Tissue-specific metabolism of benzo[a]pyrene in rainbow trout (*Oncorhynchus mykiss*) – a comparison between liver and immune organs

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Abbreviations:

ANF: alpha-naphthoflavone; BaP: benzo[a]pyrene; BPDE: Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide; CYPs: cytochrome P450-dependent monoxygenases; EROD: 7-ethoxyresorufin-O-deethylase; fu: arbitrary fluorescence units; HPLC: high performance liquid chromatography; PAHs: polycyclic aromatic hydrocarbons
Abstract

Polycyclic aromatic hydrocarbons (PAHs) are immunotoxicants in fish. In mammals, phase I metabolites are believed to be critically involved in the immunotoxicity of PAHs. This mechanism has been suggested for fish as well. The present study investigates the capacity of immune organs (head kidney, spleen) of rainbow trout, *Oncorhynchus mykiss*, to metabolize the prototypic PAH, benzo[a]pyrene (BaP). To this end, we analyzed (i) induction of enzymatic capacity measured as 7-ethoxyresorufin-<i>O</i>-deethylase (EROD) activity in immune organs compared to liver, (ii) the organ profiles of BaP metabolites generated <i>in vivo</i>, and (iii) rates of BaP metabolite production determined microsomes <i>in vitro</i>. All measurements were done for control fish and for fish treated with an intraperitoneal injection of 15 mg BaP/kg body weight. In exposed trout, liver, head kidney and spleen contained similar levels of BaP, whereas EROD induction differed significantly between the organs, with liver showing the highest induction factor (132.8 times), followed by head kidney (38.4 times) and spleen (1.4 times). Likewise, rates of microsomal metabolite formation experienced the highest induction in the liver of BaP-exposed trout, followed by head kidney and spleen. Microsomes from control fish displayed tissue-specific differences in metabolite production. In contrast, in BaP-exposed trout microsomes of all organs produced the potentially immunotoxic BaP-7,8-dihydrodriol as the main metabolite. The findings from this study show that PAHs like BaP are distributed into immune organs of fish and they provide the first evidence that immune organs possess inducible PAH metabolism leading to <i>in situ</i> production of potentially immunotoxic PAH metabolites.
Introduction

The immune system is critical for survival and fitness of organisms in that it enables to distinguish between self, non-self (e.g. pathogens) and altered self. In teleost fish, a major immune organ is the anterior portion of the kidney (head kidney). This organ is a primary site of hematopoiesis in teleost, containing both myeloid and lymphoid leukocytes as well as endocrine cells (Powell, 2000; Carlson and Zelikoff, 2008). A second important immune organ of teleost fish is the spleen, which is believed to function similar to the mammalian spleen (Carlson and Zelikoff, 2008). The immune cells in these organs are closely associated with the blood system, partly acting as filtering system for the circulatory system, and therefore they are highly accessible to toxicants. In fact, a wide variety of chemicals has been reported to compromise immune functions of fish and to modulate susceptibility to infectious pathogens (Rice, 2001; Carlson and Zelikoff, 2008; Segner et al., 2013). One class of chemicals that has been repeatedly shown to affect immune functions of fish are polycyclic aromatic hydrocarbons (PAHs) (Arkoosh et al., 2001; Carlson and Zelikoff, 2002; Carlson et al., 2004b; Hutchinson et al., 2003; Reynaud et al., 2004; Kennedy and Farrell, 2008; Danion et al., 2011).

Many of the toxic effects of PAHs such as mutagenicity and cancerogenicity are not caused by the parent compounds but by the metabolites. PAH biotransformation is initiated by cytochrome P450-dependent monooxygenases (CYPs) (Xue and Warshawsky, 2005). The monooxygenases convert PAH parent compounds into dihydrodiols, phenolics and epoxide intermediates, which then are further metabolized and eventually excreted (Shimada, 2006). The question is whether PAH metabolites are also responsible for the immunotoxic effects of PAHs. For mammals, there exists indeed strong evidence that PAH immunotoxicity is not caused by the parent compounds but by their metabolites. Although the main site for PAH metabolism is the liver, the immunotoxic metabolites appear to be generated directly in the immune cells, i.e. by in situ biotransformation (Burchiel and Luster, 2001; Ioannides et al., 2004; Yusuf et al., 2007). Also for teleost fish, it has been suggested that in situ metabolism of PAHs is causatively involved in PAH immunotoxicity. Carlson et al. (2004a,b) showed that in vivo treatment of medaka (Oryzias latipes) with the prototypic PAH, benzo[a]pyrene (BaP),
resulted in immunotoxic effects, which could be ameliorated by co-treatment with alpha-naphthoflavone (ANF), an inhibitor of CYP1A activity. While this result might still be explained by metabolism of BaP in the liver and subsequent transport of the metabolites into the immune organs, the finding of Carlson et al. (2004b) that ANF ameliorated BaP immunotoxicity also in isolated medaka immune cells points to an involvement of PAH metabolism directly in the immune cells. As concluded by Carlson et al. (2004b), “results of this study suggest that (i) BaP-induced suppression of medaka humoral immunity relies upon the CYP1A-catalyzed production of immunotoxic BaP metabolites and (ii) BaP metabolites may be created in situ, directly by specific cells within the kidney lymphoid tissue”. What is lacking to date is the final piece in the picture, that is the demonstration that fish immune organs are capable of in situ PAH metabolism.

The present study examines the capability of fish immune organs for in situ metabolism of BaP. More specifically, it investigates (i) patterns and relative levels of BaP metabolites in fish immune organs, both in the intact fish and in microsomal preparations in vitro, (ii) the rates at which BaP metabolites are produced, and (iii) how rates and patterns change after exposure of the fish to BaP. The fish model used for this purpose was rainbow trout (Oncorhynchus mykiss). The immune organs examined included head kidney and spleen, with the liver used as benchmark.

Material and methods

Reagents

BaP and all other chemicals were purchased from Sigma-Aldrich. BaP metabolites (Benzo[a]pyrene-trans-7,8-dihydrodiol, Benzo[a]pyrene-trans-4,5-dihydrodiol, Benzo[a]pyrene-trans-9,10-dihydrodiol, 3-Hydroxybenzo[a]pyrene and 9-Hydroxybenzo[a]pyrene) were purchased from the NCI Chemical Carcinogen Reference Standards Repository (Kansas City, MO). Acetonitrile, methanol, ethyl acetate and acetone were purchased from Carl Roth. MS-222 was purchased from Sandoz LTD (Basel, Switzerland).
Experimental animals

Rainbow trout (average weight: 300 g) were obtained from a local hatchery (cantonal fish farm Kandersteg, Switzerland). Experiments were carried out according to the Swiss Animal Welfare regulations. Fishes had an average size of 28 cm ± 3.3 and an average weight of 260 g ± 85. Classification of maturity stages was performed on histological sections for female and male trout according to Körner et al. (2007). Maturity was stage 1 (immature oocytes) to 2 (presence of cortico-alveolar oocytes) in female trout and at stage 2 (all maturity stages of spermatocytes equally present) to 3 (mature spermatocyte stages are dominant) in male trout. One animal had undifferentiated gonads. During the experiments, each individual fish was kept in a separate 120 L glass aquarium supplied with tap water in a flow-through system. Tanks were aerated and water temperature during experiments was 15°C ± 1°C.

Experimental treatments and sampling

Fishes were treated by intraperitoneal injection either with corn oil (control) or with 15 mg/kg BaP in corn oil (BaP exposure). The selected BaP concentration is in the lower range of dosing concentrations that are found in literature (2-200 mg/kg). During the exposure, trout were fasted. Five days after dosing, trout were sacrificed by an overdose of MS-222, followed by a blow to the head. The tissues collected from these fish were used for the following investigations:

- Bile fluid was collected from five control and five BaP-treated fish and analyzed for BaP metabolites in order to verify that BaP injection successfully induced BaP metabolism and excretion.

- Liver (n=5) and immune tissues (n=3) of control and BaP-exposed fish were collected and analyzed for patterns of BaP metabolites, in order to estimate tissue-specific BaP metabolism in vivo.
Microsomes were prepared from liver and immune tissues of control and BaP-exposed fish, and were incubated in vitro with 7-ethoxyresorufin in order to analyze EROD activity (n=3).

Microsomes were prepared from liver and immune tissues of control and BaP-exposed fish, and were incubated in vitro with BaP in order to (i) compare BaP metabolite patterns and (ii) BaP metabolic rate between the organs (liver: n=1, immune tissues: n=2).

Tissue and bile samples were stored at -80°C until further processing. Microsomes were prepared immediately from freshly dissected tissues. Preparation was done according to Harris et al. (2009), and aliquots were stored frozen at -80°C until use for experiments. Microsomal protein content was determined according to the method of Bradford (1976). In addition to liver and immune organs, gonadal tissue was fixed in formaldehyde 4 % for histological determination of sex and maturity.

**Bile analysis**

Aliquots of bile fluid of five fish were used to determine the sum of BaP metabolites. Determination of the sum of BaP metabolites in the bile was achieved by the fluorescence method of Aas et al. (2000) and Lin et al. (1996), with the excitation/emission wavelength pair of 380/430 nm. Bile fluid was diluted 1:2000 with double distilled water, and 200 µL of the diluted bile were placed into individual wells of an opaque 96 well plate and measured for fluorescence using a fluorescence plate reader (EnSpire 2300 Multilabel Reader, Perkin Elmer). Fluorescence values were expressed as arbitrary fluorescence units (fu) and data is expressed as mean fu value ± SD.

Other aliquots of the bile fluid were used to determine individual BaP metabolites. To this end, bile samples were extracted using a protocol adapted from Willett et al. (2000). To 50 µL bile, 1250 µL sodium acetate buffer were added containing 1000 U β-glucuronidase and 19 U arylsulfatase (from Helix pomatia, Sigma-Aldrich). Mixtures were incubated overnight at 40°C for deconjugation. Afterwards, they were extracted as described by Willett et al. (2000) and individual metabolites were analyzed using HPLC (see below). Data is expressed as mean ng substance per µL bile ± SD.
Tissue preparation and extraction for HPLC analysis

Tissue samples were extracted using a modified method of Ramesh et al. (2001) and Willett et al. (2000). Livers were collected from five animals each of the control and of the BaP-exposed group, spleen and head kidneys from three trout per group. Livers were perfused with PBS to remove the blood. Organ samples (1 g) from individual animals were homogenized in two volumes of sodium acetate buffer (0.4 M, pH 5.0). Metabolite conjugates in the homogenates were deconjugated enzymatically by incubation at 40°C overnight with β-glucuronidase (1000 U) and aryl-sulfatase (19 U, both from Helix pomatia, Sigma-Aldrich). Subsequently, to precipitate proteins, sodium dodecyl sulfate (1 %) was added and mixed for 1 minute. Deconjugation products were extracted twice by liquid-liquid extraction with 2 volumes ethyl acetate:acetone (2:1). Extraction tubes were mixed (1 minute) and centrifuged (5000 × g, 20 minutes, 10°C). Organic fractions were combined and dried down under a stream of nitrogen. The residue was dissolved in 400 μL of acetonitrile. The aqueous phase was adjusted to pH 1 with 1 N HCl to deconjugate glutathionated metabolites (Beyer et al., 2010; Yu et al., 1995). This step was followed by a 3-step liquid-liquid-extraction (2 volumes ethyl acetate:acetone (2:1), 2 × 2 volumes ethyl acetate). The organic phase was dried down under nitrogen and dissolved in 200 μL acetonitrile. Acetonitrile fractions were combined and analyzed using HPLC and fluorescence detection. Values are given as mean ng substance per mg tissue ± SD.

Microsomal incubations

Microsomal incubations and extraction for metabolism studies were performed as described previously (Harris et al., 2009, for microsomal incubation; Stuchal et al., 2006, for extraction). The in vitro metabolism assay was performed with microsomal protein concentrations of 0.5 mg/mL and substrate concentration of 5 μM BaP. Control assays included incubations that were immediately stopped after BaP exposure and incubations without NADPH. Liver microsomes were incubated for 2 hours, spleen and head kidney microsomes for 3 hours, respectively, at 21°C. The extended incubation period for microsomes from immune tissues was selected to obtain sufficient amounts of metabolites.
The reactions were stopped by addition of 5 mL of ethyl acetate:acetone (2:1); afterwards they were extracted (Harris et al., 2009; Stuchal et al., 2006). The organic phase including metabolites was dried down under nitrogen, dissolved in acetonitrile and analyzed in the HPLC. Data is expressed as mean ng substance per mg protein per h ± SD for BaP-treated and control microsomes from head kidney and spleen (n = 2, respectively). One control and one BaP-treated fish were sampled for liver microsomes.

HPLC analysis of extracts from in vivo tissues and from in vitro microsomal incubations

Samples were subjected to HPLC analysis for detection of BaP and BaP metabolites. Given the differences between liver, spleen and head kidney in tissue structure, composition, and blood perfusion, we placed emphasis on comparing relative capacities for metabolite formation in liver and immune tissues. Our analytical method was based on the methods of Beyer et al. (2010) and Ramesh et al. (2001), determining the concentration of the target analyte by comparing retention times and peak areas of samples with that of standards. A dilution curve was prepared for each metabolite standard and BaP at concentrations between 0.1 and 8 ng/µL. This standard curve was linear for each substance. The limit of detection was evaluated for each substance and was defined as the concentration which resulted in a peak approximately three times above the baseline. To identify BaP metabolites in tissue extracts and microsomal incubations, individual standards were added to the biological sample matrix of BaP-treated fish and the respective peaks were assigned by the resulting peak addition.

A Dionex HPLC system (Dionex P680 HPLC pump, ASI-100 automated sample injector, RF-2000 fluorescence detector) was used for sample analysis. Before injection, the extracts were centrifuged (10 minutes, 9300 × g, 10°C). Using an automated sampler, 25 to 150 µL were injected onto a C18 reversed phase column (Supelcosil LC-PAH C18, 150 × 4.6 mm, 5 µm, Sigma-Aldrich). Separation of analytes was achieved at a flow rate of 1 mL/min as follows: 30:70 acetonitrile: H₂O was held for 5 minutes, followed by a linear gradient to 85:15 acetonitrile: H₂O in 35 minutes. This was held for
another 10 minutes, before returning to 30:70 acetonitrile: H₂O in 10 minutes. Fluorescence was monitored at excitation/emission wavelengths 320/430 nm.

**EROD activity**

7-ethoxyresorufin-O-deethylase (EROD) activity was determined in head kidney, spleen and liver microsomes to characterize organ-specific monooxygenase activity. Microsomes from BaP-treated and control fish were analyzed. Enzyme kinetic analyses were performed in black 96 well plates with 250 µL reaction volume per well using a protocol adapted from (Nakayama et al., 2008). As substrate, 7-ethoxyresorufin was used (16 µM). Conversion of 7-ethoxyresorufin into fluorescent resorufin was measured immediately after addition of the microsomes at the excitation/emission wavelength pair 544/590 nm in a fluorescence plate reader (EnSpire 2300 Multilabel Reader, Perkin Elmer). Resorufin formation was linear under conditions of the measurement. Each sample was analyzed at three different protein amounts: 2.5, 5 or 10 µL of undiluted microsomes (the microsomal protein concentration was approximately 2 mg/mL). Microsomes from head kidney, spleen and liver from three control or BaP-treated animals were used. Data is expressed as mean pmol resorufin per minute per mg protein ± SD. For reaction rate analysis, GraphPad Prism software Version 5.02 was used.

**Statistics**

Differences between treatment groups were analyzed using Student’s t test. Metabolite levels in treatment groups were analyzed for significant differences by one-way analysis of variance (ANOVA), followed by a Tukey-Kramer Multiple Comparison test. All statistical analysis was performed using GraphPad Prism software (Version 5, USA).
Results

BaP exposure of rainbow trout: verifying effectiveness of treatment

To obtain an indication whether the experimental BaP exposure was effective in inducing BaP metabolism in the liver as the main metabolic organ, we analyzed hepatic EROD activity in control and BaP-exposed fish. The fish injected with 15 mg/kg BaP and sampled 5 days after injection showed a 133 times induction of hepatic EROD levels over controls. In addition, we measured bile fluorescence by means of fixed wavelength fluorescence spectroscopy. This method, which is based on the fact that PAH metabolites are fluorescent, is frequently used as a summary parameter to indicate that the liver actively performs PAH metabolism and metabolite secretion. In control fish, 1 μL of bile displayed a mean fluorescence of 2799 ± 812 fluorescence units (fu) (n=5), whereas the same volume of bile from BaP-exposed fish showed a mean fluorescence of 120698 ± 29833 fu (n=5), which corresponds to a 43 fold increase of fu.

EROD activity in head kidney and spleen of control and BaP-exposed rainbow trout

EROD activity, which is executed by the PAH-metabolizing CYP1A, was used as proxy to assess (i) whether head kidney and spleen principally possess enzymatic capacity for PAH metabolism, (ii) how it compares to the reference metabolic organ, the liver, and (iii) whether the enzymatic capacity of spleen and head kidney for PAH metabolism is inducible by BaP exposure. Microsomes prepared from the immune organs head kidney and spleen of control fish showed EROD activities of 0.25 ± 0.016 and 0.48 ± 0.085 pmol resorufin/mg protein/min, respectively (Fig. 1). This corresponded to 3 % and 5.6 %, respectively, of the EROD activity of in the liver of control trout. Treatment of trout with 15 mg BaP/kg body weight resulted in increased microsomal EROD activities in both immune organs: 9.6 ± 0.22 pmol resorufin/mg protein/min in head kidney, and 0.65 ± 0.29 pmol resorufin/mg protein/min in spleen. These levels correspond to 0.8 % and 0.06 % respectively, of EROD activity in the liver of BaP-exposed fish. Induction factors were higher in the liver (132.8) than in head kidney (38.4) and spleen (1.4). The treatment-related increases of EROD activities were significant in the
liver and head kidney, but not in the spleen. Differences between microsomal EROD levels in liver and immune tissues of BaP-exposed fish were more pronounced than in control fish, with hepatic EROD activities being 119 times higher than EROD activities in the head kidney, and 1758 times higher than in the spleen.

Levels of BaP and BaP metabolites in head kidney, spleen, liver and bile of rainbow trout in vivo: tissue analysis

Liver (n=5), head kidney and spleen tissues (n=3, respectively) from control and BaP-exposed trout were extracted and analyzed for the presence of BaP and BaP metabolites. Additionally, bile of control and exposed fish was analyzed. Data is presented as absolute numbers (ng substance/mg tissue or µL bile, Fig. 2).

Control fish had neither detectable BaP concentrations nor detectable metabolite concentrations. In exposed fish, the parent compound BaP was detected in all three organs. In BaP-treated fish, the highest amount of BaP was found in the head kidney (1.3 ng/mg ± 1.1), followed by the liver (0.4 ng/mg ± 0.48) and the spleen (0.25 ng/mg ± 0.16). Regarding the total sum of BaP metabolites, there was a different ranking of the three organs (Fig. 2). Most metabolites were present in the liver (0.38 ng/mg ± 0.2), followed by the spleen (0.017 ng/mg ± 0.0072). In the head kidney, none of the analyzed BaP metabolites was found. The sum of all analyzed metabolites plus BaP (in the following referred to as “total substance level”) was 0.78 ± 0.4 ng substance/mg tissue in liver, 0.27 ± 0.2 ng substance/mg tissue in spleen and 1.3 ± 1.1 ng substance/mg tissue in head kidney. In bile, 13.27 ± 2.8 ng substance/µL were present. Total substance levels of head kidney, spleen and liver tissue were not significantly different.

HPLC analysis of liver tissue showed that the major hepatic metabolites were 3-hydroxy-BaP (0.27 ± 0.19 ng/mg tissue) and BaP-7,8-dihydrodiol (0.1 ± 0.05 ng/mg), a precursor to BPDE (Fig.2). Furthermore, BaP-9,10-dihydrodiol (0.0013 ± 0.0014 ng/mg) and 9-hydroxy-BaP (0.005 ± 0.0035 ng/mg) were present in lower amounts. BaP-4,5-dihydrodiol was not detected, neither in the liver nor
in any other tissue or in bile. The bile displayed a similar metabolite distribution pattern as the liver, with BaP-7,8-dihydrodiol and 3-hydroxy-BaP being the main metabolites (6.1 ng/µL ± 1.7 and 6 ng/µL ± 1.8, respectively), while 9-hydroxy-BaP was present only at low concentrations (0.3 ng/µL ± 0.07). Neither BaP-9,10-dihydrodiol nor BaP could be detected in the bile.

The spleen had a metabolite profile different to the liver (Fig. 2). Whereas we detected BaP-dihydrodiols, neither 3-hydroxy-BaP nor 9-hydroxy-BaP was present. The main BaP metabolite in the spleen was BaP-7,8-dihydrodiol (0.02 ng/mg ± 0.007), whereas BaP-9,10-dihydrodiol was present at a concentration that was one order of magnitude lower (0.002 ng/mg ± 0.0008). In the head kidney, no metabolites were found (Fig. 2).

For each of the three tissues, the ratios of metabolites and BaP concentrations were estimated. To this end, metabolite and BaP concentrations were converted from ng substance into pmol to account for the differences in molecular weight. The sum of BaP and metabolite concentrations (in pmol/mg tissue) in each tissue was taken as 100% and the metabolite concentrations expressed as percent of this sum (Table 1). This calculation shows that the liver tissue contained a clearly higher percentage of metabolites than the two immune organs; while in the liver, the sum of BaP plus metabolites contained 46.6 % metabolites, the metabolites contributed only 7.2 % and 0 % in spleen and head kidney, respectively.

**Rates of BaP metabolite formation in head kidney, spleen and liver of rainbow trout: in vitro microsomal incubations**

Microsomes prepared from liver, head kidney and spleen of untreated and BaP-exposed trout were incubated with BaP to analyze in vitro rates of BaP metabolite formation and metabolite patterns. Microsomal preparations of all three organs were capable to metabolize BaP, both in control fish and in BaP-exposed fish (Fig. 3). Liver microsomes of untreated fish produced 3-hydroxy-BaP (106.8 ng/mg protein/h), BaP-7,8-dihydrodiol (68.1 ng/mg protein/h), 9-hydroxy-BaP (27.9 ng/mg protein/h) and BaP-9,10-dihydrodiol (4.1 ng/mg protein/h). Liver microsomes of BaP-treated fish produced the
same metabolites, but at higher levels and in a different order: 1245.1 ng/mg protein/h of BaP-7,8-dihydrodiol, 602 ng/mg protein/h of 9-hydroxy-BaP, 572.7 ng/mg protein/h of 3-hydroxy-BaP and 238.6 ng/mg protein/h of BaP-9,10-dihydrodiol (Fig. 3).

Spleen microsomes from control fish produced BaP-7,8-dihydrodiol (12.2 ± 0.8 ng/mg protein/h) and 9-hydroxy-BaP (4.2 ± 6.0 ng/mg protein/h) but neither 3-hydroxy-BaP nor BaP-9,10-dihydrodiol. Spleen microsomes from exposed fish produced BaP-7,8-dihydrodiol (20.7 ± 10 ng/mg protein/h), 9-hydroxy-BaP (8.6 ± 0.2 ng/mg protein/h) and BaP-9,10-dihydrodiol (2.4 ± 3 ng/mg protein/h). The difference of BaP-7,8-dihydrodiol and 9-hydroxy-BaP levels in microsomal incubations from control and BaP-treated fish was not significant. 3-hydroxy-BaP was not formed in any spleen incubation in detectable amounts.

Head kidney microsomes of control fish produced 3-hydroxy-BaP (13.9 ± 19 ng/mg protein/h) and BaP-7,8-dihydrodiol (9.9 ± 0.2 ng/mg protein/h), but neither 9-hydroxy-BaP nor BaP-9,10-dihydrodiol. In head kidney microsomes from BaP-exposed fish, the metabolite formed at the highest rate was BaP-7,8-dihydrodiol (72.7 ± 2.3 ng/mg protein/h), followed by 3-hydroxy-BaP (53.9 ± 17.8 ng/mg protein/h). In contrast to control fish, head kidney microsomes of BaP-treated fish produced also 9-hydroxy-BaP (33.1 ± 8.3 ng/mg protein/h) and BaP-9,10-dihydrodiol (13.1 ± 1.5 ng/mg protein/h). The formation of BaP-7,8-dihydrodiol was increased significantly in microsomes of BaP-exposed fish over control microsomes.

When summing up the metabolic rates of the individual metabolites for each organ, rates of liver microsomes were found to be one to two orders of magnitude higher than in head kidney and spleen. The sum of metabolites produced by liver microsomes of control fish was 206.9 ng substance/mg protein/h, whereas head kidney showed a total rate of 23.9 ± 19.5 ng substance/mg protein/h and spleen 16.5 ± 6.8 ng substance/mg protein/h. In microsomes of BaP-treated fish, total metabolite formation rates showed the same ranking: they were highest in the liver (2658.4 ng substance/mg protein/h), followed by head kidney (172.8 ± 22.2 ng substance/mg protein/h) and spleen (31.7 ± 13.9
ng substance/mg protein/h). None of the microsomal preparations formed BaP-4,5-dihydrodiol. Induction of metabolic rates of control fish to BaP-treated fish was highest in the liver (12.9 times) followed by head kidney (7.2 times) and spleen (1.9 times). The treatment-related increases of total metabolite formation were significant in liver and head kidney, but not in the spleen.

One unknown peak occurred both in spectra from BaP treated liver and head kidney incubations at 35-36 min, which was not present in any control incubation (Supplemental Figure 1).

BaP metabolites in head kidney, spleen and liver of rainbow trout: comparison of metabolite patterns from tissue analysis and microsomal incubations

In the liver of control fish, ranking of metabolites on the basis of their tissue concentrations was 3-hydroxy-BaP > BaP-7,8-dihydrodiol > 9-hydroxy-BaP > BaP-9,10-dihydrodiol. The rate of metabolite formation by microsomes of control liver showed an identical ranking, i.e. the rates were highest for 3-hydroxy-BaP, followed by BaP-7,8-dihydrodiol, 9-hydroxy-BaP and BaP-9,10-dihydrodiol. In contrast, liver tissue and microsomes from BaP-exposed fish differed in their ranking, with microsomes producing BaP-7,8-dihydrodiol as main metabolite while 3-hydroxy-BaP was the dominant metabolite in the intact liver. The main metabolite in the spleen of control and BaP-exposed fish, was BaP-7,8-dihydrodiol and spleen microsomes also showed the highest rate of formation for this metabolite. The second highest metabolic rate in spleen microsomes from control and BaP-exposed fish were observed for 9-hydroxy-BaP, but this metabolite was not found in spleen tissue in vivo. Differences between the results of the microsomal incubations and tissue analysis were prominent in the head kidney, where no metabolites at all were found in the intact tissue, whereas microsomes generated BaP metabolites. Head kidney microsomes from BaP-exposed fish produced all four metabolites, with the ranking of BaP-7,8-dihydrodiol > 3-hydroxy-BaP > 9-hydroxy-BaP > BaP-9,10-dihydrodiol. Head kidney microsomes of control fish produced 3-hydroxy-BaP at the highest rate, followed by BaP-7,8-dihydrodiol. The relative importance of the individual metabolites in
microsomes from liver, head kidney and spleen, expressed as percent of total metabolite levels, is shown in Table 2.
Discussion

In mammals, the importance of tissue-specific PAH metabolism for organ-related adverse effects is well known (Ioannides et al., 2004; Miller and Ramos, 2001). The capability of mammalian immune organs to generate PAH metabolites is thought to be critically involved in PAH immunotoxicity (Burchiel and Luster, 2001; Ioannides et al., 2004), and tissue or cell-dependent differences in amount or spectrum of the produced metabolites can greatly influence the toxicological outcome. Also for fish, it has been shown that tissue-specific PAH metabolism is an important determinant in PAH toxicity (e.g., James et al., 1997), as it has been suggested with respect to PAH immunotoxicity (Carlson et al., 2004a; Reynaud and Deschaux, 2006; Reynaud et al., 2008). However, the actual capability of piscine immune organs to metabolize PAHs has not been demonstrated to date. Here, we investigated whether immune organs of rainbow trout have the capacity for in situ metabolism of PAHs and whether metabolism is inducible by exposure to BaP.

The findings from the in vivo part of this study indicate that distribution of the parent compound, BaP, to the immune organs appears to be similar to distribution to the liver, as BaP concentrations in head kidney and spleen tissue were not significantly different from liver. Given the location of fish immune organs in the vascular system, and their blood-filtering role (Press and Evensen, 1999), substantial exposure of immune organs to circulating xenobiotics is to be expected. In fact, Valdez Domingos et al. (2011) showed in a toxicokinetic study on PAH distribution in medaka that a substantial fraction is distributed to the immune organs. Medaka head kidney tended to accumulate higher amounts of PAH than the spleen (Valdez Domingos et al., 2011), what is in agreement with our observation on rainbow trout. The difference between the two immune organs might be related to their role and position in the blood system of fish: while the head kidney receives a direct supply from the dorsal aorta close to its origin, the more distant location of the spleen as well as its function as an encapsulated plasma filter imply lower blood flow rate and supply. As shown by Barron et al. (1987) for rainbow trout, the kidney receives a clearly higher blood flow distribution (measured as percent of cardiac output) and organ perfusion (mL blood per gram of tissue per hour) than does the spleen.
Do the immune organs of rainbow trout possess a capacity for BaP metabolism, and is it inducible?

We approached this question by analyzing rates of BaP metabolite formation by microsomes prepared from head kidney and spleen of control and BaP-exposed trout. These rates of the immune organs were compared to rates of BaP metabolite formation in liver microsomes as a benchmark. In parallel, we measured microsomal EROD activity - as proxy of the overall enzymatic capacity of a tissue for BaP metabolism - in the immune organs and the liver.

In control fish, liver microsomes showed significantly higher rates of BaP metabolite formation, and higher EROD activity than the immune organs. The head kidney possessed 3% of the hepatic EROD activity and the metabolite formation rate of the microsomes was 12% of the hepatic one. With these activities, the head kidney is clearly less active than trout liver, but also less active than the excretory kidney of trout (Pesonen et al., 1987). The spleen reached 8% of the metabolite formation rate of the liver and had 5.6% of the hepatic EROD activity. After BaP exposure, levels of BaP metabolites were highest in the liver, and liver microsomes displayed the strongest induction in the rate of metabolite production and of EROD activity. In the head kidney of BaP-exposed fish, microsomal metabolite levels reached 7% of liver metabolite levels, BaP metabolism rates were accelerated by a factor of 7.2, and EROD activity increased by a factor of 38.4. The spleen of BaP-exposed fish contained BaP metabolites at 1% of the hepatic level, its EROD activity was elevated by a factor of 1.4, and the rates of microsomal metabolite formation were increased by a factor of 1.9. Notably, whereas BaP treatment led to a significant increase of metabolite production rates in liver and head kidney, it failed to do so in spleen. This was paralleled by EROD activities, which increased significantly in liver and head kidney of BaP-exposed fish, but not in spleen. The main conclusions from these findings are that (i) the immune organs possess metabolic capacity for BaP transformation, (ii) there exists good agreement between the tissue levels of EROD activity and the rates of BaP metabolite formation, (iii), the metabolic capacity is inducible in the head kidney but not in the spleen, and (iv) that the metabolic capacities of the immune organs, both in control and BaP-exposed trout, are clearly lower than in the liver.
Does the BaP metabolite spectrum obtained in the present study from liver and immune organ microsomes agree with published data on BaP metabolites in fish? A comparison is possible only for the liver, as almost no information exists on metabolite spectra of other organs. For this organ, our findings largely agree with literature reports. BaP-incubation of hepatic microsomes or isolated hepatocytes were reported to produce BaP-9,10-dihydrodiol, BaP-7,8-dihydrodiol, 9-hydroxy-BaP and 3-hydroxy-BaP (Sikka et al., 1990; Stegeman et al., 1984; Yuan et al., 1997). A more controversial case appears to be BaP-4,5-diol. In our study, this metabolite was not detected, neither in the microsomal incubations nor in the tissues. In contrast, Williams and Buhler (1984), using purified cytochrome P450 LM4a and LM4b (CYP1As) from rainbow trout liver, observed the conversion of BaP to several quinones, BaP-3-OH, BaP-9-OH, BaP-7,8-diol, BaP-9,10-diol as well as BaP-4,5-diol. Interestingly, Miranda et al. (2006) when studying BaP metabolism in trout liver microsomes also did not observe the production of BaP-4,5-diol. Non-production of BaP-4,5-diol by fish tissues was reported by other authors as well (Stegeman et al., 1984; Yuan et al., 1997). Miranda et al. (2006) suggested that BaP-4,5-diol might be detectable in microsomal preparations only if huge amounts of microsomes are used and the products are concentrated prior to HPLC analysis.

In addition to analysing BaP metabolites in microsomal preparations, which allows a determination of the rate of metabolite formation, we also analysed BaP metabolites in the intact tissues of exposed fish in order to examine whether we would find the same type of metabolites as in the microsomal assays. Good agreement between tissue analyses and microsomal assays existed in the case of liver and spleen. In both organs, the same metabolite spectrum was observed in the microsomal assays and in the tissues, with the levels of metabolite being substantially lower in the spleen than in the liver. However, a discrepancy was observed for the head kidney: although the microsomal assays clearly indicated the capacity of this tissue to produce BaP metabolites, the head kidney of BaP-exposed fish contained no detectable levels of BaP metabolites. This is particularly surprising in comparison to the spleen, which contained BaP metabolites, despite significantly lower rates of BaP metabolite formation and EROD activity. Therefore, the absence of BaP metabolites from the head kidney in vivo
cannot be explained by a lack of metabolic capacity of this tissue. We speculate that, due to the higher blood perfusion of the head kidney compared to the spleen (Barron et al., 1987; Schultz et al., 1999; see also above), metabolites are rapidly removed via the blood stream, so that the metabolite concentrations remaining in the tissue were below the detection limits of our analytical method.

With respect to the immunotoxicity of PAHs, it is not only important to know if the immune organs are capable of in situ PAH metabolism, but also if they produce a metabolite spectrum specific to these organs. Organ differences in BaP metabolite patterns might be related to tissue-specific expression of CYPs. Here, we studied only CYP1A, but multiple CYP forms participate in BaP metabolism, including CYP1A1, CYP1A2, CYP1B1, and several members of the CYP2 subfamilies and CYP3A4 (Gautier et al., 1996; Bauer et al., 1995; Scornaienchi et al., 2010). The activities of these enzymes can vary between organs and even between specific immune cell types (Baron et al., 1998; Okano et al., 1979; Kapitulnik and Strobel, 1999). For fish, the available database on tissue-specific expression of distinct CYP enzymes is limited. Best documented is the presence of CYP1A in the head kidney (e.g., Lorenzana et al., 1988; Pesonen et al., 1990), spleen (Taysse et al., 1998) or in distinct immune cell populations (Nakayama et al., 2008). Particularly the work in Buhler's group showed expression of a series of other CYP transcripts in the kidney of trout, mainly within the CYP1 and CYP3 families, both of which could be responsible for tissue differences in BaP metabolites (reviewed in Buhler and Wang-Buhler, 1998). Tissue-specific differences of BaP metabolites, in fact, have been observed in the present study. For instance, 3-hydroxy-BaP was produced only by head kidney and liver but not by spleen microsomes. Importantly, during BaP exposure, liver and head kidney showed a shift in metabolite ranking from 3-hydroxy-BaP as main metabolite produced by control microsomes, to BaP-7,8-dihydrodiol as the main metabolite formed by microsomes of BaP-treated fish. As discussed above, this may indicate a BaP-induced alteration of the relative expression of CYP isoenzymes. Similar results were obtained in turbot (Telli-Karakoc et al., 2002). The dominant formation of BaP-7,8-dihydrodiol under BaP-exposure is toxicologically of interest. This metabolite suppresses T cell proliferation in mammals (Davila et al., 1996) and induces apoptosis in human B cells (Salas and
Burchiel, 1998). Further metabolism of this compound may lead to BPDE, an intermediate of BaP that binds to DNA and had immunosuppressive effects in murine splenocytes (Kawabata and White, 1989). In medaka, a single intraperitoneal injection of BaP led to immunosuppressive effects in kidney lymphocytes that were not observed after concurrent exposure to BaP and ANF – a finding that points to a role of metabolism in the immunotoxic effects of BaP (Carlson et al., 2004a). This interpretation is corroborated by the finding of the present study that BaP-7,8-dihydrodiol, which is a known immunotoxic compound, is the dominant metabolite in the immune organs of BaP-exposed trout.

In conclusion, this study provides an answer to an important gap in our understanding of PAH immunotoxicity in fish. It has been suggested that the production of PAH metabolites in the immune cells is a mechanism through which PAHs cause immunotoxicity, but it had not been demonstrated yet whether immune organs indeed are capable of PAH metabolism. This study now provides strong evidence that rainbow trout immune organs possess an (inducible) capacity for \textit{in situ} metabolism of the prototypic PAH, BaP.
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Author contributions

*Participated in research design*: Möller, Hermsen, Lamoree, Segner

*Conducted experiments*: Möller, Hermsen, Floehr

*Performed data analysis*: Möller, Hermsen, Floehr

*Wrote or contributed to the writing of the manuscript*: Möller, Segner, Lamoree
DMD #53777

References


Footnote

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Figure legends

**Fig. 1.** EROD activity in microsomes of liver, head kidney and spleen isolated from rainbow trout treated with BaP in corn oil solvent or with corn oil alone (control), sampled after 5d of exposure (n=3). **p<0.01, ***p<0.001, significantly different from control group. Incubations were carried out with 16 µM 7-ethoxyresorufin. Note the break in the y-axis to accommodate liver values.

**Fig. 2.** Benzo[a]pyrene metabolites in A) liver (n=5), B) bile (n=5), C) head kidney (n=3) and D) spleen (n=3) 5 days after fish were treated with 15 mg BaP/kg. Values are given as mean concentrations of ng substance + SD. Note the different scale of y-axis.

**Fig. 3.** Benzo[a]pyrene metabolite formation of A) liver (n=1), B) spleen (n=2) and C) head kidney (n=2) microsomes. Microsomes of both BaP-treated (5 days after injection of 15 mg BaP/kg body weight) and control fish were incubated with BaP for 1 h. Afterwards, the reaction was stopped and BaP metabolites were extracted and analyzed using HPLC. Values are given as mean + SD. *p<0.05, ***p<0.001, significantly different from control group. The data of liver microsomal incubations was not applied for statistical analysis.
**Tables**

**Table 1**

Percentage of benzo[a]pyrene metabolites in 1 mg of liver (n=5), spleen (n=3) and head kidney (n=3) tissue of BaP treated fish. The amount of BaP plus metabolites that were extracted per mg tissue from each organ correlates to 100%. Values in brackets are amount of pmol substance per mg tissue. n.d.: not detected

<table>
<thead>
<tr>
<th></th>
<th>liver</th>
<th>spleen</th>
<th>head kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-OH</td>
<td>34% (1.01 pmol)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>7,8-diol</td>
<td>11.8% (0.35 pmol)</td>
<td>6.5% (0.07 pmol)</td>
<td>n.d.</td>
</tr>
<tr>
<td>9,10-diol</td>
<td>0.16% (0.0046 pmol)</td>
<td>0.7% (0.007 pmol)</td>
<td>n.d.</td>
</tr>
<tr>
<td>9-OH</td>
<td>0.64% (0.019 pmol)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>total metabolites</td>
<td>46.6% (1.4 pmol)</td>
<td>7.2% (0.077 pmol)</td>
<td>n.d.</td>
</tr>
<tr>
<td>BaP plus metabolites</td>
<td>100% (2.97 pmol)</td>
<td>100% (1.07 pmol)</td>
<td>100% (5.1 pmol)</td>
</tr>
</tbody>
</table>
Table 2

Relative distribution of benzo[a]pyrene metabolites in liver, spleen and head kidney microsomes of control and untreated trout. The sum of metabolites, that were extracted from microsomal incubations, correlates to 100%. Values in brackets are amount of pmol substance per mg protein/h. n.d.: not detected

<table>
<thead>
<tr>
<th>metabolite</th>
<th>liver control</th>
<th>liver BaP-treated</th>
<th>spleen control</th>
<th>spleen BaP-treated</th>
<th>head kidney control</th>
<th>head kidney BaP-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-OH</td>
<td>52.7% (0.39)</td>
<td>22.2% (2.1)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>59.6% (0.05)</td>
<td>32.1% (0.2)</td>
</tr>
<tr>
<td>7,8-diol</td>
<td>31.7% (0.24)</td>
<td>45.7% (4.4)</td>
<td>73.1% (0.04)</td>
<td>64.1% (0.073)</td>
<td>40.4% (0.035)</td>
<td>40.8% (0.26)</td>
</tr>
<tr>
<td>9,10-diol</td>
<td>1.9% (0.02)</td>
<td>8.7% (0.8)</td>
<td>n.d.</td>
<td>7.5% (0.009)</td>
<td>n.d.</td>
<td>7.4% (0.046)</td>
</tr>
<tr>
<td>9-OH</td>
<td>13.7% (0.1)</td>
<td>23.4% (2.2)</td>
<td>26.9% (0.016)</td>
<td>28.4% (0.03)</td>
<td>n.d.</td>
<td>19.7% (0.12)</td>
</tr>
<tr>
<td>total</td>
<td>100% (0.75)</td>
<td>100% (9.5)</td>
<td>100% (0.056)</td>
<td>100% (0.112)</td>
<td>100% (0.085)</td>
<td>100% (0.63)</td>
</tr>
</tbody>
</table>
Figure 1

The graph shows the EROD activity (pmol/min/mg protein) for different tissues: head kidney, spleen, and liver. The x-axis represents the different tissues, and the y-axis represents the EROD activity. The bars are divided into two categories: control (white) and BaP-treated (black).

- **Head Kidney**: The activity is very low, with no significant difference between control and BaP-treated groups.
- **Spleen**: The activity is slightly higher than in the head kidney, but no significant difference is observed.
- **Liver**: The activity is significantly higher in the BaP-treated group compared to the control group, with a marked increase in EROD activity.

The graph includes error bars indicating variability in the data. The significance of the difference between the control and BaP-treated groups is indicated by stars: one star for a significant difference, two stars for a very significant difference.