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Glutathione (GSH) and the GSH synthesis gene *Gclm* modulate plasma redox and vascular responses to acute diesel exhaust inhalation in mice

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Abstract

Context—Inhalation of fine particulate matter ($PM_{2.5}$) is associated with acute pulmonary inflammation and impairments in cardiovascular function. In many regions, $PM_{2.5}$ is largely derived from diesel exhaust (DE), and these pathophysiological effects may be due in part to oxidative stress resulting from DE inhalation. The antioxidant glutathione (GSH) is important in limiting oxidative stress-induced vascular dysfunction. The rate-limiting enzyme in GSH synthesis is glutamate cysteine ligase and polymorphisms in its catalytic and modifier subunits (GCLC and GCLM) have been shown to influence vascular function and risk of myocardial infarction in humans.

Objective—We hypothesized that compromised de novo synthesis of GSH in $Gclm^{-/+}$ mice would result in increased sensitivity to DE-induced lung inflammation and vascular effects.

Materials and methods—WT and *Gclm*$^{-/+}$ mice were exposed to DE via inhalation (300 μg/m^3) for 6 h. Neutrophil influx into the lungs, plasma GSH redox potential, vascular reactivity of aortic rings and aortic nitric oxide ($NO^{\bullet}$) were measured.

Results—DE inhalation resulted in mild bronchoalveolar neutrophil influx in both genotypes. DE-induced effects on plasma GSH oxidation and acetylcholine (ACh)-relaxation of aortic rings were only observed in $Gclm^{-/+}$ mice. Contrary to our hypothesis, DE exposure enhanced ACh-induced relaxation of aortic rings in $Gclm^{-/+}$ mice.

Discussion and conclusion—These data support the hypothesis that genetic determinants of antioxidant capacity influence the biological effects of acute inhalation of DE. However, the acute effects of DE on the vasculature may be dependent on the location and types of vessels involved. Polymorphisms in GSH synthesis genes are common in humans and further investigations into these potential gene-environment interactions are warranted.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Keywords

Diesel exhaust; glutathione; lung inflammation; nitric oxide; oxidative stress

Introduction

There is substantial evidence that exposure to fine ambient particulate matter ($PM_{2.5}$) is associated with an increased risk of cardiopulmonary mortality (Dockery et al., 1993; Pope et al., 2002, 2004). From these initial observations, there have been further epidemiological investigations to understand the role of $PM_{2.5}$ in eliciting acute myocardial events with both chronic exposures (Miller et al., 2007) and acute exposures resulting from transient excursions in ambient $PM_{2.5}$ levels (Peters et al., 2001, 2000; Rich et al., 2005, 2006).

In many urban regions, ambient $PM_{2.5}$ is largely derived from diesel exhaust (DE) emissions (Lewtas, 2007), thus many investigators have utilized controlled DE exposure facilities to investigate the pulmonary and cardiovascular effects of $PM_{2.5}$ exposure from DE. Although the exact pathways by which $PM_{2.5}$ and DE inhalation cause adverse effects are not fully elucidated, numerous studies investigating $PM_{2.5}$ and DE toxicity have highlighted the role of oxidative stress in both the onset of inflammation and on bioavailable nitric oxide ($NO^{\bullet}$) within the vasculature, both of which are implicated as principle components in the observed impairments in vascular function and increased risk of acute myocardial infarction (Cherng et al., 2009, 2011; Kampfrath et al., 2011; Knuckles et al., 2008; Mudway et al., 2004; Pourazar et al., 2005; Weldy et al., 2011a,b). In addition, there is limited but supportive evidence to suggest that oxidative stress occurs in humans following inhalation of $PM_{2.5}$ (Baccarelli et al., 2007; Bräuner et al., 2007; Chuang et al., 2007; Romieu et al., 2005, 2008; Sørensen et al., 2003; Vinzents et al., 2005). Together, evidence from *in vitro, in vivo*, and epidemiological studies support the notion that oxidative stress plays an important physiological role in the onset of cardiovascular toxicity following $PM_{2.5}$ and DE inhalation, and thus the status of antioxidants is an important factor in mitigating these adverse effects.

We recently demonstrated that the *de novo* synthesis of the antioxidant glutathione (GSH) plays an important role in mediating pulmonary inflammation resulting from intranasal instillation of diesel exhaust particulate (DEP) (Weldy et al., 2011a). This finding was in support of our previous *in vitro* observation whereby increased GSH synthesis occurred in endothelial cells following both direct DEP exposure and following exposure to soluble factors released from DEPtreated macrophage cells (Weldy et al., 2011b). These findings suggest that the *de novo* synthesis of GSH plays a protective role in antioxidant defense and proinflammatory responses following DEP exposure. Taken together, these findings suggest that individuals who have compromised *de novo* GSH synthesis may be more sensitive to the adverse pulmonary and cardiovascular effects of $PM_{2.5}$ inhalation.

GSH is a tripeptide thiol that is present in millimolar concentrations in certain cells such as hepatocytes. Due to its stereochemistry and bioavailable cysteine, GSH participates in redox cycling to maintain a reduced state in the cell through the action of GSH peroxidases (GPxs) and glutathione disulfide (GSSG) reductase (GRx) (Franklin et al., 2009). In many tissues GSH is the principal determinant of the intracellular redox state (Dalton et al., 2004), and numerous reports from Loscalzo, Stamler, and their colleagues have shown the clinical importance of GSH redox in vascular disease (Espinola-Klein et al., 2007; Jin et al., 2011; Leopold et al., 2007; Maron et al., 2009; Stamler et al., 1988; Weiss et al., 2001). GSH is synthesized in a two-step process; the first and rate-limiting step is carried out by the heterodimeric enzyme glutamate cysteine ligase (GCL), which is composed of catalytic (GCLC) and modifier (GCLM) subunits. Synthesis of GSH is determined by the total GCL

activity within the cell, which is regulated by both GCL enzyme level, by *GCLC* and *GCLM* transcription, and by holoenzyme formation (Bea et al., 2003, 2009; Dickinson et al., 2004; Franklin et al., 2009; Krejsa et al., 2010; Lu, 2009; Shenvi et al., 2012; Wild & Mulcahy, 2000).

Because the activity of GCL and the promoters of both *GCLC* and *GCLM* will influence GSH content, and since we have previously demonstrated that GSH synthesis plays an important protective role in mediating DEP-induced inflammation, it is important to investigate the potential of genetic variations in GSH synthesis genes to influence cardiovascular response to DE inhalation. It has been previously demonstrated that single nucleotide polymorphisms in the 5′ promoter regions of both *GCLC* and *GCLM* lead to compromised promoter activity and are associated with an increased risk of myocardial infarction, impaired vasomotor function and dilated cardiomyopathy in a Japanese population (Koide et al., 2003; Nakamura et al., 2002, 2003; Watanabe et al., 2013). Although the effects of these polymorphisms are relatively small (one copy of the −588T *GCLM* allele results in ~2-fold increased risk of MI), their frequency (e.g. ~20% of the population have at least one *GCLM* −588T allele) highlights their importance to public health in the context of air pollution and cardiovascular disease.

Our previous studies have provided evidence that the $Gclm^{-/+}$ mouse is a unique model for investigating the role of GSH and its *de novo* synthesis in mediating biological responses to toxicant exposures (McConnachie et al., 2007; Weldy et al., 2011a, 2012). As opposed to $Gclm^{-/-}$ mice, which can have GSH levels within tissues that range from 5% to 15% of wild type (WT) mice, $Gclm^{-/+}$ mice typically have GSH levels that range from 70% to 90% of WT. Paradoxically, $Gclm^{-/-}$ mice appear to be protected from certain oxidative challenges, such as DEP (Weldy et al., 2011a) and ozone (Johansson et al., 2010). This lack of sensitivity of $Gclm^{-/-}$ mice appears to be due to dramatic compensatory responses that are still being investigated (Haque et al., 2010; Weldy et al., 2012). Alternatively, $Gclm^{-/+}$ mice do not appear to upregulate the same compensatory response genes (or at least to the same magnitude as $Gclm^{-/-}$ mice), and are thus more sensitive to certain challenges such as DEP-induced lung inflammation (Weldy et al., 2011a). This heightened sensitivity is likely due to an inability to further upregulate *Gclm* and to engage in *de novo* GSH synthesis at a rate that is adequate to provide sufficient antioxidant protection. Importantly, we propose that $Gclm^{-/+}$ mice are more similar to humans with GCL polymorphisms than $Gclm^{-/-}$ mice, as individuals with these polymorphisms have only modest reductions in GSH, as opposed to the dramatic loss of GSH that is present in $Gclm^{-/-}$ mice. Accordingly, investigating the effects of DE inhalation on $Gclm^{-/+}$ mice provides a valuable opportunity to not only understand the mechanisms of DE-induced pathophysiology, but also provides insight into the potential susceptibility of humans with GCL polymorphisms to $PM_{2.5}$ exposures.

In this report, we investigated the effect of a 6 h DE inhalation (300 μg/m^3) on neutrophilic airway inflammation, plasma GSH, GSSG and GSH reduction potential, aortic ring vascular reactivity by wire myography, as well as aortic $NO^{\bullet}$ production by spin trap and electron spin resonance (ESR) in WT and $Gclm^{-/+}$ mice. Since acute DE inhalation studies have been previously observed to cause pulmonary inflammation and impair vascular $NO^{\bullet}$ production in a manner that is consistent with the generation of reactive oxygen species (ROS) and subsequently oxidative stress, we hypothesized that mice heterozygous for *Gclm* would be more sensitive to DE-induced effects due to a compromised ability to maintain sufficient GSH stores and thus be more sensitive to oxidative challenge, as measured by enhanced pulmonary inflammation, oxidation of the plasma GSH redox, and impaired aortic $NO^{\bullet}$ generation and vascular reactivity.

Materials and methods

Mice and filtered air or diesel exhaust exposure

Gclm wild-type (WT) and *Gclm*$^{-/+}$ mice were backcrossed for at least 10 generations onto the C57BL/6 background and were bred and housed in a modified specific pathogen-free (SPF) vivarium at the University of Washington. All animal experiments were approved by the University of Washington Institutional Animal Care and Use Committee. Male and female littermates were genotyped as previously described (McConnachie et al., 2007) and randomly assigned to either filtered air (FA) or diesel exhaust (DE) exposure treatments. For studies on lung inflammation, an equal number of male and female mice were used in both WT and *Gclm*$^{-/+}$ genotypes, whereas in all additional studies on vascular reactivity, plasma GSH, and aortic $NO^{\bullet}$, only male mice were used. Mice were transferred to our diesel exposure facility where exposures to either FA or DE were conducted simultaneously in an Allentown caging system (Allentown, NJ) under SPF conditions. DE (300 μg/m^3) was generated from a single cylinder Yanmar diesel engine (model YDG5500EV-6EI) operating on 75% load as previously described (Gould et al., 2008). A DE exposure concentration of 300 μg/m^3 was chosen as this is a relevant exposure concentration in certain occupational settings such as mining (Janisko & Noll, 2010), and is consistent with prior studies conducted investigating the vascular effects of acute DE inhalation (Campen et al., 2010; Cherng et al., 2009, 2011; Knuckles et al., 2008).

Bronchial alveolar lavage, cell staining and flow cytometry

Mice were sacrificed by Isoflurane narcosis followed by cervical dislocation immediately after a 6-h exposure to either FA or DE. To perform bronchoalveolar lavage (BAL), the peritoneal, thoracic and cervical areas were carefully opened and the trachea was surgically isolated. A small incision was made in the trachea just below the larynx and an 18 G catheter attached to a 1 ml syringe was inserted to perform the lavage. One milliliter of phosphate-buffered saline (PBS) was slowly instilled into the lungs and subsequently withdrawn. This rinsing action was repeated 3× per wash, and three 1 ml washes were performed for each mouse. The lavage sample from the first wash was collected independently and placed into a 1.5 ml microcentrifuge tube, while the lavage from the second and third washes were combined. Cells in the lavage samples were then pelleted by centrifugation at 200 × G for 15 min at 4 °C. The supernatant from the first wash was collected for future analyses, whereas the supernatants from the 2nd and 3rd washes were discarded. Cells from all three washes were combined, treated with a red blood cell (RBC) lysis buffer (ammonium chloride lysing solution; 1.5 M NH_4Cl, 10 mM $NaHCO_3$, 1 mM disodium EDTA, in dH_2O) at room temperature for 5 min, blocked for 30 min with 1% bovine serum albumin and 5% rat serum, and then subsequently stained for 15 min with primary antibodies directed against F4/80 antigen conjugated with Alexafluor 488 (eBioscience, San Diego, CA; Cat# 53-4801-80), phycoerythrin conjugated anti-mouse Ly-6 G/Ly6C (Gr1; BioLegend, San Diego, CA; Cat# 108404), and biotinylated anti-mouse CD11b. Subsequently, streptavidin Alexafluor 350 (Invitrogen, Carlsbad, CA; Cat# S11249) was added. Cells were analyzed on a Beckman-Coulter Altra fluorescence activated cell sorter (FACS) (Beckman-Coulter, Miami, FL), and 10 000 cells were examined for each animal. Neutrophils were identified as cells expressing low F4/80, high Gr1, and very high CD11b (F4/80^{lo}/Gr1^{hi}/CD11b^{vhi}). A total of 48 mice were used in the assessment of neutrophil influx, 24 male and 24 female, equal numbers in each genotype (12 WT-FA, 12 WT-DE, 12 *Gclm*$^{-/+}$-FA, 12 *Gclm*$^{-/+}$-DE).

To determine if DE exposure resulted in uptake of DEP by alveolar macrophages, we performed BAL on male WT mice following a 6-h exposure to either FA or DE as described above. Fifty microliter samples of washed resuspended cells were further diluted with 450

μL PBS, placed into Shandon Cytospin cartridges (ThermoScientific, Waltham, MA), and the cartridges were centrifuged at 600 rpm for 10 min. Deposited cells were stained using a Diff-Quik Fixative (Siemens Healthcare Diagnostics, Newark, DE). Bright field images of representative alveolar macrophages were taken at 40× magnification using a Nikon Optiphot microscope (Nikon USA, Melville, NY).

Assessment of plasma GSH reduction potential by HPLC

Whole blood was collected directly into a heparin-coated plasma collection tube and inverted three times to prevent clotting. Blood was immediately centrifuged at 6000 rpm for 2 min in a microcentrifuge to separate out plasma from RBCs, and 100 μL of plasma was removed and diluted 1:1 with 10% 5-sulfosalicylic acid (SSA) to stabilize GSH and precipitate proteins. The plasma and acid mix was incubated on ice for 10 min, and then centrifuged at 15 600 × Gin a microcentrifuge for 2 min to obtain deproteinated supernatants. These were transferred to a cryotube, purged with argon and stored in liquid N_2 for two weeks. Practice samples were measured immediately after collection and after two weeks of storage to ensure oxidation of plasma GSH did not take place. Concentrations of GSH and GSSG present in the original homogenate were determined by high-pressure liquid chromatography (HPLC) using a modification of previously described methods (Eaton & Hamel, 1994; Thompson et al., 1999). Briefly, for GSH measurements, deproteinized supernatant was mixed with monobromobimane (MBB) to derivatize GSH and measured by HPLC with fluorescence detection. For GSSG measurements, 2-vinylpiridine was added to a portion of the supernatant to react with GSH. Residual 2-vinylpiridine was then removed with chloroform extraction, and the remaining GSSG was reduced to GSH with *tris*(2-carboxyethyl)phosphine (TCEP; 10 μM), derivatized with MBB and measured by HPLC as above. Glutathione reduction potential ($\Delta E_{GSSG/2GSH}$) was calculated using the Nernst Equation, assuming a pH of 7.2 and 37 °C as previously described (Dalton et al., 2004). Twentyfour male mice were used for this experiment (6WT-FA, 6WT-DE, 6 *Gclm*$^{-/+}$-FA, 6 *Gclm*$^{-/+}$-DE).

Vascular reactivity

Following FA or DE exposures, aortas from male WT and *Gclm*$^{-/+}$ mice were cut into 3-mm rings and transferred to an organ bath containing 6 ml of physiological saline solution (119 mM NaCl, 4.7 mM KCl, 2.4 mM $MgSO_4$, 1.2 mM KH_2PO_4, 3.3 mM $CaCl_2$, 25 mM $NaHCO_3$, 30 μM EDTA, 6 mM dextrose), equilibrated with 95% O_2 and 5% CO_2. Buffer was maintained at 37 °C, pH 7.4. Aortic rings were hung with wire to a force transducer (Model 610 M, Danish Myo Technology, Aarhus, Denmark), and the transducer was interfaced to a Powerlab 8/26 recorder for measurement of isometric force. Rings were placed under an initial tension of 20 mN and equilibrated for 1 h. Ring contraction was measured using PE hydrochloride (Sigma-Aldrich, St. Louis, MO), and endothelium-dependent and -independent relaxations were measured using ACh and sodium nitroprusside, respectively. Relaxation curves were determined following ring pre-contraction with 3×10^{-7}M PE. A total of 50 male mice were used for these vascular reactivity studies ($n = 8$ for both WT FA and WT DE; $n = 17$ for both *Gclm*$^{-/+}$ FA and *Gclm*$^{-/+}$ DE).

Detection of aortic NO• production by $Fe(DETC)_2$ spin trap and ESR

Aortic NO• production was detected in male WT and *Gclm*$^{-/+}$ mice following 6-h FA or DE inhalation by methods previously described (Khoo et al., 2004). Aortas were quickly removed after sacrifice (as above), and the aortic vessel, along with the perivascular adipose tissue was incubated in a Krebs/HEPES buffer (99 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$; 1 mM KH_2PO_4, 1.9 mM $CaCl_2$, 25 mM $NaHCO_3$, 11.1 mM glucose, 20 mM HEPES) adjusted to pH 7.4. Colloidal iron diethyldithiocarbamate $(Fe/DETC)_2$ spin trap

was prepared by dissolving sodium DETC (3.6 mg) and $FeSO_4$ $7H_2O$ (2.25 mg) under argon gas in 10 ml of ice cold Krebs-HEPES buffer. Aortas were then incubated at 37 °C in the $(Fe/DETC)_2$ spin trap for 60 minutes. Immediately after incubation, three aortas of the same genotype and treatment were combined and placed into a 1 ml syringe (with the end cut off) and frozen in liquid nitrogen. The frozen pellet was then pressed out of the syringe and stored at −80 °C until $NO^{\bullet}$ detection by electron spin resonance (ESR) spectroscopy. ESR studies were performed on a table-top x-band spectrometer Miniscope (Magnettech, Berlin, Germany). Measurements were taken on samples placed in a Dewar tube and kept in liquid nitrogen. Instrument settings were: biofield 3275, sweep 115 G, microwave frequency 9.78 GHz, microwave power 20 mW, and a kinetic time of 10 min. A total of 54 mice were used for this study (n = 3, 4, 5 and 6 for WT FA, WT DE, *Gclm*$^{-/+}$ FA and *Gclm*$^{-/+}$ DE, respectively, where each n represents aortic $NO^{\bullet}$ measured from 3 mouse aortas of the same genotype and treatment).

Statistical analyses

Data were analyzed using Prism (Graphpad Software, La Jolla, CA). Differences were determined by ANOVA followed by a Dunnett's *post-hoc* test. All error bars in figures represent standard error of the mean (SEM). *, **, *** = Significant difference from the matched control at p values of <0.05, 0.01 and 0.001, respectively. Vascular reactivity was analyzed by repeated-measurement 2-way ANOVA. Concentration-response curves were fitted with a nonlinear regression program (GraphPad Prism) to obtain effective concentration of 50% (EC50) and maximal effect values, which were compared by 1-way ANOVA.

Results

BAL, alveolar macrophage uptake of DEP, and neutrophilic lung inflammation

Images of alveolar macrophages isolated from BAL following acute exposure to DE (6 h, 300 μg/m^3) show apparent uptake of DEP (Figure 1). After the washing steps taken during the preparation of cytospins, macrophages from DE exposed mice clearly show black DEP still associated with the cells, whereas such material was not observed in macrophages collected from FA-exposed mice.

DE exposure produced a small but significant increase in neutrophils within the lungs of both WT and *Gclm*$^{-/+}$ mice (Figure 2). There is a trend whereby *Gclm*$^{-/+}$ mice had slightly less neutrophilic inflammation, but this did not reach statistical significance (p = 0.18). As we had performed this experiment with both male and female mice, we stratified our data to see if there was a sex-dependent response to DE exposure. No evidence for a differential effect in males versus females was found (Figure 3), and the trend of fewer BAL neutrophils in *Gclm*$^{-+}$ mice after DE inhalation, remained present for both sexes.

Plasma GSH, GSSG, %GSSG and GSH reduction potential ($\Delta E_{GSSG/2GSH}$)

To investigate the effects of DE on systemic oxidative stress, we measured the concentrations of reduced (GSH) and oxidized (GSSG) glutathione in the plasma of male WT and *Gclm*$^{-/+}$ mice. DE exposure did not have any significant effect on plasma GSH in WT mice. Although there was an apparent decrease in plasma GSH in *Gclm*$^{-/+}$ mice, statistical significance was not achieved (p = 0.09) (Figure 4A). Similarly, we observed slight trends for a DE-induced decrease in GSSG in both WT and *Gclm*$^{-/+}$ mice, but again, this effect did not reach statistical significance (WT, p = 0.39; *Gclm*$^{-/+}$, p = 0.19) (Figure 4B). Nonetheless, DE inhalation did significantly increase %GSSG, resulting in a more oxidized redox poise (i.e. a less negative $\Delta E_{GSSG/2GSH}$ reduction potential) in the plasma of *Gclm*$^{-/+}$ mice (Figure 4, panels C and D). We did not observe this effect in the plasma of

WT mice. Interestingly, when comparing across FA control groups, $\Delta E_{GSSG/2GSH}$ was not oxidized in the plasma of *Gclm*$^{-/+}$ mice compared to WT mice (Figure 4, panel D).

Vascular reactivity of aortic rings measured by wire myography

To investigate the effect of DE inhalation on aortic vascular reactivity, we exposed male WT and *Gclm*$^{-/+}$ mice to either FA or DE and analyzed aortic ring vascular reactivity by wire myography. We exposed both WT and *Gclm*$^{-/+}$ mice to either FA or DE for 6 h and assessed aortic ring vascular reactivity immediately thereafter. Stimulation with PE did not reveal any effect of DE inhalation or *Gclm* genotype on contractility (Figure 5). ACh-stimulated relaxation of aortic rings in WT mice showed that DE exposure caused no observable impairment in relaxation; however, there was a trend of increasing ACh-stimulated relaxation, but this trend did not reach significance (Figure 6, panel A). When assessing ACh-relaxation of aortic rings from *Gclm*$^{-/+}$ mice, DE did not produce any impairment in relaxation, but, it did significantly increase ACh-stimulated relaxation (Figure 6, panel B) (nonlinear regression log EC50: *Gclm*$^{-/+}$ FA = −7.287 (5.160×10^{-8}M ACh) 95%CI −7.380 to −7.195; *Gclm*$^{-/+}$ DE = −7.613 (2.438×10^{-8}M ACh) 95%CI −7.683 to −7.543). This effect equates to DE producing a reduction in the ACh-EC50 by roughly 2-fold in the *Gclm*$^{-/+}$ mouse. When comparing the response to ACh between the FA-exposed WT and *Gclm*$^{-/+}$ mice, we observed that aortic rings from *Gclm*$^{-/+}$ mice had a small but significant impairment in relaxation (Figure 6, panel C) (nonlinear regression log EC50: WT FA = −7.538 (2.896×10^{-8} M ACh) 95%CI −7.619 to −7.457; *Gclm*$^{-/+}$ FA = −7.287 (5.160×10^{-8} M ACh) 95%CI −7.380 to −7.195). This observation is consistent with our previously reported findings (Weldy et al., 2012). When comparing aortic rings from WT and *Gclm*$^{-/+}$ DE-exposed mice, we observed no difference in ACh-stimulated relaxation (Figure 6, panel D). Assessment of sodium nitroprusside (SNP)-stimulated aortic ring relaxation revealed no changes across genotype or treatment (data not shown), further suggesting that observations of enhanced or impaired ACh-relaxation are endothelium dependent.

Aortic NO• production as measured by $Fe(DETC)_2$ spin trap and ESR

To determine if acute DE inhalation causes any effect on aortic NO• production or bioavailability, we measured aortic NO• by spin trap and ESR. We isolated the aortas of male WT and *Gclm*$^{-/+}$ mice following either FA or DE inhalation, and measured the total NO-$Fe(DETC)_2$ accumulated over 60 min. An acute 6-h exposure to DE in WT or *Gclm*$^{-/+}$ mice did not cause any reduction in NO• production (Figure 7, panel A). However, there was a trend whereby DE appeared to increase aortic NO• production in both WT and *Gclm*$^{-/+}$ mice compared to FA controls (Figure 7, panel A), with p values of 0.11 and 0.07 for WT and *Gclm*$^{-/+}$ comparisons, respectively. When genotypes were combined, and the comparison was made between all FA and all DE-exposed mice, DE significantly increased aortic NO• production (Figure 7, panel B). Regarding the effect of DE on NO• produced across genotypes, we observed a trend for increased NO• in both WT mice (17% higher) and *Gclm*$^{-/+}$ mice (26% higher) after DE exposures compared to FA controls (Figure 7, panel C). Although DE appeared to increase aortic NO• production more in *Gclm*$^{-/+}$ mice than WT mice (Figure 7, panel C), this difference was not statistically significant.

Discussion

We investigated the effects of acute DE inhalation and *Gclm* status on neutrophilic lung inflammation, plasma GSH redox status, aortic vascular reactivity and aortic NO• production in mice. The major observations of our studies were: (1) 6-h DE inhalation produced a measurable increase in neutrophilic lung inflammation, but this effect was not altered by genotype; (2) DE inhalation caused an oxidation of the plasma GSH reduction potential in

mice heterozygous for *Gclm* but not in WT; (3) DE inhalation did not cause an impairment in ACh-stimulated relaxation or PE-stimulated contraction in aortic rings from either WT or $Gclm^{-/+}$ mice; rather, there was an increase in ACh-stimulated relaxation in aortic rings from $Gclm^{-/+}$ mice, (4) DE inhalation caused a significant increase in aortic $NO^{\bullet}$ production, an effect that appeared to be enhanced in mice heterozygous for *Gclm*. These observations suggest that an acute 6-h DE (300 μg/m^3) inhalation in WT C57BL/6 mice does not cause a direct impairment in aortic vascular reactivity, aortic $NO^{\bullet}$ production or oxidation of plasma GSH redox status, but this acute exposure does produce a modest but measurable increase in neutrophilic airway inflammation. Alternatively, in this mouse model of compromised GSH synthesis, acute DE inhalation results in minimal lung inflammation that does not exceed the inflammation seen in WT mice, but does result in oxidation of plasma GSH redox status. Moreover, DE exposure enhanced ACh-stimulated relaxation of aortic rings, shifting the relaxation curve to the left of aortic rings from FA-exposed $Gclm^{-/+}$ mice. This observation was in the opposite direction of what we had hypothesized, and the physiological relevance of this observation is currently unknown. However, these observations provide a platform for future investigations into the mechanisms of DE-induced changes in vascular function, and further support the hypothesis that *Gclm* genotype and *de novo* synthesis of GSH play important roles in mediating the effects of acute DE inhalation in mice.

Understanding the genetic determinants of DE-induced effects on cardiovascular function will be critically important to predict and reduce the adverse effects of $PM_{2.5}$-inhalation within sensitive human populations. Previously published studies of controlled DE exposures have consistently shown DE to have adverse effects on vascular function, both in humans (Brook et al., 2002; Cosselman et al., 2012; Mills et al., 2005, 2007; Peretz et al., 2008) and animals (Campen et al., 2005; Cherng et al., 2009, 2011; Knuckles et al., 2008; Nurkiewicz et al., 2004). Although oxidative stress has been strongly suggested to play a key role in these observed effects, there have been relatively few investigations into genetic determinants of susceptibility due to compromised antioxidant synthesis. We have proposed that GSH and its *de novo* synthesis play an important role in modulating the adverse effects of DE inhalation. By investigating the effect of DE inhalation in mice with compromised GSH synthesis, our aim was to determine the role of *Gclm* genotype in DE-induced pulmonary inflammation and vascular dysfunction. If *Gclm* genotype and GSH synthesis were to play a key role, this would have implications for susceptible populations who may be especially sensitive to DE inhalation, i.e. those with functional genetic polymorphisms in *GCLM* and *GCLC*. We in fact did find that *Gclm* status in mice modifies certain responses to DE inhalation, but our observations further suggest that changes in vascular function may not be directly related to pulmonary inflammation, or be consistent within all systemic arteries.

The presence of neutrophils in BAL is taken as a measure of acute, innate immune response to DE inhalation, and we have previously demonstrated that neutrophilic airway inflammation in response to DEP is highly correlated to the production of proinflammatory cytokines IL6 and TNFα (Weldy et al., 2011a). Although we had previously shown neutrophilic lung inflammation to be enhanced in $Gclm^{-/+}$ mice following intranasal instillation of DEP, we did not observe this effect in our current study. The percentage of neutrophils was significantly increased in both WT and $Gclm^{-/+}$ mice following DE inhalation compared to FA controls, but this effect was not enhanced by *Gclm* heterozygosity. In fact, our results actually suggested a mild protective effect (although not statistically significant), which would be contrary to our hypothesis. Intranasal instillation of DEP, which is delivered as a single bolus treatment, is obviously a very different exposure scenario than whole DE inhalation. This suggests that in a single large exposure to DEP, the rapid *de novo* synthesis of GSH is necessary and requires two functional alleles of *Gclm* to

maintain GSH content and limit DEP-induced inflammation. In the current inhalation study, DE exposure is over a 6-h time period, and the lack of enhanced inflammation suggests that this exposure scenario does not 'max out' the *Gclm* promoter capability and thus does not reveal any enhanced sensitivity to DE-induced lung inflammation. These observations may further highlight the complexities of extrapolating instillation studies to inhalation studies. But, our observation of mild, low-grade pulmonary inflammation following only a 6-h DE inhalation supports previous observations that DE inhalation results in neutrophilic airway inflammation (Salvi et al., 1999; Sunil et al., 2009).

Although we did not observe an effect of *Gclm* genotype on neutrophilic lung inflammation, DE did cause a significant oxidation of the plasma glutathione reduction potential in *Gclm*$^{-/+}$ mice but not WT mice (Figure 4). By measuring this, we are able to quantitate the systemic oxidant/antioxidant balance following DE inhalation (Jones et al., 2000). In addition to a significantly oxidized GSH plasma reduction potential, measures of %GSSG were also significantly increased in the plasma of *Gclm*$^{-/+}$ mice following DE inhalation. This effect seemed to be due to a loss of reduced GSH within the plasma, where we observed a strong trend of a lower level of GSH in the plasma following DE ($p = 0.09$). This observation would suggest that acute DE inhalation accelerates the oxidation of GSH to GSSG within the plasma, or it causes enhanced oxidation at various tissue locations, resulting in GSH being oxidized to GSSG within the cell and then exported to the plasma. Either would result in a subsequent drop in plasma GSH and increased plasma GSSG. As the *Gclm*$^{-/+}$ mice do not appear to have increased plasma GSSG following DE inhalation, the drop in plasma GSH may have resulted from less export by tissues such as the liver, an enhanced breakdown by γ-glutamyltransferase in tissues, or participation in covalent modifications of electrophiles (such as reactive aldehydes) mediated by glutathione-S-transferase (GST) activity.

Overall, our observation of enhanced sensitivity to DE-induced oxidation of plasma GSH in *Gclm*$^{-/+}$ mice suggests that *Gclm*$^{-/+}$ mice are unable to appropriately balance oxidant generation with antioxidants following DE inhalation. Although not necessarily causative, this observation further suggests that the *de novo* synthesis of GSH plays a key role in balancing this oxidative effect. This observation suggests that individuals with *GCLM* polymorphisms may also have increased sensitivity to DE-induced effects on systemic oxidant/antioxidant balance.

There is an extensive body of evidence indicating a strong role for vascular oxidative stress in endothelial dysfunction where the generation of excessive ROS in addition to loss of antioxidant capacity results in the inactivation of NO$^{\bullet}$ and oxidation of tetrahydrobiopterin (BH4) a vital cofactor necessary for proper eNOS function (Brocq et al., 2008). It is therefore our hypothesis that in a model of compromised antioxidant synthesis, i.e. the *Gclm*$^{-/+}$ mouse, the ability of DE to increase oxidative stress should be exacerbated, thus resulting in impaired endothelial function and NO$^{\bullet}$ generation. However, DE did not cause any impairment in ACh-stimulated vessel relaxation or PE-stimulated vessel contraction in aortic rings from either WT or *Gclm*$^{-/+}$ mice (Figures 5 and 6). Rather, there was an unexpected effect whereby DE inhalation increased ACh-stimulated vessel relaxation in the aortic rings from *Gclm*$^{-/+}$ mice (Figure 6, panel B). A trend for this effect was also observed in WT mice but this did not reach statistical significance. When comparing the ACh-stimulated relaxation across genotype in the FA controls only, we observe a slight but significant impairment in ACh-response in aortic rings from the *Gclm*$^{-/+}$ mice compared to WT (Figure 7, panel C). This observation is consistent with our previous report, where we observed this effect in combination with increased aortic 3-nitrotyrosine protein modifications and compromised NO$^{\bullet}$ generation (Weldy et al., 2012), suggesting that these *Gclm*$^{-/+}$ mice do have a mild phenotype of impaired aortic ring relaxation in response to

ACh. Thus, our observation that DE did not exacerbate this impairment, but rather produced an increase in relaxation, is counterintuitive.

We anticipated that DE exposure would be associated with a reduction in aortic $NO^{\bullet}$ in either WT or *Gclm*$^{-/+}$ mice. Contrary to our expectations, DE inhalation tended to produce an increase in aortic $NO^{\bullet}$ production in both WT and *Gclm*$^{-/+}$ mice (Figure 7, panel A). While these were only trends that approached statistical significance, when the data from both genotypes are combined, we did observe DE exposure to produce a significant increase in $NO^{\bullet}$ production (Figure 7, panel B).

Although measuring aortic ring function can provide valuable insight into vascular reactivity in mice, our observations suggest that acute DE inhalation produces no effect in aortic ring vascular reactivity in WT mice; and in *Gclm*$^{-/+}$ mice an effect opposite to what had been previously observed in either coronary arteries or mesenteric arteries in other studies of rodents exposed to DE. For example, Cherng and colleagues measured the acute (6 h exposure) effects of DE-inhalation on vascular reactivity of coronary arteries in rats (Cherng et al., 2009, 2011). They observed enhanced contraction from ET-1 and impaired ACh-stimulated relaxation, and these effects were attributed to uncoupling of eNOS and loss of bioavailable $NO^{\bullet}$ due to oxidation of its cofactor BH4. In a study of chronic exposure to $PM_{2.5}$ in rats, Sun and colleagues (Sun et al., 2008) demonstrated similar effects in the aorta, whereby impaired ACh-stimulated relaxation and enhanced PE-stimulated contraction was attributed to vascular oxidative stress, loss of bioavailable $NO^{\bullet}$ and activation of the Rho/ROCK pathway. Although we hypothesized a similar effect would occur in the aorta following acute exposures in mice, our observations might highlight the dramatic differences in physiological function between the coronary arteries and the aorta, as well as the vascular response to PM following acute as opposed to chronic exposures. Nurkiewicz and colleagues examined the effects of intratracheal instillation of PM on microvascular function and observed that PM caused marked effects on microvascular dilation (Nurkiewicz et al., 2004, 2006). This observation highlights a potentially crucial part of the mechanism of $PM_{2.5}$-induced effects on cardiovascular function whereby acute effects may be limited to the microvasculature, and large conductance vessels such as the aorta may show opposite effects following acute exposures.

The observation that aortic rings from *Gclm*$^{-/+}$ mice have enhanced ACh-mediated relaxation was unexpected. However, because *Gclm*$^{-/+}$ mice have elevated aortic oxidative stress and impaired $NO^{\bullet}$ synthesis at baseline (Weldy et al., 2012), an increased relaxation following DE inhalation suggests either activation of antioxidant response pathways (thus preventing oxidative stress and increasing bioavailable $NO^{\bullet}$), or increased NOS activity by either upregulating eNOS/iNOS expression or post-translational modifications (i.e. phosphorylation or glutathionylation of eNOS). Using whole mRNA transcriptome analysis, we previously observed the Nrf2-antioxidant response pathway to be slightly increased in the aortas of *Gclm*$^{-/+}$ mice (Weldy et al., 2012). If antioxidant response pathways within the aorta were increased following acute DE inhalation, we would expect $NO^{\bullet}$ bioavailability to return to that of WT-FA controls, as the antioxidant capacity would reduce ROS and prevent the loss of bioavailable $NO^{\bullet}$, but not necessarily increase it beyond that present in WT mice. This appeared to be the case, as aortic rings from *Gclm*$^{-/+}$-DE mice responded to ACh in a manner similar to that of WT-FA mice. This observation suggests that compensatory antioxidant responses following DE inhalation in the *Gclm*$^{-/+}$ mice may explain the enhanced ACh relaxation. If enhanced antioxidant protection did explain this observation, then we would also expect baseline aortic $NO^{\bullet}$ (as measured by ESR) to be similar to that of WT-FA. Since aortic $NO^{\bullet}$ was greater in the aortas of DE-exposed *Gclm*$^{-/+}$ mice than that of WT-FA, it seems likely that acute DE inhalation causes a modest increase in $NO^{\bullet}$

formation possibly due to increased expression or activity of NOS isoforms, and this will be pursued in future studies.

Conclusions

In the studies reported here, we examined the effects of acute DE inhalation on neutrophilic airway and lung inflammation, plasma GSH, GSSG, %GSSG, and GSH reduction potential, aortic ring vascular reactivity and aortic $NO^{\bullet}$ production in both WT and *Gclm*$^{-/+}$ mouse models. Polymorphisms in *GCLM* are highly frequent in humans (~20% of the public) and have been demonstrated to influence clinical outcomes of cardiovascular disease. The observations reported here indicate that *Gclm* genotype in mice influences their biological response to acute DE inhalation, but our observations of enhanced ACh-stimulated vessel relaxation of aortic rings suggest that previously observed impairments in ACh-relaxation and $NO^{\bullet}$ generation are likely limited to coronary arteries, the mesenteric arteries and the microvasculature. The observations reported here provide the impetus to further investigate genetic determinants of antioxidant capacity as factors influencing sensitivity to $PM_{2.5}$ inhalation on vascular function at the microvascular level.

Acknowledgments

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References

Baccarelli A, Zanobetti A, Martinelli I, et al. Air pollution, smoking, and plasma homocysteine. Environ Health Perspect. 2007; 115:176–181. [PubMed: 17384761]

Bea F, Hudson FN, Chait A, et al. Induction of glutathione synthesis in macrophages by oxidized low-density lipoproteins is mediated by consensus antioxidant response elements. Circulation Res. 2003; 92:386–393. [PubMed: 12600891]

Bea F, Hudson FN, Neff-Laford H, et al. Homocysteine stimulates antioxidant response element-mediated expression of glutamate-cysteine ligase in mouse macrophages. Atherosclerosis. 2009; 203:105–111. [PubMed: 18691715]

Bräuner EV, Forchhammer L, Møller P, et al. Exposure to ultrafine particles from ambient air and oxidative stress-induced DNA damage. Environ Health Perspect. 2007; 115:1177–1182. [PubMed: 17687444]

Brocq ML, Leslie SJ, Miliken P, Megson IL. Endothelial dysfunction: from molecular mechanisms to measurement, clinical implications, and therapeutic opportunities. Antioxid Redox Signal. 2008; 10:1631–1673. [PubMed: 18598143]

Brook RD, Brook JR, Urch B, et al. Inhalation of fine particulate air pollution and ozone causes acute arterial vasoconstriction in healthy adults. Circulation. 2002; 105:1534–1536. [PubMed: 11927516]

Campen MJ, Babu NS, Helms GA, et al. Nonparticulate components of diesel exhaust promote constriction in coronary arteries from ApoE−/− mice. Toxicol Sci. 2005; 88:95–102. [PubMed: 16093524]

Campen MJ, Lund AK, Knuckles TL, et al. Inhaled diesel emissions alter atherosclerotic plaque composition in ApoE−/− mice. Toxicol Appl Pharmacol. 2010; 242:310–317. [PubMed: 19891982]

Cherng TW, Campen MJ, Knuckles TL, et al. Impairment of coronary endothelial cell ETB receptor function after short-term inhalation exposure to whole diesel emissions. Am J Physiol Regul Integr Comp Physiol. 2009; 297:R640–R647. [PubMed: 19535675]

Cherng TW, Paffett ML, Jackson-Weaver O, et al. Mechanisms of diesel-induced endothelial nitric oxide synthase dysfunction in coronary arterioles. Environ Health Perspect. 2011; 119:98–103. [PubMed: 20870565]

Chuang K-J, Chan C-C, Su T-C, et al. The effect of urban air pollution on inflammation, oxidative stress, coagulation, and autonomic dysfunction in young adults. Am J Respir Crit Care Med. 2007; 176:370–376. [PubMed: 17463411]

Cosselman KE, Krishnan MR, Oron AP, et al. Blood pressure response to controlled diesel exhaust exposure in human subjects. Hypertension. 2012; 59:943–948. [PubMed: 22431582]

Dalton TP, Chen Y, Schneider SN, et al. Genetically altered mice to evaluate glutathione homeostasis in health and disease. Free Radic Biol Med. 2004; 37:1511–1526. [PubMed: 15477003]

Dickinson DA, Levonen A-L, Moellering DR, et al. Human glutamate cysteine ligase gene regulation through the electrophile response element. Free Radical Biol Med. 2004; 37:1152–1159. [PubMed: 15451055]

Dockery DW, Pope CA, Xu X, et al. An association between air pollution and mortality in six U.S. cities. N Engl J Med. 1993; 329:1753–1759. [PubMed: 8179653]

Eaton DL, Hamel DM. Increase in gamma-glutamylcysteine synthetase activity as a mechanism for butylated hydroxyanisole-mediated elevation of hepatic glutathione. Toxicol Appl Pharmacol. 1994; 126:145–149. [PubMed: 7910419]

Espinola-Klein C, Rupprecht HJ, Bickel C, et al. Glutathione peroxidase-1 activity, atherosclerotic burden, and cardiovascular prognosis. Am J Cardiol. 2007; 99:808–812. [PubMed: 17350371]

Franklin CC, Backos DS, Mohar I, et al. Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase. Mol Aspects Med. 2009; 30:86–98. [PubMed: 18812186]

Gould T, Larson T, Stewart J, et al. A controlled inhalation diesel exhaust exposure facility with dynamic feedback control of PM concentration. Inhalation Toxicol. 2008; 20:49–52.

Haque JA, Mcmahan RS, Campbell JS, et al. Attenuated progression of diet-induced steatohepatitis in glutathione-deficient mice. Lab Invest. 2010; 90:1704–1717. [PubMed: 20548286]

Janisko, S.; Noll, JD. Field evaluation of diesel particulate matter using portable elemental carbon monitors. In: Hardcastle, S.; McKinnon, DL., editors. Sudbury, Ontario, Canada: Proceedings of the 13th U.S./North American Mine Ventilation Symposium; 2010. 4752. MIRARCO Mining Innovation [Online]. Available from: http://www.cdc.gov/niosh/mining/UserFiles/works/pdfs/feodp.pdf. [Last accessed: 3 Jun 2013]

Jin RC, Mahoney CE, Coleman Anderson L, et al. Glutathione peroxidase-3 deficiency promotes platelet-dependent thrombosis in vivo. Circulation. 2011; 123:1963–1973. [PubMed: 21518981]

Johansson E, Wesselkamper SC, Shertzer HG, et al. Glutathione deficient C57BL/6J mice are not sensitized to ozone-induced lung injury. Biochem Biophys Res Commun. 2010; 396:407–412. [PubMed: 20417186]

Jones DP, Carlson JL, Mody VC, et al. Redox state of glutathione in human plasma. Free Radic Biol Med. 2000; 28:625–635. [PubMed: 10719244]

Kampfrath T, Maiseyeu A, Ying Z, et al. Chronic fine particulate matter exposure induces systemic vascular dysfunction via NADPH oxidase and TLR4 pathways. Circulation Res. 2011; 108:716–726. [PubMed: 21273555]

Khoo JP, Alp NJ, Bendall JK, et al. EPR quantification of vascular nitric oxide production in genetically modified mouse models. Nitric Oxide. 2004; 10:156–161. [PubMed: 15158695]

Knuckles TL, Lund AK, Lucas SN, Campen MJ. Diesel exhaust exposure enhances venoconstriction via uncoupling of eNOS. Toxicol Appl Pharmacol. 2008; 230:346–351. [PubMed: 18455212]

Koide S-I, Kugiyama K, Sugiyama S, et al. Association of polymorphism in glutamate-cysteine ligase catalytic subunit gene with coronary vasomotor dysfunction and myocardial infarction. J Am Coll Cardiol. 2003; 41:539–545. [PubMed: 12598062]

Krejsa CM, Franklin CC, White CC, et al. Rapid activation of glutamate cysteine ligase following oxidative stress. J Biol Chem. 2010; 285:16116–16124. [PubMed: 20332089]

Leopold JA, Dam A, Maron BA, et al. Aldosterone impairs vascular reactivity by decreasing glucose-6-phosphate dehydrogenase activity. Nat Med. 2007; 13:189–197. [PubMed: 17273168]

Lewtas J. Air pollution combustion emissions: characterization of causative agents and mechanisms associated with cancer, reproductive, and cardiovascular effects. Mutat Res. 2007; 636:95–133. [PubMed: 17951105]

Lu SC. Regulation of glutathione synthesis. Molec Aspects Med. 2009; 30:42–59. [PubMed: 18601945]

Maron BA, Zhang Y-Y, Handy DE, et al. Aldosterone increases oxidant stress to impair guanylyl cyclase activity by cysteinyl thiol oxidation in vascular smooth muscle cells. J Biol Chem. 2009; 284:7665–7672. [PubMed: 19141618]

McConnachie LA, Mohar I, Hudson FN, et al. Glutamate cysteine ligase modifier subunit deficiency and gender as determinants of acetaminophen-induced hepatotoxicity in mice. Toxicol Sci. 2007; 99:628–636. [PubMed: 17584759]

Miller KA, Siscovick DS, Sheppard L, et al. Long-term exposure to air pollution and incidence of cardiovascular events in women. N Engl J Med. 2007; 356:447–458. [PubMed: 17267905]

Mills NL, Tärnqvist H, Gonzalez MC, et al. Ischemic and thrombotic effects of dilute diesel-exhaust inhalation in men with coronary heart disease. N Engl J Med. 2007; 357:1075–1082. [PubMed: 17855668]

Mills NL, Törnqvist H, Robinson SD, et al. Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. Circulation. 2005; 112:3930–3936. [PubMed: 16365212]

Mudway IS, Stenfors N, Duggan ST, et al. An in vitro and in vivo investigation of the effects of diesel exhaust on human airway lining fluid antioxidants. Arch Biochem Biophys. 2004; 423:200–212. [PubMed: 14871482]

Nakamura S-I, Kugiyama K, Sugiyama S, et al. Polymorphism in the 5′-flanking region of human glutamate-cysteine ligase modifier subunit gene is associated with myocardial infarction. Circulation. 2002; 105:2968–2973. [PubMed: 12081989]

Nakamura S-I, Sugiyama S, Fujioka D, et al. Polymorphism in glutamate-cysteine ligase modifier subunit gene is associated with impairment of nitric oxide-mediated coronary vasomotor function. Circulation. 2003; 108:1425–1427. [PubMed: 12975258]

Nurkiewicz TR, Porter DW, Barger M, et al. Particulate matter exposure impairs systemic microvascular endothelium-dependent dilation. Environ Health Perspect. 2004; 112:1299–1306. [PubMed: 15345343]

Nurkiewicz TR, Porter DW, Barger M, et al. Systemic microvascular dysfunction and inflammation after pulmonary particulate matter exposure. Environ Health Perspect. 2006; 114:412–419. [PubMed: 16507465]

Peretz A, Sullivan JH, Leotta DF, et al. Diesel exhaust inhalation elicits acute vasoconstriction in vivo. Environ Health Perspect. 2008; 116:937–942. [PubMed: 18629317]

Peters A, Dockery DW, Muller JE, Mittleman MA. Increased particulate air pollution and the triggering of myocardial infarction. Circulation. 2001; 103:2810–2815. [PubMed: 11401937]

Peters A, Liu E, Verrier RL, et al. Air pollution and incidence of cardiac arrhythmia. Epidemiology. 2000; 11:11–17. [PubMed: 10615837]

Pope CA, Burnett RT, Thun MJ, et al. Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution. JAMA. 2002; 287:1132–1141. [PubMed: 11879110]

Pope CA, Burnett RT, Thurston GD, et al. Cardiovascular mortality and long-term exposure to particulate air pollution: epidemiological evidence of general pathophysiological pathways of disease. Circulation. 2004; 109:71–77. [PubMed: 14676145]

Pourazar J, Mudway IS, Samet JM, et al. Diesel exhaust activates redox-sensitive transcription factors and kinases in human airways. Am J Physiol Lung Cell Mol Physiol. 2005; 289:L724–L730. [PubMed: 15749742]

Rich DQ, Mittleman MA, Link MS, et al. Increased risk of paroxysmal atrial fibrillation episodes associated with acute increases in ambient air pollution. Environ Health Perspect. 2006; 114:120–123. [PubMed: 16393668]

Rich DQ, Schwartz J, Mittleman MA, et al. Association of short-term ambient air pollution concentrations and ventricular arrhythmias. Am J Epidemiol. 2005; 161:1123–1132. [PubMed: 15937021]

Romieu I, Garcia-Esteban R, Sunyer J, et al. The effect of supplementation with omega-3 polyunsaturated fatty acids on markers of oxidative stress in elderly exposed to PM(2.5). Environ Health Perspect. 2008; 116:1237–1242. [PubMed: 18795169]

Romieu I, Téllez-Rojo MM, Lazo M, et al. Omega-3 fatty acid prevents heart rate variability reductions associated with particulate matter. Am J Respir Crit Care Med. 2005; 172:1534–1540. [PubMed: 16210665]

Salvi S, Blomberg A, Rudell B, et al. Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. Am J Respiratory Crit Care Med. 1999; 159:702–709.

Shenvi SV, Smith E, Hagen TM. Identification of age-specific Nrf2 binding to a novel antioxidant response element locus in the Gclc promoter: a compensatory means for the loss of glutathione synthetic capacity in the aging rat liver? Aging Cell. 2012; 11:297–304. [PubMed: 22212472]

Sørensen M, Daneshvar B, Hansen M, et al. Personal PM2.5 exposure and markers of oxidative stress in blood. Environ Health Perspect. 2003; 111:161–166. [PubMed: 12573899]

Stamler J, Cunningham M, Loscalzo J. Reduced thiols and the effect of intravenous nitroglycerin on platelet aggregation. Am J Cardiol. 1988; 62:377–380. [PubMed: 3137795]

Sun Q, Yue P, Ying Z, et al. Air pollution exposure potentiates hypertension through reactive oxygen species-mediated activation of Rho/ROCK. Arterioscler Thromb Vasc Biol. 2008; 28:1760–1766. [PubMed: 18599801]

Sunil VR, Patel KJ, Mainelis G, et al. Pulmonary effects of inhaled diesel exhaust in aged mice. Toxicol Appl Pharmacol. 2009; 241:283–293. [PubMed: 19729031]

Thompson SA, White CC, Krejsa CM, et al. Induction of glutamate-cysteine ligase (gamma-glutamylcysteine synthetase) in the brains of adult female mice subchronically exposed to methylmercury. Toxicol Lett. 1999; 110:1–9. [PubMed: 10593589]

Vinzents PS, Møller P, Sørensen M, et al. Personal exposure to ultrafine particles and oxidative DNA damage. Environ Health Perspect. 2005; 113:1485–1490. [PubMed: 16263500]

Watanabe Y, Watanabe K, Kobayashi T, et al. Chronic depletion of glutathione exacerbates ventricular remodeling and dysfunction in the pressure-overloaded heart. Cardiovasc Res. 2013; 97:282–292. [PubMed: 23129588]

Weiss N, Zhang YY, Heydrick S, et al. Overexpression of cellular glutathione peroxidase rescues homocyst(e)ine-induced endothelial dysfunction. Proc Natl Acad Sci USA. 2001; 98:12503–12508. [PubMed: 11606774]

Weldy CS, Luttrell IP, White CC, et al. Glutathione (GSH) and the GSH synthesis gene Gclm modulate vascular reactivity in mice. Free Radic Biol Med. 2012; 53:1264–1278. [PubMed: 22824862]

Weldy CS, White CC, Wilkerson H-W, et al. Heterozygosity in the glutathione synthesis gene Gclm increases sensitivity to diesel exhaust particulate induced lung inflammation in mice. Inhal Toxicol. 2011a; 23:724–735. [PubMed: 21967497]

Weldy CS, Wilkerson H-W, Larson TV, et al. DIESEL particulate exposed macrophages alter endothelial cell expression of eNOS, iNOS, MCP1, and glutathione synthesis genes. Toxicol In Vitro. 2011b; 25:2064–2073. [PubMed: 21920430]

Wild AC, Mulcahy RT. Regulation of gamma-glutamylcysteine synthetase subunit gene expression: insights into transcriptional control of antioxidant defenses. Free Radical Res. 2000; 32:281–301. [PubMed: 10741850]

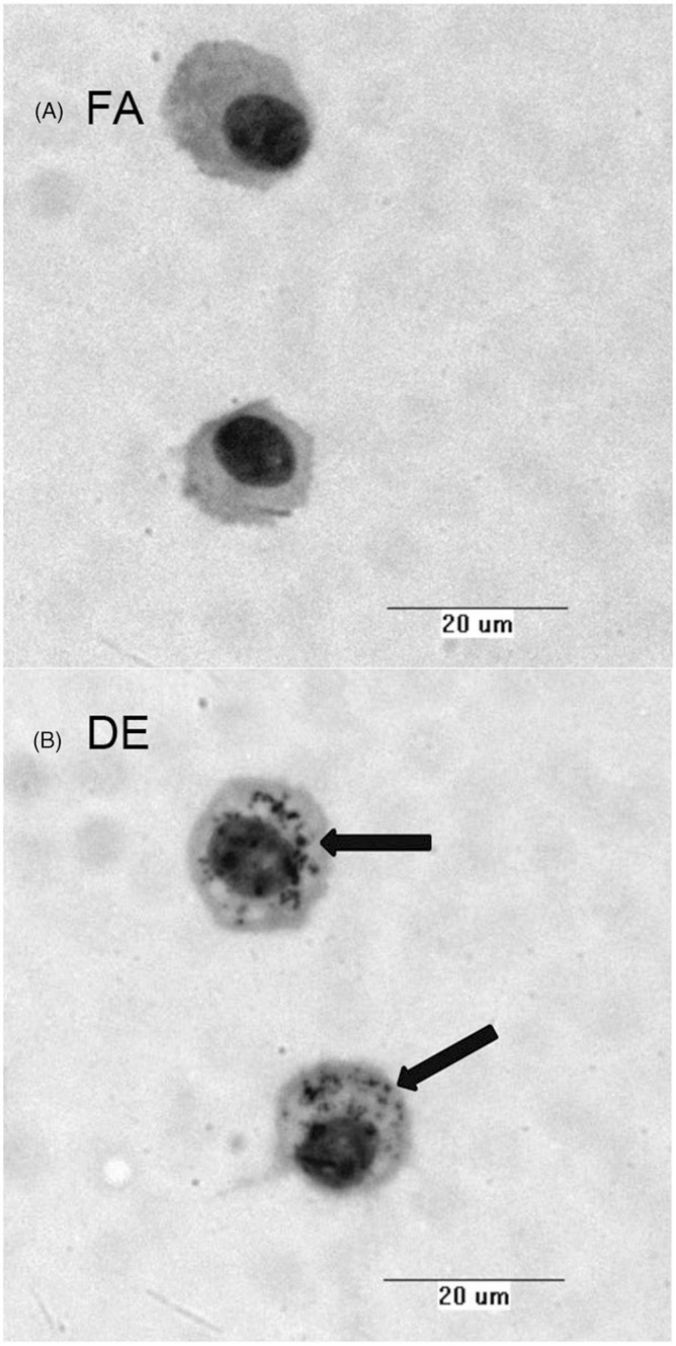

Figure 1.
Alveolar macrophages collected by BAL of mice treated with either filtered air (FA) or diesel exhaust (DE) for 6 h. Images reveal diesel exhaust particulate taken up into the cell in DE-exposed mice.

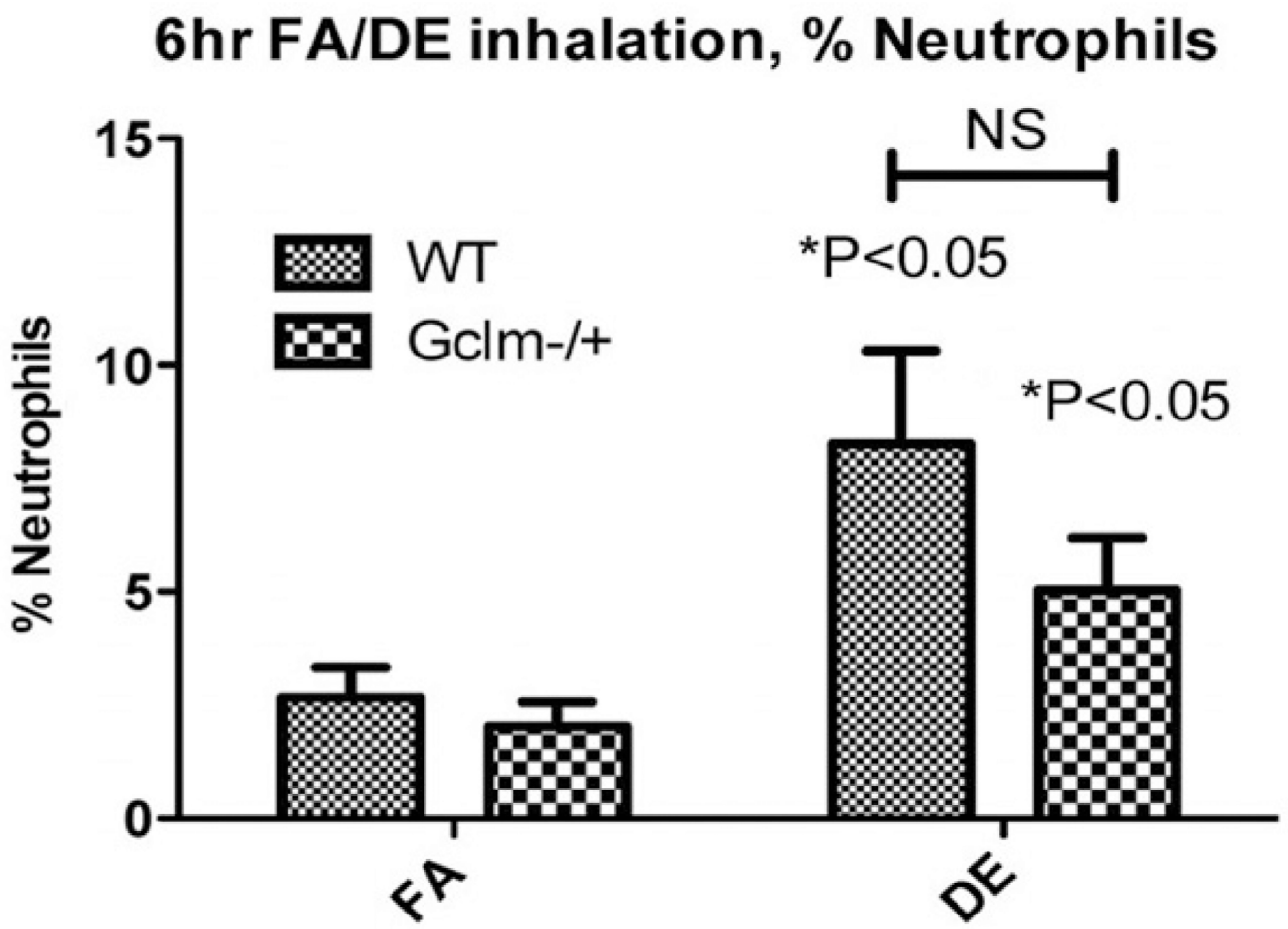

Figure 2.

Percent neutrophils ($Gr1^{hi}/F4/80^{lo}/CD11b^{vhi}$) as measured by the FACS analysis of BAL cells collected from FA and DE-exposed male and female mice. $N = 12$ for each genotype and treatment with equal number of each sex in each group. *Significantly different ($p < 0.05$) compared to genotype specific FA control by ANOVA and Dunnett's *t*-test.

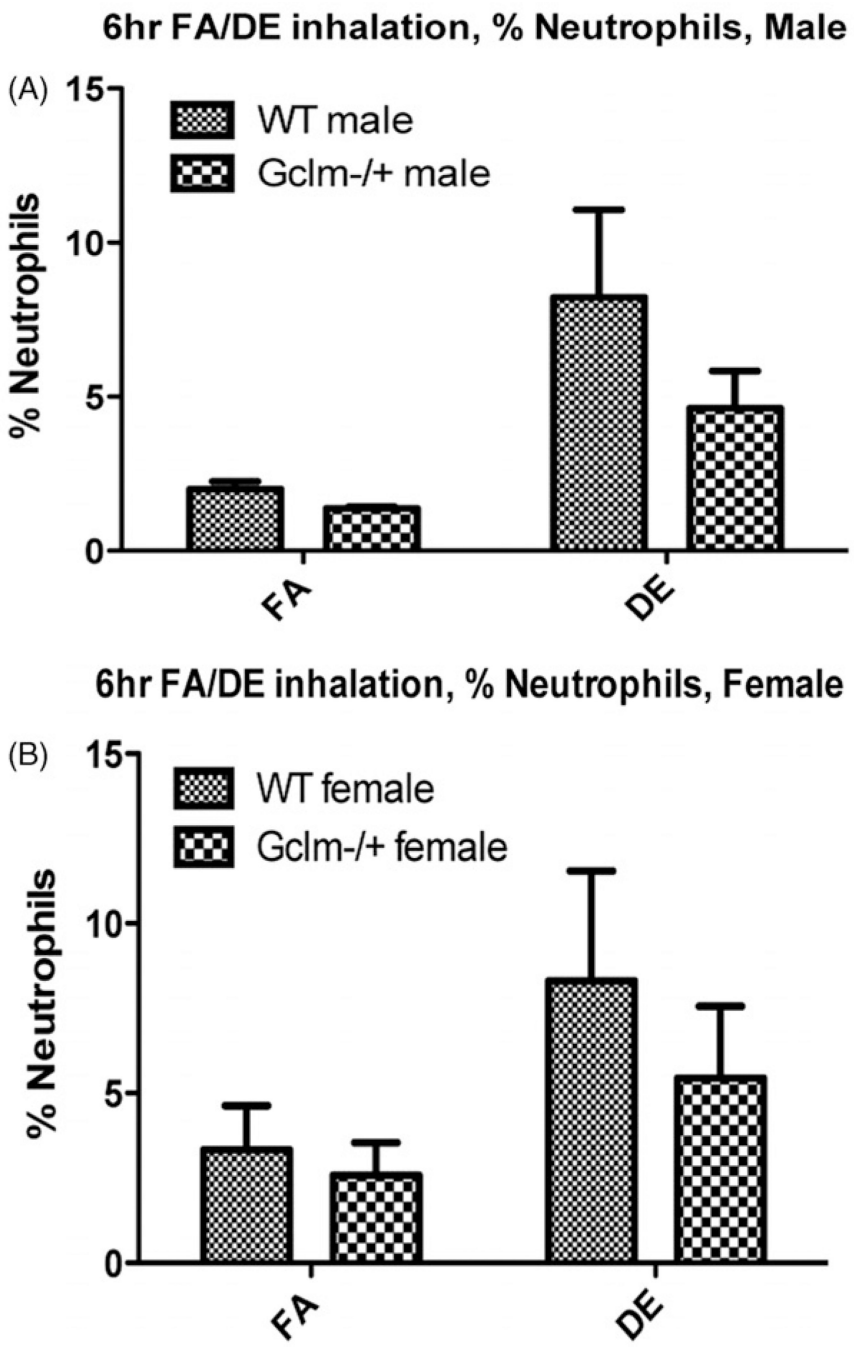

Figure 3.
Stratification of percent neutrophils in BAL by sex. The data reveal nearly identical trends for each sex.

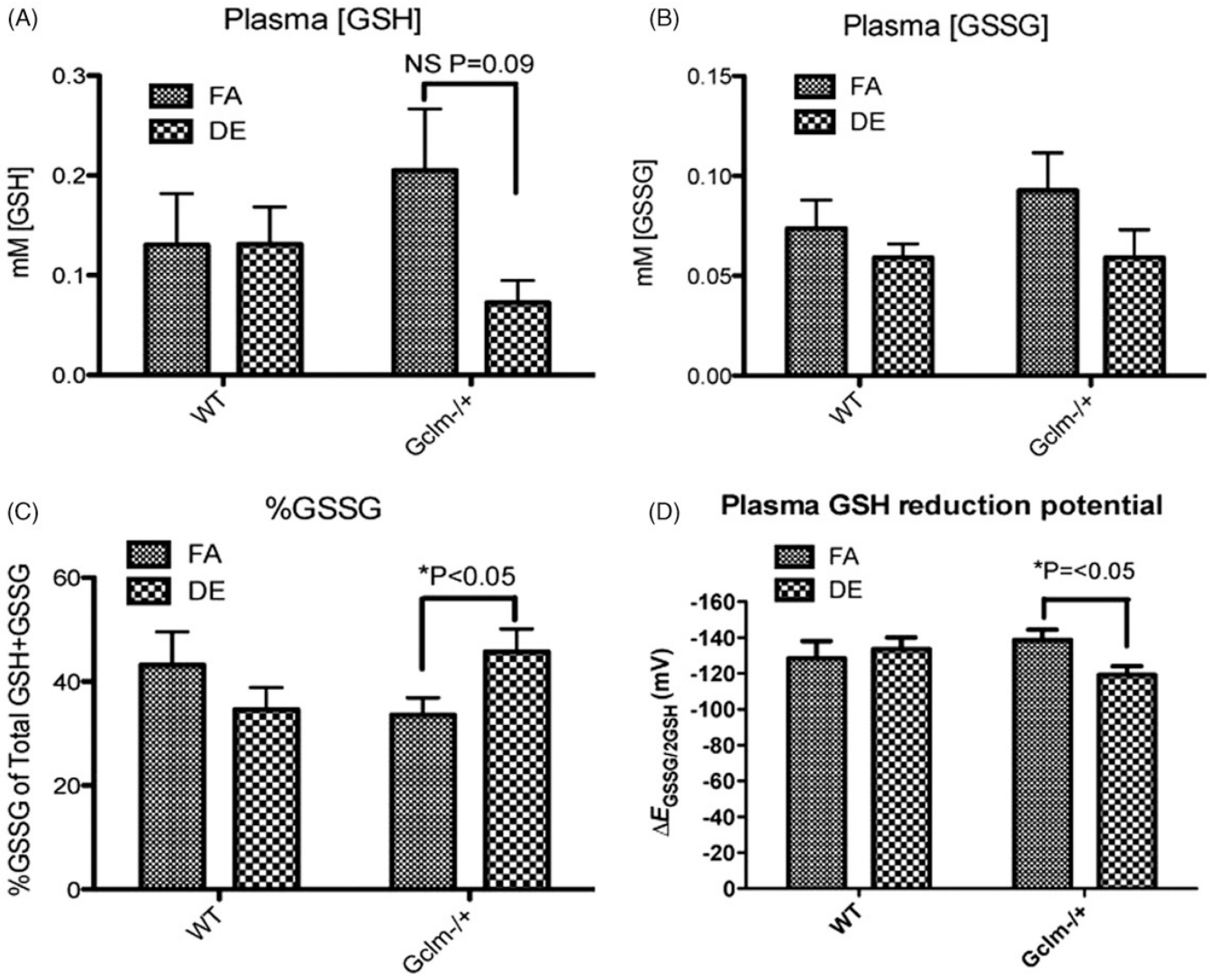

Figure 4.

Plasma [GSH] (A), plasma [GSSG] (B), %GSSG of total glutathione (C), and GSH reduction potential (D) in male FA or DE exposed mice. $N = 6$ for each genotype and treatment. *Significantly different ($p < 0.05$) compared to genotype specific FA control by ANOVA and Dunnett's *t*-test.

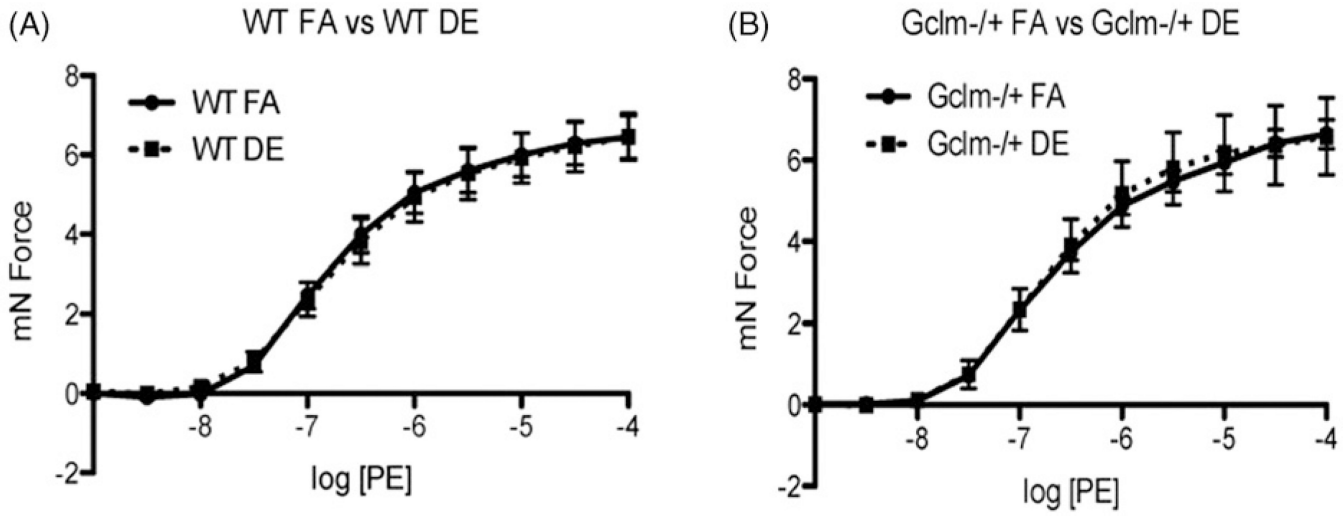

Figure 5.
Measurement of phenylephrine (PE)-stimulated contraction of aortic rings from WT and *Gclm*$^{-/+}$ mice following 6 h FA or DE inhalation. WT FA and DE: $N = 8$, *Gclm*$^{-/+}$ FA and DE: $N = 9$.

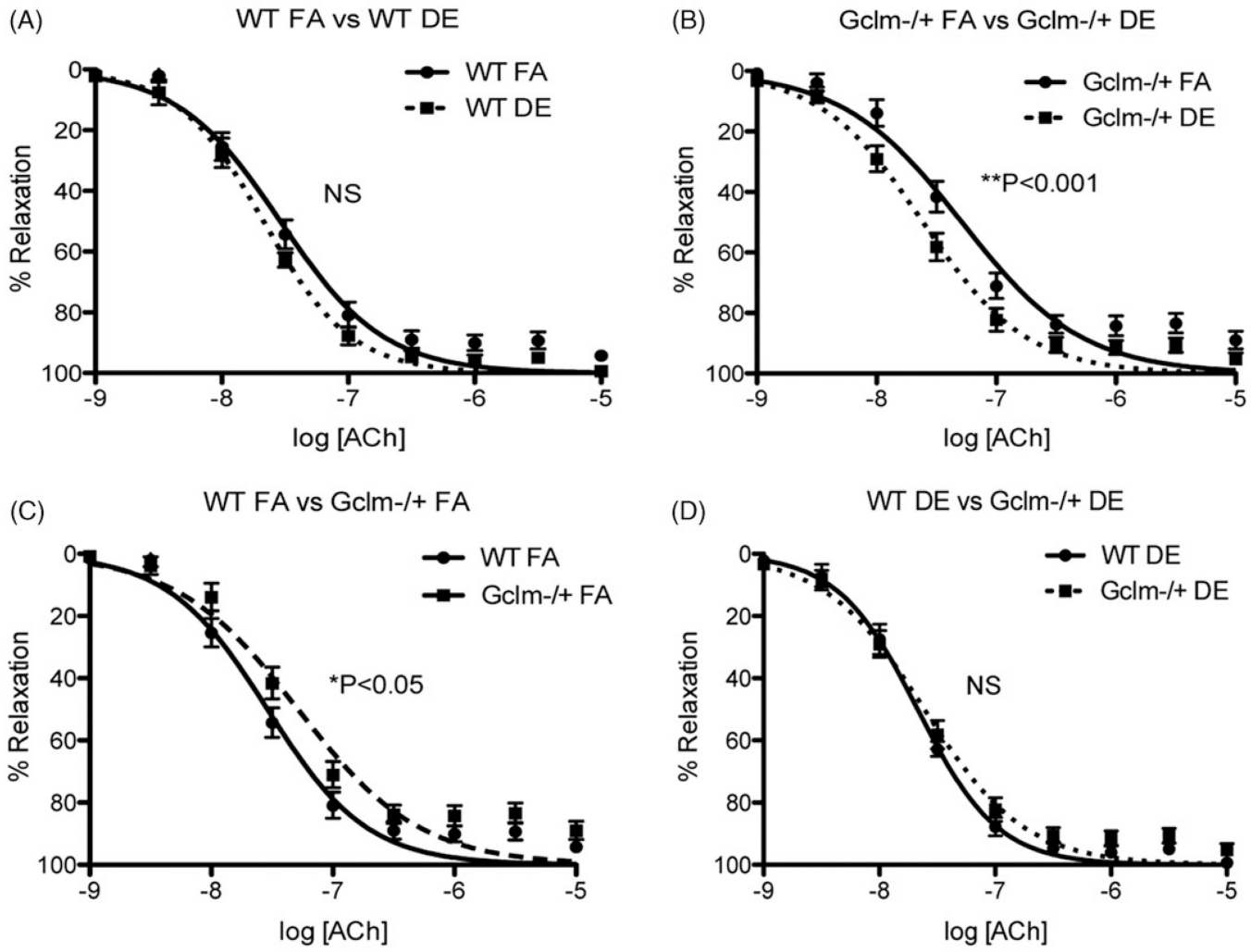

Figure 6.

Measurement of acetylcholine (ACh)-stimulated relaxation of aortic rings from WT and *Gclm*$^{-/+}$ mice following 6 h FA or DE inhalation. (A) WT FA versus WT DE, (B) *Gclm*$^{-/+}$ FA versus *Gclm*$^{-/+}$ DE, (C) WT FA versus *Gclm*$^{-/+}$ FA, (D) WT DE versus *Gclm*$^{-/+}$ DE. Curves represent nonlinear regression model used for EC50 and significance testing. Significance (*$p < 0.05$, **$p < 0.001$) determined by nonlinear regression fit and EC50 determination, as well as by two-way ANOVA. WT FA and DE: $N = 8$, *Gclm*$^{-/+}$ FA and DE: $N = 17$.

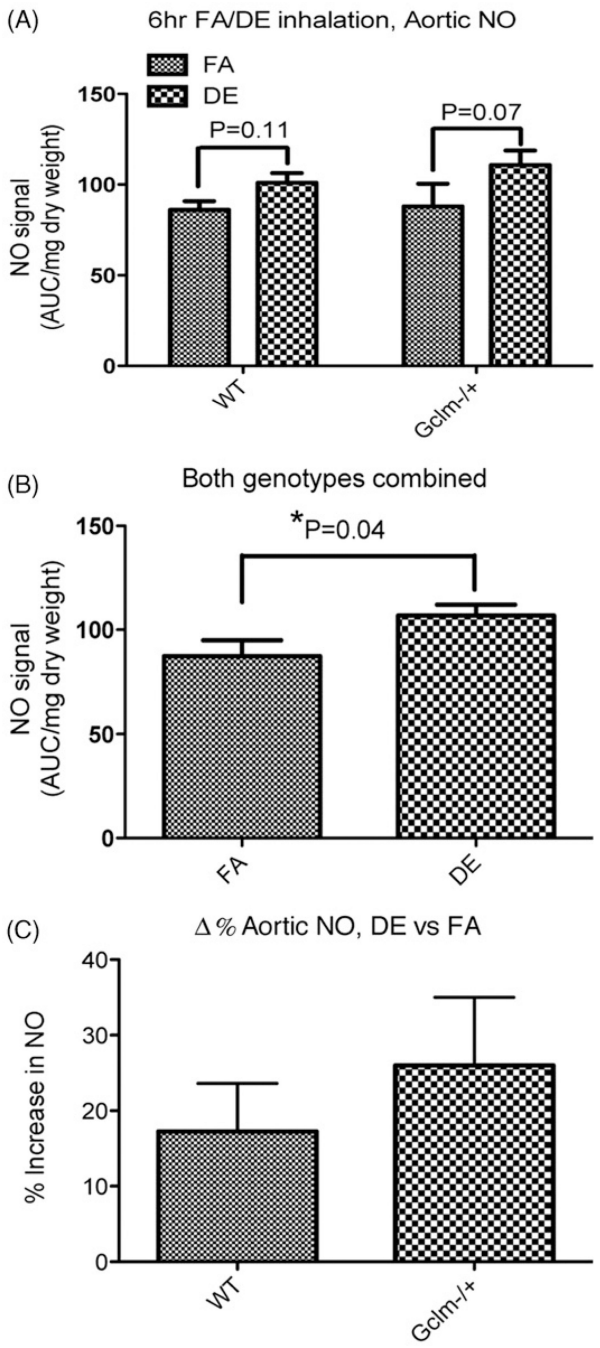

Figure 7.

Measurement of aortic NO• production by $Fe(DETC)_2$ spin trap and ESR. NO-$Fe(DETC)_2$ signal relative to dry weight of aortic tissue (A) within genotypes, (B) with both genotypes combined, (C) delta of percentage increase in NO signal of DE versus FA in WT and $Gclm^{-/+}$ mice. *Significantly different ($p < 0.05$) compared to genotype specific FA control by ANOVA and Dunnett's *t*-test.