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Original Contribution

# 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 NF-κB activation. Pharmacological inhibition of NF-κB activation or mutation of the *IL-8* promoter at the 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 NF-κB activation and IL-8 production. Consistently, an 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, NF-κB activation, and IL-8 production. Taken together, *GSTM1* deficiency enhances ozone-induced IL-8 production, which is mediated by generated ROS and subsequent NF-κB activation in human bronchial epithelial cells.



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 level by photochemical reactions involving nitrogen dioxide, oxygen, hydrocarbons, and ultraviolet (UV) radiation. Human activities are major sources of ozone precursors, although these are also generated by nonanthropogenic processes [5]. Increased outdoor levels of ozone have been associated with an increased risk of admissions for asthma and chronic obstructive pulmonary disease and increased mortality in patients with asthma and the general population [6–10]. Controlled inhalation studies of both asthmatic and nonasthmatic 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 the tissue of the airway wall. The production of proinflammatory 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 proinflammatory 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

*Abbreviations:* GSTM1, glutathione *S*-transferase Mu 1; IL-8, interleukin 8; ROS, reactive oxygen species; EGF, epidermal growth factor; BALF, bronchoalveolar lavage fluid; 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; m.o.i., multiplicity of infection; fLCF, firefly luciferase; IκBα, inhibitory protein κBα; NF-κB, nuclear factor κB; UV, ultraviolet.

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damage and increased release of proinflammatory mediators including IL-8 in bronchoalveolar lavage fluid (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 coexistence 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 Mu 1 (*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 supplementation with the antioxidant 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 were similar between these groups [34]. It should be noted that these in vivo studies investigated only the association of *GSTM1* genotype with pollutant-induced lung inflammation, and they cannot exclude the contribution of other genetic factors in the 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, USA). SDS–PAGE supplies such as molecular mass standards and buffers were from Bio-Rad (Richmond, CA, USA). 5-(and-6)-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-$H_2$DCFDA) was purchased from Invitrogen (Carlsbad, CA, USA). Bay11-7082 was obtained from EMD Chemicals (Gibbstown, NJ, USA). IκBα antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). β-Actin antibody was purchased from USBiological (Swampscott, MA, USA). Lactate dehydrogenase (LDH) cytotoxicity detection kit was obtained from TaKaRa Bio (Mountain View, CA, USA). QuantiChrom glutathione (GSH) assay kit was obtained from BioAssay Systems (Hayward, CA, USA). Rabbit anti-human GSTM1 antibody and GST activity assay kit were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). IL-8 ELISA kit was purchased from eBioscience (San Diego, CA, USA). Chemiluminescence reagents, expression-arrest GIPZ lentiviral *GSTM1* short-hairpin RNA (shRNA) particles, and nonsilencing control shRNA particles were obtained from Thermo Scientific (Huntsville, AL, USA).

### *Cell culture and ozone exposure*

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 μg/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). BEAS-2B cells ($3\times10^5$) were placed on collagen-coated filter supports (transwell insert) with a 0.4-μm pore size (Costar, Cambridge, MA, USA) 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 U.S. 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 main-stem bronchus using a cytology brush during fiber-optic 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 μg/ml hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 0.5 μg/ml epinephrine, 6.5 ng/ml tri-iodothyronine, 50 μg/ml gentamycin, 50 ng/ml amphotericin-B, 52 μg/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 MES (2-[N-Morpholino]ethanesulfonic acid) (pH 7) and 1 mM EDTA. The cell lysates were centrifuged at 10,000*g* 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 the 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.

### *Enzyme-linked immunosorbent assay*

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 the cell lysates were subjected to SDS–PAGE. Proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk, washed briefly, and incubated with primary antibody at 4 °C overnight, followed by incubation with the corresponding HRP-conjugated secondary antibody for 1 h at room temperature. Immunoblot images were detected using chemiluminescence