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HYDROPEROXIDE-DEPENDENT EPOXIDATION OF 3,4-DIHYDROXY-3,4-DIHYDROBENZO[a]ANTHRACENE BY RAM SEMINAL VESICLE MICROSOMES AND BY HEMATIN

Thomas A. Dix, Joseph R. Buck, and Lawrence J. Marnett

Department of Chemistry, Wayne State University
Detroit, MI 48202

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SUMMARY Addition of arachidonic acid to ram seminal vesicle microsomes oxidizes 3,4-dihydroxy-3,4-dihydrobenzo[a]anthracene (BA-3,4-diol) to five more polar products. Four of the products are identified by chromatographic and spectroscopic analysis as tetrahydrotetraols, which are solvolysis products of dihydrodiolepoxides. The fifth product is a 10-methyl ether formed by methanolysis of the *anti*-diolepoxide. Quantitation of the individual products indicates that *anti*-diolepoxides predominate over *syn*-diolepoxides by approximately 2:1. Identical product profiles are detected from the reaction of BA-3,4-diol with hematin and 13-hydroperoxy-octadecadienoic acid in the presence of Tween 20. No other products are detected in either system, which indicates that peroxyl radicals oxidize BA-3,4-diol exclusively by epoxidation of the 1,2-double bond. The stereochemical and regiochemical differences between oxidation of BA-3,4-diol by peroxyl radicals and cytochrome P-450 are dramatic and suggest that BA-3,4-diol is uniquely suited as a probe to quantitate peroxyl radical-dependent epoxidation *in vitro* and *in vivo*.

Oxygenation of unsaturated fatty acids by prostaglandin H (PGH) synthase triggers the generation of potent oxidizing agents capable of reacting with many structurally diverse organic molecules (1-3). A class of important molecules oxidized is polycyclic aromatic hydrocarbons (4-7), which undergo transformations leading in certain cases to the formation of metabolites possessing mutagenic and carcinogenic potencies orders of magnitude greater than the parent molecules. Benzo[a]pyrene is cooxidized by PGH synthase to quinones (4), whereas 7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene is cooxidized to dihydrodiolepoxides (6,7). The latter reaction represents the terminal activation step of BP carcinogenesis and is catalyzed by cytochrome P-450 as well as PGH synthase (8,9). Stereochemical differences in epoxidation by the two enzymes provide useful diagnostic probes for the involvement of one or the other enzyme in cellular or subcellular oxidation of BP-7,8-diol (9,10). By exploiting these probes, we and others

Abbreviations: PGH, prostaglandin H; BP-7,8-diol, *trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene; BA-3,4-diol, *trans*-3,4-dihydroxy-3,4-dihydrobenzo[a]anthracene; RSVM, ram seminal vesicle microsomes; 13-OOH-18:2, 13-hydroperoxy-9-*cis*, 11-*trans*-octadecadienoic acid; *anti*-BA-diolepoxide, 3β,4α-dihydroxy-1β,2β-epoxy-1,2,3,4-tetrahydrobenzo[a]anthracene; syn-BA-diolepoxide, 3β,4α-dihydroxy-1α,2α-epoxy-1,2,3,4-tetrahydrobenzo[a]anthracene; HPLC, high pressure liquid chromatography; BHA, 2(3)-t-butylhydroxyanisole; MDA malondialdehyde.

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have been able to demonstrate non-cytochrome P-450-dependent epoxidation of BP-7,8-diol by a number of hydroperoxide and peroxyl radical generating systems.[1] In fact, it is possible to detect peroxyl radical-dependent epoxidation in systems containing an active cytochrome P-450 (11).

The extent to which PGH synthase-dependent cooxidation contributes to metabolic activation of drugs and carcinogens *in vivo* is of considerable interest. Stereochemical differences such as those described above, or unique DNA adducts are extremely useful in such studies, but the biochemical characteristics of each cooxidation must be established prior to the initiation of *in vivo* studies. A molecule with significant potential as *in vivo* probe of cooxidation is 3,4-dihydroxy-3,4-dihydrobenzo[a]anthracene. BA-3,4-diol and its diolepoxide derivatives are weak tumor initiators on mouse skin (12,13). Guthrie *et al* have shown that BA-3,4-diol is activated to a mutagen by PGH synthase but no products were identified (14). BA-3,4-diol is unique among dihydrodiols of polycyclic hydrocarbons in its oxidation by cytochrome P-450 (15). Despite extensive cytochrome P-450-dependent metabolism dihydrodiolepoxides only constitute 15% of the total oxygenated products. The major products are bis-dihydrodiols that result from epoxidation in different regions of the molecule (15). Peroxyl radicals epoxidize isolated double bonds much more efficiently than aromatic double bonds so one anticipates that 3,4-diol-1,2-epoxides will represent major products of BA-3,4-diol cooxidation by PGH synthase and other peroxyl radical generating systems. If so, stereochemical and regiochemical differences in metabolism would make BA-3,4-diol an exquisitely sensitive discriminator of oxidizing agents generated *in vitro* and *in vivo*. For this reason, we have undertaken a detailed investigation of BA-3,4-diol oxidation by PGH synthase and a peroxyl radical-generating system consisting of hematin and 13-hydroperoxy-octadecadienoic acid (13-OOH-18:2). The results are described in this communication.

MATERIALS AND METHODS

RSVM were prepared as previously described (16) from seminal vesicles obtained at a local slaughterhouse. BA-3,4-diol and BA-diolepoxides were from the National Cancer Institute Chemical Carcinogen Repository. Standards of BA-tetraols were prepared by hydrolysis of BA-diolepoxides (17) and purified by HPLC before use. 13-OOH 18:2 was prepared and purified by the method of Funk *et al* (18). All biochemicals were obtained from Sigma at the highest available purity with the exception of the fatty acids (Nu Chek Prep) and hematin (Calbiochem). Distilled, deionized water was used in the preparation of all buffers.

Hematin/13-OOH-18:2-dependent epoxidation of BA-3,4-diol was performed under conditions identical to those previously utilized for BP-7,8-diol epoxidations (19). Incubations contained in 5.0 ml total volume, 0.1 M phosphate, pH 7.8, 200 μM Tween 20, 0.5 μM hematin, 18 μM BA-3,4-diol and were initiated by the addition of 50 μM 13-OOH-18:2. After a 10 min stirred incubation at 25°C, products were extracted into 4 vol ethyl acetate, solvent removed *in vacuo*, and residue dissolved in 50 μl methanol in preparation for HPLC analysis. Metabolism of BA-3,4-diol by RSVM was performed in total volumes of 5.0 and 50 ml for HPLC and product identification experiments, respectively. Incubations contained 0.1 M phosphate, pH 7.8, 1.0

1. PGH synthase biosynthesizes fatty acid hydroperoxides, which partially decompose to peroxyl radicals. The latter are responsible for PGH synthase - dependent epoxidation. Theoretically, any biochemical system capable of generating peroxyl radicals should be capable of epoxidizing BP-7,8-diol. This has been verified experimentally (summarized in (3)).

mg/ml microsomal protein, 20 μM BA-3,4-diol, and were initiated by the addition of a given amount of arachidonic acid. After a 10 min stirred incubation at 25°C, products were extracted and worked up as described above. PGH synthase activity in RSVM was assayed by monitoring $\Delta[O_2]$ with a Clark oxygen electrode. Microsomal lipid peroxidation was determined with the thiobarbituric assay for malondialdehyde equivalents (20).

The four tetraol products of BA-diolepoxide hydrolysis were separated on a Dupont Zorbax ODS 4.6 x 250 mm column utilizing a Varian 5000 HPLC with a Varian Varichrome absorbance monitor set at 256 nm, the λ_{max} of the BA-tetraols. The gradient utilized was 40 to 100% methanol/water over 60 min, at a flow rate of 1.2 ml/min, which enabled baseline separation of the BA-tetraols (15). Metabolism was quantitated by the peak height ratio of tetraol incubation products to known amounts of tetraol standards separated by HPLC under identical conditions. Product identity was confirmed by a) co-chromatography with standards on HPLC, b) UV spectrometry and, in certain cases c) direct probe mass spectrometry of tetracetyl derivatives of BA-tetraols that were prepared and analyzed as previously described for BP-tetraols (19).

RESULTS

BA-diolepoxides are unstable in aqueous solution so their formation is inferred and quantitated from the amount of hydrolysis products formed. Each diolepoxide hydrolyzes to *cis* and *trans*-tetraols (Figure 1). HPLC separation of the four tetraols on reverse phase columns constitutes the basis of the assay for diolepoxide formation. Ultraviolet absorbance at 256 nm was utilized to detect and quantitate products eluting from the column.

PGH synthase and hematin/13-OOH-18:2-dependent epoxidation of BP-7,8-diol share the following mechanistic criteria: a) each is inhibited by antioxidants, b) each results in the formation of *anti*-vs *syn*-diolepoxides and c) each leads to incorporation of molecular (*vs* hydroperoxide) oxygen as the epoxide moiety (6,7,19,21). Hence BA-3,4-diol was examined as a substrate for epoxidation by both oxidation systems. Figure 2A and B show HPLC separations of tetraol standards and the product profile detected from the hematin/13-OOH-18:2-dependent oxidation of BA-3,4-diol. Peaks co-chromatographing with all four tetraol standards were noted. A similar profile was detected from RSVM/arachidonic acid-dependent BA-3,4-diol metabolism (not shown). Tetraol 1 (*trans-anti*), the major product of the reaction was converted to its peracetyl derivative and

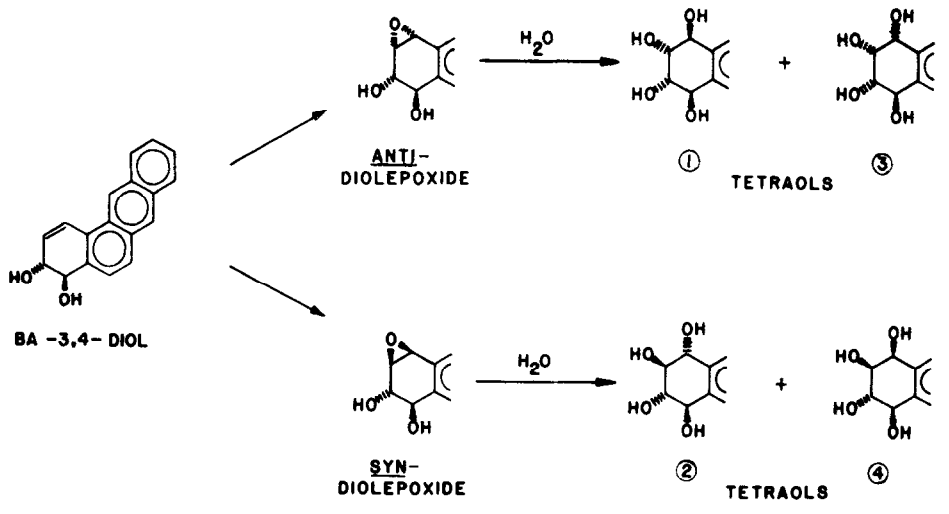

Figure 1. Oxidation of BA-3,4-diol to diolepoxides and their tetraol hydrolysis products.

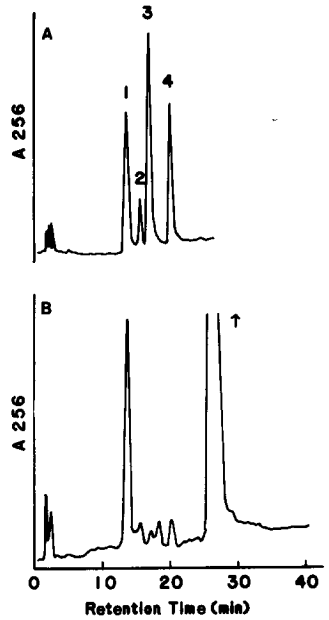

Figure 2. HPLC separation of tetraol standards derived from BA-3,4-diol-1,2-epoxides (A) and the products of BA-3,4-diol oxidation by hematin and 13-OOH-18:2 (B).
Conditions are described in **MATERIALS AND METHODS.**

analyzed by direct probe mass spectrometry. The mass spectrum exhibited a molecular ion at m/e 464 and major fragment ions at 404,344, and 228. The spectrum of the metabolite was identical to that of an authentic standard of the *trans-anti*-tetraol.

Inspection of Figure 2B reveals that the four tetraols account for virtually all of the products of BA-3,4-diol metabolism (the large peak at 28 min is BA-3,4-diol). A small peak was detected at 18 min, which cochromatographs with 10-methoxy-7,8,9-trihydroxy-7,8,9,10-tetrahydro benzo[a]-anthracene. This product forms by methanolysis of extracted *anti*-diolepoxide on the HPLC column. An analogous product is detected from PGH synthase-dependent oxidation of BP-7,8-diol (21). BA-3,4-diol and its metabolites possess intense absorbances at 256 nm so it is unlikely that other oxidation products are formed that are transparent to our detection system. It appears that peroxyl radicals oxidize BA-3,4-diol exclusively by epoxidation of the 1,2-double bond.

Table 1 lists relative percentages of tetraols formed by both systems. Tetraols 1 and 3, derived from the *anti*-diolepoxide, form with an 1.9 to 1 excess over *syn*-diolepoxide derived tetraols 2 and 4. The total amount of metabolism to tetraols (3.5%) is lower than the previously reported for oxidation of BE-7,8-diol under identical conditions (19).

Table 2 demonstrates that BA-3,4-diol epoxidation in RSVM is PGH synthase-dependent. A direct relationship between the amount of arachidonic acid oxygenated and tetraol formation was observed. In addition, the PGH synthase inhibitor indomethacin completely blocked BA-3,4-diol epoxidation. Inhibition by the phenolic antioxidant BHA was also noted, in common within the hematin/13-OOH 18:2 system.

Table 1. **Tetraol Products of RSVM- and Hematin-Dependent Epoxidation of BA-3,4-diol**

Tetraol[a]	Amount (pmol)	
	RSVM[b]	Hematin[c]
trans-anti- (1)	198	153
trans-syn- (2)	45	63
cis-anti- (3)	27	54
cis-syn- (4)	63	45

a. Numbers assigned to tetraols based on chromatographic profile in Figure 2 Structures given in Figure 1. b. Total isolated yield of tetraols was 3.7% of the starting BA-3,4-diol. c. Total isolated yield of tetraols was 3.5%.

We previously demonstrated that microsomal lipid peroxidation results in epoxidation of BP-7,8-diol (11). This suggests that BA-3,4-diol epoxidation in RSVM could be a result of PGH synthase-generated free radicals that induce microsomal lipid peroxidation. We tested this hypothesis by analyzing each incubation listed in Table 2 for the formation of malondialdehyde equivalents, which are produced in lipid peroxidation and as a result of PGH synthase-catalyzed oxygenation of unsaturated fatty acids (20). A concentration dependence was established between the formation of MDA equivalents and the amount of arachidonic acid added to the microsomes

Table 2. **Effects of Substrates and Inhibitors on BA-3,4-diol epoxidation by RSVM**

Incubation Conditions[a]	Tetraols (pmol)	MDA[b]
Arachidonate (0 μM)	35	0.70
" (25 μM)	142	1.39
" (100 μM)	218	1.97
" (250 μM)	291	2.98
" (100 μM) + BHA (100 μM)	13	2.11
" (100 μM) + Indomethacin (100 μM)	11	0.46
" (100 μM) + Boiled RSVM	72	0.58
13-OOH-18:2 (100 μM)	372	nd[c]
13-OOH-18:2 (100 μM) + BHA (100 μM)	42	nd

a. All incubations contained 0.1 M phosphate, pH 7.8, 1.0 mg microsomal protein, and 20 μM BA-3,4-diol in a total volume of 5 ml. Values are the means of duplicate experiments; b. nmol/mg microsomal protein; c. n.d. = not detected.

(Table 2). Addition of BHA, a potent inhibitor of microsomal lipid peroxidation, inhibited BA-3,4-diol epoxidation but did not inhibit the formation of malondialdehyde equivalents. It appears the MDA was derived from breakdown of prostaglandin endoperoxides rather than non-specific lipid peroxidation. BHA did not inhibit oxygenation of arachidonate to prostaglandin endoperoxides. Interestingly, addition of 13-OOH-18:2 to microsomes also caused BA-3,4-diol epoxidation but not formation of MDA equivalents. Taken with the results of BHA experiments, this observation suggests that fatty acid hydroperoxides, generated enzymatically from arachidonic acid or added directly to the microsomes, do not epoxidize BA-3,4-diol by inducing lipid peroxidation.

DISCUSSION

The present manuscript establishes that microsomal PGH synthase and hematin/13-OOH-18:2 epoxidize BA-3,4-diol. The major product detected is the *trans-anti*-tetraol, which is the major solvolysis product of the *anti*-diolepoxide. Similar results are obtained with BP-7,8-diol although the extent of epoxidation of the BP derivative is somewhat greater than that of the BA derivative (approximately three-fold) (19). This is probably due to the higher reactivity of the isolated double bond of BP-7,8-diol. BA-3,4-diol is intermediate in reactivity between BP-7,8-diol and aflatoxin B_1, which is epoxidized very poorly by peroxyl radical-generating systems (22). The only products detected in the peroxyl radical-dependent oxidation of BA-3,4-diol are derived from diolepoxides. Oxidation does not appear to occur at other positions in the molecule. This is in marked contrast to the oxidation of BA-3,4-diol by cytochrome P-450, which produces diolepoxides as only 15% of the products of oxidation (15). The stereoselectivity of BA-3,4-diol epoxidation by peroxyl radical-generating systems and cytochrome P-450 is also different. *Anti*-diolepoxides are the major products from peroxyl radical-dependent oxidation and based on the stereoselectivity of BP-7,8-diol epoxidation one anticipates that both enantiomers are epoxidized at equal rates (10,19). In contrast, Thakker *et al* reported that cytochrome $P\text{-}450_c$ only epoxidizes the (-)-enantiomer of BA-3,4-diol to *anti*-diolepoxide (15). The (+)-enantiomer is not converted to diolepoxides at all (15). Although the studies of the cytochrome P-450-dependent oxidation were conducted with enzyme preparations from rat liver microsomes, recent results establish that the stereoselectivity of epoxidation of BP-7,8-diol by cytochromes P-450 (control and ß-naphthoflavone-induced) in mouse skin, a target organ for BP-7,8-diol and BA-3,4-diol carcinogenesis, is the same as rat liver (23). Therefore, the combination of regiochemical and stereochemical differences established in the present work suggests that the (+)-enantiomer of BA-3,4-diol will be be an especially powerful tool to differentiate pathways of epoxidation *in vivo*.

Although the number of compounds studied in detail is small, it appears that peroxyl radical-dependent epoxidation constitutes a general pathway for metabolic activation, governed primarily by the reactivity of the double bond oxidized. Several systems that generate peroxyl radicals have been described and each of these epoxidizes BP-7,8-diol (5,6,11,21,24,25). An attractive hypothesis is that the systems cause lipid peroxidation and lipid peroxyl radicals are the common oxidizing agents. However, the results summarized in Table 2 indicate that epoxidation of BA-3,4-diol by RSVM preparations is independent of lipid peroxidation, which implies a common peroxyl radical oxidizing agent for all peroxyl radical-dependent epoxidations does not exist. It is,

therefore, possible that compounds may be developed to chemically differentiate among different peroxyl radical oxidants *in vitro* and *in vivo*.

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