IDENTIFICATION OF CYP1A5 AS THE CYP1A ENZYME MAINLY RESPONSIBLE FOR UROPORPHYRINOGEN OXIDATION INDUCED BY AH RECEPTOR LIGANDS IN CHICKEN LIVER AND KIDNEY

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ABSTRACT:

Uroporphyrinogen is an intermediate of the heme biosynthetic pathway. The oxidation of uroporphyrinogen to uroporphyrin (UROX) has been demonstrated to be catalyzed by mammalian CYP1A2. This reaction has an important role in uroporphyria caused by halogenated aromatic compounds. Two CYP enzymes induced by Ah receptor ligands were purified recently from chick embryo liver. One, designated CYP1A5, was preferentially active in arachidonic acid epoxygenation and the other, designated CYP1A4, in 7-ethoxyresorufin deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH), reactions mainly catalyzed by CYP1A1 in rodents. The amino acid sequences of both CYP1A5 and CYP1A4 are more similar to CYP1A1 than to 1A2, and neither can be classified as an ortholog of mammalian CYP1A1 or 1A2. Here we report that reconstituted purified CYP1A5 was eight times more active than CYP1A4 in catalyzing UROX. The stimulation of UROX by 3,4,3’,4’-tetrachlorobiphenyl that has been observed in microsomes was also observed with the reconstituted enzymes. Similar dose response relationships were found for induction of UROX and EROD in both chick embryo liver microsomes and in cultured chick hepatocytes, indicating coinduction of CYP1A5 and CYP1A4. UROX was induced by the Ah receptor ligand, 3-methylcholanthrene, in chicken kidney as well as liver. The findings reported here and other evidence that CYP1A4 and CYP1A5 tend to exhibit CYP1A1 and 1A2-like enzyme activities, respectively, indicate that the division of some enzyme activities among CYP1A enzymes applies to different vertebrate classes.

Ah receptor ligands, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), planar polychlorinated biphenyls (PCBs) and 3-methylcholanthrene induce enzymes in the CYP1A subfamily, CYP1A1 and CYP1A2, in rodents and other mammals (1). These enzymes exhibit catalytic differences. For example, 7-ethoxyresorufin deethylase (EROD) activity is commonly used as an index of CYP1A1 activity in rat liver because it has been found to be catalyzed principally by CYP1A1 with only about 20% of the activity being catalyzed by CYP1A2 (2). CYP1A2 is preferentially active in different activities, including the 2-hydroxylation of estradiol (3).

Recently the oxidation of uroporphyrinogen to uroporphyrin (UROX) was shown to be preferentially catalyzed by rat and mouse CYP1A2 rather than 1A1 (2, 4). Uroporphyrinogen, an intermediate in heme biosynthesis, is normally converted to coproporphyrinogen by the enzyme, uroporphyrinogen decarboxylase (5). The oxidation of uroporphyrinogen prevents its conversion to coproporphyrinogen and results in accumulation of uroporphyrin. UROX activity is increased by inducers of the CYP1A subfamily in rodents and is considered to be an essential participant in the massive accumulation of hepatic uroporphyrin (URO) caused by these compounds (2, 5). Ah receptor ligands induce UROX in chick embryo hepatocytes (2, 6, 7), but the question of which CYP enzymes are involved has not been clarified.

In contrast to rodent liver, previous investigations indicated that in chicken liver there was only one P450 form in the IA subfamily induced by 3-methylcholanthrene and similar compounds (2). This one form appeared to catalyze both EROD and UROX activities, based on the effects of an antibody prepared with a single 3-methylcholanthrene-induced form (8). Two TCDD-induced CYP enzymes from chick embryo liver have recently been identified and purified (9–11). One enzyme, which has been designated CYP1A4, was found to be responsible for EROD and aryl hydrocarbon hydroxylase (AHH) in chick embryo. The other CYP enzyme, CYP1A5, was found to be responsible for TCDD-induced arachidonic acid epoxygenation and the 4-hydroxylation of tamoxifen in chick embryo liver (10, 12) but was inactive in EROD or AHH activities (9, 11). The cDNA-derived amino acid sequences of each of these chick CYP enzymes permit both to be classified in the CYP1A subfamily (13), but the sequences of both are more similar to CYP1A1 than to 1A2 and neither can be said to be the direct ortholog of CYP1A1 or 1A2 (13).

To better understand the function of CYP1A enzymes in nature, we therefore examined the abilities of the two avian CYP1A enzymes to carry out UROX, an activity preferentially catalyzed by mammalian CYP1A2. We examined the abilities of each of the TCDD-induced chick CYP enzymes to catalyze UROX in reconstitution assays and

Abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; CYP, cytochrome P450; EROD, ethoxyresorufin deethylase; UROX, uroporphyrinogen oxidation; TCB, 3,4,3’,4’-tetrachlorobiphenyl; CYP1A5 and CYP1A4, CYP1A enzymes isolated from TCDD-treated chick embryos previously known as TCDD1 and TCDD2, respectively (11); AAH, aryl hydrocarbon hydroxylase; AA, arachidonic acid.

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the effect of TCB in stimulating reconstituted UROX activity. We also investigated the dose-response relationships for the induction of UROX and EROD in chick embryo liver microsomes and cultured hepatocytes and compared the induction of UROX and EROD in chick embryo liver and kidney.

Materials and Methods

Materials. Sources of chemicals were as follows: 3-methylcholanthrene, ketocozanole and dilaurylphosphadlycholines, Sigma Chemical (St Louis, MO); 3,4,3',4'-tetrachlorobiphenyl (TCB) and 3,4,5,3',4',5'-hexachlorobiphenyl, Ultrascietific (North Kingston, RI); dimethyl sulfoxide, Fluka (Haupt- page, NY); uroporphyrin, Porphyrin Products (Logan, UT). The CYP enzymes, CYP1A5 and CYP1A4, were purified from the livers of TCDD-treated em- byros as described previously (9, 11). Rat liver NADPH-cytochrome P450 reductase used for the reconstitution studies was purified as described (9). Recombinant rat liver NADPH-cytochrome P450 reductase used for micro- some supplementation was a gift from Drs. C. Fisher and R. Estabrook, University of Texas Southwestern Medical Center, Dallas, TX.

Chick Embryo Hepatocyte Cultures. Cultures were prepared as described previously (14). Microsomes were prepared by centrifuging sonicates of the cells at 10,000g for 10 min, and then the supernatant was centrifuged at 100,000g for 60 min. The pellet was resuspended in 0.25 M sucrose/0.05 M HEPES/1 mM EDTA, pH 7.4 and stored at −60°C. There was no loss of activity for up to 6 months.

Treatment of Animals. Twelve-day-old chickens were treated by intraper- itoneal injection with 3-methylcholanthrene (50 mg/kg) in corn oil (20 mg/ml). After 48 hr, chickens were killed by decapitation and liver and kidney micro- somes were prepared by differential centrifugation as described (4). Sixteen- day-old chick embryos were treated with increasing doses of TCB in 0.012 ml acetone pipetted through a hole in the shell onto the air sac or were treated with 3-methylcholanthrene (0.5 mg/egg) in dimethyl sulfoxide (0.1 ml) injected into the embryo. After 48 hr, liver microsomes were prepared and stored as previously described (4). Each set of data presented reflects experiments performed at least twice.

Uroporphyrinogen Oxidation (UROX) by Microsomes or Purified
P450s. UROX activity was monitored by appearance of uroporphyrin fluores- cence at room temperature in a Perkin-Elmer 650–10S spectrophotofluorim- eter (excitation wavelength, 400 nm; emission wavelength, 618 nm) with excitation and emission slit widths at 3.5 nm and 20 nm, respectively (2). Uroporphyrinogen I was used as a standard. The assay mixture contained 0.21 ml of assay buffer (0.25 M sucrose/0.05 M HEPES/1 mM EDTA, pH 7.4), 0.032 ml of a microsomal suspension or reconstitution complex containing 32 pmol of cytochrome P450, 0.005 ml of a NADPH-generating system (125 mg of sodium isocitrate, 40 mg of nicotinamide, 35 mg of NADPH, 70 mg of MgCl2, 0.6H2O, and 0.2 ml of isoteric dehydrogenase (10 units) dissolved in 0.75 ml of assay buffer) and TCB at 7–14 μM, in a total volume of 0.25 ml. The assay mixture was essentially the same as in the UROX assay, except for the addition of 1 mg bovine serum albumin/ml (final concentration) (2). The reaction was started by the addition of 0.003 ml of 7-ethoxyresorufin in DMSO to give a final concentration of 1 μM, and the fluorescence was measured for 2–3 min. Resorufin was used as a standard. The data for reconstituted EROD presented in fig. 2 were deter- mined previously (11) using the same purified P450 preparations used in the present study.

Other Assays. Uroporphyrin in cultured cells was measured spectrofluorim- etrically (15). Protein concentrations were determined by the method of Lowry et al. (16), using bovine serum albumin as a standard. Cytochrome P450 concentrations were determined by the method of Omura and Sato (17).

Results

Stimulation of UROX Activity in Chick Embryo Liver Micro- somes. Fig. 1 shows that treatment of chick embryos with the Ah receptor ligand, 3-methylcholanthrene, increased liver microsomal UROX activity 3-fold as compared with the untreated controls. In- clusion of TCB in the reaction mixtures had no effect on the UROX activity of the control microsomes but stimulated UROX by 8-fold in microsomes from 3-methylcholanthrene-treated chick embryos. The degree of UROX induction and the effects of TCB are similar to results previously reported (2, 6, 7). The mechanism of the enhance- ment of induced UROX by TCB is not understood (18).

UROX Activity of Purified Chick CYP1A5 and CYP1A4. Fig. 2 compares the ability of the two purified chick enzymes to carry out UROX activity in reconstitution assays. CYP1A5 was eight times more active than CYP1A4 in catalyzing UROX activity. In contrast, the EROD activity of the same preparation of CYP1A5 was less than 5% of the activity of CYP1A4 (11).

Table 1 shows that CYP1A5 was eight times more active than CYP1A4 in catalyzing UROX activity in the presence of TCB and six times more active in the absence of TCB. The addition of TCB increased UROX catalyzed by reconstituted CYP1A5 by 3-fold and by CYP1A4 by 2.6-fold, indicating that the activation of UROX by TCB in microsomes could be a direct effect on the enzyme. Further- more, the preferential activity of CYP1A5 in UROX activity seemed to be independent of the activation of that reaction by TCB. Keto- cozaole, a nonspecific CYP inhibitor, decreased CYP1A5-catalyzed UROX activity by 94% and CYP1A4-catalyzed UROX activity by 89%, supporting the involvement of a cytochrome P450 rather than the reductase in each reaction. Consistent with these findings, UROX activity in the reconstituted system in the absence of added P450 was less than 5% of that in the presence of P450 (data not shown).

Dose Responses for Induction of UROX and EROD in Chick
Embryo Liver and Cultured Chick Hepatocytes. Treatment of chick embryos in ovo with TCB produced almost identical dose- dependent increases in hepatic microsomal UROX and EROD (r² = 0.98) (fig. 3A). In other experiments using 3-methylcholanthrene as an inductor, nearly superimposable dose response relationships for UROX and EROD were also found (data not shown).
The dose-response relationships for induction by 3,4,5,3\textsuperscript{9},4\textsuperscript{9},5\textsuperscript{9}\textsuperscript{-}-hexachlorobiphenyl, another Ah receptor ligand, in cultures of chick embryo hepatocytes were also examined. Parallel dose-response relationships were found for UROX and EROD induction (Fig. 3B) in the cultured cells, just as in chick embryo liver microsomes. In other experiments using 3-methylcholanthrene or TCB as an inducer, nearly superimposable dose response relationships for UROX and EROD were also found (data not shown).

Effect of Added NADPH Cytochrome P450 Reductase on Microsomal UROX Activity. It was shown previously that in sonicates of cultured hepatocytes the addition of NADPH cytochrome P450 reductase increased CYP1A5 mediated arachidonic acid metabolism \textsuperscript{11}-fold, but EROD activity only by 20\% (19) and that the addition of extra reductase to microsomes increased tamoxifen hydroxylation about 3-fold (12). To exclude the possibility that the results of the dose-response experiments were being affected by limitations in reductase, we examined the effects of added reductase on UROX and EROD activities in liver microsomes of 3-methylcholanthrene-treated chick embryos. Only small effects, an increase of 40\% in UROX and a decrease of 20\% in EROD, were observed, indicating that UROX activity, unlike some other CYP1A5-mediated activities, was not much affected by extra reductase. In addition, the limitations in reductase activity did not affect the dose-response results.

Induction of UROX and EROD in Chicken Liver and Kidney. Previous studies showed that CYP1A5 and CYP1A4 were both increased in liver and kidney by Ah receptor ligands. The capacity of 3-methylcholanthrene to induce UROX activity in kidney of 12-day old chickens was also examined. Table 2 shows that treatment of chickens with 3-methylcholanthrene increased UROX and EROD in kidney as well as in liver. The activities and the degree of increase were lower in kidney than liver, as previously observed for CYP1A5.

**Fig. 2.** Reconstitution of uroporphyrinogen oxidation (UROX) (A) and 7-ethoxyresorufin O-deethylase (EROD) (B) catalyzed by purified chicken CYP1A5 and CYP1A4.

Details of the assays for reconstituted purified CYP enzymes are described in Materials and Methods. For UROX, the reaction mixture contained 7 \(\mu\)M TCB.

**TABLE 1**

<table>
<thead>
<tr>
<th>Purified P450</th>
<th>Uroporphyrinogen Oxidation\textsuperscript{a}</th>
<th>No addition</th>
<th>TCB\textsuperscript{b}</th>
<th>TCB + Ketoconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmole uroporphyrin/min/nmol P450</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1A5</td>
<td>64</td>
<td>205</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>CYP1A4</td>
<td>10</td>
<td>26</td>
<td>3</td>
<td></td>
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\textsuperscript{a}Uroporphyrinogen oxidation was assayed in duplicate as described in Methods (range of values \textless;10\%).

\textsuperscript{b}TCB concentration was 14 \(\mu\)M and ketoconazole 50 \(\mu\)M.

The dose-response relationships for induction by 3,4,5,3\textsuperscript{9},4\textsuperscript{9},5\textsuperscript{9}\textsuperscript{-}-hexachlorobiphenyl, another Ah receptor ligand, in cultures of chick embryo hepatocytes were also examined. Parallel dose-response relationships were found for UROX and EROD induction (Fig. 3B) in the cultured cells, just as in chick embryo liver microsomes. In other experiments using 3-methylcholanthrene or TCB as an inducer, nearly superimposable dose response relationships for UROX and EROD were also found (data not shown).

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**Fig. 3.** Dose responses for effects of planar halogenated biphenyls on induction of UROX and EROD in livers of 15-day-old chick embryos (A) and in hepatocyte cultures (B) prepared from 15-day-old chick embryos.

*A* Chick embryo liver microsomes: Increasing doses of 3,4,3\textsuperscript{9},4\textsuperscript{9},5\textsuperscript{9}-tetrachlorobiphenyl (TCB) were administered in 0.012 ml acetone on the air sac of each embryo. After 18 hr, liver microsomes were prepared. Each point represents the mean of duplicate assays of pools of 5–6 livers with the range indicated by a vertical bar. Where no bar is indicated the range fell within the symbol. (\(\odot\)) UROX; (\(\bigtriangleup\)) EROD. B. Chick hepatocyte cultures: Cultures were prepared in 10-cm dishes as described in Methods. Cultures were treated on the second day of the culture with increasing concentrations of 3,4,5,3\textsuperscript{9},4\textsuperscript{9},5\textsuperscript{9}-hexachlorobiphenyl (HCB) for 18 hr. Cells from three dishes were pooled for each HCB concentration. The 100,000 g membrane fractions were prepared and assayed for UROX and EROD activities as described in Materials and Methods. UROX activity in untreated cells was 4.6 pmol URO/min/mg protein and this value has been subtracted from the values obtained for the treated cells. (\(\odot\)) UROX; (\(\bigtriangleup\)) EROD.
and CYP1A4 in chick embryo kidney (11). The ratio of induced EROD:UROX was 21:1 in liver and 8:1 in kidney, indicating that the relative proportions of CYP1A4 and CYP1A5 differ in liver and kidney and that there was relatively more CYP1A5 in kidney than in liver. The low EROD activity in kidney was not a result of limiting reductase since cytochrome c reductase activities of liver and kidney microsomes were comparable (0.14 and 0.11 nmol cytochrome c per micromole P450). Nor was it a result of a lower affinity for the substrate since the Km's for ethoxyresorufin of liver and kidney microsomes were about the same (0.29 and 0.21 μM, respectively.)

**Discussion**

Although TCDD induces two CYP1A enzymes in avian as well as in mammalian liver, the amino acid sequences of the chick enzymes do not permit either form to be classified as a 1A1 or a 1A2 ortholog (13). Nevertheless, CYP1A4 seems to exhibit more CYP1A1-like activities based on its selectivity for EROD and AHH while the other form, CYP1A5, seems to behave in some, but not all respects, more like CYP1A2 (11). Thus CYP1A5 shares an immunologic epitope form, CYP1A5, seems to behave in some, but not all respects, more active in estradiol like CYP1A2 (11). Thus CYP1A5 shares an immunologic epitope in mammalian liver, the amino acid sequences of the chick enzymes is isolated in a low spin iron state and is insensitive to induction respects in which CYP1A5 does not behave like CYP1A2. CYP1A5 exhibits the CYP1A2-selective activity, UROX. Notably, the relative proportions of CYP1A5 and CYP1A4 shown here, 8:1, were the same as found for mouse CYP1A2 and 1A1 (4). Considering that CYP1A5 and CYP1A4 are present in approximately equal amounts in microsomes from TCDD-treated chick embryos (11), our findings lead to the conclusion that CYP1A5 is the main CYP enzyme responsible for the induced UROX expressed in chick embryo liver microsomes. CYP1A5 does not appear to be a CYP1A2-like ortholog. Its amino acid sequence is more like CYP1A1 than 1A2 (13), and there are some respects in which CYP1A5 does not behave like CYP1A2. CYP1A5 is isolated in a low spin iron state and is insensitive to induction by isosafrole (11).

The finding that UROX and EROD were co-induced in the studies with both chick embryo microsomes and cultured cells (fig. 3) further distinguishes the avian CYP1A enzymes from mammalian CYP1A1 and 1A2. The mammalian enzymes exhibit different dose responses to various Ah receptor ligands in vivo (20–22). The coincident dose responses for EROD and UROX found here are in agreement with the findings for TCDD-induction of EROD and AA epoxygenation in liver of chick embryos (11) and suggest that the regulation of the two TCDD-induced chicken CYP1A enzymes differs in some respects from the regulation of mammalian CYP1A1 and 1A2.

Similarly, the finding of induced UROX activity in kidney from 3-methylcholanthrene-induced chickens is not characteristic of mammalian CYP1A2. CYP1A2 is scarcely expressed in rat kidney (2). The expression in chicken kidney of induced UROX is consistent with the observations that CYP1A3-mediated arachidonic acid epoxygenation is expressed in chick embryo kidney as well as liver (11). The expression of CYP1A5 in kidney and the evidence for relative differences in the amounts of CYP1A5 and CYP1A4 in liver and kidney provide further evidence supporting differences in the regulation of mammalian CYP1A1 and 1A2 and avian CYP1A4 and CYP1A5.

Both CYP1A5 and CYP1A4 were shown previously to be expressed and catalytically active in cultured chick embryo hepatocytes using arachidonic acid metabolism for CYP1A5 and EROD activity for CYP1A4 (19). The present results confirm the presence of both TCDD-induced enzymes in primary chick hepatocyte cultures, using EROD as an index of CYP1A4 and UROX as an index of CYP1A5. The results indicate that uroporphyrin accumulation in these cultures after treatment with Ah receptor ligands (15) was due to the ability of CYP1A5 to catalyze UROX and not to a single form of 3-methylcholanthrene-induced P450 which catalyzed both EROD and UROX, as was thought previously (2). The fact that chick embryo liver cultures express both avian CYP1A enzymes underscores the utility of the chick embryo culture system to study reactions involving both forms of P450 as well as the regulation of both forms. In contrast, primary rat hepatocytes, unlike chick embryo hepatocytes, do not accumulate uroporphyrin when treated with CYP1A inducers (7) because in the rat cultures CYP1A2 protein is not expressed (23). Mouse hepatocyte cultures accumulate uroporphyrin, but only after several days of exposure to CYP1A inducers, coincident with increases in CYP1A2 (24, 25). TCB-induced accumulation is also found in a fish hepatoma line (26), indicating that those cells express a P450 which can catalyze UROX.

In summary, chicken CYP1A5 preferentially catalyzes UROX, an activity which has been previously shown to be a CYP1A2 activity in mammals. Thus, even though there are structural differences among mammalian and avian CYP1A enzymes, there is some conservation of the division of catalytic activities across different vertebrate classes. The challenge is to understand the basis for the division of function among CYP1A enzymes, and in particular, the selective relegation of UROX activity, an apparently toxic response, to one of two CYP1A enzymes.

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**References**


12. D. Kupfer, C. Mani, C. A. Lee, and A. B. Rifkind: Induction of tamoxifen-4-hydroxylase by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), β-naphthoflavone (βNF), and phenobarbital (PB) in avian liver: identification of P450TCDD α as catalyst of 4-hydroxylation induced by TCDD and βNF. *Cancer Res.* 54, 3140–3144 (1994).


27. H. G. Morrison, M. F. Oleksiaik, N. W. Cornell, M. L. Sogin, and J. J. Stegeman: Identification of cytochrome P450 1A (CYP1A) genes from two teleost fish, toadfish (Opsanus tau) and Scup (Stenotomus chrysops), and phylogenetic analysis of CYP1A genes. *Biochem. J.* 308, 97–104 (1995).