Malformation of some brain blood vessels caused by TCDD activation of Ahr2/Arnt1 signaling in developing zebrafish

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) causes various signs of toxicity in early life stages of vertebrates through activation of the aryl hydrocarbon receptor (Ahr). The AHR also plays important roles in normal development in mice, and $AHR^{-/-}$ mice show abnormal development of vascular structures in various blood vessels. Our previous studies revealed that Ahr type 2 (Ahr2) activation by TCDD and β-naphthoflavone (BNF) caused a significant decrease in blood flow in the dorsal midbrain of zebrafish embryos. Here we report effects of TCDD exposure on the morphology of some blood vessels in the head of developing zebrafish. TCDD caused concentration-dependent anatomical disarrangements in the prosencephalic artery in zebrafish larvae. In contrast, no major vascular defects were recognized in the trunk and tail regions following exposure to TCDD at least at the concentrations used. Essentially, the same observations were also confirmed in BNF-exposed larvae. Knock-down of either Ahr2 or Ahr nuclear translocator type 1 (Arnt1) by morpholino oligonucleotides (MOs) protected larvae against abnormal prosencephalic artery formation by TCDD and BNF. On the other hand, knock-down of Ahr2 or Arnt1 in vehicle-exposed control zebrafish larvae showed no clear effect on the morphology of the prosencephalic artery or trunk vessels. Ascorbic acid, an antioxidant, protected against the TCDD-induced decrease in blood flow through the prosencephalic artery, but not the abnormal morphological changes in the shape of this artery. These results indicate that activation of Ahr2/Arnt1 pathway by TCDD and to a lesser extent by BNF affects the formation of certain blood vessels in the brain of developing zebrafish.

Keywords:

antioxidant, circulation failure, *Danio rerio*, developmental toxicology**,** TCDD, vascular formation

1. Introduction

Fish embryos are among the most sensitive organisms to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity. Exposure of fish larvae to TCDD causes cardiovascular toxicities such as edema and circulation failure, craniofacial malformation, and growth retardation resulting in mortality (Walker and Peterson, 1994; Henry et al., 1997; Teraoka et al., 2002). Among these, the cardiovascular system is one of the most characteristic and important targets in developmental toxicity by TCDD and by polycyclic aromatic hydrocarbons in a variety of fish larvae (Guiney et al., 1997; Cantrell et al., 1996; 1998; Wassenberg and Di Giulio, 2004). In zebrafish (*Danio rerio*), a model fish for environmental toxicology (Teraoka et al., 2003a; Hill et al., 2005), TCDD exposure disrupts heart development with a reduction in the number of cardiac myocytes, a reduction in cardiac output, and hemorrhage (Teraoka et al., 2002; Antkiewicz et al., 2005; 2006). The cardiovascular system is a common target of TCDD also in other vertebrates, including rodents and chick, which show edema, hemorrhage and heart malformation upon exposure (Ishimura et al., 2009).

It is well established that TCDD binds the AHR, a ligand-activated basic-helix-loop-helix transcription factor, and the complex further dimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT) to induce the expression of a battery of genes (the AHR gene battery), including cytochrome P4501A (CYP1A) (Nebert et al., 2000). Studies in AHR knock-out mice ($AHR^{-/-}$ mice) have established that TCDD causes various developmental toxicities by way of AHR activation (Fernandez-Salguero et al., 1996; Mimura et al., 1997). Whereas mammalian species have a single AHR,

there are multiple Ahr isoforms in teleosts, including zebrafish, which has Ahr1a, Ahr1b and Ahr2 (Hahn et al., 1997; Karchner et al., 2005). Knock-down studies with morpholino antisense oligonucleotides (MOs) indicate that pericardial edema as well as other endpoints of TCDD toxicity is mediated by Ahr2 and Arnt1 in zebrafish (Antkiewicz et al., 2006; Prasch et al., 2003; Teraoka et al., 2003b).

In addition to adaptive responses to environmental xenobiotics, AHR plays important roles in normal development in mice, and $AHR^{-/-}$ mice show abnormal development of some vascular structures. $AHR^{-/-}$ mice exhibit portocaval shunting of blood within the liver parenchyma (Lahvis et al., 2000; Walisser et al., 2005). In adult $AHR^{-/-}$ mice, more than half of the portal blood that flows to the liver bypasses the liver sinusoids (Lahvis et al., 2000), suggesting the role of AHR in vasculogenesis in rodents.

Previously, we reported that TCDD causes a transient decrease in blood flow through the mesencephalic vein by about 50 hpf, which precedes the occurrence of pericardial edema, and that the associated ischemia is a possible cause of TCDD-induced apoptosis in the dorsal midbrain (Dong et al., 2001; 2002). Using an Ahr2-MO knock-down approach, Dong et al. (2004) suggested that Ahr was involved in mesencephalic circulation failure. Mesencephalic circulation failure caused by TCDD is also sensitive to various chemicals, such as antioxidants, inhibitors of CYP or cyclooxygenase 2 (COX2), and thromboxane receptor (TP) antagonists, suggesting the involvement of oxidative stress, CYP and the prostaglandin pathway (Dong et al., 2002; Teraoka et al., 2009).

During a survey of mesencephalic circulation in zebrafish embryos, we recognized that some vessels in the head showed morphological variation in some

embryos exposed to TCDD. In the present study, we report that disarrangement of certain blood vessels in the brain is caused by TCDD and BNF, and that this involves activation of the Ahr2/Arnt1 pathway in developing zebrafish.

2. Materials and methods

2.1. Chemicals

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from Cambridge Isotope Laboratories (98% purity: Andover, MA); β-naphthoflavone (BNF) was purchased from Sigma (St. Louis, MO). Other chemicals were obtained from Kanto Chemical (Japan).

2.2. Zebrafish and TCDD treatment

Fertilized eggs were obtained from natural mating of adult zebrafish (long fin) in our laboratory according to the *Zebrafish Book* (Westerfield, 1993). Adult fish and developing fish were maintained at 28.5 ^{o}C with a lighting schedule of 14 hr light and 10 hr dark. Eggs were collected within 1 hr of spawning, rinsed, and placed into a clean petri dish. At 24 hr after spawning, fertilized embryos were exposed to either the TCDD vehicle, dimethyl sulfoxide (DMSO, 0.1%) or an apparent concentration of waterborne TCDD of 0.3 to 2.0 parts per billion (ppb) dissolved in 0.1% DMSO in 3 ml of Zebrafish Ringer solution (38.7 mM NaCl, 1.0 mM KCl, 1.7 mM HEPES-NaOH pH 7.2, 2.4 mM $CaCl_2$) in 3 cm polystyrene petri dishes (Asahi Techno, Japan) until 48 hpf (hr post fertilization; n = 10 embryos/dish). After that, developing fish were maintained in

Zebrafish Ringer solution until evaluation of vascular structure at 55 hpf. When the effect of antioxidant was investigated in some experiments, 10 mM ascorbic acid was included in the Zebrafish Ringer solution together with TCDD.

2.3. Knock-down with morpholino antisense oligonucleotides

Morpholino antisense oligonucleotides (MOs) against translation of Ahr2 (Ahr2-MO), and their respective negative controls having four different nucleotides (4mis-Ahr2-MO), were synthesized by Gene Tools (Philomath, OR), as described previously (Teraoka et al., 2003b). Translational inhibition type of MO against Arnt1 (Arnt1-MO) was used as previously reported (Prasch et al., 2006). Each morpholino was injected into the yolk of embryos at one to four cell stages with a fine glass needle connected to an automatic injector (IM-300: Narishige, Japan). 2 nL of 50 μM MOs in Ca^{2+}-free Zebrafish Ringer solution were injected.

2.4. Evaluation of vascular morphology and blood flow

Using inverted microscopy (IX71, Olympus, Japan), blood flow in vessels in the head and other regions of zebrafish larvae were examined at 55 hpf by monitoring red blood cells passing through them. Some larvae were subjected to microangiography to visualize blood vessels under fluorescent microscopy (IX71, Olympus), just after injection of fluorescent polystyrene microspheres (FluoSpheres, Invitrogen, Carlsbad, CA) into the sinus venosus (Isogai et al., 2001). Blood flow in the prosencephalic artery was evaluated by time-lapse recording using a high-speed digital video camera (LRH1601BL, Digimo, Osaka, Japan), as originally described for measuring blood flow

in the mesencephalic vein (Teraoka *et al*., 2002). Larvae were suspended in 200 μL of 3% methyl cellulose/Zebrafish Ringer solution in a hand-made plastic bath mounted on the stage of an inverted microscope (IMT-2: Olympus, Japan). Temperature of the suspension solution was maintained at 28.5ºC with a PDMI-2 Micro-Incubator (Harvard Apparatus, Holliston, MA).

2.5. Statistics

Results are presented as mean ± SEM. Significance of differences between groups was determined by Tukey-Kramer's test ($p < 0.05$).

3. Results

3.1. Abnormal brain blood vessel morphology induced by TCDD exposure

We observed striking TCDD-induced alterations ~~also~~ in the prosencephalic artery at first. At 55 hpf, two types of prosencephalic arteries were observed in control larvae; i.e., one with a typical arch (Typical; Fig. 1A, B) and the other with a meandering arch (Meandering; Fig. 1C, D). Prominent abnormal shapes of the prosencephalic artery found in TCDD-exposed larvae included a vessel with a split arch with both parts ending the anterior cerebral vein (Split arch; Fig. 1E, F) and a vessel with new branch ending an unspecified blood vessel other than the anterior cerebral vein (New vessel; Fig. 1G, H). In addition, prosencephalic arteries with a small arch (Small arch) or that end at the prosencephalic artery on the opposite side (Opposite end), were also observed

in the TCDD-exposed group (Fig. 2).

At 55 hpf, control zebrafish had developed a beautifully arched mesencephalic vein (Fig. 1I, J), while some control zebrafish exhibited a mesencephalic vein with a curved arch (Curved; Fig. 1K, L). Different shapes of mesencephalic veins were observed in zebrafish exposed to TCDD (1 ppb). These include a vein with a sigmoid arch (Sigmoid) as compared to the curved arch in a normal mesencephalic vein (Fig. 1M, N), and a vein with a new branch ending at different region (New vessel; Fig. 1O, P). Mesencephalic veins with these shapes were never seen in control larvae and are regarded as abnormal.

The morphology of other vessels in the head was more complex and could not be rigorously assessed for effects of TCDD.

In contrast to vasculature in the head region, we could not recognize clear alterations in the arteries and veins in the trunk and tail region, including dorsal aorta, caudal artery and vein, intersegmental artery and vein and posterior cardinal vein, at least at the concentrations of TCDD used in this study. In a few cases, however, a relatively smaller arch in the posterior cardinal vein and some alteration such as bifurcation with the same ending in intersegmental vessels were observed.

3.2. Concentration-dependent effects of TCDD on prosencephalic artery

In our preliminary experiments, the incidence of abnormal morphological changes caused by TCDD in the mesencephalic vein was relatively low and there was no clear TCDD concentration dependency. Thus we focused on the effects of exposure to graded concentrations of TCDD on the morphological changes observed in the prosencephalic

artery.

Fig. 2 shows the percent incidence of each type of prosencephalic artery morphology observed in control and TCDD-treated larvae at 55 hpf (N=81-193 for each concentration of TCDD). The incidence of each type of artery abnormality was increased by TCDD exposure in a concentration-dependent manner. Generally, there was no preferential increase among the four types of abnormal artery formed. On the other hand, Meandering prosencephalic artery, one of the normal types observed in control larvae, was increased in frequency, instead of a gradual decrease of frequency of the typical artery shape caused by TCDD.

Typical and Meandering were regarded as normal, and Opposite end, Split arch, New vessel and Small arch caused by TCDD were counted as abnormal in Figs. 2 and 3. In assessing the occurrence of these abnormal prosencephalic arteries, we used 18-20 larvae for each treatment group to determine the percentage of malformation (Fig. 3). We repeated the experiment five **to sixteen** times, and the mean and SEM were calculated for statistical analysis. As shown in Fig. 3, TCDD at doses from 0.3 to 2 ppb resulted in a distinct concentration-dependent increase in abnormal vessel formation in developing zebrafish larvae at 55 hpf.

3.3. Effects of BNF on brain blood vessel morphology

The effects of β-naphthoflavone (BNF), another AHR agonist, on vascular formation were studied. As shown in Table 1, BNF (0.1-1.0 μM) caused the same types of malformations in prosencephalic artery as were caused by TCDD. However, BNF was less efficacious than TCDD in causing morphological changes in the artery (Table

2). The highest BNF concentration used caused a 19% incidence of prosencephalic artery malformation compared to 44% when TCDD was used (Table 2 and Fig. 3). BNF also induced abnormalities in the mesencephalic vein, producing new vessel and sigmoid arch deformities, in addition to the loss of blood flow through the vein. Alterations in the morphology of trunk and tail vessels were not observed in BNF-exposed larvae. Thus, BNF caused similar malformation responses to TCDD in the mesencephalic vein and prosencephalic artery, qualitatively.

3.4. Effects of morpholino knock-down of Ahr2 and Arnt1

Knock-down of Ahr2 translation with antisense morpholino oligonucleotides was carried out to address possible roles for Ahr in normal brain blood vessel formation, as well as in abnormalities of the brain vasculature induced by TCDD and BNF. First, the effect of Ahr2 knock-down (Ahr2-KD) in vehicle-exposed larvae was studied. We examined 203 control larvae injected with Ahr2-MO at a concentration that strongly blocked the circulation failure in TCDD-exposed larvae (Prasch et al., 2003; Dong et al., 2004). Alterations were rarely observed in these vehicle-exposed larvae in morphology of the prosencephalic artery, mesencephalic vein or other vessels in the brain, trunk and tail. A few changes in mesencephalic veins and prosencephalic arteries were seen in Ahr2-KD larvae (Ahr2 morphant), i.e., there were 3 larvae with a loss of blood flow in the mesencephalic vein and 2 larvae having either a new vessel or a split arch in the prosencephalic artery. A similar very low incidence of malformation was also observed in larvae injected with the negative Ahr2-MO homologs (4mis-Ahr2-MO, 4mis-MO) in a similar ratio (1/178 and 2/178 for mesencephalic vein and prosencephalic artery,

respectively), suggesting a non-specific effect of these MO molecules, rather than an effect due to knock-down of Ahr2.

Fig. 4 indicates the effects of Ahr2-KD on disarrangement of the prosencephalic artery by TCDD. Ahr2-KD markedly inhibited abnormal vessel formation by both concentrations of TCDD tested (0.5 and 1 ppb), while 4mis-Ahr2-MO did not alter the TCDD response significantly. Similarly, the BNF-induced formation of abnormalities in the prosencephalic artery was effectively blocked by Ahr2-KD, but not by the negative control MO homologs (Table 3). Ahr2-KD was also effective to block malformation by TCDD in mesencephalic vein completely (1 ppb TCDD 13.9 ± 4.6%, AHR2-KD + 1 ppb TCDD 0%).

As shown in Table 3, morpholino knock-down of Arnt1 expression (Arnt1-KD) significantly inhibited the prosencephalic artery malformations induced by TCDD (1 ppb) and BNF (0.5 μM). Prosencephalic artery morphology was rarely affected by Arnt1-KD alone, although the incidence of the abnormal prosencephalic artery in the Arnt1-KD morphants (8/159) was slightly higher than that seen in the Ahr2-KD morphants described above.

3.5. Effects of antioxidants on TCDD-induced increases in prosencephalic artery malformations and decreases in blood flow

Previously we reported that some antioxidants protected against TCDD-induced decreases in blood flow through the mesencephalic vein of developing zebrafish (Dong et al., 2002). In the present study we sought to investigate the possible involvement of oxidative stress in the TCDD-evoked abnormal prosencephalic artery formation. As

shown in Fig. 5A, TCDD (1 ppb) decreased blood flow through the prosencephalic artery at 55 hpf, which was before disruption of erythropoiesis in later of 3 days post fertilization (Belair et al., 2001). Exposure to ascorbic acid (10 mM) effectively protected against this effect of TCDD by restoring blood flow to almost the control level. On the other hand, the same application of ascorbic acid did not affect the abnormal prosencephalic artery formation caused by TCDD (Fig. 5B).

4. Discussion

The present results show that TCDD and BNF can affect the morphology of ~~specific~~ **some** blood vessels, the mesencephalic vein and prosencephalic artery in the head of developing zebrafish. Vascular effects of TCDD have been seen in fish; retarded regression of the common cardinal vein at an early stage of development by TCDD was reported in zebrafish (Bello et al., 2004) and red seabream (Yamauchi et al., 2006). The alteration of morphology of a vein and an artery in the brain, in some cases including neovascularization, is an endpoint of TCDD developmental toxicity in zebrafish of which we were previously unaware. This effect of TCDD exposure was essentially absent in Ahr2 morphants and in Arnt1 morphants, suggesting that hyperactivation of Ahr2/Arnt1 signaling is involved, similar to other TCDD-induced responses in zebrafish (Antkiewicz et al., 2006). Our finding that TCDD caused neovascularization (new branches) as one type of malformation in the mesencephalic vein and prosencephalic artery in zebrafish larvae is particularly striking. It has been reported that TCDD

treatment significantly exacerbates photocoagulation-induced choroidal neovascularization in mice (Takeuchi et al., 2009). TCDD also induces remodeling of the placental vascular network in rats which is a possible cause of fetal death (Ishimura et al., 2009). In the chick embryo, TCDD induced aortic arch anomalies in combination with conotruncal malformations and ventricular septal defects (Cheung et al., 1981). Thus, hyperactivation of AHR/ARNT signaling by TCDD has profound effects on vascularization in a variety of vertebrates.

A role of AHR in normal mammalian vascular formation has been inferred from studies using $AHR^{-/-}$ mice. In our study of vehicle-exposed zebrafish, however, Ahr2-KD alone had no major impact on development of the vasculature throughout the body. However, knockdown of Ahr2 did effectively block both the TCDD-induced decrease in blood flow and increase in malformation in the prosencephalic artery, as well as in the mesencephalic vein (Dong et al., 2002). Since we used a transient knock-down technique with an Ahr2-MO, the small amount of remaining Ahr2 protein might still be sufficient for normal vascular formation to occur generally in zebrafish. Thus, further study, with zebrafish in which Ahr2 protein is completely absent, will be required to determine if Ahr2 has any role in normal vasculogenesis throughout the animal, like AHR has in mice (Lahvis et al., 2000).

On the other hand, Meandering prosencephalic artery, the frequency of which was increased by TCDD in an Ahr2-dependent manner, was also present in normal control larvae. This observation could signal a physiological role of endogenous Ahr2 protein in some aspect of vascular development. Zebrafish has two Ahr1 genes, Ahr1a and Ahr1b (Karchner et al., 2005) in addition to Ahr2. The normal physiological

functions of these three Ahrs are largely unknown, at least at the *in vivo* level. Ahr1a might be involved in CYP1A induction by some polycyclic aromatic hydrocarbons in zebrafish larvae (Incardona et al., 2006). However, at all stages of vascular development it is possible that one or more of the Ahrs in zebrafish could be involved. Continued research is needed to identify those processes in vasculogenesis and vascular remodeling that are dependent on certain forms of the Ahr in zebrafish, and to determine to what extent AHR-mediated effects on vascular development in zebrafish and mice are similar or different. An approach using Ahr2, Ahr1a, and Ahr1b knock-out zebrafish, prepared by the TILLING (Targeting Induced Local Lesions In Genomes) method (Wienholds et al., 2003), would be helpful in this regard.

Our finding that malformed blood vessels caused by TCDD exposure in zebrafish larvae appear to be restricted to the head will need to be rigorously assessed given the complexity of the vascular system throughout the body. In addition, it is recognized that although vascular disarrangement was observed only in the prosencephalic artery and mesencephalic vein, other vessels in the head and brain might also be affected. Identifying additional malformed vessels was beyond the scope of this study. In general, however, mechanisms of blood vessel formation have been extensively studied almost exclusively in the trunk, but seldom in the head and we found no malformed blood vessels in the trunk. Thus, it is suggested that malformation by Ahr activation could be restricted to brain vessel at least. There are potentially two explanations for the lack of defects in trunk vessels. First, the major vessels in the trunk region are set up earlier than TCDD exposure from 24 hpf in this study, so it may be that a sensitive window is past (Isogai et al., 2001). Alternatively, because cranial vessel

patterning is intertwined with neural development, there are other mechanisms involved not present in the trunk, such as the interaction between vessels and neural tissue. This is not surprising, since it is generally accepted that molecular, structural and functional specializations are different among various vascular beds. For instance, the zebrafish *bubblehead* (*bbh*) mutant exhibits hydrocephalus and severe cranial hemorrhage during early embryogenesis, whereas blood vessels in other regions of the embryo appear intact (Liu et al., 2007). Understanding differences in the response of zebrafish larval blood vessels to TCDD is an important first step in identifying the genes and signaling pathways that are being disrupted by hyperactivation of Ahr2/Arnt1 signaling leading to these blood vessel-specific malformations.

The downstream mechanism by which TCDD and BNF elicit these effects is unclear. The production of oxidative stress by TCDD has been extensively studied in various systems (Reichard et al., 2006; Goldstone and Stegeman, 2006). In developing zebrafish and medaka, antioxidants blocked edema and apoptosis in blood vessels caused by TCDD or 3,3',4,4',5-pentachlorobiphenyl exposure (Cantrell et al., 1996; Na et al., 2009). Previously, we reported that mesencephalic vein circulation failure caused by TCDD in zebrafish larvae could be inhibited by the antioxidants ascorbic acid and N-acetylcysteine (Dong et al., 2002). In the present study we confirmed that blood flow through the prosencephalic artery also was reduced by TCDD, and that this inhibitory effect of dioxin was protected against by ascorbic acid treatment. In contrast, ascorbic acid was without effect on TCDD-evoked deformities of the prosencephalic artery and mesencephalic vein. Hemodynamic changes play an important role in blood vessel formation and changes in blood flow can lead to severe vascular distortion (Yashiro et

al., 2007). The ascorbic acid protection against the TCDD-induced decrease in blood flow in these particular blood vessels but not the various types of malformations that TCDD caused in the same blood vessels, suggests it is unlikely that TCDD-induced circulation failure is the cause of the cephalic vessel anomalies in TCDD-exposed larvae in the present study.

Hypoxia-inducible factor 1α (Hif1α) is important in vasculogenesis and competition of AHR and Hif1α for ARNT might be a mechanism by which TCDD exposure could affect blood vessel formation (Ema et al., 1997). However, some of our results show that TCDD induced new vessel formation rather than regression. This cannot be explained by AHR and Hif1α competing for ARNT. Furthermore, TCDD-induced vascular malformations in the prosencephalic artery disappeared in Arnt1 morphants, similar to other endpoints of TCDD toxicity (Prasch et al., 2006). The possible role of Arnt2 in the production of TCDD-induced blood vessel malformations will require further study. Reductions in peripheral blood flow were not protected in *arnt2*$^{-/-}$ null mutant zebrafish (Prasch et al., 2004) and it seems likely that this will also be the case for prosencephalic artery malformations caused by TCDD. However, blood vessel deformities caused by TCDD exposure have yet to be examined in *arnt2* null larvae. It was recently reported that TCDD increased photocoagulation-induced choroidal neovascularization in association with enhanced VEGF mRNA expression in mice (Takeuchi et al., 2009). On the other hand, TCDD has been reported to inhibit VEGF expression in other studies (Ishimura et al., 2009). Obviously further research is needed to elucidate the mechanism of the **some** brain vessel malformations caused by TCDD and BNF.

In summary, the present study found that activation of Ahr2/Arnt1 signaling triggered by TCDD and, to a lesser extent, by BNF caused concentration-dependent increases in blood vessel malformation in the prosencephalic artery and mesencephalic vein in early life stages of zebrafish. This anatomical disarrangement of **some** brain blood vessels caused by Ahr2/Arnt1 activation is a novel endpoint that might be used as a sensitive marker for Ahr2-mediated toxicities during zebrafish development.

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Table 1. Incidence of morphological changes of prosencephalic artery in developing zebrafish exposed to graded concentration of β-naphthoflavone

		Classification of observed arteries					
		Typical	Meandering	Opposite end	Split arch	New vessel	Small arch
Control	(48)	97.9%	2.1%	0.0%	0.0%	0.0%	0.0%
β-naphthoflavone 0.1 μM	(74)	82.4%	8.1%	2.7%	2.7%	2.7%	1.4%
0.3 μM	(95)	66.3%	17.9%	3.2%	6.3%	5.3%	1.1%
0.5 μM	(94)	60.6%	17.0%	6.4%	3.2%	12.8%	0.0%
1.0 μM	(93)	54.8%	16.1%	9.7%	40.8%	8.6%	0.0%

Embryos were exposed to various concentrations of β-naphthoflavone (BNF) or vehicle from 24 to 48hpf. Prosencephalic arteries in control and BNF-treated larvae could be classified into 6 types at 55hpf. These are typical and meandering arteries also observed in control larvae (Typical, Meandering). The rest is abnormal arteries never observed in control larvae, including arteries with the opposite end (Opposite end), arteries with split arch (Split arch), arteries with newly formed vessel (New vessel) and arteries with small arch (Small arch). The percent incidence of these types was calculated with 48 to 95 larvae for each treatment, as indicated by numericals in parenthesis.

Table 2. Abnormal prosencephalic arteries in developing zebrafish exposed to graded concentrations of β-naphthoflavone

		Concentration of β-naphthoflavone			
	Control	0.1 μM	0.3 μM	0.5 μM	1 μM
Abnormal vessel	0.0± 0.0 % (6)	6.8±4.3% (5)	12.6±2.2%(6)	16.0±4.5 % (6)	19.4±4.5 % (6)

Embryos were exposed to various concentrations of β-naphthoflavone (BNF) or vehicle to evaluate prosencephalic artery at 55 hpf. Arteries with opposite end (Opposite end), split arch (Split arch), new vessel formation (New vessel) and small arch (Small arch) were counted as abnormal, as these were never recognized in control larvae, as shown in Fig. 1. Ten to 20 larvae were used for each group and the experiment was repeated for 5 or 6 times (n =5 or 6). Results are expressed as mean ± SEM.

Table 3. Effects of gene knock-down of Ahr2 and Arnt1 on percent incidence of abnormal prosencephalic artery formation caused by TCDD and BNF.

	Control	TCDD	Control	BNF
No treatment	0.0 ± 0.0% (5)	23.3 ± 6.3% (5)	0.0 ± 0.0% (7)	37.0± 4.0% (7)
4mis-Ahr2-MO	0.0 ± 0.0% (5)	33.7 ± 7.5% (5)	1.4 ± 1.5% (6)	20.3 ± 4.1% (6)
Ahr2-MO	0.0 ± 0.0% (5)	1.1 ± 1.2% (5)[a]	3.1 ± 1.6% (7)	5.1 ± 3.5% (7)[a]
No treatment	0.0 ± 0.0% (4)	37.2 ± 5.0% (4)	0.0 ± 0.0% (4)	33.3 ± 4.9% (4)
Arnt1-MO	3.7 ± 4.1% (4)	11.4 ± 2.9% (4)[a]	1.3 ± 1.4% (4)	11.5 ± 4.7% (4)[a]

Embryos were injected with morpholino antisense oligos against aryl hydrocarbon receptor 2 (Ahr2), the negative homologue (4mis-Ahr2-MO) and Arnt1 (Arnt1-MO) at one to four cell stages. After these treatments, the embryos were exposed to 1 ppb TCDD (TCDD), 0.5 μM β-naphthoflavone (BNF) or vehicle only (DMSO: Control), from 24 to 48 hpf. At 55 hpf, the number of abnormal vessels was determined in 17-30 larvae as a group for each treatment to express as percentage. Average of 4-7 groups was presented with SEM (N=4-7). a: ($p < 0.05$)

Figure legends

Fig. 1. Malformation of blood vessel induced by TCDD in the brain of developing zebrafish. Embryos were exposed to vehicle or 1 ppb TCDD from 24 to 48 hpf. Blood vessels, focusing on prosencephalic artery (A-H, bold line) and mesencephalic vein (I-P, bold line), were visualized by fluorescent beads injected into sinus venosus at 55 hpf. Photographs and their schemes are presented as a set for each. Dashed lines in panels B, D, F, H and in panels J, L, N, P indicate mesencephalic vein and prosencephalic artery, respectively. Panels A, B and I, J are control (vehicle) and the others are TCDD-treated larvae. A, B: Typical, C, D: Curved, E, F: Sigmoid, G, H: New vessel for prosencephalic artery. I, J: Typical, K, L: Meandering, M, N: Split, O, P: New vessel for mesencephalic vein. Detailed explanation is given in the text. Bars = 250 μm.

Fig. 2. Percent incidence of different types of abnormal vessel caused by exposure to a graded concentration of TCDD in prosencephalic artery. Embryos were exposed to vehicle or graded concentrations of TCDD (0.3, 0.5, 1 and 2 ppb) from 24 to 48 hpf. At 55 hpf, abnormal prosencephalic arteries could be divided into 4 groups: vessels with split arch (Split arch), branch of new vessel (New vessel) as indicated in Fig. 1 and vessels, which ended at prosencephalic artery at other side (Opposite end) and vessels with small arch (Small arch). Meandering prosencephalic arteries as well as Typical ones (Typical) were also observed in control larvae. Eighty one to 193 larvae were assessed at each concentration of TCDD.

Fig. 3. Concentration-dependent formation of abnormal vessel induced by TCDD in prosencephalic artery. Prosencephalic arteries with split arch, small arch, branch of new vessel and the end at the opposite side were defined as abnormal vessels (Abnormal vessel), since those were never observed in vehicle-treated control larvae, as described in Fig. 2. Abnormal vessels were counted in 18-22 larvae exposed to graded concentrations of TCDD (0.3, 0.5, 1 and 2 ppb) for each group. Average of the five to sixteen groups was presented with SEM (n = 21 for control, n = 5 for 0.3 and 2 ppb TCDD, n = 12 for 0.5 ppb TCDD, N = 16 for 1 ppb TCDD).

Fig. 4. Involvement of Ahr2 in TCDD-induced abnormal vessel formation in prosencephalic artery. After injection of either a morpholino antisense oligonucleotide against Ahr2 (Ahr2-MO) or its negative homolog (4mis-Ahr2-MO or 4mis-MO), embryos were exposed to vehicle (Control) and 0.5 ppb or 1 ppb TCDD from 24 to 48 hpf. At 55 hpf, the number of abnormal vessels was determined in 17-24 larvae as a group for each treatment. Average of the five groups was presented with SEM (n = 5). * $P < 0.05$, compared to respective controls (TCDD 0.5 ppb and 1 ppb).

Fig. 5. Differential effect of antioxidant treatment on the reduced blood flow and abnormal vessel formation in prosencephalic artery by TCDD. Embryos were exposed to vehicle, ascorbate (10 mM), TCDD (1 ppb) or their combination (Ascorbate + TCDD) from 24 to 48 hpf. At 55 hpf, the number of red blood cells passing through the prosencephalic artery per 15 sec (RBC/15 sec) was determined as an index of blood

flow (A), in addition to the percent incidence of abnormal vessel formation in prosencephalic artery (B). A: Fifteen to 19 larvae were evaluated for each treatment. B: Seventeen to 21 larvae were observed for each group and 4 groups were used for each treatment (n = 4). Results are expressed as mean ± SEM. * $P < 0.05$, compared to control.

Fig. 1

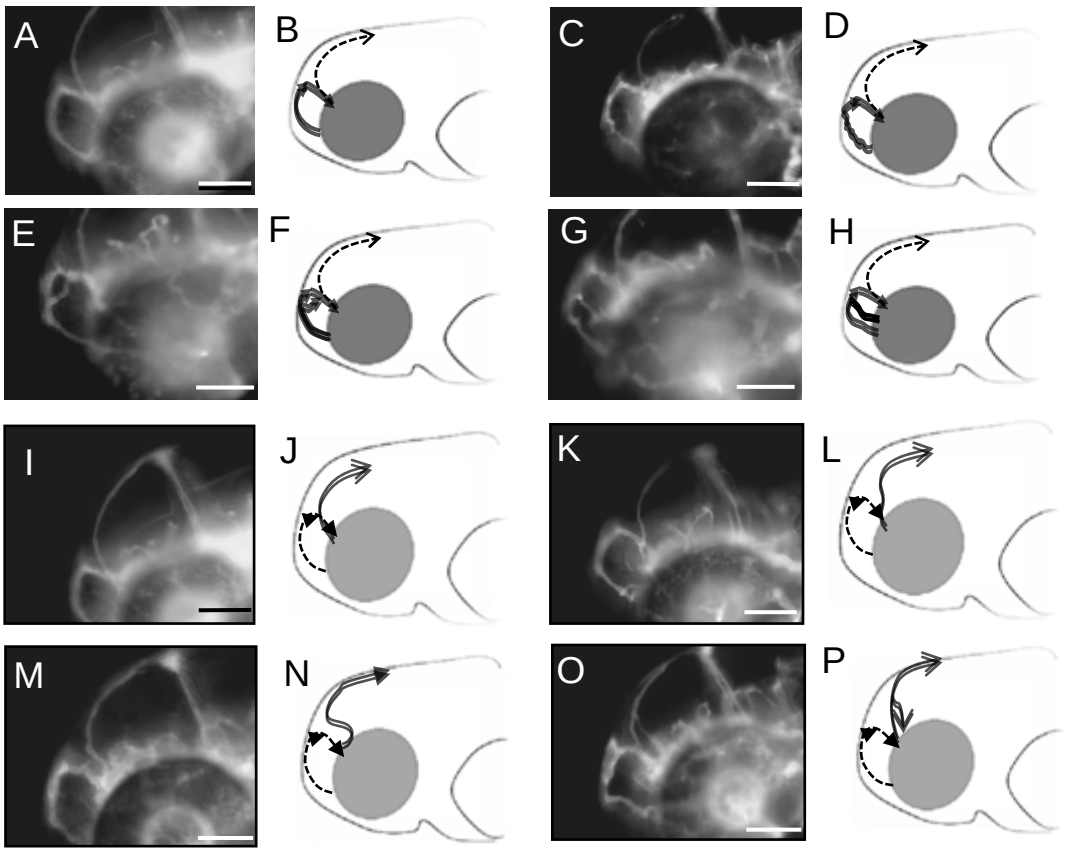


Fig. 2

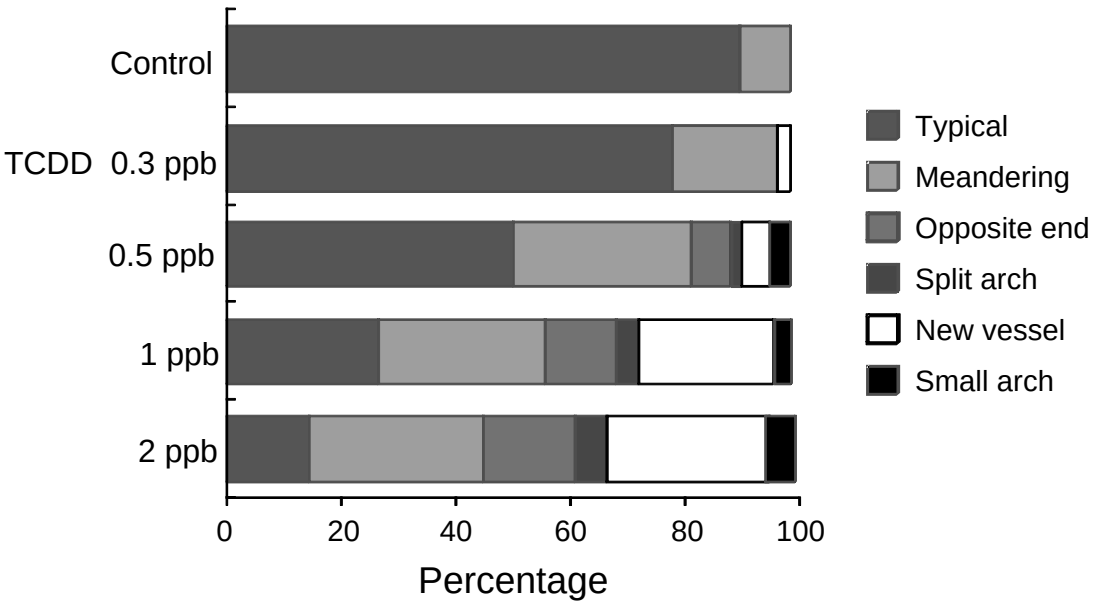

Control
TCDD 0.3 ppb
0.5 ppb
1 ppb
2 ppb
0
20
40
60
80
100
Percentage
Typical
Meandering
Opposite end
Split arch
New vessel
Small arch

Fig. 3

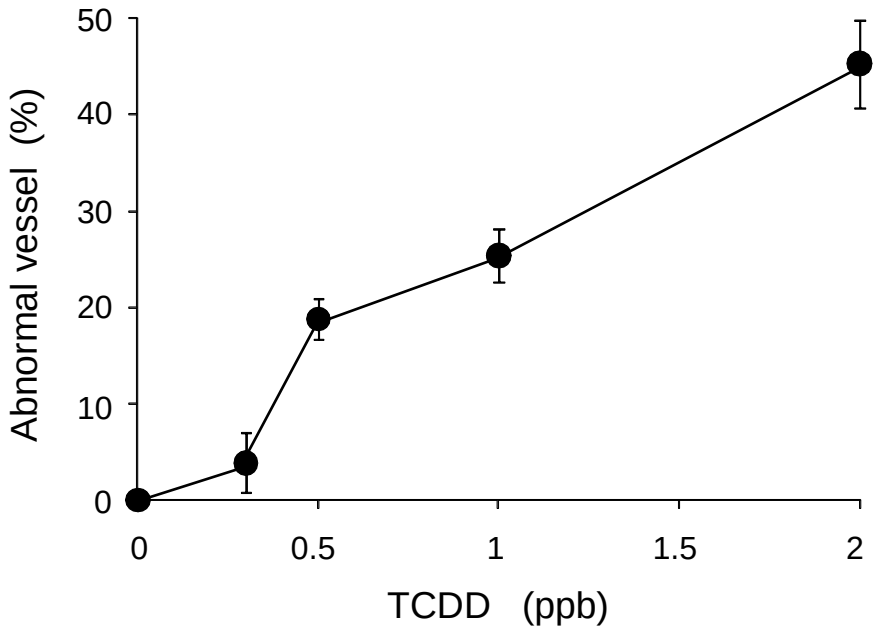


Fig. 4

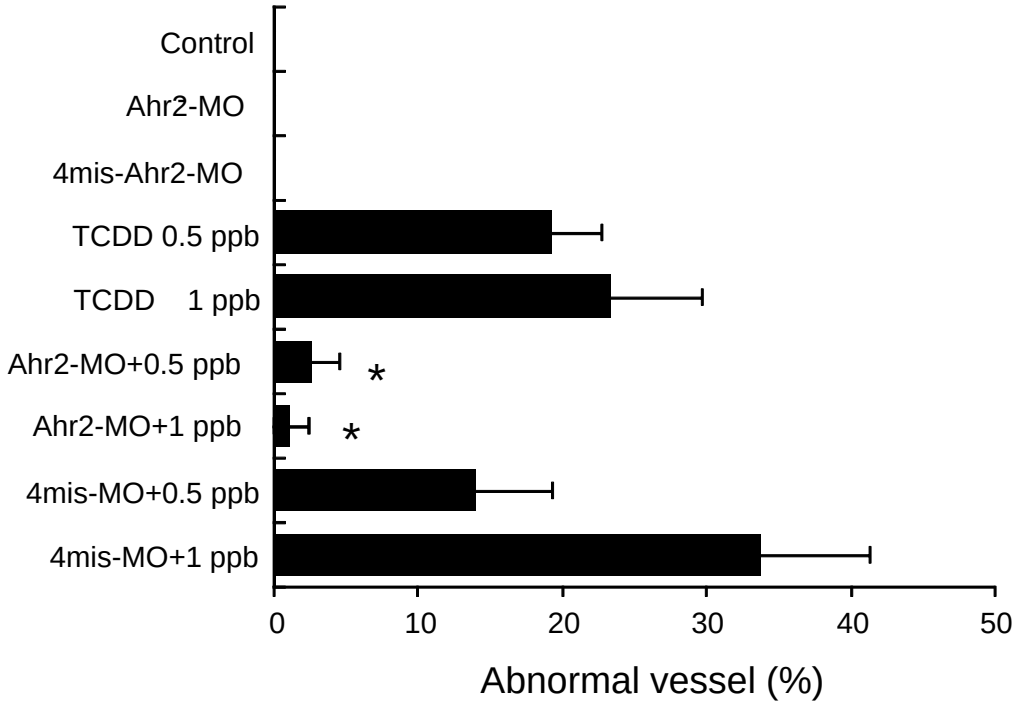
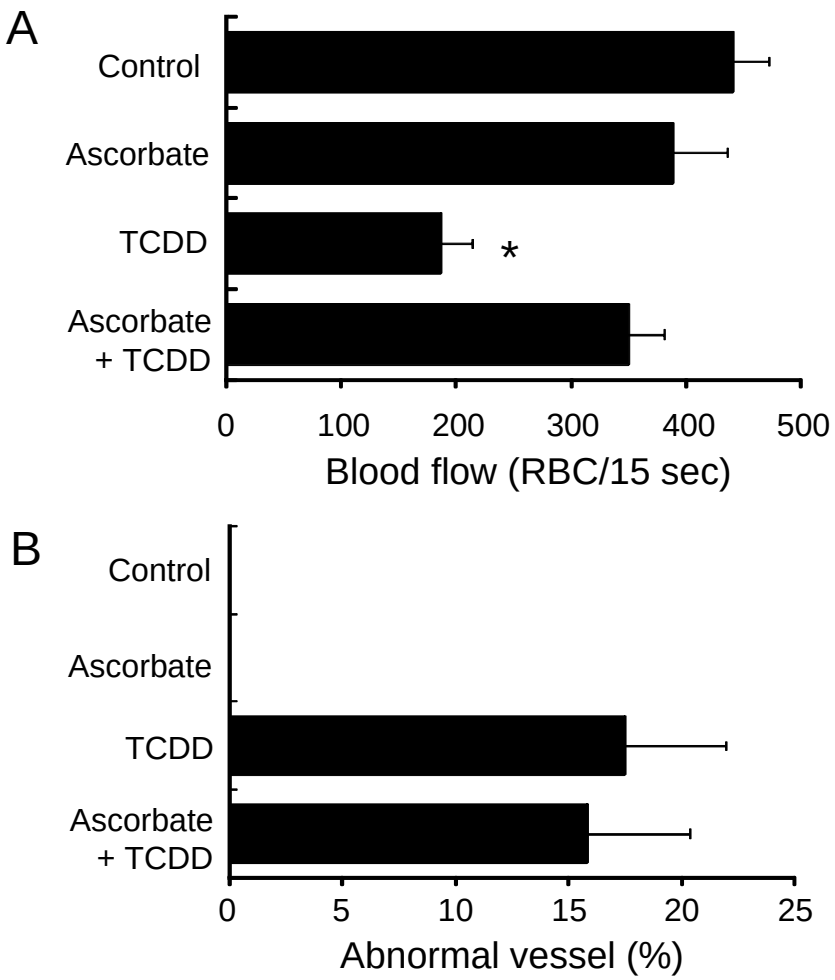

Control
Ahr2-MO
4mis-Ahr2-MO
TCDD 0.5 ppb
TCDD 1 ppb
Ahr2-MO+0.5 ppb
Ahr2-MO+1 ppb
4mis-MO+0.5 ppb
4mis-MO+1 ppb
*
*
0
10
20
30
40
50
Abnormal vessel (%)

Fig. 5


A
Control
Ascorbate
TCDD
*
Ascorbate
+ TCDD
0
100
200
300
400
500
Blood flow (RBC/15 sec)
B
Control
Ascorbate
TCDD
Ascorbate
+ TCDD
0
5
10
15
20
25
Abnormal vessel (%)