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# Aryl Hydrocarbon Receptor is Required for Prevention of Blood Clotting and for the Development of Vasculature and Bone in the Embryos of Medaka Fish, *Oryzias latipes*

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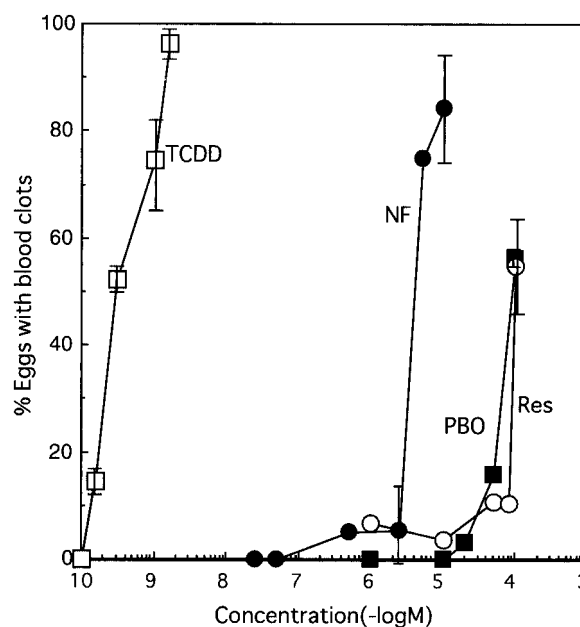
**ABSTRACT**—The aryl hydrocarbon receptor (AHR) is a member of ligand-activated transcription factors and conserved among vertebrates. To investigate the role of AHR in fish development, medaka embryos were treated with agonist (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), antagonists ( $\alpha$ -naphthoflavone and resveratrol), and inhibitor (piperonyl butoxide) of cytochromes (Cyts) P450 encoded by a battery of target genes. These embryos were found to have similar abnormal phenotypes. Among the most consistent phenotypes were blood clotting and malformation of bone that were associated with vascular damages. These results thus indicate that control of AHR is important for proper development of fish embryos. AHR may control levels of Cyts P450 that are responsible for synthesis and metabolism of a toxic compound that caused the abnormal phenotypes. Complementary DNA fragments encoding AHR homologs were cloned from medaka embryos. AHR-specific mRNA was ubiquitously expressed in embryos and adult tissues.

**Key words:** aryl hydrocarbon receptor, blood clotting, bone formation, cytochrome P450, dioxin.

## INTRODUCTION

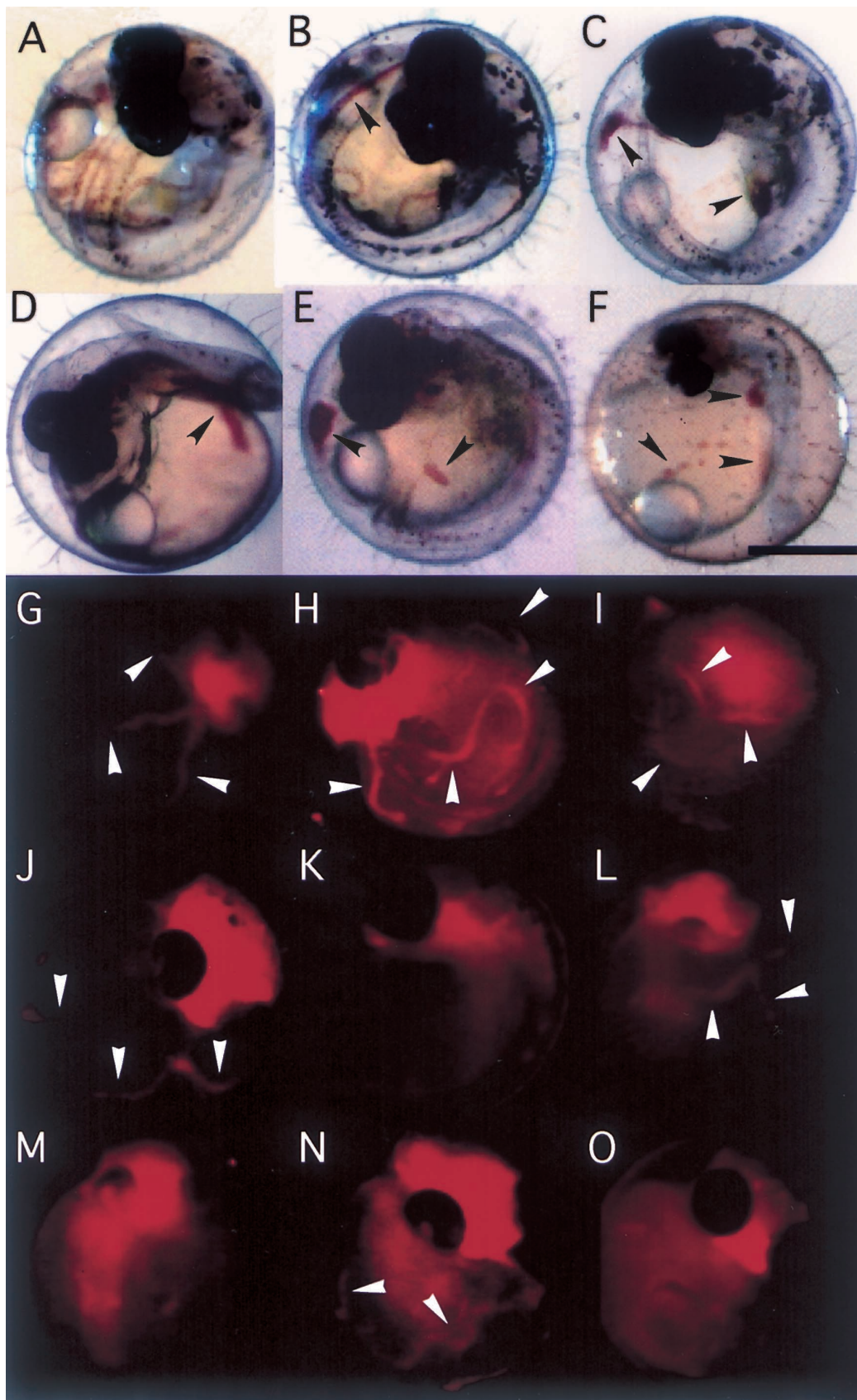
Planar halogenated hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are notorious environmental pollutants that are extremely toxic to early stages of vertebrate development (Peterson *et al.*, 1993). Hallmark signs of TCDD toxicity in fish sac fry are yolk sac edema, slowed blood flow, hemorrhage, and growth retardation culminating in mortality (Cantrell *et al.*, 1996; Henry *et al.*, 1997; Hornung *et al.*, 1999). Vascular damage, as assessed by TCDD-induced apoptotic cell death, is a key physiological mediator of the embryo toxicity (Cantrell *et al.*, 1996; Cantrell *et al.*, 1998). These chemicals bind to a ligand-dependent transcriptional factor called the aryl hydrocarbon receptor (AHR), resulting in the activation of a battery of genes encoding various cytochromes (Cyts) P450 that are responsible for degradation of the environmental contaminants (Hankinson, 1995; Guiney *et al.*, 1997; Guiney *et al.*, 2000). AHR is conserved among vertebrates, thus, may have arisen in an ancestral vertebrate as a detoxification system.

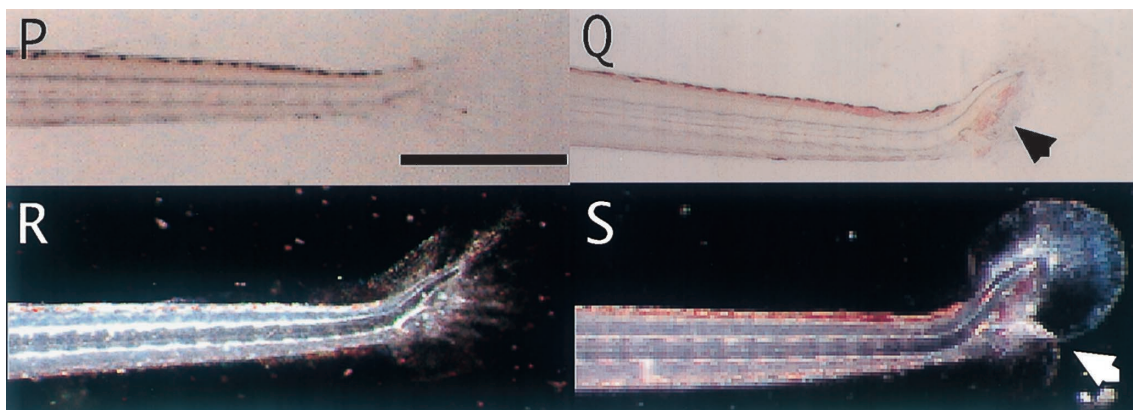
Although, to date, an endogenous ligand for AHR has not been found, AHR is ubiquitously expressed in most



**Fig. 1.** Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD),  $\alpha$ -naphthoflavone (NF), resveratrol (Res), and piperonyl butoxide (PBO) on blood clotting during the embryo stage. Eggs were treated with TCDD, NF, Res, or PBO at the indicated concentrations until 6, 4, or 5 dpf, respectively, and counted for blood clots.

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**Fig. 2.** Photographs of blood clots, yolk vein, and fin. Eggs and fry were treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD),  $\alpha$ -naphthoflavone (NF), resveratrol (Res), or piperonyl butoxide (PBO) as follows and photographed for blood clots (A–F), yolk vein under green fluorescence (G–O), and fin (P–S): (A) mock-treated, 5 dpf; (B, C) 1.55 nM TCDD, 5 and 7 dpf; (D) 10  $\mu$ M NF, 5 dpf; (E) 100  $\mu$ M PBO, 5 dpf; (F) 100  $\mu$ M Res, 4 dpf; (G–I) mock-treated at 3, 5, and 7 dpf; (J, K) 1.55 nM TCDD, 5 and 7 dpf; (L, M) 10  $\mu$ M NF, 3 and 5 dpf; (N, O) 100  $\mu$ M PBO, 4 and 5 dpf; (P, R) mock-treated, 5-day post-hatching; and (Q, S) 0.155 nM TCDD, 5-day post-hatching. Arrows indicate blood clots (B–F, and Q), yolk veins (G–J, L, and N), and the constricted fin (S). Bar, 0.5 mm.

organs and cells in the body (Rowlands and Gustafsson, 1997). However, there is only a limited knowledge of developmental and physiological functions of AHR in the mouse (Gonzalez and Fernandez-Salguero, 1998), although the role of AHR in detoxification of environmental aryl hydrocarbons has been extensively studied *in vitro* (Hankinson, 1995). AHR-null mice were resistant to the acute toxicity (Fernandez-Salguero *et al.*, 1996) of and the teratogenic response (Mimura *et al.*, 1997) to TCDD, and found to have a number of abnormal phenotypes such as decreased accumulation of lymphocytes in the spleen and lymph nodes and reduction in liver size that are associated with accelerated rates of apoptosis (Fernandez-Salguero *et al.*, 1995), and difficulties in reproduction (Abbott *et al.*, 1999; Robles *et al.*, 2000). Thus, AHR is involved in the toxicity of and the teratogenesis by TCDD *in vivo*, and plays an important role in the development of the liver and the immune system, and in reproduction. However, no such function has been elucidated in other vertebrates.

Here we re-evaluated the role of AHR in chemical toxicity of TCDD in medaka fish embryos because there have been no pharmacological studies in fish using antagonist and also examined for any possible developmental and physiological function of AHR in medaka fish embryos using antagonists and Cyts P450 inhibitor. We found that AHR mediates TCDD toxicity such as blood clotting, malformation of bone, and regression of blood vessels, and that AHR is required for the embryonic development of vasculature and bone. To our knowledge, this is the first report of the developmental role of AHR in lower vertebrates.

## MATERIALS AND METHODS

### Fish and embryo culture

We used the d-rR strain of medaka fish, *O. latipes* (Kawahara and Yamashita, 2000). The fish were maintained at 25–26°C under

artificial photo-period of 14L:10D, and fed by powdered Tetramin (Tetra). Eggs were collected within 12 hr postfertilization (hpf), rinsed with tap water, and immersed in Yamamoto's salt solution (Yamamoto, 1969) with or without test chemicals. At least 30 eggs were used in each experiment. TCDD was purchased from Cambridge Isotope Laboratories, Inc. Antagonists,  $\alpha$ -naphthoflavone (NF) (Gasiewicz and Rucci, 1991; Merchant *et al.*, 1993) and resveratrol (Res) (Ciolino *et al.*, 1998; Casper *et al.*, 1999; Singh *et al.*, 2000), were from Sigma. Cyts P450 inhibitor, piperonyl butoxide (PBO) (Dahl and Hodgson, 1979; Testa and Jenner, 1981; Adams *et al.*, 1993), was from Tokyo Kasei Kogyo Co. These reagents were dissolved in acetone. The stock solutions were diluted over 1,000-fold with Yamamoto's solution and added to eggs of 12 hpf for NF, Res, and PBO or of 24 hpf for TCDD. The solvent was added to the mock-treated eggs as a control. The reducing agent, N-acetyl cysteine (NAC) (Sigma), was dissolved in Yamamoto's solution and added to 12 hpf eggs. Eggs and fry were cultured under the same condition as above (except without feed) and inspected for blood clotting under a dissecting microscope. Eggs and fry in which blood clots formed were counted.

Data are presented as mean  $\pm$  SEM. Statistical significance between values of control and experiment was assessed by Student's *t*-test.

### Observation of blood vessels

In order to observe the development of blood vessels, eggs were fixed with 4% paraformaldehyde for 3 days and observed under green fluorescence with a filter set (excitation filter, 546/10 nm; barrier filter, 590 nm) in Leica MZ FLIII stereo-fluorescence microscope. The fixed eggs were also dechorionated with forceps and stained with hematoxylin.

### Bone staining

In order to observe the bone development, calcified bone was stained with alizarin S essentially as described (Takeuchi, 1960). In brief, fish were anesthetized with 0.015% phenylurethane, skinned with forceps, treated with 2% KOH for 24 hr, and finally stained with 0.1% alizarin S solution. After washing in tap water, the fish were successively transferred to 50% and 70%, and finally embedded in 100% glycerin. Anesthetized fry were directly treated with 2% KOH for 2 h, fixed in 4% paraformaldehyde for 24 hr, then stained with alizarin S.

### Isolation of cDNAs encoding medaka AHR homologs

As PAS domain of AHR is highly conserved among vertebrates (Rowlands and Gustafsson, 1997), a corresponding region of cDNA was amplified with degenerated oligonucleotides (AhR-A1 and AhR-B1) as described (Hahn and Karchner, 1995) using total RNA from 6-day postfertilization (dpf) medaka embryos. The cDNA fragment was cloned in plasmid and sequenced. Based on the sequence, nested oligonucleotides were designed and 5' and 3' RACEs (rapid amplification of cDNA ends) were performed on the same RNA by using 5' and 3' RACE Systems (GIBCO BRL), yielding the remainder of the coding sequence, 5' and 3' untranslated regions, and polyadenylation sequence.

### RNA analysis

Total RNA was extracted from embryos and adult tissues as described (Kawahara *et al.*, 2000). RT-PCR (reverse transcription-polymerase chain reaction) analysis was done as described (Kawahara *et al.*, 2000) with the primers as follows for generation of the 437-bp cDNA encoding a part of PAS domain: poly(dT) oligonucleotide used for RT, and 5'-CCAGCAGGAGTTCAGGAGGA and 5'-ATTTTACCCTTTGCGTCACA for PCR. Amplified DNA was electrophoresed in 1% agarose gel and stained with ethidium bromide.

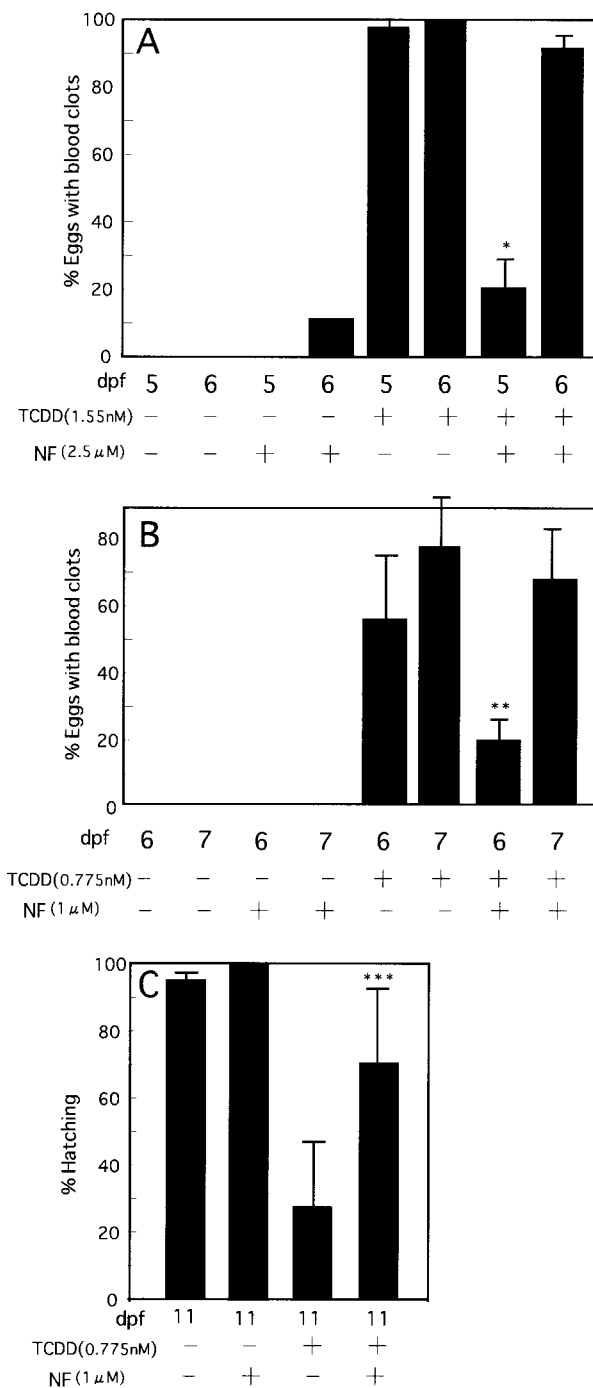
## RESULTS

### AHR mediates the toxic effects of TCDD on vascular development

We re-evaluated the toxic effects of TCDD on medaka embryos. To do this, embryos (1 dpf) were immersed in saline solution for medaka containing increasing concentrations of TCDD, and observed for any abnormal phenotype under a dissecting microscope (Fig. 1). Clearly visible signs of blood clotting were apparent after 4 days in caudal veins of TCDD-treated embryos (Fig. 2B), although blood cells were circulating in vasculature (Fig. 2J) but at a reduced rate. Blood clots were also found in yolk veins after 6 days (Fig. 2C), at that time, vascular structure was almost absent (Fig. 2K). In control embryos, yolk veins were apparent at 3 dpf (Fig. 2G) and developed progressively in a curve structure (Fig. 2A, H and I). Very small blood clots were occasionally found in yolk veins of normal embryos (less than 3%), but not scored in this study. These results are consistent with the previous observation that TCDD induces apoptosis of blood vessels (Cantrell *et al.*, 1996).

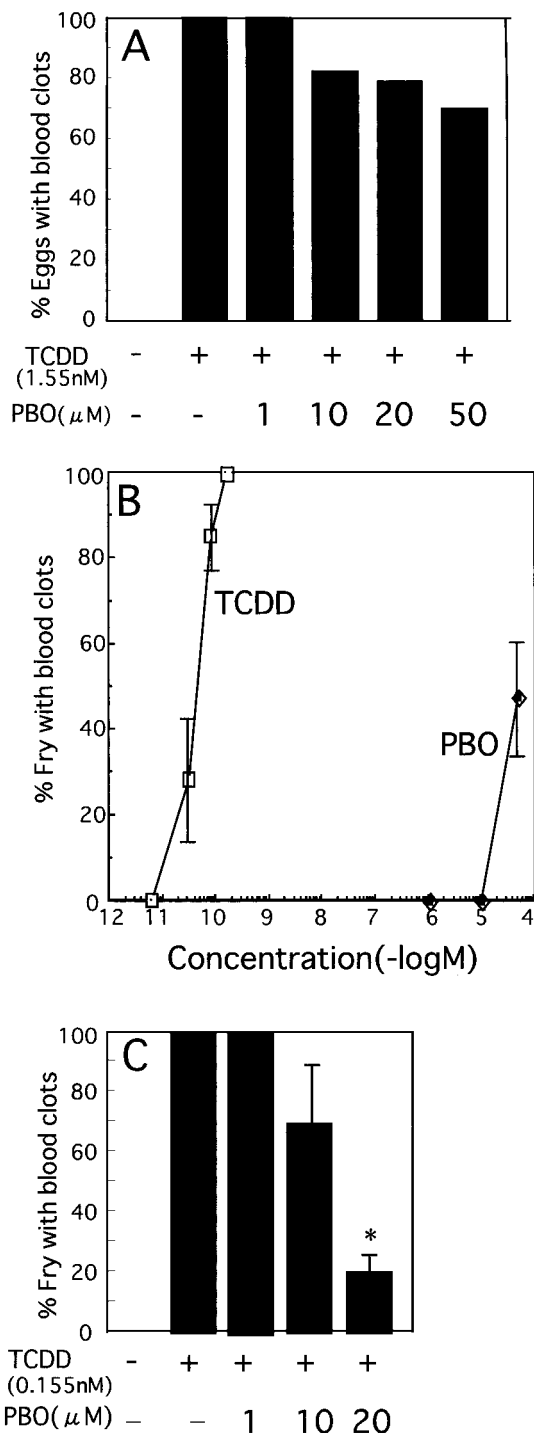
If TCDD induced the vascular damage through activation of AHR, the antagonist (NF) would reduce the extent to which blood clotting was detected. For this purpose, two different experiments were done, in which embryos were treated with high (1.55 nM) or medial (0.775 nM) concentration of TCDD (Fig. 3A or B, respectively). For both cases, addition of NF effectively suppressed blood clotting but only transiently (Fig. 3A and B). However, in the latter case, NF markedly enhanced the hatching success of TCDD-treated embryos, giving rise to almost complete hatching (Fig. 3C). These results indicate that TCDD-induced vascular damage is mediated through activation of AHR.

It is well known that TCDD-bound AHR activates transcription of a battery of genes encoding Cyts P450. If these



**Fig. 3.** Suppression by  $\alpha$ -naphthoflavone (NF) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced blood clotting and mortality. **(A)** Eggs were treated with 1.55 nM TCDD and 2.5  $\mu$ M NF until 5 and 6 dpf as indicated, and examined for blood clotting. \* $P$ <0.01. **(B)** Eggs were treated with 0.775 nM TCDD and 1  $\mu$ M NF until 6 and 7 dpf as indicated, and examined for blood clotting. \*\* $P$ <0.2. **(C)** Eggs were treated as described in **(B)**, and examined for hatching rate at 11 dpf. \*\*\* $P$ <0.05.

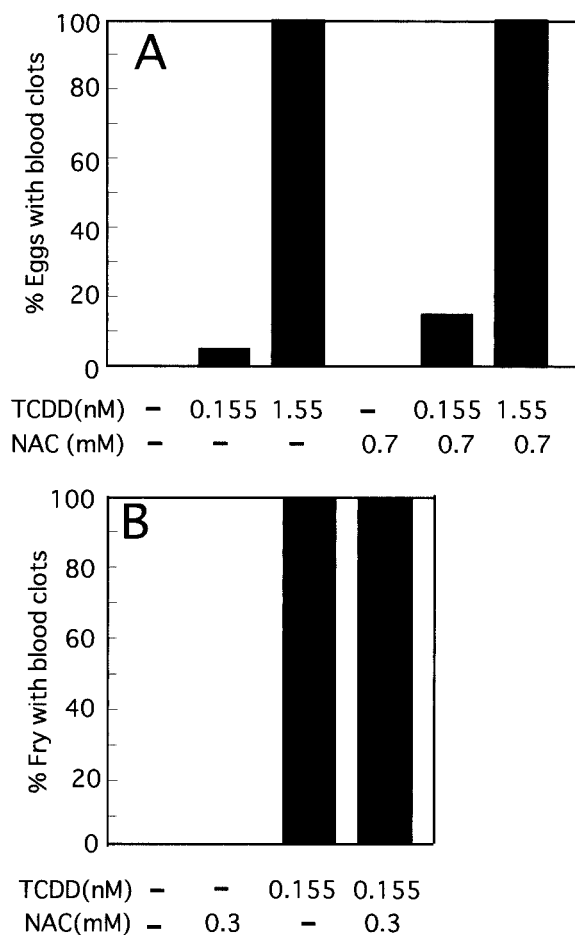
enzymes were involved in the TCDD-induced toxicity, an inhibitor of P450 would reduce the rate of TCDD-induced blood clotting. We therefore examined the ability of PBO to provide protection against high concentration (1.55 nM) of



**Fig. 4.** Suppression by piperonyl butoxide (PBO) of 2,3,7,8- tetra-chlorodibenzo-*p*-dioxin (TCDD)-induced blood clotting. (A) Eggs were treated with 1.55 nM TCDD and increasing concentrations (μM) of PBO as indicated until 5 dpf, and examined for blood clotting. (B) Eggs were treated with TCDD and PBO at the indicated concentrations until 5-day post-hatching, and examined for blood clotting in the caudal fin. (C) Eggs were treated with 0.155 nM TCDD and increasing concentrations of PBO as indicated until 5-day post-hatching, and examined for blood clotting in the caudal fin. \**P*<0.05.

TCDD (Fig. 4A). Unexpectedly, PBO reduced the blood clotting rate only slightly; we cannot use higher concentrations of PBO because PBO itself induced blood clotting (described below). We therefore tried to seek for conditions under which lower concentrations of TCDD induce blood clotting effectively. We found that blood clots formed in the caudal fin (Fig. 2Q) after immersing embryos until 5-day post-hatching at subnanomolar concentrations of TCDD (Fig. 4B). Blood clots did not form in the control fin (Fig. 2P). Under the above condition, PBO effectively suppressed the adverse effect of TCDD (Fig. 4C). These results suggest that the TCDD-induced toxicity was caused by elevated expression of a certain Cyt P450.

Previous reports conclude that oxidative stress caused by TCDD-induced expression of Cyts P450 contributes to embryotoxicity and vascular damage associated with apoptosis, because the reducing agent, NAC, partially recovers



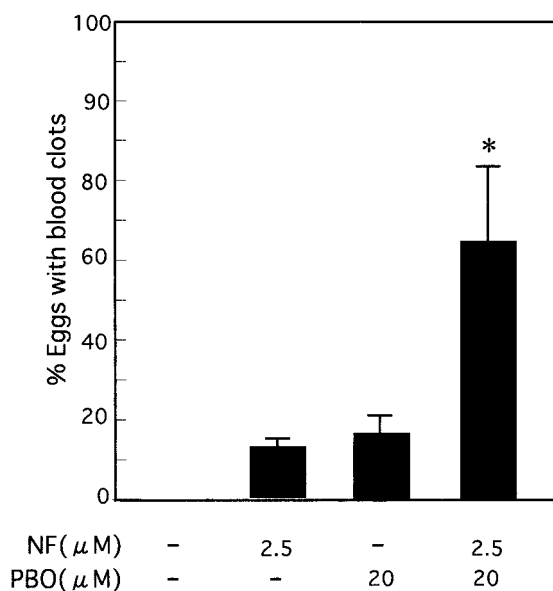
**Fig. 5.** N-acetyl cysteine (NAC) fails to suppress the 2,3,7,8- tetra-chlorodibenzo-*p*-dioxin (TCDD)-induced blood clotting. (A) Eggs were treated with TCDD (nM) and NAC (mM) at the indicated concentrations until 5 dpf, and examined for blood clotting. (B) Eggs were treated with 0.155 nM TCDD and 0.3 mM NAC as indicated until 5-day post-hatching, and examined for blood clotting in the caudal fin.

the TCDD-induced embryotoxicity (Cantrell *et al.*, 1996): they observed 41% survival of the embryos that had been treated with 28 nM TCDD for 2 hr and released in 0.1 mM NAC until 3 days posthatch, in contrast to 2% survival of the embryos that had been treated with TCDD and released in water. The ability of NAC to inhibit TCDD-induced toxicity was re-assessed by adding 0.7 mM (Fig. 5A) or 0.3 mM (Fig. 5B) NAC to eggs before and during the treatment with TCDD. NAC could not inhibit the blood clotting induced by 0.155 or 1.55 nM TCDD. NAC itself induced blood clotting at more than 0.9 mM (data not shown). These results suggest that general oxidative stress is not responsible for the TCDD-induced blood clotting.

#### Vascular damage induced by antagonists (NF and Res) and Cyts P450 inhibitor (PBO)

At the initial experiments determining the concentrations of reagents used, we found that NF, Res, and PBO induced blood clotting at higher concentrations than those used for suppression of TCDD-induced toxicity (Fig. 1). Blood clots formed in caudal and yolk veins (Fig. 2D-F). Yolk veins developed normally at the early time of incubation (up to 4 dpf) (Fig. 2L and N), but their regression was apparent at the time when blood clots formed in yolk veins (at 5 dpf) (Fig. 2M and O). These results suggest that either inactivation of AHR by NF and Res or inhibition of certain Cyts P450 by PBO caused vascular damage and blood clotting.

If the hypothesis were true, antagonist of AHR and Cyts P450 inhibitor would act synergistically to cause toxicity. We examined the synergy between low concentrations of NF (2.5  $\mu$ M) and PBO (20  $\mu$ M) that alone did not show any effect. Combination of these chemicals clearly increased the



**Fig. 6.** Synergistic effects of  $\alpha$ -naphthoflavone (NF) and piperonyl butoxide (PBO) on blood clotting. Eggs were treated with NF and PBO at the indicated concentrations ( $\mu$ M) until 6 dpf, and examined for blood clotting. \* $P < 0.2$ .

rate of blood clotting (Fig. 6). We therefore conclude that control of AHR activity and levels of Cyts P450 is required for proper development of vasculature in fish.

#### Malformation or degeneration of bone induced by TCDD, NF, and PBO

During the experiments by incubating eggs with lower concentrations of TCDD (less than 80 pM) until 7 dpf, most eggs developed normally in appearance and blood clots did not form. The eggs were transferred to Yamamoto's solution, then to aquaria after hatching, and reared to adult by normal diet as usual. Unexpectedly we found that these fish were deformed in shape like wavy mutants (Takeuchi, 1960). We examined the bone development by staining with alizarin S. The vertebral column of TCDD-treated fish curved dorso-ventrally and laterally (Fig. 7A and B). Neural and haemal spines were short in length and deformed (Fig. 7B). NF also suppressed the TCDD-induced toxicity on bone formation (Fig. 7C), indicating the involvement of AHR.

We examined the effect of TCDD on the embryonic bone formation by incubating eggs with TCDD until 5 days post-hatching. The staining of the fry with alizarin revealed the absence of calcification in the posterior region of spinal cord and in spines (Fig. 7D and E). We also found that caudal fins were round in shape and constricted (indicated by arrow in Fig. 2S) in the TCDD-treated fry.

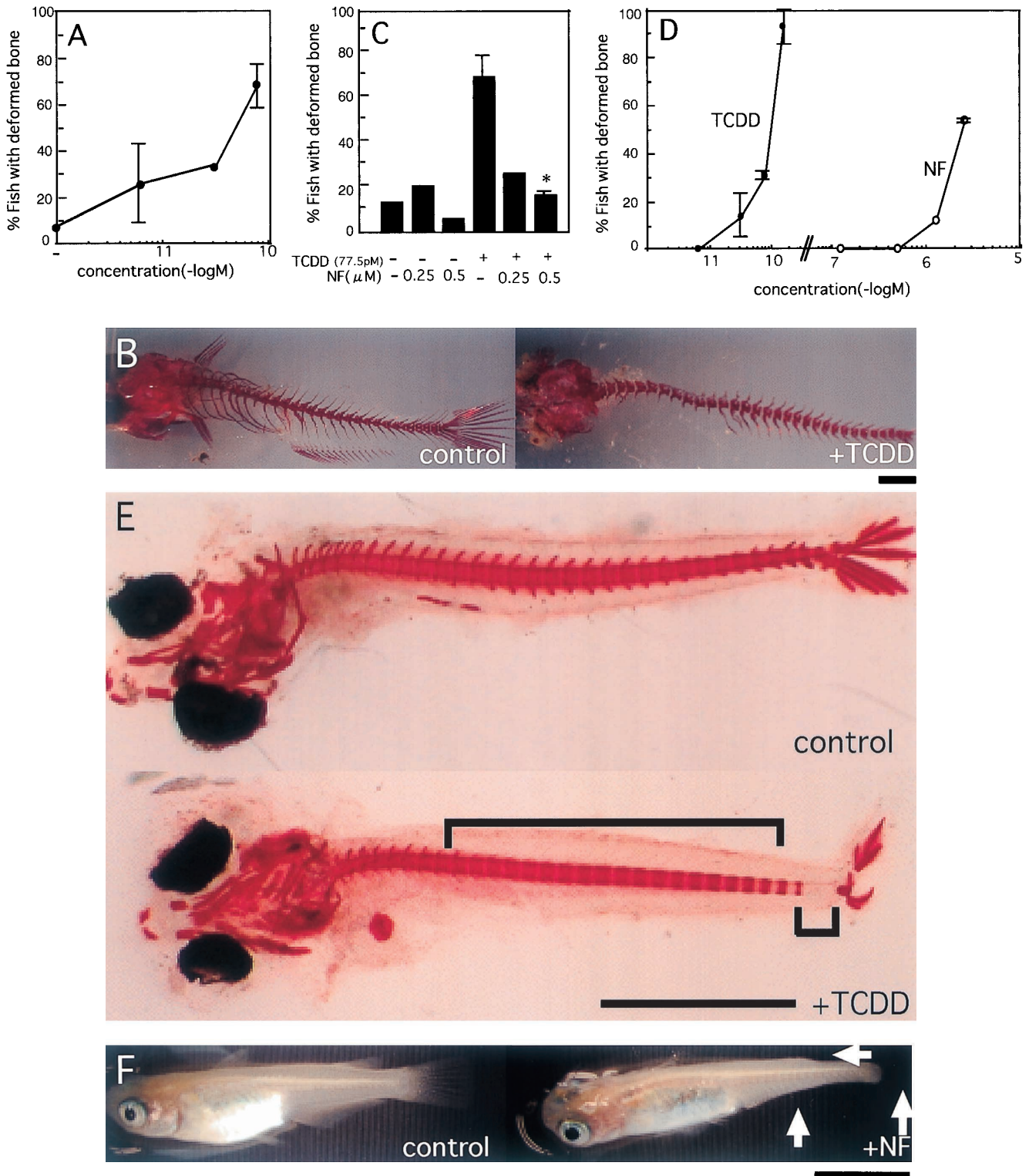
In order to examine the possible function of AHR and Cyts P450 in the embryonic bone formation, eggs were treated with NF or PBO until 5 days post-hatching. The treatment with NF (2.5  $\mu$ M) did not cause blood clotting in any portion of the fry (data not shown), which was different from the result with TCDD (Figs. 2Q and 4B). However, the treatment also caused degeneration of the posterior end of the spinal cord, but with normal development of spines (Fig. 7D, data not shown). PBO (50  $\mu$ M) also caused the same defect in bone formation as that NF did (data not shown).

We further examined whether NF affects homeostasis of adult fish. To do this, adult fish which had been reared by normal diet for 2 months were fed by NF-containing diet (2 mg NF/g diet) for 2 months. During the cultivation, population of fish lacking posterior fins including anal, caudal, and dorsal fins appeared after a month and became increasing near to 100% by two months (Fig. 7F).

Taken together, these results suggest that hyperactivation of AHR by TCDD is toxic to the embryonic development of bone and caudal fin, that AHR is required for proper development of bone and homeostasis of posterior fins, and that a certain Cyt P450 is also required for bone development.

#### Isolation and characterization of cDNAs encoding AHR homologs of medaka fish, and ubiquitous expression of AHR mRNA

We first obtained four independent cDNA clones (clones 1, 2, 3 and 4) corresponding to PAS domain (Fig. 8A). These clones were found to be identical by sequencing.



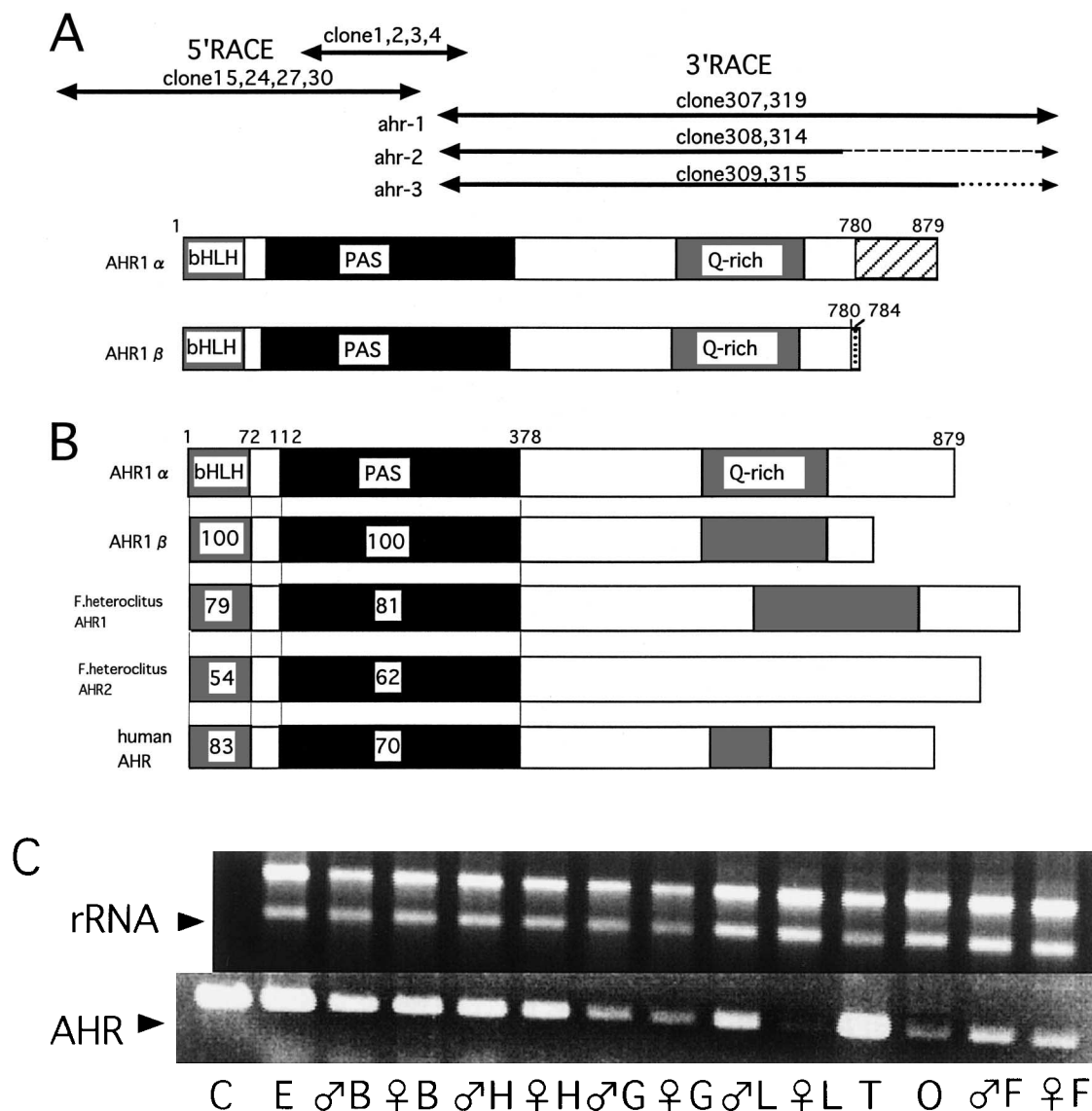
**Fig. 7.** Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and  $\alpha$ -naphthoflavone (NF) on bone formation. (A) Eggs were treated with TCDD at the indicated concentrations until 7 dpf, and reared to adult under TCDD-free condition. The adult fish were examined for bone formation after staining with alizarin. (B) Alizarin-stained bone of mock-treated (control) and TCDD (77.5 pM)-treated fish in (A). (C) Eggs were treated with 77.5 pM TCDD and NF at the indicated concentrations ( $\mu$ M) until 7 dpf, reared to adult under normal condition, and examined for bone formation. \* $P < 0.05$ . (D) Eggs were treated with increasing concentrations of TCDD and NF until 5-day post-hatching, and examined for bone formation. (E) Alizarin-stained bone of mock- (control) and TCDD (0.155 nM)-treated fish in (D). Spines and posterior spinal bone are absent in the TCDD-treated fry as noted. (F) Normal adult fish were fed by NF-containing diet (2 mg NF/g diet) for 2 months, and photographed. Arrows indicate the degenerated fins. Bar, 1 mm in (B) and (E), and 5 mm in (F).



Next, 5' and 3' RACEs were performed, yielding four (clones 15, 24, 27 and 30) and six (clones 307–309, 314, 315, and 319) independent clones, respectively (Fig. 8A). Four clones from 5' RACE were identical. Six clones from 3' RACE were subdivided into three identical pairs, which differ from each other only in the 3' proximal sequences denoted by broken and dotted lines in Fig. 8A. Thus, we obtained three different cDNAs, named *ahr-1*, -2, and -3 (DDBJ accession numbers AB065092, AB065093, and AB065094, respectively). However, *ahr-1* and *ahr-3* encoded the same protein (AHR1 $\alpha$ ), and *ahr-2* encoded another homolog (AHR1 $\beta$ ). AHR1 $\alpha$  and

AHR1 $\beta$  differ from each other in the C-terminal peptides (amino acid 780–879 and 780–784) denoted by shaded and dotted boxes (Fig. 8A).

AHR1 $\alpha$  and AHR1 $\beta$  are composed of 879 and 784 amino acids with calculated molecular weights of 95.5 and 85.3 kDa, respectively. Both proteins may be classified into a type of AHR1 because they are most homologous to AHR1 of the teleost *Fundulus heteroclitus* (Karchner *et al.*, 1999) (Fig. 8B). The medaka AHR1 $\alpha$  and AHR1 $\beta$  are also composed of three conserved domains such as basic-helix-loop-helix (bHLH), Per-ARNT-Sim (PAS), and glutamine-rich



**Fig. 8.** Schematic drawing of the cDNAs cloned and the deduced proteins, and ubiquitous expression of AHR mRNA. **(A)** Inserts in the plasmid clones are shown on the deduced proteins (AHR1 $\alpha$  and AHR1 $\beta$ ). Plasmid numbers are marked on the corresponding inserts. The three cDNAs which differ from each other only in the 3' terminal sequences (denoted by broken and dotted lines) are named *ahr-1*, -2, and -3. AHR1 $\alpha$  and AHR1 $\beta$ , in which three conserved domains are marked by bHLH, PAS, and Q-rich, differ from each other only in the C-terminal short peptides marked by shaded and dotted boxes. **(B)** Identity (%) of amino acid sequence among bHLH and PAS domains of AHRs from medaka, *F. heteroclitus* (killifish), and human (Dolwick *et al.*, 1993). **(C)** RT-PCR analysis of total RNAs from medaka embryos (6 dpf) and adult tissues. Symbols: B, brain; C, the control band amplified from the cDNA; E, embryo; F, caudal fin; G, gill; H, heart; L, liver; O, ovary; and T, testis. Ribosomal RNAs in the RNA samples are also shown.

(Q) domains (Rowlands and Gustafsson, 1997) (Fig. 8B).

Expression of AHR mRNA was analyzed by RT-PCR on total RNAs prepared from medaka embryos and adult tissues such as brain, fin, gill, heart, liver, ovary, and testis. AHR mRNA was detected in all samples tested, and in large amounts in embryos and testis (Fig. 8C).

## DISCUSSION

### TCDD-induced vascular and bone damages through hyperactivation of AHR

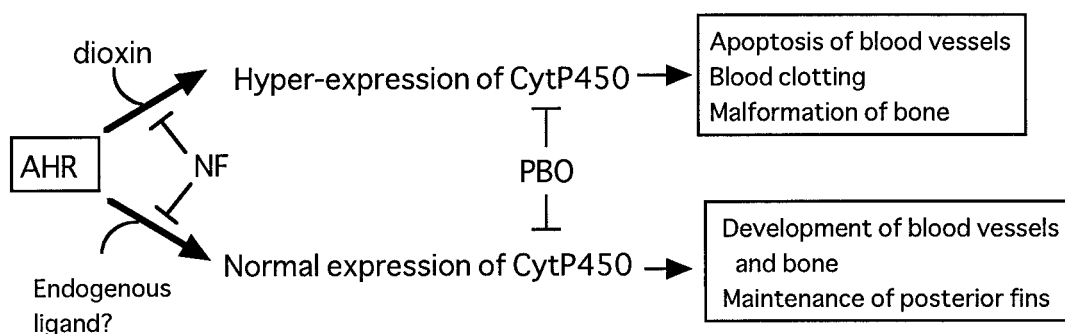
TCDD is the most potent toxicant for vertebrate species. Exposure of vertebrate embryos to TCDD can result in various acute and chronic toxicities such as reproductive failure, teratogenic abnormalities, and immunological dysfunction (Peterson *et al.*, 1993). In fish, vascular damage is the most pronounced adverse effects of TCDD exposure during embryonic development. Vascular hemorrhaging, regression of blood vessels, pericardial sac edema, and reduced circulation are hallmark indicators that vascular function is compromised in the developing embryos (Cantrell *et al.*, 1996; Henry *et al.*, 1997; Hornung *et al.*, 1999; Guiney *et al.*, 2000). The vascular lesions have been demonstrated to be associated with apoptosis and induced expression of Cyt P450 1A in blood vessels of medaka embryos (Cantrell *et al.*, 1998). In the present study, we re-examined the TCDD-induced vascular damage in medaka embryos by observing blood clotting and regression of blood vessels. We found that these vascular damages can be suppressed, but transiently, by antagonist, NF (Fig. 3), giving a convincing evidence that the TCDD-induced vascular damage is mediated through hyperactivation of AHR. The transient suppression may be explained by the fact that TCDD, but not NF, is very stable *in vivo* against catabolic activities of Cyts P450 (Miniero *et al.*, 2001). Although the damage can also be suppressed by Cyts P450 inhibitor, PBO (Fig. 4C), general oxidative stress caused by Cyts P450-mediated oxidative reactions may not be responsible for the

TCDD-induced damage, in inconsistent with the previous conclusion (Cantrell *et al.*, 1996), because reducing agent, NAC, could not recover the damage in vasculature (Fig. 5) or also in bone (data not shown). We assume that a toxic compound that may be accumulated *in vivo* by elevated levels of Cyt P450 is responsible for the TCDD-induced pathology (Fig. 9).

We also found that embryonic treatment with picomolar concentrations of TCDD causes malformation of bone in adult fish (Fig. 7). The treatment did not give apparent complications including blood clotting in the hatching fry, thus, the bone staining is the most sensitive method for detecting TCDD toxicity. The bone deformity could also be recovered by co-treatment with the antagonist (Fig. 7C), implying the role of hyperactivated AHR. TCDD may directly act on bone, because it inhibits osteogenesis in bone-forming cultures of chicken and rat cells (Gierthy *et al.*, 1994; Singh *et al.*, 2000). Treatment of medaka fish with TCDD from the egg stage to post-hatching also caused developmental defects in bone formation at the posterior region of vertebral column and at spines (Figs. 7D, E). However, it may be possible that these defects occurred secondarily to vascular damage, because blood clots formed at the base of the caudal fin under the same condition (Fig. 2Q).

### AHR is required for prevention of blood clotting and for proper development of vasculature and bone in medaka fish

AHR is conserved among vertebrates, and ubiquitously expressed in embryos and adult tissues. In the present study, we have cloned three different cDNAs encoding two AHR homologs from medaka fish, *O. latipes* (Fig. 8). The two homologs obtained may belong to a type of AHR1 by amino acid sequence similarity, thus named AHR1 $\alpha$  and AHR1 $\beta$ . They differ from each other only in C-terminal short peptide, and may be derived from alternative splicing. AHR1 mRNA was also ubiquitously expressed in medaka embryos and adult tissues, suggesting developmental and physiolog-



**Fig. 9.** Model for the role of AHR in the TCDD (dioxin)-induced toxicity, the development of blood vessels and bone, and the maintenance of posterior fins in the medaka fish, *O. latipes*. TCDD-bound AHR induces hyper-expression of a certain Cyt P450, resulting in the toxicities such as apoptosis of blood vessels, blood clotting, and malformation of bone. Either the antagonist (NF) or the Cyts P450 inhibitor (PBO) can suppress the TCDD-induced toxicity. An endogenous ligand is bound to and constitutively activates AHR. The activated AHR is responsible for normal expression of a certain Cyt P450 that is required for the development of blood vessels and bone and homeostasis of posterior fins. *In vivo* inhibition of AHR and Cyt P450 by NF and PBO, respectively, causes developmental abnormalities in vasculature and bone.

ical roles in medaka fish.

To investigate the role of AHR in fish development and physiological homeostasis, medaka embryos (12 hpf) were treated with the antagonists, NF and Res. These compounds did not cause any apparent defects until 4 dpf, but displayed developmental toxicities such as blood clotting and regression of blood vessels at 5 dpf (Figs. 1 and 2). Blood clotting may be caused by regression of blood vessels, because platelet adhesion to subendothelial collagens and activation by components of the extracellular matrix are crucial for blood coagulation (Nieswandt *et al.*, 2001). NF also caused the malformation of bone at 5-day post-hatching (Fig. 7D) and the regression of posterior fins such as anal, caudal, and dorsal fins at the adult period (Fig. 7F). These results suggest the presence of an endogenous ligand for AHR and that constitutive activation of AHR is specifically required for the development of blood vessels and bone and for the maintenance of posterior fins (Fig. 9).

Ligand-bound AHR activates transcription of a battery of genes including various Cyp1A50. If levels of a certain Cyt P450 were controlled by AHR bound to an endogenous ligand and required for proper development of blood vessels and bone, the well-known inhibitor (PBO) of the enzymatic activity of Cyp1A50 would induce the same developmental defect as did the antagonist. Treatment of embryos with PBO specifically induced blood clotting, regression of blood vessels (Figs. 1 and 2), and degeneration of the posterior end of spinal cord (data not shown) at the same developmental stage as did the antagonist, suggesting the importance of Cyt P450, the identity of which is, however, unknown (Fig. 9). The synergistic effects exerted by NF and PBO (Fig. 6) also support the hypothesis. We assume that a certain Cyt P450 is responsible for degradation (or catabolism) of a toxic compound that caused the developmental abnormalities.

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## REFERENCES

- Abbott BD, Schmid JE, Pitt JA, Buckalew AR, Wood CR, Held GA, Diliberto JJ (1999) Adverse reproductive outcomes in the transgenic Ah receptor-deficient mouse. *Toxicol Appl Pharmacol* 155: 62–70
- Adams NH, Levi PE, Hodgson E (1993) Regulation of cytochrome P-450 isozymes by methylenedioxyphenol compounds. *Chem Biol Interact* 86: 255–274
- Cantrell SM, Joy-Schleizinger J, Stegeman JJ, Tillitt DE, Hannink M (1998) Correlation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced apoptotic cell death in the embryonic vasculature with embryotoxicity. *Toxicol Appl Pharmacol* 148: 24–34
- Cantrell SM, Lutz LH, Tillitt DE, Hannink M (1996) Embryotoxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD): The embryonic vasculature is a physiological target for TCDD-induced DNA damage and apoptotic cell death in medaka (*Orizias latipes*). *Toxicol Appl Pharmacol* 141: 23–34
- Casper RF, Quesne M, Rogers IM, Shirota T, Jolivet, A, Milgrom E, Savouret JF (1999) Resveratrol has antagonist activity on the aryl hydrocarbon receptor: Implications for prevention of dioxin toxicity. *Mol Pharmacol* 56: 784–790
- Ciolino HP, Daschner PJ, Yeh GC (1998) Resveratrol inhibits transcription of CYP1A1 *in vitro* by preventing activation of the aryl hydrocarbon receptor. *Cancer Res* 58: 5707–5712
- Dahl AR, Hodgson E (1979) The interaction of aliphatic analogs of methylenedioxyphenyl compounds with cytochromes P-450 and P-420. *Chem Biol Interact* 27: 163–175
- Dolwick KM, Schmidt JV, Carver LA, Swanson HI, Bradfield CA (1993) Cloning and expression of a human Ah receptor cDNA. *Mol Pharmacol* 44: 911–917
- Fernandez-Salguero PM, Hilbert DM, Rudikoff S, Ward JM, Gonzalez FJ (1996) Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced toxicity. *Toxicol Appl Pharmacol* 140: 173–179
- Fernandez-Salguero P, Pineau T, Hilbert DM, McPhail T, Lee SS, Kimura S, Nebert DW, Rudikoff S, Ward JM, Gonzalez FJ (1995) Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. *Science* 268: 722–726
- Gasiewicz TA, Rucci G (1991) Alpha-naphthoflavone acts as an antagonist of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by forming an inactive complex with the Ah receptor. *Mol Pharmacol* 40: 607–612
- Gierthy JF, Silkworth JB, Tassinari M, Stein GS, Lian JB (1994) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin inhibits differentiation of normal diploid rat osteoblasts *in vitro*. *J Cell Biochem* 54: 231–238
- Gonzalez FJ, Fernandez-Salguero P (1998) The aryl hydrocarbon receptor. *Drug Metab Dispos* 26: 1194–1198
- Guiney PD, Smolowitz RM, Peterson RE, Stegeman JJ (1997) Correlation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induction of cytochrome P4501A in vascular endothelium with toxicity in early life stages of lake trout. *Toxicol Appl Pharmacol* 143: 256–273
- Guiney PD, Walker MK, Spitsbergen JM, Peterson RE (2000) Hemodynamic dysfunction and cytochrome P4501A mRNA expression induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin during embryonic stages of lake trout development. *Toxicol Appl Pharmacol* 168: 1–14
- Hahn ME, Karchner SI (1995) Evolutionary conservation of the vertebrate Ah (dioxin) receptor: amplification and sequencing of the PAS domain of a teleost Ah receptor cDNA. *Biochem J* 310: 383–387
- Hankinson O (1995) The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* 35: 307–340
- Henry TR, Spitsbergen JM, Hornung MW, Abnet CC, Peterson RE (1997) Early life stage toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in zebrafish (*Danio rerio*). *Toxicol Appl Pharmacol* 142: 56–68
- Hornung MW, Spitsbergen JM, Peterson RE (1999) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin alters cardiovascular and craniofacial development and function in sac fry of rainbow trout (*Oncorhynchus mykiss*). *Toxicol Sci* 47: 40–51
- Karchner SI, Powell WH, Hahn ME (1999) Identification and functional characterization of two highly divergent aryl hydrocarbon receptors (AHR1 and AHR2) in the teleost *Fundulus heteroclitus*. *J Biol Chem* 274: 33814–33824
- Kawahara T, Okada H, Yamashita I (2000) Cloning and expression of genomic and complementary DNAs encoding an estrogen receptor in the medaka fish, *Oryzias latipes*. *Zool Sci* 17: 643–649
- Kawahara T, Yamashita I (2000) Estrogen-independent ovary formation in the medaka fish, *Oryzias latipes*. *Zool Sci* 17: 65–68
- Merchant M, Krishnan V, Safe S (1993) Mechanism of action of alpha-naphthoflavone as an Ah receptor antagonist in MCF-7

- human breast cancer cells. *Toxicol Appl Pharmacol* 120: 179–185
- Mimura J, Yamashita K, Nakamura K, Morita M, Takagi TN, Nakao K, Ema M, Sogawa K, Yasuda M, Katsuki M, Fujii-Kuriyama Y (1997) Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. *Genes Cells* 2: 645–654
- Miniero R, De Felip E, Ferri F, di Domenico A (2001) An overview of TCDD half-life in mammals and its correlation to body weight. *Chemosphere* 43: 839–844
- Nieswandt B, Brakebusch C, Bergmeier W, Schulte V, Bouvard D, Mokhtari-Nejad R, Lindhout T, Heemskerk JWM, Zirngibl H, Faessler R (2001) Glycoprotein VI but not  $\alpha 2\beta 1$  integrin is essential for platelet interaction with collagen. *EMBO J* 20: 2120–2130
- Peterson RE, Theobald HM, Kimmel GL (1993) Developmental and reproductive toxicity of dioxins and related compounds: cross-species comparisons. *Crit Rev Toxicol* 23: 283–335
- Robles R, Morita Y, Mann KK, Perez GI, Yang S, Matikainen T, Sherr DH, Tilly JL (2000) The aryl hydrocarbon receptor, a basic helix-loop-helix transcription factor of the PAS gene family, is required for normal ovarian germ cell dynamics in the mouse. *Endocrinol* 141: 450–453
- Rowlands JC, Gustafsson JA (1997) Aryl hydrocarbon receptor-mediated signal transduction. *Crit Rev Toxicol* 27: 109–134
- Singh SUN, Casper RF, Fritz PC, Sukhu B, Ganss B, Girard Jr B, Savouret JF, Tenenbaum HC (2000) Inhibition of dioxin effects on bone formation *in vitro* by a newly described aryl hydrocarbon receptor antagonist, resveratrol. *J Endocrinol* 167: 183–195
- Takeuchi K (1960) A study of the mutant (*wavy*) in the medaka, *Oryzias latipes*. *Annotationes Zoologicae Japonenses* 33: 124–131
- Testa B, Jenner P (1981) Inhibitors of cytochromes p450 and their mechanisms of action. *Drug Metab Rev* 12: 1–117
- Yamamoto T (1969) Sex differentiation. In "Fish Physiology 3" Ed by WS Hoar, DJ Randall Academic Press, New York, pp 117–175

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