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METABOLISM AND MACROMOLECULAR COVALENT BINDING OF BENZO[*a*]PYRENE IN CULTURED FISCHER-344 RAT LUNG TYPE II EPITHELIAL CELLS*†

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Abstract—Pulmonary biotransformation of many xenobiotics may be important for the mutagenic, carcinogenic and/or toxic response of lung tissue to these compounds. Recently, a lung epithelial cell line (designated LEC), with morphological characteristics suggestive of type II cell origin, was developed in our laboratory. When LEC cells were co-cultivated with Chinese hamster ovary (CHO) cells in a cell-mediated mutagenesis assay, LEC metabolized promutagens to metabolites mutagenic to the CHO cells [A. P. Li, A. L. Brooks, J. M. Benson and F. F. Hahn, *Environ. Mutagen.* **4**, 407 (1982)]. In the present investigation, rates of benzo[*a*]pyrene (BaP) metabolism in type II lung cells were determined, and the effects of various pollutants on rates of BaP metabolism and covalent binding of BaP to LEC macromolecules were measured. Cultures of LEC cells were incubated for 24 hr with 5 μM [^{14}C]BaP, and the culture medium was analyzed for organic- and water-soluble metabolites. LEC cells metabolized BaP to BaP-7,8-diol and BaP-9,10-diol with total rates of formation of these metabolites measured at 500–600 pmoles per 10^6 cells per 24 hr. BaP-9,10-diol was the major metabolite accounting for about 80% of the total BaP metabolized. Enzyme hydrolysis studies revealed the presence of small quantities (less than 20% of BaP metabolized) of the glucuronide conjugates of BaP-7,8-diol and 9-hydroxy-BaP. Pretreatment of LEC cells with benz[*a*]anthracene, coal gas condensate, or diesel exhaust particle extract (DEP) prior to incubation with BaP resulted in a 2- to 5-fold increase in overall rates of BaP metabolism. The largest increase in covalent binding of [^{14}C]BaP equivalents to LEC macromolecules was seen after LEC cells were pretreated with DEP (3-fold). The data suggest that lung epithelial cells may play an important role in the biological fate of inhaled xenobiotics.

Respiratory tract tissue, which serves as a major site of deposition of inhaled xenobiotics, is a target for inhaled circinogens [1, 2]. Most human lung cancers arise from bronchiolar epithelial cells of the upper airways [3–5]; these cells may be particularly sensitive to inhaled carcinogens. Accordingly, research has focused on the role that bronchiolar epithelial cell metabolism plays in the generation of ultimate mutagenic, carcinogenic, and/or toxic species of inhaled xenobiotics which may be responsible for the initiation of cell damage [6, 7].

There have been several reports on the metabolic capability of pulmonary alveolar cells [8–10]. Freshly isolated rabbit pulmonary epithelial type II cells, for example, contain cytochrome P-450 isozymes capable of metabolizing benzo[*a*]pyrene (BaP), 7-ethoxycoumarin, and *N,N*-dimethylaniline [8, 11]. Cell preparations used in these studies were "enriched" with type II cells which contained other cell types, including pulmonary alveolar macrophages (PAM) and Clara cells. Both Clara cells and PAM contain several xenobiotic metabolizing enzymes, including the cytochrome P-450-dependent monoxygenases [12–15]. Jones *et al.* [10] have described recently a method for both the isolation and purification of rat Clara cells and type II cells. The type II cells, which were isolated on the basis of size and density gradient centrifugation, were found to be slightly contaminated with neutrophils. These isolated type II cells contained minimally detectable aryl hydrocarbon hydroxylase activity (BaP as substrate) which was induced by pretreating cells with β-napthoflavone. These studies did not report on the metabolites of BaP formed after incubation of type II cells with BaP.

Recently, a rat lung epithelial cell line (LEC), with morphological characteristics suggestive of type II cell origin, was developed in our laboratory [16, 17]. These LEC cells have been cultured for

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over 18 months *in vitro* and found to retain the lamellar inclusions and the ability to synthesize and secrete surfactant lipids, characteristics of type II cells. When LEC cells were co-cultivated with Chinese hamster ovary cells, which lack xenobiotic metabolizing enzymes, in a cell-mediated mutagenesis assay (LEC-CHO/HGPRT assay), LEC cells activated benzo[*a*]pyrene, 3-methylcholanthrene, and dimethylnitrosamine to mutagenic metabolites [18, 19].

The objective of this investigation was to study the rates of formation of BaP metabolites in cultured LEC cells. The effects of pretreatment of LEC with benz[*a*]anthracene, diesel exhaust particle extract, and low Btu coal gasifier condensate on both metabolism (rates and spectrum of BaP metabolites) and macromolecular covalent binding of BaP to LEC macromolecules were measured, and the results indicate that cultured LEC cells metabolize BaP to several metabolites, the quantities of which can be increased by prior exposure of LEC to environmental pollutants.

MATERIALS AND METHODS

Chemicals. [7,10-^{14}C]BaP (29.7 μCi/μmole) was purchased from Amersham-Searle, Inc., Arlington Heights, IL, and purified (preparative high-performance liquid chromatography) to a radiochemical purity of >99% as determined by high-performance liquid chromatography (HPLC). Radiolabeled BaP was diluted with unlabeled BaP to yield a final specific activity of 20 μCi/μmole. BaP (99% pure) and benz[*a*]anthracene (BA) (99% pure) were purchased from the Aldrich Chemical Co., Milwaukee, WI. β-Glucuronidase, (EC 3.2.1.31), aryl-sulfatase (EC 3.1.6.1), D-saccharic acid 1,4-lactone, and EDTA were purchased from the Sigma Chemical Co., St. Louis, MO. BaP-9,10-diol, BaP-4,5-diol, BaP-7,8-diol, BaP-4,5-oxide, 3-OH-BaP, 9-OH-BaP, BaP-1,6-dione, BaP-3,6-dione, BaP-6,12-dione and BaP-tetrols were obtained from Dr. David G. Longfellow of the National Cancer Institute. All other chemicals utilized were of the highest purity commercially available.

Diesel exhaust particles were collected in dilution tunnels at the Bartlesville Energy Technology Center, Bartlesville, OK, from a light-duty, eight-cylinder diesel-powered car. Details of the collection and extraction of diesel exhaust particles have been described previously [20]. Gasifier samples were collected from a low Btu coal gasifier process stream (Morgantown Energy Technology Center, Morgantown, WV). The low-Btu coal gasifier material used in this study represents the dichloromethane-soluble compounds in relatively clean process gas and chiefly contained compounds of molecular weight < 200 daltons. Higher molecular weight materials had been removed from the process stream by a humidifier, tar trap, and Venturi scrubber.

LEC cell culture and exposure. The LEC strain developed in our laboratory was originally derived from the lungs of an adult male Fischer-344 rat [16, 17]. These cells have been cultured for over 18 months and found to retain lamellar inclusions and the ability to synthesize and secrete surfactant lipids. No evidence of PAM or other cell types in the LEC cultures were noted throughout the experiments.

LEC cells at passages 12–14 were used for these studies. LEC cells were cultured in Ham's F-12 growth medium (Flow Laboratories, McLean, VA) supplemented with 10% newborn calf serum (K. C. Biological Inc., Lenaxa, KN), penicillin (100 units/ml), and streptomycin (100 μg/ml) and incubated at 37° in a humidified atmosphere consisting of 95% air and 5% CO_2. One million cells were seeded in 75 cm^2 culture flasks containing 10 ml of growth medium. The medium was changed 7 days later to one containing 5 μM (1.0 μCi) [^{14}C]BaP in dimethyl sulfoxide (DMSO) (0.5%, v/v) in 10 ml culture medium without serum. Preliminary studies demonstrated that this concentration of BaP was not cytotoxic to LEC. Triplicate flasks containing LEC cells were incubated with [^{14}C]BaP on a rocking platform (10 oscillations/min) for 24 hr. Preliminary studies indicated that BaP metabolite formation in LEC cells was linear for at least 24 hr. After the 24-hr incubation, culture medium was removed and immediately added to 20 ml of ice-cold (4°) ethyl acetate. LEC cells were rinsed twice with an additional 2.5 ml of serum-free medium which then was added to the original 20 ml of medium. Culture medium from individual flasks was extracted twice, and organic layers were removed, pooled, and dried under a gentle flow of nitrogen in subdued light. Dried organic extracts were reconstituted in methanol–acetone (1:1) and analyzed by HPLC (described below).

When the effects of various modifiers on LEC cell metabolism were tested, BA (0 to 2.5 μg/ml), diesel exhaust particle extract (0 to 12.5 μg/ml), or low Btu coal gasifier condensate (0 to 12.5 μg/ml) in DMSO (0.5%, v/v) was added to the culture medium and incubated with LEC cells for 48 hr prior to addition of [^{14}C]BaP. After 24 hr of incubation, fresh serum and modifier were added and allowed to incubate for an additional 24 hr with LEC cells. Control cells were incubated for 48 hr with DMSO (0.5%, v/v). Following the 48-hr incubation, LEC cells were then washed twice with 10 ml of growth medium after which 5 μM (1.0 μCi) [^{14}C]BaP was added.

In all experiments, one of the three culture flasks from each group of incubations was used to determine cell number. Following incubations with BaP the number of cells per flask was determined by enzymatic detachment of cells from the culture flasks using trypsin-EDTA. Cell suspensions were counted using an electronic particle counter (Coulter Electronics, Inc., Hialeak, FL). In all experiments, cell counts did not differ between control and treated LEC cells.

High-performance liquid chromatography of organic extracts of culture medium. Fifteen microliters of the reconstituted organic extract was injected along with a mixture of authentic BaP standards into a Spectra Physics SP 8100 high-performance liquid chromatograph (Spectra Physics Inc., San Jose, CA). The metabolites were separated at ambient temperature on a 4.6 mm (i.d.) × 10 cm analytical column (RP-18; 5 μm) (Brownlee Labs, Santa Clara, CA). A constant flow rate of 1.8 ml/min and a 35-min linear gradient from 55% methanol in water to 100%

methanol was used. Fractions (30 sec, 0.9 ml) of eluate were collected directly into mini-liquid scintillation-counting vials. Scintillation fluors (Aquasol, New England Nuclear Corp., Boston, MA) were added, and the samples were counted in a Packard model 460 liquid scintillation counter. Quench correction was performed by the automatic external standard method with counting efficiencies of >88%. Sufficient counts were accumulated for each peak to provide for <5% error with 95% confidence intervals. Retention times of the radioactive metabolites were compared with standard BaP and metabolites. Quantities of BaP metabolites formed after incubation of BaP with LEC cells are expressed as pmoles product formed per million cells per 24 hr after subtraction of background radioactivity (40 dpm/fraction).

Enzyme hydrolysis. The remaining aqueous portion after the two ethyl acetate extractions was analyzed for conjugated BaP metabolites. Aqueous samples were lyophilized in a Vitris Unitrap (Vitris Co., Gardiner, NY), reconstituted in 500 μl methanol–water (1:2), and 10 μl portions were incubated at 37° for 16 hr with β-glucuronidase and aryl sulfatase as previously described [21]. D-Saccharic acid-1,4-lactone, an inhibitor of β-glucuronidase, was included in some incubations with β-glucuronidase. Additional portions of reconstituted solution were incubated as described above, but in the absence of any enzymes. Following incubation, aliquots of samples were analyzed by HPLC. Portions of incubations were injected along with a mixture of authentic BaP standards into a Spectra Physics SP 8100 high-performance liquid chromatograph. Metabolites were separated as described above with the exception that a constant flow rate of 1.5 ml/min and a 30-min linear gradient from 30% methanol in water to 100% methanol was used. Fractions were collected and analyzed as previously described above.

Macromolecular covalent binding. Following removal of culture medium, LEC cells were scraped from culture flasks with a rubber policeman. Five milliliters of TCA (10%, w/v) was added to the cells, and the pellet obtained by centrifugation was exhaustively extracted [22] to determine the amount of ^{14}C covalently bound to LEC cell macromolecules. Control assays were performed in which [^{14}C]BaP was added to LEC cells which were maintained at 4°. Results are expressed as pmole equivalents of BaP bound per one million cells per 24-hr incubation after subtraction of counts from controls.

RESULTS

LEC cells metabolized BaP to oxidized and conjugated metabolites (Figs. 1 and 2). The major metabolite produced after incubation of LEC cells with BaP was BaP-9,10-diol, which accounted for nearly 80% of the total BaP metabolized (450 pmoles per 10^6 cells per 24 hr). Lesser quantities of BaP-7,8-diol and conjugated metabolites were detected (10–20% of total BaP metabolized; 100 pmoles per 10^6 cells per 24 hr). Enzyme hydrolysis studies revealed the presence of small quantities (<20% of BaP metabolized) of the glucuronide conjugate of BaP-7,8-diol (BaP-7,8-DG) and 9-OH-BaP (BaP-9-G) (Fig. 2). No evidence of sulfate conjugates was detected in any experiments, nor was there any significant extractable radioactivity remaining in the cells.

A dose-dependent increase in the formation of BaP-7,8,diol, BaP-9,10-diol, and conjugated metabolites (BaP-7,8-DG; BaP-9-G) was observed when LEC cells were pretreated with different concentra-

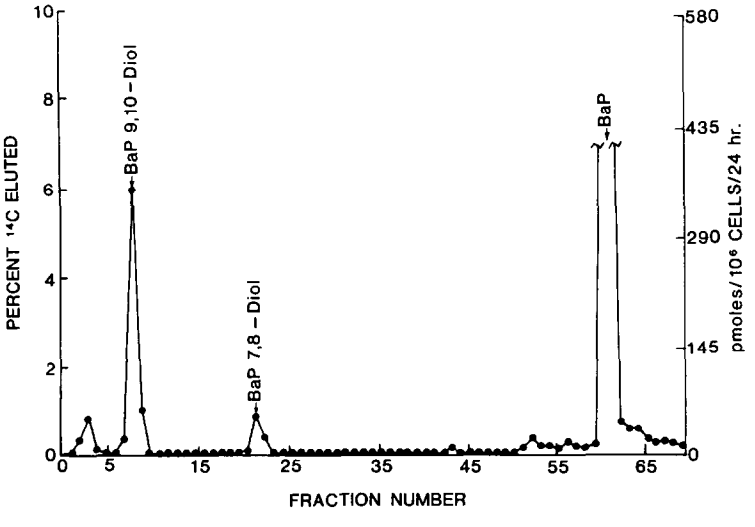

Fig. 1. High-performance liquid chromatogram of BaP metabolites formed after incubation of [^{14}C]BaP with LEC cells. Cells were incubated for 24 hr with 5 μM (1.0 μCi) [^{14}C]BaP. Culture medium was removed and extracted, and organic extracts were dried under N_2. Samples were reconstituted in methanol–acetone (1:1) and aliquots were injected into a high-performance liquid chromatograph for analysis as described in Materials and Methods. Arrows represent the peak fraction in which the BaP metabolite eluted as detected by u.v. absorption (254 nm).

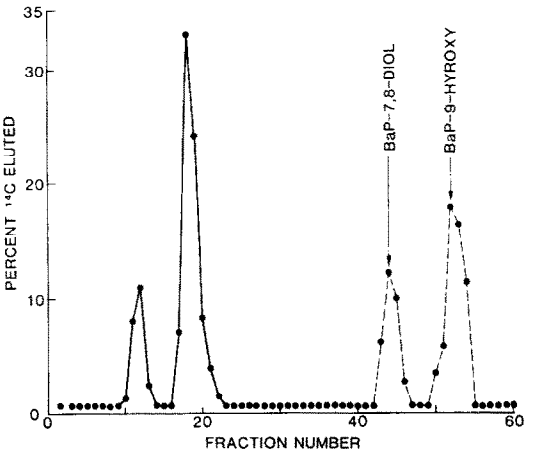

Fig. 2. High-performance liquid chromatogram of BaP conjugated metabolites before and after incubation with β-glucuronidase. Aqueous extracts obtained from culture medium in which LEC cells were incubated for 24 hr with 5 μM (1.0 μCi) [^{14}C]BaP were treated with β-glucuronidase for 16 hr. Portions of enzyme incubations were injected into a high-performance liquid chromatograph for analysis as described in Materials and Methods. Key: (——) BaP conjugates incubated for 16 hr without β-glucuronidase or in the presence of β-glucuronidase and D-saccharic acid-1,4-lactone, and (---) metabolites released upon incubation of aqueous extract with β-glucuronidase. Arrows represent the peak fraction in which the BaP metabolite elutes as detected by u.v. absorption (254 nm).

tions of BA (0 to 2.5 μg/ml culture medium) prior to incubation with [^{14}C]BaP. Maximal increases in the formation of all detectable BaP metabolites were on the order of four to five times that seen when LEC cells were incubated with DMSO only (Fig. 3). Neither higher concentrations of BA nor longer preincubation times resulted in a further increase in LEC cell metabolism of BaP (data not shown).

When the effects of diesel exhaust particle extract and low Btu gasifier condensate on LEC cell metabolism of BaP were tested, a dose-dependent increase in the formatioin of BaP-7,8-diol, BaP-9,10-diol, BaP-7,8-DG, and BaP-9-G was observed with maximal increase in the formation of all detectable BaP metabolites seen at concentrations of 12.5 μg extract/ml culture medium (Fig. 3). Higher concentrations of either diesel exhaust particle extract or the gasifier condensate did not result in further increases in the formation of BaP metabolites (data not shown).

Pretreatment of LEC cells with diesel exhaust particle extract at concentrations (12.5 μg/ml) that yielded maximal rates of BaP metabolism, resulted in a 2- to 3-fold increase in the amount of pmole equivalents of BaP bound to LEC cellular macromolecules when compared to controls (Fig. 3). However, concentrations of BA or low Btu gasifier condensate that maximally increased BaP metabolism by nearly five times that of controls resulted in no significant increase in covalent binding.

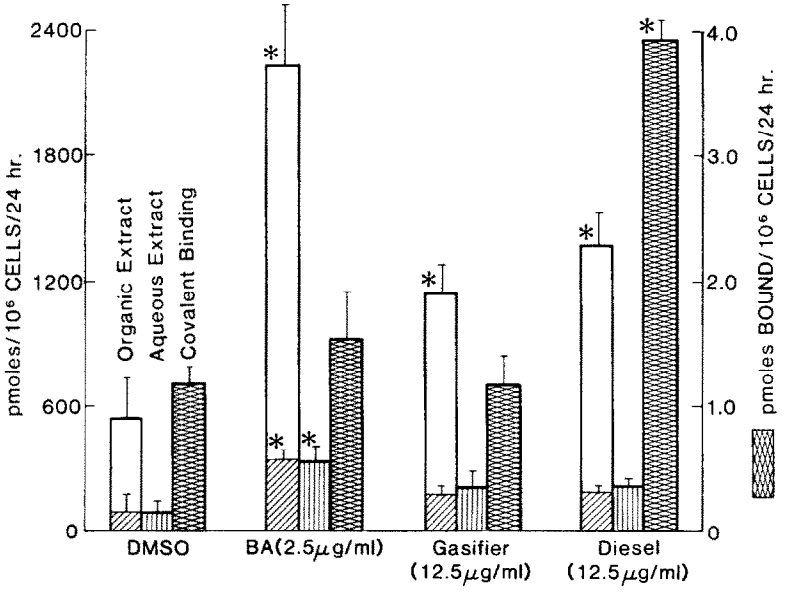

Fig. 3. Effect of benz[*a*]anthracene, low Btu gasifier condensate, and diesel exhaust particle extract on LEC cell metabolism and macromolecular covalent binding of BaP. Cells were incubated with benz[*a*]anthracene (in DMSO), low Btu coal gasifier condensate (in DMSO), and diesel exhaust particle extract (in DMSO) for 48 hr prior to incubation with [^{14}C]BaP. After 48 hr of incubation with extract, cells were incubated for 24 hr with 5 μM (1.0 μC) [^{14}C]BaP after which time the culture medium was analyzed for BaP metabolites. Maximal increases in BaP metabolism observed after treatment with extract are shown in this graph. Key: (▨) organic extract; the hatched portion of the organic extract bar represents BaP-7,8-diol; the open portion represents BaP-9,10-diol; (▩) aqueous extract; (■) macromolecular covalent binding. Error bars represent the standard error of the mean. Asterisks indicate significantly different ($P < 0.05$) from DMSO treatment (Student's *t*-test).

DISCUSSION

The data presented in this study indicate that cultured rat LEC cells have the capacity to metabolize BaP to both oxidized and conjugated metabolites. These data are consistent with reports from other laboratories in which it was shown that alveolar type II cells are capable of metabolizing several xenobiotics including BaP [8, 10, 15, 23]. In contrast to data from other laboratories in which freshly isolated alveolar type II cells were found to metabolize BaP to phenols [8, 10, 23], the cultured LEC cells used in this study metabolized BaP primarily to dihydrodiols and only small quantities of phenol (9-OH-BaP) as determined from the enzyme hydrolysis studies.

Consistent with other reports, LEC cells displayed very low rates of metabolism when compared to the more active Clara cells [10]. There is evidence that cytochrome P-450-I is present in type II cells, and it has been suggested that this cytochrome is responsible for alveolar type II cell metabolsim of BaP [11].

BaP metabolism in LEC cells was increased markedly (2- to 5-fold) after prior treatment of cells with BA, diesel exhaust particle extract, or low Btu gasifier condensate. This observation is similar to previous studies [9, 10] in which induction of BaP metabolism in primary cultures of type II alveolar cells was demonstrated. A concomitant increase in the macromolecular covalent binding of [^{14}C]BaP equivalents was seen only after pretreatment with diesel exhaust particle extract. Previous reports have demonstrated that both the diesel exhaust particle extract and low Btu coal gasifier condensate used in these studies contain a complex mixture of chemicals, including polycyclic aromatic hydrocarbons [24, 25,*]. The most likely candidates in the extracts for induction in the LEC cells are the polycyclic aromatic hydrocarbons; however, due to the nature of the complex mixtures, it is possible that several chemicals are responsible for the overall induction observed. The observation that neither BA nor gasifier condensate increased the macromolecular covalent binding of [^{14}C]BaP suggests that these inducers may also increase other enzyme activities which may be responsible for the detoxification of reactive BaP intermediates produced by LEC cells.

Reports from our laboratory have shown that LEC cells metabolize BaP to mutagens in the CHO/HGPRT mutation assay [18], suggesting that LEC cells are capable of forming highly reactive mutagenic BaP metabolites. The data presented in this study indicate that LEC cells can metabolize BaP to metabolites which covalently bind to LEC cellular macromolecules and that the quantities of ^{14}C bound are increased after pretreatment of LEC cells with diesel exhaust particle extract. These results are consistent with the observation that diesel exhaust particle extract enhances the mutagenicity of BaP in the CHO/HGPRT assay [26]. LEC cells from our laboratory have the capacity to form reactive epoxides as suggested by the observation that LEC metabolize BaP to BaP-7,8-diol.

Very little importance has been attached to the xenobiotic-metabolizing capacity of type II lung cells, primarily because their metabolic capability appears to be considerably less than that measured in other lung cells, particularly the Clara cells. There are good data which implicate Clara cells in the formation of reactive intermediates that may be responsible for the production of lung tumors [14]. Similar evidence for type II alveolar cells has not been demonstrated. Type II cell metabolism, however, may be particularly important for determining the metabolic fate of inhaled xenobiotics which reach the deep lung since many environmental pollutants contain known carcinogens adsorbed onto particles of sizes which deposit through the respiratory tract [27, 28]. Thus, ultrafine particles (<0.3 μm aerodynamic diameter) would tend to reach the alveolar cells of the deep lung and be acted upon by enzymes located within the cells. The data presented in this report indicate that epithelial cells of type II cell origin may play an important role in the biological fate of inhaled xenobiotics.

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