ARACHIDONIC ACID-DEPENDENT METABOLISM OF 7,8-DIHYDROXY-7,8-DIHYDRO-BENZO[a]PYRENE BY RAM SEMINAL VESICLES

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Received 25 June 1979

1. Introduction

Certain polycyclic aromatic hydrocarbons can be converted to carcinogenic derivatives during metabolism in animal tissue [1,2]. Identification of the carcinogenic derivatives and elucidation of the enzymatic pathways of their formation have been the focus of intensive study for a number of years [3–5]. A substantial body of recent evidence implicates bay region diolepoxides as likely candidates for the ultimate carcinogenic forms of a number of polycyclic aromatic hydrocarbons [6]. In the case of BP, 7,8-diol-9,10-epoxides have been found to be more mutagenic and carcinogenic than any of the other reported or suspected metabolites of the parent hydrocarbon [7–9]. In vitro experiments have demonstrated that NADPH-dependent mixed-function oxidases possessing cytochrome *P*-450 as the terminal oxidase will catalyze the oxidation of BP-7,8-diol to BPDE [10–12].

We have reported that the addition of 20:4 to PES preparations from ram seminal vesicles causes the cooxidation of BP-7,8-diol to derivatives which are strongly mutagenic to *Salmonella typhimurium* strain TA 98 [13]. It has also been reported that PES preparations from guinea pig lung cooxidize BP-7,8-diol to derivatives which covalently bind to protein and nucleic acid [14]. In neither study were the actual products of BP-7,8-diol metabolism identified. We report here the results of our study of the arachidonic acid-dependent metabolism of [^{14}C]BP-7,8-diol which indicate that a diolepoxide is produced as an intermediate. We also present evidence that cytochrome *P*-450 plays no role in this cooxidation, which implies that enzyme systems besides the mixed-function oxidases can catalyze the terminal activation step in BP carcinogenesis.

Abbreviations: BP, benzo[a]pyrene; BPDE, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene; 20:4, 5,8,11,14-eicosatetraenoic acid; PES, prostaglandin endoperoxide synthetase; BP-7,8-diol, 7,8-dihydroxy-7,8-dihydro-benzo[a]-pyrene; [^{14}C]BP-7,8-diol, [7,10-^{14}C]BP-7,8-diol; BP-tetraol, 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydro-benzo[a]pyrene; HPLC, high performance liquid chromatography; 15-HPETE, 15-hydroxyperoxy-5,8,11,13-eicosatetraenoic acid; BHA, butylated hydroxyanisole; *anti-trans*-BP-tetraol, 7β, 8α, 9α, 10β-tetrahydroxy-7,8,9,10-tetrahydro-benzo[a]pyrene; PGG_2, 15α-hydroxy-9α-11α-peroxido-5-*cis*-13-*trans*-prostadienoic acid; indo, indomethacin

2. Materials and methods

20:4 was generously provided by Dr John Paulsrud, Hoffman-LaRoche and was purified by open column chromatography on silicic acid. Unlabeled BP-7,8-diol, the *syn* and *anti* isomers of the diol-epoxides, and [^{14}C]BP-7,8-diol were obtained from the National Cancer Institute Carcinogenesis Research Program. The [^{14}C]BP-7,8-diol was purified by reverse-phase HPLC immediately prior to use. Authentic standards of the BP-tetraols were prepared by hydrolysis of the *syn* and *anti* diolepoxides in 100 mM sodium phosphate (pH 7.8) followed by extraction and purification by HPLC. The structures were assigned by the correspondence of relative yield and HPLC mobility to [10,11]. 15-HPETE was prepared according to [15]. Ram seminal vesicle microsomes and the Tween-20 solubilized enzyme were prepared as in [16].

Preparative scale metabolism of [^{14}C]BP-7,8-diol was carried out at 37°C in 200 ml of 100 mM sodium phosphate (pH 7.8) containing solubilized enzyme (110 mg protein) and 1 mg [^{14}C]BP-7,8-diol (8 μCi/μmol). After a 3 min preincubation, 9 mg 20:4 was added. The reaction was terminated after 15 min by the addition of 180 ml acetone and the products were extracted with 360 ml ethyl acetate. Following drying ($MgSO_4$), the solvent was evaporated and the residue was taken up in 500 μl ethyl acetate for HPLC analysis.

The incubations described in table 1 were performed at 37°C in 1.0 ml 100 mM Tris·HCl (pH 7.4) containing microsomes (1 mg protein), 15 μmol $MgCl_2$, and 20 nmol [^{14}C]BP-7,8-diol (4.5 μCi/μmol). After a 3 min preincubation, reaction was initiated by the addition of the appropriate substrate and allowed to proceed for 3 min. One series of experiments were performed with NADPH for 15 min. BHA (200 nmol) was added to terminate cooxidation and the incubation mixtures were allowed to stand at 0°C for 30 min to complete solvolysis of BPDE. After ethyl acetate extraction, the samples were chromatographed on 20 cm silica gel 60 plates (EM labs) with chloroform/ethanol (70/30, v/v). Radioactive zones were detected using a Berthold LB-2760 radiochromatogram scanner and the percentage of radioactivity cochromatographing with authentic BP-tetraols determined by liquid scintillation counting.

Optical difference spectra of the solubilized enzyme preparation were performed on a Cary 118. Solubilized enzyme (6.0 ml, 800 μg protein/ml) was bubbled with CO for 2 min, divided between 2 cuvettes, and a baseline spectrum was recorded. Solid $Na_2S_2O_4$ was added to the sample cuvette, the contents were mixed, and the difference spectrum recorded.

3. Results and discussion

Figure 1b is the HPLC profile of the radioactive products obtained when [^{14}C]BP-7,8-diol is incubated with a solubilized PES preparation and 20:4. The major radioactive product (~70% of the total metabolism) cochromatographs with an authentic standard of the *anti-trans*-BP-tetraol prepared by hydrolysis of *anti*-BPDE. The ultraviolet spectrum of

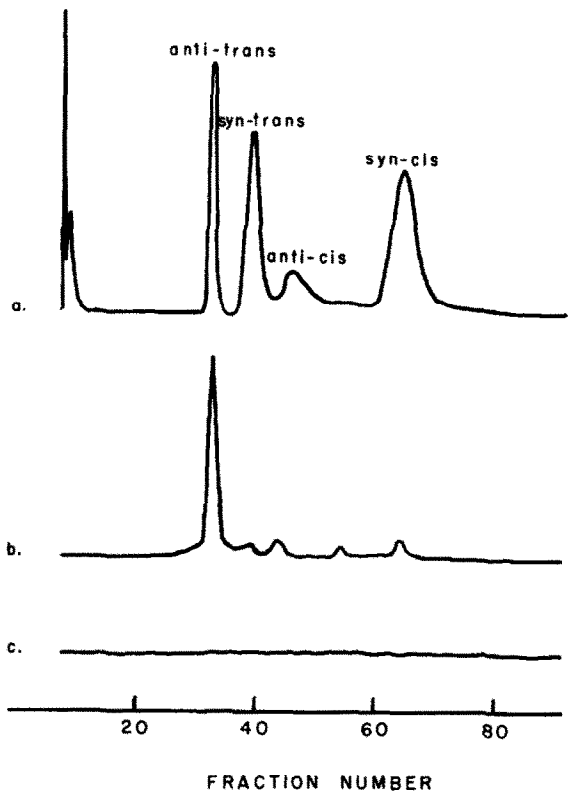
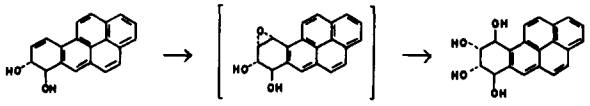


Fig.1. HPLC analysis of the products of BP-7,8-diol oxidation by ram seminal vesicles. Chromatography was performed on a column of Lichrosorb RP-18 (4 × 250 mm) using 45% methanol in water at 1.5 ml/min. (a) Ultraviolet profile of a mixture of authentic BP-tetraol standards; (b) radioactivity profile of the products of BP-7,8-diol oxidation by PES and 20:4; (c) same as (b) except a boiled enzyme preparation was used for the incubation.

the major metabolite is identical to the ultraviolet spectrum of the *anti-trans*-BP-tetraol and is typical of a BP derivative with a fully saturated benzo ring. This indicates that metabolism of BP-7,8-diol has occurred at the 9,10-double bond. The high resolution mass spectrum of the major metabolite exhibits a molecular ion which is the base peak at *m/e* 320.1046 (calculated for $C_{20}H_{16}O_4$, 320.1044). The remaining radioactive peaks in fig.1b cochromatograph with the other known tetraol metabolites of BP, but we have been unable to obtain sufficient quantities of these compounds for complete spectral analysis.

The chromatographic characteristics, ultraviolet spectrum, and mass spectrum of the major metabolite

Scheme 1



of BP-7,8-diol indicate that it is the *anti-trans*-BP-tetraol. This compound is not mutagenic which suggests that the mutagen produced during the PES-dependent metabolism of BP-7,8-diol is an intermediate which undergoes non-enzymatic conversion to the observed tetraol. The most likely possibility for the intermediate is the *anti*-BPDE which is highly mutagenic and hydrolyzes spontaneously to a mixture of the *trans* and *cis* isomers of the tetraols (predominantly *trans*) [10,11,17,18] (scheme 1). We cannot completely rule out the possibility that a highly mutagenic intermediate, distinct from BPDE, is produced which undergoes conversion to the same tetraol. However, we have no evidence for the formation of any intermediate besides BPDE.

Previous studies have shown that PES-dependent cooxidations are peroxidatic and utilize the hydroperoxy endoperoxide, PGG_2, as the hydroperoxide substrate [19]. Incubation of [^{14}C]BP-7,8-diol with PES and 15-HPETE, a model for PGG_2, followed by HPLC analysis generates a chromatographic profile identical to fig.1b (data not shown). Equal amounts of 20:4 and 15-HPETE trigger equal amounts of BP-7,8-diol oxidation when incubated with intact microsomes (table 1). In contrast, 15-HPETE causes a substantially higher level of diol oxidation than 20:4 when added to boiled microsomes. This indicates that a significant non-enzymatic hydroperoxide-dependent oxidation occurs. This non-enzymatic component is also observed with BP as substrate and suggests that a major role of PES in cooxidation is as a generator of lipid hydroperoxides [20].

The fact that the cooxidation is peroxidatic and partially enzymatic raises questions about the nature of the peroxidase. PES, purified to homogeneity, contains a reconstitutable peroxidase activity [21]. However, this may not be the sole peroxidase operative in crude enzyme preparations. In particular, one must consider the possible involvement of cytochrome *P*-450. Figure 2 is the dithionite reduced–

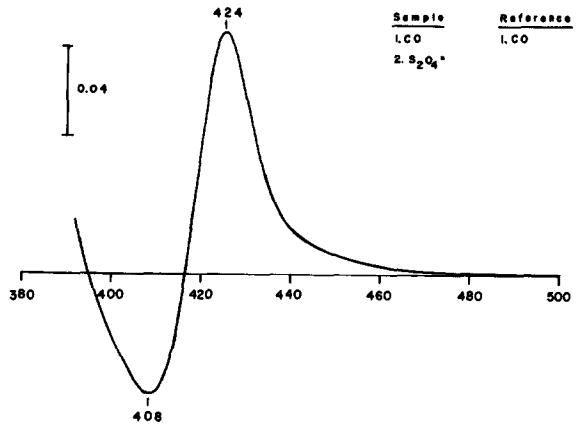


Fig.2. Dithionite-reduced–carbon monoxide difference spectrum of the Tween-20 solubilized preparation from ram seminal vesicles. Procedure described in section 2. Protein was 800 μg/ml.

Table 1
Metabolism of BP-7,8-diol by Ram seminal vesicle microsomes

Additive	Tetraols[a] (pmol)		
	Intact enzyme	Boiled enzyme	Net metabolism[b]
None – Control[c]	393 ± 20	178 ± 20	215
1 mM NADPH	295 ± 7	156 ± 40	139
1 mM NADPH (15 min)	348 ± 80	137 ± 16	211
0.1 mM 20 : 4	551 ± 25	233 ± 16	318
0.1 mM 20 : 4 + 0.1 mM indo	251 ± 24	240 ± 20	11
0.1 mM 15-HPETE	554 ± 110	412 ± 3	142

[a] Unless indicated, all incubations were performed for 3 min. The results are expressed as total metabolism during the incubation period
[b] Difference between intact enzyme and boiled enzyme columns
[c] Incubation conditions are described in section 2

carbon monoxide difference spectrum of the Tween-20 solubilized PES from ram seminal vesicles. A peak at 424 nm and a trough at 408 nm are evident. These spectral features may be related to the reduced and oxidized forms of PES reported [22]. Figure 2 contains no A_{450} band indicating that the cytochrome *P*-450 content of this preparation is below the limit of spectral detection.

Since cytochrome *P*-450 is spectrally undetectable in the solubilized preparation, we have attempted to detect it using the NADPH-dependent oxidation of BP-7,8-diol as an enzymatic assay. For these experiments, we used a microsomal enzyme in order to avoid possible complications introduced by the solubilization procedure. The addition of 1 mM NADPH to ram seminal vesicle microsomes gives rise to no BP-7,8-diol oxidation above controls – in fact, there is slight inhibition (table 1). The addition of the mixed-function oxidase inhibitors, carbon monoxide (3 min bubbling) or metyrapone (1 mM) has no inhibitory effect on any of the incubations listed in table 1. In contrast, the PES inhibitor, indomethacin, completely inhibits BP-7,8-diol oxidation in the presence of 20:4 or in its absence. The latter observation suggests that the oxidation of BP-7,8-diol observed in the control incubations, performed in the absence of exogenous 20:4, is due to cooxidation triggered by endogenous 20:3 or 20:4 released from membrane lipids during the course of the incubations.

We have been unable to detect any NADPH-dependent activation of BP-7,8-diol to mutagenic derivatives in the presence of solubilized ram seminal vesicle enzyme preparations (Reed, G. A., unpublished observations). Therefore, spectral, metabolic, and mutagenicity experiments strongly suggest that cytochrome *P*-450 is not the peroxidase involved in the PES-dependent oxidation of BP-7,8-diol in the ram seminal vesicle. Work is in progress to determine if the peroxidase operative in crude enzyme preparations is the hydroperoxidase component of PES. Regardless of the outcome of these experiments, the present work indicates that, in vitro, enzyme systems besides the mixed-function oxidases can catalyze the terminal activation step in benzo[a]pyrene carcinogenesis.

Acknowledgements

This work was supported by the American Cancer Society (BC-244a). We are grateful to Mr John DeFever for mass spectral analysis.

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