PROSTAGLANDIN SYNTHETASE DEPENDENT BENZO(A)PYRENE OXIDATION: PRODUCTS OF THE OXIDATION AND INHIBITION OF THEIR FORMATION BY ANTIOXIDANTS

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SUMMARY

The products of the arachidonic acid dependent oxidation of benzo(a)pyrene by enzyme preparations from sheep seminal vesicles are the 1,6-, 3,6-, and 6,12- quinones. The metabolites were identified by high performance liquid chromatography and visible spectroscopy. The amount of benzo(a)pyrene converted to quinones by a Tween 20 solubilized preparation during a 15 min period is 43 μM/mg protein. The relative yields of the individual quinones are 1,6 - 25%, 3,6 - 30%, and 6,12 - 45%. Arachidonate dependent benzo(a)pyrene oxidation is strongly inhibited by butylated hydroxyanisole, vitamin E, and diethyldithiocarbamate and moderately inhibited by butylated hydroxytoluene and vitamin C. Epinephrine and lipoic acid are also inhibitors.

INTRODUCTION

Current theories of chemical carcinogenesis propose that initiators react as electrophiles with critical macromolecular nucleophiles in an event fundamental to cell transformation (1-3). BP*, like many carcinogens, is not appreciably electrophilic but is converted to reactive derivatives during oxidative metabolism (4). Of the many known metabolites of BP only a few possess the mutagenic and carcinogenic potency to be considered metabolically activated forms (5-8).

The BP activating systems most commonly studied are the NADPH dependent mixed-function oxidases possessing cytochrome P-450 as the terminal oxidase (9,10). We have recently demonstrated that during prostaglandin biosynthesis in sheep seminal vesicle microsomes a number of xenobiotics, among them BP,

*Abbreviations: BP, benzo(a)pyrene; BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole; EDTA, ethylenediaminetetraacetic acid disodium salt; Hb, hemoglobin; HPLC, high performance liquid chromatography; DDC, diethyldithiocarbamate, sodium salt; DMBA, 7,12-dimethylbenzanthracene; Indo, indomethacin.

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undergo significant cooxidation (11). This raises the possibility that prostaglandin synthetase contributes to the metabolic activation of BP. We now report that the products of arachidonic acid dependent BP oxidation in sheep seminal vesicles are quinones and that their formation is strongly inhibited by antioxidants. The latter finding is of potential interest with regard to reports that antioxidants inhibit polycyclic aromatic hydrocarbon initiated tumorigenesis in certain tissues (12-14).

MATERIALS AND METHODS

Chemicals: 7,10-[^{14}C]-BP (61 μCi/μmol) was obtained from Amersham/Searle. Arachidonic acid was generously provided by Dr. John Paulsrud of Hoffman-La Roche. L-(+)-Ascorbic acid was from Eastman, BHT from Aldrich, and all solvents and scintillation chemicals were from Fischer. Silica gel plates (Silica gel 60/F-254, 20 x 20 cm, .250 mm) were from E. Merck. BP metabolite standards were provided by the Standard Compound Reference Bank of the National Cancer Institute. All other chemicals were from Sigma.

Enzyme preparations: The post-mitochondrial supernatant and microsomal fraction were prepared as previously described (15). Solubilization was by the method of Miyamoto et al. (16). Solubilized enzyme in 20 mM $NaPO_4$, pH 7.4 containing 1% Tween-20(v/v) was at a concentration of 1 g tissue/ml. All enzyme preparations were assayed for oxygen uptake and used in BP incubations within 2 hours of preparation. Oxygen uptake was determined using either a Gilson Model KM or a Yellow Springs Instruments Model 53 oxygraph, both equipped with a Clark electrode from Yellow Springs Instrument Co.

Protein was determined by the method of Lowry et al using bovine serum albumin as a standard (17).

Incubations: Routine BP oxidation was carried out in 15 x 100 mm test tubes. The total volume of 1.0 ml contained 100 mM $NaPO_4$, pH 7.8, 125 μM 7,10-[^{14}C]-BP (0.35 μCi/μmol), enzyme, and 1 μM Hb. Hb was added to ensure maximum prostaglandin synthetase activity, as shown by other workers (18-21). BP was added in 25 μl acetone, BHA, BHT, and vitamin E were added in 5-20 μl acetone, and indomethacin and lipoate were added in 5-20 μl ethanol. All operations were carried out under subdued light. Following a 3 minute preincubation, the reaction was initiated by the addition of arachidonate and continued for 15 minutes at 25° C in a shaking water bath. The reaction was terminated by the addition of 1.0 ml acetone and brief vortexing, followed by the addition of 4.0 ml ethyl acetate and further vortexing for 2 x 1 min. The organic layer was dried over $MgSO_4$, the volume reduced by a stream of nitrogen, and applied to silica gel plates which were developed with benzene-ethanol (99:1). The plates were scanned on a Berthold LB 2760 radiochromatogram scanner. Radioactivity profiles served as a guide for dividing the entire plate into 5 zones: one containing unreacted BP one each for the two separated quinone bands, and two for more polar metabolites. Each zone was scraped and counted in a dioxane-water cocktail by a Packard 2002 scintillation counter. Samples were standardized by the External Standard method.

Preparative scale incubations were carried out in 10 ml of 80 μM $NaPO_4$ containing 6 mg solubilized protein, 1 μM Hb, and 125 μM 7,10-[^{14}C]-BP (0.02 μCi/μmol) at 25°. After a 3 min preincubation 1 mg arachidonic acid

TABLE 1. Arachidonate Dependent BP Oxidation by Enzyme Preparations from Sheep Seminal Vesicles

	μM Quinones[a]	μM Total Oxidation[a]
9,000 x g supernatant[b]	1.2	2.0
" " + Indo[c]	0.1	0.4
" " + NADPH[d]	0.3	0.6
Microsomal fraction[b]	4.0	4.9
" " + Indo[c]	0.8	1.2
" " + NADPH[d]	0.9	1.3
Solubilized enzyme[b]	11	14
" " + Indo[c]	0.5	0.8

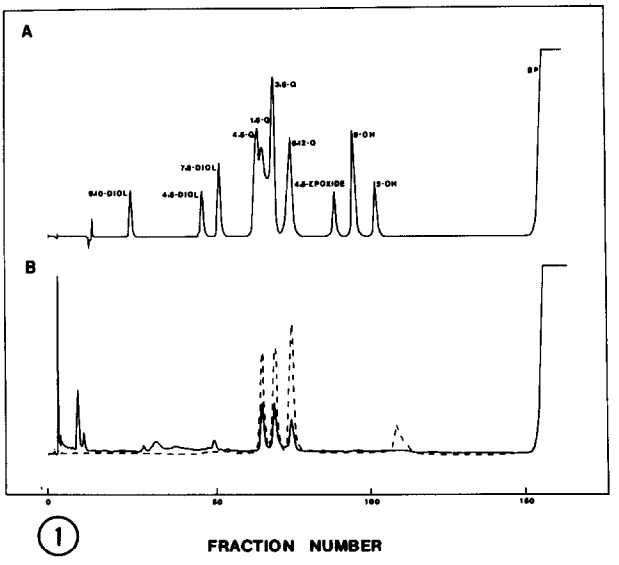
Conditions described in MATERIALS AND METHODS. (a) All values are reported as conversion per 100 mg wet weight tissue; (b) [Arachidonate] = 300 μM; (c) [Arachidonate] = 300 μM, [Indo] = 100 μM; (d) [Arachidonate] = 0 μM, [NADPH] = 1 mM.

was added and shaken for 15 min. The reaction was terminated by the addition of 10 ml acetone and 40 ml ethyl acetate and the organic layer separated. The extracts from 10 incubations were pooled, dried over $MgSO_4$, and the solvent evaporated. The residue was dissolved in chloroform and stored at -20° prior to analysis by HPLC.

HPLC analysis was performed on Waters Model 6000 Liquid Chromatograph equipped with a Model 660 Solvent Programmer. A 25 cm x 4 mm Waters Associates μ Bondapak C_{18} reverse phase column was employed and the flow rate was 1.5 ml/min. A 35 min linear program was run from 50 to 70% methanol/water and 20 drop fractions were collected. Radioactivity was determined by counting active fractions in 5 ml Bray's solution. Control experiments showed less than a 10% variation in counting efficiency through the entire program. Visible spectra were recorded on a Cary 118 spectrophotometer.

RESULTS

The data in Table 1 demonstrate that significant arachidonic acid dependent oxidation of BP occurs in the post-mitochondrial supernatant, microsomal fraction, and a solubilized preparation from the sheep vesicular gland.



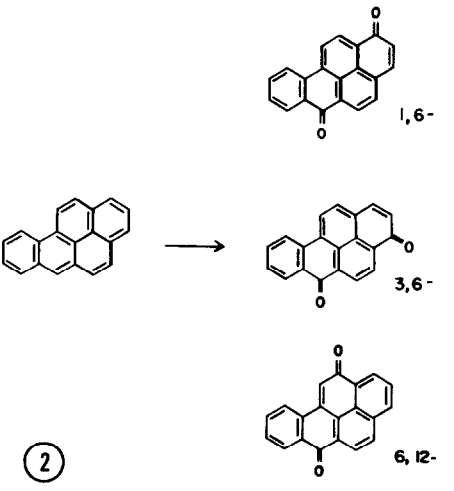


Fig. 1. HPLC Profile of Arachidonate Dependent BP Oxidation Products. (A) Mixture of authentic samples of BP metabolites; (B) Aliquot from incubation mixture. Ordinates are in arbitrary units and are not defined. (—) UV profile, (- -) radioactivity profile.

Fig. 2. Quinone Products of Arachidonate Dependent BP Oxidation.

As previously reported the oxidation by all preparations is strongly inhibited by the prostaglandin synthetase inhibitor indomethacin (11). NADPH dependent BP oxidation is lower than arachidonate dependent oxidation. Attempts to spectrally detect cyt P-450 in the microsomal preparations following $Na_2S_2O_4$ reduction and CO bubbling revealed no detectable absorbance at 450 nm at a protein concentration of 1.7 mg/ml. Since the solubilized preparation gives higher yields of oxidized products we have used it for subsequent studies.

The products of arachidonate dependent BP oxidation by all the enzyme preparations in Table I cochromatograph with standard samples of BP quinones when analyzed by thin layer chromatography. Fig 1 (B) shows the chromatographic profile observed when the oxidation products are subjected to HPLC

TABLE 2. Inhibition of Arachidonate Dependent BP Oxidation by Antioxidants.

Additive	Conc(μM)	% Inhibition
BHA	50	45
"	100	86
Vit E	50	39
"	100	72
Vit C	500	67
BHT	500	83
DDC	50	52
"	100	72
Epinephrine	50	16
"	100	61
Lipoate	100	27
"	200	57

Conditions described in MATERIALS AND METHODS

on a Waters μ Bondapak C_{18} reverse phase column employing a linear gradient of 50 to 70% methanol-water. The three major peaks of radioactivity co-chromatograph with standard samples of BP 1,6-, 3,6-, and 6,12-quinones, respectively. The visible spectra of the metabolites isolated by HPLC are identical to those of the standard quinones in both 50% aqueous methanol and in benzene (22). The percentage of each metabolite in the quinone fraction is 1,6 - 25%; 3,6 - 30%; 6,12 - 45%.

The effect of antioxidants on oxidation by the Tween 20 solubilized preparation is shown in Table 2. BHA, vitamin E, and DDC are effective at 50 or 100 μM while higher concentrations are required for comparable inhibition by BHT and vitamin C. In fact, vitamin C slightly stimulates oxidation at 50 and 100 μM. Also included in Table 2 are data for epinephrine and lipoic acid which have been reported to stimulate prostaglandin biosynthesis (15,23). Both of these compounds inhibit cooxidation of BP. Preliminary investigations have shown that all of the compounds in Table 2 sti-

mulate prostaglandin endoperoxide biosynthesis by solubilized preparations.

DISCUSSION

On the basis of their chromatographic properties and visible spectra we have assigned the structures of the three major arachidonate dependent metabolites to the 1,6-, 3,6-, and 6,12-quinones, shown in Fig. 2. These compounds are also produced by the mixed-function oxidases but usually in relatively small amounts (24). They have been suggested to occur as spontaneous decomposition products of unstable phenols, especially 6-hydroxy-BP (22,25). Ts'o has shown that 6-hydroxy-BP autoxidizes to a mixture of 1,6-, 3,6-, and 6,12-quinones with a half-life of 2.4 min in rat liver homogenates (25) and approximately 2 hr in 1:1 ethanol:phosphate buffer (22). If 6-hydroxy-BP is produced in our incubations it would, therefore, probably not survive the workup.

BP - 1,6-, 3,6-, and 6,12-quinones are not mutagenic in Salmonella typhimurium or in Chinese hamster V 79 cells (26) nor do they bind covalently to DNA (27). Therefore, although they are moderately cytotoxic (26) and induce strand scission of bacteriophage T 7 DNA (27), they are not considered to be activated forms of BP. However, it is possible that they arise via decomposition of the unstable 6-hydroxy-BP which is mutagenic in Salmonella typhimurium and V79 cells (26), carcinogenic when injected subcutaneously into rats (28), and binds covalently to DNA (28). This raises the possibility that an activated form of BP is produced during arachidonate dependent BP oxidation as an unstable intermediate. In this regard, the recent work of Eling et al. is of interest**. These workers studied the binding of BP to protein and DNA induced by guinea pig lung microsomes which contain both NADPH and arachidonate dependent BP oxygenase systems. As in the sheep vesicular gland the products of arachidonate dependent BP oxidation appear to be exclusively quinones by TLC. They find that the amounts of BP covalently bound to DNA are comparable whether initiated by NADPH or ara-

**T. Eling, personal communication

chidonate and that the amounts of BP covalently bound to protein are greater when initiated by arachidonate than by NADPH. This suggests that an electrophilic derivative of BP is formed during arachidonate dependent oxidation which is capable of reaction with macromolecular nucleophiles.

A number of recent papers have described the prophylactic effect of antioxidants on polycyclic aromatic hydrocarbon initiated tumorigenesis. BHA and BHT inhibit BP and DMBA carcinogenesis of the mouse forestomach and rat mammary gland (12). Dimethyldithiocarbamate inhibits mammary tumor formation by DMBA (13) and BHA, BHT, vitamin C, and vitamin E inhibit DMBA skin tumor initiation (14). In the latter case, the same antioxidants do not inhibit aryl hydrocarbon hydroxylase whether administered in vivo or in vitro nor do they induce this enzyme (14). Antioxidants do not inhibit aryl hydrocarbon hydroxylase activity in liver microsomes when added to the diet (29), but they do inhibit when added directly to the assay mixture (30). BHA, BHT, vitamin E, vitamin C, and DDC inhibit prostaglandin synthetase dependent oxidation of BP in enzyme preparations from the sheep vesicular gland. BHA, vitamin E, and DDC are particularly effective (Table 2) and cause complete inhibition of quinone formation at a concentration of 200 μM (data not shown). Therefore, the production of a metabolically activated form of BP by an arachidonate dependent pathway could be totally inhibited by these agents.

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