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Title:

Passive smoking impairs histone deacetylase-2 in children with severe asthma

(Short title: Passive smoking impairs HDAC2 in pediatric asthma)

Yoshiki Kobayashi (MD)¹, Cara Bossley (MD)^{1,2}, Atul Gupta (MD)^{1,2}, Kenichi Akashi (MD)¹, LEMONIA Tsartsali (MD)^{1,2}, Nicolas Mercado (PhD)¹, Peter J. Barnes (FRS)¹, Andrew Bush (MD)^{1,2} and Kazuhiro Ito (PhD)¹

1: Airway Disease Section, National Heart and Lung Institute, Imperial College London, London, United Kingdom, 2: Department of Respiratory Paediatrics, Royal Brompton Hospital, London, United Kingdom

Correspondence and requests for reprints should be addressed to Kazuhiro Ito, Ph.D., Airway Disease Section, National Heart and Lung Institute, Imperial College London, Guy Scadding Building, Royal Brompton Campus, Dovehouse Street, London SW3 6LY, UK. E-mail: k.ito@imperial.ac.uk.

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Conflict of Interest:

KI is currently an employee of RespiVert Ltd and has honorary contract with Imperial College. PB has served on Scientific Advisory Boards of AstraZeneca, Boehringer-Ingelheim, Chiesi, Daiichi-Sankyo, GlaxoSmithKline, Novartis, Nycomed, Pfizer, RespiVert, Teva and UCB and has received research funding from Aquinox Pharmaceuticals, AstraZeneca, Boehringer-Ingelheim, Chiesi, Daiichi-Sankyo, GlaxoSmithKline, Novartis, Nycomed, Pfizer, and Prosonix. The other authors have declared that no competing interests exist for this research.

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50 **Abstract**

51

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

58 **Methods:** Bronchoalveolar lavage fluid (BALF) was obtained from 19 children with uncontrolled severe
59 asthma (10 non-passive smoking subjects and 9 passive smoking subjects), and HDAC2
60 expression/activity, Akt/HDAC2 phosphorylation levels and corticosteroid responsiveness in alveolar
61 macrophages were evaluated.

62 **Results:** Parental smoking reduced HDAC2 protein expression by 54% and activity by 47% with
63 concomitant enhancement of phosphorylation of Akt1 and HDAC2. In addition, phosphorylation levels of
64 Akt1 correlated well with HDAC2 phosphorylation levels positively and negatively with HDAC2 activity.
65 Furthermore, passive smoke exposure reduced the inhibitory effects of dexamethasone on TNF α -induced
66 CXCL8 release in alveolar macrophages. There was relatively higher neutrophil count and CXCL8
67 concentration in BALF and lower ACT score compared with non-passive smoke exposed children with
68 uncontrolled severe asthma.

69 **Conclusions:** Passive smoking impairs HDAC2 function via PI3K signalling activation, which could
70 contribute to corticosteroid insensitive inflammation in children with severe asthma. This novel
71 mechanism will be a treatment target in children with severe asthma and stresses the need for a smoke-free
72 environment for asthmatic children.

73

74 **Keywords**

75 Corticosteroid insensitivity, histone deacetylase-2, passive smoking, severe asthma, phosphorylation, Akt1,
76 PI3K

77

34 **Abbreviations**

35 ACT: asthma control test,

36 AM: alveolar macrophage,

37 BALF: bronchial alveolar lavage fluid,

38 FeNO: Exhaled nitric oxide fraction,

39 FEV1: forced exhaled volume in 1 second,

40 GR: glucocorticoid receptor,

41 HDAC: histone deacetylase,

42 IP: immunoprecipitate,

43 MDA: malondialdehyde,

44 Non-PS: non-passive smoking,

45 PI3K: phosphoinositide 3 kinase,

46 PS: Passive smoking

47 RIPA: Radio-Immunoprecipitation Assay

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49

Introduction

Asthma is the commonest inflammatory disease, and its prevalence is increasing throughout the world. Although corticosteroids are the most effective anti-inflammatory agents for the treatment of asthma¹, adult patients with asthma who currently smoke have relative steroid resistance². Furthermore, their asthma becomes more severe and lung function decreases more rapidly compared with non-smoking asthmatics^{3,4}. Passive smoking 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 one of the risk factors for the persistence of asthma in later childhood⁵. However, the molecular mechanisms of the effects of passive smoke 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 receptor GR β ⁷. Recently, histone deacetylase-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 airways and alveolar macrophages from adults with severe asthma¹¹⁻¹³ and chronic obstructive pulmonary disease (COPD)^{14,15}. Even more importantly, in smoking asthmatics, there is a significantly greater reduction of HDAC activity in bronchial biopsies than in non-smoking asthmatic patients¹⁶. Further analysis revealed that oxidative stress such as cigarette smoke impairs HDAC2 via phosphoinositide-3-kinase δ (PI3K δ)/Akt activation^{9,17}.

Here we have tested the hypothesis that passive exposure to tobacco smoke is associated with reduced HDAC2 in alveolar macrophages in children with severe and refractory asthma.

Methods

Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), phorbol 12-myristate 13-acetate (PMA), the rabbit polyclonal HDAC1 antibody and the mouse monoclonal HDAC2 antibody were purchased from Sigma-Aldrich (Poole, UK). The rabbit polyclonal antibody to phospho-HDAC2 (Ser 394) and the mouse monoclonal antibody to β -actin were obtained from Abcam (Cambridge, UK). Protein A/G plus-agarose immunoprecipitation reagent was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The mouse monoclonal anti-phospho-Akt1/PKB α (Ser 473) antibody and the rabbit polyclonal anti-Akt1/PKB α antibody were obtained from Millipore (Billerica, MA). Recombinant Human TNF α was purchased from R&D Systems Europe (Abingdon, UK).

Patients

Nineteen children with severe asthma were recruited for bronchoscopy as part of the work-up for severe, therapy-resistant asthma¹⁸. All children were under regular follow-up at Royal Brompton Hospital. Asthma was diagnosed according to the American Thoracic Society criteria and the severity was defined based on the Global Initiative for Asthma (GINA) criteria. All had undergone a detailed evaluation to exclude as far as possible reversible factors such as poor adherence to therapy¹⁹. Subjects were classified into two groups (non-passive smoking and passive smoking). Exposure to passive smoking was assessed on the basis of information reported by parents concerning their smoking habits. Cotinine levels in saliva or urine were also measured to support their statement. This study was conducted in accordance with the amended "Declaration of Helsinki (<http://www.wma.net/en/30publications/10policies/b3/>), and 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

Fractional exhaled nitric oxide (FeNO) was measured according to current guidelines²⁰ using a NIOX chemiluminescence analyzer (Aerocrine, Solna, Sweden) at a flow rate of 50 mL/s.

Bronchoalveolar Lavage and Macrophage Processing

Bronchoalveolar lavage (BAL) using fiberoptic bronchoscopy was performed under general anaesthetic, as previously described²¹. Cells were centrifuged and washed with Hanks' balanced salt solution (HBSS). 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 incubated in Macrophage Serum Free Medium (Invitrogen Ltd., Paisley, UK).

Cells

The human monocytic cell line U937 were differentiated into an adherent macrophage-like morphology by exposure to PMA (50 ng/ml) for 48 h.

Cytokine ELISA and Corticosteroid Sensitivity

CXCL8 concentrations were determined by sandwich ELISA (R&D Systems Europe). Alveolar macrophages or U937 cells were treated with dexamethasone (10^{-6} M), followed by the TNF α stimulation (10 ng/ml) for 2hrs. Dexamethasone's ability to inhibit TNF α -induced CXCL8 release was analyzed as a marker of corticosteroid sensitivity.

TBARS assay

As a marker of oxidative stress, malondialdehyde (MDA) was measured as thiobarbituric acid reactive substances (TBARS) using a TBARS Assay Kit (Cayman Chemical Company, Ann Arbor, MI).

Protein extraction and detection

Whole cell protein extracts were prepared using Radio-Immunoprecipitation Assay (RIPA) buffer as previously described⁸. Immunoprecipitation was conducted with 2 μ g of anti-HDAC2 antibody (Sigma-Aldrich) overnight 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, Chalfont St. Giles, UK) as previously reported²².

Total HDAC and HDAC2 Activity

To measure In-cell HDAC activity, cells were incubated with *Fluor de Lys*TM substrate (200 μ M) for 1 hr before cell lysis using RIPA buffer. Total HDAC activity was measured using the HDAC Fluorimetric Assay/Drug Discovery Kit (BIOMOL[®] International, Inc., Plymouth Meeting, PA). Immunoprecipitated (IP) HDAC2 was resuspended in HDAC assay buffer and the activity was measured as shown above. HDAC activity was expressed as μ M of fluorescence standard provided in the kit.

Immunocytochemistry

HDAC2 in BAL macrophage cytopins was immunostained with HDAC2 antibody (Santa Cruz Biothechnology; diluted 1:25) using the VECTASTAIN[®] kit (Vector Laboratories, Peterborough, UK), as previously described⁸.

Statistical analysis

Statistical analysis between non-passive smoke group and passive smoke group was conducted with Mann Whitney U-test. If applicable in the *in vitro* test, Student t-test was used for the comparison in paired groups. Correlation coefficients were calculated with the use of Spearman's rank method. The results were expressed as medians (first and third quartiles) or means $\pm$ standard error of the mean (SEM). GraphPad Prism[®] (GraphPad Software, San Diego, CA) was used for all statistical analyses, and P-values of less than 0.05 were considered to be statistically significant.

Results

Patient Characterization

Characteristics of patients are summarized in Table 1. All patients were receiving regular inhaled corticosteroids (ICS) (median dose 1600 µg BDP equivalent in patients without passive smoking (PS) and 1600 µg in those with PS, $P = 0.78$) and a long-acting β_2 -adrenoceptor agonist. There were no differences in age, sex, FEV₁ % predicted, IgE level, atopic status and use of any other medications between non-PS and PS groups. FeNO (51.2 ppb in non-PS vs. 44.0 ppb in PS, $P = 0.60$) and Asthma Control Test (ACT) score (16.0 in non-PS vs. 11.0 in PS, $P = 0.095$) were relatively lower in PS, but there was no significant difference between the groups. Median year exposure to passive smoking in passive-smoke group was 10.0. Consistent with information reported by the patients' parents, all non-PS subjects had little or no cotinine detected, while PS group showed relatively higher cotinine levels (> 2 µg/L in urine or > 0.2 µg/L in saliva).

BAL analysis

There was no difference in total BAL cell counts, or numbers of macrophages and lymphocytes between PS and non-PS group. PS subjects had relatively higher number of neutrophils than non-PS subjects who had more eosinophils in BALF (Table 2). In line with the increase of neutrophils, higher levels of CXCL8 were found in BALF from PS subjects (Table 2) and furthermore there was a positive correlation between CXCL8 concentrations and the percentage of neutrophils ($r = 0.71$, $P < 0.001$).

HDAC2 expression and activity

In alveolar macrophages (AM), HDAC2 protein level normalized to β actin, but not HDAC1, was significantly reduced in PS subjects compared with non-PS subjects (Fig 1, A and B). This reduction of HDAC2 was confirmed by immunocytochemistry (Fig 1. C) as positive brown signals in AMs were reduced in PS. Although there was no significant difference in total HDAC activities in AMs between two groups (Median (1st and 3rd quartile): 30.6 (10.8-45.0) AFU/µg in non-PS vs. 22.4 (13.8-28.6) AFU/µg in PS), the activity of IP-HDAC2 was significantly reduced in PS group compared with non-PS group (5.8 (4.5-8.8) AFU/µg in non-PS vs. 3.7 (1.4-5.4) AFU/µg in PS) (Fig 1. D and E). Moreover, there was a

positive correlation between HDAC2 expression and IP-HDAC2 activity ($r = 0.59$, $P = 0.0072$) (Fig 1. *F*), suggesting that reduction in IP-HDAC2 activity was due to the reduction of HDAC2 protein expression.

Akt1 and HDAC2 phosphorylation

As shown in Figure 2A, HDAC2 was highly phosphorylated in AMs in PS compared with that in non-PS. The levels of phosphorylation of Akt1, a surrogate marker of PI3K signalling activation, were also significantly increased by 2.4 fold in AM of PS compared with that in non-PS (Fig 2. *B*), when normalized to total Akt1 protein expression. Furthermore, Akt1 phosphorylation levels was positively correlated with HDAC2 phosphorylation levels ($r = 0.71$, $P < 0.001$) (Fig 2. *C*) and negatively correlated with HDAC2 activity ($r = -0.54$, $P = 0.018$) (Fig 2. *D*).

Corticosteroid sensitivity of CXCL8 production in AMs

Dexamethasone (Dex) at 10^{-6} M significantly inhibited TNF α -induced CXCL8 production by 40% in AMs from non-PS subjects, but there was no significant inhibition in AMs from PS-subjects (Fig 3. *A*). Next, PMA-differentiated macrophage like U937 cells were exposed to BALF obtained from PS group or non-PS group. BALF from PS group significantly increased Akt1 phosphorylation (Fig 3. *B*), increased HDAC2 phosphorylation (Fig 3. *C*) and reduced IP-HDAC2 activity (336.6 (310.1-358.4) AFU/ μ g in non-PS, 299.1 (277.0-316.7) AFU/ μ g in PS) in U937 cells (Fig 3. *D*). Pre-treatment of Dex (10^{-6} M) completely inhibited TNF α -induced CXCL8 release in U937 exposed to the BALF from non-PS, but the inhibitory action was limited in U937 exposed to the BALF from PS (Fig 3. *E*). Furthermore, the level of MDA, a marker of oxidative stress, was found to be significantly higher in BALF in PS than that in non-PS (Fig 3. *F*).

Discussion

This manuscript reports that asthmatic children passively exposed to tobacco smoke had the same molecular abnormalities leading to *in vitro* steroid resistance as adults who actively smoke⁸. Children with severe asthma, who were exposed to PS had higher CXCL8 levels (Table 2), in agreement with previous reports^{14,23}, and higher neutrophil count and percentage in BALF (Table 2), and the neutrophil percentage of total cell counts positively correlated with CXCL8 concentration. Even more importantly, alveolar macrophages from PS-exposed asthmatic children exhibited corticosteroid insensitivity in the *in vitro* assay, manifest by the reduced effect of dexamethasone in suppressing TNF α -induced CXCL8 production compared with non-PS children with uncontrolled severe asthma. As prescribed corticosteroid dose was the same between PS and non-PS groups, inhaled corticosteroids were not potential modifier of this response. This is the first evidence that PS affects corticosteroid sensitivity in resident lung cells in children with severe asthma *in vitro*. As there is no agreed definition of steroid sensitivity in children with asthma, we did not compare the *in vitro* steroid sensitivity and clinical response to steroids, and thus the clinical significance of our findings is speculative. However we have previously shown that responses to steroids are reduced in children exposed to passive smoking²⁴.

Recently, reduction in HDAC activity, especially HDAC2, has been reported to cause corticosteroid insensitivity^{8,25}. We found that PS is associated with lower expression of HDAC2 but not HDAC1 in AMs (Fig 1. A, B and C). AMs in BALF and peripheral lung tissue from COPD patients showed lower levels of HDAC2 expression with increasing disease severity; however HDAC1 expression was not altered¹⁵. Furthermore, in AMs from healthy cigarette smokers, HDAC2 but not HDAC1 expression was significantly decreased compared with those from healthy non-smokers⁸ although in bronchial biopsies obtained from adults with mild asthma, the expression levels of both HDAC1 and HDAC2 are slightly reduced^{11,12}. These findings suggest that HDAC2 might be more sensitive to oxidative stress, especially cigarette smoking, than HDAC1 as we previously reported^{25,26}.

The molecular mechanism of HDAC2 reduction has been extensively studied. Defects of HDAC2 were induced by oxidative stress leading to nitration and subsequent ubiquitination of HDAC2²⁶, or carboxylation/oxidation^{27,28}. A well-documented mechanism is PI3K-dependent phosphorylation of HDAC2. Akt is phosphorylated through the PI3K δ pathway during oxidative stress^{9,17}, and activated Akt dependent phosphorylation, mainly at Ser³⁹⁴, Ser⁴²² and Ser⁴²⁴²⁹ may cause degradation and inactivation of

HDAC2^{30,31}. In line with these previous reports, we found that PS increased phosphorylation of Akt1 (Fig 2. B) and HDAC2-Ser³⁹⁴ (Fig 2. A). Akt1 phosphorylation was positively correlated with HDAC2 phosphorylation and negatively with HDAC2 activity in AMs from children with severe asthma (Fig 2. C and D). This observations seem to be driven by the presence of single outlier, but when the outlier was removed, pAkt1 was still correlated with pHDAC2 ($r=0.66$, $p=0.0027$). Thus, PI3K signalling activation by PS is likely one of major causes of HDAC2 down-regulation in children with severe asthma.

Oxidative stress such as from cigarette smoke may be an important factor in inducing corticosteroid insensitivity. MDA is a product of lipid oxidation and an oxidative stress biomarker³². Previous reports showed that MDA was increased in several biological fluids with exposure to cigarette smoke^{27,33,34}. In this study, we also found higher level of MDA in BALF from PS subjects (Fig 3. F). Importantly, when BALF from PS children was added to the macrophage type U937 cell line, the cells became corticosteroid-insensitive with increased phosphorylated Akt/HDAC2 and reduced HDAC2 activity. Thus, high levels of oxidative stress and their end products may induce corticosteroid insensitivity with dysfunction of HDAC2. In contrast, the FeNO level was relatively lower in the passive-smoke group than in non-passive group, which is consistent with previous finding in adult³⁵.

In our study, more severe asthma children exposed to PS had ACT scores of ≤ 15 when compared with the severe asthma patients not exposed to PS, indicating that their asthma of PS exposed children was relatively poorly controlled as previously reported³⁶. However, there were no significant differences in ACT scores between the groups, probably due to the relatively small number of patients. In order to strengthen our findings, more clinical outcomes are needed, for example, the effect of avoidance of PS, in a longitudinal and much bigger group of children with uncontrolled severe asthma.

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 in all children, particularly those with severe asthma⁵. This study underscores on a molecular level the harm done to asthmatic children by parents who smoke.

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Author contribution

YK, AB, PJB, and KI were involved in the conception and design of this study, CB, AG, LT and AB enrolled patients, YK, KA, CB, AG, LT, NM and KI conceived the experiments, YK, CB, AG, LT, NM and KI were involved in analysis and interpretation of the data, YK, PJB, AB, and KI were involved in drafting the manuscript for important intellectual content. All authors were involved in the reviewing of the report and approved the final version.

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TABLE 1. CHARACTERISTICS OF SUBJECTS

	Non passive smoking	Passive smoking
	(n = 10)	(n = 9)
*Age; yr	9.5 (8.5–11.5)	10.0 (8.5-13.5)
Sex; M : F	7 : 3	5 : 4
*Lifetime exposure to passive smoking; yr	0	10.0 (8.5-13.5)
*FEV ₁ %predicted	71.5 (68.0-78.5)	72.0 (51.5-89.0)
*FeNO (ppb) ¹⁾	51.2 (29.6-73.7)	44.0 (26.9-61.9)
*ACT score	16.0 (10.5-17.5)	11.0 (8.5-14.5)
≤15	5/10	8/9
*Total IgE (IU/mL)	386 (150-1511)	355 (158-1462)
Atopy ²⁾	8/9	7/9
Medication		
*ICS (µg) ³⁾	1600 (1300-2000)	1600 (1200-2000)
LABA	10/10	9/9
LTRA	9/10	6/9
Theophylline	2/10	2/9

There are no significant differences in both groups. *Values are expressed as medians (1st and 3rd quartile).

¹⁾ Values in non-passive smoking represent medians of nine subjects. ACT = Asthma Control Test, ²⁾

Atopy was defined as at least one positive specific immunoglobulin (Ig)E radioallergosorbent assay

(RAST; ≥0.34 kU/L), ³⁾ ICS = Inhaled corticosteroid; beclomethasone dipropionate equivalent dose,

LABA = Long-acting β₂-adrenoceptor agonist, LTRA = Leukotriene receptor antagonist

406 **TABLE 2. CELL and CXCL8 ANALYSIS IN BRONCHOALVEOLAR LAVAGE**

Cell count ($\times 10^3/\text{ml}$)	Non passive smoking	Passive smoking	<i>p</i> -value
[% of total cells]	(n = 10)	(n = 9)	
Total	370 (265-553)	280 (205-403)	0.2110
Macrophage	287 (196-389)	236 (326-162)	0.4967
	[76.2 (70.5-78.0)]	[82.8 (81.3-85.9)]	[0.0030]
Neutrophil	13.3 (23.1-9.2)	24.1 (12.7-34.4)	0.1333
	[3.7 (3.0-4.5)]	[8.3 (6.5-9.5)]	[0.0021]
Lymphocyte	55.2 (33.3-80.9)	10.9 (7.7-38.1)	0.0101
	[14.7 (10.7-18.1)]	[5.0 (3.7-8.8)]	[< 0.001]
Eosinophil	11.6 (5.8-45.1)	2.9 (1.0-9.6)	0.0435
	[4.0 (1.2-10.4)]	[1.6 (0.5-3.1)]	[0.1333]
CXCL8 (pg/ μg protein)	0.11 (0.05-0.24)	0.78 (0.27-1.40)	0.0021

407 Values are expressed as medians (1st and 3rd quartile).

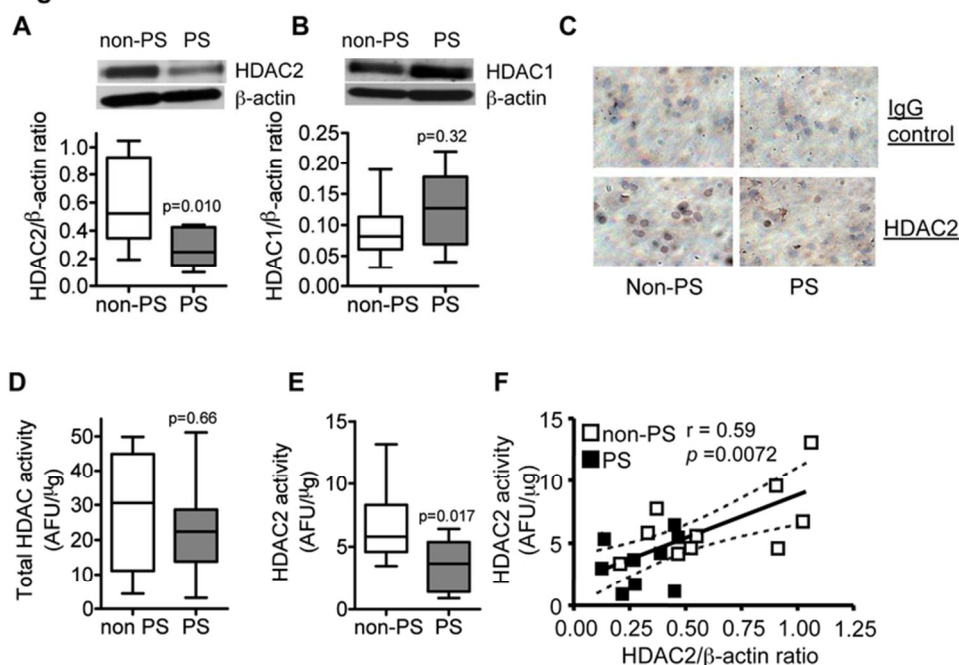
408

Figure Legends

FIGURE 1. Effects of passive smoking on HDAC2 protein expression and activity in alveolar macrophages from children with severe asthma. **A, B**, Western blotting analysis of HDAC2 and HDAC1 protein expression normalized to β -actin expression, respectively. **C**, HDAC2 protein expression detected by immunocytochemistry (original magnification 400 $\times$). Results were representative of at least 7 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 means $\pm$ SEM of 10 non-passive smoking (non-PS) subjects or 9 passive smoking (PS) subjects.

FIGURE 2. Effects of passive smoking on phosphorylation level of Akt1 and HDAC2 in alveolar macrophages from children with severe asthma. **A, B**, Phosphorylation levels of HDAC2-Ser³⁹⁴ (A) and Akt1 (B) normalized to total HDAC2 and Akt1 expression. Values represent means of 10 (in non-passive smoking (non-PS) group) or 9 (in PS group) subjects $\pm$ SEM; **C**, Correlation between HDAC2 and Akt1 phosphorylation levels. **D**, Correlation between immunoprecipitated-HDAC2 activity and phosphorylation level of Akt1.

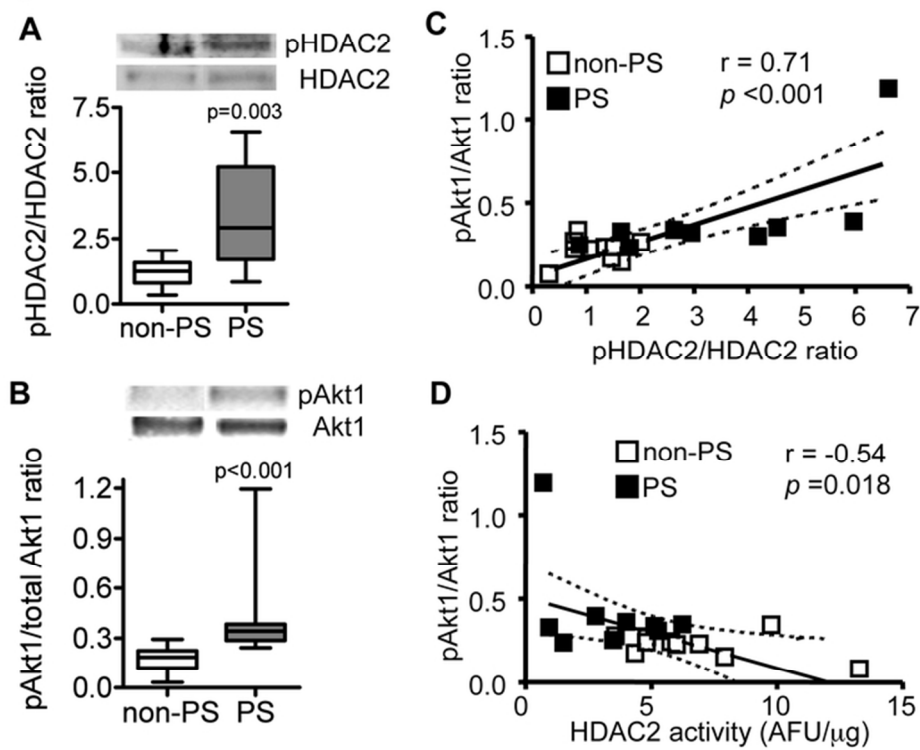
FIGURE 3. Effects of passive smoking on corticosteroid sensitivity. **A**, Effects of dexamethasone (Dex; 10⁻⁶ M) on TNF α -induced CXCL8 release in alveolar macrophages. **B-E**, Effects of BAL supernatant on Akt1 phosphorylation levels (B), HDAC2-Ser³⁹⁴ phosphorylation (C), immunoprecipitated-HDAC2 activity (D) and effects of Dex on TNF α -induced CXCL8 release (E). PMA-differentiated U937 cells were preincubated with BALF (adjusted to 2 μ g/ml of protein) overnight. **F**, MDA levels in BALF from non-PS and PS.

Figure 1

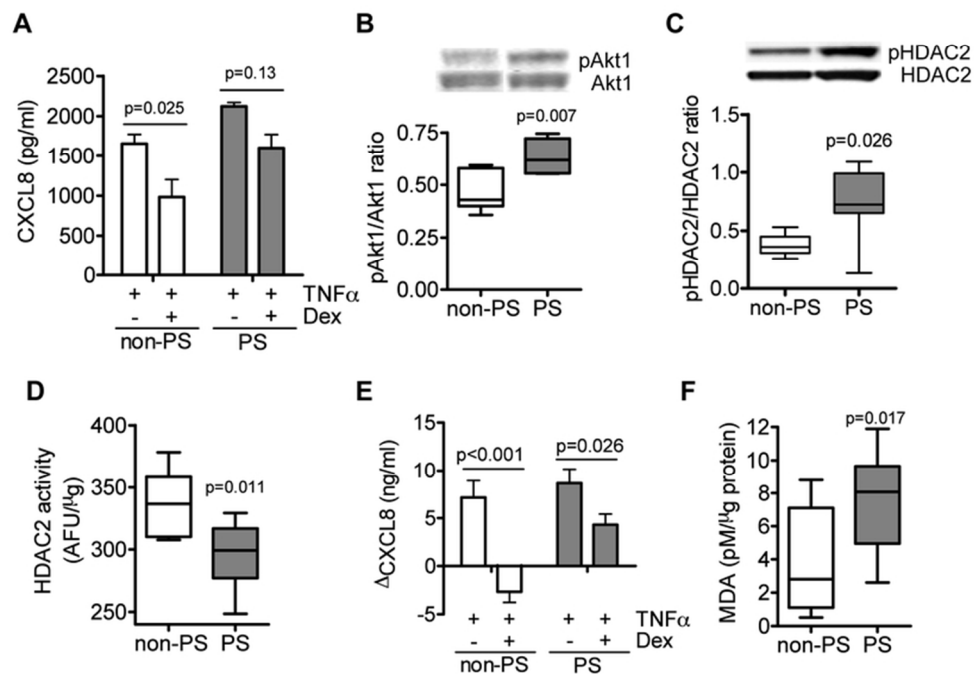
Effects of passive smoking on HDAC2 protein expression and activity in alveolar macrophages from children with severe asthma. A, B, Western blotting analysis of HDAC1 and HDAC2 Protein expression normalized to β -actin expression. C, HDAC2 protein expression detected by immunocytochemistry (original magnification 400x). Results were representative of at least 7 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 means $\pm$ SEM of 10 non-passive smoking (non-PS) subjects or 9 passive smoking (PS) subjects.

71x52mm (300 x 300 DPI)

Figure 2



Effects of passive smoking on phosphorylation level of Akt1 and HDAC2 in alveolar macrophages from children with severe asthma. A, B, Phosphorylation levels of HDAC2-Ser³⁹⁴ (A) and Akt1 (B) normalized to total HDAC2 and Akt1 expression. Values represent means of 10 (in non-passive smoking (non-PS) group) or 9 (in PS group) subjects $\pm$ SEM; C, Correlation between HDAC2 and Akt1 phosphorylation levels. D, Correlation between immunoprecipitated-HDAC2 activity and phosphorylation level of Akt1.

Figure 3

Effects of passive smoking on corticosteroid sensitivity. A, Effects of dexamethasone (Dex; 10^{-6} M) on TNF α -induced CXCL8 release in alveolar macrophages. B-E, Effects of BAL supernatant on Akt1 phosphorylation levels (B), HDAC2-Ser³⁹⁴ phosphorylation (C), immunoprecipitated-HDAC2 activity (D) and effects of Dex on TNF α -induced CXCL8 release (E). PMA-differentiated U937 cells were preincubated with BALF (adjusted to 2 μ g/ml of protein) overnight. F, MDA levels in BALF from non-PS and PS.

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