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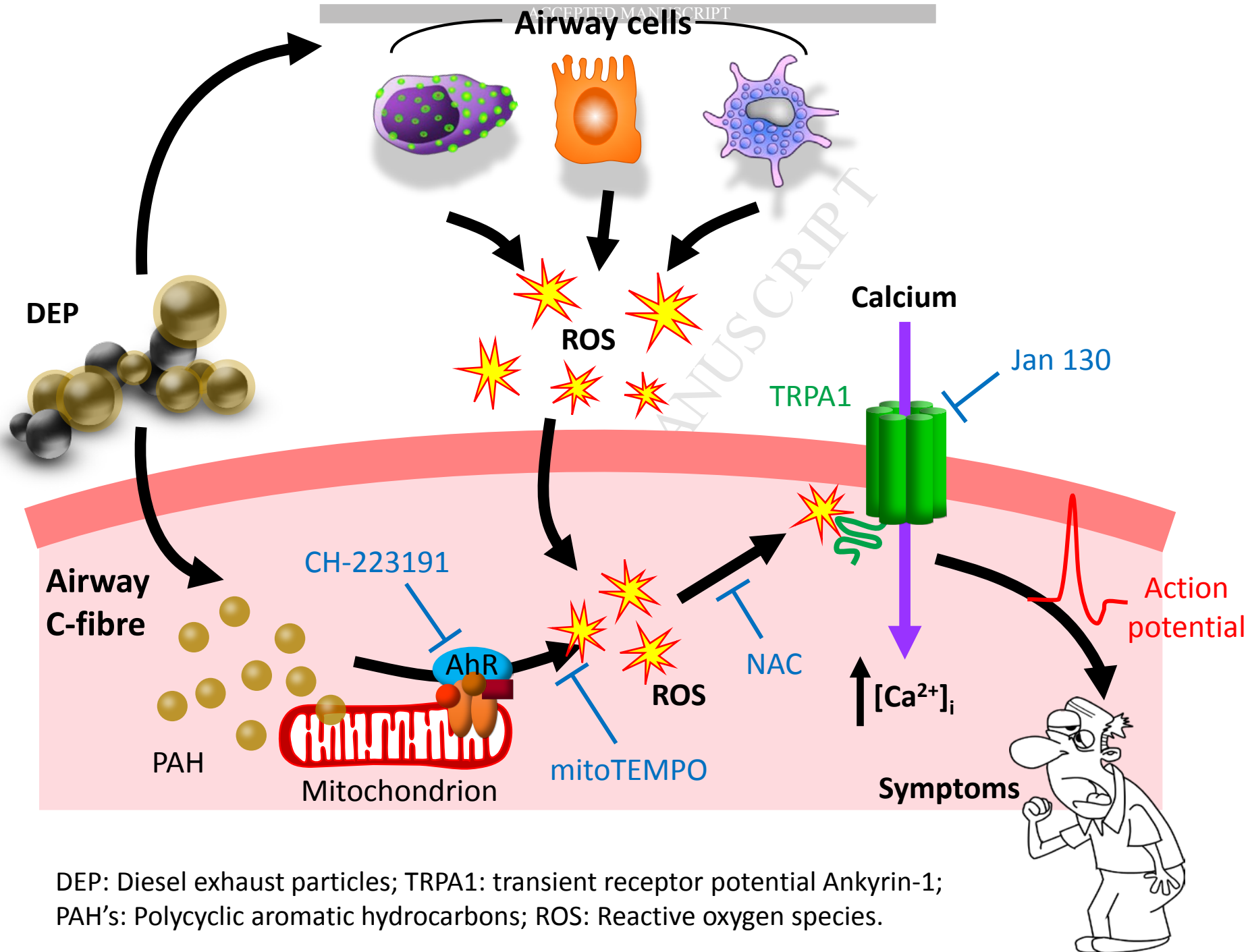
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**Mechanistic Link between Diesel Exhaust Particles and Respiratory Reflexes**

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**ABSTRACT**

**Background:** Diesel exhaust particles (DEP) are a major component of particulate matter in Europe's largest cities and epidemiological evidence links exposure with respiratory symptoms and asthma exacerbations. Respiratory reflexes are responsible for symptoms and are regulated by vagal afferent nerves which innervate the airway. It is not known how DEP exposure activates airway afferents to elicit symptoms such as cough and bronchospasm.

**Objective:** To identify the mechanisms involved in the activation of airway sensory afferents by DEPs.

**Methods:** In this study we utilize *in vitro* and *in vivo* electrophysiological techniques including a unique model which assess depolarization (a marker of sensory nerve activation) of human vagus.

**Results:** We demonstrate a direct interaction between DEP and airway C-fiber afferents. In anaesthetized guinea pigs, intratracheal administration of DEP activated airway C-fibers. The organic extract (DEP-OE), and not the cleaned particles, evoked depolarization of guinea-pig and human vagus and this was inhibited by a TRPA1 antagonist and the antioxidant N-acetyl cysteine (NAC). Polycyclic aromatic hydrocarbons (PAHs), major constituents of DEP, were implicated in this process via activation of the aryl hydrocarbon receptor (AhR) and subsequent mitochondrial ROS production, which is known to activate TRPA1 on nociceptive C-fibers.

**Conclusions:** This study provides the first mechanistic insights into how exposure to urban air pollution leads to activation of guinea-pig and human sensory nerves which are responsible for respiratory symptoms. Mechanistic information will enable the development of appropriate therapeutic interventions and mitigation strategies for those susceptible individuals who are most at risk.

**Key messages:**

- Exposure to diesel exhaust particles (DEP) is associated with respiratory symptoms but the mechanisms involved are unknown.
- Here we demonstrate a direct interaction between DEP and the activation of airway C-fiber afferents. Polycyclic aromatic hydrocarbons (PAHs) were implicated in this process via activation of the aryl hydrocarbon receptor (AhR) and subsequent mitochondrial ROS production.
- These findings explain how exposure to DEP leads to the activation of human sensory nerves which are responsible for respiratory symptoms and could explain how air pollution can cause disease exacerbations in susceptible groups such as those with asthma.

**Capsule Summary:** These findings provide the first mechanistic insights into how exposure to urban air pollution leads to the activation of human sensory nerves which are responsible for respiratory symptoms.

**Keywords:** Pollution, oxidative stress, transient receptor potential (TRP) ion channels, sensory nerves, vagus.

**Abbreviations:** Transient receptor potential (TRP), particulate matter (PM), diesel exhaust particles (DEP), aryl hydrocarbon receptor (AhR), polycyclic aromatic hydrocarbons (PAH's), reactive oxygen species (ROS)

## INTRODUCTION

Air pollution is a major global health concern especially in industrialized countries<sup>1</sup>. In urban environments exposure to traffic-derived particulate matter (PM) has been a major focus, especially with regard to primary tail pipe emissions from diesel vehicles. Smaller fractions of PM, because its size and low density are able to remain airborne, disperse widely in the environment and penetrate deep into the lungs when inhaled to distribute throughout the respiratory tract. There is currently no safe lower limit of exposure to PM. Diesel exhaust particles (DEP) represent a significant proportion of urban PM<sup>2,3</sup> especially within Europe due to the high proportion of diesel vehicles<sup>4</sup> and ongoing problems with emission compliance<sup>5</sup>. Epidemiological studies have found strong associations between exposure to DEP, or air pollution markers indicative of diesel exhaust (black and elemental carbon), and respiratory symptoms including cough, wheeze and shortness of breath<sup>6,7</sup>, hospital admissions<sup>8</sup> and mortality<sup>9</sup>. Clinical studies utilizing diesel exposure have documented increases in total symptom scores<sup>10,11</sup> and increased airway resistance<sup>12</sup>. However, information regarding the molecular mechanism linking DEP exposure and respiratory symptoms is lacking.

Respiratory reflexes are responsible for symptoms and are regulated by vagal afferent nerves which innervate the airway<sup>13-15</sup>. There are several different sensory nerve subtypes present in the lung, some are more mechanically sensitive and others more chemosensitive; namely C-fibers and A $\delta$ -fibers, respectively. Transient receptor potential (TRP) channels present on vagal nerve termini situated in and under the airway epithelium can be activated by a wide variety of stimuli to elicit reflexes leading to respiratory symptoms. These include mechanical and inflammatory stimuli, environmental irritants and changes in osmolarity, pH or temperature<sup>16, 17</sup>. Upon activation TRP channels allow the influx of calcium into the cell leading to subsequent membrane depolarization and

ultimately the generation of an action potential that propagates along the vagus nerve<sup>18</sup>. Interestingly, one publication has demonstrated DEP-induced activation of TRPV4 expressed in an epithelial cell line and another showed activation of TRPA1 on murine dorsal root ganglion cells<sup>19, 20</sup>. Our hypothesis was that DEP are able to initiate respiratory symptoms via direct activation of lung specific afferent sensory nerves. The scope of this study was to determine whether DEP can directly activate airway sensory nerves using a range of human and animal *in vitro* models and *in vivo* electrophysiological studies in an animal model. We also evaluated which component of DEP was responsible and the signaling mechanisms involved.

**METHODS - Detailed methods are provided in the online supplement.**

### ***Animals***

Male Dunkin-Hartley guinea-pigs and C57BL/6 mice were used. All experiments were performed in accordance with the U.K. Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act of 1986 and the ARRIVE guidelines<sup>21</sup>.

### ***Human Tissue and Ethics***

Human lung and trachea surplus to transplant requirements (N=3, 56-73 years old, 1 male/2 female, 1 smoker/2 non-smokers), with the vagus nerve still attached, were used to obtain translational data to complement data generated in guinea pig tissue. Tissue was provided by the International Institute for the Advancement of Medicine (IIAM, Edison, New Jersey, USA). In all cases the tissue was

approved for use in scientific research and ethical approval was obtained from the Royal Brompton & Harefield Trust.

### ***Compounds and Materials***

Diesel exhaust particles from a forklift truck (DEP - SRM-2975) and its commercial organic extract (DEP-OE - SRM-1975) were purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, USA). Generator DEP, obtained from the Air Pollution Exposure Laboratory (APEL), was obtained which has been designed for the controlled inhalation of human subjects to aged and diluted diesel exhaust to mimic “real-world” occupational and environmental conditions<sup>22</sup>. Drugs (listed in the supplementary methods) were made up in stock solutions using DMSO, with the final concentration of DMSO kept at 0.1% for experiments.

### ***Particle Suspensions***

Particle suspension solutions were freshly prepared daily. Suspensions of DEP or cleaned particulate carbon core (par-DEP) were prepared in a modified Krebs-Henseliet solution by sonication before dilution to working concentrations. For *in vivo* experiments, suspensions were prepared in PBS in a similar manner.

### ***Physicochemical characterization of DEP***

Cryo preparation was performed done using an automatic plunge freezer. Nanoparticles, dispersed in (1µg/ml in Krebs), were dropped onto a grid and frozen by rapidly plunging into liquid ethane. These were transferred in their frozen state into a cryo-rod and then into the electron microscope. For chemical analysis, DEP samples were dispersed by sonication in ethanol and then pipetted onto a grid

at room temperature. TEM and Energy Dispersive X-ray Spectroscopy (EDX) analysis was performed. The organic/inorganic ratio composition of SRM 2975 was assessed using thermogravimetric analysis (TGA). Dynamic light scattering (DLS) measurements were also carried out as described in supplementary text. DEP was separated into the organic extract (org-DEP) and cleaned particles (par-DEP) using Soxhlet extraction.

***In vivo recording of action potential firing in single-fiber afferents.***

Guinea-pigs were anaesthetized with urethane (1.5 g/kg) intraperitoneally. The trachea was cannulated and the animal artificially ventilated. The right jugular vein and carotid artery were cannulated for respectively injecting drugs and measuring systemic arterial blood pressure. Animals were paralysed with vecuronium bromide, initially administered at a dose of 0.10 mg/kg, i.v., followed every 20 min with 0.05 mg/kg, i.v. to maintain paralysis. The depth of anaesthesia was frequently assessed by monitoring the response of heart rate and blood pressure to noxious stimuli (as described below). Both cervical vagus nerves were located, via a cervical incision, and dissected free; both vagus nerves were cut at the central end. The left vagus nerve was used for sensory nerve fiber recording as previously described<sup>23</sup> (diagram of experimental set up can be found in a recent review article<sup>16</sup>). Following identification of a suitable single nerve fiber, control responses were obtained to capsaicin (100 $\mu$ M in saline, aerosolized for 15s), acrolein (10mM in saline, aerosolized for 60 s) and citric acid (300mM, aerosolized for 60s). The nerve under investigation was then challenged with either vehicle (PBS, 200 $\mu$ L) or DEP (10  $\mu$ g/ml in PBS, 200 $\mu$ L, intratracheal dose) and subsequent action potentials recorded. For antagonist studies, control responses were obtained to capsaicin (100 $\mu$ M in saline, aerosolized for 15s), acrolein (10mM in saline, aerosolized for 60s) and DEP-OE (1  $\mu$ g/ml in saline, aerosolized for 60s) prior to the introduction of Janssen 130 (30mg/kg, 1% methyl cellulose in saline)

into the animal via intravenous route 60 minutes before challenging again with capsaicin, acrolein and DEP-OE. At the end of the experiment, the conduction velocity of the single nerve fiber was measured to determine whether it was a slow conducting non-myelinated C-fiber or a fast conducting myelinated A $\delta$ -fiber. Using the same experimental set up with the vagus nerves left intact and in the absence of neuromuscular blockade we assessed tracheal pressure ( $P_T \Delta$  increase cmH<sub>2</sub>O) as a marker of airflow obstruction which was expressed as mean  $\pm$  SEM.

### ***In vitro measurement of isolated vagus nerve depolarization***

Guinea pigs and mice were sacrificed by injection of sodium pentobarbitone (200 mg/kg i.p.) and the vagus nerves were dissected and depolarization assessed as a measure of sensory nerve activation as described in previous publications<sup>24-26</sup>. Human vagus was obtained from IIAM as previously described (<http://www.iiam.org/>).

### ***Data Analysis and Statistics***

Inhibition of DEP, phenanthrene, antimycin A, H<sub>2</sub>O<sub>2</sub>, capsaicin and acrolein responses in the isolated vagus nerve preparation was analysed by a two-tailed paired t-test, comparing responses to agonist in the absence and presence of antagonist in the same piece of nerve. Data are presented as mean  $\pm$  s.e.m., with statistical significance set at  $P < 0.05$ . In the single fibre experiments, data was analysed by paired t-test, comparing responses (absolute values) after stimulus to baseline values immediately preceding the response. Data are presented as mean  $\pm$  s.e.m., with statistical significance set at  $P < 0.05$ . Inhibition of fibre firing was analysed by paired t-test comparing responses following antagonist to control values prior to antagonist administration, or using an unpaired t-test comparing responses to vehicle control as appropriate.

## RESULTS

### ***DEP-induced activation of airway sensory nerves.***

Intratracheal instillation of DEP (10µg/ml in PBS, dose-volume 200 µl) in an anaesthetized guinea-pig model<sup>23,24</sup> evoked action potential firing in chemosensitive C-fibers (Figure 1A & B), but not the mechanosensitive Aδ- fibers (Figure. 1C, Table 1). To investigate the mechanism further and to provide translational data, the effect of DEP was examined in an isolated vagal nerve preparation<sup>24-26</sup>. DEP-evoked concentration-dependent depolarization of the guinea-pig vagus (Figure 1D & E) which was completely abolished in the presence of tetrodotoxin (blocks the flow of sodium ions into nerve cells which is a necessary step in the conduction of nerve impulses in excitable nerve fibers) (TTX – 3µM; Supplementary Figure 1). DEP also depolarized isolated human vagus tissue in a similar manner to guinea-pig (Figure 1F & E, respectively).

### ***Characterization of DEP.***

DEP are made up of a carbonaceous core surrounded by an organic hydrocarbon component, however the precise size and composition of each particle can vary greatly. Typically, DEP consists of primary nanoparticles (<100nm) that can form larger agglomerates several micrometers in size. The organic components of DEP include polycyclic aromatic hydrocarbons (PAHs) and their derivatives; they also contain traces of numerous transitional metals including iron, vanadium, manganese, copper, zinc and nickel<sup>27</sup>. Cryo-electron microscopy (cryo-EM) images of the DEP used in these studies (DEP-SRM-2975, commercially-available, generated by a forklift truck) indicated that the individual

primary nanoparticles were roughly spherical, with diameters less than 100 nm; the majority were present as small, irregular agglomerates although larger agglomerates up to several microns were present (Figure 2A). Particle size was quantified by measuring the longest length of the particles and the majority were found to be below 1  $\mu\text{m}$  (mostly <600nm; Fig. 2B and Supplementary figure 2A), which was confirmed by dynamic light scattering (DLS) analysis (Supplementary Figure 2B). Thus, the DEP were in a respirable format that would be expected to deposit throughout the lower respiratory tract on inhalation. Transmission electron microscopy-Energy-dispersive X-ray spectroscopy (TEM-EDX) elemental analysis confirmed low levels of metals (Supplementary Figure 3A-D). Thermogravimetric analysis (TGA) indicated DEP were composed of approximately 15% organic material and 83% inorganic carbon, with the remainder being trace impurities (Figure 2C).

Given that DEP appeared to activate chemosensitive rather than mechanosensitive sensory nerves, the organic chemical components of DEP (org-DEP) were separated from the cleaned particulate carbon core (par-DEP) using Soxhlet extraction. In both the guinea-pig and human vagus tissue, org-DEP depolarized the vagus nerve in a similar manner to the whole DEP, whilst par-DEP did not induce a response (Figure 2D). Having established that the organic extract of DEP was responsible for its biological activity and in order to use a characterized and standardized supply we used the commercially-available extract of SRM-2975, namely SRM-1975, in the next experiments (this is referred to in this manuscript as DEP-OE). DEP-OE depolarized the guinea-pig vagus nerve in a concentration dependent manner similar to DEP (Supplementary Figure 4). These results indicate that the organic material embedded on the surface of DEP contains the key components that activate sensory nerves. Polymyxin B (300 $\mu\text{g}/\text{ml}$ ), a cyclic cationic polypeptide antibiotic is widely used to eliminate the effects of endotoxin contamination, both *in vitro* and *in vivo* but had no effect on

depolarization induced by DEP-OE (1 $\mu$ g/ml) (Control: 0.1025  $\pm$  0.009mV; Treatment: 0.1023  $\pm$  0.020mV; Recovery: 0.0920  $\pm$  0.014mV).

### ***Role of TRP channels in DEP-OE-induced sensory nerve activation.***

A submaximal concentration of DEP-OE (1 $\mu$ g/ml) was selected for antagonist studies (Supplementary Figure 4). The specific TRPA1 antagonist Janssen 130 (10 $\mu$ M) significantly inhibited DEP-OE-induced depolarization in the isolated guinea-pig vagus nerve (Figure 3A-B), whilst vehicle (0.1% DMSO), the specific TRPV1 antagonist Xention D0501 (10 nM) and the specific TRPV4 antagonist GSK2193874 (10  $\mu$ M) had no effect (Figure 3B). Janssen 130 corresponding to the 130 compound of the patent WO2010/141805 A1<sup>28</sup>, also significantly inhibited DEP-OE-induced responses in the human vagus nerve (Figure 3C). These results were confirmed using genetically modified TRP knockout mice (Figure 3D). Responses in TRPV1<sup>-/-</sup> and TRPV4<sup>-/-</sup> mice were not significantly different from WT mice. *In vivo*, Janssen 130 (300 mg/kg, i.p) significantly inhibited C-fiber firing to both the TRPA1 positive control (acrolein – 10mM) and aerosolized DEP-OE (1  $\mu$ g/ml) (Figure 3F), while vehicle had no effect (Figure 3E). Janssen 130 (300 mg/kg, i.p) also significantly inhibited the increased tracheal pressure evoked by aerosolized DEP-OE (10  $\mu$ g/ml) (two responses to DEP-OE were evoked before (20.7  $\pm$  3.02 and 19.63  $\pm$  1.73 P<sub>T</sub>  $\Delta$  increase cmH<sub>2</sub>O) and after Janssen130 (5.23  $\pm$  0.79 P<sub>T</sub>  $\Delta$  increase cmH<sub>2</sub>O, n=3; P<0.05).

### ***Mechanisms of activation of TRPA1 by DEP-OE***

The ability of DEP to generate oxidative stress has been implicated as a key mechanism driving its adverse health effects<sup>29</sup>. Oxidative stress and the production of electrophiles have been shown to activate TRPA1 through the covalent modification of cysteine residues<sup>30-32</sup>. The oxidant H<sub>2</sub>O<sub>2</sub>

depolarized the isolated guinea-pig vagus nerve in a concentration dependent manner (Supplementary Figure 5A). Janssen 130 (10  $\mu$ M), but not vehicle (0.1% DMSO) or TRPV1 antagonism, significantly inhibited H<sub>2</sub>O<sub>2</sub>-induced depolarization in the guinea-pig vagus nerve (Supplementary Figure 5B). Vagal nerve from TRPA1<sup>-/-</sup> mice had significantly reduced depolarization responses to H<sub>2</sub>O<sub>2</sub> compared to tissues from WT mice (Supplementary Figure 5C). In the presence of the antioxidant N-acetylcysteine (NAC, 1 mM), responses to H<sub>2</sub>O<sub>2</sub> (10 mM), acrolein (300  $\mu$ M) and AITC (300  $\mu$ M; TRPA1 agonists) were abolished (Figure 4A). DEP-OE-induced depolarization was also abolished by the application of NAC on guinea-pig and human vagus (Figure 4B). When referencing the wide range of electrophilic chemicals present within DEP-OE, phenanthrene (a polycyclic aromatic hydrocarbon; PAH) was identified to be present in relatively high concentrations (SRM-1975 Certificate Analysis Sheet – NIST, USA). PAHs are traditionally thought to exert their toxic effects via induction of the aryl hydrocarbon receptor (AhR), a well conserved transcription factor. The application of two specific AhR antagonists CH223191 (10  $\mu$ M) and 2', 4'-trimethoxyflavone (TMF; 10  $\mu$ M) significantly inhibited depolarization responses to both phenanthrene (1 nM) and DEP-OE (Figure 4C & supplementary Figure 6). Depolarization of the vagus nerve by DEP-OE was reduced in AhR<sup>-/-</sup> mice compared to WT (Figure 4D). Furthermore, Antimycin A, a mitochondrial electron transport chain inhibitor and generator of mitochondrial oxidant stress, depolarized the vagus nerve in a concentration dependent manner (Supplementary Figure 7A) and this response could be inhibited by the mitochondrial superoxide scavenger MitoTEMPO (2 $\mu$ M; Supplementary Figure 7B). MitoTEMPO was capable of reducing the depolarization induced by DEP-OE (1 $\mu$ g/ml) compared to vehicle controls (Figure 4E). These data suggest that electrophilic compounds, such as the PAHs present in DEP, activate TRPA1 through an oxidative stress mechanism which involves AhR and the generation of mitochondrial oxidative stress.

***Translational experiments with generator diesel***

In the studies presented we used a characterized and standardized supply of commercially available DEP (DEP-SRM-2975, generated by a forklift truck). However, we also repeated key observations with generator DEP which has been aged and diluted to mimic “real-world” occupational and environmental conditions. This generator diesel has been used in controlled human exposure studies and is associated with a range of respiratory symptoms<sup>10,11,22</sup>. In these experiments depolarizations of the guinea pig vagus evoked by generator DEP (1µg/ml) were inhibited by the TRPA1 antagonist Jan 130 (10 µM) (Figure 5A & C) or the antioxidant NAC (1 mM) (Figure 5B & C) confirming results obtained with DEP-SRM.

**DISCUSSION**

Epidemiological studies have found strong associations between exposure to DEP and respiratory symptoms including cough, wheeze and shortness of breath<sup>6, 7</sup>. In addition, links have been made between combustion-derived PM and asthma symptoms and exacerbations<sup>33</sup>. DEP exposure has also been strongly associated with acute worsening of lung function<sup>34</sup> and airway hyperreactivity in asthmatic subjects<sup>35</sup>. Although a large number of previous studies have focused on the inflammatory effects of DEP on airway epithelium and immune cells<sup>36,37</sup>, it is still not known how DEP can evoke respiratory reflexes and the associated symptoms, or by what mechanisms.

Initial studies confirmed our hypothesis that intratracheal instillation of DEP could directly activate airway sensory afferent nerves. However, contrary to our expectations that PM would activate mechanosensitive, rapidly adapting receptors (RARs) or Aδ-fibers, it was the chemosensitive C-fibers

where action potential discharge was noted in response to DEP. The isolated vagus preparation was used to confirm these observations and to investigate this mechanism further because it is an *in vitro* technique amenable to precise pharmacological study, without the complications often associated with the interpretation of *in vivo* experiments<sup>16,24-26,38</sup>. Furthermore, and importantly, the use of the human vagus nerve preparation allowed the generation of translational data and provided an early indicator that data generated in guinea-pig vagus were predictive of effects in man. The potential for DEP to directly activate airway sensory nerves has important implications, given that millions of individuals living in urban environments are exposed to DEP on a daily basis and that activation of airway sensory nerves can result in a wide range of respiratory symptoms which can be particularly debilitating for those with underlying respiratory conditions compared to healthy people.

Given its complex composition, DEP (SRM-2975) underwent physicochemical characterization so that the biological data could be more easily interpreted. Cryo-EM imaging indicated that the majority of DEP were present in small agglomerates and TEM-EDX analysis indicated that a low level of metal impurities were present, in agreement with some previous studies<sup>39</sup>. The TGA weight loss profile revealed that the DEP was composed of approximately 15% organic material, 83% inorganic carbon material and 2% of trace impurities as measured by weight. These findings are in general agreement with existing published literature<sup>40, 41</sup>. Soxhlet extraction of DEP resulted in two separated components, the cleaned carbon particle core (par-DEP) and the organic extract (org-DEP). Only the org-DEP, and not the par-DEP, activated the vagus nerve commensurate with an activity on the chemosensitive rather than the mechanosensitive airway afferents. Other experiments have also highlighted the importance of the organic components of DEP<sup>40, 41</sup> in its bio-reactivity *in vitro* and *in*

*vivo* although we acknowledge that the par-DEP may be responsible for other biological effects of diesel.

TRP channels are environmental sensors and initiate the activation of airway sensory nerves in response to exogenous and endogenous stimuli<sup>17</sup>. Utilizing pharmacological intervention and tissues from genetically modified mice, we demonstrated that DEP-OE-induced activation of airway sensory nerves and increased tracheal pressure (indicative of airflow obstruction) was through the activation of TRPA1. Although sensory afferents arising from the dorsal root ganglia (DRGs) are not airway innervating and calcium influx measurements are not assessing action potential generation our observations are consistent with data that demonstrated that DEP could activate rodent DRG neurons<sup>20</sup>. This key result was also demonstrated utilizing human vagus nerve. TRPA1 is expressed exclusively on airway C-fibers, is activated by a number of toxic environmental irritants and has been shown to cause cough in both humans and guinea-pigs<sup>25</sup>. TRPA1 is also thought to be a key channel involved in the late asthmatic response in a rat model of allergic inflammation<sup>42</sup> and TRPA1 gene polymorphisms have been associated with childhood asthma<sup>43</sup>. TRPA1 can be activated by electrophiles via the covalent modification of cysteine residues on the cytoplasmic N-terminus of the channel<sup>44, 45</sup> and this explains its sensitivity to reactive species that have an innate oxidative potential or the ability to generate intracellular oxidative stress<sup>44, 46</sup>. In this present study, we confirm that H<sub>2</sub>O<sub>2</sub> was also able to depolarize the vagus nerve in a TRPA1-dependent manner<sup>47, 48</sup> and that responses to H<sub>2</sub>O<sub>2</sub> and DEP-OE were inhibited by the general anti-oxidant N-acetyl cysteine (NAC). This is in general agreement with previous studies where the effects of DEP have been inhibited by the application of NAC<sup>11, 49</sup>. DEP, like other PM, is known to have a redox potential<sup>50</sup> and these results suggest that certain organic compounds with oxidative potential within DEP-OE are activating TRPA1.

Identification of the specific compounds that are responsible for neuronal activation is important, as it may allow for strategies to be developed to produce safer diesel emissions. Although the list of compounds present in DEP-OE is extensive certain classes of compounds appear to be likely candidates for the observed activation of TRPA1. PAHs are present on the surface of DEP and are known to possess toxic and carcinogenic properties. Phenanthrene, one of the most commonly studied PAHs, is found in higher concentrations in DEP-OE compared to other chemicals. Phenanthrene depolarized the vagus nerve in a similar manner to DEP-OE, and this depolarization was blocked by a TRPA1 antagonist suggesting that this was one of the chemicals responsible for the activation of TRPA1. However, given the number of chemicals within DEP that share similar attributes, such as other PAHs or nitro-PAHs, it is unlikely to be the only activator of this pathway.

The toxic effects of PAHs are traditionally thought to be mediated by the cytosolic aryl hydrocarbon receptor (AhR), a highly conserved and expressed transcriptional regulator<sup>51, 52</sup>. Upon ligand binding, AhR is transported to the nucleus whereby it heterodimerises to the aryl hydrocarbon nuclear translocator (ARNT) and forms a complex so that the transcription of regulatory sequences that contain xenobiotic response elements can occur<sup>53</sup>. Target genes include detoxification response enzymes such as the widely studied CYP1A1 enzyme<sup>54</sup>. After transcription has occurred, AhR is transported back to the nucleus and degraded. Previous studies in other experimental systems have shown that PAHs present within DEP can activate AhR signaling cascades<sup>55-57</sup> and DEP containing greater PAH content induces greater cytotoxic responses in a human bronchial epithelial cell line<sup>58</sup>. However, typically these transcriptional events occur over time courses that span several hours, at odds with the present study, where we have shown that inhibition of AhR (either in vagal tissue from

AhR knockout mice or using small molecule inhibitors) immediately, and significantly, reduced the depolarization that occurred in response to DEP-OE and phenanthrene. As AhR inhibition had no effect on the TRPA1 agonist acrolein, it is likely that AhR is playing a role upstream of TRPA1. Interestingly, AhR has been identified in the mitochondria where it has been associated with mitochondrial reactive oxygen species (ROS) production and these effects are thought to be independent of either CYP1A1 or CYP1A2<sup>59</sup>. Vagal sensory fibers are densely packed with mitochondria<sup>49</sup>. Antimycin A, which evokes ROS from mitochondrial complex III, has been demonstrated to evoke action potential discharge from nociceptive C-fiber terminals innervating the mouse airways in a TRPA1-dependent manner<sup>60</sup>. In this study, we confirmed that antimycin A evoked vagal sensory nerve activation and that this, and the effect of DEP-OE, were inhibited by the mitochondrial superoxide scavenger MitoTEMPO. These unique findings describe for the first time, a non-transcriptional signaling pathway for AhR and a role for mitochondrial ROS in the activation of airway sensory nerves and the initiation of respiratory reflex events evoked by urban air pollution.

In order to assess the physiological relevance of the DEP-SRM used experimentally with regard real life situations we also repeated key observations with generator DEP which has been aged and diluted to mimic “real-world” occupational and environmental conditions. In these experiments depolarization of the guinea pig vagus evoked by generator DEP was inhibited by the TRPA1 antagonist or the antioxidant NAC confirming results obtained with DEP-SRM. This generator diesel has been used in controlled human exposure studies and is associated with a range of respiratory symptoms<sup>10,11</sup>.

In conclusion, in this study we demonstrate a direct interaction between DEP and airway C-fiber afferents mediated via an oxidative stress pathway and activation of the TRPA1 ion channel expressed on airway afferents. PAHs, major constituents of DEP, are implicated in this process via activation of the AhR and subsequent mitochondrial ROS production which is known to activate TRPA1 on nociceptive C-fibers. These findings provide the first mechanistic insights into how exposure to a significant component of urban particulate air pollution might precipitate respiratory symptoms such as cough and bronchospasm. A comparison between PAH content of diesel fuels and wider pollutants and TRPA1 mediated activation of airway sensory nerves will lead to further insights into the mechanisms driving the harmful effects of air pollution on the respiratory tract and mitigation strategies for those who are affected and at risk.

#### COMPETING INTERESTS

MGB and MAB are directors of an Imperial College spinout contract research company engaged in respiratory pre-clinical work. MGB is a consultant for Ario Pharma, Aboca, Patara, NeRRe, MedImmune, Boehringer Ingelheim.

#### ACKNOWLEDGEMENTS

TRPV1<sup>-/-</sup> breeding pairs were backcrossed on to the C57BL/6 background and were obtained originally from Jackson Laboratories (Bar Harbour, ME). TRPA1<sup>-/-</sup> mice were originally developed by Professor David Julius (UCSF, USA) and supplied as backcrossed 10 generations on to C57BL/6 background by Professor Peter Zygmunt from Lund University. Homozygous breeding pairs of mice genetically modified to disrupt the TRPV4 gene (Trpv4<sup>-/-</sup>; RBRC No. 01939) were obtained from Riken

BioResource Center (Tsukuba, Japan). Vagal tissue was obtained from AhR<sup>-/-</sup> mice provided as a generous gift by Dr. Rebecca Dearman from the University of Manchester (UK) and Dr Mark Hayes (Imperial College).

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## FIGURE LEGENDS

**Figure 1. DEP activates airway sensory afferents.** **(A)** Representative trace of action potential firing induced by vehicle (PBS) or DEP (10 $\mu$ g/ml, I.T.) recorded from a guinea pig, airway C-fiber afferent. **(B-C)** Peak action potential impulses induced by vehicle (PBS) or DEP in airway C-fiber (Figure 1B; n=3) and A $\delta$ -fiber afferents (Figure 1C; n=4). \*p <0.05, paired t-test. **(D)** DEP concentration response in isolated vagus nerve (n=4) \*p <0.05, repeated measures one-way ANOVA with Dunnett post hoc test compared against vehicle. **(E)** Representative trace of depolarization induced by DEP (1 $\mu$ g/ml) in isolated guinea-pig vagus nerve. **(F)** Representative trace of depolarization induced by DEP (1 $\mu$ g/ml) in isolated human vagus nerve. Data in histograms is expressed as mean  $\pm$  SEM.

**Figure 2. Physicochemical characterization of DEP.** **(A)** Cryo-electron microscopy (cryo-EM) image of DEP (1 $\mu$ g/ml, Krebs). **(B)** Size distribution of DEP (1 $\mu$ g/ml, Krebs) as measured by longest dimension, including agglomerates, derived from cryo-EM images (particle count = 394). Note that agglomerates larger than 600 nm were also present but in low numbers (See supplementary Figure 2) **(C)** Thermogravimetric analysis (TGA) weight loss profile of DEP when heated to 850 °C in air, showing (a) organic component fraction and (b) inorganic carbon fraction **(D)** Example trace and summary data of the effects of org-DEP (1 $\mu$ g/ml), par-DEP (1 $\mu$ g/ml) and DEP (1 $\mu$ g/ml) in isolated guinea-pig (n=4) and human (n=2) vagus tissue. Data expressed as mean  $\pm$  SEM for guinea-pig. Depolarisation of human vagus was assessed in response to org-DEP (0.03mV and 0.05mV) to par-DEP (0mV, 0mV) and DEP (0.06 mV, 0.04mV).

**Figure 3. Effect of TRP antagonists on DEP-OE-induced vagal sensory nerve activation. (A)**

Trace showing the effect of the transient receptor potential ankyrin-1 (TRPA1) antagonist (Janssen 130, 10  $\mu$ M) on DEP-OE (1 $\mu$ g/ml)-induced depolarization of the guinea-pig vagus nerve **(B)** Percentage inhibition of DEP-OE by vehicle (0.1% DMSO and antagonists- TRPA1- Janssen 130 (10  $\mu$ M), TRPV1 – Xention D0501 (100 nM), TRPV4 – GSK 2193874 (10  $\mu$ M)) in guinea-pig vagal tissue (n=4-7) \*p <0.05, paired t-test comparing antagonist responses to control in the same tissue. **(C)** Percentage inhibition of DEP-OE-induced responses by Jansen 130 (10  $\mu$ M) in human vagus tissue (n=3) \*p <0.05, paired t -test comparing antagonist responses to control in the same tissue. **(D)** DEP-OE depolarization in the isolated vagus nerve of TRP KO mice (n=4-6). \*p <0.05, unpaired t-test. **(E)** Effect of vehicle (0.5% methyl cellulose and 0.2% Tween in saline) or **(F)** Janssen 130 (300 mg/kg i.p) on DEP-OE (1 $\mu$ g/ml, aerosol for 60s), acrolein (10mM, aerosol for 60s) and capsaicin (100  $\mu$ M, aerosol for 15s) (n=3) -induced firing of guinea-pig, vagal C-fibers. White bars indicate peak impulses recorded immediately prior to application of agonists \*p <0.05, paired t-test. Data expressed as mean  $\pm$  SEM.

**Figure. 4. Mechanism involved in the activation of TRPA1 by DEP-OE (1 $\mu$ g/ml). (A)**

Inhibition of H<sub>2</sub>O<sub>2</sub> (10 mM) and transient receptor potential ankyrin-1 (TRPA1) agonist (300  $\mu$ M) - induced depolarization of guinea-pig isolated vagus nerve by N-acetylcysteine (NAC, 1 mM) (N=4-7) \*p <0.05, paired t-test comparing antagonist responses to control responses in the same tissue. **(B)** Percentage inhibition of DEP-OE-induced depolarization by NAC (black bars, 1 mM) in guinea-pig (n=4). Depolarization to DEP-OE of human vagus was assessed in the presence of vehicle (0.06mV, 0.04mV before compared to 0.05mV, 0.03mV after) or NAC

(0.04mV, 0.08mV before compared to 0mV, 0mV after) (n=2). **(C)** Percentage inhibition of phenanthrene (Phen; 1nM) and DEP-OE-induced depolarization by aryl hydrocarbon receptor (AhR) antagonist CH223191 (10  $\mu$ M; N=4-6) in guinea-pig isolated vagal tissue. \*p <0.05, paired t-test comparing antagonist responses to control responses in the same tissue. **(D)** Depolarization induced by DEP-OE in isolated vagal tissue from AhR<sup>-/-</sup> mice (N=3). **(E)** Inhibition of DEP-OE-induced depolarization by MitoTEMPO (2 $\mu$ M) in guinea-pig isolated vagal tissue (N=5) \*p <0.05, unpaired t-test. Data expressed as mean  $\pm$  SEM.

**Figure 5. Effect of Jan 130 and NAC on generator DEP-induced depolarization of guinea-pig vagus nerve.** Trace showing the effect of the transient receptor potential ankyrin-1 (TRPA1) antagonist Jan 130 (10  $\mu$ M) (A) or the antioxidant NAC (1 mM) (B) on generator DEP (1 $\mu$ g/ml)-induced depolarization of the guinea-pig vagus nerve. (C) Summary graph of effect of vehicle (0.1% DMSO), Jan 130 (10  $\mu$ M) and NAC (1mM) on generator DEP (1 $\mu$ g/ml)-induced depolarization. (n=3-5). \*p <0.05, paired t-test comparing antagonist responses to control in the same tissue. Data expressed as mean  $\pm$  SEM.

**Graphical Abstract Legend: Schematic diagram illustrating how diesel exhaust particles directly stimulate airway afferent nerves.** Direct interaction between DEP and airway C-fiber afferents mediated via an oxidative stress pathway and activation of the transient receptor potential ankyrin-1 (TRPA1) ion channel expressed on airway afferents. Polycyclic aromatic hydrocarbons, major constituents of DEP, were implicated in this process via activation of the aryl hydrocarbon receptor (AhR) and subsequent mitochondrial ROS production, which is

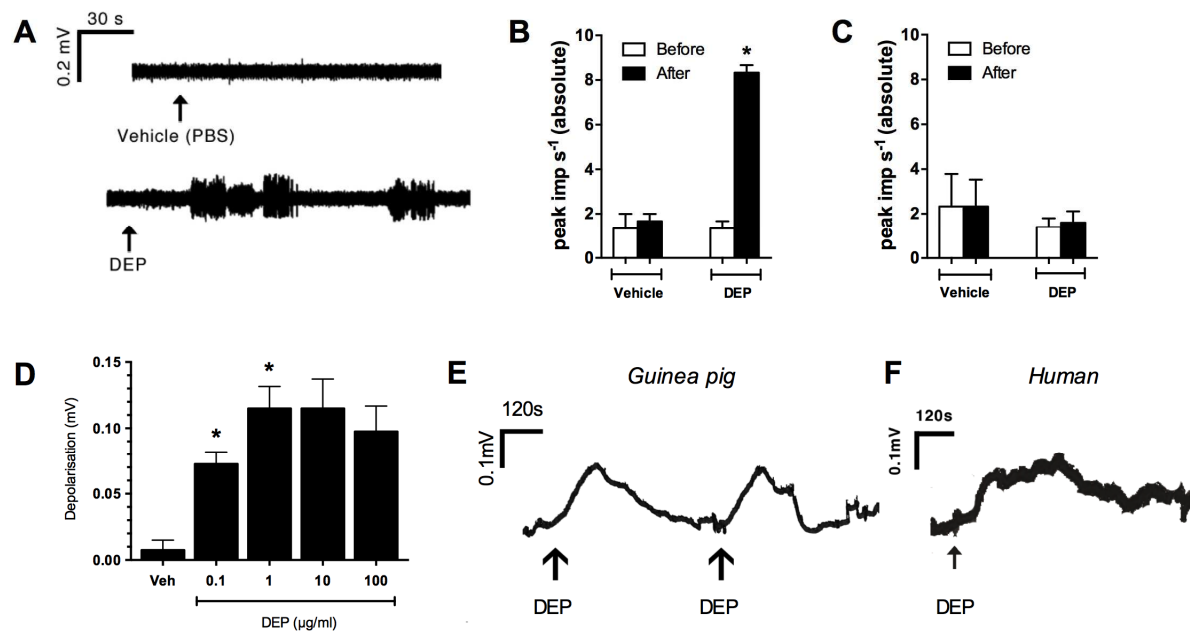
known to activate TRPA1 on nociceptive C-fibers which are known to initiate respiratory symptoms.

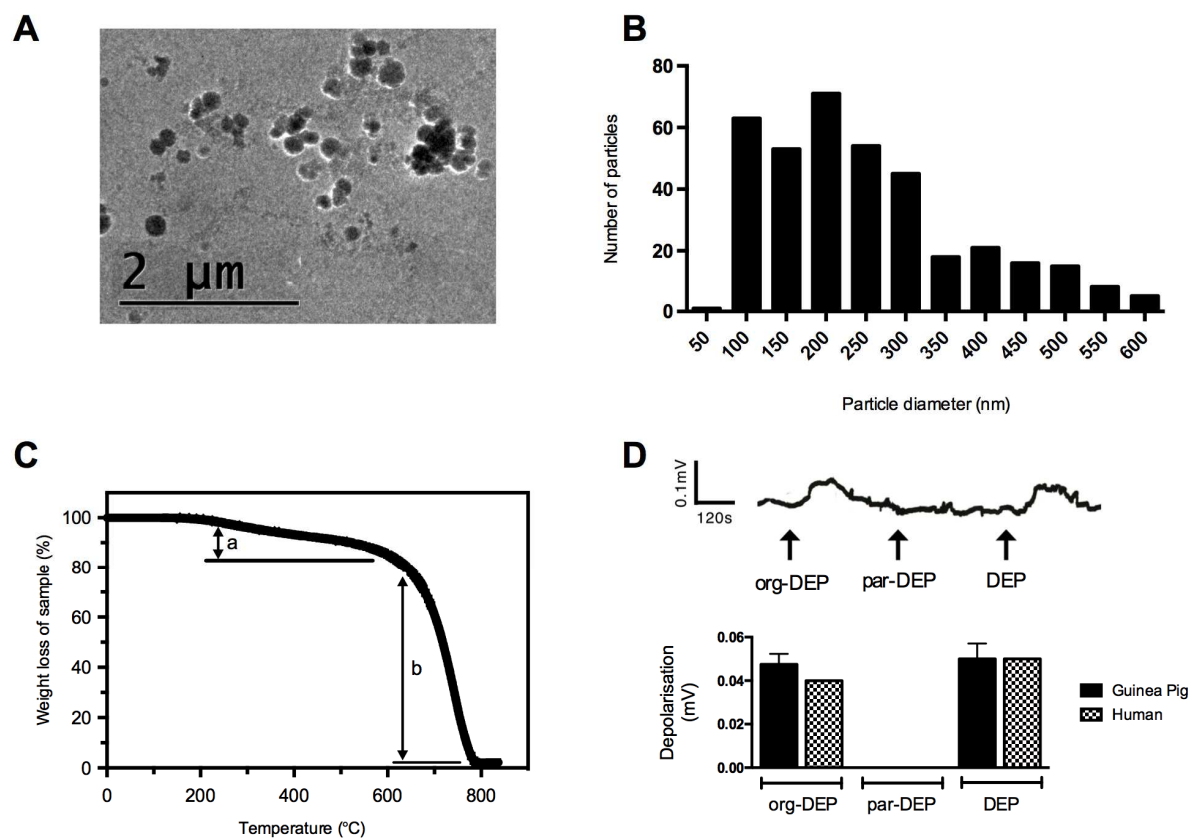
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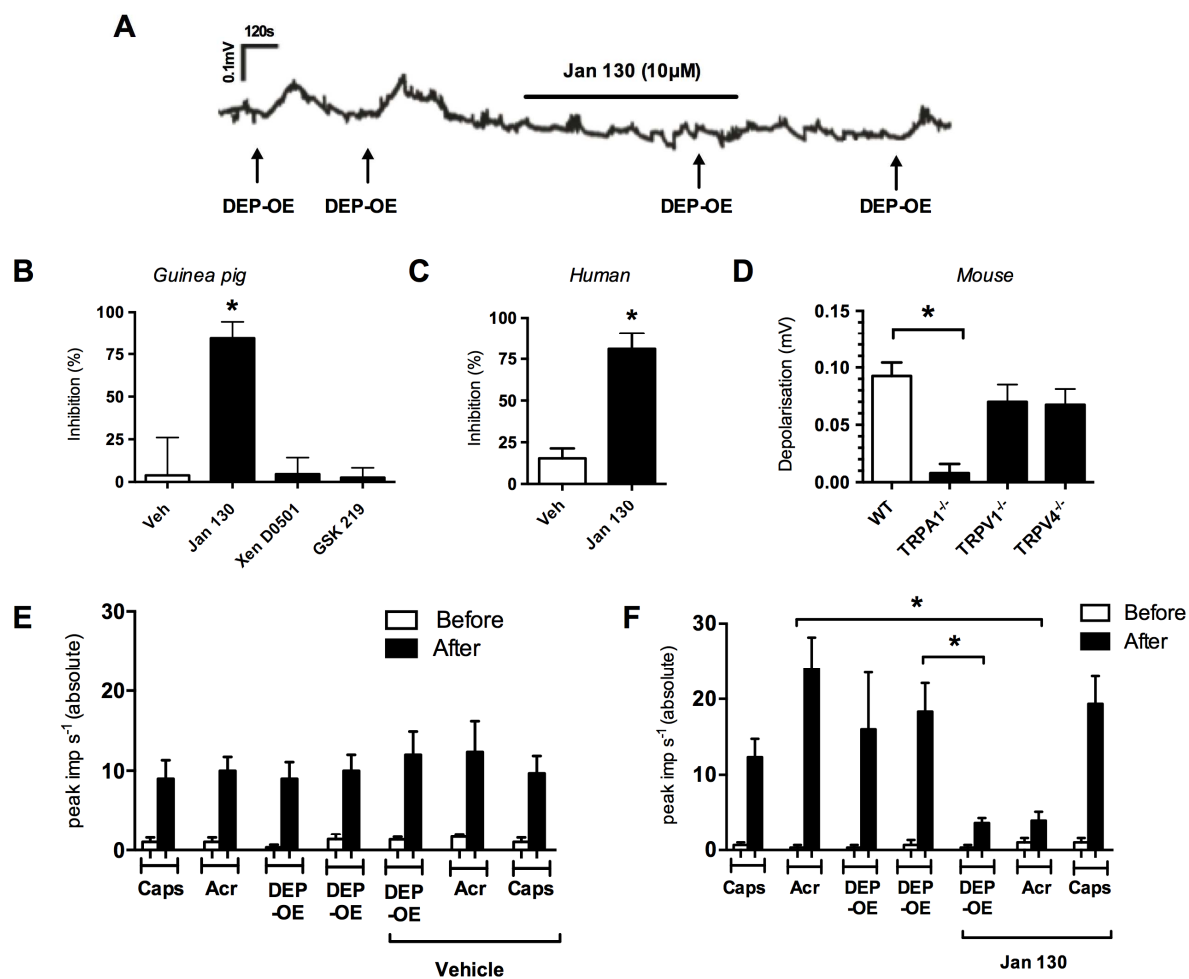
**TABLE 1****Effect of DEP on airway-specific, afferent nerve fibers *in vivo* in the guinea-pig.**

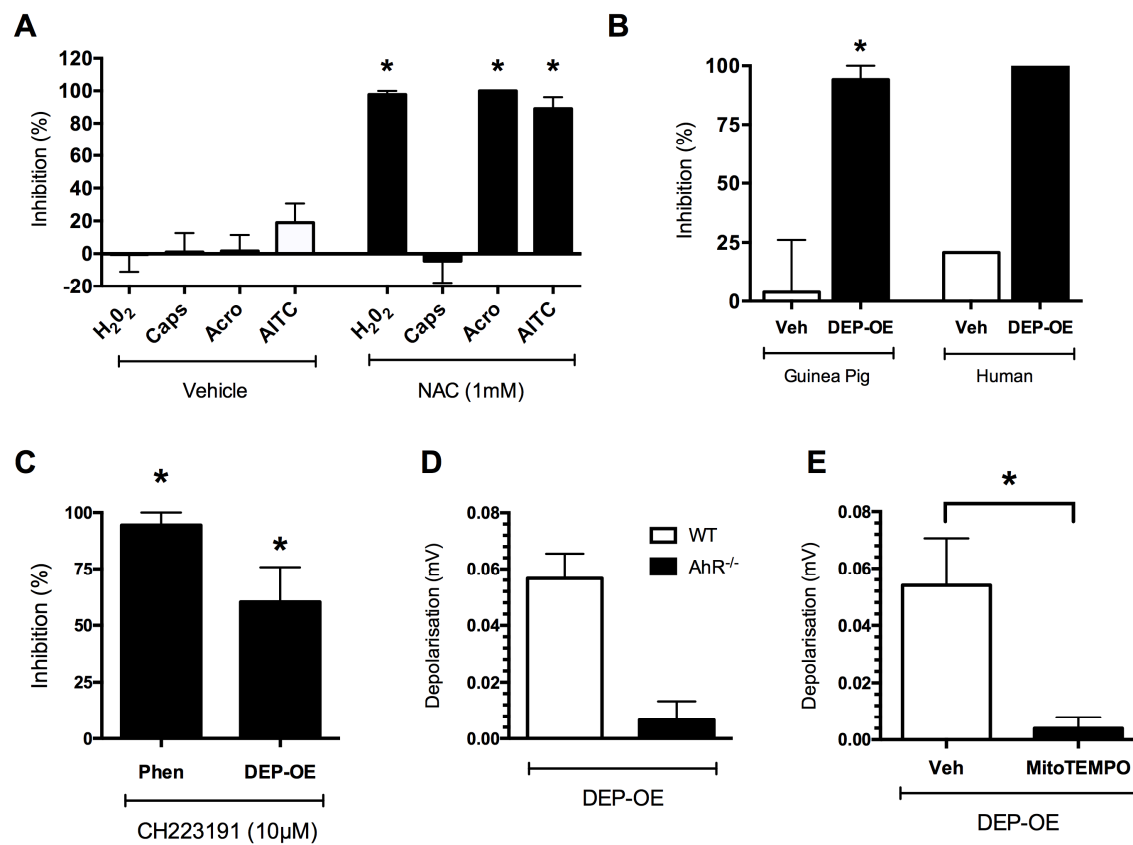
|                                     | C-Fibre<br>(CV $\leq$ 1m/s)<br>(n=3) | A $\delta$ -Fibre<br>(CV $>$ 1m/s)<br>(n=4)                                       |
|-------------------------------------|--------------------------------------|-----------------------------------------------------------------------------------|
| <b>DEP</b><br>(10 $\mu$ g/ml, I.T)  | 7.33 $\pm$ 0.88*                     | 1.6 $\pm$ 0.51                                                                    |
| Acrolein<br>(10mM, aerosol)         | 8.67 $\pm$ 1.20*                     | 1.6 $\pm$ 0.68                                                                    |
| Capsaicin<br>(100 $\mu$ M, aerosol) | 8.33 $\pm$ 0.33*                     | (Capsaicin sensitive)(Capsaicin insensitive)<br>11.3 $\pm$ 4.05* 1.27 $\pm$ 0.37* |

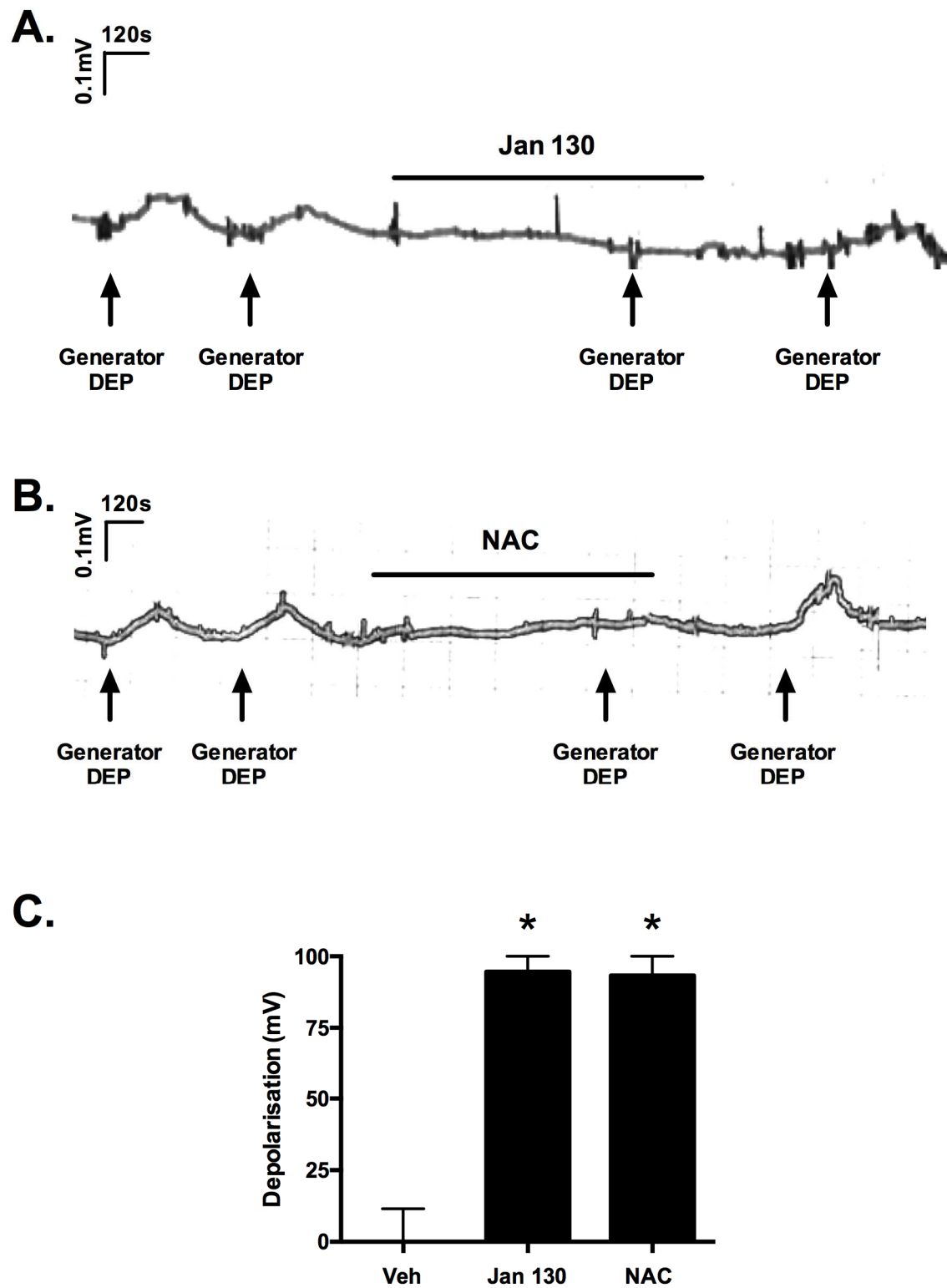
Table 1 shows peak action potential impulses (imp s<sup>-1</sup>) induced by DEP (10 $\mu$ g/ml, I.T.), acrolein (10mM in saline, aerosolized for 60s) or capsaicin (100 $\mu$ M in saline, aerosolized for 15s) recorded from airway C-fiber afferent or A $\delta$ -fiber afferents. Data was analysed by paired t-test, comparing responses (absolute values) after stimulus to baseline values immediately preceding the response. Data are presented as mean  $\pm$  s.e.m., with statistical significance set at P<0.05.











## ONLINE SUPPLEMENT

**Mechanistic Link between Diesel Exhaust Particles and Respiratory Reflexes**

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Chris Carlsten MD<sup>7</sup>, Teresa D Tetley PhD<sup>6, 8\*</sup>, Maria G Belvisi PhD<sup>1, 2\*</sup>.

## DETAILED METHODOLOGY

Male Dunkin-Hartley guinea-pigs (Harlan, Bicester, United Kingdom) were used, weighing 300–500 g for *in vitro* experiments and 400-750g for *in vivo* single fiber experiments. Male C57BL/6 mice (Harlan, United Kingdom) weighing 18-20 g were used as a background to produce wild type (WT) mice. TRPV1<sup>-/-</sup> breeding pairs were backcrossed on to the C57BL/6 background and were obtained originally from Jackson Laboratories (Bar Harbour, ME). TRPA1<sup>-/-</sup> mice were originally developed by Professor David Julius (UCSF, USA) and supplied as backcrossed 10 generations on to C57BL/6 background by Professor Peter Zygmunt from Lund University. Homozygous breeding pairs of mice genetically modified to disrupt the TRPV4 gene were obtained from Riken BioResource Center (Tsukuba, Japan)<sup>1,2</sup>. Vagal tissue was obtained from AhR<sup>-/-</sup> mice provided as a generous gift by Dr. Rebecca Dearman from the University of Manchester (UK) and Dr Mark Hayes (Imperial College). Genetic knockouts were confirmed by genotyping. All animals were housed in a climate controlled room (21 °C) with food and water freely available for at least 1 week before commencing experiments. All experiments were performed in accordance with the U.K. Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act of 1986 and the ARRIVE guidelines<sup>3</sup>.

## Human Tissue and Ethics

Human lung and trachea surplus to transplant requirements (N=3, 56-73 years old, 1 male/2 female, 1 smoker/2 non-smokers), with the vagus nerve still attached, were used to obtain translational data to compliment data generated in guinea pig tissue. Briefly, the vagus tissue was dissected from around the trachea and airways and dissected under microscope to produce sections suitable for *in vitro* experiments (see below). Tissue was provided by the International Institute for the Advancement of

Medicine (IIAM, Edison, New Jersey, USA). In all cases the tissue was approved for use in scientific research and ethical approval was obtained from the Royal Brompton & Harefield Trust.

### **Compounds and Materials**

Diesel exhaust particles from a forklift truck (DEP - SRM-2975) and its commercial organic extract (DEP-OE - SRM-1975) were purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, USA). Capsaicin (TRPV1 agonist), acrolein (TRPA1 agonist), allyl isothiocyanate (AITC – TRPA1 agonist), citric acid (CA), methyl cellulose, phosphate buffered saline (PBS), hydrogen peroxide (30% v/v; H<sub>2</sub>O<sub>2</sub>), n-acetyl cysteine (NAC), CH223191, antimycin A, (2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (Mito-TEMPO), Polymyxin B sulfate salt, dichloromethane (DCM) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Company Ltd (Dorset, UK). 6,2',4'-Trimethoxyflavone (TMF) was purchased from Tocris Bioscience (Bristol, UK). Janssen 130 (Jan 130 – TRPA1 antagonist) corresponding to the compound 130 of the patent WO2010/141805 A1 and GSK2193874 (GSK219 - TRPV4 antagonist) were synthesised by the Medicinal Chemistry Department in Almirall (Almirall S.A., Barcelona, Spain). Phenanthrene was a generous gift from Dr. Ian Mudway (Kings College London, UK). Xention D0501 (TRV1 antagonist) was provided by Ario Pharma Ltd (Cambridge, UK). Drugs were made up in stock solutions using DMSO, with the final concentration of DMSO kept at 0.1% for experiments.

### **Particle Suspensions**

Particle suspension solutions were freshly prepared daily. Suspensions of DEP or par-DEP were prepared in a modified Krebs-Henseliet solution (Krebs, in mM: NaCl 118; KCl 5.9; MgSO<sub>4</sub> 1.2;

NaH<sub>2</sub>PO<sub>4</sub> 1.2; CaCl<sub>2</sub> 2.5; glucose 6.6; NaHCO<sub>3</sub> 25.5,) by sonication in a water bath for 10 minutes, before dilution to working concentrations. For *in vivo* experiments, suspensions were prepared in PBS in a similar manner.

#### ***Physiochemical characterization of DEP***

##### ***Electron Microscopy***

The cryo preparation was done using a Leica GP automatic plunge freezer (Leica Microsystems, Milton Keynes). Nanoparticles, dispersed in (1µg/ml in Krebs), were dropped onto a grid and frozen by rapidly plunging into liquid ethane. These were transferred in their frozen state into a cryo-rod (Gatan 914) and then into the electron microscope. The electron microscopy was carried out on a Jeol 2100F equipped with a Schottky Field Emission Gun (FEG) and operated at 200kV.

For chemical analysis, DEP samples were dispersed by sonication in ethanol and then pipetted onto a grid at room temperature. TEM and Energy Dispersive X-ray Spectroscopy (EDX) analysis was performed on an FEI Titan 80–300 (S)TEM operated at 300 kV, fitted with a Cs (image) corrector and SiLi EDX spectrometer (EDAX, Leicester UK). EDX was carried out by condensing the electron beam onto the region of interest and acquiring spectra for 180 seconds.

##### ***Thermogravimetric Analysis***

The organic/inorganic ratio composition of SRM 2975 was assessed using thermogravimetric analysis (TGA) using a Perkin Elmer Pyris 1 machine (PerkinElmer Inc., Beaconsfield, UK), by heating  $1.8 \pm 0.2$  mg of DEP to 100 °C, under air flow (flow rate 10 mL/min), and holding isothermally for 30 minutes to

remove residual water and/or solvent; the temperature was then increased from 110 °C to 850 °C at a constant ramping rate of 10 °C/min under air flow (10 mL/min).

#### ***Dynamic Light Scattering***

Dynamic light scattering (DLS) measurements were carried out using a ZetaSizer Nano ZS (Malvern, UK). DEP samples were prepared in Krebs solution at 0.1µg/ml and 1µg/ml sonicated briefly in a sonicating water bath immediately before being placed into disposable micro cuvettes. Samples were warmed to 37°C and left to equilibrate for 60 seconds prior to DLS measurements, which were carried out at a 173° backscatter angle and in triplicate for each sample. Measurement run durations were automatically calculated on a per sample basis.

#### ***Component separation DEP***

1 g of DEP was loaded into an extraction thimble and extracted overnight with dichloromethane (DCM) using Soxhlet extraction equipment at 40°C. The extract solvent was then evaporated by rotary evaporator, and reconstituted in DMSO up to a volume of 10ml. This resulted in an 'equivalent concentration' of organic extract of '0.1g/ml', that represented the organic material present in 1g of DEP now being present in 10ml of DMSO. The resulting DEP organic extract (org-DEP) was centrifuged at 10,000g for 20 minutes to remove remaining particulates and supernatant was carefully removed. DEP-OE was then diluted in DMSO and stored at -80°C until needed. The remaining particles left behind in the paper filter was heated gently overnight to completely evaporate any remaining DCM, and were then kept as cleaned particles (par-DEP).

***In vivo recording of action potential firing in single-fiber afferents.***

Guinea-pigs were anaesthetized with urethane (1.5 g/kg) intraperitoneally. If required, anaesthesia was supplemented with additional urethane. The trachea was cannulated with a short length of Portex tubing and blood gases and pH were maintained at physiological levels by artificial ventilation (Ugo Basile small animal ventilator), with a tidal volume of 10 ml kg<sup>-1</sup> and 50-60 breaths min<sup>-1</sup> of laboratory air. The right jugular vein and carotid artery (passed to the ascending aorta/aortic arch) were cannulated for respectively injecting drugs and measuring systemic arterial blood pressure. Systemic arterial blood pressure and heart rate were continuously recorded using a transducer (Gould P23XL). Tracheal pressure was measured with an air pressure transducer (SenSym 647) connected to a side arm of the tracheal cannula. Body temperature was continuously monitored with a rectal thermometer and maintained at 37°C with a heated blanket and control unit (Harvard Apparatus). Animals were paralysed with vecuronium bromide, initially administered at a dose of 0.10 mg/kg, i.v., followed every 20 min with 0.05 mg/kg, i.v. to maintain paralysis. The depth of anaesthesia was frequently assessed by monitoring the response of heart rate and blood pressure to noxious stimuli. Both cervical vagus nerves were located, via a cervical incision, and dissected free from the carotid artery, sympathetic and aortic nerves; both vagus nerves were cut at the central end. The left vagus nerve was used for sensory nerve fiber recording and was cleared of its surrounding fascia. The skin and muscle in the neck at either side of the incision were lifted and tied to a metal ring to form a well, which was filled with light mineral oil. Bipolar Teflon-coated platinum electrodes (exposed at the tips) were used for recording purposes, using fascia positioned on one electrode for a reference. The vagus nerve was placed on a small black perspex plate to facilitate subsequent dissection. Thin filaments of nerve were teased from the vagus nerve, under a binocular microscope, and placed on the second electrode until a single active unit, or one of not more than two or three units, was obtained. Action

potentials were recorded in a conventional manner using electrodes connected to a pre-amp headstage (Digitimer NL100K). The signal was amplified (x1000-5000, Digitimer NL104), filtered (in the range of LF30Hz – HF8.5kHz, Digitimer NL125) and passed through a Humbug noise reducer (AutoMate Scientific) before input sampling and recording. All signals were sampled (50 kHz) and recorded using the Spike 2 software data acquisition system via a CED Micro1401 interface. The software allowed pulse train counting over selected time periods. In addition, monitoring of the input signal to the Spike software was also carried out on a digital storage oscilloscope (Tektronix DPO 2012). The input signal was also fed through an audio amplifier to a loud speaker.

Aerosols were generated by an Aerogen nebulizer (Buxco Nebulizer Control – 5) connected to the ventilator and arranged so that the inspired air passed through the medication chamber before entering the lungs of anaesthetized animals via the tracheal cannula.

Single vagal nerve fibers were identified as originating from the major groups of airway sensory nerve endings, i.e., slowly adapting stretch receptors (SARs), rapidly adapting stretch receptors, (RARs, irritant receptors, A $\delta$ -fibers – further subdivided into those which were more acid and/or less capsaicin sensitive and with CVs slower than conventional RARs), and pulmonary/bronchial C-fiber receptors using several criteria<sup>4</sup>. These include pattern of spontaneous discharge, response to hyperinflation and deflation, adaptation indices (AIs), response to capsaicin/citric acid administration and conduction velocities. As a rule, a receptor that had no obvious pattern to the spontaneous activity (often very sparse), did not respond to hyperinflation/hyperdeflation but responded to capsaicin aerosol was pursued as a C-fiber. Alternatively, and, in the first instance, a receptor that had

a spontaneous discharge with a definite rhythmical respiratory pattern and adapted rapidly/variably to hyperinflation/deflation was pursued as an A $\delta$ -fiber.

Following identification of a suitable single nerve fiber, control responses were obtained to capsaicin (100 $\mu$ M in saline, aerosolized for 15s), acrolein (10mM in saline, aerosolized for 60 s) and citric acid (300mM, aerosolized for 60s). The nerve under investigation were then challenged with either vehicle (PBS, 200 $\mu$ L) or DEP (10  $\mu$ g/ml in PBS, 200 $\mu$ L, intratracheal dose) and subsequent action potentials recorded. For antagonist studies, control responses were obtained to capsaicin (100 $\mu$ M in saline, aerosolized for 15s), acrolein (10mM in saline, aerosolized for 60s) and DEP-OE (1  $\mu$ g/ml in saline, aerosolized for 60s) prior to the introduction of Janssen 130 (30mg/kg, 1% methyl cellulose in saline) into the animal via intravenous route 60 minutes before challenging again with capsaicin, acrolein and DEP-OE.

At the end of the experiment, the conduction velocity of the single nerve fiber was measured to determine whether it was a slow conducting non-myelinated C-fiber or a fast conducting myelinated A $\delta$ -fiber. This was achieved by stimulating the vagus nerve close to the thorax using a supra threshold voltage (Grass stimulator, 0.5 ms, 1Hz). The corresponding action potential was recorded in the single nerve fiber under observation, and the time between the initial stimuli and the resulting action potential was calculated using the Spike 2 software and used with the measured distance between the two electrodes to calculate the conduction velocity. All animals were culled at the end of experiments with an overdose of pentobarbitone.

***In vitro measurement of isolated vagus nerve depolarization***

Guinea-pigs and mice were sacrificed by injection of sodium pentobarbitone (200 mg/kg i.p.) and human vagus obtained from IIAM. The vagus nerves were dissected and experiments conducted in a fully characterised isolated vagus preparation as described in previous publications<sup>5,6</sup>. The two vagus nerve trunks were then placed in Krebs bubbled with 95% O<sub>2</sub>/ 5% CO<sub>2</sub> to stay oxygenated. The vagus tissue was then cleaned of connective tissue before being cut into 15-20mm segments. These segments were then mounted into a 'grease-gap' dual recording chamber system, whereby the nerve was threaded through a narrow cylindrical gap between a test chamber and a reference chamber. The ends of the nerve exposed in each chamber were then electrically and chemically isolated by the application of petroleum jelly into the cylindrical gap through a side arm. The end of the nerve exposed in the test chamber was then constantly perfused at a rate of 2 ml/min with oxygenated Krebs heated to 37°C using a custom designed water jacket housing. Borosilicate glass electrodes mounted onto an Ag/AgCl pellet (World Precision Instruments, USA) were filled with Krebs and placed into contact with the nerve in both the test chamber and the reference chamber. Changes in the surface charges between the test and reference chamber were then recorded using an extracellular potential differential amplifier (DAM 50 Bioamplifier, World Precision Instruments, USA). Nerve depolarizations were filtered at 0.3 kHz, amplified x50, and recorded onto a chart recorder (Lectromed Multi-Trace 2). During experiments, the perfusate through the test chamber of the vagus was maintained at 37°C with a rate of 2 ml/min and a change of drug containing solution was made through a manifold. Subsequent vagal depolarization were recorded in response to various stimuli applied for 2 minutes. For general antagonist studies, two repeatable baseline responses to agonists were first obtained before pretreatment with an antagonist for 10 minutes prior to the two minute application of the agonist in the presence of the antagonist. After a ten minute wash out, a recovery

response to an agonist was then obtained to confirm nerve viability at the end of the experiment. If a recovery response could not be obtained, the data was disregarded. For mitoTEMPO antagonist studies utilising antimycin A, an irreversible binding electron transport chain inhibitor, and other agonists, nerve viability was confirmed at the beginning of the experiment using acrolein (300 $\mu$ M), before a single application of agonists with or without antagonist present. Responses were compared across different tissues from the same animal.

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## FIGURE LEGENDS

### **Supplementary Figure 1. Effect of TTX on DEP-induced depolarization of guinea-pig vagus nerve. A)**

Representative trace showing the effect of TTX (3 $\mu$ M) on DEP (1 $\mu$ g/ml) -induced depolarization of guinea-pig vagus nerve. Two control responses were obtained to DEP (1 $\mu$ g/ml), then DEP in the presence of TTX and finally recovery of the response to DEP following washout. **B)** Summary bar graph showing initial depolarization to DEP (left column), followed by response obtained in the presence of TTX (3  $\mu$ M; all values 0mV) and a recovery response following wash out (right column) (n=4). Data expressed as mean  $\pm$  SEM. \*p <0.05 as calculated by a paired t-test.

### **Supplementary Figure 2. Size characterisation of DEP. (A) Size distribution of DEP (1 $\mu$ g/ml, Krebs) as**

measured by longest dimension, including agglomerates, as analysed by images produced by cryo-EM. Particles were measured by longest length. (n=394). **(B)** Dynamic light scattering (DLS) hydrodynamic diameter measurements of DEP in Krebs solution (n=3). Data expressed as mean  $\pm$  SEM.

### **Supplementary Figure 3. Metal species characterisation of DEP. (A) Transmission electron**

microscopy–energy dispersive X-ray (TEM-EDX) spectrum showing the element analysis profile of a blank carbon film area as a control, with **(B)** the analyzed blank area of interest depicted. **(C)** TEM-EDX spectrum showing the profile of detected elements in DEP sample, with **(D)** the analyzed sample of

interest depicted. The trace of copper X-ray signals and part of the carbon signals are attributed to the holey carbon film coated copper TEM grid and sample holder.

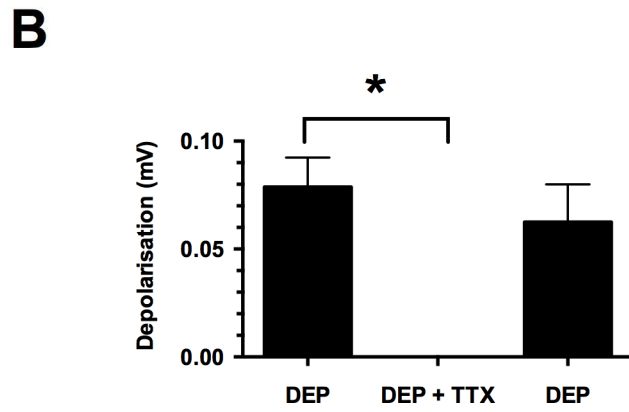
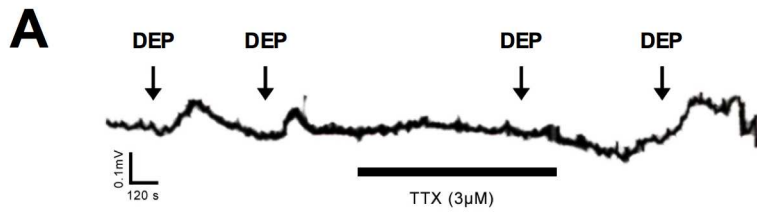
**Supplementary Figure 4. Effect of DEP-OE on isolated guinea-pig vagus.** Concentration dependent depolarization induced by DEP-OE in guinea pig isolated vagus nerve. Arrow indicates concentration selected for subsequent antagonist studies. (n=4-5).

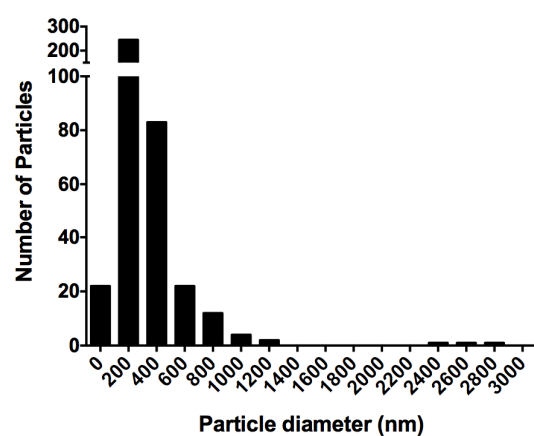
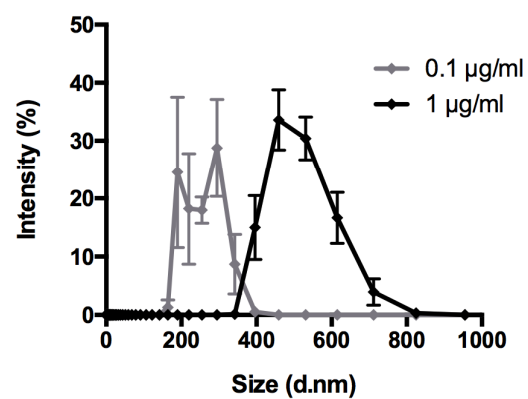
**Supplementary Figure 5. Depolarization induced by H<sub>2</sub>O<sub>2</sub> on isolated vagus nerve and effect of pharmacological interventions. A)** Concentration response showing depolarization induced by H<sub>2</sub>O<sub>2</sub> in guinea-pig isolated vagus nerve. Arrow indicates concentration selected for subsequent antagonist studies. (n=5-6). **B)** Effect of vehicle (0.1% DMSO), Janssen 130 (10 µM, all values 0mV) and Xention D0501 (100 nM) on H<sub>2</sub>O<sub>2</sub>-induced depolarization. (n=3-5). \*p <0.05, paired t test comparing antagonist responses to control responses in the same tissue. **C)** Effect of H<sub>2</sub>O<sub>2</sub> on depolarization in vagal tissue from WT (white) and TRPA1<sup>-/-</sup> mice (black) (n=2-3). Data expressed as mean ± SEM.

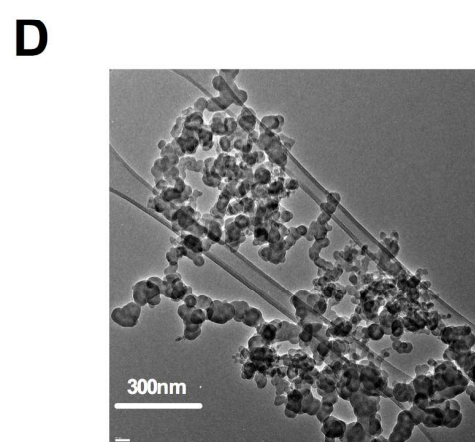
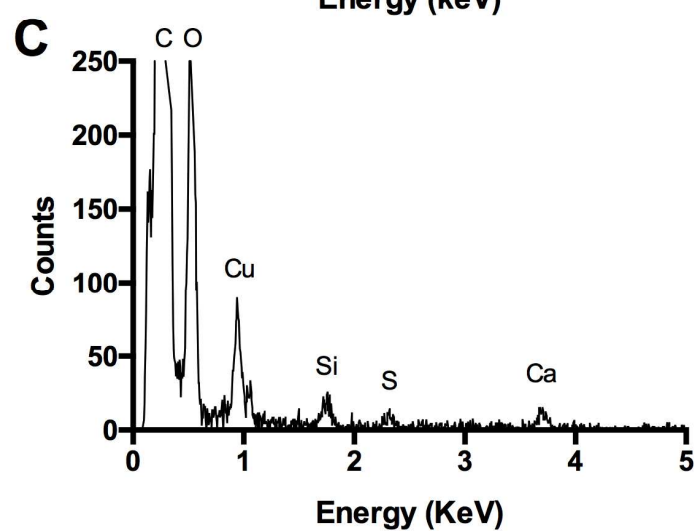
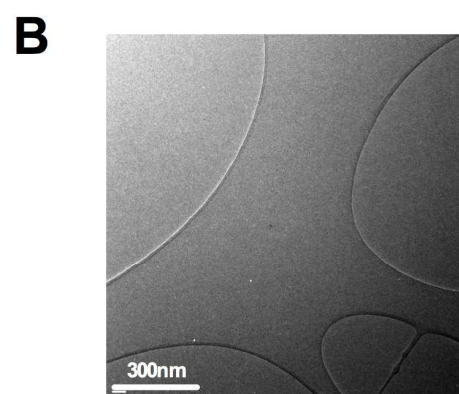
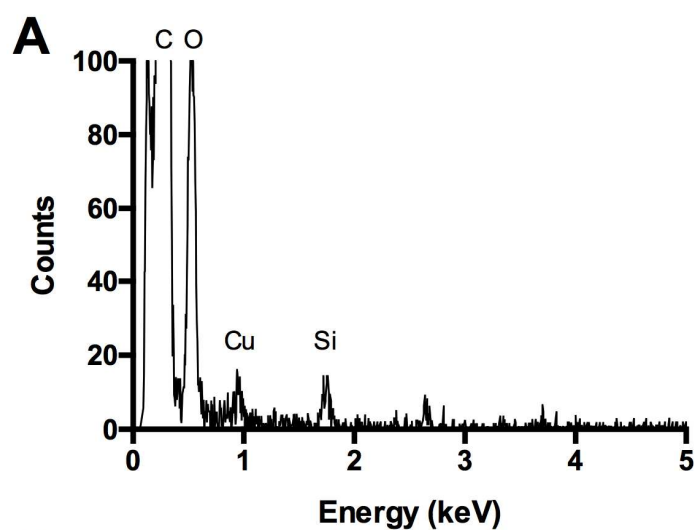
**Supplementary Figure 6. Effect of TMF on phenanthrene and DEP-OE-induced depolarisation of vagus nerve.** Effect of TMF (10µM) on phenanthrene (1nM, n=4) and DEP-OE-induced depolarisation. (n=6). \*p <0.05, paired t test comparing antagonist responses to control responses in the same tissue. Data expressed as mean ± SEM.

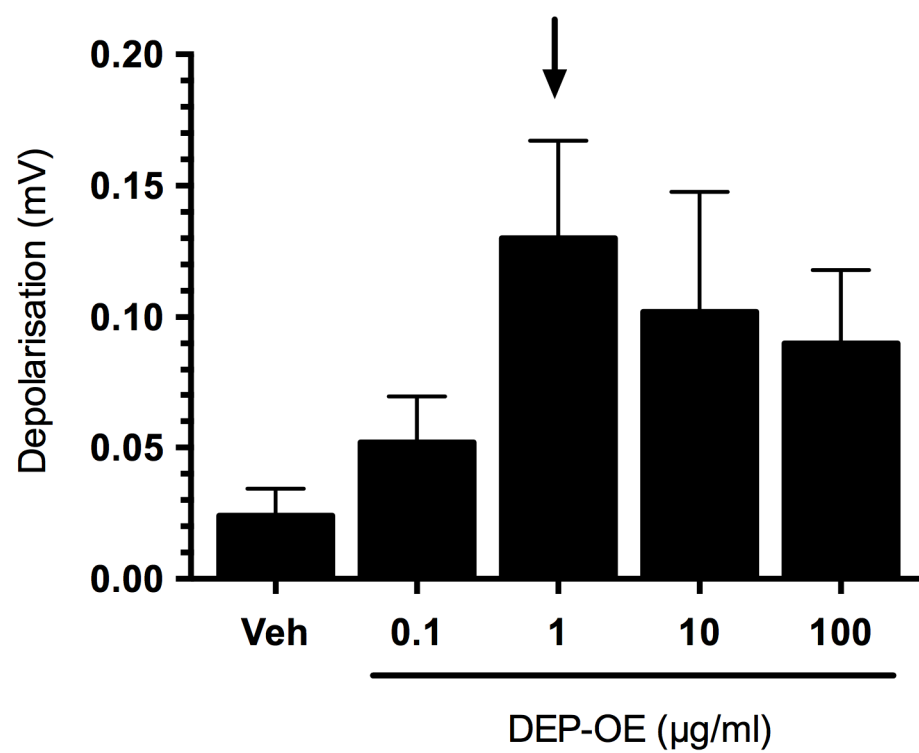
**Supplementary Figure 7. Effect of MitoTEMPO on antimycin A-induced depolarisation of vagus nerve. A)** Concentration response showing depolarization induced by antimycin A in guinea pig isolated vagus nerve. Arrow indicates concentration selected for subsequent antagonist studies. (n=5-

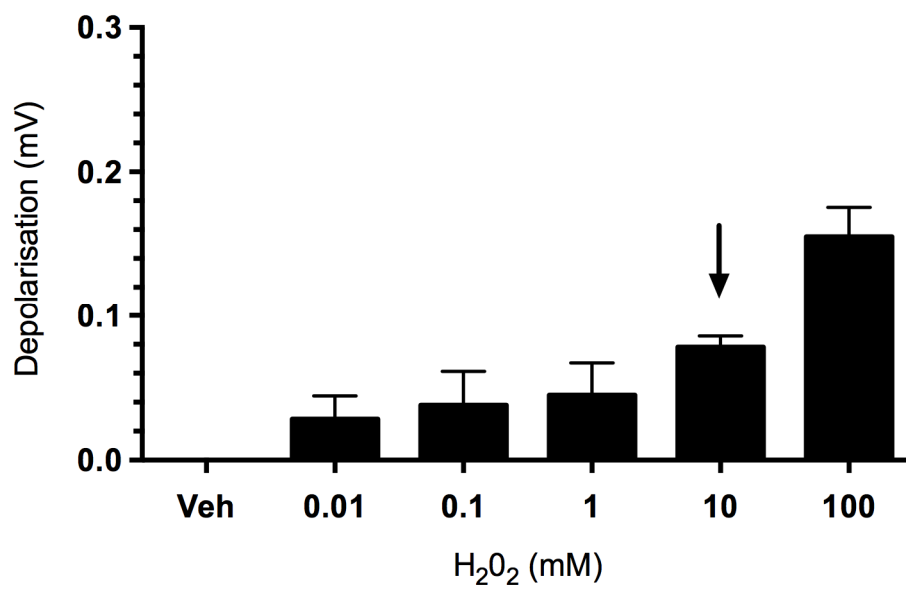
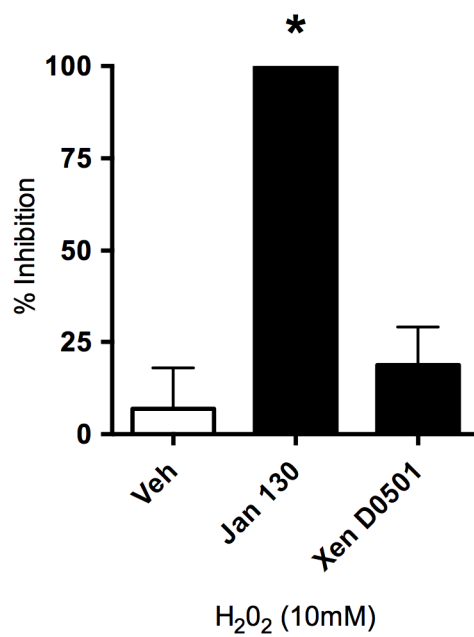
275 6). **B)** – Effect of vehicle (0.1% DMSO) and MitoTEMPO (2 $\mu$ M; all values 0mV) on antimycin A (20 $\mu$ M)-  
276 induced depolarization. (n=4). Data expressed as mean  $\pm$  SEM. \*p <0.05, unpaired two tailed t-test.



**A****B**





**A.****B.****C.**