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β -Naphthoflavone Caused Up-Regulation of AhR Regulated GFP in Transgenic Zebrafish

Seung-Hyeok SEOK¹⁾, DukWoong PARK²⁾, Jong-Hwan PARK¹⁾, Sun-A CHO¹⁾,
Min-Won BAEK¹⁾, Hui-Young LEE¹⁾, Dong-Jae KIM¹⁾, BoHwan JIN²⁾,
Doug-Young RYU²⁾, and Jae-Hak PARK¹⁾

¹⁾Department of Laboratory Animal Medicine, and ²⁾Environmental Health, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, San 56-1, Shinlim-dong, Kwanak-ku, Seoul 151-742, Republic of Korea

Abstract: A transgenic zebrafish strain was generated expressing the aryl hydrocarbon receptor (AhR)-regulated green fluorescent protein (GFP) reporter gene. Following exposure to β -naphthoflavone (β NF), the transgenic fish exhibited up-regulation of GFP in the face and vertebrae compared to vehicle controls. β NF-exposed fish exhibited gross dysmorphogenesis in vertebral development by 5 days after fertilization.

Key words: aryl hydrocarbon receptor, green fluorescent protein, zebrafish

Zebrafish are a particularly attractive fish model because they have been extensively researched in both genetics and developmental biology [13]. In addition, their short generation time (12 weeks), long life span (2–3 years), and relatively small diploid genome make the zebrafish a very attractive experimental model system.

The aryl hydrocarbon receptor (AhR) is a ligand-activated receptor that modulates the toxic actions of a class of environmental compounds including β -naphthoflavone (β NF) [17]. β NF is known for its ability to bind to AhR and thereby induce cytochrome P4501A (CYP1A). The ligand-bound AhR forms a transcriptionally active heterodimer with the AhR nuclear translocator (Arnt) protein and interacts with cis-genomic aryl hydrocarbon receptor elements (AhREs) in the 5'-promoter regions of Ah-responsive genes [4].

The objectives of the present study were to assess

the effect of β NF exposure in transgenic and wild zebrafish, and determine the spatial expression of AhR. To address these objectives, the inducible reporter system was designed to express green fluorescent protein (GFP) in response to ligand-activated AhR. Several reports in the literature have demonstrated that GFP can be used effectively to describe protein expression in live embryos and juvenile zebrafish [1, 2, 10]. GFP offers several advantages over other reporter systems in that it is nontoxic and can be detected in living animals without the addition of exogenous substrates [1, 2, 10].

AhR-regulated reporter plasmids, pAhRE-EGFP, were constructed by fusing a portion of the two consensus DRE sequences (underlined) (5'-GTTGCGTGAGAAGACTCGGAGTGTGCGTGAGAAGACTCGGAG-3') and this oligonucleotide was ligated into a pEGFP vector using the Ase I and Hind III sites (Fig. 1). Human

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Address corresponding: J.H. Park, Department of Laboratory Animal Medicine, College of Veterinary Medicine, Seoul National University, San 56-1, Shinlim-dong, Kwank-ku, Seoul 151-742, Republic of Korea

Fig. 1. AhR-regulated reporter construct. pAhR-EGFP was constructed by fusing a portion of the 5' regulatory region of the AhR-regulated gene CYP1A1 to the cDNA sequences of jellyfish GFP.

Fig. 2. The PCR analysis for the 3-month-old zebrafish injected with pAhR-EGFP. M: Marker, Lane 1–4 (injected fish), lane 5 (non-injected control fish).

HeLa cell line was purchased from the Korean Cell line Bank (Seoul, Korea). The cells were cultivated in Eagle's Minimum Essential Medium in Earle's BSS with non-essential amino acids, 90%, and heat inactivated fetal bovine serum (FBS), 10%, in an atmosphere of 5% CO₂/95% air at 28.5°C. One day before transfection, we plated 1.0×10^5 cells in 500 μ l of growth medium without antibiotics per well so that they would be 90–95% confluent at the time of transfection. Lipofectamine™ 2000 kit (Invitrogen, USA) was used for transient transfection studies according to the manufacturer's recommendations. In brief, DNA was diluted in 50 μ l of medium without serum. After mixing Lipofectamine™ 2000 with diluted DNA, the appropriate amount was diluted in 50 μ l of medium without serum and incubated for 5 min at room temperature. After the incubation, the diluted DNA was combined with the diluted Lipofectamine™ 2000 and incubated for 20 min at room temperature to allow the DNA-Lipofectamine™ 2000 complexes to form. One hundred microliters of DNA-Lipofectamine™ 2000 complexes was added to each well containing cells and medium. After mixing by rocking the plate back and forth, we incubated the cells at 37°C in a CO₂ incubator for 24 h. After 24 h, β NF, purchased from Sigma (Sigma, St. Louis, MI, USA) was added up to 1 μ M, and 6 h later the GFP expression was observed.

Adult zebrafish were raised and maintained on a 14:10 h light:dark cycle at 28.5°C and bred in tanks as described by Westerfield [16]. Mature fish were fed three times daily with a combination of Freshwater Aquarium Flakefood (TetraWerke, Melle, Germany) and live brine shrimp (San Francisco Bay Brand, Inc., Newark, CA, USA). Care and treatment of animals were conducted in accordance with guidelines established by the Seoul National University Institutional Animal Care and Use Committee. Single-cell zebrafish embryos were micro-injected with pAhR-EGFP. Eggs were transferred to agarose ramps and we injected the eggs with DNA/phenol red (2%) solution using a micropipette secured in a micromanipulator (World Precision Instruments, Inc., Sarasota, USA). Injected eggs were transferred to Petri dishes and exposed to DMSO (0.01%) or β NF (60 μ M) immediately following injection or at 48 h after fertilization for 24 h. Embryos were monitored for expression of GFP using a Olympus IX70 microscope equipped with a NIBA2 filter (λ_{ex} = 470–490 nm, λ_{em} = 510–550 nm). For the detection of AhRE insertion, PCR was performed after 3 months in F₀. The PCR primers were as follows. AhR upstream: 5'-ATTAATGTTGCGTGAGAAGACTCGGAG

Fig. 3. The GFP expression in HeLa cells following exposure to 1 μ M β NF. (A) DMSO (0.01%) treated HeLa cells. (B) pAhR-EGFP transfected HeLa cells.

Fig. 4. AhR-regulated GFP expression in developing zebrafish exposed to β NF. Microinjected zebrafish were exposed to DMSO (0.01%) or β NF (60 μ M) for 48 h, dechorionated, and analyzed for GFP expression by fluorescent microscopy. Transgenic zebrafish exposed to DMSO did not express GFP (A), β NF-exposed transgenic zebrafish expressed GFP in the vertebrae (B), and face (C).

TTGGGTGAGAAGACTCGGAGTAG-3'; pEGFP-AhR downstream: 5'-CTTTATGTTTTTGGCGTCTTCCA-3'. The template was the pAhRE-EGFP injected zebrafish tail. The gene amplification reaction conditions were as follows: 1 cycle of 94°C for 5 min; 40 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 30 s; and 1 cycle of 72°C for 7 min.

Figure 2 shows the 14(+)/48 PCR positive result (29%) in F_0 zebrafish 3 months after injection. The exposure of HeLa cells up to 1 μ M β NF for up to 24 h

increased GFP expression. By contrast, DMSO (0.01%) control showed no GFP expression (Fig. 3). Embryos were immediately exposed to DMSO (0.01%) or β NF (60 μ M) for 24 or 72 h. Zebrafish were dechorionated and examined by fluorescence microscopy. No GFP expression was observed in DMSO-exposed injected fish, whereas injected embryos exposed to β NF exhibited GFP expression. GFP expression was most consistently observed in the face and vertebrae of β NF-exposed developing zebrafish (Fig. 4). Wild-type

Fig. 5. β NF-induced dysmorphogenesis in developing zebrafish. Wild-type zebrafish embryos were exposed to DMSO (0.01%) or β NF (60 μ M) for the first 24 h of development. Following incubation for 24 h, embryos were rinsed and reared in fresh water by Mattingly's method [9]. Embryos were observed daily for morphologic aberrations. Fish shown are 3 days after fertilization. DMSO exposed zebrafish (A), β NF-exposed zebrafish exhibiting contorted tail and swollen yolk sac (B) (C).

zebrafish were exposed to DMSO (0.01%) or β NF (60 μ M) for 5 days after fertilization and were examined for gross morphologic differences. A range of vertebral abnormalities were observed (Fig. 5).

Larval fish exposed to sufficiently high tissue concentrations of AhR agonists such as the polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs) exhibit yolk sac, pericardial and meningeal edema, decreased cardiac output, reduced blood flow to most peripheral vascular beds, anemia, multifocal hemorrhage, impaired swimbladder inflation, craniofacial malformations typified by impaired lower jaw development, stunted growth, and mortality [3, 5, 7, 8, 11, 14, 15, 18]. In addition, recently Mattingly *et al.* reported that wild type zebrafish exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, 10 nM) for 5 days after fertilization showed a lack of eyes and a range of vertebral abnormalities [9].

GFP expression was detected in the face and vertebrae, suggesting that AhR may play an important physiologic role in the development of these structures. Activation of AhR indicated that β NF-induced toxicity targeted morphologic development of the vertebrae. Environmental pollutants tend to be bioconcentrated; for example, 10^{-17} M 2,3,7,8-tetra-chlorodibenzo-*p*-dioxin (TCDD) in a body of water is concentrated 100,000

times to approximately 10^{-12} M TCDD in a fish [6]. It is possible that specific receptor-regulated GFP expression systems could be useful transgenic biosensor systems to detect xenobiotic toxicants in the environment.

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