0026-895X/91/050686-06$03.00/0

MOLECULAR PHARMACOLOGY, 40:686-691

Inducible Bilirubin-Degrading System of Rat Liver Microsomes: Role of Cytochrome P450IA1

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Received May 23, 1991; Accepted August 6, 1991

SUMMARY

The ability of 3,3′,4,4′-tetrachlorobiphenyl to stimulate bilirubin degradation by liver microsomes from rats treated with a polycyclic aromatic hydrocarbon-type inducer has been confirmed and extended to another planar biphenyl, 3,3′,4,4′,5,5′-hexachlorobiphenyl. The following evidence indicates the involvement of an inducible cytochrome P450 isoenzyme in this reaction, with a role, specifically, for cytochrome P450IA1. (a) The biphenyl-dependent bilirubin degradation and 7-ethoxyresorufin *O*-deethylase (EROD) activity were both markedly inhibited by a monoclonal antibody raised against cytochrome P450IA1; the two dose-inhibition curves were essentially superimposable, with maximum inhibition observed for both activities at a ratio of antibody to total cytochrome P450 of about 1. (b) Treatment of rats with 3-methylcholanthrene increased both EROD activity and biphenyl-dependent bilirubin degradation not only in the liver (where both cytochromes P450IA1 and P450IA2 are inducible) but also in the kidney (where only induction of cytochrome P450IA1 has been reported), with similar ratios of the two enzymatic activities in both tissues. (c) With carbon tetrachloride and 3,5-diethoxycarbonyl-4-ethyl-1,4-dihydro-2,4-dimethyl pyridine as selective suicide substrates of members of the cytochrome P450IA subfamily, the biphenyl-dependent degradation of bilirubin showed a good correlation with cytochrome P450IA1, determined both as EROD activity and as an immunoreactive band on immunoblotting. These findings implicate cytochrome P450IA1 in the alternative pathway of bilirubin disposal, which can be stimulated by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in Gunn rats, and also help substantiate the hypothesis that interaction of a polyhalogenated aromatic compound with the induced cytochrome may initiate an oxidative mechanism leading to oxidation of target molecules in the cell, one of which is bilirubin.

We have recently described (1) a bilirubin-degrading system, in the hepatic microsomal fraction, that can be induced by treatment with TCDD *in vivo* and markedly stimulated by addition of a planar polyhalogenated aromatic compound (3,4-TCB) to the microsomes *in vitro*. Addition of 3,4-TCB also caused a dose-dependent inhibition of the EROD activity of the microsomal fraction from induced animals, suggesting that tight binding of the chemical to the active site of the induced cytochrome P450 might be responsible for both loss of *O*-deethylase activity and production of a bilirubin-degrading species. These findings provide a plausible mechanism for the alternative pathway of bilirubin metabolism that can be activated by TCDD treatment (2, 3) in congenitally jaundiced (Gunn) rats. However, direct evidence that cytochrome P450 is involved in these effects and that the fate of bilirubin in microsomal incubations is similar to that observed in Gunn rats *in vivo* has not yet been produced.

We have now obtained evidence that cytochrome P450IA1 plays an important role in bilirubin degradation, by using an inhibitory monoclonal antibody and selective suicide substrates directed against members of the cytochrome P450IA subfamily. Additional evidence for the involvement of cytochrome P450IA1 is provided by the finding that, like this isoenzyme, the bilirubin-degrading system is inducible not only in the liver but also in the kidney. The degradation of bilirubin by the microsomal fraction from induced animals *in vitro* leads to the loss of both the Soret absorbance and the intensity of the diazo reaction of bilirubin, as is observed upon TCDD treatment of jaundiced rats *in vivo* (1). In addition to identifying an alternative pathway of bilirubin disposal via cytochrome P450IA1, the potential importance of these studies lies in the demonstration that bilirubin, a readily oxidizable compound (1, 4), might be of use in monitoring a more general oxidative mechanism, which appears to be initiated by the direct interaction of polyhalogenated aromatics with cytochrome P450 (see Ref. 5 for a recent review).

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ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 3,4-TCB, 3,3′,4,4′-tetrachlorobiphenyl; 3,4,5-HCB, 3,3′,4,4′,5,5′-hexachlorobiphenyl; 3-MC, 3-methylcholanthrene; 4-ethyl-DDC, 3,5-diethoxycarbonyl-4-ethyl-1,4-dihydro-2,6-dimethylpyridine; PBS, phosphate-buffered saline; EROD, 7-ethoxyresorufin *O*-deethylase.

Materials and Methods

Animals. Male rats of the LAC:P Wistar-derived strain (body weight, 180–200 g) were kept on RM Expanded No. 3 breeding diet (obtained from W. Lillico & Son Ltd., Betchworth, Surrey, UK) and allowed food and water *ad libitum* until they were killed. Treatment with inducers involved a single intraperitoneal injection of TCDD (300 μg/kg of body weight) or 3-MC (80 mg/kg), both in corn oil, and the animals were killed 72 and 63 hr after injection, respectively. In addition to treatment with 3-MC, some rats also received, 1.5 hr before being killed, an intraperitoneal injection of CCl_4 (800 mg/kg) or 4-ethyl-DDC (100 mg/kg) in corn oil (10 ml/kg). Microsomal fractions from liver and kidney were isolated, washed, and stored frozen as described previously (1).

Analytical techniques. Protein was measured by the method of Lowry *et al.* (6), using crystalline bovine serum albumin as a standard, and cytochrome P450 content according to the method of Omura and Sato (7). Bilirubin in microsomal incubations was either monitored at 450 nm, to follow its rate of degradation, or measured (during the period of linear rate of loss or after an additional 24- or 48-hr incubation in the dark) by both its Soret absorbance and the intensity of the diazo reaction. The difference in absorbance between 450 and 550 nm was recorded for each sample, and, after addition of 0.25 ml of 1 N HCl to each incubation mixture (3.5 ml), a 1-ml portion was added to 1.25 ml of methanol and 0.25 ml of diazo reagent (8); absorbance at 542 nm was then recorded 30 min later, using as a blank a sample of identical composition from which the diazo reagent was omitted.

Sources of special chemicals and reagents. NADPH (type III), purified control mouse IgG, 3,4,5-HCB, and bilirubin were obtained from Sigma Chemical Co. (Poole, Dorset, UK); 7-ethoxyresorufin was from Pierce Chemical Co. (Rockford, IL); 3,4-TCB was from Ultra Scientific (Hope, RI); and TCDD and 4-ethyl-DDC were gifts from Dr. J. B. Greig and Mr. A. H. Gibbs, respectively, (both of the MRC Toxicology Unit, Carshalton, UK), and were synthesized by methods previously described (9, 10). Cytochrome P450IA1 was purified from the liver of rats treated with 3-MC (11) and was a gift from Dr. R. J. Edwards (Royal Postgraduate Medical School, London, UK). Monoclonal antibody 12/2/3/2, raised against rat cytochrome P450IA1, but reacting with both cytochromes P450IA1 and P450IA2 (11), and purified by fast protein liquid chromatography (12), was used in the microsomal inhibition studies and immunoblotting experiments described below.

Experiments with isolated microsomal fractions. The rate of metabolism of bilirubin by isolated microsomal fractions was studied as described previously (1), using EDTA (2 mM final concentration) in all experiments; liver microsomes corresponding to a concentration of cytochrome P450 of 65 pmol/ml of incubation mixture were used, except in the study of the inhibitory effect of the monoclonal antibody, when the final concentration of cytochrome P450 was 32.5 pmol/ml. Increasing concentrations of the antibody were then added, to obtain molar ratios of IgG (assumed molecular weight, 150,000) to total cytochrome P450 ranging from approximately 0.1 to 6. After incubation for 5 min, biphenyl in dimethylsulfoxide (2.8 μl/ml) was added and, 5 min later, the reaction was initiated by addition of NADPH and bilirubin. Controls received dimethylsulfoxide alone. When bilirubin degradation by kidney microsomes was studied, a final concentration of 190–220 μg of protein/ml of incubation mixture was used. The EROD activity of liver and kidney microsomes was measured by a modification (1) of the method of Burke and Mayer (13), using a final concentration of 7-ethoxyresorufin of 2 μM and similar IgG/cytochrome P450 molar ratios as used in the bilirubin degradation study for the antibody inhibition experiment.

Immunoblot analysis of microsomal fractions from induced animals was performed essentially as described by Towbin *et al.* (14). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15), using 10% (w/v) acrylamide in the gel, the samples were transferred to nitrocellulose membranes. After blocking with PBS containing 5% horse serum and 0.25% gelatin (two incubations, 30 min each), the membranes were incubated for 3 hr with 100 μg of monoclonal antibody, in the aforementioned "blocking" PBS solution. The membranes were then washed and incubated with a goat anti-mouse IgG-alkaline phosphatase conjugate, followed by staining with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris, pH 9.5, containing 100 mM NaCl and 50 mM $MgCl_2$. The intensity of the stained bands was then quantified using an LKB 2202 Ultrascan laser densitometer, with attached LKB 2220 recording integrator, and their electrophoretic mobility was compared with that of authentic cytochrome P450IA1.

Results and Discussion

Stimulation of bilirubin degradation *in vitro* by planar polyhalogenated biphenyls and effect of an antibody directed against cytochrome P450IA1. When the hepatic microsomal fraction from TCDD-treated rats was incubated with bilirubin in the presence of NADPH, there was a loss of bilirubin, which could be significantly stimulated by the addition of a planar biphenyl. However, the rate of bilirubin degradation by microsomes from control animals was insensitive to the addition of the biphenyl (Table 1). This confirms previous findings and extends the stimulary effect of 3,4-TCB, already reported (1), to another planar biphenyl, namely 3,4,5-HCB.

Additional experiments were carried out with microsomes from TCDD-treated rats, to ascertain whether their bilirubin-degrading activity was susceptible to inhibition by a monoclonal antibody raised against cytochrome P450IA1. Table 2 shows that the purified antibody inhibited the bilirubin-degrading activity of microsomes from induced animals in the presence

TABLE 1

Cytochrome P450 content, EROD activity, and bilirubin-degrading activity of liver microsomes from control and TCDD-treated rats

Male rats either were untreated controls or were treated with TCDD, as described in Materials and Methods. Hepatic microsomal fractions were isolated for the estimation of total cytochrome P450 content, EROD, and bilirubin-degrading activity, the latter in the absence and presence of a planar polyhalogenated biphenyl. Results are given as means ± standard errors of the number of observations in parentheses.

Treatment *in vivo*	Cytochrome P-450	EROD activity	Bilirubin-degrading activity	
			Compound added	Activity
	nmol/mg of protein	*pmol/min/mg of protein*		*pmol/min/mg of protein*
None	0.63 ± 0.03 (3)	13.7 ± 1.3 (3)	None	315 ± 11 (3)
			3,4-TCB (1 μg)	333 ± 8 (3)
TCDD	1.55 ± 0.06 (9)[a]	6390 ± 570 (8)[a]	None	550 ± 84 (6)
			3,4-TCB (1 μg)	1174 ± 94 (7)[b]
			3,4,5-HCB (1 μg)	1533 ± 60 (3)[b]

[a] $p < 0.001$, compared with corresponding values obtained with control microsomes.
[b] $p < 0.001$, compared with corresponding values obtained without a halogenated biphenyl.

TABLE 2

Effect of monoclonal antibody 12/2/3/2 or control mouse IgG on the bilirubin-degrading activity of liver microsomes from TCDD-treated rats

Hepatic microsomal fraction from TCDD-treated animals, corresponding to 114 pmol of cytochrome P450, was used to measure bilirubin-degrading activity in the absence or presence of a planar polyhalogenated biphenyl. Fifty micrograms of either the purified monoclonal antibody (MAb) or control mouse IgG were added, where indicated, dissolved in PBS; whereas PBS alone was added in the corresponding controls. A different batch of induced microsomes was used in each experiment. Results given are means ± standard errors of the number of observations in parentheses or are means with individual observations in parentheses.

	Compound added	Further addition	Rate of bilirubin degradation		
			A. In the absence of the biphenyl	B. In the presence of the biphenyl	C. Effect of the biphenyl (B − A)
			pmol/min/mg of protein		
Experiment 1	3,4-TCB (1 μg)	PBS	507 ± 46 (3)	1650 ± 60 (3)	1143
		MAb	484 ± 59 (3)	777 ± 49 (4)*	293
Experiment 2	3,4,5-HCB (1 μg)	PBS	369 (341, 398)	1899 ± 155 (4)	1530
		MAb	339 (328, 350)	992 ± 64 (3)*	653
Experiment 3	3,4-TCB (1 μg)	PBS	766 ± 26 (3)	1365 ± 66 (3)	599
		Control IgG	746 ± 32 (3)	1228 ± 16 (3)	482

*$p < 0.001$, compared with corresponding values with PBS.

of either 3,4-TCB or 3,4,5-HCB. In contrast, control mouse IgG was virtually without effect. It is important to note that the basal rate of bilirubin degradation, i.e., in the absence of the biphenyl, was not inhibited by the antibody. Thus, the antibody was selective for the component of bilirubin degradation stimulated by the biphenyls. To exclude the possibility that the antibody merely inhibited the uptake of biphenyl by the microsomes, in one experiment the order of addition of the antibody and of the biphenyl was inverted (with the biphenyl being added first), but the same degree of inhibition of bilirubin degradation was observed.

The effects of the antibody on EROD activity and on biphenyl-dependent bilirubin-degrading activity of microsomes from TCDD-treated animals were compared at the same antibody/cytochrome P450 molar ratios. The two dose-inhibition curves were essentially superimposible, with maximum or near-maximum inhibition at a ratio of antibody to total cytochrome P450 of about 1 (Fig. 1) and with approximately 20% of activity being resistant to inhibition in both cases.

The monoclonal antibody used in this work was also tested

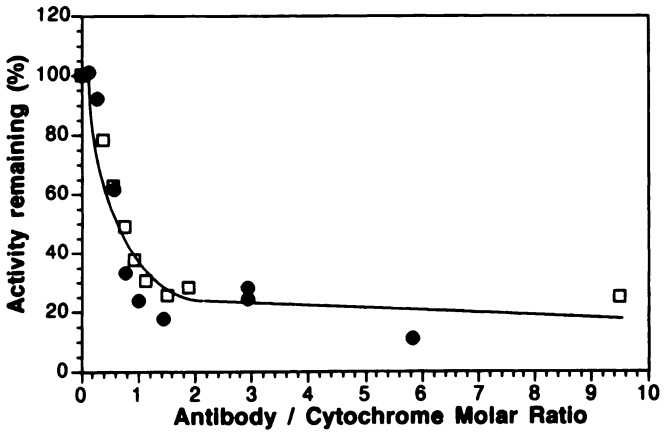

Fig. 1. Inhibition of EROD and of 3,4-TCB-dependent bilirubin degradation activities of hepatic microsomes from TCDD-treated rats caused by addition of monoclonal antibody 12/2/3/2. The two enzymatic activities were assayed as described in Materials and Methods, in the presence of increasing amounts of monoclonal antibody, so as to achieve the antibody to cytochrome molar ratios indicated. EROD activity (□) and 3,4-TCB-dependent bilirubin degradation (●) have both been expressed as a percentage of the corresponding values obtained in the absence of the antibody.

with liver microsomes obtained from chick embryos treated with β-naphthoflavone, but in this case neither the EROD activity nor the biphenyl-induced stimulation of bilirubin degradation were significantly inhibited (results not shown); therefore, the inhibitory effect of the antibody must involve recognition of a rat-specific epitope that is essential to the catalytic activity of the isoenzyme involved. The results of these studies do not, however, clarify whether cytochrome P450IA1 or cytochrome P450IA2 (a closely related member of the same subfamily that is also induced in rat liver by TCDD, β-naphthoflavone, and 3-MC) is involved in bilirubin degradation. This is because the antibody recognizes both isoforms, towards which it exhibits very similar binding affinities (11); thus, even though the inhibition of bilirubin degradation correlated closely (Fig. 1) with inhibition of EROD activity [a reaction catalyzed preferentially by cytochrome P450IA1 (16–19)], it was still possible that cytochrome P450IA2 contributed to the loss of bilirubin.

Bilirubin-degrading activity of kidney microsomes. In order to ascertain which of the two possible isoenzymes of cytochrome P450 was involved in the 3,4-TCB-dependent bilirubin degradation, studies were performed using kidney microsomes from induced animals. It has been shown previously that treatment of rats with polycyclic aromatic hydrocarbons, for example 3-MC, induces both cytochromes P450IA1 and P450IA2 in the liver but only cytochrome P4501AI in the kidney (20, 21). 3-MC treatment of rats increased both EROD activity and 3,4-TCB-dependent bilirubin degradation not only in the liver but also in the kidney, with similar ratios of the two enzymatic activities in both tissues (Table 3), thus implicating cytochrome P450IA1 in both reactions. In addition, as previously reported for TCDD- and β-naphthoflavone-induced rat liver microsomes (1), 3,4-TCB was found to inhibit markedly EROD activity of both liver and kidney microsomes from 3-MC-treated rats. This suggests that, if tight binding of 3,4-TCB to cytochrome P450IA1 is responsible for both loss of EROD activity and production of a bilirubin-degrading species, then this applies not only to liver microsomes, as previously reported (1), but also to induced kidney microsomes. These findings clearly implicate cytochrome P450IA1 and would appear to exclude a role for cytochrome P450IA2 in the 3,4-TCB-dependent bilirubin degradation.

Effect of suicide substrates of cytochrome P450 and

TABLE 3

EROD and bilirubin-degrading activities of hepatic and renal microsomes isolated from control rats and from rats treated with 3-MC

Male rats either were untreated controls or were treated with 3-MC, as described in Materials and Methods. Microsomes were prepared from liver and kidneys, and their EROD and bilirubin-degrading activities were determined *in vitro*, both in the absence and in the presence of 3,4-TCB, as indicated below. Amounts of 3,4-TCB added were 200 ng and 1 μg per total incubation in the case of EROD and bilirubin-degrading system, respectively. Results are given as means ± standard errors of the number of observations in parentheses. Note that, after 3-MC treatment, the ratios of 3,4-TCB-dependent bilirubin degradation to EROD activity are very similar in liver and kidney microsomes.

Tissue	Pretreatment of rats *in vivo*	Rate of bilirubin degradation			EROD activity	
		A. In the absence of 3,4-TCB	B. In the presence of 3,4-TCB	C. Effects of 3,4-TCB (B − A)	A. In the absence of 3,4-TCB	B. In the presence of 3,4-TCB
		pmol/min/mg of protein			*pmol/min/mg of protein*	
Liver	None	315 ± 11 (3)[a]	333 ± 8 (3)[a]	18	13.7 ± 1.3 (3)[a]	
	3-MC	505 ± 20 (4)	1300 ± 46 (4)	795	7724 ± 190 (4)	1550 ± 59 (3)[b]
Kidney	None	102.9 ± 4.5 (4)	101.8 ± 5.7 (4)	0	5.6 ± 0.8 (6)	
	3-MC	72 ± 2.9 (4)	122.9 ± 3.3 (4)[c]	50.9	473 ± 15 (7)	74.7 ± 3.9 (4)[c]

[a] Data from Table 1.
[b] $p < 0.01$, compared with corresponding value obtained in the absence of 3,4-TCB.
[c] $p < 0.001$, compared with corresponding value obtained in the absence of 3,4-TCB.

evidence for diazo-negative products of bilirubin. The effects of selective suicide substrates of cytochrome P450 were investigated. 4-Ethyl-DDC has been reported to inhibit selectively hepatic cytochrome P450IA1, causing pronounced depression of EROD activity and loss of immunoreactive cytochrome P450IA1, while having little (22) or no (23) effect on the apoprotein of cytochrome P450IA2. Carbon tetrachloride shows the opposite selectivity, causing marked loss of hepatic cytochrome P450IA2 and associated monooxygenase activity while leaving unchanged the amount and activity of cytochrome P450IA1 (24, 25).

Administration of carbon tetrachloride or 4-ethyl-DDC to rats pretreated with 3-MC resulted in a loss of total liver cytochrome P450, although this was far greater with 4-ethyl-DDC than with carbon tetrachloride (Table 4). 4-Ethyl-DDC also caused complete loss of EROD activity and of 3,4-TCB-dependent bilirubin degradation. In contrast, neither EROD activity nor 3,4-TCB-dependent bilirubin degradation was decreased following carbon tetrachloride treatment; indeed, when corrected for cytochrome P450, both activities were slightly greater than the corresponding control values (Table 4), suggesting selective enrichment of the responsible isoform (presumably cytochrome P450IA1).

Immunoblot experiments showed that carbon tetrachloride caused a significant loss of immunoreactive cytochrome P450IA2, whereas cytochrome P450IA1 was not affected. In contrast, 4-ethyl-DDC caused a decrease in the intensity of the cytochrome P450IA1 band (which may have also been slightly retarded on electrophoresis), whereas the band corresponding to cytochrome P4501A2 was significantly increased in intensity (Fig. 2). These results generally confirm previous observations (23, 25), although the increased intensity of the cytochrome P450IA2 band and retardation of the cytochrome P450IA1 band (both after 4-ethyl-DDC) have not previously been reported.

Thus, the 3,4-TCB-dependent degradation of bilirubin showed a good correlation with cytochrome P450IA1, determined both as EROD activity and as an immunoreactive band on Western blotting. It is apparent from Table 4 that this correlation applied only to that portion of bilirubin degradation that was stimulated by 3,4-TCB. Whereas both EROD activity and 3,4-TCB-dependent bilirubin degradation were completely abolished by 4-ethyl-DDC (Table 4), the loss of immunoreactive cytochrome P450IA1, although significant, was only partial (Fig. 2). The most likely explanation for this is that, because the preferential target of 4-ethyl-DDC is the heme prosthetic group of the cytochrome (10), any remaining cytochrome P450IA1 apoprotein will be devoid of heme and, hence, unable to catalyze either enzymatic activity.

Some of the reaction mixtures (Table 4) were analyzed, either during the initial linear rate of bilirubin loss or after a subsequent incubation period in the dark (so as to obtain a wide range of bilirubin values), for loss of Soret absorbance and of the typical diazo reaction of bilirubin. Fig. 3 shows that there was a good correlation between Soret absorbance and intensity

TABLE 4

Effect of treatment of 3-MC-induced rats with either carbon tetrachloride or 4-ethyl-DDC on total cytochrome P-450, EROD activity, and bilirubin-degrading activity of the microsomes isolated from their livers

Male rats were induced with 3-MC and then given carbon tetrachloride or 4-ethyl-DDC dissolved in corn oil, or corn alone, by intraperitoneal injection, 1.5 hr before killing. Bilirubin degradation was measured both in the absence and in the presence of 3,4-TCB (1 μg/incubation). Results are averages ± standard errors of the number of observations in parentheses.

Treatment	Cytochrome P-450	EROD activity	Rate of bilirubin degradation		
			A. In the absence of 3,4-TCB	B. In the presence of 3,4-TCB	C. Effect of 3,4-TCB (B − A)
	nmol/mg of protein	*pmol/min/nmol of cytochrome P-450*	*pmol/min/nmol of cytochrome P-450*		
Oil	1.26 ± 0.03 (7)	4960 ± 226 (5)	324 ± 40 (6)	883 ± 59 (6)	560 ± 32 (6)
Carbon tetrachloride	1.02 ± 0.02 (7)[a]	6750 ± 380 (5)[b]	256 ± 10 (5)	964 ± 35.5 (5)	708 ± 34 (5)[c]
4-Ethyl-DDC	0.31 ± 0.01 (5)[a]	Not detectable	441.7 ± 50 (3)	410.7 ± 34 (3)	0

[a] $p < 0.001$; [b] $p < 0.01$; [c] $p < 0.02$, compared with corresponding value obtained with rats given oil alone.

Fig. 2. Immunoblots obtained with hepatic microsomes from 3-MC-treated rats given, 1.5 hr before killing, either corn oil or a suicide substrate of the inducible cytochromes P450. Microsomal proteins (50 μg) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel, transferred to a nitrocellulose membrane, and blotted with antibody 12/2/3/2. The positions of cytochromes P450IA1 (*1*) and P450IA2 (*2*) (approximately M_r 57,000 and 54,000, respectively) are indicated, the former corresponding to the position of a purified cytochrome P4501A1 standard (not shown). *Lane A*, microsomes of rats given corn oil alone. Note that CCl_4 (*lane B*) caused a loss of cytochrome P450IA2, whereas 4-ethyl-DDC (*lane C*) induced a loss of cytochrome P450IA1 (with slightly different mobility) and, at the same time, an increase in intensity of the cytochrome P450IA2 band.

of the diazo reaction during both basal and 3,4-TCB-stimulated bilirubin degradation, regardless of the source of the microsomes and of whether measurements were made within the linear range of rate of bilirubin loss. In Gunn rats treated with TCDD, the decrease in plasma bilirubin is the same whether bilirubin is measured by the diazo reaction or by Soret absorbance (1); thus, the loss of bilirubin caused by microsomes in the present study resembles that *in vivo*, suggesting that the same mechanism may be involved. Loss of the absorbance of bilirubin at 450 nm and of its diazo reactivity are both encountered either (a) on oxidative fragmentation of bilirubin accompanied by bleaching [as in bilirubin photodegradation (26) and autooxidation (27)] or (b) on dehydrogenation of bilirubin to biliverdin, when a significant increase in absorbance at both 650 and 370 nm is also seen. In these present experiments, little or no increase in 650 and 370 nm absorbance was found,[2] indicating that bilirubin was mostly converted to metabolites other than biliverdin.

[2] F. DeMatteis and S. J. Dawson, Unpublished results.

General Conclusions

We have confirmed that 3,4-TCB, a planar polyhalogenated biphenyl, can stimulate bilirubin degradation by TCDD-induced rat liver microsomes and we have extended this observation to show that 3,4,5-HCB, another planar biphenyl, is also stimulatory and that stimulation occurs in both liver and kidney microsomes from rats treated with 3-MC. Pretreatment of the rats with a polycyclic aromatic hydrocarbon-type inducer is essential for this effect, presumably because synthesis must first be increased for a specific isoenzyme of cytochrome P450, which then interacts with a planar polyhalogenated biphenyl and binds it tightly, so as to produce a bilirubin-degrading species. We have now provided more direct evidence, using an inhibitory monoclonal antibody and selective suicide substrates of cytochromes P450IA1 and P450IA2, for the involvement of cytochrome P450 in this reaction, with a role, specifically, for cytochrome P450IA1. These findings provide a plausible molecular mechanism for the alternative pathway of bilirubin disposal that is activated by TCDD in the congenitally jaundiced Gunn rat (2, 3). They also help substantiate the proposal (reviewed in Ref. 5) that polyhalogenated chemicals activate a cytochrome P450-dependent oxidative mechanism that is capable of oxidizing several target molecules in the cell, among which is bilirubin. The hypothetical mechanism, modeled on that originally proposed to account for the hepatotoxicity of polyhalogenated aromatic compounds (28), is schematically represented in Fig. 4. It proposes that the polyhalogenated chemicals interact as poor substrates with the appropriate cytochrome P-450, leading to accelerated reduction of molecular oxygen, so that oxidative species that are potentially toxic are generated. Conclusive evidence that bilirubin is in fact oxidatively degraded by the microsomal monooxygenase system *in vitro* [as has been reported (2, 3) for unconjugated bilirubin in TCDD-induced Gunn rats *in vivo*] must await full characterization of the relevant breakdown products. For example, if propentdyopents (26) or other hydroxylated pyrrole derivatives

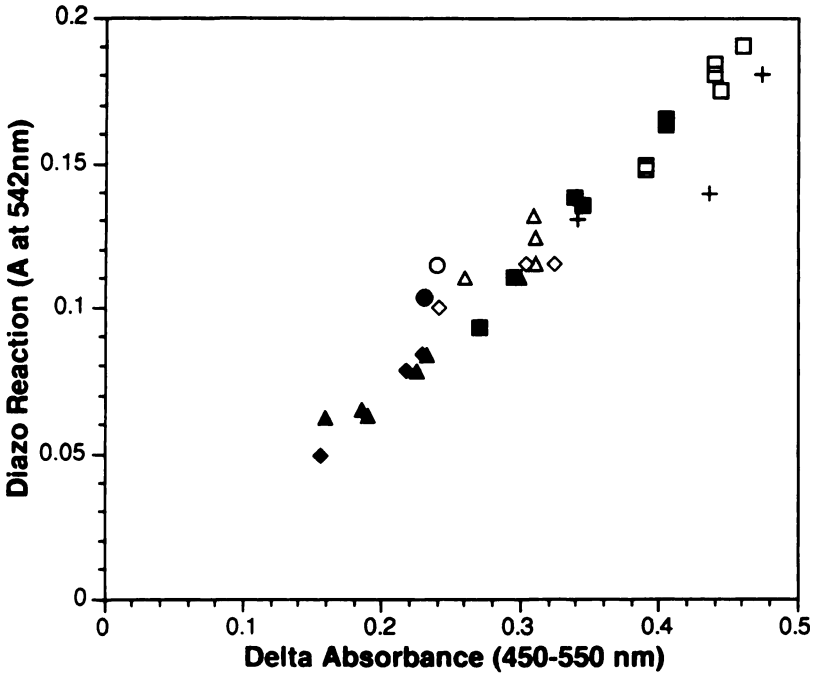

Fig. 3. Relationship between intensity of diazo reaction and Soret absorbance assays for bilirubin in bilirubin-containing incubation mixtures. Loss of bilirubin (catalyzed by microsomes from 3-MC-treated rats) was measured either during the period of linear rate of loss (*squares*) or after a subsequent incubation in the dark, with microsomes from animals treated as follows: 3-MC (*diamonds*), 3-MC and then CCl_4 (*triangles*), 3-MC and then 4-ethyl-DDC (*circles*). *Open symbols*, incubations in the absence of 3,4-TCB; *closed symbols*, results obtained in the presence of 3,4-TCB. +, Results obtained with bilirubin incubated in the absence of microsomes.

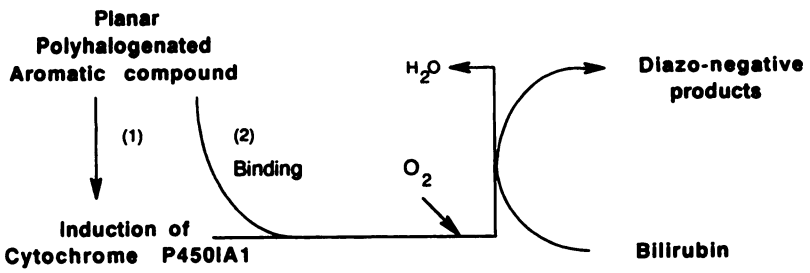

Fig. 4. Hypothetical mechanism for degradation of bilirubin by an inducible cytochrome P450IA1-dependent system. A planar polyhalogenated aromatic compound first induces cytochrome P450IA1 and then interacts with the induced isoenzyme, inhibiting its monooxygenase activity and stimulating reduction of molecular oxygen so that an oxidative species is produced. In addition to bilirubin, other molecules may serve as targets for oxidation in the cell by this mechanism, among them uroporphyrinogen and, possibly, DNA bases, with cellular iron participating, as discussed (5, 29). Note that, although hydrogen peroxide is involved in chemical and enzymic model systems for this reaction (1, 29), the oxidative species produced by the stimulated microsomal fraction has not yet been identified.

were identified as products of the reaction, this would provide strong support for an oxidative degradation mechanism.

Acknowledgments

We would like to thank Dr. J. B. Greig for treating rats with TCDD and Mr. A. H. Gibbs and Dr. R. J. Edwards for a gift of 4-ethyl-DDC and purified cytochrome P450IA1, respectively.

References

1. De Matteis, F., T. Trenti, A. H. Gibbs, and J. B. Greig. Inducible bilirubin-degrading system in the microsomal fraction of rat liver. *Mol. Pharmacol.* **35**:831–838 (1989).
2. Kapitulnik, J., and J. D. Ostrow. Stimulation of bilirubin catabolism in jaundiced Gunn rats by an inducer of microsomal mixed-function monooxygenases. *Proc. Natl. Acad. Sci. USA* **75**:682–685 (1978).
3. Cohen, A. M., J. Kapitulnik, J. D. Ostrow, and C. C. Webster. Effect of combined treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and phototherapy on bilirubin metabolism in the jaundiced Gunn rat. *Hepatology* **6**:490–494 (1986).
4. Stocker, R., Y. Yamamoto, H. F. McDonagh, A. N. Glazer, and B. N. Ames. Bilirubin is an antioxidant of possible physiological importance. *Science (Washington D. C.)* **235**:1043–1046 (1987).
5. Smith, A. G., and F. De Matteis. Oxidative injury mediated by the hepatic cytochrome P450 system in conjunction with cellular iron: effects on the pathway of haem biosynthesis. *Xenobiotica* **20**:865–877 (1990).
6. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
7. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. 1. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2370–2378 (1964).
8. Malloy, H. T., and K. A. Evelyn. The determination of bilirubin with the photoelectric colorimeter. *J. Biol. Chem.* **119**:481–490 (1937).
9. Greig, J. B., G. Jones, W. H. Butler, and J. M. Barnes. Toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Food Cosmet. Toxicol.* **11**:585–595 (1973).
10. De Matteis, F., A. H. Gibbs, and C. Hollands. *N*-Alkylation of the haem moiety of cytochrome P450 caused by substituted dihydropyridines. *Biochem. J.* **211**:455–461 (1983).
11. Sesardic, D., A. R. Boobis, J. MacQuade, S. Baker, E. A. Lock, C. R. Elcombe, R. T. Robson, C. Hayward, and D. S. Davies. Inter-relatedness of some isoenzymes of cytochrome P450 from rat, rabbit and human, determined with monoclonal antibodies. *Biochem. J.* **236**:569–577 (1986).
12. Edwards, R. J., A. M. Singleton, D. Sesardic, A. R. Boobis, and D. S. Davies. Antibodies to a synthetic peptide that react specifically with a common surface region on two hydrocarbon-inducible isoenzymes of cytochrome P450 in the rat. *Biochem. Pharmacol.* **37**:3735–3741 (1988).
13. Burke, M. D., and R. T. Mayer. Ethoxyresorufin: direct fluorimetric assay of a microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Dispos.* **2**:583–588 (1974).
14. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
15. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
16. Guengerich, F. P., G. A. Danon, S. T. Wright, M. V. Martin, and L. S. Kaminski. Purification and characterization of liver microsomal cytochrome P450: electrophoretic, spectral, catalytic and immunological properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. *Biochemistry* **21**:6019–6030 (1982).
17. Astrōm, A., and J. W. De Pierre. Metabolism of 2-acetylaminofluorene by eight different forms of cytochrome P450 isolated from rat liver. *Carcinogenesis (Lond.)* **6**:113–120 (1985).
18. Wolf, C. R., and F. Oesch. Isolation of a high spin form of cytochrome P450 induced in rat liver by 3-methylcholanthrene. *Biochem. Biophys. Res. Commun.* **11**:504–511 (1983).
19. Jacobs, J. M., P. R. Sinclair, W. J. Bement, R. W. Lambrecht, J. F. Sinclair, and J. A. Goldstein. Oxidation of uroporphyrinogen by methylcholanthrene-induced cytochrome P450. Essential role of cytochrome P-450d. *Biochem. J.* **258**:247–253 (1989).
20. Goldstein, J. A., and P. Linko. Differential induction of two 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-inducible forms of cytochrome P450 in extrahepatic versus hepatic tissues. *Mol. Pharmacol.* **25**:185–191 (1984).
21. Sesardic, D., K. J. Cole, R. J. Edwards, D. S. Davies, P. E. Thomas, W. Levin, and A. R. Boobis. The inducibility and catalytic activity of cytochrome P450c (P450IA1) and P450d (P450IA2) in rat tissues. *Biochem. Pharmacol.* **39**:499–506 (1990).
22. Tephly, T. R., K. A. Black, M. D. Green, B. L. Coffman, G. A. Dennan, and F. P. Guengerich. Effect of the suicide substrate 3,5-diethoxycarbonyl-2,6-dimethyl-4-ethyl-1,4-dihydropyridine on the metabolism of xenobiotics and on cytochrome P450 apoproteins. *Mol. Pharmacol.* **29**:81–87 (1986).
23. Riddick, D. S., S. S. Park, H. V. Gelboin, and G. S. Marks. Effects of 4-alkyl analogues of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine on hepatic cytochrome P450 heme, apoproteins, and catalytic activities following *in vivo* administration to rats. *Mol. Pharmacol.* **37**:130–136 (1990).
24. Degawa, M., A. Ohta, M. Namiki, T. Masuko, and Y. Hashimoto. *In vivo* selection of a low spin form cytochrome P-448 from 3-methylcholanthrene-induced rat cytochrome P450 isozymes by carbon tetrachloride. *Biochem. Pharmacol.* **36**:3315–3317 (1987).
25. Sesardic, D., K. J. Rich, R. J. Edwards, D. S. Davies, and A. R. Boobis. Selective destruction of cytochrome P450d and associated monooxygenase activity by carbon tetrachloride in the rat. *Xenobiotica* **19**:795–811 (1989).
26. Bonnett, R., and S. Ioannou. Phototherapy and the chemistry of bilirubin. *Mol. Aspects Med.* **9**:457–471 (1987).
27. Lightner, D. A., A. Cu, A. F. McDonagh, and L. A. Palma. On the autooxidation of bilirubin. *Biochem. Biophys. Res. Commun.* **69**:648–657 (1976).
28. De Matteis, F., and M. Stonard. Experimental porphyrias as models for human hepatic porphyrias. *Semin. Hematol.* **14**:187–192 (1977).
29. De Matteis, F. Role of iron in the hydrogen peroxide-dependent oxidation of hexahydroporphyrins (porphyrinogens): a possible mechanism for the exacerbation by iron of hepatic uroporphyria. *Mol. Pharmacol.* **33**:463–469 (1988).

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