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Effect of Polycyclic Aromatic Hydrocarbons on Generation and Efflux of Glutathione Conjugates in Primary Cultured Alveolar Epithelial Cells

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Summary: This study was initiated to functionally characterize multidrug resistance associated protein (MRP)-mediated transport across the lung epithelium. Alveolar type II cells were isolated from rabbit lung and cultured on Transwell until forming a tight monolayers. After the cell monolayer was preloaded with monochlorobimane (mBCl) that is metabolized to a fluorescent glutathione conjugate (mBCl-SG), amount of mBCl-SG exported to apical and basal compartments were measured periodically. mBCl-SG was more preferentially exported in the apical direction than in the basolateral direction. Efflux of mBCl-SG from alveolar epithelial cells was significantly inhibited by a MRP inhibitor MK-571. Pharmacokinetic analysis of efflux profiles revealed that increased efflux of mBCl-SG by B[a]P is not due to enhanced MRP activity but simply due to an elevated level of mBCl-SG in the cells. Elevation of the intracellular level of mBCl-SG corresponded well to that of reduced GSH caused by B[a]P pretreatment.

Key words: benzo[a]pyrene; glutathione conjugate; alveolar type II cells; multidrug resistance-associated protein; transport

Introduction

The lung epithelium, which is directly exposed to an air, possesses metabolic pathways for detoxification of inhaled substances. These metabolic pathways include the phase I reactions catalyzed by the cytochrome P-450 mono-oxygenase system and the phase II reactions catalyzed by enzymes such as glutathione S-transferase and quinone oxidoreductase. Of thousands of compounds inhaled during respiration, there are compounds that undergo metabolic activation through xenobiotic-metabolizing CYP enzymes. Some of polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (B[a]P) are contained in car exhaust fumes, cigarette smoke, and volatile fuels, and activated metabolically through CYPIA enzymes.1,2) Enzymatic activation of PAHs has been implicated in the incidence of lung cancers.1,2) PAHs are also known to induce both phase I and II drug-metabolizing enzymes. These inductors elevate CYP1A1 expression via a xenobiotic response element (XRE),3,4) and are subsequently converted by CYP1A1 to electrophiles that induce phase II enzymes (GST Ya, glucuronosyl transferase, aldehydehydrogenase and NQO1) via an electrophilic response element/antioxidant response element (ARE/EqRE).5) It has been reported that PAHs containing cigarette smoke would affect pharmacokinetics of drugs due to induction of drug metabolizing enzymes.6,7)

On the other hands, ABC transporters including multidrug resistance-associated proteins (MRPs) have been known to be responsible for active efflux of xenobiotics and their metabolites.8,9) Some of MRPs are ubiquitously expressed in the body: MRP1 and MRP5 are detected in the lung.8,9) These MRPs appear to be responsible for active export of xenobiotics and metabolites in the lung epithelium, but the details are yet to be known. In addition, inducers of xenobiotic metabolizing enzymes, that appear to increase the intracellular level of metabolites, may affect the expression of the transporters responsible for export of metabolites. Alteration of functional activity of the transporters might modulate pharmacokinetics and efficacy of inhaled drugs.

This study was initiated to functionally characterize...
MRP-mediated transport across the lung epithelium and examine whether PAHs modulate the transporter activity. When alveolar type II epithelial cells are cultured, they form a tight monolayer that morphologically and biochemically resembles native lung epithelium. The primary cultured alveolar epithelial cell monolayers are suitable for evaluating vectorial transport of drugs. This might be able to elucidate the directionality of efflux of detoxified metabolites, i.e., either the air space side or the blood side. In this study, we examined efflux of glutathione-bimane (mBCl-SG), as a model substrate and effect of B[a]P on generation and efflux of the glutathione conjugate in rabbit alveolar cell monolayers cultured on Transwell.

**Materials and Methods**

**Materials:** Porcine pancreatic elastase and pure Griffonia simplicifolia lectin (GS-I) were obtained from Worthington Biochemical Corporation (Freehold, NJ) and EY Laboratories, INC. (San Mateo, CO), respectively. Triethanolamine and 2-vinylpyridine were obtained from Sigma Aldrich. Soybean trypsin inhibitor, bovine serum albumin (BSA), hydrocortisone, HEPES, Dulbecco’s modified minimum Eagle’s medium nutrient mixture F-12 HAM (DMEM/F12), Eagle’s minimum essential medium Joklik, L-buthionine-(S,R)-sulfoximine, Amphotericin solubilized, 7-ethoxy-3H-Phenoxazin-3-one (ethoxyresorufin) and benzo[a]pyrene were obtained from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Equitech-Bio (Kerrville, TX). ITS + Premix, type I rat tail collagen, and recombinant human fibronectin were obtained Becton Dickinson Biosciences (Bedford, MA). DNase I was obtained from Roche. Human recombinant ighestron were obtained Becton Dickinson (Bedford, MA). DNase I was obtained from Roche. Human recombinant epidermal growth factor (EGF) was obtained from AUSTRAL Biologicals (San Ramon, California). GSSG standard and DTNB were obtained from Cayman Chemical (Ann Arbor, MI). Nonessential amino acids, penicillin and streptomycin were obtained from Gibco BRL (Gaithersburg, MD). Verapamil hydrochloride was purchased from Nacalai Tesque (Kyoto, Japan). Tetraethylammonium bromide and p-aminophippuric acid were obtained from Pure Chemical Industries, Ltd (Osaka, Japan). Monochlorobimane [syn-(ClCH2,CH3)-1,5-diazabicyclo[3.3.0]-octa-3,6-dione-2,8-dione; mBCl] was obtained from Molecular Probes (Eugene, OR). MK-571 was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Other cell culture reagents were from Invitrogen (Grand Island, NY).

**Primary culture of alveolar epithelial cell monolayers:** For isolation of alveolar epithelial type II cells, three in-house balanced salt solutions were prepared: one balanced salt solution A (BSSA) was composed of 137 mM NaCl, 5.0 mM KCl, 0.7 mM Na2HPO4, 10 mM Hepes, 5.5 mM glucose, and 3 mM EDTA at the pH adjusted to 7.4, and the other balanced salt solution (BSSB) was BSSA supplemented with 1.8 mM CaCl2 and 1.2 mM MgSO4 instead of 3 mM EDTA.

Alveolar epithelial type II cells were isolated from the rabbit lung using a method of Shen et al. Animals were first injected with heparin (1000 U/kg) and then euthanized by a rapid injection of sodium pentobarbital (1.5 mg/kg), both via a marginal ear vein. While the lung was ventilated manually through tracheal cannula with a 60 mL syringe, it was perfused with BSSA via the pulmonary vein. Afterwards, the lung was excised and lavaged several times with BSSA and once with SMEM containing 2 U/mL elastase. About 40 mL of a 2 U/mL elastase solution was instilled through the trachea and the lung was incubated in BSSB at 37°C for 35 min. After the trachea and bronchi were removed, the lung pieces were minced in SMEM containing 1.67 mg/mL trypsin inhibitor, suspended in SMEM, and sequentially filtered through gauze, a 45 μm cell strainer, and a 15 μm nylon mesh. The cell suspension was centrifuged at 200 x g for 8 min at 4°C. The cell pellets were resuspended in SMEM, incubated with 16 mg/mL GS-I lectin at room temperature for 30 min and subsequently filtered through a 15 μm nylon mesh. The filtered cell suspension was centrifuged and the cell pellet was resuspended in DMEM/F12 containing 10% FBS. Cell viability was estimated to be more than 95% by the trypan blue dye exclusion method.

Purified type II cells were plated onto Transwell (Corning, Corning, NY) at a density of 0.88 × 10⁶ cells/cm². The plastic plates were pretreated for 4 h with 0.25 mL/cm² DMEM/F12 containing 30 μg/mL collagen, 10 μg/mL fibronectin, and 10 μg/mL BSA. From day 3 in culture onward, the culture medium was changed to serum-free DMEM/F12 supplemented with ITS+, 10 ng/mL EGF and 1 μM hydrocortisone. The cultures were fed every other day with a serum-free defined medium. The volume of apical and basal media was 0.5 and 1.5 mL, respectively. The cultures were maintained in a humidified 5% CO₂ incubator at 37°C.

Transepithelial electric resistance (TEER, kΩ) was measured with an EVOM device. The rabbit alveolar epithelial cells in primary culture formed tight cell monolayers from day 3 onward, of which transepithelial electric resistance (TEER) was approximately 1.5 kΩcm². The rabbit alveolar epithelial cells monolayers on day 6 in culture were used to have experiments.

**Transport studies in primary cultured rabbit alveolar epithelial cells:** Rabbit alveolar epithelial cells cultured on 12-well Tranwells for 6 days were washed twice with ice-cold HBSS. The cells were treated with 100 μM mBCl in 0.5 mL HBSS as an apical bathing fluid for 60 min at 4°C. 1.5 mL HBSS was placed as a basal bathing fluid. It is known that mBCl is freely permeable across the cell membrane and conjugated to GSH by GST to
form a fluorescent product (mBCl-SG) in the cytosol.\textsuperscript{13)} After pretreatment with mBCl, the cells were washed twice with cold HBSS and incubated in 0.5 mL apical HBSS and 1.5 mL basal HBSS. Then, the efflux of mBCl-SG to the apical and basal bathing fluid was monitored by taking a 200 \( \mu \)L aliquot from both sides periodically. The corresponding volume of fresh HBSS was added to maintain total volume of bathing fluids constant during the experiment. The mBCl-SG content in the samples was measured by determining the fluorescence intensity using the luminescence spectrometer with excitation wavelength set to 485 nm and emission wavelength of 535 nm. A preliminary experiment found that the sum of fluorescent intensities effluxed and associated with the cells at 60 min (3760 \pm 460 fluorescent intensity unit (f.i.u.)) was not different from fluorescent intensity associated with the cells at 0 min (3590 \pm 590 f.i.u.).

In some cases, 100 \( \mu \)M B[a]P was added to the apical bathing medium 24 h prior to the transport experiment. Effect of 24-h treatment with B[a]P on TEER was not observed (1.58 \pm 0.06 and 1.44 \pm 0.12 1.5 k\Omega \cdot \text{cm}^2 for control and B[a]P treatment, respectively).

**Estimation of mBCl-SG efflux profile:** The efflux profiles of mBCl-SG were analyzed based on one-compartment model, where apical and basal efflux processes were considered. Assuming that apical and basal compartments were under sink condition and that production of mBCl-SG was negligible during the efflux phase, the equations expressing the amounts effluxed are expressed as,

\[
X_a = \frac{k_aX_0}{k_a+k_b}(1-\exp(-(k_a+k_b)t))
\]

\[
X_b = \frac{k_bX_0}{k_a+k_b}(1-\exp(-(k_a+k_b)t))
\]

where \( X_0 \) is the initial amount of mBCl-SG in cells; \( k_a \) and \( k_b \) are apical and basal efflux rate constants, respectively.

These parameters were estimated by fitting the equations to the efflux profiles using a nonlinear regression program MULTI.\textsuperscript{14)}

**Cellular GSH and GSSG measurement:** Alveolar epithelial cells were plated on 12-well plastic plates at a density of \( 0.88 \times 10^5 \) cells/cm\(^2\) and cultured in 2 mL culture medium. On day 5, B[a]P was added to yield a final concentration of 100 \( \mu \)M. Twenty four hours later, the cells were washed twice with cold PBS and lyzed by hypotonic shock with 2 mL cold distilled water. The lysate was homogenized in a glass homogenizer and centrifuged (10,000 \( \times \) g, 10 min, 4\(^\circ\)C). Total GSH in the supernatant was immediately determined using the DTNB-GSSG reductase recycling assay.\textsuperscript{15)} To determine the GSSG contents, 1 mL of the supernatant was mixed with 50 \( \mu \)L of 4M triethanolamine and 50 \( \mu \)L of 2-vinylpyridine, incubated for 1 h, and subjected to the DTNB-GSSG assay.\textsuperscript{15)}

**Results**

**Time-dependent Efflux of mBCl-SG in alveolar epithelial cell monolayers:** Figure 1 shows the efflux of mBCl-SG from alveolar epithelial cell monolayers grown on Transwell to apical and basal compartments. The efflux rate of mBCl-SG to the apical compartment was significantly greater than to the basal compartment. When intracellular GSH was depleted by pretreating the cells for 48 h with 1 mM L-buthionine-(S,R)-sulfoximine (BSO),\textsuperscript{16)} the efflux to both compartments was smaller than a detection limit. On the other hand, lowering temperature from 37 to 4\(^\circ\)C decreased the apical and basolateral effluxes to 33.7 and 35.1\% of the control, respectively.

The efflux of mBCl-SG from alveolar epithelial cells was measured in the presence or absence of inhibitors for various transporters (Fig. 2). A MRP inhibitor MK-571\textsuperscript{17)} significantly decreased the efflux of mBCl-SG to both compartments, whereas verapamil, tetraethylammonium and \( p \)-aminohippuric acid failed it.

**Effect of B[a]P on efflux of mBCl-SG in alveolar epithelial cell monolayers:** When alveolar epithelial cells were pretreated with 100 \( \mu \)M B[a]P for 24 h, the amount of mBCl-SG effluxed was significantly elevated (Fig. 3). To evaluate the effect of B[a]P quantitatively, pharmacokinetic analysis of efflux profiles were carried out. Table 1 summarizes the efflux rate constants of apical and basal compartments (\( k_a \) and \( k_b \)), and an initial amount of mBCl-SG in the cells (\( X_0 \)). When the cells were pretreated with B[a]P, \( X_0 \) was remarkably larger than that of control whereas \( k_a \) and \( k_b \) were not so
much changed.

Pretreatment of the cells with B[a]P increased the intracellular level of reduced GSH from \(5.72 \pm 0.81\) to \(14.7 \pm 2.3\) nmol/well. The increase in reduced GSH would result in elevating the level of glutathione conjugates including mBCl-SG.

**Discussion**

A family of MRPs is members of the ATP binding cassette (ABC) superfamily of transport proteins. This superfamily is among the largest and most widespread protein superfamilies known and its members are responsible for the active transport of a wide variety of compounds across biological membranes including phospholipids, ions, peptides, steroids, polysaccharides, amino acids, organic anions, drugs and other xenobiotics\(^8,9\). Some of MRPs such as MRP1 and MRP5 are known to be ubiquitously expressed throughout the body, including the lung\(^8,9\). The present study demonstrated that the efflux of the conjugate mBCl-SG was inhabitable by a MRP inhibitor MK-571. Therefore, it is likely that MRP is functionally expressed in alveolar epithelium and responsible for efflux of glutathione conjugates. Interestingly, the present study using primary cultured cell monolayer demonstrated that MRP-mediated export from alveolar epithelial cells occurred primarily in the apical direction. Lehmann *et al.*\(^18\) confirmed an expression of immunoreactive MRP1 protein in cultured human peripheral lung epithelial cells, as well as human bronchial epithelial cells. However, it has generally been known that, with specific antibodies, MRP2 is located in apical, and MRP1 and MRP3 in basolateral membranes of tissues\(^8\). MRP1 has been detected immunohistochemically in the basal membrane

### Table 1. Efflux rate constants \((k)\) and initial fluorescence intensity \((X_0)\) of mBCl-SG in primary cultured rabbit alveolar epithelial cell monolayers\(^a\).

<table>
<thead>
<tr>
<th></th>
<th>(k) (min(^{-1}))</th>
<th>(X_0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>apical</td>
<td>basolateral</td>
</tr>
<tr>
<td>Control</td>
<td>0.0944 ± 0.0197</td>
<td>0.0373 ± 0.0198</td>
</tr>
<tr>
<td>100 (\mu)M B[a]P(^b)</td>
<td>0.107 ± 0.001</td>
<td>0.0506 ± 0.0141</td>
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\(a\) Each parameter was expressed as average ± S.D. of three experiments.

\(b\) Statistical difference between B[a]P treatment group and control group was evaluated by Student t test (**P < 0.01).
of bronchial and bronchiolar epithelium of normal human lung.\(^{19}\) On the other hand, MRP2 was not detected in the lung.\(^{19}\) It remains to be elucidated what molecular species is responsible for apical-dominant efflux transport observed in this study. Unfortunately, no information on rabbit MRPs are available at this moment.

Kauffmann et al.\(^{20}\) investigated the inducibility of the drug conjugate transporter genes MRP1 and MRP2. The human MRP2 gene was inducible in HepG2 cells by rifampicin, clotrimazol, arsenite and tertiary butylated hydroquinone (tBHQ), while human MRP1 gene was induced in MCF-7 cells by only tBHQ and quercetin. They demonstrated in reporter gene assays that proximal promoter regions of the genes contribute to the induction, the deletion of binding sites supposed to mediate the induction process (a PXR-tribute to the induction, the deletion of binding sites for the inducer tBHQ) did not result in a significant decrease in the induction factor. Recently, Hayashi et al.\(^{20}\) revealed that nuclear factor-E2 p45-related factor (Nrf) 2 is involved in both the constitutive and inducible mRNA and protein expression of MRP1, using mouse embryonic fibroblasts prepared from wild type and Nrf2 knockout mice. It remains unclear whether induction of MRPs by redox-active compounds occurs in the lung epithelium.

However, pharmacokinetic analysis revealed that mBCi-SG efflux by B[a]P treatment is augmented simply by an increase in intracellular level of the metabolite. In addition, we found that the level of reduced GSH was increased by B[a]P treatment. Liu et al.\(^{20}\) demonstrated that 5,10-dihydroindeno[1,2-\(\beta\)]indole (DII) increased GSH levels in mouse hepatoma cell lines (Hepa-1c1c7) whereas it was not observed in aryl hydrocarbon (Ah) receptor-deficient mutant. This suggested that \(\gamma\)-glutamylcysteine synthetase, the rate-limiting enzyme of GSH synthesis, be induced via a functional Ah receptor. The same mechanism might be responsible for elevation of intracellular GSH by B[a]P, an Ah receptor ligand.

In conclusion, we revealed functional presence of MRP in primary cultured rabbit alveolar epithelial cell monolayers. The transporters are expressed in the apical membrane more than in the basolateral membrane. B[a]P, an inducer of several xenobiotic metabolizing enzymes, elevated intracellular reduced GSH and glutathione conjugates, although it did not alter functional activity of MRP.

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