RESEARCH PAPER

Influence of inflammation and nitric oxide upon platelet aggregation following deposition of diesel exhaust particles in the airways

Correspondence Dr Michael Emerson, Molecular Medicine Section, National Heart and Lung Institute, Imperial College London, Sir Alexander Fleming Building, Exhibition Road, London SW7 2AZ, UK. E-mail: m.emerson@imperial.ac.uk

Received 24 November 2016; Revised 7 April 2017; Accepted 13 April 2017

E Smyth1, A Solomon1, M A Birrell2, M J Smallwood3, P G Winyard3, T D Tetley4 and M Emerson1

1Platelet Biology Group, National Heart and Lung Institute, Imperial College London, London, UK, 2Respiratory Pharmacology, National Heart and Lung Institute, Imperial College London, London, UK, 3Inflammation Research Group, University of Exeter Medical School, Exeter, UK, and 4Lung Cell Biology Group, National Heart and Lung Institute, Imperial College London, London, UK

BACKGROUND AND PURPOSE
Exposure to nanoparticulate pollution has been implicated in platelet-driven thrombotic events such as myocardial infarction. Inflammation and impairment of NO bioavailability have been proposed as potential causative mechanisms. It is unclear, however, whether airways exposure to combustion-derived nanoparticles such as diesel exhaust particles (DEP) or carbon black (CB) can augment platelet aggregation in vivo and the underlying mechanisms remain undefined. We aimed to investigate the effects of acute lung exposure to DEP and CB on platelet activation and the associated role of inflammation and endothelial-derived NO.

EXPERIMENTAL APPROACH
DEP and CB were intratracheally instilled into wild-type (WT) and eNOS−/− mice and platelet aggregation was assessed in vivo using an established model of radio-labelled platelet thromboembolism. The underlying mechanisms were investigated by measuring inflammatory markers, NO metabolites and light transmission aggregometry.

KEY RESULTS
Platelet aggregation in vivo was significantly enhanced in WT and eNOS−/− mice following acute airways exposure to DEP but not CB. CB exposure, but not DEP, was associated with significant increases in pulmonary neutrophils and IL-6 levels in the bronchoalveolar lavage fluid and plasma of WT mice. Neither DEP nor CB affected plasma nitrate/nitrite concentration and DEP-induced human platelet aggregation was inhibited by an NO donor.

CONCLUSIONS AND IMPLICATIONS
Pulmonary exposure to DEP and subsequent platelet activation may contribute to the reports of increased cardiovascular risk, associated with exposure to airborne pollution, independent of its effects on inflammation or NO bioavailability.

Abbreviations
BALF, bronchoalveolar lavage fluid; CB, carbon black; DEP, diesel exhaust particles; eNOS, endothelial NOS; eNOS−/−, endothelial NOS knockout mice; MI, myocardial infarction; PM, particulate matter
Introduction

Exposure to ambient particulate matter (PM) is associated with thrombotic events such as myocardial infarction (MI) and stroke (Peters et al., 2001). The PM 0.1 fraction of air pollution, which includes diesel exhaust particles (DEP) and carbon black (CB), is principally combustion-generated from vehicle emissions and is strongly implicated as a causative risk factor for these cardiovascular events (Andersen et al., 2010). Platelet activation and aggregation are important drivers of the thrombotic events associated with exposure to PM 0.1 (Nemmar et al., 2003a; Lucking et al., 2008; Solomon et al., 2013).

Lung exposure to DEP has been shown to enhance experimental thrombosis and platelet activation in both animal models (Nemmar et al., 2003a,b) and man (Lucking et al., 2008). Exposure to both DEP and CB has been associated with inflammation (Nemmar et al., 2003a,b; Gilmour et al., 2004; Lucking et al., 2008) and initiation of pulmonary and systemic inflammation has been proposed as a mechanism by which PM 0.1 inhalation may promote platelet aggregation and thrombosis (Oberdorster et al., 1992; Mills et al., 2005). IL-6 has been particularly associated with diesel exhaust (DE) or DEP exposure in both rodents (Robertson et al., 2012; Conklin, Kong, and Committee, 2015) and humans (Krishnan et al., 2013). In addition, leukocyte infiltration in the lungs is widely reported following exposure to DEP (Oberdorster et al., 1992; Nemmar et al., 2003b; Robertson et al., 2012; Xu et al., 2013). In contrast, DEP was able to accelerate thrombosis in rats without evidence of pulmonary or systemic inflammation (Tabor et al., 2016) so that the role of inflammation in driving DEP-associated cardiovascular risk remains unclear.

As an alternative to the inflammation mechanism, it has been reported that nanoscale materials can translocate across the pulmonary epithelial barrier and enter the circulation. Thus, nanoparticles may interact directly with blood components including platelets (Nemmar et al., 2001; Oberdorster et al., 2002; Kreyling et al., 2009). Previous work by us showed that DEP can physically interact with isolated platelets leading to activation and aggregation as well as enhancement of aggregation at lower concentrations (Solomon et al., 2013). Furthermore, systemic administration of DEP by the i.v. route, to mimic a scenario in which PM 0.1 had traversed the lung epithelium, caused enhanced platelet aggregation in vivo (Solomon et al., 2013) and thrombogenesis (Tabor et al., 2016). Binding of DEP to glycoprotein VI (GPVI) and/or C-type lectin-like receptor-2 (CLEC-2) has been suggested to be an underlying mechanism behind DEP-induced platelet aggregation (Alshehri et al., 2015).

The in vivo experimental models of thrombosis primarily used to date are vascular injury models and ex vivo preparations which involve multifactorial thrombotic processes and do not distinguish specific impacts on platelets. Although DEP administered i.v. has been shown to increase platelet aggregation in vivo, the distinct effect on platelet aggregation following administration to the airways remains unclear.

It is reported that DEP can reduce the bioavailability of NO possibly via uncoupling of endothelial NOS (eNOS) or increased oxidative stress (Knuckles et al., 2008; Langrish et al., 2013; Wauters et al., 2013). As NO generated in the vascular endothelium is a major endogenous negative regulator of platelet activation (Emerson et al., 1999; Moore et al., 2010; Moore et al., 2011), reduced NO bioavailability following exposure to DEP could hypothetically lead to enhanced platelet activation.

We hypothesized that acute exposure of the mouse airways to DEP or CB would modulate platelet aggregation in vivo. We used an established mouse pharmacological model of radiolabelled (with [111In]) platelet aggregation to explore our hypothesis and evaluated whether any observed effects on platelet aggregation were associated with pulmonary leukocyte infiltration, IL-6-mediated inflammation or related to NO activity.

Methods

Preparation of washed human platelets

Informed consent was obtained from all donors and the procedures were approved by the NHS National Research Ethics Service. Washed platelet suspensions were prepared from citrated blood from consenting, healthy, aspirin-free, male and female human donors (23–55 years) as previously described (Jones et al., 2010).

Light transmission aggregometry

Human platelets were stimulated with either DEP at concentrations previously shown to induce concentration-dependent aggregation (12–50 μg·mL⁻¹) (Solomon et al., 2013), collagen (5 μg·mL⁻¹) or HEPES-buffered Tyrode’s solution (THB; composition: 134 mM NaCl, 2.9 mM KCl, 12 mM NaHCO₃, 0.34 mM Na₂HPO₄, 20 mM HEPES, 10 mM glucose and 1 mM MgCl₂; pH 7.4) and aggregation was measured in an optical aggregometer (Chrono-log Corporation, Haventown, PA, USA) for 3 min as previously reported (Solomon et al., 2013). The NO donor sodium nitroprusside (SNP) (10 μM) was incubated with platelets for 5 min prior to agonist stimulation. Having consulted the ARRIVE guidelines (Kilkenny et al., 2010), it was considered unnecessary to conduct in vivo experiments in mice on the grounds that it could be reasonably concluded that observations made in humans would apply to mice. Given that the human platelet response is the endpoint of interest, in vivo experiments in mice could not be justified from a 3Rs perspective.

Animals

All animal care and experimental procedures were performed in accordance with our Home Office licence and with authorization from the Imperial College London Ethical Review Panel. Protocols were refined in association with the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3RS) (Tymvios et al., 2008) and are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny et al., 2010; McGrath & Lilley, 2015). Male C57BL/6 mice (Harlan, Bicester, UK) between 20–25 g were used. eNOS knockout mice (eNOS⁻/⁻, strain 0026847) (18–25 g) were purchased.
Preparation of nanoparticles
DEP and CB particles were prepared in sterile saline (NaCl 0.9%) or THB and sonicated prior to use using techniques previously described and shown to generate nanoparticulate suspensions (Solomon et al., 2013).

Intratracheal instillation of diesel exhaust particles and carbon black
WT and eNOS−/− mice were anaesthetised with isoflurane (4%), and a 50 μL bolus of either saline (0.9% w/v), CB or DEP (25 μg/mouse) was administered through a Hamilton syringe® attached to an oral gavage steel feeding tube. Mice were left to recover in a surgical recovery box, for 4 h prior to the experiments.

Bronchoalveolar lavage and plasma collection and cell counting
Mice were terminally anaesthetised with urethane (10 μL·g−1 25% w/v i.p.), bronchoalveolar lavage fluid (BALF) was collected as previously described (Raemdonck et al., 2016). Total and differential cell counts were performed as previously described (Raemdonck et al., 2016). Citrated blood was collected via cardiac puncture from mice terminally anesthetized with urethane (10 μL·g−1 25% w/v i.p.). Blood was centrifuged at 300 rcf for 3 min and plasma stored at −80°C until further analysis.

In vivo platelet aggregation
A previous model of in vivo platelet aggregation was employed (Tymvios et al., 2008). Citrated blood was collected via cardiac puncture from mice (WT or eNOS−/−) terminally anesthetized with urethane (2 kg−1 i.p.). Platelets were isolated and radiolabelled with [111In] as previously described (Tymvios et al., 2008). Terminally anesthetized mice (1.5 g·kg−1 urethane i.p.) were i.v. infused with radiolabelled platelets prior to i.v. administration of collagen (50 μg·kg−1). Entrapment of platelet aggregates in the pulmonary vasculature following aggregation in the systemic circulation was quantified by recording changes in radioactive counts for 5 min with a Single Point Extended Area Ratio probe (eV products, Saxonburg, PA) positioned over the pulmonary vasculature and custom software (Mumed systems, London, UK) as previously described (Tymvios et al., 2008).

Histological analysis of nanoparticle pulmonary deposition following intratracheal instillation
Immediately following i.t. instillation mice were killed via cervical dislocation and their lungs were dissected, briefly washed in PBS and fixed in formalin overnight. Following this, samples were embedded in wax, sectioned (longitudinally) and stained (haematoxylin and eosin). The samples were viewed using a light microscope (×20 and ×40).

ELISA
WT mouse cytokines (IL-6) were quantified in cell free BALF and plasma using a standard sandwich ELISA (DuoSet ELISA kits, R&D Systems, USA) as per the manufacturer’s instructions. All samples were run in triplicate with the appropriate controls and the standard curve was run in duplicate. Cytokine concentrations were calculated using a standard curve.

Ozone chemiluminescence for measurement of plasma nitrate
Nitrate/nitrite concentrations were measured in plasma from mice treated with DEP or CB (25 μg per mouse) via i.t. instillation, using ozone chemiluminescence with a Sievers NO analyser (280; Analytix, Boldon, UK, as previously reported (Apostoli et al., 2014).

Experimental design, data and statistical analysis
Data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). For most protocols, blinding was not feasible as experiments were conducted by an individual experimenter; however, for histological analyses, blinding was used. Unless otherwise stated, data is presented as box-and-whisker plots; the horizontal lines inside the box indicate the median. The box edges extend from the 25th to the 75th percentiles, and the whiskers represent the minimum and maximum values. Statistical analyses were performed on raw data using Prism 5 Graphpad software. Two-way analyses were conducted using a Mann–Whitney test and multiple comparisons were conducted using a Kruskal–Wallis test with Dunn’s comparison. Post hoc tests were run only if there was no significant variance in homogeneity. Power calculations were conducted (at level 0.8) to determine appropriate n numbers. A P value <0.05 was indicative of statistical significance.

Materials
Materials used were supplied as follows: [111In]indium oxine (Mallinckrodt Radiopharmacy Services, London, UK); collagen (Nycomed, Munich, Germany); DEP (SRM 2975; National Institute of Standards and Technology, Gaithersburg, MD, USA); CB (Printex-90; Degussa GmbH, Hanau, Germany); cytokine ELISA DuoSet (R&D systems, Minnesota, USA). All other materials were purchased from Sigma-Aldrich (Poole, UK) and were of analytical grade.

Nomenclature of targets and ligands
Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015a,b).
Results

Diesel exhaust particles and carbon black were deposited in the conducting airways
Because a significant proportion of PM 0.1 inhaled by humans is deposited in the distal airways (ICRP, 1994), the location of DEP (Figure 1C,F) and CB (Figure 1B,E) following i.t. instillation was examined in mice. The lung microarchitecture was similar between the saline control, DEP and CB exposed airways (Figure 1). Aggregates of CB and DEP were observed in contact with the pulmonary epithelial cells (Figure 1E,F) and conducting airways (Figure 1B,C). These data confirm that i.t. instillation of nanoparticles leads to their deposition deep in the airways.

Diesel exhaust particle deposition in the airways enhanced platelet aggregation in vivo
Having shown deposition of DEP and CB in the conducting airways, we explored whether this was associated acutely (4 h later) with an enhancement of agonist-induced platelet aggregation in vivo using a model of radiolabelled platelet thromboembolism. There was a significant increase in collagen-induced platelet aggregation in vivo measured as AUC (P < 0.05 compared to saline control) 4 h following i.t. administration of DEP (Figure 2A,C). In contrast, there was no significant enhancement of the AUC (Figure 2B,D; P > 0.05 compared to saline control) following administration of CB.

Carbon black exposure induced pulmonary and systemic inflammation
Having shown contrasting impacts of DEP and CB upon in vivo platelet aggregation, we investigated whether these effects were associated with changes in inflammatory markers. A significant increase in both neutrophils and IL-6 (P < 0.05 compared to saline; Figure 3A,B) was detected in BALF following exposure to CB. In contrast, no significant increases in neutrophils (Figure 3A) or IL-6 (Figure 3B; P > 0.05 compared to saline) were observed in BALF following administration of DEP. Plasma levels of IL-6 were also significantly increased following administration of CB but not DEP (Figure 3C). Neither DEP nor CB caused a detectable change in other leukocytes in BALF (data not shown). DEP therefore enhanced in vivo platelet aggregation in the absence of an effect upon the inflammatory parameters measured whereas CB produced a detectable inflammatory response without impacting platelet aggregation.

Diesel exhaust particles enhanced platelet aggregation in the absence of eNOS
We explored the hypothesis that DEP exerted effects via eNOS by employing eNOS-/-/ mice. A lack of effect of DEP upon platelet aggregation in eNOS-/-/ would confirm this hypothesis. Following i.t. instillation of DEP into eNOS-/-/ mice, a significant enhancement of the AUC (P < 0.05 compared to saline control) in response to collagen was observed after 4 h (Figure 4A,B). No significant changes in AUC were detected for CB exposed eNOS -/- mice.

Figure 1
Deposition of DEP and CB in mouse lungs following i.t. instillation. Anaesthetised WT mice were i.t. instilled with DEP (C,F) and CB (B,E; 25 μg per mouse) or saline (SAL) (NaCl 0.9%) (A,D). Mice were killed via cervical dislocation immediately after i.t. instillation, and their lungs were removed and fixed in formalin. Samples were embedded, sectioned and stained with haematoxylin and eosin. Slides were visualized on a light microscope at x20 000 and x40 000. Sections from five mice exposed to each treatment were observed, and representative images are shown.
Thus, the effect of DEP upon platelet aggregation in vivo is not dependent upon the presence of eNOS.

We also analysed the fold-increase in AUC following DEP or CB exposure relative to saline responses in order to determine whether platelet responses were proportionally different in eNOS−/− compared to WT. Although no significant differences were observed, there was a non-significant trend towards an increased proportional response following DEP exposure in eNOS−/− versus WT (Figure 4C).
DEP and NO-mediated regulation of platelets

Neither DEP nor CB instillation caused any significant alteration in plasma nitrate levels (Figure 5A) indicating a lack of a detectable effect on NO bioavailability. Nitrite levels were below the detection limit (50 nM) of the assay and therefore data are presented as nitrate concentrations (A). Isolated human platelets were exposed to DEP (12–50 μg·mL⁻¹) or collagen (Coll; 5 μg·mL⁻¹) following a 5 min incubation with either a NO donor sodium nitroprusside (SNP; 10 μM) or Tyrode’s buffer (Tyr; B). Changes in light transmission were measured for 3 min. Data are presented as box-and-whisker plots (A) and as median ± interquartile range (B), NS = non-significant compared using Kruskal–Wallis test with Dunn’s comparison (A), *P < 0.05, significantly different from time matched controls using Wilcoxon signed rank test (B). n = 6 (A) or 5 (B).

Discussion and conclusions

In this study, we explored the hypothesis that introduction of DEP into the airways could lead to an enhancement of subsequent platelet aggregation in an acute time frame. One of the principal reasons why inhaled PM 0.1, such as DEP, is considered to be responsible for the harmful effects of ambient PM is its deposition within the lower airways including the alveolar gas exchange units (ICRP, 1994). In the present study, agglomerates of DEP and CB were observed...
both in the conducting airways and the distal alveoli confirming appropriate lung penetration following i.t. administration. I.t. instillation therefore, to some extent, mimics inhalation of nanoparticles into the airways. It is not, however, possible from the histological analyses conducted to observe individual nanoparticles nor to assess their translocation across the lung epithelium. It is important to address the relevance of the doses of nanoparticles employed in this study with reference to human exposure. More than 20 mg of particulates can be inhaled by humans in polluted cities every 24 h (Nemmar et al., 2003a). Our dosing of 25 μg equates to a total burden of 2 mg·kg⁻¹ body weight so although our study was not intended to mimic human exposure, the levels used are probably relevant if one considers an exposure period of a few days.

The thrombotic events associated with inhalation of PM 0.1 are known to involve platelet activation and aggregation but, currently, there is limited information regarding the influence of DEP and CB on platelet function in vivo. The present study showed that DEP, but not CB, administered to the lungs of mice via the instillation route caused a significant increase in agonist-induced platelet aggregation in vivo 4 h post-exposure. These results indicate that the thrombotic events associated with acute pulmonary exposure to PM 0.1 may be driven mechanistically by platelets.

Supportive to the present study, acute exposure to DEP by i.t. instillation has been demonstrated to enhance thrombosis and platelet activation in rodent models after 30, 60 (Nemmar et al., 2003a) or 120 min (Tabor et al., 2016). Similarly, thrombosis was enhanced in vivo following a 24 h exposure of lungs to DEP (Nemmar et al., 2009). Although there are limited clinical studies, increased thrombosis has been reported in human subjects using ex vivo perfusion techniques following an acute inhalation exposure (2–4 h) to DE (Lucking et al., 2008). In the current study, DEP has been shown to affect platelet aggregation independent of the other components of the thrombotic response as the model employed has been shown to be driven entirely by platelet aggregation in vivo (Tymvios et al., 2008) and in the presence of an intact endothelium (Tymvios et al., 2008; Emerson, 2010) suggesting that DEP may affect platelets in the absence of endothelial dysfunction or injury.

Inflammation is associated with increased cardiovascular risk (Mameli et al., 2009). One hypothesis regarding how PM 0.1 may promote thrombosis is therefore by induction of pulmonary inflammation, spill-over of inflammatory cytokines into the systemic circulation and consequential platelet activation (Seaton et al., 1995). In particular, exposure to PM has been linked with thrombotic events driven by IL-6 (Mutlu et al., 2007) and leukocyte influx to the airways (Nemmar et al., 2003a; Nemmar et al., 2009). No increases in neutrophils or IL-6 were observed following DEP exposure in the current study. There was therefore no evidence of an inflammatory response to DEP in our model as no measured hallmarks of pulmonary inflammation were detected. This finding conflicts with some published data in which DEP has been reported to cause increases in pulmonary neutrophils 1 and 6 h following pulmonary delivery in hamsters (Nemmar et al., 2003a) and rats (Robertson et al., 2012). In contrast, other reports support our findings, demonstrating dissociation of the thrombogenic effects of DEP from pulmonary and systemic inflammation (Tabor et al., 2016). Differences in the inflammatory impacts of DEP reported in various studies may be due in part to differences in species studied. Regardless of this, it is apparent that enhanced platelet activation following exposure to DEP can occur independently of quantifiable changes in the inflammatory response in the context of the parameters assessed in the current study.

In terms of human studies, acute exposure to DE caused changes in thrombosis formation but no alterations in systemic cytokines or acute phase proteins (Lucking et al., 2011). Additionally, no changes in systemic inflammatory markers were detected in healthy men and men with coronary artery disease exposed to DEP for 1 h with intermittent exercise (Mills et al., 2005; Mills et al., 2007) or 2 h after exposure to concentrated ambient particles (Mills et al., 2008). However, increases in systemic IL-6 and TNF-α 24 h following exposure to DEP have been reported in humans (Tornqvist et al., 2007), animals (Robertson et al., 2012) and at longer >24 h time intervals (Nemmar and Inuwa, 2008). Based on the current data, it appears that DEP may not necessarily induce systemic inflammation following acute exposure and therefore may not be the underlying mechanism behind the enhanced platelet aggregation observed in the present work. The possibility that inflammation may drive platelet activation in other models and at different exposure periods cannot be excluded. Since nanoparticles have been suggested to translocate the lung to enter the blood (Kreyling et al., 2009) and DEP can directly enhance platelet activation platelets following physical interaction (Solomon et al., 2013), an alternative or additional hypothesized mechanism is translocation of DEP across the lung epithelium and subsequent exposure and activation of circulating platelets. The ultimate objective in proving this hypothesis would be visualization of DEP in contact with activating platelets following i.t. administration or inhalation of DEP, but this has not yet been demonstrated and is technically challenging.

In contrast to DEP, administration of CB to mice caused significant increases in neutrophils and IL-6 in the BALF and IL-6 in the plasma. Systemic inflammation has similarly been reported following a single 7 h exposure of rats to unfractionated CB (Gilmour et al., 2004). The release of pro-inflammatory cytokines by CB further supports the conclusion that systemic inflammation was not the primary underlying mechanism behind the DEP-induced enhanced platelet response, as CB did not alter platelet aggregation in vivo but was associated with systemic inflammation. Thus, PM 0.1-associated inflammation was not linked to platelet aggregation in our study. The contrasting effects of DEP and CB may suggest the constituents present on the surface of DEP to be responsible for the effects on platelet aggregation since the presence of these surface compounds and metals distinguishes the two pollutants. Further work to identify the biological effects of these surface components of DEP will provide valuable information on the mechanisms underlying the health impact of combustion derived air pollutants.

A hypothesized mechanism driving DEP-enhanced platelet aggregation that was explored in this study was that DEP can reduce the bioavailability of endogenous NO and thereby reduce the inhibitory influence NO has on platelets.
Acute exposure to DE has been reported to induce endothelial dysfunction via uncoupling of eNOS (Knuckles et al., 2008). DEP has also been demonstrated to enhance vasoconstriction, potentially due to reduced NO bioavailability (Langrish et al., 2013). If reduced NO bioavailability were responsible for the effects of DEP on platelet aggregation then DEP would be expected to have no effect on platelet aggregation in eNOS−/− mice. Since DEP caused an increase in platelet aggregation in eNOS−/− mice and did not appear to influence plasma nitrate and nitrite, there was no evidence of reduced NO bioavailability as an underlying mechanism driving increased platelet aggregation. Since NO negatively regulates platelet activation, we explored whether loss of eNOS in fact led to enhanced platelet aggregation. DEP had no significant effect on platelet aggregation in eNOS−/− relative to in WT so that an effect of endogenous NO upon DEP-enhanced platelet aggregation could not be inferred. To explore whether, mechanistically, DEP-induced platelet aggregation could be impacted by NO, we conducted studies in vitro. DEP-induced platelet aggregation was significantly inhibited by a NO donor. The extent of inhibition observed was similar to that achieved with collagen and is in keeping with the proposed GPVI-mediated mechanism by which DEP is proposed to activate platelets (Alshehri et al., 2015). The finding of an inhibitory effect of NO in regulating DEP-mediated platelet aggregation in vitro, although not supported by the in vivo studies in this paper, is sufficient to hypothesize that when platelets are directly exposed to DEP, potentially following translocation across the lung epithelium, NO may act to oppose platelet activation. This suggestion is in keeping with epidemiological data that demonstrates the greatest cardiovascular risk in individuals with risk factors associated with endothelial dysfunction (Brook et al., 2002; Mills et al., 2005; Brauner et al., 2008; Wauters et al., 2013). Further investigation is warranted to ascertain whether NO acts in a physiologically cardioprotective manner to reduce DEP-driven cardiovascular risk.

In conclusion, acute airways exposure to DEP can enhance platelet aggregation responses in vitro and this may provide a mechanism for the thrombotic effects that are associated with acute exposure to PM. Additionally, the mechanisms underlying this enhanced platelet response do not appear to involve the initiation of systemic inflammation or alterations in NO bioavailability. Our study does, however, generate a hypothesis that healthy individuals may be protected from the harmful cardiovascular effects of DEP that comes into direct contact with platelets, through the inhibition of DEP-Induced platelet activation by NO.

Acknowledgements

This work was supported by a Project Grant from the British Heart Foundation (PG/10/80/28605) awarded to M Emerson and T D Tetley.

Author contributions

E.S.: design, conduct and analysis of experiments and drafting of manuscript; A.S., M.A.B. and M.J.S.: design and conduct and analysis of experiments; P.G.W.: concept and design of experiments; T.D.T.: concept and design of experiments and drafting of manuscript and revising for critically important intellectual content; M.E.: concept and design of experiments, drafting of manuscript and revising for critically important intellectual content and final approval of manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

References

Alexander SP, Davenport AP, Kelly E, Marrion N, Peters JA, Benson HE et al. (2015a). The concise guide to PHARMACOLOGY 2015/16: G protein-coupled receptors. Br J Pharmacol 172: 5744–5869.

Alexander SP, Fabbro D, Kelly E, Marrion N, Peters JA, Benson HE et al. (2015b). The concise guide to PHARMACOLOGY 2015/16: Enzymes. Br J Pharmacol 172: 6024–6109.

Alshehri OM, Montague S, Watson S, Carter P, Sarker N, Manne BK et al. (2015). Activation of glycoprotein VI (GPVI) and C-type lectin-like receptor-2 (CLEC-2) underlies platelet activation by diesel exhaust particles and other charged/hydrophobic ligands. Biochem J 468: 459–473.

Andersen ZJ, Olsen TS, Andersen KK, Loft S, Ketzel M, Raaschou-Nielsen O (2010). Association between short-term exposure to ultrafine particles and hospital admissions for stroke in Copenhagen, Denmark. Eur Heart J 31: 2034–2040.

Apostoli GL, Solomon A, Smallwood MJ, Winyard PG, Emerson M (2014). Role of inorganic nitrate and nitrite in driving nitric oxide-cGMP-mediated inhibition of platelet aggregation in vitro and in vivo. J Thromb Haemost 12: 1880–1889.

Brauner EV, Forchhammer L, Moller P, Barregard L, Gunnarsen L, Afshari A et al. (2008). Indoor particles affect vascular function in the aged: an air filtration-based intervention study. Am J Respir Crit Care Med 177: 419–425.

Brook RD, Brook JR, Urch B, Vincent R, Rajagopalan S, Silverman F (2002). Inhalation of fine particulate air pollution and ozone causes acute arterial vasoconstriction in healthy adults. Circulation 105: 1534–1536.

Conklin DJ, Kong M, Committee HEIHR (2015). Part 4. Assessment of plasma markers and cardiovascular responses in rats after chronic exposure to new-technology diesel exhaust in the ACES bioassay. Res Rep Health Eff Inst 184: 111–139. discussion 141-171.

Curtis MJ, Bond RA, Spina D, Ahiwuwalia A, Alexander SP, Giembycz MA et al. (2015). Experimental design and analysis and their
in vivo approaches to in vivo cardiovascular research. Br J Pharmacol 161: 749–754.

Emerson M (2009). Al-Salam S, Dhanasekaran S, Sudhadevi M, Ali BH (2009). Pulmonary exposure to diesel exhaust particles promotes cerebral microvesSEL thrombosis: protective effect of a cysteine prodrug l-2-oxothiazolidine-4-carboxylic acid. Toxicology 263: 84–92.

Emerson M, Momii S, Paul W, Alberti PE, Page C, Gresele P (1999). Pulmonary and systemic effects of short-term inhalation exposure to ultrafine carbon black particles. Toxicol Appl Pharmacol 195: 35–44.

ICRP (1994). Human respiratory tract model for radiological protection. A report of a task group of the international commission on radiological protection. Ann ICRP 24: 1–482.

Jones S, Solomon A, Sanz-Rosa D, Moore C, Holbrook L, Cartwright EJ et al. (2010). The plasma membrane calcium ATPase (PMCA) modulates calcium homeostasis, intracellular signalling events and function in platelets. J Thromb Haemost 8: 2766–2774.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Knuckles TL, Lund AK, Lucas SN, Campen MJ (2008). Diesel exhaust exposure enhances venoconstriction via uncoupling of eNOS. Toxicol Appl Pharmacol 230: 346–351.

Kreyleing WG, Semmler-Behnke M, Seitz J, Szymczak W, Wenk A, Mayer P et al. (2009). Size dependence of the translocation of inhaled iridium and carbon nanoparticle aggregates from the lung of rats to the blood and secondary target organs. Inhal Toxicol 21: 977–982.

Krishnan RM, Sullivan JH, Carlsten C, Wilkerson HW, Beyer RP, Bammller T et al. (2013). A randomized cross-over study of inhalation of diesel exhaust, hematological indices, and endothelial markers in humans. Part Fibre Toxicol 10: 7.

Langrish JP, Unosson J, Bosson J, Barath S, Muala A, Blackwell S et al. (2013). Altered nitric oxide bioavailability contributes to diesel exhaust inhalation-induced cardiovascular dysfunction in man. J Am Heart Assoc 2: e004309.

Lucking AJ, Lundback M, Barath SL, Mills NL, Sidhu MK, Langrish JP et al. (2011). Particle traps prevent adverse vascular and prothrombotic effects of diesel engine exhaust inhalation in men. Circulation 123: 1721–1728.

Lucking AJ, Lundback M, Mills NL, Faratian D, Barath SL, Pourzay J et al. (2008). Diesel exhaust inhalation increases thrombus formation in man. Eur Heart J 29: 3043–3051.

Mameli A, Barcellona D, Marongiu F (2009). Rheumatoid arthritis and thrombosis. Clin Exp Rheumatol 27: 846–855.

McGrath JC, Lilley E (2015). Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJPh. Br J Pharmacol 172: 3189–3193.

Mills NL, Robinson SD, Fokkens PH, Leseman DL, Miller MR, Anderson D et al. (2008). Exposure to concentrated ambient particles does not affect vascular function in patients with coronary heart disease. Environ Health Perspect 116: 709–715.

Mills NL, Tornqvist H, Gonzalez MC, Vink E, Robinson SD, Soderberg S et al. (2007). Ischemic and thrombotic effects of dilute diesel-exhaust inhalation in men with coronary heart disease. N Engl J Med 357: 1075–1082.

Mills NL, Tornqvist H, Robinson SD, Gonzalez M, Darmley K, MacNee W et al. (2005). Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. Circulation 112: 3930–3936.

Moore C, Sanz-Rosa D, Emerson M (2011). Distinct role and location of the endothelial isoform of nitric oxide synthase in regulating platelet aggregation in males and females in vivo. Eur J Pharmacol 651: 152–158.

Moore C, Tymvios C, Emerson M (2010). Functional regulation of vascular and platelet activity during thrombosis by nitric oxide and endothelial nitric oxide synthase. Thromb Haemost 104: 342–349.

Mutlu GM, Green D, Bellmeyer A, Baker CM, Burgess Z, Rajamannan N et al. (2007). Ambient particulate matter accelerates coagulation via an IL-6-dependent pathway. J Clin Invest 117: 2952–2961.

Nemmar A, Al-Salam S, Dhanasekaran S, Sudhadevi M, Ali BH (2009). Pulmonary exposure to diesel exhaust particles promotes cerebral microvesSEL thrombosis: protective effect of a cysteine prodrug l-2-oxothiazolidine-4-carboxylic acid. Toxicology 263: 84–92.

Nemmar A, Hoet PH, Dinsdale D, Vermilyen J, Hoyaerts MF, Nemery B (2003a). Diesel exhaust particles in lung acutely enhance experimental peripheral thrombosis. Circulation 107: 1202–1208.

Nemmar A, Inuwa IM (2008). Diesel exhaust particles in blood trigger systemic and pulmonary morphological alterations. Toxicol Lett 176: 20–30.

Nemmar A, Nemery B, Hoet PH, Vermilyen J, Hoyaerts MF (2003b). Pulmonary inflammation and thrombogenicity caused by diesel particles in hamsters: role of histamine. Am J Respir Crit Care Med 168: 1366–1372.

Nemmar A, Vanbilloen H, Hoyaerts MF, Hoet PH, Verbruggen A, Nemery B (2001). Passage of intratracheally instilled ultrafine particles from the lung into the systemic circulation in hamster. Am J Respir Crit Care Med 164: 1665–1668.

Oberdörster G, Ferin J, Gelein R, Soderholm SC, Finkelstein J (1992). Role of the alveolar macrophage in lung injury: studies with ultrafine particles. Environ Health Perspect 97: 193–199.

Oberdörster G, Sharp Z, Atudorei V, Eldaer A, Gelein R, Lunts A et al. (2002). Extrapulmonary translocation of ultrafine carbon particles following whole-body inhalation exposure of rats. J Toxicol Environ Health A 65: 1531–1543.

Peters A, Dockery DW, Muller JE, Mittleman MA (2001). Increased particulate air pollution and the triggering of myocardial infarction. Circulation 103: 2810–2815.

Raemdonck K, Baker K, Dale N, Dubuis E, Shala F, Belvisi MG et al. (2016). CD4(+) and CD8(+) T cells play a central role in a HDM driven model of allergic asthma. Respir Res 17: 45.

Robertson S, Gray GA, Duffin R, McLean SG, Shaw CA, Hadoke PW et al. (2012). Diesel exhaust particulate induces pulmonary and systemic inflammation in rats without impairing endothelial function ex vivo or in vivo. Part Fibre Toxicol 9: 9.

Seaton A, MacNee W, Donaldson K, Godden D (1995). Particulate air pollution and acute health effects. Lancet 345: 176–178.

Solomon A, Smyth E, Mitha N, Pitchford S, Vydyananth A, Luther PK et al. (2013). Induction of platelet aggregation after a direct physical interaction with diesel exhaust particles. J Thromb Haemost 11: 325–334.

Southan C, Sharman JL, Benson HE, Fascenda E, Pawson AJ, Alexander SP et al. (2016). The IUPHAR/BPS guide to
PHARMACOLOGY in 2016: towards curated quantitative interactions between 1300 protein targets and 6000 ligands. Nucleic Acids Res 44: D1054–D1068.

Tabor CM, Shaw CA, Robertson S, Miller MR, Duffin R, Donaldson K et al. (2016). Platelet activation independent of pulmonary inflammation contributes to diesel exhaust particulate-induced promotion of arterial thrombosis. Part Fibre Toxicol 13: 6.

Tornqvist H, Mills NL, Gonzalez M, Miller MR, Robinson SD, Megson IL et al. (2007). Persistent endothelial dysfunction in humans after diesel exhaust inhalation. Am J Respir Crit Care Med 176: 395–400.

Tyvmios C, Jones S, Moore C, Pitchford SC, Page CP, Emerson M (2008). Real-time measurement of non-lethal platelet thromboembolic responses in the anaesthetized mouse. Thromb Haemost 99: 435–440.

Wauters A, Dreyfuss C, Pochet S, Hendrick P, Berkenboom G, van de Borne P et al. (2013). Acute exposure to diesel exhaust impairs nitric oxide-mediated endothelial vasomotor function by increasing endothelial oxidative stress. Hypertension 62: 352–358.

Xu Y, Barregard L, Nielsen J, Gudmundsson A, Wierzbicka A, Axmon A et al. (2013). Effects of diesel exposure on lung function and inflammation biomarkers from airway and peripheral blood of healthy volunteers in a chamber study. Part Fibre Toxicol 10: 60.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

https://doi.org/10.1111/bph.13831

Figure S1 Original, unmodified typical in vitro isolated platelet aggregation traces showing changes in light transmission (A) in response to collagen (5 μg mL⁻¹) following a 5 min incubation with either Tyrode’s buffer (Coll) or the NO donor sodium nitroprusside (SNP, 10 μM). (B-C) Response to diesel exhaust particles (DEP) following Tyrode’s or SNP at (B) 25 μg mL⁻¹ or (C) 50 μg μl⁻¹. Typical traces of n = 5 are shown.