Prostaglandin Synthetase and Cytochrome P-450-dependent Metabolism of (±)Benzo(a)pyrene 7,8-Dihydrodiol by Enriched Populations of Rat Clara Cells and Alveolar Type II Cells

Kandiah Sivaramajah,1 Kenneth G. Jones, James R. Fouts, Theodora Devereux, Jill E. Shirley, and Thomas E. Eling


ABSTRACT

The metabolism of (±)-trans-7,8-dihydroxy-7,8-dihydrbenzo(a)pyrene (BP-7,8-diol) by prostaglandin synthetase and cytochrome P-450-dependent monooxygenases was studied using enriched fractions of Clara cells and alveolar type II cells from rat lung. Arachidonic acid-fortified fractions enriched in Clara cells and alveolar type II cells metabolized BP-7,8-diol to the 7,10/8,9-tetrol of benzo(a)pyrene and the 7/8,9,10-tetrol of benzo(a)pyrene. These tetrols are formed upon solvolysis of (±)-7,8a-dihydroxy-9a,10a-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BP diol-epoxide I). Arachidonic acid-dependent metabolism of BP-7,8-diol to BP diol-epoxide I in enriched Clara cells and alveolar type II cells was completely inhibited by indomethacin, a classical inhibitor of prostaglandin synthetase. Enriched Clara cells and alveolar type II cells also metabolized BP-7,8-diol to BP diol-epoxide I in the presence of NADPH.

Amounts of BP diol-epoxide I-derived tetrols formed from BP-7,8-diol by the prostaglandin synthetase-dependent and the cytochrome P-450-dependent pathways varied significantly between the two pulmonary cell fractions examined. In fractions enriched in Clara cells, cytochrome P-450-dependent BP-7,8-diol oxidation was higher than was prostaglandin synthetase-dependent BP-7,8-diol oxidation; while in fractions of alveolar type II cells, prostaglandin synthetase-dependent BP-7,8-diol oxidation to BP diol-epoxide I predominated. Pretreatment of rats with β-napthoflavone resulted in a 2- to 3-fold increase in BP diol-epoxide I formation by prostaglandin synthetase and cytochrome P-450-dependent monooxygenases in both enriched Clara cells and alveolar type II cells. These increases in BP-7,8-diol oxidation to BP diol-epoxide I appear to be due to induction of the two enzymatic pathways in both pulmonary cell types. No qualitative changes in the pattern of BP-7,8-diol metabolism by either enzymatic pathway in enriched Clara cells or alveolar type II cells were observed following β-napthoflavone treatment. The results suggest that pulmonary prostaglandin synthetase may serve as either an additional or an alternative bioactivating enzyme to the cytochrome P-450-dependent monooxygenases for the formation of reactive chemical carcinogens in the lung.

INTRODUCTION

The mammalian lung is one of several extrahepatic organs involved in the biotransformation of xenobiotics. Most of the enzymatic pathways responsible for foreign compound metabo-

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chrome P-450-dependent monoxygenase present in the same pulmonary fractions. These results suggested that pulmonary prostaglandin synthetase could serve as either an alternate or an additional enzyme to the cytochrome P-450-dependent monoxygenase for the formation of BP diol-epoxide I from BP-7,8-diol in the lung. However, the localization of prostaglandin synthetase in different types of lung cells is not known, but prostaglandin synthetase is present in isolated type II cells of the rat (28). In the present study, we have compared BP-7,8-diol oxidation by prostaglandin synthetase and cytochrome P-450 using enriched fractions of Clara cells and alveolar type II cells prepared from either control or β-NF-treated rats. Our results indicated that: (a) BP-7,8-diol is oxidized exclusively to BP diol-epoxide I in fractions enriched in Clara cells or in alveolar type II cells by both prostaglandin synthetase and cytochrome P-450-dependent monoxygenase; (b) oxidation of BP-7,8-diol by prostaglandin synthetase and cytochrome P-450-dependent monoxygenase differs with the different cell fractions; and (c) BP-7,8-diol metabolism to BP diol-epoxide I by both enzyme systems in these 2 cell preparations is inducible by β-NF treatment.

MATERIALS AND METHODS

Chemicals. AA (99% pure) was obtained from Nu-Chek Prep, Elysian, Minn. [1,4C]AA was purchased from New England Nuclear, Boston, Mass. NADPH and indomethacin were obtained from the Sigma Chemical Co., St. Louis, Mo. [3H]BP-7,8-diol (280 mCi/mmol), [14C]BP diol-epoxide I (anti isomer; 29.8 mCi/mmol), and BP diol-epoxide II (syn isomer; 29.7 mCi/mmol) were obtained from Dr. David Longfellow, Chemical Research Section, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Md.

Animals. Male Sprague-Dawley rats weighing from 150 to 250 g or male New Zealand rabbits weighing from 2 to 3 kg were used for experiments. Rats treated with β-NF were given a single i.p. dose (50 mg/kg) dissolved in corn oil (133 mg/ml) and were killed 48 hr later. Control animals received a corresponding amount of corn oil alone.

Preparation of Lung Cells. Lung tissue was digested with 0.1% protease type I in 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer which was instilled via the trachea. Enriched fractions of Clara cells and alveolar type II cells were then prepared by centrifugal elutriation and density gradient centrifugation (for rat) according to the method of Jones et al. (12) and, for rabbit, according to method of Devereux and Fouts (5). Cell counts were made using a hemocytometer. Rat pulmonary macrophages were isolated by lavaging lungs according to established procedures (10). Lymphocytes were separated from erythrocytes by layering whole blood from rats on a 50% Percol solution [in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline] and centrifuging at 800 x g for 20 min. Cell viability was determined by the trypan blue (0.04%) dye exclusion method (20). Identification and enumeration of Clara cells were determined by staining with nitro blue tetrazolium (12). Type II cells, macrophages, and polymorphonuclear lymphocytes were identified by modified Papanicolaou stain without acid alcohol (14). Electron microscopy examination was also used to confirm the identity of the cells. The Clara cell preparations were found to contain 30 to 35% of this cell type with no cross-contamination of type II cells. Similarly, the final alveolar type II cell preparations were found to contain 75 to 80% of this cell type with no cross-contamination of Clara cells (12). The remaining 20 to 25% of the cells were lymphocytes. Whole-lung cell digest contained approximately 0.8% Clara cells and 18% type II cells. ACE was estimated by the method of Friedland and Silverstein (8).

Incubation Conditions. One to 2.5 x 10^6 cells (1 to 2 mg cellular protein) were sonicated and then suspended in 1 ml of 0.1 M sodium phosphate buffer containing 20 nmol [3H]BP-7,8-diol. The reactions were initiated with either 0.2 μmol AA (in 10 μl of ethanol) or 2.5 μmol NADPH and were carried out for 5 min at 37°. When used, indomethacin (final concentration, 200 μM) was preincubated with the sonicated cell fractions for 3 min at 37° before the addition of AA. The reactions were stopped by adding 4 ml of ethyl acetate:acetone (2:1 v/v) and vortexed vigorously, and the organic layer was removed. After repeating this extraction procedure twice more, the organic layers were pooled, dehydrated with anhydrous magnesium sulfate, and evaporated to dryness with nitrogen gas. Residues were then solubilized with 100 μl of acetone and subjected to metabolite analysis by HPLC.

Metabolite Analysis. Analysis of BP-7,8-diol metabolites was performed using a Waters Associates ALC/GLC liquid chromatograph equipped with a M6000A pumping system, a U6K universal injection system, and a RCM-100 column chromatography module. The radial compression module was fitted with a Radial Pak A (C18) cartridge (5 x 100 mm). Samples were eluted isocratically at room temperature. The initial eluting solvent was methanol:water (11:9, v/v) at a flow rate of 1.5 ml/min for 20 min and was followed by absolute methanol at a flow rate of 2.0 ml/min for 20 min. Fractions were collected every 30 sec directly into scintillation vials using a Gilson B-200 fraction collector. Radioactivity in the fractions was quantitated using liquid scintillation techniques. The UV absorption of the column eluates was monitored simultaneously at 254 nm to confirm peak localization. Authentic BP-tetrols, prepared as described previously (26), were used as reference standards in the above HPLC system.

RESULTS

Arachidonic acid metabolism to prostaglandins by cell fractions enriched in rabbit or rat Clara cells or type II cells was first examined. Clara and type II cell fractions from rabbit were essentially devoid of prostaglandin synthetase activity while these cell fractions from rat converted AA to prostaglandins (data not shown). Therefore, we chose to study cell fractions from the rat lung, since these cell fractions contained both prostaglandin synthetase and cytochrome P-450 monoxygenase activities. Rat alveolar type II cell fractions contained 75 to 80% type II cells and 20 to 25% lymphocytes. The Clara cell-enriched fractions contained 30 to 35% Clara cells, 5 to 12% lymphocytes, 10 to 15% alveolar macrophages, 5 to 10% ciliated cells, and 20 to 25% small cells which were probably endothelial cells. To assess the contribution of these contaminating cells to the metabolism of BP-7,8-diol, alveolar macrophages and lymphocytes were prepared and incubated with BP-7,8-diol as described in "Materials and Methods." No detectable oxidation to the tetrolys was observed with macrophages or lymphocytes; thus, contamination of these cells in the enriched fractions should not contribute to any observed metabolism of the BP-7,8-diol. Metabolism of BP-7,8-diol by cytochrome P-450-dependent monoxygenases results primarily in the epoxidation of the adjacent 9,10 double bond and gives rise to BP diol-epoxide I and BP diol-epoxide II (29). The diol-epoxides are highly unstable in an aqueous solution and undergo solvolysis to the BP-tetrols, trans I and cis I, and trans II- and cis II-tetrol; the former 2 tetrols arise from BP diol-epoxide I, and the latter 2 arise from BP diol-epoxide II. Metabolism of BP-7,8-diol by prostaglandin synthetase produces only BP diol-epoxide I (25).

The Clara cell-enriched fraction also contained 20 to 25% unknown small cells which might be endothelial cells and therefore contribute to the cooxidation observed in this cell fraction. Therefore, ACE activity was measured as a biochemical marker for endothelial cells (8) in an attempt to correlate cooxygenation activity in the Clara cell fraction to endothelial cell contamination.

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ACE activity in rat pulmonary cell fractions is shown in Table 1.

The small cells of the lung in elutriator Fraction 1 showed the highest ACE activity, whereas the Clara cell-enriched fraction had quite low ACE activity, as did the macrophages and the type II cells. When cells in elutriator Fraction 1 were incubated with BP-7,8-diol, no detectable oxidation to diol-epoxides was observed. These results suggest that small-cell contamination in the enriched Clara cell fraction would not contribute significantly to oxidation of BP 7,8-diol. Although it is not possible to rule out entirely a contribution of the small cells (contaminants) to the metabolism of BP-7,8-diol, our data suggest that this problem is at a minimum with fractions enriched in type II or Clara cells.

HPLC analysis of the products formed when BP-7,8-diol was incubated with AA-fortified enriched alveolar type II cells revealed 2 major peaks (Chart 1); the larger peak cochromatographed with authentic trans I-tetrol, and the smaller peak cochromatographed with authentic cis I-tetrol. As shown in Chart 1, alveolar type II cells fortified with NADPH formed the same 2 tetrrols, although in smaller amounts than did the AA-dependent reaction. Similarly, fractions enriched in Clara cells fortified with either AA or NADPH also formed primarily trans I-tetrol along with smaller amounts of cis I-tetrol (Chart 2). In Clara cell-enriched fractions, the BP-tetrrols formed from BP-7,8-diol by the NADPH-dependent system were greater than those formed by the AA-dependent system. In the case of both cell types, no BP-7,8-diol-related products cochromatographing with either trans II- or cis II-tetrols were detected regardless of the cofactor used. The cell digest (unseparated cells), when incubated with BP-7,8-diol and AA or NADPH, also formed only trans I- and cis I-tetrols (data not shown). These results show that rat pulmonary Clara and alveolar type II cell-enriched fractions as well as total pulmonary cell digests metabolize BP-7,8-diol exclusively to BP diol-epoxide I, whether catalyzed by either the prostaglandin synthetase-dependent or the cytochrome P-450-dependent pathway.

As mentioned above, AA-dependent and NADPH-dependent BP-7,8-diol metabolism to tetrrols differed substantially between fractions enriched in Clara cells or alveolar type II cells. With Clara cell-enriched fractions, cytochrome P-450-dependent trans I and cis I formations were 2- to 3-fold greater than prostaglandin synthetase-dependent formation (Table 2). In contrast, prostaglandin synthetase-dependent metabolism of BP-7,8-diol was greater than cytochrome P-450-dependent metabolism in enriched alveolar type II cell fractions. This difference between the 2 cell types can be accounted for entirely by the cellular differences in the rates of NADPH-dependent BP-7,8-diol oxidation, since AA-dependent tetrol formation was almost identical in Clara cells and alveolar type II cells. Cytochrome P-450-dependent BP-tetrol formations were also higher than the prostaglandin synthetase-dependent generation of tetrrols in pulmonary cell digests. With all 3 types of cellular preparations, preincubation with indomethacin dramatically inhibited AA-dependent formation of trans I- and cis I-tetrrols but had no significant effect on this reaction in the presence of NADPH (Table 1).

Effects of β-NF Pretreatment on BP-7,8-Diol Metabolism by Enriched Pulmonary Cells. Treatment of rats with β-NF was found to have pronounced quantitative effects on the oxidation of BP-7,8-diol to BP-tetrrols by isolated pulmonary cell fractions. In Clara cell-enriched fractions from β-NF-treated rats, prostaglandin synthetase-dependent metabolism was increased by 130%, while cytochrome P-450-dependent BP-7,8-diol oxidation to tetrrols was increased by 93% (Table 3). In alveolar type II cells from β-NF-treated animals, AA-dependent and NADPH-dependent BP-tetrol formations were increased by 120 and

Table 1

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Histidyl-L-leucine (nmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell digest</td>
<td>38.9 ± 13.7 (2)</td>
</tr>
<tr>
<td>Elutriator Fraction 1</td>
<td>49.6 ± 26.9 (3)</td>
</tr>
<tr>
<td>Type II cells</td>
<td>2.0 ± 2.4 (3)</td>
</tr>
<tr>
<td>Clara cells</td>
<td>5.8 ± 5.3 (3)</td>
</tr>
<tr>
<td>Macrophages (from lavage)</td>
<td>0.6 ± 1.0 (3)</td>
</tr>
</tbody>
</table>

a Mean ± S.D.
b Numbers in parentheses, numbers of different experiments performed.
c Elutriator Fraction 1 contains most of the small cells of the lung.
Metabolism of BP 7,8-Diol by Isolated Pulmonary Cells

Comparison of the AA- and NADPH-dependent metabolism of BP-7,8-diol by enriched pulmonary cell fractions

BP-7,8-diol (20 μM) was added to a 1-ml incubation mixture consisting of 1 million to 3.5 million sonicated cells in 0.1 M phosphate buffer (pH 7.8). Reactions were initiated with the addition of 0.2 μmol of AA and incubated at 37°C for 5 min. When added, indomethacin (200 μM) was preincubated with the reaction mixture for 5 min at 4°C before the addition of AA. BP-tetrol formation was determined as described under "Materials and Methods."

When added, indomethacin (200 μM) was preincubated with the reaction mixture for 5 min at 4°C before the addition of AA. BP-tetrol formation was determined as described under "Materials and Methods."

Table 2

Comparison of the AA- and NADPH-dependent metabolism of BP-7,8-diol by enriched pulmonary cell fractions

<table>
<thead>
<tr>
<th>Cell preparation</th>
<th>Indo- methacin</th>
<th>AA-dependent</th>
<th>NADPH-dependent</th>
<th>NADPH:AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enriched Clara cells</td>
<td>−</td>
<td>9.2 ± 1.1</td>
<td>21.6 ± 2.7</td>
<td>2.3</td>
</tr>
<tr>
<td>Enriched type II cells</td>
<td>+</td>
<td>8.8 ± 2.0</td>
<td>5.1 ± 1.0</td>
<td>0.58</td>
</tr>
<tr>
<td>Cell digest</td>
<td>+</td>
<td>4.2 ± 0.7</td>
<td>8.5 ± 1.2</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Table 3

Effect of animal pretreatment on the metabolism of BP-7,8-diol by the AA- and NADPH-dependent systems by enriched pulmonary cell fractions

<table>
<thead>
<tr>
<th>Cell preparation</th>
<th>NADPH-dependent</th>
<th>AA-dependent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pmol tetrols/min/10⁶ cells)</td>
<td>(pmol tetrols/min/10⁶ cells)</td>
</tr>
<tr>
<td>Enriched Clara cells</td>
<td>20.0 ± 2.6</td>
<td>7.2 ± 1.1</td>
</tr>
<tr>
<td>Control</td>
<td>38.72 ± 5.1</td>
<td>16.6 ± 2.9</td>
</tr>
<tr>
<td>Treated</td>
<td>4.8 ± 0.8</td>
<td>9.6 ± 0.6</td>
</tr>
<tr>
<td>Enriched type II cells</td>
<td>13.1 ± 2.1</td>
<td>21.0 ± 3.2</td>
</tr>
<tr>
<td>Control</td>
<td>9.1 ± 1.1</td>
<td>4.2 ± 0.8</td>
</tr>
<tr>
<td>Treated</td>
<td>22.2 ± 2.6</td>
<td>11.6 ± 1.7</td>
</tr>
</tbody>
</table>

As discussed in a previous report, the metabolism of BP-7,8-diol to BP diol-epoxide I during the biosynthesis of prostaglandins is peroxidative (25). The 9,10-epoxidation of this dihydrodiol occurs during the reduction of prostaglandin G₂ (a hydroperoxy endoperoxide derived from AA) to prostaglandin H₂; this latter reaction is catalyzed by the hydroperoxidase component of prostaglandin synthetase. Marnett and Bienkowski (16) have suggested that conversion of BP-7,8-diol to BP diol-epoxide I during prostaglandin G₂ reduction proceeds via a free radical autoxidation-type mechanism. The oxidizing agent may be either a prostaglandin G₂-derived peroxy radical or a compound l-type derivative of the hydroperoxidase. In addition to AA, polyunsaturated lipid hydroperoxides such as 15-hydroperoxyarachidonic acid can initiate prostaglandin synthetase-dependent BP-7,8-diol oxidation to BP diol-epoxide I (16). Interestingly, Dix and Marnett (7) demonstrated recently that BP-7,8-diol is converted to BP diol-epoxide I in a system containing only polyunsaturated lipid hydroperoxides and hematin. Although the mechanism of prostaglandin synthetase-dependent BP-7,8-diol metabolism to BP diol-epoxide I is apparently very different from that of cytochrome P-450, the oxygen inserted into the 9,10 position of BP-7,8-diol during metabolism by either enzymatic pathway is derived from the same source, namely, molecular oxygen (16).

170%, respectively, when compared to control values. Significant increases in cis I and trans I formation from BP-7,8-diol by both enzyme systems were also observed using lung cell digests from treated animals. While β-NF treatment caused increases in the rates of AA-dependent and NADPH-dependent BP-7,8-diol oxidation to BP-tetrols in all 3 cell preparations, no qualitative changes in the pattern of pulmonary BP-7,8-diol metabolism were observed following treatment with this microsomal enzyme-inducing agent.

DISCUSSION

The results presented in this study demonstrated that enriched preparations of Clara cells and alveolar type II cells from rat lung can metabolize BP-7,8-diol to the ultimate carcinogen BP diol-epoxide I by cytochrome P-450 monoxygenase and prostaglandin synthetase in pulmonary tissue (25). In previous studies from this laboratory, we found that pulmonary microsomes from various animal species and humans metabolize BP-7,8-diol to BP diol-epoxide I via a cytochrome P-450-independent process. This other pathway of BP diol-epoxide I formation was shown to be mediated by prostaglandin synthetase, since it exhibited a requirement for AA and was inhibited by classical prostaglandin biosynthesis inhibitors (25). Because the pathological relevance of pulmonary prostaglandin synthetase-dependent BP diol-epoxide I formation was then unclear, our studies were extended here to examine this enzymatic process within the proposed target cells themselves. Prostaglandin synthetase-dependent oxidation of BP-7,8-diol to BP diol-epoxide I was found to occur in both Clara cell- and alveolar type II cell-enriched preparations. In fact, rates of prostaglandin synthetase-dependent BP diol-epoxide I formation were found to be very similar to the cytochrome P-450-dependent rates in both cell preparations. These data clearly indicate that prostaglandin synthetase may serve as either an additional or an alternative enzyme to the cytochrome P-450-dependent monoxygenase in the bioactivation of primary BP oxidative metabolites to ultimate carcinogens in these lung cells.

As discussed in a previous report, the metabolism of BP-7,8-diol to BP diol-epoxide I during the biosynthesis of prostaglandins is peroxidative (25). The 9,10-epoxidation of this dihydrodiol occurs during the reduction of prostaglandin G₂ (a hydroperoxy endoperoxide derived from AA) to prostaglandin H₂; this latter reaction is catalyzed by the hydroperoxidase component of prostaglandin synthetase. Marnett and Bienkowski (16) have suggested that conversion of BP-7,8-diol to BP diol-epoxide I during prostaglandin G₂ reduction proceeds via a free radical autoxidation-type mechanism. The oxidizing agent may be either a prostaglandin G₂-derived peroxy radical or a compound l-type derivative of the hydroperoxidase. In addition to AA, polyunsaturated lipid hydroperoxides such as 15-hydroperoxyarachidonic acid can initiate prostaglandin synthetase-dependent BP-7,8-diol oxidation to BP diol-epoxide I (16). Interestingly, Dix and Marnett (7) demonstrated recently that BP-7,8-diol is converted to BP diol-epoxide I in a system containing only polyunsaturated lipid hydroperoxides and hematin. Although the mechanism of prostaglandin synthetase-dependent BP-7,8-diol metabolism to BP diol-epoxide I is apparently very different from that of cytochrome P-450, the oxygen inserted into the 9,10 position of BP-7,8-diol during metabolism by either enzymatic pathway is derived from the same source, namely, molecular oxygen (16).

As was observed in this study, other investigations (16, 25, 26) concerning prostaglandin synthetase-dependent oxidation of BP-7,8-diol have shown that this compound is metabolized exclusively to BP diol-epoxide I. This has been the case regardless of the sources of enzyme which have included rat, guinea pig, and human lung; rabbit kidney medulla; mouse skin; and ram seminal vesicle. Rat, guinea pig, and human lung microsomes, when fortified with NADPH, also metabolize (±)-BP-7,8-diol mainly to BP diol-epoxide I; insignificant amounts of BP diol-epoxide II are formed by pulmonary cytochrome P-450-dependent monoxygenases. These results suggest that BP-7,8-diol is metabolized primarily to BP diol-epoxide I in the lung whether conversion occurs via the prostaglandin synthetase-dependent or the cytochrome P-450-dependent pathway. In contrast, liver microsomes from all animal species examined as well as reconstituted monoxygenase systems using rat liver cytochrome P-450 isozymes metabolize racemic BP-7,8-diol to both BP diol-epoxide I and BP diol-epoxide II (29, 30). In the case of rat liver monoxygenases, the ratio of BP diol-epoxide I to BP diol-epoxide II formed ranges from 1.7:1 to 0.4:1 as determined from the amounts of trans I, trans II, cis I, and cis II-tetrols detected by HPLC (29). Therefore, rat pulmonary cytochrome P-450-dependent monoxygenases appear to exhibit a different metabolic stereospecificity for racemic BP-7,8-diol than do cytochrome P-


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