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The Effect of Some Mixed Function Oxidase Inducers on Aryl Hydrocarbon Hydroxylase and Epoxide Hydrase in Nuclei and Microsomes from Rat Liver and Lung. The Effect of Cigarette Smoke*

P. M. DANSETTE,†§ K. ALEXANDROV,‡ R. AZERAD† and CH. FRAYSSINET‡

†*Institut de Biochimie, Université d' Orsay*, 91400 *Orsay, France*
‡*Institut de Recherches Scientifiques sur le Cancer*, 94800 *Villejuif, France*

Abstract—*The activities of aryl hydrocarbon hydroxylase (AHH) and epoxide hydrase (EH) in rat lung microsomes and nuclei were compared to those of liver from untreated (C) rats and rats pretreated with phenobarbital (PB). methylcholanthrene (MC) and cigarette smoke (CS).*

The pretreatment of rats with 3 different types of mixed function oxidase (MFO) inducers produced different effects on the induction of AHH and EH in rat liver microsomes and nuclei. With MC a 6-fold increase in AHH enzyme activity was observed in both microsomes and nuclei from liver, while the PB and cigarette smoke did not increase AHH activity in these fractions. Epoxide hydrase in liver microsomes and nuclei was increased 4-fold by PB while MC and cigarette smoke inhalation by rats showed no effect. The pretreatment of rats with MC increased lung nuclear and microsomal AHH 6–9 fold and cigarette smoke induced these activities 3-fold. Phenobarbital did not cause any significant induction. Lung nuclear and microsomal epoxide hydrase were not affected by pretreatment of rats with MC or PB. The only significant induction of epoxide hydrase in lung tissue was observed in the microsomal fraction and was caused by inhalation of cigarette smoke. This activity increased rapidly within 2 hr of exposure to the cigarette smoke, reached a value 2–3 times that of controls and subsequently began to decline 6 hr after inhalation.

INTRODUCTION

THE TWO major enzymes systems that metabolize polycyclic hydrocarbons are epoxide hydrase (EH) [1] and the microsomal NADPH-dependent mixed-function oxygenase system, including cytochromes P-448 and P-450, and otherwise known as aryl hydrocarbon hydroxylase (AHH) [2–4]. In rat tissues at least 2 forms of AHH which are capable of hydroxylating benzo(a)pyrene (BaP) have been found. These can be differentially induced *in vivo* by administration of phenobarbital (PB) or methylcholanthrene (MC) to rats [5].

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§Address for reprint requests: Laboratory of Chemistry, École Normale Superieur, 75231 Paris, Cedex 05, France.

These enzymes play an important role in determining the steady-state levels of various arene oxides of polycyclic hydrocarbons in mammalian tissues and consequently may also play an important role in the etiology of cancer initiated by these compounds.

For many years, the mixed function oxidases, AHH in particular, were considered to be exclusively microsomal enzymes [2, 6]. Recently, however, we [3, 5, 7, 8] and others [9–15] have reported AHH activity in the nucleus. Similarly, epoxide hydrase has been found in microsomes from both liver and lung [14–16] and also in nuclei [15, 17].

The lung is an organ directly exposed to varying concentrations of polycyclic aromatic hydrocarbons and is believed to be one of the primary target organs for hydrocarbon induced carcinogenesis. Clinical studies show a

direct relationship between lung cancer and cigarette smoking habits. However, basic research to determine which factors in cigarette smoke contribute to lung cancer is still in progress.

Furthermore, cigarette smoke produced by machines designed to imitate human smoking conditions, has also caused an increase in AHH in lungs of animals [18–21]. Increased AHH activity has been demonstrated in pulmonary alveolar macrophages and placental tissues of humans who smoke [21, 22]. Cigarette smoke induces lung AHH activity in both the inducible and non-inducible liver AHH strain of mice [18, 23, 24]. The ability of mouse lung microsome to metabolize BaP varies with the strain of mouse used to prepare microsomes. Microsomes from A/HeJ mice, which have a high incidence of spontaneous lung tumors, show extensive metabolism of BaP [25].

Tissue and cell fraction comparisons are essential for evaluating the relationship between enzyme activity and the susceptibility of certain tissues to carcinogenesis induced by aromatic hydrocarbons. We have examined the effect of two different types of MFO inducers (phenobarbital and methylcholanthrene) on aryl hydrocarbon hydroxylase and epoxide hydrase induction in nuclei and microsomes obtained from liver and lung. Furthermore, we have extended this study to compare the effect of cigarette smoke upon these activities. In this manuscript we report the presence of epoxide hydrase in liver and lung nuclei.

MATERIALS AND METHODS

Chemicals

The source of most of the chemicals has been indicated elsewhere [5]. NADPH was purchased from Boehringer Corp., London, England. Bovine albumin powder was obtained from Armour Pharmaceutical Co., Kankee, Ill. 3-OH-BP was gift of Dr. H. V. Gelboin and B (a) P-4, 5-oxide was prepared according [26].

Treatment of animals

Male Wistar rats (WAG_1) strain weighing 120–150 g. were used in all studies. MC in 0.5 ml corn oil was injected i.p. (25 mg/kg daily) for 2 days. Controls animals received corn oil only. Sodium PB (0.1%) was placed in the rats drinking water for 10 days. Inhalation studies were performed in the Tobacco Service (Institut de Recherche Scientifique sur le Cancer, Villejuif) using a special inhalation apparatus. (We thank Dr. I. Chouroulinkov for assistance with the inhalation studies). The smoke was delivered in the form of a 30 ml puff diluted with a variable volume of air to 1/15 cigarette smoke/air dilution. Every 2 sec the air/smoke mixture was blown into the closed area in which the rats were located. The stress induced by the experimental conditions was not responsible for the enzyme induction [27]. After 15 min of inhalation, the animals were returned to their cages and killed at different times according to the experiment.

Preparation of the subcellular fractions

The microsomes and nuclei from rat liver were isolated as previously described [3, 7] and used immediately without storage. The lungs of 10 rats were used for each preparation of lung microsomes and nuclei. The lungs were homogenized with a Waring Blender in a buffer containing 0.25% sucrose, 0.05 M Tris–HCl (pH 7.5), 25 mM KCl, 5 mM $MgCl_2$ and 20% glycerol.

Subsequently, the isolation of nuclei and microsomes from the resulting homogenate was carried out by the same procedures used in the preparation of liver fractions [7]. All nuclear preparations were examined routinely by light and electron microscopy and appeared to be normal, intact and essentially free of contamination from organelles such as microsomes [5, 7].

Enzyme assays

The AHH activity in nuclei and microsomes was determined according to a method described by Hayakawa and Udenfriend [28]. The nuclear pellet was resuspended in 0.05 M Tris–HCl buffer (pH 7.5) containing 0.003 M $MgCl_2$, NADPH (1.3×10^{-4} M), 0.25% sucrose and 20% glycerol, so that 1 ml contained 1–2 mg of protein. In the case of microsomes the concentration was 0.2–0.5 mg protein/ml. Blanks comprised an incubation medium to which an equal volume of acetone was added at zero time. The mixtures were incubated at 37°C as follows: 10 min for liver microsomes, 30 min for liver nuclei, 15 min for lung microsomes and 30 min for lung nuclei.

The epoxide hydrase was determined by direct fluorometric measurement of the BaP-4,5-diol formed according to a method described by Dansette and Jerina [29]. The incubation mixture (2 ml) contained a 0.1 M

phosphate buffer (pH 9), 20% glycerol (preparations of nuclei), an appropriate amount of microsomal (0.05–1 mg) or nuclear protein (1–2 mg) and 20 nmole BaP 4-5 oxide. Time dependent formation of BaP 4,5-diol at 37°C was followed directly on the spectrofluorimeter (λ exc. = 310 nm, λ em. = 385 nm).

NADPH-cytochrome-c-reductase was measured by monitoring the reduction of cytochrome-c spectrophotometrically at 550 nm as described by Johannesen [30].

RESULTS

NADPH-cytochrome-c-reductase in nuclei and microsomes obtained from rat liver and lung

Previous studies demonstrated that epoxide hydrase is localized in the endoplasmic reticulum of rat lung and is distributed in the same manner as NADPH–cytochrome-c-reductase [16].

Electron microscopic studies show that our nuclear preparations were not contaminated with endoplasmic reticulum (unpublished results, C. Lafarge-Frayssinet *et al.*) To confirm our observation we measured NADPH-cytochrome-c-reductase activities in liver and lung nuclear preparations in comparison to those found in microsomes (Table 1). The results show that NADPH-cytochrome-c-reductase was present in very small quantities in rat liver nuclei (5–6 nmole/min/mg) compared to liver microsomes (112–132 nmole/min/mg). Phenobarbital caused a 2 fold induction of NADPH-cytochrome-c-reductase activity (223 nmole/min/mg) compared to the other MFO inducers. The NADPH-cytochrome-c-reductase in rat lung nuclei was detected with difficulty, (0.3–0.8 nmole/min/mg) while the amount in rat lung microsomes was about 2.7–7.5 nmole/min/mg. These results show that the nuclear preparations from rat lung and liver contained a low but not negligible amount of the supposed endoplasmic reticulum marker, NADPH-cytochrome-c-reductase.

Induction of rat liver microsomal and nuclear aryl hydrocarbon hydroxylase (AHH) and epoxide hydrase (EH)

It has been shown that benzo(a)pyrene hydroxylase can be induced 10 fold in liver and lung microsomes by i.p. administration of methylcholanthrene to rats [3, 5, 14, 19, 23, 25, 30] whereas phenobarbital induced this activity in liver to a much lesser extent [31]. Conversely epoxide hydrase activity was observed to increase significantly in liver microsomes after PB treatment of rats. We confirm these previous observations and extend the studies to include liver nuclei (Table 2).

The pretreatment of rats with PB, MC and

Table 1. NADPH-cytochrome-c-reductase activity in nuclei and microsomes obtained from liver and lung of rats treated with different MFO inducers

Treatment of animals	Fraction	NADPH-cytochrome-c-reductase nmole/min/mg Liver	Lung
Control	Nuclei	5.2±0.3*	0.32±0.12
Phenobarbital		5.6±0.2	0.84±0.22
Methylcholanthrene		6.2±0.4	0.86±0.14
Cigarette smoke		6.1±0.6	0.53±0.08
Control	Microsomes	112±6	2.7±0.9
Phenobarbital		223±12	7.0±0.3
Methylcholanthrene		118±14	7.5±0.7
Cigarette smoke		132±8	6.2±0.6

*Mean ±S.D.

Twenty four hours prior to sacrifice, rats were given an injection of 25 mg/kg of 3-methylcholanthrene (MC) in 0.5 ml corn oil. Sodium phenobarbital (PB) was placed in the drinking water (0.1%) of 120–150 g male Wistar rats (WAG strain) for 10 days. Rats inhaled cigarette smoke for 15 min and were sacrificed 4 hr later. In each experiment the lungs of 10 rats and livers of 3 rats were pooled and the microsomes and nuclei were isolated as described in Material and Methods. Data represent the average of values obtained in at least 3 separate experiments.

Table 2. The effect of some MFO inducers on aryl hydrocarbon hydroxylase (AHH) and epoxide hydrase (EH) activities in rat liver nuclei and microsomes

Treatment of animals	Fraction	Aryl hydroxylase			Epoxide hydrase $\times 10^{-3}$		
		Mean	Range	I/C*	Mean	Range	I/C*
C	Nuclei	23	10–62	1.0	0.17	0.11–0.26	1.0
PB		39	24–87	1.7	0.58	0.38–0.64	3.4
MC		155	110–232	6.7	0.15	0.09–0.22	0.9
CS		26	12–48	1.1	0.18	0.12–0.29	1.1
C	Microsomes	273	142–406	1.0	14.2	12.4–18.2	1.0
PB		421	182–722	1.5	60.0	48.2–86.6	4.3
MC		1467	908–2020	5.4	16.4	12.6–19.8	1.1
CS		266	152–386	0.9	14.8	10.8–19.2	1.0

*I/C = inducer/control: C = controls; PB = phenobarbital pretreated rats; MC = MC pretreated rats; CS = cigarette smoke-treated rats.

Three treatment groups of rats consisted of 5 animals pooled per group. Specific activities in terms of pmole/min/mg were determined. Assay conditions were fully described under Materials and Methods. Average values are reported for each treatment group.

Table 3. Aryl hydrocarbon hydroxylase (AHH) and epoxide hydrase (EH) activities in rat lung nuclei and microsomes

Treatment of animals	Fraction	Aryl hydroxylase			Epoxide hydrase		
		Mean	Range	I/C*	Mean	Range	I/C*
C	Nuclei	1.6	0.9–2.8	1.0	56	42–68	1.0
PB		1.4	1.0–2.2	0.9	60	40–76	1.1
MC		9.6	4.0–6.6	6.0	89	72–98	1.6
CS		4.6	4.1–5.4	2.9	85	76–102	1.5
C	Microsomes	11	8–18	1.0	180	140–240	1.0
PB		18	12–36	1.6	250	180–290	1.4
MC		102	86–132	9.3	180	130–260	1.0
CS		29	22–46	2.6	500	400–600	2.8

*I/C = inducer/control; C = controls; PB = phenobarbital pretreated rats; MC = MC pretreated rats; CS = Cigarette smoke treated rats.

Specific activities (pmole/min/mg) were determined in lung nuclei and microsomal incubations. The microsomal protein concentration was 1.0 mg/ml and the nuclear protein concentration was 2.0 mg/ml. The nuclear incubation buffer contained 20% glycerol. Pretreatment was as described in Table 2 except that each group represented the lungs of 10 animals pooled per group. Average values are reported for each group. Assay conditions are fully described under Materials and Methods.

cigarette smoke affected the induction of AHH and EH in rat liver microsomes and nuclei differently (Table 2). MC had the strongest effect causing a 6-fold increase in AHH enzyme activity in both liver microsomes and nuclei. The PB pretreatment resulted in a small induction of AHH, while cigarette smoke had no effect. Epoxide hydrase was increased 4 fold by PB in rat liver nuclei and microsomes while MC and cigarette smoke inhalation by rats (Table 2) showed no effect.

Lung nuclear and microsomal aryl hydrocarbon hydroxylase and epoxide hydrase

The pretreatment of rats with MC increased lung nuclear and microsomal AHH 6–9 fold, cigarette smoke increased both activities 3 fold, while PB had no effect (Table 3). These results are similar to those observed with liver microsomes and nuclei (Table 2). Epoxide hydrase activity in rat lung microsomes has been measured in previous studies using (^{3}H) styrene oxide [16, 32]

according to the procedure essentially developed by Oesch *et al.*, for liver microsomes [33]. P. Dansette and D. Jerina [29] developed a new assay for epoxide hydrase, based on the fluorescence measurement of BaP 4,5-diol. Our studies are based on their method. The activity of lung nuclear and microsomal epoxide hydrase was not affected by pretreatment of rats with MC. Similar results were reported previously by Seidegard *et al.* [16]. Lung epoxide hydrase did not show significant induction in either nuclear or microsomal fractions, with the single exception of the activity in microsomes, which was enhanced nearly 3-fold by cigarette smoke.

The ratio of induced AHH to control AHH activity (I/C) was similar for nuclei and microsomes and for both liver and lung (Tables 2 and 3). Bresnick *et al.* [14, 15] obtained similar results for the ratio of induced to control in both nuclei and microsomes in mice. The ratio (I/C) for epoxide hydrase was similar for liver nuclei and microsomes but not for lung preparations. Cigarette smoke induced lung microsomal epoxide hydrase to a significant extent such that I/C was 3 times higher than for the other MFO inducers.

Time-dependent activities of AHH and EH in nuclei and microsomes from liver and lung treated with cigarette smoke

The exposure of rats to cigarette smoke (15 min) induced AHH activity in lung microsomes (Fig. 1) but not in liver microsomes. No induction of AHH was observed in lung and liver nuclei. This confirms data from Van Cantfort [27] showing that even 1 min exposure to the usual 1/15 dilution of cigarette smoke dose was sufficient to induce the lung enzyme significantly. The epoxide hydrase activity in lung microsomes increased rapidly and reached an activity 2–3 times that of the enzyme from untreated rats within 2–4 hr after inhalation. There after the activity began to decline. Cigarette smoke did not affect nuclear lung epoxide hydrase.

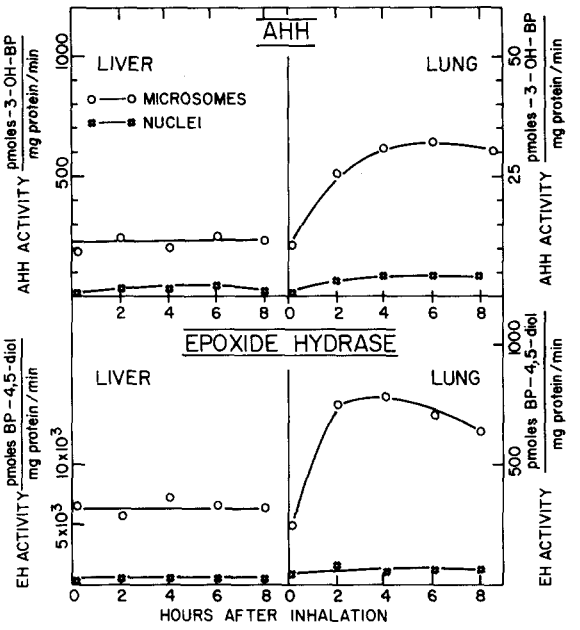

Fig. 1. Effect of cigarette smoke inhalation (15 min) *on AHH and EH activities in microsomes* (○—○) *and nuclei* (×—×) *from the livers and lungs. Each group of* 10 *rats was killed at a different time after the cigarette smoke treatment.*

DISCUSSION

Previous studies have shown that exposure to cigarette smoke increases AHH activity in lungs of animals [18–20, 24, 31, 34] and pulmonary alveolar macrophages of humans [21]. Studies on the activities of AHH and EH in rat lung microsomes and nuclei, compared to those of liver, from MC and cigarette smoke treated rats are important in understanding the metabolism of some carcinogens and its possible relationship to lung cancer. Oesch *et al.* [33] suggested that epoxide hydrase is localized on the endoplasmic reticulum in the liver and therefore could be used as a marker for the endoplasmic reticulum. Seidegard *et al.* [15] showed that epoxide hydrase is also localized on the endoplasmic reticulum in the lung. However, the present study provides strong evidence that epoxide hydrase is also present in the liver and lung nuclei and therefore should not be used only as a marker for endoplasmic reticulum. Phenobarbital induced epoxide hydrase activity in the liver nuclei and microsomes but not in the lung nuclei and microsomes. Similarly phenobarbital increased NADPH-cytochrome-c-reductase (Table 1) and AHH activity (Table 2) in rat liver while not affecting the same enzymes in rat lung nuclei and microsomes. Both epoxide hydrase and AHH activities were substantially lower in rat lung tissue than in rat liver. The distribution of AHH and EH between the nuclear and microsomal fractions of both lung and liver tissues was not equal. Nuclear AHH in both tissues was approximately 10% of the activity in the corresponding microsomes. In contrast the liver nuclear EH represented only 1% of the activity of liver microsomal EH whereas lung nuclear EH represented 20%–30% of

the microsomal activity. Thus, of the total EH in each tissue type there was a higher nuclear localization in lung (20%) than liver (1%).

Although the specific activity of epoxide hydrase was generally much higher in liver compared to lung, both in mice [25] and rats (Tables 2 and 3) an important factor to be taken into account is the ratio of specific activities for epoxide hydrase and AHH. Epoxide hydrase activity has been shown to be essential in the formation of the highly mutagenic BaP diol–epoxide which is considered to be the ultimate carcinogenic metabolite [35], and a higher ratio of EH to AHH would conduct to increased formation of dihydrodiols and that possible formation of diol-epoxide. The capability of nuclear metabolism of BaP to the very reactive diol epoxide in proximity to macromolecules such as DNA, conceivably could be an important prerequisite for the initiation of carcinogenesis in lung tissue. Recently they are studies [36–38] suggesting alternative activation pathway of BaP other than diol-epoxide.

The effect of inducers upon the relative activities of the two enzymes, AHH and EH, in various cell fractions and tissues was variable. The pretreatment of animals with MC decreased the EH/AHH ratio in nuclei and microsomes from both liver and lung when compared to controls. Conversely, PB increased this ratio in both liver nuclei and microsomes. Cigarette smoke decreased this ratio in the lung nuclei but not in lung microsomes (Tables 2 and 3). The ratio of microsomal epoxide hydrase to microsomal aryl hydrocarbon hydroxylase activity in rat liver is about 3–6 times higher than in rat lung, while this factor (EH/AHH) in the lung nuclei is 3–10 times higher than in rat liver nuclei. The lung epoxide hydrase responded differently to MC and cigarette smoke. Probably its induction is under different genetic control.

We found an increase of lung epoxide hydrase activity (using BaP-4,5-oxide as substrate) during the first hours after a single 15 min exposure to cigarette smoke (Fig. 1), while Uotila *et al.* [34] noticed that the activity of epoxide hydrase decreased (using styrene oxide as substrate) after one day of cigarette exposure, despite the fact that BaP-4,5-dihydrodiol formation increased. This controversial result points out the need for further studies on the effect of cigarette smoke on the induction of lung epoxide hydrase, especially as there is evidence that BaP-4,5-oxide and styrene oxide are metabolized by the same EH enzyme [34].

It is unlikely that benzo(a)pyrene is involved in the observed lung enzyme activity after exposure of rats to cigarette smoke for the following reasons. The inhaled smoke is estimated to contain about 10–20 ng of benzo(a)pyrene (0.1–1 mg/kg) [39, 40]. This quantity of BaP is too small to induce the enzyme activity [27]. It was reported [41] that 10–100 mg/kg intratracheal administration of MC to mice was necessary to induce lung AHH activity and even at this high concentration 24 hr were required for a maximal induction of hydroxylase activity. The inducing agents in the cigarette smoke might not be polycyclic hydrocarbons at all. Previous studies showed that lung AHH activity could be induced by cigarette smoke in non-inducible strains of mice [18, 24] and that certain fractions of cigarette smoke which do not contain polyclic hydrocarbons can also induce mouse lung AHH [41].

The responses of different strains of mice and rats to carcinogenic action of polycyclic hydrocarbons and cigarette smoke cannot be explained at the present time by the formation of a metabolite or by specific tissue enzyme induction. Although we observed differences in the induction of the enzymes by different types of MFO inducers, the profiles of BaP metabolites, obtained as a result of metabolism by MC and cigarette smoke induced enzymes, were slightly different [34]. Probably genetic and biological factors such as DNA repair or differences in immune response are important in explaining the differential susceptibility to lung hydrocarbon induced carcinogenesis.

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