Asthma is the most common inflammatory disease, and its prevalence is increasing throughout the world. Although corticosteroids are the most effective antiinflammatory agents for the treatment of asthma, adult patients with asthma who currently smoke have relative steroid resistance. Furthermore, their asthma becomes more severe and their lung function decreases more rapidly compared with non-smoking patients with asthma. Passive smoking (PS) also worsens asthma symptoms and causes poor asthma control in both adults and children. Exposure to parental smoking is related to exacerbation of asthma symptoms in children and can be a risk factor for the persistence of asthma in later childhood. However, the molecular mechanisms of the effects of PS exposure in childhood are currently unknown.

There are several possible mechanisms for corticosteroid resistance in asthma, including overexpression of proinflammatory transcription factors, phosphorylation of glucocorticoid receptors, and increases in the decoy glucocorticoid receptor-β. Histone deacetylase (HDAC)-2 (HDAC2) has been shown to be a prerequisite molecule for corticosteroids to switch off activated inflammatory genes. Oxidative stress, such
as tobacco smoke, impairs HDAC2 function, leading to corticosteroid insensitivity in vitro and in vivo.⁶⁻¹⁰ HDAC2 expression and activity are reduced in the airways of, and alveolar macrophages (AMs) from, adults with severe asthma¹¹⁻¹³ and COPD.¹⁴,¹⁵ Even more importantly, in patients with asthma who smoke, there is a significantly greater reduction of HDAC activity in bronchial biopsy specimens than in patients with asthma who do not smoke.¹⁶ Further analysis revealed that oxidative stress such as tobacco smoke impairs HDAC2 via phosphoinositide-3-kinase (PI3K) δ (PI3Kδ)/Akt activation.⁸,¹⁷ In this study, we tested the hypothesis that passive exposure to tobacco smoke is associated with reduced HDAC2 in AMs in children with severe and refractory asthma.

MATERIALS AND METHODS

Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, dimethyl sulfoxide, phorbol 12-myristate 13-acetate (PMA), the rabbit polyclonal antibody to HDAC1 (HC10) antibody, and the mouse monoclonal HDAC2 antibody were purchased from Sigma-Aldrich. The rabbit polyclonal antibody to phospho-HDAC2 (Ser²⁹⁰) and the mouse monoclonal antibody to β-actin were obtained from Abcam. Protein A/G plus-agarose immunoprecipitation reagent was obtained from Santa Cruz Biotechnology, Inc. The mouse monoclonal anti-phospho-Akt1/PKBα (Ser⁴⁷³) antibody and the rabbit polyclonal anti-Akt1/PKBα antibody were obtained from Millipore. Recombiant human tumor necrosis factor (TNF)-α was purchased from R&D Systems Europe Ltd.

Patients

Nineteen children with severe asthma were recruited for bronchoscopy as part of the workup for severe, therapy-resistant asthma.¹⁵ All the children were under regular follow-up at Royal Brompton Hospital. Asthma was diagnosed according to American Thoracic Society criteria, and the severity was defined based on GINA (Global Initiative for Asthma) criteria. All had undergone a detailed evaluation to exclude as far as possible reversible factors such as poor adherence to therapy.¹⁹ Subjects were classified into two groups (non-PS and PS). Exposure to PS was assessed on the basis of information reported by parents concerning their smoking habits. Cotinine levels in saliva or urine were measured to support their statements. The study was conducted in accordance with the amended Declaration of Helsinki (http://www.wma.net/en/30publications/10policies/b3/) and was approved by the ethics committee of the Royal Brompton and Harefield NHS Trust (Ethics approval number 08/H0708/3). All carers gave written informed consent, with age-appropriate assent from the children.

Nitric Oxide Measurement

Fraction of exhaled nitric oxide (FENO) was measured according to current guidelines.¹⁰ A NIOX chemiluminescence analyzer at a flow rate of 50 mL/s was used for analysis of FENO.

BAL and Macrophage Processing

BAL using fiber-optic bronchoscopy was performed under general anesthetic, as described previously.²¹ Cells were centrifuged and washed with Hanks’ balanced salt solution. Cytospins were prepared and stained with Diff-Quick for differential cell count. Cell viability was assessed using the Trypan blue exclusion method. BAL macrophages were isolated by plastic adhesion and were incubated in Macrophage Serum Free Medium (Invitrogen Ltd).

Cells

The human monocytic cell line U937 was purchased from LGC Standards. The cells were differentiated into an adherent macrophage-like morphology by exposure to PMA (50 ng/mL) for 48 h.

Cytokine Enzyme-Linked Immunosorbent Assay and Corticosteroid Sensitivity

CXCL8 concentrations were determined by sandwich enzyme-linked immunosorbent assay (R&D Systems Europe Ltd). AMs or U937 cells were treated with dexamethasone (10⁻⁶ M), followed by TNF-α stimulation (10 ng/mL) for 2 h. The ability of dexamethasone to inhibit TNF-α-induced CXCL8 release was analyzed as a marker of corticosteroid sensitivity.

Thiobarbituric Acid Reactive Substances Assay

As a marker of oxidative stress, malondialdehyde (MDA) was measured as thiobarbituric acid reactive substances using a TBARS Assay Kit (Cayman Chemical Company). The levels were calculated using a standard curve.

Protein Extraction and Detection

Whole cell protein extracts were prepared using a radioimmuno-precipitation assay (RIPA) buffer as described previously.⁶ Immunoprecipitation was conducted overnight with 2 μg of anti-HDAC2 antibody (Sigma-Aldrich) in RIPA buffer. Cell lysates or immunoprecipitates were analyzed by SDS-PAGE (Invitrogen Ltd) and detected with Western blot analysis by chemiluminescence (ECL Plus; GE Healthcare) as reported previously.²⁶

Total HDAC and HDAC2 Activity

To measure in-cell HDAC activity, cells were incubated with Fluor de Lys substrate (200 μM) for 1 h before cell lysis using...
Table 1—Characteristics of Subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Non-Passive Smoking (n = 10)</th>
<th>Passive Smoking (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>9.5 (8.5-11.5)</td>
<td>10.0 (8.5-13.5)</td>
</tr>
<tr>
<td>Sex, male (female)</td>
<td>7 (3)</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Lifetime exposure to passive smoking, y</td>
<td>0</td>
<td>10.0 (8.5-13.5)</td>
</tr>
<tr>
<td>FEV₁, % predicted</td>
<td>71.5 (68.0-78.5)</td>
<td>72.0 (51.5-89.0)</td>
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<tr>
<td>FENO, ppb</td>
<td>51.2 (29.6-73.7)</td>
<td>44.0 (26.9-61.9)</td>
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<tr>
<td>ACT score</td>
<td>16.0 (10.5-17.5)</td>
<td>11.0 (8.5-14.5)</td>
</tr>
<tr>
<td>≤ 15</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Total IgE, IU/mL</td>
<td>386 (150-511)</td>
<td>355 (158-462)</td>
</tr>
<tr>
<td>Atopy</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>ICS, µg</td>
<td>1,600 (1,300-2,000)</td>
<td>1,600 (1,200-2,000)</td>
</tr>
<tr>
<td>LABA</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>LTRA</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Theophylline</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Data are presented as median (first and third quartile) or No., unless indicated otherwise. There were no significant differences between the groups. ACT = Asthma Control Test; FENO = fraction of exhaled nitric oxide; ICS = inhaled corticosteroid; IU = International Units; LABA = long-acting β₂-adrenoceptor agonist; LTRA = leukotriene receptor antagonist; ppb = parts per billion.

Values in non-passive smoking represent medians of nine subjects.

Values in non-passive smoking represent medians of nine subjects.

P values < .05 were considered to be statistically significant.
Akt1 and HDAC2 Phosphorylation

As shown in Figure 2A, HDAC2 was highly phosphorylated in AMs of the PS group compared with the non-PS group. The levels of phosphorylation of Akt1, a surrogate marker of PI3K signaling activation, were also significantly increased by 2.4 fold in AMs of the PS group compared with the non-PS group (Fig 2B) when normalized to total Akt1 protein expression. Furthermore, Akt1 phosphorylation levels were positively correlated with HDAC2 phosphorylation levels \((r = 0.71, P < .001)\) (Fig 2C) and negatively correlated with HDAC2 activity \((r = -0.54, P = .018)\) (Fig 2D).

Table 2—Cell and CXCL8 Analysis in BAL

<table>
<thead>
<tr>
<th>Readout</th>
<th>Non-Passive Smoking (n = 10)</th>
<th>Passive Smoking (n = 9)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Count, (\times 10^3/mL)</td>
<td>% of Total Cells</td>
<td>Cell Count, (\times 10^3/mL)</td>
<td>% of Total Cells</td>
</tr>
<tr>
<td>Total</td>
<td>370 (265-553)</td>
<td>...</td>
<td>280 (205-403)</td>
</tr>
<tr>
<td>Macrophage</td>
<td>287 (196-389)</td>
<td>76.2 (70.5-78.0)</td>
<td>236 (326-162)</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>13.3 (9.2-23.1)</td>
<td>3.7 (3.0-4.5)</td>
<td>24.1 (12.7-34.4)</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>55.2 (33.8-80.9)</td>
<td>14.7 (10.7-18.1)</td>
<td>10.9 (7.7-38.1)</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>11.6 (5.8-45.1)</td>
<td>4.0 (1.2-10.4)</td>
<td>2.9 (1.0-9.6)</td>
</tr>
<tr>
<td>CXCL8, pg/(\mu)g protein</td>
<td>0.11 (0.05-0.24)</td>
<td>...</td>
<td>0.78 (0.27-1.40)</td>
</tr>
</tbody>
</table>

Data are presented as median (first and third quartile).

Figure 1. Effects of PS on HDAC2 protein expression and activity in alveolar macrophages from children with severe asthma. A and B, Western blotting analysis of HDAC2 and HDAC1 protein expression, respectively, normalized to \(\beta\)-actin expression. C, HDAC2 protein expression detected by immunocytochemistry (original magnification \(\times 400\)). Results were representative of at least seven subjects in each group. D, Total in-cell HDAC activity. E, Immunoprecipitated HDAC2 activity. F, Correlation between HDAC2 protein expression and activity. Values in A, B, D, and E represent mean ± SEM of 10 non-PS group subjects or nine PS group subjects. AFU = arbitrary fluorescence units; HDAC = histone deacetylase; PS = passive smoking.
from subjects in the non-PS group. However, there was no significant inhibition in AMs from subjects in the PS group (Fig 3A).

Next, PMA-differentiated macrophage-like U937 cells were exposed to BALF obtained from the PS group or the non-PS group. BALF from the PS group significantly increased Akt1 phosphorylation (Fig 3B) and HDAC2 phosphorylation (Fig 3C), and reduced IP-HDAC2 activity (336.6 ± 310.1-358.4 AU/μg in the non-PS group, 299.1 ± 277.0-316.7 AU/μg in the PS group) in U937 cells (Fig 3D). Pretreatment of dexamethasone (10⁻⁶ M) completely inhibited TNF-α-induced CXCL8 release in U937 exposed to BALF from subjects in the non-PS group, but the inhibitory action was limited in U937 exposed to BALF from PS group subjects (Fig 3E). Furthermore, the level of MDA, a marker of oxidative stress, was found to be significantly higher in BALF in the PS group than in the non-PS group (Fig 3F).

**Discussion**

This article reports that children with asthma who are passively exposed to tobacco smoke had the same molecular abnormalities leading to in vitro steroid resistance as do adults who actively smoke.⁸ Children with severe asthma who were exposed to PS had higher CXCL8 levels (Table 2), in agreement with previous corticosteroid sensitivity of CXCL8 production in AMs

Dexamethasone at 10⁻⁶ M significantly inhibited TNF-α-induced CXCL8 production by 40% in AMs

![Figure 2](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/929712/)  
Figure 2. Effects of PS on phosphorylation level of Akt1 and HDAC2 in alveolar macrophages from children with severe asthma. A and B, Phosphorylation levels of HDAC2-Ser³⁹⁴ and Akt1, respectively, normalized to total HDAC2 and Akt1 expression. Values represent means of 10 (in non-PS group) or nine (in PS group) subjects ± SEM; C, Correlation between HDAC2 and Akt1 phosphorylation levels. D, Correlation between immunoprecipitated HDAC2 activity and phosphorylation level of Akt1. See Figure 1 legend for expansion of abbreviations.

![Figure 3](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/929712/)  
Figure 3. Effects of PS on corticosteroid sensitivity. A, Effects of Dex (10⁻⁶ M) on TNF-α-induced CXCL8 release in alveolar macrophages. B-D, Effects of BAL supernatant on (B) Akt1 phosphorylation levels, (C) HDAC2-Ser³⁹⁴ phosphorylation, and (D), immunoprecipitated HDAC2 activity. E, Effects of Dex on TNF-α-induced CXCL8 release. PMA-differentiated U937 cells were preincubated overnight with BAL fluid (BALF) (adjusted to 2 μg/mL of protein). F, MDA levels in BALF from non-PS and PS group subjects. Dex = dexamethasone; MDA = malondialdehyde; PMA = phorbol 12-myristate 13-acetate; TNF = tumor necrosis factor. See Figure 1 legend for expansion of other abbreviations.
HDAC2 expression with increasing disease severity; AMs from patients with COPD showed lower levels of expression of HDAC2, but not HDAC1, in AMs from BALF and peripheral lung tissue. We found that PS is associated with a lower level of both HDAC1 and HDAC2 activity. In line with these previous reports, we found that PS-exposed children were induced by oxidative stress, leading to nitration and subsequent ubiquitination of HDAC2, or carboxylation/oxidation. A well-documented mechanism is PI3K-dependent phosphorylation of HDAC2. Akt is phosphorylated through the PI3K/Akt pathway during oxidative stress, and activated Akt-dependent phosphorylation, mainly at Ser364, Ser422, and Ser424, may cause degradation and inactivation of HDAC2. In line with these previous reports, we found that PS increased phosphorylation of Akt1 (Fig 2B) and HDAC2-Ser364 (Fig 2A). Akt1 phosphorylation was positively correlated with HDAC2 phosphorylation and negatively correlated with HDAC2 activity.

Conclusions
We have demonstrated the molecular and cellular basis of an important adverse effect of PS exposure in children with severe asthma. PS exposure impairs HDAC2 function via PI3K activation, which may contribute to a more steroid-resistant phenotype. Clearly, the avoidance of PS exposure is of paramount importance to all children, particularly those with severe asthma. This study underscores on a molecular level the harm done to asthmatic children by parents who smoke.

Acknowledgments
Author contributions: Drs Kobayashi and Ito had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Dr Kobayashi contributed to the conception and design of the study, conception of the experiments, analysis and interpretation of the data, drafting of the manuscript, review of the report, and approval of the final version.
Dr Bossley: contributed to patient enrollment, conception of the experiments, analysis and interpretation of the data, review of the report, and approval of the final version.

Dr Max: contributed to patient enrollment, conception of the experiments, analysis and interpretation of the data, review of the report, and approval of the final version.

Dr Akashi: contributed to the conception of the experiments, review of the report, and approval of the final version.

Dr Taatsals: contributed to patient enrollment, conception of the experiments, analysis and interpretation of the data, review of the report, and approval of the final version.

Dr Mercado: contributed to the conception of the experiments, analysis and interpretation of the data, review of the report, and approval of the final version.

Dr Barnes: contributed to the conception and design of the study, drafting of the manuscript, review of the report, and approval of the final version.

Dr Bush: contributed to the conception and design of the study, patient enrollment, drafting of the manuscript, review of the report, and approval of the final version.

Dr Ito: contributed to the conception and design of the study, conception of the experiments, analysis and interpretation of the data, drafting of the manuscript, review of the report, and approval of the final version.

Financial/nonfinancial disclosures: The authors have reported to CHEST the following conflicts of interest: Dr Barnes has served on scientific advisory boards for AstraZeneca; Boehringer-Ingelheim; Chiesi Pharmaceuticals; Daiichi Sankyo, Inc; GlaxoSmithKline; Novartis; Nycomed; Pfizer Inc; RespiVert; Teva Pharmaceutical Industries, Ltd; and UCB and has received research funding from Aquinox Pharmaceuticals; AstraZeneca; Boehringer-Ingelheim; Chiesi Pharmaceuticals; Daiichi-Sankyo, Inc; GlaxoSmithKline; Novartis; Nycomed; Pfizer Inc; and Prosonix. Dr Ito is currently an employee of RespiVert and has an honorary contract with Imperial College. Drs Kobayashi, Bossley, Gupta, Akashi, Tsartsali, Mercado, and Bush have reported that no potential conflicts of interest exist with any companies/organizations whose products or services may be discussed in this article.

Role of sponsors: The sponsors had no role in the design of the study; the collection and analysis of the data, or in the preparation of the manuscript.

Other contributions: We are grateful to all the patients and parents for agreeing to take part in our study. We gratefully acknowledge the following people for their invaluable help performing the bronchoscopies: S. Saglani, MD; M. Rosenthal, MD; I. Balfour-Lynn, MD; C. Hogg, MD; and J. Davies, MD.

REFERENCES


