Published in final edited form as:

Inhal Toxicol. 2011 July ; 23(8): 486–494. doi:10.3109/08958378.2011.584919.

Increased transforming growth factor beta 1 expression mediates ozone-induced airway fibrosis in mice

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Abstract

Ozone (O₃), a commonly encountered environmental pollutant, has been shown to induce pulmonary fibrosis in different animal models; the underlying mechanism, however, remains elusive. To investigate the molecular mechanism underlying O₃-induced pulmonary fibrosis, 6- to 8-week-old C57BL/6 male mice were exposed to a cyclic O₃ exposure protocol consisting of 2 days of filtered air and 5 days of O₃ exposure (0.5 ppm, 8 h/day) for 5 and 10 cycles with or without intraperitoneal injection of IN-1233, a specific inhibitor of the type 1 receptor of transforming growth factor beta (TGF-β), the most potent profibrogenic cytokine. The results showed that O₃ exposure for 5 or 10 cycles increased the TGF-β protein level in the epithelial lining fluid (ELF), associated with an increase in the expression of plasminogen activator inhibitor 1 (PAI-1), a TGF-β-responsive gene that plays a critical role in the development of fibrosis under various pathological conditions. Cyclic O₃ exposure also increased the deposition of collagens and alpha smooth muscle actin (α-SMA) in airway walls. However, these fibrotic changes were not overt until after 10 cycles of O₃ exposure. Importantly, blockage of the TGF-β signaling pathway with IN-1233 suppressed O₃-induced Smad2/3 phosphorylation, PAI-1 expression, as well as collagens and α-SMA deposition in the lung. Our data demonstrate for the first time that O₃ exposure increases TGF-β expression and activates TGF-β signaling pathways, which mediates O₃-induced lung fibrotic responses *in vivo*.

Keywords

Ozone; TGF-β; PAI-1; airway fibrosis

Introduction

Ozone (O₃) is an extremely reactive gas formed in the troposphere when nitrogen oxides and volatile organic compounds, released mainly from fossil fuel combustion, react in the presence of sunlight. O₃ is one of the most abundant ambient urban pollutants with over half of the population in the United States (56%) living in areas with unhealthful levels of ozone

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Declaration of interest

The work was supported by grants from National Institute of Environmental Health Sciences R01 ES011831 (RML) and P01 ES011617 (EMP, MF, and CB), National Heart, Lung, and Blood Institute R01 HL088141 (RML) and R01 HL54696 (EMP), and the American Lung Association (RML).

(American Lung Association State of the Air 2010). In addition, intentionally generated O₃ 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. Therefore, many workers in different industries including pulp mill workers, outdoor construction workers, copy machine operators, and steel mill welders are exposed to relatively high concentrations of O₃ through their work environment.

Although O₃ exposure causes a myriad of health effects involving different organ systems, the lung is considered to be the major target. Both clinical and epidemiological studies have shown that O₃ induces airway obstruction, noninfectious rhinitis, adult-onset asthma, and decreased lung function. Animal studies have also shown that O₃ exposure increases the expression of various inflammatory cytokines and chemokines, causes hyperplasia and hypertrophy in airway and alveolar epithelial cells, and disrupts normal lung development. Collagens are the major components of extracellular matrix (ECM) and increased collagen production/ deposition is a pathological hallmark of fibrosis. Several studies have shown that O₃ increases collagen synthesis and deposition in the lung, indicating that O₃ induces lung fibrosis (Last et al., 1994; Parks and Roby, 1994; Chang et al., 1995; Stockstill et al., 1995). Importantly, it has been reported that O₃-induced lung fibrotic changes persist several months after exposure ceased (Last et al., 1981; Pickrell et al., 1987; Reiser et al., 1987; Sun et al., 1988), suggesting a long-term health impact of O₃ exposure. Nonetheless, although it has been well-documented that O₃ induces lung fibrosis, the underlying mechanism is unclear. It should also be pointed out that in most of these studies, animals (rats in most cases) were exposed to ozone continuously for many months (Last et al., 1984; Shima and Adachi, 1991; Dormans et al., 1999; van Bree et al., 2001) or 8 h a day for almost their entire life (20–24 months) (Last et al., 1984, 1994; Chang et al., 1995; Stockstill et al., 1995). Such exposure regimens do not resemble environmental or occupational exposure scenarios. Whether exposure to ozone under conditions that mimic environmental/ occupational exposure situations can also induce lung fibrosis and the molecular mechanism underlying O₃-induced lung fibrosis, however, remain to be determined.

Transforming growth factor beta (TGF- β) is the most potent and ubiquitous profibrogenic cytokine. TGF- β mRNA and protein expression are increased in various lung fibrotic diseases (Bergeron et al., 2003; Fahy et al., 2003; Tzortzaki et al., 2007) and in lung fibrosis models induced by various agents (Williams et al., 1993; Coker et al., 1997; Rube et al., 2000; Churg et al., 2009). Overexpression of TGF- β in transgenic mice or administration of TGF- β to the lung by adenovirus-mediated gene transfer techniques lead to lung fibrosis (Sime et al., 1997; Kolb et al., 2002; Lee et al., 2004; Vicencio et al., 2004). On the other hand, treatment of animals with TGF- β -binding proteins or anti-TGF- β antibodies ameliorates lung fibrosis induced by different stimuli (Yamaguchi et al., 1990; Bonniaud et al., 2005; Higashiyama et al., 2007). All these lines of evidence indicate that TGF- β plays a vital role in the development of lung fibrosis. Nonetheless, although studies have shown that O₃ exposure induces the expression of TGF- β in human blood cells and in fibroblasts co-cultured with bronchoepithelial cells (Bocci et al., 1994; Lang et al., 1998; Valacchi and Bocci, 1999), whether O₃ increases TGF- β production in the lung *in vivo* and whether TGF- β mediates O₃-induced lung fibrosis, however, are unknown.

In this study, we showed that cyclic O₃ exposure increased the expression of TGF- β 1, which was followed by deposition of collagens and alpha smooth muscle actin (α -SMA) in airway walls and in the lung. Furthermore, we showed that inhibition of TGF- β signaling with a specific inhibitor to TGF- β type 1 receptor suppressed O₃-induced Smad phosphorylation, PAI-1 expression, and airway fibrosis. The results demonstrate for the first time that O₃ induces TGF- β expression *in vivo* and that increased TGF- β -expression mediates O₃-induced airway fibrosis.

Materials and methods

Animals and O₃ exposure regimen

Six- to eight-week-old C57BL/6 male mice (Charles River, Wilmington, MA) were exposed to a series of O₃ exposure cycles consisting of 2 days of filtered air (FA) and 5 days of O₃ exposure (0.5 ppm, 8 h/day) for 5 or 10 cycles at the UAB Inhalation Exposure Facility. Animals were exposed to O₃ or FA in 0.8-m³ stainless steel chambers that employed 30 vol changes/h turnover rates. O₃ was generated from medical grade O₂ using a silent arc electrode and bled into the chamber inflow (~22°C; 50% relative humidity) using mass flow controllers. Chamber supply air (O₃ and FA chambers) was conditioned via passage through sequential coarse filter, activated charcoal, and HEPA filter units. O₃ concentrations in the Chamber were continuously monitored using a ThermoEnvironmental model 49 Photometric O₃ Analyzer. FA (unexposed) controls were treated similarly in all aspects except for O₃ in the chambers, and done in parallel. Animals were allowed free access to water, whereas food was withheld during exposure to prevent ingestion of constituents oxidized by O₃, which could introduce confounders. The animals were sacrificed after cessation of O₃ exposure at the end of the fifth and 10th cycles. All procedures involving animals were approved by the Institutional Animal Care and Use Committees at the University of Alabama at Birmingham.

To determine whether TGF- β mediates O₃-induced lung fibrosis, 6–8-week-old C57BL/6 male mice were divided into four groups: group 1 mice were exposed to FA and intraperitoneally (i.p.) injected with 100 μ L of sterile saline; group 2 mice were exposed to O₃ and i.p. injected with 100 μ L of sterile saline; group 3 mice were exposed to FA and i.p. injected with IN-1233, a selective inhibitor of TGF- β type I receptor (Kim et al., 2010), at a dose of 20 mg/kg (dissolved in 100 μ L of sterile saline); group 4 mice were exposed to ozone and i.p. injected with the same amount of IN-1233. Exposures were conducted as described above for 10 cycles of air or O₃. IN-1233 administration (i.p. injection) was conducted every morning before O₃ exposure initiated, started on the first day of O₃ exposure and continued to the end of the 10th cycle (discontinued during 2 days of FA exposure period in each cycle).

Bronchoalveolar lavage

Mice were anesthetized and tracheas cannulated with a 22G 1 1/2-in bead-tipped needle. Bronchoalveolar lavage (BAL) was performed with 0.8 mL saline and the resulting BAL fluid (BALF) was centrifuged at 500 *g* for 10 min. The supernatants were collected for ELISA analyses of TGF- β 1 and PAI-1.

Lung tissue collection and collagen staining

After lavage, the pulmonary artery was cannulated and the vascular bed was perfused using 310 mOsm PBS. The left main stem bronchus was cannulated; the lung was resected and inflated under constant pressure of 25 cm H₂O with 4% paraformaldehyde. Sections were cut 4–6 μ m thickness and used for collagen and immunohistochemical staining. Masson's trichrome staining technique was employed to reveal the collagen deposition. In brief, the sections were deparaffinized and hydrated through gradient alcohol, subsequently fixed in Bouin's mordant fluid, and the sections sequentially stained using Weigert's hematoxylin, Biebrich Scarlet-Acid Fuchsin, and Aniline Blue solutions. Quantification of collagen in the airway was done using computer-assisted stereology technique as described before (Fanucchi et al., 1997). The collagen mass (V/S) as measured by volume (μ m³) of bronchiolar epithelial cells per unit area (μ m²) of basement membrane was estimated from point and intercept counts with the cycloid grid by the equation = $(P_0 \times I/p)/(2 \times I_{bl})$, where

P_0 represents the points counted for collagen, I_{bl} represents the number of intercepts of the basal lamina, and $l/p = (\text{No. of curves on grid} \times \text{length of curve})/\text{points on grid}$.

Measurement of urea concentration in the BALF and plasma

The concentrations of urea in the plasma and BALF samples were measured using a commercially available kit from Teko Diagnostics (cat # B550-400) according to manufactures' protocol. In brief, 1 mL of the assay reagent was warmed to 37°C for 10 min. After 10 min, 10 μ L of BALF or plasma sample was added to it, and absorbance was read at 340 nm for 5 min with 30 s interval. The dilution factor of epithelial lining fluid (ELF) from each mouse was calculated by dividing plasma urea concentration with BALF urea concentration from the same mouse.

ELISA analyses of TGF- β and PAI-1 proteins in the ELF

The amount of TGF- β protein in the BALF was measured using an ELISA kit from e-Biosciences (San Diego, CA) according to the protocol provided by the manufacturer. Total TGF- β was measured after activation of latent TGF- β with HCl. PAI-1 antigen in the BALF was measured using a commercially available ELISA kit from Molecular Innovations (Southfield, MI), which detects active and inactive PAI-1 as well as PAI-1-PA complexes, as we have described previously (Vayalil et al., 2005). The amounts of TGF- β and PAI-1 proteins in ELF were calculated based on standard curves generated with each batch of samples and normalized with dilution factor of ELF as discussed above.

Western analyses of proteins

Lung tissues were homogenized in 500 μ L of 0.25 M sucrose solution containing 1 mM EDTA, 20 mM Tris-HCl (pH 7.4), and both protease and phosphatase inhibitors. The homogenates were centrifuged at 3000 g for 10 min at 4°C and the resulting supernatants centrifuged again at 13,000 g for 20 min at 4°C. Protein concentrations were measured using the commercially available BCA protein assay kit (Pierce-Thermo Scientific, Rockford, IL). Forty micrograms of protein were resolved on 10% SDS-PAGE gels. The separated proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes and blocked with 5% nonfat dry milk. The membranes were probed at 4°C overnight with the following antibodies: α -SMA Biocare Medical LLC (Concord, CA) at 1:1000 dilution, PAI-1 Molecular Innovations (Novi, MI) at 1:2000 dilution, pSmad2/3 Santa Cruz (Paso Robles, CA) at 1:200 dilution, Smad2/3 Cell Signaling (Danvers, MA) at 1:200 dilution, and β -actin Sigma (Saint Louis, Missouri) at 1: 5000 dilution, and then probed with corresponding HRP-tagged secondary antibodies. Protein bands were detected using ECL solution Pierce (Atlanta, GA). Densitometric analyses of the protein bands were performed using a Bio-Rad Chemi-Doc-XRS Imaging system and semiquantified with Quantity One software.

Immunohistochemical staining of α -SMA

Sections were deparaffinized and hydrated through graded alcohol series. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide solution in methanol. After rinsing in PBS, sections were blocked with 10% normal horse serum and probed with primary antibody (α -SMA 1:200 dilution) at 4°C overnight. Sections were subsequently washed and incubated with biotin-conjugated secondary antibody (Vector, Burlingame, CA) for 1 h at room temperature. After rinsing, the sections were incubated in avidin-biotin enzyme reagent (Vector) for 1 h at room temperature, washed, and incubated with DAB chromophoric solution (Scytek Labs, Logan, UT) for 5 min at room temperature, then counterstained with Harris's hematoxylin (Sigma). α -SMA immunostaining was

semiquantified using Axiovision and AV4 Mod Automeasure software Carl Zeiss MicroImaging, Inc (Thornwood, NY) and the results were expressed as percentage of area.

Statistical analysis

Data are presented as mean $\pm$ SE and evaluated by Student's *t*-test or two-way ANOVA followed by Fisher LSD test. *P* < 0.05 was considered significant.

Results

Ozone exposure increased TGF- β expression in the lung, which was followed by an increase in collagen deposition in airway walls.

To determine whether TGF- β was involved in O₃- induced lung fibrosis, we first assessed the amount of TGF- β protein in the ELF harvested from air and O₃- exposed mice by ELISA. The results showed that mice exposed to O₃ for five cycles had significantly higher levels of TGF- β 1 protein in the ELF than mice exposed to air (Figure 1A) and that such an increase persisted even after 10 cycles of O₃ exposure. Interestingly, trichrome staining showed that although O₃ exposure for 10 cycles increased collagen deposition in the airways (Figure 1B) no significant change in collagen accumulation in the lung was observed in mice exposed to O₃ for five cycles (data not shown). These results showed for the first time that O₃ exposure induced a sustained elevation of TGF- β expression in the lung *in vivo*. The results also suggest that increased TGF- β expression may mediate O₃-induced lung fibrosis.

Effect of ozone exposure on lung PAI-1 protein levels

Plasminogen activator inhibitor 1 (PAI-1), a physiological inhibitor of urokinase type and tissue type plasminogen activator (uPA and tPA), plays an important role in the regulation (suppression) of ECM turnover and is induced by TGF- β (Liu, 2008). Therefore, we examined the impact of cyclic O₃ exposure on lung PAI-1 protein levels by western analysis. The results showed that PAI-1 protein level was significantly increased in mouse lung after five cycles of O₃ exposure and remained elevated after 10 cycles of O₃ exposure (Figure 2). The results further suggest that O₃ exposure activates TGF- β signaling pathways and that increased PAI-1 expression may contribute to O₃-induced lung fibrosis.

Effect of ozone exposure on α -SMA deposition in the lung

One important mechanism whereby TGF- β promotes ECM production is by activation of fibroblasts, forming myofibroblasts, the major producers of ECM. To reveal fibroblast activation, expression of α -SMA, a marker of myofibroblasts, was assessed by immunohistochemical staining and western analysis. Both immunohistochemical staining and western data showed that α -SMA deposition in the lung was significantly increased in mice exposed to O₃ for 10 cycles (Figure 3A and 3B). However, no significant change in α -SMA expression was detected after five cycles of O₃ exposure (Fig 3B). The data further suggest that the increase in TGF- β expression mediates O₃-induced lung fibrosis at least in part by activating fibroblasts.

Effect of TGF- β type I receptor inhibitor on O₃-induced lung fibrosis

TGF- β signaling occurs through a heteromeric complex of types I and II receptors (T β RI and T β RII, respectively). IN-1233 is a specific inhibitor of T β RI/ALK5, which has been reported to block TGF- β signaling in different animal models (Long et al., 2009; Kim et al., 2010). To further investigate whether TGF- β mediates O₃-induced lung fibrosis, 6–8-week-old mice were exposed to O₃ for 10 cycles as described above and, at the same time, treated with IN-1233 through intraperitoneal injection. The results showed that O₃ exposure increased Smad2/3 phosphorylation in the lung (Figure 4). Administration of IN-1233, on

the other hand, blocked O₃-induced Smad2/3 phosphorylation although IN-1233 alone had no significant effect on the baseline levels of Smad2/3 phosphorylation. Most importantly, the results showed that IN-1233 administration blocked O₃-induced collagen accumulation in the airways (Figure 5), PAI-1 expression in ELF (Figure 6), and α -SMA accumulation in the lung (Figure 7). IN-1233 alone had no significant effect on collagen accumulation, PAI-1, or α -SMA expression (Figures 5, 6, and 7), nor did it block O₃-induced TGF- β expression (data not shown). However, IN-1233 inhibited the expression of collagen, PA-1, and α -SMA in O₃-exposed group more than in FA-exposed mice. The mechanism underlying such enhancement of O₃ on IN-1233 effect is unclear at moment. Nonetheless, the results further demonstrate that induction of TGF- β expression and activation of TGF- β signaling pathways mediate O₃-induced lung fibrotic responses.

Discussion

It has been reported that continuous or cyclic (8 h/day, 5 days/week) exposure to O₃, an extremely reactive gas and one of the most abundant ambient urban pollutants, for an extended period of time (up to 24 months) induces lung fibrosis in different animal models. Whether exposure to O₃ under the conditions that mimic environmental/ occupational settings also induces lung fibrosis and what is the molecular mechanism whereby O₃ induces lung fibrosis, however, are unclear. The results presented in this study showed for the first time that a cyclic O₃ exposure protocol that resembles certain environmental/ occupational exposure situations induced the expression of TGF- β 1, the most potent profibrogenic cytokine, which was followed by lung (airway) fibrosis. Most importantly, we showed that blocking the TGF- β signaling pathway with a specific inhibitor to TGF- β receptor 1 attenuated O₃-induced fibrotic responses and lung fibrosis in mice. Together, these data suggest that TGF- β 1 plays a critical role in O₃-induced lung fibrosis. This study is of significance as it not only reveals the potential adverse health effects of O₃ exposure under physiologically relevant conditions but also sheds new light on the mechanism whereby O₃ induces lung fibrosis.

TGF- β is a potent and ubiquitous profibrogenic cytokine and plays a critical role in the development of fibrosis under various pathological conditions. Although *in vitro* studies using cultured cells have shown that O₃ increases TGF- β production (Bocci et al., 1994; Lang et al., 1998; Valacchi and Bocci, 1999), whether this is the case *in vivo* is unknown. In the present study, we showed for the first time that TGF- β protein levels were significantly increased in the ELF of mice exposed to O₃ and that such induction persisted for a long time (even after 10 cycles of O₃ exposure, Figure 1). The molecular mechanism underlying the induction of TGF- β 1 by O₃ is unknown at the moment. It has been reported that reactive oxygen species (ROS) stimulate the expression and secretion of TGF- β in different types of cells (Leonarduzzi et al., 1997; Bellocq et al., 1999; Saito et al., 2005; Shvedova et al., 2008; Sullivan et al., 2008; Zhao et al., 2008). Leonarduzzi et al. (1997) reported that treatment of human and murine macrophages with 4-hydroxy-2,3-nonenal (HNE), a major aldehyde end product of membrane lipid oxidation, induced both mRNA and protein expression of TGF- β 1. It has also been reported that in human alveolar epithelial cells, ROS generated by xanthine/xanthine oxidase system induced TGF- β expression through a transcription mechanism, whereas reactive nitrogen species (RNS) increased TGF- β expression by a posttranscription mechanism (Bellocq et al., 1999). Whether O₃ increased TGF- β 1 protein level in mouse lung as shown in Figure 1 through a transcription or posttranscription mechanism is unknown. Lang et al. (1998) reported that O₃ increased the expression of TGF- β 1 mRNA in human fibroblasts that were co-cultured with human airway epithelial cells, suggesting that O₃ increases TGF- β protein level at least in part by inducing its mRNA expression.

The steady-state levels of ECM represent a net balance between its production/deposition and degradation. ECM degradation is mediated mainly by the matrix metalloproteinases (MMPs) and the PAs/plasmin system (Laurent, 1987; Davidson, 1990; Arthur, 1994; Liu, 2008). Plasmin is converted from the zymogen plasminogen by tissue type and urokinase type plasminogen activators (tPA and uPA), whereas the activities of tPA and uPA, under physiological conditions, are controlled by PAI-1. Therefore, PAI-1 plays an important role in controlling ECM degradation. PAI-1 is of particular interest because its expression is elevated in variety of fibrotic disorders and in experimental fibrosis models (Idell et al., 1989; Bertozzi et al., 1990; Günther et al., 2000a, b). It has also been reported that knockout of the PAI-1 gene reduces whereas overexpression of PAI-1 enhances the sensitivity of animals to lung fibrosis induced by various stimuli (Eitzman et al., 1996; Hattori et al., 2000; Chuang-Tsai et al., 2003), further suggesting a pivotal role of PAI-1 in the development of lung fibrosis. In previous studies, we have shown that TGF- β 1 induced PAI-1 expression in fibroblast, which contributed importantly to TGF- β 1-mediated collagen accumulation by inhibiting collagen degradation (Vayalil et al., 2005, 2007; Liu et al., 2010). In this study, we showed for the first time that PAI-1 expression was increased in the lung after five cycles of O₃ exposure, which was followed by increases in collagen accumulation in airway walls after 10 cycles of O₃ exposure, suggesting that increased PAI-1 expression may contribute to O₃-induced lung fibrosis. The molecular mechanism underlying the induction of PAI-1 in O₃-exposed mice is unclear. As TGF- β is a potent inducer of PAI-1, it is hypothesized that O₃ exposure increases PAI-1 expression through induction of TGF- β . This hypothesis is supported by the results from the TGF- β inhibitor study, in which we showed that inhibition of TGF- β signaling with TGF- β type I receptor inhibitor suppressed O₃-induced PAI-1 expression. Together, these data suggest that induction of PAI-1, probably due to increased TGF- β expression/activity, may contribute to the development of lung fibrosis in O₃-exposed animals through inhibition of ECM degradation as we have shown previously in fibroblasts.

The Smad pathway mediates the induction of many TGF- β -responsive genes including PAI-1. TGF- β signaling through the Smad pathway has been well-described in the past. Following the binding of TGF- β to the type II receptor (T β R-II), the type I receptor (T β R-I, also called ALK5) is phosphorylated, which further phosphorylates Smad2 or Smad3. Phosphorylated Smad2 and Smad3 form a heteromeric complex with Smad4, which translocates to the nucleus and binds to the promoters of TGF- β -targeted genes. In this study, we showed that ozone increases TGF- β protein level, which was followed by increases in Smad2/3 phosphorylation and PAI-1 expression. IN-1233, on the other hand, blocked ozone-induced Smad2/3 phosphorylation and PAI-1 expression. These data further suggest that IN-1233 inhibited ozone-induced lung fibrosis by blocking TGF- β signaling pathways.

In summary, this study demonstrated for the first time that exposure to a cyclic O₃ exposure protocol that resembles certain environmental/occupational exposure situations increased the expression of TGF- β and PAI-1 in mouse lung and that increased TGF- β 1 expression mediated O₃-induced airway wall fibrosis. These results shed new light on the molecular mechanism whereby O₃ induces lung fibrosis.

Acknowledgments

The authors would like to thank the following people for their technical assistance with ozone exposure and sample collection/processing: Jo Anne Balanay, Wentan Huang, and Kishor Gangani.

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Figure 1.

Ozone exposure increased transforming growth factor beta (TGF-β) protein levels in the epithelial lining fluid (ELF) and collagen deposition in the airway walls in mice. (A) Bronchoalveolar lavage fluid (BALF) was harvested after 5 and 10 cycles of air or O₃ exposure and the amounts of total TGF-β protein determined by ELISA. Results were normalized with a dilution factor to determine ELF concentrations as described in the “Materials and methods” section and expressed as the mean ± SEM. *Significantly different from filtered air exposure controls ($P < 0.05$, $n = 10$). (B) Collagen deposition (blue) in the lung after 10 cycles of O₃ or filtered air exposure was assessed by trichrome staining and the amounts of collagen in the airway walls were quantified using computer-assisted stereology technique as described in the “Materials and methods” section. *Significantly different from the filtered air-exposed control ($P < 0.05$, $n = 6$).

Figure 2.

Ozone exposure increased PAI-1 protein levels in mouse lung. PAI-1 protein levels in the lung after 5 and 10 cycles of filtered air or O₃ exposure were determined by western blot techniques. Upper panel: representative western blots; Lower panel: densitometry analysis of the PAI-1 bands using Bio-Rad Chemi-Doc-XRS Imaging system with Quantity One software. The results were normalized to β -actin. *Significantly different from the filtered air-exposed controls ($P < 0.05$, $n = 6$).

Figure 3.

Ozone exposure induced alpha smooth muscle actin (α -SMA) expression in the lung. (A) Immunohistochemistry staining of α -SMA after 10 cycles of filtered air or O₃ exposure. The results were semiquantified using Axiovision and AV4 Mod Automeasure software and expressed as percentage of area. (B) Western analysis of α -SMA protein. Intensities of the bands were semiquantified using Bio-Rad Chemi-Doc-XRS Imaging system with Quantity One software. The data were normalized to β -actin. *Significantly different from the filtered air-exposed controls ($P < 0.05$, $n = 6$).

Figure 4. Inhibitor of transforming growth factor beta (TGF- β) type I receptor blocked O₃-induced Smad2/3 phosphorylation. The levels of phosphorylated Smad2/3 (pSmad2/3) and non-phosphorylated Smad2/3 were examined by western blotting. Top panel: representative western blots; bottom panel, semiquantified data of pSmad2/3 and Smad2/3 bands. (a) Significantly different from the filtered air alone exposed control and (b) significantly different from filtered air plus IN-1233 treated group ($P < 0.05$, $n = 3$).

Figure 5. Inhibitor of transforming growth factor beta (TGF- β) type I receptor suppressed O₃-induced collagen accumulation in the airway walls. Collagen deposition in the lung was assessed by trichrome staining and the amounts of collagens in the airway walls were quantified using computer-assisted stereology technique as described in the “Materials and methods” section. Top panel, representative trichrome staining pictures; bottom panel, semiquantitative trichrome staining data. (a) Significantly different from the filtered air alone exposed control and (b) significantly different from filtered air plus IN-1233-treated group ($P < 0.05$, $n = 7$).

Figure 6.

Inhibitor of transforming growth factor beta (TGF- β) type I receptor suppressed O₃-induced PAI-1 expression. PAI-1 protein levels in epithelial lining fluid (ELF) were determined by ELISA and normalized with a dilution factor to determine ELF concentrations as described in the “Materials and methods” section. Results were expressed as the mean $\pm$ SEM.

*Significantly different from the filtered air exposure controls ($P < 0.05$, $n = 6$).

Figure 7.

Inhibitor of transforming growth factor beta (TGF- β) type I receptor suppressed O₃-induced alpha smooth muscle actin (α -SMA) expression. (A) Immunostaining of α -SMA in the lung. (B) Western analysis of α -SMA in the lung. (C) Summary of semiquantified α -SMA western data using Bio-Rad Chemi-Doc- XRS Imaging system with Quantity One software, normalized by β -actin band. (a) Significantly different from the filtered air control group and (b) significantly different from filtered air plus IN-1233-treated group ($P < 0.05$, $n = 3$).