2,3,7,8-Tetrachlorodibenzo-p-dioxin Toxicity in the Zebrafish Embryo: Altered Regional Blood Flow and Impaired Lower Jaw Development

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Received May 4, 2001; accepted October 17, 2001

The effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure on regional red blood cell (RBC) perfusion rate, as an index of blood flow, and lower jaw development were investigated quantitatively in zebrafish embryos (Danio rerio) during early development. As revealed by observation of live embryos and alcian-blue staining, TCDD retarded lower jaw development in a concentration-dependent manner with only a minor inhibitory effect on total body length. Both inhibitory effects were significant as early as 60 h postfertilization (hpf), at which time the area of goosecoid (gsc) mRNA expression was clearly reduced in the lower jaw. To examine effects of TCDD on RBC perfusion rate, time-lapse recording was performed using a digital video camera attached to a light microscope. TCDD did not show marked effects on RBC perfusion rate until 72 hpf, when vessel-specific effects emerged. TCDD severely inhibited RBC perfusion rate in intersegmental arteries of the trunk, but only modestly and slightly inhibited RBC perfusion rate in certain vessels of the head such as the central arteries and optic vein. Conversely, at both 72 and 84 hpf, TCDD significantly increased RBC perfusion rate in the hypobranchial artery branching to the lower jaw primordia, and then reduced it at 96 hpf. RBC perfusion rate in all vessels examined in TCDD-exposed embryos was inhibited at 96 hpf. The zebrafish aryl hydrocarbon receptor 2 (zfAhr2) mRNA was strongly expressed in the lower jaw primordia at 48 hpf, and expression of this transcript was augmented by TCDD treatment. Thus, TCDD exposure of the zebrafish embryo has a disruptive effect on local circulation and lower jaw cartilage growth. Initially, TCDD may act directly on the lower jaw primordia to impair lower jaw development. Reductions in hypobranchial RBC perfusion rate occurred well after the initial retardation in lower jaw development had become apparent, and may contribute further to the effect.

Key Words: Ah receptor (AhR); 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD); zebrafish; embryo; developmental toxicity; craniofacial; jaw; cardiovascular; blood flow; cartilage.

Halogenated aromatic hydrocarbons (HAHs), including polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), and dibenzofurans (PCDFs) are persistent, bioaccumulative toxicants that are widely distributed in the environment (Safe, 1994). These chemicals have caused congenital malformations such as cleft palate and hydrenephrosis in mice, heart anomaly and circulation failure with edema in chicks, and alterations in male and female reproductive tract development and function in rats (Abbott, 1997; Peterson et al., 1993). Fish embryos are also sensitive to HAH developmental toxicity with lake trout being the most sensitive fish species. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), the prototype and most potent HAH, has been investigated for its ability to cause developmental toxicity in early life stages of several fish species. In embryos of all of these fish species, TCDD exposure caused a reduction in peripheral RBC perfusion rate, edema, craniofacial malformations, growth retardation, and death (Walker and Peterson, 1994). Among these various signs of toxicity, circulation failure, edema, and craniofacial malformations characterized by impaired lower jaw growth are observed earliest in development, but their etiology remains to be determined. Hornung et al. (1999) studied the time course of these effects in early life stages of rainbow trout exposed to TCDD and suggested the possible involvement of circulation failure as a cause of edema and craniofacial malformations in the embryos of rainbow trout. However, they did not measure the reduction in blood flow or lower jaw growth observed in TCDD-treated trout embryos, quantitatively.

Zebrafish (Danio rerio) has been extensively used in developmental biology for 10 years. They have several advantages as a vertebrate toxicological model. They produce many embryos of high quality. They develop rapidly and hatch as early as 2 days after fertilization. They have a transparent body and adequate for the observation of internal organs by conventional microscopy in early stages of development. They are applicable for both forward and reverse genetics. Several thousand mutant lines are established and available (Haffter et al., 1996). Zebrafish embryos have further advantages as a toxicological model because there is a wealth of information on their genetics and developmental biology. Also zebrafish embryos are...
responsive to TCDD developmental toxicity (Henry et al., 1997) and the aryl hydrocarbon receptor (AhR) and its dimerization partner the aryl hydrocarbon receptor nuclear translocator (ARNT) have been cloned and functionally characterized in this species (Tanguay et al., 1999, 2000, 2001). In the present study, we examined, by quantitative measurement, the lower jaw growth and RBC perfusion rate as an index of local blood flow in zebrafish embryos exposed to TCDD.

MATERIALS AND METHODS

**Chemicals.** 2,3,7,8-Tetrachlorodibenzo-p-dioxin (99% purity) was purchased from Cambridge Isotope Laboratories, Inc. The other chemicals were obtained from Wako Pure Chemical (Japan) or Sigma (St. Louis, MO) unless otherwise indicated.

**Zebrafish embryos and waterborne TCDD exposure.** According to Westerfield (1993), fertilized embryos were obtained from natural mating of adult zebrafish (AB line) originally derived from the University of Oregon, Eugene, OR. Adult and juvenile fish were maintained at 28.5°C in a 14-h light and 10-h dark cycle. Newly fertilized embryos were exposed to TCDD dissolved in 1/3 Ringer solution (Zebrafish Ringer solution, ZR solution; Dong et al., 2001) in petri dishes at 28.5°C. Usually 10 embryos were kept in 3 ml of ZR solution in a 3-cm plastic petri dish (Falcon). However, for assessment of mortality, embryos were kept individually in 1 ml of ZR solution in an individual well of a 24-multiwell dish (Falcon). This was done to exclude potential harmful effects of decaying dead embryos on those that were still alive. Unless otherwise noted, embryos were continuously exposed to the waterborne vehicle (0.1% DMSO) or to graded concentrations of waterborne TCDD (0.1–1.0 ppb). The exposures started at 24-h postfertilization (hpf) and continued until the time of observation. The composition of ZR solution was as follows: 38.7 mM NaCl, 1.0 mM KCl, 1.7 mM HEPES (pH 7.2), and 2.4 mM CaCl2. Three µl of TCDD stock solution in dimethylsulfoxide (DMSO) was directly added to a 3-cm petri dish containing 3 ml of ZR solution. Thus, the final concentration of the DMSO vehicle was usually 0.1% regardless of TCDD concentration.

**Measurement of lower jaw growth.** Live embryos from 48 to 120 hpf were observed microscopically in 3% carboxymethyl cellulose sodium sulfate (CMC-ZR) solution after being anesthetized with FA100 (Tanabe, Japan) or MS 222 (Sigma). The length of the lower jaw was determined with an oculometer. We used the anterior edge of the eye as a reference point.

**Alcian-blue staining.** Zebrafish embryos were stained with alcian blue according to the method of Kelly and Bryden (1983). 10% Neutral formalin-fixed embryos were stained with 0.1% alcian blue 8GX/80% ethanol/20% acetic acid for 6 h. After a series of washes with 75% and 50% ethanol/PBS each for 1 h, embryos were incubated with PBS overnight. For clarification, embryos were treated with 1% KOH/3% H2O2 for 20 h, followed by digestion with 0.05% trypsin/saturated tetraborate for 1 h. Stained embryos were preserved and observed in 80% glycerol solution.

**In situ hybridization.** Whole mount in situ hybridization was carried out according to Barth and Wilson (1995). Four percent paraformaldehyde (PFA)-fixed embryos were treated with proteinase K (Sigma) in PBS with 0.1% Tween (PVT). After incubation with hybridization buffer (Hyb [−]) containing 50% formamide, 5× SSC, 2 mg/ml Torula RNA (Sigma), and 200 µg/ml heparin (prehybridization) (Hyb [+]), embryos were hybridized with antisense probes of zebrafish goosecoid (gsc) or zfAhR2 at 65°C overnight. To obtain the zfAhR2 probe, cDNA was cloned by PCR according to Tanguay et al. (1999). cDNA for gsc was a gift from S.W. Wilson. Following hybridization, embryos were washed with 2× SSC and 0.2× SSC for 30 min, twice, at 65°C, respectively. After blocking with a blocking buffer containing 2% blocking reagent (Roche, Germany) in 100 mM maleic acid (pH 7.5) and 150 mM NaCl, embryos were incubated with 1:4000 diluted anti-DIG antibody conjugated with alkaline phosphatase (Roche) at 4°C overnight. The color reaction was carried out by incubation in BM-purple substrate or Fast Red (Roche) after equilibration with NTMT buffer (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, and 50 mM MgCl2) at room temperature for 15 min. In the case of zfAhR2, 0.003% phenylthiourea (Sigma) was included in ZR solution from 24 hpf to prevent pigmentation of the embryos. Pigments of embryos with gsc signals were bleached by 6% H2O2 for 2 h. After PFA fixation, the stained embryos were cleared in 70% glycerol and used for observation. In some experiments, stained embryos were sectioned after embedding in paraffin.

**Measurement of RBC perfusion rate.** As an index of local circulation, RBC perfusion rate was evaluated by time-lapse recording using a digital video camera (DVL-700, Victor, Japan) connected to a CCD camera (MKC-385, Ikegami, Japan). Embryos were observed in 200 µl of 3% carboxymethyl cellulose/ZR solution in a bath on the stage of an inverted microscope (Olympus, Japan). The temperature was maintained at 28.5°C with a PDM-2 Micro-incubator (Medical Systems). Nomenclature for the vessels of zebrafish embryos was that of Weinstein et al. (http://www.dir.nichd.nih.gov/lmg/uvo/WEINSLAB.html).

**Statistics.** Results are presented as mean ± SEM. Significance was determined by one-way ANOVA followed by the Bonferroni/Dunn test (p < 0.05).

RESULTS

**Retardation of Lower Jaw Growth**

Zebrafish embryos were exposed statically to waterborne TCDD from 24-h postfertilization (hpf) until the time of observation. TCDD (0.1–1 ppb) did not affect hatching and mortality at 96 hpf. At waterborne concentrations of 0.5 and 1 ppb TCDD, there was a slight increase in mortality at 120 hpf, but at 0.3 ppb there was no effect. The pattern of these results is similar to Henry et al. (1997), who observed that TCDD at 7.4 and 4.7 ng/g embryo but not at 3.1 ng/g embryo caused a significant increase in mortality before 148 hpf.

Figure 1 shows representative photographs of live embryos treated with vehicle (control) or TCDD. Effects of TCDD are shown on total length of the body in Figure 2A and on lower jaw length in Figure 2B, respectively. Total body length was not markedly affected by TCDD. TCDD at an exposure level of >0.3 ppb caused a slight but significant reduction in total body length at 72–84 hpf, whereas TCDD at 0.1 ppb did not have any effect (Fig. 2A). The jaw of zebrafish embryos appeared from 48 hpf and grew gradually to reach the anterior edge of the eye after around 72 hpf (protruding mouth; Figs. 1 and 2). Positive value means that the mouth is placed in front of the anterior edge of the eye and negative value means that the mouth is placed behind the anterior edge of the eye (Fig. 2, inset photos). Since TCDD exposure caused a slight reduction in total body length, each value for lower jaw length (Fig. 2B) is indicated as a percentage of total body length. In examining Fig. 2B, it can be seen that TCDD reduced lower jaw growth as early as 60 hpf. This inhibitory effect was dependent on the waterborne concentration of TCDD. TCDD concentrations of 0.3, 0.5, and 1.0 ppb exerted significant effects that were first detected at 84, 72, and 60 hpf, respectively. On the other hand, 0.1 ppb TCDD did not have any effect until 120 hpf (Fig. 2B).
These results suggest that the effects of TCDD were lower jaw-specific and not merely secondary to a reduction in embryo growth.

We performed whole-mount, in situ hybridization for goosecoid (gsc), whose mRNA expresses early in certain cartilage elements including Meckel’s cartilage (Schulte-Merker et al., 1994). TCDD reduced the area of gsc mRNA expression in Meckel’s cartilage primordia at 60 hpf, although it did not affect gsc expression at 48 hpf (Fig. 3). In later stages of zebrafish development, cartilage elements in the head were stained with alcian blue (Fig. 4). It was found that TCDD affected chondrogenesis in the head. TCDD shortened the length of Meckel’s cartilage and caused a slightly wider angle against the ethmoid plate of the upper jaw. These inhibitory effects did not appear to be restricted to cartilage components of the lower jaw, but to all cartilage in the head, albeit to a lesser extent than Meckel’s cartilage. Also, the ceratobranchial cartilage lost their angles with the opposite ones in later stages (Figs. 4G and 4H). Thus, TCDD did not abolish the generation of the various cartilage components in the head. Instead it caused an inhibitory effect on cartilage growth, especially in Meckel’s cartilage.

To investigate a possible critical period of exposure for the effects of TCDD to be manifested, we treated embryos with TCDD beginning at progressively later times after fertilization (0, 12, 24, 36, 48, 60, 72, and 84 hpf) and assessed lower jaw length at the fixed time of 96 hpf. When embryos were exposed to TCDD either at 0 or 24 hpf, the extent of the inhibitory effect of TCDD on lower jaw length did not change significantly. However, the magnitude of the inhibitory effect decreased gradually with successively later times of TCDD exposure from 24 to 84 hpf (Fig. 5). Thus, the inhibitory effect of TCDD on lower jaw growth did not have a critical period. Because zebrafish neural crest cells, which play a key role in lower jaw formation as well as in other primordia formation, finish migration within 24 hpf (Akimenko et al., 1994), the migration process of neural crest cells might not be a target of TCDD developmental toxicity.

TUNEL or acridine-orange staining of 1 ppb TCDD-treated embryos at designated times from 48 – 96 hpf did not reveal any apoptotic cells in the jaw primordia (data not shown), suggesting that the shortened lower jaw is probably not the result of apoptosis or necrosis but rather growth retardation.

Local Circulation Failure Induced by TCDD

The reduction of RBC perfusion rate in various vascular beds was consistently observed in TCDD-exposed embryos of many fish species including zebrafish (Henry et al., 1997; Hornung et al., 1999; Walker and Peterson, 1994). However, in all cases these have been subjective measurements of the RBC perfusion rate that was scored by the observer. In the present study, local circulation was quantitatively evaluated by counting the number of RBCs passing through certain vessels with time-lapse recording. Before RBC perfusion rate was measured, we assessed effects of TCDD exposure on heart rate and pericardial edema. Regardless of the concentration of TCDD used (0.3 – 1 ppb), heart rate did not change until 96 hpf when most of the embryos showed severe pericardial edema (Fig. 1E). In control embryos, pericardial edema was not observed. After treatment with 1 ppb TCDD, pericardial edema was first observed in 35% of the embryos at 60 hpf and increased gradually to reach almost 100% of the embryos at 96 hpf (Fig. 6). A significant finding was that the heart was flattened under pressure of the extensive pericardial edema by 96 hpf.

For all vessels of the body, no circulation was observed at 36 hpf except around the heart (Figs. 7A – 7D). In intersegmental arteries, vigorous blood flow was detected at 50 hpf and was maintained at least until 96 hpf (Fig. 7A). Although TCDD at all concentrations tested failed to reduce RBC perfusion rate in the intersegmental arteries at 60 hpf, at TCDD concentrations of 0.3 ppb and higher, RBC perfusion rate in the intersegmental vessels was markedly reduced at 72, 84, and 96 hpf. As for other trunk vessels such as dorsal aorta and posterior cardinal vein, RBC perfusion rate decreased very similarly to that in the intersegmental arteries, although we did not evaluate it by video recording. We did evaluate RBC perfusion rate in 2 vessels in the head region. One was the central artery that

FIG. 1. Representative zebrafish embryos exposed to vehicle (control) or TCDD. Embryos were exposed statically to ZR solution containing 0.1% DMSO or a TCDD concentration of 1 ppb starting at 24 hpf until observation at 60 hpf (A and B) and 84 hpf (C–F). Arrows (A and B) show the mouth; (E) arrow shows pericardial edema, respectively. (A), (C), and (D) are control and (B), (E), and (F) are TCDD-treated embryos. (A and B), ventral view; (C and E) lateral view; (D and F), dorsal view. Bar = 200 μm.
branched from the primordial hindbrain channel at the level of the otic vesicles and perfused the anterior hindbrain region. The other is the optic vein that collected blood perfusing the basal brain and optic region. As shown in Fig. 7B, TCDD did not affect RBC perfusion rate in the central artery at any time up to 60 hpf. After that, however, circulation was significantly inhibited in the central artery. The optic vein did not show a significant retardation of RBC perfusion rate in TCDD-exposed embryos up to 72 hpf (Fig. 7C). Since measurement of optic vein blood flow was very difficult after 80 hpf because of frequent jaw movements by the embryo, it was not measured beyond 72 hpf. The hypobranchial artery is the main vessel branching at the lower jaw primordia after 60 hpf. Unexpectedly RBC perfusion rate in this vessel was markedly increased by TCDD treatment from 72 to 84 hpf (Fig. 7D). At 96 hpf, however, RBC perfusion rate in this artery also was reduced by the 2 highest TCDD concentrations. Taken together, these observations suggest that blood flow, in all vessels studied, was not affected by TCDD at 60 hpf. At 72 hpf, RBC perfusion rate was decreased in several vessels with the notable exception of the hypobranchial artery, where it was increased. Significantly, when lower jaw chondrogenesis was first significantly inhibited by TCDD at 60 and 72 hpf (Fig. 2B), blood flow in the hypobranchial artery was either unaffected or increased (Fig. 7D).

AhR mRNA Expression in the Lower Jaw Primordia

Two forms of zebrafish AhR (zfAhR1 and zfAhR2) have been cloned and functionally characterized (Tanguay et al., 1999, 2001; Wang et al., 1998). Since zfAhR2 is functional in TCDD-induced AhR signaling, whole-mount in situ hybridiza-
tion of zfAhR2 was performed to study the spatial and temporal patterns of its mRNA expression. The zfAhR2 message was strongly expressed in the lower jaw primordia, head, pectoral fin bud, and other branchiogenic primordia (Fig. 8). Comparison of mRNA expression of zfAhR2 and gsc showed that zfAhR2 expression included gsc expression in Meckel’s cartilage, suggesting that expression of zfAhR2 protein may occur in chondroid tissue. This was confirmed by sectioning embryos prepared for whole-mount in situ hybridization of zfAhR2 (Fig. 8D). zfAhR2 mRNA expression was observed from 30 hpf onward. Also contrary to mammalian species, zfAhR2 mRNA expression was enhanced by TCDD exposure, supporting the Northern-blot results of Tanguay et al. (1999).

FIG. 4. Alcian-blue staining of zebrafish embryos exposed to vehicle (control) or TCDD. Embryos were exposed to vehicle or 1 ppb TCDD starting at 24 hpf and fixed at 72 hpf (A–D) and 96 hpf (E–H). (A, C, E, and G), representative vehicle controls; (B, D, F, and H), representative TCDD-exposed embryos. (A, B, E, and F), lateral views; (C, D, G, and H), ventral views. Abbreviations: cb (I–V), first to fifth ceratobranchial; ch, ceratohyal; ep, ethmoid plate; mk, Meckel’s cartilage; pf, pectoral fin; pq, palatoquandrate; t, trabeculae cranii. Bar = 400 \( \mu \)m.

FIG. 5. Inhibitory effects of TCDD exposure on lower jaw growth at various onsets of exposure. Embryos were treated with a concentration of 1 ppb TCDD, starting at approximately 12-h intervals from 0–84 hpf as revealed on the abscissa. Relative lower jaw length was determined at 96 hpf as described in the Figure 2 legend. Each symbol represents the mean ± SEM of 10 embryos.

DISCUSSION

Lower Jaw Development

Many reports have described the adverse effects of TCDD on the circulation and craniofacial development characterized by lower jaw growth retardation in embryos of various fish species. However, most of the studies provided only subjective data for these effects. The present experiments provide the first quantitative measurements of lower jaw growth and blood flow around the lower jaw primordia of zebrafish embryos during early development. We found that the effects of TCDD on jaw growth and blood flow could be dissociated. First, TCDD significantly retarded lower jaw growth beginning at 60 hpf in embryos exposed to 1 ppb TCDD and beginning at 72 hpf in

FIG. 6. Time course of development of pericardial edema in zebrafish embryos exposed to TCDD. Embryos were exposed to 1 ppb TCDD starting at 24 hpf until the time indicated on the abscissa when they were fixed for conventional histology. Each column represents the percent of TCDD-exposed embryos that developed pericardial edema (n = 20). Control embryos did not exhibit edema throughout the experiment.
those exposed to 0.3 and 0.5 ppb TCDD. Yet local circulation in the head was not inhibited by these concentrations of TCDD before 72 hpf. In the hypobranchial artery, a major vessel that perfused the lower jaw primordia after 60 hpf TCDD exposure, increased RBC perfusion rate until 84 hpf. We do not know why RBC perfusion rate in this vessel was increased by TCDD. It might be a compensatory response for retarded lower jaw growth, or a result of interrupted local circulation in other vessels. Also, vessels perfusing the lower jaw are near the heart, and this location may compensate for the inhibitory effects of TCDD exposure on RBC perfusion rate in other tissues. The increased RBC perfusion rate in this branch of the hypobranchial artery is quite different from the early circulation failure caused by TCDD exposure in the intersegmental arteries, where only inhibition of RBC perfusion rate is seen. Second, even though a TCDD concentration-dependent inhibition of lower jaw growth (0.3 to 1 ppb TCDD) was observed at 60–72 hpf, it is significant that this was not accompanied by a TCDD concentration-related decrease in RBC perfusion rate to this region. Third, despite only 35% of the TCDD-exposed embryos showing signs of pericardial edema at 60 hpf, all of the embryos in the 2 highest TCDD exposure groups were similarly affected by reduced lower jaw growth. When all of these findings are considered, they suggest that the mechanism by which TCDD disrupts lower jaw growth is independent of circulation failure to the lower jaw at least from 60 to 72 hpf. Circulation failure should have preceded the jaw growth retardation for the period required for chondrogenesis, if it was the primary factor responsible for the retarded lower jaw growth. Hornung et al. (1999) found decreased RBC perfusion rate in various vessels including the hypobranchial artery, which they called the mandibular vessel, a reduction in heart size and retardation of lower jaw growth in TCDD-treated rainbow trout embryos. Their results suggested a possible relationship between decreased blood flow in the hypobranchial artery and jaw growth retardation in trout. Henry et al. (1997) reported that RBC perfusion rate was reduced in zebrafish embryos at

FIG. 7. Effects of TCDD exposure on RBC perfusion rate in different blood vessels of the zebrafish embryo. Embryos were treated with graded concentrations of waterborne TCDD (0.3–1 ppb) from 24 hpf until the time indicated on the abscissa. The number of RBCs that passed through a vessel per 15 s were counted in the last intersegmental artery (A), central artery (B), optic vein (C), and hypobranchial artery (D). Each symbol represents the mean ± SEM of 10 embryos. Asterisks show significant difference from control (p < 0.05).

FIG. 8. Expression of zfAhR2 mRNA in the lower jaw primordia of the zebrafish embryo. Following exposure to 1 ppb TCDD starting at 24 hpf, embryos were fixed for whole-mount in situ hybridization at 48 hpf. Views are dorsal (A), ventral (B), and lateral (C). (D), image of a section of a 48 hpf embryo prepared for whole-mount in situ hybridization of zfAhR2. The section was counter-stained by eosin. For all panels, arrows indicate zfAhR2 mRNA expression in the lower-jaw primordia. The arrowhead (C) indicates the heart ventricle. Bar = 300 μm.
77 hpf and that the magnitude of the reduction in RBC perfusion rate was severe and occurring in all vessels examined at 96 hpf. This latter result was confirmed by the present study. Finally, as suggested by Henry et al. (1997), circulation failure could contribute to the disruption in jaw development after 96 hpf.

Expression of AhR and CYP1A

AhR mRNAs were strongly expressed in the lower jaw primordia during its extensive growth. The AhR transcript expression area covers Meckel’s cartilage and can be detected as early as 30 hpf, which is before the general circulation failure that occurs in TCDD-exposed embryos. Therefore, TCDD may act directly to impair chondrogenesis in lower jaw primordia via an AhR-dependent mechanism that is independent of circulation failure. AhR expression in the lower jaw primordia has also been reported in mammals including human (Abbott et al., 1994, 1998). Also CYP1A mRNA and protein were expressed constitutively in the lower jaw primordia of control fathead minnow and other fish species including zebrafish, and was markedly induced by TCDD in all of these species (Iwata and Stegeman, 2000; Teraoka et al., unpublished observations).

Edema and Local Circulatory Failure

The mechanisms involved in TCDD cardiovascular toxicity in the zebrafish embryo remain to be determined. Pericardial edema occurred in 35% of TCDD-exposed embryos at 60 hpf, but at this time RBC perfusion rate in the vessels examined in the present study did not change, and this also appears to include the cardinal vessels. A marked reduction of RBC perfusion rate was first detected at 72 hpf in vessels of the trunk. However, whether the early edema is a primary event or is secondary to reduced trunk blood flow remains to be determined. The magnitude of RBC perfusion rate reduction was different for different vessels, but the reason for this differential effect of TCDD on blood flow at 72 hpf is unknown. It may be that TCDD exposure produces differential effects on endothelial cells in different blood vessels of the zebrafish embryo. Cantrell et al. (1996, 1998) reported a possible relationship between CYP1A expression and TUNEL signals in endothelial cells in the yolk sac vein of medaka embryos exposed to TCDD. However, Guiney et al. (2000) failed to find evidence of enhanced apoptosis of vascular endothelial cells in lake trout embryos but found instead a slight gap between endothelial cells, suggesting an increase in vascular permeability. In cultured porcine endothelial cells, exposure to the AhR agonist PCB 77 augmented albumin permeability, but only produced apoptosis when endothelial cell cultures were treated with PCB 77 and a glutathione synthesis inhibitor, buthionin-sulfoxamine (Slim et al., 2000; Toborek et al., 1995). In TCDD-exposed zebrafish embryos, the cause of the severely reduced RBC perfusion rate that occurs in all vessels later in development at 96 hpf, is probably related to decreased cardiac output caused by the progressively extensive pericardial edema that severely flattens the heart. A reduction of circulating RBCs in TCDD-exposed zebrafish and Xenopus embryos has also been reported to occur and would further enhance the hypoxic insult caused by the circulatory failure at 96 hpf (Belair et al., 2001; Sakamoto et al., 1997). The progressive circulatory failure in zebrafish embryos exposed to TCDD could also be due to an effect on the heart. In the chick embryo heart, AhR and ARNT are expressed (Walker et al., 1997) and CYP1A is induced by AhR agonists (Gannon et al., 2000). TCDD causes cardiac malformations in the chick embryo (Cheung et al., 1981; Walker et al., 1997; Walker and Catron, 2000) as well as functional changes in cardiac myocytes of TCDD-treated chick embryos (Canga et al., 1988). Therefore, extensive studies of TCDD effects on the embryo heart are also needed to understand the mechanism of the TCDD-induced circulatory failure in zebrafish, especially for the later stage.

ACKNOWLEDGMENTS

This work was supported by grants from the Japanese Ministry of Education, Science, Sports and Culture, Hokkaido Foundation for the Promotion of Scientific and Industrial Technology, and Kurabayashi Foundation. We deeply thank the late Professor N. Holder and Dr. S. W. Wilson for providing technical training in UCL.

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