Polycyclic aromatic hydrocarbons impair β_2AR function in airway epithelial and smooth muscle cells

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Methods

Primary mouse tracheal epithelial cell (mTEC) isolation. Use of animals for the studies described herein was approved by the Columbia University Animal Care and Use Committee. Mice for these studies were specific-pathogen free C57BL/6 mice obtained from Charles River Laboratories (Wilmington, MA). MTEC monolayers were grown on semi-permeable supports with an apical air-liquid interface to recapitulate the *in vivo* phenotype of a high-resistance epithelium with functional cilia as described (1). Briefly, tracheas isolated from C57b6 mice were treated with overnight digestion with pronase and purified by differential adherence to plastic as described by Brody and colleagues (1). MTECwere plated on Transwellfilter inserts (Corning, Inc., Corning, NY) and grown in DMEM–Ham's F-12 medium with 30 mM HEPES, 4 mML-glutamine, 3.5 mM NaHCO3, 100 U/ml penicillin, and 100 mg/ml streptomycin, supplemented with 10 μg/ml insulin, 10 μg/ml transferrin, 0.1 μg/ml cholera toxin, 25 ng/ml epithelial growth factor (Becton Dickinson, Bedford, MA), 30 μg/ml bovine pituitary extract, 0.01 μM retinoic acid, 5% fetal bovine serum, amphotericin B (0.25 μg/ml), penicillin, gentamicin. Medium was providedinitially in the upper and lower chambers. When transmembrane resistance reached 1,000Ω · cm^{-2}, medium in the apical chamber was removed to create an air liquid interface and the lower compartment medium was changed to basic medium supplemented with 2% Nuserum (BD BioSciences, San Diego,CA) and retinoic acid, this typically was at day 2 or 3 after harvest. Cells were studied at 7-10 days in culture.

Primary human airway smooth muscle (HASM)cell isolation. Tracheas were obtained from unused human lung allographs in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving HumanBeings. A segment of the trachealis muscle just proximal to the carina wasdissected under sterile conditions, minced, centrifuged, and resuspended in 0.2 mM $CaCl_2$, 640 U/ml of collagenase, 10mg of soybean trypsin inhibitor, and 10 U/ml of elastase.Enzymatic dissociation of the tissue was performed for 90min in a shaking water bath at 37°C. The cell suspension wasfiltered through 125-μm Nytex mesh, and washed with cold Ham's F-12 mediumwith 10% fetal bovine serum. Aliquots of thecell suspension were plated at a density of $1.0x10^4$cells/cm^2.Ham's F-12 medium supplemented with 10% fetal bovineserum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and100 μg/ml of amphotericin B was replaced every 72 hours. Detailsregarding the characterization of this cell line have been reported (2-4).

PAH mixture. In prior work from the Columbia Center for Childrens Environmental Health (CCCEH), pregnant Dominican and African-American women age 18-35 years living in the Washington Heights, Central Harlem, and South Bronx

neighborhoods of New York, NY wore personal air sampling devices for 48 hours during the third trimester of pregnancy. Study participants wore the devices during the daytime and placed them adjacent to their beds at night. These devices operated continuously over this period collecting vapors and particles ≤2.5 μm in diameter in a pre-cleaned quartz microfiber filter and a pre-cleaned polyurethane foam cartridge backup. Samples thus collected were analyzed by the Southwest Research Institute for 8 carcinogenic PAHs: benz[a]anthracene, chrysene, benzo[b]fluroanthene,benzo[k]fluroanthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene,disbenz[a,h]anthracene, benzo[g,h,i]perylene plus pyrene as described previously (5, 6). The mixture for cellular assays was composed at Lovelace Respiratory Research Institute to represent proportionally levels measured from CCCEH participants (Table 1). This mixture was suspended in DMSO (100%) at a total stock PAH concentration of 55.9 ng/ml.

MTEC and HSAM cells were exposed to varying concentrations of the PAH mixture diluted with serum free medium for 24 hours prior to assessment of β_2AR function (procaterol-induced cAMP production). These concentrations were based on preliminary dose finding experiments that used β_2AR function as a physiologic end-point.

Whole cell membrane isolation, western analysis, real time PCR.To gauge if changes in β_2AR function in MTEC and HASM cells correlate with membrane bound receptor number, β_2AR expression was evaluated via western analysis of whole cell membrane fractions. Membrane proteins were obtained by homogenizing cells in situ in homogenization buffer (300 mMmannitol, 10 mMHepes-Tris (pH 7.4) with 3mM EGTA/1mM EDTA, 0.1mM PMSF (all from Sigma, St. Louis, MO) and protease inhibitor cocktail (Roche Diagnostics)(7). Cell debris was removed by centrifugation at 10,000xg for 20 minutes at 4˚C. The resultant supernatant was centrifuged at 100,000xg at 4˚C. The pellet containing whole cell membrane fractions was resuspended in 100μl of homogenization buffer and protein content quantified (Bio-Rad protein assay, Bio-Rad, Hercules, CA). For western analysis 10 μg of whole cell membrane protein/lane was separated by 4-12% SDS-PAGE, electrophoretically transferred to nitrocellulose and probed with rabbit anti-mouse or anti-human β_2AR antibody (Santa Cruz Scientific, Santa Cruz, CA). Protein bands were visualized using peroxidase coupled secondary antibodies and a chemi-luminescent detection kit (Pierce, Rockford, IL).To verify equivalent sample loading, blots were stripped and reprobed with mouse monoclonal anti-actin antibodies (Chemicon International). Quantitative, real-time rtPCR using human and mouse β_2AR primers (Taqman, Applied Biosystems, Foster City, CA) and Applied Biosystem reverse transcriptase reagents were used to evaluate steady-state β_2AR mRNA expression. Copy numbers were calculated for β_2ARfor each sample and normalized to copy numbers of GAPDH.

Measurement of cellular cAMP production(β_2AR function).β_2AR function was assessed by measuring whole cell cAMP concentrations after treatment of cells with the β_2AR specific agonist procaterol (10^{-6}M, 15 minutes @ 37°C). In all experiments, cells were pretreated with 10^{-4}M IBMX for 15 minutes to inhibit phosphodiesterases. To assessadenylyl cyclase function cells were treated with forskolin ($2x10^{-5}$M) for 15 min at 37°C.Cyclic AMP was quantified using

an enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) as described previously (8). Measurements were performed in triplicate and are presented as pmolcAMP/mg protein.

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