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Hypoxia-induced Pulmonary Arterial Hypertension Augments Lung Injury and Airway Reactivity Caused by Ozone Exposure

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Abstract

Ozone (O_3)-related cardiorespiratory effects are a growing public health concern. Ground level O_3 can exacerbate pre-existing respiratory conditions; however, research regarding therapeutic interventions to reduce O_3-induced lung injury is limited. In patients with chronic obstructive pulmonary disease, hypoxia-associated pulmonary hypertension (HPH) is a frequent comorbidity that is difficult to treat clinically, yet associated with increased mortality and frequency of exacerbations. In this study, we hypothesized that established HPH would confer vulnerability to acute O_3 pulmonary toxicity. Additionally, we tested whether improvement of pulmonary endothelial barrier integrity via rho-kinase inhibition could mitigate pulmonary inflammation and injury. To determine if O_3 exacerbated HPH, male C57BL/6 mice were subject to either 3 wks continuous normoxia (20.9% O_2) or hypoxia (10.0% O_2), followed by a 4-h exposure to either 1 ppm O_3 or filtered air (FA). As an additional experimental intervention fasudil (20mg/kg) was administered intraperitoneally prior to and after O_3 exposures. As expected, hypoxia significantly increased right ventricular pressure and hypertrophy. O_3 exposure in normoxic mice caused lung inflammation but not injury, as indicated by increased cellularity and edema in the lung. However, in hypoxic mice, O_3 exposure led to increased inflammation and edema, along with a profound increase in airway hyperresponsiveness to methacholine. Fasudil administration resulted in reduced O_3-induced lung injury via the enhancement of pulmonary endothelial barrier integrity. These results indicate that increased pulmonary vascular pressure may enhance lung injury, inflammation and edema when exposed to pollutants, and that enhancement of pulmonary endothelial barrier integrity may alleviate such vulnerability.

Keywords

Ozone; hypoxia; pulmonary hypertension; chronic obstructive pulmonary disease (COPD); fasudil; inflammation

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Conflicts of Interest Statement

The authors declare no conflicts of interest with the contents of this manuscript.

INTRODUCTION

Chronic mild-to-moderate hypoxia-associated pulmonary arterial hypertension (HPH) occurs concurrently with pre-existing respiratory diseases, such as chronic obstructive pulmonary disease (COPD) (Christman *et al.*, 1992). Clinical PAH is often defined as a sustained elevation of pulmonary arterial pressure ≥ 25 mmHg and prevalence can range dramatically depending on the severity of COPD (Chaouat *et al.*, 2008). Chronic alveolar remodeling from cigarette smoking and environmental exposures leads to a decrease in gas exchange surface area and subsequent impairments in oxygenation (Tuder *et al.*, 2007). Alveolar hypoxia, in turn, causes elevated pulmonary vascular resistance and pulmonary pressure (Jeffery, 2001). HPH is frequently a complication in COPD patients and is predictive of increased frequency and severity of exacerbations, poorer quality of life, and worse overall prognosis (Kessler *et al.*, 1999; McGhan *et al.*, 2007; Terzano *et al.*, 2010; Wells *et al.*, 2012).

Importantly, patients with COPD are at greater risk for hospital admission subsequent to exposure to airborne environmental insults, such as inhaled particulates or oxidant gases (Sunyer *et al.*, 1993; Halonen *et al.*, 2008). Long-term air pollution exposure and residential proximity to a busy roadway was associated with incidence of COPD in women (Schikowski *et al.*, 2005). Additionally, multiple studies indicate that COPD-related emergency room visits increase in tandem with levels of air pollution (Sunyer *et al.*, 1993; Anderson *et al.*, 1997; Halonen *et al.*, 2008). One study examining 94 patients in London found that outdoor air pollution was specifically associated with acute symptoms in COPD patients including dyspnea (Peacock *et al.*, 2011). Unfortunately, few COPD patients report modifying their daily routine based on daily air pollution forecast, indicating alternative means to reduce environmental exacerbations are needed, aside from behavioral modifications (Dell *et al.*, 2015).

Treatment of elevated pulmonary pressure with classical vasoactive drugs such as sildenafil, bosentan, or prostenoids is complicated by the deterioration of gas exchange in the deficient COPD lung (Stolz *et al.*, 2008; Blanco *et al.*, 2010; Seeger *et al.*, 2013), however other aspects of elevated pulmonary vascular pressure, such as the risk of vascular leakage, might be valuable therapeutic targets. Sildenafil, in particular, inhibits phosphodiesterase-5, which in turn increases levels of cGMP thereby promoting smooth muscle relaxation and vasodilation. However, this potent vasodilator is ineffective in the COPD lung as its dilatory effects tend to decrease ventilation-perfusion matching and ultimately decrease gas exchange. Inhibition of the RhoA/Rho Kinase pathway has been shown to improve endothelial barrier integrity and is protective in models of vascular injury and inflammation (Gibson *et al.*, 2014). Fasudil hydrochloride is a potent Rho-kinase inhibitor that has been clinically developed to treat pulmonary hypertension (Ishikura *et al.*, 2006; Fujita *et al.*, 2010) as well as improve blood brain barrier permeability by protection of tight junction proteins and ROCK inhibition (Fujii *et al.*, 2012).

Therefore, this study was implemented to examine whether HPH contributes to exacerbated responses to ozone (O_3), which is a ground-level airborne pollutant formed when substrates

such as volatile organic compounds and nitrogen dioxide, mostly from anthropogenic sources, interact with ultraviolet light. O_3 remains a major global concern with a demonstrated cardiopulmonary health impact, especially in COPD patients and has been associated specifically with exacerbations in COPD (Desqueyroux *et al.*, 2002; Malig *et al.*, 2015). Additionally, airway hyperreactivity has been implicated following O_3 exposure, in part due to irritation of airway nerves (Fabbri *et al.*, 1984; Kasahara *et al.*, 2015; Williams *et al.*, 2015). However, it remains unknown whether elevated pulmonary vascular pressure contributes to worsened outcomes related to environmental exposures.

MATERIALS AND METHODS

Animals

C57BL/6 mice (male, 6–8 weeks old at beginning of studies; Harlan Laboratories, Indianapolis, IN) were housed four per cage and allowed to acclimate over the course of two weeks after their arrival at UNM under AAALAC housing conditions. Mice were subject to a light/dark cycle of 12-h and had access to water and standard chow *ad libitum* (Harlan). Mice were euthanized via cardiac exsanguination while under isoflurane anesthesia. Sacrifice of animals was staggered due to space limitations of the hypoxia chamber. All procedures performed were approved by the UNM Institutional Animal Care and Use Committee (IACUC).

Exposures

Mice were subject to acute hypoxia (10.0% O_2) or normoxia (20.9% O_2) 24 h a day for 3 wks, followed by a single exposure to O_3. Hypoxia (10% O_2) was monitored continuously in the exposure chambers and remained at 10% for 3 weeks. Food and water was changed on an as needed basis and cages, including bedding was changed once per week. An OREC silent arc discharge O_3 generator (Osmonics, Phoenix, AZ) was used to expose rodents to O_3, and the concentration was continuously monitored over the course of 4h; all terminal endpoints were obtained 18–20 h following the end of this O_3 exposure. Water was available for mice during the exposure, but food was withheld. Exposures took place in a chamber without bedding to prevent O_3 scavenging. The initial study involved four treatment groups: normoxia then filtered air (Nx,FA), normoxia then O_3 (Nx,O_3), hypoxia then filtered air (Hx,FA), hypoxia then O_3 (Hx,O_3). In a second study, fasudil or PBS were intraperitoneally injected at 20mg/kg at 3 timepoints: once before O_3 exposure, once after O_3 exposure, and once the following day before euthanasia (Fig. 1). A total of 8 treatment groups received either fasudil or PBS: Nx,FA,Fas; Nx,O_3,Fas; Hx,FA,Fas; Hx,O_3,Fas or Nx,FA,PBS; Nx,O_3,PBS; Hx,FA,PBS; Hx,O_3,PBS.

Bronchoalveolar lavage fluid collection and cell counts

Immediately after euthanasia, a cannula was inserted into the trachea and lungs were lavaged with 1 mL phosphate buffered saline solution (PBS). Brochoalveolar lavage fluid (BAL) was centrifuged and pelleted cells were resuspended in PBS. Total cell counts, which included macrophages, neutrophils, lymphocytes, eosinophils, and basophils, were analyzed in triplicate using a hemocytometer and trypan blue staining.

Lung resistance and methacholine reactivity measurement

Twenty-four hours after O_3 exposure, airway resistance was monitored using a methacholine (MCh) challenge using the FlexiVent system (SCIREQ, Montreal, Quebec, Canada). While under isofluorane anesthesia, a 20-gauge needle was inserted into tracheal incision. The cannula was then inserted on the Flexivent system and the mouse was artificially ventilated, as previously described (Mishra *et al.*, 2008). Lung resistance was determined at baseline and after dose-response challenges with nebulized MCh (0–50 mg/ml) to assess airway hyperreactivity (AHR). Mice used for airway reactivity measurement were not used for measurements of lung weights, BAL, and RV pressure.

Right ventricular pressure

Right ventricular pressure measurements were obtained using saline-filled catheters connected to pressure transducers (Cobe, Lakewood, CO) in an artificially ventilated, open-chest model, as previously described, with n=4–8 per treatment group (Paffett *et al.*, 2012a; Paffett *et al.*, 2012b). Continuous hemodynamic traces were recorded (AD Instruments) and right ventricular systolic pressure (RVSP) and right ventricular mean pressure (RVMP) were calculated from a stable recording of 20–30 seconds for each subject.

Right ventricular hypertrophy and lung weights

The heart and lung block was dissected and removed from the chest cavity following cardiac exsanguination. Wet lung weight was recorded and lungs were dried for 48 h at room temperature according to a previously established protocol (Campen *et al.*, 2005; Lund *et al.*, 2009). After drying, lung weights were recorded again and total lung water, as an index of edema, was calculated as the difference between wet and dry weights. The right ventricle and the left ventricle + septum were dissected under a light microscope and weighed immediately to assess the presence of right ventricular hypertrophy. Fulton's index was calculated (Fulton's index = RV weight/LVS weight) to assess right ventricular cardiac remodeling subsequent to hypoxia.

Statistics

All statistics for terminal endpoints were computed using an ANOVA with a Holm-Sidak post-hoc test. Airway hyperreactivity responses were assessed with a repeated measures 2-factor (exposure condition, MCh concentration) with Tukey's multiple comparison test. Resulting p-values ≤ 0.05 were considered significant. GraphPad Prism software (Montreal, Quebec, Canada) was utilized for all statistical analyses.

RESULTS

Chronic hypoxia induces RV hypertrophy, which is not altered by acute O_3 exposure

Mice were sacrificed 18–20 h following the end of O_3 exposure, and 22–24 h following the end of the hypoxia exposure. At that time, Fulton's index indicated substantial RV remodeling with a significantly higher RV/LVS ratio in the hypoxia-exposed groups (Fig. 2A). Additionally, significantly elevated RVSP and RVMP in hypoxia-treated mice

compared to normoxic controls were noted, as indicated by a 2-factor ANOVA (Fig. 2B, C). Acute O_3 exposure had no impact on RV remodeling or pressure in this model.

Ozone significantly exacerbates lung injury and inflammation

In normoxic mice, O_3 did not lead to significant changes in lung weight or lung water weight, despite a clear increase in inflammatory cells in the BALF (Fig 3). Hypoxia alone significantly increased lung weight/body weight, lung water weight/body weight, and lung dry weight/body weight, but had no independent effect on BALF cell counts (Fig. 3). In hypoxic mice, O_3 exposure resulted in significant elevation of lung weight/body weight, lung water/body weight and total BALF cells, although did not alter the dry lung weight/ body weight ratio (Fig. 3).

Lung resistance is increased with combined hypoxia and O_3 exposure

Hypoxia did not cause significant increases in airway hyperreactivity to MCh challenge (Fig. 4). O_3 exposure in normoxic mice did induce a significant enhancement in resistance responses to MCh challenge. However, this effect was further potentiated in mice exposed to both hypoxia and O_3 exposure, with the combined exposure resulting in a statistically significant increase in lung resistance compared to O_3 alone. Treatment with fasudil was able to completely abrogate this hyperreactivity response in both HxO_3 and NxO_3 treatment groups.

Fasudil intervention mitigates lung injury

In addition to blocking hyperreactivity changes due to O_3 and hypoxia-O_3 exposure, fasudil (20 mg/kg) i.p. administration resulted in mitigation of O_3-induced lung injury (Fig. 5). Fasudil treatment followed the 3-week hypoxia regimen, and so did not alter the development of pulmonary hypertension in these mice, as indicated by RV/LVS ratios (Fig 5A). While hypoxia resulted in a significant increase in lung weight/ body weight, dry lung weight/ body weight and lung water/ body weight (Fig. 5B–D), there was no significant alteration of these parameters by O_3 exposure.

DISCUSSION

O_3-induced lung injury was exacerbated in the presence of a moderate pulmonary hypertension induced by 3 weeks of hypoxia in a mouse model. The presence of HPH led to enhanced O_3 effects in terms of airway hyperresponsiveness and lung inflammation and edema. Additionally, i.p. injection with low-dose fasudil, administered after HPH had developed, mitigated lung injury by O_3. While this study is a preliminary investigation into the relationship between HPH and environmental exposures, the results are consistent with a potential link to pollution-induced exacerbations in COPD patients with associated pulmonary hypertension.

Respiratory effects of O_3 in mice are well-characterized, however, vascular pathology, including pulmonary hypertensive effects from O_3 exposure has not been explored (Bauer and Kleeberger, 2010). Particulate matter air pollution has been studied in a limited manner in more severe models of pulmonary hypertension, but with similar findings of exacerbated

outcomes. Gardner and colleagues explored interactions between monocrotaline-induced pulmonary hypertension and bolus intratracheal exposures to a residual oil fly ash particulate and specifically noted an interaction in terms of vascular inflammation in histological analysis (Gardner *et al.*, 2004). In a similar study, rats treated with monocrotaline exhibited greater bradycardic, arrhythmic, and hypothermic responses to residual oil fly ash instillation compared to healthy rats (Campen *et al.*, 2000), along with exacerbated lung injury outcomes (Kodavanti *et al.*, 1999). However, in all f these studies, the time frame for monocrotaline-related treatment was likely insufficient to drive significant pulmonary hypertension (<14 days post injection) and still in the inflammatory phase of the pathology. Collectively, these studies did not focus on characterizing the pulmonary vascular hemodynamics and in only one study was RV remodeling reported (Campen *et al.*, 2000).

Based on the findings of the present study, we propose that pulmonary hypertension in COPD patients may be a crucial factor conferring vulnerability to environmental pollutants. Pulmonary hypertension in COPD patients can range from mild to severe (Ppa<40mmHg) (Weitzenblum and Chaouat, 2005), and both prevalence and severity of pulmonary hypertension are associated with the severity of COPD. Most COPD patients exhibit mild-to-moderate increases in Ppa concomitant with decreases in lung function, but there are also a significant minority of COPD patients that exhibit more profound Ppa increases that may relate to arterial-alveolar oxygen gradients of simply other etiological causes of pulmonary hypertension (Thabut *et al.*, 2005; Chaouat *et al.*, 2008).

COPD exacerbations may be driven by a host of causes including viruses, bacteria, cigarette smoke, air pollution and pre-existing genetic and physiological factors (Sapey and Stockley, 2006). Air pollution exposure and exacerbation of COPD symptoms has been well documented in several studies (Sunyer *et al.*, 1993; Anderson *et al.*, 1997; Halonen *et al.*, 2008), but pulmonary arterial hypertension has not specifically been addressed as a predisposing factor. Given that pulmonary hypertension is a relatively common comorbid condition in COPD and that several rat studies with a more severe pulmonary hypertension model (monocrotaline) suggest an interaction Campen *et al.*, 2000; Kodavanti *et al.,* 1999), our findings in a model of mild HPH offer strong support for pulmonary vascular pressure having an important role in determining the outcomes of environmental exposures in COPD patients.

Under hypoxic conditions (such as in COPD), blood flow to the upper respiratory zones increases due to vasoconstriction and elevated Ppa to improve the ventilation-perfusion ratio (V/Q) (Galvin *et al.*, 2007). Therefore, clinically, PAH in COPD patients is often not treated because vasodilators may antagonize V/Q benefits and subsequently incur hypoxemia (Galiè *et al.*, 2009). Furthermore, there are currently no pharmacological treatments indicated to oppose potential action of environmental pollutants in COPD patients. Because of these issues, we tested a pharmacological agent known to improve endothelial barrier integrity, fasudil, in terms of its ability to attenuate O_3 inflammation and edema in the HPH model. Both Rac1 and RhoA of the Rho-kinase pathway are involved in endothelial cytoskeletal changes and are vital to endothelial barrier integrity in opposition to intracellular tethering forces (Wojciak-Stothard and Ridley, 2002). Activation of RhoA/Rho can cause changes in gene expression for over 300 cytoskeletal proteins involved in microfilament building,

actomyosin structure, microfilament structure and endothelial cytoskeletal reorganization (Hall, 1998).

Attenuation of pulmonary lung injury has been demonstrated using the Rho kinase inhibitor fasudil as an intervention. Tasaka and colleagues found that intravenous injection of LPS from *E.coli* induced lung injury in mice and that fasudil pretreatment attenuated endothelial cytoskeletal rearrangements, resulting in decreased neutrophil infiltration (Tasaka *et al.*, 2005). Still others that have used LPS to induce lung injury have found that fasudil decreases early stage sepsis and systemic inflammation by decreasing leukocyte transmigration and restoring endothelial barrier integrity (Ding *et al.*, 2011). In an intestinal ischemia-reperfusion model, fasudil was found to mitigate lung injury, which is often a consequence of systemic shock (Li *et al.*, 2011). Fasudil has also demonstrated improvement in stroke outcomes by improving cerebrovascular, endothelial cell barrier integrity (Satoh *et al.*, 2001; Shibuya *et al.*, 2005). Additionally, while we posit that fasudil benefits on reducing O_3-induced airway hyperreactivity relate to the protection from lung edema and injury, fasudil may also relax airways through the rho-kinase pathway in airway smooth muscle (Gosens *et al.*, 2004; Schaafsma *et al.*, 2004). Regardless, considering that ambient air pollution exacerbates COPD symptoms, this study is the first PAH mouse model to establish that O_3-induced lung injury may be prevented with prophylactic fasudil treatment.

However, inhibition of Rho kinase may also have limited the degree of pulmonary hypertension during O_3 exposure in our model, which limits the strength of our conclusions related to endothelial barrier integrity. Because we were not able to continuously measure pulmonary arterial pressure throughout the 24h period between the onset of O_3 exposure and the time of sacrifice, it is unclear if vascular pressure was reduced by fasudil in a meaningful way. Execution of such continuous pulmonary hemodynamic measurements is exceedingly challenging and infrequently reported due to technical issues (Campen *et al.*, 2005; Schwenke *et al.*, 2006); with the additional logistics of O_3 exposure, continuous pulmonary hemodynamics monitoring was considered unworkable for the present study. In clinical studies, acute administration of fasudil has also been used to treat severe PAH patients (Fukumoto *et al.*, 2005). Using intravenous administration of fasudil hydrochloride (30 mg for 30 min), it was found that mean pulmonary vascular resistance significantly decreased, while pulmonary artery pressure did not change in humans. High dose and/or long-term use of fasudil has been used to treat pulmonary hypertension in monocrotaline rodent models and, at similar concentrations to the present study, fasudil did reduce rat pulmonary arterial pressure; however, we estimate from their pharmacodynamics data that the effect in our model of 3 injections would amount to only a 10% mean reduction in pulmonary pressure over the 24h period (Jiang *et al.*, 2007). Additionally, acute fasudil administration has clinically attenuated pulmonary pressure in PAH patients (Abe *et al.*, 2006; Ishikura *et al.*, 2006; Fujita *et al.*, 2010), however in our study, we did not find a significant change in pressure after fasudil administration, despite confirmation of elevated right ventricular pressure in hypoxic mice. This may have been due to the timeframe that the pressure was obtained (24 h after the end of hypoxia). Future studies will focus on obtaining pressure immediately after the end of hypoxia.

CONCLUSIONS

The hypoxia-induced pulmonary hypertension model led to a clear exacerbation of O_3-related lung injury, consistent with the concept that elevated vascular pressures may promote edema and inflammation following oxidative lung injury. This study demonstrated that Rho kinase inhibition via fasudil may be a viable therapy to attenuate lung injury by enhancing endothelial barrier integrity and/or through the reduction in pulmonary pressure. Further studies are needed to determine if the results of this study may have clinical parallels in at-risk populations, such as COPD patients. Additionally, clinically-relevant therapies such as inhaled fasudil could be examined using this model of O_3 and HPH (Nagaoka *et al.*, 2005; Fujita *et al.*, 2010).

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Highlights

- Environmental exposures can exacerbate chronic obstructive pulmonary disease (COPD)
- It is unknown if comorbid pulmonary hypertension may influence such effects in COPD patients
- Pulmonary hypertension in a mouse model significantly exacerbated ozone-induced lung injury
- Adverse ozone outcomes were largely attenuated by a rho kinase inhibitor, fasudil
- Therapeutic benefit from rho kinase inhibition may be related to endothelial barrier integrity

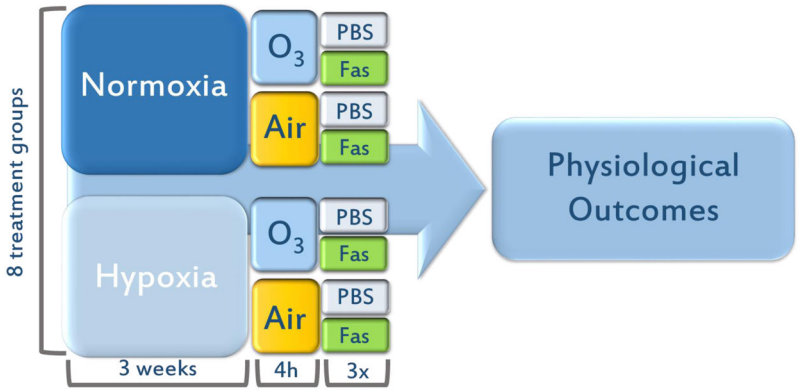

Figure 1. Study Design

C57BL/6 mice (n=6–10 per treatment group) received either 3 wks of or FA. Mice were injected with either continuous normoxia or hypoxia followed by 4 h 1ppm O_3 exposure. Data were subject Fasudil (20mg/kg) or PBS (vehicle) before, after and 24 h post-O_3 to a two-way ANOVA statistical analysis and expressed as a mean +/− SEM.

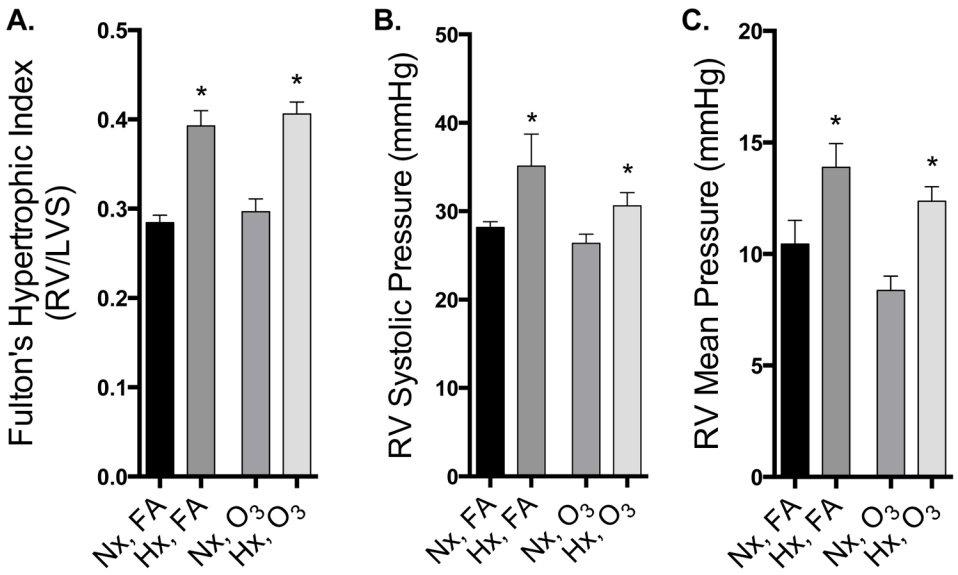

Figure 2. Right ventricular remodeling and RV pressure are elevated following 3 weeks of hypoxia, but not affected by O_3

A. Right ventricular remodeling in mice after 3 wks hypoxia (N=11–12/group). **B.** Right ventricular systolic pressure measured 24 h after cessation of hypoxia treatment (N=4–8/group). **C.** Right ventricular mean pressure measured 24 h after cessation of hypoxia treatment (N=4–8/group). Data were subject to a two-way ANOVA statistical analysis with Newman-Keuls posthoc test and expressed as a mean +/− SEM. Asterisks (*) indicate a significant effect of hypoxia treatment, regardless of O_3 exposure.

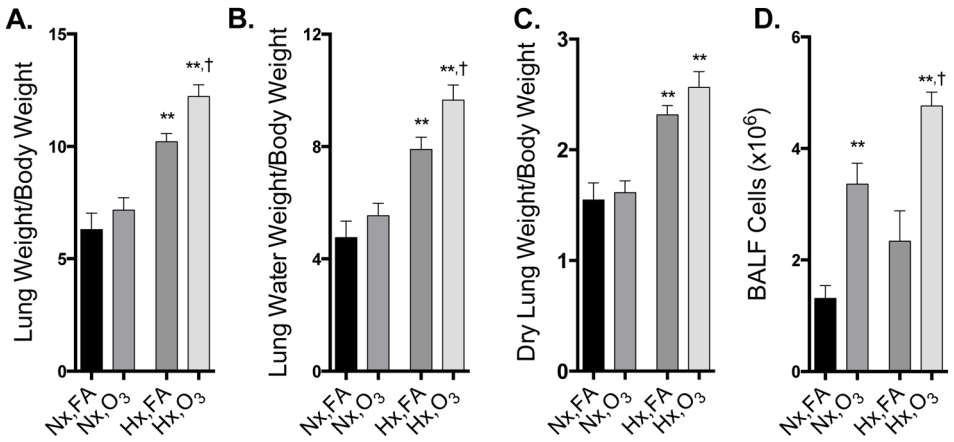

Figure 3. Ozone-induced lung injury is exacerbated in mice with HPH

A) Lung weight normalized to body weight B) Lung water weight normalized to body weight C) Dry lung weight normalized to body weight, and D) BALF inflammatory cell counts (n=5–6 per group). Group data were compared by an ANOVA with a Holm-Sidak post-hoc test and expressed as a mean +/− SEM. Asterisks (*) indicate difference from normoxia controls (*, $p<0.05$; **, $p<0.01$); crosses **(†) indicates significant enhancement of effect by the hypoxia-O_3 combination compared to hypoxia or ozone alone ($p<0.05$).**

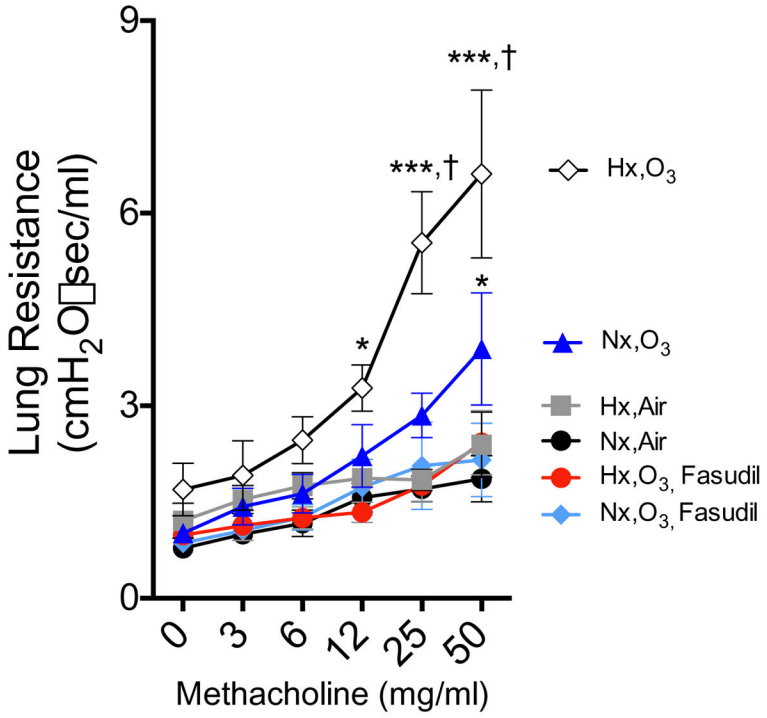

Fig. 4. Airway hyperreactivity significantly increased with combined hypoxia and O_3 after methacholine challenge

Lung resistance after sequential methacholine administration (0–50 mg/mL). Data were subject to a two-way ANOVA statistical analysis with a Tukey's multiple comparison test and expressed as a mean +/− SEM (n=6–8 per treatment group). Asterisks (*) indicate difference from normoxia-air controls (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$); crosses (†) indicates significant enhancement of effect by the hypoxia-O_3 combination compared to O_3 alone ($p<0.05$).

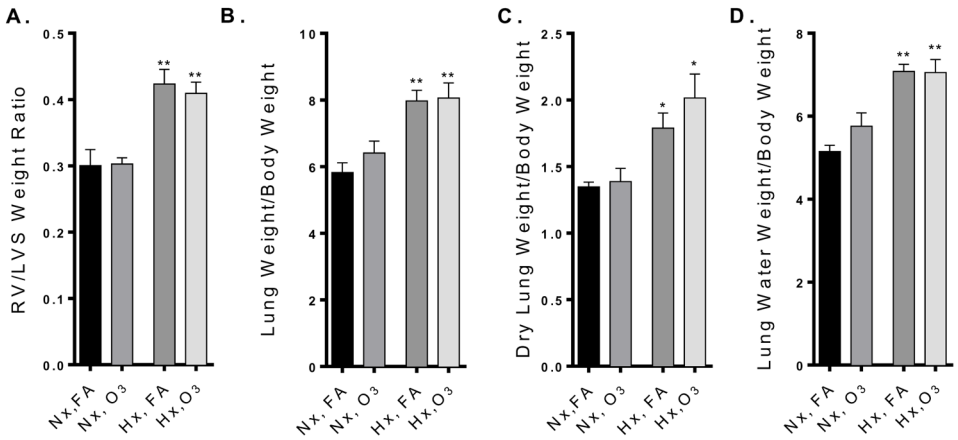

Figure 5. Fasudil intervention significantly attenuates HPH-dependent ozone-induced lung injury

A) RV/LVS weight ratio, B) lung weight normalized to body weight, C) dry lung weight normalized to body weight, and D) lung water weight normalized to body weight in mice administered a 20mg/mL i.p. Fasudil intervention after hypoxia, but before O_3 exposure. Only the 3-week hypoxia treatment induced changes to any parameter, with O_3 effects completely abrogated by the fasudil treatment. Group data were compared by an ANOVA with a Holm-Sidak post-hoc test and expressed as a mean +/− SEM. Asterisks (*) indicate difference from normoxia controls (*, $p<0.05$; **, $p<0.01$; N=4–6 per group).