CELL-SPECIFIC CYP1A EXPRESSION AND BENZO[a]PYRENE ADDUCT FORMATION IN GILLS OF RAINBOW TROUT (ONCORHYNCHUS MYKISS) FOLLOWING CYP1A INDUCTION IN THE LABORATORY AND IN THE FIELD

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Abstract—The effect of cytochrome P4501A (CYP1A) induction on cell-specific benzo[a]pyrene (BaP) adduct formation was studied in rainbow trout (Oncorhynchus mykiss) gills. Fish preexposed to β-naphthoflavone (βNF) or caged in a polluted river were exposed to waterborne [3H-benzo[a]pyrene ([3H-BaP]). The [3H-benzo[a]pyrene adducts in the gill filaments were localized by autoradiography and CYP1A protein by immunohistochemistry. Ethoxyresoru®n-O-deethylase (EROD) activity was measured using a gill filament-based ex vivo assay. Branchial [3H-BaP binding and EROD activity were enhanced by exposure to βNF or to the river water, and completely blocked by the CYP1A inhibitor ellipticine. The predominant sites of adduct formation were in the epithelium of the secondary lamellae and in epithelium of the efferent edge of the gill filament. In βNF-exposed fish, the strongest CYP1A immunoreactivity was observed in differentiating cells and in pillar cells. In fish caged in the polluted river, strong CYP1A immunoreactivity was found in most cells in the secondary lamellae, whereas the primary lamellae were almost devoid of immunoreactivity. Our results reveal a discrepancy between the localization of CYP1A protein and BaP adducts in the gill. Consequently, other factors, such as bioavailability of waterborne polycyclic aromatic hydrocarbons (PAHs) to the target cells, are important for the localization of PAH adducts in the gill.

Keywords—Gill  Cytochrome P4501A  Benzo[a]pyrene  Adduct

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widespread organic pollutants in marine and freshwater environments, mainly originating from incomplete combustion of organic material and release of petroleum and petroleum products. Many PAHs are promutagens and procarcinogens that are biotransformed into reactive intermediates via aryl hydrocarbon receptor (AhR) regulated cytochrome P450 (CYP) enzymes, such as CYP1A and CYP1B [e.g., 1–4]. By interaction with the AhR, PAHs, polychlorinated dibenzoxydioxins and dibenzofurans, and coplanar polychlorinated biphenyls induce CYP1A in various tissues in vertebrates [5,6]. Aryl hydrocarbon receptor agonists induce CYP1A in fish gill cells, as shown by increases in CYP1A immunoreactivity [e.g., 7–10] and ethoxyresoru®n-O-deethylase (EROD) activity [11–13].

Organic pollutants occur in complex mixtures in the aquatic environment. This implies that fish may be exposed simultaneously to CYP1A inducers and pollutants that are metabolized by CYP1A. The gill is a major site for absorption of waterborne organic pollutants [14], and gill cells therefore are exposed to high concentrations in polluted waters. Gill cells can metabolize PAHs such as benzo[a]pyrene (BaP) into reactive intermediates and stable water-soluble metabolites. Consequently, the gill may be a site of adduct formation, and by first-pass metabolism it may also reduce the systemic bioavailability of PAHs [15–17].

Despite the accumulating knowledge about the branchial metabolic function in fish, the cells involved in bioactivation of PAHs are still largely unidentified. The aim of this work was to study the effect of preexposure to AhR agonists on the cellular localization of CYP1A expression and BaP-adduct formation in fish gills. Cytochrome P4501A was induced in gills of rainbow trout (Oncorhynchus mykiss) by laboratory and field exposure to waterborne AhR agonists, after which the fish were exposed to waterborne [3H-BaP]. The cellular localization of [3H-BaP] adducts was determined by autoradiography. The sites of CYP1A expression were investigated by immunohistochemistry, and the EROD activity was determined in gill filaments ex vivo [13].

MATERIALS AND METHODS

Animals

Juvenile rainbow trout (weighing about 50 g) were purchased from Persbo-Klotens Fiskodling AB (Kloten, Sweden). The fish (n = 200) were kept in the aquarium facility at the Evolutionary Biology Center, Uppsala University (Uppsala, Sweden), in a 1-m³ tank continuously supplied with aerated Uppsala tap water (1.5 L/min; 11–13°C). The day/night cycle was adjusted automatically to the diurnal variations at latitude 52°N. The fish were fed pellets (EST40–4 from Aller Aqua, Christiansfeld, Denmark) once daily, at a ration corresponding to 1% of their body mass.

Chemicals

[1H-3H]Benzo[a]pyrene ([3H-BaP) and X-ray film (Hyperfilm-3H) were obtained from Amersham Biosciences UK (Little Chalfont, UK). The β-naphthoflavone (βNF), ellipticine, 7-ethoxyresoru®n, dicumarol, dimethyl sulfoxide, and 3-amino-
The bags were placed in four plastic aquaria (35 cm) and filled with 10 L of continuously aerated tap water. The bags were exposed to the CYP1A inhibitor ellipticine. The fish were sacrificed soon after arrival in Uppsala and their gill EROD activity was determined according to Joënsson et al. [13]. In brief, duplicate groups of 10 filament tip pieces (2 mm long) were placed in wells of a 12-well tissue culture plate, and 0.5 ml of reaction buffer, consisting of 7-ethoxresoruﬁn (1 μM) and dicumarol (10 μM) in HEPES-Cortland buffer (pH 7.7), was added. After 10 min of incubation (at 13°C) with continuous shaking, the buffer was replaced with 0.7 ml of fresh reaction buffer. After another 10 and 30 min of incubation, 0.2-ml aliquots were transferred from each well to a Fluoromax 96-well plate. Aliquots (0.2 ml) of resoruﬁn standard solutions (0.5–250 nM; prepared from a 0.1-mM stock solution in methanol by dilution with tap water) were placed in wells. The fluorescence was determined in a multi-well plate reader (Fluostar®P, SLT Labinstruments, Grödig, Austria) using the wavelengths 544 (ex) and 590 nm (em). The EROD activity was calculated and expressed as picomole of resoruﬁn per filament tip and minute.

9-ethylcarbazole (AEC) tablets were obtained from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal primary antibody C10-7, raised against peptide 277-294 of rainbow trout CYP1A (i.e., CYP1A1 and CYP1A3), was obtained from Biosense (Bergen, Norway), while the biotinulated secondary antibody, normal horse serum, and avidin-biotin blocking kit were from Vector Laboratories (Burlingame, CA, USA) and peroxidase-coupled streptavidin-biotin complex (ABC) was from DakoCytomation A/S (Glostrup, Denmark). Methacrylate (Technovit 7100) was purchased from Kulzer (Heraeus, Germany). Within 3 h, branchial EROD activity was determined according to Jönsson et al. [13]. In brief, duplicate groups of 10 filament tip pieces (2 mm long) were placed in wells of a 12-well tissue culture plate, and 0.5 ml of reaction buffer, consisting of 7-ethoxresoruﬁn (1 μM) and dicumarol (10 μM) in HEPES-Cortland buffer (pH 7.7), was added. After 10 min of incubation (at 13°C) with continuous shaking, the buffer was replaced with 0.7 ml of fresh reaction buffer. After another 10 and 30 min of incubation, 0.2-ml aliquots were transferred from each well to a Fluoromax 96-well plate. Aliquots (0.2 ml) of resoruﬁn standard solutions (0.5–250 nM; prepared from a 0.1-mM stock solution in methanol by dilution in reaction buffer) were included on the plate. The fluorescence was determined in a multi-well plate reader (Fluostar®P, SLT Labinstruments, Grödig, Austria) using the wavelengths 544 (ex) and 590 nm (em). The EROD activity was calculated and expressed as picomole of resoruﬁn per filament tip and minute.

### Table 1. Exposure regimen

<table>
<thead>
<tr>
<th>Exposure group</th>
<th>Day 0–2</th>
<th>Day 2.5–4.5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Benzo[a]pyrene (BaP)</strong></td>
<td>Carrier&lt;sup&gt;a&lt;/sup&gt;</td>
<td><strong>3</strong>&lt;sup&gt;a&lt;/sup&gt;-BaP&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-naphthoflavone (βNF) + BaP</td>
<td>βNF&lt;sup&gt;b&lt;/sup&gt;</td>
<td><strong>3</strong>&lt;sup&gt;a&lt;/sup&gt;-BaP&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>βNF + ellipticine/BaP</td>
<td>βNF&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Ellipticine&lt;sup&gt;e&lt;/sup&gt; and <strong>3</strong>&lt;sup&gt;a&lt;/sup&gt;-BaP&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Viskan + BaP</td>
<td>Caged in the Viskan River&lt;sup&gt;f&lt;/sup&gt;</td>
<td><strong>3</strong>&lt;sup&gt;a&lt;/sup&gt;-BaP&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nonexposed</td>
<td>Carrier&lt;sup&gt;a&lt;/sup&gt;</td>
<td>_</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each group consisted of two fish.
<sup>b</sup> Waterborne **3**<sup>a</sup>-benzo[a]pyrene (**3**<sup>a</sup>-BaP, 0.5 mCi/10L; 0.64 nM) and acetone (10 ppm).
<sup>c</sup> 1 μM βNF and 20 ppm of acetone.
<sup>d</sup> 1 μM ellipticine and 10 ppm of acetic acid. The fish were preexposed to ellipticine for 0.5 h and then **3**<sup>a</sup>-BaP was added to the water.
<sup>e</sup> Caged in the Viskan River (located in southwestern Sweden) 2 km downstream from the Borås sewage treatment plant for four weeks, and thereafter held overnight in water from the Viskan River.
Tape section autoradiography

The 3H-BaP–exposed fish were embedded in aqueous carboxymethyl cellulose (40 g/L), frozen in carbon dioxide–cooled hexane, and tape-sectioned (20 μM) for autoradiography according to Ullberg [18]. Sections were extracted by bathing in 5% (volume/volume [v/v]) trichloroacetic acid (1 min), 50% (v/v) methanol (30 s), absolute methanol (30 s), heptane (20 s), absolute methanol (30 s), 50% methanol (30 s), and running tap water (5 min). The dried sections were pressed against Hyperfilm-H and after 21 d of exposure at −20°C, the film was developed. The autoradiograms obtained were considered to represent mainly irreversibly bound 3H-BaP metabolites.

Light microscopy autoradiography

A gill arch from each 3H-BaP–exposed fish was fixed overnight in a phosphate-buffered (0.1 M, pH 7.4) mixture of 1.5% (v/v) glutaraldehyde and 1.5% (v/v) formaldehyde. The fixed

Fig. 2. (a) Scanning electron microscopic representation showing primary and secondary lamellae of gill filaments from a rainbow trout. The efferent edge (leading edge) is the side of the filament where the blood leaves the secondary lamellae and that faces the water flow. (b) Longitudinal section through a primary and a number of secondary lamellae of a gill filament. (c) Distal part of a secondary lamella (enclosed in b). (d) Basal part of a secondary lamella and an interlamellar region of the epithelium of the primary lamella (enclosed in b). CC = chloride cell, EC = endothelial cell in marginal channel, RC = respiratory cell, DC = differentiating cell, PVC = pavement cell, and PC = pillar cell.
BoP adduct formation and CYP1A in rainbow trout gills

Fig. 3. Microautoradiograms of gill filaments from a fish caged in the Viskan River (located in southwestern Sweden) for 28 d, exposed to waterborne \(^3\text{H}\)-benzo[a]pyrene (\(^3\text{H}\)-BaP) in the laboratory for 2.5 h, and then kept in clean water for 2 h. (a) Sectioned gill filaments (primary and secondary lamellae) with the efferent edges (i.e., the leading edges = the edges on the sides of the filaments that meet the water flow and where the blood leaves the secondary lamellae; see Fig. 2a) to the left, and the afferent edges (i.e., the trailing edges = the edges on the sides where the blood enters the secondary lamellae) to the right. The filaments were sectioned diagonally from one edge to the other. The silver grains (black dots) represent irreversible \(^3\text{H}\)-BaP binding. (b) Corresponding dark-field image showing the localization of silver grains. EfA = efferent artery, CVS = central venous sinus, AfA = afferent artery, EfE = efferent edge, and AfE = afferent edge.

gill arches were rinsed in 70% (v/v) ethanol and then stored for several days in 70% ethanol at room temperature. The gill filaments were dissected and then dehydrated in 95% and absolute ethanol. This procedure has previously been found to extract most unbound labeled substance in tissues from \(^3\text{H}\)-BaP- and \(^3\text{H}\)-DMBA–treated mice [19]. The dehydrated gill filaments were embedded in Technovit 7100 (Heraeus Kulzer, Hanav, Germany), sectioned (2 μM), and mounted on super-frost glass slides. The slides were dipped in liquid film emulsion (NTB-2 diluted in distilled water, 2:1, v/v) and kept in the dark at 4°C. After four months of exposure, the autoradiograms were developed, fixed, and stained with toluidine blue. Silver grains on the sections were considered to represent irreversibly bound \(^3\text{H}\)-BaP metabolites.

Fig. 4. Rainbow trout were caged for 28 d in the Viskan River (located in southwestern Sweden) and then exposed for 2.5 h to \(^3\text{H}\)-benzo[a]pyrene (\(^3\text{H}\)-BaP) via the water. (a) \(^3\text{H}\)-BaP–binding (black silver grains) in epithelial cells in the efferent edge of the primary lamella. (b) Corresponding dark-field image showing the localization of silver grains. (c) CYP1A immunoreactivity in the endothelium of an efferent artery and arterioles in the primary lamella, as well as in the epithelium of the efferent edge of the primary lamella. Note the lack of \(^3\text{H}\)-BaP binding in the endothelium despite the intense CYP1A immunoreactivity. EC = endothelial cell, PE = epithelium of the primary lamella.
Fig. 5. Microautoradiography (a, b, e, g, and i) and cytochrome P4501A (CYP1A) immunohistochemistry (c, d, f, h, and j) of gill filaments in rainbow trout following exposure to waterborne 3H-benz(a)pyrene (3H-BaP). The fish were subjected to various preexposures before exposure to 3H-BaP (2.5 h). Figures represent: (a–d) fish preexposed to 20 parts per million of acetone (carrier; 2 d); (e–f) fish preexposed to 1 μM βNF (2 d);
Immunohistochemistry

A gill arch from each fish according to Table 1 and from another six fish that had been caged in the Viskan River were fixed overnight in Saint Marie’s fixative (1% glacial acetic acid and 99% ethanol, v/v). The following day, the gill arches were washed in 70% ethanol and then stored in 70% ethanol at room temperature. The gill filaments were dissected, dehydrated in 95% and absolute ethanol, soaked in xylol, embedded in paraffin, sectioned (4 µM), and mounted as previously indicated. Following deparaffinization in xylol and rehydration in ethanol (using the graded series to 70% ethanol), the sections were treated according to the immunohistochemistry protocol summarized below.

Unless otherwise indicated, the slides were washed three times in phosphate-buffered saline (PBS; pH 7.4) between each of the following steps. Endogenous peroxidase activity was blocked with 0.3% (v/v) hydrogen peroxide in methanol (30 min) and epitopes were unmasked by treatment (30 min) in steam-heated citrate buffer (pH 6.6). The sections were treated with an avidin/biotin blocking kit, covered with 10% (v/v) horse serum in PBS (20 min), and drained (not washed in PBS) before the monoclonal antibody C10-7 (diluted 1:500 in PBS) was applied. Following incubation overnight in a humidity chamber (4°C), the sections were incubated (40 min) with the secondary antibody (diluted 1:500 in PBS including 5% horse serum, v/v), and then with peroxidase-coupled streptavidin-biotin complex (ABC; 20–30 min). Labeling was visualized using a mixture of AEC (0.24 mg/ml), Photo-Flo (0.2%, v/v), and hydrogen peroxide (0.03%, v/v) in acetate buffer (0.1 M). After the last step, the sections were washed in water and mounted.

RESULTS

The autoradiography and immunohistochemistry results presented here were consistent for the two fish in each treatment group.

Tape-section autoradiography and EROD activity

In autoradiograms of tape sections, high levels of 3H-BaP-derived radioactivity were found in gills, kidney, liver, fat, bile, and intestinal contents. Following solvent extraction of tape sections from fish only preexposed to the carrier and then exposed to 3H-BaP, a selective localization of radioactivity in the gill filaments and the liver emerged. The labeling of the gill filaments was markedly increased in fish preexposed to the CYP1A inducer βNF via the water, whereas no 3H-BaP uptake was detected in the gills of βNF-induced fish exposed to the CYP1A inhibitor ellipticine (Fig. 1). A high concentration of BaP-derived radioactivity was also found in the gill filaments of fish that had been caged in the Viskan River for four weeks before being exposed to 3H-BaP in the laboratory (Fig. 1). As shown in Figure 1, the amounts of silver grains in the autoradiograms were roughly proportional to the EROD activities measured in the corresponding gill filaments. The EROD activities in the two control fish nonexposed to 3H-BaP (carrier controls) were 0.002 and 0.013 pmol/filament tip/min. The six fish that had been caged in the Viskan River but had not been exposed to 3H-BaP had an EROD activity of 0.114 ± 0.030 pmol/filament tip/min (mean ± standard deviation of the mean).

Microautoradiography and CYP1A immunohistochemistry of gills

The various parts of the gill and the gill cell types mentioned below are depicted in Figure 2.

General distribution of 3H-BaP–binding and CYP1A immunoreactivity. There was a more intense radiolabeling in the gills of fish exposed to βNF or caged in the Viskan River than in the gills of those pretreated with the carrier. However, all fish in these groups had a similar overall distribution pattern. Intense binding was present in the epithelium at the efferent edge of the filament (Fig. 3 and 4a), whereas the afferent edge showed less binding (Fig. 3). In the epithelium of the secondary lamellae, the radiolabeling was most intense close to the efferent edge and decreased toward the afferent edge (Fig. 3). Strong CYP1A immunoreactivity was observed in the gills in βNF-exposed and caged fish, whereas the CYP1A staining in the fish exposed to 3H-BaP without pretreatment with inducer was weak. The localization of CYP1A immunostaining differed in the various exposure groups, as described below.

3H-BaP–binding and CYP1A immunoreactivity in branchial arteries, arterioles, and central venous sinus. There was no selective 3H-BaP binding in the branchial endothelia of the afferent and efferent arteries and arterioles and the central venous sinus in any fish (Fig. 3a, b, and 4a). However, a strong CYP1A staining was observed in arteries and arterioles in Viskan River-exposed (Fig. 4b) and βNF-exposed fish (not shown). The fish exposed to only 3H-BaP showed no or very weak CYP1A immunoreactivity in endothelia.

Cellular distribution of 3H-BaP–binding and CYP1A immunoreactivity in the primary and secondary lamellae. Exposure to waterborne 3H-BaP without pretreatment with inducer gave rise to binding in differentiating cells close to the basal membrane of the epithelium in the primary lamellae (Fig. 5a). In the secondary lamellae, binding was present mainly in the inner epithelial cell layer, consisting of differentiating cells (Fig. 5b). Scattered CYP1A immunoreactivity was observed in differentiating epithelial cells in the primary lamellae (Fig. 5c) and in differentiating epithelial cells and pillar cells in the secondary lamellae (Fig. 5d).

In βNF-induced fish, labeled cells were rare in the interlamellar regions of the epithelium in the primary lamellae (Fig. 5e). Intense binding of 3H-BaP–derived radioactivity was observed in the epithelium of the secondary lamellae (Fig. 5e). The radioactivity was generally most intense in differentiating cells in the inner epithelial layer, but also cells in the outer layer (respiratory cells and chloride cells) were labeled (Fig. 5e). Occasionally, pillar cells were labeled. Pronounced staining for CYP1A immunoreactivity was observed in several gill cell types. Particularly strong staining was found in cells in...
the inner epithelial layers of the primary lamellae. In the outer layer, pavement cells showed strong staining whereas chloride cells merely showed background staining (Fig. 5f). In the secondary lamellae, pillar cells, differentiating cells in the inner epithelial layer, and endothelial cells in the marginal channel were stained. Staining for CYP1A was weak or lacking in cells of the outer epithelial layer of the secondary lamellae (i.e., in respiratory cells and chloride cells; Fig. 5f).

In the βNF-induced fish exposed both to ellipticine and 1H-BaP via the ambient water, no binding of radioactivity above the background level was observed in any gill cell type (Fig. 5g). The cellular pattern of CYP1A immunostaining was similar to that described for the βNF-induced fish (Fig. 5h).

In the fish that were caged in the Viskan River before exposure to 1H-BaP in the laboratory, labeled cells were rare in the interlamellar regions of the epithelium in primary lamellae, whereas an intense labeling was found in most cells in the epithelium of the secondary lamellae (Fig. 5i). The radioactivity in the chloride cells was generally low or moderate (Fig. 5i). Cytochrome P4501A immunoreactive cells were almost completely lacking in the interlamellar regions of the epithelium in the primary lamellae (Fig. 5j), and only few scattered immunoreactive cells occurred in the afferent and efferent edges (not shown). Immunoreactivity was also lacking in the proximal parts of the secondary lamellae (Fig. 5j), whereas strong immunoreactivity was observed in cells in the distal parts, i.e., in pillar cells, cells in both layers of the epithelium, and in marginal channel endothelial cells, but not in chloride cells (Fig. 5j). A similar pattern of CYP1A immunoreactivity occurred in all six fish that had been caged in the Viskan River, but were not exposed to 1H-BaP.

**DISCUSSION**

Altogether the results of the present study confirm that the gill is a major target for irreversible BaP metabolite binding following waterborne exposure in fish [20–23]. The observations in tape-section and light microscopy autoradiograms that βNF markedly enhanced and ellipticine completely blocked branchial BaP-binding suggest that the binding was due to CYP1A-catalyzed bioactivation of BaP. These findings were supported by the EROD activity measurements.

However, there were striking differences between the cellular pattern of BaP adduct formation and CYP1A expression in the gills. In βNF-induced fish, CYP1A expression was most pronounced in endothelial cells (pillar cells and cells lining the arteries and the marginal channel), and in the inner cell layers of the epithelium of the primary and secondary lamellae (differentiating cells). It is notable therefore that among the major targets for irreversible BaP binding were cells in the outer epithelial layer of the secondary lamellae, where CYP1A protein expression was less pronounced. Furthermore, a significant BaP adduct formation was found in epithelial cells in the efferent edge of the primary lamellae, showing that epithelial cells in the primary lamellae may form BaP adducts when exposed to a bulk flow of BaP-containing water. These data suggest that the distribution of BaP adducts was not solely determined by the local CYP1A protein concentration. It appears that the sites of BaP adduct formation were largely dependent on the bioavailability of BaP to the different branchial cells. The major absorption site for waterborne organic chemicals is most likely the secondary lamellae, and therefore it seems probable that the cells in the secondary lamellae obtained the highest exposure to both βNF and BaP. The extent of first-pass metabolism in these cells would consequently determine the exposure of the underlying cells as well as the systemic bioavailability of BaP and other CYP1A-metabolized pollutants in fish. The lack of BaP adducts despite a marked CYP1A immunoreactivity observed in efferent arteries in the primary lamellae of Viskan River-exposed fish complies with an absorption of CYP1A inducers into the capillaries and a first pass metabolism of BaP in the secondary lamellae. The sites of BaP adduct formation however will be determined also by the local concentrations of epoxide hydrolases, glutathione-S-transferases, and other biotransformation enzymes. It cannot be ruled out that CYP forms other than CYP1A may contribute BaP adduct formation in the target gill cells. For instance, CYP3A is strongly expressed in gill filaments from the killifish [24] and inhibited by ellipticine in rainbow trout liver microsomes [25].

Although several previous studies reveal the endothelium (mainly pillar cells) as the primary site of CYP1A expression in the gill, expression in epithelial gill cells has also been reported [7–10]. The epithelium of the primary lamellae is multilayered and accommodates stem cells and proliferating cells [26]. The epithelium of the secondary lamellae has two layers, the inner layer containing differentiating cells derived from the primary lamella, which will replace cells in the outer layer (respiratory cells and chloride cells) [26]. Husay et al. [9] injected cod (Gadus morhua L.) with βNF (i.p.) and found CYP1A expression in pillar cells, and in cells of the inner epithelial layer, i.e., in differentiating cells of the secondary lamella, but not in the outer epithelial layer. Furthermore, EROD proved to be inducible in primary cultures of epithelial gill cells [12] and in epithelial gill cells cultured on permeable supports [27].

An interesting finding in the present study was that respiratory cells (i.e., the outer epithelial layer) showed strong CYP1A expression in all fish caged in the Viskan River but not in the βNF-induced fish. A major difference between these groups was the exposure time (four weeks in the Viskan River and 2d in βNF-containing water). Within four weeks, cells originating from the inner epithelial layer of the secondary lamella will differentiate and replace cells in the surface layer [26]. It is therefore an intriguing possibility that respiratory cells require exposure to AhR agonists before they are fully differentiated to express CYP1A. This idea is supported by the finding that moderate CYP1A expression occurs in pillar cells after 4d of waterborne (and dietary) BaP exposure in the mummichog (Fundulus heteroclitus), whereas in respiratory cells a moderate CYP1A expression was recorded first at 19 d of exposure [10]. Furthermore, Dang et al. [28] studied metallothionein induction by copper in gills of tilapia (Oreochromis mossambicus) and found that chloride cells, respiratory cells, and pillar cells expressed metallothionein only if the cells had been exposed to copper at a nondifferentiated stage. However, once expression was initiated, metallothionein remained expressed throughout the life cycle of the cell [28].

A peculiar finding in all Viskan River–exposed fish was that cells in the distal part of the secondary lamellae were strongly immunoreactive, whereas in large parts of the primary lamellae and the base of the secondary lamellae, CYP1A-immunoreactive cells were rare or completely lacking (Fig. 5j). All types of cells in these parts, with the exception of endothelial cells in the arteries and arterioles, lacked CYP1A immunoreactivity. In the βNF-exposed fish, these parts of the lamellae contained strongly immunoreactive cells. This strik-
ing difference between Viskan River-exposed and βNF-exposed fish is not presently understood.

The aqueous \(^3\)H-BaP concentration used in the present study (initially 0.16 parts per billion [ppb]) was lower than the maximum contaminant level of BaP in drinking water (0.2 ppb) set by the U.S. Environmental Protection Agency (http://www.epa.gov/safewater/dhw/c-soc/benzopyr.html). This concentration appeared high enough to induce CYP1A in gills, because gill filament EROD activity was higher in the BaP-exposed than in nonexposed fish. We have recently found EROD induction in gills after 3-h exposure to 0.5 ppb (i.e., initial concentration) of \(^3\)H-BaP (unpublished observation).

We have previously reported that gills respond rapidly to AhR agonists; gill filament EROD activity was significantly induced in rainbow trout gills after 6 hours of exposure to waterborne βNF [13]. Accordingly, we proposed gill filament EROD activity as a useful tool for the monitoring of dioxin-like pollutants in aquatic environments [13]. The EROD activity in fish exposed to βNF for two days in the present study was similar to that previously reported by Jönsson et al. [13] (about 0.3 pmol/filament tip/min). The high EROD activity recorded in gill filaments from these fish reflects the strong CYP1A expression in the primary and secondary lamellae of the filament. The observation that the EROD activities recorded in the Viskan River-exposed fish were lower than in the βNF-exposed fish could be due to the lack of CYP1A expression in the primary lamellae. Nevertheless, the strong CYP1A staining in parts of the secondary lamellae and the pronounced BaP adduct formation in the secondary lamellae suggest that the water in the Viskan River contained chemicals with a high CYP1A-inducing potency (i.e., dioxin-like pollutants).

**CONCLUSION**

The results confirm that the gill filament is an important target for CYP1A catalyzed BaP adduct formation, and suggest that BaP is able to induce CYP1A expression in the gill filament at a concentration below regulatory levels set for drinking water. Furthermore, it is proposed that the cellular localization of BaP adducts was not only dependent on the cellular pattern of CYP1A enzyme expression, but also on the bioavailability of BaP to the CYP1A-immunoreactive cells, and the local concentrations of other enzymes involved in the bio-transformation of BaP. Finally, the results suggest that the capability of respiratory cells to express CYP1A activity depends on their exposure to AhR agonists before they reach full differentiation.

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