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Induction of Electrophilic Metabolites of PAH by Placental Microsomes in Mice

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Metabolism of benzo(a)pyrene, the most thoroughly studied PAH, was studied in mouse placental microsomes incubated with ^{3}H-labeled B(a)P. B(a)P metabolites were separated using HPLC fitted with a C18-μ Bondapak column. The single major metabolite by mouse placental microsomes induced by B(a)P was 7,8-diol B(a)P, while 4,5-diol B(a)P, 3-OH and quinones constituted minor metabolites. Treatment with 3-methyl-cholanthrene to mice resulted in induction of hydroxy B(a)P and quinone compounds. Phenobarbital treated mouse placental microsomes also showed elevated level of B(a)P metabolism with 7,8-diol B(a)P as a major metabolite.

KEY WORDS: Benzo(a)pyrene, PAH, Placental microsome

The ability of PAHs (polycyclic aromatic hydrocarbon) to induce neoplastic transformation in animals has been well documented (Miller, 1979; Miller and Miller, 1981; Miller, 1978). Many PAHs such as B(a)P[Benzo(a)pyrene] are precarcinogens requiring metabolic activation prior to exhibiting the biological activities. The metabolism of PAH carcinogens including B(a)P involves microsomal cytochrome P-450-dependent mixed function oxidases. These are membrane-bound multi-component systems composed of an NADPH-cytochrome P-450 reductase and cytochrome P-450. These enzyme systems require NADPH as a cofactor and use molecular oxygen as the oxidant, and catalyze the metabolim of a wide variety of drugs, steroids, fatty acids, pesticides, chemical carcinogens and other xenobiotics (Heidelberg, 1975; Burke and Orrenius, 1980).

Although the liver has long been considered to be the major site of PAH metabolism, it has been also recognized that extrahepatic enzyme systems may also play an important role in the metabolism of some xenobiotics. The rate of extrahepatic metabolism might be lower than that in liver as a total capacity. Human exposures to habitual or occupational carcinogens, however, is often a chronic situation involving exposure to a relatively small dose over a long period of time. It is known that the placental tissue can carry out many drug metabolism reactions including oxidations, reductions, hydrolytic reactions and conjugation reactions (Juchau, 1972).

A number of studies have shown that PAH chemicals including B(a)P and dimethylbenz(a)anthracene serve as transplacental carcinogens in experimental animals (Rice *et al*, 1978; Bulay and Wattenberg, 1970). In this study, we present data on the HPLC analysis of B(a)P metabolites induced by placental mcrocomes obtained from mice.

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Materials and Methods

Animals

Female ICR mice weighing 20~30 g were used in all experiments. Pregnant ICR mice were housed in individual plastic cage and maintained on commercial animal food (Jeil Company, Korea) and

water adlibitum.

Chemicals

B(a)P was supplied from Sigma Chemical Company and 3-methylcholanthrene from Eastman Kodak, USA. ^{3}H-B(a)P (69.1 Ci/mmole) was purchased from Amersham, England. Standard B(a)P metabolites (NCI Chemical Carcinogen Reference Standard) were from National Cancer Institute, MD. USA.

Preparation of Mouse Placental Microsomes

The 22 week old pregnant ICR mice were sacrificed by cervical dislocation and placentas were obtained and used as controls. Induction of placental enzyme activity *in vivo* was done by intraperitoneal injection of 3-methyl-cholanthrene (5 μg/g body weight) or phenobarbital (2.5 μg/g body weight) for 5 days. Another group of mice was treated with B(a)P (0.5 μg/g body weight) in 0.1 ml corn oil for 5 days by oral administration for the preparation of B(a)P-induced placental microsomes. Placental samples were frozen at −70°C until used for microsomal preparation. Placental tissue was rinsed in 1.15% KCl and homogenized with a glass homogenizer by 20 strokes in 0.5 M Tris buffer (pH 7.4) containing 0.5 M KCl, 5 mM $MgCl_2$ and 0.25 M sucrose. Microsomes were isolated by centrifuging for 10 min at 9,000 × g. Microsomal suspension was assayed for the protein content as described by Bradford (1976).

Incubation Conditions

The incubation buffer (0.05 M phosphate buffer, pH 7.4) containing 3 mM $MgCl_2$, 1 mM $MnCl_2$, 0.33 mM NADP, 8 mM D,L-isocitrate and 20 μg /ml protein of isocitrate dehydrogenase was incubated for 30 min at 37°C to generate NADPH. Placental microsomes (2 to 3 mg protein), unlabeled B(a)P (30 μg) and labeled ^{3}H-B(a)P (5 μ Ci) were mixed with the incubation buffer in a final voume of 1 ml and incubated for 2 hrs at 37°C. The reaction was terminated by the addition of 2 ml of ethyl acetate. The organic phase was separated by centrifugation, and the extract was evaporated under the stream of nitrogen. The residue was dissolved in 50 μl of methanol for the analysis by HPLC.

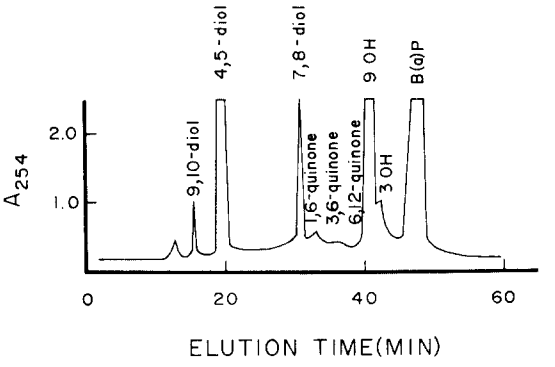

Fig. 1. The distribution of metabolites of standard B(a)P derivatives. Samples were separated by an HPLC and monitored at 254 nm with the UV detector.

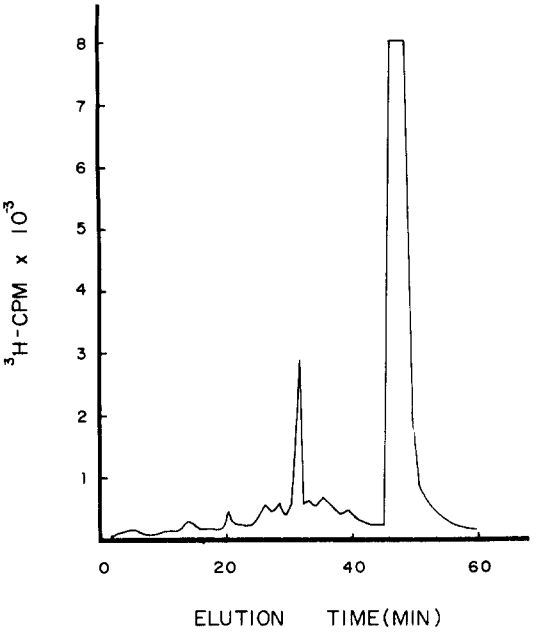

Fig. 2. An HPLC elution profile of B(a)P metabolites by negative control mouse placental microsomes (no B(a)P treatment). Tritiated B(a)P was incubated with placental microsomes for 2 hrs, followed by ethylacetate extraction. The residues were resuspended in a small volume of methanol and metabolites were resolved by HPLC.

HPLC Analysis

Metabolites were separated by HPLC on Millipore Waters Model 510 liquid chromatograph equipped with C18 μ-Bondapak column (7.8 mm × 30 cm). Metabolites were eluted with linear gra-

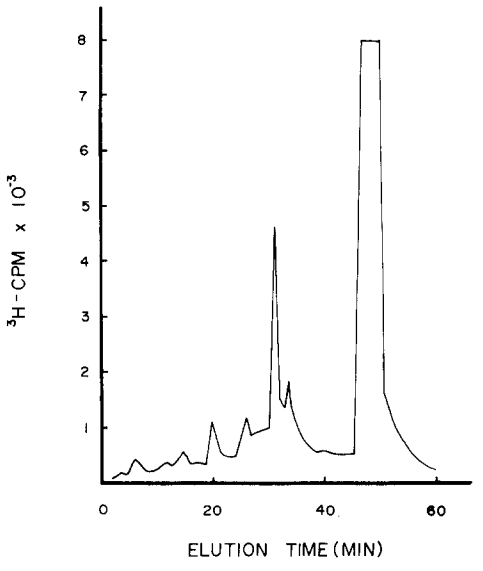

Fig. 3. An HPLC elution profile of B(a)P metabolites by B(a)P-induced mouse placental microsomes. A 12-week old ICR mouse was orally given 0.5 μg of B(a)P per g of body weight for 10 days. Prior to decapitation, the animal was fasted for 10 hrs.

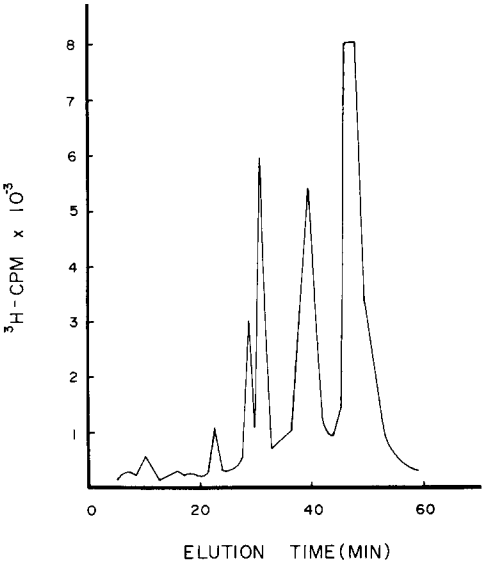

Fig. 4. An HPLC elution profile of B(a)P metabolites by phenobartital-induced mouse placental microsomes. The animal received 2 μg of phenobarbital sodium per g of body weight through intraperitoneal injection for 5 days.

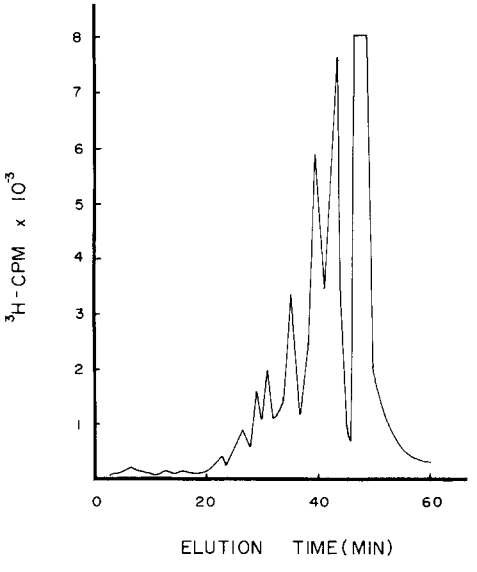

Fig. 5. An HPLC elution profile of B(a)P metabolites by 3-methylcholanthrene induced mouse placental microsomes. 5 μg of 3-methyl-cholanthrene per g of body weight was given to the animal for 5 days.

dient of 30 to 90% methanol over a period of 40 min at 50°C. The flow rate was 1 ml/min and each 1 ml fraction was collected.

Results

To isolate particular metabolites of B(a)P by mouse placental microsomes, high pressure liquid chromatogaphy technique was used. Fig. 1 shows elution position of authentic standards of B(a)P metabolites monitored by UV absorbance at 254 nm. Since the chromatography is reversed phase, the least polar compound B(a)P elutes last. The elution pattern shows that the three dihydrodiol derivatives of B(a)P elute first and three known quinones (1,6 quinone, 3,6 quinone and 6,12 quinones) appeared between 30 min and 40 min elution time, followed by 9-OH and 3-OH form of B(a)P. The results from incubation of B(a)P with control mouse placental microsomes are shown in Fig. 2. Shown in this profile is the 7,8-diol peak with the 4,5-diol, quinones and dihydroxy forms

Table 1. Benzo(a)pyrene metabolites produced *in vitro* by mouse placental microsomes.

Treatment	% of total radioactivity in each peak				
	4,5-Diol	7,8-Diol	Quinones	9-OH	3-OH
None	0.1	1.2	—	—	—
B(a)P	0.6	2	0.8	—	—
3-Methyl-cholanthrene	—	—	1.7	3.7	4.3
Phenobarbital	—	2	—	3.8	—

Inbred mouse strain was ICR mouse and total radioactivity was routinely 9 $\times$ 10^4 cpm. Each value represents average of three individual experimental samples.

presented only in trace amounts. It is readily apparent in Fig. 2 and Table 1 that oral administration of B(a)P to pregnant mice cause a striking induction of placental PAH metabolism. General induction of B(a)P metabolism was particulary noteworthy with observed 7,8-diol B(a)P, 4,5-diol B(a)P and quinones.

Elution profiles of the radioactivity of ^{3}H-B(a)P reacted with placental microsomes obtained from ICR mice pretreated with phenobarbital and 3-methyl-cholanthrene are illustrated in Fig. 4 and Fig. 5, respectively. The metabolite profiles in Fig. 4 demonstrate that major metabolites by the phenobarbital-induced mouse placental microsome were 7,8-diol B(a)P and 9-OH B(a)P. However, the metabolite profiles induced by 3-methyl-cholanthrene in Fig. 5 deviate significantly from the PAH metabolism seen in Fig. 4, with 9-OH B(a)P, 3-OH B(a)P and quinones are greatly increased. Also the diol forms of B(a)P [4,5-diol B(a)P and 7,8-diol B(a)P] were either absent or too low to be observed. The relative ratio of B(a)P metabolism by B(a)P or phenobarbital and 3-methyl-cholanthrene are listed in Table 1.

Discussion

B(a)P is one of the most extensively studied environmental carcinogens. Among more than 30 metabolites of B(a)P which have been characterized, 7,8-diol B(a)P has been identified as a proximate carcinogen and 7-β, 8α-dihydroxy-9α, 10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE) as an ultimate carcinogen (Melikian *et al.*, 1987). It has been also known that the major metabolites of B(a)P are the same in the majority of biological system (Selkirk, 1977). Studies on placental drug metabolism have shown that enzyme activities including arylhydrocarbon hydroxylase and epoxide hydrase are induced by habitual exposure to drugs or by cigarette smoking (Vaught *et al.*, 1979, Herbst *et al.*, 1974).

The formation of B(a)P-induced 7,8-diol in a large amount in the placental metabolizing system as seen in Fig. 3 is of particular interest, since 7,8-diol B(a)P is known to be a proximate carcinogen in animals (Kapitulnik *et al.*, 1977). Further indication of the ability of placental microsomes to participate in the metabolism of PAH carcinogens to an ultimate form of carcinogen is not available at present time. Our data, however, along with previous studies do suggest that placental microsome is also capable of metabolizing PAH carcinogens into electrophilic metabolites which are presumed to be potentially carcinogenic chemicals.

Many of the studies concerning B(a)P metabolism dealt with measuring AHH activity in a variety of tissues (Busbee *et al.*, 1972, Jacobson *et al.*, 1974, Jones *et al* 1978) have reported increased enzyme activity. Epoxide hydrase activity which is believed to be involved in the regulation of PAH metabolism (Miller, 1978) has been reported to be slightly induced by PAH treatment. It is known that more than two hundred xenobiotic agents such as drugs, herbicides, and pesticides stimulate microsomal enzyme induction. The inducers are of at least two types; exemplified phenobarbital, a type I inducer, and 3-methyl-cholanthrene (one of the polycyclic aromatic hydrocarbons), a type II inducer. Our data (Figs. 4 and 5) suggest that placental microsome

enzyme activities are also elevated by either type of inducer, phenobarbital or 3-methyl-cholanthrene.

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쥐의 태반조직에 의한 PAH 화합물의 대사활성화

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PAH화합물의 하나인 benzo(a)pyrene의 쥐 태반 microsome에 의한 대사활성화를 조사하였다. B(a)P대사물은 C18-μ Bondapak 컬럼을 사용하여 고압액체크로마토그래피로 분석하였다. 그 결과 B(a)P를 투여한 쥐의 태반 microsome에 의한 주 대사산물은 발암성이 강한 7,8-diol B(a)P였으며, 또한 적은량의 4,5-diol B(a)P와 quinone류가 검출되었다. 3-methyl-cholanthrene을 투여한 경우 hydroxy B(a)P와 quinone화합물이 주 대사산물이었으며 phenobarbital을 전처리했을 경우 7,8-diol B(a)P이 주 대사산물인 것으로 나타났다.