Oxidative and nitrosative stress and histone deacetylase-2 activity in exacerbations of Chronic Obstructive Pulmonary Disease

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Complete List of Authors:	Footitt, Joseph; Imperial College, Respiratory Medicine Mallia, Patrick; Imperial College, Respiratory Medicine durham, andrew; Imperial College, Ho, Eugene; Yong Loo Lin School of Medicine, Department of Pharmacology Trujillo-Torralbo, Maria-Belen; Imperial College, Respiratory Medicine Telcian, Aurica; Imperial College London, Airway Disease Infection Del Rosario, Ajerico; Imperial College, Respiratory Medicine Chang, Cheng; Yong Loo Lin School of Medicine, Department of Pharmacology Peh, Hong Yong; Yong Loo Lin School of Medicine, Department of Pharmacology Kebadze, Tatiana; National Heart and Lung Institute, Airway Disease Infection Aniscenko, J; National heart and lung institute, Imperial College, Stanciu, Luminita; Imperial College, Respiratory Medicine Essilfie-Quaye, Sarah; NHLI, Imperial College, Airway Disease Ito, Kazuhiro; NHLI, Imperial College, Airway Disease; Barnes, Peter; NHLI, ; Elkin, Sarah; Imperial College Healthcare NHS Trust, Chest & Allergy; Kon, Onn Min; Imperial College, Respiratory Medicine Wong, Fred; Yong Loo Lin School of Medicine, Department of Pharmacology Adcock, IM; Imperial College London, NHLI; Johnston, Sebastian; Imperial College, Respiratory Medicine
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Abstract 236

Oxidative and nitrosative stress and histone deacetylase-2 activity in exacerbations of Chronic Obstructive Pulmonary Disease

Short title: Oxidative stress and HDAC2 in COPD exacerbations

Joseph Footitt PhD[†1,2,3*], Patrick Mallia PhD[1,2,3*], Andrew L. Durham PhD[4*], W. Eugene Ho PhD[5], Maria-Belen Trujillo-Torralbo BSc[1,2], Aurica G Telcian PhD[1], Ajerico Del Rosario BSc[1,2], Cheng Chang PhD[5], Hong-Yong Peh MSc[5], Tatiana Kebadze MD[1], Julia Aniscenko[1], Luminita Stanciu PhD[1], Sarah Essilfie-Quaye PhD[4], Kazuhiro Ito PhD[4], Peter J Barnes FRS, FMedSci[4], Sarah L Elkin PhD[1,2], Onn M Kon[1,2,3], WS Fred Wong PhD[5], Ian M Adcock PhD[4], Sebastian L Johnston PhD[1,2,3]

[1]Airway Disease Infection Section, National Heart and Lung Institute, Imperial College, London, United Kingdom

[2]Imperial College Healthcare NHS Trust, London, United Kingdom

[3]Centre for Respiratory Infection, Imperial College, London, United Kingdom

[4]Airways Disease Section, National Heart and Lung Institute, Imperial College, London, United Kingdom

[5]Department of Pharmacology, Yong Loo Lin School of Medicine, National University Health System, Singapore 117456

[†]deceased

[*]equal contributions

Corresponding author: Sebastian L Johnston, Professor of Respiratory Medicine, Airway Disease Infection Section, National Heart and Lung Institute, Imperial College, Norfolk Place, London W2 1PG, UK.

Email: s.johnston@imperial.ac.uk.

Declaration of interests

JF, ALD, WEH, MBTT, AGT, ADR, CC, HYP, TK, JA, LS, SEQ, AT and SLE have no competing interests. PM has received honoraria and travel grants from GSK. KI is an employee of Pulmocide Ltd and has honorary contract with Imperial College. PJB has served on Scientific Advisory Boards of AstraZeneca, Boehringer-Ingelheim, Bespak, Chiesi, Daiichi-Sankyo, DeepBreeze, GlaxoSmithKline, Glenmark, Johnson & Johnson, Merck, Novartis, Nycomed/Takeda, Pfizer, Prosonix, Sun Pharmaceuticals, Teva and UCB and has received research funding from Aquinox Pharmaceuticals, AstraZeneca, Boehringer-Ingelheim, Chiesi, Daiichi-Sankyo, GSK, Novartis, Nycomed/Takeda, Pfizer, Prosonix, Sun Pharmaceuticals. He is also a cofounder of RespiVert (now part of Johnson & Johnson), which has discovered novel inhaled anti-inflammatory treatments for asthma and COPD. OMK has received travel grants from Boehringer Ingelheim, WSFW has received consultancies from Davos Life Science Pte Ltd, Singapore, IMA has received consultancies, honoraria, and travel and research grants from GSK, AstraZeneca, Johnson & Johnson, Chiesi, Pfizer, Boehringer Ingelheim, Novartis and Vectura, SLJ has received consultancies, honoraria, and travel and research grants from GSK, Johnson & Johnson, Sanofi Aventis, Chiesi, Pfizer, Boehringer Ingelheim, AstraZeneca, Novartis and Synairgen and has stock options in Synairgen.

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Abstract

Background

Respiratory virus infections are commonly associated with COPD exacerbations but little is known about the mechanisms linking virus infection to exacerbations. Pathogenic mechanisms in stable COPD include oxidative and nitrosative stress and reduced activity of histone deacetylase-2 (HDAC2) but their roles in COPD exacerbations is unknown. We investigated oxidative/nitrosative stress and HDAC2 in COPD exacerbations using experimental rhinovirus infection.

Methods

9 subjects with COPD (GOLD stage II), 10 smokers and 11 non-smokers were successfully infected with rhinovirus. Markers of oxidative and nitrosative-stress associated cellular damage, inflammatory mediators and proteases were measured in sputum, and HDAC2 activity measured in sputum and bronchoalveolar macrophages. In an *in vitro* model monocyte derived THP-1 cells were infected with rhinovirus and nitrosylation and activity of HDAC2 measured.

Results

Rhinovirus infection induced significant increases in airways inflammation and markers of oxidative and nitrosative stress in COPD subjects. Oxidative/nitrosative stress markers correlated with virus load and inflammatory markers. Macrophage HDAC2 activity was reduced during exacerbation and correlated inversely with virus load, inflammatory markers and nitrosative stress. Sputum macrophage HDAC2 activity pre-infection was inversely associated with sputum virus load and inflammatory makers during exacerbation. Rhinovirus infection of monocytes induced nitrosylation of HDAC2 and reduced HDAC2 activity, inhibition of oxidative/nitrosative stress inhibited rhinovirus-induced inflammatory cytokines.

Conclusions

Oxidative and nitrosative stress, airways inflammation and impaired HDAC2 may be important mechanisms of virus-induced COPD exacerbations. Therapies targeting these mechanisms offer potential new treatments for COPD exacerbations.

Abbreviations List

3-NT	3-nitrotyrosine
8-OHdG	8-hydroxy-2'-deoxyguanosine
ATS	American Thoracic Society
COPD	Chronic obstructive pulmonary disease
ERS	European Respiratory Society
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GM-CSF	Granulocyte-macrophage colony stimulating factor
GOLD	Global Initiative for Obstructive Lung Disease
HDAC2	Histone deacetylase-2
IL-	Interleukin
MMP-9	Matrix metalloprotease-9
NAC	N-acetylcysteine
NE	Neutrophil elastase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
$TCID_{50}$	Tissue culture infective $dose_{50}$
TNF-α	Tumour necrosis factor-alpha

Introduction

Chronic obstructive pulmonary disease (COPD) is a rapidly growing global epidemic[1]. COPD exacerbations cause impaired quality of life, accelerated loss of lung function, and enormous healthcare costs[2]. Most exacerbations are a consequence of viral and/or bacterial infections, the most common viruses identified are rhinoviruses[3] but the mechanisms of virus-induced exacerbations remain unclear[4]. Current therapies for exacerbations consist of corticosteroids and antibiotics but these are not very effective, have frequent adverse effects and are focused on bacterial exacerbations[5,6]. New treatments are urgently needed and developing these requires a better understanding of the mechanisms of COPD exacerbations.

Pathogenic mechanisms in stable COPD include oxidative and nitrosative stress and reduced expression of the anti-inflammatory enzyme histone deacetylase-2 (HDAC2)[7-9]. High levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in COPD resulting in induction of pro-inflammatory cytokines and chemokines, mucous hypersecretion, activation of proteases and damage to cellular components[10]. Histone deacetylases remove acetyl groups on histones and influence gene expression. HDAC2 suppresses inflammatory gene expression and is reduced in alveolar macrophages and lung tissue in COPD, and this is believed to be a key mechanism of corticosteroid resistance in COPD[8,11]. Nitrosylation of tyrosine residues on HDAC2 by peroxynitrite results in its inactivation[12] and ROS activate phosphoinositide 3-kinase leading to phosphorylation and inactivation of HDAC2[13]. Therefore oxidative and nitrosative stress exert pro-inflammatory effects both through direct induction of inflammatory mediators and reduction of the anti-inflammatory action of HDAC2.

The roles of oxidative and nitrosative stress and HDAC2 in COPD exacerbations are unclear. We have previously demonstrated that experimental rhinovirus infection induces symptoms, airflow obstruction and airways inflammation in COPD subjects[14-16]. We hypothesized that oxidative and nitrosative stress are increased and HDAC2 activity reduced in virus-induced COPD exacerbations.

Methods

Study design

Ethical approval for the study was obtained from St Mary's Local Research Ethics Committee (study number 07/H0712/138) and informed consent obtained from all subjects. COPD subjects (GOLD stage II) and smokers with normal lung function were recruited according the criteria established in our previous study (Supplementary Table 1)[15], together with non-smokers with normal lung function. The COPD patients were receiving no inhaled corticosteroids, long-acting regular bronchodilators or oral corticosteroids. Baseline samples of induced sputum and bronchoalveolar lavage were obtained approximately 14 days prior to infection and subjects were inoculated with 10 $TCID_{50}$ of rhinovirus 16 on day 0 as described previously[15]. Sputum sampling was repeated on subsequent visits on days 3, 5, 9, 12, 15, 21, and 42 post-infection and bronchoscopy on day 7. Respiratory symptoms were recorded daily using diary cards as per our previous studies. A COPD exacerbation was defined as an increase in the total lower respiratory score of at least 2 points over baseline for at least 2 consecutive days.

Sputum assessments

8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of hydroxyl radical damage to DNA, 8-isoprostane, a marker of lipid peroxidation and 3-nitrotyrosine (3-NT) a product of tyrosine nitration mediated by peroxynitrite were measured in sputum supernatants using commercially available enzyme immunoassays (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Sputum nitrite levels were measured using the Griess assay. The Meso Scale Discovery (MSD) platform (Maryland, USA) was used to measure inflammatory mediators in sputum and bronchoalveolar lavage supernatants. The mediators measured were the pro-inflammatory cytokines IL-1β, TNF-α, and GM-CSF, the neutrophil chemokine CXCL8/IL-8 and the protease matrix metalloprotease-9 (MMP-9). Neutrophil elastase was measured using an enzyme-linked immunosorbent assay according to the manufacturers' instructions (Immunodiagnostik, Benshein, Germany). Further details are provided in the e-Appendix.

HDAC2 immunoprecipitation and activity

The HDAC2 isoenzyme was isolated from macrophage pellets obtained from sputum and bronchoalveolar lavage samples. Immunoprecipitation using a HDAC2 antibody (Insight Biotect, UK) and PureProteome® Protein A magnetic beads (Millipore, USA) was performed. HDAC activity was measured using a HDAC activity assay (Cayman Chemicals, USA) the details of which are provided in the e-Appendix.

Details of the experimental protocols of the *in vitro* infection of THP-1 cells (a human monocyte cell line) to further investigate the effects of rhinovirus infection on HDAC2 are provided in the e-Appendix.

Statistical analysis

Data are presented as either means or median, changes from baseline were analysed using repeated measures ANOVA or Friedman's test and differences between groups using Holm-Sidak's multiple comparisons test or the Kruskal-Wallace test. Correlations between data sets were examined using Spearman's rank correlation coefficient. Correlations were examined using peak post-inoculation values as in a dynamic and evolving clinical event such as a respiratory infection the temporal relationships between the induction of the different markers was variable between individual subjects, and using set days may miss relationships where timing of events varied. Differences were considered significant for all statistical tests at P values of less than 0.05. All reported P values are two-sided. Analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego USA).

Results

Study subjects

The clinical characteristics of the subjects successfully infected with rhinovirus 16 are shown in Table 1. Subjects were age and sex matched between the COPD subjects and both control groups, but the non-smokers were older than the smoking controls. Cigarette smoke exposure between smoking controls and COPD subjects was matched for pack year history.

Clinical and inflammatory responses

Symptoms and lung function: All COPD subjects experienced an exacerbation following rhinovirus infection (e-Figure 1)[15]. All exacerbations were Level I according

to the ATS/ERS severity classification[17]. FEV_1 fell significantly from baseline in the COPD subjects on days 3, 5 (P<0.05), 9 (P<0.01), 12 (P<0.05) and 15 (P<0.01), with a maximum fall of 250mL (12.92%) on day 15. There were no significant falls from baseline in FEV_1 in the control groups (e-Figure 2).

Sputum inflammatory cells, neutrophil elastase and virus load: In the COPD subjects sputum inflammatory cells were significantly increased over baseline on day 9, and were significantly higher compared to the non-smokers on days 9 and 15 (Figure 1a). There was no significant change from baseline in the smokers and the non-smokers. Sputum neutrophils increased significantly from baseline in the COPD subjects on day 9 and were significantly higher compared to the smokers on day 12 (Figure 1b). There were no significant changes in numbers of neutrophils in either control group, nor in numbers of macrophages, lymphocytes, or eosinophils in any group. Levels of neutrophil elastase in sputum increased from baseline on days 9 and 15 in the COPD subjects and were significantly higher in the COPD group compared to controls on day 5(Figure 1c).

Sputum virus load was increased from baseline on days 3–15 in the COPD subjects, on days 5 and 9 in the smokers and on days 3 and 9 in non-smokers (Figure 1d). Sputum virus load was significantly higher in the COPD group compared to both control groups on day 12 and compared to the non-smokers on day 15.

Sputum inflammatory mediators: In the COPD subjects there were significant increases from baseline in IL-1β, GM-CSF, CXCL8/IL-8, TNF-α, and MMP-9 in sputum (Figure 2). There was no significant induction of inflammatory mediators in the non-COPD subjects apart from IL-1β on day 9 and GM-CSF on day 21 in the smokers. Sputum mediator levels were significantly higher in the COPD subjects compared to the non-smokers on days 9 and 15 and for some mediators on days 5,

12, and 21. Levels of inflammatory mediators in bronchoalveolar lavage both at baseline and post-inoculation were generally lower than in sputum and are shown in the Supplementary Index. Correlations between sputum inflammatory mediators, inflammatory cells and virus load in the COPD subjects are shown in Table 2.

Oxidative and nitrosative stress: Prior to infection sputum 8-OHdG levels were higher in the COPD group compared to the smokers (P<0.05) and 3-NT levels were higher in the COPD group compared to both non-smokers (P<0.05) and smokers (P<0.001), with no differences between the groups in baseline levels of nitrite or 8-isoprostane (Figure 3). Markers of oxidative and nitrosative stress were all significantly induced following rhinovirus infection in COPD subjects with much smaller induction seen in the smokers and were significantly higher in the COPD subjects compared to the non-COPD subjects (Figure 3 a-d).

Macrophage HDAC2 activity

At baseline there were no significant differences between the groups in HDAC2 activity in sputum (data not shown) or bronchoalveolar lavage macrophages (Figure 3f). Following infection HDAC2 activity in the smoking controls and non-smoking controls did not change significantly from baseline, but tended to increase (Figure 3e and f). In the COPD subjects there was a trend towards reduced HDAC2 activity in both sputum (P=0.064) and bronchoalveolar lavage macrophages (P=0.098, Figure 3e and f). Sputum HDAC2 activity was significantly lower in the COPD subjects compared to non-smokers on days 5 and 21 (P<0.05), and there was a trend towards lower levels of bronchoalveolar lavage HDAC2 activity during exacerbation compared to the non-smokers (P=0.095) and smokers (P=0.059).

Correlations

Baseline HDAC2: Sputum macrophage HDAC2 activity at baseline correlated inversely with peak post-inoculation sputum virus load (*P*=0.022, r=-0.82),sputum NE (*P*=0.022, r=-0.81), CXCL8/IL-8 (*P*=0.047, r=-0.71) and TNF-α (*P*=0.028, r=-0.79) (Table 2). There were no relationships between baseline HDAC2 activity and outcomes following infection in the non-COPD subjects.

Oxidative stress: In the COPD subjects peak sputum 8-isoprostane levels and 8-OHdG levels during exacerbations correlated with each other (*P*=0.0068, r=0.82) and 8-OHdG correlated with peak sputum neutrophil elastase (*P*=0.0125, r=0.78) and sputum 3-NT levels (*P*=0.0016, r=0.83).

Nitrosative stress: Peak sputum 3-NT and sputum nitrite levels during exacerbation correlated with each other (*P*=0.0037, r=0.85). Peak sputum 3-NT correlated with peak neutrophil elastase (*P*=0.0009, r=0.9) and peak sputum nitrite correlated with peak sputum inflammatory cell numbers (*P*=0.0125, r=0.778), neutrophil elastase (*P*=0.042, r=0.68) and neutrophil numbers (*P*=0.0096, r=0.8) (Table 3).

HDAC2 activity: HDAC2 activity in bronchoalveolar lavage macrophages in COPD subjects during exacerbations correlated inversely with peak nasal lavage virus load (*P*=0.0096, r=-0.8), peak sputum GM-CSF (*P*=0.0499, r=-0.67), TNF-α (*P*=0.03, r=-0.72), neutrophil elastase (*P*=0.0499, r=-0.67) and sputum nitrite levels (*P*=0.0125, r=-0.78) (Table 2).

Rhinovirus infection of monocytes *in vitro*

Rhinovirus infection of the monocytic cell line THP-1 resulted in up-regulation of the inflammatory cytokines IL-6 and CXCL8/IL-8 (Figure 4). Rhinovirus infection reduced expression of HDAC2 (both mRNA and protein) compared to non-infected cells

(Figure 4c and 4d), and reduced HDAC2 activity (Figure 4e). Levels of nitrosylated HDAC2 were increased post-infection compared to non-infected cells (Figure 4f). THP-1 cells were treated with N-acetylcysteine (NAC) in order to inhibit the actions of peroxynitrate[18-20]. Treatment of the THP-1 cells with NAC led to a dose-dependent reduction in IL-6 and CXCL8/IL-8, with significant reductions in inflammatory mediators observed at doses over 1mM (Figures 4g and 4h).

Discussion

Acute exacerbations are important events in the natural history of COPD, but their pathogenesis remains poorly understood. Prevention of exacerbations remains a key therapeutic goal but current treatments have only modest benefits and considerable adverse effects. Previous work from our group has demonstrated that experimental rhinovirus infection in COPD subjects induces respiratory symptoms, airflow obstruction and neutrophilic inflammation[15]. In the current study we provide novel evidence of the mechanisms of virus-induced COPD exacerbations and identify new potential therapeutic targets.

Oxidative/nitrosative stress, airways inflammation and reduced HDAC activity are well described in stable COPD but their roles in COPD exacerbations are unclear. Some studies have reported increased inflammation and oxidative stress in exacerbations[3,21-24], whereas others have reported no change compared to the stable state[25-28]. Increased numbers of 3-NT positive cells have been reported in COPD exacerbations[21] and two studies reported that HDAC activity is unchanged in exacerbated COPD[28,29]. In these studies of naturally-occurring exacerbations there are numerous sources of variability that likely account for the discrepant results of these studies, including variations in exacerbation aetiology, timing of presentation/sampling after exacerbation onset, different treatments etc. that are

difficult to eliminate. Understanding the molecular pathways in COPD exacerbations is critical to developing new, more effective preventative and therapeutic strategies. Our model of COPD exacerbation permits repeated lower airway sampling in treatment-naïve subjects in a manner not possible with naturally-occurring exacerbations, thereby reducing variability and offering unique insights into the mechanisms of COPD exacerbations[15,16,30,31]. Moreover use of a human, as opposed to an animal model provides data that is more likely to be rapidly translated into new therapies. Using this model we measured markers of inflammation, oxidative and nitrosative stress and HDAC activity prior to and following rhinovirus infection.

We previously reported that virus-induced exacerbations are associated with increased neutrophils, neutrophil elastase, and CXCL8/IL-8[15]. In the current study we replicated these findings in a new cohort of subjects and, in addition, demonstrated that virus infection induces significant increases in the pro-inflammatory cytokines GM-CSF, TNF-α and IL-1β, and the protease MMP-9. Levels of 8-isoprostane (a marker of lipid peroxidation), 8-OHdG (a marker of hydroxyl radical damage to DNA), 3-NT (a product of tyrosine nitration mediated by peroxynitrite) and nitrite were significantly increased in sputum in the COPD subjects, with only small, transient increases in non-COPD smokers. There were positive correlations between markers of nitrosative stress and inflammatory mediators in sputum. These data provide new evidence of a pathogenic role of oxidative and nitrosative stress in COPD exacerbations. Exacerbations are associated with accelerated loss of lung function[32-34] and nitrosative/oxidative stress[35,36], neutrophil elastase[37] and MMP-9[38] are all associated with airway remodelling and therefore these are potential mechanisms linking exacerbations with loss of lung function in COPD.

HDAC2 levels at baseline were not lower in the COPD subjects as has been reported previously in patients with COPD GOLD stages II and III[8]. Reduced HDAC2 is most pronounced in severe COPD and therefore may not be present in subjects with GOLD stage II disease. Following rhinovirus infection there was a trend towards reduced HDAC2 activity in sputum and BAL macrophages in COPD subjects and HDAC activity correlated inversely with airways inflammation, virus load and nitrite levels. Nitrosylation of HDAC, followed by HDAC degradation is a mechanism of reduced HDAC2 activity[39] and our *in vitro* data is the first report that virus infection induces nitrosylation of HDAC2. The inverse relationship between airway HDAC2 activity and nitrite levels following virus infection suggests this is relevant *in vivo* also. Therefore nitrosylation of HDAC2 may link virus infection, oxidative/nitrosative stress, impaired HDAC2 activity and enhanced inflammation in COPD exacerbations.

These results have a number of important implications for our understanding of the pathogenesis of COPD exacerbations and future therapeutic directions. Corticosteroids are used in COPD exacerbations despite modest clinical benefits and frequent adverse effects[5,40]. Reduced HDAC2 may contribute to corticosteroid resistance in stable COPD[8] and our results suggest this is also relevant in exacerbated COPD. Thus enhancing HDAC2 activity through inhibiting virus-induced oxidative/nitrosative stress has the potential to improve the therapeutic effect of corticosteroids in COPD exacerbations, as well as having a direct anti-inflammatory effect itself. This warrants investigation in studies specifically designed to address this issue. The demonstration that oxidative/nitrosative stress and multiple inflammatory mediators and proteases are elevated in exacerbations may account for the lack of clinical benefit seen with inhibition of single mediators[41-44], and casts

doubt on this as a valid therapeutic approach. N-acetylcysteine that is both an antioxidant and a peroxynitrite inhibitor demonstrated an anti-inflammatory effect *in vitro* and may also be effective *in vivo*.

Reduced baseline HDAC2 activity in sputum macrophages was associated with greater virus loads post-infection; therefore reduced HDAC2 activity may also be linked to impaired antiviral responses. We observed significantly higher sputum virus loads in COPD subjects, confirming for the first time impaired antiviral immunity in COPD results in more severe lower respiratory virus infection. HDAC activity is required for an effective antiviral response to picornavirus infection[45], therefore reduced HDAC activity may contribute to impaired antiviral host defence and increased severity of clinical illness following virus infections in COPD.

Our study has a number of limitations that are inherent to experimental infection studies. The number of subjects was small and only subjects with moderate COPD were included, and therefore the findings may not be applicable to more severe patients. However as HDAC2 activity is further reduced[8] and exacerbations more frequent in severe COPD[2], we believe these mechanisms are likely to be even more relevant in more severe disease. Secondary bacterial infections did occur in some subjects[30] but the numbers were too small to allow an analysis of the interactions between HDAC2 activity and bacteria (6 subjects with bacterial infection, 3 without). Whether changes in HDAC2 activity affect the ability of macrophages to clear bacteria is a subject of great interest and warrants further investigation. The smokers were significantly younger compared to the non-smokers but there were no significant differences in age between the COPD group and the 2 control groups and as the key comparisons were between the COPD and non-COPD subjects, we do not believe this affected the important results. Moreover Ito et al reported that age

had no effect on HDAC activity in bronchial biopsies[8]. However, ideally subsequent studies should use age-matched subjects whenever possible to remove this confounder. We used sputum to measure inflammatory meditors and cells and these do not reflect inflammation in the small airways. The main site of rhinovirus replication is epithelial cells rather than macrophages and therefore the significance of the relationship between HDAC2 levels in macrophages and virus load is unclear. The *in vitro* work was carried out in THP-1 cells rather than alveolar macrophages as these are difficult to obtain in large enough numbers to perform the necessary experiments. However the data obtained provides valid mechanistic information as to the potential mechanisms involved in macrophage responses to viral infection in COPD.

In summary we have demonstrated induction of oxidative and nitrosative stress, reduced HDAC2 activity, increased inflammatory mediators and increased virus load in the airways following rhinovirus infection in COPD subjects. These results provide unique insights into the mechanisms of virus-induced COPD exacerbations and suggest novel therapeutic targets for the development of new targeted therapies in COPD.

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interpreted data, and revised the report. SLJ had full access to all the data in the study and had final responsibility for the decision to submit for publication. All authors have given final approval of the version to be published.

This article is dedicated to the memory of Dr. Joseph Footitt.

Figure Legends

Figure 1. Time course of inflammatory cells, neutrophil elastase and virus load in sputum during experimental rhinovirus infection. Panel A: Total sputum inflammatory cells (neutrophils, macrophages/monocytes, lymphocytes, eosinophils). Panel B: Sputum neutrophils. Panel C: Sputum neutrophil elastase. Panel D: Sputum virus load. All data are means ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. baseline. †*P*<0.05, ††*P*<0.01 COPD vs. non-smokers. #*P*<0.01, ##*P*<0.01 COPD vs. smokers.

Figure 2. Time course of inflammatory mediators in sputum during experimental rhinovirus infection in COPD. Panel A: Sputum IL-1β. Panel B: Sputum TNF-α. Panel C: Sputum GM-CSF. Panel D: Sputum CXCL8/IL-8. Panel E: Sputum MMP-9. All data are medians ± IQR. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. baseline. †*P*<0.05, ††*P*<0.01 COPD vs. non-smokers.

Figure 3. Time course of oxidative and nitrosative stress markers in sputum and HDAC2 activity in sputum and bronchoalveolar lavage macrophages during experimental rhinovirus infection. Panel A: Sputum 8-isoprostane. Panel B: Sputum 8-hydroxy-2'-deoxyguanosine. Panel C: Sputum 3-nitrotyrosine. Panel D: Sputum nitrite. Panel E: Sputum macrophage HDAC2 activity –change from baseline. Panel F: Bronchoalveolar lavage macrophage HDAC2 activity. All data are medians ± IQR. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. baseline. †*P*<0.05, ††*P*<0.01, †††*P*<0.001 COPD vs. non-smokers. $^{\#}$*P*<0.05, $^{\#\#}$*P*<0.01, $^{\#\#\#}$*P*<0.001 COPD vs. smokers.

Figure 4. Rhinovirus infection of THP-1 cells *in vitro*. Panel A: Induction of IL-6 by rhinovirus in THP-1 cells. Panel B: Induction of CXCL-8 by rhinovirus in THP-1 cells.

Panel C: HDAC2 protein in THP-1 cells infected with rhinovirus. Panel D: HDAC2 RNA in THP-1 cells infected with rhinovirus protein. Panel E: HDAC2 activity in THP-1 cells infected with rhinovirus. Panel F: HDAC2 nitrosylation in THP-1 cells infected with rhinovirus. Panel G: Inhibition of rhinovirus-induced IL-6 by N-acetylcysteine (NAC). Panel H: Inhibition of rhinovirus-induced CXCL8/IL-8 by NAC. *P<0.05 Data shows mean ± standard error of at least 3 independent experiments.

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Tables

	Non-smokers (N=11)	Smokers (N=10)	COPD (N=9)	P values
Age	62.18 (±1.62)	52.50 (±2.23)	60.44 (±3.17)	P<0.05 SMK vs. NS
Sex M:F	4:7	4:6	6:3	NS
Current smokers	0	10/10	8/9	
Smoking history (pack years)	0	32.1 (±3.02)	39.44 (±3.25)	NS
FEV_1 (Litres)	3.17 (±0.17)	3.24 (±0.21)	2.31 (±0.13)	P<0.05 COPD vs. NS, P<0.01 COPD vs. SMK
FEV_1 (% predicted)	102.2 (±3.34)	96.60 (±3.28)	68.11 (±1.58)	P<0.0001 COPD vs. SMK and NS
FEV_1/FVC	77.62 (±1.09)	78.04 (±2.18)	61.58 (±1.80)	P<0.0001 COPD vs. SMK and NS

Table 1. Baseline clinical characteristics of study subjects. All data mean ± SEM.

	Baseline sputum HDAC2	Post-inoculation BAL macrophages HDAC2
Peak sputum virus load	P=0.022, r=-0.82	/
Peak sputum neutrophil elastase	P=0.022, r=-0.81	P=0.0499, r=-0.67
Peak sputum CXCL8/IL-8	P=0.047, r=-0.71	/
Peak sputum TNF-α	P=0.028, r=-0.79	P=0.03, r=-0.72
Peak nasal lavage virus load	/	P=0.0096, r=-0.8
Peak sputum GM-CSF	/	P=0.0499, r=-0.67
Peak sputum nitrite	/	P=0.0125, r=-0.78

Table 2. Correlations between baseline sputum HDAC2 levels and post-inoculation BAL macrophages HDAC2 levels and peak levels of sputum inflammatory mediators and virus load.

Inflammatory mediators	Virus load	Inflammatory cells and neutrophil markers			Oxidative stress		Nitrosative stress	
		Total cell count	Neutrophils	Neutrophil elastase	8-IP	8-OHdG	3-NT	Nitrite
IL-1β	NS	NS	*P*=0·043 r=0·7	NS	NS	NS	*P*=0·031 r=0·73	*P*=0·017 r=0·78
TNF-α	NS	*P*=0·031 r=0·73	*P*=0·016 r=0·77	*P*=0·026 r=0·75	NS	NS	*P*=0·017 r=0·78	*P*=0·.0007 r=0·93
GM-CSF	NS	*P*=0·021 r=0·77	*P*=0·02 r=0·75	NS	NS	NS	NS	*P*=0·0053 r=0·83
CXCL8 /IL-8	P=0.0096 r=0.8	*P*=0·0096 r=0·8	*P*=0·0072 r=0·82	NS	NS	NS	NS	*P*=0·05 r=0·68
MMP-9	NS	*P*=0·0031 r=0·88	*P*=0·0025 r=0·87	NS	NS	NS	*P*=0·0083 r=0·83	*P*=0·0004 r=0·95

Table 3. Correlations between peak levels of sputum inflammatory mediators, virus load, sputum cell counts and markers of oxidative and nitrosative stress in the COPD subjects. 8-IP – 8-isoprostane, 8-OHdG – 8-hydroxy-2'-deoxyguanosine, 3-NT – 3-nitrotyrosine.

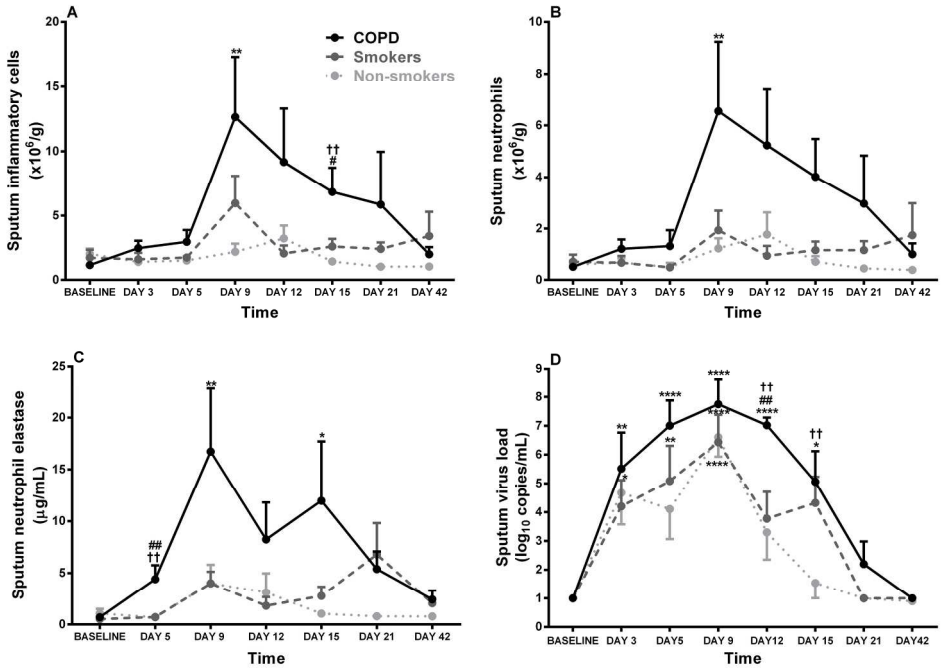

Figure 1
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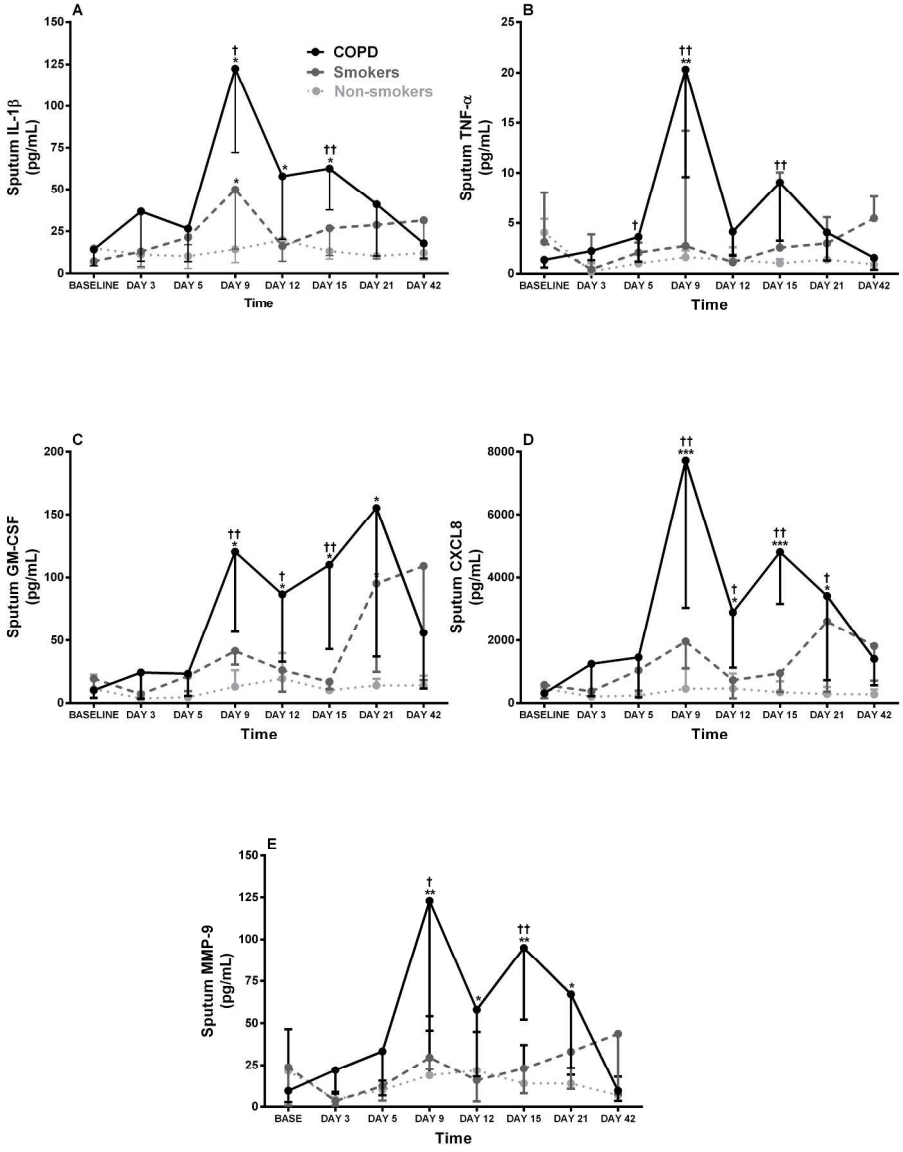

Figure 2
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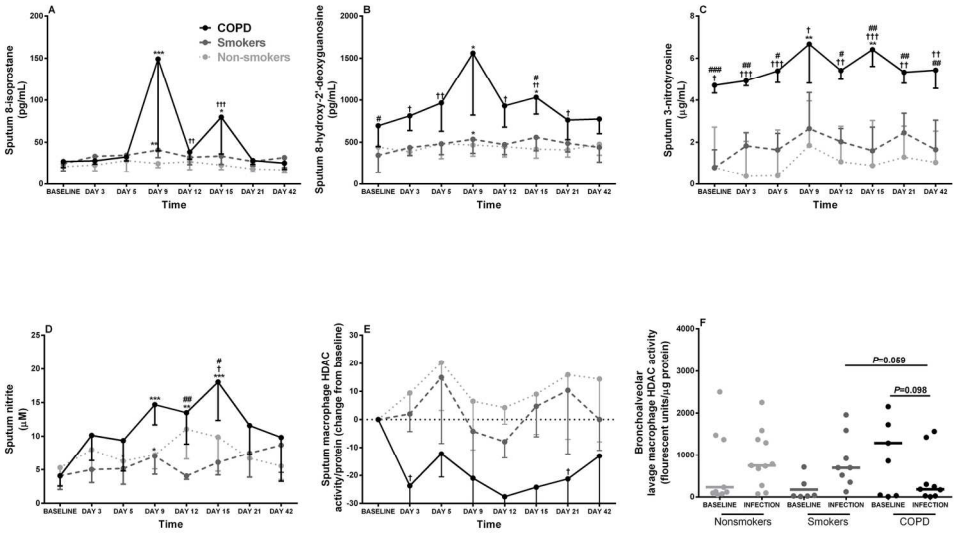

Figure 3
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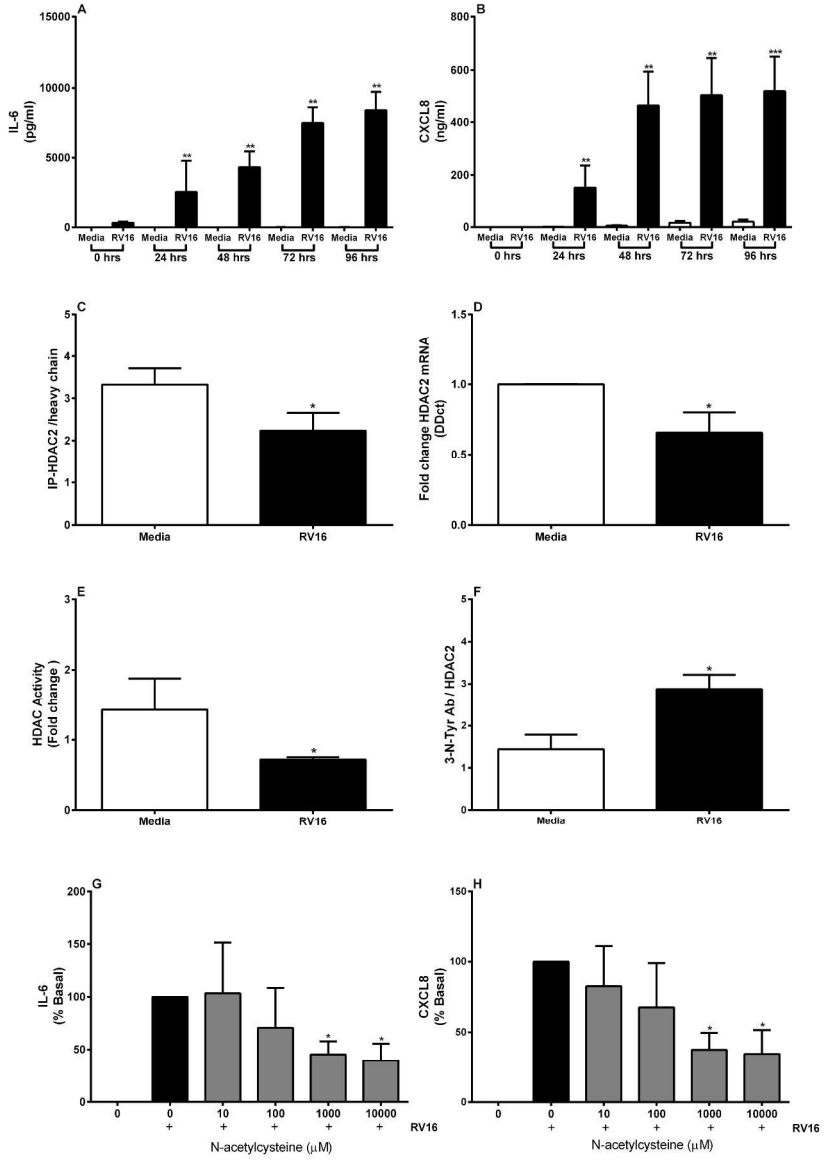


Figure 4
295x412mm (300 x 300 DPI)

Supplementary Appendix

Oxidative and nitrosative stress and histone de-acetylase-2 activity in exacerbations of Chronic Obstructive Pulmonary Disease

Joseph Footitt[†1,2,3*], Patrick Mallia[1,2,3*], Andrew L. Durham[4*], W. Eugene Ho[5], Maria-Belen Trujillo-Torralbo[1,2], Aurica G Telcian[1], Ajerico Del Rosario[1,2], Chang Cheng[5], Hong-Yong Peh[5], Tatiana Kebadze[1], Julia Aniscenko[1], Luminita Stanciu[1], Sarah Essilfie-Quaye[4], Kazuhiro Ito[4], Peter J Barnes[4], Sarah L Elkin[1,2], Onn M Kon[1,2,3], WS Fred Wong[5], Ian M Adcock[4], Sebastian L Johnston[1,2,3]

[1]Airway Disease Infection Section, National Heart and Lung Institute, Imperial College, London, United Kingdom; [2]Imperial College Healthcare NHS Trust, London, United Kingdom; [3]Centre for Respiratory Infection, London, United Kingdom; [4]Airways Disease Section, National Heart and Lung Institute, Imperial College, London, United Kingdom; [5]Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117456

†deceased

*equal contributions

Materials and Methods

Symptom scores

Symptoms were assessed using diary cards that were completed on a daily basis from screening until 6 weeks post-inoculation. The scoring system for the lower respiratory symptoms of shortness of breath, cough, wheeze, sputum quantity and sputum quality was that used in our previous experimental infection studies and is shown in e-Table 1. The daily lower respiratory score was summated from the individual scores and a COPD exacerbation was defined as an increase in the lower respiratory score of at least 2 points over baseline for at least 2 consecutive days. For both upper and lower respiratory daily symptom scores the mean scores on days -6 to 0 were calculated and subtracted from subsequent daily scores to correct for baseline symptoms.

Induced sputum

Sputum was induced and processed using protocols used in our previous studies. A cell pellet was obtained and total inflammatory cell numbers (neutrophils, macrophages/monocytes, lymphocytes and eosinophils) counted. Cytospins were prepared and counted blind to study status to obtain differential cell counts. Cell counts were expressed as a percentage of at least 400 inflammatory cells.

Measurement of oxidative and nitrosative stress

For each subject all the treatment days were coded and assayed blind on a single 96 well plate, and all groups were assayed together in three plates, so the observed differences between the different groups are not linked to plate-to-plate variation. All the samples were assayed in a total of 3 plates over 3 days per target and there

were no observable variations between those 3 plates for standard curves and blank read-outs. Sputum nitrite levels were measured using the Griess assay which is based on formation of an azo dye by reaction of nitrite with the Griess reagent. All analysis was performed with the same batch of reagents, made in-house, stored at 4°C in light protective containers. A nitrate standard curve was prepared using sodium nitrite (Sigma, UK) at concentrations from 0-1000μM (PBS alone). Briefly an equal volume of Griess reagents 1 (1% sulphanilamide (Sigma, UK) and 5% phosphoric acid (H_3PO_4)) & 2 (0.5% of napthylethyl-endiamide dihydrochloride (Sigma, UK)) were mixed and allowed to equilibrate, 100μL of standard or sample was added to duplicate wells of a 96 well plate before addition of 100μL complete (combined) Griess reagent and colorimetric values measured at 550nm read on a standard plate reader. The lower limit of detection was 1μM. Data was analysed by linear regression against the standard curve.

HDAC2 immunoprecipitation and activity

A cell pellet as obtained from sputum and BAL and diluted with 3ml of macrophage media, distributed across wells of a modified polystyrene flat bottom adhesive culture plate (Falcon, Becton Dickinson, USA) and incubated for 2 hours at 37°C and 5% CO2 to facilitate macrophage separation by adhesion to the well base. After 2 hours adherent cells were washed from the well base using a cell scraper and centrifuged (18.8g for 7 minutes) to obtain a cell pellet which was stored immediately at –80°C. HDAC activity was measured using an HDAC activity assay (Cayman Chemicals, USA). Briefly the protocol involved addition of radioimmunoprecipitation (RIPA) assay buffer to the macrophage pellet obtained from cellular adhesion. This was incubated before combination with the HDAC2 antibody and overnight mixing on a

sample roller. Magnetic beads were then added for 2 hours of incubation prior to bead removal. Analysis of HDAC2 activity was performed in the protein sample and the supernatant used to calculate total protein concentration using the bicinchoninic acid assay performed according to published methods[1] and results of HDAC2 activity corrected for sample protein levels.

MesoScale Discovery

The technique enables quantitative detection of between 1 and 9 mediators per well in a 96 well plate format using a Multi-spot® technique. This technology utilises a capture antibody attached to each spot within the well which enables measurement of multiple mediators simultaneously. Electrochemiluminescence of detection antibody is then recorded with an internal high sensitivity camera (Sector imager). Briefly, the protocol requires addition of 25µL of blocking solution before incubation. Following plate washing either sample or standard was added to the plate, followed by incubation and washing and addition of detection antibody. Finally read buffer was added and the plate passed through the Sector imager for reading. The lower limits of detection of the individual analytes were as follows: IL-1β (1.17pg/ml), CXCL8/IL-8 (0.6pg/ml), TNF-α (0.376pg/ml), CXCL10 (12pg/ml), GM-CSF (1.12pg/ml), MMP-9 (0.0988ng/ml).

Rhinovirus *in vitro* infection of THP-1 cells

THP-1 cells (a human monocytic cell line) were sub-cultured overnight in media containing 2% foetal calf serum. Rhinovirus 16 was prepared as previously described[2] and cells were incubated with rhinovirus 16 for 1 hour. This was then replaced with fresh media and cells were collected at 24, 48, 72 and 96 hours post-

infection. The levels of the cytokines IL-6 and CXCL8/IL-8 were measured in the supernatant using ELISA kits following manufacturer's instructions (R&D systems, UK). HDAC2 protein was extracted using RIPA buffer and immunoprecipitated (IP) as described above. HDAC2 levels were quantitated by Western blot and standardised against the heavy chain of the (IP) antibody. 3-nitrotyrosine HDAC2 levels were measured in IP-HDAC2 by Western Blot (antibody from Sigma, UK) with the 3-nitrotyrosine band standardised to HDAC2 levels.

Total RNA was isolated from THP-1 cells using the RNeasy RNA extraction kit (Qiagen, UK). cDNA was made from quantified RNA by reverse transcription. *HDAC2* mRNA expression levels were quantified by Real-Time PCR protocol using TaqMan (Applied Biosystems, UK). Each transcript was analysed by delta (Δ)CT method (against baseline) and variations in cDNA concentrations between different samples was corrected using the housekeeping gene *GNB2L1*.

Supplementary Tables

All subjects

- Age 40-75 years.
- No history of asthma or allergic rhinitis and not atopic on skin testing.
- Absence of a current or previous history of bronchiectasis, carcinoma of the bronchus or other significant respiratory disease (other than COPD).
- Absence of significant systemic disease.
- No COPD exacerbation or respiratory tract infection within the previous eight weeks.
- Serum antibodies to rhinovirus 16 at screening in a titre <1:2.
- No treatment with antibiotics, oral, inhaled or nasal topical steroids, long-acting β-agonists or tiotropium in the previous three months.

COPD group

- FEV_1 50% - 79% predicted normal value and β-agonist reversibility <12%.
- $FEV_1/FVC<70\%$.
- Current or ex-smokers with at least 20 pack years cumulative smoking

Smokers

- $FEV_1 \geq 80\%$ predicted normal value.
- $FEV_1/FVC>70\%$.
- Current or ex-smokers with at least 20 pack years cumulative smoking

Non-smokers

- $FEV_1 \geq 80\%$ predicted normal value.
- $FEV_1/FVC>70\%$.
- Non-smokers

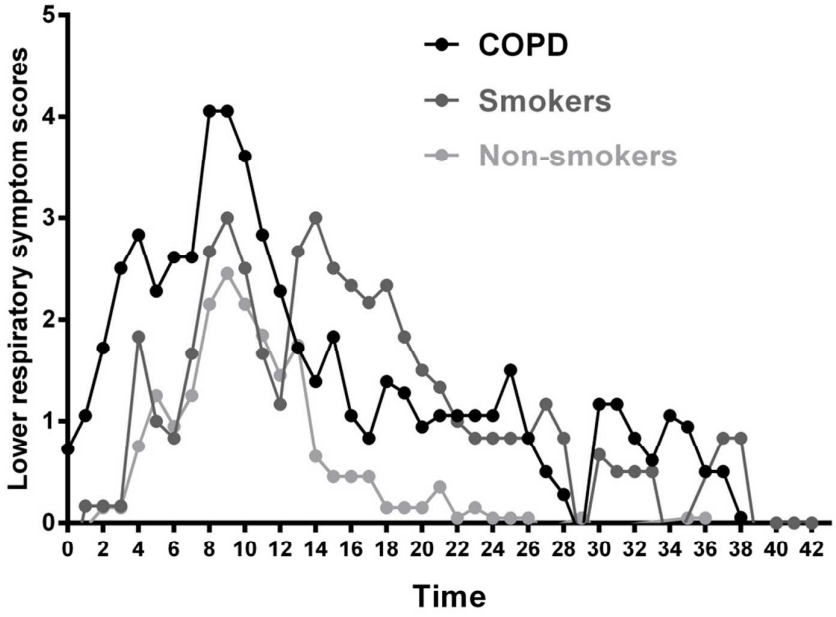
e-Table 1. Inclusion and exclusion criteria for study subjects.

SYMPTOM	SCORE				
	0	1	2	3	4
SHORTNESS OF BREATH	Not breathless	On moderate exertion	On mild exertion	On minimal exertion	At rest
WHEEZE	No wheeze	On moderate exertion	On mild exertion	On minimal exertion	At rest
COUGH	No cough	Mild	Moderate	Severe	/
SPUTUM QUANTITY	None	Minimal (<30mL)	Moderate (30-100mL)	Large (>100mL)	/
SPUTUM QUALITY	None	Mucoid (clear)	Mucopurulent (yellow)	Purulent (green)	/

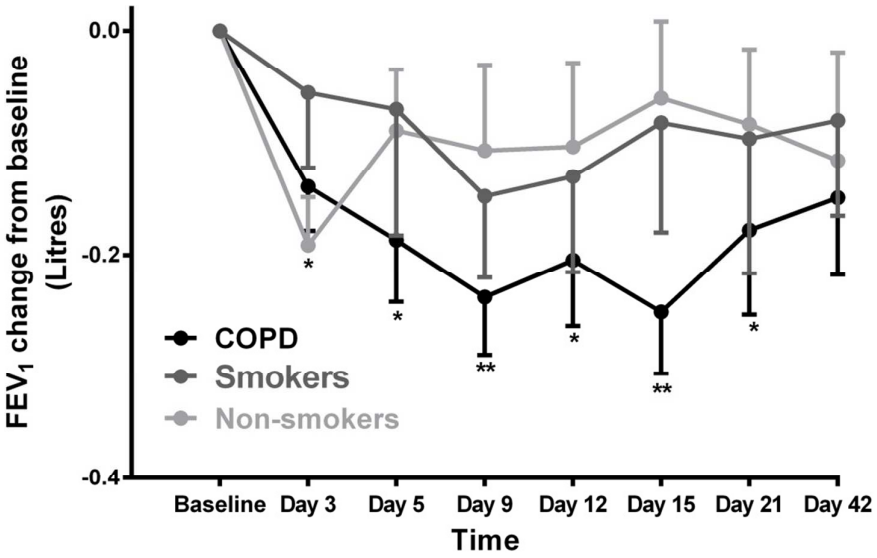
e-Table 2. Scoring system for lower respiratory symptoms.

Results

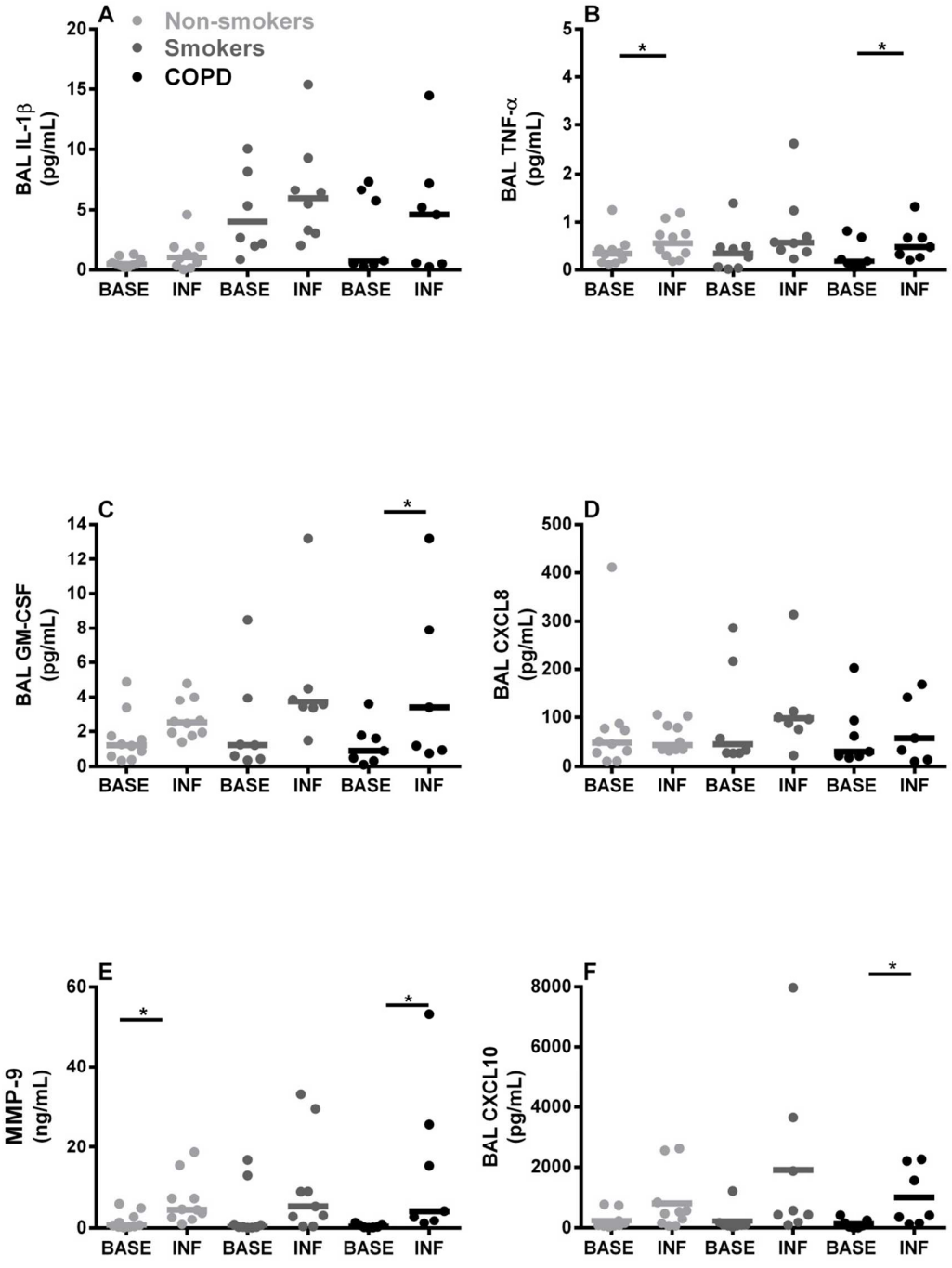
Supplementary Figures



e-Figure 1. Time course of total lower respiratory scores following rhinovirus infection



e-Figure 2. Time course of FEV_1 following rhinovirus infection. All data are means ± SEM. *P<0.05, **P<0.01, vs. baseline.



e-Figure 3. Inflammatory mediators in bronchoalveolar lavage during experimental rhinovirus infection.

	Baseline	Day 3	Day 5	Day 9	Day 12	Day 15	Day 21	Day 42
Non-Smokers	2.02 ± 0.4	1.41 ± 0.19	1.51 ± 0.32	2.18 ± 0.64	3.22 ± 1.0	1.43 ± 0.24	1.03 ± 0.19	1.04 ± 0.15
Smokers	1.72 ± 0.63	1.61 ± 0.5	1.74 ± 0.27	5.96 ± 2.13	2.06 ± 0.61	2.60 ± 0.59	2.41 ± 0.51	3.42 ± 1.9
COPD	1.16 ± 0.21	2.47 ± 0.58	2.96 ± 0.92	12.65 ± 4.66	9.17 ± 4.14	6.85 ± 1.89	5.86 ± 4.1	1.99 ± 0.56

e-Table 3. Total inflammatory cell numbers in sputum (x 10^6/g sputum, mean ± SEM)

	Baseline	Day 7
Non-Smokers	79.28 (73.81 - 143)	182.2 (146.1 - 524.4)
Smokers	318.8 (146.1 - 524.4)	359.2 (174.2 - 698.4)
COPD	144.7 (88.03 - 630.9)	353.4 (141.6 - 511.6)

e-Table 4. Total inflammatory cell numbers in bronchoalveolar lavage (x10^6/L, median and IQR)

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Text 2948

Abstract 236

Oxidative and nitrosative stress and histone deacetylase-2 activity in exacerbations of Chronic Obstructive Pulmonary Disease

Short title: Oxidative stress and HDAC2 in COPD exacerbations

Joseph Footitt PhD[†1,2,3*], Patrick Mallia PhD[1,2,3*], Andrew L. Durham PhD[4*], W. Eugene Ho PhD[5], Maria-Belen Trujillo-Torralbo BSc[1,2], Aurica G Telcian PhD[1], Ajerico Del Rosario BSc[1,2], Cheng Chang PhD[5], Hong-Yong Peh MSc[5], Tatiana Kebadze MD[1], Julia Aniscenko[1], Luminita Stanciu PhD[1], Sarah Essilfie-Quaye PhD[4], Kazuhiro Ito PhD[4], Peter J Barnes FRS, FMedSci[4], Sarah L Elkin PhD[1,2], Onn M Kon[1,2,3], WS Fred Wong PhD[5], Ian M Adcock PhD[4], Sebastian L Johnston PhD[1,2,3]

[1]Airway Disease Infection Section, National Heart and Lung Institute, Imperial College, London, United Kingdom

[2]Imperial College Healthcare NHS Trust, London, United Kingdom

[3]Centre for Respiratory Infection, Imperial College, London, United Kingdom

[4]Airways Disease Section, National Heart and Lung Institute, Imperial College, London, United Kingdom

[5]Department of Pharmacology, Yong Loo Lin School of Medicine, National University Health System, Singapore 117456

[†]deceased

[*]equal contributions

Corresponding author: Sebastian L Johnston, Professor of Respiratory Medicine, Airway Disease Infection Section, National Heart and Lung Institute, Imperial College, Norfolk Place, London W2 1PG, UK.

Email: s.johnston@imperial.ac.uk.

Declaration of interests

JF, ALD, WEH, MBTT, AGT, ADR, CC, HYP, TK, JA, LS, SEQ, AT and SLE have no competing interests. PM has received honoraria and travel grants from GSK. KI is an employee of Pulmocide Ltd and has honorary contract with Imperial College. PJB has served on Scientific Advisory Boards of AstraZeneca, Boehringer-Ingelheim, Bespak, Chiesi, Daiichi-Sankyo, DeepBreeze, GlaxoSmithKline, Glenmark, Johnson & Johnson, Merck, Novartis, Nycomed/Takeda, Pfizer, Prosonix, Sun Pharmaceuticals, Teva and UCB and has received research funding from Aquinox Pharmaceuticals, AstraZeneca, Boehringer-Ingelheim, Chiesi, Daiichi-Sankyo, GSK, Novartis, Nycomed/Takeda, Pfizer, Prosonix, Sun Pharmaceuticals. He is also a cofounder of RespiVert (now part of Johnson & Johnson), which has discovered novel inhaled anti-inflammatory treatments for asthma and COPD. OMK has received travel grants from Boehringer Ingelheim, WSFW has received consultancies from Davos Life Science Pte Ltd, Singapore, IMA has received consultancies, honoraria, and travel and research grants from GSK, AstraZeneca, Johnson & Johnson, Chiesi, Pfizer, Boehringer Ingelheim, Novartis and Vectura, SLJ has received consultancies, honoraria, and travel and research grants from GSK, Johnson & Johnson, Sanofi Aventis, Chiesi, Pfizer, Boehringer Ingelheim, AstraZeneca, Novartis and Synairgen and has stock options in Synairgen.

Funding

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Abstract Presentations

British Thoracic Society 2011 S14. American Thoracic Society 2013 A2167. European Respiratory Society 2013 P1880, P1892. British Thoracic Society 2013 S112.

Abstract

Background

Respiratory virus infections are commonly associated with COPD exacerbations but little is known about the mechanisms linking virus infection to exacerbations. Pathogenic mechanisms in stable COPD include oxidative and nitrosative stress and reduced activity of histone deacetylase-2 (HDAC2) but their roles in COPD exacerbations is unknown. We investigated oxidative/nitrosative stress and HDAC2 in COPD exacerbations using experimental rhinovirus infection.

Methods

9 subjects with COPD (GOLD stage II), 10 smokers and 11 non-smokers were successfully infected with rhinovirus. Markers of oxidative and nitrosative-stress associated cellular damage, inflammatory mediators and proteases were measured in sputum, and HDAC2 activity measured in sputum and bronchoalveolar macrophages. In an *in vitro* model monocyte derived THP-1 cells were infected with rhinovirus and nitrosylation and activity of HDAC2 measured.

Results

Rhinovirus infection induced significant increases in airways inflammation and markers of oxidative and nitrosative stress in COPD subjects. Oxidative/nitrosative stress markers correlated with virus load and inflammatory markers. Macrophage HDAC2 activity was reduced during exacerbation and correlated inversely with virus load, inflammatory markers and nitrosative stress. Sputum macrophage HDAC2 activity pre-infection was inversely associated with sputum virus load and inflammatory makers during exacerbation. Rhinovirus infection of monocytes induced nitrosylation of HDAC2 and reduced HDAC2 activity, inhibition of oxidative/nitrosative stress inhibited rhinovirus-induced inflammatory cytokines.

Conclusions

Oxidative and nitrosative stress, airways inflammation and impaired HDAC2 may be important mechanisms of virus-induced COPD exacerbations. Therapies targeting these mechanisms offer potential new treatments for COPD exacerbations.

Abbreviations List

3-NT	3-nitrotyrosine
8-OHdG	8-hydroxy-2'-deoxyguanosine
ATS	American Thoracic Society
COPD	Chronic obstructive pulmonary disease
ERS	European Respiratory Society
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GM-CSF	Granulocyte-macrophage colony stimulating factor
GOLD	Global Initiative for Obstructive Lung Disease
HDAC2	Histone deacetylase-2
IL-	Interleukin
MMP-9	Matrix metalloprotease-9
NAC	N-acetylcysteine
NE	Neutrophil elastase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
$TCID_{50}$	Tissue culture infective $dose_{50}$
TNF-α	Tumour necrosis factor-alpha

Introduction

Chronic obstructive pulmonary disease (COPD) is a rapidly growing global epidemic[1]. COPD exacerbations cause impaired quality of life, accelerated loss of lung function, and enormous healthcare costs[2]. Most exacerbations are a consequence of viral and/or bacterial infections, the most common viruses identified are rhinoviruses[3] but the mechanisms of virus-induced exacerbations remain unclear[4]. Current therapies for exacerbations consist of corticosteroids and antibiotics but these are not very effective, have frequent adverse effects and are focused on bacterial exacerbations[5,6]. New treatments are urgently needed and developing these requires a better understanding of the mechanisms of COPD exacerbations.

Pathogenic mechanisms in stable COPD include oxidative and nitrosative stress and reduced expression of the anti-inflammatory enzyme histone deacetylase-2 (HDAC2)[7-9]. High levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in COPD resulting in induction of pro-inflammatory cytokines and chemokines, mucous hypersecretion, activation of proteases and damage to cellular components[10]. Histone deacetylases remove acetyl groups on histones and influence gene expression. HDAC2 suppresses inflammatory gene expression and is reduced in alveolar macrophages and lung tissue in COPD, and this is believed to be a key mechanism of corticosteroid resistance in COPD[8,11]. Nitrosylation of tyrosine residues on HDAC2 by peroxynitrite results in its inactivation[12] and ROS activate phosphoinositide 3-kinase leading to phosphorylation and inactivation of HDAC2[13]. Therefore oxidative and nitrosative stress exert pro-inflammatory effects both through direct induction of inflammatory mediators and reduction of the anti-inflammatory action of HDAC2.

The roles of oxidative and nitrosative stress and HDAC2 in COPD exacerbations are unclear. We have previously demonstrated that experimental rhinovirus infection induces symptoms, airflow obstruction and airways inflammation in COPD subjects[14-16]. We hypothesized that oxidative and nitrosative stress are increased and HDAC2 activity reduced in virus-induced COPD exacerbations.

Methods

Study design

Ethical approval for the study was obtained from St Mary's Local Research Ethics Committee (study number 07/H0712/138) and informed consent obtained from all subjects. COPD subjects (GOLD stage II) and smokers with normal lung function were recruited according the criteria established in our previous study (Supplementary Table 1)[15], together with non-smokers with normal lung function. The COPD patients were receiving no inhaled corticosteroids, long-acting regular bronchodilators or oral corticosteroids. Baseline samples of induced sputum and bronchoalveolar lavage were obtained approximately 14 days prior to infection and subjects were inoculated with 10 $TCID_{50}$ of rhinovirus 16 on day 0 as described previously[15]. Sputum sampling was repeated on subsequent visits on days 3, 5, 9, 12, 15, 21, and 42 post-infection and bronchoscopy on day 7. Respiratory symptoms were recorded daily using diary cards as per our previous studies. A COPD exacerbation was defined as an increase in the total lower respiratory score of at least 2 points over baseline for at least 2 consecutive days.

Sputum assessments

8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of hydroxyl radical damage to DNA, 8-isoprostane, a marker of lipid peroxidation and 3-nitrotyrosine (3-NT) a product of tyrosine nitration mediated by peroxynitrite were measured in sputum supernatants using commercially available enzyme immunoassays (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Sputum nitrite levels were measured using the Griess assay. The Meso Scale Discovery (MSD) platform (Maryland, USA) was used to measure inflammatory mediators in sputum and bronchoalveolar lavage supernatants. The mediators measured were the pro-inflammatory cytokines IL-1β, TNF-α, and GM-CSF, the neutrophil chemokine CXCL8/IL-8 and the protease matrix metalloprotease-9 (MMP-9). Neutrophil elastase was measured using an enzyme-linked immunosorbent assay according to the manufacturers' instructions (Immunodiagnostik, Benshein, Germany). Further details are provided in the e-Appendix.

HDAC2 immunoprecipitation and activity

The HDAC2 isoenzyme was isolated from macrophage pellets obtained from sputum and bronchoalveolar lavage samples. Immunoprecipitation using a HDAC2 antibody (Insight Biotect, UK) and PureProteome® Protein A magnetic beads (Millipore, USA) was performed. HDAC activity was measured using a HDAC activity assay (Cayman Chemicals, USA) the details of which are provided in the e-Appendix.

Details of the experimental protocols of the *in vitro* infection of THP-1 cells (a human monocyte cell line) to further investigate the effects of rhinovirus infection on HDAC2 are provided in the e-Appendix.

Statistical analysis

Data are presented as either means or median, ~~and~~ changes from baseline were analysed using repeated measures ANOVA or Friedman's test and differences between groups using Holm-Sidak's multiple comparisons test or the Kruskal-Wallace test. Correlations between data sets were examined using Spearman's rank correlation coefficient. Correlations were examined using peak post-inoculation values as in a dynamic and evolving clinical event such as a respiratory infection the temporal relationships between the induction of the different markers was variable between individual subjects, and using set days may miss relationships where timing of events varied. Differences were considered significant for all statistical tests at P values of less than 0.05. All reported P values are two-sided. Analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego USA).

Results

Study subjects

The clinical characteristics of the subjects successfully infected with rhinovirus 16 are shown in Table 1. Subjects were age and sex matched between the COPD subjects and both control groups, but the non-smokers were older than the smoking controls. Cigarette smoke exposure between smoking controls and COPD subjects was matched for pack year history.

Clinical and inflammatory responses

Symptoms and lung function: All COPD subjects experienced an exacerbation following rhinovirus infection (e-Figure 1)[15]. All exacerbations were Level I according

to the ATS/ERS severity classification[17]. FEV_1 fell significantly from baseline in the COPD subjects on days 3, 5 (P<0.05), 9 (P<0.01), 12 (P<0.05) and 15 (P<0.01), with a maximum fall of 250mL (12.92%) on day 15. There were no significant falls from baseline in FEV_1 in the control groups (e-Figure 2).

Sputum inflammatory cells, neutrophil elastase and virus load: In the COPD subjects sputum inflammatory cells were significantly increased over baseline on day 9, and were significantly higher compared to the non-smokers on days 9 and 15 (Figure 1a). There was no significant change from baseline in the smokers and the non-smokers. Sputum neutrophils increased significantly from baseline in the COPD subjects on day 9 and were significantly higher compared to the smokers on day 12 (Figure 1b). There were no significant changes in numbers of neutrophils in either control group, nor in numbers of macrophages, lymphocytes, or eosinophils in any group. Levels of neutrophil elastase in sputum increased from baseline on days 9 and 15 in the COPD subjects and were significantly higher in the COPD group compared to controls on day 5(Figure 1c).

Sputum virus load was increased from baseline on days 3–15 in the COPD subjects, on days 5 and 9 in the smokers and on days 3 and 9 in non-smokers (Figure 1d). Sputum virus load was significantly higher in the COPD group compared to both control groups on day 12 and compared to the non-smokers on day 15.

Sputum inflammatory mediators: In the COPD subjects there were significant increases from baseline in IL-1β, GM-CSF, CXCL8/IL-8, TNF-α, and MMP-9 in sputum (Figure 2). There was no significant induction of inflammatory mediators in the non-COPD subjects apart from IL-1β on day 9 and GM-CSF on day 21 in the smokers. Sputum mediator levels were significantly higher in the COPD subjects compared to the non-smokers on days 9 and 15 and for some mediators on days 5,

12, and 21. Levels of inflammatory mediators in bronchoalveolar lavage both at baseline and post-inoculation were generally lower than in sputum and are shown in the Supplementary Index. Correlations between sputum inflammatory mediators, inflammatory cells and virus load in the COPD subjects are shown in Table 2.

Oxidative and nitrosative stress: Prior to infection sputum 8-OHdG levels were higher in the COPD group compared to the smokers (P<0.05) and 3-NT levels were higher in the COPD group compared to both non-smokers (P<0.05) and smokers (P<0.001), with no differences between the groups in baseline levels of nitrite or 8-isoprostane (Figure 3). Markers of oxidative and nitrosative stress were all significantly induced following rhinovirus infection in COPD subjects with much smaller induction seen in the smokers and were significantly higher in the COPD subjects compared to the non-COPD subjects (Figure 3 a-d).

Macrophage HDAC2 activity

At baseline there were no significant differences between the groups in HDAC2 activity in sputum (data not shown) or bronchoalveolar lavage macrophages (Figure 3f). Following infection HDAC2 activity in the smoking controls and non-smoking controls did not change significantly from baseline, but tended to increase (Figure 3e and f). In the COPD subjects there was a trend towards reduced HDAC2 activity in both sputum (P=0.064) and bronchoalveolar lavage macrophages (P=0.098, Figure 3e and f). Sputum HDAC2 activity was significantly lower in the COPD subjects compared to non-smokers on days 5 and 21 (P<0.05), and there was a trend towards lower levels of bronchoalveolar lavage HDAC2 activity during exacerbation compared to the non-smokers (P=0.095) and smokers (P=0.059).

Correlations

Baseline HDAC2: Sputum macrophage HDAC2 activity at baseline correlated inversely with peak post-inoculation sputum virus load (*P*=0.022, r=-0.82),sputum NE (*P*=0.022, r=-0.81), CXCL8/IL-8 (*P*=0.047, r=-0.71) and TNF-α (*P*=0.028, r=-0.79) (Table 2). There were no relationships between baseline HDAC2 activity and outcomes following infection in the non-COPD subjects.

Oxidative stress: In the COPD subjects peak sputum 8-isoprostane levels and 8-OHdG levels during exacerbations correlated with each other (*P*=0.0068, r=0.82) and 8-OHdG correlated with peak sputum neutrophil elastase (*P*=0.0125, r=0.78) and sputum 3-NT levels (*P*=0.0016, r=0.83).

Nitrosative stress: Peak sputum 3-NT and sputum nitrite levels during exacerbation correlated with each other (*P*=0.0037, r=0.85). Peak sputum 3-NT correlated with peak neutrophil elastase (*P*=0.0009, r=0.9) and peak sputum nitrite correlated with peak sputum inflammatory cell numbers (*P*=0.0125, r=0.778), neutrophil elastase (*P*=0.042, r=0.68) and neutrophil numbers (*P*=0.0096, r=0.8) (Table 3).

HDAC2 activity: HDAC2 activity in bronchoalveolar lavage macrophages in COPD subjects during exacerbations correlated inversely with peak nasal lavage virus load (*P*=0.0096, r=-0.8), peak sputum GM-CSF (*P*=0.0499, r=-0.67), TNF-α (*P*=0.03, r=-0.72), neutrophil elastase (*P*=0.0499, r=-0.67) and sputum nitrite levels (*P*=0.0125, r=-0.78) (Table 2).

Rhinovirus infection of monocytes *in vitro*

Rhinovirus infection of the monocytic cell line THP-1 resulted in up-regulation of the inflammatory cytokines IL-6 and CXCL8/IL-8 (Figure 4). Rhinovirus infection reduced expression of HDAC2 (both mRNA and protein) compared to non-infected cells

(Figure 4c and 4d), and reduced HDAC2 activity (Figure 4e). Levels of nitrosylated HDAC2 were increased post-infection compared to non-infected cells (Figure 4f). THP-1 cells were treated with N-acetylcysteine (NAC) in order to inhibit the actions of peroxynitrate[18-20]. Treatment of the THP-1 cells with NAC led to a dose-dependent reduction in IL-6 and CXCL8/IL-8, with significant reductions in inflammatory mediators observed at doses over 1mM (Figures 4g and 4h).

Discussion

Acute exacerbations are important events in the natural history of COPD, but their pathogenesis remains poorly understood. Prevention of exacerbations remains a key therapeutic goal but current treatments have only modest benefits and considerable adverse effects. Previous work from our group has demonstrated that experimental rhinovirus infection in COPD subjects induces respiratory symptoms, airflow obstruction and neutrophilic inflammation[15]. In the current study we provide novel evidence of the mechanisms of virus-induced COPD exacerbations and identify new potential therapeutic targets.

Oxidative/nitrosative stress, airways inflammation and reduced HDAC activity are well described in stable COPD but their roles in COPD exacerbations are unclear. Some studies have reported increased inflammation and oxidative stress in exacerbations[3,21-24], whereas others have reported no change compared to the stable state[25-28]. Increased numbers of 3-NT positive cells have been reported in COPD exacerbations[21] and two studies reported that HDAC activity is unchanged in exacerbated COPD[28,29]. In these studies of naturally-occurring exacerbations there are numerous sources of variability that likely account for the discrepant results of these studies, including variations in exacerbation aetiology, timing of presentation/sampling after exacerbation onset, different treatments etc. that are

difficult to eliminate. Understanding the molecular pathways in COPD exacerbations is critical to developing new, more effective preventative and therapeutic strategies. Our model of COPD exacerbation permits repeated lower airway sampling in treatment-naïve subjects in a manner not possible with naturally-occurring exacerbations, thereby reducing variability and offering unique insights into the mechanisms of COPD exacerbations[15,16,30,31]. Moreover use of a human, as opposed to an animal model provides data that is more likely to be rapidly translated into new therapies. Using this model we measured markers of inflammation, oxidative and nitrosative stress and HDAC activity prior to and following rhinovirus infection.

We previously reported that virus-induced exacerbations are associated with increased neutrophils, neutrophil elastase, and CXCL8/IL-8[15]. In the current study we replicated these findings in a new cohort of subjects and, in addition, demonstrated that virus infection induces significant increases in the pro-inflammatory cytokines GM-CSF, TNF-α and IL-1β, and the protease MMP-9. Levels of 8-isoprostane (a marker of lipid peroxidation), 8-OHdG (a marker of hydroxyl radical damage to DNA), 3-NT (a product of tyrosine nitration mediated by peroxynitrite) and nitrite were significantly increased in sputum in the COPD subjects, with only small, transient increases in non-COPD smokers. There were positive correlations between markers of nitrosative stress and inflammatory mediators in sputum. These data provide new evidence of a pathogenic role of oxidative and nitrosative stress in COPD exacerbations. Exacerbations are associated with accelerated loss of lung function[32-34] and nitrosative/oxidative stress[35,36], neutrophil elastase[37] and MMP-9[38] are all associated with airway remodelling and therefore these are potential mechanisms linking exacerbations with loss of lung function in COPD.

HDAC2 levels at baseline were not lower in the COPD subjects as has been reported previously in patients with COPD GOLD stages II and III[8]. Reduced HDAC2 is most pronounced in severe COPD and therefore may not be present in subjects with GOLD stage II disease. Following rhinovirus infection there was a trend towards reduced HDAC2 activity in sputum and BAL macrophages in COPD subjects and HDAC activity correlated inversely with airways inflammation, virus load and nitrite levels. Nitrosylation of HDAC, followed by HDAC degradation is a mechanism of reduced HDAC2 activity[39] and our *in vitro* data is the first report that virus infection induces nitrosylation of HDAC2. The inverse relationship between airway HDAC2 activity and nitrite levels following virus infection suggests this is relevant *in vivo* also. Therefore nitrosylation of HDAC2 may link virus infection, oxidative/nitrosative stress, impaired HDAC2 activity and enhanced inflammation in COPD exacerbations.

These results have a number of important implications for our understanding of the pathogenesis of COPD exacerbations and future therapeutic directions. Corticosteroids are used in COPD exacerbations despite modest clinical benefits and frequent adverse effects[5,40]. Reduced HDAC2 may contribute to corticosteroid resistance in stable COPD[8] and our results suggest this is also relevant in exacerbated COPD. Thus enhancing HDAC2 activity through inhibiting virus-induced oxidative/nitrosative stress has the potential to improve the therapeutic effect of corticosteroids in COPD exacerbations, as well as having a direct anti-inflammatory effect itself. This warrants investigation in studies specifically designed to address this issue. The demonstration that oxidative/nitrosative stress and multiple inflammatory mediators and proteases are elevated in exacerbations may account for the lack of clinical benefit seen with inhibition of single mediators[41-44], and casts

doubt on this as a valid therapeutic approach. N-acetylcysteine that is both an antioxidant and a peroxynitrite inhibitor demonstrated an anti-inflammatory effect *in vitro* and may also be effective *in vivo*.

Reduced baseline HDAC2 activity in sputum macrophages was associated with greater virus loads post-infection; therefore reduced HDAC2 activity may also be linked to impaired antiviral responses. We observed significantly higher sputum virus loads in COPD subjects, confirming for the first time impaired antiviral immunity in COPD results in more severe lower respiratory virus infection. HDAC activity is required for an effective antiviral response to picornavirus infection[45], therefore reduced HDAC activity may contribute to impaired antiviral host defence and increased severity of clinical illness following virus infections in COPD.

Our study has a number of limitations that are inherent to experimental infection studies. The number of subjects was small and only subjects with moderate COPD were included, and therefore the findings may not be applicable to more severe patients. However as HDAC2 activity is further reduced[8] and exacerbations more frequent in severe COPD[2], we believe these mechanisms are likely to be even more relevant in more severe disease. Secondary bacterial infections did occur in some subjects[30] but the numbers were too small to allow an analysis of the interactions between HDAC2 activity and bacteria (6 subjects with bacterial infection, 3 without). Whether changes in HDAC2 activity affect the ability of macrophages to clear bacteria is a subject of great interest and warrants further investigation. The smokers were significantly younger compared to the non-smokers but there were no significant differences in age between the COPD group and the 2 control groups and as the key comparisons were between the COPD and non-COPD subjects, we do not believe this affected the important results. Moreover Ito et al reported that age

had no effect on HDAC activity in bronchial biopsies[8]. However, ideally subsequent studies should use age-matched subjects whenever possible to remove this confounder. We used sputum to measure inflammatory meditors and cells and these do not reflect inflammation in the small airways. The main site of rhinovirus replication is epithelial cells rather than macrophages and therefore the significance of the relationship between HDAC2 levels in macrophages and virus load is unclear. The *in vitro* work was carried out in THP-1 cells rather than alveolar macrophages as these are difficult to obtain in large enough numbers to perform the necessary experiments. However the data obtained provides valid mechanistic information as to the potential mechanisms involved in macrophage responses to viral infection in COPD.

In summary we have demonstrated induction of oxidative and nitrosative stress, reduced HDAC2 activity, increased inflammatory mediators and increased virus load in the airways following rhinovirus infection in COPD subjects. These results provide unique insights into the mechanisms of virus-induced COPD exacerbations and suggest novel therapeutic targets for the development of new targeted therapies in COPD.

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interpreted data, and revised the report. SLJ had full access to all the data in the study and had final responsibility for the decision to submit for publication. All authors have given final approval of the version to be published.

This article is dedicated to the memory of Dr. Joseph Footitt.

Figure Legends

Figure 1. Time course of inflammatory cells, neutrophil elastase and virus load in sputum during experimental rhinovirus infection. Panel A: Total sputum inflammatory cells (neutrophils, macrophages/monocytes, lymphocytes, eosinophils). Panel B: Sputum neutrophils. Panel C: Sputum neutrophil elastase. Panel D: Sputum virus load. All data are means ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. baseline. †*P*<0.05, ††*P*<0.01 COPD vs. non-smokers. #*P*<0.01, ##*P*<0.01 COPD vs. smokers.

Figure 2. Time course of inflammatory mediators in sputum during experimental rhinovirus infection in COPD. Panel A: Sputum IL-1β. Panel B: Sputum TNF-α. Panel C: Sputum GM-CSF. Panel D: Sputum CXCL8/IL-8. Panel E: Sputum MMP-9. All data are medians ± IQR. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. baseline. †*P*<0.05, ††*P*<0.01 COPD vs. non-smokers.

Figure 3. Time course of oxidative and nitrosative stress markers in sputum and HDAC2 activity in sputum and bronchoalveolar lavage macrophages during experimental rhinovirus infection. Panel A: Sputum 8-isoprostane. Panel B: Sputum 8-hydroxy-2'-deoxyguanosine. Panel C: Sputum 3-nitrotyrosine. Panel D: Sputum nitrite. Panel E: Sputum macrophage HDAC2 activity –change from baseline. Panel F: Bronchoalveolar lavage macrophage HDAC2 activity. All data are medians ± IQR. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. baseline. †*P*<0.05, ††*P*<0.01, †††*P*<0.001 COPD vs. non-smokers. #*P*<0.05, ##*P*<0.01, ###*P*<0.001 COPD vs. smokers.

Figure 4. Rhinovirus infection of THP-1 cells *in vitro*. Panel A: Induction of IL-6 by rhinovirus in THP-1 cells. Panel B: Induction of CXCL-8 by rhinovirus in THP-1 cells.

Panel C: HDAC2 protein in THP-1 cells infected with rhinovirus. Panel D: HDAC2 RNA in THP-1 cells infected with rhinovirus protein. Panel E: HDAC2 activity in THP-1 cells infected with rhinovirus. Panel F: HDAC2 nitrosylation in THP-1 cells infected with rhinovirus. Panel G: Inhibition of rhinovirus-induced IL-6 by N-acetylcysteine (NAC). Panel H: Inhibition of rhinovirus-induced CXCL8/IL-8 by NAC. *P<0.05 Data shows mean ± standard error of at least 3 independent experiments.

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Tables

	Non-smokers (N=11)	Smokers (N=10)	COPD (N=9)	P values
Age	62.18 (±1.62)	52.50 (±2.23)	60.44 (±3.17)	P<0.05 SMK vs. NS
Sex M:F	4:7	4:6	6:3	NS
Current smokers	0	10/10	8/9	
Smoking history (pack years)	0	32.1 (±3.02)	39.44 (±3.25)	NS
FEV_1 (Litres)	3.17 (±0.17)	3.24 (±0.21)	2.31 (±0.13)	P<0.05 COPD vs. NS, P<0.01 COPD vs. SMK
FEV_1 (% predicted)	102.2 (±3.34)	96.60 (±3.28)	68.11 (±1.58)	P<0.0001 COPD vs. SMK and NS
FEV_1/FVC	77.62 (±1.09)	78.04 (±2.18)	61.58 (±1.80)	P<0.0001 COPD vs. SMK and NS

Table 1. Baseline clinical characteristics of study subjects. All data mean ± SEM.

	Baseline sputum HDAC2	Post-inoculation BAL macrophages HDAC2
Peak sputum virus load	P=0.022, r=-0.82	/
Peak sputum neutrophil elastase	P=0.022, r=-0.81	P=0.0499, r=-0.67
Peak sputum CXCL8/IL-8	P=0.047, r=-0.71	/
Peak sputum TNF-α	P=0.028, r=-0.79	P=0.03, r=-0.72
Peak nasal lavage virus load	/	P=0.0096, r=-0.8
Peak sputum GM-CSF	/	P=0.0499, r=-0.67
Peak sputum nitrite	/	P=0.0125, r=-0.78

Table 2. Correlations between baseline sputum HDAC2 levels and post-inoculation BAL macrophages HDAC2 levels and peak levels of sputum inflammatory mediators and virus load.

Inflammatory mediators	Virus load	Inflammatory cells and neutrophil markers			Oxidative stress		Nitrosative stress	
		Total cell count	Neutrophils	Neutrophil elastase	8-IP	8-OHdG	3-NT	Nitrite
IL-1β	NS	NS	*P*=0·043 r=0·7	NS	NS	NS	*P*=0·031 r=0·73	*P*=0·017 r=0·78
TNF-α	NS	*P*=0·031 r=0·73	*P*=0·016 r=0·77	*P*=0·026 r=0·75	NS	NS	*P*=0·017 r=0·78	*P*=0·.0007 r=0·93
GM-CSF	NS	*P*=0·021 r=0·77	*P*=0·02 r=0·75	NS	NS	NS	NS	*P*=0·0053 r=0·83
CXCL8 /IL-8	P=0.0096 r=0.8	*P*=0·0096 r=0·8	*P*=0·0072 r=0·82	NS	NS	NS	NS	*P*=0·05 r=0·68
MMP-9	NS	*P*=0·0031 r=0·88	*P*=0·0025 r=0·87	NS	NS	NS	*P*=0·0083 r=0·83	*P*=0·0004 r=0·95

Table 3. Correlations between peak levels of sputum inflammatory mediators, virus load, sputum cell counts and markers of oxidative and nitrosative stress in the COPD subjects. 8-IP – 8-isoprostane, 8-OHdG – 8-hydroxy-2'-deoxyguanosine, 3-NT – 3-nitrotyrosine.