EVIDENCE FOR THE INVOLVEMENT OF CYTOCHROME P-450 IN REDUCTION OF BENZO(a)PYRENE 4,5-OXIDE BY RAT LIVER MICROSOMES

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SUMMARY : Benzo(a)pyrene 4,5-oxide is reduced to benzo(a)pyrene by microsomes in the presence of NADPH. Carbon monoxide and oxygen inhibit this reduction. The liver has highest activity which is almost lackng in new-born rats. Phenobarbital as well as 3-methylcholanthrene pretreatment increases the epoxide reduction. Additions of FMN or methylviologen stimulate the epoxide reduction ; dimethylaniline N-oxide and cumene hydroperoxide are inhibitory. These results indicate that benzo(a)pyrene 4,5-oxide is reduced by the reduced form of cytochrome P-450.

Introduction

In previous papers, we have reported that reduction of tertiary amine N-oxides in liver microsomes is catalyzed by the reduced form of cytochrome P-450 (1,2). The reduction of tertiary amine N-oxide was almost completely inhibited by CO and the inhibition reversed by light exposure.

Booth et al. (3) recently reported the NADPH-dependent reduction of aromatic hydrocarbon epoxides by rat liver microsomes. This may be important since aromatic hydrocarbon epoxides are generally considered as more toxic compounds than the parent hydrocarbons and could possibly act as proximate or ultimate carcinogens (4,5).

This prompted us to investigate the character and mechanism of the reduction of aromatic hydrocarbon epoxides in liver microsomes.

Materials and Methods

Male rats of Sprague-Dawley strain of 7 weeks age were used unless otherwise specified. Benzo(a)pyrene 4,5-oxide was synthesized by Prof. T. Watabe (Tokyo College of Pharmacy) and kindly donated for the present experiments. Benzo(a)pyrene was purchased from Nakarai Chemicals Co. Ltd. (Kyoto). Dimethylaniline N-oxide and tiaramide

N-oxide were synthesized in our laboratories as reported in previous papers (1,2).

NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co., St. Louis.

Liver microsomes were prepared as described in a previous paper (1). The microsomes were resuspended in 1.15 % KCl and recentrifuged to remove traces of contaminating hemoglobin. Standard incubation mixtures (2.5 ml) contained microsomal fraction (equivalent to 0.5 g of liver), NADP (5 μmole), glucose 6-phosphate (25 μmole), and glucose 6-phosphate dehydrogenase (7.5 I.U.) and pH 7.4 phosphate buffer (150 μmole). Benzo(a)pyrene 4,5-oxide (0.25 μmole) was added after dissolving in 0.1 ml acetone. Incubations were carried out for 10 min at 37° in an atomosphere of nitrogen.

The aqueous and organic phases were separated by centrifugation and 4 ml portions of the ethyl acetate phases were evaporated to dryness under reduced pressure. The residues were dissolved in ethanol (100 μl) and the solutions (20 μl) were applied to a Eastman 13181 silica gel and developed in a petroleum ether (b.p. 45-60°)-benzene mixture (4 : 1, v/v). After development the area (Rf 0.42) of the chromatogram containing the metabolically formed benzo(a)pyrene (locate in u.v. light) was cut out, the hydrocarbon eluted with ethanol (3 ml) and the fluorescence recorded with an Aminco-Bowman Spectrophotofluorometer 768-H (excitation wavelength, 290 nm ; emission wavelength, 410 nm).

Results and Discussion

NADPH was the preferential cofactor for the reduction of benzo(a)-pyrene 4,5-oxide. NADH showed only about 15 % the activity of NADPH (Table 1). Both NADPH and NADH together seemed to exhibit a synergic effect on the epoxide reduction. Nonenzymatic reduction of the epoxide by boiled microsomes was negligible. The reduction rate was approximately linear for 10 min.

Carbon monoxide markedly inhibited the reduction of benzo(a)pyrene 4,5-oxide as shown in Table 2 and Fig. 1. The carbon monoxide concentration which inhibits 50 % reduction of benzo(a)pyrene 4,5-oxide in microsomes from untreated rats was about 2.3 μM. The epoxide reduction in microsomes from 3-methylcholanthrene induced rats seemed to be more resistent to CO, and 50 % inhibition occurred at about 10 μM.

Recently we have observed that additions of FMN or methylviologen markedly stimulate the reduction of tertiary amine N-oxides and the stimulated activity was completely inhibited by CO (3,6,7). In the present experiments, the reduction of benzo(a)pyrene 4,5-oxide was stimulated by both FMN and methylviologen and the stimulation was almost completely abolished by carbon monoxide as observed in the

Table 1. Coenzyme requirement for benzo(a)pyrene 4,5-oxide reduction by liver microsomes

Coenzyme	Activity (pmoles/mg/min)	Percent
NADPH generating system	21.3	100
NADH (2 mM)	3.5	16
NADPH generating system + NADH	34.0	160
No coenzyme	1.7	8
Boiled microsomes	0.3	1

Standard incubation mixtures (2.5 ml) contained microsomal fraction (12.5 mg protein), $MgCl_2$ (25 μmole), NADP (5 μmole), glucose 6-phosphate (25 μmole) and glucose 6-phosphate dehydrogenase (7.5 I.U.) and pH 7.4 phosphate buffer (150 μmole). Benzo(a)pyrene 4,5-oxide (0.25 μmole) was added after dissolving in 0.1 ml acetone. Incubation was for 10 min at 37° under a nitrogen atomosphere. The activity was expressed as pmoles of benzo(a)pyrene formed per min per mg microsomal protein. The results were averages of two experiments.

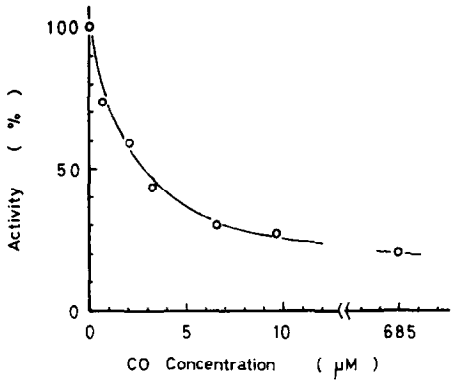

Fig. 1 The inhibitory effect of carbon monoxide on the reduction of benzo(a)pyrene 4,5-oxide by rat liver microsomes. The incubations were carried out in an atomosphere of various concentrations of CO as described in Table 1.

previous experiment with tertiary amine N-oxides (Table 2). The mechanism of stimulation by FMN or methylviologen is likely due to a reduction of FMN or methylviologen by a microsomal flavoprotein.

Subsequently the reduced form of FMN or methylviologen could addi-

Table 2. Characteristics of benzo(a)pyrene 4,5-oxide reduction by liver microsomes

	Addition*)	Gas phase	Activity (pmoles/mg/min)	Percent
Exp. I	No	N_2	11.9	100
		CO	2.5	21
		Air	4.4	37
	+ FMN	N_2	33.0	277
		CO	2.7	23
	+ Methylviologen	N_2	43.4	365
		CO	3.6	30
Exp. II	No	N_2	14.3	100
	+ SKF 525-A	N_2	22.9	157
	+ DMANO	N_2	2.3	16
	+ TRNO	N_2	5.6	36
	+ Cumene hydroperoxide	N_2	0.7	4

*) Concentrations of various added compounds were as follows : FMN 50 μM, methylviologen 100 μM, SKF 525-A 100 μM, dimethylaniline N-oxide (DMANO) 1 mM, tiaramide N-oxide (TRNO) 1 mM and cumene hydroperoxide 1 mM.

tionally reduce cytochrome P-450 leading to an increased rate of reduction of tertiary amine N-oxides or the benzo(a)pyrene 4,5-oxide.

SKF 525-A is known as an inhibitor of cytochrome P-450 dependent monooxygenases interacting with oxidized cytochrome P-450. However, addition of SKF 525-A did not inhibit but rather stimulated the reduction rate of benzo(a)pyrene 4,5-oxide, probably through an acceleration of NADPH dependent cytochrome P-450 reduction (2,8).

Dimethylaniline N-oxide, tiaramide N-oxide and cumene hydroperoxide are assumed to interact with the reduced form of cytochrome P-450 at the heme region (2,9,10). The additions of both N-oxides or cumene hydroperoxide produced marked inhibitions of benzo(a)pyrene 4,5-oxide reduction. On the other hand, the additions of benzo(a)pyrene 4,5-oxide or cumene hydroperoxide markedly reduced the reduction of the tertiary amine N-oxide (2,7).

Table 3. Reduction of benzo(a)pyrene 4,5-oxide by liver microsomes from different aged rats

Age (days)	Microsomal cytochrome (nmoles/mg protein) Cyt. b_5	Cyt. P-450	Activity (pmoles/mg/min)
1	0.10	0.11	n.d.
28 (Male)	0.41 ± 0.01	0.70 ± 0.03*	12.4 ± 1.0*
49 (Female)	0.41 ± 0.01	0.80 ± 0.03*	20.3 ± 3.6
49 (Male)	0.43 ± 0.02	1.09 ± 0.05	26.1 ± 2.3

The results were given as averages ± S.E. from 4-7 rats. Pooled livers from 4-5 new-born rats were used for one determination.
*: $p < 0.05$ compared to 49 days male rats.

It is known that livers of new-born rats have low contents of cytochrome P-450 as well as no or very low activity in drug oxidations (11, 12). The reduction of benzo(a)pyrene 4,5-oxide in livers from one day old rats was not detected and in 28 days old rats the reduction rate was about 50 % of that in adult rats (Table 3). A slight sex difference was noted between male and female rats.

The administration of phenobarbital or 3-methylcholanthrene increased the cytochrome P-450 content as well as the reduction of benzo(a)pyrene 4,5-oxide. The reduction activity per content of cytochrome P-450 in microsomes from 3-methylcholanthrene treated rats was clearly higher than that from controls (Table 4). This result therefore suggests that benzo(a)pyrene 4,5-oxide may be reduced preferentially by cytochrome P-448 (13). As shown in Table 5, benzo(a)pyrene 4,5-oxide reductase activity is very low or apparently lacking in brain, lung, kidney and muscle. After administration of 3-methylcholanthrene a measurable activity was found in kidney and muscle.

All results reported in the present investigation were similar to those reported in previous papers on the reduction of tertiary amine N-oxides by rat liver microsomes (2) and suggest that the reduction of benzo(a)-pyrene 4,5-oxide is catalyzed through reduced form of cytochrome P-450. Since liver microsomes generally contain high activity of epoxide hydrase

Table 4. Effect of phenobarbital and 3-methylcholanthrene treatment on benzo(a)pyrene 4,5-oxide reduction

Treatment	Microsomal cytochromes (nmoles/mg protein)		Activity*) (pmoles/mg/min)
	Cyt. b_5	Cyt. P-450	
Saline	0.37 ± 0.01	0.64 ± 0.02	14.3 ± 2.6
Phenobarbital	0.45 ± 0.00	1.75 ± 0.11	28.7 ± 3.3
Olive oil	0.35 ± 0.01	0.67 ± 0.06	12.3 ± 3.5
3-Mc	0.45 ± 0.02	1.32 ± 0.03	69.7 ± 4.7

Female rats (4 weeks old) were received intraperitoneally, phenobarbital (80 mg/kg) 72, 48 and 24 hours and 3-methylcholanthrene (3-Mc) (50 mg/kg) 72 hours prior to sacrifice, respectively. The results were given as averages ± S.E. from 4 rats.

*) The activity was expressed as pmoles of benzo(a)pyrene formed per mg microsomal protein per min.

Table 5. Effect of 3-methylcholanthrene treatment on benzo(a)-pyrene 4,5-oxide reduction in various tissues

Treatment		Tissue					
		Brain	Lung	Liver	Kidney	Muscle	Intestinal Mucosa
-	-	n.d.	n.d.	301	n.d.	n.d.	46
3-Mc	-	n.d.	n.d.	969	69	34	151
3-Mc	FMN 50 μM	n.d.	3	1560	99	46	151

Male rats (4 weeks old) were received 3-methylcholanthrene 50 mg/kg intraperitoneally 72 hours prior to sacrifice. All values were expressed as pmoles of benzo(a)pyrene formed per g tissue per min. The results were given as means of two determinations from 5 rats.

(14), further studies will be required to establish the functional role of reduced cytochrome P-450 in the in vivo reduction of benzo(a)pyrene 4,5-oxide or related epoxides under various conditions.

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