Published in final edited form as:
Clin Immunol. 2013 April ; 147(1): . doi:10.1016/j.clim.2013.02.004.

Hypoxia Potentiates Allergen Induction of HIF-1α, Chemokines, Airway Inflammation, TGF-β1, and Airway Remodeling in a mouse model

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Abstract

Whether hypoxia contributes to airway inflammation and remodeling in asthma is unknown. In this study we used mice exposed to a hypoxic environment during allergen challenge (simulating hypoxia during an asthma exacerbation) to investigate the contribution of hypoxia to airway inflammation and remodeling. Although neither hypoxia alone, nor OVA allergen alone, induced significant neutrophil influx into the lung, the combination of OVA and hypoxia induced a synergistic 27 fold increase in peribronchial neutrophils, enhanced expression of HIF-1α and one of its target genes, the CXC-family neutrophil chemokine KC. The combination of hypoxia and OVA allergen increased eotaxin-1, peribronchial eosinophils, lung TGB-β1 expression, and indices of airway remodeling (fibrosis and smooth muscle) compared to either stimulus alone. As hypoxia is present in >90% of severe asthma exacerbations, these findings underscore the potential of hypoxia to potentiate the airway inflammatory response, remodeling, and accelerate the decline of lung function in asthma exacerbations.

Keywords

hypoxia; neutrophil; eosinophil; KC; eotaxin-1

1. INTRODUCTION

Exacerbations of severe asthma are associated with hypoxemia that can persist for several days in approximately 90% of subjects as assessed by arterial blood gas analysis [1]. The

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cause of the hypoxemia in the majority of asthma exacerbations is due to altered ventilation perfusion ratios [1]. In addition, laboratory studies in asthmatics have demonstrated that hypoxia impairs the perception of symptoms including difficulty breathing, chest tightness, and breathlessness, all of which may contribute to treatment delay during asthma exacerbations [2]. Asthma exacerbations are also associated with neutrophilic airway inflammation in adults [3,4,5], eosinophilic and neutrophilic inflammation in children [6], and a greater decline in lung function [7,8]. At present there is limited information regarding whether hypoxia during exacerbations of asthma contributes to neutrophilic and/or eosinophilic airway inflammation and subsequent remodeling or decline in lung function. In this study we have used a mouse model to investigate whether mice exposed to a hypoxic environment during allergen challenge (to simulate hypoxia during an asthma exacerbation) have evidence of increased neutrophilic and/or eosinophilic airway inflammation and enhanced airway remodeling.

Hypoxia induces the transcription factor hypoxia-inducible factor (HIF) which regulates expression of over 100 genes, many of which are potentially relevant to inflammation and remodeling in asthma [9, 10, 11]. For example, hypoxia induces expression of pro-inflammatory cytokines (IL-1β, TNFα, IL-8, VEGF)[9–11], which have been detected at increased levels in the airway of asthmatics [12, 13, 14]. IL-8 in particular is a chemokine regulating neutrophil recruitment that may contribute to the neutrophilic airway inflammation noted during exacerbations of asthma [3–5]. Hypoxia in asthma exacerbations may also contribute to airway remodeling as neonatal calves exposed to chronic hypoxia develop increased airway fibrous tissue and smooth muscle [15], mice exposed to chronic hypoxia develop increase lung type III fibrillar and type IV basement membrane collagen after ten days of hypoxia [16], and hypoxia can increase the proliferation of rat airway smooth muscle cells in vitro [17].

The ubiquitously expressed and best-studied form of HIF is HIF-1, a heterodimer consisting of the oxygen-regulated alpha subunit (HIF-1α) and a constitutively expressed beta subunit HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator protein or ARNT)[9–11]. Less well studied isoforms HIF-2 and HIF-3 exhibit more restricted tissue expression [9]. In previous studies we have demonstrated, using conditional myeloid HIF-1α knockout mice and pharmacologic HIF-1α inhibitors, that myeloid cell expression of HIF plays an important role in the development of airway hyperresponsiveness under normoxic conditions [18]. Interestingly, HIF may also be induced by local tissue hypoxia as opposed to systemic hypoxia in inflamed tissues that are often hypoxic as a result of decreased perfusion, edema, vascular insult and/or influx of oxygen-consuming immune cells or pathogens [19]. These localized areas of lung tissue hypoxia may be pertinent not only to severe asthma, but may also occur in mild and moderate asthmatics. Thus, activation of HIF-1α in the context of inflammation can occur in both normoxic as well as hypoxic external environments. Additional studies using mouse models of asthma have demonstrated under normoxic conditions that HIF-1 pharmacologic inhibitors [20, 21], HIF siRNA knockdown [21], and conditional HIF-1β deficient mice [22] influence levels of airway inflammation and/or airway remodeling. Human studies have also demonstrated under normoxic conditions increased levels of HIF-1α in lung tissue and bronchial fluid of patients with asthma, and in the nasal fluid of patients with rhinitis after allergen challenge [22].

In this study we have used a mouse model of allergen induced asthma studied under hypoxic conditions (to simulate severe asthmatics having hypoxic asthma exacerbation) to determine the influence of hypoxia on levels of airway inflammation and remodeling. These studies under hypoxic conditions differ critically from prior studies investigating the role of HIF in allergen-induced inflammation and remodeling under normoxic conditions [18, 20, 21, 22]. Overall our studies demonstrate that in a mouse model simulating hypoxia during an asthma exacerbation, the combined hypoxia and allergen stimulus significantly enhanced HIF-1α expression, airway inflammation (in particular neutrophilic but also eosinophilic), as well as lung levels of KC (the murine equivalent of IL-8), eotaxin-1, and TGF-β1, with resultant increased airway remodeling. As hypoxia is present in >90% of severe asthma exacerbations, these findings underscore the potential of hypoxia to potentiate the airway inflammatory response, increase levels of remodeling, and contribute to the decline in lung function in severe asthmatic exacerbations.

2. MATERIAL AND METHODS

2.1 Mouse model of acute OVA challenge and/or hypoxia exposure

The following four groups of BALB/C mice aged 6–8 weeks (n= 8 mice/group)(Jackson Labs) were studied. 1) No hypoxia + no OVA; 2) No hypoxia + OVA; 3) Hypoxia + no OVA; and 4) Hypoxia + OVA. The hypoxia and allergen exposures as well as all procedures involving experimental animals were approved by the Animal Care and Use Committee of the University of California San Diego.

2.2 Hypoxia exposure

Mice in the hypoxia groups (group 3 and 4) were placed in a plexiglass chamber maintained at 10% O2, while normoxic groups (group 1 and 2) were placed in a control chamber open to room air (21% O2). The duration of the hypoxia or normoxia exposure was 7 days based on pilot time course studies. CO2, water vapor, and ammonia were removed from the chambers by Drierite (anhydrous calcium sulfate)(Fisher Scientific, Atlanta, GA).

2.3 Acute OVA protocol

BALB/C mice were immunized s.c. on days 0, 7, 14, and 21 with 25 μg of OVA (OVA,grade V; Sigma) adsorbed to 1 mg of alum (Aldrich) in 200 μl normal saline as previously described[23]. OVA-challenged mice received intranasal OVA (20 μg OVA in 50μl PBS) on days 27, 29 and 31 under isoflurane (Vedco, Inc. St Joseph, MO) anesthesia. The non-OVA control mice (age and sex matched) were sensitized but not challenged with OVA.

2.4 Combined hypoxia and acute OVA protocol

Mice exposed to either hypoxia or normoxia started their chamber exposures on day 33, two days after the mice had received their last intranasal OVA challenge. The protocol was designed to address whether addition of hypoxia to an ongoing episode of allergic inflammation (to simulate the development of hypoxia after the initiation of the exacerbation) would influence levels of inflammation and remodeling 7 days later (a sufficient time period to allow remodeling to develop).

2.5 Processing of lungs for immunohistology

Mice were sacrificed 24 hours after the final OVA and/or hypoxia challenge and broncho-alveolar lavage (BAL) fluid and lungs were analyzed as previously described [23, 24, 25]. In brief, BAL was obtained by flushing 0.8ml PBS through an intratracheal tube.Lungs in the different groups of mice were equivalently inflated with an intratracheal injection of a similar volume of 4% paraformaldehyde solution (Sigma Chemicals, St Louis, MO) to preserve the pulmonary architecture. Lungs from the different experimental groups were processed as a batch for either histologic staining or immunostaining under identical conditions. Stained and immunostained slides were all quantified under identical light microscope conditions, including magnification (20 X), gain, camera position, and background illumination. The quantitative histologic and image analysis of all coded slides was performed by blinded research associates.

2.6 Quantitation of peribronchial eosinophils and neutrophils

The number of peribronchial major basic protein (MBP) positive eosinophils and neutrophil elastase positive neutrophils were quantitated as previously described [23–26]. In brief, lung sections were processed for MBP immunohistochemistry using an anti-mouse MBP Ab (kindly provided by James Lee PhD, Mayo Clinic, Scottsdale, Arizona) or anti-neutrophil elastase antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The number of individual cells staining positive for either MBP or neutrophil elastase in the peribronchial space were counted using light microscopy. Results are expressed as the number of peribronchial cells staining positive for either MBP or neutrophil elastase per bronchiole with 150–200 μm of internal diameter. At least ten bronchioles were counted in each slide.

2.7 Lung levels of chemokines/cytokines that regulate recruitment of neutrophils and eosinophils

Levels of neutrophil chemoattractant KC the murine equivalent of human IL-8 [26], eosinophil chemoattractant eotaxin-1, Th2 cytokines (IL-5, IL-13), and innate cytokines (IL1β) were measured in BAL by ELISA (R&D System Inc, Minneapolis, Minn). The IL-1β, IL-5, IL-13, KC, and eotaxin-1 assays had a sensitivity of 15 pg/ml.

2.8 Detection of peribronchial cells expressing KC

Lung sections were immunostained with both a KC primary Ab (R&D systems, Minneapolis, MN) and an anti-MBP Ab to determine whether MBP+ peribronchial cells express KC. The two different primary antibodies were detected using two different horseradish peroxidase (HRP) enzyme-labeled secondary antibodies with tyramide signal amplification (Molecular Probes) according to the manufacturer’s instructions as previously described [25]. The anti-MBP Ab was detected with Alexa 546 (red), while the anti-KC Ab was detected with Alexa 488 (green). Cells co-expressing MBP and KC had a merged yellow color.

2.9 Peribronchial fibrosis

The area of peribronchial trichrome staining (as an index of collagen deposition) in paraffin embedded lung was outlined and quantified using a light microscope (Leica DMLS, Leica Microsystems Inc., NY) attached to an image analysis system (Image-Pro plus, Media Cybernetics, MI) as previously described [25]. Results are expressed as the area of trichrome staining per μm length of basement membrane of bronchioles 150–200 μm of internal diameter.

2.10 Thickness of the peribronchial smooth muscle layer

Lung sections were immunostained with an anti-α-smooth muscle actin primary antibody (Sigma-Aldrich). The area of α-smooth muscle actin staining was outlined and quantified using a light microscope attached to an image analysis system as previously described[25].Results are expressed as the area of α-smooth muscle actin staining per μm length of basement membrane of bronchioles 150–200 μm of internal diameter.

2.11 Mucus

The number of PAS positive and PAS negative, airway epithelial cells in individual bronchioles were counted as previously described in this laboratory [25]. At least ten bronchioles were counted in each slide. Results are expressed as the percentage of PAS-positive cells per bronchiole which is calculated from the number of PAS-positive epithelial cells per bronchus divided by the total number of epithelial cells of each bronchiole.

2.12 Peribronchial TGF-β1+ cells

The number of peribronchial cells expressing TGF-β1 were assessed in lung sections processed for immunohistochemistry using an anti-TGF-β1 primary Ab (Santa Cruz Biotechnology,Inc, Santa Cruz, CA), the immunoperoxidase method, and image analysis quantitation as previously described [25].Results are expressed as the number of TGF-β1 positive cells/bronchus [25].

2.13 HIF-1α expression

Levels of lung HIF-1α mRNA were quantitated by qPCR. Total RNA obtained from lung tissue was prepared with RNeasy®Mini Kit (Qiagen) according to the manufacturer's protocol. Synthesis of cDNA was performed from 1ug of total RNA with Superscript®II (Invitrogen) according to the manufacturer's recommendations. qPCR was performed on a Max 3000s (Stratagene) using RT²SYBR®Green Rox™qPCR master mix (Qiagen). Mouse HIF1α primers for qPCR were purchased from Origene Technologies. Levels of lung GAPDH in each sample were used as controls.

To identify which lung cells express HIF-1α, lung sections were immunostained with an anti-HIF-1α Ab (Novus Biologicals) using the immunoperoxidase method and a TSA detection kit as previously described in this laboratory [25].

2.14 Statistical analysis

Results in the different groups of mice were compared by a Student t test. All results are presented as mean + SEM. A statistical software package (Graph Pad Prism, San Diego, CA) was used for the analysis. P values of < 0.05 were considered statistically significant.

3. RESULTS

3.1 Effect of hypoxia on HIF-1α expression in the lung

Exposure of mice to hypoxia alone induced a 3.2 fold induction of lung HIF-1α mRNA (hypoxia vs normoxia)(Fig 1A). In contrast, exposure of mice to allergen alone in the absence of hypoxia induced a slight 1.3 fold induction of lung HIF-1α mRNA (Fig 1A). However, the combination of hypoxia and OVA allergen induced a significant 5.3 fold increase in levels of lung HIF-1α mRNA compared to OVA and normoxia (p=0.03)(Fig 1A), and a 2.2 fold increase compared to hypoxia and no OVA (p=0.05)(Fig 1A). Immunohistochemistry studies demonstrated that epithelial cells were the predominant cell expressing increased levels of HIF-1α following exposure to hypoxia (Fig 1C). In contrast, following either OVA allergen or OVA + hypoxia exposure, peribronchial inflammatory cells as well as airway epithelium expressed increased levels of HIF-1α (Fig 1D, E).

3.2 Effect of hypoxia on OVA induced eosinophil and neutrophil airway inflammation

In the absence of allergen, peribronchial eosinophils did not differ significantly between mice exposed to hypoxia (2.2 + 0.1 MBP+ cells/bronchus) and their normoxic controls (0.7 + 0.2) (p=NS, Fig 2A). Likewise, peribronchial neutrophils were similar in the hypoxia (3.9 + 1.2) and normoxia (0.3 + 0.04) groups without OVA challenge (p = NS, Fig 2B). In normoxia OVA-challenged mice had greatly increased peribronchial eosinophils (86.9 + 5.4 vs. 0.7 + 0.2 MBP+cells/bronchus, OVA vs No-OVA, p<0.0001, Fig 2A), and a slight but statistically insignificant change in peribronchial neutrophils (p=NS, Fig. 2B).

Synergy was observed in combination of hypoxia with allergen challenge. During OVA-challenge, mice exposed to hypoxia had 1.8 fold more peribronchial eosinophils (155.9 + 7.5) compared to normoxia controls (86.9 + 5.4) ($p<0.001$, Fig 2A). An even more dramatic effect was observed with OVA allergen induced recruitment of peribronchial neutrophils, which increased approximately 27.3-fold in hypoxia (62.7 + 4.1) compared to normoxia (2.3 ± 0.9) ($p<0.0001$, Fig 2B).

3.3 Effect of hypoxia on levels of lung neutrophil chemokine KC

As the combination of hypoxia and OVA allergen synergistically increased levels of peribronchial neutrophils, we examined whether hypoxia influenced levels of lung KC a major neutrophil chemoattractant [26]. Exposure of mice to hypoxia alone in the absence of OVA allergen challenge did not induce a significant increase in BAL KC (hypoxia vs normoxia in the absence of OVA)(p=NS)(Fig 3A), which corresponded to the observation that hypoxia alone did not induce significant peribronchial neutrophil recruitment (Fig 2B). Similarly, mice challenged with OVA allergen alone in normoxic conditions had a slight but statistically insignificant increase in levels of BAL KC (OVA vs no OVA in normoxia) (p=NS) (Fig 3A) and peribronchial neutrophils (p=NS) (Fig 2B). However, hypoxia in combination with OVA allergen challenge induced a large synergistic increase in levels of BAL KC (hypoxia + OVA vs normoxia + OVA)($p=0.03$) (Fig 3A) which corresponded to the synergistic increase in peribronchial neutrophils in mice exposed to hypoxia + OVA (Fig 2B).

To determine the cellular source of lung KC in hypoxia and OVA allergen challenged mice, we immunostained lung sections and demonstrated that peribronchial cells rather than epithelial cells were the predominant source of KC (Fig 3B). Double label immunofluorescence microscopy studies demonstrated that MBP+ eosinophils were one of the peribronchial inflammatory cells expressing KC (Fig 3B–D).

3.4 Effect of hypoxia on levels of chemokines (eotaxin-1), Th2 cytokines (IL-5 IL-13), and innate cytokines (IL-1β) that regulate eosinophilic inflammation

As hypoxia also increased OVA induced peribronchial eosinophil recruitment (Fig 2A) we examined whether hypoxia influenced levels of mediators known to influence levels of lung eosinophils, including chemokines (eotaxin-1)[27], Th2 cytokines, (IL-5, IL-13) [25, 28], or innate cytokines (IL-1β)[29]. Exposure of mice to hypoxia in the absence of OVA allergen induced a significant increase in levels of BAL IL1β (78.7 ±18.3 vs 23.8 ± 10.7 pg/ml) (hypoxia vs normoxia in the absence of OVA)($p=0.04$), but not other chemokines/cytokines studied including eotaxin-1 (p=NS), IL-13 (p=NS), or IL5 (p=NS). OVA allergen challenge alone induced significant increases in BAL IL-1β (109.5 ± 15.8 vs 23.8 ± 10.7 pg/ml)(OVA vs no OVA in normoxia)($p=0.003$), eotaxin-1 (35.6 ±11.7 vs 15.8 ± 8.4 pg/ml) ($p=0.04$), IL13 (151.2 ± 28.1 vs 16.3 ± 4.1 pg/ml) ($p=0.001$) and IL-5 (113.7 ± 24.8 vs 4.0 ± 1.4 pg/ml) ($p= 0.003$). The combination of hypoxia and OVA allergen challenge induced a significant increase in eotaxin-1 compared to OVA alone (52.8 ± 16.2 vs 35.6 ± 11.7 pg/ml) (hypoxia + OVA vs normoxia + OVA)($p=0.02$) but did not induce significant increases in BAL IL-1β (p=NS), IL-13 (p=NS), or IL-5 (p=NS).

3.5 Effect of hypoxia on OVA induced peribronchial fibrosis

In the absence of OVA allergen, exposure of mice to hypoxia alone challenge induced a significant increase in peribronchial fibrosis (0.25 ± 0.06 vs 1.07 ± 0.03 $\mu m^2/\mu m$ trichrome stained peribronchial area)(normoxia vs hypoxia)($p<0.001$)(Fig 4 A ,C). Likewise, OVA allergen challenge alone under normoxic conditions induced a significant increase in peribronchial fibrosis (0.25 ± 0.01 vs 1.86 ±0.03)(no OVA vs OVA in normoxia)($p<0.001$) (Fig 4 A,B,C). Hypoxia in combination with OVA allergen challenge significantly increased levels of peribronchial fibrosis compared to either hypoxia alone ($p<0.001$)(Fig 4 A ,E),or compared to OVA allergen alone ($p<0.001$)(Fig 4 A).

3.6 Effect of hypoxia on OVA induced changes in peribronchial smooth muscle layer

Exposure of mice to hypoxia alone in the absence of OVA allergen induced significant increases in peribronchial smooth muscle as assessed by the α-smooth muscle actin (α-SMA) immunostained area (1.0 ± 0.1 vs 1.8 ± 0.2 $\mu m^2/\mu m$ α-SMA immunostained peribronchial area)(normoxia vs hypoxia in the absence of OVA)($p=0.004$)(Fig 5 A–C). OVA allergen challenge alone also induced a similar increase in the peribronchial α-SMA immunostained area (OVA vs no OVA in normoxia)($p<0.001$)(Fig 5 A,B,D). The combination of hypoxia and OVA allergen significantly increased the area of peribronchial α-SMA staining compared to either hypoxia alone ($p< 0.01$)(Fig 5 A,C,E) or OVA alone ($p<0.05$)(Fig 5 A, D, E).

3.7 Effect of hypoxia on the number of cells expressing the remodeling mediator TGF-β1

OVA allergen challenge alone induced an increase in the number of peribronchial TGF-β1 positive cells (2.4 ± 0.4 vs 31.8 ± 3.5 TGF-β1 positive cells/bronchus)(OVA vs No OVA in normoxia)($p=0.003$)(Fig 6). In contrast, exposure of mice to hypoxia alone did not increase the number of peribronchial TGF-β1 positive cells (hypoxia vs normoxia in the absence of OVA allergen (p=NS)(Fig 6). However, the combination of hypoxia and OVA allergen significantly increased the number of peribronchial TGF-β1 positive cells in a synergistic fashion as compared to OVA allergen alone (31.8 ± 3.5 vs 101.7 ±14.9 TGF-β1 positive cells/bronchus)(OVA + normoxia vs OVA + hypoxia)($p<0.0001$)(Fig 6).

3.8 Effect of hypoxia on OVA induced changes in mucus production

OVA allergen challenge alone induced a significant increase in mucus production compared to the non-OVA challenged group under conditions of normoxia (63.4 + 1.2 vs 2.0 + 0.1 PAS+ cells/bronchus)(OVA vs no OVA in normoxia)($p<0.0001$). Exposure of mice to hypoxia alone in the absence of OVA allergen induced a very slight but statistically significant increase in mucus production (5.4 + 1.9 vs 2.0 + 0.2 PAS+ cells/bronchus) (hypoxia vs normoxia in the absence of OVA)($p=0.03$). However, the level of mucus production induced by hypoxia alone (5.4 + 1.9 PAS+ cells/bronchus) was significantly less than that induced by OVA alone (63.4 + 1.2 PAS+ cells/bronchus)(OVA vs hypoxia) ($p<0.0001$). The combination of hypoxia and OVA allergen (75.3 + 1.8 PAS+ cells/bronchus) induced a significant increase in levels of mucus production compared to hypoxia alone (5.4 + 1.9 PAS+ cells/bronchus) ($p= 0.03$) but not compared to OVA (p=NS).

4 DISCUSSION

Using a mouse model simulating hypoxia in an allergen induced asthma exacerbation, we have demonstrated that the combination of hypoxia and allergen is a stronger stimulus than either stimulus alone in inducing increased lung HIF-1α expression, peribronchial inflammation (neutrophilic and eosinophilic), expression of lung chemokines (KC, eotaxin-1), and lung remodeling cytokines (TGF-β1) with resultant increased airway remodeling (peribronchial fibrosis, peribronchial smooth muscle). As hypoxia is present in over 90% of severe asthma exacerbations [1], these findings underscore how hypoxia can not only potentiate the airway inflammatory response, but also increase remodeling in severe asthma exacerbations associated with hypoxia. As exacerbations are common in severe asthmatics [30, 31], and are associated with an accelerated decline in lung function [7,8], further studies are needed to determine whether early institution of oxygen therapy in an asthma exacerbation associated with hypoxia could reduce levels of airway inflammation and subsequent remodeling which lead to decline in lung function.

We found that the combination of hypoxia and allergen is stronger than either stimulus alone in enhancing HIF-1α expression in the lung. Previous studies in mouse models undergoing allergen challenge have demonstrated under conditions of normoxia that inhibiting HIF reduces levels of inflammation and remodeling [19–22]. As HIF is induced under normoxic conditions by allergen in these [19–22] and our current study, it suggests that allergen induced inflamed tissues may be hypoxic as a result of decreased perfusion, edema, vascular insult and/or influx of oxygen-consuming immune cells [19]. Human studies have also demonstrated under normoxic conditions increased levels of HIF-1α in lung tissue and bronchial fluid of patients with asthma, and in the nasal fluid of patients with rhinitis after allergen challenge [22]. Our study extends these observations to demonstrate that under hypoxic conditions allergen challenge induces a much greater increase in HIF-1α expression compared to either stimulus alone. In addition, we demonstrate that while hypoxia predominantly induces HIF-1α expression in airway epithelium, the combination of allergen and hypoxia induces HIF-1α expression in peribronchial inflammatory cells and airway epithelium.

The importance of our demonstration of increased HIF-1α expression in response to allergen and hypoxia is underscored by the detection of increased expression of the HIF-1α regulated chemokine KC and the associated highly significant 27 fold increase in peribronchial neutrophils. This hypoxia induced potentiation of allergen induced neutrophilic inflammation may be particularly important to adults with asthma [3–5] as well as approximately 50% of children [6] in whom exacerbations are frequently associated with neutrophilic airway inflammation. In life threatening severe exacerbations of asthma (i.e. intubation for management of status asthmaticus), high levels of neutrophils and IL-8 have been detected in BAL fluid [4]. The importance of the large number of neutrophils and neutrophil derived pro-inflammatory mediators to the pathogeneses of either asthma exacerbations or remodeling awaits studies with neutrophil specific anatagonists. Studies of neutrophilic inflammation in human asthma exacerbations are also frequently confounded by variables including corticosteroid administration (which can increase neutrophilic inflammation by reducing neutrophil apoptosis)[32], or infection (which can independently provoke neutrophilic inflammation). In this mouse model, we were able to demonstrate that the combination of hypoxia and allergen (in the absence of confounding variables such as corticosteroid therapy or infection) significantly induces neutrophilic inflammation. Further studies in human asthma exacerbations are needed to determine the relative contribution of hypoxia to neutrophilic airway inflammation during an exacerbation.

Exacerbations of asthma are also known to be associated with eosinophilic inflammation in children [6] and adults [33–36]. For example asthmatics with refractory eosinophilic inflammation have significantly higher exacerbation rates [33], while eosinophil targeted therapies such as anti-IL-5 significantly reduce asthma exacerbation rates [33–36]. In this study, the absolute number of peribronchial eosinophils (155.9 + 7.5) was greater than the absolute number of peribronchial neutrophils (62.7 + 4.1), even though the combined hypoxia and allergen stimulus had its most dramatic effect on increasing peribronchial neutrophils. Studies by the Severe Asthma Network have identified that each of the five severe asthma phenotypes based on cluster analysis have high rates of asthma exacerbations (3.4-4.6 exacerbations/year) [37], compared to meta-analysis studies of mild asthmatics (0.2 exacerbations/year in mild asthmatics)[38]. Interestingly, early onset atopic severe asthma with sputum eosinophilia had the highest exacerbation rate (4.6/year)[37].

We demonstrated that hypoxia also increases levels of peribronchial eosinophilic inflammation and that this is associated with increased levels of eotaxin-1. Since eotaxin-1 is not one of the >100 genes known to be directly regulated by HIF-1α [10,11], the increased levels of eotaxin-1 may be a result of activation of either HIF-1α target genes which through downstream cascades subsequently regulate eotaxin-1 expression, and/or a HIF-1α independent effect on eotaxin-1 expression. HIF-1α is expressed in eosinophils and regulates eosinophil chemotaxis and survival [19,39]. Deletion of HIF-1α in eosinophils decreased their chemotaxis under normoxia [19]. In addition, in vitro studies of human peripheral blood eosinophils have demonstrated that hypoxic eosinophils upregulated HIF-1α expression as well as anti-apoptotic Bcl-XL protein levels more than pro-apoptotic Bax levels, and were more viable than normoxic eosinophils [39]. Thus, the increased eosinophils we have detected in lungs of mice exposed to hypoxia and allergen may be mediated by the increased eotaxin-1 we have detected, as well as by potential alternate pathways including HIF effects on eosinophil chemotaxis and survival. Increased eosinophilic inflammation may itself contribute to increased neutrophilic inflammation (through eosinophil expression of KC) as well as to airway remodeling (through increased expression of TGF-β1)[25]. We have demonstrated that the combination of hypoxia and allergen significantly increased levels of the pro-remodeling cytokine TGF-β1 as well as indices of airway remodeling, including increased peribronchial fibrosis and increased smooth muscle. We have also previously demonstrated the importance of eosinophil expressed TGF-β1 to airway remodeling in mouse models of asthma [25, 40]. Similar results have also been observed in humans with asthma in which airway eosinophil expression of TGF-β1 and indices of airway remodeling are significantly reduced by eosinophil targeted anti-IL-5 therapy [41].

In summary, this study demonstrates that hypoxia may significantly potentiate allergen induced lung HIF-1α expression, chemokine expression, airway inflammation (neutrophilic, eosinophilic), expression of the pro-remodeling cytokine TGF-β1, and indices of airway remodeling (fibrosis, and smooth muscle). Studies have shown that synergism between asthma triggers (viral infection, allergen exposure, and atopic sensitization) conferred the greatest risk of triggering severe asthma exacerbations [42]. As over 90% of severe asthma exacerbations are associated with hypoxia [1], hypoxia may be another asthma trigger contributing to synergism with allergen or viral infection. Although exposure of mice to 10% hypoxia is a standard model of investigating hypoxia in mouse models of lung disease [43], how this level of hypoxia in mice translates to in vivo studies in humans with severe asthma and hypoxia is unknown. Further study is thus needed to determine whether hypoxia contributes to increased neutrophilic and/or eosinophilic airway inflammation in human subjects with asthma. Moreover, as exacerbations of asthma are associated with an accelerated decline in lung function, further study is needed to determine whether the hypoxia induced increased remodeling in our mouse model is evident in asthmatics with exacerbations associated with hypoxia and contribute to previously observed enhanced decline in lung function associated with severe asthma exacerbations [7,8]

Acknowledgments

This research was supported by NIH AI 38425, AI 70535, and AI 72115 (D Broide), a Veterans Affairs Career Development Award-2 (LE Crotty Alexander) and a Stephen Silberstein Senior Fellowship Award from the American Asthma Foundation (V Nizet)

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HIGHLIGHTS

- OVA and hypoxia induced a 27 fold increase in peribronchial neutrophils.
- OVA and hypoxia increased KC a HIF-1α regulated CXC chemokine
- OVA and hypoxia increased peribronchial eosinophils
- OVA and hypoxia increased airway remodeling
- Hypoxia may accelerate lung function loss in asthma exacerbations

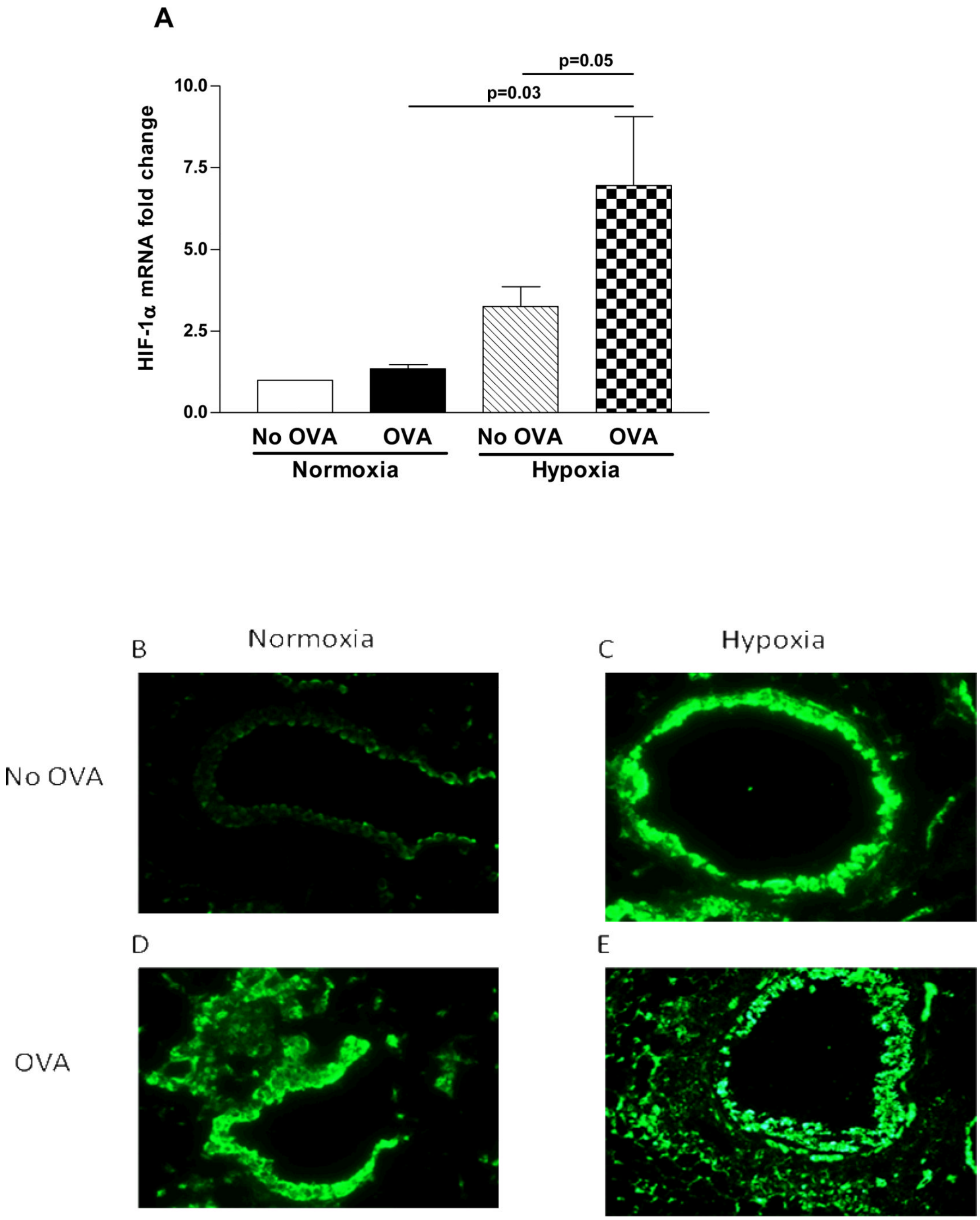

FIGURE 1. Effect of hypoxia and OVA allergen on lung HIF-1α expression

Different groups of BALB/c mice (n=8 mice/group) were sensitized and challenged with OVA allergen. Mice were then exposed to normoxia or hypoxia in exposure chambers. Mice were sacrificed 24 hours after the final OVA and/or hypoxia challenge. Lungs were processed for qPCR (to detect HIF-1α and GAPDH)(Fig 1A) and immunofluoresence microscopy (Fig 1 B–E) to detect HIF-1α expression. The combination of hypoxia and OVA allergen induced a significant 5.3 fold increase in levels of lung HIF-1α mRNA compared to OVA and normoxia (p=0.03)(Fig 1A), and a 2.2 fold studies demonstrated that epithelial cells were the predominant cell expressing increased levels of HIF-1α following exposure to hypoxia (Fig 1B). In contrast, following either OVA allergen or OVA + hypoxia exposure, peribronchial inflammatory cells as well as epithelium expressed increased levels of HIF-1α (Fig 1D–E)

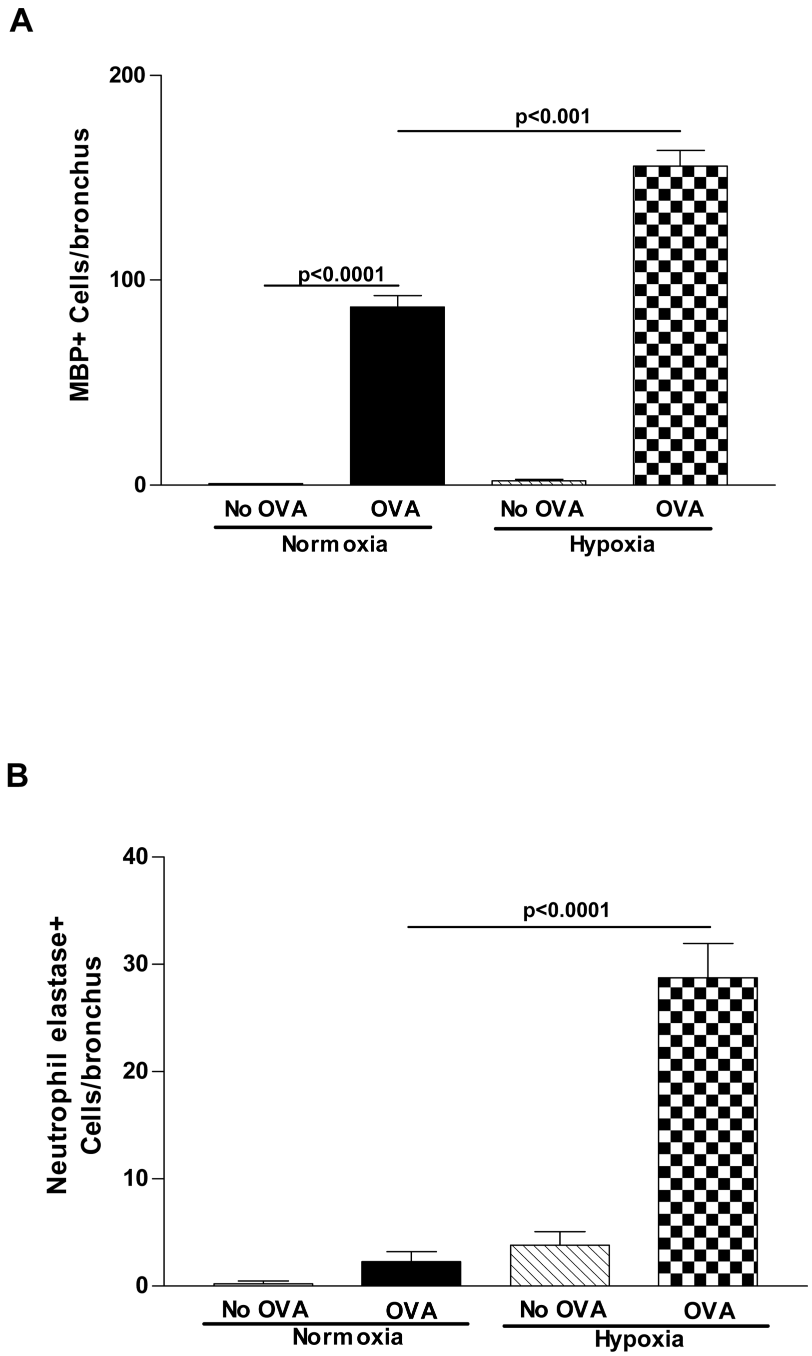

FIGURE 2. Effect of hypoxia and OVA allergen on peribronchial eosinophils and neutrophils
Different groups of BALB/c mice (n=8 mice/group) were sensitized and challenged with OVA allergen. Mice were then exposed to normoxia or hypoxia in exposure chambers. Mice were sacrificed 24 hours after the final OVA and/or hypoxia challenge. Lungs were processed for immunohistochemistry using either an anti-MBP Ab to detect eosinophils (Fig 2A) or an anti-neutrophil elasatase Ab to detect neutrophils (Fig 2B). OVA allergen challenge in normoxic conditions induced a significant increase in peribronchial eosinophils (no OVA vs OVA in normoxic conditions)($p<0.0001$)(Fig 2A), and a very slight but statistically insignificant increase in peribronchial neutrophils (p=ns)(Fig 2B). Hypoxia in combination with OVA allergen challenge increased peribronchial eosinophils (OVA in

normoxia vs OVA in hypoxia)(p<0.001)(Fig 2A) and peribronchial neutrophils (OVA in normoxia vs OVA in hypoxia) (p<0.0001)(Fig 2B)

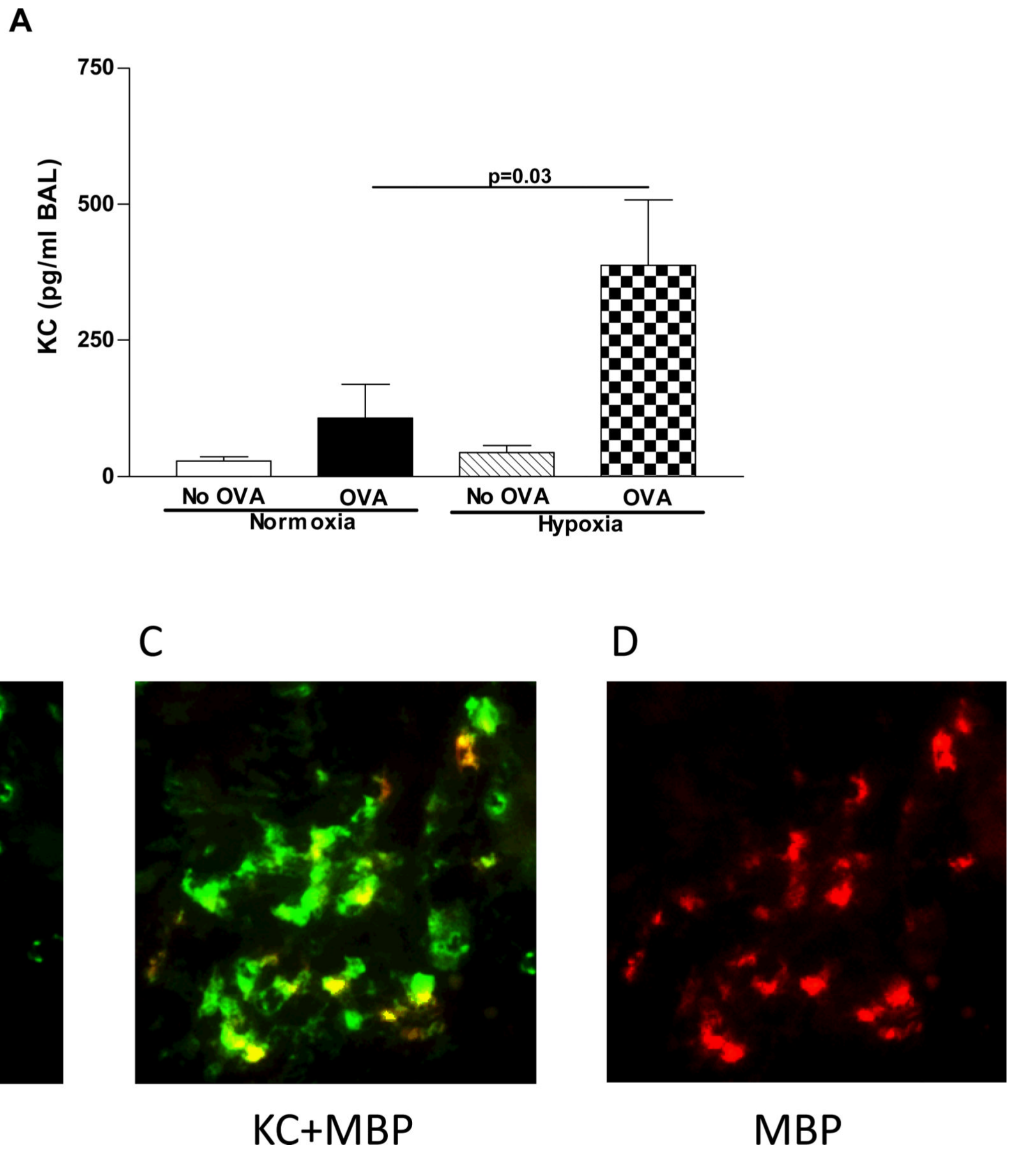

FIGURE 3. Effect of hypoxia and OVA allergen on lung KC expression

Different groups of BALB/c mice (n=8 mice/group) were sensitized and challenged with OVA allergen. Mice were then exposed to normoxia or hypoxia in exposure chambers. Mice were sacrificed 24 hours after the final OVA and/or hypoxia challenge. BAL was used to measure levels of KC by Elisa (Fig 3A), and lungs were processed for immunoflurescence microscopy using anti-KC and anti-MBP Abs (Fig 3 B–D). Hypoxia in combination with OVA allergen induced a synergistic increase in levels of BAL KC (hypoxia + OVA vs normoxia + OVA)(p=0.03)(Fig 3A).Double label immunofluorescence microscopy studies demonstrated that MBP+ eosinophils were one of the peribronchial inflammatory cells expressing KC (merged yellow)((Fig 3B).

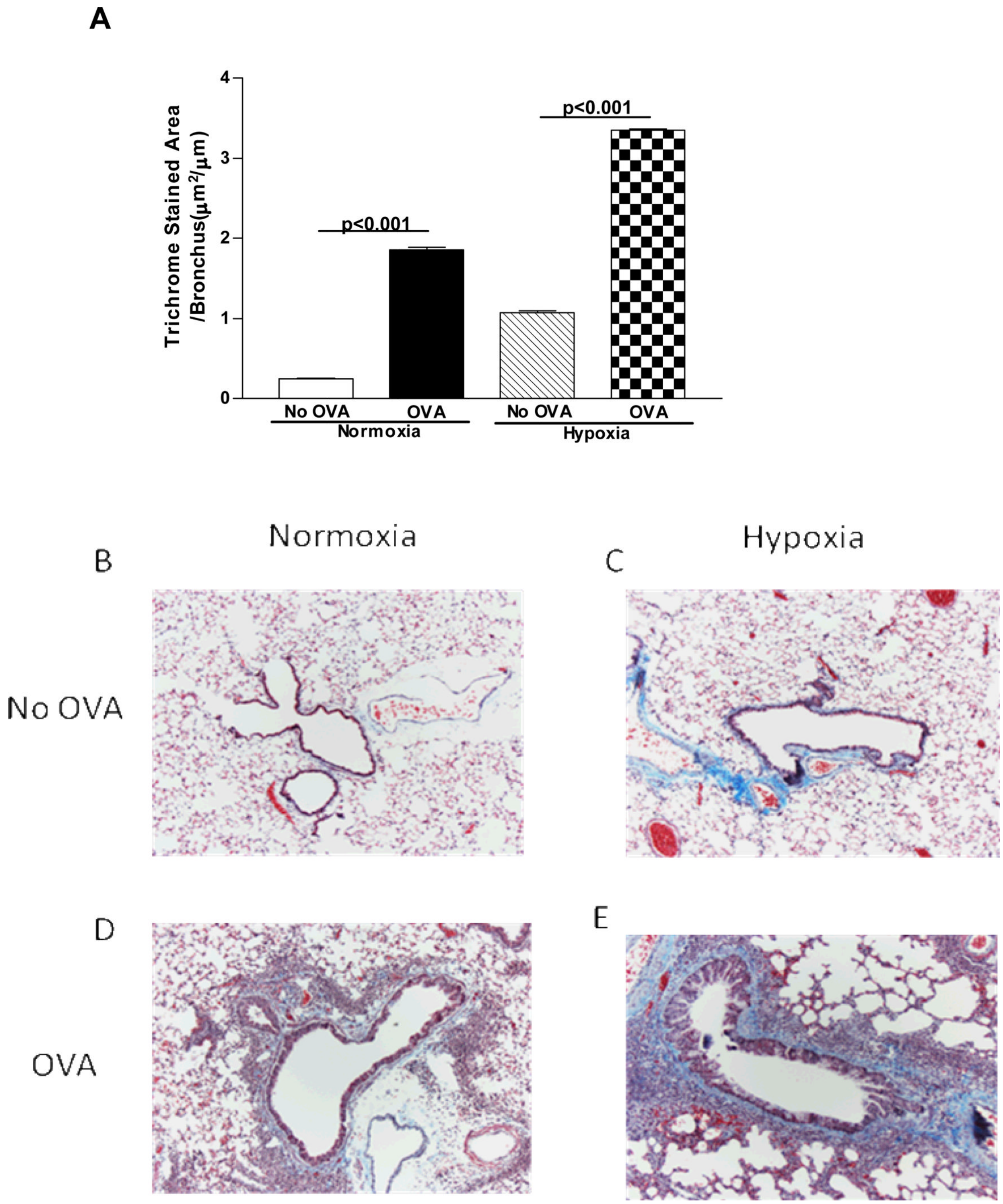

FIGURE 4. Effect of hypoxia and OVA allergen on peribronchial fibrosis

Different groups of BALB/c mice (n=8 mice/group) were sensitized and challenged with OVA allergen. Mice were then exposed to normoxia or hypoxia in exposure chambers. Mice were sacrificed 24 hours after the final OVA and/or hypoxia challenge. Lungs were stained with trichrome to detect the area of peribronchial trichrome staining as an index of collagen deposition. Exposure of mice to hypoxia alone in the absence of OVA allergen challenge induced a significant increase in peribronchial fibrosis (normoxia vs hypoxia)($p<0.001$)(Fig 4 A,C). OVA allergen challenge alone in normoxic conditions induced a significant increase in peribronchial fibrosis (no OVA vs OVA in normoxia)($p<0.001$)(Fig 4 A,B,C). Hypoxia in combination with OVA allergen challenge significantly increased levels of peribronchial fibrosis compared to either hypoxia alone ($p<0.001$)(Fig 4 A,E), or compared to OVA allergen alone ($p<0.001$)(Fig 4 A)

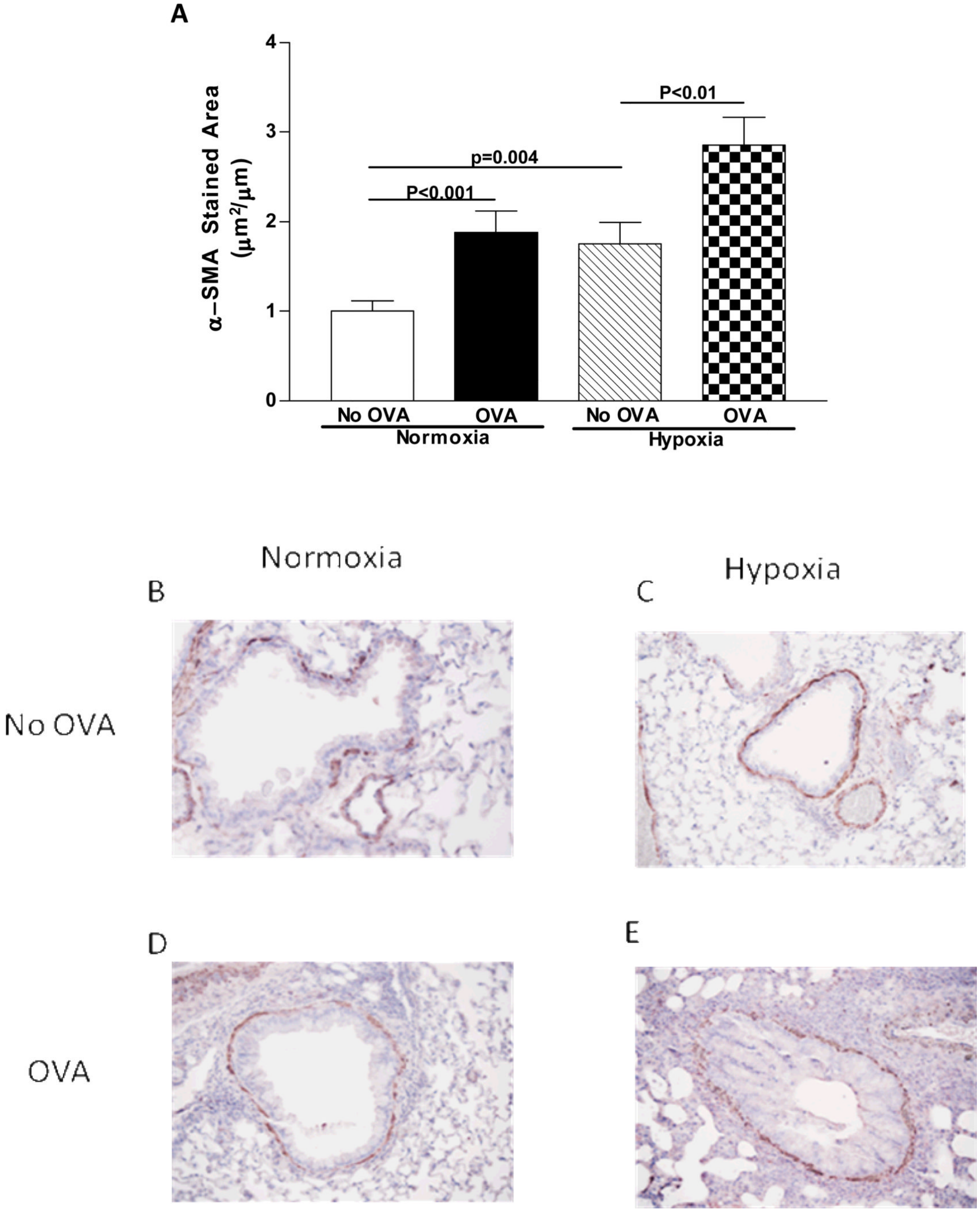

FIGURE 5. Effect of hypoxia and OVA allergen on peribronchial smooth muscle

Different groups of BALB/c mice (n=8 mice/group) were sensitized and challenged with OVA allergen. Mice were then exposed to normoxia or hypoxia in exposure chambers. Mice were sacrificed 24 hours after the final OVA and/or hypoxia challenge. Lungs were processed for immunohistochemistry with an anti-α-smooth muscle actin Ab to detect the area of peribronchial α-smooth muscle actin (α-SMA) staining. Exposure of mice to hypoxia alone in the absence of OVA allergen induced significant increases in the peribronchial α smooth muscle actin immunostained area (normoxia vs hypoxia in the absence of OVA)(p=0.004)(Fig 5 A–C). OVA allergen challenge alone also induced a similar increase in the peribronchial α-SMA immunostained area (OVA vs no OVA in normoxia)($p<0.001$) (Fig 5 A, B, D). The combination of hypoxia and OVA allergen significantly increased the area of peribronchial α-SMA staining compared to either hypoxia alone ($p<0.01$)(Fig 5 A, C, E) or compared to OVA alone ($p=0.05$)(Fig 5 A, D, E)

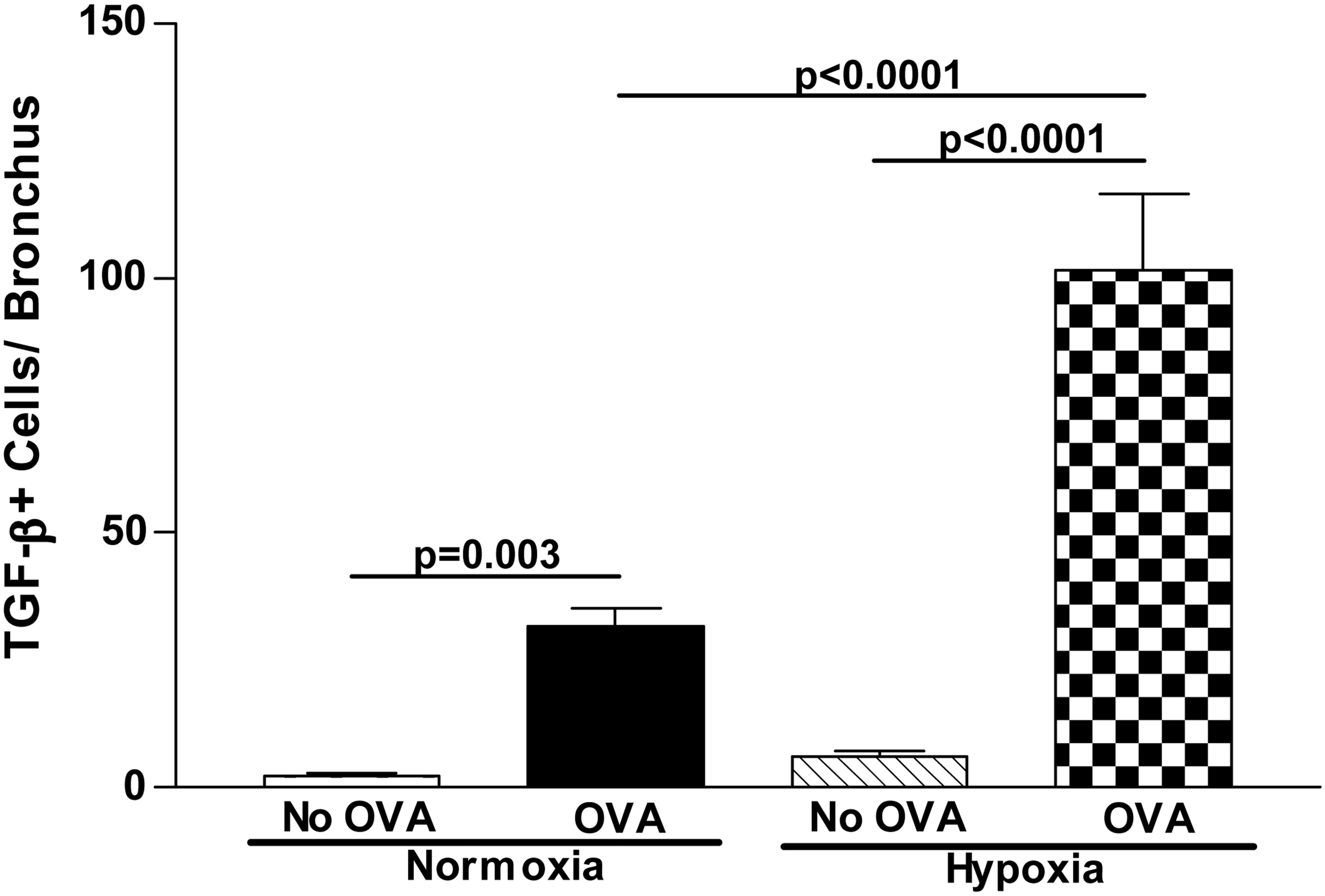

FIGURE 6. Effect of hypoxia and OVA allergen on peribronchial TGF-β1+ cells

Different groups of BALB/c mice (n=8 mice/group) were sensitized and challenged with OVA allergen. Mice were then exposed to normoxia or hypoxia in exposure chambers. Mice were sacrificed 24 hours after the final OVA and/or hypoxia challenge. Lungs were processed for immunohistochemistry to using an anti-TGF-β1 Ab to quantitate the number of peribronchial TGF-β1+ cells. OVA allergen challenge alone induced an increase in the number of peribronchial TGF-β1 positive cells (OVA vs No OVA in normoxia)(p=0.003). Exposure of mice to hypoxia alone did not increase the number of peribronchial TGF-β1 positive cells (hypoxia vs normoxia in the absence of OVA allergen (p=NS). The combination of hypoxia and OVA allergen significantly increased the number of peribronchial TGF-β1 positive cells in a synergistic fashion as compared to OVA allergen alone (OVA + normoxia vs OVA + hypoxia)(p<0.0001)