Association between Exposure to Diesel Exhaust Particles and Glutathione in Epithelial Lining Fluid of the Lung in Glutathione-Deficient Mice

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Abstract

Inhalation of diesel exhaust particles (DEPs) has been associated with adverse health effects. An important and abundant antioxidant in the lungs is glutathione (GSH), which can attenuate oxidative stress induced by DEP exposure. Glutamate cysteine ligase (GCL) carries out the rate-determining step in GSH synthesis. GCL consists of modifier (Gclm) and catalytic (Gclc) subunits. In a previous study investigating the effects of DEP on lung inflammation, it was shown that Gclm knock-out (KO) (-/-) mice were not more sensitive to DEP exposure than Gclm wild type (WT) (+/+) mice. Thus, it was hypothesized that Gclm KO (-/-) mice have likely adapted by up-regulating other genes involved in protecting the lungs from DEP-induced oxidative stress. While the exact mechanisms are not known, one possible explanation is that GSH transporters might be exporting GSH into the epithelial lining fluid (ELF). Four candidate GSH transporters include ATP-binding cassette (ABC) subfamily C protein 2 (ABCC2, also known as multidrug-associated resistance protein), ABCC12, ABC subfamily G protein (ABCG2, also known as breast cancer-related protein), and ABCC7 (also known as cystic fibrosis

transmembrane regulator). This study was aimed to measure ELF GSH levels in mice with different GCLM genotypes (*Gclm* wild-type (+/+), *Gclm* heterozygous (+/-), and *Gclm* knock-out (-/-) mice) following DEP exposure and to measure candidate GSH transporter mRNA expression levels in these mice. The mice were exposed to DEPs via intranasal instillation and sacrificed after 6 hours. Total GSH levels in bronchoalveolar lavage fluid (BALF) were measured using a plate assay in which GSH reacts with naphthalene-2, 3-dicarboxaldehyde (NDA), forming a fluorescent derivative compound. The mRNA levels of the candidate GSH transporter genes were analyzed using quantitative real-time polymerase chain reaction (qRT-PCR). The results showed that the total ELF GSH found in BALF increased in response to DEP exposure in *Gclm*-/- mice. It was observed that *ABCC2* mRNA and *ABCC12* mRNA in the lungs were statistically significantly upregulated following exposure to DEP in *Gclm*+/+ and *Gclm*+/- mice, but they had low expression. Furthermore, it was shown that among PBS-treated controls, CFTR expression was higher in *Gclm*-/- mice than in *Gclm*+/+ or *Gclm*+/- mice, suggesting that CFTR is likely responsible for maintaining ELF GSH levels in the lungs in *Gclm*-/- mice. In summary, exposure to DEP can increase levels of ELF GSH in *Gclm*-/- mice and this may be the basis for an adaptive response that can oppose oxidative stress and inflammation.

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1. Introduction

Exposure to fine particulate matter (PM) in air is associated with pulmonary symptoms and diseases (Pope et al., 2002). Fine PM is easily deposited in the deeper airways, reaching terminal bronchioles and alveoli. Protective responses toward PM exposure can range from natural reflex responses such as coughing, to complex biochemical responses by lung macrophages which are cells associated with the immune system. When either coarse particles or fine particles enter into the lungs, they can be removed by these and other mechanisms. However, smaller particles are eliminated from the lungs with difficulty, which can cause health problems. It is well known that inhalation of fine PM results in cardiovascular and respiratory complications such as asthma, airway inflammation, myocardial ischemia, and myocardial infarction (Dockery and Pope, 1994). Therefore, further research into the health effects of fine and ultrafine PM is necessary and important for public health.

In urban areas, diesel exhaust particles (DEPs) are a major component of ambient airborne fine PM (Lewtas, 2007; Schauer et al., 1996). DEPs are produced when an engine burns diesel fuel. DEPs contain a multitude of compounds that include known or suspected cancer-causing pollutants, such as benzene, cadmium and formaldehyde. Inhalation of DEPs containing such compounds contributes to cell mutation and inflammation, potentially resulting in cancer (Sun Y et al., 2014). The mean DEP concentration in ambient air in the United States is approximately 2 $\mu g/m^3$, but levels adjacent to major streets and freeways are estimated to reach 25 $\mu g/m^3$ (Gilman, 2002; Corfa et al. 2004). Furthermore, mechanics, bus or truck drivers, and coal miners can be exposed to greater levels of DEPs than the general population. Ambient diesel

emissions have been gradually decreasing by changing to newer engines, but diesel nanoparticle emissions still may be a health problem and need further research.

When DEPs enter the body, they cause disruption of the cellular redox system, thus leading to adverse health effects often manifested as oxidative stress. Inhaled DEP activates neutrophilic inflammation and associated release of reactive oxygen species (ROS), and this is associated with induction of antioxidant response pathways in the lungs (Salvi S et al., 1999; Mudway et al., 2004; Weldy et al., 2011).

The tripeptide glutathione (GSH) is one of the most important and prevalent intracellular antioxidants (Fig. 1), preventing the uncontrolled formation of free radicals and ROS. The cysteine thiol group (-SH) in GSH acts as a reducing agent, which donates electrons to activated species or free radicals.

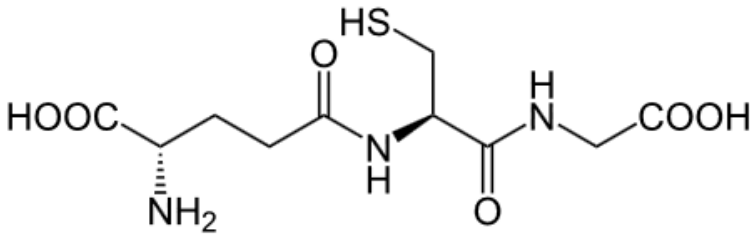

Figure 1. Structure of Glutathione (GSH).

The reaction of GSH with oxidants converts it to either an oxidized glutathione form, glutathione disulfide (GSSG), or a mixed disulfide (RSSG). Consequently three types of GSH may be present in a cell. GSSG is reduced to GSH by endogenous glutathione reductase (GR), which utilizes NADPH. The mixture of GSH and GSSG is a principle redox buffer in the cell. Under healthy physiological conditions, the ratio of GSH:GSSG is estimated to be between 30:1 to 100:1 (Hwang et al., 1992). In fact, using a green fluorescent protein (GFP) reporter that is

sensitive to glutathione redox status, it has recently been reported that the ratio of GSH:GSSG may be closer to 50,000:1, meaning that the vast majority of cellular glutathione is found in the reduced form (Morgan et al., 2013). GSH is found in all tissues, and also in various body fluids including serum/plasma, bile, glomerular filtrate, and lung epithelial lining fluid (ELF) recovered by bronchoalveolar lavage (BAL) (Cantin et al., 1987). While intracellular GSH is maintained at very high (in the range of millimolar concentrations), extracellular concentrations vary depending on location and pathophysiological status. The extracellular concentration of GSH present in ELF is critical to lung health, because low levels of alveolar GSH have been associated with respiratory diseases (Day et al., 2004).

The lungs serve as an important interface between the environment and organism, through which environmental contaminants can enter into body. Thus, the lungs have evolved protective mechanisms against ambient toxic contaminants. GSH in the lungs and ELF plays a critical role in protecting the lungs from oxidative damage. It is thought that the ELF GSH is maintained at high concentrations compared to the plasma, because there is a greater chance for the lungs to be damaged by inhaled gases or particles such as DEP. Among rodents exposed to DEP, extracellular GSH level increased in a dose-dependent manner (Rahman et al., 1999). Rats exposed to high oxygen tension increased extracellular GSH concentrations as well (Rahman et al., 1999). In asthma patients, increased levels of GSH found in BALF were observed (Smith et al., 1993). The primary source of GSH in ELF is not yet established, but there is evidence that certain lung cells play a role in transporting GSH into the apical space in the lungs in response to environmental stress such as cigarette smoke (Gould et al., 2012).

GSH is synthesized in two sequential ATP-dependent steps. Glutamate and cysteine are ligated together to form γ-glutamylcysteine (γ-GC), catalyzed by glutamate cysteine ligase

(GCL). Next, γ-GC is combined with glycine to form GSH, catalyzed by glutathione synthase (GS). The first step is the rate-determining step; thus, GCL is important for GSH synthesis. GCL consists of two subunits, modifier (GCLM) and catalytic (GCLC). While GCLC carries out all the catalytic activity, GCLM functions to enhance the efficiency of the GCLC by decreasing the Km for glutamate and ATP, and attenuating feedback inhibition by GSH. In previously published work from our laboratory (McConnachie et al., 2006; Weldy et al., 2011) and that of others (Johansson et al, 2010), it was observed that *Gclm* knock-out (KO; -/-) mice had low level (~5-15%) of GSH in the lung compared to *Gclm* wild type (WT; +/+) and *Gclm* heterozygous (HT; +/-) mice, as shown in Fig. 2. Interestingly, *Gclm*-/- mice were not more sensitive to the DEP-induced lung injury as compared to *Gclm*+/+ mice, when expressed as %neutrophils in the BALF (Fig. 2). Furthermore, Johansson et al. (2010) demonstrated that *Gclm*-/- mice are not sensitized to ozone-induced lung injury compared to *Gclm*+/+ mice and observed that *Gclm*-/- mice had relatively normal levels of GSH in their ELF, and upregulated expression of metallothionein 1 and 2, α-tocopherol transporter, and solute carrier family 23 member (sodium-dependent vitamin C transporter) in response to ozone more than *Gclm*+/+ mice, suggesting that GSH deficient mice have compensatory mechanisms that can protect them from various environmental stressors. These studies therefore suggested that although *Gclm*-/- mice have dramatically reduced intracellular GSH levels, their ELF GSH levels may not be similarly decreased under basal conditions or in response to oxidative stress.

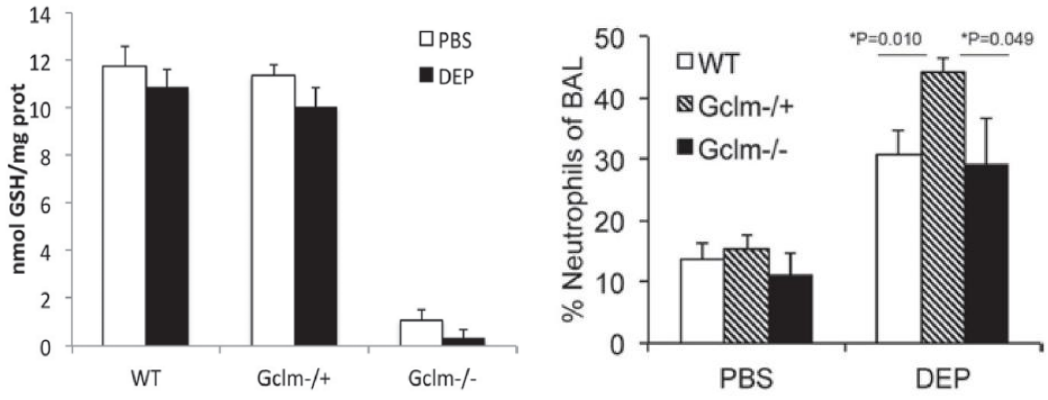

Figure 2. Total GSH levels in whole lung homogenate in GCLM wild type (WT), heterozygous (HT), and knockout (KO) mice 6h after either PBS or DEP intranasal instillation (left). Percentage neutrophils from 5000 cells collected in BAL (right). Data represented as mean ± SEM. N=6-15 mice per genotypes and treatments. *Significantly different, $p < 0.05$
Data are from Weldy et al., 2011.

Recent studies have shown that several proteins are responsible for transporting GSH and its conjugates into the extracellular medium (Gould et al., 2012; Day et al., 2004; Bachhawat et al., 2013; Ballatori et al., 2009). They are classified as ATP-binding cassette (ABC) subfamily C proteins (ABCC), commonly known as the multidrug-associated resistance proteins (MRPs), and ABC subfamily G proteins (ABCG), one of which is known as the breast cancer-related protein (BCRP). The cystic fibrosis transmembrane regulator (CFTR) protein is a member of the ABCC protein family members. Gould et al. (2012) demonstrated that mice deficient in CFTR had reduced ELF GSH. However, CFTR knock-out mice can still increase their ELF GSH levels, meaning that CFTR is not the only protein involved in GSH transport (Day et al., 2004). For many years, researchers have attempted to define the transporters responsible for pumping GSH into the extracellular spaces. However, a clear mechanism has not yet been confirmed.

The goals of this study are, first, to measure GSH in lung ELF following DEP exposure in *Gclm*+/+, *Gclm*+/- and *Gclm*-/- mice. For my first goal, I hypothesized that ELF GSH would

be increased to protect lungs following the DEP exposure in *Gclm*-/- mice. This is because the defense mechanisms against environmental stressors are necessary, and it was observed previously that *Gclm*-/- mice had fewer BALF neutrophils after exposure to DEP when compared to *Gclm*+/- mice. Also, Fig. 2 shows that the GSH levels in the lungs slightly decreased when mice were exposed to DEP, and that this was proportionately greater in *Gclm*+/- and *Gclm*-/- mice than in *Gclm*+/+ mice. This may be due to an adaptive response, whereby GSH in lung tissue is lower with DEP exposure because more of it is pumped into the ELF in response to DEP challenge. Thus, the second goal of this study is to examine which GSH transporters might be responsible for this proposed adaptive response to DEP. For my second goal, I hypothesized that one or more of the candidate GSH transporter genes would be upregulated in response to DEP so that GSH synthesized in the lung cells would be pumped into the bronchoalveolar space in *Gclm*-/- mice.

In summary, the objectives of this study are to measure GSH levels in lung ELF induced by exposure to DEPs in *Gclm*+/+, *Gclm*+/-, and *Gclm* -/- mice, and to investigate the GSH transporters involved in exporting GSH out into the epithelial space in the lungs as a possible adaptive response.

2. Materials and Methods

2.1 DEP Collection

DEPs with a diameter less than 2.5 μm (PM 2.5) were generated from a Cummins diesel engine and collected from the outflow duct at the University of Washington diesel exhaust exposure facility (Weldy et al., 2011). The collected fine particle size distributions closely

resemble DEPs a few hundred meters away from a major roadway. The DEPs were suspended in dimethyl sulfoxide (DMSO) (2.5%) and then further diluted in phosphate buffered saline (PBS) (97.5%) to 10 mg/mL. The DEP solutions were sonicated for 1 min before dosing the mice. An equivalent volume of PBS-DMSO mixture was given to the vehicle treated control groups. (Weldy et al. 2011)

2.2 Animals

The BALF and lung tissue RNA samples used in this study were previously collected by Dr. Chad Weldy as part of a previously published study from our laboratory (Weldy et al, 2011). All animal experiments were approved by the University of Washington Institutional Animal Care and Use Committee. All mice (C57BL/6) were bred and housed in a modified specific pathogen free (SPF) vivarium at the University of Washington. They were all male, aged 8-12 weeks. Mice were genotyped for *Gclm* as either wild type (+/+), heterozygous (-/+), or knock-out (-/-).

2.3 Intranasal Instillation of DEP

Mice were randomly selected for either DEP or phosphate buffered saline (PBS) vehicle treatments. The exposure was achieved via intranasal instillation. First, the mice were lightly anesthetized (0.01 mL/g body weight of a 0.44 mg/mL Xylazine, 6.5 mg/mL Ketamine solution in sterile saline) via intraperitoneal injection. During anesthesia, 10 µL of a 10 mg/mL solution of DEP was slowly pipetted into each nostril with a total dose of 20 µL/mouse (~200 µg DEP, ~6.7 mg/kg in a 30 g mouse).

2.4 Bronchoalveolar Lavage

Six hours after being exposed to DEP, mice were sacrificed by CO_2 narcosis. The trachea was isolated, and a small incision was made just below the larynx. An 18 G catheter attached to a

1mL syringe was inserted into the incision to perform the lavage. One mL of PBS was slowly instilled into the lungs and subsequently withdrawn. This rinsing action was repeated three times per lavage, and three 1 mL lavages were conducted for each mouse. Cells in the lavage samples were pelleted by centrifugation at 200 x g for 15 minutes at 4 °C. The first cell-free lavage supernatant sample was used for this study.

2.5 RNA Isolation

Lung tissue from each mouse was taken 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. RNA was subsequently isolated using Qiagen RNAeasy Kit (Qiagen, Valencia, CA, USA). Purity and quantity of the total RNA were determined using the Nanodrop UV-visible spectrophotometer.

2.6 Total GSH Measurement in BALF

GSH was measured by reaction with naphthalene-2, 3-dicarboxaldehyde (NDA) (Fig. 3) which forms a fluorescent adduct. First, the BALF GSH was reduced by adding 1 mM triscarboxyethylphosphine (TCEP) and incubating for 15 minutes at room temperature (volume ratio of TCEP/BALF equaled 0.4). To stabilize the reduced glutathione, a solution of 5% sulfosalicyclic acid (SSA) was then added to the mixture (for a volume ratio of SSA solution/BALF = 1), which was then incubated on ice for 15 minutes. To obtain deproteinated supernatants, the mixture was centrifuged at 12,000 rpm for 2 minutes at 4 °C. Thirty-five µl aliquots of the supernatants were added to a 96-well black microtiter plate in triplicate followed by addition of 100 µl of 0.1 M N-ethylmorpholine/0.2 M potassium hydroxide (KOH). Following the addition of 50 µl of 0.5 N NaOH, GSH was derivatized by the addition of 10 µl of 10 mM NDA followed by a 30 minute incubation in the dark at room temperature. The

fluorescence intensity of the NDA-GSH conjugate (Fig. 4) was measured in a spectrofluorometer using 472 nm excitation and 528 nm emission, with a cut-off at 495 nm (White et al., 2003).

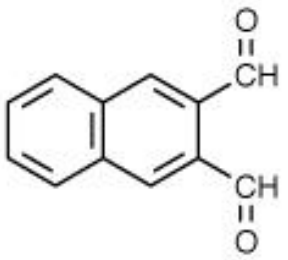

Figure 3. Structure of naphthalene-2, 3-dicarboxaldehyde (NDA).

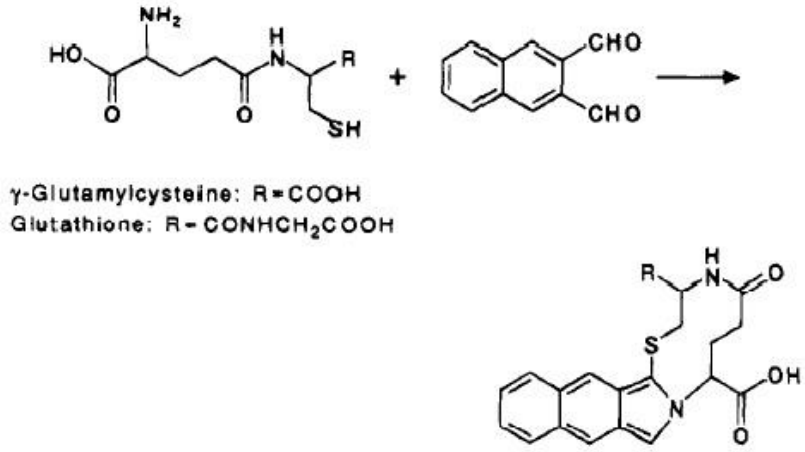

Figure 4. Reaction scheme showing the structure of the proposed predominant reaction product between NDA and GSH (from Orwar et al., 1995).

For generating the standard curves, GSH was dissolved in dispersion medium diluted 1:7 (v/v) with PBS because dispersion medium has characteristics similar to lung ELF. The dispersion medium consists of 0.6 mg/ml mouse serum albumin and 10 µg/ml 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine surfactant in ethanol (0.1% v/v) in PBS.

2.7. Urea Measurement in BALF and Serum

To determine the degree of ELF dilution in the lavage, GSH values measured in BALF should be normalized to relative levels of urea in serum and BALF. Because urea diffuses easily through the body, the urea concentrations in ELF and serum are presumed to be the same; thus, the ELF dilution factor can be calculated (Rennard et al. 1986). In order to measure urea concentrations in BALF and serum, a Quantichrom Urea Assay Kit (BioAssay Systems, Hayward, CA) was used. The chromogenic reagent in the kit forms a colored complex specifically with urea. First, 5µL of the distilled water for the blank, the urea standard, the BALF samples, and the serum samples (diluted 1:10 with distilled water) were loaded onto a flat-bottom 96-well plate in triplicate. Then 200 µL of the chromogenic working reagent was added to each well, mixed thoroughly, and incubated for 20 min at room temperature. The optical density was measured at 520 nm using a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA). The serum urea values were divided by the BALF urea values to give the dilution factor, which was then multiplied by the raw BALF GSH values to obtain normalized GSH levels in ELF.

2.8 Quantitative Real-Time PCR

To synthesize cDNA, reverse transcription was performed using total RNA and the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). First, all total RNA samples were adjusted to 1 µg in a total of 9.5 µL, and 0.5 µL of DNaseI was added to remove genomic DNA. The solutions were incubated for 20 minutes at 37 °C, then for 5 minutes at 70 °C using a PTC-100 ™ Programmable Thermal Controller (MJ Research Inc., St. Bruno, Canada). The next steps were according to the manufacturer's established protocol. Random hexamers were added to each sample as primers. For gene expression measurements

with high sensitivity, cDNA targets were pre-amplified using TaqMan PreAmp Master Mix (Applied Biosystems Inc., Foster City, CA) in order to detect cDNA targets with low expression levels. Specifically, each gene specific assay mix was combined with DNA suspension buffer first (pooled assay mix), and then added to the PreAmp Master Mix. The cDNA targets were amplified using a 2720 Thermal Cycler (Applied Biosystems Inc., Foster City, CA) with the reaction as follows: 1 cycle of 95 °C for 10 minutes, 10 cycles of 95 °C for 15 seconds, and 10 cycles of 60 °C for 4 minutes. Preamplified samples were diluted 1:5 with nuclease-free water. Then a 2 µL aliquot of preamplified cDNA was added to a 12 µl PCR reaction mix. The PCR reaction mix consisted of the primers, probes, and the TaqMan Gene Expression Master Mix (Applied Biosystems Inc., Foster City, CA). The primers and probes for the specific genes of the candidate glutathione transporters were designed by Applied Biosystems Inc., and the amplification and detection of the genes were performed using the ABI PRISM 7900 system (Applied Biosystems Inc., Foster City, CA). The PCR reaction cycles were as follows: 1 cycle of 95 °C for 10 minutes, 40 cycles of 95 °C for 30 seconds, and 40 cycles of 60 °C for 1 minute. The expression levels for β-actin from the reference samples with serial dilution (1, 1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000) were used to create a linear regression equation for back-calculation of the target gene mRNA expression levels. The mRNA expression levels were then normalized to β-actin mRNA expression levels in each sample

2.9 Data Analysis and Statistical Methods

All data are represented as the mean ± SEM, and each treatment group has an n=4-6. Differences among groups was determined by two-way analysis of variance (ANOVA), followed by Bonferonni correction for multiple comparisons. Significance was set at $p < 0.05$. All data

were analyzed using Prism software (GraphPad, La Jolla, CA). In some cases, log transformation was performed if the data were not normally distributed.

3. Results

3.1 GSH in BALF

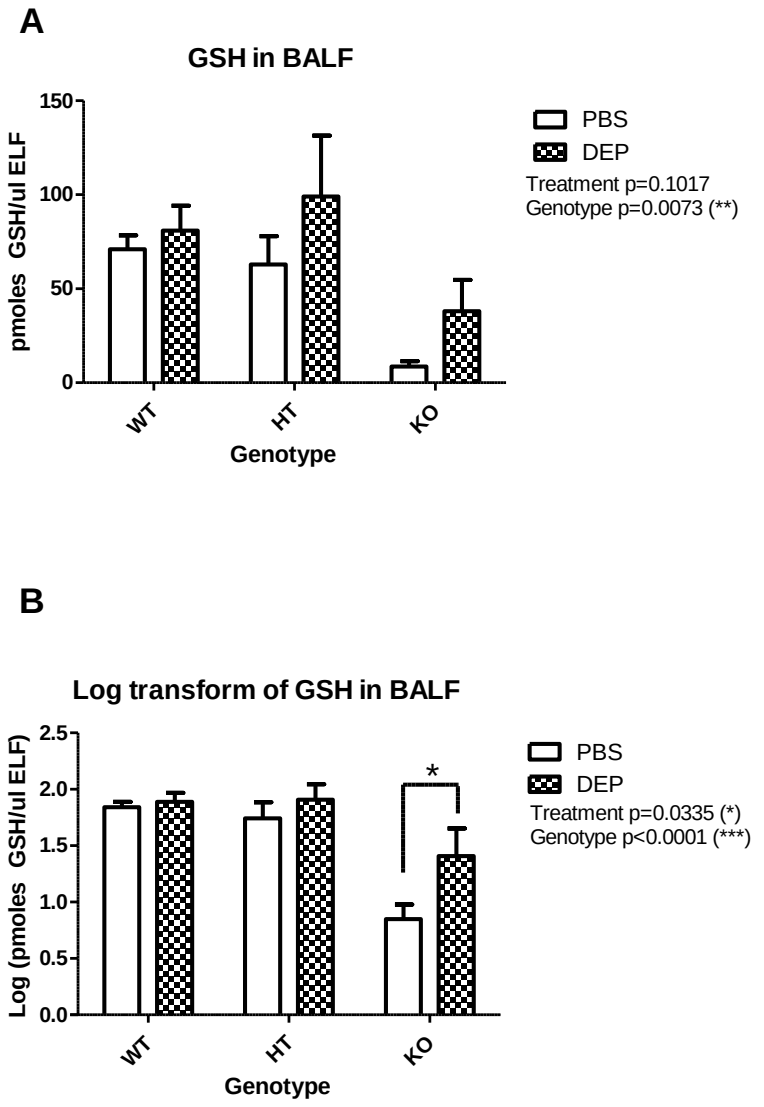

Figure 5. BALF Glutathione levels in in GCLM WT, HT, and KO mice 6 h after either PBS or DEP intranasal instillation. (A) Two-way analysis of variance (ANOVA) with a Bonferroni post-hoc test performed. (B) Log transformation performed before analysis. All data represented as mean ± SEM. N=4-6 mice per genotypes and treatments. *Significantly different, $p < 0.05$

The GSH levels found in BALF increased following exposure to DEP in *Gclm*-/- mice (Fig5; $p < 0.05$). Because the sample size was small (n=4-6 per group) and the results were not normally distributed, the statistical analysis was performed on log-transformed data. Statistical significance was observed for the effect of treatment after performing log transformation ($p = 0.0335$).

3.2 Ratio of GSH in BALF to GSH in Lung Tissue

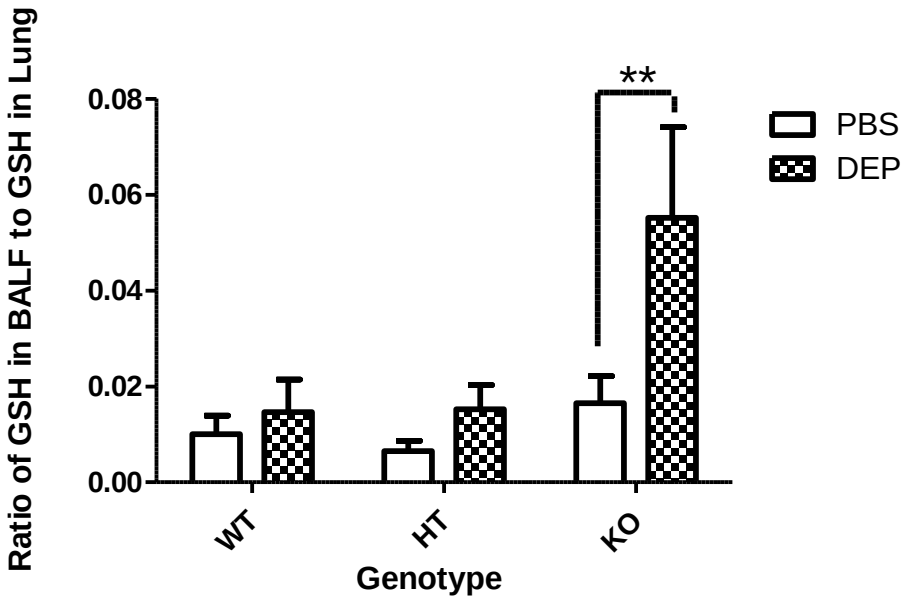

Figure 6. Ratio of GSH in BALF to GSH in Lung in GCLM WT, HT, and KO mice 6 h after either PBS or DEP intranasal instillation. Data represented as mean ± SEM. N=4-6 mice per genotypes and treatments. **Significantly different, $p < 0.01$

The ratio of BALF GSH to lung GSH in Gclm-/- mice was dramatically increased when mice were exposed to DEP (Fig 6; $p < 0.01$). Because the ELF levels of GSH were already quite low in Gclm-/- (Fig. 5), it was possible that these mice were pumping out of their lung cells proportionately more of the GSH present, in order to protect the lungs from oxidative stress induced by the presence of DEPs.

3.3 Gene Expression Analysis of Candidate GSH Transporters

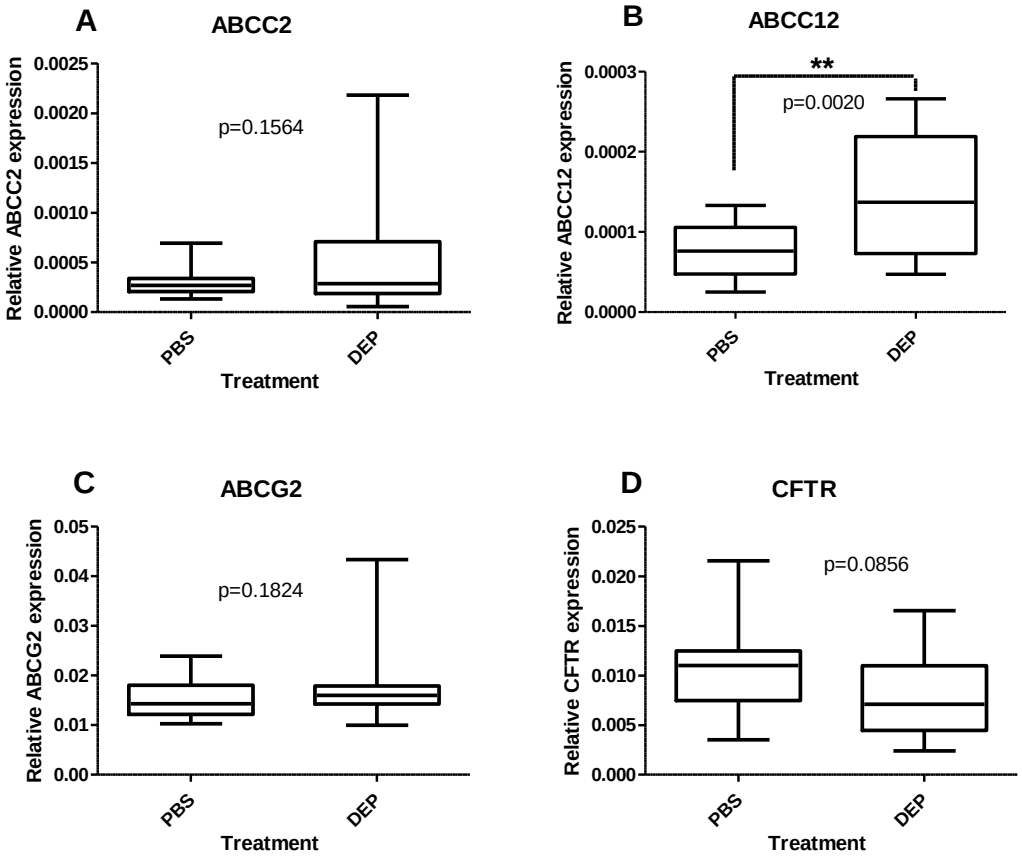

Figure 7. Real-time PCR assessment of GSH transporter mRNA normalized to β-actin in the lung 6 h after either PBS or DEP intranasal instillation (unpaired t-test). Unpaired t-test performed. Data represented as mean ± SEM. N=4-6 mice per genotypes and treatments.
****Significantly different, $p < 0.01$**

Using quantitative real-time PCR, the mRNA expression for potential GSH transporter genes was assessed. Regardless of genotype, ABCC12 mRNA was significantly upregulated following DEP exposure (Fig. 7). It was observed that mRNA expression levels for ABCC2, ABCG2, and CFTR genes were not statistically different between treatments. There was a trend in the expression of ABCC2 mRNA, with GCLM WT and HT mice showing increased expression following exposure to DEP (Fig. 8). However, these differences were not statistically

significant. When the GCLM WT and HT were pooled together for analysis, a statistically significant treatment effect was detected in ABCC2 mRNA expression (Fig. 9). Also, after pooling GCLM WT and HT, ABCC2 mRNA was upregulated in GCLM KO mice compared WT and HT under the PBS control conditions (Fig. 9). Expression of ABCC12 mRNA increased, with statistically significant differences detected between treatments ($p = 0.0005$) and genotype ($p = 0.0198$). Also, ABCC12 mRNA was upregulated in response to DEP exposure in *Gclm*+/+ mice (Fig. 8; $p < 0.01$). However, the expression of ABCG2 mRNA was not significantly different for either treatment or genotype.

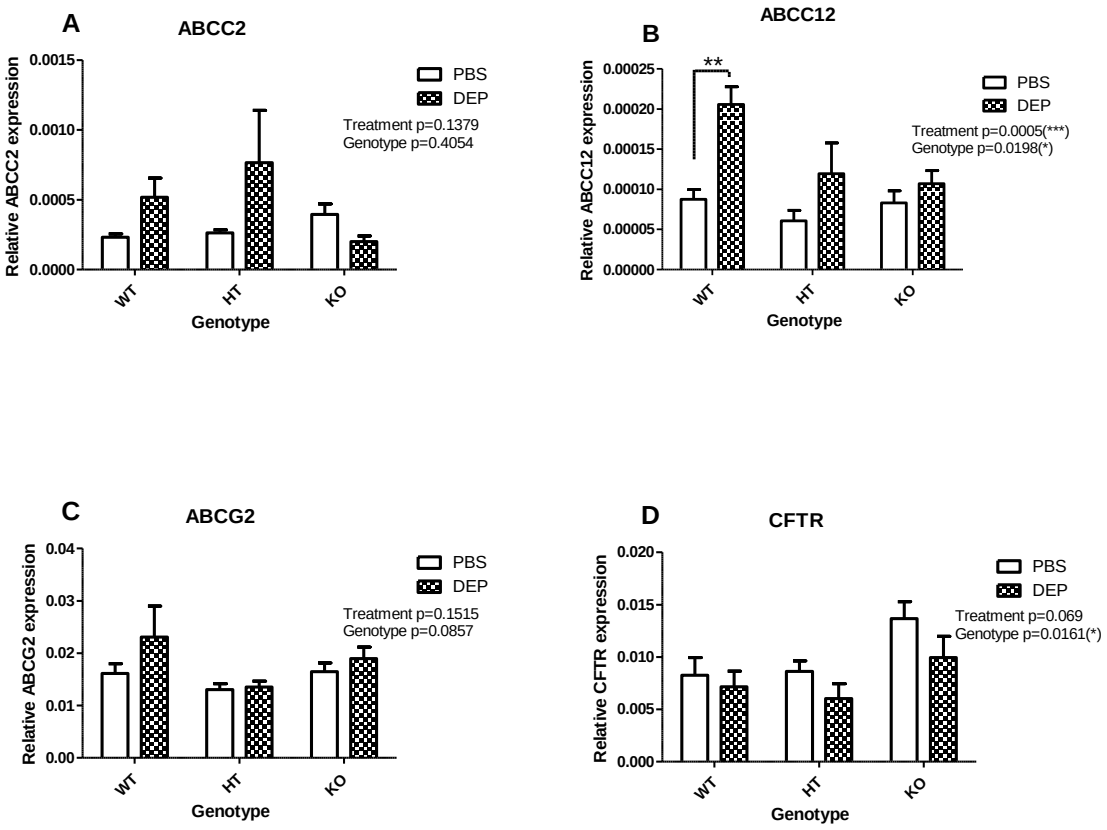

Figure 8. Real-time PCR assessment of GSH transporter mRNA normalized to β-actin in the lungs of GCLM WT, GCLM HT, and GCLM KO mice 6 h after either PBS or DEP intranasal instillation. Two-way ANOVA performed with a Bonferroni post-hoc test. Data represented as mean ± SEM. N=4-6 mice per genotypes and treatments. **Significantly different, $p < 0.01$

Interestingly, there was a different trend for CFTR expression (Fig. 8, panel D). In response to DEP exposure, CFTR expression decreased. For controls, CFTR expression was high in *Gclm*-/- mice compared to *Gclm*+/+ or *Gclm*+/- mice (Fig. 9).

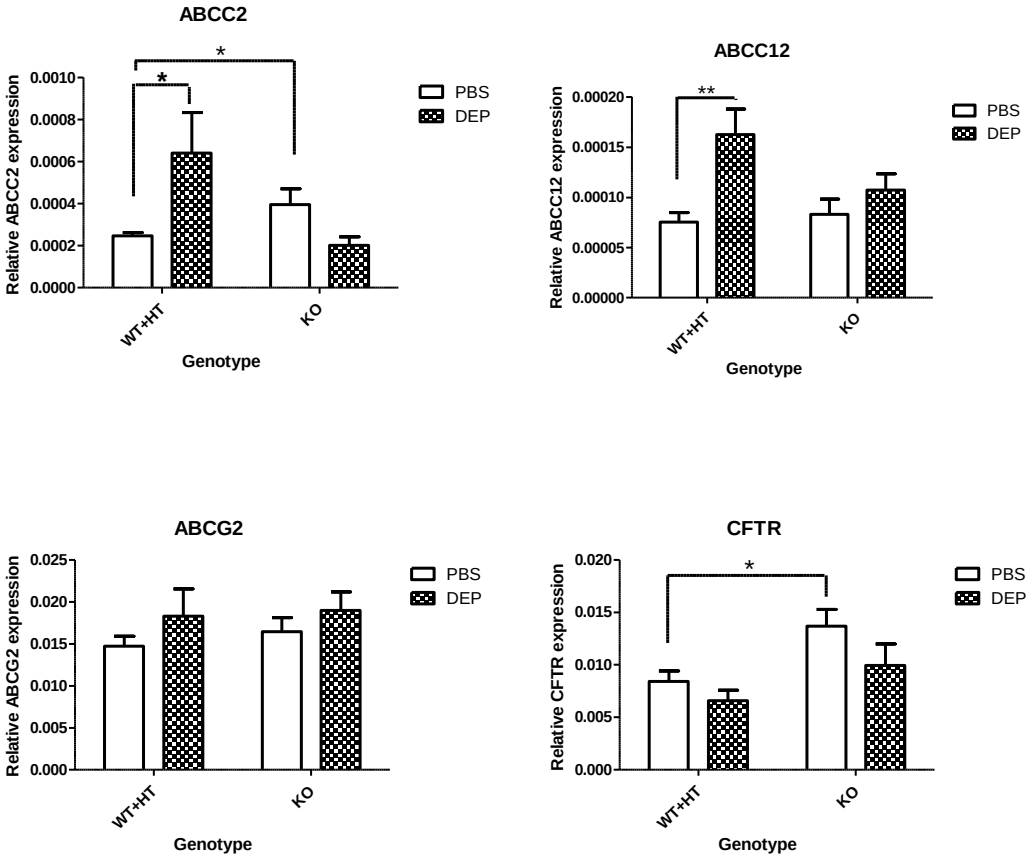

Figure 9. Real-time PCR assessment of GSH transporter mRNA normalized to β-actin in the lungs of GCLM WT, GCLM HT, and GCLM KO mice 6 h after either PBS or DEP intranasal instillation. GCLM WT and HT were pooled together for statistical analysis. Two-way ANOVA performed with a Bonferroni post-hoc test. Data represented as mean ± SEM. N=4-6 mice per genotypes and treatments. *Significantly different, $p < 0.05$, and **$p < 0.01$

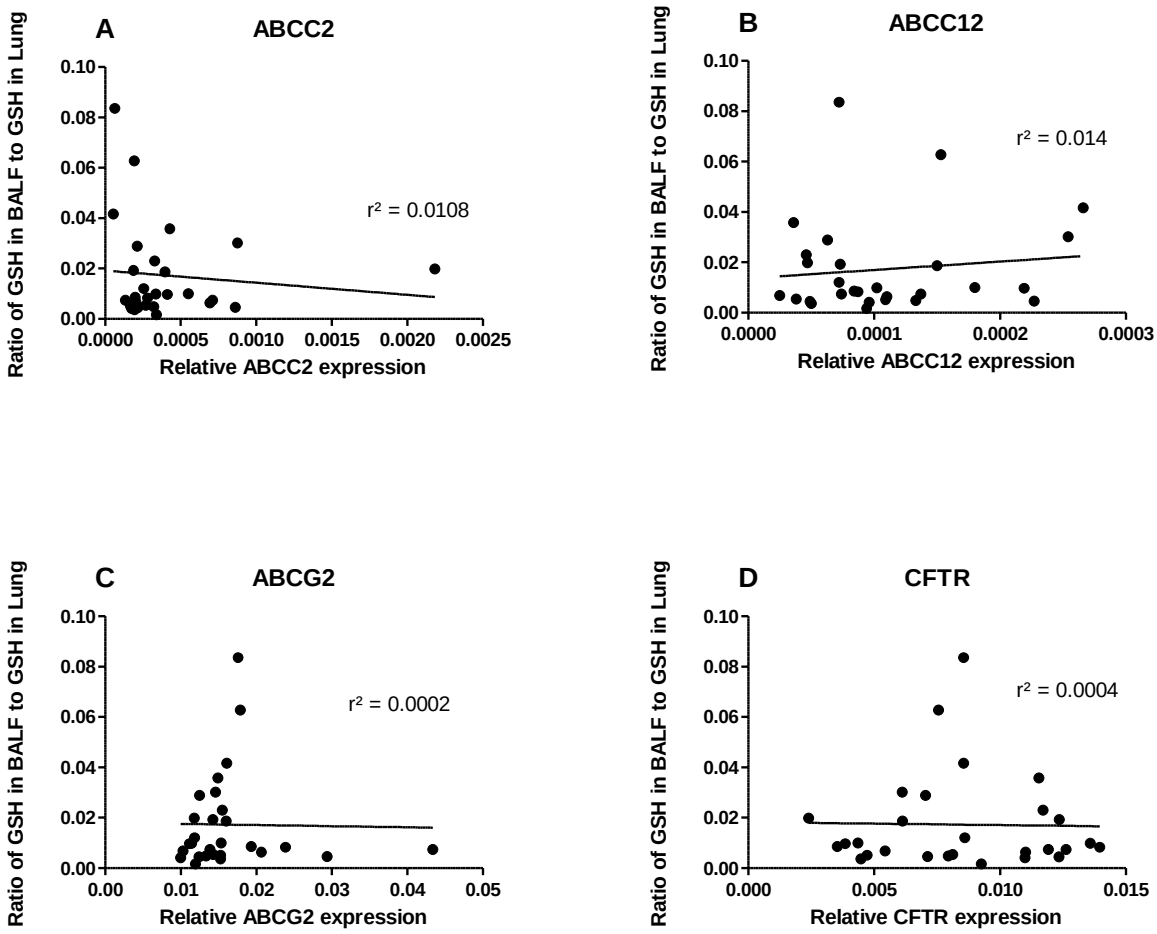

Figure 10. Linear regression between ratio of GSH in BALF to GSH in lung and relative expression levels of each GSH transporter mRNA. N=4-6 mice per genotypes and treatments.

A linear regression was performed examining the ratio of BALF GSH to lung GSH and relative mRNA expression levels for each gene (Fig. 10). There was weak linear relationship only for the ABCC12 gene ($r^2 = 0.014$). After separating Gclm+/+ and Gclm+/- mice from Gclm-/- mice, there was a slightly stronger correlation between the GSH ratio and relative expression of ABCC12 mRNA (Fig. 11; $r^2 = 0.2157$).

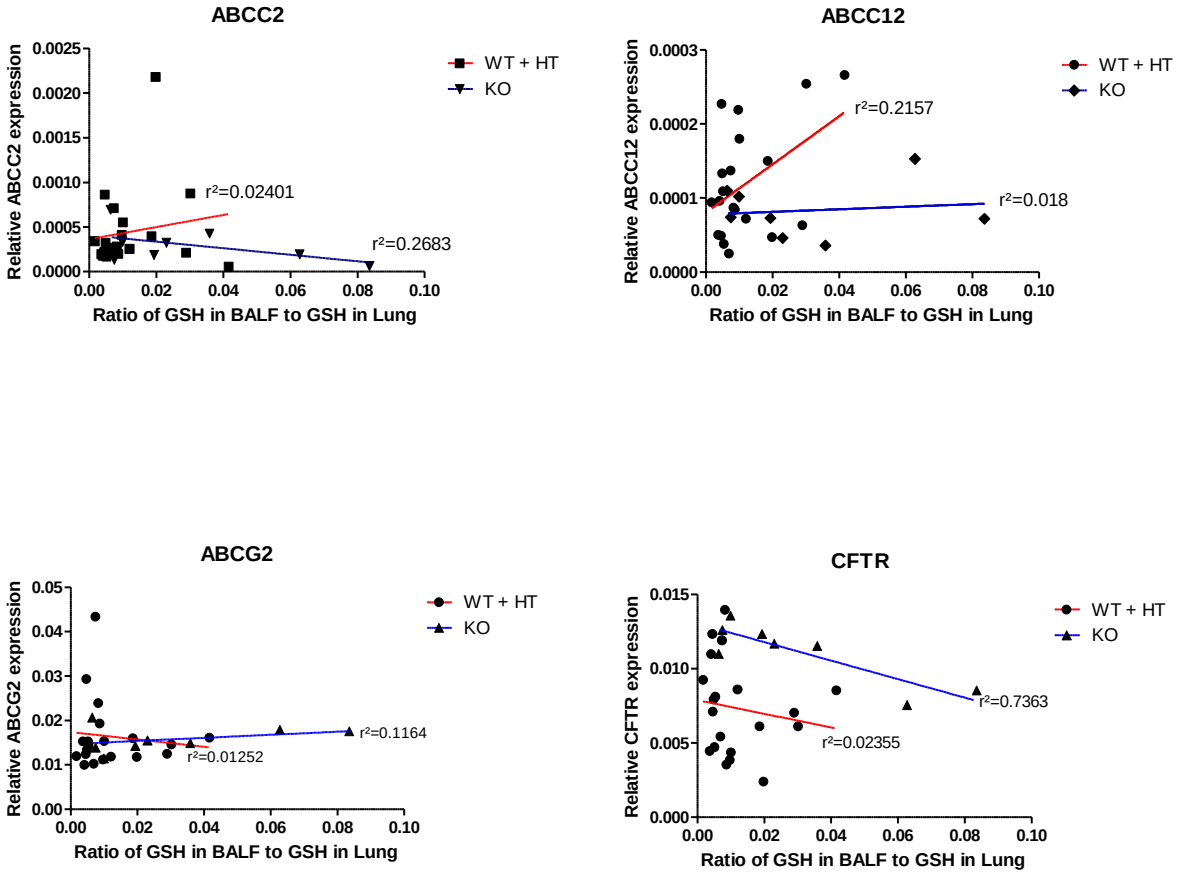

Figure 11. Linear regression between ration of GSH in BALF to GSH in lung and relative expression level. GCLM WT and GCLM HT mice were pooled together for statistical analysis. N=4-6 mice per genotypes and treatments.

Regardless of treatment, the mRNA expression levels of the four candidate GSH transporters are shown on the same scale (Fig. 12). ABCC2 and ABCC12 mRNA expression was very low compared to the expression of ABCG2 and CFTR mRNAs. After pooling *Gclm*+/+ and *Gclm*+/- mice for statistical analysis, CFTR mRNA expression was significantly upregulated in *Gclm*-/- mice compared to the other genotypes pooled.

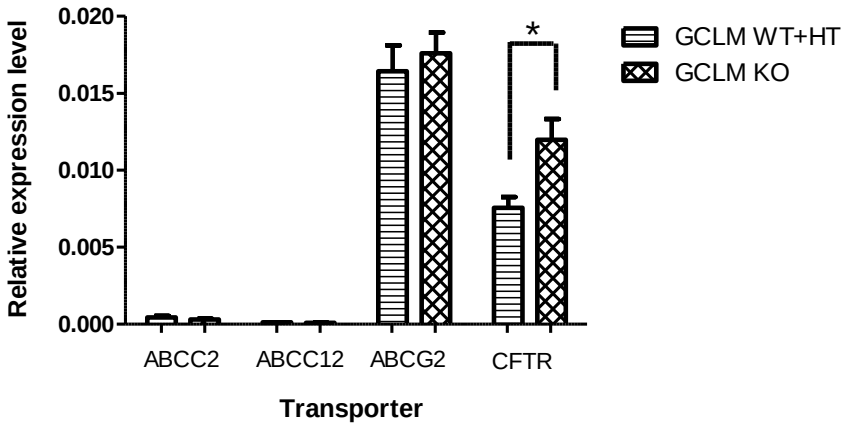

Figure 12. GSH transporter mRNA expression analysis normalized to β-actin in the lungs of GCLM WT, GCLM HT, and GCLM KO mice 6 h after either PBS or DEP intranasal instillation. GCLM WT and HT were pooled together for statistical analysis. Two-way ANOVA performed with a Bonferroni post-hoc test. Data represented as mean ± SEM. N=4-6 mice per genotypes and treatments. *Significantly different, $p < 0.05$

In summary, in response to DEP exposure, it was observed that ELF GSH increased in *Gclm*-/- mice. ABCC2 and ABCC12 genes were significantly upregulated following the DEP exposure in *Gclm*+/+ and *Gclm*+/- mice, but they had low expression. CFTR was highly expressed in *Gclm*-/- mice compared to *Gclm*+/+ and *Gclm*+/- mice.

4. Discussion

Glutathione (GSH) is a prominent intracellular antioxidant important for combating oxidative stress and plays a critical role in maintaining redox balance in the body. GSH is synthesized in the cell, so ELF GSH must be maintained by mechanisms which export it into the bronchoalveolar space. While ELF GSH can be elevated in response to oxidative stress induced by environmental stressors, the responsible GSH transporting mechanisms are not clearly established.

In this study, it was observed that GSH found in BALF was elevated when exposed to diesel exhaust particles (DEP) in *Gclm*-/- mice. Surprisingly, *Gclm*-/- mice were not sensitive to the DEP exposure compared to *Gclm*+/- mice (Fig. 2). The result that ELF GSH increased following the DEP exposure in *Gclm*-/- mice generally supports the finding that they were not more sensitive to DEP exposure. We hypothesized that in *Gclm*-/- mice GSH transporters play an important role in exporting GSH into the ELF following the DEP exposure as a compensatory mechanism. To attempt to identify the responsible GSH transporters, four candidate transporter genes (ABCC2, ABCC12, ABCG2, and CFTR) were selected based on previous studies (Gould et al., 2012; Day et al., 2004). However, we did not observe that these GSH transporters were upregulated following the DEP exposure in *Gclm*-/- mice Statistically significant treatment differences were found in the expression of ABCC2 and ABCC12 mRNAs in *Gclm*+/+ and *Gclm*+/- mice; however, this didn't result in increases of ELF GSH levels in *Gclm*+/+ and *Gclm*+/- mice, most likely because of the very low expression of these mRNAs.

A possible transporter responsible for exporting proportionately more GSH into the ELF in *Gclm*-/- mice is CFTR because it was highly expressed in *Gclm*-/- mice compared to *Gclm*+/+

and *Gclm*+/- mice. Although it was not shown to be upregulated in response to the DEP exposure in *Gclm*-/- mice, this result suggests that CFTR could be responsible for partially maintaining ELF GSH in *Gclm*-/- mice.

CFTR mRNA expression followed a different trend than the three other investigated genes. It was observed that CFTR mRNA expression decreased following exposure to DEP. CFTR is a member of the ABC cassette family of transporter proteins, and is involved in chloride conductance in secretory epithelial cells (Knowles et al., 1983). Cystic fibrosis is a genetic disorder that involves defective CFTR protein. CFTR is an apical transporter in the lung with has a role in exporting GSH; Gould et al. (2011) demonstrated that inhibition or deletion of CFTR decreased extracellular levels of GSH by more than 50%, even following exposure to cigarette smoke. They concluded that CFTR is the primary glutathione transporter protein in response to an environmental stressor such as cigarette smoke. However, the current study showed different results. CFTR mRNA expression decreases slightly in response to DEP exposure. This result corresponds with another study (Cantin et al., 2006), which demonstrated that the expression of CFTR was suppressed in cigarette smokers. These discrepancies between studies may be due to measurements taken at different time points. The Gould et al. study showing increases in CFTR expression in response to environmental oxidants measured mRNA expression 48 hours after the exposure. However, in the current study, mice were euthanized 6 hours after the exposure to measure acute effects of DEP exposure. It is possible that CFTR mRNA expression can be initially suppressed by acute exposure to oxidants, but upregulated after 48 hours after cells have had a chance to adapt to the exposure by subsequently activating protective pathways.

In our study, the basal CFTR mRNA expression in the control group is intriguing. It is known that *Gclm*-/- mice have low GSH due to the deficiency in glutathione synthesis. It was observed that *Gclm*-/- mice have the highest level of CFTR expression among the three *Gclm* genotypes. This result means that CFTR might be responsible for maintaining ELF GSH when GSH levels are low.

Another gene investigated as a potential GSH transporter in the lung was ABCC2. ABCC2 is also a member of the ABC cassette family, and is known as multidrug-associated resistance protein 2 (MRP2). ABCC2 is expressed in hepatocytes and renal proximal tubular cells, where it transports GSH for detoxification (Jedlitschky et al., 2006; Ballatori et al., 1998). ABCC2 is also a multispecific organic anion transporter. This study showed that ABCC2 expression slightly increased in response to DEP, but not to a statistically significant level. Even though a statistically significant treatment effect was detected after pooling *Gclm*+/+ and *Gclm*+/- mice, the mRNA expression levels were probably too low to result in elevating ELF GSH. One interesting study has suggested that CFTR could enhance ABCC2 function by forming a complex together (Li et al., 2010). Thus, the decrease in CFTR expression seen with DEP exposure might affect MRP2 expression/function.

ABCC12 is a member of the MRP family, but has not been investigated as extensively as the other three genes. Until recently, the function of ABCC12 was not known. Our results show that ABCC12 (MRP9) was upregulated following DEP exposure in GCLM WT mice with statistical significance, but similar to ABBC2, the expression level was probably too low to elevate ELF GSH.

ABCG2 is a member of the ABC superfamily G protein subfamily. It is also known as breast cancer-related protein (BCRP). A recent study (Brechbuhl et al., 2010) has shown that

overexpression of ABCG2 in both yeast and human epithelial cells resulted in an increase in basal extracellular levels of GSH. Furthermore, silencing ABCG2 mRNA was associated with decreasing extracellular levels of GSH. Our results also suggest that ABCG2 plays a role in maintaining basal GSH concentrations in ELF, but ABCG2 mRNA expression did not dramatically increase as an adaptive response to DEP exposure (Fig. 8). Also, ABCG2 mRNA expression did not show any significant differences among the three genotypes. However, the overall expression level of ABCG2 mRNA was high compared to that of ABCC2 and ABCC12 (Fig. 12), so ABCG2 might be responsible for maintaining basal ELF GSH levels in the lungs.

In summary, we observed that ELF GSH increased following DEP exposure in *Gclm*-/- mice, supporting our previous data that inflammatory responses (as determined by the %neutrophils present in BALF) were similar between *Gclm*+/+ and *Gclm*-/- mice (Weldy et al., 2011). Furthermore, this result suggests that GSH transporters responsible for maintaining ELF GSH may act as a compensatory mechanism in *Gclm*-/- mice allowing the lung to adapt to the presence of DEPs. Even though CFTR was not up-regulated in response to DEP exposure, the fact that its expression is already quite high in *Gclm*-/- mice suggests this transporter might be responsible for maintaining ELF GSH in response to DEP exposure in *Gclm*-/- mice.

Investigating GSH transporter genes and mechanisms involved is challenging because multiple transporters and mechanisms exist. Nevertheless, it is important to uncover the responsible GSH transporters because respiratory diseases are associated with low ELF GSH levels (Day et al., 2004). It is well known that DEPs have been long associated with respiratory diseases. For susceptible people including asthmatic children, pregnant women, and elderly people with cardiovascular symptoms, DEP exposure may be especially harmful. If GSH transporters responsible for maintaining or increasing ELF GSH in response to DEP or other

environmental stressors could be identified, these could be targeted by drug developers to reduce the risk of respiratory diseases related to air pollution, cigarettes or other lung toxicants.

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