Inhibition of the Carcinogenic Action of Benzo(a)pyrene by Flavones

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SUMMARY

A study of the effect of feeding flavone inducers of increased benzo(a)pyrene (BP) hydroxylase activity on pulmonary adenoma formation resulting from p.o. administration of BP to A/HeJ mice has been carried out. Three flavones have been used: β-naphthoflavone, a highly potent inducer; quercetin pentamethyl ether, intermediate in inducing potency; and rutin, a weak inducer. Administration of β-naphthoflavone resulted in almost total inhibition of pulmonary adenoma formation; with quercetin pentamethyl ether approximately 50% inhibition occurred, and with rutin there was no inhibition. In a second investigation, the effect of inducing increased BP hydroxylase activity in the skin of Ha/ICR mice on epidermal neoplasia initiated by BP was studied. Topical application of β-naphthoflavone resulted in slightly more than 50% inhibition of skin tumor formation.

INTRODUCTION

The objective of the present study was to determine whether induction of increased BP hydroxylase activity by flavones will protect against the carcinogenic effects of BP. In previous studies, several inducers of increased polycyclic hydrocarbon hydroxylase activity have been shown to inhibit carcinogenesis resulting from administration of 7,12-dimethylbenz(a)anthracene to mice and rats (7, 13, 16). 7,12-Dimethylbenz(a)anthracene is an unusual polycyclic hydrocarbon carcinogen, particularly in relationship to members of this chemical class occurring as environmental pollutants. It is an exceedingly potent carcinogen and has unusual toxic features, and hydroxylation of its methyl groups constitutes a principle pathway of its metabolism (3, 8, 12). Accordingly, it would be desirable to determine whether increased polycyclic hydrocarbon hydroxylase activity will protect against the carcinogenic effects of more typical polycyclic hydrocarbon carcinogens. For this purpose BP was chosen for study. BP occurs widely as an environmental pollutant and in its carcinogenic potency and metabolism it is similar to a number of other polycyclic hydrocarbons found in the environment. In addition, a technique for determining BP hydroxylase activity is available (14, 15).

The inducers of increased BP hydroxylase activity which have been selected for use in the present work are 3 flavones with widely differing capacities to induce increased BP hydroxylase activity (15). The most active of the 3 is β-naphthoflavone (5,6-benzoflavone), a synthetic compound. Of all flavones tested thus far, β-naphthoflavone is the most potent inducer of increased BP hydroxylase activity. The 2nd flavone selected is quercetin pentamethyl ether (3,3',4',5,7-pentamethoxyflavone), which is of intermediate potency as an inducer. This compound has an inducing capacity similar to tangeretin (5,6,7,8,4'-pentamethoxyflavone) and nobiletin (5,6,7,8,3',4'-hexamethoxyflavone), which are the most potent of the naturally occurring flavone inducers of increased BP hydroxylase activity which have been studied. Like these 2 compounds, it is totally methylated. The 3rd flavone chosen for study is rutin (3,3',4',5,7-pentahydroxyflavone-3-rutinoside), a naturally occurring compound which is a very weak inducer.

Two experimental carcinogenesis systems have been studied. The first of these is pulmonary adenoma formation in mice receiving BP by p.o. intubation subsequent to administration of inducers. In this experimental system, the carcinogen traverses tissues with BP hydroxylase activity prior to reaching the target organ in which neoplasms develop. The second experimental system studied is epidermal tumor formation in the mouse. In the procedure used, topical application of BP was used to initiate tumor formation (1) subsequent to administration of inducer. In this instance, there is direct contact of carcinogen with the target tissue.

MATERIALS AND METHODS

In the studies of pulmonary adenoma formation, female A/HeJ mice obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine, were used. At 7 weeks of age, the mice were placed on a diet consisting of a powdered form of Purina rat chow (Ralston Purina Company, St. Louis, Mo.) to which had been added 4% sesame oil USP. In mice receiving inducers, the compounds were dissolved in the sesame oil. The animals were maintained on these diets for 16 days. They were then placed on Purina rat chow without any additions. At this time, 4 groups of mice received BP and 2 remaining groups, 1 of which had been on the control diet and 1 of which had received β-naphthoflavone in the diet, were maintained as "nocarcinogen" controls, to determine pulmonary tumor incidence in mice not receiving carcinogen. The mice receiving BP were given 2 doses administered 2 hr apart by p.o.

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2 The abbreviation used is: BP, benzo(a)pyrene.

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intubation. Each dose consisted of 3 mg of BP in 0.25 ml of sesame oil. The initial administrations were started at 9 a.m. and the 2nd administrations were started at 11 a.m. When the mice were 11 weeks old, they were again placed on diets containing the same inducers (or the control diet) as before. As in the initial procedure, this was continued for 16 days, after which the animals were returned to a normal diet and p.o. administrations of BP were carried out a 2nd time with the same dosage and schedule as for the 1st administrations. Thus, the mice received 2 courses of inducers (or control diet), each followed by BP administration. The morning following each course, 4 mice from each group were sacrificed for BP hydroxylase determinations in the mucosa of the proximal 6 cm of the small intestine, liver, and lung, according to procedures previously described (14, 15). One unit of BP hydroxylase activity is equivalent to the formation of 100 μg of 8-hydroxy-BP/min. After sacrifice of animals for determination of BP hydroxylase activity, all groups of mice ultimately to be used for pulmonary adenoma counts consisted of 13 to 16 animals. Subsequently, 1 death occurred. For the pulmonary adenoma counts, all mice were sacrificed at 30 weeks of age, and gross counts were carried out with the method of Shimkin (11).

For the investigations of skin tumor formation, female Ha/ICR mice obtained from the Millerton Farms, New York, N. Y., were used. At 60 days of age, an area of skin approximately 2 x 4 cm was shaved on the upper portion of the back. Three days later, topical applications of β-naphthoflavone or vehicle were begun. Only animals in the resting stage of hair growth were used. The group of mice in which induction was carried out received a topical application of β-naphthoflavone, 6 mg/ml sesame oil, to the shaved area. Control animals received sesame oil only. A No. 7 camel’s hair brush was used. These applications were carried out each morning for 3 days. On the next day, 4 mice from each group were sacrificed for BP hydroxylase determinations of the skin and the remaining mice were used for the carcinogenesis study. Four groups of mice were formed (Table 1). The first of these was from the mice which had received sesame oil and the second was from those which had received β-naphthoflavone. Both of these groups now received topical administrations of BP. An initial administration was given at 10 a.m. and a 2nd administration was at 2 p.m. At both times, 5 μg of BP in 2 drops of acetone with 1% mineral oil were dropped onto the shaved area of the back. The 3rd group of mice was from the original group which had received sesame oil and the 4th was from those which had received β-naphthoflavone. Both of these groups now received topical applications of acetone with 1% mineral oil, but no BP, according to the same schedule as the first 2 groups. Two weeks after the application of BP or solvent, croton oil administrations were begun. Two drops of croton oil 1% in acetone with 1% mineral oil were dropped onto the backs of all mice twice a week. This was continued until the termination of the experiment.

The procedure for determining BP hydroxylase activity in skin differs from that used previously for other tissues. A piece of skin approximately 2 x 4 cm dissected from the upper back was used. It included the full thickness of the skin, containing both epidermis and dermis. The specimen was frozen at −40° and made into a pellet with a handpress. The frozen pellet had a diameter of 1 cm, and was 1 to 2 mm in thickness. It weighed 150 to 300 mg. Each specimen (frozen pellet) was homogenized in 20 ml of isotonic KCl for 90 sec in a VirTis “45” homogenizer at 0—2°. It then was filtered through a 2 x 2-cm square of cotton gauze. Incubation was carried out for 40 min at 37°. An aliquot of 2 ml of the organic-solvent phase was extracted with 1 ml of N NaOH for the fluorescence assay. The BP hydroxylase activity is expressed as units/mg protein. Protein concentrations were determined by the method of Lowry et al. (9) with bovine serum albumin used as a standard.

### Table 1

**Induction of increased BP hydroxylase activity by flavones**

<table>
<thead>
<tr>
<th>Additions to diet</th>
<th>Diet sequence No.a</th>
<th>BP hydroxylase activity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Small intestine</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Units/mg of wet weightb</td>
<td>Ratio test/ controlc</td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>7 ± 1</td>
<td>73 ± 3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8 ± 1</td>
<td>68 ± 7</td>
</tr>
<tr>
<td>β-Naphthoflavone, 3 mg/g of diet</td>
<td>1</td>
<td>370 ± 20</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>250 ± 26</td>
<td>31</td>
</tr>
<tr>
<td>Quercetin pentamethyl ether, 5 mg/g of diet</td>
<td>1</td>
<td>68 ± 18</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>87 ± 12</td>
<td>11</td>
</tr>
<tr>
<td>Rutin 5 mg/g of diet,</td>
<td>1</td>
<td>23 ± 3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24 ± 5</td>
<td>3</td>
</tr>
</tbody>
</table>

a Sequence 1, diet fed to 7-week-old female A/HaI mice for 16 days, at which time they were sacrificed. Sequence 2, diet fed to mice at 7 weeks of age for 16 days and then again at 11 weeks of age for an additional 16 days, at which time they were sacrificed.

b Mean ± S.D., 4 mice/group.

c Ratio of the benzpyrene hydroxylase activity of mice receiving the indicated flavones to that of the controls for the same sequence.
The induction of increased BP hydroxylase activity resulting from the addition of various flavones to the diet of A/HeJ mice is shown in Table 1. β-Napthoflavone causes a profound induction of increased BP hydroxylase activity in the small intestine, a lesser effect in lung, and a very slight effect in liver. Quercetin pentamethyl ether is a less potent inducer. Its greatest effect is on the small intestine, but it also causes a significant induction in the lung. Rutin, the least potent of the 3 inducers, causes some induction in small intestine, but none in the other tissues studied.

In Table 2, the effects of various flavones on pulmonary adenoma formation in the lung is presented. β-Napthoflavone feeding resulted in almost total inhibition. Quercetin pentamethyl ether feeding caused approximately 50% reduction in pulmonary adenoma formation. The p value for the number of mice bearing tumors in the quercetin pentamethyl ether, carcinogen group of mice versus the no inducer, carcinogen group is less than 0.01. The addition of rutin to the diet did not significantly alter the tumor count. In the 2 groups of mice that had not received BP, a very low tumor incidence occurred. Of 25 mice in both of these groups, 4 showed a single adenoma and the rest showed none.

Topical application of β-napthoflavone causes a marked induction of BP hydroxylase activity in the skin of Ha/ICR mice (Table 3). In mice in which an increase in BP hydroxylase activity had been induced by β-napthoflavone, there is a greater than 50% reduction in skin tumor formation resulting from initiation by BP and subsequent promotion by croton oil (p < 0.05). In the 2 groups of mice which had received croton oil applications but no carcinogen, no tumors were present (Table 4).

DISCUSSION

In the present work, it has been shown that induction of high levels of BP hydroxylase activity will result in inhibition of pulmonary adenoma formation resulting from the p.o. administration of BP. Administration of β-napthoflavone resulted in almost total inhibition of pulmonary adenoma formation, whereas with the less potent inducer, quercetin pentamethyl ether, approximately 50% inhibition occurred. In a 2nd study, it was found that induction of increased BP hydroxylase activity in the skin has a protective effect against BP-initiated epidermal carcinogenesis. The inhibitory effect was of a lesser magnitude in this latter study. The 2 experimental systems differ in a number of respects, 1 of which is that in the pulmonary adenoma study the carcinogen is administered at a site distant from the target tissue in which the neoplasms occur, whereas in the skin study it is applied directly to the target tissue. This latter condition would appear to be considerably less favorable for detoxification than when the carcinogen is administered at a remote site. Other tissues with BP hydroxylase activity are not interposed, and, in addition, a minimum diluting effect of carcinogen occurs. However, even under these less favorable conditions it is possible to demonstrate an inhibitory effect of increased BP hydroxylase activity on epidermal neoplasia.

BP hydroxylase is a microsomal enzyme system which converts BP to weakly carcinogenic or noncarcinogenic hydroxyl derivatives (2, 4, 5). Very little is known about the nature of the proximate carcinogen which actually produces neoplastic effects when BP is administered. Gelboin (6) has recently published in vitro studies showing binding of BP to
DNA in a reaction mixture containing rat liver microsomes and NADPH. It was also demonstrated that several-fold more binding of BP to DNA occurred with the microsomes of rats which had received 3-methylcholanthrene, a potent inducer of microsomal enzyme systems catalyzing ring hydroxylation, and also N-hydroxylation, which is an activation step (10). Whether or not microsomal systems will cause comparable and also N-hydroxylation, which is an activation step (10). Whether or not microsomal systems will cause comparable effects on BP remains to be determined. However, at the present time a number of studies including the ones presented in this paper have demonstrated that induction of increased polycyclic hydrocarbon hydroxylase activity has an overall protective effect against polycyclic hydrocarbon carcinogens (7, 13, 16). In addition, tissues such as liver and small intestine, which have high polycyclic hydrocarbon hydroxylase activity, are resistant to the carcinogenic effect of these compounds. Thus, the information currently available indicates that polycyclic hydrocarbon hydroxylases exert an overall protective function.

Inhibition of pulmonary adenoma formation can be brought about by quercetin pentamethyl ether, which has inducing potency and structural characteristics similar to the naturally occurring flavones tangeretin and nobiletin. The possibility thus exists that dietary factors might alter the response to environmental exposures to polycyclic hydrocarbons. The studies which have been carried out thus far deal with acute exposures to relatively high doses of inducer and carcinogen. For experiments which would have significance in determining a possible effect of dietary constituents on carcinogenesis resulting from environmental exposures such as occur in man, long-term studies in which low doses of carcinogen and inducer are given will be required.

REFERENCES


### Table 4

**Effect of topical application of β-naphthoflavone on BP-initiated epidermal tumors of the mouse**

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Initial topical applicationa</th>
<th>Carcinogenb</th>
<th>No. of mice at risk 9 weeks old (g)</th>
<th>No. of mice with tumorsc</th>
<th>Mice with tumors (%)</th>
<th>No. of tumors/groupc</th>
<th>No. of tumors/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>Sesame oil</td>
<td>None</td>
<td>28</td>
<td>34</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>β-Naphthoflavone no carcinogen</td>
<td>β-Naphthoflavone</td>
<td>None</td>
<td>25</td>
<td>32</td>
<td>37</td>
<td>0</td>
<td>0</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No inducer, carcinogen</td>
<td>Sesame oil</td>
<td>BP</td>
<td>30</td>
<td>34</td>
<td>36</td>
<td>12</td>
<td>40</td>
</tr>
<tr>
<td>β-Naphthoflavone carcinogen</td>
<td>β-Naphthoflavone</td>
<td>BP</td>
<td>30</td>
<td>34</td>
<td>38</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
</tbody>
</table>

a β-naphthoflavone, 6 mg/ml sesame oil, or sesame oil only, was painted on the backs of 9-week-old mice once daily for 3 days.
b Two doses of BP 5 μg in acetone with 1% mineral oil, or vehicle only, were applied to the skin of the back 24 hr after the last inducer or vehicle administration.
c Number of epidermal tumors when mice were 40 weeks old.
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