# Polycyclic Aromatic Hydrocarbons Impair Function of $\beta_2$-Adrenergic Receptors in Airway Epithelial and Smooth Muscle Cells

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**Incomplete combustion produces a pollutant mixture that includes polycyclic aromatic hydrocarbons (PAHs). Previous work by the Columbia Center for Children's Environmental Health (CCCEH) and others linked exposure to PAH with symptoms of asthma and other adverse health effects in young children. Inhaled $\beta_2$-adrenergic agonists are mainstays in the treatment of reactive airway diseases. These exogenous catecholamines engage membrane-bound $\beta_2$-adrenergic receptors ($\beta_2$AR) on airway epithelial and smooth muscle cells to cause airway dilation. We hypothesized that exposure to PAH might similarly interfere with the function of $\beta_2$AR in airway epithelial or smooth muscle cells, reducing the efficacy of a medication important for the treatment of asthma symptoms. A PAH mixture was devised, based on ambient levels measured prenatally among a cohort of pregnant women participating at the CCCEH. Primary airway epithelial and smooth muscle cells were exposed to varying concentrations of the PAH mixture, and expression, function, and signaling of $\beta_2$AR were assessed. Murine tracheal epithelial cells and human airway smooth muscle cells, after exposure to a PAH mixture, exhibited reduced expression and function of $\beta_2$AR. These findings support our hypothesis that environmentally relevant PAHs can impede $\beta_2$AR-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.**

**Keywords:** polycyclic aromatic hydrocarbons; $\beta_2$-adrenergic receptors

Incomplete combustion produces a pollutant mixture that includes high concentrations of polycyclic aromatic hydrocarbons (PAHs) (1). All of these constituents were associated with adverse respiratory effects (2–4). Although traffic emissions remain the primary source of PAHs in United States cities, PAHs are also emitted readily from industrial sources, cigarette smoke, incense burning, cooking, and space heating (5, 6). Previous work by the Columbia Center for Children's Environmental Health (CCCEH) and others linked exposure to ambient PAHs in particular with asthma-like symptoms in young children and with seroatopy (2, 7, 8). However, the mechanism responsible for these findings has not been elucidated.

(*Received in original form December 13, 2010 and in final form May 6, 2011*)

This work was supported by National Institutes of Health grants P50ES015905, P01ES09600, and R01ES008977, and Environmental Protection Agency grant RD-83214101.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

**Am J Respir Cell Mol Biol Vol 45. pp 1045–1049, 2011**
**Originally Published in Press as DOI: 10.1165/rcmb.2010-0499OC on May 26, 2011**
**Internet address: www.atsjournals.org**

Inhaled $\beta_2$–adrenergic agonists are mainstays in the treatment of asthma and other reactive airway diseases. These exogenous catecholamines engage membrane-bound $\beta_2$-adrenergic receptors ($\beta_2$ARs) 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 numbers (10). We hypothesized that exposure to traffic-related PAHs might interfere with the function of airway $\beta_2$ARs. This effect would reduce the efficacy of an important asthma medication and worsen asthma symptoms. Our approach involved exposing two different primary lung cell types that play important roles in the pathophysiology of airway obstruction in asthma to a PAH mixture before an assessment of $\beta_2$AR function. One included murine tracheal epithelial cell (MTEC) monolayers that had been grown on semipermeable supports with an apical air–liquid interface. This method recapitulates the *in vivo* phenotype of a high-resistance epithelium with functional cilia (11). In addition, human airway smooth muscle (HASM) cells were studied, using similar methods to determine whether PAHs alter the smooth muscle cell function and expression of $\beta_2$ARs. To mimic urban ambient exposure, the PAH components in these studies were proportionally constituted to resemble New York City concentrations measured during personal monitoring of airborne levels among pregnant women from the CCCEH cohort.

## MATERIALS AND METHODS

### Isolation of Primary mTECs

The use of animals for the present experiments was approved by the Animal Care and Use Committee of Columbia University. Mice for these studies were specific-pathogen free C57BL/6 mice obtained from Charles River Laboratories (Wilmington, MA). The mTECs from C57BL/6 mice were isolated and grown on semipermeable supports (Transwell filter inserts; Corning, Inc., Corning, NY), with an apical air–liquid interface as described elsewhere (11). Medium (DMEM–Ham's F-12) was provided initially in the upper and lower chambers. When transmembrane resistance reached 1,000 $\Omega \cdot cm^{-2}$, the 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 cultured for a total of 7–10 days.

### Isolation of Primary HASM Cells

HASM cells were isolated from the trachealis muscles of unused human lung alographs, in accordance with guidelines of the Committee on Studies Involving Human Beings at the University of Pennsylvania, as described elsewhere (12–14) and in METHODS in the online supplement. 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% FBS and antibiotics. The viability of HASM cells according to Trypan blue exclusion after a 24-hour treatment with 1.86 ng/ml, 2.80 ng/ml, or

5.59 ng/ml PAHs reached 96%, 90%, and 85%, respectively. Cells treated with 10% DMSO reached a viability of 82%.

### PAH Mixture

In previous work from the CCCEH, pregnant Dominican and African-American women, aged 18–35 years and living in the Washington Heights, Central Harlem, and South Bronx neighborhoods of New York City, wore continuously active personal air sampling devices for 48 hours during the third trimester of pregnancy. Their exposure to eight 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, and benzo[g,h,i]perylene plus pyrene) were determined as described elsewhere (2, 15). A PAH mixture that reproduced the PAH exposure of the CCCEH participants (Table 1) was produced for our cellular experiments. This PAH mixture was suspended in DMSO at 55.9 ng/ml. We then calculated the dose of PAH mixture. The concentration of ambient PAHs ranged from 1–50 ng/m$^3$, with pyrene at the highest concentration of 50 ng/m$^3$. The inhalation dose of pyrene was determined based on a breathing rate of 15 L/minute, exposure for 8 hours per day, a deposition of inhaled pyrene to the lung at 30% of the inhaled dose, and a lung internal surface area of 135 m$^2$. The resulting calculated dose was approximately 1 pg pyrene/cm$^2$ lung.

### Whole-Cell Membrane Isolation, Western Blot Analysis, and Real-Time PCR

The expression of membrane-bound $\beta_2$AR was evaluated via Western blot analysis of whole-cell membrane fractions, as described previously (16). For Western blot analysis, 10 μg of whole-cell membrane protein/lane were 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 murine monoclonal anti-actin antibodies (Chemicon International, Temecula, CA). Quantitative, real-time RT-PCR, using human and murine $\beta_2$AR primers (Taqman; Applied Biosystems, Foster City, CA) and Applied Biosystem reverse transcriptase reagents, was used to evaluate the steady-state expression of $\beta_2$AR mRNA. $\beta_2$AR copy numbers were normalized to copy numbers of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

### Measurement of Cellular Cyclic Adenosine Monophosphate Production ($\beta_2$AR Function)

$\beta_2$AR function was assessed by measuring concentrations of whole-cell cyclic adenosine monophosphate (cAMP) after treating cells with the $\beta_2$AR-specific agonist procaterol ($10^{-6}$ M; 15 minutes at 37°C). In all experiments, cells were pretreated with $10^{-4}$ M 3,7-dihydro-1-methyl-3-(2-methylpropyl)-1*H*-purine-2,6-dione for 15 minutes to inhibit phosphodiesterases. To assess the function of adenylyl cyclase, cells were treated with forskolin ($2 \times 10^{-5}$ M) for 15 minutes at 37°C. We used an enzyme immunoassay (Cayman Chemical, Ann Arbor, MI), as described previously (17), to quantify cAMP.

## RESULTS

### Treatment with PAHs Reduces the Function of $\beta_2$ARs (Procaterol-Induced cAMP Production) and Expression in mTECs

The production of cAMP by cells treated with 9.32 ng/ml of the PAH mixture was reduced by 95% ($P = 0.001$, versus control cells treated with vehicle, i.e., 16.7% DMSO, $n = 6$ inserts/condition) (Figure 1). The production of cAMP by cells treated with 4.66 ng/ml of PAHs for 24 hours did not differ from that in vehicle-treated control cells (8.4% DMSO).

As seen in Figure 2, membrane-bound $\beta_2$AR protein concentrations in cells exposed to 9.32 ng/ml of PAH were reduced by 61%, compared with vehicle-treated control cells ($n = 6$ filters/condition, $P = 0.001$, versus control cells treated with 16.7% DMSO). The expression of membrane-bound receptor was not different from that of control samples in cells treated with 4.66 ng/ml of PAHs for 24 hours ($n = 6$ filters/condition, $P = 0.2$ for 4.66 ng/ml PAHs, versus control cells treated with 8.4% DMSO). We also assessed concentrations of $\beta_2$AR mRNA, normalized to GAPDH, in cells treated with 9.32 and 4.66 ng/ml PAHs for 24 hours. The higher concentration of PAHs reduced the steady-state $\beta_2$AR message by more than 90% ($P < 0.01$, versus all other groups; $n = 6$ filter supports/condition) (Figure 3).

**TABLE 1. AMBIENT CCCEH PAH CONCENTRATIONS MEASURED DURING 48-HOUR PERSONAL MONITORING (N = 645), AND ASSOCIATED CONTRIBUTIONS TO *IN VITRO* MIXTURE**

| PAH | Proportion (%) | Mean (ng/m$^3$) | *In Vitro* Stock Mixture (ng/ml) |
|---|---|---|---|
| Benzo[a]anthracene | 3.99 | 0.27 | 2.3 |
| Benzo[a]pyrene | 5.13 | 0.42 | 2.9 |
| Benzo[b]fluoranthene | 7.62 | 0.59 | 4.3 |
| Benzo[k]fluoranthene | 1.79 | 0.15 | 1.1 |
| Benzo[g,h,i]perylene | 13.02 | 1.12 | 7.6 |
| Chrysene | 4.82 | 0.35 | 2.8 |
| Dibenzo[a,h]anthracene | 0.89 | 0.06 | 0.5 |
| Indeno[c,d]pyrene | 7.42 | 0.64 | 4.4 |
| Pyrene | 52.59 | 3.69 | 30.0 |

*Definition of abbreviations*: CCEH, Columbia Center for Children's Environmental Health; PAH, polycyclic aromatic hydrocarbon.

$\beta_2$AR is a G-protein–coupled receptor that affects concentrations of cAMP through the activation of adenylyl cyclase. The changes in concentrations of whole-cell cAMP shown in Figure 1 could reflect the reduced functional $\beta_2$AR in the cell membrane, as suggested by the data shown in Figure 2, or the impairment of the $\beta_2$AR signal transduction pathway. To test for the effects of PAHs on the $\beta_2$AR signal transduction pathway, cells were treated with an established concentration of the adenylyl cyclase activator forskolin ($2 \times 10^{-5}$ M for 15 minutes) (16). As seen in Figure 4, the forskolin-induced production of cAMP was reduced by 68% in cells treated with 9.32 ng/ml of PAHs for 24 hours, compared with control cells treated with vehicle (16.7% DMSO). These data imply that a proximal component of the $\beta_2$AR signal transduction pathway is affected by PAHs.

### Treatment with PAHs Reduces the Function of $\beta_2$AR (Procaterol-Induced Production of cAMP) and Expression in HASM Cells

The function of $\beta_2$AR was assessed in HASM cells by measuring whole-cell concentrations of cAMP after treatment with the $\beta_2$AR-specific agonist procaterol. As shown in Figure 5, cells treated with 1.86 ng/ml of PAHs showed no reduction in $\beta_2$AR function, whereas cells treated with 2.80 ng/ml of PAHs demonstrated a $\beta_2$AR function at 41% of control cells ($P = 0.02$, versus controls treated with 5% DMSO; $n = 6$ dishes/condition). HASM cells exposed to 5.59 ng/ml of PAHs demonstrated a $\beta_2$AR function that was less than 1% of that in control cells treated with 10% DMSO ($P = 0.001$, versus control cells; $n = 6$ dishes/condition). The PAH concentrations used in these experiments were 10-fold lower than those used in mTECs, implying that the function of HASM $\beta_2$AR is more sensitive to PAHs than to mTECs.

The function of β-receptor in HASM cells treated with the highest concentration of PAHs (5.59 ng/ml) was reduced by 34% ($P = 0.04$, versus controls treated with 10% DMSO; $n = 6$ dishes/condition) (Figure 6). Lower concentrations exerted no measurable effect on the function of $\beta_2$AR in these cells. Despite repeated attempts, $\beta_2$AR mRNA could not be measured, using a variety of methodologies in HASM cells.

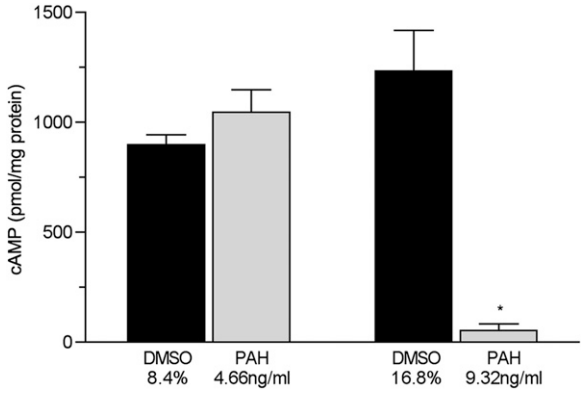


*Figure 1.* Concentrations of whole-cell, procaterol-induced cyclic adenosine monophosphate (cAMP) in primary murine tracheal epithelial cells exposed to the indicated concentrations of polycyclic aromatic hydrocarbons (PAHs) in DMSO or DMSO-only vehicle in complete medium for 24 hours ($n = 6$ filters/condition). *$P < 0.001$, versus all other groups.

Finally, the forskolin treatment of HASM cells treated with 5.59 ng/ml of PAHs produced whole-cell concentrations of cAMP that were reduced by 84%, compared with those in vehicle-treated control cells (Figure 7) ($P = 0.01$, versus control cells). The reduced production of cAMP in response to forskolin treatment indicates the reduced function of adenylyl cyclase, a proximal component of the $\beta_2$AR signal transduction pathway in HASM cells.

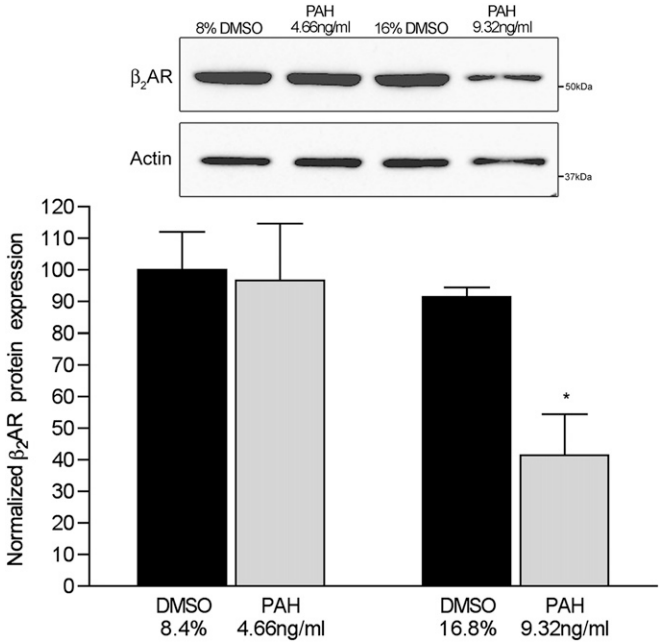


*Figure 2.* Concentrations of membrane-bound $\beta_2$–adrenergic receptors ($\beta_2$ARs) in murine tracheal epithelial cells exposed to PAHs for 24 hours. Western blot represents whole-cell membrane fractions probed with an anti-human $\beta_2$AR antibody. Graphical data represent optical density of a 52-kD band normalized to same sample actin concentrations to control for interlane loading variations, and then to control cells exposed to vehicle only (8.4% and 16.8% DMSO in complete medium). Band intensity was arbitrarily set at 100 for control cells treated with 8.4% DMSO, and normalized to actin. *$P < 0.01$, versus all other groups.

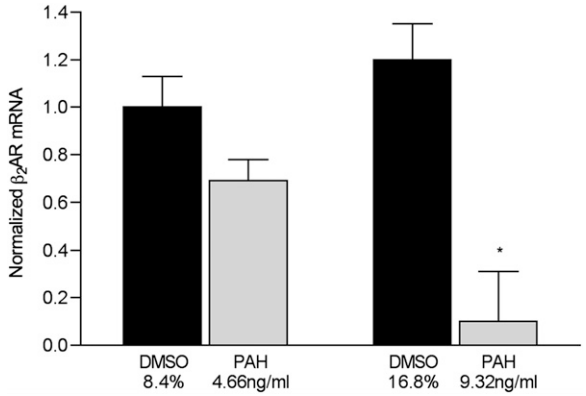


*Figure 3.* Expression of normalized $\beta_2$AR mRNA in murine tracheal epithelial cells exposed to the indicated concentrations of PAHs in DMSO or DMSO-only vehicle in complete medium for 24 hours. Messenger RNA was quantified according to real-time RT-PCR, as described in MATERIALS AND METHODS. The data are normalized to same-cell glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Signal intensity was arbitrarily set at 100 for control cells treated with 8.4% DMSO, to permit comparisons among groups. *$P < 0.01$ versus all other groups ($n = 6$ samples/condition).

## DISCUSSION

These results indicate that an environmentally relevant mixture of PAHs impairs multiple aspects of the $\beta_2$AR 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 cell (18–22) and inflammatory cell (22) $\beta_2$ARs can be desensitized by β-agonists (23, 24). The regular use of inhaled β-agonists increases bronchial hyperresponsiveness, diminishes β-agonist

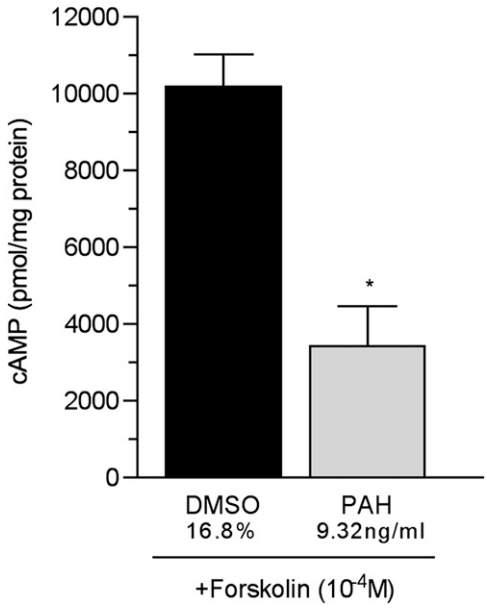


*Figure 4.* Concentrations of whole-cell cAMP in murine tracheal epithelial cells exposed to 9.32 ng/ml PAHs for 24 hours. cAMP was measured after treatment with the adenylyl cyclase activator forskolin ($2 \times 10^{-5}$ M) for 15 minutes, to test for adenylyl cyclase function. The data represent cAMP in pmol/mg protein. Control samples were treated with the same concentration of vehicle (16.8% DMSO in complete medium) as the 9.32 ng/ml PAH–treated cells. *$P = 0.002$, versus vehicle-treated control samples.

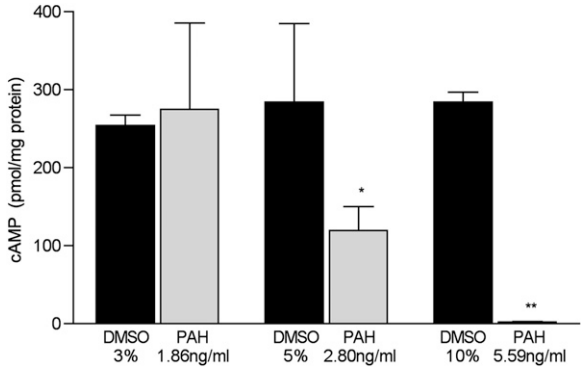


*Figure 5.* Concentrations of whole-cell cAMP in human airway smooth muscle cells treated with the indicated concentrations of PAH for 24 hours. The data represent pmol of cAMP/mg protein measured in cell lysates after 24-hour treatment with PAHs or vehicle (Control). Control cells were treated with 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 PAHs, respectively; $n = 6$ dishes/condition). *$P = 0.05$, versus same dilution of vehicle-treated control samples. **$P = 0.001$, versus same dilution of vehicle-treated control samples.

protection from antigenic stimuli, and causes tolerance to β-agonists, possibly via agonist-induced receptor desensitization (25). The present novel results suggest that the function of $\beta_2$AR also may be impaired after exposure to traffic-related air pollution, and to PAHs specifically. The loss of receptor function can be attributed to either decreased numbers of receptors in the cell membrane (down-regulation) or a loss of receptor responsiveness to its ligands, because of changes in the receptor or any portion of its signal transduction pathway (desensitization). studying this study, we noted fewer receptors in the cell membrane according to Western blot analysis, and decreased function of adenylyl cyclase, a key member of the $\beta_2$-receptor signal transduction pathway. Thus both receptor down-regulation and desensitization appear to play a role in the PAH-mediated loss of $\beta_2$AR function in HASM cells and mTECs. How PAHs affect other pathways of receptor desensitization, such as receptor phosphorylation by

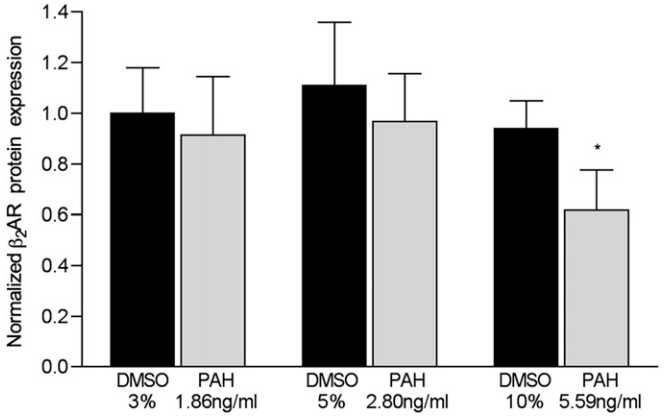


*Figure 6.* Concentrations of membrane-bound $\beta_2$AR in human airway smooth muscle (HASM) cells treated with the indicated concentrations of PAHs for 24 hours. The expression of protein was quantified according to the immunoblot band density, which was then normalized to same-sample actin band density. Control cells were treated with the indicated concentrations of DMSO in complete medium for 24 hours ($n = 6$ dishes/condition). *$P = 0.04$, versus control HASM cells treated with 10% DMSO vehicle.

β–adrenergic receptor kinases or G-protein expression and function, remains to be established.

The biological mechanisms responsible for the development of asthma symptoms after exposure to air pollution are complex. Several pathways appear important. In one, air pollutants such as diesel, particulate matter, 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 pathway, exposure to diesel may provide a strong adjuvant for allergic sensitization, and such exposure was shown to up-regulate allergic immune mechanisms in the airways (4, 30–33). PAHs, which are components of exhaust from the incomplete combustion of diesel fuel, were linked directly with the development of respiratory disease by our group (2, 7). Mechanistic experiments so far suggest that the 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 such as diesel was associated with a significantly enhanced up-regulation of proallergic cytokine and immunoglobulin production *in vivo* (8, 35, 36).

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

We acknowledge several limitations to this study. First, like other work in cell systems, the results are not necessarily translatable to what occurs *in vivo*. The choice of primary cells, alogn with our implementation during the culture of mTECs of the semipermeable supports for an apical air–liquid interface, was

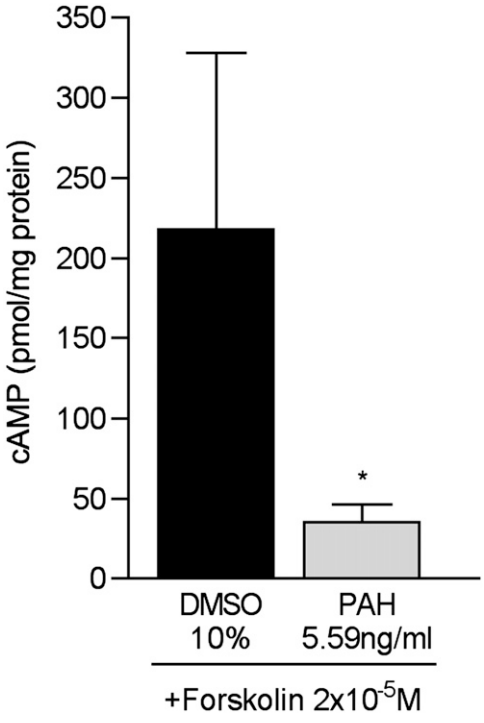


*Figure 7.* Whole-cell cAMP concentrations in human airway smooth muscle cells treated with 5.59 ng/ml PAHs in 10% DMSO for 24 hours, and then treated with the adenylyl cyclase activator forskolin ($2 \times 10^{-5}$ M) for 15 minutes before the determination of [cAMP]. The data represent cAMP in pmol/mg protein ($n = 6$ dishes/condition). *$P = 0.01$, versus control samples treated with 10% DMSO alone.

intended to improve the translation of these results. In addition, the PAHs studied here comprised 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 8–12% (37, 38).

***Author Disclosure***: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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