OZONE AND LUNG FIBROSIS

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MS in Public Health in the Department of Environmental Health and Toxicology

ABSTRACT

Ozone is an extremely reactive gas molecule composed of three oxygen atoms. The ground-level ozone is formed when nitrogen oxides and volatile organic compounds, released mainly from burning of gasoline or coal, come into contact with sunlight. Ozone is also generated and used in industrial settings such as purification of drinking water, waste treatment, deodorization of gases, bleaching of wood pulp. Therefore, many industrial workers are exposed to relatively high concentrations of ozone through their working environment. Although governmental organizations have set up the exposure limit no more than 0.1ppm for 8 hour work, the concentrations of ozone in the many work environments can reach much higher levels than the standards.

Ozone exposure is associated with many health effects in human, involving different organ systems; however, the major target of ozone toxicity is the respiratory system. Both clinical studies and epidemiological studies have shown that ozone exposure induces
various health problems in respiratory system. Studies show that industrial workers have a higher risk of developing adult-onset asthma and decreased pulmonary function as compared with general population. Although no pathological data are available regarding whether ozone exposure induces lung fibrosis in human, decreased forced vital capacity, is observed among ozone exposure workers, indicating that ozone exposure causes restrictive lung diseases.

In addition to clinical and epidemiology studies, many studies have been conducted using different animal models to address health effects of ozone exposure. Numerous studies have shown that ozone increases collagen and α-smooth muscle actin (α-SMA) and plasminogen activator inhibitor 1 (PAI-1) synthesis and deposition in the lungs indicating that ozone induces lung fibrosis. Although it has been well documented that ozone exposure induces lung fibrosis, the underlying mechanism remains unclear.

In this study we demonstrate that transforming growth factor-β (TGF-β) has a pivotal role in the pathogenesis of ozone induced lung fibrosis. Inhibition of TGF-β signaling attenuates the ozone induced lung fibrosis. These findings provide new insights into the molecular mechanism whereby ozone induces lung fibrosis, which will enable the design of more efficacious therapeutic agents for the treatment of ozone-related occupational diseases.

Keywords: fibrosis; transforming growth factor-β; collagen, smooth muscle actin; plasminogen activator inhibitor 1
Acknowledgements

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INTRODUCTION

Air pollution is an important public health concern throughout the world, especially in industrialized cities. Of the many toxic pollutants causing air pollution, ozone is of particular interest as it is a very potent respiratory irritant and is considered as one of the six criteria pollutants [1]. The six criteria pollutants are ground level ozone, particulate matter, carbon monoxide, sulfur dioxide, nitrogen oxides and lead. Of these pollutants, ground level ozone is most widespread health threat. It is a highly reactive gas composed of three oxygen atoms. The ground level ozone is called the tropospheric ozone which is formed when nitrogen oxides and volatile organic compounds, released mainly from the burning of gasoline or coal, come in contact with UV rays of sunlight [2]. Ozone is also generated purposely and is used widely in many industrial settings such as purification of drinking water, waste treatment, deodorization of air and sewage gases, bleaching of waxes, oils, wet paper, and textile, or used as a bactericide and therapeutic agent in clinics for some disease conditions.

Based on scientific findings, the Environmental Protection Agency (EPA) has set the 8 hour National Ambient Air Quality standard as 0.075 ppm of ozone to protect public health, including sensitive population such as children, elderly and asthmatics. The American Conference of Governmental Industrial Hygienists has set the standard for industrial workers as follows; up to the 8-hour Threshold Limit Value for ozone exposure is
0.05 ppm for heavy work and 0.1 ppm for light work. But the workers in many industries such as pulp-mill workers [3-6], outdoor workers such as mail delivery workers [7], copying machine operators [8], and steel mill welders [9] are exposed to relatively high concentrations of ozone through their working environment.

Exposure to ozone causes various health problems such as respiratory tract irritation, cough, and decrements in lung function including airway hyper-responsiveness [3, 5]. Elevated ambient ozone levels have been related to increased hospital visits with respiratory symptoms. In particular, individuals with preexisting respiratory diseases such as asthma or children and elderly are vulnerable to ozone [10]. It has been documented that elevated ozone concentrations have led to increased emergency room visits [11]. Compared to ambient ozone levels, occupational exposure to ozone in some settings could be very high. Workers in certain industrial settings could be working in high background ozone area more frequently than expected e.g. average amount of ozone generated during steel arc welding is 0.15 ppm. During arc welding, ozone is generated immediately and remains in high concentrations for more than 15 minutes. Welders have been reported to be exposed to ozone concentrations of 0.15 ppm on a regular basis [33]. These values are far higher than designated by EPA rules. Exposure to such high levels of ozone has been reported to cause pulmonary congestion and obstructive pulmonary disease [3-5, 12]. A 20 percent reduction in timed vital lung capacity was also reported in occupational exposure to average concentrations of ozone of 1.5 ppm. Another industry where there is evidence of pulmonary injury due to ozone exposure is pulp mills [3, 5]. The concentrations used for the bleaching of papers are in the digits of 9000 ppm [5]. These workers can be
exposed to accidental leakages of 0.9 ppm of ozone. Studies have shown that workers in pulp mills have a higher risk of developing adult-onset asthma, wheezing, and decreased pulmonary function as compared with general population [5]. Decreased forced vital capacity (FVC) found in workers exposed to ozone indicate that ozone exposure causes restrictive lung disease [13]. However, no pathological data are available regarding whether ozone exposure induces lung fibrosis in human.

In addition to clinical and epidemiology studies, many studies have been conducted using different animal models to address the health effects of ozone exposure. Studies have shown that ozone exposure increases the expression of various inflammatory cytokines and chemokines and induces hyperplasia and hypertrophy in airway and alveolar epithelial cells. Numerous studies have shown that ozone increases collagen synthesis and deposition in the lung parenchyma and in airway wall, indicating that ozone induces lung fibrosis [14]. Chronic exposure to high ambient levels of ozone (0.12-1.0 ppm) has also been shown to induce bronchiolarized metaplasia and interstitial fibrosis in rats [15-18]. Most importantly, it has been reported that ozone-induced lung fibrotic changes persisted several months after exposure ceased [19-23].

Nonetheless, although it has been well documented that ozone exposure induces lung fibrosis in animals, the underlying mechanism remains unclear. A shift in the balance of growth factors and cytokines that promote extracellular matrix (ECM) deposition and proteases that degrade matrix contributes to the progression of fibrotic disease. The myo-fibroblasts are the prominent cells producing extracellular matrix components such as
type 1 collagen and α−smooth muscle actin (α-SMA)[24]. Accumulation and contraction of these fibroblasts produce excess amounts of ECM. Plasminogen-activator inhibitor (PAI-1) is also a key prognostic marker for fibrotic disease. Plasmin, a broad-spectrum protease that is generated from plasminogen by the activity of urokinase plasminogen activator (uPA), is one of the proteases that degrades matrix and activates other proteases to clear fibrotic lesions. Since uPA activity is inhibited by PAI-1, the over expression of PAI-1 results in ECM accumulation [25].

Transforming growth factor-beta (TGF-β) has a pivotal role in the regulation of a variety of physiological processes. Deregulation of TGF-β has been implicated in the pathogenesis of various diseases including fibrosis, atherosclerosis, and cancer [26]. TGF-β promotes the production of ECM which is mainly represented by collagens and fibronectin and increased collagen production is a pathological hallmark of fibrosis. Rats exposed to 0.8 to 1.5 ppm ozone for 7 days show increased collagen synthesis and deposition in the lungs [44]. TGF-β also transforms fibroblasts to myofibroblasts which are recognized by their α-SMA production. Upregulation of TGF-β has been shown to amplify the production of PAI-1 [25]. However, whether TGF-β mediates ozone-induced lung fibrosis is not known.

TGF-β signals through a transmembrane receptor serine/threonine complex that comprises the type I and type II receptor kinases. Once activated, TGF-β binds to the constitutively active type II receptor, and the type I receptor kinase activin receptor-like kinase (ALK)5 is subsequently recruited into the complex and is activated by TGF-β type II re-
ceptor-mediated phosphorylation. Phosphorylation of serine/threonine residues in the ALK5 subsequently phosphorylates the major downstream signaling molecules Smad2 and 3 proteins. Phosphorylated Smad2 (pSmad2) and 3 form a complex with Smad4. This complex translocates into the nucleus and regulates the transcription of specific genes involved in cell growth, differentiation, development, and immune response [26, 27].

Attempts to block the effects of TGF-β have contributed to the development of molecules that inhibit TGF-β binding to its receptor [26]. The extensive knowledge regarding TGF-β-mediated ALK5-dependent signaling pathway has highlighted the therapeutic potential of TGF-β signaling antagonist. Recent studies have shown that several small molecule adenosine triphosphate (ATP)-competitive ALK5 inhibitors inhibit or retard progressive fibrosis in kidney, lung, and liver [26]. Recently a novel small molecule inhibitor of TβRI/ALK-5, designated IN-1130, has been reported to inhibit renal fibrosis. Therefore in the current study we used IN-1130 to inhibit TGF-β signaling in mice and to test whether TGF-β is involved in ozone induced lung fibrosis.

The primary aim of this study was to determine whether ozone-induced lung fibrosis is mediated by TGF-β. In the first series of experiments we determined whether ozone exposure increases TGF-β production and consequently causes lung fibrosis in a mouse model. In the second series of experiments, we tested whether inhibition of TGF-β signaling will attenuate ozone-induced lung fibrosis. Our results demonstrated for the first time that inhibition of TGF-β signaling attenuates ozone-induced lung fibrosis.
These studies provide new insights into the molecular mechanism whereby ozone induces lung fibrosis, which will enable the design of more efficacious therapeutic agents for the treatment of the ozone-related occupational diseases.
MATERIALS AND METHODS

**Ozone exposure and TGF-β inhibitor treatment:** Six to eight-week old C57BL male mice, a fibrosis prone strain, were exposed to a series of ozone exposure cycles consisting of 5 days of ozone exposure (0.5 ppm, 8 hours/day), followed by 2 days of filtered air exposure (recovery) for 5 or 10 cycles (see the diagram below) at UAB Inhalation Exposure Facility directed by Dr. Postlethwait (Professor and Chair of Environmental Health Sciences department and a consultant for the project). Filtered air (unexposed) controls were treated similar in all aspects except for ozone in the chambers, and done in parallel. Briefly, mice were housed within the confines of space specifically dedicated to the Facility until necropsy. During the exposure protocols (8 hrs/day; 5 day/wk) animals were left in the chambers during non-exposure periods to help assure infection control and minimize non-essential human contact. During the 2 day recovery periods, animals were housed in micro isolators in a dedicated room contiguous within the Facility. Animals were allowed free access to water during exposures but, food was withheld to prevent ingestion of constituents oxidized by ozone, which could introduce confounders. The room lighting was kept on a 12 on/off cycle. Chamber flow rate was approximately 30 volume changes/hr that prevented buildup of CO₂ and ammonia and assured that chamber oxidant concentrations are well mixed and maintained.
To determine whether TGF-β mediates ozone-induced lung fibrosis, IN-1130, a selective inhibitor of TGF-β type I receptor kinase (a gift from Dr. Dae-Kee Kim, Professor, College of Pharmacy, Ewha Woman’s University, Korea) was given to mice by intraperitoneal injection at dose of 20 mg/kg (dissolved in saline) once a day starting on the first day of ozone exposure and continuing to the end of the experiment. The control group mice were injected with the same amount of saline. Ten mice were used for each treatment group and each time point.

**Experimental Setup figure 1**

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<td>Air exposure (n=10)</td>
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<tr>
<td>6 week old male mice</td>
<td>Ozone exposure (n=10)</td>
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1 cycle = 5 days of ozone (0.5ppm for 8hrs a day) + 2 days of filtered air
Filtered air exposure throughout 7 days period.
There were 2 groups for 5 cycle’s exposure period. Total of 6 groups of mice were studied for 10 cycle’s exposure period. Detailed protocol is explained in the figure 2.

**Exposure groups Figure 2**

<table>
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<th>Exposure Groups</th>
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<tr>
<td>Air exposure + IN-1130 (TGF-β receptor 1 inhibitor)</td>
<td>Ozone exposure + IN-1130 (TGF-β receptor 1 inhibitor)</td>
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<tr>
<td>Air exposure + Saline</td>
<td>Ozone exposure + Saline</td>
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IN-1130 / Saline administration: IP injection once a day, start at the first day of ozone exposure and continue till the end of exposure

**Sample Collection:** The mice were anesthetized by intraperitoneal injection of Nembutal (50mg/kg) and placed in a supine position to collect the samples. All the samples were collected within two hours after removing mice from the exposure chambers using following procedures. After sample collection, mice were killed by exsanguinations.

**Bronchoalveolar lavage fluid (BALF):** After anesthetization, mice tracheas were cannulated with a 22G 1 ½-in bead-tipped needle and room temperature normal saline con-
containing 0.45% glucose (0.8 ml) was gently instilled by syringe until resistance was felt. After 1-2 min, the saline was withdrawn. This procedure was repeated three times and then lavage solution was collected. The resulting BALF was spun down at 400 g for 10 min to collect cells and supernatant. The supernatant solution was used for further experiments.

**Protein Concentration:** The protein concentration of BALF was determined using commercially available BCA protein assay (Pierce-Thermo scientific, Rockford, IL, USA). The assay was performed according to manufactures’ protocol and was analyzed using the Sigma plot software.

**Differential cell count:** The pelleted cells from BALF samples were uniformly suspended in BALF buffer, and the number and type of cells present in each lavage fluid were determined as follows. A well-mixed sample of BALF cells from each mouse was centrifuged onto a separate microscope slide, air dried, and stained with Protocol HEMA3 (Protocol Cat # 123-869) stain according to manufacture’s protocol. Total of 500 cells were counted on each slide using OIL immersion (100X) lens of Zeiss microscope and differential count for macrophages, neutrophils, lymphocytes and basophils was performed.

**Antioxidant Measurement in BALF:** In the study of the effect of ozone on the antioxidants in the lung lining fluid, 180-µl aliquots of BALF were each mixed with 26 µl of 80% m-phosphoric acid (final 10% m-phosphoric acid), centrifuged (13,000 rpm at 4°C
for 30 min), and the supernatant was collected and stored at -80°C for assay by HPLC. Lavage samples were normalized using the Urea Assay to determine the concentration of antioxidants in the lung lining fluid.

**HPLC method:** For HPLC analysis of low molecular weight antioxidants, we used a Shimadzu LC-10Ai HPLC with a Phenomenex Luna reversed phase column (5μ C18 (2) 250 x 4.6 mm) provided with a Phenomenex guard column (ODS, 4 mm L x 3.0 mm ID). The mobile phase consisted of an isocratic mixture of 50 mM phosphate buffer, pH 3.1, containing 50 μM octanesulfonic acid and methanol (95:5). Using an 8-channel ESA CoulArray Model 5600A electrochemical detector, we simultaneously detected ascorbate, glutathione, and uric acid and oxidized glutathione.

**Urea Assay:** The concentration of urea was measured in plasma samples as well as BALF samples. The commercially available kit for measurement of urea from Teko Diagnostics (cat # B550-400) was used. The assay was performed according to manufacturers’ protocol. Briefly, 1ml of the assay reagent was warmed to 37°C for 10 minutes. After 10 minutes 10 μl of BALF or plasma sample was added to it, and absorbance was read at 340 nm for 5 minutes with 30 seconds interval. The dilution of lung lining fluid was calculated by dividing plasma urea amount by BALF urea amount from the same mouse. Further calculations were performed according to manufacturer.
**ELISA analysis of active and latent TGF-β in BALF:** The amounts of TGF-β in BALF and was measured using an ELISA kit (cat # 84-7344-88) from e-Biosciences, according to the protocol provided by the manufacturer, which measures total amount of TGF-β. The total TGF-β was measured after activation of samples by treating the BALF samples with 1 N HCl for 15 minutes at room temperature and then neutralized with 1 N NaOH. The amount of TGF-β was calculated based on a standard curve, which was run each time with samples.

**Western analysis of protein content.** Western analysis was performed using the following protocol. The lung tissue was homogenized in 500 µl of 0.25 M sucrose solution containing 1mM EDTA, 20mM Tris-HCL (pH 7.4), 2.5 µl protease inhibitor cocktail (Sigma cat # P8340 ) and 5 µl phosphatase inhibitor cocktail (Sigma cat # P5726). The homogenates were centrifuged at 3,00g for 10 minutes at 4°C and the supernatant was centrifuged at 13,000 rpm for 20 minutes at 4°C. The protein concentration of the individual samples was measured using the BCA assay as described earlier. 40 µg of proteins were used for analysis. The proteins were resolved by 10% SDS-PAGE and transferred to a PVDF membrane, which was probed with the following antibodies: α-SMA (Biocare Medical LLC, Cat# CM001B) 1:1000, PAI-1 (Molecular Innovations, Cat# ASMPAIGF) 1:2000, Collagen 1A (Santa-Cruz Cat# SCBT 8788 ) 1:100 and β-actin (Sigma cat # A5441) 1:5000 at 4°C overnight and then probed with corresponding HRP-tagged secondary antibodies; anti-mouse (Jackson Immuno-research cat # 315-035-044) 1:5000, anti-rabbit (Sigma cat #A9161) 1:10,000 or anti-goat (Santa-Cruz cat # SCBT 2350 )1:5000 for 2 hours at room temperature. The protein bands were detected using ECL solution (Pierce
Densitometric analysis of the protein bands was performed using Bio-Rad Molecular imager, Chemi-Doc-XRS Imaging system with Quantity One software.

**Lung histopathology:** The pulmonary artery was cannulated and the vascular bed was perfused using 310 mOms PBS containing 4g% ficoll as the oncotic agent. The right main stem bronchus was cannulated; the lung was resected and inflated (constant 25 cm H₂O) with 4% paraformaldehyde. Tissues were immediately fixed in 10% buffered formalin for 24 hours and paraffin embedded. Sections were cut 4-6 μM thickness and were further used for trichrome staining for collagen staining and immunohistochemistry for α-SMA.

**Collagen staining:** Masson’s Trichrome staining method was performed to detect the deposition of collagen in the lung tissue. The sections were deparaffinized and hydrated through gradient alcohol. Sections were fixed in Bouin's mordant fluid and stained sequentially in Weigert's hematoxylin, Biebrich Scarlet-Acid Fuchsin, and Aniline Blue, followed by 1% acetic acid treatment, dehydration, and permanent mounting. The collagen deposition areas were stained blue while cytoplasm, muscles and erythrocytes were red and nuclei were stained black.

**Immunohistochemistry:** Sections were deparaffinized and hydrated through percentage graded alcohol series. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution in methanol. After rinsing in PBS, sections was blocked with 10% normal horse serum in PBS and then probed with primary antibody (α-SMA 1:200 dilu-
tion) at 4°C overnight. After the incubation, sections were washed and incubated with biotin conjugated secondary antibody (Vector Biolabs cat # BA2000) for 1 hour at room temperature. After rinsing the slides were covered with HRP-substrate (Vector Biolabs cat # A2704) for 1 hour at room temperature which was followed by rinsing with PBS and staining with DAB chromophoric solution (Scytek Labs cat #2010-01/02) for 5 minutes at room temperature. Slides were rinsed off with distilled water and stained for Harris’s hematoxylin (Sigma cat # HHS- 16) for 15 seconds. After washing off hematoxylin with distilled water, slides were dehydrated through a series of percentage graded alcohol and xylene. Sections were mounted with paramount and examined under microscope.

**Statistical analysis:** All the data were analyzed using Sigma plot 9.0 software. Data are expressed as mean ± SEM. Intergroup comparisons were assessed by student’s t-test or one way ANOVA. A $P$ value of less than 0.05 was considered as significant.
RESULTS

1. Ozone exposure leads to increased protein level and inflammatory cell numbers in the BALF

Elevated protein concentration in BALF is an indication of altered airway permeability due to lung injury [28]. Therefore, in the first series of experiments we assessed whether sustained exposure to ozone leads to increased protein concentration in the BALF. Compared to control mice that received filtered air, ozone treated mice had higher protein content in BALF after both 5 and 10 exposure cycles but the increase was not significant (Fig. 3). The result suggests that ozone exposure may have damaged normal tight junctions of airway epithelium resulting in movement of proteins across the epithelium.
Groups of mice (n=10) were exposed to filtered air or ozone (0.5 ppm) over a period of 5 or 10 cycles. 1 cycle consists of 5 days of 0.5 ppm of ozone exposure, 8 h per day, followed by 2 days of exposure to filtered air. Control mice were exposed to filtered air throughout the period of the experiment. After 5 and 10 cycles, BALF from individual mice was harvested and the protein concentration was determined by BCA. Results are expressed as the mean ± SEM.

**Fig. 3: Protein concentration in BALF of mice exposed to air or ozone.** Groups of mice (n=10) were exposed to filtered air or ozone (0.5 ppm) over a period of 5 or 10 cycles. 1 cycle consists of 5 days of 0.5 ppm of ozone exposure, 8 h per day, followed by 2 days of exposure to filtered air. Control mice were exposed to filtered air throughout the period of the experiment. After 5 and 10 cycles, BALF from individual mice was harvested and the protein concentration was determined by BCA. Results are expressed as the mean ± SEM.
In addition to an increased protein concentration in the BALF, altered airway permeability is also associated with inflammation of the lung tissue [28]. Therefore we next examined whether exposure to ozone leads to lung inflammation by determining the number of inflammatory cells in the BALF of mice exposed to ozone. BALF cells from individual mice were centrifuged on slides and differentially stained using hematoxylin-eosin for counting. Consistent with the protein results, mice exposed to ozone had a significantly higher ($P < 0.05$) number of lymphocytes in the BALF compared to air exposed control mice (Table 1 and Fig. 4). However, the differences in percentage of primary immune cells such as neutrophils and alveolar macrophages were not significant. Taken together these results provide evidence that exposure to ozone leads to lung injury in mice as the protein content and inflammatory cells were increased in the BALF of mice exposed to ozone.
Table. 1: Differential count of inflammatory cells in BALF.

Groups of mice (n=10) were exposed to filtered air or ozone (0.5 ppm) over a period of 5 or 10 cycles as described in Fig. 1. After 5 and 10 cycles, BALF cells from individual mice were harvested, spun on slides and differential cell count performed. *, Significantly different from air exposed controls ($P < 0.05$, n=4). Results are expressed as the mean ± SEM of counts from 15 different microscopic fields.

<table>
<thead>
<tr>
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<th>5 cycles</th>
<th>10 cycles</th>
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<tr>
<td></td>
<td>Air</td>
<td>Ozone</td>
</tr>
<tr>
<td>%Lymphocytes</td>
<td>4.0 ± 0.235 (n=4)</td>
<td>7.94 ± 0.3536 * (n=4)</td>
</tr>
<tr>
<td>% Macrophages</td>
<td>94.79 ± 0.279 (n=4)</td>
<td>90.64 ± 0.328 (n=4)</td>
</tr>
<tr>
<td>% Neutrophils</td>
<td>1.24 ± 0.04 (n=4)</td>
<td>1.44 ± 0.117 (n=4)</td>
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</table>
Fig. 4: Lymphocytes percentage in BALF of mice exposed to air or ozone.

Groups of mice (n=10) were exposed to filtered air or ozone (0.5 ppm) over a period of 5 or 10 cycles as described in Fig. 1. After 5 and 10 cycles, BALF from individual mice was harvested, spun on slides and differential cell count performed. *, Significantly different from air exposed controls ($P < 0.05$, n=4). Results are expressed as the mean ± SEM of counts from 15 different microscopic fields.
2) Ozone exposure leads to oxidative stress in the lung

Ozone is a powerful oxidant and is believed to cause damage to biological tissues either by direct reaction and/or through the formation of free radicals and reactive oxygen intermediates. Production of anti-oxidants such as ascorbic acid (AH2), glutathione (GSH), oxidized form of glutathione (GSSG) and uric acid (UA) is a natural response against oxidative stress and inflammation and can promote the repair of injured cells and tissues, enabling the lung to withstand further injury [29, 30]. Therefore in the next series of experiments we assessed the effects of ozone exposure on the levels of AH2, GSH, GSSG and UA in the BALF using HPLC. The results show that mice exposed to ozone for 10 cycles had a significant increase in the levels of AH2 ($P < 0.01$) and GSSG ($P < 0.05$) compared to air exposed control mice (Table. 2).

Additionally, there was a notable increase in the level of GSH upon exposure to ozone but not statistically significant. Importantly, the amount of GSSG was significantly increased in ozone-exposed mice suggesting an oxidative stress in lungs. A modest increase in the levels of AH2, GSH and GSSG was also observed after 5 cycles of ozone exposure, although difference was not significant (Table. 2). UA concentrations in the lung-lining fluid were similar between ozone and control mice after both 5 and 10 cycles. These results suggest that exposure to ozone causes oxidative damage to the lung, which induces antioxidant defense system.
Table. 2: Ozone exposure leads to oxidative stress in the lung. Groups of mice (n=10) were exposed to filtered air or ozone (0.5 ppm) over a period of 5 or 10 cycles as described in Fig. 1. After 5 and 10 cycles, BALF from individual mice was harvested and assessed for various antioxidants by HPLC. *, Significantly different from air exposed controls ($P < 0.01, n=10$). Results are expressed as the mean ± SEM.

<table>
<thead>
<tr>
<th>Antioxidants (µmoles)</th>
<th>5 Cycles</th>
<th>10 cycles</th>
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<tbody>
<tr>
<td></td>
<td>Air</td>
<td>Ozone</td>
</tr>
<tr>
<td>AH2</td>
<td>230.8±20.47</td>
<td>289.40± 43.6(n=10)</td>
</tr>
<tr>
<td></td>
<td>(n=10)</td>
<td>(n=10)</td>
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<tr>
<td>GSH</td>
<td>91.08 ± 9.03</td>
<td>122.48 ± 20.02(n=10)</td>
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<tr>
<td></td>
<td>(n=10)</td>
<td>(n=10)</td>
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<tr>
<td>GSSG</td>
<td>2.13 ± 0.19</td>
<td>2.54 ± .34</td>
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<td>(n=10)</td>
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<td>UA</td>
<td>38.33± 3.82</td>
<td>37.66 ± 6.2</td>
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<td></td>
<td>(n=10)</td>
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3) Ozone exposure leads to an increased TGF-β production in the lung

TGF-β is a multifunctional cytokine playing a critical role in the development of fibrosis [31]. Upregulation of TGF-β has been documented in several inflammatory disorders and in the case of lung diseases including pulmonary fibrosis [32]. To understand the molecular mechanism underlying ozone-induced lung injury, we assessed whether TGF-β is increased in ozone-exposed mice. Total amount of TGF-β, including active and latent form, was assessed in the BALF harvested from air exposed control mice and ozone exposed mice after 5 and 10 cycles. Indeed, after 5 cycles, mice that were exposed to ozone had significantly higher level of TGF-β ($P < 0.05$) than air exposed control mice. Similarly, after 10 cycles, TGF-β was higher in mice exposed to ozone than air exposed control mice but the difference was not significant.
Fig. 5. Level of TGF-β in BALF of mice after air or ozone exposure. Groups of mice (n=10) were exposed to filtered air or ozone (0.5 ppm) over a period of 5 or 10 cycles as described in Fig. 1. After 5 and 10 cycles, BALF from individual mice was harvested and assessed for total amount of TGF-β by ELISA. *, Significantly different from air exposed controls ($P < 0.05$, n=10). Results are expressed as the mean ± SEM.
4) Ozone exposure induces fibrotic responses in the lungs of mice

Over expression of TGF-β promotes the excessive production of ECM by activation of fibroblasts to myofibroblasts [31] and by increasing collagen production [14]. Therefore, in the next series of experiments we wanted to determine if exposure to ozone leads to an increased production and/or deposition of ECM. As type 1 collagen is a major type of collagen expressed in fibrotic tissue, type 1 collagen levels in the lung tissue of mice exposed to ozone or air were evaluated by western blot analysis. Densitometric analysis of the western blots showed only a slight increase in amount of type 1 collagen in the lungs of ozone exposed mice as compared to air exposed controls for both 5 and 10 cycle’s (Fig. 6.A and 6.B). Similar to the western blot data, collagen staining data show that after 5 exposure cycles, there was a slight but not significant increase in the deposition of type I collagen in the lungs of mice exposed to ozone compared to air-exposed control mice (data not shown). However, after 10 exposure cycles, mice that were exposed to ozone had a clearly increased deposition of type I collagen in the lung tissue compared to air exposed control mice (Fig. 7). These results indicate that there might be a gradual production of excessive ECM upon exposure to ozone and this starts becoming more evident after 10 exposure cycles.
Fig. 6. **A) Level of collagen 1 in lung tissue.** Groups of mice (n=10) were exposed to filtered air or ozone (0.5 ppm) over a period of 5 or 10 cycles as described in Fig. 1. At the end of the exposure period, lung tissues were harvested and collagen deposition was assessed by western blot analysis. β-actin was used as a loading control. **B) Quantitation of the mean band density ± SEM (n=10).** Results were normalized to β-actin.
Fig. 7 **Collagen deposition in lung tissue.** Groups of mice (n=4) were exposed to filtered air or ozone (0.5 ppm) over a period of 5 or 10 cycles as described in Fig. 1. At the end of the exposure period, the right lobe of mice was fixed in paraformaldehyde and embedded in paraffin. Fixed and embedded tissue was then sectioned and assessed for collagen deposition (blue) using trichrome staining. (Original magnification X 20. Inside box original magnification X40)
In addition to type 1 collagen, excessive production of α-SMA and PAI-1 in the lung tissue are also well known markers of fibrosis [26]. Therefore, to further investigate if ozone induces fibrotic responses, we monitored the expression of α-SMA and PAI-1 in the lungs of mice exposed to ozone and controls that were exposed to air. Western blot analysis demonstrated an increase in the level of α-SMA upon ozone exposure compared to air exposed control mice (Fig. 8.A). As seen with collagen, increase in α-SMA levels were also most evident after 10 exposure cycles. These results were further corroborated using immunohistochemical analysis of α-SMA expression in the lungs of mice upon 10 ozone exposure cycles (Fig. 8.B). Additionally, PAI-1 expression was also upregulated after exposure to ozone after 10 exposure cycles (Fig. 9). Interestingly, unlike collagen and α-SMA, increase in PAI-1 expression was even evident after 5 exposure cycles to ozone. Taken together these results confirm that ozone induces fibrotic responses in mice.
Fig. 8: Amount of α-SMA levels in the lung tissue. A) Groups of mice (n=10) were exposed to filtered air or ozone (0.5 ppm) over a period of 5 or 10 cycles as described in Fig. 1. At the end of the exposure period, lung tissues were harvested and α-SMA deposition was assessed by western blot analysis. β-actin was used as a loading control. B) Immunohistochemical staining for α-SMA. At the end of the exposure period, the right lobe of mice was fixed in paraformaldehyde and embedded in paraffin. Fixed and embedded tissue was then sectioned and assessed for α-SMA deposition. (Original magnification X 40)
**Fig. 9: Amount of PAI-1 levels in mouse lung tissue.** Groups of mice (n=10) were exposed to filtered air or ozone (0.5 ppm) over a period of 5 or 10 cycles as described in Fig. 1. At the end of the exposure period, lung tissues were harvested and PAI-1 amounts were assessed by western blot analysis. β-actin was used as a loading control.
5) Inhibition of TGF-β signaling attenuates ozone induced lung fibrosis

Numerous studies have consistently indicated the role of TGF-β as a potent fibrogenic cytokine causing fibrosis in various organs. Therefore, in these sets of experiments we determined whether ozone-induced lung fibrosis is mediated by TGF-β and whether inhibition of TGF-β signaling will attenuate ozone-induced lung fibrosis. IN-1130, a selective inhibitor of TGF-β type I receptor kinase, was given to mice by intraperitoneal injection at dose of 20 mg/kg in saline once a day starting day one of ozone exposure and continuing till the end of 10 cycles of ozone exposure. The control group mice were injected with the same amount of saline.

5A) IN-1130 does not affect the production of TGF-β

IN-1130, a selective inhibitor of TGF-β type I receptor kinase which inhibits the binding of activated TGF-β to its receptor and thus affects the downstream signaling of TGF-β. Therefore in these sets of experiments we determined whether ozone induced TGF-β production is affected by administration of IN-1130. Total amount of TGF-β, including active and latent form, was assessed in the BALF harvested from air exposed control mice, ozone exposed mice and ozone exposed but IN-1130 administered mice after 10 cycles. As seen earlier, mice that were exposed to ozone had higher level of TGF-β than air exposed control mice. TGF-β levels did not differ in ozone exposed mice and the mice which were given IN-1130 along with ozone exposure. (Fig.10) No significant change
was observed in ozone exposed and saline administered ozone exposed controls. (Data not shown)
Fig.10: Production of TGF-β in presence of IN-1130. Groups of mice (n=10) were exposed to ozone (0.5 ppm) with and without the administration of IN-1130. Mice exposed to air served as controls. At the end of the exposure period, BALF from individual mice was harvested and assessed for total amount of TGF-β by ELISA. Results are expressed as the mean ± SEM.
5B) **IN-1130 inhibits the signaling of TGF-β**

IN-1130, a selective inhibitor of TGF-β type I receptor kinase which inhibits the binding of activated TGF-β to its receptor and thus affects the downstream signaling of TGF-β. PAI-1 protein is regulated by TGF-β signaling. Therefore in these sets of experiments we assessed whether inhibition of TGF-β signaling will attenuate PAI-1 expression levels. PAI-1 protein levels were assessed in BALF as well as in the lung tissue of mice exposed to air, ozone and ozone exposed but IN-1130 administered mice. Significant increase in PAI-1 level was observed in the BALF of ozone exposed mice as compared to air exposed controls. Significantly lower levels of PAI-1 protein were found in the BALF of mice exposed to ozone but IN-1130 administered as compared to ozone exposed mice. (Fig.11 A) Similar trend of PAI-1 expression level was observed in western blot analysis of the lung tissue. (Fig.11 B)
**Fig. 11** A) Production of PAI-1 protein upon inhibition of TGF-β. Groups of mice (n=10) were exposed to ozone (0.5 ppm) with and without the administration of IN-1130. Mice exposed to air served as controls. At the end of the exposure period, BALF from individual mice was harvested and assessed for amount of PAI-1 protein by ELISA. Results are expressed as the mean ± SEM. B) PAI-1 protein levels in the lung tissue homogenates assessed by western blot analysis. β-actin was used as a loading control.
5 C) Inhibition of TGF-β signaling attenuates ozone induced fibrosis.

Among the key marker of fibrosis is the up-regulation of α-SMA in the lung tissue. Therefore using western blotting, the expression of α-SMA was monitored in the lung homogenates of mice that were exposed to ozone with or without the administration of IN-1130. Mice exposed to air served as controls. Ozone exposure caused a prominent increase in the expression of α-SMA compared to mice that received only air (Fig.12). Importantly, administration of IN-1130 reduced the levels α-SMA in ozone exposed mice. Moreover, levels of α-SMA in control groups receiving air with or without IN-1130 were not different from one another (Data not shown). Taken together, results indicate that TGF-β plays a prominent role in ozone-induced lung fibrosis and that inhibition of TGF-β signaling using IN-1130 effectively attenuates lung fibrosis.
Fig. 12 A): Amount of α-SMA in the lung tissue upon inhibition of TGF-β. Groups of mice (n=6) were exposed to ozone (0.5 ppm) with or without the administration of IN-1130. Mice exposed to air served as controls. At the end of the exposure period, lung tissues were harvested and α-SMA deposition was assessed by western blot analysis. B) Immunohistochemical staining for α-SMA. At the end of the exposure period, the right lobe of mice was fixed in paraformaldehyde and embedded in paraffin. Fixed and embedded tissue was then sectioned and assessed for α-SMA deposition. (Original magnification X 40)
DISCUSSION

In the present study, we investigated whether TGF-β is involved in mediating ozone-induced lung fibrosis in mice. Our results demonstrate that exposure to ozone causes increased TGF-β production and fibrosis in the lungs of mice. Importantly, we show that inhibition of TGF-β signaling using IN1130 attenuates ozone-induced lung fibrosis. This is the first report showing an important role of TGF-β in ozone-induced lung fibrosis. Our results uncover a novel mechanism underlying ozone-induced lung fibrosis and offers TGF-β as a promising therapeutic target for preventing ozone-mediated lung diseases.

Ozone exposure causes airway inflammation, leading to lung injury [28]. One of the characteristic features of lung injury is the accumulation of protein and inflammatory cells in BALF due to perturbations in epithelial tight cell junctions allowing serum proteins and albumin in air spaces [44]. Indeed, our results also show an increased protein content in the BALF of ozone exposed mice as compared to air exposed control mice suggesting that chronic exposure to ozone causes lung injury.

As the lung injury may result from persistent inflammation, we examined the presence of
inflammatory cells in the BALF of ozone-exposed mice. Our studies show that repeated exposure to ozone leads to a significantly increased lymphocyte count in the lungs of mice as compared to air exposed control mice. This result is consistent with manifestations of various fibrotic diseases in which lungs have excessive pulmonary accumulation of lymphocytes [33, 34]. Inflammatory cells are very few in numbers in normal lungs but this population expands in association with inflammation and fibrosis [35, 36]. Last et al. and Neuhaus et al. have reported that chronic ozone exposure elicits Th2 response in BALB/c mice [37, 38]. The Th2 lymphocytes produce cytokines such as IL-4 and IL-13 which induce airway epithelial cells to produce excess of amounts of TGF-β and also modulate the behavior of fibroblasts [39].

Even though ozone leads to an increase in lymphocyte recruitment to the lungs, we did not observe any significant differences in neutrophil recruitment upon chronic ozone exposure. One of reason for this observation is that neutrophils are the first type of cells to populate the site of injury and infection and are typically found only at the initial stages of inflammation. Long N et al. have reported that single time exposure to ozone elicits neutrophilic influx in lung tissue [40]. Therefore, neutrophils seem to be recruited to the lungs upon exposure to ozone but do not persist over a long period of time.

In the next series of experiments we found that chronic exposure to ozone caused increased antioxidant levels in BALF as compared to air exposed control mice. Ozone is a
A highly oxidizing agent which reacts with the antioxidants present in BALF and depletes their concentration during acute exposure [41]. However, ozone may induce adaptation-mechanism in which body produces excess amounts of antioxidants in response to sustained insults of oxidizing agents. Ballinger et al. have reported that ozone reacts with BALF antioxidants in the following order; AH2 > UA > GSH > proteins > unsaturated lipids [42]. In our studies we particularly found significantly higher amounts of AH2 in ozone exposed mice which indicate that AH2 might have an important protective role against inflammatory oxidative stress produced by ozone. Our results are in line with earlier reports showing that ozone injury leads to increased production of AH2 in ozone exposed rats [30]. Mudway et al. have also shown AH2 is the first antioxidant reacting with oxidizing agents like ozone in the lung lining fluid [41]. A significant increase in the levels of oxidized glutathione (GSSH) levels in 10 cycles ozone exposed mice suggests an increased oxidative stress even though ozone increases antioxidant production.

Numerous studies have consistently indicated the role of TGF-β as a potent fibrogenic cytokine evoking fibrosis in various organs including liver, kidney and lungs. It has been reported that ozone exposure induces the expression of TGF-β in human blood cells and in the co-cultured bronchiole-fibroblasts [43-45]. But whether TGF-β is increased in the lungs upon ozone exposure is not known. Our results show for the first time an upregulation of TGF-β upon exposure to ozone suggesting that it may have an important role in causing ozone-mediated fibrosis in the lung. We found that levels of total form of TGF-β were significantly higher in the BALF of ozone exposed mice as opposed to air exposed controls. The active form of TGF-β was increased in the ozone exposed mice as com-
pared to air exposed control mice, however the difference was not significant indicating the probability that active form may be cell surface bound or might be internalized by fibroblasts or other cells.

Long term exposure to ozone has shown to cause lung fibrosis in animal models through increased ECM production by fibroblasts [44]. TGF-β promotes the production of ECM by activating fibroblast to myofibroblasts transition [31]. Myofibroblasts are shown to be predominant source of collagen, α-SMA and PAI-1 [25], which collectively produce fibrotic lesions in the organ. Our results show that ozone strongly induced the production of collagen, α-SMA and PAI-1 in the lungs of mice. However, there was a difference in the expression pattern of collagen and α-SMA. Even though TGF-β was increased after 5 exposure cycles, there was not a significant increase in the levels of collagen and α-SMA. This could be explained by the fact that inflammatory responses elicited by ozone may act to limit the progression of fibrosis by clearing collagen producing cells [46]. However, the healing mechanisms might not be able to sustain injury caused by long term exposure to ozone leading to fibrotic responses. Indeed, expression of collagen and α-SMA was prominently increased after 10 ozone exposure cycles. An increase in collagen and α-SMA levels was not evident by western blot analysis. However, there was a prominent collagen deposition in the lungs after 10 ozone exposure. Thus it appears that even though ozone exposure leads to TGF-β production after 5 cycles the development of fibrotic responses is gradual and most evident at 10 exposure cycles. Further studies looking into longer time points will give additional insight into the increased production of fibrotic responses upon exposure to ozone.
Unlike collagen and α-SMA, expression of PAI-1 was evidently increased after both 5 and 10 exposure cycles, indicating its production may be differently regulated. TGF-β is known to induce PAI-1 expression [25]. However, whether production of PAI-1 is increased by ozone, directly or indirectly via other mechanisms is not known.

To demonstrate that TGF-β mediates ozone-induced lung fibrosis, it was important to show attenuation of lung fibrosis upon inhibition of TGF-β and ozone exposure. Therefore in the final series of experiments, we inhibited downstream TGF-β signaling using IN-1130. As stated earlier, ozone exposure leads to a drastic increase in α-SMA and PAI-1 in the lungs of mice. Importantly, we further showed that inhibition of TGF-β signaling prevented α-SMA and PAI-1 increase in the lungs of mice upon exposure to ozone. These results are in line with earlier reports where inhibition of TGF-β in rat unilateral ureteral obstruction (UUO) model was shown to prevent renal fibrosis. However, Moon JA et al. also reported that IN-1130 did not confer complete protection from renal fibrosis in UUO kidneys at a later stage of fibrosis. Our studies show that IN-1130 was able to limit fibrotic responses up to 10 exposure cycles, but further experiments need to be done to assess whether inhibition of TGF-β could prevent chronic fibrosis upon longer ozone exposures.

Our results indicate that inhibition of TGF-β signaling before injury caused by ozone exposure could be an important therapeutic target. However, it should be pointed out that the clinical use of TGF-β inhibitors is complicated due to a unique property of TGF-β, ie.
it is a tumor suppressor for early stage cancer and it promotes tumor progression at late stages of malignancy [26].

Due to the lack of efficacious treatment, lung fibrosis imposes a major health concern on the workers exposed to high levels of ozone. The overall results of this project indicate that ozone exposure leads to pulmonary fibrosis and this is mediated by TGF-β, offering new ways to treat ozone-induced lung diseases.
REFERENCES


APPENDIX:
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: June 17, 2009
TO: Liu, Rui-Ming
RPHB 317 0022
934-7028

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Glutathione and Lung Fibrosis
Sponsor: NIH
Animal Project Number: 090568499

On June 17, 2009, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
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<th>Species</th>
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<th>Number in Category</th>
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<tr>
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<td>B</td>
<td>360</td>
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Animal use is scheduled for review one year from June 2009. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 090568499 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.