Carcinogenicity of 2-Hydroxybenzo(a)pyrene and 6-Hydroxybenzo(a)pyrene in Newborn Mice


ABSTRACT

Benzo(a)pyrene (BP), 2-hydroxybenzo(a)pyrene (2-HOBP), and 6-hydroxybenzo(a)pyrene (6-HOBP) were tested for tumorigenicity by i.p. injection into newborn mice. The mice were treated sequentially with 200, 400, and 800 nmol of compound on the first, eighth, and eleventh day of life, and the animals were killed at 24 weeks of age. Treatment with 2-HOBP caused about 4-fold more pulmonary tumors than BP, while 6-HOBP had little or no tumorigenic activity. Newborn mice treated with 2-HOBP, BP, and 6-HOBP had a 98, 81, and 11% incidence of pulmonary adenomas with an average of 24, 6.4, and 0.11 adenomas per mouse, respectively. In the control group, 7.5% of the animals had pulmonary adenomas with an average of 0.08 adenoma per mouse. When 25, 50, or 100 nmol of BP or 2-HOBP was applied to mouse skin once every 2 weeks for 60 weeks, both compounds had about the same carcinogenic activity. These results demonstrate the importance of evaluating the carcinogenic potential of chemicals in more than one system. BP and 2-HOBP were tested for mutagenicity towards two strains of Salmonella typhimurium and towards Chinese hamster V79 cells in the presence of hepatic microsomes from rats pretreated with Aroclor 1254. The products formed during the metabolism of 2-HOBP or BP by liver microsomes had significant mutagenic activity.

INTRODUCTION

When the 12 possible isomeric phenols of BP4 were tested for carcinogenicity by chronic topical application to mouse skin, 2-HOBP was found to have potent carcinogenic activity at the one high dose tested (18). This was the first example of a phenolic polycyclic aromatic hydrocarbon with high carcinogenic activity. Studies with the initiation-promotion model also revealed that 2-HOBP was a good tumor initiator on mouse skin (16). Although 6-HOBP has been proposed as a possible carcinogenic metabolite of BP (10, 12), this compound was inactive as a complete carcinogen (7) on mouse skin at several dose levels. In addition, we have compared the mutagenic activities of BP and 2-HOBP in the presence of liver microsomal enzymes in strains TA98 and TA100 of Salmonella typhimurium and in Chinese hamster V79 cells.

MATERIALS AND METHODS

Materials

BP (Sigma Chemical Co., St. Louis, Mo.) 8-azaguanine (Calbiochem, San Diego, Calif.), Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.), fetal calf serum (Reheis Chemical Co., Kankakee, Ill.), and Aroclor-1254 (Monsanto Co., St. Louis, Mo.), were obtained from the indicated sources. 6-HOBP and 2-HOBP were synthesized as previously described (22) and were of analytical purity.

Newborn Mouse Experiments. Pregnant Swiss Webster mice, BLU:Ha(ICR), were obtained from Blue Spruce Farms, Altamont, N. Y., and were housed in plastic cages on corn cob bedding. They delivered their litters from 2 to 6 days after arrival in our laboratory. Within 24 hr of birth, 10 of the healthiest pups of each litter were given i.p. injections of 200 nmol of the compound in 5 μl of DMSO. At 8 and 15 days of age, the mice were given i.p. injections of 400 and 800 nmol of the compound in 10 and 20 μl of DMSO, respectively. Each mouse was treated with a total dose of 1400 nmol. Control mice were given injections of DMSO alone. After weaning at 25 days of age, the mice were fed Purina laboratory chow (Ralston Purina Co., St. Louis, Mo.) and water ad libitum and were kept in plastic cages with corn cob bedding. Animals that died during the course of the experiment or were killed when the experiment ended at 24 weeks of age were autopsied. Lungs and other organs which appeared abnormal were fixed in formalin. Macroscopic pulmonary tumors were counted after formalin fixation, and malignant lymphomas and lung adenomas were confirmed histopathologically. The diameter of the lung tumors ranged from 3 to less than 0.5 mm. The induction of lung tumors by chemicals in the mouse and the progression of these tumors to adenocarcinomas are described elsewhere (15).

Mouse Skin Experiments. Female C57BL/6J mice (4 to 5 weeks old) were obtained from The Jackson Laboratory, Bar Harbor, Maine and were fed the above commercial diet and water ad libitum. Application of BP or 2-HOBP was begun after an equilibration period of 4 weeks to ensure that the mice were in good health and gaining weight. The dorsal region of the animals was shaved with electric clippers 24 hr before the test compounds were applied. The shaving was done while the...
mice were under light ether anesthesia and was repeated whenever necessary. The compounds were dissolved in acetone and were applied in 25 µl of acetone once every 2 weeks for 60 weeks. Control mice received acetone.

Each treatment group originally consisted of 30 mice. Deaths among non-tumor-bearing animals never exceeded 8 animals per group. Progress of skin tumor formation was recorded every 3 weeks, and tumors greater than 2 mm in diameter were included in the cumulative total only if they persisted for 3 weeks or longer. Most of the skin tumors observed after chronic topical application of BP or 2-HOBP were squamous cell carcinomas.

**Microsomal Enzymes.** Hepatic microsomes were obtained from immature male Long Evans rats pretreated with the polychlorinated biphenyl mixture Aroclor-1254 as described previously (13). The microsomes were stored at −90°C.

**Metabolic Activation Assays with Mammalian Cells**

Chinese hamster V79-6 cells were kindly provided by Dr. E. H. Y. Chu, University of Michigan, Ann Arbor, Mich. The cells were grown in complete MEM6 supplemented with 10% fetal bovine serum. The metabolic activation assay was adapted from the procedure of Kuroki et al. (9). The V79 cells were plated in 35-mm culture dishes at a concentration of 5 x 10^5 cells/dish and were cultured for 18 hr. The medium was removed, the cell monolayer was washed once with PBS, and an incubation mixture was added that contained 0.5 ml of PBS (pH 7.4), 0.25 ml of cofactors (2.0 µmol NADP, 2.5 µmol of glucose 6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase) in 0.2 µmol phosphate buffer (pH 7.4) along with 0.25 ml of a suspension of microsomes containing 1 nmol of cytochrome P-450. BP or 2-HOBP was then added in 20 µl of DMSO. After 30 min of incubation at 37°C, the reaction mixture was removed, and the cell monolayer was washed twice with 1 ml PBS before a 3-hr incubation in the culture medium. The cells were then suspended with trypsin as described previously (9). For each determination, 4 replicate 60-mm culture dishes seeded with 10^6 cells were used to evaluate toxicity, and 16 replicate 60-mm culture dishes seeded with 10^5 cells were used to score 8-azaguanine-resistant colonies. The culture conditions and procedures used were described previously (20).

**Metabolic Activation Assays with Bacteria**

Strains TA98 and TA100 of histidine-dependent S. typhimurium were obtained from Dr. B. Ames, University of California, Berkeley, Calif., and were cultured as described (11, 20). Mutagenesis experiments with microsomes as the source of the monoxygenase activity were based on the procedure described by Ames et al. (11). NADP (2.0 µmol), glucose 6-phosphate (2.5 µmol), and glucose-6-phosphate dehydrogenase (1 unit) were added to a 13- x 100-mm culture tube in 0.25 ml of pH 7.4 buffer containing 50 µmol of sodium phosphate, 4 µmol of MgCl2, and 16.5 µmol of KCl. After addition of microsomes containing 0.60 nmol of cytochrome P-450 (in 0.25 ml of 0.15 M KCl), the bacteria (2 x 10^8) were added in 0.1 ml of 0.15 M NaCl buffered to pH 7.0 with 5 mm sodium phosphate. Reactions were started by the addition of BP or 2-HOBP in 15 µl of solvent (acetone/DMSO, 90:10), and the complete incubation mixtures were incubated at 37°C for 5 min before the addition of 2 ml of top agar. Each incubation mixture was then poured onto a Petri dish that contained histidine-deficient agar. Mutations to histidine-independent growth were assessed by counting the macroscopic colonies of bacteria after a 2-day incubation of the plates at 37°C. All experiments were performed in triplicate.

**RESULTS**

Ninety-eight% of newborn mice treated with a total dose of 1400 nmol of 2-HOBP had lung adenomas when the mice were killed at 24 weeks of age. These mice had an average of 24 lung adenomas per mouse (Table 1). Mice treated with a total dose of 1400 nmol of BP had an 81% incidence of lung adenomas with an average of 6.4 lung adenomas per mouse. Mice treated with the same dose of 6-HOBP and those in the control group had an 11 and 8% incidence of lung adenomas with an average of 0.11 and 0.08 adenoma per mouse, respectively. One mouse in each of the groups treated with 2-HOBP and 6-HOBP developed malignant lymphoma, and one mouse treated with 6-HOBP had an endometrial stromal sarcoma of the uterus.

The chronic application of 25, 50, or 100 nmol of BP or 2-HOBP on mouse skin once every 2 weeks for 60 weeks revealed that BP had about the same or slightly more tumorigenic activity than 2-HOBP (Chart 1). Previous studies (19) have shown that concentrations of 2-HOBP from 4 to 40 nmol/plate did not induce mutations above background in strains TA98, TA100, or TA1538 of S. typhimurium. Similarly, BP is not intrinsically active as a mutagen in these strains of bacteria. Chart 2 illustrates the number of mutations induced in strain TA98 or TA100 of S. typhimurium when these target cells and from 1 to 10 nmol of BP or 2-HOBP were incubated with an NADPH-generating system and hepatic microsomes from Aroclor 1254-pretreated rats. In strain TA98, the products formed from 2-HOBP were 4 to 5 times more mutagenic than were the metabolic products from BP. In strain TA100, the products formed from 2-HOBP were more mutagenic than those formed from BP at the low substrate concentrations but less active than those formed from BP at the higher substrate concentrations (Chart 2). In the same experiment, the mutagenic activities of BP and 2-HOBP at a concentration of 2 nmol/incubation were completely dependent on and increased with increasing amounts of microsomes. In Chinese hamster V79 cells, the metabolic activation of BP to mutagens occurred to a slightly greater extent than did the metabolic activation of 2-HOBP (Chart 3). In 2 additional experiments with Chinese hamster cells, 25 and 50 nmol of BP or 2-HOBP had no intrinsic cytotoxic (≥98% cell survival) or mutagenic (<0.7 8-azaguanine-resistant colonies/10^6 surviving cells) activities when microsomes were omitted from the incubation. Addition of microsomes containing 1.0 nmol of cytochrome P-
Table 1

Tumorigenicity of BP, 2-HOBP, and 6-HOBP in newborn mice

Swiss-Webster mice (BLU:Ha(ICR)) were given i.p. injections of 200, 400 and 800 nmol of BP or the BP phenols in DMSO on the 1st, 8th, and 15th days of life, respectively. Control animals received injections of DMSO. The animals were killed at 24 weeks of age, and the number of lung tumors was determined as described in "Materials and Methods." One mouse treated with 6-HOBP had an endometrial stromal sarcoma of the uterus.

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<th>Compound</th>
<th>No. of mice given injections on day 1</th>
<th>Sex</th>
<th>No. of mice alive at weaning</th>
<th>No. of mice with pulmonary adenoma</th>
<th>No. of adenomas</th>
<th>% of mice with pulmonary adenomas</th>
<th>No. of adenomas/mouse</th>
<th>No. of mice with malignant lymphomas</th>
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Chart 1. The development of skin tumors in C57BL/6J mice treated topically with BP or 2-HOBP. Thirty mice were treated topically with 25, 50, or 100 nmol of compound in acetone once every 2 weeks for 60 weeks. The cumulative percentage of animals with tumors was calculated from surviving mice with and without tumors and from tumor-bearing mice that died during the 60 weeks of treatment.

450 resulted in the induction of 5 and 21 colonies that were 8-azaguanine resistant per 10⁸ surviving cells at the 25- and 50-nmol dose, respectively, of 2-HOBP. Similar results were obtained with BP. These data indicate that the biological activities of 2-HOBP and BP shown in Chart 3 are mediated by a metabolite(s) of these compounds.

DISCUSSION

The data presented in Table 1 demonstrate that 2-HOBP is more tumorigenic than BP in the newborn mouse, and these results are the first demonstration that a phenolic polycyclic aromatic hydrocarbon can have greater tumorigenicity than the parent hydrocarbon. Since the results in Chart 1 indicate that 2-HOBP possesses the same or slightly less carcinogenic activity than does BP on mouse skin, the relative tumorigenicity of BP and 2-HOBP depends on the animal model used. Previous studies in which 400 nmol of each of the 12 isomeric phenols of BP were applied to the skin of mice once every 2 weeks for 60 weeks indicated that 2-HOBP and BP had high carcinogenicity, 11-hydroxybenzo(a)pyrene was weakly active, and the other 11 BP phenols were inactive (7, 18). Since the amount of BP used in these earlier studies was at the top of the dose-response curve, it was not possible to accurately assess the relative activities of BP and 2-HOBP. Equal tumorigenicity of BP and 2-HOBP was also found when a single high dose of 400 nmol of these compounds was used in tests for initiating activity in the initiation-promotion model on mouse skin (16). In

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Chart 2. Microsomal-mediated metabolism of BP and 2-HOBP to products mutagenic to strains TA98 and TA100 of S. typhimurium. Each reaction mixture contained 0.6 nmol of cytochrome P-450, the indicated amounts of substrate, and an NADPH-generating system. Background mutation frequencies of 32 and 75 His+ revertants per plate have been subtracted from the data in strains TA98 and TA100, respectively. No mutagenic response was observed in the absence of microsomes.

Chart 3. Microsomal-mediated metabolism of BP and 2-HOBP to products mutagenic to Chinese hamster V79 cells. The cells were incubated with liver microsomes containing 1.0 nmol of cytochrome P-450, the indicated amounts of substrate, and an NADPH-generating system. In control experiments in which either hydrocarbon or microsomal protein were omitted, the mutagen frequency was less than 0.7 colony that was 8-azaguanine resistant per 10^5 surviving cells.

indicate that 6-HOBP has little or no tumorigenic activity in newborn mice. Similarly, 6-HOBP was inactive as a complete carcinogen on mouse skin (7), and 6-HOBP had little or no activity as an initiator in the initiation-promotion model on mouse skin (16). The results of these studies indicate that 6-HOBP does not play an important role in the carcinogenicity of BP on mouse skin or in the newborn mouse.

Studies of the intrinsic mutagenicity of 2-HOBP and 6-HOBP indicated that 6-HOBP possessed weak mutagenic activity in S. typhimurium strains TA98 and TA100 (19) and in Chinese hamster V79 cells (5, 19), whereas 2-HOBP was inactive as a bacterial mutagen in strains TA98, TA100, and TA1538 of S. typhimurium (19). In Chinese hamster V79 cells, 2-HOBP showed very weak inherent mutagenic activity at a total dose of 200 to 300 nmol. As indicated in the present manuscript, doses of 25 to 50 nmol had no intrinsic mutagenic activity. Studies on the metabolic activation of 2-HOBP and 6-HOBP demonstrated that both compounds can be metabolically activated to mutagens by a highly purified cytochrome P-450 system from rat liver (21), but both phenols were significantly less active than BP when the purified monooxygenase system was used. The present study, which used liver microsomes from Aroclor-treated rats, indicated that the products formed during the metabolic activation of 2-HOBP were more mutagenic towards S. typhimurium strain TA98 than are the products formed from the metabolic activation of BP. In strain TA100, the relative mutagenic activity of 2-HOBP and BP after metabolism was dependent on the substrate concentration used. When a low substrate concentration was used, 2-HOBP was more mutagenic than BP, whereas BP was more active than 2-HOBP when a high substrate concentration was used. BP was slightly more active than 2-HOBP when both compounds were metabolized to mutagens in the presence of mammalian V79 cells. It would be of interest to determine how 2-HOBP is metabolized in order to identify the metabolic intermediates of this compound that cause mutations in bacterial and mammalian cells and that participate in the initiation of cancer in mice. A recent study indicated that metabolism of benzo(a)pyrene 7,8-dihydriodiol or 2-HOBP by liver microsomes in the presence of calf thymus DNA resulted in the formation of DNA-bound adducts with similar fluorescence properties (4). These results suggest metabolism of 2-HOBP to appreciable amounts of a 7,8-diol-9,10-epoxide or a 9,10-diol-7,8-epoxide. Although the former diol-epoxide would be expected to have a greater biological activity (17), formation of the latter metabolite would be expected, from chemical considerations, to be enhanced by the presence of a hydroxyl group at the 2-position (18).

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