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THE STRONG HEPATOCARCINOGENICITY OF THE ELECTROPHILIC AND MUTAGENIC METABOLITE 6-SULFOOXYMETHYLBENZO[*a*]PYRENE AND ITS FORMATION OF BENZYLIC DNA ADDUCTS IN THE LIVERS OF INFANT MALE B6C3F$_1$ MICE

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SUMMARY: 6-Hydroxymethylbenzo[*a*]pyrene was activated to an electrophilic and mutagenic sulfuric acid ester metabolite by rat and mouse liver sulfotransferase activity. The intrinsic mutagenicity of this reactive ester, 6-sulfooxymethylbenzo[*a*]pyrene, was inhibited by glutathione and glutathione *S*-transferase. A single i.p. dose of 2.5 nmol/g body wt of 6-sulfooxymethylbenzo[*a*]pyrene in infant male B6C3F$_1$ mice induced liver tumors in 35 of 36 mice at 10 months with an average multiplicity of 4.4. A comparable dose of the parent hydrocarbon, 6-hydroxymethylbenzo[*a*]pyrene, was only a tenth as active. The electrophilic sulfuric acid ester produced high levels of benzylic DNA adducts in the livers of these mice that accounted for about 80% of the total DNA adducts. These results strongly suggest that this sulfuric acid ester is an important ultimate electrophilic and carcinogenic metabolite in carcinogenesis by 6-hydroxymethylbenzo[*a*]pyrene and possibly even by 6-methylbenzo[*a*]pyrene and benzo[*a*]pyrene in mouse liver.

Extensive studies on benzo[*a*]pyrene (BP), one of the most potent carcinogenic polycyclic aromatic hydrocarbons, have demonstrated the importance of the "bay-region" dihydrodiol-epoxide as an ultimate electrophilic and carcinogenic metabolite of this hydrocarbon (1,2). However, other reactive metabolites may also be important in the activation and carcinogenicity of BP. The two principal alternative metabolic activation pathways suggested for BP concern the *meso*-anthracenic carbon atom. Thus, one-electron oxidation of BP at the 6-position has been shown by Cavalieri and Rogan (3) to produce a reactive radical cation intermediate. Other studies by Flesher and his associates (4,5) have demonstrated a small *S*-adenosyl-L-methionine-dependent methylation of BP to 6-methyl

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ABBREVIATIONS: BP, benzo[*a*]pyrene; MBP, 6-methylbenzo[*a*]pyrene; HMBP, 6-hydroxymethylbenzo[*a*]pyrene; AMBP, 6-acetoxymethylbenzo[*a*]pyrene; SMBP, 6-sulfooxymethylbenzo[*a*]pyrene; CMBP, 6-chloromethylbenzo[*a*]pyrene; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; GSH, glutathione.

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benzo[*a*]pyrene (MBP) by rat liver and lung cytosols. The enzymatic hydroxylation at the methyl groups of MBP and further metabolism to reactive benzylic esters such as sulfuric, phosphoric and acetic acid esters were proposed to generate a benzylic carbonium ion capable of covalently binding to the target cell DNA, thereby initiating the malignant transformation (6, briefly reviewed in 7). The supporting data on the metabolic formation of a reactive ester of 6-hydroxymethylbenzo[*a*]pyrene (HMBP) have been provided by our recent studies (8,9). Thus, incubation of HMBP with rat or mouse liver cytosols and the sulfo-group donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) produced an electrophilic sulfuric acid ester metabolite which covalently bound to the amino groups of deoxyguanosine and deoxyadenosine residues in calf thymus DNA to form benzylic adducts. These adducts were also produced in the livers of infant rats injected i.p. with HMBP (8,9). The chemically synthesized sulfuric acid ester of HMBP was reported to be directly mutagenic toward *Salmonella typhimurium* TA98 (10). Repeated topical applications of this reactive sulfuric acid ester to female Swiss mice produced high incidences of skin tumors (11,12).

The present report deals with the potent hepatocarcinogenicity of 6-sulfooxymethylbenzo[*a*]pyrene (SMBP), the electrophilic sulfuric acid ester metabolite of HMBP, in mouse liver. The sulfotransferase-mediated mutagenicity of HMBP was also investigated in this study.

MATERIALS AND METHODS

Chemicals HMBP and 6-acetoxymethylbenzo[*a*]pyrene (AMBP) were synthesized as reported previously (9). SMBP was prepared as a sodium salt according to the method for the synthesis of other electrophilic benzylic sulfuric acid esters (13). [^{3}H-CH_2]SMBP (97% radiochemical purity) was prepared from [^{3}H-CH_2]HMBP (9) in a manner similar to the synthesis of unlabelled SMBP. BP, PAPS, dehydroepiandrosterone, 5β-cholanic acid-3-one, and bromosulfophthalein were all products of Sigma Chemical Co. (St. Louis, MO). 2,6-Dichloro-4-nitrophenol was supplied by Fluka Chemical Corp. (Ronkonkoma, NY). Glutathione (GSH) and rat liver GSH *S*-transferase were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) and Sigma Chemical Co., respectively. Trioctanoin was purchased from Pfaltz and Bauer, Inc. (Stamford, CT). Other chemicals used were of reagent grade.

Bacterial mutagenicity assays The mutagenicity tests were performed with *Salmonella typhimurium* TA98 as a tester strain as previously described (13). The cytosolic fraction employed for sulfotransferase-mediated activation was prepared from the livers of 12-day-old male Sprague-Dawley rats.

Induction of hepatic tumors in mice Female C57BL/6J and male C3H/HeJ mice from the Jackson Laboratory (Bar Harbor, ME) were bred in our laboratory to give B6C3F_1 offspring. Male 12-day-old B6C3F_1 mice received a single intraperitoneal injection of BP, HMBP, SMBP or AMBP in 10 μl of 5% DMSO in trioctanoin/g body wt. Control animals were treated with only the vehicle. The mice were weaned at 4 weeks of age and killed at 10 months for enumeration of hepatomas.

Hepatic DNA adduct formation from SMBP in mice Infant male B6C3F$_1$ mice were injected i.p. with 0.1 μmol of [^{3}H-CH$_2$]SMBP (sp. act., 11 mCi/mmol) in 5 μl of DMSO/g body wt. Mice were killed 6 hr later and their livers were removed and immediately stored in -70°C. Isolation and digestion of hepatic nucleic acids and HPLC analysis of benzylic nucleic acid adducts were performed as previously described (9).

RESULTS AND DISCUSSION

Previous studies by Rogan *et al.* (10) reported the direct mutagenicity of synthetic SMBP in *S. typhimurium* TA98. We have recently demonstrated the sulfotransferase activity for HMBP in rat and mouse liver cytosols (8,9). These results imply that HMBP can become mutagenic through metabolic sulfuric acid esterification. The data from the present studies indicate that HMBP, which has no intrinsic mutagenicity, does induce a large increase in the number of His$^+$ revertants in the TA98 strain of *Salmonella typhimurium* when incubated with these bacteria in the presence of the sulfotransferase system (Table I). Heat-denaturation of the cytosolic proteins or omission of PAPS from the incubation mixtures resulted in no appreciable mutagenicity, indicating the involvement of sulfotransferase activity in converting HMBP to a mutagenic metabolite. This sulfotransferase-mediated mutagenicity of HMBP was markedly reduced by dehydroepiandrosterone (Table I). Dehydroepiandrosterone, a typical substrate

Table I. Sulfotransferase-dependent mutagenicity of HMBP in *Salmonella typhimurium* TA98

Experimental conditions	His$^+$ revertants/plate
Complete*	738 ± 87
Boiled cytosol replacement	47 ± 5
– PAPS	27 ± 10
+ 2,6-Dichloro-4-nitrophenol	832 ± 111
+ Dehydroepiandrosterone	43 ± 8
+ 5β-Cholanic acid-3-one	93 ± 10

*The complete incubation mixture contained HMBP (3 μM), $MgCl_2$ (3 mM), EDTA (0.1 mM), PAPS (0.2 mM), rat liver cytosol (equivalent to 1.4 mg of protein) and bacteria (3 x 10^9 cells) in a final volume of 0.5 ml phosphate buffer (0.1 M), pH 7.4. The concentration of each sulfotransferase inhibitor was 50 μM. The average number of spontaneous revertants in this experiment was 70 per plate and the data shown are corrected for this base level of revertants.

for hydroxysteroid sulfotransferases (14), has previously been demonstrated to strongly inhibit the hepatic sulfotransferase activity for HMBP (8,9). The phenol sulfotransferase inhibitor 2,6-dichloro-4-nitrophenol (15) was not effective in this regard. 5β-Cholanic acid-3-one, an inhibitor of the bile salt sulfotransferase I (16), was also found to strongly inhibit the sulfotransferase-mediated mutagenicity of HMBP (Table I). The data suggest that the bile salt sulfotransferase I may also catalyze the sulfonation of HMBP to SMBP. The identity of this enzyme with hydroxysteroid sulfotransferase II has recently been suggested (16).

As reported previously (10), the chemically synthesized sulfuric acid ester of HMBP was directly mutagenic without metabolic activation. The intrinsic mutagenicity of SMBP was significantly inhibited by GSH plus GSH *S*-transferase activity (Fig. 1); GSH alone was not effective in inactivating the mutagenic sulfuric acid ester. Heat-denaturation of the GSH *S*-transferase resulted in the loss of its catalytic activity for detoxification of SMBP by GSH. Bromosulfophthalein, an inhibitor of GSH *S*-transferases, preserved the mutagenic activity of SMBP, which otherwise would have been considerably inhibited by GSH and GSH *S*-transferase activity (Fig. 1). Similar findings were demonstrated with the

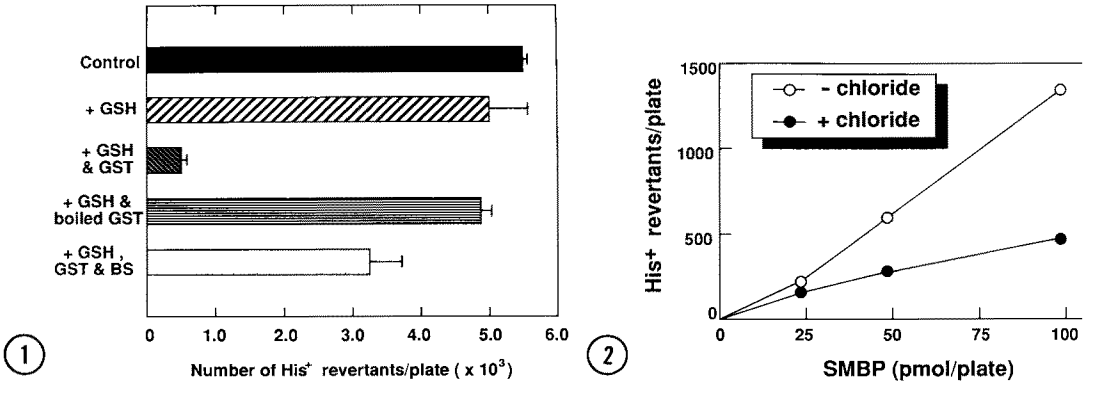

Fig. 1. Effect of GSH and GSH *S*-transferase on the bacterial mutagenicity of SMBP. The control incubation mixture in 0.5 ml of 0.1 M phosphate buffer (pH 7.4) contained SMBP (3 μM) and *Salmonella typhimurium* TA98 (3 x 10^9 cells). The other incubation mixtures included one or more of the following additional ingredients: GSH (2 mM), 0.5 mg of rat liver GSH *S*-transferase (GST), and bromosulfophthalein (BS; 0.1 mM).

Fig. 2. The effect of chloride ion on the intrinsic mutagenicity of SMBP. The indicated doses of SMBP were incubated with *S. typhimurium* TA98 in 0.5 ml sterilized water at 37°C for 30 min in the presence and absence of KCl (0.154 M). The bacterial cells were centrifuged prior to use. The supernatant was then discarded and the cells were rinsed with distilled water to remove most of the chloride ion originally derived from the culture medium.

sulfuric acid esters of other hydroxymethyl hydrocarbons including 9-hydroxymethyl-10-methylanthracene and 1-hydroxymethylpyrene (13).

It was recently shown that the chloride ion enhanced the bacterial mutagenicity of 1-sulfooxymethylpyrene by forming a less polar and highly mutagenic chloromethyl derivative (17,18). Similar chloride ion enhancements of mutagenicity were later observed with 9-sulfooxymethyl-10-methylanthracene (13) and 7-sulfooxymethyl-12-methylbenz[*a*]anthracene (Y.-J. Surh *et al.*, manuscript in preparation). By contrast, the mutagenicity of SMBP as determined in the present study was greatly reduced by a physiological concentration of chloride ion (Fig. 2). This appears to be due to the extremely short life span in water of the 6-chloromethylbenzo[*a*]pyrene (CMBP) formed by interaction of chloride ion with SMBP. The half-life of CMBP determined in 50% acetone in water at room temperature was reported to be only 0.7 sec (19). The half-life of CMBP in our assay is expected to be even shorter than this value since the assay was performed at 37°C in water. Thus, the rapid decomposition of CMBP would make it difficult for this hydrocarbon to penetrate the bacterial cell in sufficient amounts to interact with cellular DNA. This may account for the relatively weak mutagenicity of CMBP

Table II. Hepatocarcinogenicities of BP, HMBP, SMBP, and AMBP in male B6C3F$_1$ mice

Compound	Dose (μmol/g body wt)	Mice with hepatomas	Average no. of hepatomas/mouse
BP	0.015	12/32	0.5 ± 0.8
	0.005	7/29	0.3 ± 0.5
HMBP	0.015	23/28	2.6 ± 2.1
	0.005	14/31	0.6 ± 0.8
	0.0025	10/33	0.4 ± 0.6
SMBP*	0.0025	35/36	4.4 ± 2.8
AMBP	0.005	11/29	0.7 ± 1.1
	0.0025	10/33	0.3 ± 0.5
Vehicle only	-	4/30	0.2 ± 0.5

Groups of 30-40 male B6C3F$_1$ mice at 12 days of age received single i.p. doses of BP, HMBP, SMBP or AMBP in 10 μl per gram body wt of DMSO-trioctanoin (5:95, v/v). Control animals were treated with the vehicle alone. Experiments were terminated after 10 months for enumeration of hepatomas.

*A dose of 0.005 μmol/g body weight of SMBP was too toxic to permit an assessment of tumor formation.

previously noted by Ball *et al.* (19). Therefore, chloride ion may not necessarily enhance the mutagenic activity of all benzylic sulfooxymethyl aromatic hydrocarbons. The stability of the respective chloromethyl derivatives in water and their intrinsic chemical reactivities would appear to be important factors in determining the effect of chloride ion on the mutagenicity of electrophilic benzylic sulfuric acid esters in bacteria.

When the carcinogenicity of SMBP was assessed in B6C3F_1 mice, it was found to be a strong hepatocarcinogen in these animals (Table II). The parent hydroxymethyl hydrocarbon HMBP showed a dose-dependent hepatocarcinogenicity, but the activity was much weaker than that of the sulfuric acid ester. In this assay BP was even less carcinogenic than HMBP. It is striking that SMBP produced almost an 100% incidence of liver tumors at a single i.p. dose of only 2.5 nmol per gram body weight (Table II). The multiplicity of tumors induced by this ester was also higher than those of other hydrocarbons tested in this study. An i.p. dose of 5 nmol/g body wt of SMBP was toxic, producing severe adhesions in the peritoneal cavity, and the survival of the mice which received this dose of SMBP was quite low (~13%). The acetic acid ester AMBP was not more carcinogenic than HMBP (Table II). The low carcinogenic potency of the acetic acid ester of HMBP may be attributable to its low chemical reactivity as compared with that of the sulfuric acid ester (9). The results are in good agreement with those previously reported by Cavalieri *et al.* (11,12) on comparisons of the activities of multiple doses of benzylic esters of HMBP and the parent hydrocarbons including BP, MBP and HMBP in inducing skin tumors in mice.

Intraperitoneal injection of [^{3}H-CH_2]SMBP to infant male B6C3F_1 mice produced high levels of benzylic DNA adducts in the livers of these mice which accounted for approximately 80% of the total hepatic DNA adducts. The potent carcinogenicity of SMBP and the high levels of DNA adducts derived *in vivo* from this reactive ester strongly suggest that it plays an important role as an ultimate electrophilic and carcinogenic metabolite in carcinogenesis by HMBP and possibly even by MBP and BP.

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