**Microsomal activation of benzo[a]pyrene by Alligator mississippiensis: mechanisms, mutagenicity and induction**

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**Introduction**

Owing to careful management practices, the American alligator, *Alligator mississippiensis*, has rebounded remarkably from its previous status as an endangered species to one that now requires regulated hunting to prevent it from overgrazing its habitats. Because of this renewed status, the alligator is increasingly recognized for its potential as an agricultural commodity and is a crop of significant economic potential to the burgeoning aquaculture industry [1]. In light of its growing use as a food source concern has been raised over the anthropogenic impact from the numerous petrochemical industries that discharge into its habitats [1, 2].

We have previously reported on the concomitant induction by 3-methylcholanthrene (3MC) of cytochrome *P*-450 specific content, ethoxyresorufin and ethoxyresorufin 0-deethylase activities (EROD and PROL), respectively, were measured according to the methods of Phillips and Langdon [6]. 7-Ethoxyresorufin and 7-pentoxyresorufin O-deethylase activities (EROD and PROD, respectively), were measured according to the methods of Burke et al. [7]. Lauric acid hydroxylase activity was determined essentially as described by Parker and Orton [8]. Glutathione-S-transferase and DT-diaphorase activities were determined as described in Habig and Jakoby [9] and Ernster [10], respectively.

**Methods**

**Animals, treatment and preparation of microsomes**

Alligators (120–1260 g) obtained from Rockefeller Wildlife Refuge (Grand Chenier, LA) and male Sprague–Dawley rats (180–240 g) from Hill Top Lab Animals Inc. (Scottsdale, PA) were maintained essentially as described in Jewell et al. [3]. Treatments were similar for both species consisting of intraperitoneal injections of either 3MC or 2,2',4,4'-tetrachlorobiphenyl (TCB) in corn oil (45 mg/kg body weight; days 1, 3 and 4 of exposure); clobibrate (200 mg/kg, days 1 and 3 of exposure) or phenobarbital (80 mg/kg, days 1 through 5 of exposure). All animals were decapitated 24 h after the final injection. Microsomes were prepared from both species by differential centrifugation [3].

**Determination of cytochrome P-450 content, NAD(P)H-cytochrome c reductase activity and mono-oxygenase activities**

Cytochrome *P*-450 contents were determined by the method of Omura and Sato [5] with slight modifications for the alligator system as described in [3]. NAD(P)H-cytochrome *c* reductase activities were measured according to Phillips and Langdon [6]. 7-Ethoxyresorufin and 7-pentoxyresorufin O-deethylase activities (EROD and PROD, respectively), were measured according to the methods of Burke et al. [7]. Lauric acid hydroxylase activity was determined essentially as described by Parker and Orton [8]. Glutathione-S-transferase and DT-diaphorase activities were determined as described in Habig and Jakoby [9] and Ernster [10], respectively.

**Determination of BaP metabolism by h.p.l.c.**

BaP metabolism was determined by reverse-phase h.p.l.c. following initial incubations of microsomal protein with [1H]BaP (approximately 20 mcg/ml). Final incubation volumes of 1 ml contained 0.7 mg of microsomal protein, 80 μg-BaP, 100 mM-potassium phosphate buffer (KPi), pH 7.4, 10 mM-MgCl2, and 0.1 mM-EDTA. Reactions were started with 2 mM-NADPH and following 20 min of incubation at 37°C, stopped with 1 ml ice-cold acetone. BaP metabolites and unmetabolized BaP were extracted into ethyl acetate, evaporated to dryness under argon and the metabolites resuspended in 100 μl of methanol. An aliquot (20 μl) of this was injected onto a C18, 3 μm Microsorb column and analytes eluted with a linear gradient of 10% methanol:90% water/acetonitrile/glacial acetic acid...
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(60/40/0.3) to 100% methanol (1 ml/min for 20 min beginning 5 min after injection). Eighteen-second fractions were collected into 5 ml scintillation fluid and metabolites quantified on a Beckman LS 250 liquid scintillation counter. In some cases when peaks could not be base-line resolved by radiometric analysis quantification was achieved with external standard curves of metabolite peak area versus concentration (measured at 254 nm).

**SDS-PAGE and Western blotting analysis**

SDS-PAGE and Western blotting were conducted essentially as described in Laemmli [11] and Towbin et al. [12], respectively.

**Umu mutagenicity assay**

The activation of BaP to mutagenic products was determined by measuring the expression of the *umu* gene in *Salmonella typhimurium* TA1535/pSK1002 according to Shimada and Nakamura [4].

**Results and discussion**

The present communication corroborates a previous report from our laboratory on the induction of alligator liver cytochrome *P*-450 by 3MC [3]. Evidence is now presented indicating that this induction, similar to what has been observed in rats, results in the enhanced catalytic capability of alligator liver microsomes to convert BaP to mutagenic products.

**SDS-PAGE and Western blotting analysis**

Figure 1 (panel A) shows marked induction of several protein bands in the 50 kDa molecular mass range in liver microsomes from 3MC-pretreated alligators compared with untreated controls. Panel B is a typical Western blot obtained from such gels and shows that in both 3MC-pretreated rat and alligator liver microsomal fractions a protein band corresponding to about 50 kDa is strongly cross-reactive with a goat polyclonal antibody to rabbit liver cytochrome *P*-450LM4 (IA2). No such protein was recognized by this antibody in liver microsomes from untreated rat or alligator. Thus, at least one major cytochrome *P*-450 isoform, presumably an orthologue of that found in rabbit and rat liver, is induced in alligator liver by 3MC.

Induction of cytochrome *P*-450 by the hypolipidaemic agent clofibrate, a selective inducer of cytochrome *P*-450La6o (IVA1) in rats [13], and TCB, a known potent inducer of cytochrome *P*-450b (IIB1) [14], the major phenobarbital-inducible isoform, was studied. In contrast to the rat, in which both pretreatments increased cytochrome *P*-450 specific content as measured by CO-binding spectra and SDS-PAGE, no apparent induction by either pretreatment was noted in the alligator (data not shown). This apparent lack of induction was confirmed by the observation that neither lauric acid hydroxylase nor PROD activities were increased in clofibrate- and TCB-treated alligators, respectively, whereas these activities were markedly increased in the rat by the same respective pretreatments. In contrast, 3MC-pretreatment was shown to induce EROD activity by about 10-fold in the alligator (126 compared with 13 pmol min⁻¹ mg⁻¹) and 25-fold in the rat (3500 compared with 142 pmol min⁻¹ mg⁻¹). None of the pretreatments had any effect on NAD(P)H-cytochrome *c* reductase activity in alligator liver microsomes.
Microsomal metabolism of BaP

We have previously reported that arylhydrocarbon (BaP) hydroxylase activity of alligator liver microsomes of 3MC-pretreated alligators is increased more than 10-fold over untreated control levels when assayed fluorimetrically (which measures predominantly 3-OH-BaP) [3]. To further characterize this induction, BaP metabolite profiles were determined by h.p.l.c. for both untreated and 3MC-pretreated alligators and compared both with each other, and with those profiles obtained from equivalently-treated rats. Table 1 indicates that despite lower overall activities in alligators, the extent of increase of metabolite formation was considerably greater in 3MC-pretreated alligators than rats. For example, the fold increase in 3-OH-BaP formation was considerably greater relative to control levels in 3MC-pretreated alligators (14.7-fold) than in rats (1.8-fold). On the other hand, the increase in 7,8-dihydrodiol-9,10 BaP epoxide (DBPE), the putative ultimate mutagen, was approximately equivalent for both species. Interestingly, the relative increase in 7,8-dihydrodiol-BaP, the precursor of DBPE, was also considerably greater in the alligator than the rat (10.5-fold compared with 5.3-fold). Metabolite profiles from TCB-pretreated alligators were found not to differ from those of untreated alligators, indicating no induction of BaP oxidation and supporting the above assertion that this polychlorinated biphenyl has no inductive effect in this species (data not shown).

Activation of BaP to mutagens

The absolute identity and nature of the 3MC-inducible alligator cytochrome P-450 isoforms responsible for the increased metabolism of BaP await further elucidation; however, it is clear that their presence in microsomes leads to enhanced formation of mutagenic products of BaP. The significance of this induced metabolism in vivo also remains to be established. In this regard, it should be noted that current aquaculture practices employ various regimens of antibiotics which may be subject to altered metabolism upon induction of the microsomal mixed-function oxidase system. Owing to the complex nature of the BaP molecule, its oxidation by microsomes results in the formation of various products which can either facilitate its detoxification via phase I metabolism or its activation via formation of mutagenic intermediates. The latter is believed to proceed via formation of DBPE. Fig. 2 shows that the metabolism of BaP by rat and alligator liver microsomes forms products that are mutagenic in the umu gene induction assay and, further that the mutagenic expression is markedly greater when the reaction is catalysed by microsomes from 3MC-pretreated rats and alligators. The activation of BaP by 3MC-pretreated alligator leads

Table 1

Metabolism of benzo[a]pyrene by liver microsomes from untreated and 3MC-pretreated rats and alligators

Values are given as nmol·mg⁻¹ protein·20 min⁻¹ incubation and represent single determinations on pooled microsomes from three animals. H.p.l.c. conditions are described in the methods section. Abbreviations used: 3-OH and 9-OH, 3-hydroxy- and 9-hydroxy-benzo[a]pyrene; 1,6-, 3,6- and 6,12-dione, benzo[a]pyrene 1,6-, 3,6- and 6,12-quinone; 4,5-, 7,8- and 9,10-dihydriodoil, DBPE, 7,8-dihyddriodoil-9,10-benzo[a]pyrene epoxide; tetrals, benzo[a]pyrene tetrals.

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<th>BaP metabolite</th>
<th>untreated</th>
<th>3MC-pretreated</th>
<th>fold increase</th>
<th>untreated</th>
<th>3MC-pretreated</th>
<th>fold increase</th>
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<td>3-OH</td>
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<td>11.53</td>
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<td>0.34</td>
<td>4.98</td>
<td>14.65</td>
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<td>0.32</td>
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Activation of benzo[a]pyrene to mutagenic products by liver microsomes from untreated versus 3MC-pretreated rats (A) and alligators (B): effects of varying the microsomal protein concentration.

Assay conditions were as described in [4]. Microsomes, Salmonella typhimurium 1535/pSK1002 and 25 μM-BaP were incubated in the presence of an NADPH-generating system. The reaction was stopped after 2 h and β-galactosidase activity determined at 420 nm with o-nitrophenyl β-D-galactoside as substrate. Key to symbols: - - - , untreated; ----, 3MC-pretreated.

Fig. 2

Partial purification of 3MC-inducible cytochrome P-450 from alligator liver

Partial purification of 3MC-inducible cytochrome P-450 from alligator liver has been achieved by column chromatography of cholate-solubilized alligator liver microsomes on cyanogen bromide-activated p-chloramphetamine-Sepharose 6B. Most of the microsomal cytochrome P-450 was found to bind to the top 2 or 3 cm of the column and SDS-PAGE analysis of the flow-through wash (30 mM-KPi, pH 7.4, containing 20% (v/v) glycerol, 0.2% Emulgen 911, 0.5% cholate and no NaCl). Fractions (5 ml) were collected; the salt wash eluted a peak of low specific content cytochrome P-450 (approximately 3 nmol/mg, fractions 5–9) that yielded a CO-binding spectrum with a maximum at 447 nm (peak A), and the second wash yielded a peak of higher specific content (5–6 nmol/mg, fractions 25–30) with an absorbance maximum in the CO-difference spectrum of 448 nm (peak B). Western blot analysis of the pooled fractions that comprised peaks A and B against, individually, a rabbit polyclonal antibody to rat cytochromes P-450c (IA1) and P-450d (IA2) was performed. The blot of peak A protein revealed the presence of two bands in the 50 kDa region, one of lower molecular mass which strongly cross-reacted with the anti-P-450c antibody, and one of slightly higher molecular mass which cross-reacted less strongly. The blot of peak B protein also revealed two bands corresponding in molecular masses to those of peak A. In this case however, the higher molecular mass band was of significantly greater intensity than that seen in the peak A eluent. The antibody to rat cytochrome P-450d recognized the same two proteins in peak A eluent as did the anti-rat cytochrome P-450c antibody; in this instance however, the higher molecular mass band was preferentially recognized. In peak B only the higher molecular mass protein was recognized by anti-rat cytochrome P-450d. SDS-PAGE also indicated a markedly higher purity of cytochromes P-450 in peak B than in peak A. That alligator liver expresses at least two proteins in microsomes that are inducible by 3MC and that cross-react with antibodies raised against rat cytochrome P-450c (IA1) and rat P-450d (IA2) suggests that these proteins might be the alligator orthologues of rat cytochromes P-450c and d. Of particular interest is the fact that a rabbit polyclonal antibody to trout liver P-450LM4, under the same conditions as those employed for the rat anti-cytochrome P-450 studies, recognized only a single protein in peak B and nothing in the peak A eluent. Both goat anti-rabbit P-450LM4 and rabbit anti-trout (Bella) P-450LM4, recognize at least one protein in 3MC-induced alligator liver microsomes, suggesting that one of the alligator proteins is the orthologue of the rat, rabbit and trout proteins and corresponds to gene product 1 of the cytochrome P-450 gene family 1 [15].

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In mammalian systems, induction of cytochrome P-450 by 3MC is regulated by the Ah locus [16] and is generally accompanied by induction of certain cytosolic enzymes involved in phase II xenobiotic metabolism e.g. glutathione S-transferases and DT-diaphorase(s) [15, 17, 18]. In our laboratory, both of these enzymes were found to be markedly elevated in rat liver cytosol but not in that of alligator following 3MC-pretreatment (data not shown). These data suggest that unlike the situation in mammalian species such as the rat, the regulation of cytochrome P-450 induction by 3MC in the alligator is independent of these phase II enzymes.

Studies of the comparative biochemistry of the cytochrome P-450-dependent mixed-function oxygenase system certainly are important in the quest to understand the molecular evolutionary trends of this remarkable gene family. A more altruistic rationale for such studies might relate to the fact that although A. mississippiensis is no longer an endangered species, it may prove to be a useful model for understanding xenobiotic risk for the many endangered species of crocodilians and perhaps other reptiles that do exist precariously.

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