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Induction Time Course of Cytochromes P450 by Phenobarbital and 3-Methylcholanthrene Pretreatment in Liver Microsomes of *Alligator mississippiensis*

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ABSTRACT. *Alligator mississippiensis* has at least two classes of inducible hepatic microsomal cytochromes P450 (CYP): (1) those induced by 3-methylcholanthrene (3MC), and (2) those induced by phenobarbital (PB). The rates of induction by these xenobiotic compounds are significantly slower than those reported for mammals. Carbon monoxide binding, western blots, and enzymatic activity measurements indicated that at least 48–72 hr are required to reach full induction. A methoxy-, ethoxy-, pentoxy-, and benzyloxyphenoxazone (resorufin) O-dealkylation (MROD, EROD, PROD, and BROD) profile was indicative of substrate selectivity typical of 3MC- and PB-induced P450s. MROD and BROD showed the greatest ability to discriminate between alligator hepatic microsomes induced by 3MC and PB, respectively. This is in contrast to mammals, in which EROD is a biomarker of polycyclic aromatic hydrocarbon exposure because of its ability to discriminate the induction of CYP 1A. In a similar manner, PROD is a highly preferred activity of CYP 2B in mammals; thus, it is used to indicate CYP 2B induction. The induction of P450 by PB is a general phenomenon in mammals and birds. To the best of our knowledge, this is the first report demonstrating PB induction of P450 activities typical of the mammalian CYP 2 family isoforms in alligator or any reptilian liver. The importance of this finding to the evolution of CYP 2 family regulation by PB is heightened by the fact that induction by this xenobiotic is not common to fish and other lower vertebrates (Ertl RP and Winston GW, *Comp Biochem Physiol*, in press). Although indicating the presence of CYP 1A- and CYP 2B-like isoforms in alligator, it remains to be established how closely related these alligator P450s are to mammalian isoforms. BIOCHEM PHARMACOL **55**;9:1513–1521, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. alligator; cytochromes P450; induction time course; 3-methylcholanthrene; mixed function oxidase; phenobarbital

The American alligator (*Alligator mississipiensis*) has become an important agricultural commodity for both its meat and its hide since its removal from the endangered species list. It is the most economically important cultured reptilian species in Louisiana, with 158,760 wild and farm-raised alligators harvested in 1995 [1]. In the same year, the United States, as a whole, harvested 208,332 alligators. Based on Louisiana commodity prices, the annual gross farm value is about $17,000,000 and $24,000,000 in Louisiana and the U.S., respectively.

The effect and metabolism of drugs used in aquaculture of this organism are of particular importance in light of the mandate of the U.S. Food and Drug Administration calling for the investigation and development of minor animal use drugs and alteration of the guidelines to encourage application for drug approval in minor agricultural species [2–8]. The absence of currently approved drugs stems from the comparatively low gross market value of these minor species, which precludes the amount of research previously required in the approval process. Diseases associated with the aquaculture of alligators and the effects of some classic major use drugs are starting to be investigated [9–13]. The lack of approved, safe, and effective drugs for alligator aquaculture poses direct and indirect human health risks in light of the increasing use of these animals as agricultural commodities [5].

Understanding how this reptile metabolizes pollutants and other xenobiotics from farm runoff and chemical industry discharge into its environment [14, 15] is of concern, as exposure not only threatens this organism, but, as a food commodity it is also a potential source of human exposure. Reproductive failure in alligator populations in Florida has been linked to chlorinated hydrocarbon pesticide exposure [16–19]. Such chlorinated hydrocarbons are P450 inducers and are estrogenic molecules that may

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compete with steroid hormones in regulating reproductive cycles in alligators and other reptiles [16–19]. Furthermore, specific P450 isoforms are important regulators of temperature-dependent sex determination in various reptiles, including alligators [20, 21].

The mixed function oxidase system is the primary pathway of oxidative metabolism of drugs and other xenobiotics, and is composed of a monooxygenase (CYP§) and a reductase (cytochrome b_5 and CYP reductases). P450 is involved in the biosynthesis of endogenous compounds such as steroids and fatty acids, and over 500 constitutively and nonconstitutively expressed P450 isoforms exist.

Herein, we report on studies of the temporal patterns of induction of P450 proteins in *A. mississippiensis*. Two P450 isoforms often studied for their involvement in xenobiotic metabolism are the members of the CYP 1 and CYP 2 families. The mammalian CYP 1A1 isoform is induced by PAHs, for example 3MC, among other xenobiotics. The CYP 2B isoforms are induced by various nonplanar xenobiotics including PB. In this study, 7-alkyl-substituted phenoxazones (resorufins) were chosen as substrates because of their preference for certain isoforms [22–24]. The induction time and corresponding activities expressed are important when considering the holistic effects of xenobiotic exposure in this reptile. Because of the slow metabolic rate of alligators [25–29], the time course of induction was studied over 72 hr, which is considerably longer than the 16–24-hr period that characterizes maximal induction of activity in mammals [30–34]. 3MC pretreatment of alligators caused induction of a protein with an apparent MW of 52,000 that was not detected in microsomes of untreated control alligators. Similarly, pretreatment with PB caused induction of two of three constitutive CYP, having apparent MWs of 49,000, 51,000, and 53,000. We showed that while the induction of alkoxyresorufin *O*-dealkylase activities by 3MC and PB is, in many respects, qualitatively similar to that observed in mammals, the induced catalytic activity is 10 or 30 times lower. The longer time course of induction and the lower specific activities in drug-metabolizing pathways in alligators as compared with mammals may be an important consideration in determining the amount and duration of antibiotic treatments in farm-raised reptiles. Moreover, the observed differences in induction and substrate specificity of alligator P450s as compared with mammalian P450s discussed herein are indicative of the phylogenetic differences in these enzymes.

MATERIALS AND METHODS

Animals

Juvenile alligators (~1 kg) obtained from the Rockefeller Wildlife Refuge were induced with one of the following i.p. injection regimes: animals were multiply injected with either 3MC in corn oil, 45 mg/kg of body weight on days 1, 3, and 4; or PB in buffered saline, 20 mg/kg of body weight on days 1 and 4 and 40 mg/kg of body of weight on days 2 and 3. All animals were killed on the day following the last injection. Animals used in the induction time course were injected at time zero with either 45 mg/kg of body weight of 3MC in corn oil or 40 mg/kg of body weight of PB in buffered saline and killed at appropriate time intervals thereafter. Both untreated and vehicle-treated controls were used. Microsomes were prepared by differential centrifugation and solubilized as described by Jewell *et al.* [14, 15].

Specific Content

Specific content was determined at 25° by differential spectroscopy of reduced microsomes compared with and without carbon monoxide in a 50 mM of KP_i, 20% glycerol (pH 7.4) buffer and quantified from the extinction coefficient of 91 mM^{-1} cm^{-1} [35]. Protein concentration was determined by a modified fluorescamine assay using a Millipore cytofluor 2350 plate reader as described by Böhlen *et al.* [36] and modified by Lorenzen and Kennedy [37].

Enzymatic Assays

7-Ethoxy-, 7-methoxy-, 7-benzyloxy- and 7-pentoxyphenoxazone (resorufin) O-dealkylation (EROD, MROD, BROD, and PROD, respectively) assays were performed according to Burke and coworkers [22, 23, 38] as adapted to the micro-plate system by Kennedy *et al.* [39]. The reaction mixture contained microsomal protein in 50 mM of Tris buffer (pH 7.2), 5 mM of $MgCl_2$, 5 μM of substrate. Reactions were initiated with NADPH, incubated at 37°, and stopped after 15 min with 1.5 vol. of ice-cold methanol. The assay was measured in a Millipore cytofluor 2350 microplate reader at 530- and 590-nm excitation and emission, respectively. Activity is expressed as product produced per minute per milligram of microsomal protein (pmol/min/mg) or divided by the specific content and expressed as a turnover number (pmol/min/nmol total P450). Degree of induction is the activity of the sample (pmol/min/mg) divided by the activity of the appropriate control and expressed as a percentage.

Statistical Analysis

Statistical analysis was performed by a two-sample *t*-test assuming unequal variances comparing treated animals with zero-time controls. Statistical calculations were done with Microsoft Excel 5.0 for the Macintosh, utilizing the add-in analysis tool package for statistical and engineering analysis.

Western Blot Analysis

SDS–PAGE and Western blots were done by standard techniques described by Laemmli [40] and Towbin *et al.*

§ *Abbreviations:* CYP, cytochromes P450; MFO, mixed-function oxygenase; 3MC, 3-methylcholanthrene; PB, phenobarbital; (M,E,B,P)ROD, methoxy, ethoxy, benzyloxy, pentoxy resorufin *O*-dealkylase; PAH, polycyclic aromatic hydrocarbon; and DF, discrimination factor.

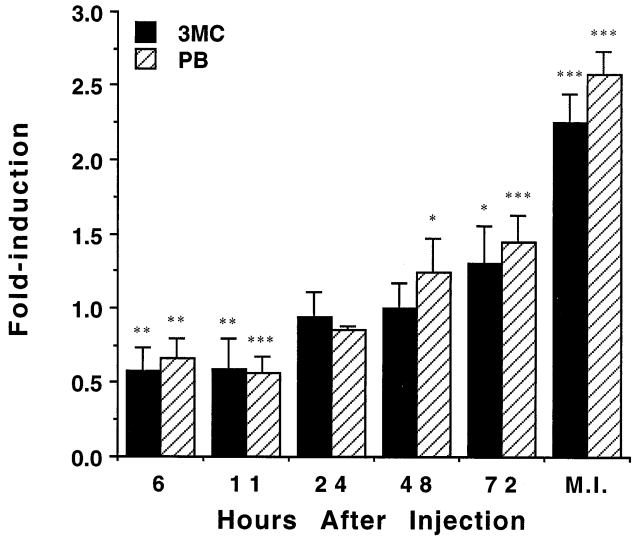

FIG. 1. Fold-induction of CYP specific content by PB and 3MC. The time-dependent changes in P450 specific content in alligator liver microsomes were determined from CO-bound, dithionite-reduced difference spectra and shown as fold-induction of P450 specific content of the treated groups over controls. The range of specific content was from 0.2 nmol/mg at 6 hr to 0.95 nmol/mg after 5 days with multiple injections (M.I.). Values (expressed as means ± SD, N = 3) at various time intervals were significantly different from the appropriate control as indicated: *0.01 < $P \leq 0.05$, **0.001 < $P \leq 0.01$, and ***$P \leq 0.001$.

[41], respectively. The primary antibodies used to probe the blots were mouse-anti-scup CYP 1A1, goat-anti-rabbit CYP 2B1 and 2 (Oxford Biomedical), or rabbit-anti-scup P450B (putative CYP 2B antibody). The secondary antibody was the appropriate biotin-conjugated anti-IgG. The blot was then treated with an alkaline phosphatase-conjugated extra-avidin followed by staining with nitroblue tetrazolium and bromochloroindol phosphate in a 100 mM of sodium carbonate buffer containing 1 mM of $MgCl_2$, pH 9.8.

RESULTS

Induction of CYP Specific Content

The induction time course of total P450 specific content in alligator liver microsomes by 3MC or PB pretreatment was determined by difference spectroscopy of dithionite-reduced, carbon monoxide liganded minus unliganded microsomes (Fig. 1). The data are presented as fold-induction above control levels over a period of 72 hr following a single i.p. dose of 45 mg/kg of 3MC or 40 mg/kg of PB. The data from the time courses are compared with those obtained following a standard multiple injection protocol. At the 6- and 11-hr time points, the specific content of P450 was actually lower than the basal levels (fold-induction was significantly less than 1 in both 3MC- and PB-pretreated alligators) (Fig. 1). The content of P450 returned to approximately basal levels by 24 hr and then increased to a level of about 1.4-fold over basal by 72 hr. In alligators that received the multiple injections of 3MC or PB, the P450 levels were induced to a significantly greater level (~2.5-fold) than in animals given a single injection.

O-Dealkylation of Alkoxy Phenoxazones

Alligator liver microsomal O-dealkylation of substituted resorufins occurred with a pattern of substrate preference for 3MC- and PB-pretreated animals similar to those reported by Burke and Mayer [23]; EROD was induced markedly in 3MC-treated and PROD was induced in PB-treated animals. Over the PB induction time course studied, BROD and PROD did not increase substantially until about 48 hr, at which point both activities increased markedly up to 72 hr (Fig. 2, A and B, respectively). As with the pattern of induction of P450 specific content, BROD and PROD were induced to a greater extent in alligators given multiple injections than in those given a single injection. Closer scrutiny of panels A and B of Fig. 2 revealed an attenuation of the specific activities of these O-dealkylases at 6 hr postinjection, similar to that observed in P450 content. This trend was also seen in the time course of induction of EROD activity in PB-treated alligators (Fig. 2C), i.e. attenuated activity at 6 and 24 hr postinjection, with a small but significant induction observed in the singly-injected alligators at 72 hr and in the multiply-injected alligators. The highest rate of O-dealkylase activity observed in untreated alligators was EROD (compare control activities, Fig. 2, A–D). Despite the greater induction of PROD compared with EROD (Table 1), the rates of induced PROD and EROD activity at 72 hr post PB treatment were similar (Fig. 2, B and C) due to the lower basal EROD rates. While the trend in MROD activity along the time course (Fig. 2D) for PB-treated alligators was similar to the trend in EROD, the response was much lower and did not appear significantly induced over the control except in multiply-injected animals (2-fold).

Burke and Mayer [23] defined a DF to indicate the isoform preference for a specific alkoxyresorufin O-dealkylase activity. The DF is defined as the degree of induction by the agent giving the greater induction divided by the degree of induction by the agent giving the lesser induction. In the alligator, BROD was the best discriminator of PB-induced isoforms, both for multiply-injected animals (DF = 24) and along the time course (DF = 14.3) (Table 1). PROD activity appeared to discriminate P450 induction by PB from induction of P450s by 3MC in the multiply-injected animals (DF = 10.6). However, this distinction between PB and 3MC induction was not as great along the time course (DF = 4.7). The temporal profiles of induction also differed. A lag phase was noted in the BROD induction time course although, once expressed, BROD increased more rapidly than PROD (compare activities at the 72–hr time point with those of multiply-injected animals). Similar to mammalian models, multiply-injected alligators exhibited greater induction than animals receiving only a single injection. The greatest specific activities and highest degrees of induction by PB pretreatment were observed for

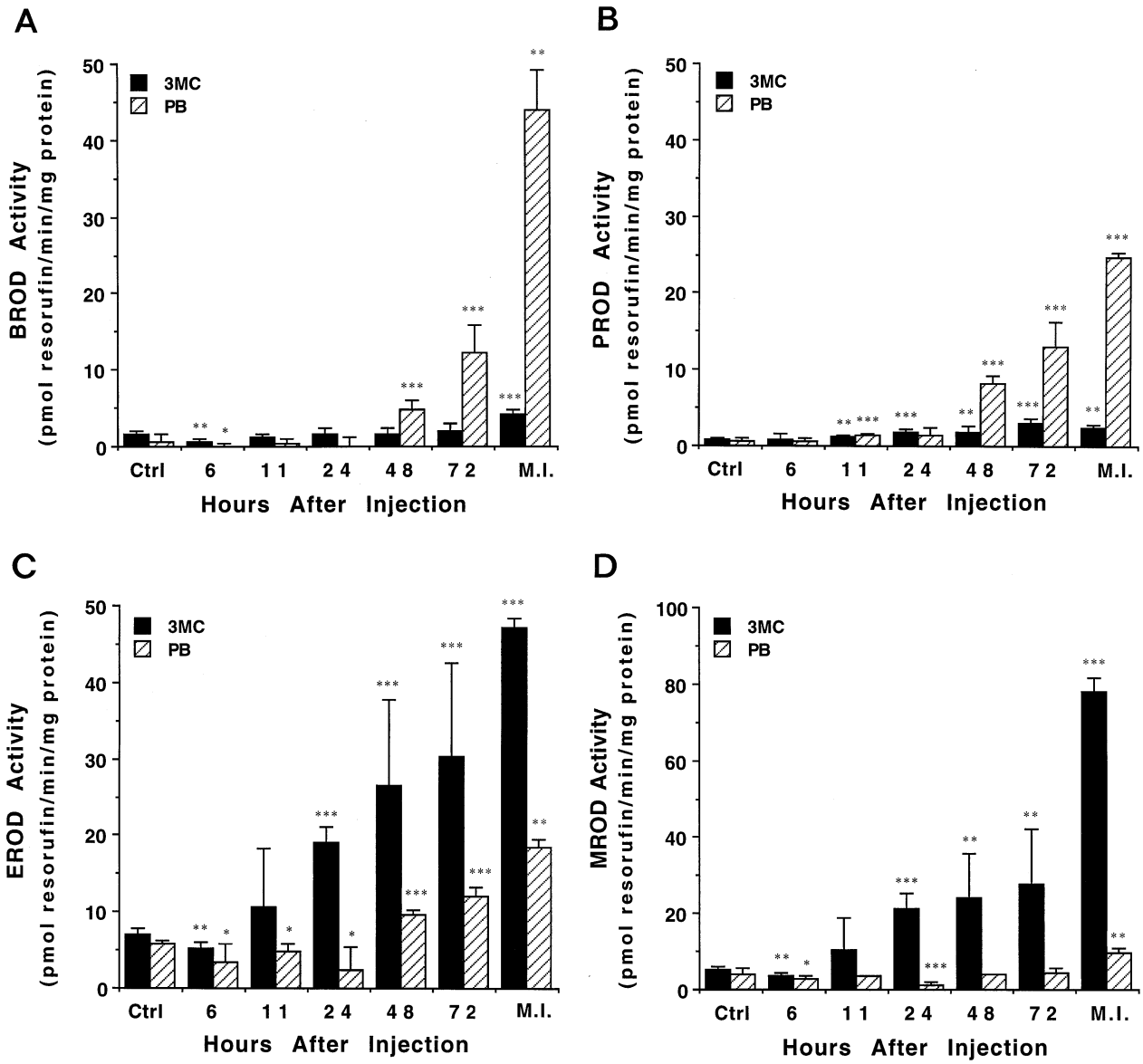

FIG. 2. Time course of induction of alkoxyresorufin O-dealkylase activity in alligator liver microsomes after 3MC or PB pretreatment. Alligators were measured at various time intervals after a single injection of either 3MC (45 mg/kg) or PB (40 mg/kg). A standard multiple injection regime over 5 days is presented in the last column (M.I.). Note that the scale for MROD is two times larger than for the other O-dealkylase activities. Values (expressed as means ± SD, N = 3) at various time intervals were significantly different from the appropriate control as indicated: *0.01 < $P \leq 0.05$, **0.001 < $P \leq 0.01$, and ***$P \leq 0.001$.

BROD (67-fold) and PROD (36-fold) in multiply-injected animals (Table 1).

In 3MC-treated alligators, a marked increase in the specific activities of EROD and MROD was observed over the 72-hr time course (Fig. 2, C and D). BROD was not induced significantly by 3MC pretreatment over the time course, while the degree of induction of PROD increased to about 4-fold at 72 hr (Table 1). At 72 hr after a single injection or in animals multiply-injected, MROD had the highest discrimination factor (DF = 4.8 and 6.6, respectively) for 3MC induction (Table 1). EROD, a classical biomarker of mammalian exposure to CYP 1A inducers, did not discriminate as well (DF = 2.1) between PB and 3MC induction in the alligator (see Discussion). The greatest degree of induction by 3MC pretreatment was in MROD of multiply-injected animals (15-fold).

Western Blots

Three antibodies were used to probe isoform-specific protein induction over the time course. Mouse anti-scup CYP 1A antibody was used to probe the isoform-specific induction of protein by 3MC pretreatment (Fig. 3). A protein band with an apparent MW of 52,000 was induced by 3MC; this band increased during the time course and plateaued at 48 hr. No proteins in the 52-kDa range were detected at zero time in control alligator liver microsomes (10 μg/lane) by antibody to CYP 1A (Fig. 3). To probe the time course

TABLE 1. Discrimination factors (DF) for alkoxyphenoxazone O-dealkylases of liver microsomes from PB- or 3MC-pretreated alligators

Activity	Degree of induction*		Discrimination factor†
	PB	3MC	
Time course			
BROD, 72 hr	18.6	1.3	14.3
PROD, 72 hr	19.1	4.1	4.7
EROD, 72 hr	2.1	4.4	2.1
MROD, 72 hr	1.1	5.3	4.8
Multiple injections			
BROD, 5 days	67.2	2.8	24
PROD, 5 days	35.9	3.4	10.6
EROD, 5 days	3.2	6.8	2.1
MROD, 5 days	2.3	15.1	6.6

*Degree of induction is the activity of the sample (pmol/min/mg) divided by the activity of the appropriate control.

†Discrimination factor is the degree of induction by the agent giving the greater induction divided by the degree of induction by the agent giving the lesser induction [23].

of induction by PB, anti-scup P450B (putative CYP 2B by sequence homology) and anti-rabbit CYP 2B1 and 2 antibodies were used (Fig. 4). Qualitatively, these antibodies yielded very similar induction profiles for the PB time course; however, recognition of reptilian protein by the mammalian antibody was much less intense than by the fish antibody (compare the top panel of Fig. 4 with the bottom panel). These antibodies recognized induction of two prominent bands of approximately 49 and 52 kDa. Both of these bands were present in the control animals, but their content increased over the time course to maximum levels at 48–72 hr. Another band of MW between these bands was observed in the immunoblots, but the content of that band did not change over the time course studied. The profiles of induction of the expressed isoforms detected in the western blots paralleled the enzymatic O-dealkylation of the preferred substrates. For example, induction of protein recognized by anti-CYP 2B tracked predominantly PROD and BROD activities, and that recognized by anti-CYP 1A tracked mainly EROD and MROD activities.

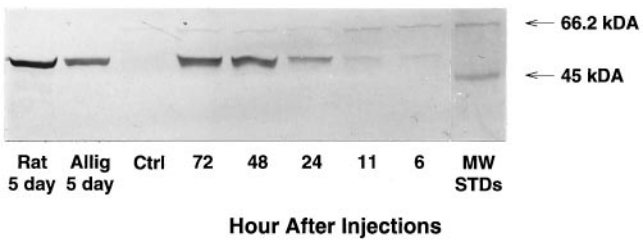

FIG. 3. Western blots of microsomes from the time course of alligators pretreated with 3MC. Alligators and rats were treated with a single injection of 3MC (45 mg/kg), and the blots were performed on microsomes prepared at the indicated time intervals, or with microsomes prepared after three injections (45 mg/kg) over 4 days, as described in Materials and Methods. Ten micrograms of protein was loaded per lane. The blot was probed with mouse-anti-scup CYP 1A1.

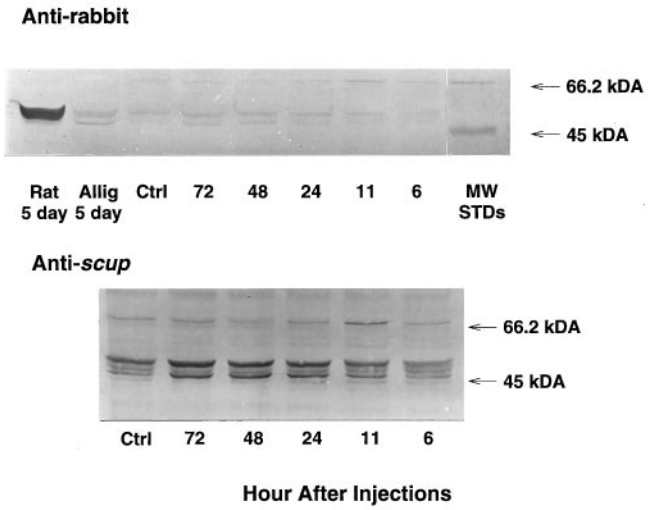

FIG. 4. Western blots of microsomes from the time course of alligators pretreated with PB. Alligators and rats were treated with a single injection of PB (40 mg/kg), and the blots were performed on microsomes prepared at the indicated time intervals, or with microsomes prepared after four injections over 5 days, as described in Materials and Methods. Ten micrograms of protein was loaded per lane. The top blot was probed with goat-anti-rabbit CYP 2B1 and 2. The bottom blot was probed with rabbit-anti-scup P450B (putative CYP 2B) antibody.

DISCUSSION

The central role of CYP in xenobiotic metabolism is a cogent rationale for identifying their constitutive and inducible isoform profiles, the circumstances under which they are induced, and the extent and duration of induction. This study addresses the extent and duration of induction of two major CYP families by the benchmark inducers PB and 3MC in a class of vertebrates (Reptilia) underrepresented in studies of the fate and disposition of xenobiotics in the environment. The present research underscores the need to investigate directly organisms from different taxa as mammalian models alone are insufficient to predict the environmental consequences of pollutants in other groups of organisms [42]. The importance of studying alligator MFO is heightened because of the alligator's significance as an aquacultural commodity. Drugs used in aquaculture and pollutants discharged into the environment may be altered or accumulated by these animals, thereby potentially entering the human food chain. Furthermore, reproductive failure in alligator populations in Florida has been linked to chlorinated aromatic hydrocarbon exposure [16–19]. These chemicals are inducers of CYP, and many are endocrine disrupters. Alligator research may also be of heuristic value in protecting closely related endangered crocodilians.

Pretreatment with 3MC in mammals or fish induces non-constitutive CYP 1A isoforms with preference for metabolism of flat planar polycyclic aromatic hydrocarbons. Western blots indicated that 3MC pretreatment of alligators induces proteins with epitope homology to known CYP 1A isoforms. Alligators pretreated with 3MC have induced liver microsomal P450s that preferentially catalyze O-

dealkylation of flat planar ethoxy- and methoxy-substituted phenoxazones rather than of the bulkier pentoxy- and benzyloxy-substituted derivatives. Similar to rats [14], the greatest enzymatic activity observed in control alligators was EROD. However, this EROD activity did not appear to be associated with CYP 1A, as evidenced by the lack of detectable (<50 pg) CYP 1A expression in the western blots of control (uninduced) alligator liver microsomes. EROD activity is often used as a biomarker of PAH and planar halogenated aromatic hydrocarbon pollution in mammals, as it can discriminate induction of CYP 1A in response to these contaminant classes. The relatively low ability of EROD to discriminate (DF = 2.1) between PB and 3MC induction in alligators as compared with the rat (DF = 58; [23]) suggests that caution should be exercised when applying mammalian paradigms across classes or phyla. In the alligator, MROD activity appears to be a better indicator of PAH exposure and concomitant induction of CYP 1A isoforms (DF = 6.6; Table 1).

Data from CO-binding spectra, enzymatic activity, and western blots were consistent with a time course of induction that plateaus at ~48 hr, i.e. much later than in the rat, which plateaus at 16–24 hr [30–34]. When plotted in terms of activity units per nanomole of P450, the rates of alligator microsomal *O*-dealkylases appeared to plateau earlier in the time course than did the rates when expressed per milligram of protein or than did the P450 content (nmol P450/mg of protein), which continued to increase up to 48 hr post 3MC treatment (Figs. 1, 2C, 2D, and 3). In light of the isoform preferences toward the alkyl-substituted substrates, these data suggest that the ratio of the various isoforms expressed relative to one another plateaus by 12 hr post–3MC–treatment (Fig. 5), whereas at least 48 hr is required for maximal expression of the total P450 content. The slow time course of induction in response to 3MC treatment may reflect the low metabolic rates and physiological processes of the alligator.

The degree of induction in alligators is significantly lower than in mammals. The final level of EROD induction after multiple 3MC injections was 7-fold in the alligator as compared with 50- to 100-fold [14, 22, 23, 43] in the rat. Compared with induction patterns observed in the alligator, the rat shows a higher degree of induction, higher constitutive EROD activity (~23-fold), and higher 3MC-induced activity (~130-fold). Low EROD rates in induced animals have been reported for other reptiles [42, 44]. This may reflect the lower specific content of P450 in the microsomes or an altered active-site as shown in the turtle *Chrysemys picta picta*, which has low EROD activity but relatively high levels of aryl hydrocarbon hydroxylase (AHH), another preferred activity of CYP 1A [44]. We reported results of experiments in which a trout, *Oncorhynchus mykiss*, CYP 1A1 cDNA probe was used to examine if P450 induction by 3MC in alligators was transcriptionally regulated [42]. Although these studies were inconclusive due to large standard deviations obtained from cross-hybridization of the probe with several isoforms, transcriptional regulation is probable in light of a cytosolic protein of ~120 kDa recognized in alligator liver by an anti-mouse Ah-receptor antibody [42]. This receptor is normally associated with transcriptional regulation of the *CYP 1A* gene in mammals.

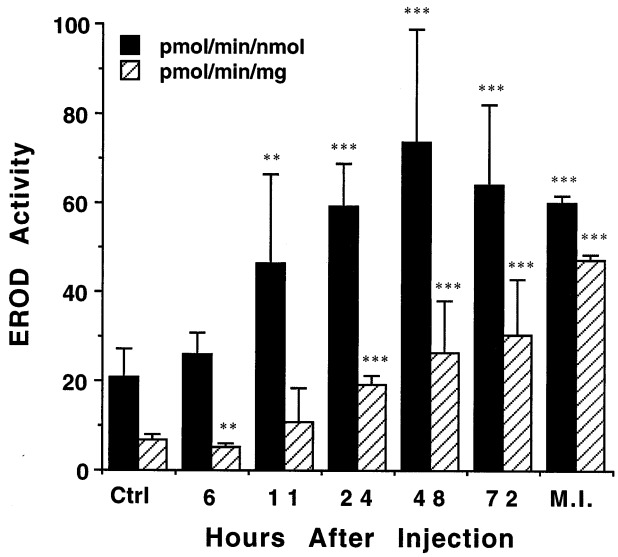

FIG. 5. Time course of induction of EROD activity in alligator liver microsomes after 3MC pretreatment. EROD activity was measured at the indicated time intervals after a single injection of 3MC (45 mg/kg). EROD activity after a standard multiple injection regime over 5 days is presented in the last column (M.I.) for comparison. EROD activity is shown as pmol/min/mg of microsomal protein (hatched bars) or as pmol/min/nmol total P450 (solid bars). Values (expressed as means ± SD, N = 3) at various time intervals were significantly different from the appropriate control as indicated: **0.001 < *P* ≤ 0.01, and ******P* ≤ 0.001.

Our results are consistent with induction of CYP 1A-type activities in other reptiles. The snake *Thamnophis sp.* exhibited maximal induction (~2-fold) of hepatic microsomal benzo[*a*]pyrene hydroxylase activity on or before day 4 after pretreatment with 40 mg of 3MC/kg with induction still visible 12 days post-pretreatment [45]. While the rates of activity in the snake *Thamnophis sp.* are much lower than in mammals, a nonconstitutive, transcriptionally regulated P450 is indicated by the undetectable mRNA levels in controls and the induced levels of mRNA 22 hr post-treatment with 100 mg/kg of β-naphthoflavone detected with a rainbow trout CYP 1A cDNA probe [46]. In the same study, the turtle *Chrysomyces picta* exhibited a similar trend in mRNA levels, which lacked statistical significance due to large standard deviations [46]. Induction of EROD and benzo[*a*]pyrene hydroxylation by classic Ah-receptor agonists was observed in the turtle *Chrysemys picta picta* [44].

PB pretreatment in mammals induces several isoforms in the CYP 2 family. These isoforms preferentially metabolize PROD and BROD. Similarly, alligators pretreated with PB displayed a significant induction of liver microsomal *O*-dealkylation of the non-planar substrates benzyloxy- and

pentoxyphenoxazone. Western blots indicated that three proteins were recognized by CYP 2B antibodies and that two of those were induced by PB. Differences in the ability of BROD and PROD to discriminate PB-induced isoforms along with differences between BROD and PROD induction profiles (discussed earlier) are consistent with induction of multiple isoforms having different but overlapping substrate preferences. Induction of certain cytochromes P450 by PB is a general phenomenon in mammals and birds but is not so in fish and other lower vertebrates [42]. To the best of our knowledge, our results with the alligator are the first to show significant induction of P450 activities typical of the mammalian CYP 2 family isoforms by PB in a reptile. To date the exact identity of the major PB-inducible P450 isoform from alligator liver remains unresolved. The 50 amino acid N-terminal sequence determined for a PB-inducible P450 purified from alligator liver in our laboratory clearly indicates that it is a member of the CYP 2 family but exhibits similar homology with several CYP 2 subfamilies [42]. The full-length cDNA sequence of this alligator CYP 2 family isoform is currently under investigation by us. The presence of PB induction in alligators, in conjunction with recent reports of P450 induction by PB in turtle as detected by Western blots [44], suggests that this type of P450 regulation evolved in the ancestors of reptiles. Comparing the points in phylogeny where PB induction occurs may help to elucidate the significance of this evolutionary pattern. While PB induction of P450 is absent in most amphibians, fish, and marine invertebrates tested to date, a few reports have indicated possible minor induction of CYP 2 family isoforms. The newt *Pleurodeles waltl* [47], the lizard *Agama lizard* [48], and the fish *Carpus carpus* [48] appear to display a minor temperature-dependent induction of P450s by PB or PB-type inducers. These reports combined with the absence of PB induction in the snake, *Thamnophis sp.* [45], emphasize the importance of not generalizing about PB-type induction in lower vertebrates. Molecular biological approaches to the study of these and other lower vertebrates will be required to accurately resolve the origins of cytochrome P450 regulation by PB and related PB-like inducers.

In alligators, BROD activity appears to be the better indicator of PB-induced CYP 2 family isoforms (DF = 24; Table 1) with PROD performing adequately in multiply-injected animals (DF ~11). The preference of PB- and 3MC-induced alligator liver microsomes for BROD and MROD, respectively, though different from the rat, is consistent with previous studies in avians (quail) that found similar alterations in the AROD substrate preference in xenobiotic-induced liver microsomes [49]. CO-binding spectra, Western blots, and enzymatic O-dealkylation activities indicated that induction of the total P450 content of alligator liver microsomes by PB, as discussed above for 3MC, plateaus much later than does the ratio of the various P450 isoforms (Fig. 6). The response to PB pretreatment in the alligator was not only slow, but the corresponding

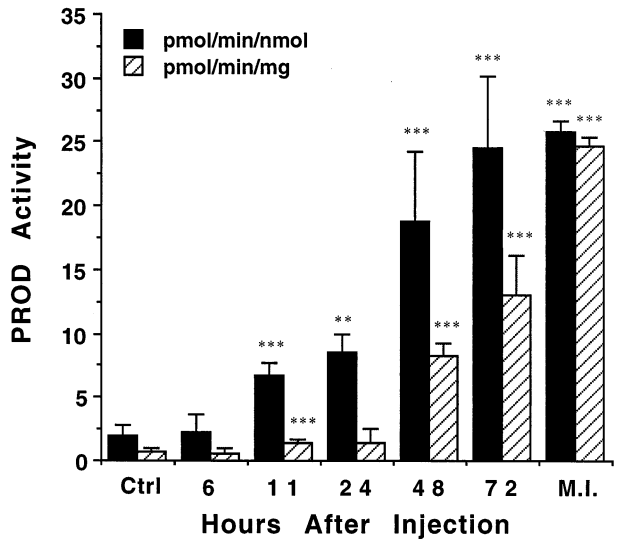

FIG. 6. Time course of induction of PROD activity in alligator liver microsomes after PB pretreatment. PROD activity was measured at the indicated time intervals after a single injection of PB (40 mg/kg). PROD activity after a standard multiple injection time regime over 5 days is presented in the last column (M.I.). PROD activity is shown as pmol/min/mg of microsomal protein (hatched bars) or as pmol/min/nmol total of P450 (solid bars). Values (expressed as means ± SD, N = 3) at various time intervals were significantly different from the appropriate control as indicated: **$0.001 < P \leq 0.01$, and ***$P \leq 0.001$.

enzyme activities also were lower than in mammals. The final level of PROD induction after multiple PB injections in the alligator was 36-fold as compared with 121-fold induction in similarly treated rats [23, 24, 43]. As compared with alligators, mammals exhibit a higher degree of induction of total P450 content and higher basal and induced levels of P450 activity. The PROD activity of control rat liver microsomes is 10-fold higher, and the PB-induced activity is 64-fold higher, than in the alligator [14, 24, 43]. Similar to the CYP 1A Northern blot analysis, experiments with human CYP 2B and 2E cDNAs to probe the PB induction time course yielded large SDs due to cross-hybridization of the probe with several isoforms [42].

The CO binding assays and Western blots corroborate the induction profiles indicated by the enzymatic assays for both 3MC and PB pretreatment. The reason for the initial decrease observed in the induction profiles is not understood. The maximal induction time of 48–72 hr and the low activity levels with either 3MC or PB are consistent with the slow metabolic rate of alligators compared with mammals [25, 26]. The fact that induction times in the alligator are significantly longer than in mammals, combined with the relatively low activities catalyzed by alligators, should affect our view of how drug dosages are defined in the aquaculture of this and other reptiles. The present study, in conjunction with recent studies showing low EROD but relatively high AHH activity [44], both preferred activities of CYP 1A in mammals, underscores the need to evaluate the effects of chemicals in the organism of

interest and to avoid extrapolation of paradigms across taxa. The slower rate of induction and the lower overall activities and content of P450 in the alligator may require significantly lower therapeutic doses of drugs, which are metabolized by these routes for this and other reptiles. It also suggests that certain classes of pollutants discharged into the environment of the alligator would take longer to be metabolized, and short-term exposures may not give as large a response as observed in the mammalian system.

The findings reported herein should enhance our understanding of the effects of pollution on *A. mississippiensis* and other endangered crocodilians. The reptilian class of vertebrates is currently under-represented with respect to data on the P450 system. The observed differences in induction and substrate specificity of alligator P450 as compared with that of mammals are indicative of the underlying phylogenetic differences in P450 metabolism. Finally, our results, in conjunction with those of other studies of CYP in submammalian vertebrate and invertebrate systems, represent important steps toward elucidating the evolutionary patterns of the MFO system. MFO studies of reptiles will fill gaps in our current understanding of the phylogeny of CYP between fish and mammals. While this study indicates that the alligator has a slower rate of induction by 3MC and PB pretreatment along with an altered DF as compared with the rat, it remains to be established whether the induced isoforms, and associated activities, are homologues/orthologues of mammalian CYP 1A and CYP 2 family isoforms, respectively. Determination of the position and identity of these isoforms in the overall evolution of the P450 supergene family and the mechanism of induction will have to await further sequence analysis of this protein and its DNA promoter regions.

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