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Airway antioxidant and inflammatory responses to diesel exhaust exposure in healthy humans

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ABSTRACT: **Pulmonary cells exposed to diesel exhaust (DE) particles *in vitro* respond in a hierarchical fashion with protective antioxidant responses predominating at low doses and inflammation and injury only occurring at higher concentrations. In the present study, the authors examined whether similar responses occurred *in vivo*, specifically whether antioxidants were upregulated following a low-dose DE challenge and investigated how these responses related to the development of airway inflammation at different levels of the respiratory tract where particle dose varies markedly.**

A total of 15 volunteers were exposed to DE (100 μg·m^{-3} airborne particulate matter with a diameter of <10 μm for 2 h) and air in a double-blinded, randomised fashion. At 18 h post-exposure, bronchoscopy was performed with lavage and mucosal biopsies taken to assess airway redox and inflammatory status.

Following DE exposure, the current authors observed an increase in bronchial mucosa neutrophil and mast cell numbers, as well as increased neutrophil numbers, interleukin-8 and myeloperoxidase concentrations in bronchial lavage. No inflammatory responses were seen in the alveolar compartment, but both reduced glutathione and urate concentrations were increased following diesel exposure.

In conclusion, the lung inflammatory response to diesel exhaust is compartmentalised, related to differing antioxidant responses in the conducting airway and alveolar regions.

KEYWORDS: **Air pollution, airway inflammation, antioxidants, diesel exhaust, glutathione, particulate matter**

Time-series studies demonstrate that short-term increases in ambient particle concentrations are associated with hospital admissions [1] and deaths from respiratory and cardiovascular disorders [2, 3]. In urban areas, motor vehicles can contribute to 25–35% of total airborne particulate matter (PM) with a diameter of <2.5 μm ($PM_{2.5}$) [4, 5], with concentrations near busy roads up to 30% greater than background levels. Numerous studies have examined the relationship between exposure to motor vehicle emissions and respiratory health [6–14] with recent evidence demonstrating that children living near busy roads have an increased prevalence of chronic respiratory symptoms [7, 8, 13, 14]. Whilst the majority of these studies have been unable to single out specific traffic components associated with the measured health effects, some studies utilising automated traffic counts have concluded that symptoms are associated with heavy, predominantly diesel-powered vehicles [7, 8, 13].

Whilst the biological mechanisms underlying these health effects are poorly understood, emerging data suggest that the capacity of particles to cause inflammation *via* the induction of oxidative stress is important. In this scenario, particles activate redox sensitive transcription factors, promoting the transcription of pro-inflammatory cytokines [15]. This capacity to cause oxidative stress appears to be related to the presence of oxidants or oxidant-generating components on the particle surface. Consistent with this view, diesel particles have been shown to contain redox-cycling quinones [16], polycyclic aromatic hydrocarbons (PAHs) [16] and transition metals capable of catalysing oxidation reactions [17, 18].

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Furthermore, reactive oxygen species have been detected in airway epithelial cells exposed to diesel exhaust particles (DEP) or their organic extracts [19, 20].

DEP and their organic components have been shown to upregulate pro-inflammatory cytokines in macrophage and bronchial epithelial cell lines [21–23], and to trigger neutrophilic inflammation in laboratory animals [24–26] and humans [27–34] exposed to diesel exhaust (DE). Consistent with the oxidative stress hypothesis, treatment of epithelial cells and macrophages with antioxidants has been shown to reduce particle-induced cytokine production through the down-regulation of redox-sensitive signalling pathways [25, 26]. The importance of antioxidants in modulating the inflammatory response to particles has also been demonstrated by the upregulation of endogenous antioxidant defences including: reduced glutathione [17, 35], haem oxygenase-1 [23], catalase, metallothionein [36] and glutathione reductase [35]. XIAO *et al*. [36] have proposed that a response hierarchy exists in cells exposed to diesel particles, with low concentrations stimulating protective antioxidant responses, and inflammation and injury only occurring at higher concentrations once endogenous defences have been overwhelmed.

Previous human exposure studies examining the capacity of inhaled particles (concentrated ambient particles [32–34], resuspended DEP [31] and fresh DE [27–29]) to elicit inflammation have yielded somewhat inconclusive findings, with evidence of airway inflammation at high particle concentrations [28, 33], but with less clear-cut responses to lower dose exposures [29, 34]. In the current study, the authors examined inflammatory and antioxidant responses in healthy volunteers exposed to a low-dose diesel inhalation challenge. Under question was whether the attenuated inflammatory responses reported with the low-dose exposures could be related to an upregulation of endogenous antioxidant defences. By examining the responses in the bronchial airways and alveoli it was possible to investigate the balance between antioxidant and inflammatory processes at sites experiencing significantly different tissue doses of particles. Hence, two tissue-dose scenarios could be investigated in a single inhalation study.

METHODS

Experimental exposure and particle characterisation

DE was generated using an idling Volvo diesel engine (Volvo TD45, 4.5 L, four cylinders, 680 $r \cdot min^{-1}$, model 1991; Volvo, Gottenburg, Sweden) as previously described [27, 29]. The steady state concentration of PM_{10}, gases and semi-volatiles during the diesel exposures were 100 ± 4.9 $\mu g \cdot m^{-3}$ (PM_{10}), 10.4 ± 1.7 parts per million (ppm; carbon monoxide), 1.3 ± 0.04 ppm (nitrogen monoxide), 0.4 ± 0.02 ppm (nitrogen dioxide), 1.8 ± 0.03 ppm (oxides of nitrogen) and 1.3 ± 0.49 ppm (total gaseous hydrocarbons: C_3H_8-equivalents), expressed as mean $\pm$ SD.

The PM mass in the exposure chamber was dominated by fine particles (<1 μm) in the accumulation mode, with a mass median particle diameter for the sub-micrometer sized PM of 0.18 μm. Quartz fibre filters were used to sample PAHs on particles. A polyurethane foam plug was used for collecting semi-volatile compounds. A total of 45 specific PAH compounds were analysed by gas chromatography–mass spectrometry. Elemental composition of the fine particle fraction was assessed using inductively coupled plasma-mass spectroscopy.

Subjects

In total, 15 healthy volunteers (seven female and eight male) with a mean age of 24 yrs were recruited. They were all never-smokers with normal lung function and negative skin-prick tests (table 1). No anti-inflammatory or other drugs, including antioxidant supplements, were permitted for the duration of the study. All participants gave informed written consent and the local ethics committee (Umeå University, Umeå, Sweden) approved the study.

Exposure protocol

Each subject was exposed to diluted DE with a particulate concentration (total PM) of 100 $\mu g \cdot m^{-3}$ and filtered air for 2 h in a specially built exposure chamber as previously described [27, 28]. The exposures took place on two separate occasions, in a randomised sequence, $\geqslant$3 weeks apart. The subjects were blinded to the actual exposure. Challenge dates were rescheduled if subjects experienced any form of respiratory infection within a 6-week period prior to scheduled exposure date. During the exposures subjects alternated between 15-min intervals of exercise (minute ventilation=20 $L \cdot min^{-1} \cdot m^{-2}$ body surface area) and rest, to model a moderate level of outdoor activity. Exercise was performed using a bicycle ergometer situated within the exposure chamber.

Bronchoscopy and processing of samples

Bronchoscopy was performed 18 h post-exposure, as previously described, using a flexible video bronchoscope (Olympus BF IT200; Olympus, Tokyo, Japan) [28, 29]. Bronchial biopsies were taken from proximal cristae. Bronchial wash (BW;

TABLE 1 Characteristics of subjects

Subject	Sex	Age yrs	Height cm	Weight kg	FVC L	FEV_1 L	FEV_1 % pred
1	M	22	181	72	4.9	4.3	87
2	M	23	176	69	4.2	4.1	87
3	F	24	171	61	3.9	3.5	94
4	M	25	195	92	5.8	4.6	86
5	M	23	183	80	5.2	4.4	88
6	M	27	180	79	5.8	5.1	107
7	F	21	162	67	3.0	2.9	83
8	F	22	168	66	4.0	3.8	106
9	F	27	152	49	2.9	2.7	87
10	F	26	164	62	4.0	3.8	110
11	F	27	158	63	3.5	3.2	99
12	F	23	158	59	3.8	3.4	101
13	M	24	181	82	5.2	4.5	92
14	M	26	177	76	5.3	4.9	103
15	M	21	178	71	5.2	5.0	103
Mean $\pm$ SD		24±2	172±12	70±11	4.4±1	4.0±0.8	96±9

FVC: forced vital capacity; FEV_1: forced expiratory volume in one second; % pred: percentage of predicted; M: male; F: female.

2×20 mL) and bronchoalveolar lavage (BAL; 3×60 mL) were carried out in the contra-lateral lung. The aspirates recovered from the first and second 20 mL instillations of the BW and the pooled BAL were collected into separate siliconised containers and immediately placed on ice. All lavage samples were filtered through nylon (pore diameter=100 μm) and centrifuged at 400 *g* for 15 min. The supernatants were separated from the cell pellet and analysed for albumin, total protein, interleukin (IL)-6, IL-8 and myeloperoxidase (MPO). Cell pellets were resuspended in PBS at a cell concentration of 10^6 cells·mL^{-1}. Differential cell counts were performed on cyto-centrifuge preparations stained with May-Grünwald Giemsa and 400 cells per slide were counted. IL-6 and IL-8 were measured using commercially available ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA). MPO was analysed using an MPO radio-immunoassay (Pharmacia AB, Uppsala, Sweden).

Lung function tests

Standard lung function tests, forced vital capacity and forced expiratory capacity in one second were performed at inclusion using a spirometer (Vitalograph R, Buckingham, UK).

Immunohistochemistry

Mucosal biopsies were processed into glycolmethacrylate resin, as previously described [28]. Sections (2 μm) were cut and stained immunohistochemically using the streptavidin biotin-peroxidase technique with monoclonal antibodies (mAb) directed against specific cellular markers to detect inflammatory cells in the bronchial mucosa.

Stained inflammatory cells were counted in the epithelium and submucosa excluding glands, blood vessels and muscle. The counts were expressed as cells·mm^{-1} in the epithelium and cells·mm^{-2} in the submucosa and counted using a light microscope. The length of the epithelium and the area of the submucosa were calculated using a computer-assisted image analyser (Zeiss KS400 software; Image Associates, Bicester, UK). Endothelial adhesion molecules (P-selectin, E-selectin, vascular adhesion molecule (VCAM)-1 and intracellular adhesion molecule (ICAM)-1) in the vessels were quantified by expressing the number of vessels stained with specific mAb as a percentage of the total number of blood vessels stained with the pan-endothelial mAb EN4 in adjacent 2-μm sections.

Antioxidant determinations

Reduced glutathione (GSH) and oxidised glutathione (GSSG) were measured using the GSSG reductase-5,5′-dithiobis(2-nitrobenzoic acid) recycling assay as described previously [17]. Vitamin C (ascorbate+dehydroascorbate) and urate concentrations were determined using reverse phase HPLC with electrochemical detection, as outlined previously [17], following sample reduction with dithiothreitol.

Permeability and cellular injury marker determinations

Albumin concentrations were measured using a commercial kit (Boehringer-Mannheim, Mannheim, Germany). Total protein determinations were made using the bicinchoninic acid method. DNA concentrations were determined using bisbenzimadazole (Hoechst 33258; Sigma, Poole, UK) according to established protocols [17].

Statistical analysis

All data were nonparametric and are therefore described throughout using medians with 25th and 75th percentiles. Comparisons between post-air and diesel values were performed using the Wilcoxon signed-rank test. Correlations between diesel-induced responses were performed using Spearman's rank-order correlation.

RESULTS

Particle composition

A total of 64% of the PM sampled in the DE consisted of carbonaceous material with the major part (94.5%) present as organic carbon and only 5.5% as elemental carbon. The major part (96.5%) of the PAH consisted of semi-volatile gaseous compounds with only a minor fraction (3.5%) present as particulate-associated material, 0.04% of total PM and 0.06% of the PM organic fraction. The dominating PAHs were phenanthrene, fluorene, 2-methylfluorene, dibenzothiophene and different methyl-substituted phenanthrenes accounting for ~90% of the total PAH. Of the metals measured in the diesel particle fraction (Pb, Al, Zn, Cu, Ni, Fe, Mn, Cr, V and Ti), only appreciable concentrations of the redox-inactive metals Al and Zn were detected.

Inflammatory responses

Following DE exposure, there was a 1.7-fold increase in median neutrophil numbers in the BW compared with post-air samples (0.85×10^4 post-air *versus* 1.41×10^4 cells·mL^{-1} post-DE; p=0.02). All other cell types remained unchanged (table 2). In contrast, no cell changes were detected in the alveolar lavage sample 18 h after DE exposure (table 2). Immunohistochemical analysis of the bronchial biopsies revealed that DE induced a significant increase in submucosal neutrophils (45.0 post-air *versus* 65.9 cells·mm^{-2} post-DEP; p=0.01) as well as a small increase in submucosal mast cell numbers (13.3 post-air *versus* 20.1 cells·mm^{-2} post-DE; p=0.02; table 3). No changes were found in the numbers of eosinophils or lymphocytes (CD3+, CD4+ or CD8+) in the submucosa after DE exposure (table 3). The expression of the vascular endothelial adhesion molecules, P-selectin, E-selectin, ICAM-1

TABLE 2 Differential cell counts in the bronchial wash (BW) and bronchoalveolar lavage (BAL) following a 2-h exposure to air and diesel exhaust (DE)

Cell type cells × 10^4·mL^{-1}	Air	DE	p-value
BW neutrophils	0.85 (0.51–1.68)	1.41 (1.05–2.24)	0.02
BAL neutrophils	0.09 (0.07–0.23)	0.10 (0.07–0.11)	0.50
BW macrophages	7.7 (4.9–8.6)	7.4 (5.2–8.3)	0.80
BAL macrophages	9.2 (8.0–10.2)	9.4 (5.9–11.4)	0.72
BW lymphocytes	0.28 (0.17–0.36)	0.31 (0.09–0.49)	0.93
BAL lymphocytes	1.05 (0.82–1.83)	1.18 (0.83–1.98)	0.76
BW eosinophils	0.0 (0.0–0.04)	0.02 (0.0–0.03)	0.68
BAL eosinophils	0.02 (0.0–0.05)	0.02 (0.0–0.04)	0.84
BW mast cells	0.0 (0.0–0.002)	0.0 (0.0–0.002)	0.74
BAL mast cells	0.0 (0.0–0.003)	0.0 (0.0–0.00)	0.31

Data are presented as median (interquartile range). n=15.

TABLE 3 Inflammatory cells in the bronchial tissue following a 2-h exposure to air and diesel exhaust (DE)

Inflammatory cells cells·mm^{-2}	Air	DE	p-value
Neutrophils	45.0 (24.7–71.4)	65.9 (57.0–118.9)	0.01
Mast cells	13.3 (9.4–20.3)	20.1 (15.4–32.8)	0.02
Eosinophils	0.0 (0.0–0.0)	0.0 (0.0–0.0)	1.00
CD3+	42.1 (23.7–57.0)	36.6 (30.7–45.0)	0.80
CD4+	22.5 (16.9–27.8)	18.2 (9.5–29.7)	0.39
CD8+	14.3 (7.0–21.4)	11.8 (8.6–17.6)	0.45

Data are presented as median (interquartile range). n=15.

and VCAM-1 were unchanged 18 h after DE exposure (data not shown). This increase in bronchial airway lumen and submucosal neutrophils after DE was associated with a significant increase in IL-8 protein (35.0 post-air *versus* 45.0 pg·mL^{-1} post-DE; p=0.01), and MPO (5.1 post-air *versus* 11.2 μg·L^{-1} post-DE; p=0.03) in the BW along with a trend toward an increase in the concentration of IL-6 protein in BW (table 4). Notably, a strong association was observed between the magnitude of the BW-MPO and BW-IL-8 responses (r=0.74; p=0.002). The concentrations of these pro-inflammatory cytokines (IL-6 and IL-8) and the neutrophil degranulation product MPO were unaltered in the alveolar lavage sample 18 h after DE (table 4).

Epithelial cell injury and altered permeability

No increase was observed in airway permeability (total protein and albumin) or cell injury (DNA) in either the bronchial (BW) or alveolar (BAL) lavage fluid samples after DE (data not shown).

Antioxidant responses

A total of 18 h after the end of the DE exposure, significant increases were observed in both urate and reduced glutathione in alveolar lavage (0.78 post-air *versus* 1.07 μM post-DE (p=0.03) and 0.33 post-air *versus* 0.46 μM post-DE (p=0.03), respectively), whereas no effect in vitamin C (ascorbate and dehydroascorbate) was observed (table 5). In contrast, no change was seen in the concentration of urate, glutathione (GSH and GSSG) or vitamin C in bronchial lavage (table 5).

DISCUSSION

In the current study, the authors investigated the hypothesis that inflammatory responses in humans exposed to PM could be modulated by upregulation of antioxidant defences at the air–lung interface. Subjects were exposed to 100 μg·m^{-3} of DE. Under the exposure conditions employed, the present authors observed neutrophil and mast cell recruitment into the bronchial airways 18 h after DE challenge. These cell influxes were not associated with any upregulation of VCAMs, suggesting the response had progressed beyond the recruitment phase. Increased concentrations of bronchial wash MPO were also observed, indicating neutrophil degranulation following the diesel challenge. In contrast, no alveolar inflammation was observed. Instead the alveolar response was characterised by the movement of GSH and urate into the airway lumen. These data demonstrate that DE causes bronchial inflammation, but suggest that the movement of GSH and urate onto the lung surface protects against inflammation in the alveolar region.

Human ambient particle exposure studies fall into two broad types, using either concentrated ambient particles (CAP) [32–34] or DE, which may be either freshly generated DE (particles and associated gaseous components) [27–29] or re-aerosolised DEP [31]. Studies in which human volunteers have been exposed to CAP have yielded inconsistent results, with evidence that systemic inflammation may be more pronounced than pulmonary responses [32–34]. In contrast, pulmonary and systemic inflammatory responses have been reported in volunteers exposed to high concentrations of fresh DE (300 μg·m^{-3} PM_{10}, 1.6 ppm nitric dioxide, 4.3 ppm total hydrocarbons for 1 h with intermittent exercise) [28] or resuspended diesel particles (200 μg·m^{-3} for 2 h at rest) [31]. In both studies, an early neutrophilia was seen 4–6 h post-exposure in induced sputum and bronchial lavage, respectively [28, 31]. However, in another study where alveolar lavage

TABLE 4 Soluble inflammatory mediators in bronchial wash (BW) and bronchoalveolar lavage (BAL) 18 h after a 2-h exposure to air and diesel exhaust (DE)

Inflammatory mediators	Air	DE	p-value
BW IL-6 pg·mL^{-1}	4.0 (1.4–4.9)	4.9 (2.6–7.0)	0.06
BAL IL-6 pg·mL^{-1}	1.3 (0.8–2.0)	1.0 (0.9–1.7)	0.28
BW IL-8 pg·mL^{-1}	35 (15–65)	45 (35–140)	0.01
BAL IL-8 pg·mL^{-1}	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.25
BW MPO μg·L^{-1}	5.1 (3.2–9.9)	11.2 (4.3–20.7)	0.03
BAL MPO μg·L^{-1}	0.0 (0.0–1.4)	1.7 (0.0–2.1)	0.11

Data are presented as median (interquartile range). IL: interleukin; MPO: myeloperoxidase. n=15.

TABLE 5 Antioxidant concentrations in bronchial wash (BW) and bronchoalveolar lavage (BAL) 18 h after a 2-h exposure to air and diesel exhaust (DE)

Antioxidants μM	Air	DE	p-value
BW vitamin C	0.59 (0.44–0.75)	0.65 (0.58–0.85)	0.57
BAL vitamin C	0.51 (0.37–0.70)	0.62 (0.44–0.74)	0.23
BW urate	0.55 (0.39–1.06)	0.75 (0.51–0.93)	0.56
BAL urate	0.78 (0.64–0.85)	1.07 (0.87–1.36)	0.03
BW GSH	0.16 (0.05–0.35)	0.24 (0.10–0.47)	0.52
BAL GSH	0.33 (0.20–0.45)	0.46 (0.24–0.74)	0.04

Data are presented as median (interquartile range). GSH: reduced glutathione. n=15.

was also performed a similar neutrophil response was not seen in the alveolar region [28]. When these responses were examined at a lower concentration (100 $\mu g \cdot m^{-3}$), the only significant changes seen 6 h post-exposure were increases in bronchial airway IL-6 and IL-8 protein, and upregulation of the vascular endothelial adhesion molecule P-selectin [29]. Despite the absence of increased airway neutrophils, these changes were interpreted as being indicative of an early inflammatory response. The present authors also observed evidence for a movement of GSH onto the bronchial and nasal airway surfaces, but not in alveolar lavage [17].

To distinguish whether the diminished response seen 6 h after the 100 $\mu g \cdot m^{-3}$ DE challenge was related to the evolution of a protective antioxidant response, the current authors repeated this exposure but examined airway responses after 18 h. The rationale for this was to establish whether the response at 6 h was attenuated or simply delayed. As the PM in the chamber during the DE exposure was dominated by fine particles with a median mass diameter of 0.18 μm, the fractional particle deposition would have been low, but similar in conducting airways and alveoli [37]. However, due to the large surface area of the alveoli, the particle dose per unit surface area would be significantly less than in bronchial airways. Consequently, examining the balance between antioxidant and pro-inflammatory processes in these two regions permitted a comparison at different tissue doses.

A considerable body of evidence exists demonstrating that DEP are capable of inducing oxidative stress *in vivo*, largely related to their content of redox-active metals [17, 18] and quinones [16]. Mechanistically, this oxidative capacity has been proposed to drive the upregulation of pro-inflammatory cytokines through the activation of nuclear factor κB [38, 39]. Consistent with this view, treatment of particle-challenged cells with antioxidants abolishes the production of a range of pro-inflammatory cytokines [19, 21, 38, 39]. Recent work has also illustrated that cells challenged with increasing concentrations of DEP elicit a hierarchical response, with protective antioxidant responses predominating at low concentrations, and inflammation and injury only occurring at high particle concentrations [36].

At the 6-h time point, the present authors observed a significant increase in GSH concentrations in both nasal and bronchial airway lavage samples after DE [17]. Despite this early mobilisation of GSH to the airway surface [17], inflammation was established (neutrophilia, degranulation and mastocytosis) 18 h after DE. In contrast, no inflammatory or antioxidant responses were seen 6 h post-diesel in the peripheral lung. Even 18 h post-exposure, no evidence of an increase in pro-inflammatory cytokines or inflammatory cells was seen in alveolar lavage, but GSH and urate concentrations were significantly increased. It seems unlikely that this reflects a delayed response in the alveoli as these antioxidant responses were seen in the absence of any upregulation of pro-inflammatory signals. Hence, the current authors conclude that the dose of particles penetrating to the peripheral lung in a diesel challenge of 100 $\mu g \cdot m^{-3}$ was insufficient to elicit inflammation due to the predominance of protective antioxidant responses.

Similar increases in alveolar lavage urate have been reported following ozone exposures in humans [40, 41] and animals [42]. This may reflect a movement of urate from the plasma pool, but no evidence of altered airway permeability was found to support this view. This increase in urate could also reflect increased synthesis by pulmonary cells, either as a consequence of increased adenosine metabolism or increased xanthine oxidase activity. Notably, increased lavage fluid urate concentrations have been reported in rats after intratracheal challenge with iron salts, associated with an increase in pulmonary xanthine oxidase activity [43]. Elevated concentrations of airway GSH have also been reported in numerous air pollutant studies [17, 44]. It has been speculated that this increase may reflect release from necrotic or apoptotic cells. However, the current authors saw no evidence of a reduction in any alveolar cell population. Therefore, it seems likely that the observed increase reflects an increased rate of glutathione synthesis, allied to increased export to the cell surface. Furthermore, *in vitro* incubation of macrophages with DEP has been shown to result in time-dependent increases in intracellular GSH and cysteine concentrations, as well as increased glutathione reductase activity [35].

In conclusion, these results, placed in the context of the earlier findings obtained 6 h after diesel exhaust [17], indicate clear regional differences in the response of the airway to diesel exhaust. Although reduced glutathione concentrations were increased in the bronchial airways 6 h after diesel exhaust, this response was insufficient to prevent the development of neutrophilia or mastocytosis 18 h post-exposure. In contrast, in the alveoli, where tissue particle doses were lower, no inflammation was apparent at either time point, while the response was characterised by protective antioxidant movements onto the airway surface. These responses lend *in vivo* support to the hierarchical response model proposed by XIAO *et al*. [36] and provide mechanistic data supporting the existence of a threshold for acute airway responses to diesel exhaust in humans.

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