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Metabolism of Aflatoxin B_1 by Rat Hepatic Microsomes Induced by Polyhalogenated Biphenyl Congeners

MICHAEL R. HALVORSON,[1] TIMOTHY D. PHILLIPS,[1]* STEVEN H. SAFE,[2] AND L. W. ROBERTSON[2]

Departments of Veterinary Public Health[1] and Veterinary Physiology and Pharmacology,[2] Texas A&M University, College Station, Texas 77843

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The metabolism of aflatoxin B_1 to aflatoxins M_1 and Q_1 by rat liver microsomes from animals pretreated with polychlorinated or polybrominated biphenyl congeners depended on the structure of the halogenated biphenyl inducers. Microsomes from rats treated with phenobarbital (PB) or halogenated biphenyls that exhibit PB-type activity preferentially enhanced the conversion of aflatoxin B_1 to aflatoxin Q_1. In contrast, microsomes from rats treated with 3-methylcholanthrene (MC) or halogenated biphenyls that exhibit MC-type induction activity increased the metabolism of aflatoxin B_1 to aflatoxin M_1. The coadministration of PB and MC produced microsomes that exhibited both types of induction activity (mixed type) in catalyzing the oxidative metabolism of diverse xenobiotic agents. However, PB-plus-MC-induced hepatic microsomes from immature male Wistar rats preferentially increased the metabolism of aflatoxin B_1 to aflatoxin M_1 but did not enhance the conversion of aflatoxin B_1 to aflatoxin Q_1. Comparable results were observed with microsomes from rats pretreated with halogenated biphenyls classified as mixed-type inducers; moreover, in some cases there was a significant decrease in the conversion of aflatoxin B_1 to aflatoxin Q_1 (compared with that of controls treated with corn oil).

Aflatoxin B_1 (AFB_1) is a mold metabolite that has been characterized as a potent hepatotoxin and hepatocarcinogen in several animal species. As with many other toxins, the evidence suggests that AFB_1 requires oxidative metabolic activation to elicit the observed toxic and carcinogenic effects (7, 27). The active metabolite of AFB_1 is presumed to be 2,3-epoxy-2,3-dihydro-AFB_1. This metabolite is formed by the introduction of oxygen at the C-2–C-3 position via the mixed-function oxidase system (3, 9, 27). 2,3-Epoxy-2,3-dihydro-AFB_1 can alkylate critical cellular macromolecules or form a number of other metabolic products, including 2,3-dihydroxy-2,3-dihydro-AFB_1 and metabolic conjugates (3, 17).

Some reports demonstrate that the cytochrome P-450-dependent oxidation of AFB_1 can also occur at several other positions to produce a wide spectrum of metabolites that still retain their 2,3-olefinic functionality (4, 9, 11). Some of these compounds include aflatoxin M_1 (AFM_1) (oxidation at C-4), aflatoxin Q_1 (AFQ_1) (oxidation at C-9), and aflatoxin P_1 (O-dealkylation). Many of these AFB_1 metabolites are highly toxic and carcinogenic. AFM_1 is a major fungal and mammalian metabolite of AFB_1 and has been identified in animal livers, urine, and milk, including milk from cows (10). AFQ_1 is about 18 times less toxic than AFB_1 is to chicken embryos, and it was not mutagenic in a bacterial assay that included rat liver microsomes (10). In vitro studies indicate that AFM_1, 2,3-dihydroxy-2,3-dihydro-AFB_1, and AFQ_1 are the major metabolic products formed after incubation with microsomal monooxygenases (10, 13, 17).

Several reports show that xenobiotic agent-induced microsomal monooxygenases can markedly alter the metabolic profile of AFB_1 (8, 9, 13). The preferential induction of specific cytochrome P-450 isozymes catalyzes the oxidation of AFB_1 at different rates or sites or both, depending on the specific isozyme induced. Both polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs) are highly persistent pollutants which have been widely identified in human and animal tissues and are potent inducers of cytochrome P-450-dependent monooxygenases (1, 19, 20, 26). Thus, the interactive effects of these environmental pollutants (PCBs and PBBs) and AFB_1 may be an important consideration in populations exposed to both types of toxins.

PCB and PBB congeners have recently been classed into three major categories (1, 2, 19, 22, 23). One category is typified by phenobarbital (PB)-induced, cytochrome P-450-dependent monooxygenase activities. Another category, typified by 3-methylcholanthrene (MC), induces cytochrome P-448-dependent monooxygenases. A third category represents a mixed pattern of induction such as is produced by coadministering PB and MC. In the present study, we report the effects of several PCB- and PBB-induced rat hepatic microsomal enzyme preparations on the metabolism of AFB_1 to the toxic metabolite AFM_1 and the less toxic metabolite AFQ_1.

MATERIALS AND METHODS

Chemicals. AFB_1, AFM_1, and AFQ_1 were obtained from Sigma Chemical Co., St. Louis, Mo. Tritium-labeled AFB_1 was purchased from Moravek Chemicals, Brea, Calif. A working solution of radiolabeled AFB_1 was prepared by mixing 2.0 mg of unlabeled AFB_1 with 100 μl of [^{3}H]AFB_1 (specific activity, 15.0 Ci/mmol) and diluting with 0.9 ml of methanol. The solutions were stored at −80°C until ready for use. Sodium PB (Harvey Laboratories, Inc., Philadelphia, Pa.) was diluted in saline to a concentration of 30 mg/ml. Firemaster BP-6 and Aroclor 1254 were generous gifts from the Michigan Chemical Corp., St. Louis, Mich., and the Monsanto Chemical Co., St. Louis, Mo., respectively.

Individual PCB and PBB congeners were synthesized from the appropriate precursors and purified by column chromatography on alumina-Florisil and by silica gel thin-layer chromatography (1, 2). The purities were determined by gas-liquid chromatography with a chromatograph (model 5710; Hewlett-Packard Co., Avondale, Calif.) equipped with a ^{63}Ni electron-capture detector and a glass column (0.6 cm

* Corresponding author.

TABLE 1. Effects of microsomal enzyme inducers on cytochrome P-450-mediated metabolism of AFB_1[a]

Animal pretreatment	AFB_1 remaining[b] (nmol/mg of protein)	AFQ_1 (nmol/mg of protein)	AFM_1 (nmol/mg of protein)	4-(Dimethylamino)-antipyrine N-demethylase activity[c]	Benzo[a]-pyrene hydroxylase activity[d]
Corn oil (control)	2.66 ± 0.28	0.07 ± 0.02	0.15 ± 0.03	8.52 ± 0.90	0.43 ± 0.03
PB	2.18 ± 0.26[e]	0.13 ± 0.02[e]	0.15 ± 0.01	14.18 ± 0.59[e]	0.47 ± 0.02
MC	0.65 ± 0.15[e]	0.03 ± 0.00[e]	1.03 ± 0.15[e]	6.69 ± 0.38	5.53 ± 0.65[e]
PB + MC	1.17 ± 0.22[e]	0.07 ± 0.01	0.81 ± 0.25[e]	11.58 ± 0.94[e]	4.71 ± 0.33[e]
Aroclor 1254	0.97 ± 0.12[e]	0.04 ± 0.00[e]	1.10 ± 0.20[e]	12.68 ± 0.78[e]	3.76 ± 0.21[e]
Firemaster BP-6	1.42 ± 0.20[e]	0.07 ± 0.02	0.85 ± 0.26[e]	13.01 ± 0.73[e]	3.94 ± 0.10[e]

[a] Three animals per group. Values are expressed as mean ± standard deviation.
[b] 64 nmol of AFB_1 per incubation tube. AFB_1 remaining = AFB_1 extracted after a 30-min incubation.
[c] Nanomoles of HCHO formed per milligram of protein per minute.
[d] Nanomoles of benzo[a]pyrene metabolized per milligram of protein per minute.
[e] Significantly different from control group mean (Student's t test, $P < 0.05$).

by 1.2 m) packed with 3% OV101 on Ultrabound Carbowax 20 M (80/100 mesh; RFR Corp., Hope, R.I.). Structures of purified PCB and PBB congeners were confirmed by their nuclear magnetic resonance and their mass spectra.

NADP, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride, and benzo[a]pyrene were obtained from Sigma. 4-(Dimethylamino)antipyrine was supplied by Aldrich Chemical Co., Inc., Milwaukee, Wis. [^{3}H]benzo[a]pyrene was obtained from New England Nuclear Corp., Boston, Mass., and was purified by Florisil column chromatography.

Animal treatment and isolation of microsomes. One-month-old male Wistar rats (Timco Breeding Laboratories, Houston, Tex.), approximately 100 g in weight, were housed in stainless steel or plastic cages and allowed free access to feed (Purina Rat Chow no. 5002; Ralston Purina Co., St. Louis, Mo.) and water. Animals were maintained on a diurnal cycle of 12 h of light and 12 h of darkness. Animals were injected intraperitoneally with the compounds to be tested at a level of 150 μmol/kg of body weight on days 1 and 3 as previously described (19, 20). The compounds were administered in corn oil solution, and the animals were killed by cervical dislocation on day 6. PB (400 μmol/kg, dissolved in isotonic saline) and MC (100 μmol/kg, dissolved in corn oil) were administered individually as well as coadministered to the animals on days 1 and 2, and the animals were killed on day 3. These animals served as positive controls. Another control group was injected with corn oil (0.5 ml) on days 1 and 3 and then killed by cervical dislocation on day 6. To lower liver glycogen levels, all animals were fasted for 24 h before being killed.

Microsomes were prepared as described by Parkinson and Safe (21). The rat livers were perfused with ice-cold isotonic saline via the hepatic portal vein. The livers were removed and placed in preweighed beakers of ice-cold 0.25 M sucrose–0.1 mM EDTA solution for the determination of liver weights. The livers were then homogenized, and the homogenate was centrifuged at 10,000 × g for 20 min. The microsomal fraction was collected as a 100,000 × g pellet by further centrifugation (1 h) of the 10,000 × g supernatant fraction. The protein content of the microsomal fraction was determined by the method of Lowry et al. (12).

Assays. The metabolism of AFB_1 by isolated microsomes was carried out under conditions similar to those used by Roebuck and co-workers (24), using 12.5 mg of microsomal protein per tube. Negative controls were prepared by heating selected control tubes in boiling water for 10 min. To each tube, 10 μl of a 2-mg/ml solution of AFB_1 in methanol was added, and the tubes were transferred to a water bath and incubated with gentle shaking for 30 min at 37°C (16). The total volume in each tube was 5 ml. The reaction was terminated by adding 2.0 ml of ice-cold methanol to each tube.

The rate of oxidative N-demethylation of 4-(dimethylamino)antipyrine was measured by quantifying the production of formaldehyde (6). The formaldehyde, trapped as the semicarbazone, was developed in double-strength Nash reagent (14). The rate of benzo[a]pyrene hydroxylation was measured by the radiometric assay of DePierre et al. (5) as improved by Nesnow et al. (18), by quantifying the base-soluble metabolites after hexane extraction of the unreacted benzo[a]pyrene.

Aflatoxin analysis. Aflatoxins were extracted from in vitro incubations by the following procedure. Hexane (2 ml) was added to each test tube to remove residual lipids. The hexane and the aqueous phases were mixed well and allowed to separate, and the upper hexane layer was discarded. The aflatoxins were extracted from the reaction tubes with three successive 5-ml portions of chloroform. The chloroform extracts were combined and evaporated to dryness under nitrogen. The extracts were redissolved in 200 μl of the high-pressure liquid chromatography mobile phase (25).

The chloroform extracts were analyzed by normal-phase high-pressure liquid chromatography by a previously reported method (25). Aflatoxin peaks were detected by UV absorbance at 365 nm. Fluorescence detection (365-nm excitation; 425-nm emission) was used for confirmation.

Statistics. Statistical comparisons between control and treatment groups were made by using the Student t test. Differences from controls were considered significant at $P < 0.05$.

RESULTS

Table 1 summarizes the in vitro metabolism of AFB_1 by rat hepatic microsomes from animals pretreated with corn oil, PB, MC, PB plus MC (coadministered), and commercial PCB and PBB (Aroclor 1254 and Firemaster BP-6, respectively). Compared with microsomes from the corn oil controls, hepatic microsomes from all the xenobiotic agent-treated animals increased the amount of AFB_1 metabolized (as determined by the AFB_1 remaining). PB-induced microsomes increased the amount of AFQ_1 formed but did not alter the AFM_1 levels; in contrast, MC-induced microsomes increased the amount of AFM_1 formed but decreased AFQ_1 formation. Microsomes from rats treated with the mixed-type inducers (PB plus MC, Aroclor 1254, and Firemaster

TABLE 2. Effects of polyhalogenated biphenyl congeners on cytochrome P-450-mediated metabolism of AFB_1[a]

Animal pretreatment	AFB_1 remaining[b] (nmol/mg of protein)	AFQ_1 (nmol/mg of protein)	AFM_1 (nmol/mg of protein)	4-(Dimethylamino)antipyrine *N*-demethylase activity[c]	Benzo[*a*]pyrene hydroxylase activity[d]
Corn oil (control)	2.66 ± 0.28	0.07 ± 0.02	0.15 ± 0.03	8.52 ± 0.90	0.43 ± 0.03
2,2',4,4'-Tetrachlorobiphenyl	2.31 ± 0.59	0.14 ± 0.05[e]	0.24 ± 0.06[e]	10.86 ± 0.88[e]	0.44 ± 0.02
2,2',4,5,5,'-Pentabromobiphenyl	2.54 ± 0.42	0.11 ± 0.02[e]	0.21 ± 0.15	11.09 ± 0.82[e]	0.47 ± 0.02
3,3',4,4'-Tetrachlorobiphenyl	0.08 ± 0.39[e]	0.04 ± 0.01[e]	1.74 ± 0.45[e]	5.83 ± 0.63[e]	5.03 ± 0.44[e]
3,3',4,4'-Tetrabromobiphenyl	0.79 ± 0.29[e]	0.05 ± 0.02	1.96 ± 0.44[e]	3.67 ± 0.48[e]	4.40 ± 0.16[e]
2,3,4,4',5-Pentachlorobiphenyl	0.69 ± 0.12[e]	0.03 ± 0.00[e]	1.30 ± 0.17[e]	8.30 ± 0.35	5.11 ± 0.18[e]
2,3,4,4',5-Pentabromobiphenyl	0.76 ± 0.38[e]	0.04 ± 0.00[e]	1.77 ± 0.31[e]	7.76 ± 0.07	4.82 ± 0.16[e]

[a] Three animals per group. Values are expressed as mean ± standard deviation.
[b] 64 nmol of AFB_1 per incubation tube. AFB_1 remaining = AFB_1 extracted after a 30-min incubation.
[c] Nanomoles of HCHO formed per milligram of protein per minute.
[d] Nanomoles of benzo[*a*]pyrene metabolized per milligram of protein per minute.
[e] Significantly different from control group mean (Student's *t* test, $P < 0.05$).

BP-6) all increased the amount of AFM_1 formed but did not affect (or slightly decreased) the levels of AFQ_1 produced.

Table 2 and Fig. 1 summarize the metabolism of AFB_1 by hepatic microsomes from rats pretreated with several PCB and PBB congeners. 2,2',4,4'-Tetrachlorobiphenyl and 2,2',4,5,5'-pentabromobiphenyl resemble PB in their mode of induction of cytochrome P-450-dependent monooxygenases. The microsomes from rats pretreated with these two compounds did not significantly increase the amount of AFB_1 metabolized, but they markedly increased the production of AFQ_1. 3,3'4,4'-Tetrachloro- and tetrabromobiphenyl resemble MC in their mode of induction of cytochrome P-450-dependent monooxygenases. Microsomes from 3,3',4,4'-tetrachloro- and tetrabromobiphenyl-pretreated rats enhanced AFB_1 metabolism, slightly reduced the levels of AFQ_1 produced, and increased the amount of AFB_1 converted to AFM_1. Microsomes from rats pretreated with 2,3,4,4',5-pentachloro- and pentabromobiphenyl gave results similar to those of microsomes from rats pretreated with 3,3',4,4'-tetrachloro- and tetrabromobiphenyl. 2,3,4,4',5-Pentachloro- and pentabromobiphenyl are classified as mixed-type inducers.

Tables 1 and 2 also compare the in vitro metabolism of AFB_1 to AFM_1 and AFQ_1 with that of two other cytochrome P-450-dependent monooxygenases, 4-(dimethylamino) antipyrine *N*-demethylase and benzo[*a*]pyrene hydroxylase. The *N*-demethylase is a PB-inducible enzyme, whereas benzo[*a*]pyrene hydroxylase is primarily induced by MC. Microsomes from rats pretreated with PB and PB-type inducers increased the levels of hepatic microsomal 4-

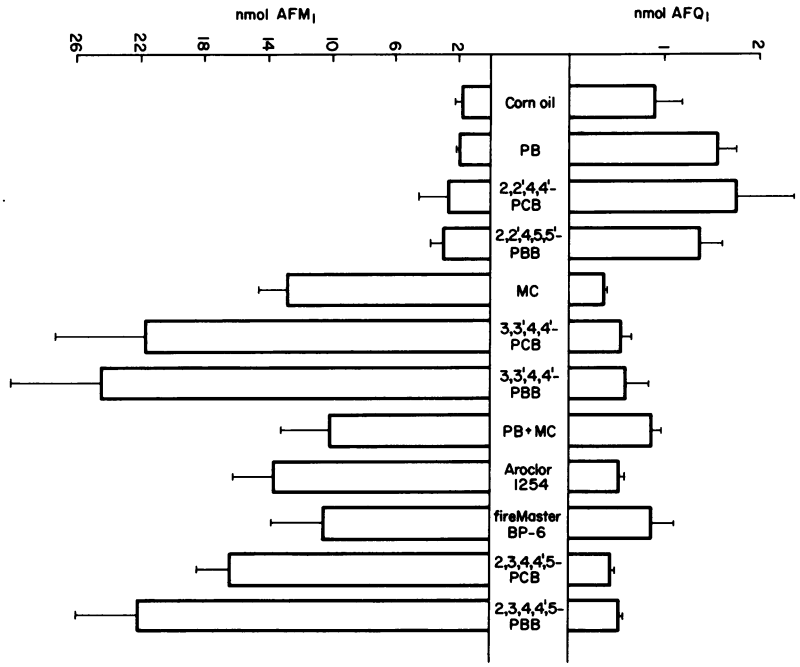

FIG. 1. Effect of various microsomal enzyme inducers on the in vitro metabolism of AFB_1 to AFM_1 and AFQ_1. The inducers are classified as PB and PB type (2,2',4,4'-PCB and 2,2',4,5,5'-PBB), MC and MC type (3,3',4,4'-PCB and -PBB), and mixed type (PB plus MC, Firemaster BP-6, Aroclor 1254, and 2,3,4,4',5-PCB and -PBB). Results are reported as nanomoles produced per milligram of protein. A total of 64 nmol of AFB_1 was added per incubation tube. Results are the mean ± the standard deviation (bars) of independent analysis of microsomes from different animals, each assayed in triplicate.

(dimethylamino)antipyrine *N*-demethylase. Microsomes from rats pretreated with MC or MC-type inducers showed increased levels of benzo[*a*]pyrene hydroxylase. Mixed-type inducers increased the levels of both enzymes. These results are consistent with those of earlier studies (1, 19, 23, 26).

DISCUSSION

Previous reports have shown that microsomes from animals pretreated with MC exhibit a 10-fold increase in the formation of AFM_1 from AFB_1 without affecting the levels of AFQ_1 formed (13, 15). Not surprisingly, the AFB_1-4-hydroxylase activities of microsomes from 3,3',4,4'-tetrachlorobiphenyl- and 3,3',4,4'-tetrabromobiphenyl-pretreated rats and MC-pretreated rats were similar. All three xenobiotic agents induce a comparable pattern of cytochrome P-450 isozymes (22). Both 2,2',4,4'-tetrachlorobiphenyl and 2,2',4,5,5'-pentabromobiphenyl resemble PB in their mode of induction of rat hepatic cytochrome P-450 isozymes and cytochrome P-450-dependent monooxygenases (22). This study confirms that PB and the two PB-type inducers preferentially enhance the conversion of AFB_1 to AFQ_1 (8, 9, 13).

The effects of coadministered PB and MC as monooxygenase enzyme inducers are additive, and similar activities have been observed after the administration of Aroclor 1254, Firemaster BP-6, 2,3,4,4',5-pentachlorobiphenyl, 2,3,4,4'5-pentabromobiphenyl, and several other halogenated biphenyls (1, 19, 22, 23). This mixed-type induction activity is characterized by the induction of the microsomal monooxygenase enzymes and the cytochrome P-450 isozymes induced by both PB and MC. Thus, the mixed-type inducers used in this study induced both 4-(dimethylamino)antipyrine *N*-demethylase and benzo[*a*]pyrene hydroxylase. However, the additivity expected for the formation of the metabolites AFM_1 and AFQ_1 was not observed. Microsomes from animals treated with all the mixed-type inducers significantly increased (5 to 12-fold) the amount of AFB_1 converted into AFM_1 but they did not increase the formation of AFQ_1. Moreover, microsomes from rats pretreated with Aroclor 1254, 2,3,4,4',5-pentachlorobiphenyl, and 2,3,4,4',5-pentabromobiphenyl exhibited significantly lower levels of AFQ_1 formed after incubation with AFB_1. Although mixed-type and MC-type inducers elicit markedly different microsomal monooxygenase enzyme induction responses for most substrates, their effects on AFB_1 conversion into AFM_1 and AFQ_1 were similar.

Our results show that approximately 40% of the AFB_1 metabolized by microsomes from rats pretreated with MC and the MC-type inducers 3,3',4,4'-tetrachloro- and tetrabromobiphenyl was accounted for by the metabolites AFQ_1 and AFM_1. Approximately 30% of the AFB_1 metabolized by microsomes from rats pretreated with PB plus MC, Aroclor 1254, Firemaster BP-6, and 2,3,4,4',5-pentachloro- and pentabromobiphenyl was accounted for by AFQ_1 and AFM_1. With microsomes from rats pretreated with PB, 2,2',4,4'-tetrachlorobiphenyl, and 2,2',4,5,5'-pentabromobiphenyl, only about 13% of the metabolized AFB_1 was accounted for by AFQ_1 and AFM_1. The remainder of the AFB_1 metabolized is probably involved in binding to microsomal macromolecules and in the formation of minor metabolites (13, 15, 17).

The results of this study show that rat microsomes induced with mixed-type inducers significantly increased the conversion of AFB_1 to AFM_1 and caused little change in AFQ_1 formation. In a recent study (E. C. Shepherd, Ph.D. dissertation, Texas A&M University, College Station, 1982), AFB_1 was administered to lactating rats pretreated with halogenated biphenyls. The results showed that AFM_1 is the major metabolite excreted in the milk. The suppression of AFQ_1 formation may also be related to the in vivo mixed-type activity of the induced microsomes. The rationale for these unique observations is not apparent, and current research in our laboratories is focused on understanding the interactive effects of PCBs, PBBs, and aflatoxins.

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