HEPATIC MICROSOMAL MIXED FUNCTION OXYGENASE: ENZYME MULTIPLICITY FOR THE METABOLISM OF CARCINOGENS TO DNA-BINDING METABOLITES

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SUMMARY:

Microsome-mediated metabolic activation of aflatoxin B_1 (AFB_1) and benzo[a]-pyrene (BP), as determined by the in vitro formation of DNA binding metabolites, was studied, using hepatic microsomes from untreated, phenobarbital (PB)-treated and 3-methylcholanthrene (MC)-treated male rats. Contrasting results were obtained for the two substrates: in the case of AFB_1, microsomes from PB-treated rats were twice as active as microsomes from untreated and MC-treated rats, whereas, in the case of BP, microsomes from MC-treated rats were several fold more active than microsomes from untreated and PB-treated rats. These data strongly suggests enzyme multiplicity of microsomal mixed function oxygenase for the activation of carcinogens, especially AFB_1 and BP whose reactive metabolites are believed to be epoxides.

Hepatic endoplasmic reticulum contains a group of enzymes commonly referred to as microsomal mixed function oxygenase (MMFO). This enzyme system is involved in the oxidation of drugs, carcinogens and other chemicals (1). More than 200 drugs, insecticides and carcinogens, widely differing in structure and in biological and pharmacological activities, are known to modify both in vivo and in vitro the activity of various enzymes in the MMFO complex (1). In this regard, foreign compounds have been divided into two general categories: phenobarbital type, causing an increase in the content of cytochrome P_{450} and an enhancement in the activities of several enzymes, and polycyclic aromatic hydrocarbon type (e.g., 3-methylcholanthrene and benzo [a] pyrene (BP)), increasing the content of a different cytochrome, P_{448}, and also the activities of only a specific group of enzymes, one of which is arylhydrocarbon hydroxylase (AHH).

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AHH catalyzes the conversion of a number of polycyclic aromatic hydrocarbons into various metabolites (2, 3). Some polycyclic aromatic hydrocarbons, benzo[a]-pyrene being one them, are converted by AHH into epoxides which then undergo one of the following fates (4-6): (a) rearrange non-enzymatically to phenols, (b) are acted upon by microsomal epoxide hydrase to form dihydrodiols, (c) are conjugated with glutathione enzymatically or non-enzymatically, and (d) alkylate macromolecules, DNA, RNA and proteins, in the cell (7, 8). It is the latter reaction which may be critical in the initiation of the neoplastic process (9).

Aflatoxins, produced by some strains of the mold, Aspergillus flavus, are potent hepatotoxic and hepatocarcinogenic agents in a number of animal species (10). Participation of MMFO in the metabolism of aflatoxin B_1 (AFB_1), the most potent of all aflatoxins (11), is well known (12). However, it is only recently that some aflatoxins have been reported to be converted by MMFO into reactive metabolites which alkylate DNA, RNA and proteins (13, 14), kill bacteria in culture (15) and are mutagenic (16, 17). Examinations of structure-activity relationships among various aflatoxins have suggested that the reactive metabolites are produced by the oxidation of C_2-C_3 double bond in the first furan ring of some aflatoxins and related compounds (14). Since MMFO enzymes are known to catalyze the oxidation of double bonds in various compounds (5), it was of interest to see whether the same enzyme system or different enzyme systems are involved in the formation of reactive metabolites from AFB_1 and BP.

In this paper we present evidence to show the enzyme multiplicity of MMFO in relation to the activation of AFB_1 and BP.

MATERIALS AND METHODS:

Chemicals: Chromatographically pure AFB_1 was obtained from Calbiochem. Its purity was verified by TLC and spectroscopically as described previously (12). It was dissolved in purifed dimethyl sulfoxide (DMSO) and stored at -20° under conditions detailed elsewhere (12). ^3H-AFB_1 was obtained from New England Nuclear, purified by TLC, dissolved in DMSO and used within a week. Various details have been described (12). Benzo[a]-pyrene (3-4-benzopyrene) was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin, purified by recrystallization from benzene or ethanol and

then by TLC on silica gel plates (0.25 mm, obtained from E. Merck, Darmstadt, Germany) developed with benzene:hexane (1:15 v/v) solvent system. Generally ^{3}H-labeled BP was obtained from Amersham/Searle; it was purified by TLC, as described above, and diluted with unlabeled-BP to a required specific activity (6.2μ Ci/μmole). Cyclohexene oxide (98%) was obtained from Aldrich Chemical Company, and the sources of other chemicals have been described (12).

Preparation of Microsomes: Hepatic microsomes were isolated from untreated, phenobarbital-and 3-methylcholanthrene-treated male rats (190-260 grams) of the Sprague-Dawley strain. Both phenobarbital sodium (40 mg/kg/injection, i.p.) and 3-methy cholanthrene (15 mg/kg/injection i.p.) treated animals were given a total of seven injections of either compound over a period of four consecutive days, giving a single injection on day 1 and two injections daily on days 2 through 4. Rats were killed by decapitation on day 5, livers removed, pooled (2 livers in each pool) and used for the isolation of microsomes by the method reported earlier (12). Protein in the microsomes was assayed according to Lowry, et al. (18). The functional integrity of microsomes and the inductive effect of chemicals was verified in preliminary experiments by assaying the microsomes for aminopyrine demethylase (19), AHH (20), zoxazolamine hydroxylase (21) and cytochrome C reductase activities and the contents of cytochromes P_{450} (P_{488} for microsomes from methylcholanthrene-treated rats) and b_5(22). In general, phenobarbital was found to increase aminopyrine demethylase and cytochrome C reductase activities and the cytochrome P_{450} content by about two-fold. The effect of 3-methylcholanthrene on the activities of microsomal enzymes was evidenced by the presence of cytochrome P_{488} instead of P_{450}, by the increase in the activity of AHH and zoxazolamine hydroxylase and by a lack of an effect on the activities of aminopyrine demethylase and cytochrome C reductase.

Binding of the Reactive Metabolites of AFB_1 and BP to DNA: In a standard assay, incubation mixture (4.5 ml), containing ^{3}H-labeled AFB_1 or ^{3}H-BP, microsomes from untreated, phenobarbital-or 3- methylcholanthrene-treated rats, and DNA (native calf thymus DNA),was incubated in the presence and the absence of an NADPH-generating system (NADP, 0.81 mM; DL-isocitrate, 17 mM; isocitrate dehydrogenase, 150 μg

protein), in potassium phosphate buffer (0.07 M, pH 7.4) containing $MgCl_2$ (3.3 mM) and ethylenediamine tetraacetic acid disodium (EDTA) salt (0.2 mM). Incubation mixture devoid of DNA, microsomes and AFB_1 or BP was preincubated for 10 min at 37° to insure the presence of an adquate amount of NADPH (where needed) and to achieve thermoequilibration; this was followed by the addition of DNA, microsomes and AFB_1 (in 20 μl DMSO) or BP (50 μl acetone) in that order. Incubation was continued for an additional 1 hr and then sodium lauryl sulfate (0.02%) was added followed by the extraction of DNA, essentially according to the method described by Kuroki and Heidelberger (8). The reisolated DNA was dissolved in buffer and used for counting the incorporated radioactivity. Counting of the radioactivity was done as described previously (13). Molar extinction coefficient of 6600 at 260 nm was used for estimating the concentration of reisolated DNA (23). The concentration of the DNA was also estimated colorimetrically (24, 25) and it was found to be over 97% pure. Optical density 260/280 ratio for the DNA was about 1.9.

RESULTS AND DISCUSSION:

As shown in Table I, it was found that MMFO-mediated metabolites of ^{3}H-AFB_1 and ^{3}H-BP bind to DNA. In the absence of microsomes or NADPH no such binding was observed. These results are in agreement with results previously reported from this and other laboratories (7, 13, 15). The magnitude of the binding also differed, at least 10^3-fold more AFB_1 metabolite became bound to DNA than the metabolite(s) from BP. The binding of the AFB_1 and BP metabolites to DNA appears to be covalent as the resulting complexes were found to be resistant to separation on repeated precipitation, repeated solvent extraction with diethyl ether and $CHCl_3$ and on Sephadex (G-25) gel filtration.

In catalyzing the formation of the complex between AFB_1 and DNA, microsomes from phenobarbital-treated rats were at least twice as effective as microsomes from untreated and 3-methycholanthrene -treated rats. By contrast, when ^{3}H-BP was used as the substrate, microsomes from 3-methylcholanthrene-treated rats were about 9-fold more active than microsomes from untreated rats and about 5-fold more active than microsome from phenobarbital-treated rats. The effects of various pretreatments of rats on the in vitro formation of the reactive metabolite(s) from BP parallels their reported effects on

TABLE I

Comparison of the binding to DNA of the reactive metabolites from aflatoxin B_1 and benzo[a]pyrene in the presence of hepatic microsomes from different sources.

Native calf thymus DNA (4 mg/incubation flask) was incubated with hepatic microsomes (2.5 mg protein) from different sources and ^{3}H-aflatoxin B_1 (0.25 mM; specific activity, 0.41 μCi/μmole) or ^{3}H-benzo[a]pyrene (0.12 mM; specific activity, 6.18 μCi/μmole) in the presence of an NADPH-generating system as described in the text. An aliquot of reisolated DNA (>O.D. of 5 at 260 nm) was counted as described in the text.

Conditions of Incubation Prior to Reisolation of DNA	Aflatoxin B_1 n-moles metabolite / μ-mole DNA-P	Stochiometry of binding (Metabolite:DNA-P)	Benzo[a]pyrene p-moles metabolite / μ-mole DNA-P	Stochiometry of binding (Metabolite:DNA-P)
-MCS[a]	0.04 (2)[b]	-----	0.00 (2)[b]	-----
-NADPH	0.17 (2)[b]	-----	0.00 (2)[b]	-----
Control, MCS[c]	2.17 ± 0.14 (4)[b]	1:460	4.79 ± 2.16 (3)[b]	1;210,000
MC, MCS[d]	1.79 ± 0.38 (6)[b]	1:558	43.26 ±19.44 (3)[b,f]	1:20,000
PB, MCS[e]	7.13 ± 2.2 (4)[b,f]	1:140	8.10 ± 1.84 (3)[b]	1:120,000
	Control mcs used		Mc, mcs used	
+ Cyclohex. oxide (0.66 mM)	1.41[g]		96.11[h]	

a) hepatic microsomes; b) number of experiments given in the parentheses; (c, d, and e) hepatic microsomes from untreated, 3-methylcholanthrene-treated and phenobarbital-treated rats; f) significantly different from the corresponding control microsomes at $p < 0.05$; g) similar results were obtained with microsomes from phenobarbital-treated rats; h) results are representative of several experiments in which different concentrations of microsomal protein and BP were used.

the activity of AHH (26), in which assay the terminal phenolic metabolites (predominantly 3-hydroxy BP [20]),believed to be arising from intermediate epoxides (5), are measured. This observation tends to support the contention that epoxides might be intermediates in the formation of phenolic metabolites of aromatic hydrocarbons (5,27).

The concentration of the reactive metabolite(s) of BP, as measured by increased binding to DNA, was increased in the presence of cyclohexene oxide, an epoxide hydrase inhibitor (Table I); this observation taken together with several other reports (4-9) suggests that the reactive BP metabolites which bind to DNA are most likely epoxides. Recent studies on structure-activity relationships among various aflatoxins suggest that the reactive metabolite(s) of AFB_1 which binds to DNA and RNA is derived from MMFO-mediated oxidation of C_2-C_3 double bond (13,14). Swenson, et al. (28) have isolated 2,3-dihydrodiol of AFB_1 following the hydrolysis of RNA-AFB_1 metabolite adduct and have suggested that the reactive metabolite of AFB_1 is most probably 2,3-epoxide. Garner (29) has reported some metabolites of AFB_1 with chromatographic properties similar to the derivatives of 2,3-dihydrodiol of AFB_1. From the knowledge available concerning the mechanisms of MMFO-catalyzed oxidation of double bonds in various organic compounds (5,27), metabolic formation of 2,3-dihydrodiol of AFB_1 can be explained only in terms of 2,3-epoxide as the metabolic intermediate, which in the presence of epoxide hydrase is converted to 2,3-dihydrodiol or which alkylates RNA or DNA and the adduct on acid hydrolysis yields the dihydrodiol. Although cyclohexene oxide, by inhibiting the enzyme epoxide hydrase, caused increased accumulation and subsequent binding of "BP epoxide" to DNA, it failed to produce a similar effect with AFB_1 (Table I). Garner and Wright (17) have recently reported that cyclohexene oxide failed to enhance the microsome-mediated mutagenesis of AFB_1. If epoxide is the reactive intermediate of AFB_1, then the ineffectiveness of the epoxide hydrase inhibitor, cyclohexene oxide, to increase the concentration of the reactive AFB_1 metabolite in the DNA-binding assay could be explained in several ways: (a) AFB_1 epoxide may be very reactive and unstable, this suggestion derives support from the data showing 10^3-10^4 greater binding of AFB_1 metabolite to DNA than BP metabolite; and (b) epoxide hydrase involved in the catalysis of "BP-epoxide(s)" to the corresponding dihydrodiol may be ineffective for "AFB_1 - epoxide,"; the multiplicity of microsomal epoxide hydrases is well documented (30). Finally, a possibility remains

that the reactive metabolite of AFB_1, like reactive metabolites from some polycyclic aromatic hydrocarbons (31), might be a free radical formed by the microsome-mediated oxidation of C_2-C_3 double bond in AFB_1, although at present no data are available to support this hypothesis.

Whatever the nature of this reactive AFB_1 metabolite, our data concerning the effect of MMFO-inducers (Table I) clearly suggest that at least two different enzyme systems in MMFO complex are involved in the conversion of polycyclic compounds to DNA-binding metabolites: AFB_1 serves as a substrate for one enzyme system and BP for a different enzyme system.

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