Characterization of Pulmonary Arene Oxide Biotransformation Using the Perfused Rabbit Lung

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ABSTRACT

Rapid arene oxide biotransformation has been suggested as a protective mechanism against the effects of polycyclic aromatic hydrocarbons. This is a report on benzo(a)pyrene 4,5-oxide biotransformation by perfused rabbit lungs. Benzo(a)pyrene 4,5-oxide (5 μmol) was conjugated with glutathione and hydrated at rates of 23.8 ± 2.3 (S.D.) and 7.60 ± 1.14 nmol/min/g of tissue, respectively. The total rate for benzo(a)pyrene 4,5-oxide biotransformation by perfused lungs was about 3 times the estimated rate of arene oxide formation from benzo(a)pyrene found in previous studies. A high-pressure liquid chromatographic system was utilized to resolve the benzo(a)pyrene 4,5-oxide metabolites, which were tentatively identified by cochromatography with compounds of established chemical structure. It was discovered with this chromatographic system that perfused rabbit lungs produce a cysteine conjugate from benzo(a)pyrene 4,5-oxide but lacked the ability to conjugate trans-4,5-dihydro-4,5-dihydroxybenzo(a)pyrene with sulfuric or glucuronic acid at detectable rates. The trans-4,5-dihydro-4,5-dihydroxybenzo(a)pyrene could pass freely from the tissue into the perfusion medium but remained at a relatively high concentration in the lungs, possibly due to the lipophilic nature of this metabolite. The glutathione conjugate, which diffused slowly from the tissue into the perfusion medium, showed no tendency to accumulate in the tissue. Benzo(a)pyrene 4,5-oxide-derived radioactivity was covalently bound to pulmonary DNA, RNA, and protein. The relative activities of toxification/detoxification pathways, the apparent inability of pulmonary tissue to conjugate trans-4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene with sulfuric or β-glucuronic acid, the tendency of some benzo(a)pyrene metabolites to accumulate in the tissue, and the relationship of these parameters with the susceptibility of lung to the effects of polycyclic aromatic are discussed.

INTRODUCTION

Lung cancer is responsible for a large percentage of human mortality in industrialized nations, and PAHs present in cigarette smoke (39) and urban air (13) are believed to contribute to the incidence of this disease. Although the development of pulmonary tumors may result from selective exposure of this organ to airborne carcinogens, the susceptibility of this tissue may also have a biochemical basis. A considerable body of evidence suggests that PAHs require metabolic activation by the cytochrome P-450-dependent mixed-function oxidase system to ultimate carcinogens such as arene oxide (epoxides) or diol-epoxide derivatives (18, 19, 37). Furthermore, epoxides are generally degraded by at least 2 enzymatic mechanisms, hydration by epoxide hydrolase (EC 4.2.7.63) (28) or conjugation with glutathione by glutathione S-transferases (EC 2.5.1.19) (17). It follows that the balance between the ability of an organ (or of a cell type within a complex organ) to generate, to detoxify, and to eliminate reactive intermediates may be a critical factor in determining the susceptibility or resistance of that organ to PAH-induced carcinogenesis. The liver, for example, can rapidly metabolize a wide variety of exogenous substrates and is generally not a target organ for tumors initiated by PAH (7); the liver is also known to be very efficient in degrading and eliminating arene oxides (33, 34). In contrast, rabbit lung is susceptible to PAH-induced carcinogenesis (12) and has in vitro microsomal mixed-function oxidase specific activities with several substrates that are equal to or greater than those of rabbit liver (2, 14, 23) but has a much lower capacity than does hepatic tissue to metabolize epoxides by hydration or conjugation with glutathione (14, 16).

The purpose of this investigation was to evaluate the epoxide-metabolizing capacity of pulmonary tissue using a perfused organ system which simulates in vivo conditions. The isolated perfused rabbit lung preparation retains tissue integrity and cellular architecture and maintains the organ in a near-physiological state (27). This system also allows pulmonary metabolism to be studied in the absence of the liver, the total metabolic contribution of which is much greater than that of the lung. In addition, the isolated organ system allows quantitation of metabolites eliminated from the tissue. Two different forms of cytochrome P-450 have been purified from rabbit lung (41), and both metabolize BP to products arising through arene oxide intermediates (40). Only one form of the pulmonary cytochromes, however, facilitated binding of BP-derived radioactivity to DNA (40). In accord with these in vitro results, the perfused rabbit lung can produce metabolites from BP that are known to occur through arene oxide intermediates (4, 36). The perfused rabbit lung has also been shown to have epoxide hydrolase and glutathione S-transferase activities (17, 32, 35), 2 enzymes that are responsible for further biotransformation of epoxides (17, 28).

Administration of a xenobiotic via the pulmonary artery simulates in vivo exposure of lung tissue to metabolically generated arene oxides arising from extrapulmonary sources such as the liver, since the lungs contain the first capillary bed encountered by all venous blood (excluding that carried by the portal vein). In addition, this route of substrate administration assures that...
the lung alveolar cells, which have been reported as a site for rabbit lung carcinogenesis (12), are exposed to the test material. Accurate assessment of the ability of the pulmonary epoxide-metabolizing enzymes to degrade arene oxides was further facilitated by using BP 4,5-oxide as a substrate. BP 4,5-oxide is a weakly carcinogenic reactive intermediate (10, 21) formed during oxidative metabolism of BP (11, 31); it is a well-characterized substrate for epoxide-metabolizing enzymes (3, 25) and is relatively stable under physiological conditions (6) so that nonenzymatic conversion during studies of this duration is minimal. BP 4,5-oxide was an obligate intermediate which accounted for at least 6% of the metabolites produced by perfused rabbit lungs given BP (1).

MATERIALS AND METHODS

Animals. Male Dutch Belt rabbits [1800 ± 200 (S.D.) g] were obtained from Dutchland Laboratory Animals Inc. (Denver, Pa.) and maintained on NIH Feed A and tap water ad libitum.

Chemicals. [G-3H]BP 4,5-oxide (10 μCi/μmol), unlabeled BP 4,5-oxide, BP 4,5-dihydrodiol, and 5-hydroxybenzo(a)pyrene were synthesized by Midwest Research Institute (Kansas City, Mo.) under National Cancer Institute Contract N01-CB-33387 supplemented by the National Institute of Environmental Health Sciences. Benzo(a)pyrene 4,5-dihydrodiol; β-glucuronide (24), benzo(a)pyrene 4,5-dihydrodiol:sulfate (26), and BP 4,5-oxide:glutathione conjugate (34) were produced biosynthetically as previously reported. BP 4,5-oxide:cysteine conjugate was prepared by hydrolysis (25 min at room temp in 1 N HCl) of the BP 4,5-oxide:glutathione conjugate (30). These materials were used as chromatographic markers. Reagents and solvents were of the highest grade commercially available. HPLC-grade solvents were obtained from Fisher Scientific Co. (Fairlawn, N. J.).

Lung Perfusion. Lungs were surgically prepared from CO2-anesthetized rabbits and perfused (recirculating system; flow rate 140 ml/min) using the procedures and apparatus reported earlier (36). Five μmol of BP 4,5-oxide in dimethyl sulfoxide (200 μl) were infused during a 3-min period into the perfusion medium in the upper reservoir of the apparatus through a 27-gauge needle.

Extraction of Metabolites. The perfusion medium was sampled (0.5 ml) at various times during the experiment. BP 4,5-oxide and BP 4,5-dihydrodiol were recovered from the perfusion medium aliquots by extracting 3 times (once with 1 volume, twice with 0.5 volume) with ethyl acetate. The extracts were analyzed by TLC as described below. The radioactivity remaining in the aqueous fraction following extraction was quantitated to measure the conjugated metabolites. At the end of the experiment, the perfusion medium (approximately 190 ml) was extracted with ethyl acetate (1 volume, 0.5 volume, 0.5 volume). These extracts were combined, concentrated by flash evaporation, and analyzed chromatographically to characterize the extractable metabolites as described below. The conjugated metabolites, which were not extracted into ethyl acetate, were removed from the perfusion medium by adsorption on an XAD-2 column (1.5 x 20 cm) (34). The metabolites were recovered from the column by elution with methanol (300 ml), concentrated by flash evaporation, and characterized by HPLC as described below. In some cases, perfusion medium samples (100 μl) were chromatographed directly without work up.

The lungs were weighed at the end of each experiment and minced. BP 4,5-oxide-derived radioactivity covalently bound to tissue protein, RNA, and DNA in 1 g of lung was determined only for the 60-min experiments by methods reported elsewhere (33). Immediately following the perfusion period (10, 30, or 60 min), the remaining tissue was homogenized in water:acetone (9 volumes, 4:1, v/v). Ten ml of the homogenate were extracted with ethyl acetate (3 x 15 ml). The organic phases were combined, concentrated by flash evaporation, and analyzed by TLC. After removal of soluble protein by precipitation with methanol, the metabolites contained in the aqueous phase were concentrated to dryness by flash evaporation. The metabolites were taken up in methanol:H2O (0.5 ml, 5:1, v/v) and analyzed by HPLC (see below).

Enzymatic Hydrolyses. Conjugated metabolites were subjected to the hydrolytic activities of β-glucuronidase (bovine liver; Sigma Chemical Co., St. Louis, Mo.) or arylsulfatase (type H-2; Sigma) as reported previously (33).

Radioimetric Determinations. Radioactivity in liquid samples was determined in an LS-9000 liquid scintillation counter (Beckman Instruments, Irvine, Calif.) using Instagel scintillator (Packard Instruments, Downers Grove, Ill.). Metabolites isolated by TLC were quantitated by scraping the silica gel containing the radioactive materials from the chromatograms into scintillation vials containing 3 ml of water. Ten ml of Instagel were added to each sample, which was subsequently shaken vigorously in a Vortex Genie (Scientific Instruments Inc., Springfield, Mass.) for 30 sec. After 30 min in the dark, the radioactivity in the samples was determined as described above.

All sample counts were corrected for quenching by external standardization and were counted for a sufficient period of time to allow a counting error of less than ±2%.

Chromatographic Analyses. HPLC analyses were carried out using a Spectrophysics SP 8000 instrument fitted with a 25-cm Lichrosorb 10 μm RP-8 column (Altex Instruments, Berkley, Calif.). The column was eluted with a gradient composed of water:acetic acid (99:1, v/v; Solvent A) and acetonitrile:acetic acid (99:1, v/v; Solvent B). The 3-step gradient profile was as follows: 0 to 30 min, 80% Solvent A (20% Solvent B) to 40% Solvent A (60% Solvent B); 30 to 40 min, 40% Solvent A (60% Solvent B) to 100% Solvent B and holding for an additional 10 min. Fractions for radiometric determinations were collected at 30-sec intervals into scintillation vials using a Model B-100 fraction collector (Gilion Medical Electronics, Middleton, Wis.).

Metabolites extracted from the tissue and perfusion medium were routinely analyzed by TLC, since this method allowed the analysis of a greater number of samples and because the unchanged BP 4,5-oxide was unstable in the HPLC solvent gradient. The TLC system utilized silica gel multichannel, preadsorbent plates (LODF; Kontes, Pineland, N. J.) and benzene:ethanol (19:1, v/v) as the mobile phase. Unlabeled BP 4,5-oxide (Rf 0.75) and BP 4,5-dihydrodiol (Rf 0.27) were included in all samples as chromatographic standards.

RESULTS

The metabolites produced from BP 4,5-oxide by isolated perfused rabbit lungs were tentatively identified by HPLC. A typical chromatographic profile from a perfusion medium sam-
Pulmonary Metabolism of BP 4,5-Oxide

The pulmonary metabolism at BP 4,5-oxide after a 60-min experiment is shown in Chart 1. The major metabolites were 2 thioether conjugates and BP 4,5-dihydrodiol. A small amount of 4(5)-hydroxybenzo(a)pyrene was detected, which probably occurred through spontaneous BP 4,5-oxide rearrangement. Several minor radioactive peaks were eluted, which accounted for less than 2% of the total metabolites, but these were not characterized. There were no observable differences between the results obtained from perfusion medium samples chromatographed directly and those obtained after sample clean-up with XAD-2.

The 2 thioether derivatives produced from BP 4,5-oxide by perfused rabbit lungs were identical in this HPLC system to the biosynthetic BP 4,5-oxide:glutathione and BP 4,5-oxide:cysteine standards. These metabolites were also chromatographically identical to the BP 4,5-oxide:glutathione and BP 4,5-oxide:cysteine conjugates recently identified as biliary metabolites from rats given BP 4,5-oxide (30).

It was determined in the absence of lung tissue that less than 2% of the BP 4,5-oxide (5 μmol) decomposed or became covalently bound to the bovine serum albumin in the perfusion medium during 60 min at 37°. The decomposition product was 4(5)-hydroxybenzo(a)pyrene.

Prolonged incubation of water-soluble metabolites with sulfatase or β-glucuronidase produced no free BP 4,5-dihydrodiol or any other BP 4,5-oxide derivatives. Additionally, there was no chromatographic evidence of benzo(a)pyrene 4,5-dihydrodiol:glucuronide or benzo(a)pyrene 4,5-dihydrodiol:sulfate. These results indicate that neither BP 4,5-dihydrodiol nor BP 4,5-oxide itself underwent conjugation with sulfuric or glucuronic acid in the rabbit lung. During the 60-min perfusion period, BP 4,5-oxide-derived radioactivity was covalently bound to lung DNA, RNA, and protein (3.59 ± 0.81, 29.6 ± 14.7, and 16.3 ± 3.9 pmol/mg dry weight, respectively; N = 3). There was no evidence that BP 4,5-oxide was reduced to BP during perfusion through the rabbit lung. Exchange of the tritium label from the BP 4,5-oxide or its metabolites with water did not occur.

The total extent of BP 4,5-oxide biotransformation by pulmonary glutathione S-transferase and epoxide hydrase in lungs perfused for 10, 30, or 60 min was determined by quantitating the total thioether conjugates and BP 4,5-dihydrodiol produced (Chart 2). The initial glutathione S-transferase and epoxide hydrase activities were calculated from the metabolites produced by the 2 pathways during 10 min. Since the tissue contained a large portion of the metabolites, those contained in the lungs were also included in the determination. The glutathione S-transferase and epoxide hydrase activities, shown in Table 1, were in a 3:1 ratio. The total metabolites produced by the 2 pathways during 60 min, however, showed only a 2:1 ratio (Table 1). This difference was due to a diminished rate for the glutathione S-transferase reaction with time, while the hydration rate remained linear for 30 min (Chart 2).

The concentration of metabolites in the perfusion medium during 60 min following the BP 4,5-oxide infusion is shown in Chart 3. Rates of thioether conjugate and BP 4,5-dihydrodiol release per g of lung were calculated from these data (Table S 2).
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DISCUSSION

Although the principal pathways for epoxide metabolism (hydration and conjugation with glutathione) occurred in the intact lung, the ability of the isolated perfused rabbit lung to metabolize BP 4,5-oxide was far below that of the perfused rat liver (34). This difference in epoxide-metabolizing ability of these organs was also found in in vitro studies using subcellular fractions (16). Substantial amounts of a cysteine derivative were formed in addition to the BP 4,5-oxide:glutathione conjugate by the perfused rabbit lungs. To our knowledge, this is the first documentation of cysteine conjugate biosynthesis by mammalian lung. We do not know at this time whether the cysteine derivative was formed through biotransformation of the glutathione conjugate (which seems likely) or by direct conjugation of cysteine with BP 4,5-oxide. We did not detect the intermediate cysteinylglycine derivative, which suggests that deglutamylation is followed by rapid deglycination of the cysteinylglycine conjugate. Glutathione and cysteine derivatives of BP 4,5-oxide.

medium increased accordingly (Chart 4). Unlike BP 4,5-dihydrodiol, the thioether conjugates had little tendency to remain concentrated in the tissue compared to the perfusion medium (28.5 ± 6.3 nmol/g versus 13.3 ± 1.5 nmol/ml, respectively; $N = 3$).

Although the metabolites were released at rates which reflected enzymatic activities for the 2 pathways, product formation from BP 4,5-oxide occurred about 3 times faster than when the metabolites were removed from the tissue (Table 1).

A comparison of the total BP 4,5-dihydrodiol present in the lungs and in the perfusion medium after 10, 30, or 60 min (Chart 4) revealed that the concentration of this metabolite in the tissue did not significantly decrease after its formation had stopped (30 min). This finding demonstrates that BP 4,5-dihydrodiol, which diffused from the tissue into the perfusion medium during the period of its formation, remained 13 times more concentrated in the tissue than in the perfusion medium after 60 min (66.1 ± 9.6 nmol/g versus 5.04 ± 0.57 nmol/ml, respectively; $N = 3$). The high concentration of BP 4,5-dihydrodiol in the tissue increased accordingly (Chart 4). Unlike BP 4,5-dihydrodiol, the thioether conjugates had little tendency to remain concentrated in the tissue compared to the perfusion medium (28.5 ± 6.3 nmol/g versus 13.3 ± 1.5 nmol/ml, respectively; $N = 3$).

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| Metabolite production and release by perfused rabbit lungs treated with BP 4,5-oxide (5 μmol) |
|---|---|---|
| Rate of formation | Rate of release | Total nmol formed in 60 min |
| BP 4,5-dihydrodiol | 7.60 ± 1.04 | 2.77 ± 0.42 | 1500 ± 140 |
| Thioether conjugates | 23.8 ± 2.3 | 7.44 ± 1.70 | 2880 ± 310 |

*a Initial rates based on nmol product formed per min per g of lung during the first 10 min.

*b nmol product released into the perfusion medium per min per g of lung.

*c Mean ± S.D., $N = 3$.

*d Glutathione and cysteine derivatives of BP 4,5-oxide.
somes in vitro under anaerobic conditions (20), has been suggested as a possible detoxication mechanism in vivo (8). We found no evidence for BP 4,5-oxide reduction in our study, which suggests this pathway has little importance in the perfused rabbit lung.

The rate of BP 4,5-oxide metabolism in this study was at least 3-fold greater than was the estimated rate at which arene oxides were formed from BP by perfused rabbit lungs (36). This indicates that the lung should have no difficulty in enzymatically detoxifying any such epoxides that are suitable substrates for epoxide hydrase or the glutathione S-transferases likely to be formed in vivo. It must be remembered, however, that the 4,5-oxide is only one of several arene oxides involved in the metabolism of BP, and epoxide hydrase and glutathione S-transferase may independently have greater or lesser activities to these compared with the 4,5-oxide (17). Enzyme induction is another parameter which may influence the rates of arene oxide formation and further biotransformation. However, pulmonary tissue in this rabbit strain is generally refractory to the usual increase in epoxide hydrase, glutathione S-transferase, and oxidative drug-metabolizing activities following animal treatment with PAH (14, 15, 29). We found in a previous study that perfused lungs from rabbits treated with 3-methylcholanthrene showed a 2-fold increase in dihydrodiol production from BP compared to lungs from control animals, but the extent of BP conjugation with glutathione and the overall BP biotransformation rate were unchanged (36).

In addition, results obtained from intact lungs or whole-lung subcellular fractions may not accurately demonstrate the metabolic properties of a small but important cell population. Clara cells, for example, make up a small percentage of the total lung mass, but they are believed to be the principal location for the cytochrome P-450-dependent metabolism of ipomeanol and for the initial cytotoxic event associated with the reactive ipomeanol metabolite (5).

The capacity of perfused rabbit lungs to degrade BP 4,5-oxide exceeded their ability to eliminate the products from the tissue. The distribution of BP 4,5-oxide and its metabolites between the tissue and perfusion medium with respect to time suggests that uptake and elimination occurred principally by diffusion and that the extent of elimination was dependent on the lipophilicity of the compound in question. The BP 4,5-dihydrodiol showed a tendency to remain in the lungs, although this effect may have partially resulted from the lack of opportunity for the metabolite to become redistributed to other organs. This tendency was exacerbated by the apparent inability of rabbit lungs to conjugate this metabolite with sulfuric or glucuronic acids. These characteristics of the metabolites and of the tissue may have toxicological consequences in the intact animal; the primary oxidative metabolites of certain chemicals might accumulate in the lung where they would be available for further metabolism to other possibly more toxic compounds. This situation does not appear to apply to BP 4,5-dihydrodiol since secondary metabolism was not detected within the lung during the 60-min period investigated, but BP 7,8-dihydrodiol, a related metabolite from BP, is carcinogenic (21), and this activity is believed to arise from the benzo(a)pyrene 7,8-dihydrodiol-9,10-oxide generated by oxidative metabolism of the BP 7,8-dihydrodiols (19, 22, 37). The release of metabolically formed BP dihydrodiols into the circulation, which occurred in the perfused rabbit lung, may have similar toxicological consequences in other organs to which the diols may be transported, accumulated, and further biotransformed. In contrast to the rabbit lung, the perfused rat liver was able to remove the BP 4,5-dihydrodiol from the tissue and from the circulation by converting it to the more readily excretable glucuronide conjugate, which appeared in bile (34).

Pulmonary uptake and activation of metabolites released into the circulation by the liver may also have toxicological significance, inasmuch as the first capillary bed receiving blood from the liver lies within the lungs. Perfused rabbit lungs have been shown to convert circulating BP 7,8-dihydrodiol to reactive metabolites which become covalently bound to tissue macromolecules.

Our findings suggest that the susceptibility of rabbit lung to PAH-induced carcinogenesis may result from causes other than differences between rates of oxide formation and oxide degradation. It appears that conjugation, excretion, or accumulation of metabolites, as well as the solubility characteristics of the hydrocarbon and its metabolites, may be the most important metabolism-related parameters in the carcinogenesis process. Of course, the ability of a cell to successfully repair genetic damage is also a very important aspect determining susceptibility to carcinogenesis (38).

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