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Aryl Hydrocarbon Hydroxylase and Polycyclic Hydrocarbon Tumorigenesis: Effect of the Enzyme Inhibitor 7,8-Benzoflavone on Tumorigenesis and Macromolecule Binding

(benzo(a)pyrene/7,12-dimethylbenz(a)anthracene/DNA, RNA, and protein binding/mouse skin)

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ABSTRACT Aryl hydrocarbon hydroxylase is present and is inducible in mouse skin. 7,8-Benzoflavone, an inhibitor of the enzyme, markedly inhibits tumorigenesis by 7,12-dimethylbenz(a)anthracene, but has either no effect on or stimulates benzo(a)pyrene tumorigenesis. Thus, the role of aryl hydrocarbon hydroxylase appears highly specific for each polycyclic hydrocarbon, in respect to detoxification and/or activation of the hydrocarbon to a carcinogenic form. In parallel studies, we found that 7,8-benzoflavone significantly reduces the amount of 7,12-dimethylbenz(a)anthracene binding to mouse skin DNA, RNA, and protein, and the binding of benzo(a)pyrene to RNA and protein of mouse skin. 7,8-Benzoflavone exhibited a markedly lesser effect on the binding of benzo(a)-pyrene to DNA.

Polycyclic hydrocarbons are carcinogenic in various experimental animals and are known components of both cigarette smoke and polluted air. The major enzymes that metabolize polycyclic hydrocarbons are the microsomal mixed-function oxidases that also metabolize steroids and xenobiotics such as drugs, pesticides, and food additives (1, 2). Aryl hydrocarbon hydroxylase converts polycyclic hydrocarbons to phenols, dihydrodiols, quinones, and epoxides (3–9). The enzyme complex is present and is highly inducible in mouse skin (10) as well as in most mammalian tissues (4, 11, 12) and in some cells grown in culture (13–15). Although the enzyme complex clearly functions as a detoxification agent (1, 2, 16), we have suggested that this enzyme complex is also responsible for activation of polycyclic hydrocarbons to toxic and carcinogenic metabolites (10, 17–21). This hypothesis is supported by the following: The amount of enzyme in various mammalian cells is positively correlated with susceptibility to benzo(a)pyrene cytotoxicity (19, 22, 23). The enzyme catalyzes the formation of covalently bound complexes of hydrocarbon with DNA (17, 18, 24), RNA (17, 18), and protein (24), and the binding of carcinogenic aminoazo dyes to protein (25). A strong inhibitor of the enzyme, 7,8-benzoflavone (7, 26, 27) prevents the cytotoxicity of polycyclic hydrocarbons (26) and inhibits 7,12-dimethylbenz(a)anthracene (DMBA) tumorigenesis in mouse skin (10).

DMBA is one of the most potent polycyclic-hydrocarbon carcinogens known, and benzo(a)pyrene is a major hydrocarbon component of smoke. In this paper, we study the role of the enzyme complex in induction of skin tumorigenesis by DMBA and benzo(a)pyrene by studying the effect of the aryl hydrocarbon hydroxylase inhibitor, 7,8-benzoflavone (10, 26, 27) on tumorigenesis and on the binding of the carcinogens to skin macromolecules. Preliminary results have been reported (28, 29).

Abbreviation: DMBA, 7,12-dimethylbenz(a)anthracene.

MATERIALS AND METHODS

Animals. NIH (general purpose) Swiss male mice that were random-bred and that weigh 18–22 g were generally used.

Chemicals. DMBA and benzo(a)pyrene were obtained from Eastman Organic Chemicals (Rochester, N.Y.). 7,8-Benzoflavone was purchased from Aldrich Chemical Co., Inc. (Cedar Knolls, N.J.). Each compound was recrystallized from 95% ethanol before use. [^{3}H]DMBA (27 Ci/mmol) and [^{3}H]-benzo(a)pyrene (11 Ci/mmol) were obtained from Amersham/Searle (Des Plaines, Ill.). All radiochemicals were purified by thin-layer chromatography.

Tumorigenesis Experiments. Male mice weighing 18–22 g were shaved with electric clippers 1 day before application of the chemicals. 30 Mice were used in each group in the tumorigenesis experiments. The indicated amount (generally 100 nmol) of DMBA or benzo(a)pyrene in 0.2 ml of acetone was applied topically to the backs of the mice. The 7,8-benzoflavone was dissolved in the carcinogen solution or in 0.2 ml of acetone and applied to the skin immediately thereafter. Two different types of tumorigenesis experiments were performed. In one type, the carcinogen with or without the inhibitor was applied one time only. This single treatment was followed by a weekly application of 1% croton oil. In the second type, the carcinogen and inhibitor were applied twice per week for the duration of the experiment. Papilloma formation was recorded weekly; and is reported (see tables) 20 weeks after application of carcinogen with or without the inhibitor.

Preparation of DNA, RNA, and Protein from Mouse Skin. Nucleic acids and proteins were isolated and purified according to the procedures of Diamond *et al.* (30) and Colburn and Boutwell (31) that are the modified methods of Kirby *et al.* (32–35). The skins of five mice treated with carcinogen, with or without inhibitor, were scraped as described above and homogenized with 30 strokes in 5 ml of 6% solution of the sodium salt of *p*-aminosalicyclic acid, in a tight-fitting Potter–Elvehjem glass–glass homogenizer. 0.5 ml of a 10% solution of sodium dodecyl sulfate was added, and the

mixture was shaken for 1 hr. The resulting highly viscous solution was extracted with an equal volume of a phenol:*m*-cresol:8-hydroxyquinoline:water (500:70:0.5:55, by weight).

DNA was precipitated from the aqueous layer by the addition of an equal volume of cold 2-ethoxyethanol. DNA was removed on a glass rod and two volumes of cold ethanol were added to the remaining aqueous phase to precipitate RNA. The phenol layer was extracted once again with 6% solution of the sodium salt of *p*-aminosalicyclic acid and then added to excess methanol that precipitated the protein. The DNA was dissolved in 2 ml of 1 mM K_2HPO_4 (pH 7.0), incubated at 37° for 15 min with RNase, extracted again with the phenol reagent, and precipitated as before with 2-ethoxyethanol. Carbohydrates were removed by the extraction of DNA dissolved in 1.25 M K_2HPO_4 with 2-methoxyethanol. The DNA was precipitated from the upper layer by the addition of a half volume of 1% cetyltrimethylammonium bromide, and was converted back to the sodium salt with 70% aqueous ethanol containing 2% sodium acetate. The DNA was washed with ethanol and then with ether, and dried. The RNA was freed of carbohydrates by the same procedure used for the DNA. The protein was washed four times with large volumes of methanol, once with acetone, then with ether, and dried.

Radioactivity Assays. The concentration of nucleic acid dissolved in 1 mM K_2HPO_4 (pH 7.0) was calculated from the absorbance at 260 nm, with the assumption that at this wavelength $E_{1cm}^{1\%}$ = 200 for DNA and 250 for RNA. The protein was dissolved in 200 μl of 0.5 N NaOH at 90° for 15 min; 20 μl of the solution was used to measure the concentration. The protein content of the homogenate was measured by a modification of the method of Lowry *et al.* (36); ribonuclease A was used as standard. A Beckman liquid scintillation counter (model LS-100) was used for measurement of radioactivity. 100–150 μl of aqueous solution of sample was treated with 1 ml of NCS (solubilizer obtained from Amersham/Searle), and the clear solution obtained was counted in 10 ml of toluene solution of Liquifluor. A correction for quenching was made by use of automatic external standard ratios. The amount of DMBA and benzo(a)pyrene bound was calculated from the specific activity of the isolated protein and nucleic acids.

RESULTS

Tumorigenesis initiated by a single application of DMBA or benzo(a)pyrene: effect of 7,8-benzoflavone

A single low dose of 100 nmol of DMBA applied to the backs of mice, followed by weekly application of croton oil, an essentially noncarcinogenic promoting agent, produces multiple tumors on the backs of the mice. In three experiments, 20 weeks after application of DMBA, the average number of tumors per mouse ranged from about 12 to 17 (Table 1). As we have reported (10, 21), a single simultaneous application of equimolar amounts of 7,8-benzoflavone and DMBA inhibits tumorigenesis to a marked extent. In the three experiments shown in Table 1, the observed inhibition was 80, 55, and 74%. In contrast to the inhibition of DMBA-initiated tumorigenesis, 7,8-benzoflavone had little effect on benzo(a)pyrene-initiated tumorigenesis. Benzo(a)pyrene is a considerably weaker tumorigenesis-initiating agent than is DMBA. In three experiments in which benzo(a)pyrene and 7,8-benzoflavone were applied once simultaneously, we observed a 40% inhibition of tumorigenesis in one experiment (Exp. 6) and essentially no inhibition in two other experiments (Exps. 4 and 7). In another experiment in which the benzo(a)pyrene was applied twice, at a 3-day interval, the 7,8-benzoflavone stimulated tumor formation by about 2-fold. Thus, although 7,8-benzoflavone is an effective inhibitor of DMBA initiation of tumorigenesis, it is essentially ineffective as inhibitor of benzo(a)pyrene-initiated tumorigenesis.

TABLE 1. *The effect of 7,8-benzoflavone (BF) on tumorigenesis initiated by a single application of 7,12-dimethylbenz(a)anthracene or benzo(a)pyrene**

Exp.	Compounds (nmol)†	Survivors	Mice with tumors	Total no. of tumors	No. of tumors per mouse	% Control
1	DMBA (100)	29	28	348	12.0	—
	DMBA (100) + BF (100)	29	8	69	2.4	20
2	DMBA (100)	22	22	368	16.7	—
	DMBA (100) + BF (100)	29	23	217	7.5	45
3	DMBA (100)	28	28	387	13.8	—
	DMBA (100) + BF (100)	17	14	61	3.68	26
4	BP (100)	24	8	10	0.42	—
	BP (100) + BF (100)	26	9	10	0.38	90
5	BP (100, twice)	28	10	20	0.71	—
	BP (100, twice) + BF (100, twice)	28	8	35	1.25	176
6	BP 100)	23	12	34	1.5	—
	BP(100) + BF (100)	21	7	18	0.9	60
7	BP (300)	23	12	27	1.17	—
	BP (300) + BF (600)	21	10	22	1.05	90

* The experiment was terminated 20 weeks after application of carcinogen with or without BF.

† Each compound was applied once only, except in Exp. 5, where it was applied twice weekly. In all experiments this treatment was followed by the weekly application of 1% croton oil.

Tumorigenesis induced by repeated applications of DMBA or benzo(a)pyrene: effect of 7,8-benzoflavone

In three experiments, tumor formation caused by the repeated application of DMBA was inhibited by 74, 69, and 30% by the simultaneous application of 7,8-benzoflavone (Table 2). The effect of 7,8-benzoflavone on benzo(a)pyrene tumorigenesis was again markedly different from its effect on DMBA tumorigenesis. In three experiments, the 7,8-benzoflavone had either no effect (Exp. 6) or markedly stimulated tumor formation by benzo(a)pyrene. In two experiments, there was a 3-fold and 6-fold enhancement of tumor formation when 7,8-benzoflavone was applied simultaneously with the benzo(a)pyrene. 7,8-Benzoflavone alone was essentially nontumorigenic.

TABLE 2. *The effect of 7,8-benzoflavone (BF) on tumorigenesis induced by repeated application of 7,12-dimethylbenz(a)-anthracene or benzo(a)pyrene**

Exp.	Compounds (nmol)†	Survivors	Mice with tumors	Total no. of tumors	Tumors per mouse	Control
1	DMBA (100)	29	29	531	18.3	—
	DMBA (100) + BF (100)	21	14	99	4.7	20
2	DMBA (50)	27	27	505	18.7	—
	DMBA (50) + BF (50)	26	24	201	7.7	41
3	DMBA (100)	22	22	550	25.0	—
	DMBA (100) + BF (100)	21	21	360	17.1	68
4	BP (100)	29	11	15	0.5	—
	BP (100) + BF (100)	28	20	80	2.9	580
5	BP (100)	27	9	18	0.7	—
	BP (100) + BF (100)	27	20	63	2.3	328
6	BP (100)	19	16	52	2.7	—
	BP (100) + BF (100)	19	14	50	2.6	96
7	BF (100)	21	0	0	0	—

* The experiment was terminated 20 weeks after first application of carcinogen with or without BF.

† Each compound was applied twice weekly. No croton oil was applied.

The effect of 7,8-benzoflavone on the covalent binding of DMBA and benzo(a)pyrene to mouse skin DNA, RNA, and protein

The application of carcinogen and inhibitor in the experiments shown in Table 3 was similar to that described in the tumorigenesis experiment (Table 1). A single application of the carcinogen was applied with or without 7,8-benzoflavone. In two experiments, the 7,8-benzoflavone inhibited the binding of DMBA to all three macromolecules to about the same extent. Thus, the binding to DNA, RNA, and protein was inhibited by 59, 68, and 52%, respectively. The finding that the binding of DMBA to macromolecules was inhibited by the enzyme inhibitor suggests that enzymatic activation is a requirement for binding of DMBA to macromolecules. Similarly, the 7,8-benzoflavone inhibited the binding of benzo(a)pyrene to RNA and protein by 55 and 46%, respectively, about the same extent observed with DMBA. In marked contrast, the 7,8-benzoflavone inhibited the binding of benzo(a)pyrene to DNA by only 18%. Although the specific locus of binding of carcinogen to DNA, RNA, or protein is not known, the carcinogenic effect of the hydrocarbon most closely parallels its binding to DNA. Thus, the lack of inhibitory effect of 7,8-benzoflavone on benzo(a)pyrene tumorigenesis was paralleled by its relatively weak effect on the binding of benzo(a)pyrene to DNA.

DISCUSSION

In the animals we used (37, 38), the carcinogens and inhibitor of aryl hydrocarbon hydroxylase are applied directly to the target tissue, the skin. The single dose of carcinogen is sufficiently low so that systemic metabolism resulting in redistribution of carcinogen among different tissues plays no role in tumorigenesis in the skin. This conclusion is supported by the finding that the single intraperitoneal injection of the dose of carcinogen that we used does not initiate skin tumors (Kinoshita and Gelboin, in press). A single simultaneous application of the enzyme inhibitor, 7,8-benzoflavone, with the carcinogen markedly inhibits DMBA tumorigenesis but does not inhibit benzo(a)pyrene tumorigenesis. In fact, in some experiments, 7,8-benzoflavone enhanced tumorigenesis by benzo(a)pyrene (28, 29). The overall metabolism of both DMBA and benzo(a)pyrene is inhibited by 7,8-benzoflavone (ref. 27; unpublished results). The metabolism of these carcinogens, however, is complex. Each carcinogen is converted to multiple products that include phenols, diols, quinones, epoxides, and conjugated derivatives (3–9). The complete profile of metabolite formation for each carcinogen by the control and induced enzyme complex is not known How this profile of metabolites is altered in the presence of 7,8-benzoflavone, the enzyme inhibitor, would seem most relevant to the interpretation of our results. It is likely that with DMBA, the 7,8-benzoflavone reduces the formation of the active carcinogenic form. With benzo(a)pyrene, the 7,8-benzoflavone may reduce the formation of detoxification products to a greater degree relative to the formation of the active carcinogenic form. This may in fact enhance the formation of the active form. Another possibility is that the enzyme complex for benzo(a)pyrene is primarily a detoxification system, and its inhibition increases the amount of nonenzymatic activation of the benzo(a)pyrene to an active carcinogenic form.

The number and nature of the reactive carcinogenic forms for each hydrocarbon have not been conclusively established. Some postulate that the reactive forms of the hydrocarbons are radical cations (39) or carbonium ions (40). Several reports have demonstrated that microsomal enzymes catalyze the binding of polycyclic hydrocarbons to DNA (17, 18), RNA (17, 18), and protein (24). Nagata *et al.* have reported that benzo(a)pyrene forms free radicals when mixed with skin homogenates (41). Lesko *et al.* have reported that 6-OH-benzo(a)pyrene, a known microsomal metabolite of benzo(a)pyrene, forms covalent linkages to DNA (42). Jerina *et al.* (6) demonstrated an epoxide intermediate in the microsomal hydroxylation of naphthalene, and Selkirk *et al.* (7) found that 1:2,5:6-dibenzanthracene undergoes epoxidation by the microsomal enzymes. Grover *et al.* (43) found that an epoxide of dibenz(a,h)anthracene exhibits greater transforming activity on cells in culture than does the parent hydrocarbon. DiPaolo *et al.* (44) have suggested that the toxic and transforming effects of polycyclic hydrocarbons *in vitro* can be differentiated. Thus, numerous studies suggest that different pathways of metabolism result in either inactive, toxic, or transforming metabolites.

Cavalieri and Calvin (45) have postulated that the hydroxylating enzymes generate different types of active centers in DMBA and benzo(a)pyrene. This is consistent with our experimental finding in which the enzyme inhibitor, 7,8-benzoflavone, exerts markedly different effects on DMBA and benzo(a)pyrene tumorigenesis. Our results also point out the complexities of approaches to the control of polycyclic hydrocarbon carcinogenesis that are based on altering carcinogen

TABLE 3. *The in vivo binding of [³H]DMBA and [³H]benzo(a)pyrene(BP) to mouse skin macromolecules: the effect of 7,8-benzoflavone (7,8-BF)**

Compounds (100 nmol of each)	Time (hr)	DNA μmol per mol of phosphorus	% Inhibition†	RNA μmol per mol of phosphorus	% Inhibition†	Protein μmol per 100 g	% Inhibition†
[³H]DMBA	0	0.35 ± 0.03	—	0.08 ± 0.01	—	4.51 ± 0.22	—
[³H]DMBA	24	4.60 ± 0.41	—	2.26 ± 0.02	—	15.51 ± 0.60	—
[³H]DMBA + 7,8-BF	24	2.08 ± 0.20	59	0.78 ± 0.12	68	9.78 ± 1.76	52
[³H]BP	0	0.46 ± 0.12	—	0.11 ± 0.06	—	2.56 ± 0.81	—
[³H]BP	24	1.71 ± 0.14	—	1.35 ± 0.02	—	20.19 ± 1.42	—
[³H]BP + 7,8-BF	24	1.49 ± 0.16	18	0.67 ± 0.07	55	12.05 ± 0.56	46

* The table represents the average of values obtained in two separate experiments, in which groups of mice were treated with labeled carcinogens in a manner identical to the procedure used in the tumorigenesis experiment, in which the carcinogen was applied once only. See *Methods* for details.

† Percentage of inhibition was calculated after zero time controls were subtracted.

metabolism by either the induction or inhibition of the aryl hydrocarbon hydroxylase complex. The enzymes may uniquely detoxify or activate a specific hydrocarbon, and modification of the enzyme may yield different end products for each hydrocarbon, in respect to its ultimate carcinogenic activity.

The formation of polycyclic hydrocarbons that are covalently bound to macromolecules of mouse skin has been observed during tumorigenesis (46–49), although the relevancy of these bound forms to carcinogenesis remains to be clarified. Some correlative results suggest that these interactions may be important to polycyclic-hydrocarbon tumorigenesis. Binding of hydrocarbons to DNA may induce genetic changes, while binding to RNA or protein may induce epigenetic changes, leading to an altered gene expression that can characterize the tumor (2, 16, 50). Thus, methylcholanthrene has been shown to alter genetic expression (51). Inhibition of the aryl hydrocarbon hydroxylase complex with 7,8-benzoflavone reduces the amount of DMBA binding to DNA, RNA, and protein by about 50%. Similarly, the binding of benzo(a)pyrene to skin RNA and protein is inhibited to about the same extent. In contrast, the 7,8-benzoflavone exhibits a markedly lesser effect on the binding of benzo(a)pyrene to skin DNA. This suggests that the reactions involved in the binding of the benzo(a)pyrene to skin DNA may have a special character defined by their relative insensitivity to inhibition by 7,8-benzoflavone. The basis for the insensitivity is unknown but may relate to the loci of DNA and enzyme, the stability of the active intermediate, or the possibility of nonenzymatic reactions. Our findings also suggest that the binding of the hydrocarbon to DNA may be the interaction most relevant to carcinogenesis, since 7,8-benzoflavone has relatively little effect on both the binding of benzo(a)pyrene to DNA and on benzo(a)pyrene-initiated tumorigenesis.

Aryl hydrocarbon hydroxylase, is clearly the prime biological receptor for polycyclic hydrocarbons. Their metabolism, which may include both detoxification and activation reactions, is clearly of paramount importance to their activity as carcinogenic agents. In liver and other tissues examined, there are at least two forms of the enzyme (27) that can be distinguished by their sensitivity to 7,8-benzoflavone inhibition. The role of the enzyme complex in carcinogenesis may be influenced by various factors, such as the amount of each type of enzyme, the amount of basal and induced enzyme, the hydrocarbon concentration, the cofactor or inhibitor concentration, and the relative activity of enzymes related to the subsequent metabolism of the products of aryl hydrocarbon hydroxylase.

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