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Focus

Nervous system disruption and swimming abnormality in early-hatched pufferfish (*Takifugu niphobles*) larvae caused by pyrene is independent of aryl hydrocarbon receptors

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ABSTRACT

Pyrene, a member of the polycyclic aromatic hydrocarbons (PAHs), contributes to abnormality in the size of the brain and the swimming behavior of pufferfish (*Takifugu niphobles*) larvae. We hypothesized that the aryl hydrocarbon receptor (AHR) may mediate pyrene-induced toxic effects because AHR is assumed to be a candidate for the downstream target of PAHs in many cases. To identify the contribution of AHR on developing pufferfish, we performed exposure experiments using β-naphthoflavone, an agonist of AHR. We found that the toxic effects of pyrene and β-naphthoflavone in pufferfish larvae are fundamentally different. Pyrene specifically induced problems in the developing midbrain and in swimming behavior, while β-naphthoflavone affected the heartbeat rate and the size of the yolk. These results suggest that the behavioral and morphological abnormality caused by pyrene exposure is mediated by an AHR-independent pathway. Alternatively, defects caused by pyrene may be attributed to the inhibition of the FGF signal.

1. Introduction

Heavy oil (HO) spills in oceans have an adverse effect on marine life (Carro et al., 2006; Lee and Page, 1997; Nakayama et al., 2008; Ortiz-Zarragoitia et al., 2011; Perez-Cadahia et al., 2004; Wake, 2005). In a previous study, we demonstrated that fertilized eggs of pufferfish (*Takifugu niphobles*) exposed to HO could induce nervous system disruption and swimming abnormalities in early-hatched larvae (Kawaguchi et al., 2011). We also found that pyrene, a member of the polycyclic aromatic hydrocarbons (PAHs), contributes to abnormalities in the size of the brain and in swimming behavior (Sugahara et al., 2014), indicating that pyrene contributes to the toxicity caused by exposure to HO. However, which signaling pathway dominates the series of toxicological effects caused by pyrene remains poorly understood. In the present study, we hypothesized that the aryl hydrocarbon receptor (AHR) may mediate pyrene-induced toxic effects because many studies have shown that PAHs have agonistic effects on AHRs (Barron et al., 2004; Reynaud and Deschaux, 2006: Wolz et al., 2010; Incardona et al., 2011). Therefore, to identify the contribution of AHR on developing pufferfish, we performed exposure experiments using β-naphthoflavone (BNF), an agonist of AHR (Smeets et al., 1999). We studied heartbeat rate, body morphology, and swimming behavior in BNF-treated larvae and compared phenotypes with those of pyrene-exposed larvae.

On the other hand, it is well known that some signaling molecules secreted from developing neural tubes contribute to the formation of brain regions. Among these, fibroblast growth factor 8 (FGF8) plays a crucial role in the formation of the midbrain (Crossley et al., 1996; reviewed by Wurst and Bally-Cuif, 2001). Since pyrene-exposed larvae show a reduction in the midbrain, FGFs may contribute to the signaling pathway of pyrene. Therefore, to identify whether FGF signaling contributes to the toxicological pathway of pyrene, we treated embryos with SU5402, an inhibitor of FGF signaling, and studied brain morphology and swimming behavior.

2. Materials and methods

2.1. Fish

Pufferfish (*Takifugu niphobles*) were collected and fertilized eggs obtained as described in our previous study (Sugahara et al., 2014). Fertilized eggs were then placed in seawater filtered through coral sand and

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treated with ultraviolet radiation. During the experimental period, continuous aeration was supplied to all experimental groups.

2.2. Chemicals

Pyrene (>99% purity, Sigma–Aldrich, St. Louis, MO, USA) was dissolved in ethanol (>99% purity), and stock solutions of 500, 800 and 1000 mg/L were prepared and 25, 50, 80 and100 μg/L of pyrene were used (see also Sugahara et al., 2014). To identify the exposure concentration of BNF, the toxic equivalency factor (TEF) was compared between chemicals based on previous studies (Nisbet and LaGoy, 1992). BNF was dissolved in ethanol, and stock solutions of 500 and 1000 mg/L were prepared. In this study, 0.1, 1, 10, and 100 μg/L of BNF were used. Based on these data, it is assumed that a TEF of 1–10 μg/L BNF-exposed larvae is 10 or 100 times higher than 100 μg/L pyrene-exposed larvae. The exposure concentration of SU5402 (Calbiochem), an inhibitor of FGF signaling, was identified from previous literature (Sugahara et al., 2011), and 5, 10, and 20 μM of SU5402 dissolved in phosphate buffer were selected.

2.3. Experiments

Fertilized eggs ($n = 30$) at 24 h post-hybridization were exposed to pyrene, BNF, or SU5402. For solvent control experiments, we used seawater with 0.01% acetone for pyrene and BNF, and seawater with 0.2% DMSO for SU5402. They were exposed during their embryonic period in a glass dish containing 5 mL seawater (salinity, 3.5%). Eggs were incubated at 17 °C under fluorescence (light/dark cycle, 12 h/12 h; for pyrene and BNF) or dark (for SU5402) conditions until 8 days post-fertilization (dpf). Three independent series of experiments were performed and triplicate experiments were conducted in each series of experiments. For the hatching rate analysis, 30 eggs were examined per treatment (Table 1). A day before hatching (most eggs hatch at 9 dpf), eggs were placed on a dish. The heartbeat rate per 15 s was observed under a stereoscopic microscope. Fifteen eggs were examined per treatment. The number of hatched larvae was then determined. Larvae at 11 dpf were used for behavioral and morphological analyses. The number of hatched larva from all treatment groups was shown in Table 1.

Behavioral analyses using early-hatched larvae at 2 dph were performed as described (Kawaguchi et al., 2011) with minor modifications. A hatched larva was transferred to a Petri dish (Nihon Pharmaceutical Co. Ltd., Tokyo, Japan) on a light board, and its swimming behavior was recorded for 3000 frames (0.03 s/frame) using a video camera above the dish (HIMAWARI GE60, Library Co. Ltd., Tokyo, Japan). Ten or fifteen organisms were examined per treatment. The swimming trajectory was visualized on a personal computer, and the swimming speed and distance were measured using Move-tr/2D7.0 software (Library Co. Ltd). To calculate the swimming pattern, the maximum and the minimum values of the swimming trajectory in both the x- and the y-axis were obtained as the swimming area (see also Kawaguchi et al., 2011). Evaluations are performed by double blind method. Four independent series of experiments were performed.

For the morphological analysis, PFA-fixed specimens were observed under a stereomicroscope (Lumar V12, Carl Zeiss SMT GmbH, Oberkochen, Germany). The body length, curvature of the body axis and surface area of the eyes were measured using Image J software (version 1.47). Ten larvae were examined in each measurement.

Statistical analysis was performed using R software (version 3.2.1). We initially performed Bartlett's test to identify the homoscedasticity. If the result was not homoscedastic, P value was obtained by Steel's or Steel-Dwass' test. If the result was homoscedastic, P value was obtained by Dunnett's test or Turkey's test.

For detailed observation of external morphology, larvae postfixed with 1% OsO_4 were dehydrated through a graded ethyl alcohol series and dried with a CO_2 critical point dryer (LEICA CPD300). The dried specimens were glued to a tip of a 0.3 mm cupper wire and coated with platinum in an ion sputter (JEOL Ion Sputter JFC-1600). The copper wire was mounted on a double-sided adhesive conductive carbon tape on a rotatable pivot of a specimen holder designed by Pohl (2010) and modified by JEOL to make full-dimensional observations possible. And then, specimens were observed under a scanning electron microscope (JEOL JSM-5600).

Table 1
The number of hatched larva from all treatment groups.

Treatments		Number of hatched larva			
		1	2	3	Total
Solvent control		30/30	28/30	26/30	84/90
Pyrene	25 μg/L	30/30	30/30	25/30	85/90
	50 μg/L	29/30	28/30	28/30	85/90
	80 μg/L	29/30	27/30	25/30	81/90
	100 μg/L	30/30	30/30	25/30	85/90
BNF	0.1 μg/L	30/30	27/30	24/30	81/90
	1 μg/L	30/30	30/30	29/30	89/90
	10 μg/L	30/30	27/30	26/30	83/90
	100 μg/L	16/30	16/30	12/30	44/90
Solvent control		30/30	29/30	28/30	87/90
Pyrene	80 μg/L	29/30	28/30	27/30	84/90
Solvent control		30/30	29/30	27/30	86/90
SU5402	5 μM	27/30	26/30	25/30	78/90
	10 μM	29/30	29/30	27/30	85/90
	20 μM	30/30	30/30	28/30	88/90

3. Results

3.1. Hatching rate

We found that most of the transferred eggs hatched at 9 dpf in both the BNF- and pyrene-exposed group, whereas in the 100 μg/L BNF-exposed group, the number of hatched larva was significantly decreased ($P < 0.01$; Fig. 1A).

3.2. Heartbeat rate

We found the group exposed to 1–100 μg/L BNF had a significantly decreased heartbeat rate compared with that of the control group, while the pyrene-exposed group appeared to be unaffected (Fig. 1B).

3.3. Swimming behavior

Swimming trajectories were measured in the solvent control and in 25–100 μg/L pyrene- or 0.1–100 μg/L BNF-exposed larva at 11 dpf (Fig. 1C, D, E). The swimming trajectory (red lines in Fig. 1E) was described, and the swimming distance and area were measured from the recorded data of swimming patterns. Abnormal swimming distances and areas were observed in 80 and 100 μg/L pyrene- and 100 μg/L BNF-exposed groups, in which larvae swam in small areas compared with the control group (Fig. 1C, D, E).

3.4. Body morphology

Morphologies of the whole body and the oral apparatus (maxillary and mandibular regions) of the 100 μg/L BNF-exposed larvae were severely abnormal compared with that of the other experimental groups (Fig. 2A, B). Although the body surface size was significantly decreased in 100 μg/L pyrene- and 100 μg/L BNF-exposed larvae, 100 μg/L BNF-exposed larvae showed a more severe reduction in body size compared with the 100 μg/L pyrene-exposed larvae (Fig. 2C). Furthermore, BNF-exposed larvae had enlarged yolks, an abnormal distribution of pigments, and cardiac edema (Fig. 2A, B, C, D), while in pyrene-exposed larvae, the pigments were normally distributed and the sizes of the yolk appeared to be unaffected in the concentrations studied (Fig. 2A, B, D).

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