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Surface Marker-Defined Head Kidney Granulocytes and B Lymphocytes of Rainbow Trout Express Benzo[*a*]pyrene-Inducible Cytochrome P4501A Protein

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Polycyclic aromatic hydrocarbons (PAHs) such as benzo[*a*]pyrene (BaP) are immunotoxic to fish. Metabolism of PAHs in immune cells has been implicated in PAH immunotoxicity in mammals, but for fish the presence of metabolic enzymes in immune cells is less clear. The objective of this study was to examine localization and induction of the BaP-metabolizing biotransformation enzyme, cytochrome P4501A (CYP1A), in head kidney immune cells of rainbow trout (*Oncorhynchus mykiss*). In the first step, we measured induction of CYP1A-dependent 7-ethoxyresorufin-*O*-deethylase (EROD) activity and CYP1A protein in head kidney of rainbow trout treated with a single intraperitoneal (ip) injection of 25 mg BaP/kg body weight. From days 3 to 10 post-injection, the BaP treatment led to a significant elevation of EROD and CYP1A protein in head kidney and liver, with CYP1A expression levels in the head kidney being much lower than in the liver. Next, we examined the cellular localization of CYP1A protein in the head kidney cell types: vascular endothelial, endocrine and lymphoid cells. CYP1A immunoreactivity was detectable only in BaP-treated trout, where it was localized in endothelial and lymphoid cells. Finally, we aimed to clarify which of the hematopoietic cell types possess CYP1A protein. Using double immunostaining for CYP1A and surface markers of rainbow trout immune cells, we identified B lymphocytes and granulocytes expressing inducible CYP1A protein and being the likely sites of BaP metabolism in the head kidney.

Key Words: **rainbow trout; benzo[*a*]pyrene; immunotoxicity; cytochrome P4501A; granulocytes; B lymphocytes.**

The immune system of fish is responsive to toxicants (Rice, 2001; Segner *et al*., 2006; Zelikoff, 1994). Field studies have shown that fish from polluted sites show decreased immune responses compared with fish collected from less polluted or pristine areas (e.g., Arkoosh *et al*., 1998; Faisal *et al*., 1991; Secombes *et al*., 1995; Weeks *et al*., 1990). Also in the laboratory, an impact of toxic chemicals on the fish immune system has been demonstrated (e.g., Aaltonen *et al*., 2000; Baier-Anderson and Anderson, 2000; Hoeger *et al*., 2004; Karrow *et al*., 1999). Because the immune system is vital for the fish's defense against infectious agents, impairment of immune functions by environmental pollutants can critically affect fish health and subsequently population growth (Springman *et al*., 2005; Spromberg and Meador, 2005).

One class of environmental contaminants that modifies the fish immune system are polycyclic aromatic hydrocarbons (PAHs). PAH exposure has been shown to be associated with immunosuppressive effects such as reduced phagocytic and respiratory burst activity of macrophages (Weeks and Warinner, 1984, 1986; Weeks *et al*., 1986), reduced total white blood cell count and impaired mitogen-stimulated T- and B-lymphocyte proliferation (Carlson, 2001; Carlson *et al*., 2002a; Hart *et al*., 1998; Lemaire-Gony *et al*., 1995; Reynaud *et al*., 2001). These PAH-induced immune functional changes can be associated with decreased resistance of the fish against pathogen infection (Carlson *et al*., 2002b; Palm *et al*. 2003). The mechanisms underlying the PAH modulatory effects on the fish immune system are essentially unknown.

To exert their toxic effects, most PAHs require activation by biotransformation enzymes, particularly by members of the cytochrome P4501 family. In fact, in mammals the ability of immune cells to metabolize PAHs has been implicated in PAH immunotoxicity (Krovat *et al*., 2000; Galván *et al*., 2006; Miller and Ramos, 2001; Mounho *et al*., 1997), although additional mechanisms of action appear to be operating (Laupeze *et al*., 2002; Uno *et al*., 2006). Particular attention in research on PAH metabolism and immunotoxicity has been given to cytochrome P4501A, which is mainly expressed in the liver but also in immune cells (Davila *et al*., 1996; Grevenynghe *et al*., 2003). However, the role of other enzymes of the cytochrome P4501 family must not be overlooked. For instance, PAH toxicity to the bone marrow that leads to death of progenitor B cells and, as a consequence, to impaired humoral immunity

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appears to depend on the cytochrome P4501B1–dependent PAH metabolism (Galván *et al*., 2003, 2005).

For teleost fish, published findings on the expression of biotransformation enzymes in immune cells and hematopoietic tissues are conflicting. Two recent immunohistochemical studies described the presence of teleostean cytochrome P4501A (CYP1A) in immune cells of the excretory kidneys of European flounder, *Platichthys flesus* (Grinwis and van den Brandhof, 2001; Grinwis *et al*., 2000) and medaka, *Oryzias latipes* (Carlson *et al*., 2004a), whereas studies on scup, *Stenostomus chrysops* (Smolowitz *et al*., 1991; Stegeman *et al*., 1991), zebrafish, *Danio rerio* (Buchmann *et al*., 1993), and cod, *Gadus morhua* (Husøy *et al*., 1994) found no CYP1A-positive immune cells in the excretory kidneys. Immune cells in the spleen of medaka were negative for CYP1A (Carlson *et al*., 2004a). Whether immune cells in the head kidney, which is a major immune organ of fish, express CYP1A is not clear yet. Biochemical studies have demonstrated PAH-inducible CYP1A activity in homogenates of head kidney (Carlson *et al*., 2004a; Marionnet *et al*., 1997; Pesonen *et al*., 1990; Quabius *et al*., 2002; Taysse *et al*., 1998), however, because the head kidney is a heterogenous tissue containing endocrine (interrenal and chromaffin) and vascular endothelial cells in addition to lymphoid cells, this does not prove that CYP1A is localized in the immune cells. Husøy *et al*. (1994) who studied cellular distribution of CYP1A in the head kidney, found that CYP1A immunoreactivity was restricted to the interrenal cells, whereas vascular endothelia and lymphoid cells were negative.

Given the controversial findings on CYP1A expression in piscine immune cells, the present study aims to characterize localization and induction of CYP1A in immune cells of the head kidney of rainbow trout, *Oncorhynchus mykiss*. More specifically, we address the following questions: (1) how do basal and induced levels of CYP1A activity and CYP1A protein in head kidney compare to liver as reference organ for CYP1A induction? To answer this question, we exposed rainbow trout to benzo[*a*]pyrene (BaP) as a prototypic metabolizable and immunotoxic PAH. Time-dependent induction of CYP1A enzyme activity in liver and head kidney was determined by measuring CYP1A-associated 7-ethoxyresorufin-*O*-deethylase (EROD) activity, whereas CYP1A protein levels were estimated using Western Blot. (2) Which cell types of trout head kidney—endocrine cells, vascular endothelial cells, lymphoid cells—express CYP1A protein? The cellular CYP1A localization was assessed by means of immunohistochemistry. (3) Which immune cell population in trout head kidney expresses CYP1A protein? To study this question, we employed double immunostaining on surface marker-defined leukocytes isolated from head kidney of control and BaP-exposed rainbow trout.

MATERIALS AND METHODS

Fish and BaP exposure experiment. Adult female rainbow trout (*O. mykiss*) of 440 ± 86 g body weight were selected from the stock population reared at the Centre for Fish and Wildlife Health, University of Berne. One week before the experiment fish ($n = 56$) were transferred and distributed randomly to eight glass aquaria of 200 l volume (seven fish per 200 l). The fish were kept under flow-through conditions (0.5 l/min) in air-saturated tap water (total hardness ($CaCO_3$): 2.13 ppm; chlorine dioxide: <0.01 mg/l) and at a water temperature of 15 ± 1°C. During the acclimatization and the experimental period, the fish were fasted.

After the acclimatization period of one week, one fish per aquarium was sampled ($n = 8$ total) for histological and biochemical analyses (day 0). Afterward, from each aquarium three fish received a single ip injection of 25 mg BaP per kg body weight. The BaP (98% high performance liquid chromatography purity, 99.5 μmol BaP/kg; Fluka, Switzerland) was dissolved in 50°C warm corn oil at a concentration of 50 mg/ml BaP. The three remaining fish per tank received an injection of carrier corn oil only. For the injection, the fish were slightly anesthetized, and after injection, they passed through a bath tank in order to remove traces of the contaminant, which may leak out from the injection site. The BaP-injected and the corn oil-injected fish were discriminated by means of fin incisions. Three, 6 and 10 days postinjection (p.i.), one BaP-treated and one control fish were sampled from each of the eight replicate tanks ($n = 8$ fish per treatment and day).

Sampling of tissues. The fish were anesthetized for 2 min in buffered 3-aminobenzoic acid ethyl ester (MS-222, Sandoz LTD, Basel, Switzerland). The anesthetized fish were killed by a blow to the head, and head kidney as well as liver were dissected. Bile was collected from the gall bladder with a syringe and stored in light-protected tubes at −80°C until analysis. For biochemical analyses, tissue specimens were shock-frozen in liquid nitrogen before storage at −80°C. For histological and immunohistochemical analyses, tissues were fixed in Bouin's solution (without glacial acetic acid). After 24 h of fixation at room temperature, tissues were transferred into 70% ethanol and stored at 4°C before routine processing for paraffin embedding and sectioning.

EROD activities. EROD activities of head kidney and liver were determined as described by Scholz and Segner (1999). In brief, tissue pieces (0.8 g) were homogenized on ice using a potter in 6 ml of homogenization buffer (50mM Tris, 0.25M saccharose, 2mM Na_2-ethylenediaminetetracacetic acid, 150mM KCl, 1mM dithiotreitol, 0.25mM phenylmethylsulfonyl fluoride; pH 7.8). The homogenate was centrifuged at 10,000 × g at 4°C for 20 min in a Beckman low speed centrifuge (J2-MC). The supernatant was carefully removed and centrifuged for 1 h at 100,000 × g at 4°C in a Centrikon ultracentrifuge (T-2070). The pellet containing the microsomal fraction was resuspended in EROD buffer (80mM Na_2HPO_4, 20mM KH_2PO_4, 150mM KCl, 20% glycerine; pH 7.8). The buffer volume was 100 μl for head kidney microsomes and 400 μl for liver microsomes. The microsomes were used for EROD analysis as well as Western blotting (see below).

EROD activities were measured in a fluorescence plate reader (Multilable Counter 1420-011 VICTOR^2). In the wells of opaque microtiter plates, 250 μl of EROD buffer containing 47 μM beta-nicotinamide adenine dinucleotide phosphate (reduced) (Sigma, Switzerland) and 0.5 μM ethoxyresorufin (7-ethoxy-3*H*-phenoxazin-3-one-resorufin-ethylether, Sigma) were mixed with either 2.5, 5, or 10 μl sample and the amount of resorufin produced during 3 min at 21°C was determined at the excitation wavelength of 544 nm and at the emission wavelength of 590 nm. Each sample was analyzed at three protein concentrations (2.5-, 5-, and 10-μl sample volume) and each sample volume was measured in duplicate. The mean value was used for following calculations. The reaction velocity was calculated from the linear portion of the reaction curve and was transformed into pmol resorufin using a resorufin standard curve established by determining the fluorescence of serial resorufin concentrations (7-hydroxy-3*H*-phenoxazin-3-one, Sigma; 62.5–500 nM). EROD activity was expressed as pmol of resorufin formed per minute per milligram microsomal protein. Microsomal protein was determined by AMIDO BLACK Protein Assay according to the recommendations of the manufacturer (Merck, Darmstadt, Germany).

Quantification of BaP metabolites in bile. Bile was diluted 1:2000 with double distilled water in light-protected tubes. In the wells of opaque microtitre

plates, 300 µl of the dilution were analyzed at the excitation wavelength of 380 nm and at the emission wavelength of 430 nm (Aas *et al*., 2000). Measurements were made in a fluorescence plate reader (Multilable Counter 1420-011 VICTOR^2).

Western blot analysis of CYP1A in head kidney and liver. Microsomal samples obtained as described above were dissolved in 6× sodium dodecyl sulfate (SDS) sample buffer (Laemmli, 1970) and heated at 95°C for 5 min. Equal amounts of protein were separated on a one-dimensional 10% polyacrylamide gel under standard denaturing conditions according to the method of Laemmli (1970). As protein weight marker Broadrange Standard (SDS-PAGE Standards, range 21.5–200 kDa, Bio-Rad, Hercules, CA) was used. The proteins were then electroblotted onto nitrocellulose membranes and incubated overnight at 4°C with a mouse monoclonal antibody (mAb) against rainbow trout CYP1A (peptide 277–294, C10-7, Biosense Laboratories AS, Norway) diluted 1:200. After several washes in TBS-T buffer, the blots were incubated for 45 min with a 1:2000 dilution of horseradish peroxidase–conjugated rabbit anti-mouse IgG as secondary antibody (Amersham international, Amersham, UK). After intensification with horseradish peroxidase–conjugated anti-rabbit IgG (Amersham), diluted 1:500, the blots were visualized on preflashed X-ray film with a Super Signal Substrate Western Blotting (Pierce, Rockford, IL).

CYP1A immunohistochemistry on liver and head kidney tissue sections. Five-micrometer paraffin sections were rehydrated and endogenous peroxidase activity was blocked by treatment with 2% H_2O_2 (in ethanol) for 5 min at room temperature. Then, sections were transferred for 5 min to phosphate buffered saline (PBS) with Triton X-100 (PBS-T) and saturated in PBS-T with 0.5% casein for 30 min. Sections were incubated overnight in a moist chamber at room temperature with the C10-7 mAb directed against CYP1A diluted 1:200 in PBS-T containing 0.5% casein and 1% DMSO. For the further steps, a peroxidase ABC-staining kit (Vectastain, Vector Labs, Burlingame, CA) was used. In brief, after several washes in PBS-T, sections were incubated for 1 h at room temperature with biotinylated anti-mouse IgG (ABC-kit) diluted 1:200 in PBS-T–1% horse serum. Sections were washed again and then incubated for 1 h at room temperature with an avidin-biotinylated horseradish peroxidase complex (ABC-kit). Finally, sections were washed with PBS-T followed by the application of peroxidase substrate (3,3′ diaminobenzidine, Sigma) dissolved in Tris–HCl (0.05M, pH 7.4). Negative controls were incubated with PBS instead of the primary antibody. Counterstaining was done using acid hemalaun.

Double immunostaining for CYP1A and immune cell surface markers on isolated head kidney leukocytes. For double immunofluorescence staining of isolated head kidney cells, additional rainbow trout were injected with either corn oil or BaP (25 mg/kg body weight) dissolved in corn oil. Six days p.i., the fish were sacrificed and head kidney leukocytes were prepared using Ficoll separation solution (Biochrom AG, Berlin, Germany). The resulting leucocyte suspension was adjusted to 1×10^7 cells/ml. The leukocytes were incubated for 20 min at 4°C with a mouse mAb directed either against rainbow trout granulocytes (mAbQ4E; Kuroda *et al*., 2000) or against rainbow trout B lymphocytes (mAb4C10, Thuvander *et al*., 1990; mAbN2, Fischer and Köllner, 1994). After repeated washes by centrifugation with PBS, leukocytes were incubated with Texas red-conjugated anti-mouse IgG (Molecular Probes Europe, Juro Supply AG, Switzerland) for 1 h and fixed with 3% paraformaldehyde and 2% sucrose in PBS. For CYP1A immunostaining, cytospins were prepared using 80 µl of the cell suspension, and the cells permeabilized using microwave (160 W, 10 min). Nonspecific staining was blocked by incubation in PBS containing 3% bovine serum albumin for 1 h. Intracellular labeling of CYP1A was achieved by incubating the cells with a rabbit polyclonal antibody against fish CYP1A (1:100 diluted, CP 226, Biosense Laboratories AS) in a humid chamber at 4°C overnight. After repeated washing in PBS, visualization of CYP1A was achieved by staining with fluorescence isothiocyanate (FITC)-labeled anti-rabbit IgG (1:1500 diluted, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h. In addition to immunohistochemical staining, nuclear staining was done by means of 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, 0.5 µg/ml) (Roche, Mannheim, Germany).

Statistics. The data were initially tested for normality and homogeneity with the one-sample Kolmogorov–Smirnov test. Because the data were not normally distributed, results were calculated with the Mann–Whitney *U* test (nonparametric tests of Kruskal–Wallis, one-way analysis). Correlation analyses were made by the Pearson correlation test.

RESULTS

EROD Activity in Head Kidney and Liver Microsomes

Catalytic EROD activities in the head kidney of untreated control fish were 0.66, 0.50, 0.50, and 0.35 pmol resorufin/min/mg protein at days 0, 3, 6, and 10, respectively. There was no statistically significant difference of control values between the different days of sampling. In fish injected with BaP (25 mg/kg body weight) EROD activities of the head kidney (Fig. 1A) were significantly increased over the control group at days 3, 6, and 10 p.i. A significant influence of treatment time on EROD induction in the head kidney did not exist, that is, EROD levels in the head kidneys of BaP-injected fish did not significantly change from day 3 to day 10 p.i. The EROD activities in head

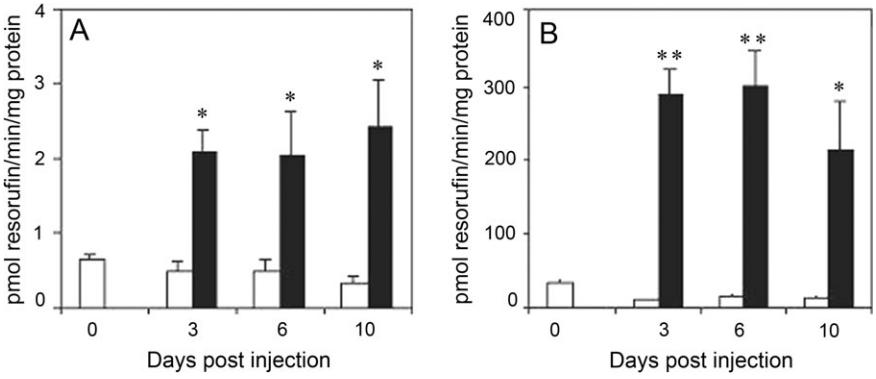

FIG. 1. EROD activities in microsomes of head kidney (A) and liver (B) from rainbow trout treated with BaP (25 mg/kg body weight, black columns) or corn oil alone (carrier control, white columns), sampled at days 0, 3, 6, and 10 p.i. The values shown are mean values ± SE (bars) of eight animals per group. *, **Significantly different from control group at a time (*$p \leq 0.01$, **$p \leq 0.001$) (Mann–Whitney *U* test).

kidneys of BaP-treated fish at days 3, 6, and 10 p.i. were 4.2-, 4.1-, and 6.9-fold higher than those in control fish.

In liver, EROD activities of control fish accounted for 32.7, 9.7, 14.6, and 13.4 pmol resorufin/min/mg protein at days 0, 3, 6, and 10, respectively. There was no statistically significant difference between these values. Control levels of hepatic EROD activities were significantly higher than in the head kidney. BaP treatment resulted in a significant induction of hepatic EROD activities over control values at 3 (30-fold induction over control), 6 (20-fold), and 10 (16-fold) days p.i. (Fig. 1B). As in head kidney, a significant influence of treatment time on EROD induction in liver did not exist, although head kidney EROD activities showed an increase from day 3 to day 10 p.i., whereas hepatic EROD activities decreased during the same period.

BaP Metabolites in Bile

To estimate the extent of BaP metabolism in the injected trout, bile metabolites of BaP were analyzed by means of fixed wavelength fluorescence spectroscopy. Although in control fish, no BaP metabolites were detected in the bile, in BaP-treated fish the amount of fluorescent BaP metabolites accumulated in the bile continuously over the 10 days p.i. Mean values were 459.3 fluorescence units (fu), 571.8 fu and 963.0 fu at days 3, 6, and 10 p.i., respectively. The calculated mean daily increase of biliary BaP metabolites from day 0 to day 3 was 153.1 fu per day (Δfu/day). This rate was approximately four times stronger than the rate found for the period from day 3 to day 6 (Δfu/day = 37.5), and about 1.5 times stronger than in the period from day 6 to day 10 (Δfu/day = 97.8). Significant correlations existed between the hepatic EROD activities and the amount of BaP metabolites in the bile of individual animals, both for each sampling day (Fig. 2) and over the whole experimental period (Pearson correlation; $n_{\text{EROD liver}} = 24$, $n_{\text{BaP metabolites}} = 22$; $p = 0.012$). No correlation was found between the EROD activities in the head kidney and the BaP metabolites in the bile.

CYP1A Immunoblotting on Head Kidney and Liver Microsomes

Immunoblotting was performed in order a) to confirm the specificity of the C10-7 antibody against CYP1A of rainbow trout, and b) to clarify if the BaP-induced increase of CYP1A at the EROD catalytic level is accompanied by a corresponding increase of CYP1A protein. In microsomes prepared from head kidneys and livers of control trout, no immunoreactive protein was detected. However, when microsomes from BaP-injected fish were used, the C10-7 antibody detected one single protein band of approximately 60 kDa weight (Fig. 3). The CYP1A-positive protein was expressed in both tissues at 3, 6, and 10 days p.i. (Fig. 3). Although microsomal protein applied to the Western Blots was fivefold higher from the head kidney than from the liver, the CYP1A-positive protein bands obtained with the head kidney microsomes were much smaller than those of the liver. This agrees with the finding that EROD activities in liver are much higher than in head kidney (see Fig. 1).

Immunohistochemical Localization of CYP1A in Liver and Head Kidney Tissues

Using the C10-7 antibody, CYP1A immunostaining in the liver of control trout was either nonexistent or very weak; immunoreactivity was found in the cytoplasm of the hepatocytes, and in bile ductular cells (Fig. 4A). In BaP-treated trout, strong CYP1A immunoreactivity was present in the hepatocytes, whereas an increase of CYP1 staining response in the bile ductular cells was not evident (Fig. 4B). In the head kidney of control fish no CYP1A immunoreactivity was observed (Fig. 5A). Strong immunoreactivity, however, was found in the head kidneys of BaP-treated fish from day 3 p.i. onwards (Fig. 5B). The staining was present in cells of the vascular endothelia and in lymphoid cells, but not in endocrine (interrenal and chromaffin) cells. Two lymphoid cell types exhibited the highest immunoreactivity for CYP1A, on the one hand cells with a circular nucleus surrounded by a thin perinuclear layer of cytoplasm (Fig. 6A), and on the other hand larger cells with a

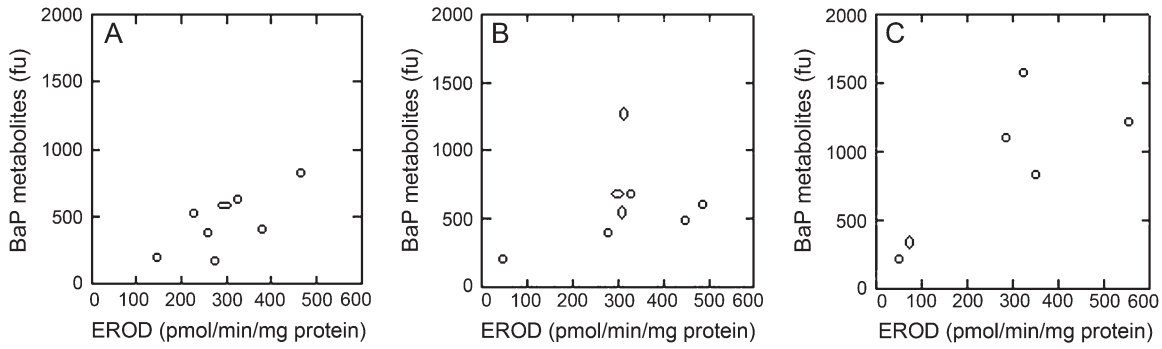

FIG. 2. Amount of BaP metabolites (expressed as arbitrary fu) in bile of trout treated with BaP (25 mg/kg body weight) compared with hepatic EROD activities of the same animals. Several days before as well as during the experiment the fish were not fed so that bile was not released but accumulated in the gall bladder. Samples were obtained at 3 (A), 6 (B), and 10 days p.i. (C). The correlations between biliary BaP metabolites and EROD activities are significant.

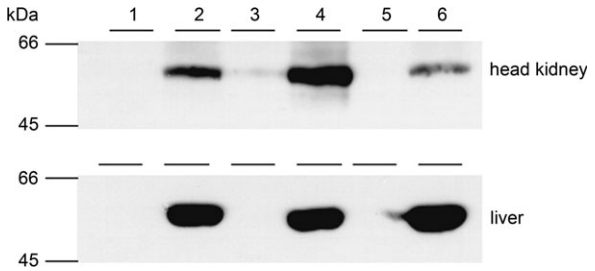

FIG. 3. Immunoblotting of CYP1A protein in head kidney and liver tissue of rainbow trout. The bands show CYP1A-immunoreactive protein of head kidney and liver microsomes from rainbow trout treated with BaP (25 mg/kg body weight, lanes 2, 4, 6) or corn oil (lanes 1, 3, 5) and sampled at 3 (lanes 1, 2), 6 (lanes 3, 4), and 10 (lanes 5, 6) days p.i., respectively. One hundred and thirty-six micrograms microsomal protein of head kidney and 24 μg microsomal protein of liver were added to each well, electrophoresed and blotted as described in ''Material and Methods.'' The antibody used was the mAb C10-7 against peptide 277–294 of rainbow trout CYP1A (Biosense).

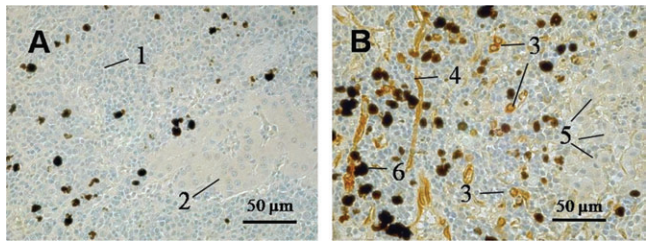

FIG. 5. Immunohistochemical localization of CYP1A by mAb C10-7 in head kidney of (A) control rainbow trout, and (B) rainbow trout treated with BaP (25 mg/kg body weight), 3 days p.i. (A). In control trout, no immunoreactivity is visible in lymphoid (1) and endocrine cells (2). (B) In BaP-treated fish, strong immunoreactivity is present in several cells of lymphoid cells (3), vascular endothelial cells (4), whereas no immunoreactivity is visible in endocrine cells (5). Pigmented cells (black spots) are also clearly visible (6).

circular or oval nucleus and a larger cytoplasmic area (Fig. 6B). The diameters of the first cell type ranged between 5 and 12 μm, and of the second cell type up to a maximum size of 15 μm.

Double Immunostaining for CYP1A and Immune Cell Surface Markers on Isolated Head Kidney Leukocytes

Head kidney leukocytes were prepared from control rainbow trout and subjected to nuclear staining using DAPI (Figs. 7A and 8A), staining for granulocyte or B-cell surface markers (Figs. 7B and 8B, respectively), and CYP1A staining (Figs. 7C and 8C, respectively). Isolated leukocytes of control rainbow trout expressed no detectable CYP1A protein except for nonspecific surface staining of some immune cells. This finding was corroborated by the doublestaining (Figs. 7D and 8D). Head kidney leukocytes isolated from rainbow trout exposed to BaP (Figs. 7E–H and 8E–H) showed strong CYP1A immunoreactivity (Figs. 7G and 8G). As evident from double immunostaining, CYP1A was localized in leukocytes reacting with the granulocyte surface marker (Fig. 7H) as well as in leukocytes reacting with the B-cell marker (Fig. 8H). These cells indeed displayed the typical morphology of granulocytes or lymphocytes. The results provide strong evidence that head kidney granulocytes and B cells of rainbow trout possess inducible CYP1A. Importantly, the head kidney leukocyte preparations of BaP-exposed trout contained cells that reacted neither with the granulocyte nor with the B-cell marker, but stained for CYP1A (Fig. 7H, arrowhead). This indicates that additional leukocyte populations other than granulocytes and B lymphocytes express inducible CYP1A. Further, among the B cells and granulocytes, there appear to exist CYP1A-negative subpopulations as not all B cells and granulocytes stained positive for CYP1A (Figs. 9A–D).

DISCUSSION

The aims of this study were to examine (1) the (time-dependent) induction of CYP1A catalytic activity and protein by BaP in the head kidney of rainbow trout in comparison to the liver as main organ of xenobiotic metabolism, (2) the distribution of CYP1A protein among the various cell types constituting the head kidney of rainbow trout, and (3) the identification of CYP1A-positive leukocyte populations

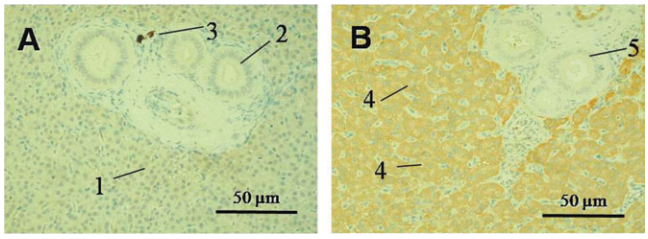

FIG. 4. Immunohistochemical localization of CYP1A by mAb C10-7 in liver of (A) control rainbow trout, and (B) rainbow trout treated with BaP (25 mg/kg body weight), 3 days p.i. (A) In control fish, no or weak CYP1A immunoreactivity is observed in hepatocytes (1) and epithelial cells of bile duct (2). Note pigmented cells (3) within the periportal field. (B) Strong CYP1A immunoreactivity is visible in hepatocytes (4), but no or very weak immunostaining is present in epithelial cells of bile ducts (5).

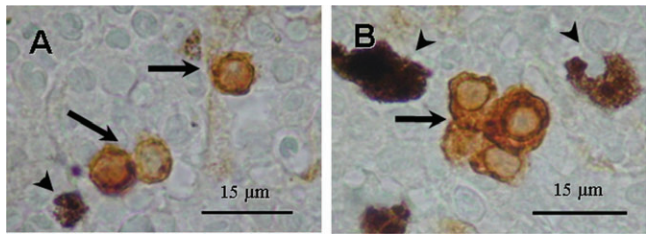

FIG. 6. Immunohistochemical staining of CYP1A by mAb C10-7 in head kidney of rainbow trout treated with BaP (25 mg/kg body weight) at 3 days p.i. (A, B) Two types of lymphoid cells exhibit the highest immunoreactivity for CYP1A, one cell type with a circular nucleus surrounded by a thin perinuclear layer of cytoplasm (A, arrows), and the other cell type with a circular or oval nucleus and a relatively high amount of cytoplasm (B, arrows). In all sections pigmented cells are clearly visible (arrowheads).

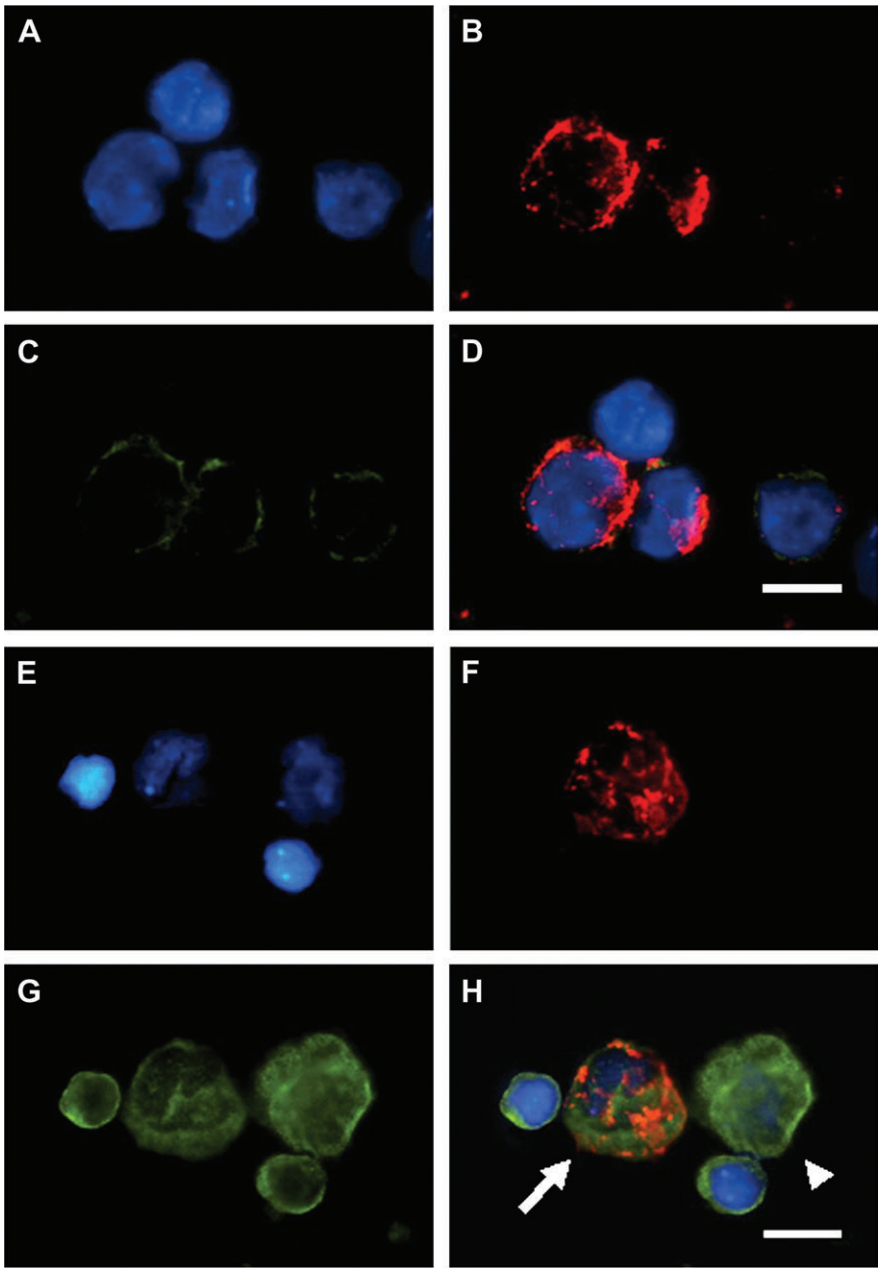

FIG. 7. Localization of CYP1A in head kidney granulocytes of rainbow trout. Control fish (A–D) and BaP-exposed fish (E–H) are shown. Nuclear staining with DAPI (A, E). Specific immunoreactivity (Texas red) to granulocyte surface marker using mAb Q4E (B, F) and strong CYP1A immunoreactivity (FITC) in the cytoplasm of leukocytes from BaP-treated fish (G). No CYP1A immunoreactivity is visible in leukocytes isolated from the head kidney of control fish (C). Overlay of DAPI nuclear staining and double immunostaining of control (D) and BaP-exposed fish (H) confirms that granulocytes of BaP-exposed rainbow trout express immunoreactive CYP1A (H, arrow). Some leukocytes did not react with the granulocyte marker, but stained for CYP1A (H, arrowhead) (scale bar: 10 μm).

of rainbow trout. The results from the BaP experiment clearly show that head kidney tissue possesses inducible CYP1A, both at the catalytic level and at the protein level. Time-dependency of EROD induction did not significantly differ between liver and head kidney, although in the liver maximum EROD values were reached 6 days p.i. and decreased thereafter, whereas in the head kidney, EROD levels continued to increase slightly until 10 days p.i. Importantly, basal and induced CYP1A expression levels in the head kidney were by an order of magnitude lower than CYP1A levels in the liver, and also the induction was clearly lower. These findings on CYP1A in the head kidney of rainbow trout agree well with previous observations from the cyprinid species, medaka and carp (Carlson *et al*., 2004a; Marionnet *et al*., 1997; Taysse *et al*., 1998). When comparing microsomal xenobiotic metabolism between trunk (excretory) and head kidney, Pesonen *et al*.

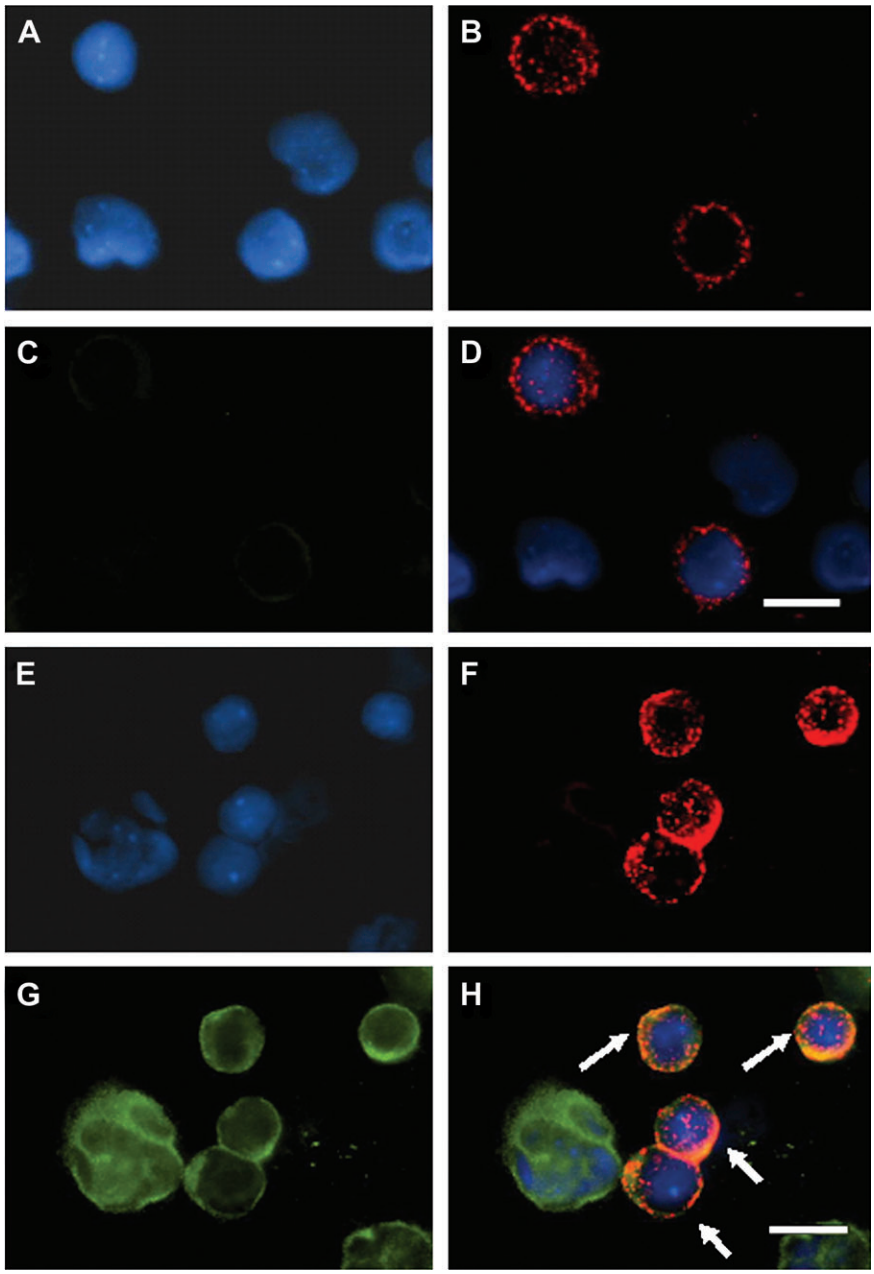

FIG. 8. Localization of CYP1A in head kidney B lymphocytes of rainbow trout. Control fish (A–D) and BaP-exposed fish (E–H) are shown. Nuclear staining with DAPI (A, E). Specific immunoreactivity (Texas red) for surface marker of B lymphocytes using both mAbs 4C10 and N2 (B, F) and strong CYP1A immunoreactivity (FITC) in the cytoplasm of leukocytes prepared from BaP-treated fish (G). No CYP1A immunoreactivity is visible in leukocytes isolated from the head kidney of control fish (C). Overlay of DAPI nuclear staining and double immunostaining of control (D) and BaP-exposed fish (H, arrows) confirms that B lymphocytes of BaP-exposed rainbow trout express immunoreactive CYP1A (scale bar: 10 μm).

(1990) found that cytochrome P450 monooxygenase activities were higher in the trunk kidney than in the head kidney, but the activities of cytochrome P450–dependent enzymes of steroid metabolism were higher in the head kidney.

Fish head kidney is a major hematopoietic and lymphoid organ of fish. However, it is a heterogenous tissue containing, in addition to the hematopoietic cells, also endocrine cells (interrenal and chromaffin cells) and vascular endothelial cells. Thus, the detection of CYP1A activity and protein in microsomes prepared from head kidney homogenates leads to the question in which particular cell type(s) CYP1A is localized. The results of the present study show that CYP1A protein is expressed in the endothelia and in the hematopoietic part of the rainbow trout head kidney. The vascular endothelial system is

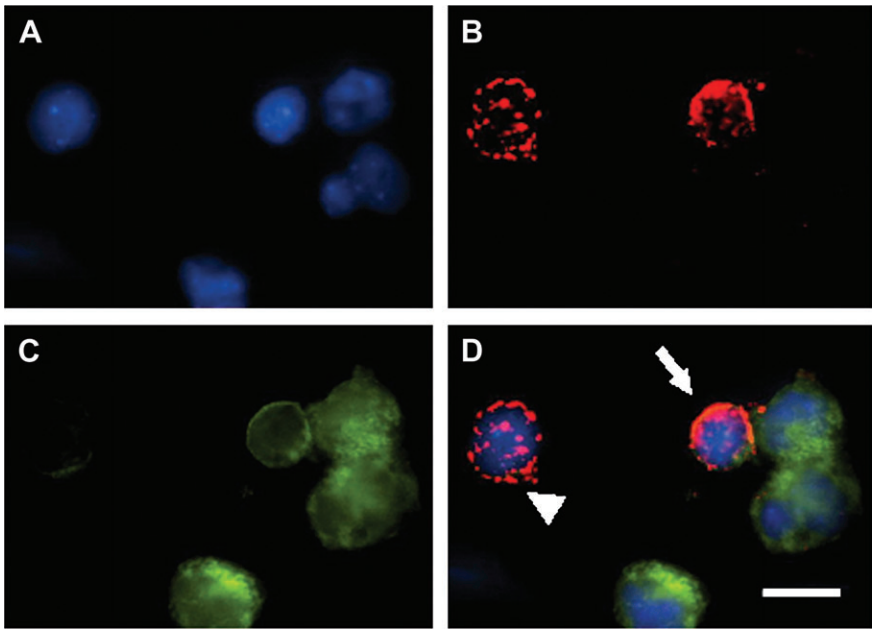

FIG. 9. Localization of CYP1A in head kidney B lymphocytes of BaP-exposed rainbow trout. Nuclear staining with DAPI (A), specific immunoreactivity (Texas red) in B lymphocytes using both mAbs 4C10 and N2 (B), strong CYP1A immunoreactivity (FITC) in the cytoplasm of leukocytes (C), and the overlay (D) are shown. There are two B lymphocytes, one of them expressing CYP1A (arrow), whereas the other one does not express CYP1A (arrowhead) (scale bar: 10 μm).

known as a prominent site of CYP1A expression in fish tissues (Sarasquete and Segner, 2000), what is in agreement with the findings from the present study. More controversial is the CYP1A expression in the endocrine cells. Although we observed no CYP1A immunoreactivity in head kidney endocrine cells of rainbow trout, neither in control nor in exposed fish, Husøy *et al.* (1994) reported the presence of CYP1A immunostaining in interrenal cells of beta-naphtoflavone-treated cod, *G. morhua*. Whether this difference represents a species difference or has other reasons remains to be resolved. Also the findings on CYP1A expression in immune cells of fish are conflicting. Absence of CYP1A immunoreactivity from lymphoid cells was reported for the excretory kidney of scup (Smolowitz *et al.*, 1991; Stegeman *et al.*, 1991), zebrafish (Buchmann *et al.*, 1993), and cod (Husøy *et al.*, 1994), whereas CYP1A-positive immune cells were reported to be present in the hematopoietic tissue of the excretory kidney of European flounder (Grinwis and van den Brandhof, 2001; Grinwis *et al.*, 2000) and medaka (Carlson *et al.*, 2004a). The latter authors found that CYP1A was present in subpopulations of mononuclear cells of the trunk kidney, but did not identify the precise nature of those subpopulations. With respect to the head kidney, only Husøy *et al.* (1994) examined CYP1A expression in hematopoietic cells, but did not observe CYP1A immunoreactivity, neither in control fish nor in beta-naphthoflavone–treated cod. The results of the present study, however, provide strong evidence for the presence of BaP-inducible CYP1A in lymphoid cells of the rainbow trout head kidney. These findings agree with the known expression of CYP enzymes in hematopoietic tissues of mammals (Krovat *et al.*, 2000; Laupeze *et al.*, 2002).

Having demonstrated that immune precursor cells of the head kidney express inducible CYP1A protein, we aimed to identify the nature of those cells. In mammalian leukocytes, the presence of CYP1A has been demonstrated in peripheral blood T and B lymphocytes, spleen lymphocytes, and lymphoblastoid cells as well as in circulating monocytes and tissue macrophages (Baron *et al.*, 1998; Grevenynghe *et al.*, 2003; Hukkanen *et al.*, 1997; Krovat *et al.*, 2000; Nohara *et al.*, 2006; Thurmond *et al.*, 2000; Van den Heuvel *et al.*, 1993; Waithe *et al.*, 1991). Morphology of the CYP1A-positive lymphoid cells in head kidney sections of rainbow trout resembled phagocyte- and lymphocyte-type cells (Lehmann and Stührenberg, 1975). However, because morphological criteria are not conclusive enough, we employed surface markers for rainbow trout leukocytes to identify the CYP1A-positive immune cells. Using double immunostaining with antibodies against piscine CYP1A and against rainbow trout granulocytes (mAbQ4E; Kuroda *et al.*, 2000) and B lymphocytes (mAb4C10, Thuvander *et al.*, 1990; mAbN2, Fischer and Köllner, 1994) we could demonstrate that granulocytes and B lymphocytes of rainbow trout express inducible CYP1A.

Importantly, the immune cell suspensions prepared from trout head kidney contained CYP1A-positive leukocytes, which reacted neither with the granulocyte marker nor with the B-lymphocyte marker. Based on theri morphology and the absence of staining by both antibodies (mAB4C10, mABN2) against B-cells, tehse cells may represent sIgM-negative lymphocytes. This could be T-cells

or Natural Killer (NK) cells. However, since antibodies to stain markers expressed on T- or NK-cells are not available, we cannot unequivocally prove this assumption. Vice versa, we observed immune cells staining for either the granulocyte or the B-cell markers, but not with the CYP1A antibody. Thus, on the one hand, granulocytes and B lymphocytes are not the only immune cells of rainbow trout containing inducible CYP1A, and, on the other hand, not all granulocytes and B lymphocyte are CYP1A-positive but there appear to exist CYP1A-negative subpopulations. In rainbow trout it was reported that head kidney granulocytes are mainly neutrophils (Moritomo *et al*., 2003). Because the vast majority of surface marker-defined granulocytes were CYP1A-positive, we speculate that neutrophils constitute the CYP1A-inducible granulocyte subpopulation of rainbow trout head kidney. The presence of CYP1A-positive and -negative subpopulations of B lymphocytes might be related to the presence of different maturation stages of B lymphocytes in the head kidney (Zwollo *et al*., 2005).

For mammalian systems, there exists clear evidence that lymphoid cells possess inducible BaP metabolism (White *et al*., 1994), and that the immunotoxic effects of BaP are related to BaP metabolism and activation of oxidative and electrophilic signaling pathways (Burchiel and Luster, 2001; Uno *et al*., 2004, 2006). Our results confirm previous findings (Carlson *et al*., 2004a, b; Grinwis and van den Brandhof, 2001; Grinwis *et al*., 2000) that immune cells in fish hematopoietic tissues possess inducible CYP1A protein and should be able to metabolize BaP. Further evidence for the presence of biotransformation capabilities in fish immune cells comes from the observation of DNA adducts in lymphoid and peripheral immune cells of BaP-treated fish (Malmström *et al*., 2000; Rose *et al*., 2001) as well as from the finding that enriched leukocyte preparations from head kidney and spleen of spot, *Leiostomus xanthurus*, were able to convert BaP into reactive intermediates (Rutan and Faisal, 1994). The results from the present study extend previous findings in that for the first time - by means of double staining- the nature of CYP1A-positive immune cells has been identified. This information can contribute to better understand the mechanisms underlying PAH immunotoxicity in fish. For instance, a typical immunotoxic response of fish to BaP exposure is a change in reactive oxygen generation by granulocytes (Carlson *et al*., 2002b, 2004a; Holladay *et al*., 1998; Karrow *et al*., 2001; Reynaud and Deschaux, 2006), an effect that could be caused by the xenobiotic induction of CYP1A and associated alterations in the prooxidant status of the granulocytes. Another hallmark of BaP immunotoxicity in fish is reduced lymphocyte proliferation and suppressed number of antibody forming cells (Carlson *et al*., 2002a, b, 2004b), which may be related to cytotoxicity arising from the BaP metabolism in B lymphocytes. Similarly, for mammals it has been shown that immune cell metabolism of PAHs causes a decrease of progenitor B lymphocytes and, consequently, impaired humoral immunity (Galván *et al*., 2003, 2006).

In conclusion, this study shows that CYP1A is inducible in rainbow trout head kidney by BaP treatment. CYP1A is localized in head kidney vascular endothelia and in immune cells of the hematopoietic part of the head kidney, more specifically in granulocytes and B lymphocytes. The advancement of the present work, that is, the allocation of CYP1A to specific immune cell types, identifies those immune cell populations that are able to execute local PAH metabolism, and, at the same time, should be the immediate targets of PAH immunotoxic action. Another interesting perspective for future investigations arising from the findings of the present study is the observation of CYP1A-negative and CYP1A-positive B lymphocytes and granulocytes in rainbow trout, pointing to the existence of functionally different leukocyte subpopulations—an aspect that has been hardly ever studied in fish.

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