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Glutathione deficient C57BL/6J mice are not sensitized to ozone-induced lung injury

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Abstract

In this study we examined the role of the antioxidant glutathione (GSH) in pulmonary susceptibility to ozone toxicity, utilizing GSH deficient C57BL/6J mice that lack the expression of glutamate-cysteine ligase modifier subunit (GCLM). *Gclm*($-/-$) knockout mice had 70% GSH depletion in the lung. *Gclm*($+/+$) wild-type and *Gclm*($-/-$) mice were exposed to either 0.3 ppm ozone or filtered air for 48 hours. Ozone-induced lung hyperpermeability, as measured by total protein concentration in bronchoalveolar lavage fluid, was surprisingly lower in *Gclm*($-/-$) mice than in wild-type mice. Lung hyperpermeability did not correlate with the degree of neutrophilia or with inflammatory gene expression. Pulmonary antioxidant response to ozone, assessed by increased mRNA levels of metallothionein 1 and 2, alpha-tocopherol transporter protein, and solute carrier family 23 member 2 (sodium-dependent vitamin C transporter) was greater in *Gclm*($-/-$) mice than in *Gclm*($+/+$) mice. These results suggest that compensatory augmentation of antioxidant defenses in *Gclm*($-/-$) mice may confer increased resistance to ozone-induced lung injury.

Keywords

ozone; glutathione; mice; antioxidant genes; lung hyperpermeability

Introduction

Ambient ozone is a secondary pollutant formed through a complex series of photochemical reactions involving nitrogen oxides and sunlight. The target organ of ozone is the lung, and numerous studies using both animals and human subjects have demonstrated that ozone causes decreased lung function, airway inflammation, and direct epithelial injury [1]. Ozone is highly reactive and oxidizes proteins and lipids in the fluid lining compartment of the lung, which initiates signaling processes that lead to inflammation and increased lung permeability. The inflammatory response to ozone exposure is initiated by the release of proinflammatory

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cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1 β , and IL-6, leading to the influx of neutrophils, which accumulate in the lung interstitium and alveolar compartments.

The epithelial lining fluid (ELF) contains antioxidants and antioxidant enzymes that are generally believed to protect the underlying epithelial cell layer against oxidative stress. The most important non-enzymatic antioxidants in the ELF are believed to be uric acid, ascorbic acid, tocopherols, and glutathione (GSH) [2]. Uric acid, which is particularly abundant in the upper airways, is a potent scavenger of reactive oxidants with very high reactivity towards ozone [3]. The powerful lipophilic antioxidant α -tocopherol, is secreted into ELF from type II epithelial cells along with surfactants [4], and is notable for its ability to terminate lipid peroxidation and protect biological membranes. Ascorbic acid also possesses high reactivity towards ozone, and has been implicated in ozone adaptation in mice [5].

GSH is the most abundant soluble thiol in mammalian cells, with a wide range of functions [6;7]. As a potent antioxidant, GSH protects cells from oxidative stress-induced damage by acting as a co-substrate for glutathione peroxidase and by directly scavenging free radicals. The first and rate-limiting step in GSH synthesis is catalyzed by glutamate cysteine ligase, which in higher eukaryotes consists of a catalytic subunit (GCLC) and a modifier subunit (GCLM). While GCLC possesses all the catalytic activity, GCLM has been shown to enhance the activity of the holoenzyme by lowering K_m for glutamate and ATP, increasing K_i for GSH, and increasing K_{cat} for γ -glutamylcysteine (γ -GC) synthesis [8]. Thus, loss of GCLM not only leads to a decrease in cellular resting GSH levels by 60–90% but also dramatically affects GSH level recovery after insult. GCLM null mice are more sensitive to acetaminophen-induced hepatotoxicity, and *Gclm*($-/-$) cells are dramatically more sensitive to hydrogen peroxide [9] and arsenite [10] toxicity. Furthermore, studies in human populations have shown that polymorphisms in the *GCLM* gene are associated with increased risks for myocardial infarction, schizophrenia, and body burden of methyl mercury, which demonstrates that fairly modest reductions in GSH synthesis can have profound health implications [11–13].

GSH is abundant in ELF, and human GSH levels in ELF have been reported to be 100 times higher than that in plasma [14], suggesting a potential role for GSH in protecting the underlying epithelium from oxidative stress induced by oxygen or inhaled pollutants.

The evidence is mixed with regard to the protective role of GSH and other low molecular weight antioxidants in the defense against environmentally relevant levels of ozone. In a study of ascorbate-depleted human subjects, ascorbate and α -tocopherol supplementation reduced ozone-induced loss of pulmonary function, but had no effect on inflammatory markers [15]. An intervention study of Dutch bicyclists demonstrated that ascorbic acid and α -tocopherol confer partial protection from the acute effects of ozone on pulmonary function [16]. Other controlled studies in humans have failed to demonstrate a protective effect of antioxidants [17] or did not find a correlation between concentrations of antioxidants in ELF and ozone-induced lung function decrements [18]. In this study, we used *Gclm*($-/-$) mice as a model of GSH deficiency to evaluate the role of GSH in the pulmonary susceptibility to environmentally relevant concentrations of ozone. Parameters of pulmonary response to ozone exposure included hyperpermeability, neutrophilia, antioxidants, and expression of inflammatory and antioxidant genes.

Materials and methods

Mice

The generation of *Gclm*($-/-$) knockout mice on a C57BL/6J background has been described earlier [9]. All procedures involving mice were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati. Litter mates of three-month old mice (4–5

mice/group) were used for the experiments, and genotyping was done by multiplexed PCR using 5'-CAAATCCTGGTTAAAGGCTA-3' as the forward primer for the wild-type *Gclm* (+) allele, 5'-ATATGCGAAGTGGACCTG-3' as the primer for the knockout *Gclm*(-) allele, and 5'-AACGTTGCAAGCTACTGC-3' as the reverse primer for both alleles. The wild-type allele was detected as a 354-bp fragment, with the forward primer located in the deleted region of intron 1. The targeted knockout allele was detected as a 250-bp fragment, with the forward primer located in the inserted *Neo* gene.

Ozone generation and exposure

Ozone was generated from 100% (Wright extra-dry O₂ Brothers, Cincinnati, OH) using a Model V1-0 ultraviolet ozone generator (OREC, Phoenix, AZ) and monitored continually with a Dasibi Model 1008-PC direct reading instrument (Dasibi, Glendale, CA). This instrument has an internal calibration system, which was routinely checked and calibrated against a US-EPA transfer standard (Hamilton County Environmental Services, Cincinnati, OH). Mice were placed in stainless steel cages inside a 0.32-m³ inhalation chamber (Bertke Manufacturing, Cincinnati, OH) capable of complete air exchange every 2 minutes, and exposed to HEPA-filtered room air containing 0.3 ppm ozone continuously for 48 hours. This exposure regime has been chosen to represent subchronic exposure to environmentally relevant dose of ozone [19].

Bronchoalveolar Lavage

After exposure to ozone, mice were euthanized and the lungs were lavaged twice with 1-ml aliquots of phosphate buffered saline (PBS) without Ca²⁺ and Mg²⁺ pre-warmed to 37°C. Recovered bronchoalveolar lavage (BAL) fluid was immediately placed on ice and pooled for each animal. BAL fluid was centrifuged at 500g for 5 min at 4°C, and supernatants were collected for protein concentration and biochemical analysis. Cells were resuspended in 500 µl of PBS supplemented with 2% fetal bovine serum. Aliquots were cytocentrifuged and stained with Hema3 cell staining for differential cell analysis by counting at least 300 cells. Total cell counts were determined with a hemocytometer.

Biochemical measurements

Levels of GSH and GSSG in tissue homogenates and BAL fluid were measured fluorometrically after *o*-phthalaldehyde derivatization [20]. Levels of ascorbate in tissue homogenates and BAL fluid were determined spectrophotometrically following ascorbate-dependent reduction of ferric iron [21]. Levels of uric acid in BAL fluid were determined by HPLC. Briefly, BAL fluid was mixed 4:1 (v/v) with 0.25 perchloroacetic acid and centrifuged 10 min at 12000g. The supernatant was injected into a Waters Nova-Pak[®] C₁₈ reverse phase column, 3.9 × 150 mm, at a flow rate of 0.8 ml/min. The mobile phase was 0.04 M phosphate buffer, pH 2.5. Peaks were detected with a Perkin Elmer LC290 UV/VIS spectrophotometric detector. Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Reverse transcription and quantitative PCR (qPCR)

Total RNA was isolated from frozen tissue using TRI Reagent[®] (Molecular Research Center, Inc., Cincinnati, OH) according to manufacturer's instructions. cDNA was synthesized using Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to manufacturer's instructions, using 2 µg of total RNA in a 20-µl reaction volume. For qPCR, reaction mixtures contained 0.4 µl of cDNA, 1x iQ SYBR Green Supermix (BioRad, Hercules, CA), and 0.15 µM of each primer in a total volume of 25 µ. Sequences for primers were as follows: chemokine (C-C motif) ligand 3 (CCL3 a.k.a Macrophage inflammatory protein alpha: MIP1α)-forward 5'-TTGAAACCAGCAGCCTTTGCTC-3', CCL3 (MIP1α)-reverse 5'-

TCTCAGGCATTTCAGTTCCAGGT-3', tumor necrosis factor (TNF)-forward 5'-AATGGCCTCCCTCTCATCAGTT-3', TNF-reverse 5'-TCCTCCACTTGGTGGTTTGCTA-3', chemokine (C-C motif) ligand 11 (CCL11 a.k.a eotaxin)-forward 5'-TCACGGTCACTTCCTTACCT-3', CCL11-reverse 5'-GCTTTCAGGGTGCATCTGTTGT-3', MT1-forward 5'-CACGACTTCAACGTCC-3', MT2-forward 5'-ACTCCTAGAACTCTTCAAAC-3', MT1/2-reverse 5'-CAGCAGGAGCAGCAGCTCTTCTTGACG-3', tocopherol (alpha) transfer protein (TTPA)-forward 5'-TCACAGACGCTTTCCTGCT-3', TTPA- reverse 5'-AATTCTGGGCATTCTGCTCG-3', superoxide dismutase 2 (SOD2)-forward 5'-GCTGGCTTGGCTTCAATAAGGA-3', SOD2-reverse 5'-CAGCGGAATAAGGCCTGTTGTT-3', solute carrier family 23 (nucleobase transporters), member 1 (SLC23A1: a.k.a. sodium-dependent vitamin C transporter 1: SVCT1)-forward 5'-TTCGGCTTGCTCCATTCTCCTGAT-3', SLC23A1-reverse 5'-ATCAGTTCGAGCCTGGAAGCCATA-3', SLC23A2-forward 5'-GCTCCACGGAAGGCAAATATGA-3', SLC23A2-reverse 5'-TGTAGATTGCCATGAGCTCCGT-3', beta-2 microglobulin (B2M)-forward 5'-CATGGCTCGCTCGGTGACC-3', B2M-reverse 5'-AATGTGAGGCGGGTGGAACTG-3', toll-like receptor 4 (TLR4)-forward 5'-TCGTCATGTTCTCTCATGGCCT-3', and TLR4-reverse 5'-AGGGATTCAAGCTTCCTGGTGT-3'. Reactions were run using the DNA Engine Opticon 2 Continuous Fluorescence Detector System (BioRad, Hercules, CA). Expression of β -2-microtubulin was used for normalization of C_T data according to the $\Delta\Delta C_T$ method [22]. mRNA levels of individual genes in non-exposed wild-type mice were set as controls (100%), and relative mRNA levels were reported as % of control.

Statistical analysis

Statistical analysis was performed using SigmaStat software (SPSS Inc., Chicago, IL). Group means were compared by 2-way analysis of variance, with the variables being genotype and ozone exposure, followed by the Holm-Sidak post-hoc test for significance. Data were reported as means $\pm$ S.E. of 4–5 mice per group, with P values of <0.05 considered statistically significant.

Results

Total protein levels and differential cell count in BAL fluid

Gclm(+/+) wild-type and *Gclm*(−/−) knockout mice were assigned to non-exposed and ozone-exposed groups (5/group) for each genotype. No difference in protein levels between non-exposed *Gclm*(+/+) (136 ± 15 μ g/ml) and *Gclm*(−/−) mice (160 ± 9 μ g/ml) was detected (Figure 1A). In both genotypes, there was a significant increase in protein levels after ozone exposure, and the protein concentration was significantly higher in *Gclm*(+/+) mice (1160 ± 185 μ g/ml), than in *Gclm*(−/−) mice (735 ± 47 μ g/ml). Differential cell analysis was performed to evaluate the inflammatory infiltration (Figure 1B). In non-exposed mice of both genotypes, the total cell count in BAL fluid was $6.6 \pm 1.6 \times 10^4$ /ml and the percentage of neutrophils was $< 1\%$. Following ozone exposure, the total cells in BAL fluid increased in both *Gclm*(+/+) and *Gclm*(−/−) mice, to 20.4 ± 2.0 and $29.3 \pm 10 \times 10^4$ /ml, respectively. The percentage of neutrophils also increased after ozone exposure in both genotypes. However, due to variability in responses, the difference between *Gclm*(+/+) and *Gclm*(−/−) mice was not statistically significant ($16.5 \pm 5.6\%$ versus $9.2 \pm 4.7\%$, respectively).

mRNA levels for genes involved in inflammation

To examine the inflammatory response, the mRNA transcript levels of ozone-induced inflammatory marker genes [23–25], were measured by qPCR using cDNA from extracts of whole lung (Figure 2). CCL11 mRNA was the only inflammatory marker to increase more in

the lungs of *Gclm*(+/+) mice (2.5 ± 0.2 -fold) than in the lung of *Gclm*(-/-) mice (1.5 ± 0.2 -fold). Expression of TNF mRNA in lung was induced by ozone exposure in both genotypes to a similar level [*Gclm*(+/+): 2.4 ± 0.4 -fold and *Gclm*(-/-): 2.0 ± 0.2 -fold]. There was a trend toward increased TLR4 mRNA after ozone exposure, but no difference was observed between the two genotypes. The CCL3 (a.k.a. macrophage inflammatory protein 1 α) mRNA after ozone treatment showed great inter-individual variability in both genotype groups, but the difference between the two groups was not statistically significant. A closer examination of ozone-exposed mice of both genotypes revealed a strong positive correlation between increased CCL3 and the percentage of neutrophils in BAL fluid, with $p < 0.0005$ (data not shown). No such correlation was seen for any of the other genes examined.

Antioxidants in lung tissue and BAL fluid

Antioxidant levels were measured in both lung tissue and BAL fluid (Table 1). In non-exposed groups, the lung GSH was significantly decreased (~29%) in *Gclm*(-/-) mice compared to that in *Gclm*(+/+) mice. Following ozone exposure, lung GSH levels increased significantly in *Gclm*(+/+) but not in the lung of *Gclm*(-/-) mice. Following ozone, lung GSSG levels significantly increased in both genotypes. In non-exposed groups, the GSH/GSSG ratio, an indicator of oxidant stress, was reduced (~50%) in *Gclm*(-/-) mice compared to that in *Gclm*(+/+) mice. After ozone exposure, GSH/GSSG ratio decreased in both genotypes. Interestingly, lung ascorbate of non-exposed *Gclm*(-/-) mice was significantly greater (~35%) than that in non-exposed *Gclm*(+/+) mice. Following ozone exposure, lung ascorbate levels increased in both genotypes; however, the difference was statistically significant only for *Gclm*(+/+) mice.

In spite of the lower GSH and higher ascorbate levels in lungs from *Gclm*(-/-) mice relative to *Gclm*(+/+) mice, levels of these two antioxidants in BAL fluid were not different between the two genotypes in the non-exposed groups. Levels of GSH, ascorbate, and uric acid expressed as nmoles/mg protein were significantly lower in ozone exposed mice in both genotypes (Table 1). Because a constant volume was used for lavage, we calculated the total amount of BAL antioxidants as expressed in μ M. In BAL fluid, GSH levels expressed as μ M were unchanged after exposure in both genotypes, whereas uric acid levels were significantly increased. Ascorbate levels expressed as μ M were increased after exposure only in *Gclm*(+/+) mice. Differences in BAL fluid antioxidant levels between the two genotypes were mostly non-significant, the only exception being ascorbic acid, which, when expressed as μ M in BAL fluid, was lower after ozone exposure in *Gclm*(-/-) mice compared to *Gclm*(+/+) mice. GSSG levels in BAL fluid were below the limits of detection for the OPA assay (<100 pmol GSSG/ml BAL).

Antioxidant defense system mRNA levels

MT1 and MT2 mRNA increased after exposure between the two genotypes (Figure 3). Following ozone lung MT1 and MT2 mRNA increased 2.2 ± 0.3 and 1.9 ± 0.2 , respectively, in *Gclm*(+/+) mice, whereas the increase was 3.3 ± 0.5 and 5.1 ± 0.6 , respectively in *Gclm*(-/-) mice. In contrast, SOD2 mRNA increased ~2-fold after ozone exposure without any significant difference between the two genotypes. Lung TTPA and SLC23A2 mRNA increased by 2.8- and 2.6-fold, respectively, after ozone exposure in *Gclm*(-/-) animals, but remained unchanged in the ozone-exposed group relative to the non-exposed group in *Gclm*(+/+) mice. Lung SLC23A1 mRNA increased ~1.7-fold in both genotype groups following ozone.

Discussion

It is generally assumed that glutathione, by virtue of its antioxidant capacity, is important in the defense against ozone-induced lung damage by reacting either directly with ozone, or with its secondary cytotoxic products [26]. However, we found that *Gclm*(-/-) mice, with 70% depletion of lung GSH, were surprisingly more resistant than *Gclm*(+/+) mice to low dose

ozone-induced pulmonary epithelial hyperpermeability as indicated by a lower protein concentration in BAL fluid.

In this study, the concentration of GSH in BAL fluid, when expressed as nmol/mg protein, was substantially lowered following ozone exposure; the same pattern of changes was also observed for ascorbate and uric acid. This may reflect the dilution of BAL antioxidants caused by hyperpermeability. The total amount of BAL antioxidants as expressed in μM , however, was unaltered by ozone exposure. It is noteworthy that, in spite of substantially decreased lung GSH in *Gclm*($-/-$) mice, GSH levels in BAL fluid did not differ significantly between the two genotypes either in non-exposed or in ozone-exposed group. This may suggest a compensatory mechanism for maintaining GSH levels in ELF when systemic GSH levels are compromised. Studies have identified the cystic fibrosis transmembrane conductance regulator protein (CFTR) as an important GSH transporter across lung secretory epithelium, and adaptive increases of ELF GSH to counter oxidative stress are severely blunted in *Cftr* KO mice [27; 28]. It is thus conceivable that GSH transport across the lung epithelium maintains ELF GSH at an adequate level in *Gclm*($-/-$) mice. Future studies are needed to investigate a possible role for CFTR in this process. Taken together, these observations suggest that the capacity of *de novo* GSH synthesis in the lung tissue is not a critical determinant of the susceptibility to low dose ozone-induced epithelial hyperpermeability, an indicator of lung injury.

In addition to small molecule antioxidants, certain antioxidant proteins also participate in the pulmonary response to oxidant stress. MT1 and MT2 are cysteine-rich small proteins that play roles in maintaining zinc homeostasis, detoxifying toxic metals, and scavenging free radicals *in vitro* and *in vivo* [29], and expression of MT1 and MT2 is increased in response to agents that cause oxidative stress and/or inflammation [30]. The protective role of MT1/2 in pulmonary response to ozone exposure is evidenced in recent studies [31;32], which have shown that MT1/2 null mice are more susceptible to ozone-induced inflammation and increased epithelial permeability in the lung. Similarly, *Ttpa*($-/-$) mice on a tocopherol-deficient diet has been shown to be more sensitive to hypoxia-induced hyperpermeability and lung damage [33]. In agreement with these studies, *Gclm*($-/-$) mice, which showed a greater induction of MT1/2 and TTPA transcripts following ozone exposure, exhibited a lesser degree of pulmonary epithelial hyperpermeability when compared with *Gclm*($+/+$) mice.

The observed enhanced upregulation of MT transcripts in ozone-exposed *Gclm*($-/-$) mice may in part be explained by their redox-sensitive regulation. Accordingly, we observed a lower GSH/GSSG ratio (1.8 ± 0.11 versus 0.84 ± 0.05) in lungs from ozone-exposed *Gclm*($-/-$) mice compared to ozone-exposed *Gclm*($+/+$) mice. It should be mentioned that a smaller GSH/GSSG ratio in non-exposed lungs from *Gclm*($-/-$) mice, when compared with that in *Gclm*($+/+$) mice, does not correlate with higher mRNA levels of MT1/2, which may suggest the complexity and stimulus-specificity of mechanism(s) underlying the redox regulation of MT.

In addition to its antioxidant properties, GSH is believed to act also as a regulator of inflammation via redox signaling [34]. Many inflammatory lung diseases, such as idiopathic pulmonary fibrosis, acute respiratory distress syndrome, cystic fibrosis and lung allograft, are associated with disrupted GSH homeostasis [35], although the causal relationship between GSH levels and disease status is unclear. Ozone exposure in mice increases pulmonary expression of a number of inflammatory genes, such as TNF, IL1B, IL6, CCL3, CXCL2 (a.k.a. MIP-2), and CCL11 [36]. In the present study, TNF mRNA was increased following ozone exposure, but no difference between *Gclm*($-/-$) and *Gclm*($+/+$) mice was observed. Ozone-induced increases in CCL11, on the other hand, seems to be impaired in *Gclm*($-/-$) mice. It is unclear how this observation relates to a previous study showing that increased GSH/GSSG ratios are associated with decreased levels of CCL11 in BAL fluid [37]. Finally, we found a

correlation between CCL3 mRNA and the percentage of neutrophils in BAL fluid in individual animals, suggesting a role of this cytokine in mediating ozone-induced neutrophil infiltration.

Although extensively studied, the consequences of inflammation in the response to ozone exposure are still in large part unknown. Neutrophils recruited to ELF and the lung interstitium are often believed to contribute to epithelial cell damage through the release of large amounts of reactive oxygen species. However, in our study there was no correlation at the individual level between lung permeability, as measured by the total protein concentration in BAL fluid, and the percentage of neutrophils. Our results agree with previous studies in mice that have suggested dissociation between neutrophil recruitment and lung injury during oxidative stress [38;39].

Conclusions

Our study provides new evidence that GSH in the ELF, not the pulmonary GSH *de novo* synthesis, plays an important role in the defense against inhaled ozone. Furthermore, we hypothesize that compensatory increases of some redox-sensitive genes confers added protection against oxidative damage in *Gclm*($-/-$) mice compared to *Gclm*($+/+$) mice. Further experimental investigations are required to provide direct evidence for a causal relationship between antioxidant genes and protection against ozone-induced lung hyperpermeability in *Gclm*($-/-$) mice.

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Figure 1.

Effects of ozone exposure on total protein concentration (A) and percentage of neutrophils (B) in BAL fluid. Ozone-exposed groups were exposed to air containing 0.3 ppm ozone continuously for 48 hrs. As a marker of lung permeability, total protein concentrations in cell-free BAL fluid were measured. Data are presented as means $\pm$ SEM. * $p < 0.05$, when compared with non-exposed mice for the same genotype. # $p < 0.05$, when compared with ozone-exposed *Gclm*(+/+) mice.

Figure 2.

Effects of ozone exposure on mRNA expressions of inflammation-related genes. mRNA levels were measured by qPCR. Relative mRNA levels of individual genes are expressed as the percentage of control levels in non-exposed *Gclm*(+/+) animals after normalization with β -2-microglobulin, and reported as means $\pm$ SEM. * $p < 0.05$, when compared with non-exposed mice for the same genotype. # $p < 0.05$, when compared with ozone-exposed *Gclm*(+/+) mice.

Figure 3.

Effects of ozone exposure on mRNA expressions of antioxidant genes. mRNA levels were measured by qPCR. Relative mRNA levels of individual genes are expressed as the percentage of control levels in non-exposed *Gclm*(+/+) animals after normalization with β -2-microglobulin, and reported as means $\pm$ SEM. * $p < 0.05$, when compared with non-exposed mice for the same genotype. # $p < 0.05$, when compared with ozone-exposed *Gclm*(+/+) mice.

Table 1

Levels of major antioxidants in lung and BAL fluid

	<i>Gclm</i> (+/+)		<i>Gclm</i> (-/-)	
	non-exposed	ozone-exposed	non-exposed	ozone-exposed
Lung (μmol/g tissue)				
GSH	0.45 ± 0.01	0.51 ± 0.03 [†]	0.13 ± 0.01 *	0.16 ± 0.01 *
GSSG	0.16 ± 0.01	0.29 ± 0.04 [†]	0.11 ± 0.01 *	0.19 ± 0.01 * [†]
GSH/GSSG	2.9 ± 0.29	1.8 ± 0.11 [†]	1.3 ± 0.15 *	0.84 ± 0.05 *
Ascorbate	0.52 ± 0.05	0.64 ± 0.02 [†]	0.70 ± 0.05 *	0.75 ± 0.03
BAL fluid (nmol/mg protein)				
GSH	96 ± 17	11 ± 0.9 [†]	74 ± 16	17 ± 2.5 [†]
Ascorbate	65 ± 8.6	12 ± 1.9 [†]	59 ± 5.4	10 ± 0.7 [†]
Uric acid	17 ± 2.4	6 ± 0.7 [†]	21 ± 4.2	9 ± 1.0 [†]
BAL fluid (μM)				
GSH	12 ± 2.0	12 ± 1.6	12 ± 1.8	10 ± 1.8
Ascorbate	8.7 ± 1.1	13 ± 1.1 [†]	9.6 ± 1.0	6.8 ± 1.1 *
Uric acid	2.2 ± 0.9	6.7 ± 0.8 [†]	3.4 ± 0.7	6.6 ± 0.7 [†]

Results were reported as means ± S.E. per treatment group.

* $p < 0.05$, when compared with *Gclm*(+/+) mice in the same treatment group.[†] $p < 0.05$, when compared with non-exposed mice for the same genotype.