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Heterozygosity in the glutathione synthesis gene *Gclm* increases sensitivity to diesel exhaust particulate induced lung inflammation in mice

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Abstract

Context—Inhalation of ambient fine particulate matter (PM_{2.5}) is associated with adverse respiratory and cardiovascular effects. A major fraction of PM_{2.5} in urban settings is diesel exhaust particulate (DEP), and DEP-induced lung inflammation is likely a critical event mediating many of its adverse health effects. Oxidative stress has been proposed to be an important factor in PM_{2.5}-induced lung inflammation, and the balance between pro- and antioxidants is an important regulator of this inflammation. An important intracellular antioxidant is the tripeptide thiol glutathione (GSH). Glutamate cysteine ligase (GCL) carries out the first step in GSH synthesis. In humans, relatively common genetic polymorphisms in both the catalytic (Gclc) and modifier (Gclm) subunits of GCL have been associated with increased risk for lung and cardiovascular diseases.

Objective—This study was aimed to determine the effects of *Gclm* expression on lung inflammation following DEP exposure in mice.

Materials and methods—We exposed *Gclm* wild type, heterozygous, and null mice to DEP via intranasal instillation and assessed lung inflammation as determined by neutrophils and inflammatory cytokines in lung lavage, inflammatory cytokine mRNA levels in lung tissue, as well as total lung GSH, Gclc, and *Gclm* protein levels.

Results—The *Gclm* heterozygosity was associated with a significant increase in DEP-induced lung inflammation when compared to that of wild type mice.

Discussion and conclusion—This finding indicates that GSH synthesis can mediate DEP-induced lung inflammation and suggests that polymorphisms in *Gclm* may be an important factor in determining adverse health outcomes in humans following inhalation of PM_{2.5}.

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Declaration of interest

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Keywords

Diesel exhaust particulate; lung inflammation; glutathione; oxidative stress; glutamate cysteine ligase

Introduction

The inhalation of fine ambient particulate matter (PM_{2.5}) has long been associated with increased risk of adverse pulmonary and cardiovascular events such as the exacerbation of asthma symptoms, airway inflammation, myocardial ischemia, and myocardial infarction (MI) (Dockery et al., 1993; Hoek et al., 2001; Hoek et al., 2002; Pope et al., 2002). Ambient PM_{2.5} has a wide range of sources, but in many urban areas, the majority of PM_{2.5} is derived from diesel exhaust particulate (DEP) (Lewtas, 2007). Thus, DEP has been widely used as a model toxicant to investigate PM_{2.5} toxicity. The pathophysiological effects observed with controlled human and animal DEP exposures are wide ranging and include neutrophilic inflammation (Nordenhäll et al., 2000; Salvi et al., 1999; Stenfors et al., 2004) and activated antioxidant response pathways (Mudway et al., 2004; Pourazar et al., 2005) within the airway. Many researchers have suggested that PM_{2.5}-induced pulmonary inflammation is influencing observed systemic changes such as inflammation, oxidative stress, and compromised vasomotor function (Stenfors et al., 2004; Sun et al., 2005; Sun et al., 2008; Törnqvist et al., 2007). DEP is largely considered a prooxidant and it is widely believed that it produces pulmonary inflammation at least in part by causing oxidative stress following particle uptake by alveolar macrophages and respiratory epithelial cells (Banerjee et al., 2009; Finkelstein et al., 1997; Pourazar et al., 2005).

Glutathione (GSH) is an antioxidant tripeptide that can reach millimolar levels within certain cells such as hepatocytes. The GSH plays a critical role in maintaining intracellular redox status with GSH-dependent enzymes including GSH reductase and GSH peroxidases. The GSH is synthesized in a two-step process: (1) glutamate and cysteine are combined together to form γ -glutamylcysteine (γ -GC), catalyzed by glutamate cysteine ligase (GCL) and (2) γ -GC is combined with glycine to form GSH, catalyzed by glutathione synthase (GS) (Franklin et al., 2009). The rate-limiting step in GSH synthesis is the formation of γ -GC by GCL. The GCL is a two-subunit enzyme composed of modifier (Gclm) and catalytic (Gclc) subunits and it has been previously demonstrated that Gclm null (*Gclm*^{-/-}) mice are dramatically compromised in GCL activity and GSH synthesis, and thus provide an excellent tool to investigate the role of GSH in mediating oxidative stress and toxicant induced injury (Botta et al., 2008; McConnachie et al., 2007; Yang et al., 2002).

Several prior studies have examined the role of the transcription factor Nrf2, which governs multiple antioxidant pathways, as a susceptibility factor for DEP-induced lung injury and inflammation. The Nrf2 is an important determinant of Gclm and Gclc expression (Bea et al., 2003; Bea et al., 2009). While oxidative stress and Nrf2-regulated antioxidant status have been pointed to as major factors mediating the observed responses with in vivo exposures to DEP (Li et al., 2010; Li et al., 2008), there have been no studies published to date specifically investigating susceptibility to DEP-induced inflammation in an animal model of compromised GSH synthesis. The GSH synthesis and redox status have been implicated in the pathogenesis of many clinical conditions. In particular, relatively common single nucleotide polymorphisms (SNPs) present within the 5' promoter regions of Gclm and Gclc have been associated with increased risk of MI, compromised vascular reactivity, and decreased lung function (Koide et al., 2003; Nakamura et al., 2002; Nakamura et al., 2003; Siedlinski et al., 2008). Nakamura et al. (2002) demonstrated that the presence of a SNP within only a single allele of Gclm (~20% of control group) was associated with a two-

fold increase in the risk of MI in humans, providing strong support for the hypothesis that *Gclm* expression, GCL activity, and GSH are important factors in mediating the risk of cardiovascular disease. In addition to vascular effects, it was demonstrated that SNPs within the 5' promoter region of *Gclc* are associated with accelerating the decrease in lung function resulting from smoking (Siedlinski et al., 2008). Although these SNPs within *Gclc* were not found to be associated with increased risk of developing chronic obstructive pulmonary disease (COPD) within a smoking cohort (Chappell et al., 2008), observations that these SNPs accelerate lung function decline in smokers suggest that GSH and GCL activity are important factors that influence pulmonary function under conditions of oxidative stress. Due to a relatively high frequency of these polymorphisms and the relatively large effect on health outcome, there is a need for increased research on the potential of these SNPs to produce gene-environment interactions following exposure to common toxicants, including PM2.5. Although *Gclm*^{-/-} mice have a dramatic reduction in GSH, these mice are not overtly sensitive to all agents that produce oxidative stress. It was recently demonstrated that many alternative antioxidant enzymes are induced in the livers of these mice, and this was associated with protection from steatohepatitis induced by a methionine and choline deficient diet (Haque et al., 2010). Similarly, *Gclm*^{-/-} mice are not particularly sensitive to ozone-induced lung injury, apparently for similar reasons (Johansson et al., 2010). However, *Gclm* heterozygous (*Gclm*^{+/-}) mice do not have a dramatic reduction in GSH, and thus would not be expected to have upregulated many compensatory antioxidant enzymes that could provide protection from oxidant injury. In humans having the —588C/T polymorphism within the 5' promoter region of *Gclm*, the total plasma GSH levels are not dramatically reduced (Nakamura et al., 2002), but it is possible that these people have a compromised ability to upregulate *Gclm* expression during periods of oxidative stress. This condition is highly similar to that of *Gclm*^{+/-} mice, and thus this mouse model is a potentially valuable to investigate susceptibility to environmental toxicants where compromised ability to upregulate *Gclm* may predispose to oxidative stress-induced disease. In this report, we exposed C57BL/6 *Gclm* wild type (WT), *Gclm*^{+/-}, and *Gclm*^{-/-} mice to DEP via intranasal instillation. Animals were sacrificed 6 h after instillation, and a series of measures were used to gauge the degree of lung inflammation. We hypothesized that although *Gclm*^{+/-} mice will not have dramatically lower GSH, they will be more susceptible to injury due to compromised ability to fully upregulate *Gclm* expression in response to DEP exposure. In addition, we hypothesized that *Gclm*^{-/-} mice may be still more sensitive to DEP than *Gclm* WT mice, but not overtly sensitive as they have compensated for the decrease in GSH by upregulating many alternative antioxidant enzymes.

Materials and methods

DEP collection

The PM2.5 was collected from a Cummins diesel engine operating under 75% maximum load. Particles were collected from the outflow duct at the University of Washington diesel exhaust exposure facility. The fine particulate matter size distributions are very similar to aged diesel exhaust particles a few hundred meters away from a major roadway; these particles and exposure facility characteristics have been previously described (Gould et al., 2008). The DEP were suspended in DMSO (2.5%) then further diluted in PBS (97.5%) to 10 mg/mL. These DEP stock solutions were sonicated for 1 min prior to all dosing. All control animals were dosed with an equivalent volume of the 2.5% DMSO, 97.5% PBS solution as a solvent control.

Mice and intranasal instillation

The *Gclm* WT, *Gclm*^{+/-}, and *Gclm*^{-/-} mice backcrossed for at least 10 generations onto the C57BL/6 background 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 littermates were genotyped, as previously described (McConnachie et al., 2007), and randomly assigned to either saline or DEP treatments. Mice between the ages of 8 and 12 weeks were subjected to light anesthesia by I.P. injection of 0.01 mL/g body weight of a 0.44 mg/mL Xylazine, 6.5 mg/mL Ketamine solution in sterile saline. During anesthesia, 10 μ L of a 10 mg/mL solution of DEP was slowly pipetted into each nostril for a total dose of 20 μ L/mouse (~200 μ g DEP, ~6.7 mg/kg in a 30 g mouse).

Bronchial alveolar lavage, cell staining, and flow cytometry

Mice were sacrificed by CO₂ narcosis followed by cervical dislocation 6 h after being exposed to DEP. This 6 h time-point was chosen as it allowed us to assess the acute inflammatory response (e.g. neutrophil influx into the alveolar space), as well as changes in mRNA expression for various stress responsive genes. 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. The PBS was used as the lavage medium, and following catheter insertion into the trachea, 1 mL of PBS was slowly instilled into the lungs and subsequently withdrawn. This rinsing action was repeated 3 $\times$ 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 $\times$ g for 15 min at 4°C. The supernatant from the first wash was collected for cytokine analysis, whereas the supernatant from the second and third washes was discarded. Cells from all three washes were combined, treated with a red blood cell lysis buffer (ammonium chloride lysing solution; 1.5 M NH₄Cl, 10 mM NaHCO₃, 1 mM disodium EDTA, in dH₂O) 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, USA; Cat# 53-4801-80) and biotinylated anti-mouse Ly-6G/Ly6C (Gr1) (BioLegend, San Diego, CA, USA; Cat# 108404). Subsequently, streptavidin Alexafluor 350 (Invitrogen, Carlsbad, CA, USA; Cat# S11249) was added. Cells were analyzed on a Beckman-Coulter Altra fluorescence activated cell sorter (FACS) (Beckman-Coulter, Miami, FL, USA), and 5000 cells were examined for each animal. Neutrophils were identified as cells expressing low F4/80 and high Gr1 levels as indicated by green versus blue fluorescence intensity, respectively. A total of 62 mice were used in the assessment of neutrophil influx (13 WT-PBS, 10 WT-DEP, 12 *Gclm*^{-/-}-PBS, 10 *Gclm*^{-/-}-DEP, 8 *Gclm*^{-/-}-PBS, and 9 *Gclm*^{-/-}-DEP).

RNA isolation and fluorogenic 5' nuclease-based assay and quantitative RT-PCR

Lung tissue from each animal was collected from the base of the inferior lobe of the right lung. Tissue samples were placed in RNA stabilizing solution (Trizol; Invitrogen, Carlsbad, CA, USA), and immediately homogenized. The RNA isolation was subsequently carried out using a Qiagen RNeasy kit (Qiagen, Valencia, CA, USA). The Center for Ecogenetics Functional Genomics Laboratory at the University of Washington developed fluorogenic 5' nuclease-based assays to quantitate the mRNA levels of specific genes. Briefly, reverse transcription was performed according to the manufacturer's established protocol using total RNA and the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). For gene expression measurements, 2 μ L of cDNA containing eluant were included in a PCR reaction (12 μ L final volume) that also consisted of the appropriate forward (FP) and reverse (RP) primers, probes, and TaqMan Gene Expression Master Mix (Applied Biosystems Inc., Foster City, CA, USA). The PCR primers and the dual-labeled probes for

the genes were designed using ABI Primer Express version 1.5 software (Applied Biosystems Inc., Foster City, CA, USA). The expression levels of several genes were assessed using the ABI inventoried TaqMan® Gene Expression Assays (Applied Biosystems Inc., Foster City, CA, USA) mix according to the manufacturer's protocol. Amplification and detection of PCR amplicons were performed with the ABI PRISM 7900 system (Applied Biosystems Inc., Foster City, CA, USA) with the following PCR reaction profile: 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 30 s, and 62°C for 1 min. The β -actin amplification plots derived from serial dilutions of an established reference sample were used to create a linear regression formula in order to calculate expression levels, and β -actin gene expression levels were utilized as an internal control to normalize the data. Six mice from each genotype and treatment were used to analyze differential expression of the selected genes.

Bronchoalveolar lavage fluid cytokine measurement

Bronchoalveolar lavage fluid (BALF) samples from the first wash were analyzed for IL6 and TNF α concentration by enzyme-linked immunosorbent assay (ELISA) using "Ready-Set-Go!" ELISA kits following the manufacturer protocol (eBioscience, San Diego, CA, USA; Cat#s 88-7064-22 and 88-7324-22). Moreover, 100 μ L of sample were used for each measurement, which were performed in duplicate. Six mice from each genotype and treatment were used for cytokine analysis.

Myeloperoxidase ELISA

Lungs were homogenized in tissue lysis buffer (Sigma-Aldrich) with a proteinase inhibitor cocktail pill (Roche Applied Science, Indianapolis, IN, USA), aliquoted, and snap frozen in liquid nitrogen. Wells of a 96-well plate were incubated with capture antibody overnight at room temperature (1 μ g/mL anti-human/mouse myeloperoxidase (MPO), AF3667, R&D Systems, Minneapolis, MN, USA) and then washed three times with PBS and 0.05% Tween (PBST). Wells were blocked with 5% BSA in PBS for 1 h, washed with PBST, and incubated with sample or standard (0.4 to 250 ng/mL) overnight at 4°C. Wells were again washed with PBST and then incubated with detection antibody (0.25 μ g/mL anti-mouse MPO, HM1051BT, Hycult Biotechnology, B.V., Uden, The Netherlands). After 2 h, wells were washed with PBST and then incubated with Streptavidin conjugated to horseradish peroxidase for 20 min. Wells were washed with PBST before TMB Substrate (Sigma-Aldrich) was added to each well and incubated for 5–20 min. About 100 μ L of a 0.5 M solution of H₂SO₄ was used to stop the reaction, and plates were read at 450 nm using a Synergy 4 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Six mice from each genotype and treatment were used for MPO analysis.

Metalloproteinase activity

Matrix metalloproteinases (MMP) activity was measured as previously reported (Gill et al., 2010). Briefly, the OmniMMP™ fluorogenic substrate (P-126, Biomol International, Plymouth Meeting, PA, USA) was used to analyze total metalloproteinase activity in lung homogenates. Samples (25 μ L) were added to 96-well plates and warmed to 37°C. Substrate was added to each well, and the plate was read immediately (λ_{ex} 328; λ_{em} 393) and then again at 1.5, 3, 6, 12, 30, 60, 120, and 240 min with a Synergy 4 (BioTek, Winooski, VT, USA). The increase in fluorescence was plotted against time, and the slope of the line, which indicates metalloproteinase activity, determined using GraphPad Prism for Macintosh version 4.0c (GraphPad Software, Inc., San Diego, CA, USA). Six mice from each genotype and treatment were used for MMP analysis.

GSH and Gclm/Gclc protein level

The Gclm and Gclc protein levels were detected with the use of rabbit polyclonal antisera raised against ovalbumin conjugates of peptides specific to each subunit, using previously described procedures (Thompson et al., 1999). The optical density of Gclm and Gclc specific bands on X-ray films were determined by Image J software and normalized to β -actin levels in six animals from each genotype and treatment. For total GSH measurements, clarified tissue homogenates prepared in TES/SB (20 mM Tris, pH 7.4, 1 mM EDTA, 250 mM sucrose, 20 mM serine, and 1 mM boric acid) were diluted 1:1 with 10% 5-sulfosalicylic acid, incubated on ice for 10 min, and then centrifuged at $15,600 \times g$ in a microcentrifuge for 2 min to obtain deproteinated supernatants. About 25 μ L aliquots of the supernatants were added to a 96-well black microtiter plate in triplicate followed by addition of 100 μ L of 0.2 M N-ethylmorpholine/0.02 M NaOH. Following the addition 50 μ L of 0.5 N NaOH, GSH was derivatized by the addition of 10 μ L of 10 mM naphthalene-2,3-dicarboxaldehyde (NDA). The reaction was incubated at room temperature for 30 min and the fluorescence intensity of NDA-GSH conjugate was measured at $\lambda_{\text{ex}}472$ and $\lambda_{\text{em}}528$, and quantified by interpolation on a standard curve constructed with NDA-conjugated GSH in TES/SB:10% sulfosalicylic acid (1:1). A total of 58 mice were used in the assessment of lung GSH content (9 WT-PBS, 8 WT-DEP, 13 *Gclm*^{-/+}-PBS, 15 *Gclm*^{-/+}-DEP, 6 *Gclm*^{-/-}-PBS, and 7 *Gclm*^{-/-}-DEP).

Statistical analysis

Data were analyzed using Prism (Graphpad Software, La Jolla, CA, USA). Differences were determined by ANOVA followed by a Dunnett's post-hoc test. All error bars in figures represent SEM; *, **, *** = Significant difference from the matched control at *p*-values of <0.05, 0.01, and 0.001, respectively.

Results

Intranasal instillation of DEP

In order to evaluate the ability of the DEP used in this study to reach the airways and lungs of mice after intranasal instillation, 10 μ L of a 10 mg DEP/mL solution was instilled into each nostril of three wild-type C57BL/6 mice. One hour later, mice were euthanized, a tracheotomy was performed, and 1 mL of Optimal Cutting Temperature compound (OCT; Tissue-Tek, Torrance, CA, USA) was used to inflate the lungs. Both lungs were then removed from the animal and embedded in OCT and frozen on dry ice. Cross sections of these lungs (7 μ m thick) were cut on a cryomicrotome and mounted on a glass slide for imaging. Images from lung cross sections clearly indicate that intranasally instilled DEP reached the airways and lungs of mice and were freely available to interact with resident cells (Figure 1A). Although most of the DEP was found in the larger airways, smaller particle agglomerates were able to reach the alveolar spaces (Figure 1B).

Bronchial alveolar lavage fluid analysis

To determine if Gclm modulation and GSH synthesis influences the pulmonary inflammatory response following intranasal instillation of DEP, *Gclm* WT, *Gclm*^{-/+}, and *Gclm*^{-/-} mice were put under light anesthesia and 10 μ L of a 10 mg DEP/mL solution or PBS was instilled into each nostril. Six hours after the DEP instillation, mice were euthanized by CO₂-narcosis followed by cervical dislocation. Measurement of neutrophil influx into the airways by flow cytometric analysis of cells present in the BALF indicated that DEP instillation caused a robust increase in the numbers of neutrophils relative to PBS treated mice for all three genotypes (Figure 2). Although we hypothesized that *Gclm*^{-/-} mice would be more sensitive to DEP instillation than WT mice, our results indicate that *Gclm*^{-/-} mice do not respond any differently than WT mice with regard to neutrophil influx.

Alternatively, as we hypothesized, *Gclm*^{-/+} mice were significantly more sensitive to the treatment (Figure 2). There were no significant differences for the percentage of neutrophils among PBS-treated WT, *Gclm*^{-/+}, and *Gclm*^{-/-} mice (14%, 15%, and 12% neutrophils, respectively), but the percentage of neutrophils reached an average of 44% in *Gclm*^{-/+} DEP-treated mice, roughly 15% higher than that observed for both *Gclm* WT and *Gclm*^{-/-} mice treated with DEP. Although FACS analysis showed that *Gclm*^{-/+} mice were more sensitive to DEP-induced neutrophil influx compared to *Gclm* WT and *Gclm*^{-/-} mice, the production of inflammatory cytokines in the lungs of DEP-exposed mice was also a concern since these factors can influence further downstream events. Two cytokines that play a role in downstream events are TNF α and IL6. Therefore, we analyzed TNF α and IL6 concentrations in the BALF of PBS- and DEP-treated *Gclm* WT, *Gclm*^{-/+}, and *Gclm*^{-/-} mice. Consistent with our previous finding of increased sensitivity to neutrophil influx, TNF α and IL6 concentrations within BALF were significantly higher in DEP-treated mice than PBS-treated mice for all three *Gclm* genotypes (Figure 3). Also consistent with our previous findings, DEP-treated *Gclm*^{-/+} mice had significantly higher BALF TNF α and IL6 compared to DEP-treated WT mice (Figure 3). Although not significantly different from either the DEP-treated WT or *Gclm*^{-/+} mice, DEP-treated *Gclm*^{-/-} mice had an intermediate response as the levels of both TNF α and IL6 were between DEP-treated WT and DEP-treated *Gclm*^{-/+} mice (Figure 3). The TNF α and IL6 BALF concentrations were very low in all three genotypes of the PBS-treated mice, where averages of TNF α concentrations were less than 20 pg/mL and averages of IL6 concentrations were roughly 30 pg/mL. In comparison, DEP treatment produced an increase in TNF α to roughly 70, 150, and 120 pg TNF α /mL in *Gclm* WT, *Gclm*^{-/+}, and *Gclm*^{-/-} mice, respectively. The IL6 concentrations increased to 120, 240, and 220 pg IL6/mL in *Gclm* WT, *Gclm*^{-/+}, and *Gclm*^{-/-} mice, respectively. These robust responses indicated that DEP instillation produced overt pulmonary inflammation and that *Gclm*^{-/+} mice were more sensitive to DEP-induced inflammation. To determine if there was a correlation between neutrophil influx and cytokine levels in the BAL, we performed regression analysis (Figure 4). This analysis indicated a strong correlation between the percentage of neutrophils and cytokine concentrations for both TNF α and IL6 in DEP-treated mice (R^2 value = 0.432 and 0.492 for TNF α and IL6, respectively). Although this correlation was strong for DEP-treated animals, there was no correlation for PBS-treated animals (R^2 values <0.01) (Figure 4). These data indicated that although there was some variability for the percentage of neutrophils in the PBS-treated mice, this variability was not associated with an increase in TNF α and IL6 production. Taken together, these data demonstrate that increases in TNF α and IL6 were only found in DEP-treated animals and that the mild neutrophil influx observed in PBS-treated mice was not associated with increased cytokine production.

Gene expression analysis of lavaged lung tissue

To further investigate whether *Gclm* genotype can modulate DEP-induced lung inflammation, we analyzed the expression of five proinflammatory cytokine mRNAs (TNF α , IL6, GM-CSF, IL1 β , and MCP1) in PBS- and DEP-treated *Gclm* WT, *Gclm*^{-/+}, and *Gclm*^{-/-} mouse lung tissues by quantitative real time PCR (qPCR). As mentioned above, assessment of BALF TNF α and IL6 proteins demonstrated that *Gclm*^{-/+} mice were more sensitive to DEP treatment than WT mice, whereas *Gclm*^{-/-} mice appeared to have an intermediate response (Figure 3). However, mRNA expression levels for these inflammatory cytokines were not consistent with this trend. Rather, *Gclm*^{-/-} mice appeared to be most sensitive following DEP treatment across all genes analyzed, although this only approached statistical significance ($p = 0.06$; Figure 5). Overall, DEP-treated *Gclm*^{-/-} mice had nearly a two-fold increase in expression compared to *Gclm*^{-/+} or WT DEP-treated mice for all genes analyzed, and this response was particularly robust for TNF α and IL6. Whereas DEP treatment produced roughly a four-fold increase in TNF α expression in *Gclm*^{-/+} mice

relative to WT PBS-treated mice, DEP-treated *Gclm*^{-/-} mice had an eight-fold increase. The expression of IL6 was increased to seven- and four- fold over WT-PBS mice for DEP-treated *Gclm* WT and *Gclm*^{+/-} mice, respectively, while DEP-treated *Gclm*^{-/-} mice had nearly a 15-fold increase. Although this increase in IL6 in *Gclm*^{-/-} mice was not significant due to high variability, the trend across all genotypes indicates that *Gclm*^{-/-} mice are most sensitive to DEP with respect to induction of inflammatory cytokine mRNAs. To further examine the relationship between lung tissue mRNA expression of inflammatory cytokines and inflammatory cytokines found in the BALF, we performed a linear regression analysis comparing BALF TNF α and IL6 protein levels and TNF α and IL6 mRNA expression in DEP-treated *Gclm* WT, *Gclm*^{+/-}, and *Gclm*^{-/-} mice relative to WT PBS-treated controls (Figure 6). We stratified linear regression based on genotype, and found that although there is a positive correlation between gene expression and BALF cytokines across all genotypes, the slope for DEP-treated *Gclm*^{-/-} mice is dramatically steeper for TNF α (Figure 6A), possibly indicating that a greater amount of TNF α mRNA expression was required in these mice to reach a similar BALF TNF α concentration. The linear slopes for the TNF α correlation with DEP-treated *Gclm* WT and *Gclm*^{+/-} mice were nearly identical, further pointing out the uniqueness of *Gclm*^{-/-} mice. Although the trend was similar when the analysis was performed for IL6, the trend was not as dramatic (Figure 6B). Together, these data suggest that *Gclm*^{-/-} mice were unable to effectively translate the mRNAs for these proinflammatory cytokines, at least at the time point measured, and under these DEP exposure conditions.

Investigation of MMP and MPO within lavaged lung tissue

The observation that *Gclm*^{+/-} mice are more sensitive to DEP-induced inflammation compared to *Gclm* WT and *Gclm*^{-/-} mice is important as it further indicates that GCL activity and GSH synthesis may influence inflammatory responses following environmental diesel exhaust exposure. Although we hypothesized that modulation of *Gclm* would lead to increased sensitivity, we believed that *Gclm*^{-/-} mice may not be the most sensitive as they likely have compensatory upregulation of alternative antioxidant enzymes. Our findings indicate that this was probably true with regard to neutrophil influx and cytokine production, but interestingly, gene expression profiles of five proinflammatory cytokines suggested that null mice might be more sensitive to DEP treatment. Furthermore, the correlation between TNF α gene expression and BALF TNF α concentration suggested there was an altered relationship between lung tissue gene expression and subsequent BALF cytokine concentrations in these mice. These findings suggested that in *Gclm*^{-/-} mice, the ability to mount an inflammatory response and recruit neutrophils to the lung and translocate from the lung interstitium into the alveolar space was compromised such that they are unable to form and/or secrete these cytokines. The MMP activity can promote neutrophil influx by shedding surface proteins and affecting chemokine activity and presentation (Gill & Parks, 2008). Because MMP activity can be regulated by oxidation, and because overt oxidation leads to inactivation of some MMPs (Fu et al., 2001), we hypothesized that *Gclm*^{-/-} mice may have compromised metalloproteinase activity, and that even though neutrophils may have been recruited to the lung they might be incapable of translocating into the alveolar space. Thus, we analyzed MPO protein level of homogenized lung tissue as a surrogate marker of neutrophil content (Figure 7A). We also determined if *Gclm* genotype influenced metalloproteinase activity to see if it might explain the decrease in neutrophil response seen in *Gclm*^{-/-} mice following DEP treatment (Figure 7B). We found no significant differences in MPO level or total metalloproteinase activity, irrespective of genotype or DEP treatment. There was a suggestion of a genotype effect across both treatments, in that *Gclm*^{-/-} mice had lower metalloproteinase activity, while *Gclm*^{+/-} mice had higher metalloproteinase activity (Figure 7B), but this trend was minor if present at all. Although not a significant effect, if this trend is real, it would be consistent with our findings of elevated neutrophil

influx into the lungs of *Gclm*^{-/+} mice and lower neutrophil influx into the lungs of *Gclm*^{-/-} mice.

Analysis of total lung GSH and *Gclm*/*Gclc* protein levels

Because oxidative stress is a critical component of DEP-induced pulmonary inflammation, it is likely that the levels of intracellular antioxidants, such as GSH, play a major role in mediating such effects. To determine if total GSH and its regeneration are influenced by DEP treatment, we measured total GSH in lung homogenates from *Gclm* WT, *Gclm*^{-/+}, and *Gclm*^{-/-} mice. Consistent with previously reported values (McConnachie et al., 2007), we found that total lung GSH content in PBS-treated mice was approximately 90% and 5% of WT mice for *Gclm*^{-/+} and *Gclm*^{-/-} mice, respectively (Figure 8). These data illustrate that under non-stressed conditions, complete deficiency in *Gclm* results in severely compromised GSH synthesis, whereas one copy of *Gclm* is sufficient to maintain relatively normal GSH levels. Across all genotypes of the DEP-treated mice there was a suggestion that DEP causes a slight reduction in total GSH, but these differences only approached significance ($p = 0.08$; Figure 8). The *Gclm*^{-/-} mice had even lowered GSH levels after DEP treatment, nearly eliminating any available GSH with levels beneath the limit of detection of our assay. The observation that there is little change in whole lung GSH level following DEP treatment in WT and *Gclm* mice may suggest that alteration in GSH levels in the epithelial lining fluid (ELF) may be of more significance. To investigate the levels of *Gclc* and *Gclm* proteins following DEP treatment in both PBS- and DEP-treated WT, *Gclm*^{-/+}, and *Gclm*^{-/-} mice, we analyzed the expression of these proteins by western immunoblot (Figure 9). As previously reported (McConnachie et al., 2007), *Gclc* protein is elevated in *Gclm*^{-/-} mouse lung (Figure 9A), and interestingly, DEP treatment produced a significant decrease in the *Gclc* protein. Although DEP treatment did not produce a significant effect on *Gclc* in either WT or *Gclm*^{-/+} mice, there was a suggestion that *Gclc* was slightly increased in the WT mice ($p = 0.10$), but not in the *Gclm*^{-/+} mice. Regarding *Gclm* protein levels, we confirmed that *Gclm* was not present in the *Gclm*^{-/-} mice. Similar to the response seen with *Gclc*, there is a suggestion of an increase in *Gclm* in WT mice ($p = 0.09$) with DEP treatment compared to the PBS control, but in *Gclm*^{-/+} mice, *Gclm* expression was not altered (Figure 9B).

Discussion

In this study, we demonstrate for the first time that genetic alteration of the GSH synthesis gene *Gclm* can exacerbate the inflammatory response of the lung following intranasal instillation of DEP. The dose of 200 μ g DEP was chosen in order to produce an inflammatory response in the lung, and to be consistent with other studies investigating pulmonary inflammation following instillation of particulate matter into the respiratory tract of mice (Happo et al., 2010; Yokota et al., 2008). Although this dose is comparatively high relative to what might be expected for ambient exposures in humans, it does allow for a comparative assessment of the role of *Gclm* in mediating DEP-induced lung inflammation in mice.

Previous studies have indicated that DEP induces lung inflammation in part via oxidative stress and that GSH plays an important role in mediating this inflammatory response (Banerjee et al., 2009). Although our study failed to directly support this contention in that we did not observe significant DEP-induced GSH depletion in WT or *Gclm*^{-/+} mice, or exacerbated inflammation in already GSH-depleted *Gclm* null mice, the finding that *Gclm* heterozygosity exacerbates DEP-induced lung inflammation does support the hypothesis that *Gclm* can influence the inflammatory response to DEP. It is reasonable to suggest that this is likely mediated via compromised GCL activity and GSH synthesis, although alternative mechanisms cannot be ruled out. Regardless of mechanism, as *Gclm* is highly polymorphic

in people, our study provides additional evidence that Gclm may be an important gene influencing diesel exhaust-induced lung inflammation in humans.

Short-term periods of elevated PM_{2.5} have been associated with a greater frequency of emergency room visits (Katsouyanni et al., 1997; Peters et al., 1997; Peters et al., 2001; Seaton et al., 1995; Zanobetti et al., 2004), and although many of these visits are due to the exacerbation of existing asthma symptoms, these reports have indicated that PM_{2.5} exposure is associated with acute cardiovascular events such as MI and myocardial ischemia. It has long been demonstrated that the elevation of systemic and vascular inflammation has a major impact on vascular function and has been suggested to be a strong influencing factor for the risk of MI and myocardial ischemia. In particular, the proinflammatory cytokine TNF α has been demonstrated to influence vascular reactivity by activation of the superoxide producing NADPH oxidases within the vasculature (Gao et al., 2007). Since oxidative stress plays a role in PM_{2.5}-induced lung inflammation, it is likely these local inflammatory factors can influence systemic vascular function by the induction of vascular oxidative stress. Individuals that are sensitive to PM_{2.5}- induced inflammation due to compromised antioxidant synthesis will likely be at increased risk for these adverse cardiovascular events.

Our finding that Gclm heterozygosity increases sensitivity to DEP-induced lung inflammation is a significant finding since Gclm is a highly polymorphic gene that has been already shown to influence the risk of MI and vasomotor dysfunction (Nakamura et al., 2002; Nakamura et al., 2003). Nakamura (2002) reported that in a Japanese sample of healthy controls, at least one copy of the —588C/T SNP within the 5' promoter region of Gclm was present in 20.3% of individuals, this number increased to 31.5% in individuals presented in the clinic with an established MI. This SNP results in a greater than 50% reduction in promoter activity, slightly decreased serum GSH, and roughly a two-fold increase in the risk of MI (Nakamura et al., 2002). Moreover, these researchers found that individuals containing two copies of the polymorphism (frequency = 1%–3%) have a greater than eight-fold increase in the risk of MI. Further work done by Nakamura and colleagues have shown that this SNP within the Gclm 5' promoter region is also associated with decreased coronary artery response to the endothelium dependent vasodilator acetylcholine (Nakamura et al., 2003).

In addition to investigations into the influence of GCL polymorphisms on vascular function, SNPs in the 5' promoter region of Gclm and Gclc have been investigated with regard to compromised lung function and development of COPD in smokers (Chappell et al., 2008; Siedlinski et al., 2008). A large amount of cigarette smoke-induced lung injury has been posited to be due to the increased production of reactive oxygen species (ROS) and resulting oxidative stress (Kirkham and Rahman, 2006; Kirkham et al., 2003; Nakayama et al., 1989). As these SNPs in Gclm and Gclc have been demonstrated to reduce oxidant-induced expression of their respective genes in endothelial cells by nearly 50% (Koide et al., 2003; Nakamura et al., 2002), it was hypothesized that these polymorphisms would accelerate lung function decline and increase the risk of COPD in smokers. Although no associations were found between COPD and SNPs within both Gclm and Gclc (Chappell et al., 2008; Siedlinski et al., 2008), it was observed that smokers who also had these SNPs in Gclc had a significant acceleration of lung function decline when measured by forced expiratory volume in 1 s (FEV₁) (Siedlinski et al., 2008). Providing further evidence that this association is due to loss of available antioxidants, this accelerated decline in lung function was found to be most significant in individuals with low intake of vitamin C. This finding suggests that polymorphisms in GCL may have a strong gene-environment interaction toward additional environmental toxicants that cause injury through an oxidative stress-mediated mechanism, such as diesel exhaust.

Our finding that *Gclm* modulation increases DEP-induced pulmonary inflammation is interesting in that the increased susceptibility is only dramatically present in mice heterozygous for *Gclm*. The *Gclm*^{-/+} mice had a significantly increased percentage of BALF neutrophils when compared to both WT and *Gclm*^{-/-} mice, while inflammatory cytokines in their BAL were elevated when compared to WT mice but not to *Gclm*^{-/-} mice. Alternatively, at the cytokine mRNA level, *Gclm*^{-/-} mice seemed to have a greater inflammatory response compared to WT or *Gclm*^{-/+} mice. In an attempt to elucidate this contradiction, we hypothesized that the lack of neutrophil infiltration in the *Gclm*^{-/-} mice was due to compromised metalloproteinase activation, but analyses of MMP and MPO failed to support the contention that *Gclm*^{-/-} mice had recruited neutrophils to the lung interstitium but were unable to mediate neutrophil translocation into the alveolar space.

It is unclear why *Gclm*^{-/-} mice would have an increased response to DEP by gene expression analysis but not have an increase in BALF neutrophils or inflammatory cytokines. As we know that *Gclm*^{-/-} mice have many genes dysregulated, it is possible that the expression of certain classes of genes responsible for post-translational cytokine release, such as *Tumor Necrosis Factor α Converting Enzyme* (*TACE*), or genes responsible for regulating neutrophil influx (*Gro1/KC* or *Tissue Inhibitors of Metalloproteinases*) are also dysregulated. Alternatively, enzyme activity or protein function within the *Gclm*^{-/-} mice may be influenced by compensatory increases in other antioxidant enzymes, decreased glutathiolation, or altered S-nitrosylation mediated by S-nitrosoglutathione, further leading to compromised cytokine release or neutrophil influx. In any case, expression of inflammatory gene mRNAs may increase, whereas the level of inflammatory cytokines or neutrophils found in the BALF may not. The reasons for these discrepancies are at present unknown and are the subject of future research.

Upon analysis of lung GSH, it is clear that *Gclm*^{-/-} mice have dramatically low GSH than WT mice (~5% of normal), but *Gclm*^{-/+} mice have a relatively normal level of GSH compared to WT mice. Because of the dramatic reduction in intracellular GSH, *Gclm*^{-/-} mice have likely adapted by dramatically upregulating other genes involved in protection from oxidant injury. In a previous report (Haque et al., 2010), we found that *Gclm* deficiency appeared to have led to metabolic adaptations that were protective toward diet-induced steatohepatitis in the liver. Some of the protective genes that are upregulated in the livers of *Gclm*^{-/-} mice include: glutathione-S-transferases (*Gsta2*, *Gstm1*, *Gstm2*, *Gstm3*, *Gstm4*), sulfotransferases (*Sult2A2*, *Sult3S1*), glutathione reductase (*Gsr1*), *Gclc*, thioredoxin reductase 1, and *PPAR γ* . In addition to these changes observed in the liver, Johansson et al. (2010) demonstrated that *Gclm*^{-/-} mice are not more sensitive to ozone-induced lung injury as compared to *Gclm* WT mice. Although they observed that *Gclm*^{-/-} mice did have dramatically reduced total of GSH at the cellular level, GSH in the ELF was not reduced in the *Gclm* null mice, suggesting a compensatory response to maintain normal GSH levels in the ELF. As well as an adaptive response in GSH level in the ELF, Johansson et al. observed that *Gclm*^{-/-} mice upregulated expression of metallothionein 1/2 (*MT1/2*) in response to ozone to a much greater extent than *Gclm* WT mice, suggesting that these mice have the capacity to respond to oxidant injury by alternative means.

The *Gclm*^{-/-} mice are unique in that they have a dramatic reduction in GSH, providing an excellent tool for investigating the biological function of GSH and the role of GSH in mediating toxicant injury (e.g. acetaminophen-induced liver injury), but as demonstrated, they are not necessarily more susceptible to general oxidants such as DEP or ozone (Johansson et al, 2010). The *Gclm*^{-/+} genotype is an interesting model in that these mice do not have dramatically low GSH compared to WT. That they respond much more robustly to DEP-induced inflammation may reflect an inability to produce GSH fast enough when under oxidative stress. Although our data only suggests this trend, analysis of *Gclm* protein levels

seemed to indicate that DEP treatment causes a slight increase in Gclm expression in WT mice where this trend is not present in *Gclm*^{-/+} mice. This finding may suggest that to maintain normal GSH level under oxidant injury, WT mice were able to upregulate the expression of *Gclm* to increase GCL activity, and due to the presence of only one functional copy of the gene in *Gclm*, *Gclm*^{-/+} mice are possibly “maxed out” in their ability to further induce this protein. Thus the lack of one *Gclm* copy in *Gclm*^{-/+} mice, although sufficient to maintain normal levels of GSH under non-stressed conditions, may not be sufficient during periods of elevated oxidative stress.

Conclusions

As inflammation has been suggested to be a critical determinant of many effects in the lung and the vascular system following exposure to PM_{2.5}, it is important to examine genetic factors that might influence the inflammatory response following diesel exhaust and PM_{2.5} exposure. We believe that our findings support the contention that DEP induces inflammation via a mechanism dictated in part by oxidative stress and that GSH plays a role in mediating the resulting inflammatory response. These findings can further suggest that individuals with compromised GSH synthesis, such as those with polymorphisms in GSH synthesis genes, may be an important population to investigate for increased sensitivity to ambient air pollution. If they are more susceptible, current regulations for PM_{2.5} should be adjusted to provide the necessary protection to these and other sensitive individuals.

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Figure 1. Images of lung cross sections 1 h after intranasal instillation of DEP (10 μ L of 10mg/mL DEP per nostril) in WT C57bl6 mice. Panels A and B are a 4x image and a 40x image of a single cross section from the upper region of the right lung.

Figure 2.

The FACS analysis of percentage neutrophils (F4/80^{lo}/Gr1^{hi}) from 5000 cells collected in BAL in *Gclm* WT, *Gclm*^{-/+}, and *Gclm*^{-/-} mice 6 h after either PBS or DEP intranasal instillation. The *n* values for treatments are: *Gclm* WT, N = 13-PBS: 10-DEP, *Gclm*^{-/+}, *n* = 12-PBS: 10-DEP and *Gclm*^{-/-}, *n* = 8-PBS: 9-DEP. Error bars represent SEM; * = Significant difference at *p*-values of <0.05.

Figure 3. Concentration of inflammatory cytokines TNF α (3a) and IL6 (3b) in BAL fluid in *Gclm* WT, *Gclm*^{-/+}, and *Gclm*^{-/-} mice 6 h after either PBS or DEP intranasal instillation. The *n* = 6 for all genotypes and treatments. Error bars represent SEM; * = Significant difference at *p*-values of <0.05.

Figure 4.

Linear regression analysis between percentage neutrophils and BAL fluid concentration of inflammatory cytokines TNF α (4a) and IL6 (4b) within all genotypes 6 h after either PBS or DEP intranasal instillation. The R^2 values are given for regression performed within treatment condition.

Figure 5.

Real-time PCR assessment of mRNA levels for $TNF\alpha$, IL6, (5a) and $GMCSF$, IL1 β , and $MCP1$ normalized to β -actin in the lung of $Gclm$ WT, $Gclm^{+/-}$, and $Gclm^{-/-}$ mice 6 h after either PBS or DEP intranasal instillation. The $n = 6$ for all genotypes and treatments. Error bars represent SEM; * = Significant difference at p -values of <0.05 .

Figure 6. Linear regression analysis between real-time PCR assessment of mRNA levels for TNF α (6A) and IL6 (6B) normalized to β -actin and BAL fluid concentration of inflammatory cytokines TNF α and IL6 in *Gclm* WT, *Gclm*^{-/+}, and *Gclm*^{-/-} mice 6 h after DEP intranasal instillation.

Figure 7.

The MPO protein level (A) and MMP activity (B) in *Gclm* WT, *Gclm*^{+/+}, and *Gclm*^{-/-} mice 6 h after either PBS or DEP intranasal instillation. The *n* = 6 for all genotypes and treatments. Error bars represent SEM.

Figure 8.

Total GSH level in whole lung homogenate in *Gclm* WT, *Gclm*^{-/+}, and *Gclm*^{-/-} mice 6 h after either PBS or DEP intranasal instillation. The *n* values for treatments are: *Gclm* WT, *n* = 9-PBS: 8-DEP, *Gclm*^{-/+}, *n* = 13-PBS: 15-DEP, and *Gclm*^{-/-}, *n* = 6-PBS: 7-DEP. Error bars represent SEM.

Figure 9.

Total GCLC and GCLM protein levels normalized to β -actin measured by western blot in *Gclm* WT, *Gclm*^{+/-}, and *Gclm*^{-/-} mice 6 h after either PBS or DEP intranasal instillation; n = 6 for all genotypes and treatments. Error bars represent SEM; * = Significant difference from the PBS-treated WT control at *p*-value of <0.05; *** = Significant difference between *Gclm*^{-/-} PBS and DEP treated mice at *p*-value of < 0.001.