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GSTM1 Modulation of IL-8 Expression in Human Bronchial Epithelial Cells Exposed to Ozone

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Abstract

Exposure to the major air pollutant ozone can aggravate asthma and other lung diseases. Our recent study in human volunteers has shown that the *glutathione S-transferase mu 1 (GSTM1)* null genotype is associated with increased airway neutrophilic inflammation induced by inhaled ozone. The aim of this study was to examine the effect of GSTM1 modulation on interleukin 8 (IL-8) production in ozone-exposed human bronchial epithelial cells (BEAS-2B) and the underlying mechanisms. Exposure of BEAS-2B cells to 0.4 ppm ozone for 4 h significantly increased IL-8 release with a modest reduction in intracellular reduced glutathione (GSH). Ozone exposure induced reactive oxygen species (ROS) production and NFκB activation. Pharmacological inhibition of NFκB activation or mutation of *IL-8* promoter at κB-binding site significantly blocked ozone-induced IL-8 production or *IL-8* transcriptional activity, respectively. Knockdown of *GSTM1* in BEAS-2B cells enhanced ozone-induced NFκB activation and IL-8 production. Consistently, ozone-induced overt increase in IL-8 production was detected in *GSTM1*-null primary human bronchial epithelial cells. In addition, supplementation with reduced GSH inhibited ozone-induced ROS production, NFκB activation and IL-8 production. Taken together, *GSTM1* deficiency enhances ozone-induced IL-8 production, which is mediated by generated ROS and subsequent NFκB activation in human bronchial epithelial cells.

Keywords

ozone; IL-8; GSTM1; human bronchial epithelial cells; ROS; NFκB

Introduction

Ozone is one of the most abundant components of air pollution in urban areas and is thought to act, in part, as an inducer of airway inflammation (1–4). This agent is generated at ground

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level by photochemical reactions involving nitrogen dioxide, oxygen, hydrocarbons, and ultraviolet (UV) radiation. Human activities are major sources of ozone precursors, although the latter are also generated by nonanthropogenic processes (5). Increased outdoor levels of ozone have been associated with an increased risk of admissions for asthma, chronic obstructive pulmonary disease and increased mortality in patients with asthma and the general population (6–10). Controlled inhalation studies of both asthmatic and non-asthmatic adults have revealed an acute decrease in lung function, enhanced allergen-induced bronchoconstriction and increases in airway inflammation typified by increased influx of neutrophils after ozone challenge (11–16). However, the mechanisms underlying ozone-induced airway inflammation have not been fully elucidated.

Airway inflammation is a central feature of many respiratory diseases. The specific characteristics of the inflammatory response in each disease and the site of inflammation differ but all involve the recruitment and activation of inflammatory cells and changes in the structural cells of the lung. These conditions are characterized by an increased expression of the inflammatory mediators including cytokines, chemokines, growth factors, enzymes, receptors and adhesion molecules (17). The airway epithelium represents the interface between the external environment and tissue of the airway wall. The production of pro-inflammatory mediators from airway epithelial cells exposed to environmental irritants plays a critical role in the pathogenesis of airway disorders, such as asthma (18). Among these pro-inflammatory mediators, interleukin 8 (IL-8) is a potent neutrophil activator and chemotaxin and often used as a biological marker of environmentally induced pulmonary inflammation (19–21). Ozone inhalation induces human airway epithelial damage and increased release of pro-inflammatory mediators including IL-8 in bronchoalveolar lavage fluids (BALF) (22, 23). *In vitro* exposure of human bronchial epithelial cells to ozone increases IL-8 production (24–26). In this study, we used IL-8 as the biomarker of ozone-induced airway inflammation and explored the regulatory events in ozone-induced IL-8 expression in human bronchial epithelial cells.

The toxicological effects of ozone are affected by many factors. In addition to the concentration and duration of exposure and co-existence of other air pollutants (4, 27–29), host susceptibility factors play a key role in determining the risk for ozone-induced airway inflammation. Ozone is a strong pro-oxidant and reacts with the epithelial lining fluid to generate free radicals (30). Genetic association studies of ozone exposure in humans have revealed an association between polymorphisms in oxidative stress genes such as *glutathione-S-transferase M1* (*GSTM1*), and exacerbation of asthma (31). Asthmatic children with *GSTM1* null and *GSTP1* valine/valine genotypes appear more susceptible to developing respiratory symptoms related to ozone exposure (32). Furthermore, a clinical trial showed that children with asthma with the low antioxidant *GSTM1* genotype had a fall in lung function with increasing ozone concentration, unless protected by antioxidant supplementation with vitamins C and E (33). We recently reported that *GSTM1* null volunteers had significantly increased airway neutrophils after ozone challenge compared to *GSTM1* sufficient volunteers, although nociceptive decreases in pulmonary function was similar between these groups (34). It should be noted that these *in vivo* studies only investigated the association of *GSTM1* genotype with pollutant-induced lung inflammation, and they cannot exclude contribution of other genetic factors in modulation of response to ozone. To our knowledge, no mechanistic studies have been conducted to examine the function of *GSTM1* protein in the pathogenesis of airway inflammation. Given the key role IL-8 plays in neutrophil chemotaxis, this study specifically examined the effect of deficient *GSTM1* protein expression on ozone-induced IL-8 expression in human bronchial epithelial cells.

Materials and methods

Reagents

Triton X-100, polyacrylamide and glutathione ethyl ester (GSH-ET) were purchased from Sigma Chemical Co. (St. Louis, MO). SDS-PAGE supplies such as molecular mass standards and buffers were from Bio-Rad (Richmond, CA). 5-(and-6)-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) was purchased from Invitrogen Corporation (Carlsbad, CA). Bay11-7082 was obtained from EMD Chemicals Inc. (Gibbstown, NJ). IkB α antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). β actin antibody was purchased from USBiological (Swampscott, MA). Lactate dehydrogenase (LDH) cytotoxicity detection kit was obtained from TAKARA Bio Inc. (Mountain View, CA). QuantiChrom™ GSH assay kit was obtained from BioAssay Systems (Hayward, CA). Rabbit anti-human GSTM1 antibody and GST activity assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI). IL-8 ELISA assay kit was purchased from eBioscience (San Diego, CA). Chemiluminescence reagents, expression arrest GIPZ lentiviral *GSTM1* shRNA particles and non-silencing control shRNA particles were obtained from Thermo Scientific (Huntsville, AL).

Cell culture and ozone exposure

BEAS-2B cell line: The BEAS-2B cell line was derived by transforming human bronchial epithelial cells with an adenovirus 12-simian virus 40 construct (35, 36). BEAS-2B cells (passages 70–80) were cultured in keratinocyte basal medium (KBM) supplemented with 30 μ g/ml bovine pituitary extract, 5 ng/ml human epidermal growth factor (EGF), 500 ng/ml hydrocortisone, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, and 5 ng/ml insulin (keratinocyte growth medium, KGM). 3×10^5 BEAS-2B cells were placed on collagen-coated filter supports (transwell insert) with a 0.4 μ m pore size (Costar, Cambridge, MA) and grown overnight. The culture medium on the apical side of the transwell was removed immediately before BEAS-2B cells were exposed to sterile air or 0.4 ppm ozone for 4 h in the exposure chambers operated by the US Environmental Protection Agency Environmental Public Health Division.

Normal human bronchial epithelial (NHBE) cells were obtained from normal adult human volunteers with the *GSTM1*-null or sufficient genotype by brush biopsy of the mainstem bronchus using a cytology brush during fiberoptic bronchoscopy, conducted under a protocol approved by the Committee on the Protection of the Rights of Human Subjects at the University of North Carolina at Chapel Hill. NHBE cells were initially plated in supplemented bronchial epithelial cell basal medium (0.5 ng/ml human EGF, 0.5 μ g/ml hydrocortisone, 5 μ g/ml insulin, 10 μ g/ml transferrin, 0.5 μ g/ml epinephrine, 6.5 ng/ml triiodothyronine, 50 μ g/ml gentamycin, 50 ng/ml amphotericin-B, 52 μ g/ml bovine pituitary extract and 0.1 ng/ml retinoic acid) on tissue culture flasks. Confluent cells were split and placed on the transwell before ozone exposure.

Measurement of intracellular reduced GSH

BEAS-2B cells were exposed to 0.4 ppm ozone for 4 h. The cells were scraped off the transwell and lysed by sonication in the cold buffer containing 50 mM MEP (pH=7) and 1 mM EDTA. The cell lysates were centrifuged at 10,000g for 15 min at 4°C. The supernatants were subjected to intracellular GSH measurement following the manufacturer's instruction.

Measurement of LDH levels in culture medium

BEAS-2B cells were exposed to 0.4 ppm ozone for 4 h. The cell culture medium from the basolateral chamber of air-liquid interface culture system was collected for the measurement of LDH concentration according to the manufacturer's instruction. Released LDH content was expressed as optical density (OD).

Enzyme linked immunosorbent assay (ELISA)

After exposure of BEAS-2B cells to 0.4 ppm ozone for 4 h, the culture medium from the basolateral compartment of the transwell unit was collected and centrifuged. Levels of IL-8 protein in the supernatants were measured with a human IL-8 ELISA kit following the manufacturer's instructions.

Immunoblotting

BEAS-2B cells exposed to sterile air or ozone were washed twice with ice-cold phosphate-buffered saline (PBS), and then lysed in RIPA buffer (1× PBS, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors: 20 µg/ml leupeptin, 20 µg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 200 µM sodium orthovanadate, and 20 mM sodium fluoride). The supernatants of cell lysates were subjected to SDS-PAGE. Proteins were transferred onto nitrocellulose membrane. Membrane was blocked with 5% nonfat milk, washed briefly, incubated with primary antibody at 4°C overnight, followed by incubating with corresponding HRP-conjugated secondary antibody for 1 h at room temperature. Immunoblot images were detected using chemiluminescence reagents and the Fujifilm LAS-3000 imaging system (Fuji Medical Systems, USA).

GSTM1 knockdown assay

5×10^4 BEAS-2B cells were placed in a 12 well plate and grown overnight. 10 moi (multiplicity of infection) of lentiviral non-silencing (scrambled) or *GSTM1* shRNA particles in 0.5 ml KBM were incubated with BEAS-2B cells for 6 h. Then 0.5 ml KGM was added into the infection medium and incubated for another 18 h. Confluent BEAS-2B cells were lysed and assayed for GSTM1 protein levels using immunoblotting.

Real-time reverse transcriptase/polymerase chain reaction (RT-PCR)

BEAS-2B cells expressing wild-type or mutated (κ B-binding site) *IL-8* promoter-conjugated *firefly luciferase (fLCF)* construct, and *enhanced green fluorescent protein (EGFP)* construct (37) were exposed to sterile air or 0.4 ppm ozone for 4 h, respectively. The cells were lysed with TRIZOL reagent (Invitrogen Corporation, Carlsbad, CA). Total RNA (100 ng), 0.5 mM NTP (Pharmacia, Piscataway, NJ), 5 µM random hexanucleotide primers (Pharmacia, NJ), 10 U/µl RNase inhibitor (Promega, CA), and 10 U/µl Moloney murine leukemia virus RT (GIBCO-BRL Life Technologies) were incubated in a 40°C water bath for 1 h in 50 µl of 1× PCR buffer to synthesize first-strand cDNAs. The reverse transcription was inactivated by heating at 92°C for 5 min. Oligonucleotide primer pairs and fluorescent probes for *fLCF* and *EGFP* were designed and obtained from Integrated DNA Technologies (Coralville, IA). Quantitative fluorogenic amplification of cDNA was performed using the ABI Prism 7500 Sequence Detection System (Perkin-Elmer, CA). The relative abundance of *fLCF* mRNA levels was calculated using the difference between the cycle threshold (CT) of the *fLCF* mRNA sequence and the reference *EGFP* mRNA sequence.

Measurement of intracellular reactive oxygen species (ROS)

The intracellular formation of ROS in BEAS-2B cells was detected using the fluorescent ROS probe carboxy- H_2DCFDA . Carboxy- H_2DCFDA is a cell-permeant indicator for ROS that is nonfluorescent until the acetate groups are removed by intracellular esterases and

oxidation occurs within the cell (38). The green fluorescence produced by BEAS-2B cells is proportional to the amount of ROS produced. Briefly, confluent BEAS-2B cells were pre-incubated with 20 μ M carboxy-H₂DCFDA at 37 °C for 1 h prior to exposure to 0.4 ppm ozone for 4 h. Cells were detached by 0.05% trypsin-EDTA, washed once with PBS, suspended in 0.5 ml PBS and put on ice before determination of green fluorescence intensity. Flow cytometry was performed with a FACSORT (Becton–Dickinson, Miami, FL, USA) by using an argon-ion laser (wavelength 488 nm). The FACSORT was calibrated with Calibrite beads before each use, and 6000 events were counted for all sample runs. Relative cell size and density/granularity were quantified by analyzing light-scatter properties using CellQuest software (Becton–Dickinson), namely forward scatter for cell size and side scatter for density/granularity, and recording the mean fluorescence intensities for each.

GSTM1 activity assay

NHBE cells from human volunteers with the *GSTM1*-null or -sufficient genotype were suspended into the sample buffer (100 mM potassium phosphates, pH6.5, containing 0.1% Triton X-100 and 1 mM glutathione). The cells were broken down with sonication and the supernatants subjected to immunoprecipitation. Specifically, 5 μ l of rabbit anti-human GSTM1 antibody was incubated with the supernatant at 4°C for 2 h prior to addition of 10 μ l Protein A-agarose and incubated at 4°C overnight. The immunoprecipitates were washed with cold PBS three times before determination of GST activity following the manufacturer's instructions.

Statistical analysis

Data were presented as means $\pm$ SE and evaluated using nonparametric paired t tests with the overall α level set at 0.05.

Results

Ozone exposure induces increased IL-8 production as well as cell damage to human bronchial epithelial cells

The overproduction of IL-8 is a biomarker of airway inflammation (39, 40). *In vivo* and *in vitro* studies have shown that ozone exposure elevates IL-8 protein levels in BALF or culture medium (22–26). Since ozone effect is concentration- and time-dependent, and cell type-specific (41), we examined IL-8 production in BEAS-2B cells exposed to 0.4 ppm ozone for 4 h referred to our recent *in vivo* study (16). Levels of IL-8 protein in the supernatant of culture medium were determined using ELISA. As shown in Figure 1A, ozone exposure significantly increased IL-8 protein release from BEAS-2B cells as compared to control (sterile air). As expected, the positive control IL-1 β (10 ng/ml) induced a pronounced increase in IL-8 production from BEAS-2B cells (Figure 1A).

Under the same exposure condition, the cytotoxicity of ozone exposure was assessed by assay of LDH activity in the culture medium. It was shown that ozone stimulation significantly elevated LDH levels in the culture medium of BEAS-2B cells compared to air (Figure 1B). This result indicated that exposure to 0.4 ppm ozone for 4 h could cause cell injury, which was consistent with previous observation (42).

Ozone exposure induces ROS production that can be inhibited by supplementation of reduced GSH

Ozone is a highly reactive gas associated with oxidative stress (43). ROS production has been shown in ozone-exposed guinea pig airway epithelial cells and human BALF cells (44, 45). To examine whether ozone challenge increased ROS production in BEAS-2B cells, the fluorogenic probe carboxy-H₂DCFDA was used. As demonstrated in Figure 2A, exposure of

BEAS-2B cells to 0.4 ppm ozone for 4 h resulted in increased ROS production in BEAS-2B cells. To determine whether ROS production could be inhibited by antioxidant, we next employed the reduced GSH (GSH-ET) to pretreat BEAS-2B cells prior to ozone exposure. As shown in Figure 2B, pre-incubation of BEAS-2B cells with 10 mM GSH-ET for 1 h modestly inhibited ozone-induced ROS production, indicating that reduced GSH is an antagonist of ROS induced by ozone stimulation.

Ozone-induced reduction of intracellular reduced GSH and its association with IL-8 production

The fact that supplementation of reduced GSH inhibited ozone-induced ROS production implied that ozone exposure might affect intracellular reduced GSH levels. To test this assumption, the intracellular reduced GSH levels were measured after ozone treatment (0.4 ppm ozone for 4 h). As shown in Figure 3A, ozone exposure induced a modest reduction in intracellular reduced GSH levels. To further examine the association of intracellular GSH with ozone-induced IL-8 expression, BEAS-2B cells were pre-incubated with 10 mM GSH-ET for 1 h before exposure to ozone. The medium containing GSH-ET on the apical side of the transwell was removed just before ozone exposure, but the GSH-ET remained in the basolateral culture medium. Levels of IL-8 protein in the culture supernatant of basolateral medium were measured as described previously. As expected, in the vehicle group ozone exposure increased IL-8 production (Figure 3B). In contrast, GSH-ET pretreatment markedly inhibited ozone-induced IL-8 release from BEAS-2B cells. These results suggested that reduction of intracellular reduced GSH could result in ozone-induced IL-8 production.

In addition, GSH-ET supplementation was also shown to inhibit ozone-induced cell toxicity (Data not shown).

GSTM1 knockdown enhances ozone-induced IL-8 production

GSTM1 has been detected in the brush border of proximal airways as well as in alveolar cells and macrophages (46). Our recent *in vivo* study has revealed that *GSTM1* null genotype is associated with increased neutrophilic airway inflammation after ozone exposure (34). Since IL-8 is a potent neutrophil chemokine we hypothesized that GSTM1 homeostasis is associated with the expression levels of IL-8 in ozone-treated human bronchial epithelial cells. To examine this hypothesis, we modulated intracellular GSTM1 levels in BEAS-2B cells with lentiviral *GSTM1* shRNA particles and then compared the difference in IL-8 production from BEAS-2B cells expressing adequate or deficient GSTM1 after ozone challenge. Infection of BEAS-2B cells with 10 moi of lentiviral *GSTM1* shRNA particles caused significant reduction of GSTM1 protein levels (by 82%) in BEAS-2B cells as compared to the cells infected with scrambled lentiviral shRNA particles (Figure 4A). GSTM1 adequate or deficient (knockdown) cells were further treated with air or 0.4 ppm ozone for 4 h and IL-8 levels in the culture supernatants measured. In the cells expressing scrambled shRNA ozone stimulation increased IL-8 production from BEAS-2B cells (Figure 4B). In the cells expressing *GSTM1* shRNA ozone-induced IL-8 production was further increased by GSTM1 knockdown (Figure 4B).

To further verify the observation in BEAS-2B cells, we next used normal human bronchial epithelial cells from volunteers with the *GSTM1*-null or -sufficient genotype to determine the association of *GSTM1* with IL-8 production after ozone exposure. GSTM1 activity was first examined in the NHBE cells with the *GSTM1*-null or sufficient genotype using immunoprecipitation. As expected, GSTM1 activity was not detected in *GSTM1*-null NHBE cells. In contrast, GSTM1 enzymatic activity was detected in *GSTM1*-sufficient NHBE cells (Figure 4C). Furthermore, Ozone exposure (0.4 ppm, 4 h) induced a significant increase in

IL-8 production from the cells with *GSTM1*-null genotype than that from the *GSTM1*-sufficient bronchial epithelial cells (Figure 4D). As shown in BEAS-2B cells, IL-1 β (10 ng/ml) significantly increased IL-8 production from normal human bronchial epithelial cells (data not shown).

NF κ B activation is required for ozone-induced IL-8 production

Activation of the transcription factor NF κ B is required for *IL-8* gene transcription in many cell types (47). To examine whether ozone stimulation activated NF κ B, degradation of the NF κ B inhibitory protein κ B α (I κ B α), an event indicative of the canonical NF κ B-activating pathway (48), was determined. BEAS-2B cells were exposed to 0.4 ppm ozone for 30 and 60 min. I κ B α levels in the cell lysates were measured using immunoblotting. As shown in Figure 5A, exposure of BEAS-2B cells to ozone for 30 min had minimal effect on I κ B α levels. However, at 60 min exposure to ozone, the I κ B α levels dropped significantly compared to those of control, indicating that ozone exposure could activate NF κ B at 60 min exposure. To further examine whether NF κ B was involved in ozone-induced IL-8 production, Bay11-7082, a specific and potent inhibitor of NF κ B activation that inhibits the phosphorylation and degradation of I κ B α and subsequent nuclear translocation of p65 and p50 (49), was used prior to ozone treatment. BEAS-2B cells were pre-incubated with 20 μ M Bay11-7082 for 60 min before further challenged with 0.4 ppm ozone for 4 h. As shown in Figure 5B, Bay11-7082 pretreatment abolished ozone-induced IL-8 production, implying that NF κ B was required for ozone-induced IL-8 production. To support this notion, BEAS-2B cells expressing wild-type or mutated (κ B-binding site) *IL-8* promoter-conjugated *fLCF*, and *EGFP* constructs were employed. Levels of *fLCF* mRNA are proportional to the transcriptional activities of *IL-8* promoter. As demonstrated in Figure 5C, ozone exposure increased *fLCF* mRNA levels in the cells encoding wild-type *IL-8* promoter (IL-8wt). However, this effect of ozone exposure was significantly reduced in the cells containing mutated (κ B-binding site) *IL-8* promoter (IL-8mNF κ B). Taken together, these results indicate that ozone exposure activates NF κ B, which is required for ozone-induced *IL-8* gene expression in human bronchial epithelial cells.

GSTM1 modulate ozone-induced NF κ B activation

As demonstrated previously, both *GSTM1* and NF κ B were involved in the regulation of ozone-induced IL-8 production from BEAS-2B cells. We next examined the possible association of *GSTM1* with NF κ B in ozone-induced IL-8 production. BEAS-2B cells were infected with lentiviral scrambled or *GSTM1* shRNA particles, respectively, as described previously. As shown in Figure 6A, in the cells expressing scrambled shRNA, ozone exposure induced marked I κ B α degradation. In contrast, in the cells encoding *GSTM1* shRNA the I κ B α levels went even lower than those in the cells containing scrambled shRNA after ozone exposure, which indicated that *GSTM1* knockdown enhanced ozone-induced NF κ B activation. Consistent with this observation, the antioxidant GSH-ET supplementation significantly inhibited ozone-induced I κ B α degradation (NF κ B activation) in BEAS-2B cells (Figure 6B).

Discussion

GSTM1 belongs to a family of dimeric phase II metabolic enzymes known to catalyze the conjugation of reduced GSH with electrophilic compounds such as products of oxidative stress (50). *GSTM1* has been mapped to the *GST mu* gene cluster on chromosome 1p13.3. Deletion variants or null alleles exist for the *GSTM1* gene present biochemically as a failure to express protein (51–55). The *GSTM* null genotype has been associated with increased risk for adverse health effects due to exposure to air pollutants (56, 57). Our recent study showed that the *GSTM1* null genotype was associated with increased airway inflammation after

ozone exposure (34). Consistent with this observation, we now report that silencing of *GSTM1* with shRNA for *GSTM1* in human bronchial epithelial cell line or *GSTM1*-null genotype of normal human bronchial epithelial cells is associated with ozone-induced overproduction of IL-8. It should be noted that the methodology used in this study is limited due to the usage of transformed epithelial cells incubated without the protection of respiratory tract lining fluids and in artificial culture media.

The results from this study indicate that *GSTM1* modulation of IL-8 production from ozone-treated BEAS-2B cells involves NF κ B and ROS. We found that silencing of *GSTM1* enhances ozone-induced NF κ B activation, which can be inhibited by supplementation with reduced GSH, the substrate of *GSTM1*, demonstrating that *GSTM1* modulates NF κ B activation. NF κ B is a family of highly regulated dimeric transcription factors that play pivotal roles in inflammatory responses and immunological reactions. Five members of the NF κ B family have been identified: NF κ B1 (p50/p105), NF κ B2 (p52/p100), RelA (p65), RelB, and c-Rel (58). In mammals, the NF κ B transcription factor consists of two subunits of either homo- or heterodimers of RelA/p65, c-Rel, and p50. The p50/RelA(p65) heterodimer is the major Rel/NF κ B complex in most cell types (59). NF κ B can be activated through two pathways, the classical (canonical) and alternative pathways. In the classic pathway, activated I κ B kinase β (IKK β) phosphorylates I κ B α , I κ B β , or I κ B ϵ , leading to their proteasomal degradation. As a result, NF κ B homodimers and heterodimers accumulate in the nucleus and regulate the expression of its target genes such as those coding for cytokines, adhesion molecules and chemokines that have a crucial role in both immune and inflammatory responses (60–62). The alternative NF κ B pathway is responsible for the activation of p100/RelB complexes and occurs during lymphoid development (63). The promoter region of human *IL-8* gene contains a number of potential binding sites for several inducible transcription factors including NF κ B, activator protein-1 (AP-1), and CAAT/enhancer-binding protein (C/EBP) (64–66). The expression of *IL-8* gene is regulated primarily at the level of transcription (66, 67). Unlike the AP-1 and C/EBP sites, the NF κ B site is essential for induction and required for maximal gene expression of *IL-8* gene (68). The data from this study shows that NF κ B is required for ozone-induced IL-8 production. First, ozone challenge causes I κ B α degradation (NF κ B activation). Second, pretreatment of BEAS-2B cells with the specific NF κ B activation inhibitor Bay11-7082 abolishes ozone-induced IL-8 production. Third, mutation of κ B-binding site on the *IL-8* promoter causes significant reduction of *IL-8* promoter transcription activity. Moreover, these results also suggest that ozone exposure induces NF κ B activation through the classic pathway since Bay11-7082, a specific and potent inhibitor of the classic NF κ B activation pathway (69), significantly blocks ozone-induced IL-8 production.

This study further indicates that *GSTM1* modulates ozone-induced NF κ B activation and subsequent IL-8 production in BEAS-2B cells through GSH-regulated ROS production although previous studies on the association of ROS generation with NF κ B activation have produced controversial results (70–75). As described previously, *GSTM1* exerts its antioxidant effect by conjugating reduced GSH with electrophilic ROS. Evidence in support of ROS-mediated NF κ B activation in ozone-exposed cells includes 1.) Observations that ozone exposure increases ROS production from BEAS-2B cells along with the reduction of intracellular reduced GST; 2.) Supplementation of antioxidant GSH blocks ozone-induced I κ B α degradation (NF κ B activation) and IL-8 production and; 3.) Knockdown of antioxidant *GSTM1* from BEAS-2B cells enhances NF κ B activation and IL-8 production. ROS include superoxide anion, hydroxyl radical, organic peroxide radicals, hydrogen peroxide, and singlet molecular oxygen (76). They are constantly generated intracellularly in eukaryotic cells as products of mitochondrial electron transfer chain reactions, and upon exposure to different environmental stimuli, such as UV light and inflammatory cytokines (77, 78). It has been speculated that increased ROS production by human bronchial epithelial cells may

result from increased activity of the microbicidal oxidase, increased mitochondrial activity, an increased oxidizing environment in the cytoplasm, or a decrease in cellular scavengers of ROS after ozone treatment (45, 79). As for the mechanisms of ROS-mediated NF κ B activation, previous studies have shown that Syk-mediated phosphorylation of I κ B α and p65 (80), and activation of IKK (81) are involved in hydrogen peroxide-induced NF κ B activation. In addition, c-Src has been shown to modulate ROS-induced NF κ B activation (82).

In summary, consistent with our recent *in vivo* study, this *in vitro* study using human bronchial epithelial cells confirms that GSTM1 is a risk factor for ozone-induced inflammatory responses. Specifically, knockdown of GSTM1 leads to enhanced IL-8 production from human bronchial epithelial cells exposed to ozone, which is verified in GSTM1-null normal human bronchial epithelial cells. Furthermore, this study demonstrates that GSTM1 modulation of IL-8 production involves ROS-regulated NF κ B activation. Since the GSTM1 null genotype occurs at high frequency in most ethnic populations (55), characterization of the GSTM1 contribution to air pollution-induced inflammatory lung diseases and underlying mechanisms provides fundamental information for the design of preventive and therapeutic measures against air pollutant-related pulmonary disorders.

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List of Abbreviations

GSTM1	glutathione S-transferase mu 1
IL-8	interleukin 8
ROS	reactive oxygen species
EGF	epidermal growth factor
BALF	bronchoalveolar lavage fluids
GSH-ET	glutathione ethyl ester
HRP	horseradish peroxidase
KBM	keratinocyte basal medium
LDH	lactate dehydrogenase
KGM	keratinocyte growth medium
ELISA	enzyme linked immunosorbent assay
PBS	phosphate-buffered saline
RT-PCR	real-time reverse transcriptase/polymerase chain reaction
EGFP	green fluorescent protein
moi	multiplicity of infection
flCF	firefly luciferase
IκBα	inhibitory protein κ B α

NFκB	nuclear factor κB
TNF	tumor necrosis factor
UV	ultraviolet

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Figure 1. Ozone exposure increases IL-8 and LDH release from BEAS-2B cells

Confluent BEAS-2B cells grown on 0.4 μ m filter supports were exposed to sterile air or 0.4 ppm ozone for 4 h, respectively. IL-8 (A) or LDH (B) levels in the culture medium of the basolateral compartment were measured. IL-1 β (10 ng/ml, 24 h) was used as the positive control. *, compared to air, $P<0.05$.

Figure 2. Ozone-induced ROS production is inhibited by GSH supplementation

A, BEAS-2B cells were pre-incubated with 20 μ M carboxy- H_2 DCFDA for 1 h, prior to stimulation with 0.4 ppm ozone for 4 h. ROS production was measured with flow cytometry. B, BEAS-2B cells were pre-incubated with 10 mM glutathione ethyl ester (GSH-ET) for 1 h, prior to stimulation with 0.4 ppm ozone for 4 h. ROS was measured as described previously. *, compared to air, $P < 0.05$.

Figure 3. Association of intracellular GSH levels with ozone-induced IL-8 production

A, BEAS-2B cells were exposed to 0.4 ppm ozone for 4 h. Intracellular reduced GSH levels were measured. B, BEAS-2B cells were pre-incubated with 10 mM glutathione ethyl ester (GSH-ET) for 1 h, prior to stimulation with 0.4 ppm ozone for 4 h. IL-8 protein levels were measured using ELISA. *, compared to air, $P < 0.05$.

Figure 4. GSTM1 modulation of IL-8 production from human bronchial epithelial cells exposed to ozone

A, BEAS-2B cells were infected with lentiviral scrambled or *GSTM1* shRNA particles (MOI=10) for 24 h, respectively. The cells were lysed and GSTM1 protein levels were measured using immunoblotting. B, BEAS-2B cells infected with lentiviral scrambled or *GSTM1* shRNA particles were challenged with 0.4 ppm ozone for 4 h. IL-8 protein levels were measured using ELISA. C, Normal human bronchial epithelial cells from volunteers with *GSTM1*-sufficient (GSTM1-WT) or *GSTM1*-null genotype (n=3 for each genotype) were assayed for GSTM1 enzymatic activity using immunoprecipitation. D, Normal human bronchial epithelial cells from human volunteers with *GSTM1*-sufficient (GSTM1-WT) or *GSTM1*-null genotype (n=5 for each genotype) were exposed to 0.4 ppm ozone for 4 h. IL-8 protein levels were measured as described previously. *, compared to air, $P<0.05$.

Figure 5. NFκB activation is required for ozone-induced IL-8 production

A, BEAS-2B cells were exposed to 0.4 ppm ozone for 30 min and 60 min. The cells were then lysed with RIPA buffer. The supernatants of the cell lysates were subjected to immunoblotting using IκBα antibody. B, BEAS-2B cells were pretreated with 20 μM Bay11-7082 for 1 h before further stimulated with 0.4 ppm ozone for 4 h. IL-8 protein levels were measured using ELISA. C, BEAS-2B cells expressing wild-type or κB-binding site mutated *IL-8* promoter-conjugated *fLCF*, and *EGFP* constructs were exposed to 0.4 ppm ozone for 4 h. Levels of *fLCF* mRNA were measured using RT-PCR and normalized with *EGFP* mRNA levels. *, compared to the cells expressing *IL-8*wt construct exposed to ozone, $P < 0.05$.

Figure 6. GSTM1 and GSH modulation of NFκB activation in ozone-treated BEAS-2B cells
 A, Infected BEAS-2B cells with lentiviral scrambled or *GSTM1* shRNA particles were challenged with 0.4 ppm ozone for 60 min. The cells were lysed with RIPA buffer and the supernatants of the cell lysates were subjected to immunoblotting using IkBα antibody. B, BEAS-2B cells were incubated with 10 mM GSH-ET for 60 min, prior to stimulation with 0.4 ppm ozone for 60 min. IkBα levels in the supernatants of the cell lysates were measured using immunoblotting.