

Effects of Chronic Intermittent Hypoxia on Allergen-Induced Airway Inflammation in Rats

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Abstract

Obstructive sleep apnea aggravates asthma, but its mechanisms are unknown. Chronic intermittent hypoxia is one hallmark feature of sleep apnea. In this study, we tested the effects of chronic intermittent hypoxia on allergen-induced inflammation in rats. Four groups ($n = 9$ – 11 /group) of ovalbumin (OVA)-sensitized Brown-Norway rats underwent intermittent hypoxia (10% oxygen, 30 cycles/h, 10 h/d) or normoxia for 30 days concurrent with weekly OVA or vehicle challenges. Lung physiology, differential leukocyte counts from bronchoalveolar lavage, and histology (Picro Sirius Red staining for collagen content) were compared between groups 2 days after the last challenge. Gene expression in bronchoalveolar lavage cells was quantified by quantitative PCR. Compared with normoxia, chronic intermittent hypoxia reduced the FEV_{0.1}/FVC ratio ($P = 0.005$), peak expiratory flow ($P = 0.002$), and mean midexpiratory flow ($P = 0.004$), predominantly in medium and large airways; decreased the baseline eosinophil number ($P = 0.01$) and amplified the effect of OVA on monocyte number ($P = 0.02$ for the interaction); in proximal airways, increased ($P = 0.008$), whereas in distal airways it decreased ($P = 0.004$), collagen density; induced qualitative emphysematous changes in lung periphery; and increased expression of the M2

macrophage marker YM-1 and augmented OVA-induced expression of plasminogen activator inhibitor-1. Chronic intermittent hypoxia alters immune response to allergen toward a more T_H-1–predominant cellular phenotype with collagen deposition and matrix degradation, leading to airflow limitation. These findings highlight the potential of sleep apnea to aggravate airway dysfunction in patients with preexistent asthma.

Keywords: airway remodeling, immunology; disease models, animal; fibrosis; lung immunology, metabolism, pathology; rats

Clinical Relevance

Obstructive sleep apnea worsens asthma control, but mechanisms are unknown. The effects of chronic intermittent hypoxia on the lower airway during allergen-induced inflammation are unknown. We show that chronic intermittent hypoxia exposure during allergen challenge induced alterations in the lower airway immune responses and heterogeneous structural changes, culminating in physiologic deficits.

Asthma is a chronic inflammatory disease of the airways that poses an increasing burden to our society owing to its rising prevalence and failure of current therapies to

reduce morbidity and mortality (1). Its pathophysiology includes lower airway inflammation, airway basement membrane thickening, and, in some cases, small airway

disease and parenchymal destruction (2). There is increased recognition that obstructive sleep apnea (OSA) is more prevalent in patients with asthma compared

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with healthy control subjects (3), particularly (88–95%) among those with difficult-to-control disease (3, 4). Furthermore, evidence is mounting that OSA worsens asthma control (5) and is a risk factor for exacerbations (6), and that its treatment with continuous positive airway pressure (CPAP) improves asthma outcomes (7).

The underlying mechanisms of OSA-related aggravation of asthma remain unstudied, but OSA may alter the asthmatic lower airway inflammation and remodeling. OSA is characterized by chronic intermittent hypoxia (CIH), and increased work of breathing against the upper airway occlusion which leads to sleep fragmentation. These features occur concurrently in humans, and completely separating their effects on the lower airway is impossible, but they all hold the potential to exacerbate the asthmatic airway. For example, CIH has been clearly demonstrated to induce inflammation and remodeling in the vasculature, which underlie the atherosclerosis and cardiovascular consequences of OSA (8). The sequence of desaturation–reoxygenation produces reactive oxygen species (ROS) and oxidative stress (8). ROS activate the NF- κ B-mediated inflammatory cascade, leading to release of systemic cytokines and chemokines (9), some of which are decreased by CPAP (8). Likewise, ROS formation and NF- κ B up-regulation lead to peripheral neutrophil activation (10). CIH also activates blood monocytes and up-regulates their ability to penetrate blood vessel walls and invade surrounding tissues (11). In the airway of patients with OSA, high concentrations of markers of oxidative stress—TNF- α , IL-6, and IL-8—have been found that correlated with OSA severity and decreased with CPAP (12, 13). Furthermore, a neutrophilic inflammation in the lower airway of patients with OSA has been confirmed with induced sputum (14, 15). This neutrophil fraction likewise correlated with OSA severity (15). However, how CIH modulates the inflammatory and remodeling processes associated with asthma remains unknown.

We hypothesized that CIH causes a shift toward TH-1 cellular airway inflammation and alters remodeling to exacerbate airway dysfunction in response to allergen in rats. Indeed, we report that CIH increased monocyte invasion and

matrix remodeling in the airways and parenchyma and led to detriments in airway function. These results underscore the potential of OSA to adversely affect pulmonary disease. Preliminary results of this study have been published in abstract form (16, 17).

Materials and Methods

All procedures were approved by the University of Wisconsin Animal Care and Use Committee.

Animals

Brown Norway (strain 091) male rats, 51 to 75 days old (9–11/group), from Charles River Laboratories (Wilmington, MA) were housed two to four per cage. Standard rat chow and drinking water were provided *ad libitum*.

Experimental Protocol

The experimental design is depicted in Figure 1A. All rats were sensitized to ovalbumin (OVA) 14 days before the start of the experiment. On Day 0, animals were challenged with nebulized OVA or vehicle (VEH) ($1 \times$ PBS) after which the CIH or normoxia (NORM) exposures were initiated, such that there were four experimental groups: NORM/OVA, CIH/OVA, NORM/VEH, and CIH/VEH. Challenges were repeated on Days 7, 14, 21, and 28. Two days after the last challenge, outcomes (pulmonary function tests, bronchoalveolar lavage [BAL], and tissue collection) were measured. This time point was chosen based on the demonstrated peak of inflammatory response to OVA (18).

OVA Sensitization and Aerosol Challenge

Details are provided in the online supplement.

CIH Exposure

CIH exposure was performed essentially as described (19). Further details are provided in the online supplement.

Pulmonary Function Tests

Rats were anesthetized by intraperitoneal injection of urethane (1.5 g/kg body weight), intubated periorally, and placed in a full-body plethysmograph (Buxco Electronics, Wilmington, NC). FEV and functional residual capacity maneuvers were performed as described (20).

BAL and Cytospin Preparation

After pulmonary physiology, the rats were killed under deep anesthesia, and an endotracheal catheter was placed. BAL was collected and processed as presented in the online supplement.

Lung Histology

Periodic acid-Schiff (PAS), Picro Sirius Red, and hematoxylin and eosin staining for goblet cell hyperplasia, collagen deposition, and assessment of parenchyma integrity, respectively, were performed. Processing of the lungs and acquired images is detailed in the online supplement.

BAL Cells Quantitative Gene Expression

BAL cells from CIH/OVA ($n = 8$), CIH/VEH ($n = 5$), normoxia (NORM)/OVA ($n = 9$), and NORM/VEH ($n = 8$) rats were lysed in 0.5 ml TRI reagent. RNA was

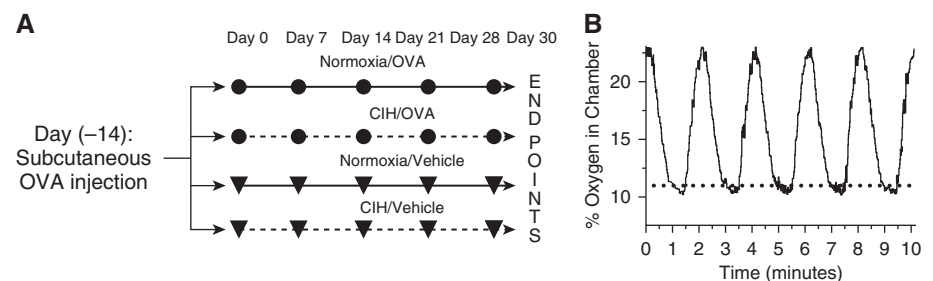


Figure 1. Experimental model. (A) Schematic of experimental design. Two weeks after ovalbumin (OVA) sensitization, rats were subjected to chronic intermittent hypoxia (CIH) (dashed line) or normoxia (solid line) for 30 days with aerosolized challenges of OVA (circles) or vehicle (triangles) on indicated days. Endpoint measurements and death occurred 48 hours after the last challenge. (B) Oxygen content in the CIH exposure chamber was measured over the course of several cycles with an oxygen sensor placed on the bottom of the chamber. Animals were exposed to 30 seconds of 10 to 11% O₂ during every 120-second cycle. Dashed line, 11% O₂.

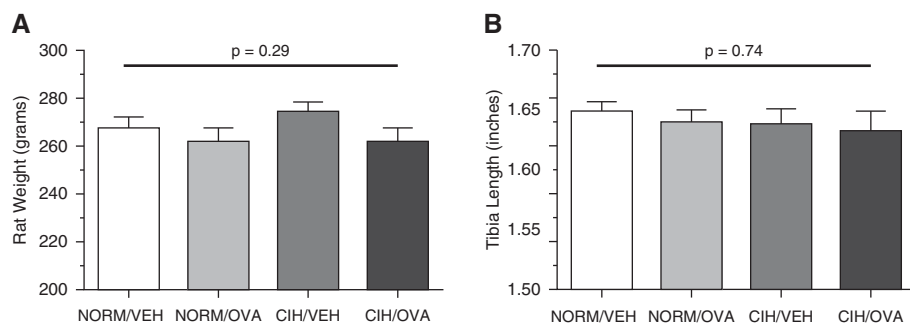


Figure 2. Experimental treatment did not affect rat weight or growth. (A) Rat weight (grams) on Day 30. (B) Length of right tibia (inches) of the rats on Day 30. Bars: mean $\pm$ SEM. *P* values describe the effect of any experimental treatment. NORM, normoxia; VEH, vehicle.

isolated according to the manufacturer's protocol and reverse transcribed with MMLV Reverse Transcriptase (Promega, Madison, WI) and random hexamer primer. Plasminogen activator inhibitor (PAI)-1, YM-1, lysyl oxidase (LOX), and cyclophilin A expression were measured by quantitative PCR using SYBR Green reagents and intron-spanning primer pairs. Fold differences in gene expression were calculated as $2^{-\Delta\Delta Ct}$ using cyclophilin A as the housekeeping gene and one of the

NORM/VEH animals as a reference sample. Gene-specific primer sequences are given in Table E1 in the online supplement.

Data Analysis

For quantitative histology, airways were grouped by size in bins of 500- μ m increments of basement membrane perimeter and analyzed within the size bins with experimental treatment as independent variable. Graphing and statistical analyses were performed with

Prizm software (GraphPad Software, La Jolla, CA). Data were analyzed with ANOVA models (one-way for effect of experimental treatment or two-way for effects of OVA, CIH, and OVA-CIH interaction) for data conforming to parametric assumptions and Kruskal-Wallis test for nonparametric data. Sidak or Dunn's *post hoc* tests were used to compare individual group means for parametric and nonparametric data, respectively. Multiple comparison-adjusted *P* values are reported. A repeated measures model was used to compare expiratory flow rate over the range of lung volumes during a FEV maneuver. The significance level was set at 0.05. Trends were noted for *P* values of 0.05 to 0.10.

Results

Experimental Treatments Did Not Affect Size or Weight of Rats

There were no group differences in weight ($P = 0.29$) (Figure 2A) or right tibia length ($P = 0.74$) (Figure 2B) at the time of outcome measurement. Because the weight of the animal may be influenced by changes in fat content without growth in other tissues, lung physiologic parameters were normalized to total lung capacity (TLC) or tibia length.

CIH Induced Airflow Limitation Independent of Allergen Exposure

CIH induced expiratory airflow limitation in OVA- and VEH-challenged rats, as evidenced by decreases in $FEV_{0.1}/FVC$ ($P = 0.004$, two-way ANOVA) (Figure 3A), peak expiratory flow (PEF) ($P = 0.002$) (Figure 3B), and maximum midexpiratory flow (MMEF) ($P = 0.001$) (Figure 3C). Allergen exposure did not have a statistically significant effect on any of these parameters ($P = 0.60$, 0.60 , and 0.80 , respectively).

To get a better idea of the size of the airways affected, expiratory flow during the FEV maneuver was plotted against lung volume expressed as a fraction of TLC. Compared with NORM/VEH-exposed rats, CIH/OVA-exposed rats had significantly reduced expiratory airflow at lung volumes of 90 to 40% of TLC (Figure 3D). In addition, CIH/VEH compared with NORM/VEH animals had significantly reduced expiratory air flow at 80 to 50% of TLC. Thus, the airway limitation in CIH-exposed rats seems to involve, primarily,

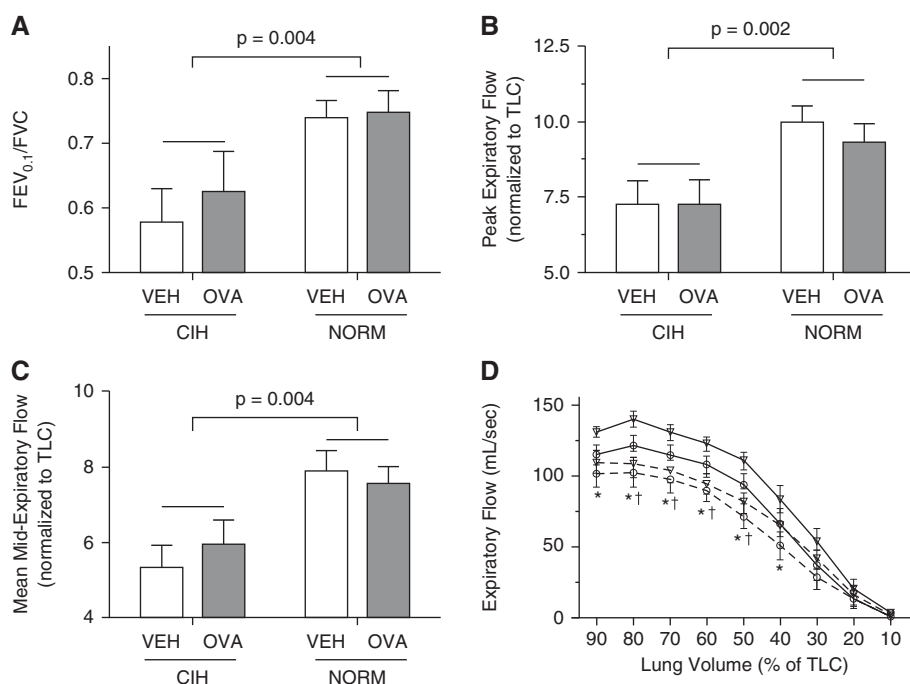


Figure 3. Chronic intermittent hypoxia (CIH) induced airflow limitation independent of allergen. (A) $FEV_{0.1}/FVC$. (B) Peak expiratory flow normalized to total lung capacity (TLC). (C) Mean midexpiratory flow normalized to TLC. Bars: mean $\pm$ SEM. Accolades: general effect of CIH versus normoxia (NORM) (two-way ANOVA). (D) Expiratory flow during forced expiration maneuver plotted against lung volume as a percentage of TLC. * $P < 0.05$, OVA/CIH versus VEH/NORM. † $P < 0.05$, VEH/CIH versus VEH/NORM (Sidak *post hoc* tests after two-way repeated measures ANOVA). Open circles: OVA; open triangles: VEH; dashed line: CIH; solid line: NORM.

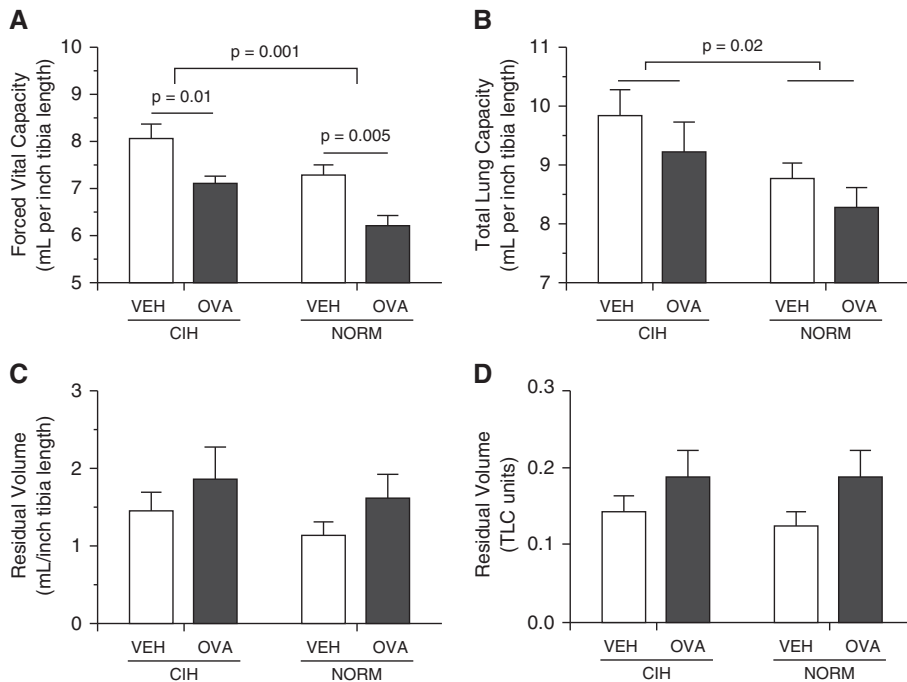


Figure 4. CIH increased FVC and TLC. (A) FVC normalized to tibia length. (B) TLC normalized to tibia length. (C) Residual volume (RV) normalized to tibia length. (D) RV normalized to TLC. Bars: mean $\pm$ SEM. Accolades: differences between group means (Sidak *post hoc* tests) or general effect of CIH (two-way ANOVA, A and B).

the proximal, large-, and medium-size airways.

CIH Increased FVC and TLC Independent of Allergen

Compared with NORM, CIH led to an increase in FVC (Figure 4A), regardless of OVA or VEH exposure ($P = 0.001$; two-way ANOVA). Likewise, TLC was increased by CIH ($P = 0.02$; two-way ANOVA) (Figure 4B). For CIH- and NORM-exposed rats, there was a trend toward a decreased TLC after OVA compared with VEH (Figure 4B), but this effect did not reach statistical significance ($P > 0.49$ for all comparisons).

Neither CIH ($P = 0.40$) nor OVA ($P = 0.20$) had a significant effect on the residual volume (RV) (Figure 4C). RV as a fraction of TLC (RV/TLC) (Figure 4D) was likewise unaffected by CIH ($P = 0.80$ for overall effect of CIH). This suggests that the expiratory flow limitation was not associated with increased air trapping.

CIH Reduced BAL Eosinophils and Amplified the OVA-Induced Increase in BAL Monocytes

CIH induced a 3-fold decrease in BAL eosinophils in VEH-challenged rats

(CIH/VEH versus NORM/VEH; $P = 0.03$) (Figure 5B). This effect was not present in OVA-challenged animals (CIH/OVA versus NORM/OVA; $P = 0.5$) (Figure 5B). Regarding BAL monocytes, we observed a significant interaction between CIH and OVA ($P = 0.02$) (Figure 5C): in CIH-exposed rats, OVA induced a 16.6-fold increase ($2.98 \pm 0.62 \times 10^4$ OVA/CIH versus $1.79 \pm 0.56 \times 10^3$ VEH/CIH [mean $\pm$ SEM]), whereas in NORM-exposed rats, OVA increased monocyte numbers only by 5.2-fold ($1.72 \pm 0.43 \times 10^4$ OVA/NORM versus $3.29 \pm 0.75 \times 10^3$ VEH/NORM). Thus, CIH exposure amplified the effect of OVA challenge on monocyte numbers by 3.2-fold on average. CIH had no effect on BAL neutrophil ($P = 0.80$) (Figure 5D), macrophage, or lymphocyte numbers (data not shown) in VEH- or OVA-challenged animals.

CIH Did Not Affect the Number of PAS-Positive Goblet Cells in Airway Epithelium

CIH did not affect PAS-positive goblet cell numbers in lower airway epithelium (Figure 6), regardless of airway size, suggesting that CIH-induced airflow limitation is not due to mucus

overproduction. OVA-challenged rats, compared with VEH-challenged rats, displayed a clear increase in the numbers of PAS-positive cells in all airway size bins, except for the largest one (1,501–3,000 μ m in perimeter), where the number of airways analyzed was ≤ 20 /group.

CIH Altered Collagen Deposition in the Airways Differentially by Airway Size and in the Parenchyma

On Picro Sirius qualitative stain images, in CIH-exposed animals, the fluorescence intensity of the airway and peribronchial alveolar septa were notably increased (Figure 7A). In quantitative analysis in medium (501–1,000 μ m perimeter) airways, CIH induced a 2- and 3-fold increase in integrated density (area $\times$ average fluorescence intensity) of the airway wall in VEH and OVA animals, respectively (Figure 7B). There was a significant effect of CIH ($P = 0.008$) on the integrated density of peribronchial collagen in medium airways. In contrast, in the small (0–500 μ m perimeter) airways, OVA induced a significant decrease in the integrated density in CIH-exposed animals but not in NORM-exposed animals (Figure 8A). There was a trend for interaction between CIH and allergen effects ($P = 0.07$).

In the lung parenchyma, CIH qualitatively increased the appearance of emphysema-like changes in alveolar size. This effect was especially pronounced in the lung periphery (Figure 8B).

These findings suggest that CIH has a profibrotic effect in more proximal, medium airways, whereas in distal lung OVA and CIH may have a synergistic effect on matrix collagen degradation.

CIH Increased the Expression of Profibrotic Effector PAI-1 in BAL Cells

CIH enabled the OVA-induced increase in PAI-1 expression. BAL cells from CIH/VEH- and NORM/OVA-treated animals did not exhibit PAI-1 expression levels different from NORM/VEH-treated animals (Figure 9B). In contrast, in CIH/OVA animals, PAI-1 expression was increased 20.04 ± 9.74 -fold (mean $\pm$ SEM) over NORM/VEH ($P = 0.04$) and 12.07 ± 0.56 -fold over NORM/OVA ($P = 0.04$).

CIH Increased the Expression of the Profibrotic Marker YM-1 in BAL Cells

Compared with NORM, regardless of allergen exposure, CIH significantly

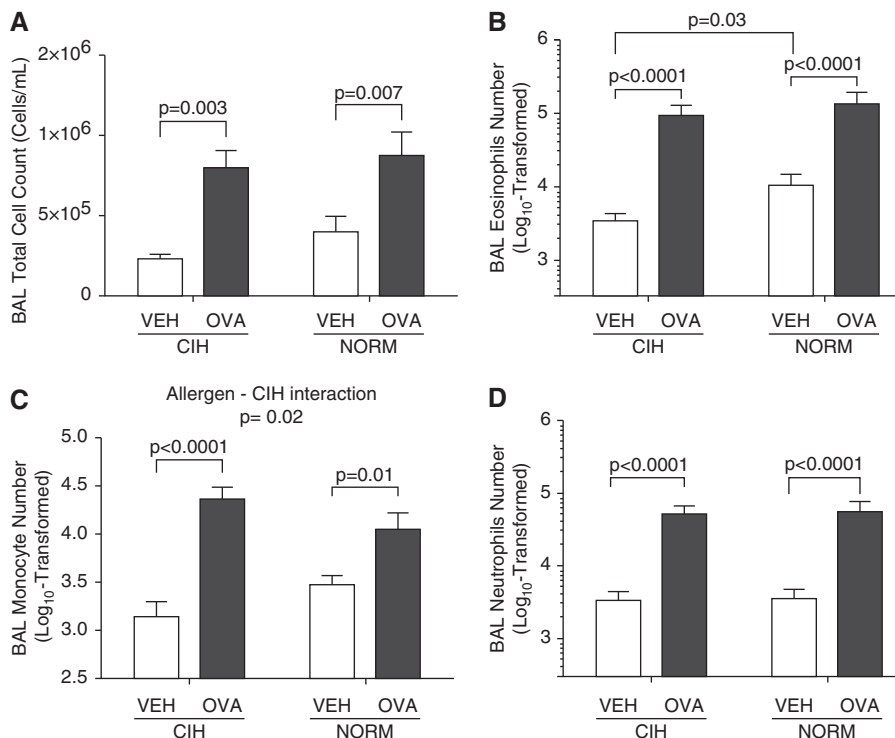


Figure 5. Differential leukocyte counts in bronchoalveolar lavage (BAL) fluid. (A) Total BAL cell counts per 1 ml of BAL fluid. (B–D) Log₁₀ of total eosinophil (B), monocyte (C), and neutrophil (D) counts. Bars: mean ± SEM. Accolades: differences between group means (Sidak *post hoc* tests). Significance of effect of allergen/CIH interaction (C) (two-way ANOVA) is specified.

increased the expression of alternatively activated macrophage marker YM-1 in BAL cells ($P = 0.04$) (Figure 9A). The effect of

CIH was more pronounced in VEH-treated ($P = 0.03$) than in OVA-treated ($P = 0.90$) groups.

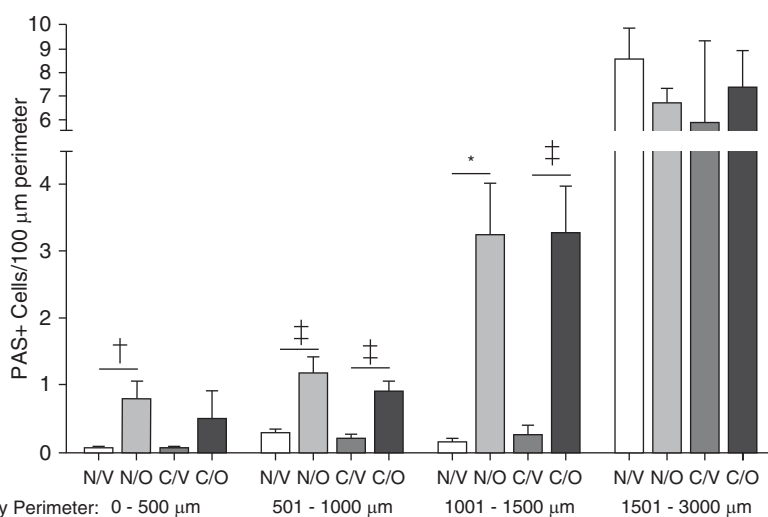


Figure 6. CIH did not increase the numbers of mucin⁺ goblet cells. Number of positively stained goblet cells (PAS stain) normalized to airway perimeter (mean ± SEM by treatment group). C/V, CIH/VEH; C/O, CIH/OVA; N/O, normoxia/OVA; N/V = NORM/VEH. Bars: mean ± SEM. * $P < 0.05$, † $P < 0.01$, and ‡ $P < 0.001$ (Dunn's multiple comparison test after Kruskal-Wallis test within airway size bin).

CIH Did Not Affect the Expression of LOX and Matrix Metalloproteinases in BAL Cells

The expression of LOX was not affected by CIH (Figure E2), contrary to previous reports of increased LOX expression in other organs (21). CIH also did not alter the expected OVA-induced increase in the expression of matrix metalloproteinases 9 and 12 (Figures E2A and E2B).

Chronic Allergen Challenge Produced the Expected Effects on Lung Physiology and BAL Cell Content

As expected, with chronic challenge (22), OVA compared with VEH resulted in a significant decrease in FVC ($P = 0.0001$) (Figure 4A). The effects of OVA and CIH did not significantly interact ($P = 0.70$). OVA compared with VEH decreased inspiratory capacity/TLC ($P = 0.02$) (Figure E3A) and tended to increase RV/TLC ($P = 0.07$) (Figure 4D). In BAL, we observed a dramatic increase in total BAL cells (Figure 5A) and in total numbers of BAL eosinophils, monocytes, neutrophils (Figures 5B–5D), lymphocytes, and macrophages (Figures E3B and E3C).

Discussion

Our study is the first to test the effects of CIH on the lower airway during allergen-induced challenge with a combination of physiological, histological, and molecular approaches. We found that CIH, independent of OVA, decreased FEV_{0.1}/FVC, PEF, and MMEF (Figures 3A–3C), predominantly in large and medium airways (Figure 3D); decreased the numbers of eosinophils and amplified the effect of OVA on monocyte numbers (Figure 5); and increased bronchial collagen density in medium-sized airways (Figure 7) coincident with matrix degradation in the distal airways and parenchyma (Figure 8). These findings suggest that CIH of OSA may alter the airway inflammation toward monocyte/macrophage-predominant phenotype and induces matrix remodeling that leads to airflow obstruction and thus may exacerbate asthma.

We found that CIH induced airflow limitation independent of allergen exposure, as denoted by the significant reduction in FEV_{0.1}/FVC, PEF, and MMEF, predominantly in the large and medium-sized airways (Figure 3). To our knowledge,

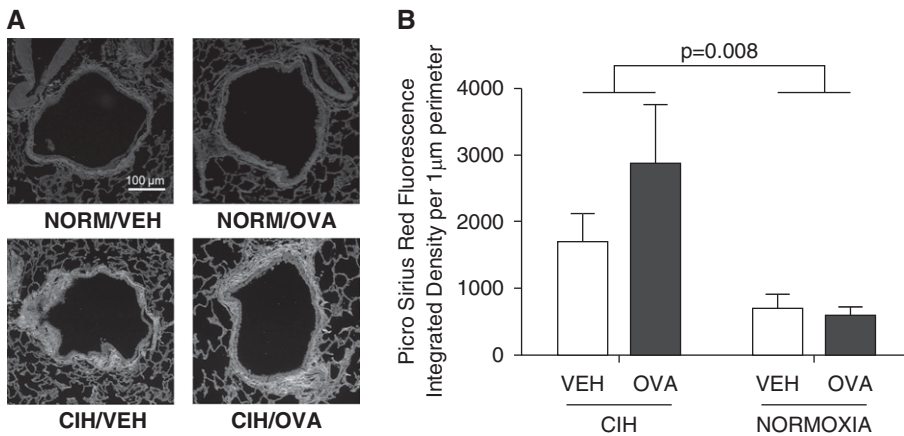


Figure 7. CIH increased bronchial collagen density in proximal 501 to 1,000 μm perimeter airways. (A) Representative images (Picro-Sirius Red fluorescence, recolored *black* and *white*) of airways in cross-section. Scale bar, 100 μm . (B) Integrated density of Picro-Sirius Red fluorescence in airway wall normalized to airway perimeter (mean $\pm$ SEM). Bars: mean $\pm$ SEM. Accolade: general effect of CIH (two-way ANOVA).

no other investigations have assessed the effects of CIH exposure on airway physiology. We reasoned that airflow limitation in our model could result from augmentation of mucus production, airway thickening/remodeling, early distal airway closure, or reduced elastic recoil due to emphysema-like formation. We found no effects of CIH on mucus cell hyperplasia on PAS staining (Figure 6). However, in the larger airways, CIH increased bronchial collagen density, whereas in the distal airways, CIH decreased the collagen density in OVA-exposed animals (Figure 8A). In addition, in the CIH-exposed animals,

more proximally in the lungs, we found qualitative evidence of thickened peribronchial septa (Figure 7A), whereas in the lung periphery we noted emphysema-like formations (Figure 8B). Although we did not see changes in the degree of air trapping with CIH (Figure 4D), these alterations may affect airway resistance and parenchymal elastance, which could be measured in future studies.

We also observed an increase in TLC (Figure 4B), consistent with the work of Reinke and colleagues, who found increased TLC (measured by water displacement) but also markers of lung growth in CIH-

exposed mice (23). Collectively, these studies provide robust evidence that CIH has a direct and detrimental effect on lung function, perhaps inducing “dysfunctional” lung growth and/or remodeling.

In our model, it appears that one mechanism of CIH-induced expiratory flow limitation is increased density of bronchial collagen in medium airways (Figures 7A and 7B). Although we are the first to show evidence of independent effects of CIH on airway fibrosis, there is a growing body of literature implicating hypoxia-induced signaling in the pathogenesis of fibrosis (24, 25). Hypoxia-inducible factor (HIF)1 α , a master orchestrator of cellular responses to hypoxia, regulates collagen secretion (26), hydroxylation (27), and cross-linking (27) in various tissues. In rodents, HIF-1 α expression coincides with the time course of fibrotic changes after paraquat-induced lung injury (28) and bleomycin-induced lung injury (29). Similarly, Sun and colleagues found that HIF-1 α expression was positively correlated with airway wall thickness in OVA-challenged mice (30). One such HIF-1 α -regulated effector is PAI-1, which plays a central role in airway remodeling (31). Knockdown of HIF-1 α inhibited TGF- β -mediated expression of PAI-1 (29) in alveolar macrophages. Our finding of markedly increased PAI-1 expression in BAL cells of CIH/OVA-exposed rats (Figure 9A) is suggestive of hypoxia modulating PAI-1 expression and supports the role of PAI-1 in CIH-induced airway fibrosis.

CIH decreased BAL eosinophils in VEH-challenged rats (Figure 5B) and amplified the OVA-induced increase in BAL monocytes (Figure 5C). Although the presence of eosinophils in the lung tissue and the mechanisms underlying reduced BAL eosinophils remain to be investigated, we speculate that CIH may up-regulate eosinophil death, either through apoptosis or degranulation, or down-regulate eosinophil chemotaxis through HIF-2 α activation (32). The mechanisms underlying the increased presence of monocytes in the BAL and their role in CIH/OVA rats are also unknown. Their enhanced recruitment likely represents a synergistic effect of allergen-induced and hypoxia-induced chemotaxis. There is ample literature on the interaction between hypoxia-induced pathways, monocyte chemotaxis, and airway remodeling/fibrosis. Hypoxia induces the expression of

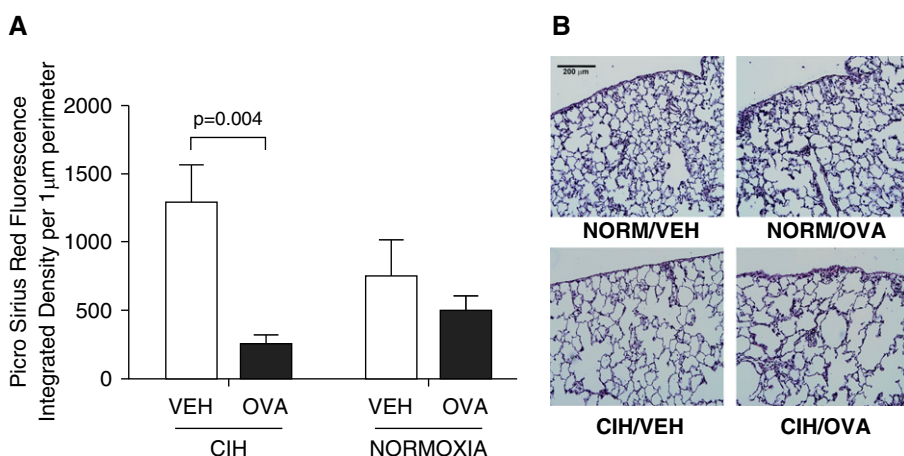


Figure 8. CIH amplified the OVA induced loss of bronchial collagen in distal (1–500 μm perimeter) airways and caused qualitative emphysema-type changes in peripheral lung. (A) Integrated density of Picro-Sirius Red fluorescence in distal airway wall normalized to airway perimeter (mean $\pm$ SEM). Bars: mean $\pm$ SEM. Accolade: difference between group means (Sidak *post hoc* test). (B) Representative images (hematoxylin and eosin stain) of lung periphery. Original magnification: $\times 20$. Scale bar, 200 μm .

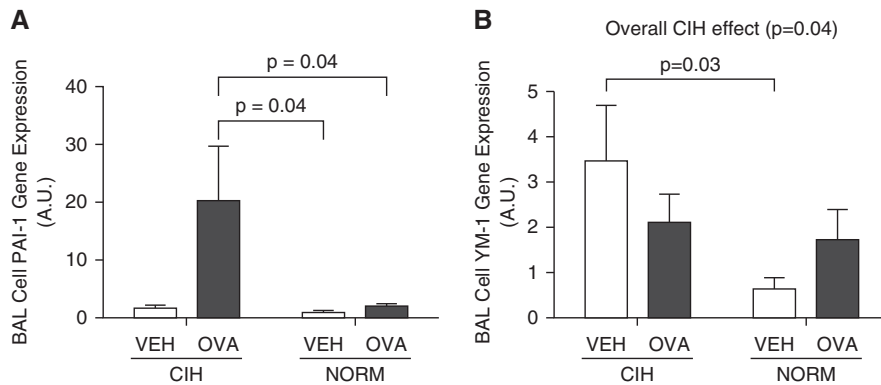


Figure 9. CIH increased profibrotic gene expression in BAL cells. Average plasminogen activator inhibitor (PAI)-1 (A) and YM-1 gene expression (B) in BAL cells. A.U., arbitrary units (fold difference in gene expression compared with one of the VEH/NORM samples). Bars: mean $\pm$ SEM. Accolades: difference between group means.

monocyte chemoattractants, such as hypoxia-induced mitogenic factor-1 (33), and macrophage migration inhibitory factor (34). Once in the lung, monocytes may differentiate into fibroblasts, which could contribute to airway wall thickening (35), or into macrophages. Macrophages can differentiate into distinct phenotypes that either inhibit or promote tissue repair and fibrosis (36). In particular, M2 or “alternatively” activated macrophages secrete a host of profibrotic effectors (37) that promote fibroblast differentiation into collagen-synthesizing myofibroblasts (38). The relative prevalence of M2 and M1 macrophages in tissue is important to the regulation of these processes. In the present study, we observed CIH-induced up-regulation in YM-1 expression (Figure 9B). YM-1 is a hallmark of M2 macrophage differentiation (39), suggesting a higher prevalence of M2 macrophages in the lungs of our CIH-treated animals. This is consistent with the recent work of Almendros and colleagues, who demonstrated a CIH-induced increase in M2 tumor-associated macrophages (40). YM-1 is a member of a highly conserved chitinase-like family of proteins, which have been postulated to regulate innate

immune responses (41) and tissue repair. Indeed, YKL-40, a homolog of YM-1, was up-regulated in sera and lung biopsies of a subgroup of patients with asthma, in relationship with asthma severity and with epithelial basement membrane thickness (42). These reports underscore the importance of elucidating the role of chitinase-like proteins in tissue remodeling and specifically in our model.

This study has several limitations. Although the CIH protocol in this study was chosen to model the severity of OSA observed in patients with asthma (3), the 30-day duration of CIH may have been too short. However, a longer duration of OVA challenges might have resulted in OVA tolerance (43). Longer (6–8 wk) studies, using allergens with less tolerogenicity, such as house dust mites (44), will enhance the clinical relevance of this model. Second, given the central-peripheral heterogeneity of structural changes observed, we were unable to quantify all of them. Likewise, we were unable to separate their individual contributions to the airway dysfunction observed. Future studies aimed at quantification of these structural abnormalities using imaging or whole lung *in vivo* morphometry (45) and

their functional correlate via compartment-specific pulmonary physiology (46) are needed. Last, our gene expression analysis focused on BAL cells. Future work needs to take into account the CIH effects on gene and protein expression in other cellular compartments, such as airway epithelium and fibroblasts.

In summary, we found that 30 days of CIH exposure in a rat model of allergen-induced airway inflammation resulted in alterations in the airway immune response, which led to heterogeneous structural effects of collagen deposition and matrix remodeling of the airways and lung parenchyma, culminating in physiologic deficits. These changes are concerning because, if they occur in patients with asthma, they would not be responsive to current standard treatments and may be irreversible. In fact, the lack of improvement in FEV₁ noted in studies of patients with asthma with OSA after treatment with CPAP (47, 48) supports this argument. This study calls for urgent, well-designed, prospective investigations aimed at understanding the reciprocal relationship of OSA with asthma and at deepening our knowledge of its mechanistic basis. In addition, these findings emphasize a need for accounting for OSA in asthma studies, which may allow better separation of asthma phenotypes and speed the discovery of more effective and targeted treatments. Moreover, these findings may be of high relevance to other common diseases featuring an interaction with OSA, such as COPD (49) and pulmonary fibrosis (50). ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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Online Data Supplement

Title: Lower Airways Effects of Exposure to Chronic Intermittent Hypoxia during Allergen Challenge in Rats

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Materials and Methods

Ovalbumin sensitization and aerosol challenge: Animals were sensitized with 1mg/mL subcutaneous ovalbumin (Grade V, Sigma-Aldrich, St. Louis, MO) and 25mg/mL ImjectAlum® (Thermo Scientific, Waltham, MA), in 0.9% sterile saline. For challenges, rats were exposed for 25 min either to aerosolized OVA (1% weight /volume ovalbumin in 1x PBS) or to sterile 1x PBS (VEH). The aerosol was generated by an Ultraneb 200 Nebulizer (Nouvag, Lake Hughes, CA) with an air flow of 2L/min and median particle size of 3.5 µm according to the manufacturer.

Chronic Intermittent Hypoxia (CIH) exposure: was similar to our previous report (1). Rats in their home cages were placed into a Plexiglass chamber and exposed to intermittent hypoxia for 10 h/day (from 0600 to 1600 h). Oxygen concentrations in the chamber were monitored using a heated zirconium sensor (Fujikura America, Pittsburgh, PA) connected to solenoid valves that controlled the flow of oxygen and nitrogen. The valves were operated by a microprocessor-controlled timer. Rats were exposed to 30 seconds of 10 - 11% inspired oxygen every 2 minutes (Figure 1B). Control rats were housed under normoxic conditions, adjacent to the hypoxia chamber. Chamber and room temperatures were maintained at 24 ± 1°F and relative humidity was maintained between 20 and 70%. Ad libitum access was provided to standard rat chow and water.

Pulmonary Function Tests: Forced expiration maneuvers and functional residual capacity (FRC) measurements were performed as described in (2). In brief, the Forced

Expiratory Volume (FEV) was obtained by inflating the rats' lungs to a pressure of 30cm H₂O, followed by a 1 second breath hold and a quick deflation to -35 cm H₂O. At least three maneuvers were obtained. FEV in the first 0.1 seconds of the maneuver (FEV_{0.1}), Mid-Maneuver Expiratory Flow (MMEF), inspiratory capacity (IC), and forced vital capacity (FVC) were calculated from the averages of the three best maneuvers. For FRC measurements, the rat's airway opening was occluded for 8 seconds in order to record airway pressure changes and thoracic volumes during inspirations. Functional residual capacity (FRC) was calculated from these data using Boyle's law method (3). Total Lung Capacity (TLC) was calculated as FRC + IC, and Residual Volume (RV) was calculated as TLC – FVC.

Bronchoalveolar Lavage (BAL) and Cytospin Slide Preparation: For the BAL, the left bronchus was clamped, and the right lung was lavaged 5 times with 4mL of 1x PBS per wash. Washes 1-2 were pooled separately from washes 3-5. BAL fluid was centrifuged at 1300 rpm for 10 minutes at 4°C. The supernatant fraction from washes 1- 2 was aliquotted and preserved at -80°C. The combined cell pellet was resuspended in fresh ice-cold 1x PBS and counted with an automatic particle counter (Beckman Coulter). An aliquot of 50,000 cells was sedimented onto a microscope slide using a cytocentrifuge preparation (Cytospin®) and stained with a modified Giemsa-Wright stain (HEMA III, Fisher Scientific). At least 400 cells were counted differentially according to their morphology.

Lung Histology: Lungs were inflated with 4% formaldehyde at a standard pressure of 20 cm H₂O. For airway histology, lungs were sectioned in a plane parallel to the hilar surface, paraffin embedded and cut at a thickness of 5 µm. Images were acquired on an Olympus BX51 Fluorescent Microscope attached to a CCD camera. From each lung section, digital images were obtained of 8-15 airways in cross section(4). Basement membrane perimeter, and the longest and the shortest diameters of each airway were measured using NIH ImageJ software (5). Airways having short to long diameter ratio >0.5 and basement membrane perimeter ≤3000 µm were analyzed(4). Goblet cells were counted from PAS-stained images and the number in each airway expressed per 100 µm of airway perimeter(6).

To visualize peribronchial collagen, red fluorescence images were acquired from Picro Sirius Red – stained sections (7). Basement membrane perimeter and airway wall area were outlined as described (8) using NIH ImageJ software (5), and the area within the airway wall stained for collagen was measured with an intensity threshold. The integrated density (mean fluorescence intensity×area) of the airway wall is expressed per 1 µm of basement membrane perimeter.

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Legends

Figure E1: *CIH did not affect the expression of lysyl oxidase (LOX). Average LOX gene expression in BAL cells. A.U.: Arbitrary Units (Fold difference in gene expression compared to one of the NORM/VEH samples). Bars: mean $\pm$ SEM.*

Figure E2: *CIH did not affect OVA-induced upregulation of matrix metalloproteinase (MMP) gene expression. A: Average MMP-9, and B: MMP-12 gene expression in BAL cells. A.U.: Arbitrary Units. Bars: mean $\pm$ SEM. Accolades: overall effect of allergen (2-way ANOVA).*

Figure E3: *Chronic OVA challenge decreased inspiratory capacity and increased BAL lymphocytes and macrophages. A: Inspiratory Capacity normalized to TLC. B,C: Log₁₀ of total lymphocyte (B) and macrophage (C) counts. Bars: mean $\pm$ SEM. Accolades: Differences between group means (Sidak post-hoc tests).*

Table E1: *Sequences of Forward and Reverse Primers (5' - 3') used for quantitative PCR.*

Gene	Forward Primer	Reverse Primer
LOX	TGGCACCGGTTACTTCCAGTAC	ACGTGGATGCCTGGATGTAGT
PAI-1	TTCCTCCACAGCCATTCTAGTCT	GAAAGGATCGGTCTAAAACCATCTC
YM-1	TTGCTGGGATGCGGAATAA	AGCTCAGTGTTCTGTCTTTC
MMP-9	CGTGGCCTACGTGACCTATGA	TGCACCGCTGAAGCAAAAG
MMP-12	GAGCTGGCACAATGAAGTTTCTC	GCCCCACAGGCAGATACCT
Cyclophilin-A	GGCTATAAGGGTTCCTCCTTTC	TTGCCACCAGTGCCATTA

Figure E1:

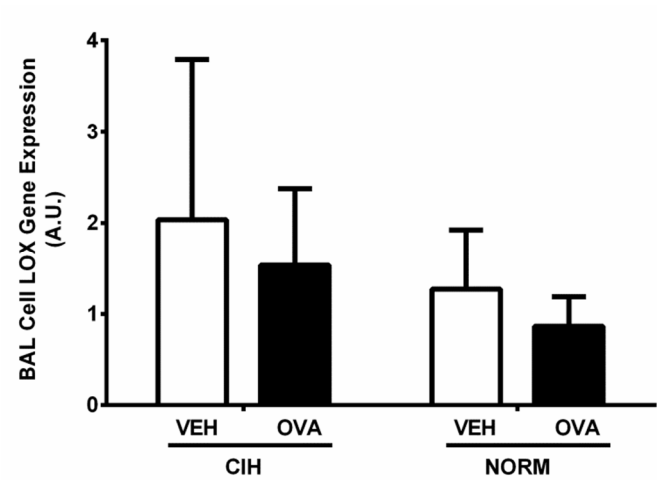
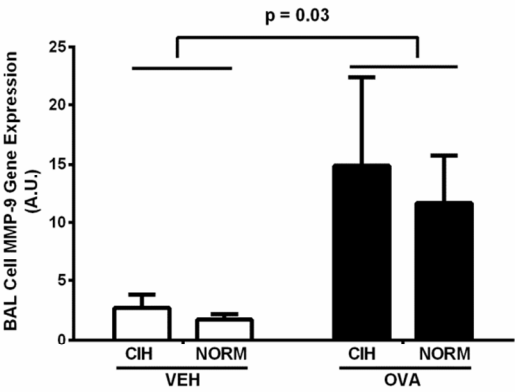


Figure E2:

A



B

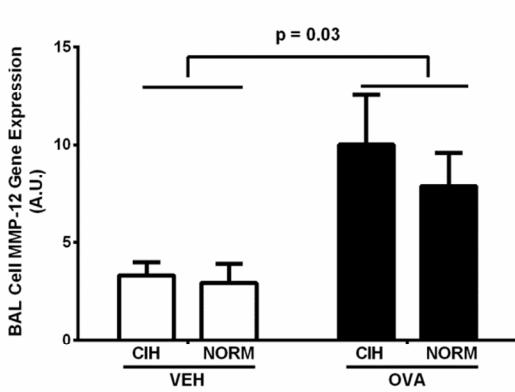
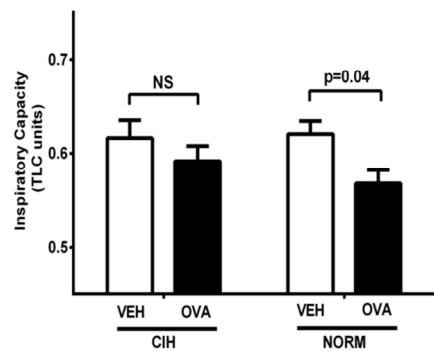
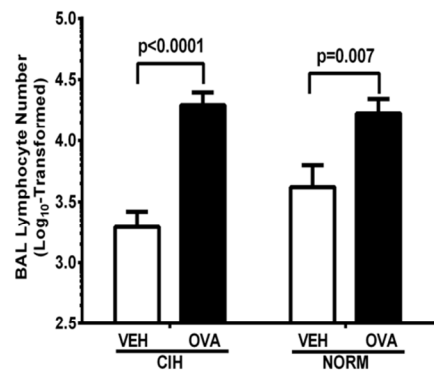


Figure E3:

A



B



C

