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Effect of Cytochrome P450 Induction on the Metabolism and Toxicity of Ochratoxin A

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ABSTRACT. Liver microsomes from rats treated with various P450 inducers were examined for their ability to metabolize the mycotoxin ochratoxin A (OTA) to 4(*R*)-4-hydroxyochratoxin A (4*R*), the major metabolite, and 4(*S*)-4-hydroxyochratoxin A (4*S*), the minor metabolite. Pretreatment of rats with phenobarbital (PB), dexamethasone (DXM), 3-methylcolcanthrene (3MC) and isosafrole (ISF) greatly induced 4*R* formation. PB, DXM, 3MC, clofibrate (CLF) and ISF treatments also induced 4*S* formation. Isoniazid (INH) pretreatment primarily induced 4*S* formation. The pH optimum for 4*R* formation was found to be 6.0 with 3MC microsomes, and 6.5 with PB and DXM microsomes. For 4*S* formation, the pH optimum was 7.0. At the optimum pH (compared with pH 7.4), 4*R* formation increased 40–50% with PB and DXM microsomes but 8.0-fold with 3MC microsomes. Studies using the inhibitors metyrapone and α-naphthoflavone as well as monoclonal antibodies against various P450s suggested that at least the P450 isoforms IA1/IA2, IIB1 and IIIA1/IIIA2 are involved in 4*R* formation. Using urinary excretion of the enzymes alkaline phosphatase and γ-glutamyl transferase as an index of renal damage, we observed that pretreatment of rats with PB, which induced hepatic P450 (P450II2B1), protected against OTA nephrotoxicity, whereas cobalt-protoporphyrin IX pretreatment, which decreased P450 levels, exacerbated OTA nephrotoxicity. Our results suggest that at least P450IIB1-dependent metabolism of OTA leads to its detoxication and that OTA itself may be toxic in some circumstances or that other pathways are responsible for its activation. BIOCHEM PHARMACOL 51;3:207–216, 1996.

KEY WORDS. ochratoxin A; metabolism; P450; inducers; inhibitors; monoclonal antibodies; toxicity

OTA§, a mycotoxin consisting of a 5′-chloro-3,4-dihydro-3-methylisocoumarin moiety linked by an amide bond to L-phenylalanine (Fig. 1), is produced by some species of the genera *Aspergillus* and *Penicillium*. The occurrence of OTA in food and feed is widespread [1], and it is known to be highly toxic to animals [2–4]. The main pathological changes associated with OTA toxicity are kidney and liver damage [5, 6]. Alterations in a variety of biochemical and immunological parameters have also been observed following OTA administration [7–10]. Dietary feeding of OTA induces renal tumors in rats [11], renal and hepatic tumors in mice [12], and DNA single-strand breaks in liver, kidney, and spleen of mice [13]. OTA is a highly suspected etiological agent in the development of Balkan endemic nephropathy and urinary tract tumors on the basis of its nephrotoxicity, the persistently observed high exposure levels in food, and the elimination of other disease risk factors [14].

A number of studies have shown that OTA is metabolized by liver microsomes from special species primarily to 4*R* and to a smaller extent to the epimeric 4*S* (Fig. 1) [15–20]. OTA metabolism has also been shown to be induced by PB and/or 3MC in Wistar rats [15], DA rats and Lewis rats [20], and in other rats (strain not mentioned) [18]. Recently, Oster *et al.* [19] showed that isolated pig liver P450 fractions are able to metabolize OTA. Hietanen *et al.* [21] showed in mice that an isozyme similar to that induced by 3MC is responsible for OTA metabolism. Here we show, through the use of various P450 inducers, inhibitors and monoclonal antibodies, that OTA is metabolized in the rat mainly by P450 isoforms IA1/IA2, IIB1 and IIIA1/IIIA2. Moreover, in *in vivo* experiments, pretreatment of rats with PB, a P450 inducer, protected rats against the nephrotoxic effects of OTA, whereas pretreatment with Co-heme, which lowers P450 levels, exacerbated OTA neophrotoxicity.

MATERIALS AND METHODS

Chemicals

CLF, isocitric acid, isocitric dehydrogenase, INH, ISF, 3MC, metyrapone, NADPH, α-naphthoflavone, OTA, phenobarbital, DXM, and TBA were purchased from the Sigma Chemical Co. (St. Louis, MO). [^{3}H]OTA was obtained from Amersham, U.K. and purified by TLC (K5F silica gel, layer thickness 250

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§ *Abbreviations:* CLF, clofibrate; Co-heme, cobalt protoporphyrin IX; P450, cytochrome P450; DXM, dexamethasone; 4*R*, 4(*R*)-4-hydroxyochratoxin A; 4*S*, 4(*S*)-4-hydroxyochratoxin A; AP, alkaline phosphatase; GGT, γ-glutamyl transferase; INH, isoniazid; ISF, isosafrole; MDA, malondialdehyde; 3MC, 3-methylcholanthrene; MAb, monoclonal antibody; OTA, ochratoxin A; PB, sodium phenobarbital; TBA, 2-thiobarbituric acid; and TCA, trichloroacetic acid.

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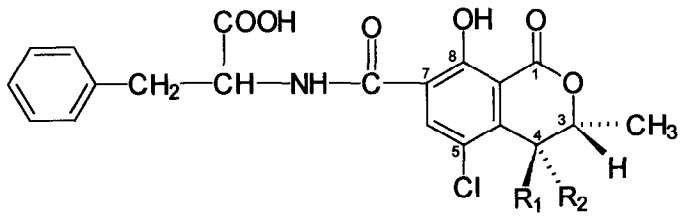

OCHRATOXIN A (OTA)	$R_1 = R_2 = H$
4 (R) - 4 - OH - OTA	$R_1 = OH$; $R_2 = H$
4 (S) - 4 - OH - OTA	$R_1 = H$; $R_2 = OH$

FIG. 1. Structure of ochratoxin A and its 4*R* and 4*S* metabolites.

μm, Whatman) using benzene:acetic acid (4:1, v/v) as eluant. OTA has an R_f of 0.47 [15]. Co-heme was obtained from Porphyrin Products (Logan, UT). All other chemicals were of the highest grade commercially available.

Animals

Male Sprague–Dawley rats (200–250 g), obtained from the Canadian Breeding Farms, Halifax, Nova Scotia, were used in all experiments. Animals were maintained on a 12-hr light and dark cycle and had free access to standard laboratory chow and water.

Treatment of Rats with P450 Inducers and Co-heme

Rats were divided into groups (N = 4/treatment group), and each group was treated with PB, 3MC, DXM, INH, ISF or CLF according to established procedures [22–27] as described in Table 1. The whole pretreatment regimen was repeated on fresh rats, and the results obtained were within 15% of those reported here. Co-heme (90 μmol/kg body wt; s.c.) was administered to rats 72 hr prior to killing [28].

Preparation of Liver Microsomes

Liver microsomes were isolated by differential centrifugation of liver homogenates as described by us earlier [29]. Isolated microsomes were resuspended in 0.1 M potassium phosphate buffer (pH 7.4) and frozen in aliquots at −80° for analysis. P450 levels were determined by the method of Omura and Sato [30]. Protein was measured as described by Lowry *et al.* [31].

Incubations

Unless otherwise indicated, incubations were carried out in duplicate or triplicate at 37° for 30 min in 0.1 M potassium phosphate buffer (pH 7.4) and contained in a total volume of 1 mL: 2 mg microsomal protein, 125 nmol OTA and an NADPH-regenerating system (consisting of 0.4 μmol $NADP^+$, 5 μmol $MgCl_2$, 5 μmol DL-isocitrate and 0.65 U isocitric dehydrogenase) [32]. At the end of 30 min, a 0.5-mL aliquot from each incubation was withdrawn for measurement of OTA metabolites, and the remaining 0.5 mL was used to measure lipid peroxidation. When only metabolites were measured, the incubation volume was 0.5 mL.

Analysis of OTA Metabolites

Aliquots (0.5 mL) were removed from incubations, and the reaction was terminated by the addition of 1 M HCl (0.1 mL) followed by saturated NaCl (0.5 mL). The mixtures were extracted with chloroform (2 × 2 mL), and the two chloroform extracts from each incubation were combined and dried under nitrogen. The residues were dissolved in 500 μL of methanol, and 50 μL of each sample was analyzed by HPLC on a Partisil 10 ODS-2 column (0.45 cm × 25 cm) using a solvent system consisting of: (i) a mixture of acetonitrile:methanol (1:1, v/v) 60%, and (ii) 5 mM sodium acetate:acetic acid (500:14, v/v) 40%. The flow rate was 1.5 mL/min. OTA and its metabolites were detected fluorimetrically at an excitation wavelength of 340 nm/emission wavelength of 465 nm (slits 5/10 nm, respectively). 4*S*, 4*R*, and OTA eluted at 4.2, 5.1, and 10.1 min, respectively [32]. Metabolites were identified and quantitated using standards that were authenticated by NMR and mass spectrophotometry.

Lipid Peroxidation

Lipid peroxidation was estimated by measuring MDA levels. For this purpose, 0.5 mL of 30% TCA and 50 μL of butylated hydroxytoluene (2% in ethanol) were added to the remaining 0.5 mL of each incubation. Finally, 0.5 mL of 50 mM TBA was added, and the mixtures were placed in a boiling water bath for 15 min. After centrifugation for 5 min at 1500 *g* (lab. top centrifuge), the absorbance of the MDA–TBA complex in the supernatant was read at 535 nm ($E_{535} = 156\ mM^{-1}cm^{-1}$) [33].

Monoclonal Antibody Experiments

Clones 1-7-1 (against P450IA1/IA2), 2-66-3 (against P450IIB1/IIB2), and 2-3-2 (against P450IIIA1/IIIA2) were used in this study. Rabbit IgG was used to determine any non-specific reaction. Microsomes (2 mg/mL) were preincubated with MAbs in 0.1 M potassium phosphate buffer (pH 7.4) at room temperature for 30 min [34] prior to initiating OTA metabolism at 37° by addition of an NADPH-regenerating system and substrate. The incubation volume was 100 μL.

In Vivo *Toxicity Experiments*

Rats (N = 6/treatment group) were administered either 0.1% PB in drinking water [22] or Co-heme (90 μmol/kg, s.c.) [28]. Control rats received no treatment. Control rats and rats on day 4 of PB treatment or 48 hr after Co-heme treatment were placed in individual metabolic cages, and urines were collected

over a 24-hr period for baseline enzyme measurements. Thereafter, Co-heme- and PB-pretreated rats received, respectively, 1.5 and 2.0 mg/kg OTA (p.o.) daily for 5 days; the corresponding control rats also received daily doses of 1.5 or 2.0 mg/kg OTA for 5 days. Urines were collected daily from individual rats for 6 days (i.e. 24 hr after the last OTA dose). After killing the rats, liver microsomes from Co-heme- and PB-treated animals had 26 and 250%, respectively, of the P450 levels of control microsomes.

Gel Filtration of Urines

Samples were centrifuged for 5 min at 2700 *g* and the clear supernatants were used for gel filtration as described by Werner *et al.* [35]. Briefly, glass columns (inner diameter 1 cm, height 30 cm) with small dead space were each filled to a height of about 18 cm with hydrated Sephadex G-50 giving a total gel bed of about 14 cm^3 in each column. Separation was performed at room temperature, and physiological saline (0.154 M NaCl) was used as eluant. Each urine sample (3 mL) was washed into a separate column with 1 mL saline followed by another 1 mL of saline. The liquid emerging from the column up to that time (5 mL) was discarded. Then, 6 mL of saline was added to each column, and the corresponding eluates were collected for enzyme analysis.

Measurement of Urinary Enzymes

AP and GGT were measured using kits (No. 104-LL and 545-A, respectively) from the Sigma Chemical Co. To avoid experimental variations due to different urine volumes, the enzyme activities were expressed as units per milligram creatinine per 24 hr (Creatinine Kit No. 555-A, Sigma Chemical Co).

[^{3}H]OTA *Distribution Experiments*

Control and PB-pretreated rats (N = 4/treatment group; as above) were treated with a single dose of [^{3}H]OTA (288 μg/kg body weight in 50 mM $NaHCO_3$; 40 μCi/kg; p.o.). Rats were kept individually in metabolic cages for 6 hr for urine collection. After ether anesthesia, rats were killed, and blood was withdrawn from the abdominal aorta. Kidneys, liver, stomach, small intestine, and cecum were excised, and the small intestine was divided into four equal parts by length. Gut segments [stomach, small intestine (Int. 1, Int. 2, Int. 3, and Int. 4), and cecum] were rinsed with 0.5% sodium taurocholate in normal saline (2 × 1 mL) followed by 50 mM $NaHCO_3$ (2 × 1 mL) to ensure complete (visual) removal of all contents. Tissue homogenates and contents were acidified to pH 2.0 with 1 N HCl and extracted three times with chloroform. After drying the combined chloroform extracts under nitrogen, residues were dissolved in methanol and counted for radioactivity. Blood samples were allowed to stand at room temperature for at least 1 hr and then centrifuged to obtain serum. Urine and serum samples were also counted for radioactivity. Serum volume was calculated assuming a blood volume (mL) of 7% of the rat's mass (g) and serum volume to be 55% of the blood volume.

RESULTS

Table 1 lists the various pretreatment procedures, the major P450 isoforms induced by them, as well as the total hepatic P450 levels measured following these treatments. The extent of OTA metabolism by these liver microsomes is shown in Fig. 2. All treatments except INH led to substantial increases in the formation of the major isomer 4*R* metabolite. Thus, PB, DXM, 3MC, ISF, CLF and INH pretreatments increased 4*R* levels by 63-, 69-, 38-, 33-, 10- and 1.6-fold, respectively (Fig. 2). Smaller increases of up to ~14-fold were also seen in 4*S* formation. It is interesting that only INH pretreatment selectively induced 4*S* formation (~8-fold). Microsomes from rats administered vehicles only (corn oil; sucrose syrup + gum arabic) gave OTA metabolites at levels comparable to those obtained from untreated rat liver microsomes, indicating that the stimulating effect of the various treatments on OTA metabolism is due to the inducers and not the vehicles (data not shown).

The effect of specific MAbs on OTA metabolism is shown

TABLE 1. List of various treatments and the major cytochrome P450 isozymes induced

Treatment	P450 induced	Method of administration	P450 content (nmol/mg protein)
Control		None	0.85 ± 0.07
PB	IIB1	0.1% in drinking water for 5 days [22]	2.15 ± 0.14
3MC	IA1	20 mg/kg in corn oil, i.p., daily for 3 days [23]	1.70 ± 0.18
CLF	IVA1	200 mg/kg in 1 mL sucrose syrup + 1% gum arabic, p.o., daily for 5 days [24]	1.40 ± 0.20
ISF	IA1/IA2	120 mg/kg in corn oil, i.p., daily for 3 days [25]	1.00 ± 0.02
DXM	IIIA1/IIIA2	50 mg/kg in corn oil, i.p., daily for 4 days [26]	2.05 ± 0.17
INH	IIE1	0.1% in drinking water for 10 days [27]	0.80 ± 0.03

Cytochrome P450 contents are means ± SD from 4 rats/treatment group.

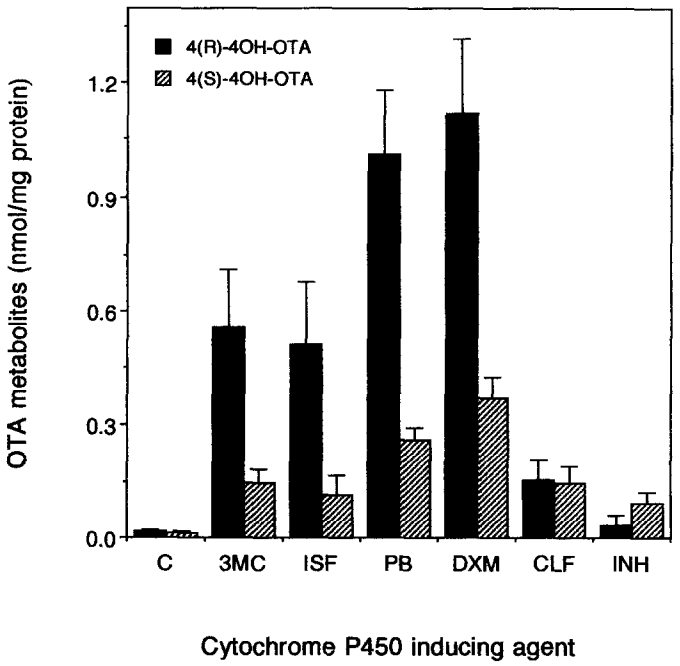

FIG. 2. Formation of 4R and 4S metabolites of OTA using liver microsomes from rats treated with different inducers of P450. Incubations were carried out in triplicate for 30 min in 1 mL of 0.1 M phosphate buffer (pH 7.4) containing 2 mg microsomal protein, 125 μM OTA and an NADPH-regenerating system. Details are described in Materials and Methods. Results are means ± SD of incubations from 8 individual rats per treatment group. C = control.

in Fig. 3. Three main monoclonal antibodies were used: clone 1-7-1 (against P450IA1/IA2), clone 2-66-3 (against P450IIB1/IIB2), and clone 2-3-2 (against P450IIIA1/IIIA2). The preincubation of 3MC liver microsomes with monoclonal antibody 1-7-1 reduced the subsequent formation of 4*R* from OTA. A maximum inhibition of 85% was seen at an MAb to microsomal protein ratio of 2 (Fig. 3; top panel). Only at an MAb to microsomal protein ratio of 5 was the formation of the 4S isomer partially inhibited (30%). Rabbit IgG was used to determine the non-specific inhibition of OTA metabolism. Preincubation of 3MC liver microsomes with rabbit IgG slightly inhibited (20%) 4*R* formation but only at an IgG to microsomal protein ratio of 5. Rabbit IgG did not inhibit 4S formation. Similarly, preincubation of PB liver microsomes with MAb 2-66-3 inhibited the subsequent formation of both the 4*R* and 4S isomers by a maximum of about 75% (Fig. 3; middle panel). As expected, OTA metabolism by PB microsomes was not inhibited by rabbit IgG. Finally, MAb 2-3-2 inhibited both 4*R* and 4S formation by DXM microsomes by a maximum of 50–55%, whereas rabbit IgG had no effect (Fig. 3; bottom panel). Table 2 shows the cross-reactivity of each of the three MAbs with microsomes from rats treated with PB, 3MC or DXM. Each MAb was used at a concentration that caused maximal inhibition of OTA metabolism with its respective microsome type (Fig. 3). With respect to the 4*R* metabolite, MAb 2-3-2 was most specific inhibiting its formation mainly by DXM microsomes (50%) and only marginally by PB or

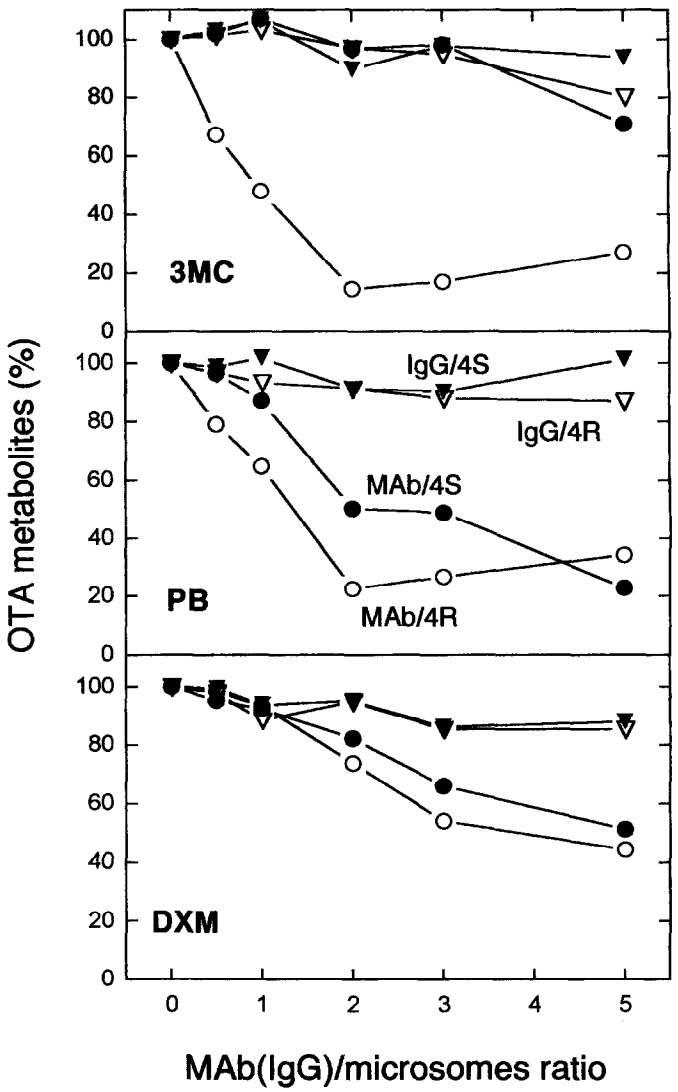

FIG. 3. Effect of MAbs or rabbit IgG on OTA metabolism by 3MC, PB, and DXM microsomes. Antibody (MAb 1-7-1 for 3MC microsomes; 2-66-3 for PB microsomes; 2-3-2 for DXM microsomes) or IgG in the amounts indicated was preincubated with microsomes in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature. Each incubation was performed in duplicate and contained 0.2 mg microsomal protein in a total volume of 100 μL. Reaction was started by adding OTA (12.5 nmol) and NADPH-regenerating system and was carried out at 37° for 30 min. Results are an average of two separate experiments that did not differ from each other by more than 15%. Key: (○–○) MAb/4R formation; (●–●) MAb/4S formation; (▽–▽) IgG/4R formation; and (▼–▼) IgG/4S formation.

3MC microsomes (4–7%). MAb 1-7-1 was less specific; it inhibited 4*R* formation by 3MC microsomes maximally (64%), but it also inhibited DXM microsomes substantially (49%) and PB microsomes to a lesser extent (23%). MAb 2-66-3 was the least specific since it inhibited 4*R* formation substantially (39–65%) with all three types of microsomes (Table 2).

α-Naphthoflavone, a known inhibitor of P450IA1/IA2 [36], specifically inhibited (90%) 4*R* formation by 3MC microsomes. With PB and DXM microsomes, the inhibition was

TABLE 2. Ability of the various antibodies to inhibit OTA metabolism by PB, 3MC, and DXM microsomes

	4R Inhibition (%)			4S Inhibition (%)		
	MAb			MAb		
Treatment	1-7-1*	2-66-3*	2-3-2†	1-7-1*	2-66-3*	2-3-2†
PB	23.2	58.3	7.0	19.2	38.0	31.0
3MC	63.8	39.0	4.2	0	15.0	0
DXM	49.0	65.0	50.4	48.6	57.4	44.7

Incubation conditions are as described in Materials and Methods. The ratios of MAb protein/microsomal protein were selected on the basis of maximum inhibition obtained in Fig. 3. Two experiments were performed, each in duplicate and on a different batch of the induced microsomes. Results shown are averages of the values obtained in the two experiments, which did not differ from each other by more than 15%. Control values for PB, 3MC, and DXM: 512, 335, and 590 pmol/mg protein, respectively.

* MAb protein:microsomal protein, 2:1.

† MAb protein:microsomal protein, 4:1.

less than 20%. On the other hand, metyrapone, a known inhibitor of P450IIB1 [37], specifically inhibited (94%) 4R formation by microsomes from PB- and DXM-treated rats while only slightly inhibiting (11%) its formation by 3MC microsomes. α-Naphthoflavone also inhibited 4S formation by PB, 3MC, and DXM microsomes by 83, 77, and 65%, respectively. In comparison, metyrapone inhibited 4S formation by 62, 14, and 87% with PB, 3MC, and DXM microsomes, respectively.

The pH optimum for 4R formation was found to be 6.0 with 3MC microsomes and 6.5 with PB or DXM microsomes. For 4S formation, the pH optimum was 7.0 with microsomes from all three pretreatments (data not shown). Table 3 compares the effect on OTA metabolism by PB, 3MC, and DXM microsomes when incubations were carried out at the optimum pH [6.0 or 6.5; for 4R formation] and the usual incubation pH of 7.4. At the optimum pH, 4R formation increased 8.0-, 1.5-, and 1.4-fold with microsomes from 3MC-, DXM-, and PB-treated rats, respectively. The corresponding decrease in 4S formation was 47, 42, and 79% with 3MC, DXM, and PB microsomes. Table 4 shows the MDA levels and P450 contents at the end of a 30-min incubation using microsomes from rats treated with 3MC, DXM, and PB at pH 6.0 (or 6.5) and 7.4. With 3MC microsomes, more MDA and lower P450 contents were observed at pH 7.4 than at pH 6.0. Thus, MDA formed at pH 7.4 was 18.5 nmol/mg protein and the P450 loss was 61%. In contrast, at pH 6.0, the MDA formed was 10.6 nmol/mg protein and the P450 loss was 28%. With DXM microsomes, there was no significant difference between pH 6.5 and 7.4 in terms of either MDA formation (~30 nmol/mg protein) or P450 destruction (~90%). With PB microsomes, there was a small reduction in MDA formation at pH 6.5 (24%) and only a marginal decrease in the level of P450 destruction. Thus, at the two pH values, the difference in P450 loss was substantial only with 3MC microsomes (~33%) but not with PB or DXM microsomes. In general, a greater loss of P450 was observed when lipid peroxidation was higher (Table 4). In support of this, addition of 30 μM EDTA to the incubation containing 3MC microsomes (pH 7.4) decreased lipid peroxidation by 93%, decreased P450 destruction from 62 to 22% and increased 4R formation by 77%.

TABLE 3. Comparison of OTA metabolism by 3MC, PB, and DXM microsomes at the optimum pH (6.0 or 6.5) and pH 7.4

Microsome type	pH	4S Metabolite (nmol/mg protein)	4R Metabolite (nmol/mg protein)
3MC	6.0	0.023 ± 0.008	5.280 ± 0.430
3MC	7.4	0.043 ± 0.029	0.650 ± 0.160
DXM	6.5	0.215 ± 0.026	1.690 ± 0.232
DXM	7.4	0.370 ± 0.124	1.147 ± 0.530
PB	6.5	0.018 ± 0.003	0.870 ± 0.110
PB	7.4	0.087 ± 0.060	0.610 ± 0.170

Details of incubation conditions are described in Materials and Methods. pH 6.0/6.5 reflects the optimum pH for the formation of the major 4R isomer by the particular microsome type. Results are means ± SD of three separate incubations (each performed in triplicate), using microsomes from three different rats.

Figure 4 shows AP and GGT levels in urines of control and PB-pretreated rats 2 days before OTA treatment (days −1 and 0) and 5 days after OTA treatment (days 2–6). Urinary AP was increased significantly in the control group on day 4 (~5-fold) and day 5 (~8-fold), while the PB group showed a much smaller but significant increase (~2-fold) on days 5 and 6 (Fig. 4A). There was also a significant difference between the control and PB groups on days 4 and 5. Compared with day 0, a small but significant decrease was also observed on day 2 in both the control and PB groups. The reason for this decrease is not clear. Urinary GGT levels were also increased significantly (up to 2-fold) on days 4 and 5 in the control group, whereas in the PB group no such increase was observed (Fig. 4B). There was also a significant difference between the control and PB groups on days 4, 5, and 6. These results indicate that PB pretreatment of rats protects them against the nephrotoxic effects of OTA. In contrast to PB treatment, Co-heme treatment substantially increased OTA nephrotoxicity. Thus, the urinary excretion of both AP (Fig. 5A) and GGT (Fig. 5B) was increased vastly in the Co-heme group as compared with the control group.

Table 5 shows the percentage of administered [^{3}H]OTA found in the serum, urine, various tissues (liver, kidney, stomach, the four portions of small intestine, and cecum) and in

TABLE 4. Effect of pH on lipid peroxidation and cytochrome P450 destruction in 3MC, DXM, and PB microsomes in the presence of OTA

Microsome type	pH	MDA levels (nmol/mg protein)	P450 levels (nmol/mg protein)		P450 loss (%)
			At 0 min	At 30 min	
3MC	6.0	10.6	1.83	1.32	27.9
3MC	7.4	18.5	1.83	0.71	61.2
DXM	6.5	28.3	2.10	0.19	91.0
DXM	7.4	32.8	2.10	0.17	92.0
PB	6.5	9.5	2.30	1.65	28.3
PB	7.4	12.5	2.30	1.54	33.0

Incubations were carried out in a total volume of 3 mL as described in Materials and Methods. At the end of 30 min, 0.5 mL was withdrawn for measurement of lipid peroxidation. The remaining volume (2.5 mL) was diluted 1:1 with buffer (pH 7.4) for the measurement of P450 levels. In the case of pH 6.0/6.5 incubations, the pH of the mixture was raised to 7.4 prior to P450 determination. Two experiments were performed, each in duplicate and on a different batch of the induced microsomes. Results shown are averages of the values obtained in the two experiments, which did not differ from each other by more than 15%.

the gut contents 6 hr later. Significantly higher OTA levels were found in the small intestinal tissue (2- to 6-fold increase) and gut contents (4- to 8-fold increase) of control rats than of PB rats (Table 5). More OTA was found in the serum of control (12.4% of administered dose) than of PB-treated rats (8.4%), while more OTA was found in the urine of PB rats (5.2%) than in the control rats (2.0%). However, these differences were not significant.

DISCUSSION

In these studies, we have examined the OTA-metabolizing ability of liver microsomes from rats pretreated with the P450 inducers PB, 3MC, CLF, DXM, and INH. The microsomal P450 contents (Table 1) of livers from the different treatment groups were in good agreement with the reported values [20, 22, 24, 38]. The large increase in OTA metabolism by the various pretreatments that are known to induce different P450 isoforms indicates that several isoforms are capable of efficiently metabolizing OTA. Most of the pretreatments gave rise to very substantial increases in both 4*R* and 4*S* formation. The notable exception was INH treatment which increased only 4*S*, suggesting that P450IIE1 is not capable of metabolizing OTA to the 4*R* isomer (Fig. 2).

We thus elected to examine further OTA metabolism by microsomes from 3MC-, PB-, and DXM-pretreated rats since these treatments induce different P450 isoforms and also lead to high levels of OTA metabolism (Fig. 2). The results of the antibody studies suggest that OTA is metabolized by several P450 isoforms including P450IA1/IA2, IIB1 and IIIA1/IIIA2 (Table 2; Fig. 3). With regard to 4*R* formation, MAb 2-3-2 was most specific towards DXM microsomes, although it inhibited the latter by only 50%. MAb 1-7-1 was less specific; it inhibited 4*R* formation by 3MC microsomes maximally, but it also inhibited DXM microsomes substantially and PB microsomes slightly. MAb 2-66-3 was the least specific since it cross-reacted with all three types of microsomes to inhibit 4*R* formation (Table 2). The specificity of MAbs differs with substrates.

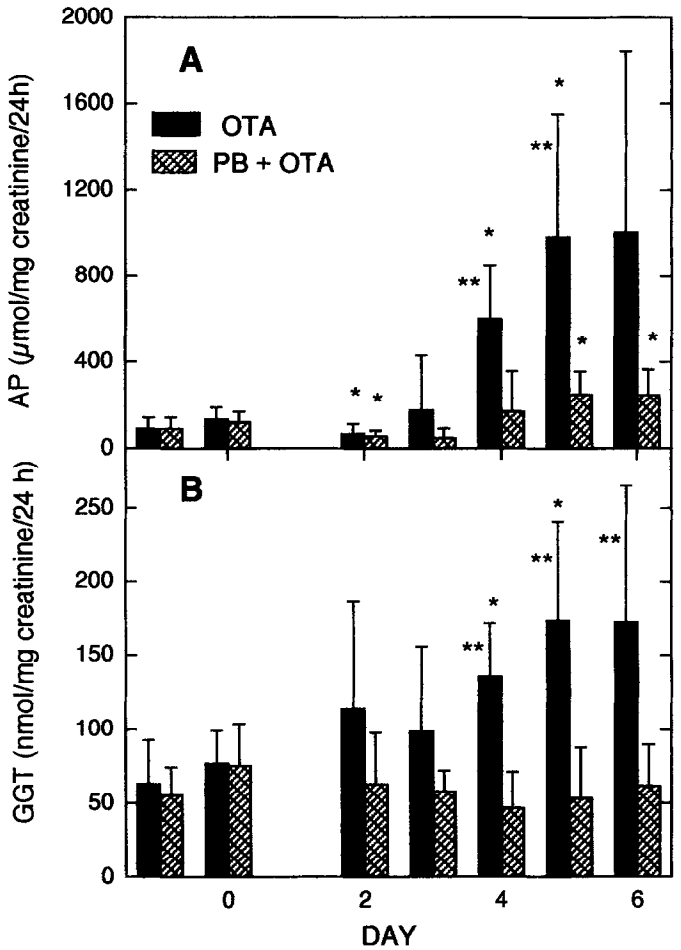

FIG. 4. AP and GGT activities in urines of control and PB-pretreated rats before and following OTA administration. Control and PB-treated rats were each given OTA daily (2 mg/kg body wt for 6 days). Details of the PB pretreatment, OTA treatment, and AP and GGT measurements are described in Materials and Methods. Results are means ± SD of 6 animals per group.

Key: (*) significantly different from day 0 within each group ($P < 0.05$); and (**) significant difference between the two groups on a given day ($P < 0.05$).

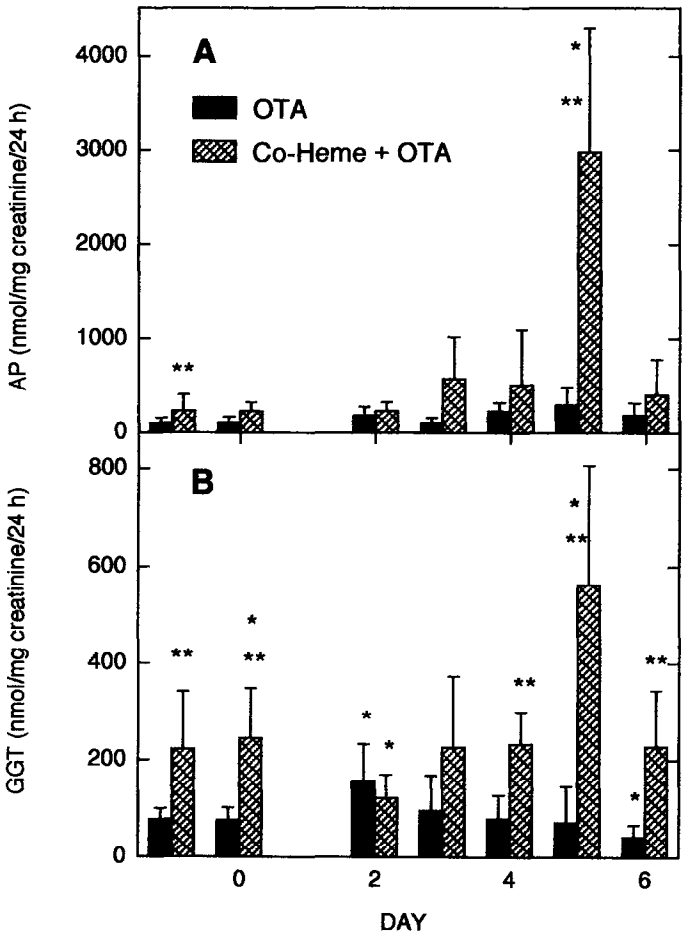

FIG. 5. AP and GGT activities in urines of control and Co-heme-treated rats before and following OTA administration. Control and Co-heme-treated rats were each given OTA daily (1.5 mg/kg body wt for 6 days). Details of the Co-heme pre-treatment, OTA treatment, and AP and GGT measurements are described in Materials and Methods. Results are means ± SD of 6 animals per group.

Key: (*) significantly different from day 0 within each group ($P < 0.05$); and (**) significant difference between the two groups on a given day ($P < 0.05$).

A given MAb may inhibit very specifically a reaction of a certain substrate, but show less or even no specificity towards another substrate [39]. Inhibition studies with α-naphthoflavone and metyrapone also indicated the involvement of P450 isoenzymes IA1/IA2, IIB1 and IIIA1/IIIA2. Microsomes from Co-heme-pretreated rats (P450 depleted by >80%) failed to metabolize OTA, indicating a requirement for P450.

The formation of 4R and 4S metabolites showed a significant difference in pH optima. This is particularly evident in the case of 3MC microsomes where lowering the pH from 7.4 to 6.0 led to an 8-fold increase in 4R formation (Table 3). The increase in 4R formation could be due to: (i) increased affinity of the substrate for P450, (ii) change in the conformation of the flavoprotein and/or P450 leading to increased substrate activation, and (iii) decreased lipid peroxidation. At pH 7.4, OTA would be quite polar since the pK_a of its phenolic hydroxyl is 7.05 [40]. Lowering the pH to 6.0 would make OTA more lipophilic and possibly favor its binding to P450. The effect of pH on the metabolism of substrates, particularly those that are ionizable, has been examined previously. The rate of metabolism of a series of arylalkylamines [41], as well as erythromycin derivatives [42], was shown to be dependent on a pH favoring the neutral form of the substrate together with its partition coefficient between the microsomal layer and the aqueous medium. A decrease in testosterone hydroxylation by increased ionic strength and pH was suggested to involve an impairment of the interaction between P450 and its reductase [43]. Also, Jefcoate [44] showed that cholesterol binding to adrenal mitochondrial $P450_{scc}$ is enhanced by decreased pH. This facilitated adrenodoxin binding and subsequent metabolism. These criteria may also apply to OTA hydroxylation. Since the various P450 isoforms have different structures, each may be affected differently by pH. In the case of P450IA1/IA2, a lowering of the pH is highly conducive to 4R formation.

We have already shown that OTA can induce lipid peroxidation, which, in turn, leads to P450 destruction [45]. Lower lipid peroxidation especially at pH 6.0 and consequently lesser destruction of P450 should therefore lead to increased OTA metabolism. This seems to be the case with 3MC microsomes (Tables 3 and 4). The correlation between lipid peroxidation and P450 destruction in 3MC microsomes is strengthened by results showing that inclusion of 30 μM EDTA in the incubation medium decreased lipid peroxidation, partially protected P450 from destruction, and increased 4R levels.

It is interesting that the pH optimum for 4S formation and lipid peroxidation with all three microsome types was 7.0 (data not shown). We showed earlier [32] that lipid peroxides are

TABLE 5. Distribution of radioactivity in liver, kidney, gut, and gut contents of control and PB-treated rats 6 hr after [^{3}H]OTA administration

Organ	Administered dose (%)	
	Control	PB
Serum	12.4 ± 2.8	8.4 ± 3.0
Urine	2.0 ± 0.7	5.2 ± 2.9
Liver	1.50 ± 0.32	1.24 ± 0.81
Kidney	0.15 ± 0.03	0.13 ± 0.04
Stomach	1.14 ± 0.63	0.68 ± 0.68
Stomach contents	4.40 ± 2.14	0.85 ± 1.03*
Intestine		
Int. 1	0.19 ± 0.06	0.16 ± 0.08
Int. 2	0.28 ± 0.09	0.13 ± 0.07*
Int. 3	0.31 ± 0.07	0.16 ± 0.05*
Int. 4	0.58 ± 0.31	0.14 ± 0.06*
Intestinal contents		
Int. 1	0.43 ± 0.22	0.09 ± 0.07*
Int. 2	0.78 ± 0.44	0.12 ± 0.05*
Int. 3	1.14 ± 0.05	0.25 ± 0.13†
Int. 4	2.50 ± 0.80	0.28 ± 0.08‡
Cecum	0.78 ± 0.47	0.91 ± 0.73
Cecum contents	7.50 ± 4.80	2.41 ± 1.76

Rats were administered [^{3}H]OTA (288 μg/kg, 40 μCi/mg; p.o.), and 6 hr later serum, urine and organ samples were processed for radioactivity counting as described in Materials and Methods. Values are means ± SD of samples from 4 individual rats per treatment group.

Statistical significance (measured by Student's *t*-test): *$P < 0.05$, †$P < 0.001$, and ‡$P < 0.01$.

not involved directly in 4S formation but that a Fe^{2+}-oxygen complex, formed via NADPH-cytochrome P450 reductase and P450-dependent reduction of free Fe^{3+} followed by oxygen binding, serves as the species inducing lipid peroxidation and 4S formation. This may explain why DXM microsomes showed a high level of lipid peroxidation and 4S formation (Tables 3 and 4) and why 4S formation was not inhibited like 4*R* by antibodies and by α-naphthoflavone and metyrapone.

Cytochrome P450-dependent metabolism of most xenobiotics leads to their detoxication and subsequent excretion; however, some compounds are also activated to intermediates that are toxic and/or carcinogenic [e.g. Refs. 46 and 47]. Urinary excretion of renal enzymes is considered to be a sensitive non-invasive indicator of renal toxicity and damage [48, 49]. Our results showing that PB pretreatment protected against the urinary excretion of AP and GGT whereas Co-heme pretreatment exacerbated the excretion of these enzymes (Figs. 4 and 5) suggest that at least P450IIB1-dependent metabolism of OTA, presumably to the 4*R* and 4S hydroxylated products, leads primarily to its detoxication. In support of this, Hutchison *et al.* [50] showed that 4-OH-OTA was non-toxic to rats at doses up to 40 mg/kg body weight. It must be noted that the protective effect of PB was observed at an OTA dose of 2 mg/kg body wt/day (Fig. 4), while the enhanced effect of Co-heme was observed at the lower OTA dose of 1.5 mg/kg body wt/day (Fig. 5). At the higher OTA dose, Co-heme-pretreated rats showed excessive kidney damage as judged by the very high levels of renal enzymes excreted (data not shown). The nephrotoxic effects of OTA were particularly evident on days 4–5 of OTA administration. Berndt and Hayes [51] have shown that multiple doses of OTA are required to observe changes in renal function. It would be interesting to determine if other inducers of P450 also protect against OTA nephrotoxicity.

Quantitation of radioactivity in different tissues and body fluids following administration of [^{3}H]OTA to control and PB-treated rats suggests that PB treatment leads to more rapid elimination of OTA and/or its metabolites (Table 5). OTA levels tended to be higher in urine and lower in serum of PB-pretreated rats than of control rats, although the results were not statistically significant. Significantly higher OTA levels were found in the gut contents of control rats (Table 5) compared with PB-treated rats, indicating that OTA was absorbed, metabolized, and excreted faster in PB-treated rats. Significantly more OTA was also found in the small intestinal tissues of control rats, indicating more unabsorbed OTA in the intestinal contents of these rats. The high OTA levels in cecum contents may represent, in part, excretion of OTA and metabolites in bile since OTA is known to undergo enterohepatic circulation [52].

From our studies, it appears that OTA itself could be toxic, or it may be metabolized by other P450 isoenzymes or via other pathways to products that are toxic. Hietanen *et al.* [20] showed that 4-OH-OTA formation by microsomes from Lewis rats was 3–4 times more than that from DA rats. Lewis and DA rats are recognized, respectively, as extensive and poor metabolizers of the drug debrisoquine. Nikolov *et al.* [53] showed that the distribution among patients with Balkan endemic nephropathy/urinary tract tumors indicated a predominance of extensive debrisoquine metabolizers and a lack of poor metabolizers. Debrisoquine is metabolized in humans by P450IID6, which shows genetic polymorphism [54]. While it is not known if P450IID6 is responsible for OTA activation in humans, the results are consistent with the belief that the efficiency of oxidative metabolism is greater in Balkan endemic nephropathy/urinary tract tumor patients and that this may be one of the key host factors determining predisposition to this disease [53]. Also, Dirheimer, Creppy and their coworkers have suggested that reactive oxygen species such as superoxide anions and H_2O_2 are involved in OTA nephrotoxicity and genotoxicity [55, 56]. The kidney is particularly rich in peroxidases (e.g. prostaglandin synthase), and these authors [56] have suggested that peroxidatic pathways are responsible for the majority of DNA adducts induced in the kidney by OTA.

In conclusion, our results show that OTA is metabolized by the main P450 isoforms IA1/IA2, IIB1, and IIIA1/IIIA2 mainly to 4*R* and to a smaller extent to 4S. Also, hepatic P450 induction by PB pretreatment reduces the nephrotoxicity of OTA, indicating that at least P450IIB1-dependent mixed-function oxidation to OTA leads to its detoxication. Furthermore, depletion of P450 by Co-heme pretreatment increased the nephrotoxic effects of OTA, again suggesting the role of P450 in OTA detoxication. Presumably other pathways, perhaps those involving peroxidases or other renal specific P450 isoforms, are involved in OTA activation.

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