Induction of Increased Benzpyrene Hydroxylase Activity by Flavones and Related Compounds

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SUMMARY

A study of relationships between the structure of flavones, flavanones, and chalcones and their capacity to induce increased 3,4-benzpyrene (BP) hydroxylase activity in the liver and lung of the rat has been carried out. Appropriate halogenation increases inducing activity. In the compounds studied, hydroxylation reduces inducing capacity, but the corresponding methoxy compounds are active. Two naturally occurring polymethoxy flavones, tangeretin and nobiletin, have been found to be active inducers of BP hydroxylase activity.

INTRODUCTION

BP2 hydroxylase is one of a group of closely related microsomal enzyme systems which have the capacity to metabolize foreign compounds (5). One of the characteristics of this group of enzyme systems is that an increase in activity can be induced by administration of an appropriate organic compound (3-5, 9, 10). Very little information is available on the relationship between the structure of a compound and its capacity to act as an inducer of increased activity of one or several of the microsomal systems. Compounds which induce increased activity of a microsomal system metabolizing one substrate vary between the structure of a compound and its capacity to act as an inducer of increased activity of one or several of the microsomal systems. Compounds which induce increased activity of a microsomal system metabolizing one substrate vary with its abilities to induce increased activity of systems metabolizing other substrates. In some instances the activity of a wide range of reactions is increased, whereas with other inducers, a more limited effect is obtained (4).

BP hydroxylase converts BP to a number of hydroxy and quinone derivatives (5). Several groups of compounds have been shown to be inducers of increased activity of this hydroxylase system. These include polycyclic hydrocarbons, phenothiazines, phenylbenzthiazoles, and symmetrically substituted 5-membered heterocyclics such as 2,5-bis(4-pyridyl)-1,3,4-thiazole (5, 12-15). In the present paper, studies of the inducing activity of a number of flavones and related compounds will be presented. These compounds would appear to be of particular interest because a large number of them occur in nature in a wide variety of plants.

MATERIALS AND METHODS

For testing inducer activity, 0.1 mmole of the compound under investigation was dissolved in 1 ml of DMSO and administered by rubber catheter into the stomach of 48-day-old female rats. The animals were sacrificed 48 hours later by cervical fracture and the liver and lungs removed. Female Sprague-Dawley rats obtained from the Simonsen Laboratories, Minneapolis, and having an average weight of 180 grams, were used throughout. Unless otherwise stated, the diet consisted of pellets of Purina Labena.

Flavone, flavanone, and beta-naphthoflavone were obtained from the Aldrich Chemical Company. Flavone was recrystallized from petroleum ether to give a melting point of 98.5-99°C. Aqueous ethanol was used to recrystallize flavanone (m.p. = 76-77°C) and beta-naphthoflavone (m.p. = 166-167°C). The syntheses of 4'-fluoroflavone (m.p. = 148.5-149.5°C) and 4'-bromoflavone (m.p. = 176.5-177.5°C) were accomplished using powdered potassium hydroxide in the method of Wheeler (6) to convert the 2-acetylphenyl-4'-halobenzoates to 2-hydroxy-4'-halodibenzoylmethanes, which were then converted to the 4'-haloflavones by the procedure used by Mozingo and Adkins (8) to prepare flavone.

3',5,7-Trihydroxy-4'-methoxyflavanone (Hesperetin), 3',3',4',5,7-pentamethoxyflavone (Quercetin), 3',5,7-trihydroxy-4'-methoxyflavanone (Hesperidin), 3',4',5,7-pentahydroxyflavone (Chrysoeriol), and 3,3',4',5,7-pentamethoxyflavone-3-L-rhamnoglucoside (Hesperidin) (m.p. = 257-260°C), and 3,3',4',5,7-pentamethoxyflavone-3-L-rhamnoside (Quercitin) (m.p. = 174-179°C) were prepared using Calbiochem. Recrystallization of hesperetin from ethyl acetate gave material melting at 232°C (dec). Quercetin was recrystallized from ethanol to give a melting point of 314-316°C. Treatment of quercetin in acetone by the methylating procedure of Baker and Robinson (1) gave 3,3',4',5,7-pentamethoxyflavone (Quercetin pentamethyl ether) (m.p. = 149-151°C).

Through the generosity of Mr. M. K. Veldhuis of the U.S. Fruit and Vegetable Products Laboratory, Winter Haven, Florida, 5,6,7,8,4'-pentamethoxyflavone (Tangeretin) (m.p. = 152-153°C) and 5,6,7,8,9',3'-4'-hexamethoxyflavone (Nobiletin) (m.p. = 136-137°C) were made available to us. Chalcone was obtained from K and K Laboratories and recrystallized twice from petroleum ether to give a melting point of 57-58°C.

2'-Hydroxychalcone (b.p. = 89-90°C), 2'-methoxychalcone (b.p. = 214-217°C at 7.5 mm Hg), and 2'-hydroxy-4-bromochalcone (m.p. = 148-149°C) were prepared using the method by which Chen et al. (2) prepared chalcones. A portion of the 2'-hydroxy-4-bromochalcone was converted to 4'-bromofla-
vanone (m.p. = 118–119°C) by the phosphoric acid method (11).

The procedure for determining BP hydroxylase activity was similar to that employed previously (12, 15). The homogenates (1.25% w/v) were prepared in cold (0–2°C) isotonic KCl. Each lung specimen was homogenized for 90 sec in a Virtis 45 homogenizer with a blade-rotor speed of approximately 20,000 rpm, and each liver specimen was homogenized for 60 sec in a glass Potter-Elvehjem homogenizer with a Kel-F® pestle attached to a mechanical stirrer. An aliquot of 100 µl of acetone containing 25 µg of BP was pipetted into each 25-ml Erlenmeyer flask containing 2 ml of homogenate in an ice bath. One ml of reaction mixture was added last to initiate the reaction. It contained the following: 1 mg NADPH, 0.5 mg NADH, 60 µmoles nicotinamide, 50 µmoles KCl, and 5,000 µmoles NaH₂PO₄ • Na₂HPO₄ buffer at pH 7.4. The flasks were incubated aerobically with constant shaking at 37°C in a covered water bath. Lungs and livers were incubated for 20 minutes and 5 minutes respectively. The reaction was stopped by the rapid addition of 3.0 ml of cold acetone, and the flasks were placed in an ice bath. To this mixture was then added 9.0 ml of petroleum ether (Skellysolve B, b.p. 66-68°C). The flasks were mechanically shaken at room temperature for approximately 5 minutes to facilitate quantitative extraction of the metabolite into the 10-ml organic solvent phase. An aliquot (1.0 ml) of the solvent was removed and extracted with 10.0 ml of 1 N NaOH. The fluorescence of the aqueous extract was determined in a Farrand photoelectric fluorometer, model A-3, equipped with a primary filter which transmits light maximally at 400 µm and a secondary interference filter with a peak wavelength at 522 µm and a half-band width of 14 µm. A quinine sulfate solution (0.3 µg/ml of 0.1 N H₂SO₄) was used as a fluorescent reference standard. Fluorescence analysis was performed within 1–5 minutes after obtaining the aqueous extracts. The compounds administered to the rats under the conditions and concentrations described did not interfere with the fluorescent assay. This was ascertained by the extraction of tissue homogenates for interfering substances to the assay system. Readings of the blank samples (zero time controls and no NADPH-NADH controls) were subtracted from those of the reaction samples to correct for the generally very slight background fluorescence. BP hydroxylase activity is expressed as units per mg wet weight of tissue. Wet weight has been used as a base for expressing activity to facilitate comparison with previous published work on other inducers and investigations giving evidence that the increases in BP hydroxylase activity is due to enzyme synthesis (5, 7, 12, 14, 15). One unit of BP hydroxylase activity is equivalent to the formation of 100 µg of 8-hydroxy BP/min.

<table>
<thead>
<tr>
<th>Compound tested*</th>
<th>Liver (units/mg wet weight)</th>
<th>Lung (units/mg wet weight)</th>
<th>Liver (ratio: test/control)*</th>
<th>Lung (ratio: test/control)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>14 ± 2</td>
<td>0.58 ± 0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle control (dimethylsulfoxide)</td>
<td>12 ± 4</td>
<td>0.51 ± 0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavone</td>
<td>48 ± 5</td>
<td>1.42 ± 0.27</td>
<td>4.0</td>
<td>2.8</td>
</tr>
<tr>
<td>4'-Fluoroflavone</td>
<td>65 ± 10</td>
<td>1.37 ± 0.44</td>
<td>5.4</td>
<td>3.7</td>
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<tr>
<td>4'-Bromoflavone</td>
<td>147 ± 26</td>
<td>3.57 ± 0.68</td>
<td>12.2</td>
<td>7.0</td>
</tr>
<tr>
<td>3,3',4',5,7-Pentahydroxyflavone-3-L-rhamnoside (Quercitrin)</td>
<td>8 ± 1</td>
<td>0.30 ± 0.07</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>3,3',4',5,7-Pentahydroxyflavone (Quercetin)</td>
<td>8 ± 1</td>
<td>0.37 ± 0.03</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>3,3',4',5,7-Pentamethoxyflavone (Quercetin pentamethyl ether)</td>
<td>39 ± 4</td>
<td>1.33 ± 0.07</td>
<td>3.2</td>
<td>2.6</td>
</tr>
<tr>
<td>5,6,7,8,4'-Pentamethoxyflavone (Tangeretin)</td>
<td>48 ± 4</td>
<td>1.49 ± 0.14</td>
<td>4.0</td>
<td>2.9</td>
</tr>
<tr>
<td>5,6,7,8,3',4'-Hexamethoxyflavone (Nobiletin)</td>
<td>76 ± 7</td>
<td>1.60 ± 0.03</td>
<td>6.3</td>
<td>3.1</td>
</tr>
<tr>
<td>β-Naphthoflavone</td>
<td>181 ± 25</td>
<td>3.74 ± 0.82</td>
<td>15.1</td>
<td>7.3</td>
</tr>
<tr>
<td>Flavanone</td>
<td>8 ± 2</td>
<td>0.75 ± 0.24</td>
<td>0.7</td>
<td>1.5</td>
</tr>
<tr>
<td>4'-Bromoflavonone</td>
<td>45 ± 6</td>
<td>1.60 ± 0.27</td>
<td>3.7</td>
<td>3.1</td>
</tr>
<tr>
<td>3',5,7-Trihydroxy-4'-methoxyflavanone (Hesperetin)</td>
<td>12 ± 1</td>
<td>0.34 ± 0.07</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>3',5,7-Trihydroxy-4'-methoxyflavanone-7-rhamnoside (Hesperidin)</td>
<td>14 ± 3</td>
<td>0.48 ± 0.03</td>
<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Chalcone</td>
<td>26 ± 1</td>
<td>0.61 ± 0.12</td>
<td>2.2</td>
<td>1.2</td>
</tr>
<tr>
<td>2'-Hydroxy-4'-bromochalcone</td>
<td>14 ± 2</td>
<td>0.51 ± 0.10</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>2'-Methoxychalcone</td>
<td>37 ± 2</td>
<td>0.92 ± 0.10</td>
<td>3.1</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Effects of flavanoid compounds on benzpyrene hydroxylase activity of rat liver and lung.

* 0.1 mmole of each compound in 1 ml dimethylsulfoxide was administered by stomach tube to 48-day-old female Sprague-Dawley rats 48 hours prior to sacrifice; there were 4-8 rats per group except for vehicle control which contained 28 animals. Trivial names are in brackets after the chemical names.

* Mean ± S.D.

* Ratio of benzpyrene hydroxylase activity of tissue from animal receiving the indicated compound divided by activity in the vehicle control.
RESULTS

In Table 1 the inducing activity of a number of flavones, flavanones, and chalcones is presented. It will be noted that flavone is a more potent inducer than flavanone or chalcone. Introduction of the bromo moiety in the 4' position of flavone more than doubles its inducing capacity in both liver and pulmonary tissue. In the case of flavanone, the unsubstituted compound shows no inducing activity for liver, whereas 4'-bromoflavanone has appreciable inducing activity. In lung tissue there is a borderline inducing activity for the unsubstituted compound; this activity increases with the bromo derivative.

All of the hydroxy derivatives which have been studied are inactive as inducers. When the corresponding methoxy compounds are tested, activities of the same order of magnitude as the unsubstituted parent structure are found. Tangeretin and nobiletin, two naturally occurring totally methoxylated compounds, have inducing activity of the same general order as that of unsubstituted flavone. The structures of these two compounds and others included in this investigation are shown in Chart 1.

In Table 2 the results of feeding diets containing various amounts of flavone are shown. A diet containing 0.01% flavone results in a BP hydroxylase activity of liver and lung slightly...
greater than twice that of control animals. Since the animals eat approximately 16 grams of diet per day, this corresponds to a daily intake of flavone of 1.6 mg. A further increase of the amount of flavone in the diet results in a considerably greater increase in BP hydroxylase activity in the liver. The effect on the lung is less pronounced.

**DISCUSSION**

In the present study, a number of flavanoid compounds and their derivatives have been investigated with regard to their capacity to induce increased BP hydroxylase activity in the liver and lung of the rat. The results presented in Table 1 offer some insight into structure-activity relationships of these compounds.

It is readily apparent that the hydroxyl derivatives studied are not successful inducers of increased BP hydroxylase activity, possibly due to their rapid conjugation and excretion or to their high polarity which prevents passage into the cells. In contrast, methoxyl groups appear to be relatively neutral substituents, in that they neither contribute to nor detract from any great extent from the inducing capacity of the basic molecule on which they are substituted. This is true in spite of the fact that the methoxyl substitutions block numerous positions, increase molecular size, and change the polarization and resonance potential of the molecule.

Halogen substitution at the para position on the phenyl ring, on the other hand, can exert a marked enhancement on induction. If this were due simply to the blocking of this position, one might expect similar results with both of the halogens studied as well as the methoxyl group. In fact, there are significant differences in the levels of activity, with bromine substitution showing a considerably greater effect than fluorine. The significant contribution made by halogen substitution may be due to the electrical effect, i.e., to an optimizing of the polarization or resonance potential of the molecule or portions thereof.

Inducers of increased BP hydroxylase activity which have previously been studied have been planar molecules (5, 7, 12, 13, 14). Flavanone, which differs from flavone by the lack of the hetero-ring double bond, is not a planar compound and is ineffective as an inducer. 4'-Bromoflavone, likewise non-planar, is an effective inducer. However, the interpretation of this fact that the methoxyl substitutions block numerous positions, increase molecular size, and change the polarization and resonance potential of the molecule or portions thereof.

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Exceedingly little is known about the mechanism of induction of increased BP hydroxylase activity other than that protein synthesis is required (5, 7, 10). A perusal of the known inducers of increased BP hydroxylase activity reveals certain common features. They all are lipid soluble; are planar, can assume a planar form, or are readily converted to a compound which can assume a planar form; have similar molecular dimensions; and finally, contain a region of electron availability. If appropriate charge distribution is a requirement for inducer activity, the ketonic group of the flavone and chalcone molecule would appear to be of particular importance. Efforts to evaluate this possibility are in progress.

In the present study, two naturally occurring flavones, tangethin and nobiletin, have been shown to have the capacity to induce increases in BP hydroxylase activity in liver and lung. These are the only two naturally occurring constituents of edible foods which have been shown to have this effect. No data are available on the amounts of these two flavones in diets consumed by either man or animals. However, the occurrence of naturally occurring inducers of increased BP hydroxylase activity in edible material opens up the possibility that dietary constituents might conceivably alter the response of animals or man to exposure to BP and other polycyclic hydrocarbons.

**REFERENCES**

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