CHEST

Original Research

ASTHMA

Passive Smoking Impairs Histone Deacetylase-2 in Children With Severe Asthma

Yoshiki Kobayashi, MD; Cara Bossley, MD; Atul Gupta, MD; Kenichi Akashi, MD; Lemonia Tsartsali, MD; Nicolas Mercado, PhD; Peter J. Barnes, DM, DSc, FCCP; Andrew Bush, MD; and Kazuhiro Ito, PhD

Background: **Parental smoking is known to worsen asthma symptoms in children and to make them refractory to asthma treatment, but the molecular mechanism is unclear. Oxidative stress from tobacco smoke has been reported to impair histone deacetylase-2 (HDAC2) via phosphoinositide-3-kinase (PI3K)/Akt activation and, thus, to reduce corticosteroid sensitivity. The aim of this study was to investigate passive smoking-dependent molecular abnormalities in alveolar macrophages (AMs) by comparing passive smoke-exposed children and non-passive smoke-exposed children with uncontrolled severe asthma.**

Methods: **BAL fluid (BALF) was obtained from 19 children with uncontrolled severe asthma (10 non-passive smoking-exposed subjects and nine passive smoking-exposed subjects), and HDAC2 expression/activity, Akt/HDAC2 phosphorylation levels, and corticosteroid responsiveness in AMs were evaluated.**

Results: **Parental smoking reduced HDAC2 protein expression by 54% and activity by 47%, with concomitant enhancement of phosphorylation of Akt1 and HDAC2. In addition, phosphorylation levels of Akt1 correlated positively with HDAC2 phosphorylation levels and negatively with HDAC2 activity. Furthermore, passive smoke exposure reduced the inhibitory effects of dexamethasone on tumor necrosis factor-α-induced CXCL8 release in AMs. There were relatively higher neutrophil counts and CXCL8 concentrations in BALF and lower Asthma Control Test scores compared with non-passive smoke-exposed children with uncontrolled severe asthma.**

Conclusions: **Passive smoking impairs HDAC2 function via PI3K signaling activation, which could contribute to corticosteroid-insensitive inflammation in children with severe asthma. This novel mechanism will be a treatment target in children with severe asthma and stresses the need for a smoke-free environment for asthmatic children.** ***CHEST 2014; 145(2):305–312***

Abbreviations: ACT = Asthma Control Test; AFU = arbitrary fluorescence units; AM = alveolar macrophage; BALF = BAL fluid; FENO = fraction of exhaled nitric oxide; HDAC = histone deacetylase; IP = immunoprecipitated; MDA = malondialdehyde; PI3K = phosphoinositide-3-kinase; PMA = phorbol 12-myristate 13-acetate; ppb = parts per billion; PS = passive smoking; RIPA = radioimmunoprecipitation assay; TNF = tumor necrosis factor

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,[1] adult patients with asthma who currently smoke have relative steroid resistance.[2] Furthermore, their asthma becomes more severe and their lung function decreases more rapidly compared with nonsmoking patients with asthma.[3,4] Passive smoking (PS) also worsens asthma symptoms and causes poor asthma control in both adults and children.[5,6] 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.[5] 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.[7] 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.[8-10] HDAC2 expression and activity are reduced in the airways of, and alveolar macrophages (AMs) from, adults with severe asthma[11-13] 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.[9,17] 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-2yr)-2-5-diphenyltetrazolium bromide, dimethyl sulfoxide, phorbol 12-myristate 13-acetate (PMA), the rabbit polyclonal HDAC-1 (HDAC1) antibody, and the mouse monoclonal HDAC2 antibody were purchased from Sigma-Aldrich. The rabbit polyclonal antibody to phospho-HDAC2 (Ser^{394}) 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 (Ser^{473}) antibody and the rabbit polyclonal anti-Akt1/PKBα antibody were obtained from Millipore. Recombinant 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.[18] 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.[20] 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 radioimmunoprecipitation assay (RIPA) buffer as described previously.[8] 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.[22]

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

Manuscript received April 6, 2013; revision accepted August 1, 2013.
Affiliations: From the Airway Disease Section (Drs Kobayashi, Bossley, Gupta, Akashi, Tsartsali, Mercado, Barnes, Bush, and Ito), National Heart and Lung Institute, Imperial College London; and the Department of Respiratory Paediatrics (Drs Bossley, Gupta, Tsartsali, and Bush), Royal Brompton Hospital, London, England.
This study was presented previously at the American Thoracic Society 2011 International Conference, May 13-18, 2011, Denver, CO.
Funding/Support: This study was funded by the Wellcome Trust [WHRD_P31768 to Dr Barnes], London, England, and by the National Institute of Health Research Respiratory Disease Biomedical Research Unit at the Royal Brompton and Harefield NHS Foundation Trust and Imperial College London (to Drs Bossley, Gupta and Bush). Dr Gupta is also the recipient of a British Medical Association, James Trust Fellowship.
Correspondence to: Kazuhiro Ito, PhD, Airway Disease Section, National Heart and Lung Institute, Imperial College London, Guy Scadding Bldg, Royal Brompton Campus, Dovehouse St, London, SW3 6LY, England; e-mail: k.ito@imperial.ac.uk

DOI: 10.1378/chest.13-0835