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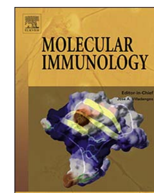

# Progesterone attenuates airway remodeling and glucocorticoid resistance in a murine model of exposing to ozone

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ABSTRACT

Airway remodeling is a vital component of chronic obstructive pulmonary disease (COPD). Despite the broad anti-inflammation effects of glucocorticoids, they exhibit relatively little therapeutic benefit in COPD, indicating the accelerating demands of new agents for COPD. We aim to explore the effect of progesterone on airway remodeling in a murine modeling of exposing to ozone and to further examine the potential effect of progesterone on glucocorticoid insensitivity. C57/BL6 mice were exposed to ozone for 12 times over 6 weeks, and were administered with progesterone alone or combined with budesonide (BUD) after each exposure until the 10th week. The peribronchial collagen deposition was measured. The protein levels of MMP8 and MMP9 in bronchoalveolar lavage fluid (BALF) and lungs were assessed. Western blot analysis was used to detect the levels of hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF), a-smooth muscle actin (α-SMA), glycogen synthase kinase-3β (GSK-3β). The expression of VEGF and histone deacetylase 2 (HDAC2) in the lung were determined by Immunohistochemical analyses. We observe that progesterone attenuates the peribronchial collagen deposition, as well as the expression of MMP8, MMP9, HIF-1α, VEGF, α-SMA, and GSK-3β in BALF or lung tissues. Progesterone or BUD monotherapy has no effect on HDAC2 production. Progesterone combines with BUD induce dramatically enhanced effects. Thus, these results demonstrate novel roles of progesterone for the pathogenesis and airway remodeling in COPD. Progesterone plus BUD administration exerts more significant inhibition on airway remodeling with dose-independent. Additionally, progesterone may, to some extent, improve the glucocorticoid insensitivity.

## 1. Introduction

Chronic obstructive pulmonary disease (COPD), which is characterized anatomically by emphysema and the remodeling of airway structure, is ranked eighth as the cause of disease burden in 2015 (Mortality and Causes of Death, 2016; Disease et al., 2016), and now is the fourth commonest cause of death worldwide. Exposure to air pollution has been associated with stimulating pulmonary immune defense responses in both animals and human subjects (Stieb et al., 2012; Billionnet et al., 2012). Ground-level ozone ($O_3$) or ambient $O_3$ exposure is an important component of air pollution that is related to adverse health impacts. Researches have shown that short-term exposure to $O_3$ was concerned in decreased lung function (Rice et al., 2013) and increased hospitalizations for COPD (Halonen et al., 2010; Arbex et al., 2009), whereas long-term exposure to $O_3$ was linked to increasing respiratory mortality (Jerrett et al., 2009) and increasing mortality among subjects with COPD (Zanobetti and Schwartz, 2011). Air pollution has been identified as significant initiating and risk factor for COPD and oxidative and carbonyl stress were found in attributing to the cell damage and death in COPD airways. Oxidative stress occurs when exposed to the overgeneration of reactive oxygen species (ROS), arising endogenously or exogenously, conduces to a major predisposing factor in the pathogenesis of COPD (Chung and Marwick, 2010; Kirkham and Barnes, 2013).

Airway remodeling can be collectively considered a process encompassing changes in structural cells and tissues of the airway in

*Abbreviations:* COPD, chronic obstructive pulmonary disease; $O_3$, ozone; BUD, budesonide; BALF, bronchoalveolar lavage fluid; MMP, matrix metalloproteinase; HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor; α-SMA, α-smooth muscle actin; GSK-3β, glycogen synthase kinase-3β; HDAC2, histone deacetylase 2; ICS, inhaled corticosteroids; ELISA, enzyme linked immunosorbent assay; WB, Western blot; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; GR, glucocorticoid receptor; NF-κB, transcription factor κB; IL, interleukin; ASM, airway smooth muscle; ECM, extracellular matrix; TGF-β1, transforming growth factor-β1; MAPK, mitogen activated protein kinase; ROS, reactive oxygen species

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obstructive disease, and the cardinal pathological features of airway remodeling are peri-bronchial fibrosis, fibroblast proliferation and conversion to myofibroblasts, smooth muscle hypertrophy, and vascular remodeling. Although airway remodeling has been profound and progressive researched in the last two decades, particularly in the context of asthma, there are few intensive studies focus on the structural airway remodeling in COPD, and little if any effective agents target to alleviate and reverse airway remodeling in COPD, which may impair lung function and affect the progression of COPD.

Despite intensive research, there are no effective agents for modifying the COPD. Inhaled corticosteroids (ICS) and long-acting bronchodilators improve quality of life without decreasing mortality, (Calverley et al., 2007) and multitudinous other agents have been experimented in clinical trials without success (Barnes, 2002; Barnes, 2013), indicating the existence of glucocorticoid resistance in COPD and the accelerating demands of new agents for COPD. Progesterone, produced by corpus luteum, is crucial for pregnancy maintenance and menstrual cycle. Recently, more pleiotropic effects of progesterone were discovered in other tissues (Csapo et al., 1971; Csapo et al., 1972). Tyler and colleagues showed that progesterone may influence breathing in the pregnant women (Tyler, 1960). Susan and colleagues observed progesterone could inhibit prostaglandin activity associated with a concomitant decrease in inflammation (Fox et al., 1993). There are also increasing evidences of progesterone in inhibiting inflammation and oxidative stress in central nervous system, traumatic brain injury, rheumatoid arthritis and autoimmune diseases (He et al., 2004; Sarkaki et al., 2013; Ostensen and Villiger, 2007; Garay et al., 2017). These studies underline a latent role of progesterone in modulating the inflammation, oxidative stress, and the structural remodeling in COPD.

Glucocorticoids are the proverbial major pillar of antiinflammation in inflammatory diseases. However, it is controversial in the effect of airway remodeling and there were increasing clinical and experimental evidences reported the insensitivity of glucocorticoids in COPD. There are few researches focus on the airway remodeling in murine models of COPD. Our previous research has observed that progesterone experts a profound anti-inflammation role in mice exposed to $O_3$ and has synergism with glucocorticoids (Fei et al., 2017). The roles of progesterone on airway remodeling in mice exposed to $O_3$ were unrecognized previously. Thus we postulated expandingly in this article whether progesterone, or synergistically with glucocorticoids, attenuated the airway remodeling in mice exposed to $O_3$, and further to explore the potential effect of progesterone on glucocorticoid insensitivity.

## 2. Materials and methods

### 2.1. Study approval and ethics statement

This study was implemented in strict accordance with the recommendations of the Shanghai Committee for Accreditation of Laboratory Animal. All the research protocols of this study were approved by the Shanghai General Hospital Institutional Review Board. All surgeries were carried out under the anesthesia of sodium pentobarbital, and all efforts were made to minimize suffering.

### 2.2. Animals, ozone exposure, drugs confecting and administration

Pathogen-free male C57/BL6 mice, 10 weeks old, were kept in filter-topped cages under constant conditions of temperature (21–25 °C) and humidity (40–60%). A strict 12 h light-dark cycle was performed and adlibitum feeding was received. Mice were divided randomly into 7 groups as follows: air + sterile saline, $O_3$ + sterile saline, $O_3$ + Budesonide (BUD) (0.2 g/L), $O_3$ + low progesterone (0.03 mg/L), $O_3$ + high progesterone (0.3 mg/L), $O_3$ + BUD + low progesterone and $O_3$ + BUD + high progesterone. Mice were exposed to normal air mixed with $O_3$, which produced by an ozoniser (Model 500; Sander Ozoniser, Germany), for 3 h at a concentration of 2.5 parts per million (ppm) in a sealed Perspex container, twice a week for 6 weeks. $O_3$ concentration was continuously monitored by $O_3$ detector (ATi Technologies, Oldham, UK). During the corresponding periods, controls were exposed to normal air only, twice a week for 6 weeks as well. Treatment groups were subsequently aerosol inhaled with BUD, low progesterone, high progesterone, BUD + low progesterone or high progesterone from day 42. BUD and progesterone, which dissolved in 1% DMSO to the final concentration of 0.03 and 0.3 mg/L, were inhaled by the ultrasonic nebulizer (PARI-BOY N037; PARI, Starnberg, Germany) in treatment mice. Controls were inhaled with atomized saline in 1% DMSO. Progesterone (Xianghe Shunda Fine Chemical Co., Ltd, Wuhan, China) was dissolved in 1% DMSO to final concentrations of 0.03 and 0.3 mg/L. BUD (AstraZeneca Pty Ltd., North Ryde, NSW2113, Australia) sodium phosphate was prepared at a concentration of 0.2 g/L. The control and model mice received solvent inhalation. BUD was aerosolized 30 min before $O_3$ exposure for 10 weeks and progesterone was operated prior 12 h to $O_3$ exposure for 10 weeks as well.

### 2.3. Masson trichrome staining

The left lung lobe was separated and fixed in 10% neutral buffered formalin and paraffin embedded for Masson Trichrome staining analysis. Sections were stained with Masson Trichrome stain to identify collagen deposition following the standard protocol recommended by the manufacturer. All histologic examination was performed in a double blind manner under 400x magnification. Results were expressed as the average area of per airway.

### 2.4. Enzyme linked immunosorbent assay (ELISA) for matrix metalloproteinase (MMP)8 and MMP9 in bronchoalveolar lavage (BALF)

MMP8 and MMP9 protein levels in the BALF were measured by ELISA. Mice were subsequently narcotized by over dose of pentobarbitone(500 mg/kg i.p) after 24 h following the last $O_3$ exposure. Then PE-60 tubing (0.72-mm inner diameter, 1.22-mm outer diameter) intubated in the exposed tracheal of mice. Three aliquots of 0.4 ml sterilized saline lavaged into the lung of mice through the tracheal and BALF was retrieved. Return volume was collected and was consistent > 80% of the instilled volume. The collected BALF samples were then centrifuged at 3000 r/min for 10 min at 4 °C, the supernatant was collected and then stored in 200 ul tubes at −80 °C for ELISA analyses.

### 2.5. Western blot analysis

Lung tissues were washed with cold PBS for three times and then lysed in RIPA (Beyotime Biotechnology, Jiangsu, China) containing 10 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min on ice. The proteins were separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary antibodies at 4 °C overnight against hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF), a-smooth muscle actin (α-SMA), glycogen synthase kinase-3β (GSK-3β) (Cell Signaling Technology, Beverly, CA) after blocking for 1 h in 5% non-fat milk in PBST buffer for blot detection. Then the membranes were washed three times for 10 min with TBS-T solution and incubated with oscillation for 1 h with the corresponding secondary antibodies (1:5000, Abgent). Chemiluminescent detection was implemented by an ECL kit (Millipore, Billerica, MA, USA) and Bio-Rad ChemiDoc MP Imaging System. All experiments were implemented in triplicate. Final protein concentration was determined by β-actin as the loading control and the expression levels of HIF-1α, VEGF, α-SMA, GSK-3β were normalized to that of β-actin.

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