Original Research

Evaluation of the pulmonary effects of short-term nose-only cigarette smoke exposure in mice

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Abstract

Much is known about the chronic effects of cigarette smoke (CS) on lung function and inflammation and development of chronic obstructive pulmonary disease. However, the underlying pathophysiological mechanisms related to the short-term exposure to CS are not fully understood. Here, we assessed the effect of CS generated by nine consecutive cigarettes per day for four days in a nose-only exposure system on airway resistance measured using forced oscillation technique, lung inflammation and oxidative stress in BALB/c mice. Control mice were exposed to air. Mice exposed to CS showed a significant increase of neutrophils and lymphocytes numbers in bronchoalveolar lavage (BAL). The total protein and endothelin levels in BAL fluid were significantly augmented suggesting an increase of alveolar–capillary barrier permeability. Similarly, airway resistance was significantly increased in the CS group compared with controls. Furthermore, reactive oxygen species and lipid peroxidation levels in lung tissue were significantly increased. The antioxidant activities of reduced glutathione, glutathione *S* transferase and superoxide dismutase were all significantly increased following CS exposure, indicating that CS could trigger adaptive responses that counterbalance the potentially damaging activity of oxygen radicals induced by CS exposure. In conclusion, our data indicate that short-term nose-only exposure to CS causes lung inflammation and increase of airway resistance mediated at least partly through the oxidative stress.

Keywords: cigarette smoke, nose-only exposure, short-term exposure, airway resistance, oxidative stress

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Introduction

Chronic obstructive pulmonary disease (COPD) is a major and increasing health global issue. Presently, it is the fifth leading cause of death worldwide and is predicted to become the third leading cause of mortality by the year 2020.[1] COPD is characterized by an airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious gases and particles.[2]

COPD patients show evidence of increased release of reactive oxygen species (ROS) leading to oxidative stress. Moreover, inflammatory processes associated with the recruitment and activation of phagocytic cell types, namely neutrophils and mononuclear cells, may also play a role in generating endogenous oxidative stress.[3]

Experimental studies reported that short-term exposure to CS causes lung inflammation and oxidative stress.[4] It has been reported that the activity profiles of enzymes (superoxide dismutase [SOD], catalase and glutathione peroxidase) contributing to oxidant–antioxidant imbalance in the lungs are affected by CS exposure in mice[5] and that nitric oxide supplementation by administration of nitroglycerine or L-arginine was able to reduce CS-induced oxidative stress.[6] Moreover, Lagente *et al.*[7] have recently demonstrated that mice in which the $p47^{phox}$ subunit of NADPH oxidase has been deleted failed to develop airway inflammation after exposure to CS, suggesting that oxidative stress is involved in the pathogenesis of airway inflammation associated with COPD.

Several exposure systems are being used to study the effect CS exposure in mice.[8–10] The smoking machines that have been used with animal models include systems that use nose-only or whole-body exposures. The drawback of using whole-body exposure is that the animals

may ingest nicotine or tar substances when cleaning their fur. The nose-only exposure system avoids this problem and most likely best resembles the human situation.[9,10] When developing any smoke exposure model, measurement of either serum cotinine (a nicotine metabolite) or blood carboxyhemoglobin is useful as a method of confirming the relative amount of smoke exposure.[8] Wright *et al.*[8] reported in a guinea pig nose-only exposure model that acute postsmoke carboxyhemoglobin of 15–20% and a chronic blood carboxyhemoglobin level of ~5%, the latter is comparable with that seen in many human smokers. Hitherto, merely limited studies have used nose-only exposure systems to study the pulmonary and systemic effect of CS.[10–12] However, as far as we are aware, no study has combined comprehensively the assessment of pulmonary inflammation, oxidative stress and airway resistance after short-term exposure to CS using a relevant type of exposure system, namely nose-only exposure.

Considerable information has been reported on the effects of chronic CS exposure on lung function and airway inflammation. Indeed, numerous experimental studies have been performed to uncover the mechanisms leading to the development of emphysema. Such experiments necessitate chronic exposure to CS lasting for several months. However, there is a scarcity of data on the short-term and effects of CS smoke. One of the reasons why it is important to investigate this effect is because repetitive short-term effects of CS may constitute the underlying causal chain of reactions leading to the ultimate chronic effects.[4] Therefore, studying the short-term effect of CS can give more specific information and mirror the early changes in the pathophysiological mechanisms of CS-induced chronic lung diseases.

The hypothesis we wanted to test here is whether and to what extent would short-term nose-only exposure to CS affect airway resistance, inflammation and oxidative stress in mice. Therefore, the specific aim of this study is to investigate the effect of short-term nose-only CS exposure on a comprehensive set of respiratory indices including (1) lung inflammation assessed by bronchoalveolar lavage (BAL) fluid analysis (including cells, total proteins used as marker of epithelial permeability and endothelin, known to increase pulmonary vascular and airway tonus) and histology; (2) airway resistance measured invasively using forced oscillations technique; (3) pulmonary levels of reactive oxygen species, lipid peroxidation and antioxidant enzymes comprising reduced glutathione (GSH), glutathione *S* transferase (GST) and SOD.

Materials and methods

Animals and treatments

This project was reviewed and approved by the Institutional Review Board of the United Arab Emirates University, Faculty of Medicine and Health Sciences, and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

CS exposure

BALB/C mice (Taconic Farms Inc, Germantown, NY, USA) were housed in a conventional animal house and maintained on a 12-hour light–dark cycle. The animals were placed in cages and supplied with pelleted food and water *ad libitum*. Following one week of acclimatization, animals were randomly divided into the control and CS-exposed groups. Mice were placed in soft restraints and connected to the exposure tower. Animals were exposed to mainstream CS generated by commercially available filtered cigarettes (Marlboro Red, 12 mg tar/1.0 mg nicotine; Philip Morris, Richmond, VA, USA) through their noses using a nose-only exposure system (InExpose System, Scireq, Canada). A computer-controlled puff was generated every minute, leading to 10 s of CS exposure followed by 50 s of fresh air. The CS-exposed group inhaled CS from nine consecutive cigarettes per day for four days as previously reported by Vlahos *et al.*[13] who investigated the inflammatory effects of CS in mice and the role of neutralizing granulocyte/macrophage colony stimulating factor thereon. The CS challenge chosen in the present study was associated with a good tolerance of mice to the CS sessions, and an acceptable level of particulate density concentration according to literature.[10,13] Control animals were treated similarly and were exposed to filtered air for the same duration. The total particulate density concentration of CS was measured daily and indicated an average of 420.5 mg total particulate matter per m^3 (TPM/m^3) in the tower.

Airway reactivity to methacholine

In separate mice ($n = 7$ in each group), airway hyperreactivity responses were measured using a forced oscillation technique (FlexiVent, SCIREQ, Montreal, Canada). Airway resistance (R) was assessed after increasing exposures to methacholine. Mice were anesthetized with an intraperitoneal injection of pentobarbital (70 mg/kg). The trachea was exposed and an 18-gauge metal needle was inserted into the trachea. Mice were connected to a computer-controlled small animal ventilator and quasi-sinusoidally ventilated with a tidal volume of 10 mL/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H_2O to achieve a mean lung volume close to that during spontaneous breathing. After measurement of a baseline, each mouse was challenged with methacholine aerosol, generated with an in-line nebulizer and administered directly through the ventilator for five seconds, with increasing concentrations (0, 0.625, 1.25, 2.5 and 5 mg/mL). Airway resistance (R) was measured using a 'snapshot' protocol each 20 s for 2 min. The mean of these five values was used for each methacholine concentration, unless the coefficient of determination of a measurement was smaller than 0.95. For each mouse, R was plotted against methacholine concentration (from 0 to 5 mg/mL).[14]

Collection and analysis of BAL fluid

The collection and analysis of BAL has been performed according to a previously described method.[15–18] In brief,

in separate animals ($n = 8$), after CS or air exposure, the mice were killed with an overdose of sodium pentobarbital. The trachea was cannulated and lungs were lavaged three times with 0.7 mL (a total volume of 2.1 mL) of sterile NaCl 0.9%. The recovered fluid aliquots were pooled. No difference in the volume of collected fluid was observed between the different groups. BAL fluid was centrifuged (1000 *g* for10 min, 4°C). Cells were counted and the differentials were microscopically performed on cytocentrifuge preparations fixed in methanol and stained with Diff Quick (Dade, Brussels, Belgium). The supernatant was stored at – 80°C until further analysis for total proteins (kit from Biorad, München, Germany) and endothelin (kit from Cayman Chemical, Ann Arbor, MI, USA).

Histopathology and morphometric analysis

On the same animals used for BAL, after the lavage, the right lung was fixed with 10% formaldehyde.[14] Five-micrometer sections were prepared from right lung paraffin blocks and stained with hematoxylin and eosin. A histologist examined slices (5 μm sections) from all lung lobes and evaluated without knowledge of the animals' treatment. The degree of interstitial infiltration by inflammatory cells, in response to CS exposure, was evaluated by counting the number of inflammatory cells in the lung at high power field ($\times$100) as previously described.[16]

Measurement of oxidative stress, lipid peroxidation and antioxidant enzyme activities in lung tissue

In separate mice ($n = 8$ in each group), following the exposure to CS or air, animals were sacrificed by an overdose of sodium pentobarbital. Lung tissues from the air-exposed control and CS-treated mice were collected and rinsed with ice-cold phosphate-buffered saline (pH 7.4) before homogenization in 0.1 mol/L phosphate buffer pH 7.4 containing 0.15 mol/L KCl, 0.1 mmol/L EDTA, 1 mol/L dithiothreitol and 0.1 mmol/L phenylmethylsulfonylfluoride at 4°C. Homogenate was centrifuged for 10 min at 3000 *g* to remove cellular debris and supernatants were used for further analysis. Protein content was measured by Bradford's method as described before.[19,20]

Measurement of ROS and lipid peroxidation: ROS in lung tissue of the control and CS-exposed mice were measured using 2′,7′-dichlorofluorescein diacetate (DCFDA; Molecular Probes, Eugene, OR, USA) as a fluorescent probe as described before.[21,22] NADPH-dependent membrane lipid peroxidation was measured as thiobarbituric acid-reactive substance using malonedialdehyde as the standard (Sigma-Aldrich Fine Chemicals, St Louis, MO, USA).[22]

Measurement of GSH and GST: GSH and GST levels (Sigma-Aldrich Fine Chemicals) were measured in the tissues from the control and CS-exposed animals by standard procedures as described in previous publications.[22]

Measurement of SOD: SOD was measured as the conversion of NBT to NBT-diformazan according to the vendor's protocol (R&D System, Minneapolis, MN, USA). The extent of reduction in the appearance of NBT-formazan was used as a measure of SOD activity present in the experimental samples.

Statistics

All statistical analyses were performed with GraphPad Prism Software version 4. To determine whether parameters were normally distributed, the D'Agostino and Pearson normality test was applied. Data on lung inflammation and oxidative stress were normally distributed and were analyzed using the unpaired *t*-test for differences between groups. Airway hyper-reactivity to methacholine data was analyzed by repeated measures analysis of variance with Bonferroni's multiple comparison test. P values <0.05 were considered as significant. All the data in figures were reported as mean $\pm$ SD.

Results

Histology and morphometry

Sections of lung from air-exposed mice showed a normal appearance under light microscopy. Lung sections from CS-exposed mice showed the presence interstitial infiltration of inflammatory cells. The number of inflammatory cells per high power field ($\times$100) in the lung of CS-exposed mice was significantly higher compared with controls ($P < 0.0005$). The inflammatory cells consisted mainly of lymphocytes (Figure 1).

Cell composition and number in BAL fluid

Compared with the control group, short-term nose-only CS exposure caused a significant increase in neutrophil ($P < 0.05$) and lymphocyte ($P < 0.05$) numbers (Figure 2b and c). However, the number of macrophages was not affected by CS exposure (Figure 2a).

Total proteins and endothelin concentrations in BAL fluid

The total proteins, used as a marker of epithelial permeability, was significantly increased ($P < 0.05$) following short-term nose-only CS exposure compared with air-exposed mice (Figure 3a). Similarly, endothelin levels, known to increase pulmonary vascular and airway tonus, were also increased ($P < 0.005$) after short-term nose-only CS exposure (Figure 3b).

Airway hyper-reactivity to methacholine

Figure 4 shows airway resistance, measured by the forced oscillations technique after increasing concentrations of methacholine, following the short-term nose-only exposure to air or CS. The baseline of airway resistance in CS-exposed mice was significantly increased compared with air-exposed mice ($P < 0.001$), indicating the occurrence of bronchoconstriction as a consequence of CS exposure. Likewise, the airway resistance following methacholine administration was significantly and dose-dependently ($P < 0.001$ at all the studied concentrations, i.e. 0.625, 1.25, 2.5 and 5 mg/mL) increased in the CS group compared with the air-exposed group. These significant effects are related to the increase of the baseline of airway resistance

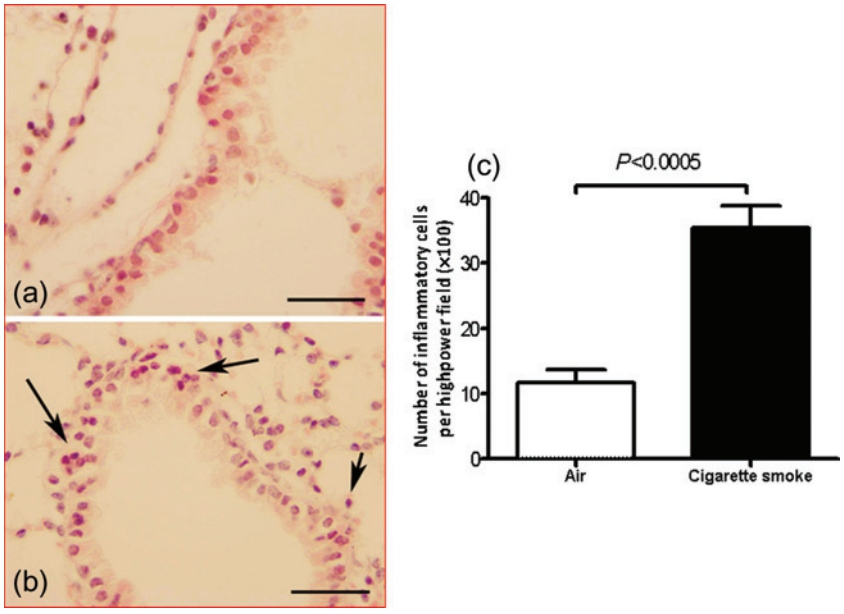

Figure 1 Representative light micrographs of lung tissues of mice following short-term nose-only air (control, a) or cigarette smoke (b) exposure. Morphometric analysis showing the number of inflammatory cells (c) following short-term nose-only air or cigarette smoke exposure. Note the presence of mainly lymphocytic inflammatory cells in the wall of terminal bronchiole (arrow). Scale bar = 25 μm. Data are mean $\pm$ SD ($n = 8$). (A color version of this figure is available in the online journal)

that resulted from CS exposure rather than the effect of methacholine *per se* because compared with their respective baselines, the degree of increase in airway resistance for the same concentration of methacholine is similar between air and CS-exposed mice.

Reactive oxygen species and lipid peroxidation levels in lung tissue

Short-term nose-only CS exposure induced a significant increase ($P < 0.005$) in the concentration of reactive oxygen species in lung tissue compared with the air-exposed group (Figure 5a). Similarly, lipid peroxidation levels were also significantly increased ($P < 0.005$) in CS-exposed compared with air-exposed mice (Figure 5b).

GSH concentration and GST and SOD activities in lung tissue

Figure 6 illustrates the effect of short-term nose-only CS exposure on the level of antioxidants in lung tissue including GSH level, GST and SOD. CS exposure caused a significant increase of GSH ($P < 0.0001$) in lung tissue compared with the control group (Figure 6a). The GST activity was also augmented ($P < 0.05$) in mice exposed to CS versus those exposed to air (Figure 6b). Similarly, the SOD activity was significantly increased ($P < 0.005$) in lung tissue of CS-exposed mice compared with the control (Figure 6c).

Discussion

The present work provides evidence that short-term nose-only exposure to CS induced pulmonary inflammation evidenced by influx of neutrophils and lymphocytes, and increased of epithelial permeliability. Both ROS and lipid peroxidation levels were increased in lung tissue. The antioxidant enzymes levels including GSH, GST and SOD were augmented by CS, suggesting the occurrence of an adaptive response against CS toxicity.

CS exposure is the most appropriate model to study emphysema in mice and several exposure systems are available.[8–10,23] Although a number of smoke-induced animal models of emphysema have been reported in the literature to investigate the pathogenesis of emphysema, the nose-only exposure system probably best mimics the human situation.[8–10,23] The novelty of our data is that we assessed the effect of short-term nose-only exposure to CS on comprehensive respiratory indices including, lung inflammation and oxidative stress, and their possible impact on a relevant physiological endpoint, namely airway resistance *in vivo*.

Here, we have assessed the short-term effect of CS exposure on airway reactivity to methacholine using forced oscillation technique. In spite of its invasiveness, evaluation of pulmonary and airway mechanics with forced oscillation technique provides precise information, and physiological variables can be measured accurately.[24,25] We have recently demonstrated that acute exposure to diesel exhaust particle causes lung inflammation and increases airway resistance in mice.[16] Our data show that the baseline of airway resistance in CS-exposed mice was significantly increased compared with air-exposed mice. Likewise, the airway resistance following methacholine administration was significantly and dose-dependently increased in the CS group compared with the air-exposed group. The number of infiltrating inflammatory cells in the lung assessed by morphometry, which consisted mainly in lymphocytes, was also significantly increased in CS-exposed

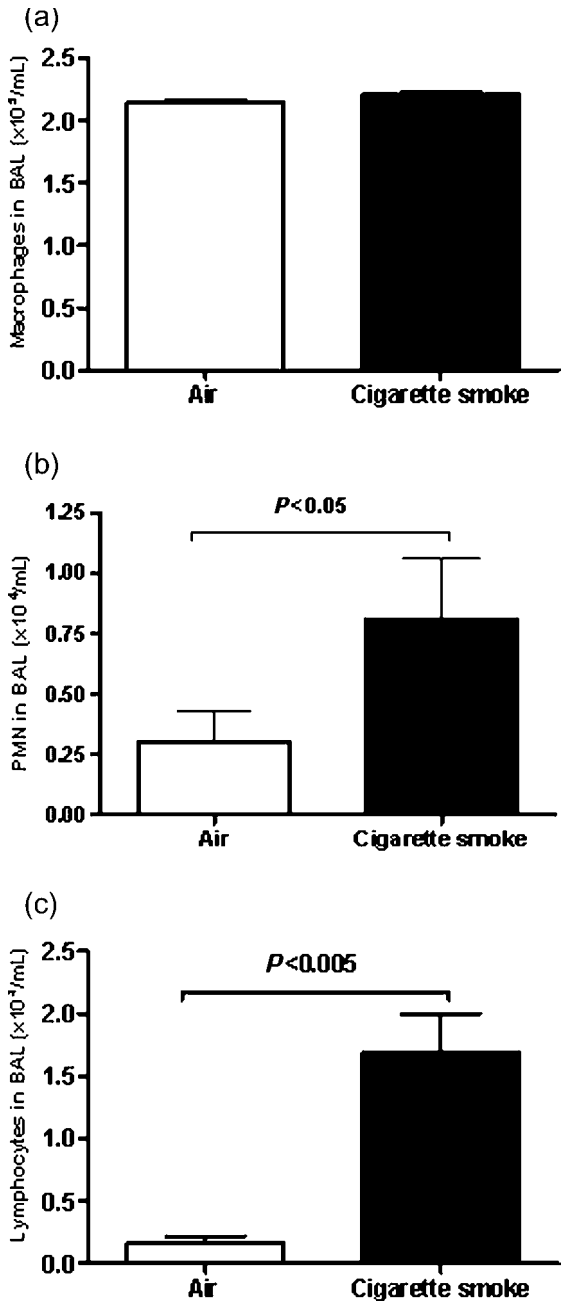

Figure 2 Number of macrophages (a), polymorphonuclear neutrophils (PMN) (b) and lymphocytes (c) in bronchoalveolar lavage (BAL) fluid, following short-term nose-only cigarette smoke exposure or air (control). Data are mean ± SD ($n = 8$)

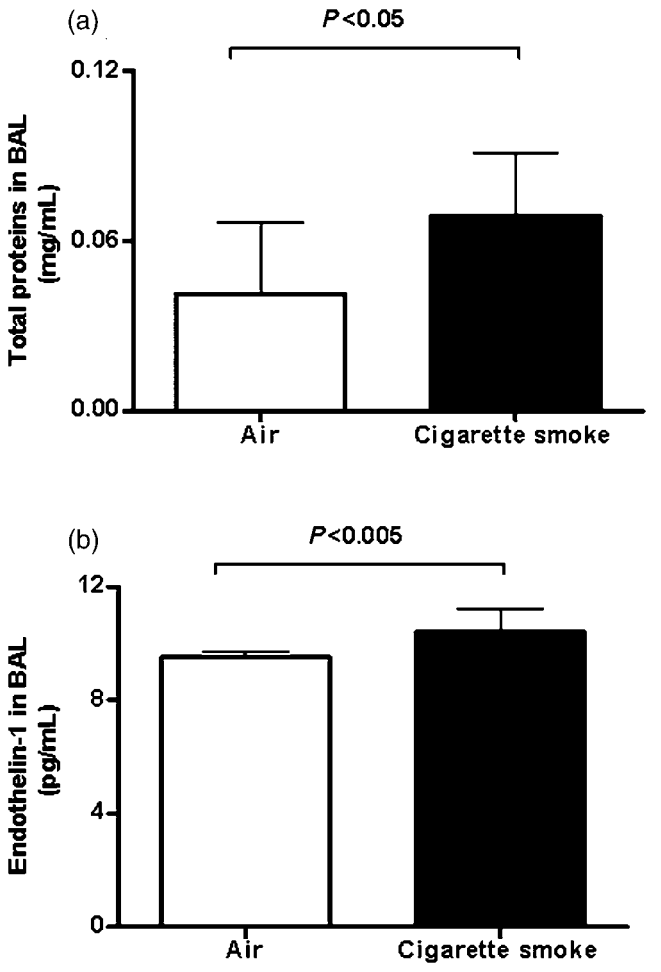

Figure 3 Total proteins (a) and endothelin (b) levels in bronchoalveolar lavage (BAL) fluid, following short-term nose-only cigarette smoke or air (control) exposure. Data are mean ± SD ($n = 8$)

mice compared with controls. In BALB/C mice subjected to whole body exposure of the smoke of 9 Winfield Red cigarettes per day (delivered as 3 cigarettes, 3 times/day) for four days, a mild inflammatory reaction comprising subtle perivascular and alveolar infiltrates was observed.[26]

Likewise, our data confirm the occurrence of lung inflammation evidenced by BAL fluid analysis. We observed an increase in the numbers of PMN and lymphocytes BAL fluid. This effect is indicative of response to short-term inflammation caused by CS. Similar observations have been reported in mice following long-term (3 months) nose-only CS exposure.[10] In-line with the increase of BAL cellularity, the total proteins, used a marker of epithelial permeability, was significantly increased following CS exposure compared with air-exposed mice. An increase of total proteins in BAL has been reported following exposure

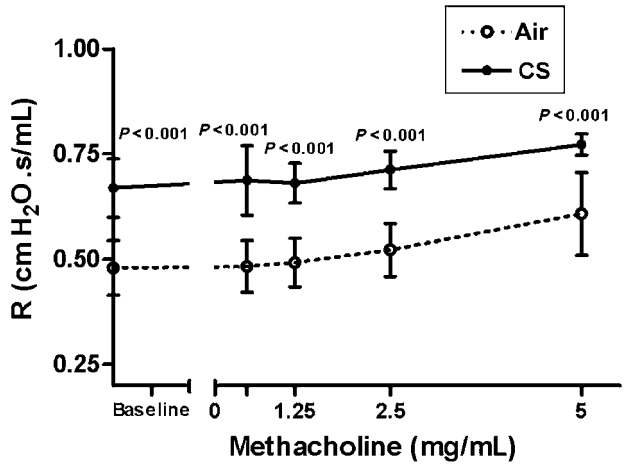

Figure 4 Airway hyper-responsiveness. The airway resistance (R), after increasing concentrations of methacholine (0–5 mg/mL), was measured via the forced oscillation technique (FlexiVent) following short-term nose-only cigarette smoke (CS) or air (control) exposure. Data are mean ± SD ($n = 7$)

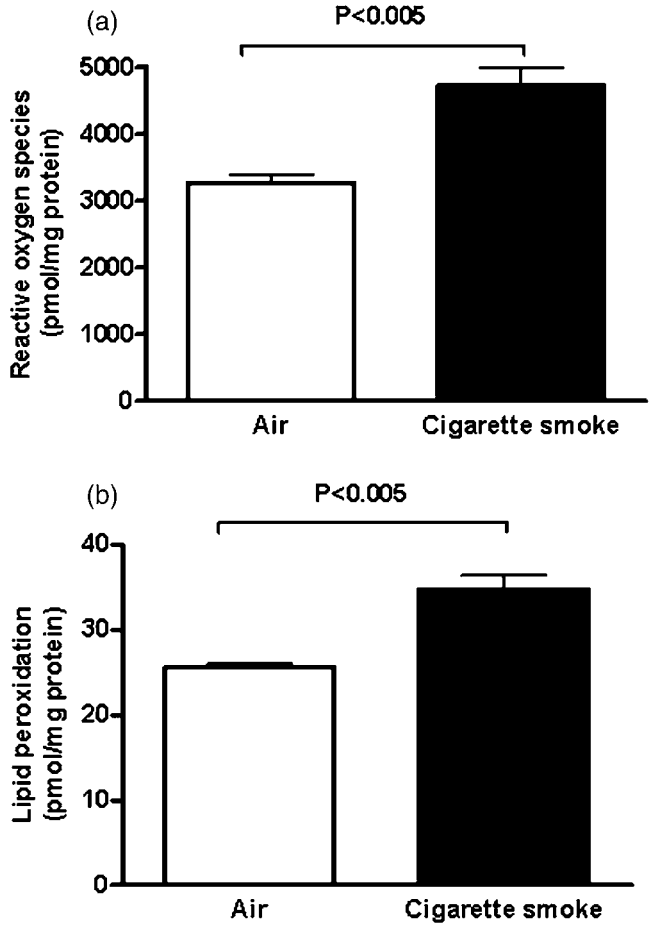

Figure 5 Reactive oxygen species (a) and lipid peroxidation (b) levels in lung tissues following short-term nose-only cigarette smoke or air (control) exposure. Data are mean ± SD ($n = 8$)

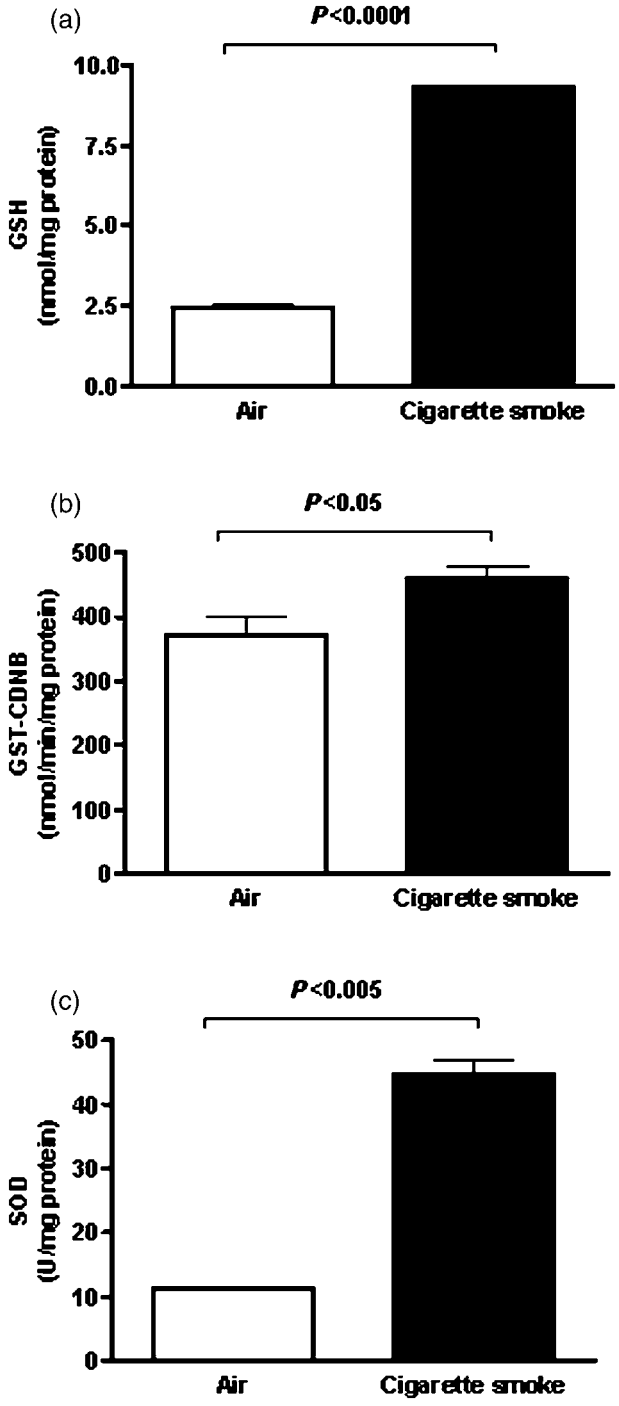

Figure 6 Reduced glutathione (GSH, c), glutathione *S* transferase (GST, b) and superoxide dismutase (SOD, c) levels in lung tissues following short-term nose-only cigarette smoke or air (control) exposure. Data are mean ± SD ($n = 8$)

to particulate air pollution in mice.[17] The endothelin peptide family consists of three isoforms, ET-1 (most predominant isoform), ET-2 and ET-3. ET-1 is a 21-amino acid peptide and is a proinflammatory mediator that causes an increase of pulmonary vascular and airway tonus.[27,28] We found an increase in endothelin concentration in BAL after CS exposure. Such an effect has not been reported so far. An increase of endothelin has been reported in BAL fluid of patient with asthma and COPD.[29,30] This rise can, at least in part explain the PMN influx in the BAL. It has been reported that endothelin-1, causes recruitment of neutrophils and that inhibition of this proinflammatory mediator may decrease short-term pulmonary inflammation associated with CS and lipopolysaccharide exposure.[27,28] The observed increase in airway resistance following CS exposure could also be explained by the augmentation of endothelin. Indeed, it has been demonstrated that direct administration of CS to lung explants produces constriction of the small airways.[31] This effect was related to endothelin in animals without previous CS exposure, and to the balance of oxidants and antioxidants in animals which have undergone smoke exposure *in vivo*.[31]

To further assess the mechanism underlying the increase of airway resistance following short-term nose-only CS exposure, we have measured the ROS and lipid peroxidation in lungs. Oxidative stress results from an imbalance between radical-generating and radical-scavenging systems leading to cell membrane impairment or DNA damage.[4,32] Our data show that exposure to CS caused oxidative stress in lung tissue evidenced by a significant increase of ROS and lipid peroxidation levels in the lung. Our data show a significant increase of antioxidant enzymes in lung tissue, including GST, GSH and SOD. We suggest that the increase in oxidative stress was

accompanied by increased antioxidant capacity, indicating that CS could trigger adaptive responses that counterbalance the potentially damaging activity of oxygen radicals and limiting further oxidant-mediated lung inflammation.[33] An increase in antioxidant activity has been reported in patient with adult respiratory distress syndrome and mice exposed to particulate air pollution.[33,34] Our findings are in-line with those reported by Valenca *et al.*[5] who found an increase in lung SOD, catalase, malondialdehyde and glutathione peroxidase in mice exposed to smoke for five days, using a smoking chamber system. On the other hand, it has been reported that acute (1 h) CS exposure of rats caused a significant depletion of GSH in lung.[35] Similarly, immediately following exposure by nose-only inhalation to mainstream CS (at varying doses 40, 120, 240 puffs), dose-dependent decreases in pulmonary GSH was observed in rats whereas, in guinea pigs, reductions in pulmonary GSH was observed only at the highest level of exposure, and that within three hours of exposure, GSH has returned to pre-exposure levels.[36] Ardite *et al.*[12] reported no reduction of GSH content in guinea pig lungs three hours and 24 h following nose-only CS exposure.

In conclusion, our data demonstrate that short-term nose-only exposure to CS, a system that best resembles the human exposure situation, induced increase in airway resistance, pulmonary inflammation and oxidative stress. Our data provide information on short-term exposure to CS which may constitute the underlying causal chain of reactions leading to the ultimate chronic pulmonary effects.

Author contributions: All authors have read and approved the manuscript. AN designed, planned, supervised all the experiments and wrote the article. HR supervised and performed the measurement of markers of oxidative stress, and contributed in the writing of the article. DS preformed the experiments. AJ performed the measurement of markers of oxidative stress. ME was involved in the animal work. BHA contributed in the design of the study and wrote the article. EA supervised and performed the histological and morphometric parts of the study, and contributed in the writing of the article.

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