Lack of Carcinogenicity of 4-, 5-, 6-, 7-, 8-, 9-, and 10-Hydroxybenzo(a)pyrene on Mouse Skin

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SUMMARY

Seven phenols of benzo(a)pyrene (4-, 5-, 6-, 7-, 8-, 9-, and 10-hydroxybenzo(a)pyrene) were tested for carcinogenicity on mouse skin by application of 0.4 μmole of compound once every two weeks for 56 weeks. None of the seven phenols tested was carcinogenic to mouse skin, while treatment with the same dose of benzo(a)pyrene produced tumors in 92% of the treated animals. The lack of carcinogenicity of 7- and 8-hydroxybenzo(a)pyrene indicates that the strong carcinogenic activity previously reported for benzo(a)pyrene 7,8-oxide was not due to either phenolic isomerization product of this arene oxide.

INTRODUCTION

The widespread occurrence of the polycyclic aromatic hydrocarbon BP2 in polluted city air, automobile exhaust, cigarette smoke, and elsewhere in man’s environment has stimulated intense interest in the carcinogenic properties of this compound (2, 4). Polycyclic hydrocarbons such as BP require metabolic activation in order to react with macromolecular cellular components (7, 8, 11, 18, 19, 24). This activation is believed to be an intermediary step in the production of mutations as well as cancer. The biotransformation of polycyclic hydrocarbons, which is catalyzed by the microsomal mixed-function oxidase system (3, 9, 11, 24, 34), results in the generation of arene oxides that can either be converted into dihydrodiols by microsomal epoxide hydrolase, conjugated with glutathione S-transferases, or can undergo nonenzymatic rearrangement to phenols.

Levin et al. (15) recently reported that BP 7,8-oxide was highly carcinogenic when applied on mouse skin (0.4 μmole once every 2 weeks), while the “K-region” BP 4,5-oxide was a very poor carcinogen, and BP 9,10-oxide was not carcinogenic. The results of the present study indicate that the 6 BP phenols corresponding to the nonenzymatic activation of the above BP oxides do not cause skin cancer in mice.

6-HOBP, another phenolic derivative of BP, has been suggested as a proximate carcinogen of BP, due to its high reactivity with nucleic acid (25) and its capacity to induce transformation of Syrian hamster embryo fibroblasts (21). In the present study, 6-HOBP did not show any carcinogenic effect when applied to mouse skin.

MATERIALS AND METHODS

Chemicals. BP was obtained from Sigma Chemical Co., St. Louis, Mo. The 4-, 5-, 6-, 7-, 8-, 9-, and 10-HOBP derivatives of analytical purity were synthesized as previously described (32). BP and the phenols of BP were dissolved in acetone to give a concentration of 0.4 μmole of compound per 25 μl of solvent. The solutions were stored at ~90° in amber glass vials. Since the phenols of BP may undergo spontaneous oxidation to quinones, their stability was checked by thin-layer chromatography before application of the solutions to mouse skin. Five μl of phenol solution were applied on Eastman Chromagram No. 6060 sheets, and chloroform:ethyl acetate:benzene (1:1:1) or chloroform:ethyl acetate:benzene (1:1:1) or chloroform:ethyl acetate (3:1) was used as the developing solvent. The phenols of BP were stable for at least 1 month when stored in acetone at ~90°.

Animals. Female C57BL/6J mice were obtained at 4 weeks of age from The Jackson Laboratory, Bar Harbor, Maine, and were fed a commercial diet (Purina laboratory chow; Ralston Purina Co., St. Louis, Mo.) and water ad libitum. Application of the chemicals began after an equilibration period of 5 weeks in order 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 performed under light ether anesthesia and was repeated whenever necessary. Twenty-five μl of each of the test solutions were applied on the exposed area of the skin once every 2 weeks for 56 weeks. Control mice were treated with 25 μl of acetone. The room was kept in subdued light during application of the chemicals, and the mice were housed in the dark for 24 hr after each application.

Each treatment group consisted originally of 30 mice. Deaths among nontumorous animals were minimal (up to 4/ group) and were similar in number for both control and treated groups. No significant differences were detected in the weights of the animals among the different groups throughout the study until animals had large tumors or other skin lesions. Progress of skin tumor formation was recorded every 2 weeks. Starting at 39 weeks after the study was initiated, selected animals with tumors or skin lesions were killed and their skin and internal organs were sub-

1 To whom requests for reprints should be addressed.
2 The abbreviations used are: BP, benzo(a)pyrene; 4-, 5-, 6-, 7-, 8-, 9-, and 10-HOBP, 4-, 5-, 6-, 7-, 8-, 9-, and 10-hydroxybenzo(a)pyrene, respectively.

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jected to histopathological examination. The study was terminated 59 weeks after initiation, and the remaining animals were killed for histopathological examination.

RESULTS

Repeated application of 0.4 μmole of 4-, 5-, 6-, 7-, 8-, 9-, or 10-HOBP on the skin of mice once every 2 weeks for 56 weeks did not cause tumors, while 92% of the BP-treated animals developed skin tumors (Table 1). The 1st tumors in the latter group were observed after 26 weeks of treatment. At the time the experiment was terminated, 59 weeks after its initiation, 24 animals treated with BP had developed a total of 34 skin tumors. The 2 BP-treated animals without skin tumors had developed gross skin ulcerations after 44 weeks of treatment. Histological examination of the skin of 16 BP-treated tumor-bearing animals revealed the presence of 17 squamous cell carcinomas. Histology on an additional BP-treated animal revealed the presence of 1 papilloma. Metastatic squamous cell carcinomas were also found in the lungs of 2 of the 16 animals with skin squamous cell carcinomas. Skin ulcerations were found in several of the BP-treated animals that developed tumors and in 1 animal treated with 4-HOBP, in 4 animals treated with 7-HOBP, and in 3 animals treated with 10-HOBP. In addition, lymphomas of the lymph nodes and/or liver were found in 2 animals treated with 6-HOBP, in 1 animal treated with 9-HOBP, and in 2 animals treated with 10-HOBP.

The stability of the BP phenols could be an important factor in determining the carcinogenicity of these compounds. Since 6-HOBP is the least stable of the 7 BP phenols included in the present study (unpublished observations), we determined the half-life of this compound. Two hundred μl of a 2 mM solution of 6-HOBP in dimethyl sulfoxide were added to 10 ml of a phosphate-buffered saline solution that consisted of 8 mM sodium phosphate, 1.5 mM potassium phosphate, 140 mM NaCl, and 3 mM KCl, pH 7.4. The mixture was incubated with gentle shaking at 37°C in the dark and then extracted with acetone:hexane (1:3), and the breakdown of 6-HOBP was determined by the method described by Wiebel (26). Fluorescence was measured in the organic phase at an excitation wavelength of 400 nm and at an emission wavelength of 454 nm. The half-life of 6-HOBP was thus found to be 25 min. The organic extracts were also chromatographed by the thin-layer system described for determining the stability of the BP phenols in acetone. Incubation of 6-HOBP for 30 min at 37°C produced new nonfluorescent spots on the chromatogram which were not observed before incubation. A half-life of 25 min was also obtained for 6-HOBP by determining the decrease in mutagenicity of this compound after incubation in phosphate-buffered saline for various times prior to addition to strain TA 98 of Salmonella typhimurium. Quinones arising by spontaneous autooxidation are the presumed breakdown products of 6-HOBP under the aerobic conditions of these experiments (14, 16).

DISCUSSION

The non-K-region BP 7,8-oxide is highly carcinogenic to mouse skin, while the K-region BP 4,5-oxide is a poor carcinogen and the non-K-region BP 9,10-oxide is not carcinogenic (15). Since arene oxides undergo spontaneous isomerization to phenolic derivatives (12), it was important to determine whether the phenolic isomerization products of the above arene oxides were responsible for their carcinogenicity to mouse skin. The results of the present study indicate that 4-, 5-, 7-, 8-, 9-, and 10-HOBP, which are the phenolic rearrangement products of BP 4,5-, 7,8-, and 9,10-oxides, do not produce skin tumors when applied topically on mice. The failure of the BP phenols to induce skin cancer is highly significant when one considers that the dose of BP phenols used (0.4 μmole) is at least 4 times greater than that required to produce a maximum number of tumors with BP (15). This finding is especially important in the case of the carcinogenic BP 7,8-oxide, which is very unstable (15, 29), and which gives rise to appreciable amounts of 8-HOBP and, especially, 7-HOBP (33). The present findings rule out the possibility that the strong carcinogenicity of BP 7,8-oxide and the weak carcinogenicity of BP 4,5-oxide are due to their phenolic isomerization products. Although noncarcinogenic, the BP phenols might possibly act as promoters or modifiers of tumor development. 5-HOBP (5) and 7-HOBP (1, 6, 22) previously have been shown to have little or no carcinogenic activity when painted onto or injected s.c. into mice.

The noncarcinogenic 4-, 5-, 7-, 8-, 9-, and 10-HOBP are nonmutagenic to strains TA 1538, TA 98, and TA 100 of S. typhimurium and to Chinese hamster V79 cells (27, 29). On metabolic activation with a highly purified cytochrome P-448-containing system, 9-HOBP shows weak mutagenic activity, while none of the other 5 phenols is metabolically activated (30).

6-HOBP is mutagenic to strains TA 98 and TA 100 of S. typhimurium (27), and these mutagenic activities were in-

<table>
<thead>
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<th>Treatment</th>
<th>Total no. of animals</th>
<th>No. of animals alive at 59 wk</th>
<th>No. of animals with skin tumors</th>
<th>Total no. of skin tumors</th>
</tr>
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<tr>
<td>Control</td>
<td>28</td>
<td>28</td>
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<td>0</td>
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<tr>
<td>BP</td>
<td>26</td>
<td>14</td>
<td>24</td>
<td>34*</td>
</tr>
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<td>4-HOBP</td>
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<td>26</td>
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<td>0</td>
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<td>26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6-HOBP</td>
<td>28</td>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7-HOBP</td>
<td>30</td>
<td>27</td>
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<td>27</td>
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<tr>
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<td>26</td>
<td>26</td>
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</tr>
<tr>
<td>10-HOBP</td>
<td>28</td>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Total number of mice alive at the end of the study, plus mice with skin tumors or ulcers that died earlier or were killed for autopsy.
* Seventeen squamous cell carcinomas of the skin, 2 metastatic squamous cell carcinomas of the lung, and 1 papilloma were found in 17 autopsied tumor-bearing animals that were treated with BP. Skin ulcers were found in 1 animal treated with 4-HOBP, in 4 animals treated with 7-HOBP, in 3 animals treated with 10-HOBP, and in 2 animals treated with BP. Lymphomas of the lymph nodes and/or liver were found in 2 animals treated with 6-HOBP, in 1 animal treated with 9-HOBP, and in 2 animals treated with 10-HOBP.
creased by metabolic activation of 6-HOBP in the presence of highly purified microsomal enzymes (30). Ts’o et al. (25) and Nagata et al. (20) recently suggested that the highly reactive phenoxy radical of BP, 6-0xy-BP, which originates enzymatically as well as nonenzymatically from 6-HOBP (14, 16, 20), might be a proximate carcinogen of BP. Under conditions that facilitate the oxidation of 6-HOBP, this compound will cause strand breakage in DNA (25) and cell transformation in Syrian hamster embryo fibroblasts (21). Nagata et al. (20) reported that the nuclear fraction of the cell has a high capacity to transform BP enzymatically into the 6-0xy-BP radical. They suggested that, since the site of formation of the above radical is in the vicinity of DNA, this might facilitate the interaction of 6-0xy-BP with nucleic acid, which in turn will cause mutations and possibly cancer. 6-HOBP was shown to induce fibrosarcomas in rats and, to a lesser degree, in mice (20), but this effect was far less potent than that of BP. Shear et al. (23) did not detect tumor formation after a single s.c. injection of 6-HOBP to mice. 6-HOBP did not induce skin tumors in the present study. This inactivity is probably not due to low stability, since 6-HOBP is at least as stable as BP 7,8-oxide which, despite its being a very unstable compound, was highly carcinogenic to mouse skin (15).

An important finding in the present study is the remarkably efficient fashion in which a single hydroxyl group, at any of the ring positions from the 4- to the 10-position, is able to block the skin carcinogenicity of BP. Although it is not known whether a phenolic hydroxyl group is capable of blocking metabolic formation of an arene oxide at the formal aromatic double bond, to which it is attached, it is known that a hydroxyl group will dramatically shorten the half-life for isomerization of an arene oxide to a phenol (13). Thus, introduction of a hydroxyl group might not only markedly alter the profile of metabolites normally obtained from BP, but could also dramatically reduce the amount of binding to cellular nucleophiles and decrease the hydration of an arene oxide by epoxide hydrase. This effect of a hydroxyl group may effectively block formation of the highly mutagenic (10, 17, 28, 31) and possibly carcinogenic stereoisomers of (7-trans-7,8-dihydoxy-9,10-epoxy-7,9,10-tetrahydrobenzo[a]pyrene) by preventing formation of trans-7,8-dihydoxy-7,8-dihydoxybenzo[a]pyrene. Another possible reason for the inactivity of the BP phenols is that they may be metabolized or removed from the skin more rapidly than BP.

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