

A comprehensive analysis of oxidative stress in the ozone-induced lung inflammation mouse model

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Abstract

Ozone is an oxidizing environmental pollutant that contributes significantly to respiratory health. Exposure to increased levels of ozone has been associated with worsening of symptoms of patients with asthma and COPD (chronic obstructive pulmonary disease). In the present study, we investigated the acute and chronic effects of ozone exposure-induced oxidative stress-related inflammation mechanics in mouse lung. In particular, we investigated the oxidative stress-induced effects on HDAC2 (histone deacetylase 2) modification and activation of the Nrf2 (nuclear factor erythroid-related factor 2) and HIF-1 α (hypoxia-inducible factor-1 α) signalling pathways. Male C57BL/6 mice were exposed to ozone (3 p.p.m.) for 3 h a day, twice a week for a period of 1, 3 or 6 weeks. Control mice were exposed to normal air. After the last exposure, mice were killed for BAL (bronchoalveolar lavage) fluid and lung tissue collection. BAL total cell counts were elevated at all of the time points studied. This was associated with increased levels of chemokines and cytokines in all ozone-exposed groups, indicating the presence of a persistent inflammatory environment in the lung. Increased inflammation and L_m (mean linear intercept) scores were observed in chronic exposed mice, indicating emphysematous changes were present in lungs of chronic exposed mice. The antioxidative stress response was active (indicated by increased Nrf2 activity and protein) after 1 week of ozone exposure, but this ability was lost after 3 and 6 weeks of ozone exposure. The transcription factor HIF-1 α was elevated in 3- and 6-week ozone-exposed mice and this was associated with increased gene expression levels of several HIF-1 α target genes including *Hdac2* (histone deacetylase 2), *Vegf* (vascular endothelial growth factor), *Keap1* (kelch-like ECH-associated protein 1) and *Mif* (macrophage migration inhibitory factor). HDAC2 protein was found to be phosphorylated and carbonylated in nuclear and cytoplasm fractions, respectively, and was associated with a decrease in DNA-binding activity and protein expression of HDAC2. Decreased HDAC2 activity, most likely a direct result of protein modification, in combination with the loss of the antioxidative stress response and activation of the HIF-1 α pathway, contribute to the inflammatory response and emphysema observed in ozone-exposed mice.

Key words: chronic obstructive pulmonary disease (COPD), histone deacetylase, hypoxia, inflammation, oxidative stress, ozone

INTRODUCTION

COPD (chronic obstructive pulmonary disease) is predominantly caused by cigarette smoking in the Western world and affects 10% of the global adult population [1] and is predicted to be the third cause of death in the world by 2020 [2]. COPD is characterized by

chronic inflammation and high levels of oxidative stress, which further enhances the inflammatory response. This leads to the development of airflow limitation following airway remodelling and destruction of lung tissue [3]. Ozone is an oxidizing environmental pollutant that contributes significantly to respiratory health. Exposure to increased levels of ozone has been associated

Abbreviations: BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; CBP CREB (cAMP-response-element-binding) protein; CI, confidence interval; COPD, chronic obstructive pulmonary disease; CXCL, CXC chemokine ligand; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; Gpx1, glutathione peroxidase 1; HAT, histone acetyl transferase; H&E, haematoxylin and eosin; HDAC, histone deacetylase; HIF, hypoxia-inducible factor; *Ho1*, haem oxygenase 1; HRE, hypoxia response element; IFN- γ , interferon- γ ; IL, interleukin; KC, keratinocyte-derived cytokine; Keap1, kelch-like ECH-associated protein 1; LPS, lipopolysaccharide; MIF, macrophage migration inhibitory factor; Nrf2, nuclear factor erythroid-related factor 2; PBMC, peripheral blood mononuclear cell; pHDAC2, phosphorylated HDAC2; PHD, prolyl hydroxylase; SOD, superoxide dismutase; TNF, tumour necrosis factor; *Txnrd1*, thioredoxin reductase 1; *Vegf*, vascular endothelial growth factor.

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with worsening of symptoms of patients with asthma and COPD [4,5] and increased morbidity and mortality rates of cardiovascular and respiratory patients [6–10]. Ozone exposure in mice induces airway inflammation associated with an increased presence of neutrophils and macrophages, high levels of cytokines and chemokines [11,12] and emphysematous changes associated with an increase in apoptotic cells in alveolar and airway epithelial cells [12].

Inflammatory gene transcription is regulated by the relative activities of transcriptional co-activators such as CBP [CREB (cAMP-response-element-binding) protein] that possesses intrinsic HAT (histone acetyl transferase) activity and co-repressors such as HDAC2 (histone deacetylase 2) [13]. HDAC2 is associated with the repression of pro-inflammatory cytokines particularly under conditions of oxidative stress such as that seen in alveolar macrophages isolated from COPD patients [14] and its expression is decreased in COPD [13,15]. HDAC2 activity and protein degradation is, at least in part, determined by post-translation modifications including phosphorylation [16], acetylation [17], nitrosylation [18] and carbonylation [19]. Cigarette smoke exposure induces CKII-dependent phosphorylation of HDAC2 resulting in ubiquitination and degradation of the protein [20]. RCS (reactive carbonyl species) target cysteine residues to cause carbonylation and might modulate HDAC proteins function. HDAC1 is carbonylated on conserved cysteine residues resulting in a reduced ability of HDAC1 to interact with its binding partners [19]. However, there is little information concerning HDAC2 carbonylation.

Enhanced oxidative stress, as seen in COPD, can be counteracted by the actions of Nrf2 (nuclear factor-erythroid 2-related factor), a transcription factor that is involved in the defence against oxidative stress [21]. However, Nrf2 expression is reduced in COPD patients [22], which might contribute to the enhanced inflammation observed in patients. Oxidative stress induces Nrf2 protein and activity *in vitro* and is regulated by the acetylation status of Nrf2 under the control of HDAC2 [23].

HIF (hypoxia-inducible factor)-1 α is a basic helix–loop–helix-PAS domain heteromeric transcription factor consisting of an α and an β subunit. Under normoxic conditions the HIF-1 α subunit is continuously expressed but degraded [24]. Under hypoxic conditions the rate of degradation is reduced and allows stabilization of HIF-1 α protein and its translocation to the nucleus and initiation of target gene transcription [24,25]. HIF-1 α DNA binding is able to reduce *HDAC2* gene transcription [26] and might therefore affect the antioxidant stress response mediated by the Nrf2 pathway.

We have developed an oxidative-stress-mediated model of COPD that reproduces many aspects of the human disease including increased inflammation and oxidative stress, airway remodelling and alveolar destruction, which occurs in a much reduced time frame compared with that observed with cigarette-smoke models (3–6 weeks compared with 6–8 months) [12]. In the present study, we investigate the effects of increasing ozone exposure on oxidative stress and inflammation in mice. We show that ozone exposure results in a persistent inflammatory environment in the lung. After 1 week of ozone exposure, the inflammation is associated with a functional antioxidative stress response

Table 1 Volunteer and patient details

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Mann–Whitney test.

	Healthy subjects	COPD patients
<i>n</i>	5	5
Gender (male/female) (<i>n</i>)	2/3	3/2
Age (years)	49.8 $\pm$ 3.8	59.5 $\pm$ 7.2
FEV ₁	2.8 $\pm$ 0.6	1.7 $\pm$ 0.2*
FEV ₁ (% predicted)	102.9 $\pm$ 6.1	73.4 $\pm$ 4.9**
FVC	3.4 $\pm$ 0.7	2.9 $\pm$ 0.2
FEV/FVC	83.6 $\pm$ 7.8	60.7 $\pm$ 3.7***

(Nrf2 recruitment). However, after 3 and 6 weeks of exposure this ability is lost and replaced by a hypoxic drive that may contribute further to the inflammatory process.

MATERIALS AND METHODS

Subjects

The present study was approved by the Ethics Committee of the Royal Brompton & Harefield Hospitals National Health Service Trust. All subjects gave written informed consent. Five healthy volunteers and five COPD patients were recruited (Table 1).

PBMC (peripheral blood mononuclear cell) isolation

PBMCs were isolated by Ficoll-Paque™ PLUS (GE Healthcare Life Sciences) as described previously [27]. Cells were starved in 0.5% FBS/RPMI medium for 2 h and subsequently stimulated for 30 min with H₂O₂ (50 μ M), treated for 30 min with sulforaphane (0–20 μ M in DMSO; Sigma–Aldrich) and 20 h with LPS (lipopolysaccharide) (1 ng/ml; Sigma–Aldrich). The medium was collected and used to measure CXCL8 (CXC chemokine ligand 8) secretion (R&D Systems).

Mice

Experiments were performed under a Project License from the British Home Office, UK, under the Animals (Scientific Procedures) Act 1986. C57BL/6 mice (6 weeks old; Harlan) were exposed to ozone (model 500 Ozoniser; Erwin Sander) mixed with air to a 3 p.p.m. concentration. Mice were exposed for 3 h a day, twice a week for a period of 1, 3 or 6 weeks ($n = 8$ mice per group). Control groups were exposed to normal air in an identical experimental setup ($n = 8$ mice per group).

BAL (bronchoalveolar lavage)

BAL samples were obtained as described previously [12]. Briefly, lungs were rinsed three times with 0.8 ml of ice-cold PBS via an endotracheal tube and retrieved as BALF (BAL fluid). BAL samples were centrifuged to isolate cells from fluid. Cell pellets were resuspended in 200 μ l of PBS and counted on a haemocytometer (total cell count). BALF was frozen at -80°C until analysis. BAL cell samples were centrifuged on to glass slides by centrifugation (30 *g* for 6 min in a Shandon Cytospin 4; Thermo Scientific). Cells slides were subsequently fixed and stained using Diff-Quick kit (Reagent) and counted under an

optical microscope (Olympus BH2). BAL supernatants were used to detect the inflammatory mediators KC (keratinocyte-derived cytokine), TNF (tumour necrosis factor)- α , IFN (interferon)- γ , MIF (macrophage migration inhibitory factor) and members of the IL (interleukin) family (IL-6 and IL-17) using commercially available ELISA kits (R&D Systems), according to the manufacturer's instructions.

Histological analysis

Mouse lungs were inflated with 4% paraformaldehyde and were processed as described previously [12]. Sections (5 μ m) were stained with H&E (haematoxylin and eosin) and Masson trichrome stain.

The severity of inflammation observed in the H&E-stained lung sections was scored on a 0–3 scale defined as: 0 = no inflammatory response; 1 = mild inflammation with foci of inflammatory cells in bronchial or vascular wall and in alveolar septa; 2 = moderate inflammation with patchy inflammation or localized inflammation in walls of bronchi or blood vessels and alveolar septa, and less than one-third of the lung cross-sectional area is involved; and 3 = severe inflammation with diffuse inflammatory cells in walls of bronchi or blood vessels and alveoli septa, and between one-third and two-thirds of the lung area is involved. Five sections of each lung were scored and plotted as means with 95% CIs (confidence intervals).

The mean linear intercept (L_m) was determined with a Thurlbeck grid (five lines, 550 μ m long) and scored for the number of times the alveolar wall intercepted each line. L_m was then calculated by dividing the length of the line by the number of alveolar wall and grid line interceptions counted [12].

RNA isolation

Total RNA was extracted from frozen lung tissue using RNase mini kit (Qiagen) as described previously [15]. RNA (5 μ g/sample) was used to synthesize single-stranded cDNA using random hexamers and an avian myeloblastosis virus reverse transcriptase (Promega). Transcript levels were determined by real-time PCR (Rotor Gene 3000; Corbett Research, Sydney, Australia) using SYBR Green PCR Master Mix Reagent (Qiagen).

Primers

Primers for inflammatory mediators were designed using the online programme OligoPerfect™ Designer on the Invitrogen website. Primers and reaction conditions used in the present study are listed in Table 2.

Protein isolation

Nuclear and cytosolic proteins were isolated using a nuclear extraction kit (Active Motif). Briefly, 30 mg of lung tissue was homogenized on ice using a homogenizer (model T10; IKA). Cytosolic proteins were extracted with hypotonic buffer (catalogue number 100505; Active Motif) and nuclear proteins were extracted using a high-salt extraction buffer [15 mM Tris/HCl, pH 7.9, 450 mM NaCl, 0.25 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol and protease inhibitors (Roche)]. The salt concentration was normalized with Tris/glycerol buffer

(15 mM Tris/HCl, pH 7.9, 0.25 mM EDTA, 10 mM mercaptoethanol, 10% glycerol and protease inhibitors) and the final protein concentrations were determined using a protein assay reagent kit (Pierce Chemical) and BSA standard (Sigma–Aldrich).

Activity assays

HDAC activity was measured using a commercially available kit (Biomol). Total HIF-1 α protein and HIF-1 α activity levels were measured using a commercially available sandwich ELISA and a DNA-binding assay respectively (R&D systems). Nrf2 activity was measured by TransAM® assay (Active Motif). SOD (superoxide dismutase) activity levels were determined by ELISA (Cell Biolabs). Carbonylation status was determined using OxiSelect™ Protein Carbonyl ELISA Kit (Cell Biolabs). All analyses were performed according to manufacturer's instructions.

Immunoblotting

Protein extracts (30 μ g) were separated on 10% (w/v) NuPAGE gels and transferred on to nitrocellulose membranes using iBlot™ (Invitrogen). Membranes were blocked for 1 h at room temperature (22°C) or overnight at 4°C with 5% (w/v) non-fat dried skimmed milk powder in TBS-T (TBS containing Tween 20; Sigma–Aldrich), prior to probing with primary antibodies. Western blotting was performed using SNAP technology (Merck Millipore) exactly as described by the manufacturer. Protein detection was performed with Luminata Western HRP (horseradish peroxidase) substrate solutions (Merck Millipore) and densitometry was performed using a Gel-Doc-It imaging system (Photon Imaging Systems).

Antibodies

The following primary antibodies were used: lamin A/C, β -actin (Santa Cruz Biotechnology), HDAC2 (Sigma–Aldrich) and phospho-HDAC2 (Signalway Antibodies). Secondary HRP-linked polyclonal immunoglobulins were obtained from Dako.

HDAC2 immunoprecipitation

Protein extracts (50 μ g, final volume 40 μ l) were incubated with 5 μ g of anti-HDAC2 antibody (Sigma–Aldrich) overnight with constant agitation at 4°C followed by isolation with Protein A/G–agarose beads (Santa Cruz Biotechnology) for 2 h at 4°C with constant agitation. Immunoblotting and protein detection were performed as described above.

Statistics

Data are expressed as means $\pm$ S.D. The Kruskal–Wallis test for ANOVA was used for multiple comparisons. The Mann–Whitney test was subsequently used when the Kruskal–Wallis test was significant. A *P* value of <0.05 was accepted as significant.

Table 2 List of forward and reverse primers and PCR conditions

Gene	Primer	Sequence (5'→3')	PCR conditions
18S	Forward	CTTAGAGGGACAAGTGGCG	20 s at 95°C; 30 s at 60°C; 30 s at 72°C × 30 cycles
	Reverse	ACGCTGAGCCAGTCAGTGTA	
Gpx1	Forward	GTCCACCGTGATGCCTTCT	20 s at 95°C; 30 s at 62°C; 30 s at 72°C × 60 cycles
	Reverse	CTCCTGGGTGCCGAAGTGAT	
Hdac2	Forward	CCGGTGTTTGATGGACTCTT	20 s at 95°C; 30 s at 62°C; 30 s at 72°C × 60 cycles
	Reverse	GCCCAATTGACAGCCATATC	
Hif1a	Forward	GTGTATCGGGCCAGTTCTA	20 s at 95°C; 30 s at 60°C; 30 s at 72°C × 60 cycles
	Reverse	TGGCCATCACTCATGTTTA	
Hif2a	Forward	CTAAGTGGCTGTGGGTGAT	20 s at 95°C; 30 s at 60°C; 30 s at 72°C × 60 cycles
	Reverse	GTGTCTTGAAGGCTTGCTC	
Ifng	Forward	GTGGTTGACACTTAGTGGTCTC	20 s at 95°C; 30 s at 60°C; 30 s at 72°C × 60 cycles
	Reverse	GGTGACATGAAAATCCTGCAGAGC	
Il6	Forward	CACAGAGGATACCACTCCCAACA	20 s at 95°C; 30 s at 62°C; 30 s at 72°C × 60 cycles
	Reverse	TCCACGATTTCCAGAGAACA	
Il17	Forward	ACTACCTCAACGGTCCACG	20 s at 95°C; 30 s at 60°C; 30 s at 72°C × 60 cycles
	Reverse	AGCTCTCAGGCTCCCTCTTC	
Fih	Forward	TTAAGCCAAGGTCCAACAGG	20 s at 95°C; 30 s at 60°C; 30 s at 72°C × 60 cycles
	Reverse	CCACGGTGTCAATTGAGTGC	
Kc	Forward	CGCTGCTGCTGCTGGCCACCA	20 s at 95°C; 30 s at 60°C; 30 s at 72°C × 60 cycles
	Reverse	GGCTATGACTTCGGTTTGGGTGCA	
Keap1	Forward	CATGAGGCATCACCTAGTG	20 s at 95°C; 30 s at 60°C; 30 s at 72°C × 60 cycles
	Reverse	GCATTCCACACTGTCCAGAA	
Mif	Forward	CAAGCCCGCACAGTACATC	20 s at 95°C; 30 s at 60°C; 30 s at 72°C × 60 cycles
	Reverse	AGGCCACACAGCAGCTTACT	
Nrf2	Forward	CCAGAAGCCACACTGACAGA	20 s at 95°C; 30 s at 60°C; 30 s at 72°C × 60 cycles
	Reverse	GGAGAGGATGCTGCTGAAAG	
Sod1	Forward	CCAGTGCAGGACCTCATTTT	20 s at 95°C; 30 s at 62°C; 30 s at 72°C × 60 cycles
	Reverse	CCAAAGGAGAGTTGCTGGAG	
Sod2	Forward	CCAAAGGAGAGTTGCTGGAG	20 s at 95°C; 30 s at 60°C; 30 s at 72°C × 60 cycles
	Reverse	GAACCTTGGSCTCCACAGA	
Sod3	Forward	CAGCTTCGACCTAGCAGACA	20 s at 95°C; 30 s at 60°C; 30 s at 72°C × 60 cycles
	Reverse	CCATCCAGATCTCCAGCACT	
Tnfa	Forward	AGTTCTATGGCCAGACCCCT	20 s at 95°C; 30 s at 62°C; 30 s at 72°C × 60 cycles
	Reverse	AGGGTCTGGGCCATAGAACT	
Txrd1	Forward	TGAAGAGCAGACCAATGTGC	20 s at 95°C; 30 s at 60°C; 30 s at 72°C × 60 cycles
	Reverse	AGGCCACAACAGCCATATTC	
Phd2	Forward	TACAGGATAAACGGCCGAAC	20 s at 95°C; 30 s at 60°C; 30 s at 72°C × 60 cycles
	Reverse	CGCATCTTCCATCTCCATTT	
Vegf	Forward	CAGGCTGCTGTAAACGATGAA	20 s at 95°C; 30 s at 60°C; 30 s at 72°C × 60 cycles
	Reverse	TATGTGATGGCTTTGGTGAG	

RESULTS

Ozone induces inflammation and emphysematous changes in lung tissue

Lung tissue sections stained with H&E showed increased numbers of infiltrating inflammatory cells and an alveolar space enlargement in lung tissue of mice exposed to ozone compared with air controls (Figure 1). The inflammation scores, expressed as the median and 95% CIs, were 0.20 (0.0009–0.40) compared with 0.93 (0.50–1.22) and L_m values, expressed as means $\pm$ S.D., were 63.6 ± 3.7 compared with 82.1 ± 8.9 ; values were increased

4.7- and 1.3-fold respectively ($P < 0.05$), indicating that chronic inflammation is associated with emphysematous changes in lung tissue in this model of COPD. This was indicated further by the correlation between the inflammation score and L_m ($r^2 = 0.97$, $P < 0.001$; results not shown).

Ozone exposure increases BAL inflammatory cell number

BAL total cell counts were increased in all ozone-exposed groups (2.7-fold, 2.8-fold and 3.4-fold in 1-, 3- and 6-week-exposed groups respectively, $P < 0.05$; Figure 2A). Differential cell counts

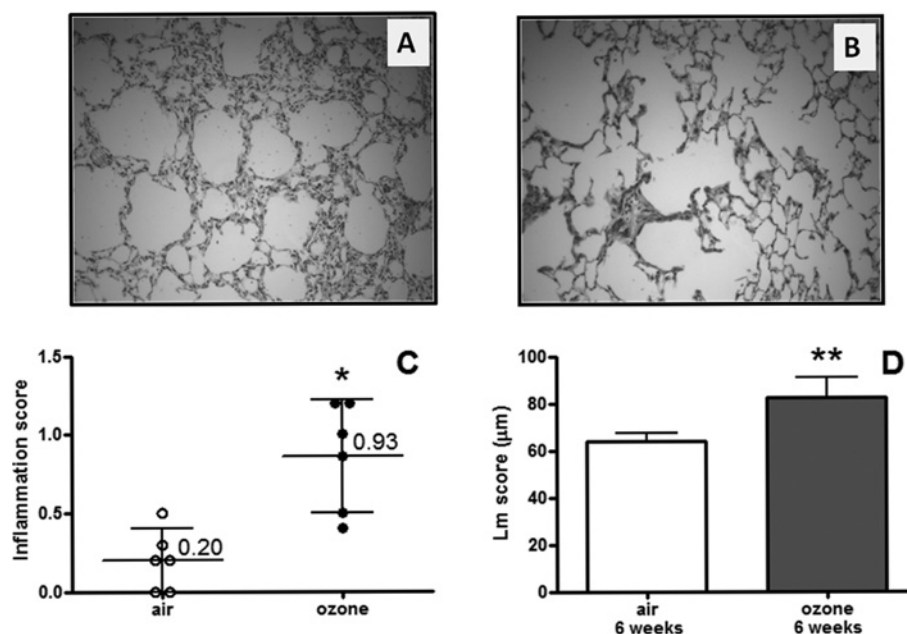


Figure 1 Effect of ozone exposure on inflammation and emphysematous changes in lung tissue

Representative histological sections of mouse lung (H&E staining) from 6-week air-exposed (A) and 6-week ozone-exposed (B) mice. Results are representative of eight animals in each group. Inflammation scores (C) and L_m measurements (D) in 6-week ozone-exposed and air control mice. Inflammation scores are shown as individual values and as medians (95% CIs), and L_m scores presented as the means $\pm$ S.D. of eight animals in each group. * $P < 0.05$ and ** $P < 0.01$ compared with the air control group, Mann–Whitney test.

indicated that this was due to increased numbers of neutrophils (Figure 2B) and macrophages (Figure 2C) at all time-points studied in ozone-exposed mice as reported previously [12]. There was no significant increase in eosinophils ($0.3 \times 10^5 \pm 0.1 \times 10^5$ compared with $0.6 \times 10^5 \pm 0.1 \times 10^5$; $0.4 \times 10^5 \pm 0.1 \times 10^5$ compared with $0.8 \times 10^5 \pm 0.2 \times 10^5$ and $0.3 \times 10^5 \pm 0.1 \times 10^5$ compared with $0.8 \times 10^5 \pm 0.2 \times 10^5$ cells after 1, 3 and 6 weeks of ozone exposure respectively) or CD3⁺ T-cells ($0.4 \times 10^5 \pm 0.1 \times 10^5$ compared with $0.6 \times 10^5 \pm 0.2 \times 10^5$; $0.4 \times 10^5 \pm 0.1 \times 10^5$ compared with $0.5 \times 10^5 \pm 0.1 \times 10^5$ and $0.3 \times 10^5 \pm 0.1 \times 10^5$ compared with $0.5 \times 10^5 \pm 0.1 \times 10^5$ cells after 1, 3 and 6 weeks of ozone exposure respectively; results not shown).

Ozone induces pro-inflammatory cytokines in the lung

Ozone exposure also increased the protein levels of several inflammatory mediators in BALF (Figures 2D–2I). KC, IFN- γ , MIF, TNF- α , IL-6 and IL-17 levels were all increased in BALF of ozone-exposed mice compared with air-exposed mice. KC levels were elevated 3.3-fold, 1.9-fold and 1.9-fold in 1-week-, 3-week- and 6-week-exposed groups respectively ($P < 0.05$; Figure 2D). IFN- γ levels were increased 7.5-fold and 3.2-fold in 1- and 3-week-exposed groups ($P < 0.05$), but levels were not significantly elevated after 6 weeks (+2.3-fold; Figure 2E). MIF levels were elevated 2.0-fold and 1.6-fold in mice exposed to ozone for 1 and 3 weeks ($P < 0.05$). MIF levels were not significantly elevated in mice exposed to ozone for 6 weeks (+1.3-fold; Figure 2F). TNF- α levels were elevated 4.2- and 2.2-fold after 1 and 3 weeks of ozone exposure respectively ($P < 0.05$), but re-

turned to normal after 6 weeks of ozone exposure (+1.4-fold; Figure 2G). IL-6 levels were elevated 3.9-, 1.9- and 2.0-fold in the 1-week-, 3-week- and 6-week-exposed groups respectively ($P < 0.05$; Figure 2H). IL-17 levels were elevated 3.3-fold, 2.5-fold and 1.8-fold by 1, 3 and 6 weeks of ozone exposure respectively ($P < 0.05$; Figure 2I). These elevated levels of inflammatory mediators and cells in BALF indicate the presence of a persisting and stable inflammatory environment in lungs of ozone-exposed mice.

Ozone enhances mRNA expression of inflammatory genes

The increases in BAL protein levels were matched by changes in mRNA expression within the lung. The expression levels of *Kc*, *Mif*, *Tnfa* and *Il6* mRNA were elevated in 1-week-exposed mice (Figure 2J–2O). The expression of *Kc* and *Mif* mRNA remained elevated for up to 6 weeks of ozone exposure ($P < 0.05$; Figures 2J and 2L) whereas that of *Tnfa* and *Il6* returned to control levels (Figures 2M and 2N). In contrast, *Ifng* and *Il17* mRNA levels were elevated in mice exposed to ozone for 3 and 6 weeks ($P < 0.05$; Figures 2K and 2O).

Ozone modulates lung antioxidant responses

SODs convert superoxide radicals into hydrogen peroxide and play a role in the protection against oxidant damage in the lung. Lung SOD activity levels (Figure 3A) were significantly elevated in 1-week (1.8-fold increase, $P < 0.05$) and 3-week (1.5-fold increase, $P < 0.05$) ozone-exposed groups, but not in 6-week exposed animals (1.2-fold increase), indicating the presence of

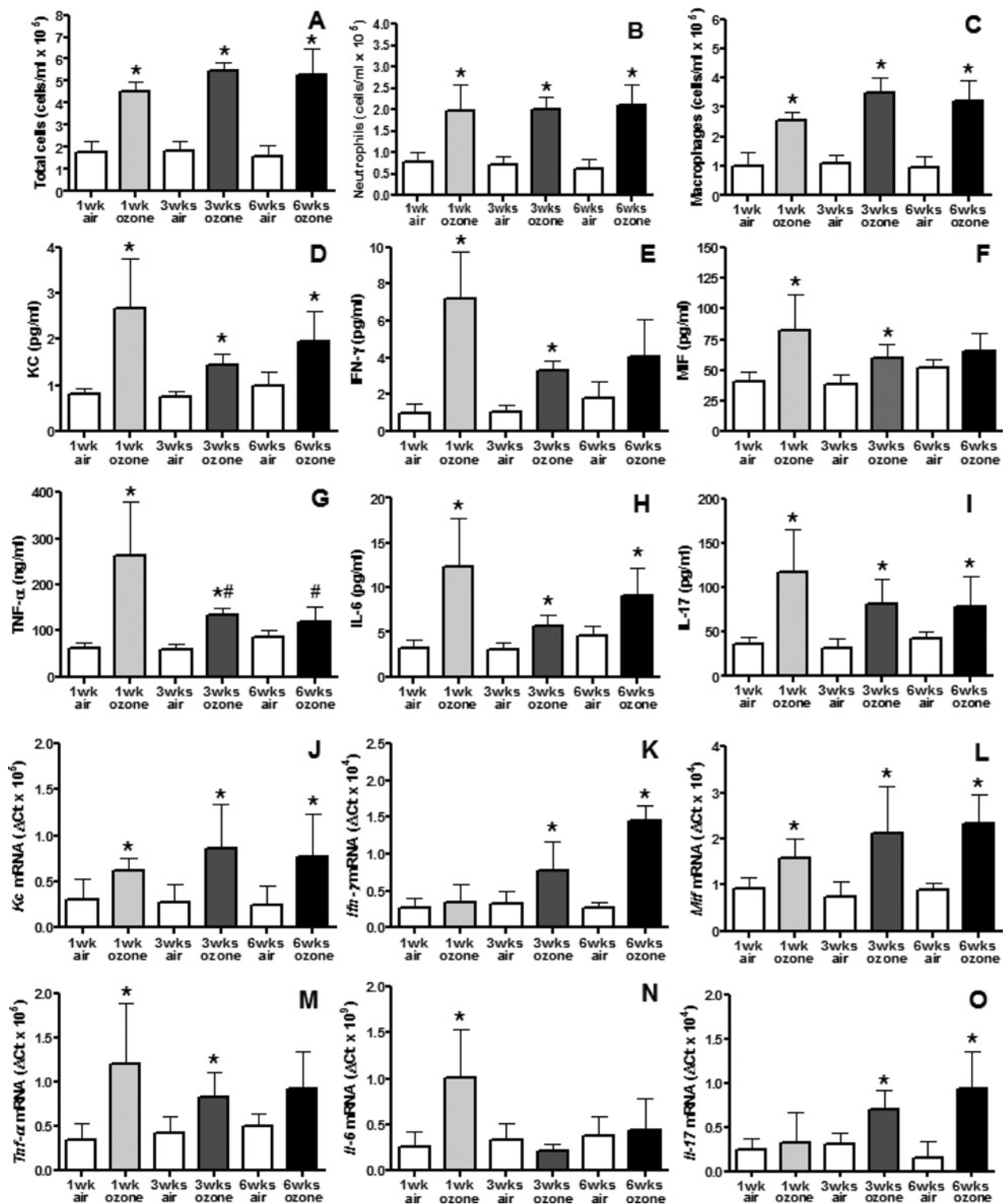


Figure 2 Inflammatory responses in BALF

Total inflammatory cell count (**A**), neutrophil numbers (**B**) and macrophage counts (**C**) in BALF of mice-exposed ozone for 1 week (grey bars), 3 weeks (dark grey bars) and 6 weeks (black bars) compared with air-exposed control groups (white bars) are shown. The expression of the inflammatory mediators KC (**D**), IFN γ (**E**), MIF (**F**), TNF α (**G**), IL-6 (**H**), IL-17 (**I**) in BALF are presented as mean $\pm$ S.D., $n = 8$. The mRNA expression of Kc (**J**), *Ifng* (**K**), *Mif* (**L**), *Tnf* (**M**), *Il6* (**N**) and *Il17* (**O**) in the lungs of mice are presented as means $\pm$ S.D. of $n = 8$ animals as determined by the ΔC_t method with the level of 18S mRNA used as control. * $P < 0.05$ compared with air control; # $P < 0.05$ compared with 1-week ozone-exposed group, Mann-Whitney test.

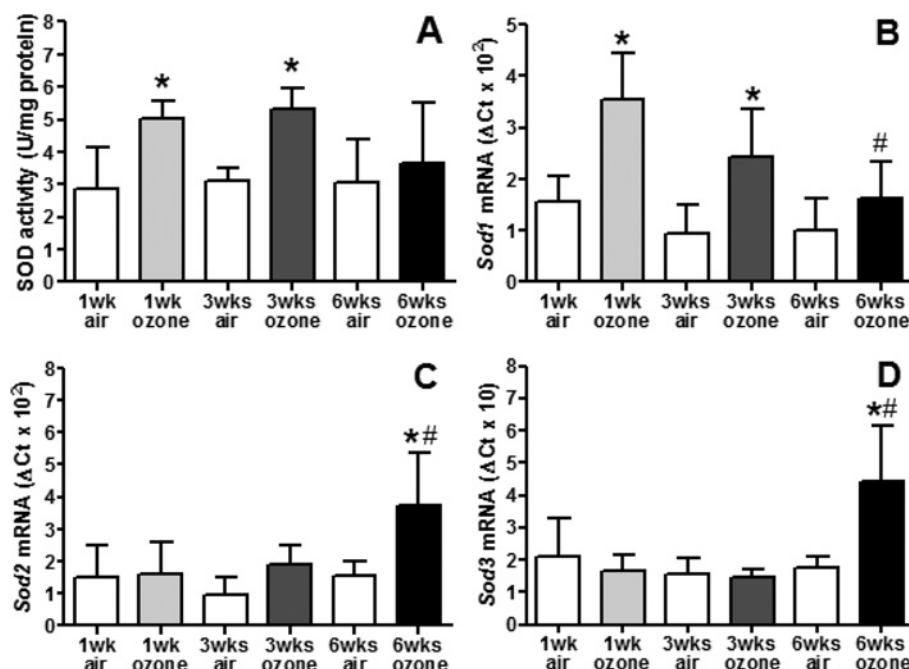


Figure 3 Effect of ozone exposure on SOD activity and mRNA levels

SOD activity levels in mouse lung protein extracts (A) and mRNA expression levels of *Sod1* (B), *Sod2* (C) and *Sod3* (D) genes in lungs of mice exposed to ozone for 1 week (grey bars), 3 weeks (dark grey bars) and 6 weeks (black bars) compared with air control groups (white bars). Results are presented as means $\pm$ S.D., $n=8$. * $P < 0.05$ compared with air control; # $P < 0.05$ compared with 1-week ozone-exposed group, Mann-Whitney test.

an antioxidant response in the lung. Elevated *Sod1* mRNA expression levels were observed after 1 week (2.3-fold increase, $P < 0.05$) and after 3 weeks (2.6-fold increase, $P < 0.05$), but not after 6 weeks (1.6-fold increase) of ozone exposure (Figure 3B). In contrast, *Sod2* and *Sod3* mRNA levels were only elevated in lung tissue after 6 weeks of ozone exposure (2.4-fold and 2.5-fold respectively, $P < 0.05$; Figures 3C and 3D).

Ozone exposure is associated with a loss of Nrf2 activation

Nrf2 is another key antioxidant molecule. Nuclear Nrf2 activity (DNA binding) was increased 3.2-fold in 1-week ozone-exposed mice ($P < 0.05$, Figure 4A). Nuclear Nrf2 protein levels were also elevated in the same group of mice (2.0-fold, $P < 0.05$; Figure 4B). In contrast, Nrf2 activity and nuclear protein expression levels were not elevated after 3 and 6 weeks of ozone exposure. Indeed, Nrf2 nuclear protein was decreased compared with air controls after 3 weeks of ozone exposure (1.8-fold) and after 6 weeks of ozone exposure (3.0-fold, $P < 0.05$). This loss of Nrf2 activity and protein in the nuclear fraction was associated with elevated cytoplasmic levels of Nrf2 (3.1-fold increase, $P < 0.05$; Figure 4C), no change in nuclear Keap1 (kelch-like ECH-associated protein 1) (Figure 4D) and a 2.3-fold increase in cytoplasmic Keap1 protein ($P < 0.05$; Figure 4E) in 6-week-exposed mice. This initial enhancement of Nrf2 activity at 1-week ozone exposure and loss of induction after 3 and 6 weeks was mimicked by the expression of Nrf2-inducible genes. *Gpx1* (glutathione peroxidase 1) and *Txnrd1* (thioredoxin reductase 1) mRNA expression levels were elevated in 1-week ozone-exposed

mice only (3.6-fold and 3.1-fold respectively, $P < 0.05$; Figures 4F and 4G).

In contrast, the expression levels of *Nrf2* and *Ho1* (haem oxygenase 1) mRNA were elevated after 3 and 6 weeks of ozone exposure (Figures 4H and 4I). *Keap1* mRNA levels were only elevated in mice exposed to ozone for 6 weeks (Figure 4J) similar to that observed for Keap1 protein levels (Figure 4E).

Ozone exposure increases HIF-1 α protein and DNA-binding activity levels

In contrast with the loss of Nrf2 activity in the lungs of mice following prolonged ozone exposure, nuclear and cytoplasmic HIF-1 α protein levels were increased in mice exposed to ozone for 3 and 6 weeks. Nuclear protein expression was elevated 1.6-fold and 1.8-fold in the 3- and 6-week-exposed groups respectively compared with air controls ($P < 0.05$; Figure 5A). Compared with 1-week-ozone-exposed mice, HIF-1 α protein levels were also elevated in the cytoplasm fractions after 3 and 6 weeks of ozone (1.7-fold and 1.5-fold increase, $P < 0.05$; Figure 5B). No differences in HIF-1 α expression was observed after 1 week of ozone exposure. These changes in protein levels were reflected by HIF-1 α activity levels. Nuclear DNA-binding activity was elevated in mice exposed to ozone after 6 weeks (2.1-fold increase, $P < 0.05$; Figure 5C). Cytoplasmic activity levels were increased after 3 and 6 weeks of ozone exposure with a significant 1.4-fold increase in both groups ($P < 0.05$; Figure 5D).

To investigate whether these changes in protein and activity levels occurred in response to alterations in gene expression, the

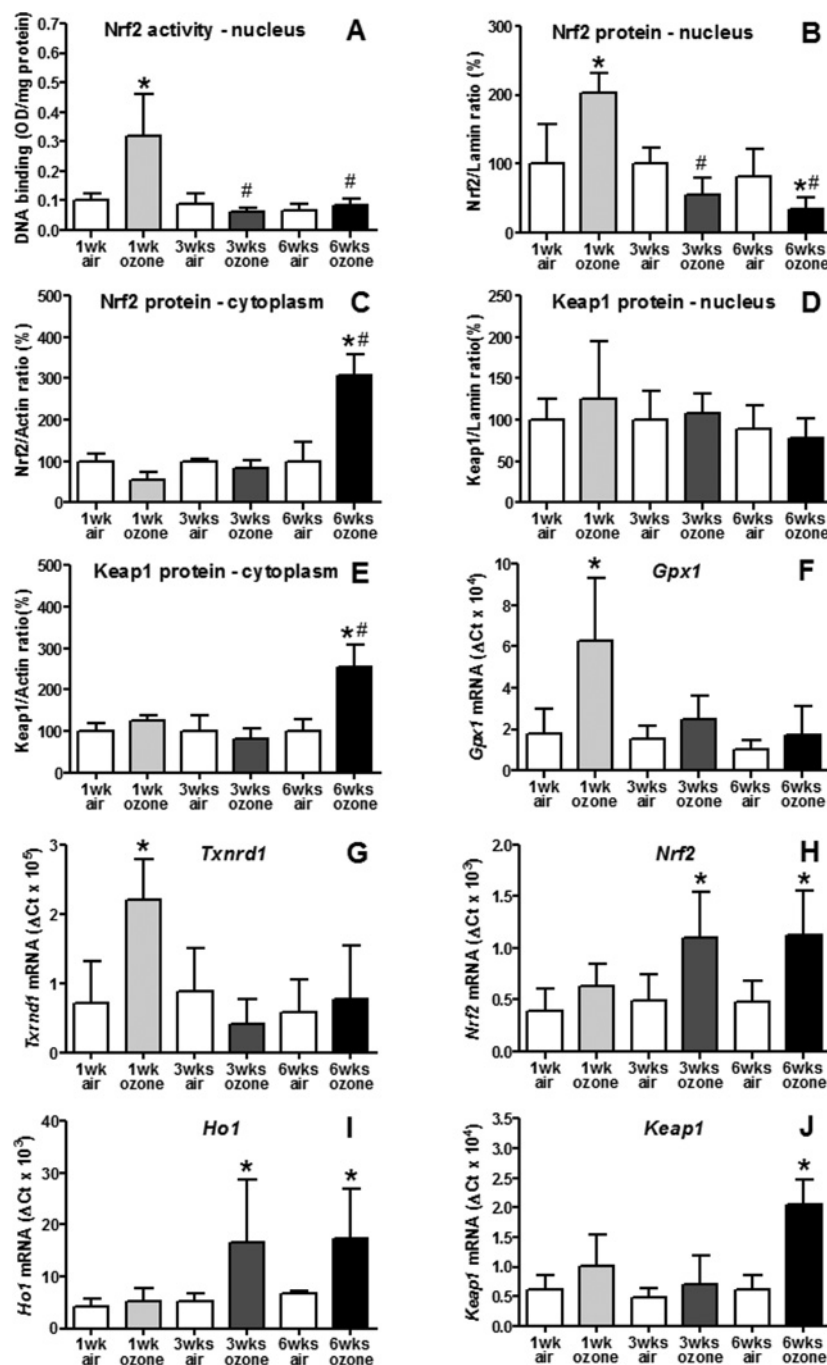


Figure 4 Effect of ozone exposure on Nrf2 protein and gene expression, and Nrf2-induced genes

Nuclear Nrf2 activity (A) and Nrf2 protein (B) expression in lung tissue from mice exposed to ozone for 1 week (grey bars), 3 weeks (dark grey bars) and 6 weeks (black bars) compared with air control groups (white bars). Results are presented as means $\pm$ S.D., $n = 8$. The levels of cytoplasmic Nrf2 protein (C), nuclear Keap1 (D), and cytoplasmic Keap1 protein (E) levels are also shown. Increased expression of the Nrf2-induced Gpx1 mRNA (F) and Txnrd1 mRNA (G) after 1 week of ozone exposure are shown. The effect of ozone exposure on Nrf2 (H), Ho1 (I) and Keap1 (J) mRNA in lung tissue is also shown. * $P < 0.05$ compared with air control; # $P < 0.05$ compared with 1-week ozone-exposed group, Mann-Whitney test.

mRNA levels of the HIFs were determined. The expression of *Hif1a* and *Hif2a* mRNAs were elevated in 3-week and 6-week-exposed groups (Figures 5E and 5F), whereas those of *Hif1b* and *Hif2b* were not affected in any of the exposed groups (results not shown). We also examined the mRNA expression levels

of the PHD (prolyl hydroxylase) genes, which are classically involved in the HIF-1 α degradation pathway. *Phd2* expression levels were decreased in all ozone-exposed groups (Figure 5G), whereas *Phd1* and *Phd3* mRNA levels were not affected by ozone exposure (results not shown). The expression of *Fih* (factor

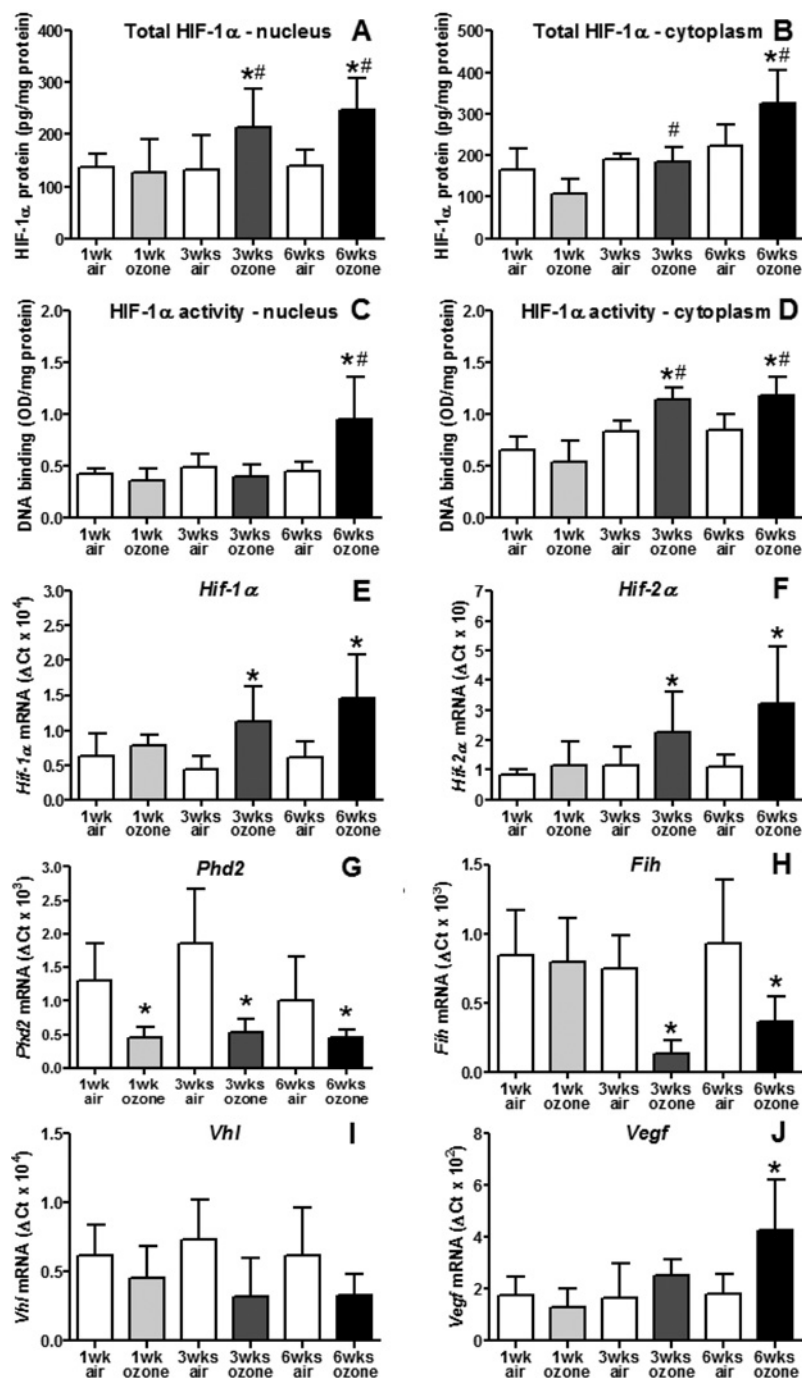


Figure 5 Effect of ozone exposure on HIF-1α protein and DNA-binding activity levels

Total HIF-1α protein in nuclear fraction (A) and cytoplasm fraction (B) and HIF-1α activity levels in nuclear fraction (C) and cytoplasm fraction (D). mRNA expression levels of *Hif1a* (E), *Hif2a* (F), *Phd2* (G), *Fih* (H), *Vhl* (I) and *Vegf* (J) in lung tissue from mice exposed to ozone for 1 week (grey bars), 3 weeks (dark grey bars) and 6 weeks (black bars) compared with air control groups (white bars). Results are presented as means ± S.D., $n = 8$. * $P < 0.05$ compared with air control; # $P < 0.05$ compared with the 1-week ozone-exposed group, Mann-Whitney test.

inhibiting HIF-1α mRNA was decreased in mice exposed to ozone for 3 and 6 weeks ($P < 0.05$; Figure 5H). In addition, the expression levels of *Vhl* (von Hippel-Lindau) mRNA were decreased in ozone-exposed mice, but this effect did not reach significance (Figure 5I).

Finally, the expression of HIF-1α target genes [genes containing HRE (hypoxia response element) sites in the promoter region] such as *Vegf* (vascular endothelial growth factor) (Figure 5J) and others, including *Hdac2* (Figure 8G), *Keap1* (Figure 4J) and *Mif* (Figure 2L), were all modulated in the 6-week ozone-exposed

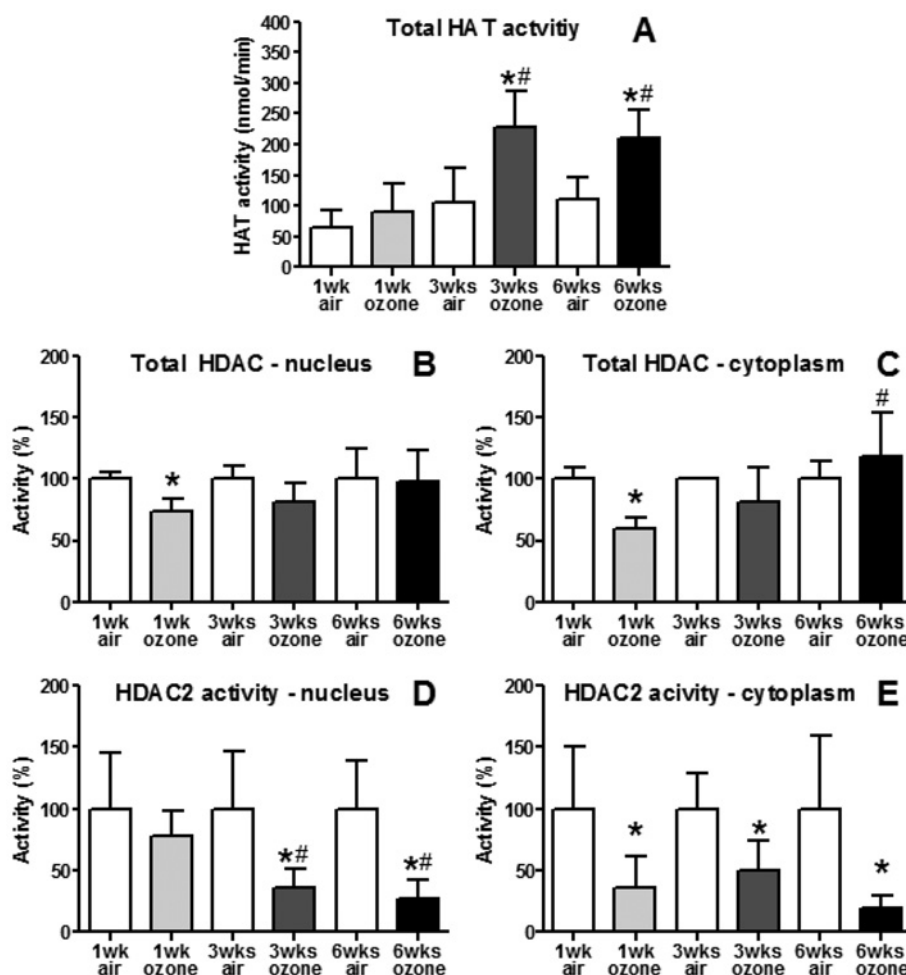


Figure 6 Effect of ozone exposure on HAT and HDAC activity

Total nuclear HAT activity (A), total nuclear and cytoplasmic HDAC activity (B and C) and nuclear and cytoplasmic HDAC2 activity levels (D and E) in lung tissue from mice exposed to ozone for 1 week (grey bars), 3 weeks (dark grey bars) and 6 weeks (black bars) compared with air control groups (white bars). Results are presented as means $\pm$ S.D., $n = 8$. * $P < 0.05$ compared with air control; # $P < 0.05$ compared with the 1-week ozone-exposed group, Mann-Whitney test.

mice, suggesting that HIF-1 α might be able to influence their expression pattern.

Ozone exposure induced HAT activity after 3 and 6 weeks

Having demonstrated that altered gene transcription of key mediators was evident after ozone exposure we measured the expression and activity of specific HAT and HDAC proteins, which control inflammatory gene transcription. Nuclear HAT activity levels were elevated in lung extracts of mice exposed to ozone for 3 weeks (2.1-fold, $P < 0.05$) and 6 weeks (1.9-fold, $P < 0.05$). No significant change was observed between air controls and mice exposed to ozone after 1 week of ozone exposure (Figure 6A).

Ozone exposure differentially modulates total HDAC and HDAC2 activity

Total nuclear HDAC activity levels were decreased in 1-week ozone-exposed mice (1.4-fold, $P < 0.05$) and returned to normal

levels after 3 weeks (1.2-fold) and 6 weeks (1.0-fold) compared with air-exposed mice (Figure 6B). Total HDAC activity levels in the cytoplasmic fraction showed a similar pattern with a decrease after 1 week of ozone exposure (1.7-fold, $P < 0.05$), but no significant changes after 3 weeks (1.2-fold) and 6 weeks (0.8-fold) of ozone exposure (Figure 6C).

In contrast, nuclear HDAC2 activity levels were decreased in all ozone-exposed groups with a 1.3-fold, 2.8-fold and 3.6-fold reduction after 1-, 3- and 6-weeks of ozone exposure respectively ($P < 0.05$; Figure 6D). Similar results were observed in the cytoplasmic fraction where HDAC2 activity levels were decreased 2.8-fold, 2.0-fold and 5.3-fold in the 1-, 3- and 6-week-exposed groups respectively ($P < 0.05$; Figure 6E). These data suggest that alterations in HDAC2 expression induced by impaired antioxidant responses to ozone may regulate inflammatory gene expression in this model of COPD. We therefore examined the mechanism(s) by which oxidative stress could modulate HDAC2 function.

Ozone exposure reduces nuclear and cytosolic HDAC2 expression

Total nuclear (Figures 7A and 7C) and cytoplasmic (Figures 7B and 7D) HDAC2 protein levels were reduced in the lung tissue of ozone-exposed mice at each time point studied. Nuclear HDAC2 protein levels were decreased 5.0-, 27.2- and 8.3-fold in the 1-week-, 3-week- and 6-week-exposed groups respectively ($P < 0.05$; Figure 7C) and cytoplasmic HDAC2 protein levels decreased by 2.1-, 2.4- and 1.9-fold in the 1-week-, 3-week- and 6-week-exposed groups respectively ($P < 0.05$; Figure 7D).

The presence of the HDAC2 degradation product (~36 kDa) increased in intensity in concordance with increased duration of ozone exposure. A nuclear HDAC2 degradation band was visible after 1 week of ozone exposure (1.6-fold) compared with controls. This increase in the HDAC2 degradation product reached significance after 3 (6.3-fold increase) and 6 (5.6-fold increase) weeks of ozone exposure (both $P < 0.05$; Figures 7A and 7E). This degradation band was also visible in the cytoplasmic fraction of lung from mice exposed to ozone for 6 weeks (2.3-fold increase, $P < 0.05$; Figures 7B and 7F).

Ozone exposure alters HDAC2 phosphorylation status

Phosphorylation of HDAC2 on Ser³⁹⁴ is associated with increased HDAC2 degradation and so we examined whether ozone affected HDAC2 phosphorylation status in murine lungs. pHDAC2 (phosphorylated HDAC2) levels were significantly elevated in the nuclear fractions of 3-week (5.1-fold, $P < 0.05$) and 6-week (5.9-fold, $P < 0.05$) ozone-exposed mice, but not after 1 week of ozone exposure (0.8-fold) (Figures 7A and 7G). In addition, cytoplasmic pHDAC2 levels were not different between the air and ozone-exposed groups at any of the time points studied (Figures 7A and 7H). This suggests that although HDAC2 phosphorylation may be associated with loss of nuclear HDAC2 expression after 3- and 6-weeks of ozone exposure it cannot account for the loss of nuclear HDAC2 after 1 week of ozone exposure nor for the loss of cytoplasmic HDAC2 expression.

Ozone exposure increases cytoplasmic HDAC2 carbonylation levels

HDAC2 carbonylation has also been associated with HDAC2 degradation. Total protein carbonylation levels in nuclear, but not cytoplasmic, fractions were significantly elevated in mice exposed to ozone for 1 and 3 weeks (1.4-fold and 1.3-fold, respectively $P < 0.05$; Figures 8A and 8C). This enhanced carbonylation was lost after exposure to ozone for 6 weeks (Figure 8E). In contrast, HDAC2 protein carbonylation was elevated in the cytoplasm, but not the nucleus, of ozone-exposed mice at 1 (2.5-fold increase, $P < 0.05$; Figure 8B) and 3 weeks (1.8-fold increase, $P < 0.05$; Figure 8D). No differences in cytoplasmic HDAC2 carbonylation levels were observed after 6 weeks of ozone exposure (Figure 8F). In contrast with the effect of ozone on HDAC2 protein expression, *Hdac2* mRNA levels were elevated in mice exposed to ozone for 3 and 6 weeks (Figure 8G, $P < 0.05$) possibly in response to HIF-1 α (see earlier).

Impaired activation of the Nrf2 pathway in PBMC isolated from COPD patients

To investigate the effects of oxidative stress in an *ex vivo* setting, we stimulated PBMCs isolated from healthy volunteers and COPD patients with LPS/H₂O₂. COPD patients were older compared with healthy controls, but this was not significant, had lower FEV₁ (forced expiratory volume in 1 s) values and FEV₁/FVC (forced vital capacity) ratio, indicating decreased lung function (Table 1). The effect of the Nrf2 pathway activator sulforaphane on oxidative stress-induced CXCL8 secretion was measured (Figure 9). LPS/H₂O₂ induced CXCL8 secretion in PBMCs from both healthy and COPD patients to a similar extent (5.1 and 4.9-fold respectively, $P < 0.05$; Figures 9A and 9B). Sulforaphane significantly reduced CXCL8 secretion in healthy PBMCs at all of the concentrations investigated. In contrast, sulforaphane had an attenuated ability to reduce CXCL8 secretion from PBMCs from COPD patients indicated by a rightward shift in the concentration–response curve. This suggests that the Nrf2 pathway is compromised in PBMCs isolated from COPD patients. This result is in line with the observed effects of chronic ozone exposure in mice, where the Nrf2 pathway is defective after 3- and 6-weeks of ozone exposure and confirms the previous data in COPD patients [22].

DISCUSSION

In the present study, we have investigated the ozone exposure mouse model in detail. We show that acute and chronic ozone exposure results in a persistent inflammatory response including neutrophilia with elevated levels of both cytokine and chemokine mRNA and protein in lung and BAL, extending our previous data [12,28,29]. This was accompanied by an increase in alveolar destruction. Prolonged ozone exposure was associated with a change in the antioxidant response. A clear antioxidant response was evident at 1 week as measured by an enhanced SOD and Nrf2 response and the expression of Nrf2-responsive genes and proteins. However, by weeks 3 and 6 of ozone exposure, SOD and Nrf2 activation was lost and there was a predominant HIF-1 α response associated with the regulation of HIF-1 α -responsive genes. Ozone exposure led to a loss of HDAC2 protein expression, which was associated, in part, with differential effects on HDAC2 phosphorylation and carbonylation status in the nuclear and cytoplasmic compartments. Overall, the loss in antioxidant response and elevated HIF-1 α resulted in reduced HDAC2 expression and activity that was associated with an enhanced inflammatory response.

We described previously changes in lung morphology in chronic ozone-exposed mice [12]. Ozone exposure for 6 weeks induces alveolar space enlargement with increased lung volume, alveolar space and L_m . In the present study, we confirm our previous findings that ozone exposure increased inflammation in the lung and induced characteristics associated with emphysemic changes in the mouse lung.

The persistent degree of neutrophilic inflammation in the lungs was associated with elevated SOD activity levels after 1 and 3 weeks but not after 6 weeks of ozone exposure. SODs

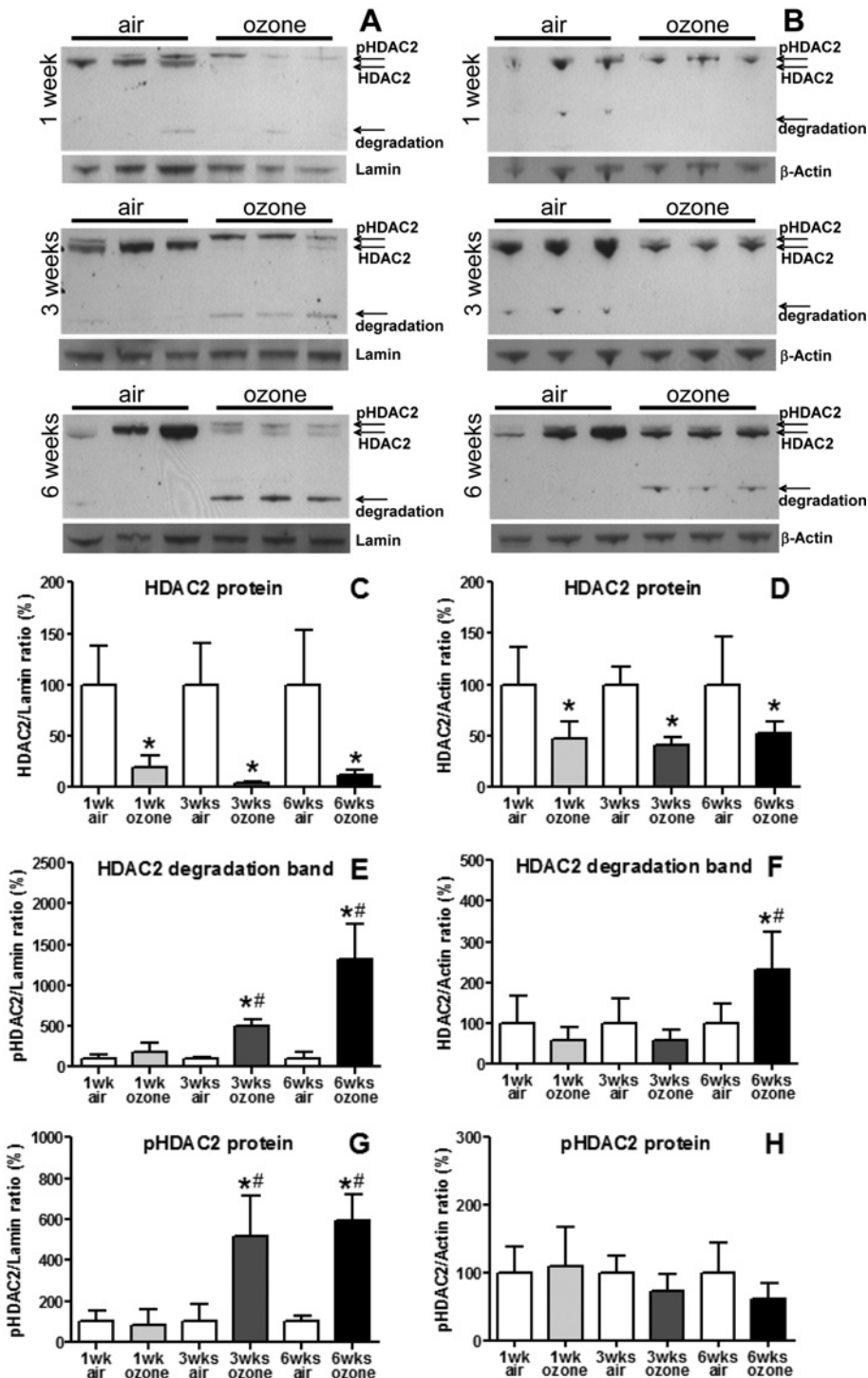


Figure 7 Effect of ozone exposure on nuclear and cytosolic HDAC2 expression

Representative Western blot analysis of HDAC2 and pHDAC2 in the nuclear fraction (A) and cytoplasmic fraction (B) of lung tissue from mice exposed to ozone for 6 weeks. The HDAC2 degradation band (~36 kDa) is clearly visible in both the nuclear and cytoplasmic fractions. Lamin and β-actin are shown as loading controls for nuclear and cytoplasm fractions, respectively. Data are representative of eight animals in each group. Densitometric analysis of Western blot data showing the expression of nuclear (C) and cytoplasmic (D) HDAC2, nuclear (E) and cytoplasmic (F) HDAC2 degradation and nuclear (G) and cytoplasmic pHDAC2 (H) after 1 week (grey bars), 3 weeks (dark grey bars) and 6 weeks (black bars) compared with air control groups (white bars). Results are presented as means ± S.D., $n = 8$. * $P < 0.05$ compared with air control; # $P < 0.05$ compared with the 1-week ozone-exposed group, Mann–Whitney test.

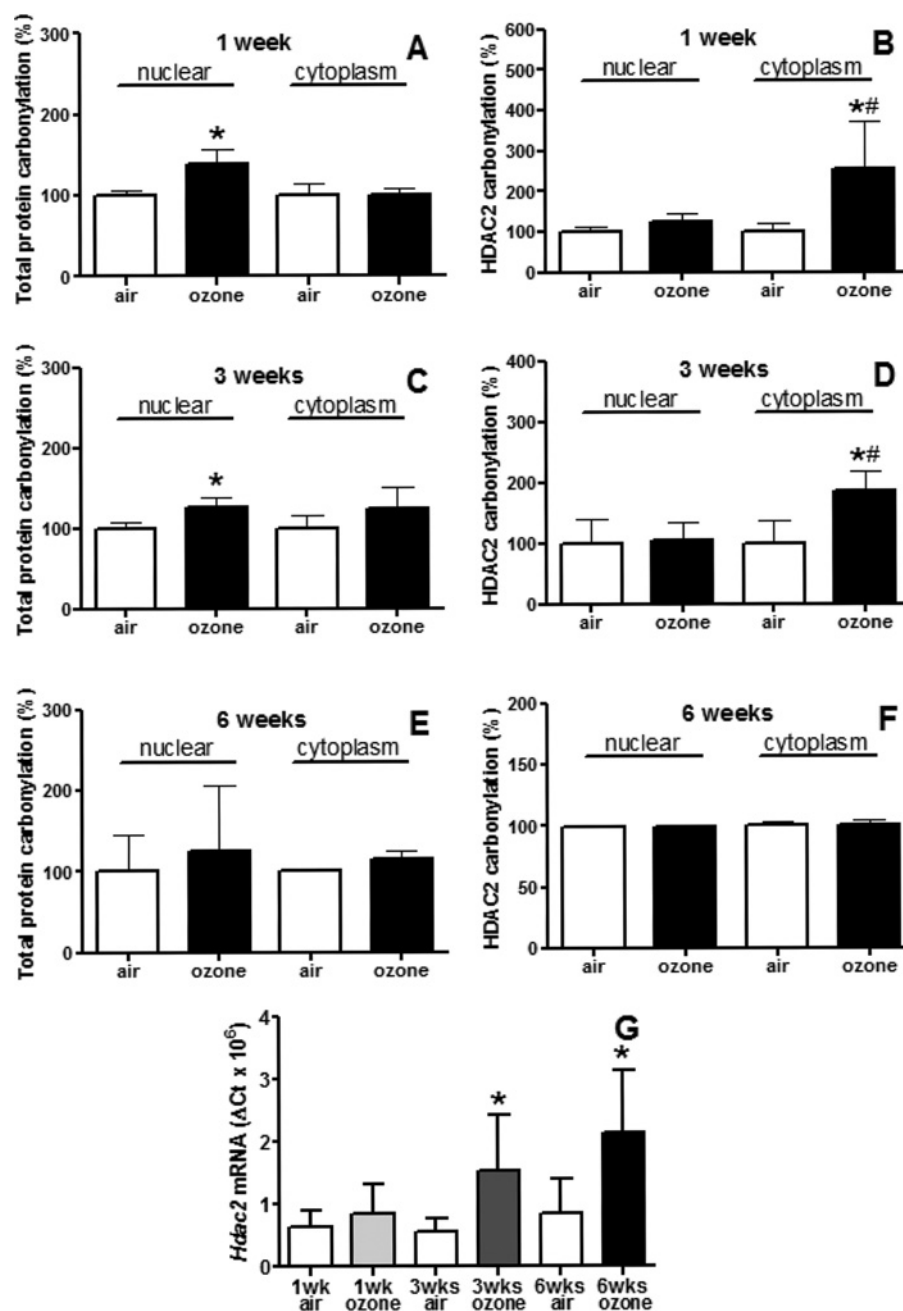


Figure 8 Effect of ozone exposure on cytoplasmic HDAC2 carbonylation levels

Total protein carbonylation in lung tissue of mice exposed to ozone for 1 week (A), 3 weeks (C) and 6 weeks (E), HDAC2 carbonylation in lung tissue of mice exposed to ozone for 1 week (B), 3 weeks (D) and 6 weeks (F) and *Hdac2* mRNA expression (G). Results are presented as means $\pm$ S.D., $n = 8$. * $P < 0.05$ compared with air control; # $P < 0.05$ compared with the 1-week ozone-exposed group, Mann-Whitney test.

convert superoxide radicals into H_2O_2 and are thought to play a role in the protection against oxidant stress in the lung [30]. There are three different mammalian SODs: intracellular Cu,ZnSOD (copper/zinc SOD) [31], mitochondrial MnSOD (manganese SOD) [32] and extracellular SOD [33] and these are encoded by *SOD1*, *SOD2* and *SOD3* genes respectively. The expression of *Sod1* mRNA was elevated in mice exposed to 1 and 3 weeks of ozone, mimicking changes in the activity levels. In contrast, *Sod2*

and *Sod3* expression levels were only elevated in 6-week ozone-exposed mice. Elevated SOD activity levels have been reported in other murine lung inflammation models, including acute cigarette smoke exposure [34,35] and ovalbumin exposure [36], but not LPS exposure [35], indicating that this antioxidant mechanism is present and activated during chronic ozone exposure.

Nrf2 and HIF-1 α are both abnormally regulated in COPD. Low levels of Nrf2 protein and function have been observed

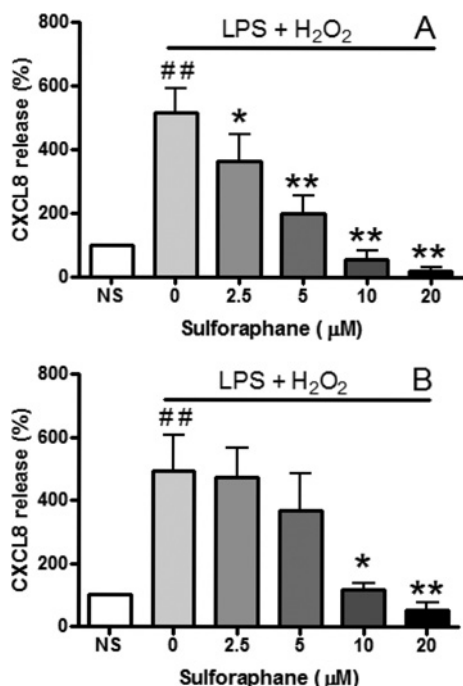


Figure 9 Effects of the Nrf2 pathway activator sulforaphane on oxidative stress-induced CXCL8 secretion from PBMCs isolated from five healthy subjects (A) and five COPD patients (B)

Results are presented as means $\pm$ S.D. * $P < 0.05$ and ** $P < 0.01$ compared with 0 μ M treatment group; ## $P < 0.01$ compared with non-stimulated cells, Mann-Whitney test.

in COPD patients [22,37] and associated with emphysema in smokers [38]. This decline of Nrf2 pathway affects the transcription of genes involved in the response to oxidative stress [38]. In accordance with the acute (1 week) SOD response, the antioxidant Nrf2 response was present at 1 week ozone-exposure but was reduced with prolonged ozone exposure and Nrf2 protein accumulated in the cytoplasmic fraction of the cell possibly under the regulation of elevated cytoplasmic Keap1 [39]. Alternatively, a decrease in HDAC2 levels and subsequently altered acetylation status of Nrf2 protein might also contribute to this loss of Nrf2 function [23].

HIF-1 α signalling has been associated with exacerbations in severe asthma [40] and was found up-regulated in pulmonary arteries [41] and bronchial epithelial cells in COPD patients [42]. Indeed, HIF-1 α signalling has been associated with goblet cell hyperplasia in COPD patients [43] indicating that this signal pathway might be involved in mucous hypersecretion. In addition, cigarette smoke-induced MUC5AC expression in human airway epithelial cell is HIF-1 α dependent [44]. Studies in other mouse models also report links between HIF-1 α signalling and allergic inflammation and airway remodelling. In the ovalbumin-sensitized mouse model of allergic asthma the combination of hypoxia and ovalbumin challenge induces HIF-1 α signalling and is associated with neutrophilia and cytokine expression [45]. In addition, ovalbumin-sensitized mice that are treated with the HIF-1 α activator EDHB (ethyl-3,4-dihydroxybenzoate) show an increased HIF-1 α pathway activation with increased CCL2 expression [46].

In the present study, HIF-1 α protein and activity levels were elevated after chronic (3 and 6 weeks), but not acute (1 week) ozone exposure. We identified *Hdac2* and *Keap1* as possible novel target genes of HIF-1 α in mice. The mouse *Hdac2* promoter region contains five HRE sites at -377 , -450 , -478 , -587 and -683 relative to the start codon. We have reported previously that deletions and point mutations in the promoter region of human *HDAC2* alters polymerase II binding to the promoter and subsequently HDAC2 expression [26]. We therefore postulated that HIF-1 α targets *Hdac2* mRNA expression and that this is able to alter gene and protein transcription levels *in vivo*. Furthermore, the mouse *Keap1* promoter region has six HRE sites (-238 , -255 , -269 , -370 , -399 and -490) which HIF-1 α potentially can bind to. Keap1 mRNA and protein expression levels were increased in chronic ozone-exposed mice similar to HIF-1 α . We hypothesize that HIF-1 α is able to activate *Keap1* gene and protein expression, resulting in a de-activated oxidative stress response seen in the form of Nrf2 capture in the cytoplasm. How the HIF-1 α signalling pathway affects these systems is currently being investigated.

Changes in inflammatory gene expression in the presence of oxidative stress are linked with altered histone acetylation status [47] and we report here that HAT activity levels are higher and HDAC2 expression and activity levels lower in mice exposed to ozone. HDAC2 protein modifications can result in increased degradation of the HDAC2 protein resulting in elevated inflammatory gene expression as reported in a smoking mouse model [20] and in human cell culture systems [18,48]. In the present study, we observed elevated phosphorylation of HDAC2 on Ser³⁹⁴ in ozone-exposed mice, which was associated with decreased total HDAC2 protein and activity levels and increased HDAC2 protein degradation as indicated by the ~ 36 kDa band observed by Western blotting. The effects of ozone exposure on HDAC2 phosphorylation was more profound in the nuclear fraction compared with the cytoplasm fraction and did not account for the loss of nuclear HDAC2 activity and expression after 1 week of ozone exposure. Therefore other mechanisms must exist to account for the early loss of HDAC2 expression.

Protein carbonylation is also able to alter target protein function and/or fate [49] and carbonylated proteins have been detected in lung tissues from patients with COPD [50]. HDAC2 carbonylation is also increased in the cytoplasm of mice exposed to ozone for 1 and 3 weeks. Carbonylation of HDAC1 on Cys²⁶¹ and Cys²⁷³ results in altered interactions with its binding partners and substrate histone but HDAC1 carbonylation does not result in loss of enzyme activity [19]. To our knowledge this is the first report describing carbonylation of the HDAC2 protein and future experiments are required to determine the effect of HDAC2 interaction with co-factors and substrates following ozone exposure.

HDAC2 modifications reported in the present study (phosphorylation and carbonylation) differed depending upon the intracellular localization. Class I HDACs, including HDAC2, are now known to shuttle between the cytoplasm and the nucleus [51] and distinct substrates for HDAC2 may exist in each compartment under the control of different post-translational modifications [51].

In contrast with the loss of HDAC2 protein, *Hdac2* mRNA expression levels were elevated in the same groups of mice. This suggests that a compensatory mechanism exists which is overcome by a greater degradation drive. More prolonged exposure may result in a persistent loss of *HDAC2* mRNA as observed in COPD [15].

Overall, exposure to ozone results in a persistent chronic inflammatory environment in the lung. This is associated with a loss of the antioxidant Nrf2 and SOD activities, enhanced intracellular oxidative stress and increased HIF-1 α signalling. We hypothesize that HIF-1 α target genes are affected by this switch in signalling and, along with reduced HDAC2 activity, contribute to the persistent inflammatory response and the pathogenesis of emphysema observed after chronic ozone exposure.

CLINICAL PERSPECTIVES

- Oxidative stress is linked to various chronic inflammation disorders. The role of oxidative stress in the aetiology, pathophysiology and disease progression of respiratory diseases such as severe asthma and COPD are now under extensive investigation.
- Chronic ozone exposure in mice results in increased inflammation, oxidative stress, airway remodelling and alveolar destruction. These changes are associated with an impaired antioxidant stress response and the activation of the HIF-1 α pathway, which contributes to the pathophysiological changes observed.
- Murine models of airway oxidative stress provide a means to better understand the molecular mechanisms and pathways involved in airway inflammation and enable the identification of potential new therapeutic targets in these diseases.

AUTHOR CONTRIBUTION

Coen Wiegman and Feng Li performed the *in vivo* experiments and analysis. Colin Clarke assisted in the *in vivo* experiments. Elen Jazrawi performed the *ex vivo* experiments. Paul Kirkham, Peter Barnes, Ian Adcock and Kian Chung were involved in study design and proofreading of the paper prior to submission.

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