ARACHIDONIC ACID-DEPENDENT METABOLISM OF (±) *TRANS*-7,8-DIHYDROXY-7,8-DIHYDRO-BENZO[a]PYRENE (BP-7,8 DIOL) TO 7,10/8,9 TETROLS

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1. Introduction

The environmental pollutant benzo[a]pyrene (BP) is not carcinogenic per se but requires metabolic activation by the cytochrome *P*-450-dependent monoxygenase system for the manifestation of carcinogenic activity [1,2]. The activation of BP results in the generation of reactive metabolites which can covalently bind to DNA. The covalent binding of some reactive BP intermediates to DNA is implicated with carcinogenesis [3,4]. Recent evidence suggests that the diol epoxides are the ultimate carcinogenic form of BP [4,5]. The pathway of diol epoxide formation involves epoxidation and hydration of BP catalyzed by the cytochrome *P*-450 monooxygenase system-dependent and epoxide hydrase.

We have reported that BP, BP-7,8-diol and 7,12-dimethyl benzanthracene are oxidized by guinea pig lung microsomes during prostaglandin (PG) biosynthesis to reactive metabolites which can covalently bind to tissue protein and nucleic acids [6]. The nature of the metabolites of BP-7,8-diol formed during PG biosynthesis is not known. The metabolites of NP-7,8-diol but not BP formed during PG biosynthesis by ram seminal vesicle microsomes are highly mutagenic to *Salmonella typhimurium* [7]. Therefore, we have investigated the nature of the metabolites generated from BP-7,8-diol during PG biosynthesis in guinea pig lung (GPL) and ram seminal vesicle (RSV) microsomes. Our studies indicate 7,10/8,9-BP tetrol as the major metabolite formed by both tissues.

2. Materials and methods

2.1. *Materials*

Arachidonic acid (AA) was purchased from Nuchek Prep., Elysian, MN. Indomethacin was obtained from Sigma Chem. Co., St Louis, MO. 7,8-[^{3}H]BP diol (298 μCi/μmol), [^{14}C]BP-7,8-diol, 9,10-oxide (*syn* isomer) (29.7 mCi/mmol) and [^{14}C]BP-7,8-diol, 9,10 oxide (*anti*-isomer) (29.8 mCi/mmol) were prepared at Midwest Res. Inst. (Kansas City, MO) under NCI contract no. 1-CP-33387.

2.2. *Preparation of enzyme fraction*

All the procedures were carried out at 4°C. Lungs from adult male guinea pigs (350—400 g body wt, obtained from Carworth Breeders (Portage, MI), were freed of extraneous tissue and the microsomes were prepared as in [6]. The microsomal protein was measured by the method in [8].

2.3. *Incubation procedures and assay for metabolism*

Incubation mixtures consisted of the following: microsomal protein (2 mg); 0.1 M phosphate buffer (pH 7.8); AA (100 μM); [^{3}H]BP-7,8-diol (20 μM) and water to 2 ml. Some incubations contained heat-denatured microsomes to correct for non-enzymatic BP oxidation. Microsomal mixtures were pre-incubated in the presence or absence of indomethacin (100 μM) for 3 min at 37°C before the addition of [^{3}H]BP-7,8-diol and AA. The reactions were initiated by the addition of [^{3}H]BP-7,8-diol (20 μM) and carried out for 5 min at 37°C. The reactions were terminated by

the addition of 2 ml ethyl acetate/acetone (2:1, v/v) and extracted with the same solvent system (3 × 2 vol.). The organic layers were pooled, dried over anhydrous Na_2SO_4, evaporated and resuspended in methanol (100 μl). For the measurement of BP-7,8-diol and its metabolites, an aliquot was applied to silica gel G plates and the plates were developed in chloroform/ethanol (19:1, v/v). Plates were scraped in 4 mm sections and the radioactivity measured by liquid scintillation techniques. PG metabolism was measured using [^{14}C]AA as in [6].

The protein in the aqueous layer was precipitated with 10% trichloroacetic acid (TCA) and washed with TCA. The protein was then exhaustively extracted with chloroform/methanol (2:1, v/v) and 80% methanol/water. The washed protein was solubilized in NaOH, and the radioactivity determined as above.

2.4. *Higher performance liquid chromatography (HPLC)*

Samples were eluted isocratically with 60% methanol for 50 min and 100% methanol for 20 min at room temperature on a Spectra-physics model 3500 high-pressure liquid chromatograph fitted with a Whatman ODS-2 column (25 cm × 4.6 mm). Eluant fractions were collected at 30 s intervals at 0.8 ml/min column flowrate. The absorption of the eluant was monitored at 254 nm while the radioactivity in the fractions was measured by liquid scintillation techniques.

2.5. *Hydrolysis of diol-epoxides*

Syn and *anti* isomers of [^{14}C]BP-7,8-diol-9,10-oxide were incubated separately at 37°C for 6 h in water (pH 7.0). The products were extracted with ethyl acetate, evaporated with N_2 and dissolved in methanol. An aliquot of the hydrolyzed diol-epoxide was used for analysis by HPLC.

3. Results

BP-7,8-diol has been shown to be metabolized by the rat liver cytochrome *P*-450 monooxygenase system to 4 tetrols [9]. Here, GPL and RSV microsomal fractions converted BP-7,8-diol in the presence of AA to polar metabolites which migrated as an apparent single peak in thin-layer chromatograms. This peak corresponded to authentic samples of BP tetrols. (Individual tetrols were not separated by this method.) As shown in table 1, 1.5% of BP-7,8-diol was metabolized by GPL microsomes. The addition of indomethacin, an inhibitor of PG synthetase, reduced the metabolism > 50%. The binding of the radioactive materials to protein was also reduced. The amount of metabolites formed by the heat-denatured tissue or in the absence of AA ranged from 3.5–5% of the metabolites formed enzymatically. Although the PG synthetase activity of ram seminal vesicle is 200-times higher than the lung, the BP-7,8-diol metabolized by this tissue is only 2.5-fold higher

Table 1
Arachidonate-dependent BP-7,8-diol oxidation by guinea pig lung and ram seminal vesicle microsomal fractions

	Relative[a] PG synthetase	Metabolism (pmol .ml^{-1} .min^{-1})	Protein bound (pmol/mg protein)	% Metabolized
AA + GPLM + [^{3}H]BP-7,8-diol	1.0	175.0 ± 20.0	11.5 ± 1.2	1.45
AA + GPLM + [^{3}H]BP-7,8-diol + IM		70.0 ± 9.0	5.3 ± 0.5	0.59
AA + GPLM (boiled) + [^{3}H]BP-7,8-diol		6.7 ± 2.5	0.1 ± 0.1	0.07
AA + RSVM + [^{3}H]BP-7,8-diol	200	411.0 ± 40.5	26.5 ± 2.4	3.4
AA + RSVM + [^{3}H]BP-7,8-diol + IM		175.5 ± 14.5	11.2 ± 1.6	1.4
AA + RSVM (boiled) + [^{3}H]BP-7,8-diol		24.6 ± 4.5	1.6 ± 0.4	0.2

[a] Total PG-like materials formed during 8 min incubation (guinea pig lung microsomes = 1.0) see [15]

AA, arachidonic acid (100 μM); BP-7,8-diol (200 μM); GPLM, lung microsomes (2 mg); RSVM, ram seminal vesicle microsomes (2 mg); IM, indomethacin (100 μM)

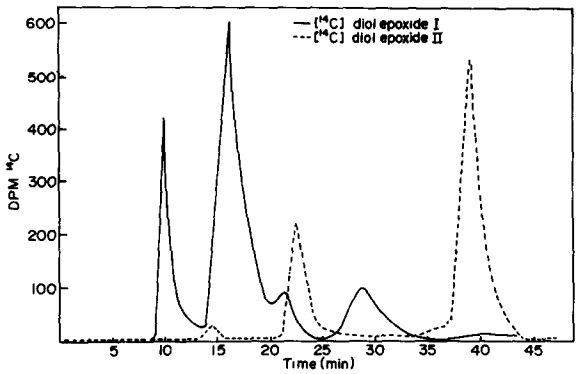

Fig.1. High pressure liquid chromatographic profile of hydrolysis products of diol-epoxide I (*anti*-isomer) (——) and diol-epoxide II (*syn*-isomer) (- - -).

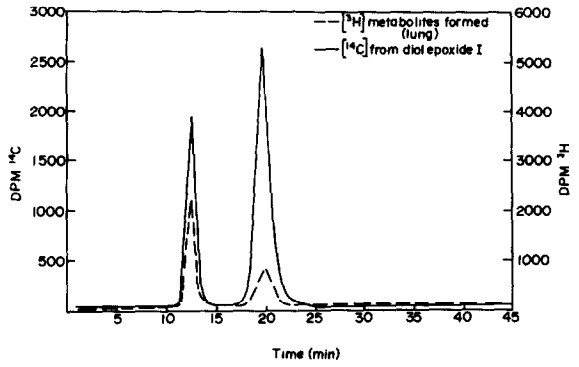

Fig.2. Metabolites of [^{3}H]BP-7,8-diol formed by incubation of guinea pig lung microsomes (- -) and the hydrolysis products of [^{14}C]diol-epoxide I.

than that metabolized by the guinea pig lung.

If the metabolites of BP-7,8-diol formed by the AA system are tetrols derived from the 2 diol-epoxides, then the formation of 4 different tetrols should be possible [9]. We prepared these tetrols by the hydrolysis of [^{14}C]diol-epoxide I and I. An HPLC method was then developed for the separation of the two major hydrolysis products (tetrols) derived from each [^{14}C]diol-epoxides (fig.1). The metabolic products of [^{3}H]BP-7,8-diol formed during incubation with AA appeared as 2 peaks on analysis by HPLC. On the basis of retention time, the peaks appeared to correspond to the tetrols derived from diol-epoxide I. The [^{14}C]tetrols, produced by hydrolysis of [^{14}C]diol-epoxide I or II, were mixed with samples obtained from incubation of [^{3}H]BP-7,8-diol with AA and lung and RSV microsomes and analyzed by HPLC (fig.2). The major tetrol produced from BP-7,8-diol by both GPL and RSV eluted with the *trans* I or 7,10/8,9-tetrol. Small amounts of 7/8,9,10 were also detected. No other major peaks apart from the unmetabolized BP-7,8-diol were observed.

4. Discussion

In this study, we have identified BP-7,10/8,9 tetrol as the major metabolite formed from BP-7,8-diol by the AA-dependent system in GPL and RSV microsomes. Other investigators have demonstrated that this tetrol is a hydrolysis product of diol-epoxide I, a highly reactive BP intermediate which can covalently bind to DNA and initiate tumors [10–12]. A recent report has shown that BP-7,8-diol is highly mutagenic to *Salmonella typhimurium* when incubated in the presence of AA and RSV microsomes; this dihydrodiol is non-mutagenic if AA is omitted [7]. Therefore, BP-7,8-diol may be metabolized to diol-epoxide I during PG formation from AA.

It is possible, however, that reactive BP intermediates other than diol-epoxides may be formed by the AA-dependent system and could result in covalent binding to DNA and mutagenicity. Evidence exists for the concurrent oxidation of various xenobiotics (oxyphenbutazone, dibenzoisofuran) during PG biosynthesis [13]. This cooxidation involves the formation of PGG_2 from AA, the conversion of PGG_2 to PGH_2, and subsequent free radical generation. Free radicals formed during PG biosynthesis may initiate the oxidation of BP-7,8-diol, resulting in the formation of a free radical BP intermediate(s) which could either covalently bind to DNA or spontaneously rearrange to form tetrols. Experiments to identify these types of reactive products formed from BP-7,8-diol by the AA-dependent system are now in progress.

We have reason to believe that AA-dependent system is integrally involved in the secondary oxidation pathways of BP metabolism.

1. We were unable to detect spectrally any cytochrome *P*-450 in ram seminal vesical microsomes which metabolized BP-7,8-diol in the presence of AA.
2. The metabolic profiles of BP obtained with the

AA-dependent system and cytochrome *P*-450-dependent monoxygenase system were completely different [6].

3. Addition of indomethacin did not alter the rate of BP metabolism by the monooxygenase system but decreased the rate in the AA- dependent system.
4. The addition of anti-oxidants (BHA) inhibited the rate of BP metabolism by the AA-dependent system but had no effect on the monooxygenase system [14].

From all the available evidence, it appears that the AA-dependent system of BP-7,8-diol metabolism is different from the monooxygenase system. However, further evidence is required to determine the nature of the oxygenase which metabolizes BP-7,8-diol. Tissues such as GPL can both synthesize PG from AA and metabolize drugs and other foreign compounds via the cytochrome *P*-450 monooxygenase system; both enzyme systems may serve as the BP-7,8-diol oxygenase. Further studies are therefore needed to determine the relative role of the AA-dependent system in the deposition of xenobiotics and in the initiation of chemical carcinogenesis.

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