Polycyclic aromatic hydrocarbons impair β2AR function in airway epithelial and smooth muscle cells

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Running Head: Polycyclic aromatic hydrocarbons impair β2AR

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Abstract
Rationale: Incomplete combustion produces a pollutant mixture that includes polycyclic aromatic hydrocarbons (PAH). Prior work by the Columbia Center for Children's Environmental Health (CCCEH) and others has linked exposure to PAH with asthma symptoms and other adverse health effects in young children. Inhaled β2-adrenergic agonists are mainstays in the treatment of reactive airways diseases. These exogenous catecholamines engage membrane bound β2-adrenergic receptors (β2AR) on airway epithelial and smooth muscle cells to cause airway dilation.

Objective: We hypothesized that exposure to PAH might similarly interfere with β2AR function on airway epithelial and/or smooth muscle cells, thereby reducing the efficacy of a medication important for treatment of asthma symptoms.

Methods: A PAH mixture was devised based on ambient levels measured prenatally among a cohort of pregnant women participating in the Columbia Center for Children's Environmental Health. Primary airway epithelial and smooth muscle cells were exposed to varying concentrations of the PAH mixture and β2AR expression, function and signaling assessed.

Results: Mouse tracheal epithelial cells and humans airway smooth muscle cells exposed to a PAH mixture experienced reduced β2AR expression and function.
Conclusions: These findings support our hypothesis that environmentally relevant PAH can impede β2AR-mediated airway relaxation and suggest a new paradigm where air pollutants not only contribute to the pathogenesis of childhood asthma but also diminish responsiveness to standard therapy.

Key words: Polycyclic aromatic hydrocarbons, β2-adrenergic receptors

Introduction

Incomplete combustion produces a pollutant mixture that includes high concentrations of polycyclic aromatic hydrocarbons (PAHs). All of these constituents have been associated with adverse respiratory effects (2-4). While traffic emissions remain the primary source of PAH in US cities, they also are emitted readily from industrial sources, cigarette smoking, incense burning, cooking and space heating (5, 6). Prior work by the Columbia Center for Children's Environmental Health (CCCEH) and others has linked exposure to ambient PAHs in particular with asthma-like symptoms in young children and seroatopy (2, 7, 8). However, the mechanism responsible for these findings has not yet been elucidated.

Inhaled β2-adrenergic agonists are mainstays in the treatment of asthma and other reactive airways diseases. These exogenous catecholamines engage membrane bound β2-adrenergic receptors (β2AR) on airway epithelial and smooth muscle cells to cause airway dilation (9). Data from adipocytes indicate that PAHs impair β-receptor function without reducing membrane bound receptor number (10). We hypothesized that exposure to traffic-related PAHs might interfere with airway β2AR function. This effect would reduce the efficacy of an important asthma medication and worsen asthma symptoms. Our approach was to expose two different primary lung cell types that play important roles in the pathophysiology of airway obstruction in asthma to a PAH mixture prior to assessment of β2AR function. One included mouse tracheal epithelial cells (MTEC) monolayers that were grown on semi-permeable supports with an apical air-liquid interface. This method recapitulates their in vivo phenotype of a high-resistance epithelium with functional cilia (11). In addition, human airway smooth muscles (HASM) cells were studied using similar methods to determine whether PAHs alter smooth muscle cell β2AR function and expression. To mimic urban ambient exposure, the PAH components used in these studies were constituted to proportionally resemble New York City levels measured during personal monitoring of airborne levels among pregnant women from the CCCEH cohort.

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 from C57BL/6 mice were isolated and grown on semi-permeable supports (Transwell filter inserts, Corning, Inc., Corning, NY) with an apical air-liquid interface as described (11). Medium (DMEM–Ham’s F-12) was provided initially 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 2% Nuserum (BD BioSciences, San Diego, CA) with retinoic acid, this was done on day 2 or 3 in culture. Cells were at 7-10 total days.

Primary human airway smooth muscle (HASM) cell isolation. HASM cells were isolated from trachealis muscles from unused human lung allografts in accordance with guidelines of the University of Pennsylvania Committee on Studies
Involving Human Beings as described elsewhere (12-14) and in the online methods that accompany this article. HASM cells were plated on tissue culture treated plastic at a density of $1.0 \times 10^4$ cells/cm$^2$ in Ham’s F-12 medium supplemented with 10% fetal bovine serum and antibiotics. HASM viability by trypan blue exclusion after 24 hours treatment with 1.86ng/ml, 2.80ng/ml, or 5.59ng/ml of PAH was 96%, 90%, and 85%, respectively. Cells treated with 10% DMSO had viability of 82%.

**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 continuously active personal air sampling devices for 48 hours during the third trimester of pregnancy. The exposure to 8 carcinogenic PAHs: benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenz[a,h]anthracene, benzo[g,h,i]perylene plus pyrene were determined as described elsewhere (2, 15). A PAH mixture that reproduces the PAH exposure of the CCCEH participants (Table 1) was produced for the cellular experiments described below. This PAH mixture was suspended in DMSO at a 55.9 ng/ml. The dose of the PAH mixture was calculated as following. Ambient PAH concentration ranged from 1-50 ng/m$^3$, with the highest concentration being Pyrene at 50 ng/m$^3$. The inhalation dose of Pyrene was determined based on a 15 L/minute breathing rate, exposure for 8 hrs per day, deposition of inhaled Pyrene to the lung at 30% of inhaled, and a lung internal surface area of 135 m$^2$. The resulting calculated dose is approximately 1 pg Pyrene/cm$^2$ lung.

**Whole cell membrane isolation, western analysis, real time PCR.** Membrane bound $\beta_2$AR expression was evaluated via western analysis of whole cell membrane fractions as described previously(16). For western analysis 10 µg of whole cell membrane protein/lane was probed with rabbit anti-mouse or anti-human $\beta_2$AR antibody (Santa Cruz Scientific, Santa Cruz, CA) and peroxidase coupled secondary antibodies. 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 $\beta_2$AR primers (Taqman, Applied Biosystems, Foster City, CA) and Applied Biosystem reverse transcriptase reagents were used to evaluate steady-state $\beta_2$AR mRNA expression. $\beta_2$AR copy numbers were normalized to copy numbers of GAPDH.

**Measurement of cellular cAMP production ($\beta_2$AR function).** $\beta_2$AR function was assessed by measuring whole cell cAMP concentrations after treatment of cells with the $\beta_2$AR 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 assess adenylyl cyclase function cells were treated with forskolin ($2\times10^{-5}$M) for 15 min at 37°C. Cyclic AMP was quantified using an enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) as described previously (17).

**Results**

**PAH treatment reduces $\beta_2$AR function (procaterol-induced cAMP production) and expression in MTEC.** Cyclic-AMP production by cells treated with a 9.32 ng/ml of the PAH mixture was reduced by 95% ($p=0.001$ vs. controls treated with vehicle (16.7% DMSO), n=6 inserts/condition)(Figure 1). Cyclic-AMP production by cells treated with 4.66 ng/ml of PAH for 24 hours did not differ from vehicle treated controls (8.4% DMSO).

As can be seen in Figure 2, membrane bound $\beta_2$AR protein levels in
cells exposed to 9.32 ng/ml of PAH were reduced by 61% as compared to vehicle treated controls (n=6 filters/condition, p=0.001 vs controls treated with 16.7% DMSO). Membrane bound receptor expression was not different from controls in cells treated with 4.66 ng/ml of PAH for 24 hours (n=6 filters/condition, p=0.2 for PAH 4.66 ng/ml vs. controls treated with 8.4% DMSO). Furthermore, β2AR mRNA levels, normalized to GAPDH, in cells treated with 9.32 and 4.66 ng/ml of PAH for 24 hours were assessed. The higher concentration of PAH reduced steady-state β2AR message by >90% (p<0.01 vs. all other groups, n=6 filter supports/condition) (Fig. 3).

The β2AR is a G-protein coupled receptor that affects cAMP levels through activation of adenylyl cyclase. The changes in whole cell cAMP levels shown in Figure 1 thus could be a reflection of reduced functional β2AR in the cell membrane as suggested by data shown in Figure 2, and/or impairment of the β2AR signal transduction pathway. To test for PAH effects on the β2AR signal transduction pathway, cells were treated with an established concentration of the adenylyl cyclase activator forskolin (2x10^-5 M for 15 mins)(16). As can be seen in Figure 4, forskolin-induced cAMP production was reduced by 68% in cells treated with 9.32 ng/ml of PAHs for 24 hours as compared to control cells treated with vehicle (16.7% DMSO). These data imply that a proximal component of the β2AR signal transduction pathway is affected by PAHs.

**PAH treatment reduces β2AR function (procaterol-induced cAMP production) and expression in HASM.** β2AR function was assessed in HASM cells by measuring whole cell cAMP levels following treatment with the β2AR specific agonist, procaterol. As shown in Figure 5, cells treated with 1.86 ng/ml of PAHs showed no reduction in β2AR function whereas cells treated with 2.80 ng/ml of PAHs had β2AR function that was 41% of controls (p=0.02 vs. controls treated with 5% DMSO, n=6 dishes/condition).

HASM cells exposed to 5.59 ng/ml of PAHs had β2AR function that was <1% of controls treated with 10% DMSO (p=0.001 vs. controls, n=6 dishes/condition). The PAH concentrations used in these experiments were 10-fold lower than those used in MTECs implying that HASM β2AR function is more sensitive to PAHs than MTECs.

β-receptor function in HASM cells treated with the highest concentration of PAHs (5.59ng/ml) was reduced by 34% (p=0.04 vs. controls treated with 10% DMSO, n=6 dishes/condition) (Figure 6). Lower concentrations had no measurable effect on β2AR function in these cells. Despite repeated attempts, β2AR mRNA could not be measured using a variety of methodologies in HASM cells.

Finally, forskolin treatment of HASM cells treated with 5.59ng/ml of PAHs had whole-cell cAMP concentrations that were reduced by 84% as compared to those in vehicle treated controls (Figure 7)(p=0.01 vs. controls). Reduced cAMP production in response to forskolin treatment indicates reduced function of adenylyl cyclase, a proximal component of the β2AR signal transduction pathway in HASM these cells.

**Discussion**

The results of these studies indicate that an environmentally relevant mixture of PAHs impairs multiple aspects of the β2AR signal transduction pathway. These findings apply to both primary lung epithelial cells and airway smooth muscle cells. Previously published data from animal and cell culture studies indicate that airway epithelial (18-22) and inflammatory cell (22) β2ARs can be desensitized by β-agonists (23, 24). Regular use of inhaled β-agonists increases bronchial hyperresponsiveness, diminishes β-agonist protection from antigenic stimuli, and causes tolerance to β-agonists possibly via agonist-induced receptor desensitization (reviewed in (25)). The novel results we present here suggest that β2AR function also may be impaired following exposure to traffic related air...
pollution, and PAHs specifically. Loss of receptor function can be due to either decreased numbers of receptors in the cell membrane (downregulation) or loss of receptor responsiveness to its ligands due to changes in the receptor or any portion of its signal transduction pathway (desensitization). In the current study we noted fewer receptors in the cell membrane by western analysis and decreased function of adenylyl cyclase, a key member of the $\beta_2$-receptor signal transduction pathway. Thus both receptor downregulation and desensitization appear to play a role in PAH mediated loss of $\beta_2$AR function in HASM and mTEC. How PAH affect other pathways of receptor desensitization such as receptor phosphorylation by $\beta$-adrenergic receptor kinases or G-protein expression and function remains to be established.

The biological mechanisms responsible for the development of asthma symptoms following exposure to air pollution exposure are complex. Several pathways appear important. In one, air pollutants such as diesel, particulate matter (PM) and metals, known triggers of asthma, may induce oxidative stress pathways causing the formation of excessive reactive oxygen species in the airways and tissue inflammation (3, 4, 26-29). In a second, exposure to diesel may be a strong adjuvant for allergic sensitization and has been shown to upregulate allergic immune mechanisms in the airways (4, 30-33). PAHs, a component of exhaust from incomplete combustion of diesel fuel, have been linked directly with the development of respiratory disease by our group (2, 7). Mechanistic experiments so far suggest that inhalation of PAHs can cause acute airway inflammation via the induction of genes associated with the aryl hydrocarbon receptor, oxidative stress and inflammation (34). In addition, exposure to PAHs like diesel, has been associated with significantly enhanced upregulation of proallergic cytokine and immunoglobulin production in vivo (8, 35, 36).

This is the first study to suggest that another mechanism for air pollution-related asthma symptoms may involve PAH-induced impairment of $\beta_2$AR signaling. The only other similar study indicated that exposure of adipocytes to PAHs impaired $\beta_2$AR function without reducing membrane bound receptor number (10).

We acknowledge several limitations of this work. First, like other work in cell systems, the results are not necessarily translatable to what occurs in vivo. The choice of primary cells, and implementation during culture of MTEC of the semi-permeable that supports an apical air-liquid interface, was intended to improve the translation of these results. In addition, the PAH studied are a selection of what may be measured in urban air as a consequence of traffic emissions and other sources of pollution. Future studies in animal models and cohort work are needed to validate these findings clinically.

In conclusion, these studies create a new paradigm for asthma morbidity. An environmentally relevant mixture of PAHs may interfere with a key regulatory molecule that is responsible for bronchomotor tone. This new paradigm offers a novel mechanism by which air pollutants may interfere with the treatment for asthma and contribute to the substantial morbidity associated with this disease. The potential public health impact is large because the prevalence of childhood asthma in urban areas ranges from 8% to 12% (37, 38).

References


**Figure Legends**

Figure 1: Whole cell procaterol-induced cyclic-AMP concentrations in primary mouse tracheal epithelial cells exposed to the shown concentrations PAH in DMSO or DMSO only vehicle in complete medium for 24 hours. N=6 filters/condition. *P<0.001 vs. all other groups.

Figure 2. Membrane bound β₂AR levels in mouse tracheal epithelial cells exposed to PAH for 24 hours. Western blot shown is whole cell membrane fractions probed with an anti-human β₂AR antibody. Graphical data are optical density of a 52 kD band normalized to same sample actin levels to control for interlane loading variation and then to control cells exposed to vehicle only (8.4% and 16.8% DMSO in complete medium). Band intensity was arbitrarily set to 100 for control cells treated with 8.4% DMSO and normalized to actin. *P<0.01 vs. all other groups.

Figure 3. Normalized β₂AR mRNA expression in mouse tracheal epithelial cells exposed to the shown concentrations of PAH in DMSO or DMSO only vehicle in complete medium for 24 hours. Messenger RNA was quantified using real-time rtPCR as described in methods. Data shown are normalized to same cell GAPDH mRNA. Signal intensity was arbitrarily set to 100 for control cells treated with 8.4% DMSO to permit comparison among groups. *P<0.01 vs. all other groups. N=6 samples/condition.

Figure 4: Whole cell cAMP concentrations in mouse tracheal epithelial cells exposed to 9.32 ng/ml of PAH for 24 hours. Cyclic-AMP was measured following treatment with the adenylyl cyclase activator forskolin (2x10⁻⁵M) for 15 minutes to test for adenylyl cyclase function. Data shown are cAMP in pmol/mg protein. Controls were treated with the same concentration of vehicle (16.8% DMSO in complete medium) as PAH 9.32 ng/ml treated cells. *P=0.002 vs. vehicle treated controls.

Figure 5: Whole cell cAMP concentrations in human airway smooth muscle cells treated with the shown concentrations of PAH for 24 hours. Data are pmol of cAMP/mg protein measured in cell lysates following 24hrs treatment with PAH or vehicle (Control). Control cells were treated with the same concentration of vehicle as PAH cells (3%, 5%, or 10% DMSO in complete medium for 1.86, 2.80, and 5.59 ng/ml PAH respectively). N=6 dishes/condition. *P=0.05 vs. same dilution vehicle treated controls. **P=0.001 vs. same dilution vehicle treated controls.

Figure 6: Membrane bound β₂AR levels in human airway smooth muscle (HASM) cells treated with the shown concentrations of DMSO in complete medium for 24 hours. Protein expression was quantified based on immunoblot band density that was then normalized to same sample actin band density. Controls were treated with the shown concentrations of DMSO in complete medium for 24 hours. N=6 dishes/condition. *P=0.04 vs. control HASM cells treated with 10% DMSO vehicle.

Figure 7: Whole cell cAMP concentrations in human airway smooth muscle cells treated with 5.59 ng/ml of PAH in 10% DMSO for 24 hours and then treated with the adenylyl cyclase activator forskolin (2x10⁻⁵M) for 15 minutes prior to [cAMP] determination. Data shown are cAMP in pmol/mg protein. N=6 dishes/condition. *P=0.01 vs controls treated with 10% DMSO alone.
Table 1 - Ambient CCCEH PAH levels measured during 48 hour personal monitoring (n=645) and associated contribution to in vitro mixture

<table>
<thead>
<tr>
<th>PAH</th>
<th>Proportion (%)</th>
<th>Mean (ng/m3)</th>
<th>In vitro stock mixture (ng/ml)</th>
</tr>
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<td>Benzo(a)anthracene</td>
<td>3.99</td>
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<td>0.42</td>
<td>2.9</td>
</tr>
<tr>
<td>Benzo(b)fluoranthene</td>
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<td>0.59</td>
<td>4.3</td>
</tr>
<tr>
<td>Benz(k)+fluoranthene</td>
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<td>1.1</td>
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<tr>
<td>Benzo(ghi)perylene</td>
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<td>1.12</td>
<td>7.6</td>
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<tr>
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<tr>
<td>Pyrene</td>
<td>52.59</td>
<td>3.69</td>
<td>30</td>
</tr>
</tbody>
</table>
Figure 1: Whole cell procaterol-induced cyclic-AMP concentrations in primary mouse tracheal epithelial cells exposed to the shown concentrations PAH in DMSO or DMSO only vehicle in complete medium for 24 hours. N=6 filters/condition. *P<0.001 vs. all other groups.
Figure 2

- **β₂AR**
  - 8% DMSO
  - PAH 4.66ng/ml
  - 16% DMSO
  - PAH 9.32ng/ml

- **Actin**
  - 50kDa
  - 37kDa

Histogram showing normalized β₂AR protein expression:
- DMSO 8.4%
- PAH 4.66ng/ml
- DMSO 16.8%
- PAH 9.32ng/ml

Note: * indicates statistical significance.
Figure 3. Normalized β2AR mRNA expression in mouse tracheal epithelial cells exposed to the shown concentrations of PAH in DMSO or DMSO only vehicle in complete medium for 24 hours. Messenger RNA was quantified using real-time rtPCR as described in methods. Data shown are normalized to same cell GAPDH mRNA. Signal intensity was arbitrarily set to 100 for control cells treated with 8.4% DMSO to permit comparison among groups. *P<0.01 vs. all other groups. N=6 samples/condition.
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106x84mm (300 x 300 DPI)
Figure 6: Membrane bound β2AR levels in human airway smooth muscle (HASM) cells treated with the shown concentrations of PAH for 24 hours. Protein expression was quantified based on immunoblot band density that was then normalized to same sample actin band density. Controls were treated with the shown concentrations of DMSO in complete medium for 24 hours. N=6 dishes/condition. *P=0.04 vs. control HASM cells treated with 10% DMSO vehicle.
Figure 7: Whole cell cAMP concentrations in human airway smooth muscle cells treated with 5.59 ng/ml of PAH in 10% DMSO for 24 hours and then treated with the adenylyl cyclase activator forskolin (2x10^{-5}M) for 15 minutes prior to [cAMP] determination. Data shown are cAMP in pmol/mg protein. N=6 dishes/condition. *P=0.01 vs controls treated with 10% DMSO alone.
Polycyclic aromatic hydrocarbons impair β2AR 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). MTEC were plated on Transwell filter inserts (Corning, Inc., Corning, NY) and grown in DMEM–Ham’s F-12 medium with 30 mM HEPES, 4 mM L-glutamine, 3.5 mM NaHCO₃, 100 U/ml penicillin, and 100 µg/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, and gentamicin. Medium was provided initially 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 allografts in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. A segment of the trachealis muscle just proximal to the carina was dissected under sterile conditions, minced, centrifuged, and resuspended in 0.2 mM CaCl₂, 640 U/ml of collagenase, 10 mg of soybean trypsin inhibitor, and 10 U/ml of elastase. Enzymatic dissociation of the tissue was performed for 90 min in a shaking water bath at 37°C. The cell suspension was filtered through 125-µm Nytex mesh, and washed with cold Ham’s F-12 medium with 10% fetal bovine serum. Aliquots of the cell suspension were plated at a density of 1.0x10⁴ cells/cm². Ham’s F-12 medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 100 µg/ml of amphotericin B was replaced every 72 hours. Details regarding the characterization of this cell line have been reported (2–4).

PAH mixture. In prior work from the Columbia Center for Children’s 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]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenz[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 HASM cells were exposed to varying concentrations of the PAH mixture diluted with serum free medium for 24 hours prior to assessment of β2AR function (procaterol-induced cAMP production). These concentrations were based on preliminary dose finding experiments that used β2AR function as a physiologic end-point.

**Whole cell membrane isolation, western analysis, real time PCR.** To gauge if changes in β2AR function in MTEC and HASM cells correlate with membrane bound receptor number, β2AR 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 mM Hepes-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 100 µl 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 β2AR antibody (Santa Cruz Scientific, Santa Cruz, CA). Protein bands were visualized using peroxidase coupled secondary antibodies and a chemiluminescent 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 β2AR primers (Taqman, Applied Biosystems, Foster City, CA) and Applied Biosystem reverse transcriptase reagents were used to evaluate steady-state β2AR mRNA expression. Copy numbers were calculated for β2AR for each sample and normalized to copy numbers of GAPDH.

**Measurement of cellular cAMP production (β2AR function).** β2AR function was assessed by measuring whole cell cAMP concentrations after treatment of cells with the β2AR 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 assess adenyl 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 pmol/cAMP/mg protein.