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Oxidative and Nitrosative Stress and Histone Deacetylase-2 Activity in Exacerbations of COPD

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e-Appendix 1.

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^{o}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*.

References

1. Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. Anal biochem 1985;150:76-85.

2. Wark PA, Johnston SL, Bucchieri F, et al. Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. Journal Exp Med 2005;201: 937-47.

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

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 2. Scoring system for lower respiratory symptoms.

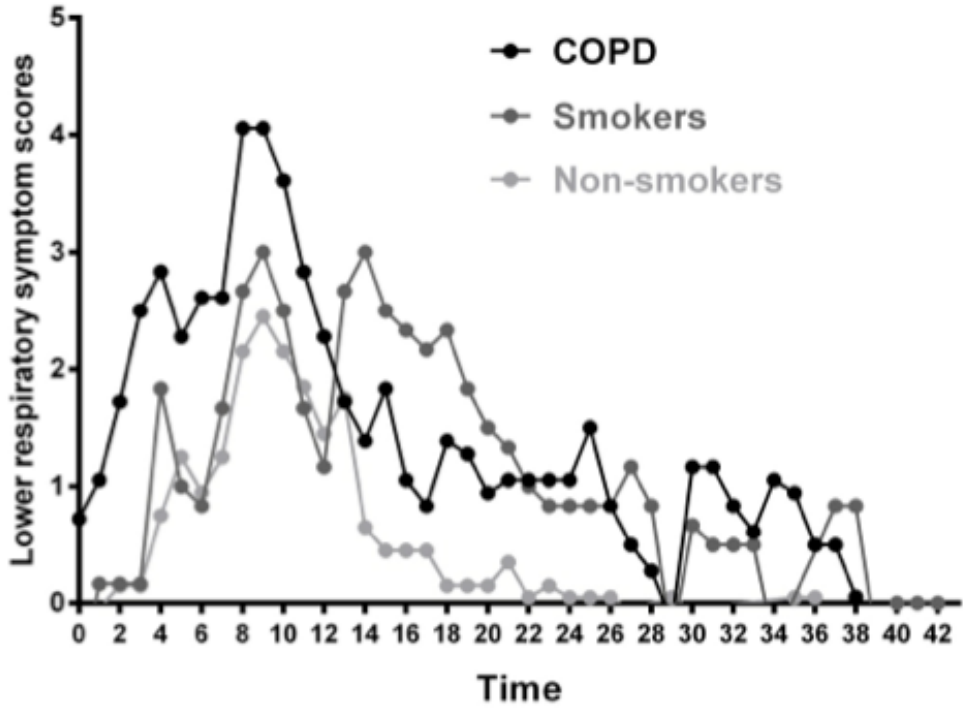
SYMPTOM	SCORE				
	0	**1**	**2**	**3**	**4**
SHORTNESS OF BREATH	Not breathless	On moderate exertion	On mild exertion	On minimal exertion	At rest
WHEEZE	No wheeze	On moderate exertion	On mild exertion	On minimal exertion	At rest
COUGH	No cough	Mild	Moderate	Severe	/
SPUTUM QUANTITY	None	Minimal (<30mL)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

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

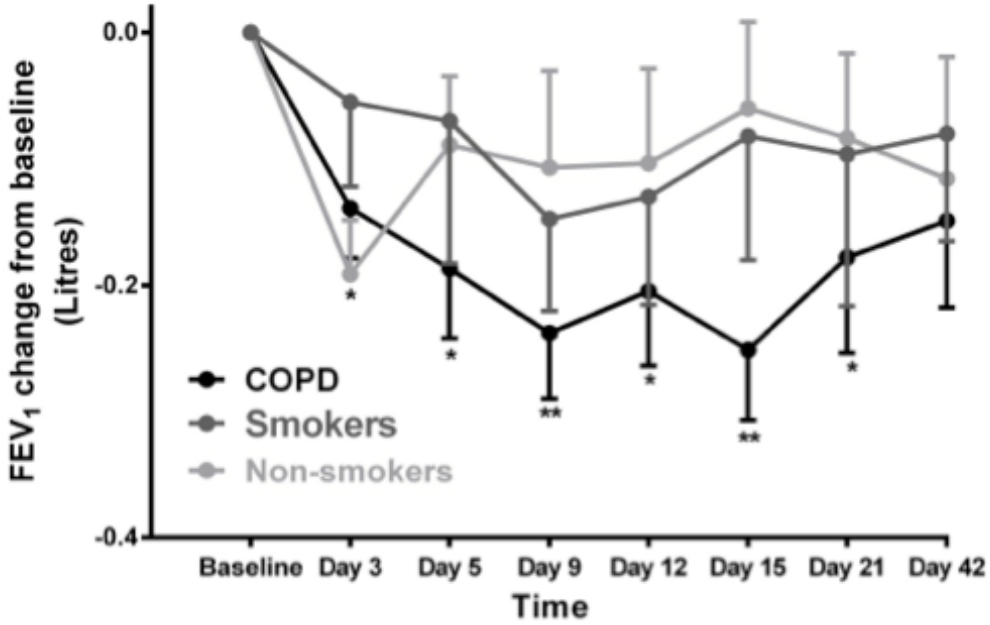
	Baseline	Day 3	Day 5	Day 9	Day 12	Day 15	Day 21	Day 42
Non-Smokers	2.02 ± 0.4	1.41 ± 0.19	1.51 ± 0.32	2.18 ± 0.64	3.22 ± 1.0	1.43 ± 0.24	1.03 ± 0.19	1.04 ± 0.15
Smokers	1.72 ± 0.63	1.61 ± 0.5	1.74 ± 0.27	5.96 ± 2.13	2.06 ± 0.61	2.60 ± 0.59	2.41 ± 0.51	3.42 ± 1.9
COPD	1.16 ± 0.21	2.47 ± 0.58	2.96 ± 0.92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

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

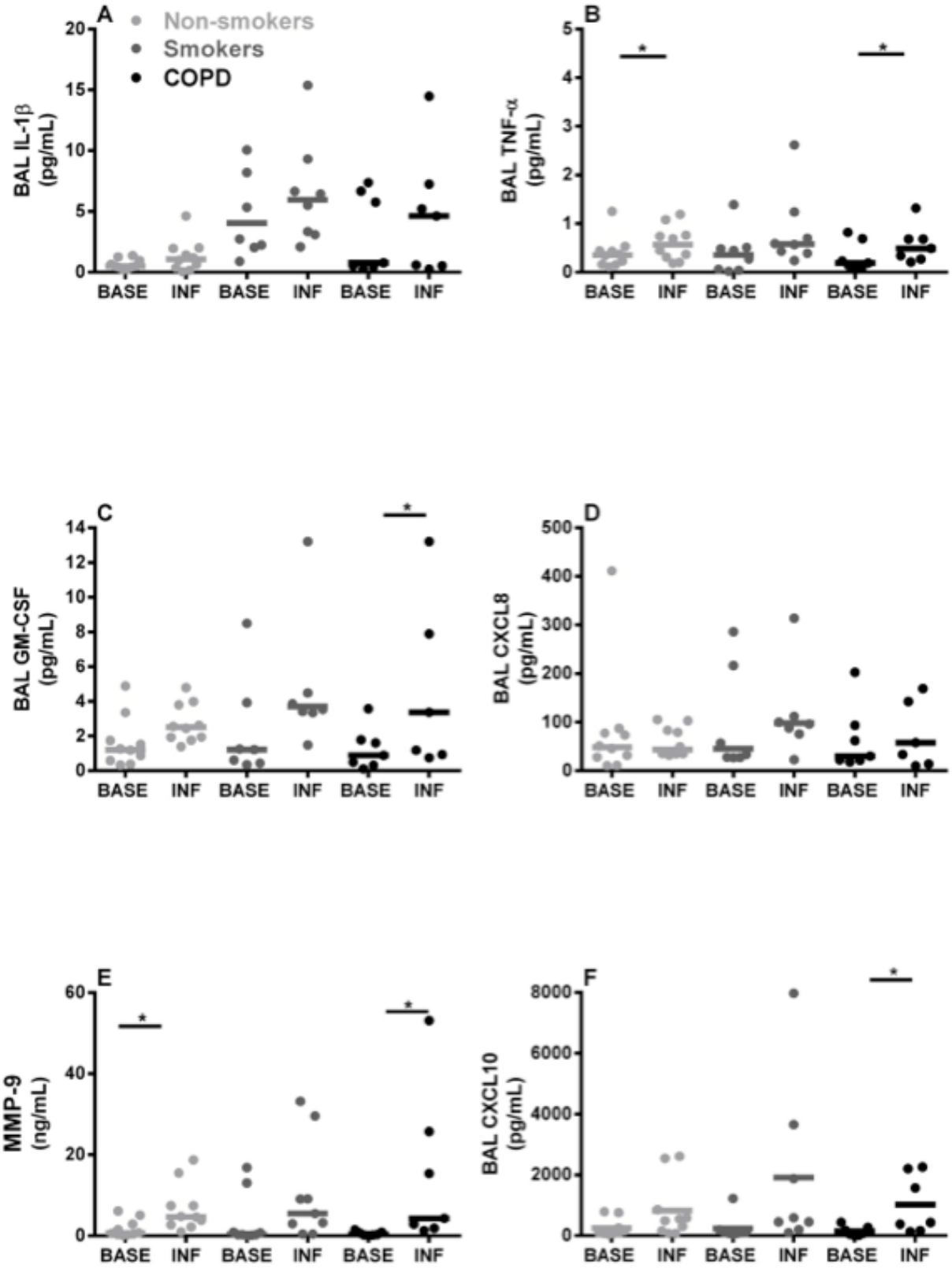
	Baseline	Day 7
Non-Smokers	79.28 (73.81 - 143)	182.2 (146.1 - 524.4)
Smokers	318.8 (146.1 - 524.4)	359.2 (174.2 - 698.4)
COPD	144.7 (88.03 - 630.9)	353.4 (141.6 - 511.6)



e-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.