0090-9556/00/2806-0701–708$03.00/0
DRUG METABOLISM AND DISPOSITION
Vol. 28, No. 6
Printed in U.S.A.

DMD 28:701–708, 2000 /1846/828099

DOSE AND INDUCER-DEPENDENT INDUCTION OF CYTOCHROME P450 1A IN ENDOTHELIA OF THE EEL, INCLUDING IN THE SWIMBLADDER RETE MIRABILE, A MODEL MICROVASCULAR STRUCTURE

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(Received November 22, 1999; accepted March 8, 2000)

This paper is available online at http://www.dmd.org

ABSTRACT:

Endothelium is a common site of cytochrome P450 1A (CYP1A) induction in vertebrates, and endothelial CYP1A could affect the distribution and toxicity of CYP1A substrates. We investigated CYP1A induction in organs rich in endothelium, gill, heart, and a microvascular model, the swimbladder rete mirabile, in the eel. Benzo[*a*]pyrene (BP) and 3,3′,4,4′-tetrachlorobiphenyl (TCB), radiolabeled and injected intraperitoneally, showed similar distribution in eels, with dose-dependent increases in concentration in heart and rete mirabile. BP [given at 0.1, 1, and 10 mg/kg (0.4, 4, and 40 μmol/kg)], TCB [given at 0.1, 1, and 10 mg/kg (0.3, 3, 30, and 60 μmol/kg)], and β-naphthoflavone (BNF) [given at 0.1, 1, 5, 10, and 100 mg/kg (0.4, 4, 20, 40, and 400 μmol/kg)] induced microsomal CYP1A and ethoxyresorufin *O*-deethylase in heart and rete mirabile. Immunohistochemical analysis confirmed that induction of CYP1A in heart and rete mirabile occurs in the endothelium. Increasing doses of each compound caused increasing penetration of induction into the vascular bed of the rete, but with BNF and BP induction penetrated further than with TCB. At high doses of BNF there also was induction in epithelial cells adjacent to endothelium in gill and kidney. CYP1A also was induced in heart and rete mirabile of eels from sites heavily contaminated by aryl hydrocarbon receptor (AHR) agonists. The penetration of CYP1A induction into capillaries of the rete mirabile reflects the penetration of the inducer itself, consistent with the idea that endothelial CYP1A can indicate the local distribution of AHR agonists. The microvascular rete mirabile in the eel provides a model system to explore further a hypothesis that endothelial CYP1A participates in removal of some AHR agonists from the circulation and to examine the consequences of CYP1A induction to the vascular system.

Endothelium, the single cell layer lining blood and lymphatic vessels, provides a physical lining, maintains blood vessel tone, and controls microvascular permeability, platelet clumping, clotting, and lymphocyte traffic (Pearson, 1991). Endothelium initiates angiogenesis essential to vessel growth supporting the development and growth of organisms and the repair of damaged blood vessels (Alberts et al., 1989). Protective functions of endothelium are prominent in the blood-tissue barriers in brain, testis, and retina. Endothelium also may be a site of xenobiotic toxicity. Activation of promutagens in the vasculature is thought to play a role in atherosclerosis (Juchau et al., 1976), and vascular defects and edema are common in developing fish, birds, and mammals exposed to planar halogenated aromatic hydrocarbons (pHAH)[1] (Peterson et al., 1993).

This research was supported in part by National Institutes of Health Grant P42-ES07381, by Environmental Protection Agency Grant R823889, by Air Force Office of Scientific Research Grant F40620-94-1039, and by the Lyons Fellowship.

[1] Abbreviations used are: pHAH, planar halogenated aromatic hydrocarbon; AHR, aryl hydrocarbon receptor; BP, benzo[*a*]pyrene; BNF, β-naphthoflavone; CYP, cytochrome P450; CYP1A, cytochrome P450 1A; CPR, cytochrome P450 reductase; PAH, polynuclear aromatic hydrocarbon; TCB, 3,3′,4,4′-tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; PCB, polychlorinated biphenyl; mAb, monoclonal antibody; EROD, ethoxyresorufin *O*-deethylase.

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Polynuclear aromatic hydrocarbons (PAH) and pHAH are toxic compounds that induce the expression of several genes, including cytochrome P4501A (CYP1A), via the aryl hydrocarbon receptor (AHR) (Hankinson, 1995). CYP1A can be induced in various cell types in many organs (Goldstein and Linko, 1984; Smolowitz et al., 1991), but endothelium is a site of induction common among organs. In mammals, induction of CYP1A1 has been detected in endothelium of aorta, umbilical vein, heart, kidney, and lung (Dees et al., 1982; Brittebo, 1994; Farin et al., 1994; Thirman et al., 1994; Stegeman et al., 1995). In fish, induction of CYP1A has been seen in endothelium in liver, heart, gill, kidney, gut, gonad, spleen, red muscle, and brain (Miller et al., 1989; Stegeman et al., 1989; Smolowitz et al., 1991; Husoy et al., 1994). Induction in endothelium can be substantial; CYP1A was estimated to account for as much as 25% of the endothelial microsomal protein in the heart of β-naphthoflavone (BNF)-treated fish (Stegeman et al., 1989).

The toxicity of AHR agonists is generally AHR-dependent (Poland and Glover, 1980) but may be mediated in part by CYP1A. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity in fish embryos correlates with CYP1A expression in endothelium, and CYP inhibitors can diminish the effect (Cantrell et al., 1996; Guiney et al., 1997). The toxicological importance of endothelial CYP1A will depend in part on the catalytic capacity and the degree of induction in endothelium. CYP1A is metabolically active in some endothelia. Bovine aorta endothelial cells and microsomes from scup heart, where CYP1A is predominantly endothelial, produce metabolites of benzo[*a*]pyrene

(BP) typical of those formed by CYP1A (Baird et al., 1980; Stegeman et al., 1982). CYP1A and binding of reactive metabolites of heterocyclic amines colocalize in endothelium of mammals and birds (Annas and Brittebo, 1998; Annas et al., 1998), and CYP1A is catalytically active in intact porcine aorta endothelial cells (Stegeman et al., 1995). CYP1A is less active in endothelium than in the liver (e.g., Stegeman et al., 1982), possibly due to a low availability of cytochrome P450 reductase (CPR). CPR mRNA was not detected in rabbit lung endothelium (Overby et al., 1992), and is expressed at low levels in porcine aorta endothelial cells (Stegeman et al., 1995).

In vivo, the patterns of induction of CYP1A in endothelium have been shown to differ with different inducers. In scup, the potent AHR agonist 2,3,7,8-tetrachlorodibenzofuran (TCDF) at 3 μg/kg (0.01 μmol/kg) and the less potent agonist 3,3′,4,4′-tetrachlorobiphenyl (TCB) at 1 mg/kg (3 μmol/kg) (both given intraperitoneally) caused similar induction of CYP1A in liver and in sites proximal to large vessels. With TCB, CYP1A induction was seen also in peripheral regions of the organism, but this did not occur with TCDF (Smolowitz et al., 1991). This suggests that CYP1A induction in endothelium of the peripheral vasculature is related to the penetration of inducer into the periphery.

In most organs, endothelium is a minor component, confounding the study of endothelial cell properties in whole organ preparations. We investigated the induction of endothelial CYP1A in extrahepatic organs but particularly in heart and in the capillary network in the rete mirabile of the swimbladder of the eel (*Anguilla rostrata*). Endothelium represents as much as 8 to 9% of the mass of the teleost heart (Stegeman et al., 1989), whereas the rete mirabile is composed predominantly of endothelial cells (Krogh, 1959). CYP1A was induced in these organs and was metabolically active in microvascular endothelium. Penetration of endothelial CYP1A induction into the capillary bed of the rete mirabile increased with increasing dose of AHR agonists. Our results with this microvascular model suggest that endothelial CYP1A can indicate local levels of inducer and possibly can affect the distribution of AHR agonists.

Materials and Methods

Chemicals. Unlabeled BP and BNF were purchased from Aldrich (Milwaukee, WI). [^{3}H]BP (55 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Unlabeled TCB (congener 77) and [^{14}C]TCB (*ring*-labeled; 52.1 mCi/mmol) were purchased from Pathfinder (St. Louis, MO). Soluble tissue dissolver was obtained from NEN Life Science Products (Boston, MA). 7-Ethoxyresorufin was obtained from Molecular Probes (Eugene, OR). MS 222 was the gift of Adria Elskus but can be purchased from Sigma (St. Louis, MO). All other reagents were obtained from Sigma.

Environmental Collections. Female American eels, *A. rostrata*, were collected from sites near Woods Hole, MA, using traps baited with horseshoe crab meat. Eels were collected from New Bedford Harbor, MA, at the Aerovox site, from Marion Harbor, MA, and from Vineyard Sound, MA. Eels in New Bedford Harbor have been shown to contain concentrations of total polychlorinated biphenyls (PCBs) as high as 700 μg/g whole body dry weight (i.e., approximately 70 mg/kg wet weight) (Lake et al., 1995). Marion Harbor is an enclosed harbor with substantial boat traffic. Vineyard Sound is an open body of water distant from New Bedford Harbor. Fish in the locations away from New Bedford have much lower levels of PCB residues (Elskus et al., 1994). Eels were transported to the laboratory and immediately sacrificed and dissected as described below.

Experimental Exposures. One hundred and twelve female eels collected in Vineyard Sound were purchased from the Marine Biological Laboratory, Woods Hole, MA. Eels were provided a diet of squid once a week. For the first experiment the eels were kept at 20°C. For all other experiments, the eels were kept for at least 3 days at 10°C in flow-through tanks until treated. A previous study in *Fundulus heteroclitus* showed that, although CYP1A induction occurs more slowly at lower temperatures, the protein turnover is reduced and thus induced protein levels persist for longer periods (Kloepper-Sams and Stegeman, 1992). We reasoned that the lower temperature also would increase retention of CYP1A induction in the eel.

Chemical Distribution Analysis. For analysis of chemical distribution, eels were anesthetized in a solution of MS 222 (6 g/4 liters of sea water) and injected intraperitoneally with corn oil or varying concentrations of [^{3}H]BP (1 and 10 mg/kg) and [^{14}C]TCB (1 and 10 mg/kg) in corn oil ($n = 6$). Eight days following injection, each eel was anesthetized in a solution of MS 222 and sacrificed by cervical transection. Portions of freshly dissected organs (0.1–0.25 g each of liver, heart, gill, spleen, gut, kidney, and muscle or 0.03–0.13 g of rete mirabile) were removed, rinsed with water, blotted, and weighed. Bile was collected via syringe, and blood was collected from the caudal vein via syringe. Tissues and bile were placed in 20-ml glass scintillation vials.

Organs placed in scintillation vials were minced with scissors, Soluble (1 ml) was added to each vial, and the vials were heated in a 50°C oven until tissue was dissolved (12–48 h). A mixture of ethanol/Soluble (1:2) was added to the blood, and the vials were warmed to 50°C for 1 h. H_2O_2 (30%, 0.75 ml) was added to all vials except those containing gut and muscle. Samples were acidified with 200 μl of 6 N HCl. Scintiverse (15 ml) was added to the vials, and the samples were held overnight before counting on a Beckman LS5000TD scintillation counter. Counts in organs from control eels were subtracted from counts in organs from treated eels, and the difference was expressed as micrograms of BP and TCB equivalents.

Dose-Response Experiments. Eels were anesthetized in a solution of MS 222 and given intraperitoneal injections with corn oil or varying amounts of BNF, BP, or TCB in corn oil. BNF was given at 0.1, 1, 5, 10, and 100 mg/kg (0.4, 4, 20, 40, and 400 μmol/kg), BP at 0.1, 1, and 10 mg/kg (0.4, 4, and 40 μmol/kg), and TCB at 0.1, 1, and 10 mg/kg (0.3, 3, 30, and 60 μmol/kg). There were three to five fish in each dose group. On day 5 (experiment 1) or day 8 (experiments 2–7) eels were anesthetized in aqueous MS 222 and sacrificed by cervical transection. Portions of heart, gill, kidney, and rete mirabile were preserved in 10% neutral buffered formalin. Heart and rete mirabile collected for microsome preparation were frozen in liquid N_2 until use.

Tissue Preparation and Enzyme Assays. Tissues were homogenized in cold buffer (0.15 M KCl, 0.05 M Tris, pH 7.2), and postmitochondrial supernatant (S9 or PMS) and microsomal fractions were prepared as before (Stegeman et al., 1979). Protein content was determined using the bicinchoninic acid method and BSA as a standard. Initial rates of ethoxyresorufin *O*-deethylase (EROD) activity were measured fluorometrically by the method of Hahn et al. (1993) using a Cytofluor 2300 (Millipore) multiwell plate reader. NADPH-CPR activity was determined as before (Stegeman et al., 1982), with a Shimadzu 210 spectrophotometer.

Western Blotting. Microsomal proteins (80 μg/lane) were resolved using 12% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to a nylon membrane. The primary antibody was the mouse monoclonal antibody (mAb) 1-12-3 (Park et al., 1986) raised against scup P450E, a CYP1A (Morrison et al., 1995). mAb 1-12-3 is an IgG that is highly specific for CYP1As in fish and CYP1A1 in mammals. The secondary antibody was alkaline phosphatase-linked goat anti-mouse IgG (Bio-Rad, Hercules, CA). The immunoreactive proteins were visualized using enhanced chemiluminescence (Tropix, Bedford, MA). CYP1A content was quantified by densitometric analysis of video images (NIH Image, 1.60b5); values are reported as scup CYP1A equivalents.

Immunohistochemistry. Fixed heart, kidney, gill, and rete mirabile were embedded in paraffin by Pathology Services, Inc. (Cambridge, MA) or by the Laboratory of Aquatic Animal Medicine and Pathology at the MBL (Woods Hole). The staining procedure used was as described by Smolowitz et al. (1991). Tissue sections (5 μm) were mounted on Superfrost Plus slides, and the sections were deparaffinated and hydrated in 1% PBS/BSA, with incubation in 3% H_2O_2 in water to block endogenous peroxidases. Sections were stained using an indirect peroxidase stain [Universal Immunoperoxidase Staining Kit (Murine), Signet Lab, Dedham, MA)] The primary antibody was mAb 1-12-3, and the secondary antibody was a peroxidase-labeled goat antibody to mouse IgG. Staining of CYP1A was scored for intensity of staining (0 = no stain, 1 = mild stain, 2 = moderate stain, 3 = strong stain, 4 = very strong stain) and for occurrence of staining (0 = no cells, 1 = rare cells, 2 = some cells, 3 = many cells, 4 = all cells staining). A stain index was computed by multiplying the intensity and occurrence scores for a scale from 0 through 16.

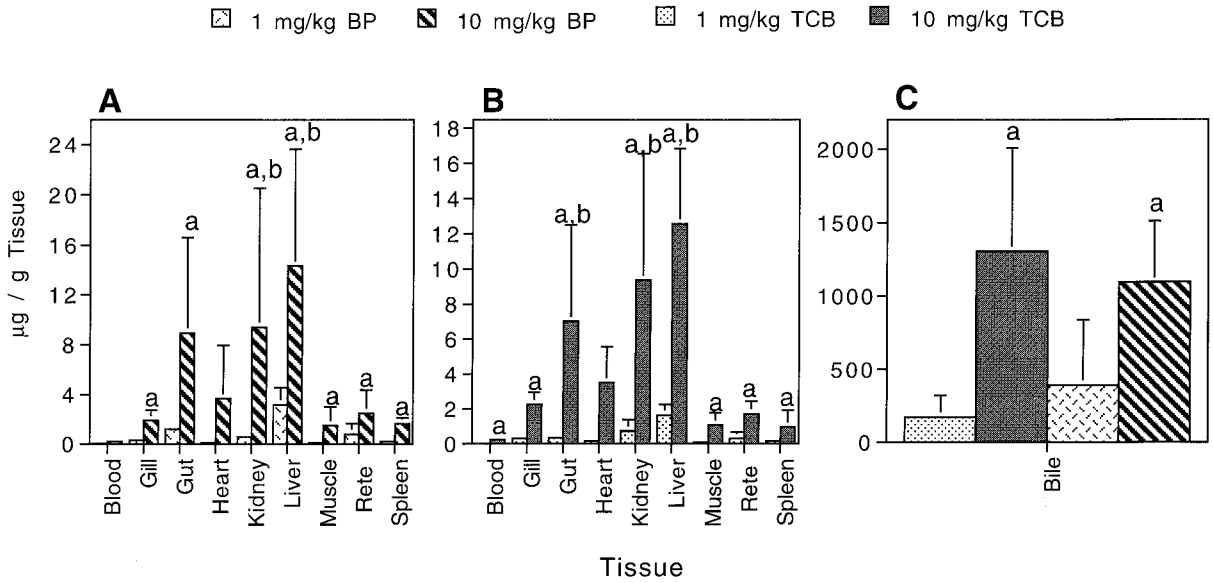

FIG. 1. *Tissue distribution of [^{3}H]BP and [^{14}C]TCB in eels.*

[^{3}H]BP or [^{14}C]TCB were injected intraperitoneally at the doses indicated. A, organs from BP-treated fish; B, organs from TCB-treated fish; C, bile from BP- and TCB-treated fish. Fish were sacrificed 8 days postinjection, and portions of freshly dissected organs were weighed, rinsed with water, blotted, digested with Soluble, minced with scissors, and placed in a 50°C oven until dissolved. Bile was collected via syringe. Blood was collected from the caudal vein via syringe and added to ethanol/Soluble (1:2). H_2O_2 was added to all vials except those containing gut and muscle. All samples containing Soluble were acidified with HCl. Scintiverse was added to the vials, and the samples were allowed to quench overnight before counting. Data are means ± S.D. of three to six individuals. For A and B: a, significantly greater than 1 mg/kg dose ($P < .05$, Student's *t* test); b, significantly greater than blood ($P < .05$, Dunnett's). Concentrations in bile were significantly greater than those in blood.

This index has been shown to correlate positively with CYP1A content measured by Western blot.

Statistics. Data were analyzed using SuperAnova for Macintosh (Abacus Concepts, Inc., Berkeley, CA). A one-factor ANOVA combined with comparisons tests or a one-tailed *t* test were used to analyze data from the environmental collections as indicated in Figures and Tables.

Results

Chemical Distribution. Initially, we examined the distribution of [^{3}H]BP and [^{14}C]TCB following intraperitoneal injection into eels (Fig. 1). Nine organs, bile, and blood were sampled 8 days after a single injection of 1 or 10 mg of BP or TCB/kg. Concentrations of BP and TCB in most of the organs corresponded to the dose given, with approximately 10-fold higher concentrations in organs of eels receiving the greater dose. The patterns of tissue distribution were similar for BP and TCB at each dose. Bile contained the highest concentration of ^{3}H or ^{14}C, whereas of the organs examined, liver, gut, and kidney contained the greatest concentrations of either BP or TCB label. At the 1 mg/kg dose, concentrations of BP were 2- to 4-fold greater than those of TCB in several organs and in bile, whereas at the high dose, tissue concentrations of the two chemicals were more similar. At both doses, BP and TCB concentrations in gill, heart, and rete mirabile were about 10-fold greater than the concentrations measured in blood, although variability prevented the differences from being significant.

Microsomal CYP1A Induction in Endothelium-Rich Organs. Microsomal preparations of heart and rete mirabile from animals treated with the different inducers were analyzed for CYP1A content and EROD activity and compared with results in liver (Table 1). In both heart and rete mirabile, CYP1A protein content and EROD activity showed dose-dependent increases in eels treated with BP (Fig. 2, Table 1). Comparing the content of CYP1A in tissues from animals given different doses of BP showed that the degree of induction in heart and rete mirabile was as great or greater than that in liver of these fish (Table 1). Comparing responses in animals given BP, BNF, or TCB at the same dose of 10 mg/kg (30–40 µmol/kg) showed that the extent of CYP1A induction in heart was less in fish given BNF than in those given BP, whereas TCB produced even less induction of CYP1A and EROD (Table 1). Likewise, induction of microsomal CYP1A and EROD activity in rete mirabile was less in animals given BNF, and particularly in those given TCB, than in the eels given BP. The lesser induction by TCB also was evident in liver (Table 1).

Microsomal EROD rates expressed per unit of CYP1A, an estimate of turnover number, were calculated for the rete mirabile, heart, and liver and averaged for animals treated with 10 mg/kg of BP, BNF, or TCB. The average of rates calculated were similar in heart and liver (7 and 8 nmol/min/nmol CYP1A, respectively), whereas in rete mirabile the average for these samples was higher, 16 nmol/min/nmol CYP1A. The comparatively high rates of EROD per unit of CYP1A in rete mirabile imply that CPR activity may not be limiting. CPR activities estimated in six pooled samples of rete mirabele microsomes averaged 106 nmol/min/mg of microsomal protein.

Immunohistochemistry. Immunohistochemical analyses were performed to evaluate the dose and inducer dependence of CYP1A induction in endothelium of heart and rete mirabile, and selected other organs. An initial examination of H&E-stained sections showed no cellular necrosis in the organs from fish given any dose of inducer.

Figure 3 shows the CYP1A staining index calculated for cells in heart, gill, kidney, and liver. Control eels (corn oil) had, at most, mild staining of CYP1A in endothelium of the heart and the kidney, in pillar cells of the gill (gill endothelium), and in hepatocytes (Fig. 3). No staining was observed in epithelial layers of gill of control eels.

BNF, BP, and TCB all induced CYP1A staining in endothelium of heart, gill, and kidney, but the dose-response pattern of this induction varied with the inducer (Fig. 3). In BNF-treated eels CYP1A immunostaining peaked at intermediate doses, at 1 mg/kg BNF in gill pillar cells and at 5 mg/kg BNF in heart and kidney endothelial cells. In contrast, in eels given BP or TCB, the staining either did not decline or continued to increase with dose. Thus, doses of 10 mg/kg or greater of BP or TCB resulted in strong induction in heart, kidney, and gill

TABLE 1

Quantification of CYP1A content and activity in eel heart and rete mirabile following treatment with model AHR agonists

Eel heart and rete mirabile were characterized for their CYP1A content by Western blot analysis and catalytic activity (EROD) as described under *Materials and Methods.*

Treatment	Liver[a]		Heart[b]		Rete Mirabile[c]	
	CYP1A	EROD	CYP1A	EROD	CYP1A	EROD
	pmol/mg	*pmol/min/mg*	*pmol/mg*	*pmol/min/mg*	*pmol/mg*	*pmol/min/mg*[d]
Control	2 ± 4	96 ± 55	ND	2 ± 2	ND	0.7
BP (0.1 mg/kg)	11 ± 5	167 ± 29	0.06	4 ± 2	ND	1.4
BP (1 mg/kg)	58 ± 12*	422 ± 234*	0.19 ± 0.10	4 ± 2	0.03	3.5
BP (10 mg/kg)	157 ± 37*	1312 ± 600*	3.32 ± 3.45	13 ± 12	0.70	6.3
BNF (10 mg/kg)	122 ± 34*	1115 ± 901*	0.97 ± 0.97	9 ± 6	0.37	6.3
TCB (10 mg/kg)	66 ± 27*	502 ± 191*	0.43 ± 0.28	3 ± 3	0.12	2.8

[a] Data are from Schlezinger and Stegeman (2000) and are shown as picomole values for ease of comparison. * Significantly different from control at $P \leq .05$ (Tukey-Kramer).
[b] Data were combined from two experiments. Values for CYP1A are means ± S.D. from two to three pools of two to three individual organs. Values for EROD are means ± S.D. from three to eight pools of two to three individual organs.
[c] For rete mirabile, values are from pools of three to five individual organs.
[d] EROD activity was measured in postmitochondrial supernatant; microsomal protein from all samples was calculated to represent 14% of the postmitochondrial supernatant. Therefore, the EROD activity in the microsomal fraction was estimated. Microsomal yield per gram of tissue was 8.0 ± 2.8 for heart ($n = 26$) and 3.0 ± 1.7 for rete ($n = 26$).

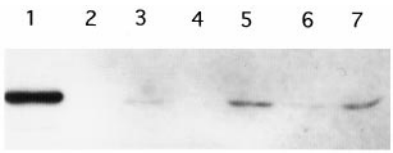

FIG. 2. *Immunoblot of CYP1A in rete mirabile microsomes from eels treated with BNF, BP, and TCB.*

Eels were sacrificed 8 days postinjection, and rete mirabile microsomal fractions were electrophoresed, transferred to nylon, immunoblotted with mAb 1-12-3 against scup CYP1A, and visualized using enhanced chemiluminescence. The treatment with BNF, BP, or TCB and the doses represented in the various lanes are: Lane 1, scup CYP1A standard; lane 2, control; lane 3, 1 mg/kg BP; lane 4, 0.01 mg/kg BP; lane 5, 10 mg/kg BP; lane 6, 10 mg/kg TCB; lane 7, 10 mg/kg BNF.

endothelia, compared with that in controls or animals given the lowest dose.

An inducer-dependent induction of CYP1A also was observed in epithelial cells of gill and kidney. In BP- and in TCB-treated eels, gill epithelial staining remained mild, even at the highest doses used. However, with BNF there was a marked transition from induction primarily in the endothelium to induction also in underlying or adjacent epithelial cells in the gill (Fig. 3), and in the kidney (data not shown). The gill epithelial staining continued to increase with increasing doses of BNF.

CYP1A Immunostaining in Rete Mirabile. Sections illustrating CYP1A staining by mAb 1-12-3 in rete mirabile are shown in Fig. 4. The rete mirabile structure has prerete arteries leading to afferent collecting vessels (heart pole) that diverge into the capillary bed of the rete. These capillaries anastomose at the site of gas secretion into the swimbladder (bladder pole) and then diverge into efferent capillaries that return blood to the body. Rete mirabile from control fish had variable staining (mild to strong) in prerete arteries only (Table 2). All treated fish showed strong to very strong staining in these prerete arteries. However, the extent to which CYP1A staining penetrated further into the body of the rete mirabile differed with inducer and dose.

With each inducer, as the dose increased, the proportion of individuals with staining of retial CYP1A increased, evident most clearly in the vessels of the heart pole (Table 2). BNF was most effective at inducing CYP1A in the heart pole endothelium (Table 2). Doses of BNF greater than 1 mg/kg resulted in penetration of CYP1A staining into the midrete region, and doses greater than 5 mg/kg resulted in CYP1A staining that extended through the entire length of afferent vessels in the rete, into the vessels of the bladder pole. As with BNF, doses of BP greater than 0.1 mg/kg induced CYP1A staining in heart-pole endothelium and the intensity of staining increased with dose. At 10 mg/kg BP, CYP1A induction extended into the midregion of the rete (Table 2). In TCB-treated eels the CYP1A stain index in endothelium of vessels in the heart-pole region of the rete mirabile showed dose-dependent induction at doses greater than 1 mg/kg TCB. However, the stain index and stain penetration into the rete of eels treated with TCB were less than in animals treated with comparable doses of BNF or BP. Even at the highest dose of TCB, CYP1A staining did not extend into the midrete region. CYP1A staining was not seen in efferent vessels of the rete mirabile of animals treated with any inducer.

Endothelial CYP1A Induction in Environmentally Exposed Eels. The above results indicate that the magnitude and penetration of CYP1A induction in vessels of the heart and rete mirabile are associated with the magnitude of exposure to AHR agonists. To determine whether induced levels of CYP1A occur in endothelium of eels exposed to CYP1A inducers in the environment, we examined CYP1A expression in heart and rete mirabile of eels from New Bedford Harbor and from two less contaminated sites, Marion Harbor and Vineyard Sound. Microsomal CYP1A content and activity were elevated significantly in heart of eels from New Bedford Harbor (Table 3). The relative levels of cardiac microsomal EROD matched the relative levels of CYP1A content in liver microsomes of these same animals (Schlezinger and Stegeman, 2000). Immunohistochemical analysis also was performed with heart, and the CYP1A stain index in cardiac endothelium corresponded to the relative levels of cardiac microsomal CYP1A content in eels from the three sites, with a strong increase in eels from New Bedford Harbor. Likewise, microsomal preparations of rete from New Bedford Harbor eels had elevated EROD rates and CYP1A content, compared with the samples from Marion Harbor or Vineyard Sound.

Discussion

Most vertebrate organs have multiple cell types in which CYP1A can be induced, however, endothelium is a common site of strong induction of CYP1A in virtually all organs of vertebrates. Yet, endothelial cells usually represent a very small proportion of organ mass, which greatly confounds the study of endothelial CYP1A. Here we investigated the dose and inducer dependence of endothelial CYP1A induction in several organs of eel, focusing on heart and the rete mirabile in the swimbladder, a countercurrent microvascular structure that increases the partial pressure of oxygen to aid its secretion into the bladder (Krogh, 1959). Fish heart has been used as a model for

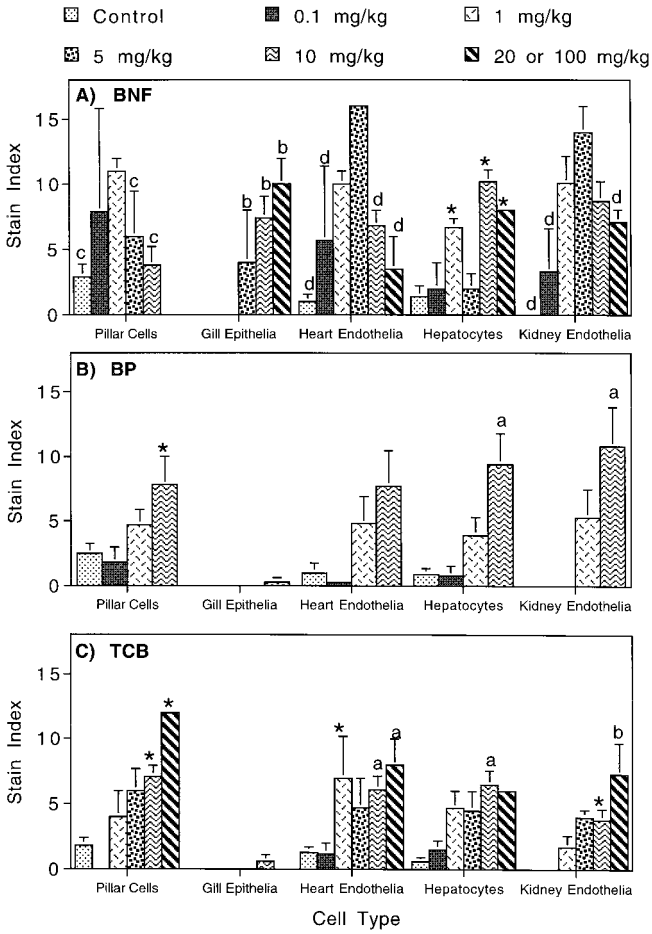

FIG. 3. *Immunohistochemical analysis of CYP1A protein in endothelium of organs from eels treated with BNF, BP, and TCB.*

A, BNF-treated eels (*N* ranged from 3 to 10). B, BP-treated eels (*N* ranged from 4 to 8). C, TCB-treated eels (*N* ranged from 3 to 15). Eels were injected intraperitoneally with BNF, BP, or TCB at the doses indicated on the figure and sacrificed 8 days postinjection. Tissues were dissected, embedded, sectioned, and stained immunohistochemically for CYP1A as described by Smolowitz et al. (1991). Staining of CYP1A was scored for intensity of staining (0 to 4) and for occurrence of staining (0 to 4), and a stain index was computed by multiplying the intensity and occurrence scores for a scale from 0 through 16. Data from experiment 1 were not significantly different from the other experiments; therefore, all data were combined. *Significantly different from control. [a]Significantly different from control and 0.1 mg/kg. [b]Significantly different from control, 0.1 and 1 mg/kg. [c]Significantly different from 1 mg/kg. [d]Significantly different from 5 mg/kg. Significance was determined as $P < .05$ (Tukey-Kramer).

CYP1A induction in endothelial cells, because in some fish endothelium represents as much as 8 to 9% of the mass of the heart (Stegeman et al., 1989). That proportion is less than in the rete mirabile, which is composed predominantly of capillary endothelium (Krogh, 1959), and which, in the eel, is relatively large and accessible. Thus, we deduced that the rete mirabile may be a unique site in which to study regulation and function of CYP1A in endothelium.

BP elicited a dose-dependent induction of microsomal CYP1A protein and EROD activity in rete mirabile and in heart, and BP, BNF, and TCB all elicited dose-dependent induction of CYP1A immunostaining in rete mirabile and heart endothelium. Eels from New Bedford Harbor, an environment highly contaminated by AHR agonists, also had induced CYP1A content and/or activity in the rete mirabile and the heart. In both experimentally and environmentally induced eels, the patterns of response observed in rete mirabile paralleled those observed in heart and liver, indicating that a similar AHR signal transduction pathway is operating in the microvascular endothelium of the rete.

Immunohistochemical staining of CYP1A induced in some endothelial cells of the heart or rete mirabile was as strong or stronger than that in hepatocytes (Fig. 3), indicating that CYP1A is as highly induced in those endothelial cells as in hepatocytes. Despite that, the content of CYP1A detected in rete or heart microsomes was much less than that in liver microsomes. In liver, the majority of cells showed strong induction, whereas in heart or rete mirabile, a smaller percentage of the cells showed strong staining.

Given the cellular localization of CYP1A, the induction of EROD activity indicates that the induced CYP1A is catalytically active in microvascular endothelium of the rete, and in endothelium of the heart. Other studies have shown that CYP1A is catalytically active in endothelial cells in culture (Stegeman et al., 1995). As with CYP1A content, EROD rates in heart and rete mirabile microsomes from induced eels were substantially less than in microsomes from liver, even of control eels (Table 1). However, the heart and rete mirabile had estimated turnover numbers for EROD (EROD per unit of CYP1A equivalent) that were similar to or greater than the value for liver. Other studies in fish heart and in mammalian endothelial cells in culture have indicated that CYP1A has a lower catalytic capacity in the endothelium than in the liver, suggested to result from a content of CPR in endothelial cells that is not sufficient to support the full catalytic capacity of induced levels of CYP1A in endothelium (Stegeman et al., 1982, 1995; Overby et al., 1992). The observation that EROD turnover in heart and rete mirabile of eel is more like that in liver suggests that the eel may differ from other species in this regard. Consistent with that, the rates of CPR in rete microsomes were as high as the CPR rates often seen in liver of fish, a novel finding for an endothelium-rich organ.

It is possible that different CYP1As are expressed in endothelium and liver of eel. Single CYP1As have been identified in most fish examined (Morrison et al., 1998). Recently, however, Itakura and colleagues identified two CYP1A genes that are expressed in a congeneric eel species, *A. japonica* (Ogino et al., 1999); there could be a divergence of CYP1As also in *A. rostrata*. mAb 1-12-3, which we used in immunoassays, recognizes an epitope conserved among all fish CYP1As sequenced thus far, and that epitope is likely to occur in multiple CYP1As in eels. Thus, whether eel endothelium expresses a distinct isoform remains to be determined.

As with induction of hepatic CYP1A in these same eels (Schlezinger and Stegeman, 2000), BP, BNF, and TCB differed in their ability to induce microsomal CYP1A protein and EROD and CYP1A staining. Induction by BNF peaked at intermediate doses, and at comparable doses TCB elicited a lesser response than BNF or BP. The dose-dependent penetration of CYP1A staining into the rete also was much less with TCB. The slighter response to TCB in rete and heart suggests that this pHAH is not as efficacious as PAH in endothelium. The basis for this difference is not understood. In the rete mirabile, the difference is not likely due to a lower tissue dose of TCB, because at 10 mg/kg the concentrations of BP and TCB in rete differed little. Thus, it appears that some inducer-specific factors are involved in the degree to which CYP1A is induced along a vessel. TCB but not BP has been shown to suppress CYP1A via oxidative inactivation following uncoupling (J. J. Schlezinger, W. D. J. Struntz, and J. J. Stegeman, submitted). TCB does inactivate CYP1A in eel liver microsomes (Schlezinger et al., 2000), but whether a similar process occurs in endothelium is not known.

Several of our results are consistent with the hypothesis that endothelial CYP1A can indicate and possibly influence the pharmacoki-

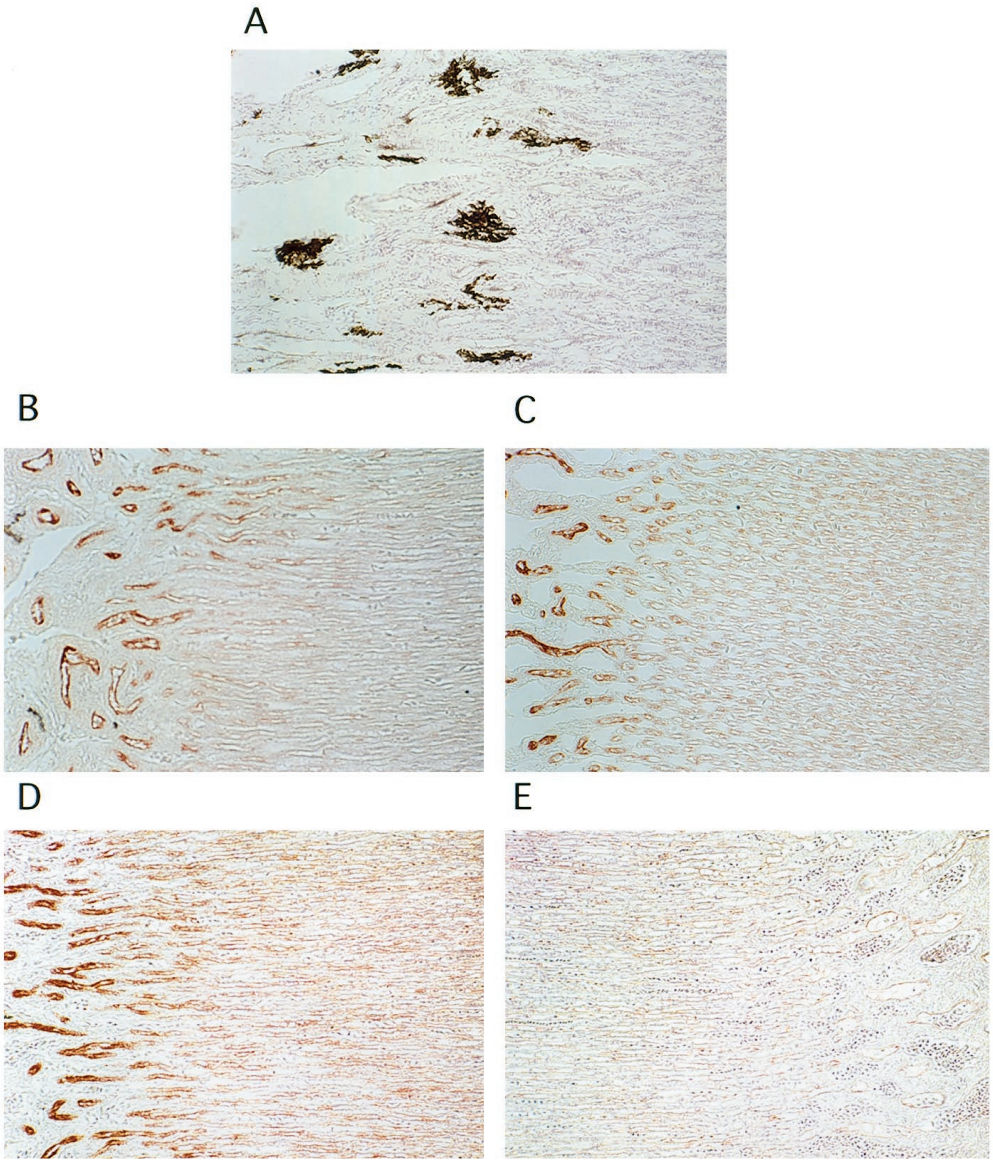

FIG. 4. *CYP1A staining in rete mirabile of eels treated with BNF.*

Eels were injected intraperitoneally with BNF and sacrificed 8 days postinjection. Dose (and sections shown) are: A, control (heart pole); B, 0.1 mg/kg (heart pole); C, 1 mg/kg (heart pole); D, 5 mg/kg (heart pole); E, 5 mg/kg (bladder pole). The immunostaining procedure used is essentially as described by Smolowitz et al. (1991).

netics of AHR agonists. The distribution of BP and TCB showed that rete mirabile, an organ composed predominantly of endothelial cells, can retain AHR agonists. The accumulation of AHR agonists from the blood and the correspondence between greater penetration of CYP1A induction and greater dose indicate that endothelial cells remove inducers from the circulation. Similar to the dose-dependent penetration seen in the rete mirabile, in a study of human placenta, endothelial cells from placenta of smokers had elevated aryl hydrocarbon hydroxylase activity, whereas endothelial cells from the umbilical vein did not (Manchester et al., 1984). The maternal portion of placenta from smokers also has significantly higher levels of CYP1A than the fetal portion (B. P. Slezak, J. Fisher, M. Alashari, J. Schlezinger, J. J. Stegeman, and J. R. Olson, in preparation). In gill, there was a dose-dependent transition of CYP1A immunostaining from staining only in endothelial cells to staining as well in epithelial cells adjacent to the endothelium, suggesting that at the higher doses inducer had crossed the endothelium.

Together, the results above provide support for the suggestion that endothelial CYP1A participates in reducing the penetration of AHR agonists to underlying tissues and into distal regions. Thus, the absence of CYP1A staining in efferent vessels of the rete mirabile of animals treated with any inducer could result if the inducers did not penetrate into those efferent structures. Yet, the possibility that there may be some difference in the responsiveness of afferent and efferent capillary endothelium cannot be excluded. Determining the extent of uptake into endothelial cells, and the clearance of CYP1A inducers in a pass through the vascular bed, will be important in resolving this issue.

Substrate binding by CYP1A could contribute to the role of endothelium as a protective cell layer in blood-tissue barriers and perhaps throughout the body. Contribution to that barrier function could occur whether or not CYP1A induced in endothelium is catalytically efficient with a particular substrate. CYP1A2, which binds but does not

TABLE 2

Immunohistochemical analysis of CYP1A in rete mirabile of eels treated with BNF, BP, or TCB

Rete mirabile from eels treated with BNF, BP, and TCB were dissected, embedded, sectioned, and immunohistochemically analyzed for CYP1A as described under *Materials and Methods.* Staining of CYP1A was scored for intensity of staining (0 = no stain, 1 = mild stain, 2 = moderate stain, 3 = strong stain, 4 = very strong stain) and for occurrence of staining (0 = no cells, 1 = rare cells, 2 = some cells, 3 = many cells, 4 = all cells staining). The stain index is computed by multiplying the intensity and occurrence scores for a scale of from 0 through 16. The average staining index is calculated only for those individuals showing staining in the indicated structures.

Chemical	Dose	Prerete Endothelium		Heart Pole Endothelium		Midrete Endothelium	Bladder Pole Endothelium
		Stain Index	+/total[a]	Intensity	+/total	+/total	+/total
	mg/kg						
BNF	0	10 ± 4	3/3	0	0/3	0/3	0/3
	0.1	13 ± 2	3/3	1 ± 1	1/3	0/3	0/3
	1	16 ± 0	3/3	3 ± 1	2/3	1/3	0/3
	5	16 ± 0	3/3	4 ± 1	3/3	3/3	2/3
	10	16 ± 0	9/9	3 ± 0	9/9	1/9	1/9
	100	16 ± 0	3/3	3 ± 0	3/3	3/3	2/3
BP	0	10 ± 6	7/12	0 ± 0	4/12	0/12	0/12
	0.1	10 ± 2	4/4	1 ± 1	3/4	0/4	0/4
	1	15 ± 2	6/7	1 ± 1	6/7	0/7	0/7
	10	16 ± 0	6/6	2 ± 1	6/6	2/6	1/6
TCB	0	10 ± 4	5/5	0 ± 0	0/5	0/5	0/5
	0.1	6 ± 3	3/3	0	0/3	0/3	0/3
	1	14 ± 3	3/3	2 ± 0	3/3	0/3	0/3
	10	15 ± 3	12/12	1 ± 1	12/12	0/12	0/12
	20	11 ± 7	3/3	2 ± 0	3/3	0/3	0/3

[a] Number of individuals showing positive staining in the parts of the rete indicated, over the total number analyzed.

TABLE 3

Microsomal CYP1A protein and activity in heart and rete mirabile of eels from the environment

Eel heart and rete mirabile microsomes from eels collected in New Bedford Harbor, Marion Harbor, and Vineyard Sound were characterized for their CYP1A content and CYP1A catalytic activity (EROD) as described under *Materials and Methods.* Values are means ± S.D.

Collection Site	Organ	EROD Activity	CYP1A	Endothelial CYP1A
		pmol/min/mg	*pmol/mg*	*Stain Index*
Vineyard Sound[a]	Heart	0.6 ± 0.5	ND[b]	0.2 ± 0.4
(n = 8)	Rete mirabile	0.3 ± 0.1	ND	NA[c]
Marion Harbor	Heart	1.0 ± 0.4	ND	1.3 ± 1.8
(n = 5)	Rete mirabile	0.2 ± 0.1	ND	NA
New Bedford	Heart	5.8 ± 2.8*	4.5 ± 1.7	10.1 ± 3.5*
(n = 11)	Rete mirabile	1.9 ± 1.2*	0.05, 0.07[d]	NA

* Significantly different from all other sites ($P < .01$, Tukey-Kramer).

[a] In liver from eels collected from Vineyard Sound, Marion Harbor, and New Bedford Harbor, the EROD activities were 77, 116, and 321 pmol/min/mg, respectively, and the CYP1A contents were 1, 13, and 190 pmol/mg, respectively (Schlezinger and Stegeman, 2000).

[b] ND, CYP1A protein was not detected. This limit of detection in the assays as performed was 0.01 pmol/mg.

[c] NA, not assayed because the entire rete was required for biochemical assays.

[d] Bands corresponding to CYP1A were visible in two of the 11 eels from New Bedford Harbor.

oxidize TCDD and other pHAH (Poland et al., 1989a,b), is responsible for sequestration and accumulation of TCDD in rat liver (Diliberto, 1997). TCB is tightly bound but is only slowly metabolized by fish CYP1A (White et al., 1997), whereas BP and BNF bind to fish (and mammalian) CYP1A1 but are rapidly metabolized. Thus, some substrates might be effectively "bound" by the CYP1A, and thereby sequestered in the endothelium. In systems where CYP1A in the endothelium is catalytically inefficient, CYP1A might then slowly metabolize PAH as well as pHAH, perhaps with a slow elimination of the compounds from the cells and potentially a slower rate of formation of reactive metabolites. Eel may provide a contrasting system for study, one in which endothelial CYP1A appears to be more active.

Overt toxicity and apoptosis caused by TCDD in lake trout embryos (Guiney et al., 1997) and in medaka embryos (Cantrell et al., 1996, 1998) correlate with CYP1A expression in endothelium, and CYP inhibitors can diminish the effect (Cantrell et al., 1996). Planar halogenated aromatic hydrocarbon exposure results in edema and cardiac dysmorphogenesis in fish embryos (Wisk and Cooper, 1990; Spitsbergen et al., 1991; Walker et al., 1991) and in other vertebrates (Brunstrom and Andersson, 1988). One potential route by which CYP1A might participate in such effects is alteration of arachidonic acid metabolism. PAH treatment of canine coronary and rabbit pulmonary arteries leads to dose-dependent increases in arachidonic acid-induced endothelium-dependent relaxations (Pinto et al., 1986), which can be reversed by general P450 inhibitors (Pinto et al., 1986) or the CYP1A-preferred substrate, 7-ethoxyresorufin (Fulton et al., 1992). Toxicity also could involve production of reactive oxygen species in endothelium; CYP1A binds but slowly metabolizes planar halogenated aromatic hydrocarbon (e.g., TCB), resulting in uncoupling and the release of reactive oxygen species (Schlezinger et al., 1999). Porcine aorta endothelial cells respond to TCB treatment with increased CYP1A1 content and activity as well as reactive oxygen species production and lipid peroxidation (Toborek et al., 1995). *N*-Acetylcysteine, an antioxidant, can prevent apoptosis of vascular cells, protecting against TCDD toxicity in fish embryos (Cantrell et al., 1996, 1998). Whether CYP1A is uncoupled by TCB or other substrates under conditions present in the endothelium must be determined.

In summary, in the eel model, CYP1A is induced by PAH and pHAH in a dose-dependent manner in microvascular endothelium, where it is metabolically active. The patterns of induction were inducer-specific in endothelium, as they were in liver (Schlezinger and Stegeman, 2000), with BNF and BP having an apparently greater potency than TCB. Penetration of CYP1A induction into the vascular bed of the rete mirabile is consistent with the idea that endothelial CYP1A can indicate local concentrations of inducers and possibly remove AHR agonists from the circulation, thereby potentially reducing their penetration peripherally. Endothelial CYP1A may both enhance and ameliorate the toxicity of AHR agonists by holding them in a sensitive cell type but also by slowing their spread to underlying tissues and distal areas. Rete mirabile in the eel swimbladder is a potential model for the study of CYP1A expression in endothelium in vivo and its role in the toxicity of AHR agonists.

Acknowledgments. We gratefully acknowledge Dr. Michael Moore and Bruce Woodin for technical assistance, Oliver Moore and the Army Corps of Engineers for assistance in trapping eels, and Dr. Mark Hahn for helpful comments on the manuscript. This is contribution number 10090 of the Woods Hole Oceanographic Institution.

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