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IMMUNOHISTOCHEMICAL LOCALIZATION OF CYTOCHROME P4501A INDUCED BY 3,3′,4,4′,5-PENTACHLOROBIPHENYL (PCB 126) IN MULTIPLE ORGANS OF NORTHERN LEOPARD FROGS, *RANA PIPIENS*

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Abstract—Monoclonal antibody 1-12-3 (MAb 1-12-3) recognizes an epitope exclusive to cytochrome P450s in subfamily 1A (CYP1A) from all vertebrates tested so far, including one amphibian species. In this study, we first tested the utility of MAb 1-12-3 for detection of presumed CYP1A proteins in hepatic microsomes of northern leopard frogs treated without or with 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126). Statistical analysis showed that ethoxyresorufin-*O*-deethylase (EROD) activities and CYP1A equivalents in treated groups were significantly increased at doses ≥2.3 mg/kg compared with the control groups ($p < 0.05$), and the increases were maintained for at least four weeks. This result confirmed that MAb 1-12-3 can be used for detection of CYP1A in northern leopard frogs and indicated that CYP1A is the primary catalyst for EROD in this species. In a subsequent experiment, sections of organs of PCB 126-treated frogs were immunohistochemically stained with MAb 1-12-3 to identify localization of the CYP1A in different cell types. The CYP1A staining was seen prominently in hepatocytes and epithelium of nephronic duct, while capillaries close to gastric epithelium and submucosal vascular epithelium in both stomach and intestine exhibited moderate to strong staining. The CYP1A was immunodetected in coronary endothelium and the vascular endothelium of lung and gonad. In skin, mild staining was seen in epithelial cells of mucous glands and serous glands and in vascular endothelium, demonstrating induction of CYP1A in the dermal layer.

Keywords—P450 enzyme Immunohistochemistry 3,3′,4,4′,5-Pentachlorobiphenyl Northern leopard frogs

INTRODUCTION

Cytochrome P450-associated monooxygenases play important roles in the activation and/or inactivation of xenobiotics obtained from ingestion or through skin, lung, and other epithelial layers in contact with the environment [1,2]. Although most prominent in the liver, P450 enzymes also occur in many extrahepatic organs of vertebrates from fish to mammals [3–5]. Differences in expression and function of P450 forms in various organs and cell types can determine the response of those cells and organs to toxic effects such as necrosis and carcinogenesis.

There have been only a few studies on amphibian P450 enzymes. Schwen and Mannering [6–8] characterized P450 enzymes of northern leopard frogs, *Rana pipiens*. Harri [9] studied P450 catalyzed enzyme activities during the seasonal life cycle of an anuran species, *Rana temporaria*. Huang et al. [10,11] and Jung [12] studied induction of cytochrome P450-associated monooxygenases in northern leopard frogs by polychlorinated biphenyls (PCBs) and conducted comprehensive field studies to evaluate the catalytic activity of hepatic cytochrome P450-associated monooxygenase as a biomarker for exposure to AhR agonists in leopard frogs. The studies to date have only examined hepatic P450 enzymes in frogs and found the insensitivity of anuran hepatic ethoxyresorufin-*O*-deethylase (EROD) activity to AhR agonists. Because metabolism of toxicants at extrahepatic sites is likely to be involved in systemic effects on reproduction, immune function, and other functions [2], it is important to investigate amphibian P450 induction at extrahepatic sites.

Monoclonal antibody 1-12-3 (MAb 1-12-3) strongly recognizes an epitope unique to CYP1A in mammals and a known or putative CYP1A in other vertebrates examined to date, including American alligator (*Alligator mississippiensis*), turtles (*Chrysemys picta*), bullfrog (*Rana catesbeiana*), and numerous fish and higher vertebrates [2]. In this study, we first established the cross-reactivity of MAb 1-12-3 with the putative CYP1A in hepatic microsomes of northern leopard frogs. We subsequently determined the localization of CYP1A in formalin-fixed, paraffin-embedded sections of organs of frogs exposed to 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126). In addition, we compared CYP1A expression with the amounts of PCB 126 in organs measured in a separate PCB 126 toxicokinetic study [13] under the assumption that the PCB 126 distribution patterns in this study were similar to those from the toxicokinetic study using radiolabeled PCB 126.

MATERIALS AND METHODS

Frog husbandry

Animals used in the present study were purchased from Nasco (Fort Atkinson, WI, USA). Frogs were maintained in large plastic aquaria with running dechlorinated water at 20°C. The aquaria were tilted so that frogs could swim in the water pool or sit on dry substrate. One week before the experiment started, frogs were individually housed in 58.4- × 42.5- × 22.9-cm Rubbermaid® tubs (Wooster, OH, USA) lined with heavy-density polyethylene plastic bags (Associated Bag, Milwaukee, WI, USA). The liners were changed every 6 d. We

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did not test whether there is leaching of AhR agonists from the high-density polyethylene bags. There is a possibility that the bags may contain AhR agonists and minute amount of them could be leached under some circumstances. However, it is unlikely that our results and discussions are biased by this concern in that PCB 126 is the most potent AhR agonist in the PCB category and very high dosages were applied in this study. To ensure adequate moisture for frogs, dechlorinated water was supplied in a polystyrene petri dish (100 × 15 mm, diameter × height). Frogs were fed mealworms sprayed with vitamin complex (Bio-Vite, Ocean Nutrition, San Diego, CA, USA) ad libitum. The room temperature was set at 20°C under a 12-h-light/12-h-dark photoperiod. In both holding regimes before and during the experiment, frogs ate normally, and no skin diseases were observed. All procedures for animal housing, handling, and dissection adhered to guidelines provided by the Animal Use Committee of Research Animal Resources Center and the Office of Biological Safety (University of Wisconsin–Madison, Madison, WI, USA).

Testing the cross-reactivity of MAb 1-12-3 with the hepatic CYP1A of northern leopard frog

The hepatic microsomes for this testing were from a previous dose–response and time-course study [10]. In that study, frogs were fasted 24 h before the intraperitoneal injection of PCB 126. Each injection volume was 2 ml/kg body weight and contained either a solution of PCB 126 in corn oil at a designated dosage or corn oil alone. For the dose–response experiment, frogs were intraperitoneally injected with 0, 0.2, 0.7, 2.3, or 7.8 mg/kg of PCB 126, and they were euthanized one week after dosing. For the time-course experiment, frogs received 7.8 mg/kg or corn oil alone and were euthanized at one, two, three, or four weeks after dosing. Each treatment group had three frogs. Preparation of hepatic microsomes and measures of enzymatic assay were as described in Huang et al. [10]. Measurement of frog liver microsomal CYP1A by protein blotting was performed as modifications of the procedure of Kloepper-Sams et al. [14]. Briefly, microsomal proteins were resolved on 8 to 16% polyacrylamide gels (Novex, San Diego, CA) and electrophoretically transferred to nylon (NYTRAN, Schleicher & Schuell, Dassel, Germany). Antibody against *Stenotomus chrysops* (scup) CYP1A, MAb 1-12-3 [15], was used as the primary antibody at 10 μg/ml in 1% blocker (NYTRAN) in tris-buffered saline; the secondary antibody was a goat antimouse IgG linked to alkaline phosphatase (Schleicher & Schuell) at the recommended dilution for immunoblotting in blocker/tris-buffered saline. Signals were generated using the RadFree™-enhanced chemiluminescence system of Scheicher & Schuel, with variable exposure times for the autoradiography film (Fuji Medical, Stamford, CT, USA). Purified scup CYP1A was used as the standard. Signal was quantified using digital images obtained with a Kodak DCS 200 Digital Camera (Eastman Kodak, Rochester, NY, USA) using the NIH Image, version 1.6065. Linearity was ensured by including standards in each run and, with multiple exposures of film, analyzing in the linear range of the standards.

The microsomes were stored in liquid nitrogen for about 1.5 years before the western blot was conducted. In other species, microsomes stored in liquid nitrogen for as long as 10 years showed no loss of EROD capacity [16]. Though the band at about 34 kD might (see Results) indicate degradation of CYP1A, lost activity during storage in liquid nitrogen should be similar for each sample and would not change the patterns of results and discussions.

Immunohistochemistry experiment

Experimental procedures. In the present study, based on each frog's body weight, a cricket was injected with an adequate amount of working solution so that a dose of 5 mg/kg would be delivered when the frog was forced fed the PCB-loaded cricket. Control frogs were force fed a cricket injected with corn oil alone. Four frogs were treated with each dosing regimen. Frogs were decapitated 9 d after treatment. Liver, heart, lung, kidney, gonad, stomach, intestine, and skin were excised immediately and then fixed in 10% neutral buffered formalin for eventual immunostaining.

Fixation and staining of tissue. Formalin fixed tissues were embedded in paraffin, and 5 μ sections were mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA) and analyzed immunohistochemically for the presence of CYP1A as before [17]. Matching serial sections were incubated using the Shandon™ coverslip system (Pittsburgh, PA, USA) for 2 h with 150-μl aliquots of 1.5 μg/ml MAb 1-12-3 in tris-buffered saline/1% bovine serum albumin added at 0 and 60 min. Blocking solutions, secondary antibodies, linker, and color developer were components of the Signet (Medford, MA, USA) murine immunoperoxidase kit. Color development was achieved as described before using 2% 3-amino-9-ethylcarbazole and 1% hydrogen peroxide. Sections were counterstained with Mayer's hematoxylin. Relative staining intensities were determined subjectively by comparing the staining of sections to that of similarly treated control and 3,3′,4,4′-tetrachlorobiphenyl-induced scup liver sections. The stain index calculated by this method shows excellent correlation (r^2 = 0.98) with the amount of CYP1A measured by western blot [18].

Statistical analyses. The EROD activity and CYP1A equivalents were square-root transformed to obtain homogeneity of variance. One-way analysis of variance and Tukey's test were used to determine differences of EROD activities or CYP1A equivalents among treatment groups. Statistical significance was set at $\alpha = 0.05$.

RESULTS

Cross reactivity of monoclonal antibody 1-12-3 with northern leopard frog CYP1A and correlation with EROD activity

The electrophoretic molecular weight of the protein detected in frog liver microsomes (~53–54 kD) was similar to that of CYP1As in other species, indicated by the nearly identical migration of the scup CYP1A standard and the major band detected in frog liver microsomes (Fig. 1). There also was a band at about 34 kD in the induced frog liver samples. Whether this was generated in vivo or in vitro is not known. This band is presumed to be reflecting degradation of CYP1A; the MAb 1-12-3 epitope is retained on fragments generated by proteolysis [19].

Ethoxyresorufin-*O*-deethylase catalytic activity and CYP1A equivalents measured in microsomes from frogs treated with different doses and sampled at times after exposure showed similar patterns in response to varying doses of PCB 126 (Table 1). In the dose–response study, one-way analysis of variance and Tukey's test showed that EROD activities and CYP1A equivalents among treatment groups were significantly

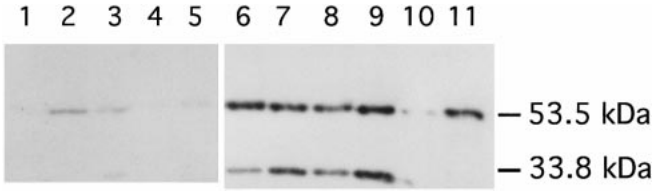

Fig. 1. Immunoblot of liver microsomes. Control and polychlorinated biphenyl (PCB)-treated frog liver microsomes were immunoblotted with monoclonal antibody 1-12-3 (MAb 1-12-3) to scup CYP1A. Lanes 1–5: control frog liver microsomes; 60 μg of microsomal protein per lane. Lanes 6–9: hepatic microsomes from frogs treated with 7.8 mg/kg PCB; 15 μg of microsomal protein per lane. Lane 10 and 11: standards; scup liver microsomes containing 0.25 and 0.75 pmol CYP1A, respectively.

increased at doses ≥2.3 mg/kg compared with the control groups ($p < 0.05$). The increased levels of EROD catalytic activity and CYP1A equivalents were maintained for at least four weeks. In the time-course study, the patterns of significant increase in EROD catalytic activity and CYP1A equivalents were similar except that EROD activity was significantly increased by week 1 but CYP1A equivalents were not increased significantly until week 2 in the time-course study ($p < 0.05$).

Immunohistochemical localization of induced CYP1A protein in organs

The location and relative amount of induced CYP1A in liver and extrahepatic organs were examined immunohistochemically (Table 2). No control frogs ($n = 4$) exhibited staining in any of the cell types examined. The staining in the PCB-treated group is described below.

Liver. Strong and specific staining occurred throughout the cytoplasm of hepatocytes (Fig. 2). No staining was seen in either vascular endothelium and bile duct epithelium in the liver.

Heart. One unidentified vascular endothelium was mildly stained (not shown). Endocardium, myocardium (cardiac muscle cell), and pericardium were not stained.

Lung. Vascular endothelium was mildly stained (not shown).

Table 1. Test of monoclonal antibody 1-12-3 in detection of hepatic CYP1A of northern leopard frogs

Treatment	EROD activity[a] (pmol/min/mg)	CYP1A equivalents[a] (pmol/mg)
Dose–response[b]		
Control	49.42 ± 2.68 A	0.64 ± 0.38 A
0.2	56.08 ± 7.67 A	0.15 ± 0.09 A
0.7	57.27 ± 4.83 A	0.00 ± 0.00[c]
2.3	185.61 ± 37.39 B	29.23 ± 1.94 B
7.8	168.07 ± 19.88 B	28.66 ± 12.32 B
Time course[d]		
Control	63.03 ± 7.90 A	2.81 ± 1.37 A
Week 1	168.07 ± 19.88 B	28.66 ± 12.32 A
Week 2	289.85 ± 21.94 C	196.18 ± 21.21 B
Week 3	384.27 ± 23.67 C	252.41 ± 21.67 B
Week 4	367.39 ± 15.33 C	305.51 ± 51.61 B

[a] Those values for ethoxyresorufin-*O*-deethylase (EROD) activity or CYP1A equivalents that share the same letters in parentheses are not significantly different. $n = 3$ for all groups.
[b] In mg/kg.
[c] Below detection level.
[d] Treatment levels are 0 and 7.8 mg/kg polychlorinated biphenyl 126.

No staining was seen in respiratory epithelium or smooth muscle.

Kidney. All treated frogs demonstrated strong, multifocal staining of CYP1A in both the proximal and distal portions of the tubular epithelium of the nephron (Fig. 3). Glomerular endothelium was mildly stained.

Stomach and intestine. Moderate to strong staining was observed in terminal arterioles in stomach, basal cells of duodenal villi, and submucosal vascular epithelium of both stomach and intestine (not shown).

Skin. Frogs exhibited mild staining of CYP1A in epithelial cells of many mucous glands and a few serous glands, which occur in the stratum spongiosum of the dermal layer. Vascular endothelium of dermal layer was mildly stained (Fig. 4).

Gonad. The vascular endothelium of testes (not shown) and ovaries (Fig. 5) exhibited mild staining. Spermatogonium, Sertoli cells, sperm, and egg showed no staining.

DISCUSSION

Ethoxyresorufin-O-*deethylase and CYP1A in northern leopard frogs*

The same patterns of significant increase in EROD catalytic activity and the content of putative CYP1A in PCB-treated microsomes support the conclusion that the protein recognized by MAb 1-12-3 is indeed CYP1A and is the catalyst for EROD. The MAb 1-12-3, which was developed against scup P4501A [20], can thus be used in detection of putative CYP1As in northern leopard frogs. This antibody has previously been used for detection of presumed CYP1As in bullfrogs [2]. Therefore, we expect that MAb 1-12-3 may recognize CYP1As in *Rana* species or other anurans.

The MAb 1-12-3 recognizes putative CYP1As in mammals, birds, reptiles, fish, and bullfrogs [2]. The epitope recognized by this antibody has been identified (Woodin and Stegeman, unpublished data), and it is highly conserved in mammalian CYP1A1 and in CYP1A in all of the nonmammalian species examined to date. The data here suggest that there is one MAb 1-12-3 cross-reacting protein in frogs. Given the specificity of the antibody and the inducibility of the protein recognized, we conclude that this is a leopard frog CYP1A. Recently, there have been two CYP1As cloned from another frog, *Xenopus laevis* [21]. Those two proteins share a high degree of identity (94%), suggesting a fairly recent divergence. Whether additional CYP1A proteins undetected by MAb 1-12-3 occur in leopard frogs is not known. In mammals, MAb 1-12-3 recognizes only CYP1A1 but not CYP1A2 proteins [22].

Immunohistochemical localization of CYP1A in multiple organs

We used the data from a previous toxicokinetic study [13] to estimate the concentrations of PCB 126 at particular putative target tissues (Table 2). Polychlorinated biphenyl concentrations were highest in fat bodies, gonads (testes and oviduct), and liver. The latter three tissues exhibited induced CYP1A protein. Other tissues that apparently had lower concentrations of PCB 126 but which also expressed considerable CYP1A protein were kidney, stomach, intestine, and skin.

The submucosal vascular endothelium of the intestine and the capillaries close to gastric epithelium were moderately stained by MAb 1-12-3. Studies in fish [23–25] and mammals [26,27] have shown that extensive metabolism of dietary benzo[*a*]pyrene and other drugs occurs in the intestine. In the

Table 2. Polychlorinated biphenyl (PCB) distribution and occurrences and intensities of P4501A staining of tissue sections of northern leopard frogs

	Control group	5.0 mg/kg group		Estimated PCB residue[a]	
Organ/cell type	Occurrence[b]	Occurrence	Intensity[c]	% of Dosage	ng/g Tissue
Liver				5.21 ± 0.94	660.5 ± 103.7
Hepatocytes	−	+++	+++		
Vascular endothelium	−	−	−		
Bile duct epithelium	−	−	−		
Heart				<0.25 ± 0.04	<86.1 ± 24.8
Endocardium	−	−	−		
Myocardium	−	−	−		
Pericardium	−	−	−		
Vascular endothelium	−	+	+		
Lung				<0.25 ± 0.04	<86.1 ± 24.8
Vascular endothelium	−	+	+		
Respiratory epithelium	−	−	−		
Smooth muscle	−	−	−		
Kidney				0.16 ± 0.04	156.6 ± 37.4
Glomerular endothelium	−	+	+		
Nephronic ducts	−	++	+++		
Stomach				0.10 ± 0.03	48.1 ± 13.0
Terminal arterioles	−	++	++		
Submucosal vascular endothelium	−	+	++		
Intestine				0.79 ± 0.17	220.6 ± 50.1
Basal cells of duodenal villi	−	+	++		
Submucosal vascular endothelium	−	++	++		
Skin				2.82 ± 0.53	115.8 ± 19.4
Epidermis	−	−	−		
Epithelial cells of mucous gland	−	+	+		
Epithelial cells of serous gland	−	+	+		
Dermal vascular endothelium	−	++	++		
Gonad (testis)				0.38 ± 0.04	1,423.9 ± 985.9
Vascular endothelium	−	+	+		
Spermatogonium and Sertoli cells	−	−	−		
Sperm	−	−	−		
Gonad (oviduct)				2.7	753.4 ± 281.3
Vascular endothelium	−	+	+		
Eggs	−	−	−		
Fat bodies (copora adiposa)	NA	NA	NA	44.68 ± 5.65	10,319.5 ± 1,367.8
Carcass[d]	NA	NA	NA	22.38 ± 3.33	294.7 ± 52.5
Muscle	NA	NA	NA	6.32 ± 1.81	122.2 ± 36.9
Other organs[e]	NA	NA	NA	0.25 ± 0.04	86.1 ± 24.8

[a] Refer to Huang and Karasov [13] for detailed experimental design of the toxicokinetic study. Animals were force fed PCB-laden crickets in both the toxicokinetic study and the present immunohistostaining experiment. In the toxicokinetic study, ^{14}C-PCB was used as a trace to estimate PCB residues in organs. Frogs received a final dose of 5.0 mg/kg body weight (n = 4) and were killed at day 9. Various organs were solubilized with soluene-350 followed by the addition of scintillation counting solution (Hionic-flour). Radioactivity in the vial then was determined with Tracor Analytic Mark III. Data were expressed as mean ± standard error.

[b] Occurrence is the relative frequency of stained cells (+ = seldom; ++ = frequent; +++ = always; − = none; NA = not available).

[c] Intensity is the density of the peroxidase cells (+ = mild; ++ = moderate; +++ = heavy; − = none; NA = not available).

[d] Carcass includes head, bone, cartilage, and residues of other tissues.

[e] Other organs includes esophagus, lung, spleen, and heart.

present study, CYP1A was expressed in the capillaries close to gastric epithelium and submucosal vascular epithelium of both stomach and intestine. Thus, it is possible that stomach and intestine of leopard frogs may be involved in metabolism of PCB 126.

We have previously reported that PCB 126 induced hepatic EROD activity in leopard frogs [10]. In the present study, a putative CYP1A was detected by MAb 1-12-3 throughout the cytoplasm of hepatocytes in all treated livers examined. Staining was not seen in hepatic vascular structure and bile duct epithelium. In other studies, rats have been shown to be capable of slowly metabolizing PCB 126 to a more soluble and more readily excreted 4′-hydroxy-3,3′,4,4′,5-PeCB [28]. Frogs, including northern leopard frogs, are capable of converting chlorinated benzene, naphthalene, and chlorobiphenyl congeners (e.g., 4,4′-dichlorobiphenyl, Aroclor 1254, Monsanto, St. Louis, MO, USA) into a range of hydroxylation products [29,30], although planar halogenated aromatic compounds have not been examined. The CYP1A in fish oxidizes PCB 77 (3,3′,4,4′-tetrachlorobiphenyl) to the same metabolites as those produced by mammalian CYP1A1 [31]. The induced frog CYP1A in liver (or other organs) is thus expected to be capable of me-

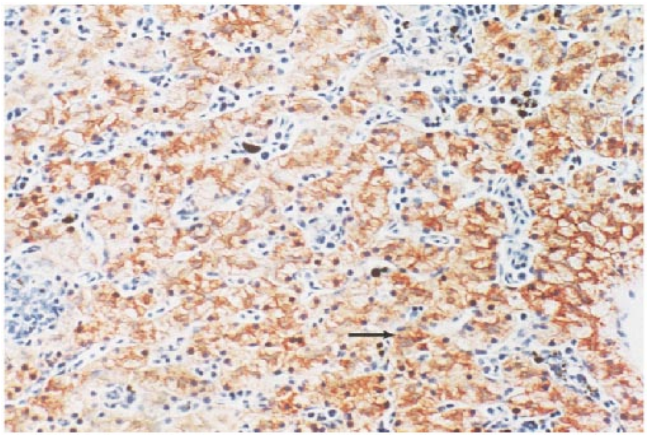

Fig. 2. Localization of CYP1A in leopard frog liver (×200). Hepatocytes were strongly stained (arrow 1). Staining was not present in vascular endothelium (arrow 2) or bile duct epithelium (not shown).

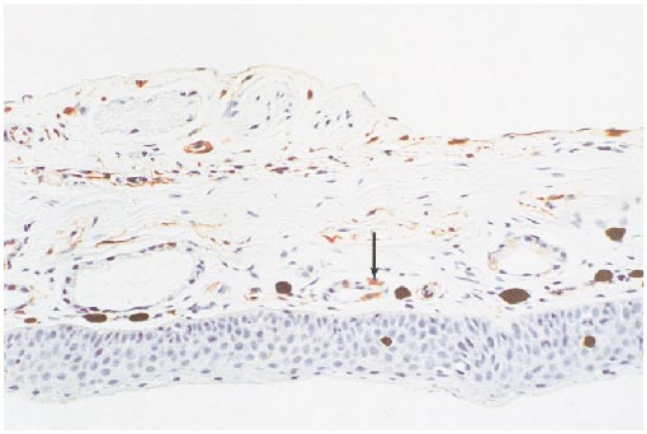

Fig. 4. Frogs exhibited mild staining of CYP1A in epithelial cells of some mucous glands (arrow 1) and serous glands (arrow 2), which occur in the stratum spongiosum of the dermal layer. Vascular endothelium of dermal layer (arrow 3) was mildly stained.

tabolizing planar PCBs, including PCB 126. In the toxicokinetic study, low, but detectable, amounts of ^{14}C-PCB 126 (0.06% ± 0.02%, n = 9) were measured in the gall bladder, consistent with the idea that liver processes this compound at a low rate. However, whether the radioactivity was associated with parent compound or metabolite(s) was not investigated. In addition, in our toxicokinetic study, liver was shown to retain the second highest concentration and the highest percentage of the total PCB dose among the organs in which CYP1A was examined. Liver is thus a major location for PCB accumulation and likely is an important site for metabolism of PCBs in leopard frogs.

Our results showed that frog skin retained 2.8 ± 0.5% of the total ingested PCB 126 and that CYP1A protein was expressed in certain types of skin cells. The stratum corneum of the epidermis is sloughed periodically. In our toxicokinetic study, molted skin contained traces of PCB 126, indicating that xenobiotics sequestered in the skin could thus be eliminated via this route. Another feature of frog integument is that highly vascularized mucous glands secrete mucus to keep the skin moist on land, to facilitate skin respiration, and to act as a lubricant in water. The PCBs might be metabolized by the induced CYP1A in the epithelial cells of mucous glands. Perhaps elimination of PCB 126 or its more hydrophilic metabolites occurred through coexcretion with water in mucus or as a bound fraction with mucopolysaccharides.

Both proximal and distal tubules of nephrons were strongly stained by MAb 1-12-3. Kidneys retained <1% of the total dose of PCB 126. However, kidney, as a major excretory organ, continuously receives and filters large amounts of blood. Thus, it would be constantly exposed to and process blood-borne PCB 126.

While CYP1A was induced intensively and extensively in endocardial and vascular endothelial cells of fish heart [20], one unidentified cardiovascular endothelium of frogs exhibited mild CYP1A staining following PCB 126 exposure. The CYP1A was induced in vascular endothelial cells of frog lungs but not in respiratory epithelium or smooth muscle. In the toxicokinetic study, only a very small fraction of PCB 126 was present in lung or heart (<0.08% of total dose). The role of lung and heart in the metabolism and elimination of PCB 126 is unclear and should be investigated further. Regardless, the results showed that endothelium was the common site of CYP1A induction in many organs of frogs, as it is in other vertebrates. The apparent level of expression was weaker in endothelium than in hepatocytes and nephronic tubular epithelium, which consistently exhibited the strongest overall staining.

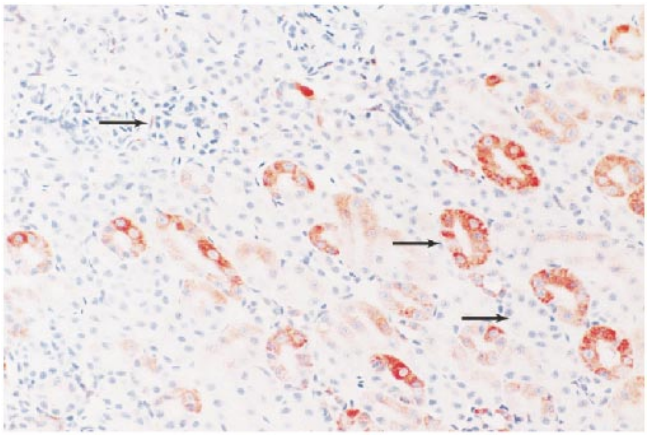

Fig. 3. Localization of CYP1A in frog kidney (×200). Staining with monoclonal antibody 1-12-3 (MAb 1-12-3) is seen in the tubular epithelium of proximal (arrow 1) and distal (arrow 2) portions of nephrons and glomerular endothelium (arrow 3).

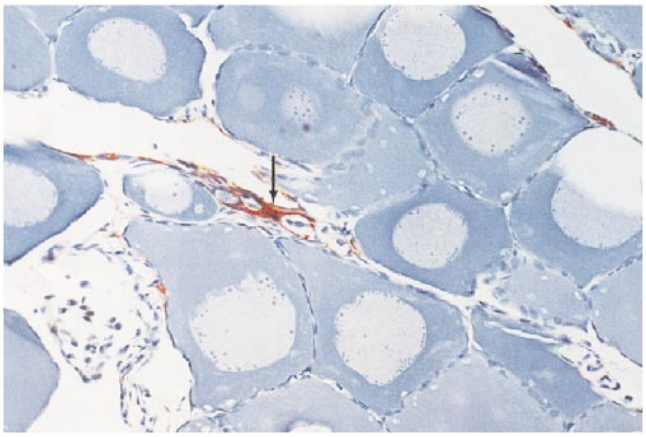

Fig. 5. The CYP1A in vascular endothelium of ovaries (arrow).

It is worth noting that, although vascular endothelial cells in stomach, intestine, skin, heart, lung, and gonad exhibited CYP1A staining, endothelial cells in liver, the organ primarily responsible for the metabolism and excretion of xenobiotics, were not stained. Endothelial cells are known to be functionally and morphologically different in various organs [32]. Therefore, the differences in CYP1A expression of endothelial cells of different frog organs might result from differential sensitivity to induction by PCB 126. One postulated function of endothelial CYP1A enzymes is the protection of underlying tissues from xenobiotics through the binding and sequestering of toxicants. In addition, protection of underlying tissues by endothelial cells could also result from metabolizing xenobiotics to less toxic and water-soluble compounds, although in other studies, the rates of metabolism attributed to CYP1A in endothelium were generally low, thought to be due to the absence or very low amounts of reduced nicotinamide adenine dinucleotide phosphate-cytochrome P450 reductase [33]. On the other hand, activation of protoxicants and procarcinogens by endothelial P450 has been reported in multiple species [34,35]. It has also been suggested that CYP1A in endothelium may be linked to early lesions that result in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced vascular derangements leading to yolk sac, pericardial, and meningial edema that is associated with lake trout sac fry mortality [36]. Whether endothelial CYP1A in frogs plays a role in protection from or activation of toxicants in amphibian species, as is it presumed to do in other species, needs to be investigated.

Insensitivity of P450 induction and discrepancy between enzyme activity and quantity

The apparent insensitivity to lower doses (0.2 and 0.7 mg/kg) and a plateau at 2.6 mg/kg imply that there may be a very steep dose–response relationship between those doses in this frog. However, the maximal response was not achieved for two weeks or longer, which suggests that there are features of the response in frogs that may differ from the responses in other species. In addition, whether there are more than CYP1A in northern leopard frogs, like the two CYP1As cloned from another frog (*Xenopus laevis*), is not known.

The PCB 126 is an inhibitor of CYP1A, and the discrepancy between EROD and CYP1A could reflect inhibition of the CYP1A by PCB 126 retained in the microsomal fraction during preparation. This phenomenon has been described in other species [37]. We did not determine the K_i for inhibition of frog EROD by PCB 126. In scup, the K_i for inhibition of CYP1A by PCB 126 is 0.2 μM. The discrepancy between the fold induction of EROD and CYP1A also could involve oxidative inactivation by the PCB 126. This process has been defined in other species treated with PCB 126 [38]. However, in scup, inactivation in vivo appears to be linked to a rapid loss of immunodetectable signal, although some inactive protein might survive longer in frogs than in fish.

Comparative utility of three different measures of CYP1A in frogs

In this study, we examined induction of presumed CYP1A in frogs using three techniques, a CYP1A-specific catalytic activity assay (i.e., EROD) and two procedures using MAb 1-12-3 monoclonal antibody against CYP1A (i.e., protein immunoblot and immunohistochemistry). The high correlation between EROD activity and CYP1A in the same hepatic microsomes demonstrates the utility of MAb 1-12-3 for localizing and quantifying CYP1As in frogs. It has been reported that high doses of slowly metabolized PCBs can competitively inhibit EROD catalytic activity in in vivo and in vitro fish studies [37,39]. Consequently, if only EROD activity is measured, an underestimation of the degree of induction of CYP1A at high doses of PCB might occur. In this situation, quantification of CYP1As by MAb 1-12-3 can provide critical information regarding the true level of induction at high doses. Though it is still not clear whether high doses of AhR agonists could cause competitive inhibition of EROD catalytic activity in amphibian species, this seems unlikely in leopard frogs given the relative insensitivity of CYP1A induction by the potent PCB 126.

Immunohistochemical staining for CYP1A has several advantages over other approaches to assess induction in frogs, as in other species. First, it may allow researchers to examine archived frog specimens collected from historically contaminated areas, given the formalin stability of the epitope recognized by MAb 1-12-3. Second, fixing samples in formalin can yield information about CYP1A induction in situations that do not permit rapid freezing (e.g., in the field) or with samples for which microsomal preparation and catalytic activity analysis are difficult (e.g., [34]). Third, CYP1A induction is generally an indication of aryl hydrocarbon receptor activation; therefore, immunohistostaining and tissue toxicant distribution studies can help to identify target cell types or organs in which toxic aryl hydrocarbon receptor agonists or CYP1A substrates may exert effects. Application of these approaches to analysis of frogs from the wild may help to discern the effects of some xenobiotics in these amphibians.

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