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CUTANEOUS METABOLISM OF BENZO[*a*]PYRENE: COMPARATIVE STUDIES IN C57BL/6N AND DBA/2N MICE AND NEONATAL SPRAGUE—DAWLEY RATS

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SUMMARY

The metabolism of the polycyclic aromatic hydrocarbon (PAH) carcinogen benzo[*a*]pyrene (BaP) was studied using microsomes prepared from the skin of the mouse and rat. Topical application of the polychlorinated biphenyl (PCB) Aroclor 1254 or the PAH 3-methylcholanthrene (3-MC) to the skin of the C57BL/6N and DBA/2N mouse and the Sprague—Dawley rat caused statistically significant enhancement of cutaneous microsomal aryl hydrocarbon hydroxylase (AHH) activity in each animal. PCB was a more potent inducer of the enzyme than was 3-MC. BaP metabolism by skin microsomes from the same animals was assessed using high performance liquid chromatography (HPLC). The skin of untreated animals metabolized BaP into 9,10-, 7,8- and 4,5-dihydrodiols, phenols and quinones. Skin application of PCB caused greater than 16—18-fold enhancement of BaP metabolism in the C57BL/6N mouse and the rat and 2—5-fold enhancement in the DBA/2N mouse. Skin application of 3-MC enhanced BaP metabolism 2—8-fold in the C57BL/6N mouse and 5—10-fold in the rat and had no effect in the DBA/2N mouse. The formation of procarcinogenic metabolite BaP-7, 8-diol was greatly enhanced (4—12-fold) by treatment with the PCB and 3-MC in the tumor susceptible C57BL/6N mouse and in the tumor-resistant neonatal Sprague—Dawley rat. In contrast, the formation of BaP-7,8-diol was either slightly enhanced (2-fold) or unaffected by treatment with the PCB or 3-MC in the tumor-resistant DBA/2N mouse. Our data indicate that neither the patterns of metabolism nor the amount of BaP-7,8-diol formation in the skin are reliable predictors of tumor susceptibility to the PAH in rodent skin.

Abbreviations: AHH, aryl hydrocarbon hydroxylase; BaP, benzo[*a*]pyrene; HPLC, high pressure liquid chromatography; 3-MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; PCB, polychlorinated biphenyl.

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INTRODUCTION

Cancer of the skin is the most common form of malignant neoplasm occurring in the human population. Of the approximately one million new cancers occurring annually in the United States, almost 1/3 develop in the skin [1]. A variety of chemicals are present in the environment which are known to cause human skin cancer [2]. Among these are coal tar products [3] which are rich in PAH such as BaP. BaP is a ubiquitous pollutant chemical generated whenever organic materials are incompletely combusted and is present in substantial amounts in polluted urban air and in a number of occupational environments [4].

The known biological activities of BaP, many of which have been shown to occur in skin or in cells cultured from skin, include cytotoxicity, tumorigenicity, and covalent binding to macromolecules including DNA, RNA and proteins [5]. Most, if not all, of these biological effects of BaP appear to require metabolism of the compound into reactive moieties that ultimately mediate the toxic response [6]. It is now known that BaP is metabolized in the skin by a cytochrome *P*-450 dependent membrane-bound enzyme system [7]. A 7,8-dihydrodiol metabolite of this reaction is further converted to 7,8-diol-9,10-epoxides of BaP which bind to cutaneous molecules, thereby, perhaps, initiating tumor formation [8].

The relationship of AHH activity and/or the inducibility of the enzyme to the tumorigenicity of the PAH has been proposed and studies by Nebert and Jensen have suggested that the responsiveness of AHH to PAH in inbred mouse strains is a genetic characteristic inherited as an autosomal dominant trait and that AHH responsiveness and skin tumorigenicity are directly related [9]. Thus, induction of skin tumors by topically applied PAH occurs much more frequently in the AHH-responsive C57BL/6N mouse strain than in the AHH-non-responsive DBA/2N mouse strain. Despite numerous studies of AHH activity and inducibility in cutaneous tissues, little information is available regarding the effect of different types of inducers on patterns of BaP metabolism in the skin and whether the patterns of metabolism correlate in any way with skin tumor susceptibility. An earlier report [10] did assess BaP metabolism in a crude whole homogenate prepared from mouse epidermis of 3-MC treated CD-1 mouse skin. The present study was designed to assess patterns of BaP metabolism in microsomes prepared from the C57BL/6N (AHH-responsive) and the DBA/2N (AHH-non-responsive) strains of mice pretreated with either a PCB (Aroclor 1254) or a PAH (3-MC). For comparative purposes the AHH-responsive and tumor-resistant neonatal Sprague—Dawley rat was also studied. BaP metabolism was assessed using HPLC.

MATERIALS AND METHODS

Chemicals

[^{14}C]BaP was purchased from New England Nuclear, Boston, MA and was purified by a silica gel (Bio-Sil A, 100—200 mesh, BioRad Laboratories) column with hexane as the eluting solvent and subsequently by reverse-phase HPLC using a DuPont Zorbax ODS column (6.2 mm × 25 cm) eluted with methanol/water (9:1, v/v). [^{14}C]BaP was diluted with unlabelled compound to a specific activity of 22.5 mCi/mmol. All solvents used were of HPLC grade and were purchased from Fisher Scientific Co., Fair Lawn, NJ. Gold Label BaP and 3-MC were obtained from Aldrich Chemical Co. All other chemicals were obtained in the purest form available. The PCB Aroclor 1254 was a gift from the Monsanto Corp., St. Louis, MO.

Animals and treatment

Female 5—6-week-old C57BL/6N and DBA/2N mice were obtained from the NCI, Frederick Cancer Research Center. For studies of neonatal Sprague—Dawley rats, pregnant animals were obtained from Holtzman Rat Farm, Madison, WI. Newborn rats were allowed to suckle until the 4th day after birth when they were treated with topical application of the inducers. The animals received 1 mg/10 g body wt. of the PCB Aroclor 1254 or 3-MC applied in 100 μl acetone on the back of the animal. Control animals received solvent alone.

Preparation of microsomes

Twenty-four hours after a single application of the inducer, the animals were sacrificed by decapitation and skin microsomes were prepared according to procedures established in this laboratory [11]. Microsomal suspensions were always incubated in the metabolic assay system on the day of preparation.

In vitro incubation system

Skin microsomes prepared from the neonatal rats or from C57BL/6N and DBA/2N adult mice were used as the enzyme source. The incubation mixture contained 1.5 mg microsomal protein, 0.05 mmol Tris—HCl (pH 7.5), 3 mmol $MgCl_2$ and 1 mg NADPH in a final volume of 0.96 ml. The reaction was initiated by the addition of 80 nmol [^{14}C]BaP in 40 μl methanol. The samples were incubated for 30 min in the dark at 37°C in a Dubnoff metabolic shaker. The reaction was terminated by adding 1 ml of acetone followed by 2 ml of ethyl acetate. The mixture was vortexed for 1 min to extract any unreacted BaP as well as the metabolites into the organic phase. The organic and aqueous layers were separated by centrifugation at 1500 rev./min for 5 min. The radioactivity in the aqueous phase ranged from $<0.05\%$ to 0.5% of the total radioactivity and was proportional to the extent of BaP metabolized. The organic phase was then dehydrated over anhydrous $MgSO_4$, dried

under a stream of N_2 and dissolved in 50 μl of methanol for HPLC analysis. All extractions were performed under yellow light.

Fluorescence assay of AHH

AHH activity was determined by a modification of the method of Nebert and Gelboin [12] as previously described [11]. The quantitation of phenolic BaP metabolites was based on comparison of fluorescence to a standard solution of 3-OH-BaP. Protein was determined according to Lowry et al. using bovine serum albumin as reference standard [13].

HPLC analysis of formation metabolites

A Waters Associates model 204 liquid chromatograph, fitted with a Whatman ODS-2 column (4.6 mm × 25 cm) was used for the analysis of radiolabeled metabolite mixtures of BaP. An unlabeled metabolite mixture prepared from a larger-scale in vitro incubation of BaP with rat liver microsomes was added as a source of UV marker for all radiolabeled samples. The column was eluted at ambient temperature with a 30-min linear gradient of methanol/water (1:1, v/v) to methanol at a solvent flow rate of 0.8 ml/min. The eluates were monitored at 254 nm, fractions of approx. 0.2 ml were collected dropwise and the radioactivity of each fraction was determined by liquid scintillation spectrometry. The counting efficiencies of the early eluted fractions, containing a higher percentage of water, were about 2% lower than those of the fractions eluted with methanol. The conversion of liquid scintillation counting data to concentration was based on the counting efficiency of the fractions eluted with methanol.

RESULTS AND DISCUSSION

The levels of AHH activity, as measured by the fluorescence assay, in skin microsomes prepared from neonatal rats, C57BL/6N and DBA/2N mice following topical application of PCB or 3-MC are presented in Table I. In all three experimental animals, PCB was a more potent inducer of the cutaneous microsomal enzyme than was 3-MC. Thus PCB treatment resulted in greater enzyme induction in the neonatal rat (16-fold) and in the C57BL/6N mice (18-fold) than in the DBA/2N mice (2-fold). 3-MC was a more effective inducer of microsomal AHH activity in neonatal rat skin (11-fold) than in the skin of either mouse strain (4-fold for C57BL/6N mice and 1.6-fold for DBA/2N mice). These results are in general agreement with those reported earlier [14].

The HPLC separation of [^{14}C]BaP metabolites obtained by incubation of the radiolabeled PAH with PCB-induced C57BL/6N mouse skin microsomes is shown in Fig. 1. The overall pattern of metabolism of BaP from neonatal Sprague—Dawley rat and the DBA/2N mouse, whether from control, PCB or 3-MC treated skin, was qualitatively similar to that depicted in Fig. 1. Under the conditions of our HPLC system, phenols are eluted earlier than quinones and no significant radioactivity was eluted prior to 12 min.

TABLE I

EFFECT OF TOPICAL APPLICATION OF AROCLOR 1254 AND 3-MC ON SKIN MICROSOMAL AHH ACTIVITY AND METABOLISM OF [^{14}C]BaP IN C57BL/6N MICE, DBA/2N MICE AND NEONATAL SPRAGUE—DAWLEY RATS

Animals were treated with a single topical application (100 mg/kg) of Aroclor 1254 or 3-MC 24 h before sacrifice. Controls received solvent alone.

AHH/Metabolite	C57BL/6N Mice			DBA/2N Mice			Neonatal Sprague—Dawley rats		
	Control	PCBs	3-MC	Control	PCBs	3-MC	Control	PCBs	3-MC
AHH activity[a]	46	821 (18×)[b]	193 (4×)	133	268 (2×)	214 (1.6×)	68	1086 (16×)	786 (11×)
trans-9,10-diol[c]	3	24	10	8	15	6	5	64	38
trans-4,5-diol	10	29	30	11	42	16	10	63	50
trans-7,8-diol	10	119 (12×)	42 (4×)	20	48 (2×)	22 (1×)	12	127 (10×)	85 (7×)
Phenol 1 (mainly 3-OH-BaP)	18	289	88	54	142	44	34	342	266
Phenol 2 (mainly 9-OH-BaP)	34	780	277	134	334	114	59	656	478
Total Phenols	52	1068 (20×)	365 (7×)	188	476 (2.5×)	158 (0.84×)	93	998 (11×)	744 (8×)
Quinones	30	127	66	30	156	38	17	109	167
Other unidentified	13	19	16	9	51	13	2	26	15
Total metabolites	118	1387 (12×)	477 (4×)	266	788 (3×)	253 (1×)	140	1420 (10×)	1100 (8×)

[a] AHH activity is expressed as pmol BaP phenols/mg protein/30 min.

[b] Fold inducibility is indicated in parentheses.

[c] Metabolite formations are expressed in pmol metabolite formed/mg protein/30 min. Numbers shown are average values of duplicate samples which agree within 10% of the values indicated and within 10—30% of the values shown from experiments using different preparations of skin microsomes.

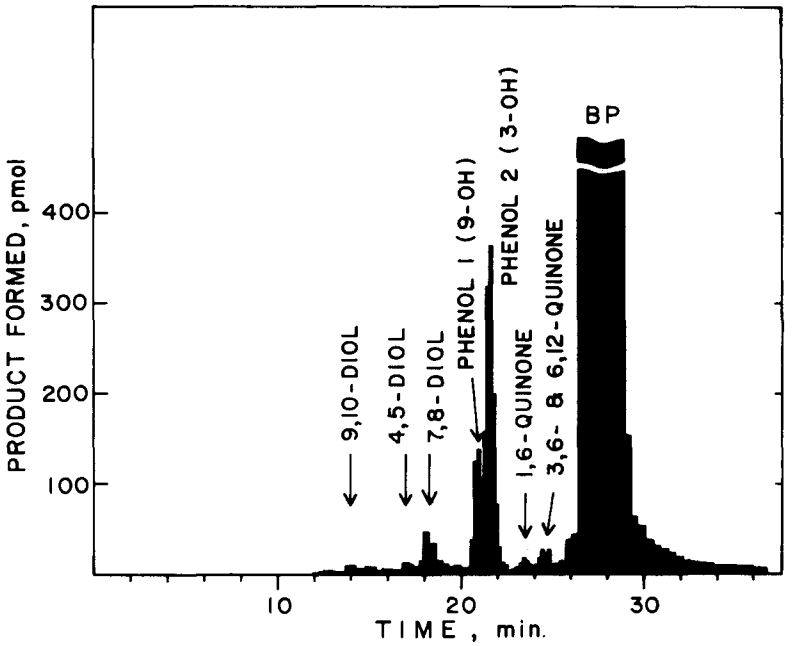

Fig. 1. BaP metabolism by skin microsomes prepared from C57BL/6N mice pretreated with topical application of Aroclor 1254 (100 mg/kg) 24 h prior to sacrifice.

The quantitation of BaP metabolism by skin microsomes from the mouse strains and from the rat are also shown in Table I. Metabolism in control skin of DBA/2N mice was substantially higher than that in the control skin of the C57BL/6N mouse or the neonatal rat. Phenol formation by skin microsomes prepared from the animals increases substantially following application of either the PCB or 3-MC. Topical treatments with the two inducers had variable effects on the amount of non-phenolic metabolites formed and this differed in each of the animals (Table I). It is of particular interest to note that the formation of *trans*-7,8-diol, a proximate carcinogenic metabolite of BaP [15] is substantially increased in the tumor-susceptible AHH-responsive C57BL/6N mice and the tumor-resistant neonatal Sprague–Dawley rats following pretreatment with either PCB or 3-MC. In contrast, the formation of this 7,8-diol increased only 2-fold following PCB treatment and was unaffected by 3-MC treatment in the tumor-resistant AHH-non-responsive DBA/2N mice.

While BaP metabolism in liver microsomal enzymes has been extensively studied [20], relatively little work has been performed in extrahepatic tissues such as the skin. Berry et al. [13] studied BaP metabolism in whole homogenate prepared from an epidermal fraction scraped from the skin of Swiss CD-1 mice. Several recent studies have shown that active BaP metabolism occurs in cultured human skin keratinocytes [21—24]. Buty et al. [25] used crude epidermal homogenates as an enzyme source and showed a correlation between the tumor-initiating capacity of a series of PAH and their enzyme-mediated binding to DNA in vitro.

The metabolic biotransformation of BaP into bay region 7,8-diol-9,10-epoxides is now thought to be an essential step in the induction of tumor formation in the skin [8,16,17]. Microsomes prepared from the liver and skin of C57BL/6N mice have been shown to catalyze the formation of adducts to DNA in vitro as a result of the metabolic formation of BaP-7,8-diol-9,10-epoxide. In tissues of the C57BL/6N mouse this was considerably greater than that occurring in the DBA/2N mouse [18]. Thus the formation of the BaP-*trans*-7,8-diol in the skin of C57BL/6N and DBA/2N mice should correlate with the known tumor susceptibility of the animals. It is interesting to point out, however, that our studies show that formation of this metabolic precursor of BaP-7,8-diol-9,10-epoxide was enhanced to a greater extent in each mouse strain by pretreatment with the PCB than with the known PAH carcinogen 3-MC. Since the PCBs are not clearly oncogenic for mammalian skin, our findings suggest that exposure to non-carcinogenic environmental pollutant chemicals which can enhance the metabolism of PAH carcinogens may be an important determinant of the risk of oncogenesis resulting from exposure to the PAH themselves.

Our findings in the neonatal rat are also difficult to interpret in the light of present-day concepts of the mechanism of chemical carcinogenesis by the PAH such as BaP. Treatment of the rat with the PCB or 3-MC stimulated the formation of BaP-*trans*-7,8-diol to the same extent observed in the tumor-susceptible C57BL/6N mouse and yet this animal is not susceptible to the induction of cutaneous tumors by the PAH. Thus the enzyme-mediated formation of the *trans*-7,8-diol metabolite per se cannot be taken as a reliable indicator of tumor susceptibility of the skin to BaP. It would be of considerable interest to measure BaP-7,8-diol-9,10-epoxide formation in the skin of this animal since prior studies by Koreeda et al. [8] in the CD-1 tumor-susceptible mouse have clearly shown considerable binding of the diol-epoxide to skin macromolecules following topical application of BaP.

In summary our data have shown that microsomal enzymes in skin are capable of metabolizing BaP and that this enzyme activity can be enhanced by topical application of at least two kinds of chemical inducers. Our data also indicate that the quantity of metabolites that are specific precursors of known ultimate tumorigenic moieties of BaP produced in skin is not in itself a reliable indicator of tumor susceptibliity to this PAH in rodent skin.

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