannitol. We observed ventricular standstill in these experiments, despite the absence of pericardial edema. Therefore, while we do not know the mechanism that causes ventricular standstill, we do not believe it to be secondary to pericardial edema.
Another effect of TCDD on heart function in embryonic zebrafish is retrograde blood flow observed as early as 72 hpf (see digital movies in Supplemental Data). At later time points, when the decrease in cardiac output and blood flow becomes more dramatic, the back-and-forth movement of blood cells between the atrium and the ventricle, as well as between the ventricle and the outflow tract can be observed in most embryos. This could be due to a defect in valve function, or a response to elevated afterload due to a block in circulation. We cannot rule out such a blockade in the vascular system at 120 hpf, when blood flow has ceased. However, we see retrograde blood flow at 72 hpf, when blood flow is still substantial and heart contractility seems unaffected. This suggests that the developing valves are not functioning properly, perhaps due to altered valve cushion development, or a loss of contractility at the sites of the nascent valves.

The Heart as a Primary Target for TCDD Toxicity

Our results suggest a model in which TCDD produces steadily diminishing cardiac output as a direct result of its effects on the heart. The resulting fall in blood flow would be expected to reduce filtration at the developing pronephros, with a consequent increase in fluid volume, leading to edema. The fact that edema is prevented by increasing the osmolarity of the water surrounding the embryo is consistent with this model. Increased osmolarity would help the developing fish compensate for decreased glomerular filtration by reducing the rate of water influx. While we have demonstrated working glomerular filtration in TCDD-exposed embryos (Hill et al., 2004), this was a qualitative assay that would not be expected to detect a quantitative decrease in filtration rate that might result from failing cardiac output.

Another possible explanation for the development of edema might be failure of a water permeability barrier, as suggested by Hill et al. (2004). Alternatively, increased vascular permeability might be responsible for the edema as suggested by Guiney et al. (2000). These mechanisms are of course not mutually exclusive. The cause of the loss of blood flow and edema in response to TCDD remains to be determined. Regardless, our results suggest that one of the very earliest responses to TCDD in developing zebrafish embryos is a progressive loss of heart function.

SUPPLEMENTAL DATA

Four short digital movies of the beating heart are included in the Supplement (accessible from the Toxicological Sciences website) to illustrate various aspects of the TCDD effects on heart function in embryonic zebrafish. Movie 1 and 2 show the beating hearts representative for the vehicle and TCDD treated embryos at 72 hpf, respectively. Valvular regurgitation of blood can be seen in the Movie 2. Movie 3 and 4 represent typical control and TCDD-exposed heart at 96 hpf. In the latter, the ventricular standstill, and changed character of atrial contraction can be observed. In addition, the complete set of sections from Figure 5 is shown in Supplemental Figure 1.

ACKNOWLEDGMENTS

We thank Dorothy Nesbit and Amy Prasch. We acknowledge P. Walker, E. Fluegel, R. Przemyk, and J. Hatfield for laboratory assistance. We also thank Patricia J. Keely, who generously provided us access to equipment. This work was supported by the University of Wisconsin Sea Grant Institute under grants from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, US Department of Commerce, Sea Grant Project Numbers R/BT-16 and R/BT-17 (W.H. and R.E.P.), and by NIH grant T32 ES07015 from the National Institute of Environmental Health Sciences (NIEHS, D.S.A., W.H., and R.E.P.). C.G.B. was supported by NIH training grant 5T32HL007208-27 awarded to Massachusetts General Hospital. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NIH. Contribution #361, Molecular and Environmental Toxicology Center, University of Wisconsin, Madison, WI 53706.

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