Interdisciplinary Studies on Environmental Chemistry—Biological Responses to Chemical Pollutants, Eds., Y. Murakami, K. Nakayama, S.-I. Kitamura, H. Iwata and S. Tanabe, pp. 61–80.

Prostanoid Signaling Mediates Circulation Failure Caused by TCDD in Developing Zebrafish

Hiroki TERAOKA, Akira KUBOTA, Yusuke KAWAI and Takeo HIRAGA

School of Veterinary Medicine, Rakuno Gakuen University, Midori-cho 582, Ebetsu 069-8501, Japan

(Received 30 April 2008; accepted 29 July 2008)

Abstract—Using zebrafish embryos, we reported that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) evoked circulation failure through activation of the aryl hydrocarbon receptor type 2 (AHR2). However, the following molecular target after AHR2 activation is largely unclear. It has been reported that TCDD induces cyclooxygenase 2 (COX2), a rate-limiting enzyme for prostaglandin synthesis in some cells. In this study, we investigated the involvement of cyclooxygenase on developmental toxicity in zebrafish exposed to TCDD. TCDD-induced mesencephalic circulation failure was markedly inhibited by selective COX2 inhibitors and by a general COX inhibitor, but not by a selective COX1 inhibitor. Gene knock-down of COX2 by morpholino antisense oligo nucleotides also recovered mesencephalic circulation failure by TCDD. The effect of TCDD was also blocked by selective antagonists for thromboxane receptor (TP). Conversely, TP agonist induced mesencephalic circulation failure, which was abolished by TP antagonist, without any effect on trunk circulation. Furthermore, gene knock-down of thromboxane A synthase 1 (TBXS) with morpholinos significantly inhibited TCDD-induced mesencephalic circulation failure. Overall similar results with selective chemicals on prostaglandin signaling were obtained for pericardial edema by TCDD. These results suggest the involvement of prostanoid synthesis pathway by way of COX2-TBXS-TP in TCDD-induced local circulation failure in developing zebrafish.

Keywords: aromatic hydrocarbon, COX-1, COX-2, CYP5A, *Danio rerio*, prostaglandin, TCDD

INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs), and coplanar polychlorinated biphenyls (Co-PCBs) are persistent, bioaccumulative, and toxic contaminants widely distributed in the environment. Fish embryos are one of the most sensitive organisms to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a prototype dioxin. Exposure of fish larvae to TCDD causes a reduction in peripheral circulation, edema, craniofacial malformation, and growth retardation resulting in mortality (Walker and Peterson, 1994; Henry *et al*., 1997; Teraoka *et al*., 2002). Among these, it has been widely recognized that the cardiovascular

system is one of the most important targets of TCDD developmental toxicity in fish larva. Recently, it has been reported that in zebrafish (*Danio rerio*) TCDD disorganized coordinated pumping movement of early developing heart (Antkiewicz *et al.*, 2006) or reduced numbers of cardiac muscle at early stages before pericardial edema (Antkiewicz *et al.*, 2005). In larva of medaka (*Oryzias latipes*), treatment with an antioxidant blocked circulation failure and mortality by TCDD (Cantrell *et al.*, 1996, 1998). Nevertheless, the mechanisms of the circulation failure and pericardial edema caused by TCDD in developing fish remain understood. Otherwise, TCDD increased water permeability of developing zebrafish, and pericardial edema could be blocked by isotonic mannitol (Hill *et al.*, 2004). On the other hand, larva of a sea fish, red seabream (*Pagrus major*), showed similar edema, despite being raised in hypertonic seawater (Yamauchi *et al.*, 2006).

TCDD is an exogenous ligand for the aryl hydrocarbon receptor (AHR) in the cytoplasm. After binding with TCDD, AHR translocates to the nucleus and dimerizes with an aryl hydrocarbon receptor nuclear translocator (ARNT) (Tanguay *et al.*, 2003). Interaction of the AHR/ARNT heterodimer with xenobiotic responsive elements (XREs) upstream of the target genes results in the induction of xenobiotic-metabolizing enzymes such as cytochrome P4501A (CYP1A) and a group of molecules called AHR gene battery (Nebert *et al.*, 2000). Unlike mammals, fish has several AHR isoforms, and AHR1a, AHR1b and AHR2 have been reported in zebrafish (Karchner *et al.*, 2005). Similar to the involvement of AHR in TCDD-induced toxicity in mice (Fernandez-Salguero *et al.*, 1996; Mimura *et al.*, 1997), circulation failure as well as other endpoints of TCDD toxicity are mediated by AHR2 in zebrafish, as revealed by gene knock-down with a morpholino antisense-oligo nucleotide against AHR2 (Prasch *et al.*, 2003; Teraoka *et al.*, 2003b). Otherwise, TCDD-induced toxicity was not recognized in ARNT2 (–/–) zebrafish or AHR2 morphant, suggesting the importance of ARNT1 against ARNT2 (Antkiewicz *et al.*, 2006). However, the following signaling pathway after interaction of AHR2/ARNT1 with XREs is largely unclear.

TCDD induces apoptosis in the dorsal midbrain of developing zebrafish (Dong *et al.*, 2001) and killifish (*Fundulus heteroclitus*) (Toomey *et al.*, 2001). In searching for the etiology, we found the concomitant decrease of blood flow before apoptosis in the mesencephalic vein, the only vessel perfusing the dorsal midbrain in early stage larvae (Dong *et al.*, 2002). Mesencephalic circulation failure was associated with an increase in albumin permeability in the mesencephalic vein of developing zebrafish (Dong *et al.*, 2004). These three endpoints of toxicity were all blocked by antioxidants, general cytochrome P450 inhibitors and AHR2 knock-down (Dong *et al.*, 2002, 2004). Thus, mesencephalic circulation failure is a sensitive and useful system as a model for circulation failure by TCDD.

Cyclooxygenases (COXs), also known as prostaglandin (PG) endoperoxide G/H synthases, are heme-containing enzymes that catalyze the rate-limiting step of the production of various kinds of PGs. It has been widely accepted that

induced COX2 contributes acute inflammatory responses by production of PGs (Tilley *et al*., 2001). In local inflammation model, rat carrageenin-induced pleurisy, PGE_2 or PGI_2 produced through COX2 enhance permeability of the vascular endothelium by histamine and bradykinin (Harada *et al*., 1996). Furthermore, a recent study using zebrafish embryos revealed that heart defects caused by aristolochic acid, a component of Chinese herbs, were attenuated by a selective COX2 inhibitor, NS398, suggesting the important role of COX2 in the cardiac toxicity to developing zebrafish (Huang *et al*., 2007). On the other hand, COX1 is expressed constitutively and is primarily responsible for PGs that maintain physiological functions especially in the stomach and the kidney. Zebrafish has two COX isozymes, COX1 and COX2 (Grosser *et al*., 2002). COX1-derived prostaglandins are required for gastrulation and segmentation in zebrafish. During gastrulation, PGE_2 signaling promotes cell motility, without altering the cell shape or directional migration of gastrulating cells (Cha *et al*., 2006). On the other hand, the role of COX2 is relatively unknown, although the relationship among COX2, adenomatous polyptosis coli (APC) and retinoic acid has been clarified (Eisinger *et al*., 2006).

There have been many reports, in which TCDD induces COX2 and produces PGs in some tissues, such as cultured Hepa-1 mouse hepatoma cells and C3H/M2 mouse fibroblast cells (Puga *et al*., 1997; Wolfle *et al*., 2000). XRE sequences have been identified in the promoter region of the mouse, rat and human COX2 (Sirois and Richards, 1993). Otherwise, c-Src could augment COX2 transcription through CCAAT-enhancer binding protein (C/EBP) responsive element in the promoter region as a XRE-independent mechanism (Vogel *et al*., 2000).

In the present study, we focused on prostaglandin synthesis pathway, especially on COX2 for its potential role in the circulation failure caused by TCDD. Furthermore, the possible involvement of downstream pathway of COX2 in TCDD-induced circulation failure was investigated. We mainly studied mesencephalic circulation failure, which is one of the earliest endpoints of toxicity caused by TCDD, occurring long before general circulation failure and pericardial edema (Teraoka *et al*., 2003a).

MATERIALS AND METHODS

Chemicals

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from Cambridge Isotope Laboratories (98% purity: Andover, MA). 9 {alpha}, 15R-dihydroxy-1ß-fluoro-15-(2,3-dihydro-1H-inden-2-yl)-16,17,18,19,20-pentanor-prosta-5Z,13E-dien-1-oic acid (AL8810), 4,5-dihydro-N-[4-[[4-(1-methyl ethoxy)phenyl] methyl]phenyl]-1H-imadazol-2-amine (CAY10441), *N*-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide (NS398), 4-[5-(4-Chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide) (SC236), 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole (SC560), 8-chloro-dibenz[b,f][1,4]oxazepine-10(11H)-carboxy-(2-acetyl)hydrazide

Table 1. Nucleotide sequences of morpholino antisense oligos.

Target	Reference number	Type	Concentration	Sequence
COX2-MO-spl	ENSDARG00000004539 (Ensembl)	splice	50 μM	5′-GATTTCATTCAACTTACACAACAGG-3′
5mis-COX2-MO-spl	—	negative control	50 μM	5′-cATTTgATTCAAcTTAgACAACAcG-3′
TBXS-MO-tra	AY398422 (Genbank)	translation	25 μM	5′-AGCTGCATGATGGGATCTGTCAATC-3′
5mis-TBXS-MO-tra	—	negative control	25 μM	5′-AGgTGgATGATGcGATgTGTgAATC-3′
TBXS-MO-spl	ENSDARG00000002249 (Ensembl)	splice	25 μM	5′-CAAACAATAACAAACCTCAGTGTCC-3′
5mis-TBXS-MO-spl	—	negative control	25 μM	5′-CAAAgAATAAgAAAgCTgAGTcTCC-3′

Reference number quoted accession number of Genbank (translation) or Ensembl Transcript ID. Modified nucleotides are indicated by small letters as for 5mis negative homologues.

(SC19220), ([1S]-1a,2b(5Z),3b,4a-7-(3-{2-[(phenylamino) carbonyl] hydrazinomethyl}-7-oxobicyclo-[2.2.1]hept-2-yl-5-heptenoic acid (SQ29,548), 9,11-dideoxy-9α,11α-methanoepoxy Prostaglandin F2α (U-46619) were from Cayman Chemical (Ann Arbor, MI). 4-(Z)-6-(2-o-Chlorophenyl-4-o-hydroxyphenyl-1,3-dioxan-cis-5-yl)hexenoic acid (ICI-192,605) was purchased from Tocris (Bristol, UK). The other chemicals were obtained from Kanto Chemical (Japan).

Zebrafish and TCDD treatment

Fertilized eggs were obtained from natural mating of adult zebrafish (Longfin) in our laboratory according to the *Zebrafish Book* (Westerfield, 1995). Adult fish and embryos were maintained at 28.5°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 of spawning, newly fertilized eggs were exposed to either the TCDD vehicle, dimethyl sulfoxide (DMSO, usually 0.1%) or an apparent concentration of waterborne TCDD of 0.3, 0.5, or 1.0 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 3.5 cm petri dishes (Asahi Techno Glass, Japan) for the duration of the experiment (10 embryos/dish).

Gene knock-down with morpholino antisense oligos

Morpholino antisense oligos against splicing of COX2 (COX2-MO-spl) and TBXS (TBXS-MO-spl) with their respective negative controls with 5 different nucleotides (5mis-COX2-MO-spl and 5mis-TBXS-MO-spl) were synthesized by Gene Tools (Philomath, OR), to block splicing of these molecules. This type of MOs makes it possible to evaluate the specificity by measuring expression levels of genes of interest. As for TBXS, morpholino antisense oligos against its translation (TBXS-MO-tra) as well as the 5mis negative control (5mis-TBXS-MO-tra) were also synthesized. The sequences of these MOs are indicated in Table 1. Each morpholino nucleotide was injected into the yolk of embryos at one to four cell stages with a fine glass needle connected with an automatic injector (IM-300: Narishige, Japan). 100 pL of 25–50 μM Ca^{2+}-free Zebrafish Ringer solution was injected (Table 1).

Blood flow

Blood flow in the mesencephalic vein was evaluated by time-lapse recording using a digital-video camera (Handycam DCR-SR100, Sony, Japan), as described previously (Teraoka *et al*., 2002). Embryos were suspended in 200 μl of 3% carboxymethyl 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 28.5°C with a PDMI-2 Micro-Incubator (Harvard Apparatus, Holliston, MA).

Evaluation of pericardial edema

The severity of pericardial edema in zebrafish fry was quantified with area analysis of the pericardial cavity using conventional image software (Adobe Photoshop). After a lateral image of the fry was obtained by digital camera (DP70, Olympus, Japan) under inverted microscope (IX71, Olympus, Japan), the pericardial space was circled on Photoshop image at the same magnification and the area was obtained in pixels (Inset photos, Fig. 7), as described previously (Teraoka *et al.*, 2003b).

Real-time RT-PCR

Real-time RT-PCR analysis was carried out to examine expression levels of COX2 as well as COX1 and TBXS. Total RNA was extracted from zebrafish embryos with Trizol (Invitrogen, Carlsbad, CA), and purified with DNase treatment using RNeasy (Qiagen, Germany) in accordance with the manufacturerss instructions. The cDNA templates for real-time RT-PCR analysis were synthesized from total RNA with Superscript III reverse transcriptase (Invitrogen) and oligo (dT) primer. Real-time RT-PCR analysis was performed in Real-Time PCR Detector (Chromo4: Bio-Rad, Hercules, CA) using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) with the reaction volume of 20 μl. Elongation factor 1-alpha) of zebrafish was used as an internal control. The primer pairs were as follows (numbers in parenthesis indicate accession number): COX1 (AY028584) sense primer 5′-CCAGTACCAGAACCGCATTT-3′, antisense primer 5′-CGATGACCCTCTCAGCAACT-3′; COX2 (COX2a: NM_153657) sense primer 5′-CGCTATATCCTGTTGTCAAG-3′, antisense primer 5′-CTTGGCATTTGGGAGATCAG-3′; TBXS (thromboxane A synthase 1: AY398422) sense primer 5′-CCTTCACTATGATCCAGAGC-3′, antisense primer 5′-TAACTCCAGAGGAATCTCAG-3′; EF1α (NM_131263) sense primer 5′-GATGCACCACGAGTCTCTGA-3′, antisense primer 5′-TGATGACCTGAGCGTTGAAG-3′.

All primer sets were confirmed to produce a single peak in melting curve as well as a single band by agarose gel electrophoresis in the preliminary study.

Statistics

Results are presented as mean ± SEM. Significance of differences between vehicle and TCDD-exposed groups was determined by one-way or two-way ANOVA followed by Scheffe's test ($p < 0.05$).

RESULTS

Effects of COX2 inhibitors on mesencephalic circulation failure

As previously reported (Dong *et al.*, 2002), blood flow in the mesencephalic vein, which is the only vessel perfusing the dorsal midbrain at this stage of development, reached a peak around 50 hpf and then gradually decreased to a lower steady level by 60 hpf. This mesencephalic circulation was markedly

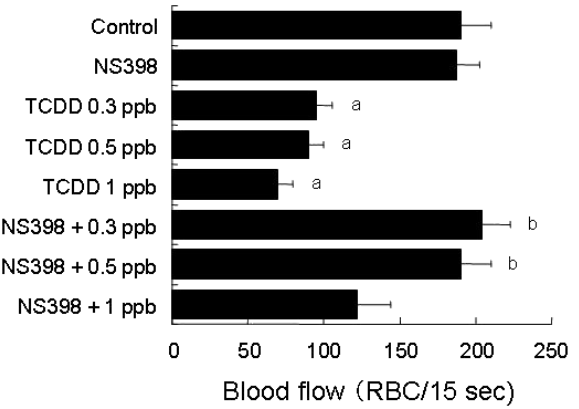

Fig. 1. Recovery of TCDD-induced mesencephalic circulation failure by a COX2 inhibitor. Embryos were treated with 10 μM NS398, a selective inhibitor for COX2, in the presence or absence of TCDD (0.3–1 ppb), beginning at 24 hpf. At 50 hpf, numbers of red blood cells passing the mesencephalic vein per 15 s (RBC/15 sec) were determined as an index of blood flow. Results are expressed as mean ± SEM. $N = 10$ per group. a; $p < 0.05$ against control. b; $p < 0.05$ against respective TCDD responses without inhibitor.

inhibited to half by TCDD between 48 and 60 hpf (Dong *et al*., 2002). As shown in Fig. 1 and Table 2, mesencephalic circulation at 50 hpf in embryos exposed to vehicle only (control), vehicle + NS398 (10 μM) and vehicle + SC236 (5 μM) were all similar. In contrast, embryos exposed to graded concentrations of TCDD (0.3, 0.5, and 1 ppb) displayed significantly lower blood flow, confirming again our previous results (Dong *et al*., 2002, 2004). Both selective inhibitors for COX2, NS398 and SC236, blocked the inhibitory effect on mesencephalic vein blood flow caused by TCDD (0.3 ppb and 0.5 ppb). Although the effects were not significant, NS398 also tended to increase circulation even after exposure to the highest concentration of TCDD, 1 ppb (Fig. 1). Since the lowest concentration (0.3 ppb) of TCDD examined was sufficient to evoke mesencephalic circulation failure at 50 hpf larva, all further experiments were performed using this concentration.

Effects of another prostaglandin synthesis enzyme inhibitor on TCDD-induced mesencephalic circulation failure

SC560 is a selective COX1 inhibitor commercially available. SC560 (2.8 μM) affected neither the basal level nor the TCDD-induced level of mesencephalic blood flow in 50 hpf zebrafish larvae (Table 2). Higher concentration of SC560 (5.6 μM) increased mortality by 48 hpf and hence was not used for the experiments. On the contrary, the general COX inhibitor, indomethacin (30 and 60 μM), mimicked the effects of NS398 and SC236 in blocking the TCDD-induced decrease in the blood flow (Table 2).

Table 2. Summary of effects of various prostaglandin synthesis inhibitors or prostaglandin receptor inhibitors on the decrease in mesencephalic vein blood flow caused by TCDD in zebrafish larva.

Target	Inhibitor	Control	Treatment	TCDD	Treatment + TCDD
COX1, 2	Indomethacin	215 ± 20 (16)	198 ± 9 (10)	100 ± 11 (10)[a]	195 ± 15 (10)[b]
COX1	SC560	211 ± 19 (10)	194 ± 18 (10)	101 ± 12 (10)[a]	101 ± 6 (10)
COX2	NS398	201 ± 16 (15)	187 ± 15 (10)	95 ± 11 (10)[a]	205 ± 18 (10)[b]
	SC236	218 ± 21 (18)	179 ± 15 (10)	118 ± 24 (10)[a]	181 ± 24 (10)[b]
EP	SC19220	191 ± 8 (10)	189 ± 8 (10)	113 ± 18 (10)[a]	105 ± 19 (10)
FP	AL8810	186 ± 11 (10)	188 ± 6 (10)	119 ± 12 (10)[a]	120 ± 11 (10)
IP	CAY10441	182 ± 8 (10)	179 ± 9 (10)	111 ± 19 (10)[a]	112 ± 14 (10)
TP	ICI-192,605	194 ± 9 (10)	184 ± 8 (10)	114 ± 11 (10)[a]	190 ± 10 (10)[b]
	SQ29,548	181 ± 9 (15)	184 ± 7 (15)	110 ± 12 (15)[a]	153 ± 11 (15)[a,b]

Embryos were treated with various inhibitors for some prostaglandin synthesis enzyme (indomethacin; 60 9M, SC560; 2.8 9M, NS398; 10 9M, SC236; 5 9M) or prostaglandin receptor inhibitors (SC19220; 60 9M, AL8810; 50 9M, CAY10441; 64 9M, ICI-192,605; 6 9M, SQ29548; 25 9M) in the presence or absence of TCDD (0.3 ppb), beginning at 24 hpf. At 50 hpf, numbers of red blood cells passing the mesencephalic vein per 15 s (RBC/15 sec) were determined as an index of blood flow. Results are expressed as mean ± SEM. Numbers in parenthesis indicate the number of individuals examined per group. The target of each inhibitor was indicated in the left column.

a; $p < 0.05$ against control. b; $p < 0.05$ against respective TCDD responses without inhibitor.

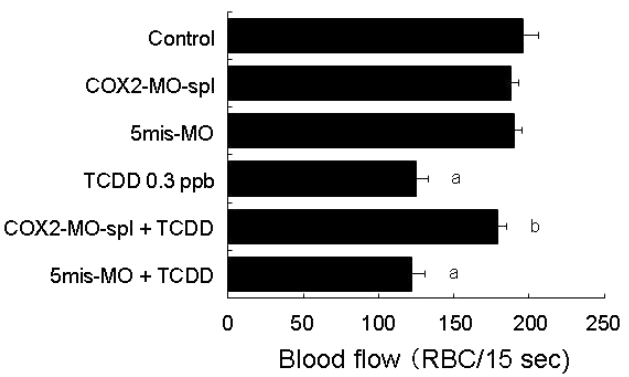

Fig. 2. Recovery of TCDD-induced mesencephalic circulation failure by COX2 knock-down. After injection of splicing-target type morpholino antisense oligonucleotide against COX2 (COX2-MO-spl) or its negative control (5mis-MO), embryos were exposed to vehicle or 0.3 ppb TCDD from 24 hpf. At 50 hpf, numbers of red blood cells passing the mesencephalic vein per 15 s (RBC/15 sec) were determined as an index of blood flow. $N = 14$ per group. a; $p < 0.05$ against control. b; $p < 0.05$ against respective TCDD responses without inhibitor.

Effects of COX2 gene knock-down on mesencephalic circulation failure caused by TCDD

We carried out gene knock-down by splice inhibition with morpholino

Table 3. Summary of effects of gene knock-down of cyclooxygenase 2 and thromboxane A2 synthase on the decrease in mesencephalic vein blood flow caused by TCDD in zebrafish larva.

Morpholinos	Control	Treatment	TCDD	Treatment + TCDD
COX2-MO-spl	196 ± 10 (14)	188 ± 5 (14)	125 ± 8 (14)[a]	179 ± 6 (14)[b]
5mis-COX2-MO-spl	—	190 ± 5 (14)	—	121 ± 10 (14)
TBXS-MO-tra	193 ± 9 (14)	190 ± 8 (14)	128 ± 6 (14)[a]	174 ± 9 (14)[b]
5mis-TBXS-tra	—	180 ± 10 (14)	—	126 ± 4 (14)
TBXS-MO-spl	195 ± 11(25)	184 ± 10 (25)	121 ± 10 (25)[a]	177 ± 12 (25)[b]
5mis-TBXS-MO-spl	—	194 ± 10 (25)	—	113 ± 11 (25)

Embryos were injected with morpholino antisense oligos against cyclooxygenase 2 (COX2) or thromboxane A2 synthase (TBXS) at one to four cell stages. The embryos were exposed to 0.3 ppb TCDD (TCDD) or vehicle only (DMSO: Control), from 24 hpf to 36 hpf. At 50 hpf, numbers of red blood cells passing the mesencephalic vein per 15 s (RBC/15 sec) were determined as an index of blood flow. Results areexpressed as mean ± SEM. Numbers in parenthesis indicate case numbers per group.

a; $p < 0.05$ against control. b; $p < 0.05$ against respective TCDD responses without inhibitor.

antisense method (COX2-MO-spl). Zebrafish embryos were injected with COX2-MO-spl at one to four cell stages and subsequently exposed to vehicle or TCDD. Mesencephalic vein blood flow was evaluated at 50 hpf. As reported by Grosser *et al.* (2002), there did not appear to be any severe alteration in normal development in COX2-MO injected embryos by 96 hpf, but some larva showed pericardial edema. As shown in Fig. 2 and Table 3, blood flow in the mesencephalic vein at 50 hpf was markedly reduced by TCDD. Injection of COX2-MO-spl almost completely prevented the TCDD-induced reduction in the blood flow (Fig. 2 and Table 3). The negative control 5mis-COX2-MO-spl morpholino (5mis-MO) was ineffective in blocking the TCDD-induced decrease in the blood flow (Fig. 2 and Table 3).

The present study could not examine the effects of COX1 knock-down, which has been reported to kill the zebrafish embryo at an early stage of development (Grosser *et al.*, 2002). However, we confirmed that the COX2-MO-spl markedly inhibited COX2 transcription without any significant effect on COX1 transcript (Table 4).

Effects of prostaglandin receptor antagonists on mesencephalic circulation failure caused by TCDD

Here the possible involvement of downstream pathway of COX2 in TCDD-evoked mesencephalic circulation failure is addressed. Although there are few antagonists for prostanoid synthase, specific antagonists for prostanoid receptors were commercially available and extensively used as useful experimental agents. Among them, SC19220 for PGE_2 receptor (EP), AL8810 for PGE_{2alpha} receptor (FP), CAY10441 for PGI_2 receptor (IP), ICI192,605 and SQ29,548 for thromboxane receptor (TP) were examined. We used only the concentrations, at

Table 4. Summary of the effects of splice type morpholino antisense oligonucleotides on trascripts.

Splice type MO	Transcripts of interest	Non-injected control		MO-injected		5mis-MO-injected	
		Trascripts/EF1α	Normalized	Trascripts/EF1α	Normalized	Trascripts/EF1α	Normalized
COX2-MO	COX1	335 ± 45	100 ± 13	379 ± 100	113 ± 31	332 ± 82	99 ± 24
	COX2	96 ± 8	100 ± 9	10 ± 3[a]	10 ± 3[a]	120 ± 28	120 ± 30
TBXS-MO	TBXS	105 ± 22	100 ± 21	52 ± 8[a]	50 ± 8[a]	112 ± 7	107 ± 6

Embryos were injected with splice inhibition type morpholino antisense oligonucleotides (MO) with their negative homologues (5mis-MO) on the relative amounts of trascripts of interest at one to four cells stage. These morphants and non-injected control embyos were euthenized at 50 hpf for total RNA extraction. cDNA converted were used for quantitative real-time PCR. Elongation factor 1α (EF1α) was used as a reference gene and each Trascrpits/EF1α indicates 1,000 times values of the simple division.

Trascripts/EF1α values were normalized as non-injected control values as unity for comparison (Normalized). Results are expressed as mean ± SEM. N = 5 per group.

a; $p < 0.05$ against control.

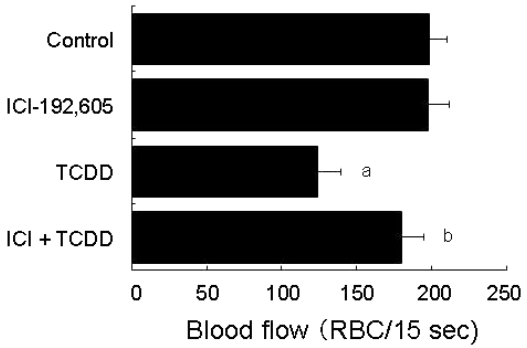

Fig. 3. Recovery of TCDD-induced mesencephalic circulation failure by thromboxane receptor blockade. Embryos, which were treated with thromboxane receptor inhibitor (6 μM ICI-192,605), were exposed to TCDD (0.3 ppb) or vehicle only (Control), beginning at 24 hpf. At 50 hpf, numbers of red blood cells passing the mesencephalic vein per 15 s (RBC/15 sec) were determined as an index of blood flow. $N = 15$ (B) per group. a; $p < 0.05$ against control. b; $p < 0.05$ against respective TCDD responses without inhibitor.

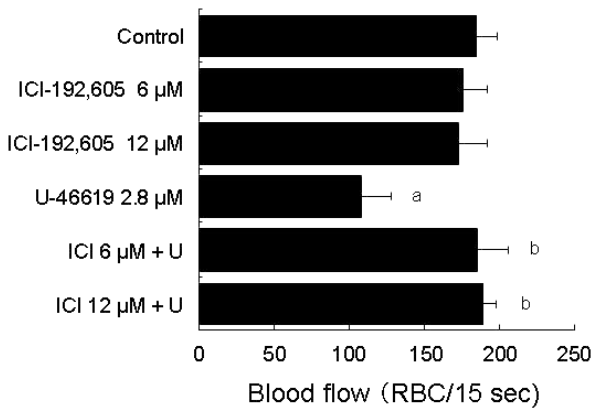

Fig. 4. Mesencephalic circulation failure caused by thromboxane receptor activation. Embryos were treated with U-46619 (2.8 μM), a selective agonist for TP receptor, beginning at 24 hpf, in the presence or absence of ICI-192,605 (ICI, 6 or 12 μM). At 50 hpf, numbers of red blood cells passing the mesencephalic vein per 15 s (RBC/15 sec) were determined as an index of blood flow. $N = 10$ per group. a; $p < 0.05$ against control. b; $p < 0.05$ against respective TCDD responses without antagonist.

which they did not cause edema and other gross morphology by themselves. As shown in Table 2, mesencephalic circulation failure caused by 0.3 ppb TCDD were not affected by single application of SC19220 (30 μM), AL8810 (25 μM), or CAY10441 (32 μM). By contrast, specific TP antagonists used, both ICI192,605

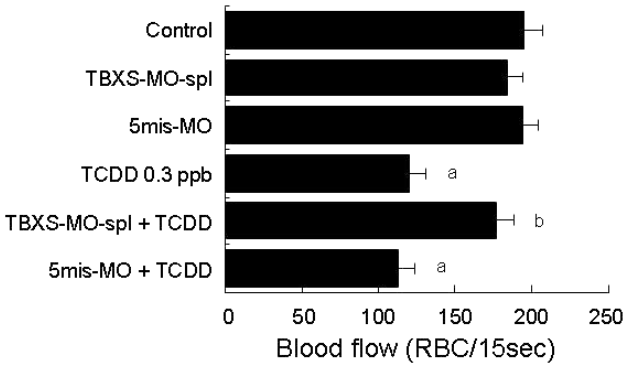

Fig. 5. Recovery of TCDD-induced mesencephalic circulation failure by TBXS knock-down. After injection of splicing-target type morpholino antisense oligonucleotide against TBXS (TBXS-MO-spl) or its negative control (5mis-MO), embryos were exposed to vehicle or 0.3 ppb TCDD from 24 hpf. At 50 hpf, numbers of red blood cells passing the mesencephalic vein per 15 s (RBC/15 sec) were determined as an index of blood flow. $N = 14$ per group. a; $p < 0.05$ against control. b; $p < 0.05$ against respective TCDD responses without morpholino injection.

(6 μM) and SQ29,548 (25 μM) effectively inhibited the effects of TCDD (Fig. 3 and Table 2).

Mesencephalic circulation failure caused by TP receptor agonist

Then we challenged TP agonist, U-46619 to verify whether mesencephalic circulation failure was mimicked by this chemical. Application of U-46619 (1.4 and 2.8 μM) significantly delayed blood flow in mesencephalic vein (Fig. 4). This effect was blocked by TP antagonist, ICI-192,605 (6 and 12 μM) almost completely. On the other hand, the TP agonist showed no significant effect on trunk circulation, when blood flow through intersegmental artery at 50 hpf was counted as an index (Control; 118 ± 18, 1.4 μM U-46619; 100 ± 15: $n = 20$ each) (Teraoka *et al.*, 2002).

Inhibitory effects of thromboxane synthase knock-down on mesencephalic circulation failure caused by TCDD

From the results above, it was assumed that production of thromboxane A2 could be involved in the effect of TCDD. Thus, the effect of gene knock-down of thromboxane A synthase (TBXS) on TCDD-induced mesencephalic circulation failure was evaluated. We intended to block translation (TBXS-MO-tra) and splicing (TBXS-MO-spl), along with their respective 5 nucleotides mismatch negative control morpholinos (5mis-MOs) (Fig. 5). Both types of TBXS-MOs and 5mis-TBXS-MOs showed no significant effect on mesencephalic circulation. Injection of TBXS-MOs but not their 5mis-MOs into one to four cell stage embryos, almost completely blocked mesencephalic circulation failure caused by TCDD at 50 hpf embryos (Fig. 5 and Table 3).

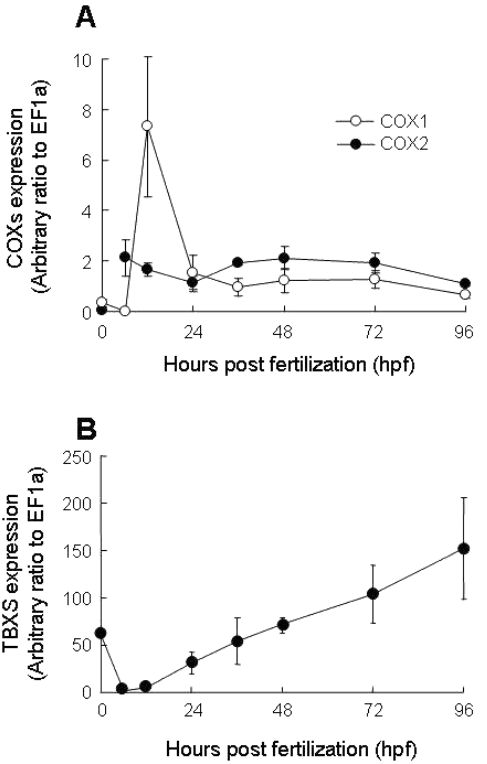

Fig. 6. Expression profiles of COXs (A) and TBXS (B) during zebrafish development. Expression levels of COXs and TBXS in various developmental stages of zebrafish just after fertilization to 96 hpf by quantitative real time RT-PCR. Results are expressed as mean ± SEM after normalization with EF1α. N = 5 per group.

Expression of transcripts of prostanoid synthesizing enzymes during zebrafish development

Although Grosser *et al*. (2002) reported spatial distribution of COX2 transcripts by *in situ* hybridization with 96 hpf larva, there was no information available for COX2 transcripts before 96 hpf, except very early stages (North *et al*., 2007). Real-time PCR was carried out to study the expression of COX2 during development, as well as for COX1. As shown in Fig. 6A, both transcripts of COX2 and COX1 were absent just after fertilization, suggesting these are completely zygotic origin. Then, the amount of COX2 transcripts increased by 6 hpf and was maintained until at least 96 hpf. By contrast, expression level of COX1 abruptly reached the maximum, decreased to the moderate value by 24 hpf, and was maintained by 96 hpf, supporting the important role of COX1 in gastrulation (Cha *et al*., 2006). Therefore, COX2 transcripts were present before and during the formation of mesencephalic circulation failure (50 hpf).

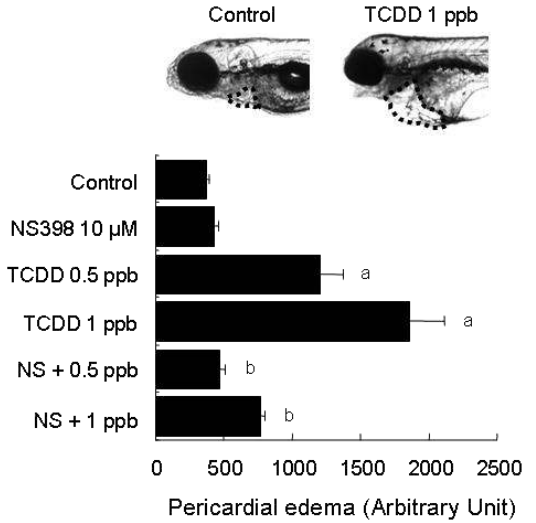

Fig. 7. Recovery of TCDD-induced pericardial edema by COX2 inhibitor. Embryos were treated with 10 μM NS398, beginning at 36 hpf, in the presence or absence of TCDD (0.5 and 1 ppb, 24–36 hpf). At 96 hpf, severity of pericardial edema was quantified with image analysis of a lateral view and was expressed in pixels. Results are expressed as mean ± SEM. $N = 8$ per group. a; $p < 0.05$ against control. b; $p < 0.05$ against respective TCDD responses without inhibitor.

As shown in Fig. 6B, TBXS transcripts were recognized just after fertilization, suggesting the maternal origin. Then the transcripts level was reduced to almost zero around 6 hpf and increased again from 24 hpf. The expression level gradually rose and reached the same level of maternal origin by 48 hpf. After that, TBXS transcripts still increased by 96 hpf.

Effects of COX inhibitors on pericardial edema by TCDD

TCDD (1 ppb) caused robust edema around the heart (pericardial edema) at 72 hpf and 96 hpf (Fig. 7), confirming our previous results (Henry *et al.*, 1997; Teraoka *et al.*, 2003b), although the effects of 0.5 ppb TCDD at 72 hpf were somehow fluctuated due to the shortening of exposure period.

A selective inhibitor for COX2, NS398 (10 μM), blocked the edema by TCDD (0.5 ppb) in 72 hpf and 96 hpf (Fig. 7). On the contrary, a selective inhibitor for COX1, SC560 (2.5 μM) was without effect for the edema evoked by 0.5 ppb and 1 ppb at both 72 and 96 hpf. We selected 2.5 μM SC560, as a higher concentration of SC560 (5 μM) per se caused circulation failure.

DISCUSSION

Our results suggest the involvement of COX2 in the mesencephalic circulation failure caused by TCDD in 50 hpf zebrafish embryos. TCDD-induced

mesencephalic circulation failure was prevented by two different selective COX2 inhibitors, NS398 and SC236, and the general COX inhibitor, indomethacin. In contrast, a selective COX1 inhibitor, SC560 showed no significant effect on the TCDD-induced circulation failure at the concentration used, although the possibility that SC560 in zebrafish embryos did not reach enough concentration to work as COX1 inhibitor in this study. The involvement of COX2 was further supported by the experiment with COX2 knock-down by splice inhibition. As COX1 has pivotal role in early embryogenesis (Cha *et al*., 2006), we could not study the effect of COX1 knock-down. However, we confirmed that the interruption of COX2 splicing effectively inhibited the production of normal COX2 transcripts without any significant effect on COX1 transcription. These results suggest that COX2 but not COX1 is involved in mesencephalic circulation failure caused by TCDD.

Recently, it was reported that zebrafish has the second COX2 gene (COX2b) (Ishikawa *et al*., 2007). COX2b was inducible in adult fish by phorbor ester exposure and functional in terms of the enzyme activity when it was expressed in COS cells (Ishikawa *et al*., 2007). Although there is no information available on the expression in zebrafish at developmental stage, COX2b transcripts were detected in the heart as well as in gill, kidney, gut and sex organs of adult zebrafish (Ishikawa *et al*., 2007). It might be assumed that general COX or COX2-specific inhibitors used in the present study affect activities of not only COX2a, but also COX2b. However, the splice site of COX2b does not have any homology with original COX2 (COX2a), and the COX2-MO-spl used here could suppress only the splicing process of COX2a gene. Thus, it can be concluded that COX2a could be involved in TCDD-induced mesencephalic circulation failure by TCDD.

In this study, several results to suggest the involvement of thromboxane pathway in the effects of TCDD were obtained. Two different specific TP antagonists (ICI-192,605 and SQ29548) were very effective to inhibit TCDD-induced mesencephalic circulation failure. Furthermore, the TCDD toxicity was mimicked by TP agonist (U-46619), and the circulation failure caused by the TP agonist was recovered by ICI-192,605. Thus, it seems that TP activation is necessary and sufficient for reduction of the blood flow in mesencephalic vein. As the detailed mRNA sequence of TP in zebrafish has not been available so far, we focused on thromboxane synthesizing enzyme (TBXS). Mesencephalic circulation failure by TCDD was significantly recovered by two different types of TBXS-MOs without any effect by their negative homologues. In our previous study (Teraoka *et al*., 2002), circulation failure by TCDD could be recognized only in mesencephalic vein, but not in the vessels in the trunk around 50 hpf in zebrafish. In accordance with this, TP agonist slowed circulation only in mesencephalic vein and possibly other vessels in the brain, but not in trunk vessels around 50 hpf in the present experiment. Thus, the response to TP agonists in mesencephalic vein is different from that in the vessels in the trunk region. This could be one of the explanations why TCDD affected mesencephalic circulation only. Collectively, these experimental facts strongly suggest that TP activation mediates TCDD-induced mesencephalic circulation failure through the production

of thromboxane A2 by TBXS under COX2 activation.

In our previous studies, TCDD-evoked mesencephalic circulation failure was blocked by several CYP inhibitors (Dong *et al.*, 2002). While most CYPs are mainly expressed in the liver, some CYPs are primarily detected in the heart and vasculature, as well as gastrointestinal tract, kidney and lung. This includes CYP1A (Stegeman *et al.*, 1995; Yamazaki *et al.*, 2002; Andreasen *et al.*, 2002). Immunohistochemistry with fish-specific antibody or *in situ* hybridization confirmed CYP1A protein and transcripts induced in fish vascular endothelium including zebrafish, upon AHR activation (Dong *et al.*, 2004). However, the role of CYP1A in TCDD toxicity in developing zebrafish is controversial (Teraoka *et al.*, 2003b; Carney *et al.*, 2004). TBXS, which emerged as a mediator of TCDD-induced circulation failure in this study, is a member of cytochrome P450 enzyme, CYP5A. Thus, TBXS could be a target of CYP inhibitors. On the other hand, other specific CYP species localized in vascular smooth muscle and endothelium contribute to the regulation of vascular tone and homeostasis (reviewed by Fleming, 2001, 2008). These CYPs include some prostanoid synthesizing enzymes, such as CYP2 family (CYP2C and CYP2J) to produce epoxyeicosatrienoic acids (EETs) and prostacyclin (PGI_2) synthase (CYP8A1) (Schildknecht *et al.*, 2004). Recently, it has been reported that CYP1C1 and CYP1C2 are highly inducible by TCDD exposure in developing zebrafish as novel CYP members (Jönsson *et al.*, 2007a, b), although there is no information available so far regarding their substrates and catalytic reactions and products, as well as their localization patterns during zebrafish development. Localization pattern and induction property of TBXS or other CYP1s should be clarified during zebrafish development in the following study.

There have been many reports on COX2 induction by TCDD, such as in hepatoma cells (Puga *et al.*, 1997) and renal epithelial cell lines (Wolfle *et al.*, 2000) as well as in spleen and lung of mice *in vivo* (Vogel *et al.*, 1998). The AHR consensus motif, XRE, has been found in the promoter region of COX2 in pancreatic beta-cell lines to stimulate transcription of COX2 (Yang and Bleich, 2004), while COX2 induction by other stimuli such as interleukin or malignant transformation is primarily due to mRNA stabilization in umbilical cord endothelial cell lines (Ristimaki *et al.*, 1994) or lung fibloblast cell lines (Dixon *et al.*, 2000). Furthermore, C/EBPα has been suggested as a key transactivator for COX2 induction (Vogel *et al.*, 2004). While TCDD is a well-known tumor promoter without significant genotoxicity, TCDD induces COX2 to cause enhancement of malignant transformation in mouse fibroblasts (Wolfle *et al.*, 2000) or lymphoma possibly by inhibition of apoptosis (Vogel *et al.*, 2007). In our preliminary study, however, TCDD did not increase COX2 transcripts when 50 hpf zebrafish larva as a whole body was used for quantitative RT-PCR (data not shown). In accordance with this, microarray analysis could not recognize COX2 as a target of TCDD in 72 hpf zebrafish whole embryos (Handley-Goldstone *et al.*, 2005; Carney *et al.*, 2006). These might indicate that COX2 induction by TCDD is localized in strictly restricted area in zebrafish larva around the stage for circulation failure. Indeed, transcripts of COX2 have been detected in only

endothelial cells of zebrafish at 36 hpf (North *et al*., 2007). It is also to be noted that typical XRE consensus motif could not be recognized in the promoter region of zebrafish COX2, according to the sequence available on NCBI database (Kubota and Teraoka, unpublished observation). The mode of COX2 involvement in the inhibitory effects of TCDD on the mesencephalic circulation failure should be clarified in further study.

Similar recovery effects by COX2 inhibition were confirmed against TCDD-induced pericardial edema, using NS398. These observations raise the possibility that prostanoid-mediated mechanism could be involved in TCDD-induced edema. In relation to our results, aristolochic acid, a natural product found in Chinese herbs, evoked an edema in developing zebrafish and this effect was attenuated by NS398 via reducing the expression of the inflammatory genes (Huang *et al*., 2007). Thus, prostanoid pathway could be a common mechanism for vasculocardiac failure by chemicals in developing zebrafish as well as in mammals. The detailed study should be carried out in the future study.

In conclusion, it is suggested that COX2-TBXS-TP pathway mediates TCDD-induced circulation failure caused by TCDD in the dorsal midbrain of developing zebrafish. As far as we know, this is the first report on the possible involvement of prostaglandin pathway in TCDD-evoked acute toxicity other than apoptosis and cancer. However, it is important to define the detailed signaling underlying these endpoints of toxicity in light of the mechanism of action of TCDD and the possible role of prostanoid signaling.

Acknowledgments—This work was supported by Grants-in-Aid for Scientific Research (H.T. and A.K.) from Japan Society for the Promotion of Science, Grant-in-Aid (H.T.) for High Technological Research Center (Rakuno Gakuen University) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, Akiyama Foundation (H.T.), and cooperative research from active research in Rakuno Gakuen University 2006 (H.T.). The award of the Postdoctoral Fellowship for Researchers from the Japan Society for the Promotion of Science to A.K. (No. 4313) is acknowledged.

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H. Teraoka (e-mail: hteraoka@rakuno.ac.jp), A. Kubota, Y. Kawai and T. Hiraga