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Induction of Aryl Hydrocarbon Hydroxylase and Forestomach Tumors by Benzo(*a*)pyrene[1]

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SUMMARY

While papillomatous tumors developed in the forestomach of female Ha/ICR mice after a 12-week chronic feeding period of benzo(*a*)pyrene (BP), no tumors developed in the glandular portion of stomach or in the lung or liver. Among all tissues examined, the forestomach showed the greatest increase of aryl hydrocarbon hydroxylase (AHH) activity following acute or chronic administration of BP. Single acute doses of BP induced AHH activity in forestomach, glandular stomach, lung, and small intestine, but not in the kidney and liver of these animals. Similarly, after chronic administration of BP, AHH activity was inducible in the forestomach, glandular stomach, and lung, but again not in the liver.

Although the formation of tumors is associated with greater inducibility of AHH activity in the forestomach after BP administration, the relationship between tissue inducibility of AHH activity and susceptibility to BP carcinogenesis is still not clear. Further studies regarding the formation of specific carcinogenic epoxides of BP in tissues both susceptible (*e.g.*, forestomach) and resistant to BP carcinogenesis would more clearly define the relationship between AHH inducibility and BP carcinogenesis.

INTRODUCTION

The susceptibility of humans to the carcinogenic action of polycyclic hydrocarbons found in the environment may be dependent on the induction of AHH.[3] This mixed-function oxidase system is essential for the metabolic activation of polycyclic hydrocarbons to form reactive epoxide metabolites. These metabolites can covalently bind *in vivo* with proteins, RNA, and DNA of target organs and are thought to be the basis for the carcinogenic potential of polycyclic hydrocarbons (6, 18).

Several reports show that there is a correlation between the inducibility of AHH in liver in certain inbred strains of mice and the susceptibility of these mice to s.c. tumors by the administration of 3-methylcholanthrene (10, 12). Other studies show a relationship between AHH induction and mouse lung squamous cell carcinoma initiated by weekly i.t. injections of 3-methylcholanthrene (11, 17). The AHH enzyme system is also present in cultured human lymphocytes and is inducible by 3-methylcholanthrene *in vitro* (7). There appears to be an association between the level of inducible AHH activity in lymphocytes and the incidence in humans of bronchiogenic carcinoma (8).

BP is a carcinogenic polycyclic hydrocarbon that has been extensively studied. This chemical was isolated first from coal tar (9), is present in tobacco smoke and charcoal-grilled foods, and occurs as an atmospheric pollutant emitted in air from the combustion of fuels and organic matter (3, 4, 13). When BP is incorporated into the diets of mice, tumors develop most often in the squamous epithelium of the forestomach (1, 23). The lower portion of the stomach is glandular and is resistant to the formation of tumors by the administration of carcinogenic polycyclic hydrocarbons (1, 20, 21). Because BP can induce AHH in many tissues (2, 16) and can selectively form tumors in the forestomach, we undertook a study to determine whether inducibility of AHH in the forestomach is associated with tumor formation at this site after BP administration. A study in mice of the relationship between the inducibility of AHH in various tissues, including 2 portions of the stomach (forestomach *versus* glandular), and the induction of tumors in the forestomach by BP administration may enhance our understanding of the mechanism of carcinogenesis by the polycyclic hydrocarbons.

MATERIALS AND METHODS

Chemicals. Practical grade BP (minimum purity, 95%) was obtained from Sigma Chemical Company, St. Louis, Mo. For measurement of tissue AHH activity, Tris buffer and the cofactors NADH and NADPH were obtained from Sigma. All control and experimental diets containing BP were prepared by Bio-Serv, Inc., Frenchtown, N. J.

Treatment of Animals. Only female Ha/ICR mice from the ARS/Sprague-Dawley Co., Madison, Wis., were used for this study. Mice (27 ± 2 g) were treated with a single dose of BP (20 mg/kg) administered p.o. in a 10% solution of ethanol in corn oil at 1% of body weight 24 hr prior to sacrifice for measurement of AHH activity. The corresponding controls were given an equivalent amount of vehicle. For the chronic studies, the mice were started at 9 weeks of age on diets to which had been added 5% corn oil (control) or 5% corn oil with 2 dosage levels of BP at 0.2 and 0.3 mg/g of Purina laboratory chow. Animals were housed in suspended mesh-wire cages and were weighed weekly. After 12 weeks

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[3] The abbreviations used are: AHH, aryl hydrocarbon hydroxylase; i.t., intratracheal; BP, benzo(*a*)pyrene; i.g., intragastric.

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of feeding on the diet, the mice were sacrificed for measurement of AHH activity and determination of tumors.

The presence of tumors was determined by expanding the stomachs with an i.g. injection of a 10% solution of formalin. The stomachs were then removed for gross macroscopic tumor count under a dissecting microscope by the method described by Wattenberg (23). In addition, livers and lungs were also observed grossly for the presence of tumor formation by BP. All specimens found to be either positive or negative for tumors were then processed for histological microscopic examination to determine cell tumor type. A thin slice of tissue was fixed in buffered 10% formalin solution and blocked in paraffin. The paraffin sections (4 μm thick) were stained with hematoxylin and eosin by standard techniques.

Tissue AHH Activity. In mice sacrificed after either a single p.o. dose or chronic feeding of BP, the stomachs were dissected into 2 parts. The pooled forestomach and glandular stomach of 3 animals were used to prepare homogenates. The homogenates of various tissues were made in ice-cold 1.15% KCl solution for assay of AHH activity by the method of Nebert and Gelboin (15). The final incubation mixture had a volume of 1.05 ml and was composed of: 0.3 ml of NADPH (0.5 μmole), 0.3 ml of NADH (0.5 μmole), 0.2 ml of 0.5 M Tris buffer at pH 7.5, 0.2 ml of 900 x *g* supernatant of tissue homogenates containing between 1.1 and 4.2 mg protein, and 0.05 ml of a methanol solution of the substrate BP (80 nmoles) which was added prior to incubation. Values for AHH activity represent the amount (in pmoles) of reference standard 3-hydroxybenzo(*a*)pyrene (provided by Dr. Harry V. Gelboin, National Cancer Institute, Bethesda, Md.) causing fluorescence equivalent to the total hydroxylated metabolites produced per mg of protein per minute of incubation. Aliquots of tissue homogenates were used for protein determination by the method of Lowry *et al.* (14).

Statistical Analysis. All data in this study were statistically analyzed by Student's *t* test, and probability values of 0.05 or less were considered significant.

RESULTS

Table 1 shows that the tumor incidence in the forestomach of female Ha/ICR mice is influenced by the concentration of BP in the diet. In mice fed diets containing 0.2 mg of BP per g, 66.6% had tumors, while, at the higher dietary level of BP, all of the mice had similar gastric tumors. More than 1 tumor was found in each tumor-bearing animal, and they were of the squamous papilloma type. The data presented show that there is a dose-effect relationship between dietary level of BP and tumor incidence when expressed as the carcinogenic index as defined in Table 1, Footnote *a*. None of the controls were found to have tumors. Gross and microscopic examination of glandular stomach, lung, and liver from control mice and those fed BP were also found to be negative for the presence of tumors. The induction of AHH activity at the site of tumor formation in forestomach, and in other tissues, is presented in Table 2. The basal level of AHH in control forestomach (0.212 ± 0.03) was significantly higher than in the glandular stomach (0.060 ± 0.01);

Table 1

Effect of various levels of BP in the diet on tumor formation in forestomach of mice

Mice were fed a diet to which had been added 5% corn oil (control) or 5% corn oil with 2 dosage levels of BP in the diet. Mice were 9 weeks old at the start of the experiment and, after 12 weeks on the diet, were sacrificed for tumor count.

BP (mg/g diet)	No. of mice	Mice with tumors (%)	No. of tumors/ mouse	Carcinogenic index[a]
0	9	0.0	0.0	0.0
0.2	9	66.6	1.8	121.9
0.3	9	100.0	4.0	400.0

[a] Percentage of mice with tumors times the mean number of tumors per tumor-bearing mice.

$p < 0.001$. After the mice were fed for 12 weeks with BP, the AHH activity was increased by 4.2- to 5.2-fold in forestomach, whereas induction of AHH was lower (2.2- to 3.9-fold) in glandular stomach. In lung, the AHH activity was significantly increased by 1.6-fold only at the higher dietary level of BP. However, in liver, where the basal level of AHH activity was found to be highest of all tissues studied, chronic feeding of BP did not cause induction of AHH activity. AHH activity was also determined in these same tissues and in small intestine and kidney 24 hr after a single p.o. dose (20 mg/kg) of BP (Table 3). Under these experimental conditions, BP was found to increase significantly this enzyme system in forestomach, glandular stomach, small intestine, and lung, but kidney and liver AHH was not significantly changed. Similar to our previous results with the chronic feeding of BP presented in Table 2, after a single acute dose of BP, AHH activity is induced to the greatest extent in forestomach (4.3-fold); this is followed by glandular stomach (2.4-fold), small intestine (2.3-fold), and lung (1.9-fold). The basal level in control mice of AHH activity was again significantly higher in forestomach than in glandular stomach ($p < 0.01$).

DISCUSSION

Previous studies (10–12, 17) using different strains of mice have shown a correlation between induction of AHH activity and the formation of tumors in various target organs by the polycyclic hydrocarbon, 3-methylcholanthrene. This report shows that the forestomach is susceptible to the development of tumors after a 12-week feeding period of the polycyclic hydrocarbon BP in the diet of mice. Glandular stomach did not form tumors, thus appearing to be resistant to the carcinogenic action of BP. The mechanism by which squamous cells of the forestomach give rise to papillomatous tumors by feeding BP is not known. Selectivity for tumor formation in forestomach has been previously reported for BP in different strains of mice (1, 23).

Studies on DNA binding (19) and mutagenic activity (5, 25, 27, 28) *in vitro* of a large number of BP derivatives suggest that the most active carcinogenic metabolite of BP is the 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene. Since the AHH enzyme system is thought to be essential for the metabolic activation of BP to form

Table 2

Effect of various levels of BP in the diet on tissue AHH activity

Mice were fed a diet to which had been added 5% corn oil (control) or 5% corn oil with 2 dosage levels of BP in the diet. Mice were 9 weeks old at the start of the experiment and, after 12 weeks, were sacrificed for enzyme assay. The results are expressed as pmoles of 3-hydroxybenzo(*a*)pyrene per mg of protein per min. Each value represents the mean ± S.D. of 4 determinations.

	AHH specific activity							
BP (mg/g diet)	Forestomach	Ratio[a]	Glandular stomach	Ratio	Lung	Ratio	Liver	Ratio
0	0.212 ± 0.03		0.060 ± 0.01		2.95 ± 0.74		60.97 ± 9.91	
0.2	0.833 ± 0.28[b]	4.2	0.131 ± 0.01[b]	2.2	3.69 ± 0.49	1.3	50.97 ± 7.00	0.8
0.3	1.100 ± 0.43[b]	5.2	0.228 ± 0.09[b]	3.9	4.57 ± 0.88[c]	1.6	55.62 ± 17.49	0.9

[a] Ratio, AHH activity of mice receiving BP divided by the activity of the control group.
[b] Significantly different from control group ($p < 0.01$).
[c] Significantly different from control group ($p < 0.05$).

Table 3

Acute effects of BP on tissue AHH activity

Mice were given BP (20 mg/kg) p.o. in a 10% solution of ethanol in corn oil of 1% of body weight 24 hr prior to sacrifice for measurement of enzyme activity. The corresponding controls were given an equivalent amount of vehicle. The results are expressed as pmoles of 3-hydroxybenzo (*a*) pyrene per mg of protein per min. Each value represents the mean ± S.D. of 3 to 4 determinations.

	AHH specific activity					
BP (mg/kg)	Forestomach	Glandular stomach	Small intestine	Lung	Kidney	Liver
0	0.241 ± 0.06	0.103 ± 0.02	8.49 ± 4.02	3.81 ± 1.36	0.297 ± 0.08	45.27 ± 4.38
20	1.037 ± 0.18[a]	0.251 ± 0.09[b]	19.48 ± 4.37[b]	7.31 ± 0.77[c]	0.463 ± 0.17	53.45 ± 6.35
Ratio[d]	4.3	2.4	2.3	1.9	1.6	1.2

[a] Significantly different from control group ($p < 0.001$).
[b] Significantly different from control group ($p < 0.05$).
[c] Significantly different from control group ($p < 0.01$).
[d] Ratio, AHH activity of mice receiving BP divided by the activity of the control group.

this reactive epoxide, it is important to know whether the basal level and/or the inducible level of AHH activity in tissues is associated with tumor formation by the administration of BP. The results presented in this study show that the basal level of AHH was higher in forestomach, a site sensitive to tumor formation, than in glandular stomach. Since no tumors were formed in tissues with higher basal AHH activity than forestomach, such as in lung, small intestine, or liver, it seems unlikely that the basal level of AHH is the major factor in the tumorigenic effects of BP.

It was further shown in this report that, in all tissues examined, the induction of AHH activity was highest in forestomach after either acute p.o. administration or chronic dietary feeding of BP. AHH activity was also inducible in glandular stomach, small intestine, and lung, but not in kidney and liver. These results on the inducibility of tissue AHH are in agreement with the work previously reported by others which showed that enzyme induction varies from tissue to tissue in a given species or strain of animal. Wiebel *et al.* (26) reported that AHH activity was inducible in lung, kidney, skin, and liver of AKR and C57 strains of mice after i.p. injection of benzanthracene. These authors found that hepatic AHH activity was not inducible in DBA/2N and N2B strains of mice under similar experimental conditions, whereas activity was significantly increased in the other extrahepatic tissues. Similarly, Thomas *et al.* (22) found, in 21 inbred strains of mice, that 10 strains responded with less than "0.5-fold" increase (noninducible) of hepatic AHH activity following the i.p. administration of 3-methylcholanthrene. Data similar to that reported in this paper on induction of AHH activity in various areas of rat stomach were reported by Wattenberg *et al.* (24). They found that rat forestomach had moderate basal AHH activity, whereas, in the glandular portion, it was barely detectable. After p.o. administration of 1,2-benzanthracene, forestomach was approximately 5 times more active in enzyme activity than glandular stomach. In our experiments with mice, the AHH activity was also approximately 3 to 5 times higher in forestomach than glandular stomach after the administration of BP.

It is generally believed that BP must be metabolically activated by the AHH enzyme system to form reactive carcinogenic epoxides. Our findings of tumor formation only in the forestomach of Ha/ICR mice associated with the highest level of AHH inducibility at this site following the administration of BP supports the hypothesis previously proposed by other investigators (8, 10, 11, 12, 17) that induction of AHH is related to the formation of tumors following the administration of various carcinogenic polycyclic hydrocarbons. An explanation as to why AHH activity was also inducible in glandular stomach, small intestine, and lung, sites that had been found to be resistant to tumor formation by the administration of BP, is not available at the present time. Further studies comparing forestomach with other tissues having

inducible AHH activity to form and/or inactivate the various carcinogenic epoxides formed from BP may resolve the question as to why the forestomach develops tumors and why other tissues with inducible AHH activity do not develop tumors.

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