

Increased vascular density is a persistent feature of airway remodeling in a sheep model of chronic asthma

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ABSTRACT

Background: Increases in blood vessel density and vascular area are now recognized as important features of remodeled airways in asthma. However, the time sequence for these vascular changes and whether they resolve in the absence of continued antigenic exposure is not well elucidated. The aim of the present study was to correlate progressive changes in airway vascularity with changes in functional airway responses in sheep chronically challenged with house dust mite (HDM) allergen, and to examine the resolution of vascular remodeling following allergen withdrawal. **Methods:** Progressive changes in vascular indices were examined in four spatially separate lung segments that received weekly challenges with HDM allergen for 0, 8, 16, or 24 weeks. Reversibility of these changes was assessed in a separate experiment in which two lung segments received 24 weeks of HDM challenges and either no rest or 12 weeks rest. Lung tissue was collected from each segment 7 days following the final challenge and vascular changes assessed by a morphometric analysis of airways immunohistochemically stained with an antibody against type IV collagen. **Results:** Blood vessel density and percent airway vascularity were significantly increased in bronchi following 24 weeks of HDM challenges compared to untreated controls ($P < .05$), but not at any of the other time-points. There was no significant correlation between vascular indices and airway responses to allergic or nonspecific stimuli. The increase in blood vessel density induced by repeated allergen exposures did not return to baseline levels following a 12-week withdrawal period from allergen. **Conclusions:** Our results show for the first time that the airways of sheep chronically exposed to HDM allergen undergo vascular remodeling. These findings show the potential of this large animal model for investigating airway angiogenesis in asthma.

KEYWORDS sheep, asthma, vascular remodeling, type IV collagen

INTRODUCTION

Neovascularization of the airway wall is now recognized as an important feature in the pathophysiology of asthma. Increased blood vessel density

and vascular area have been observed in the airway walls of asthmatic patients relative to healthy subjects [1–3]. These changes, referred to as vascular remodeling, are associated with airflow limitation in asthma [4]. In particular, an increase in bronchial vascularity may lead to an increase in the trafficking of inflammatory cells and mediators to affected airways, and contribute to vascular engorgement and airflow obstruction [5]. Concomitant with increases in airway vascularity, are increases in the levels of angiogenic factors present in bronchoalveolar lavage fluid and induced sputum of asthmatic subjects compared with the levels found in healthy subjects. These include vascular endothelial growth factor (VEGF), angiogenin, and monocyte chemoattractant protein-1 [6].

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There is evidence that some pathologic features of asthmatic airways occur early in disease progression, including basement membrane thickening, epithelial damage, and increased vascular density [7]. However, the precise temporal sequences for vascular changes and their relationship to lung function in asthma are not well understood. Very little is known about the progressive temporal stages of vascular remodeling in the airways, and less is understood about whether the remodeled vasculature in asthmatic airways can revert back to the healthy state after the airways are no longer continually exposed to allergen. Several features of airway remodeling have been shown to persist for up to 8 weeks following the withdrawal of allergen in mouse models of chronic asthma, including: subepithelial fibrosis, epithelial hypertrophy, and increased airway smooth muscle mass [8, 9]. Whether the increased vessel density and vascular area persists in asthmatic airways in the absence of continuing allergen exposure is not known, and is the subject of the present investigation.

Investigations of vascular remodeling in asthmatic patients predominantly rely on airway tissue from bronchial biopsies that is collected from large proximal airways and offers little or no information on the vascularity of the small airways [10]. Although post mortem tissues from asthmatic patients enable more in-depth studies to be performed on the bronchial vascularity in the small airways, they do not allow for the same well-controlled studies as can be performed with animal models [11, 12]. A systematic investigation of vascular remodeling in the small airways can benefit from the use of large animal models, which have an extensive microcirculation in the small airway walls. In particular, sheep have well-developed bronchial circulations that nourish wall tissues in the large and small airways [13]. In addition to the bronchial circulation, the small airways of sheep have active mast cell components, mucous glands, and smooth muscle bundles that all contribute in different ways to the pathophysiology of asthma [14, 15]. In the present study, we have taken advantage of these features, which together with the attributes of the sheep asthma model, including airway remodeling and deteriorating lung function in response to allergen challenge to investigate vascular remodeling.

The aim of this study was to determine whether the airways of sheep undergo vascular remodeling in response to repeated lung challenges with house dust mite (HDM) and to examine the resolution of vascular remodeling following allergen withdrawal. Temporal aspects of vascular remodeling, were investigated by sampling airway tissue collected from four spatially separate lung segments that have been differentially

challenged with HDM for 0, 8, 16, or 24 weeks respectively. In a separate experiment, we modified our segmental challenge design to investigate whether the allergen-induced vascular changes persist following a lengthy period without repeated allergen challenges.

MATERIALS AND METHODS

Experimental Animals and Allergen Sensitisation

Six-month-old female Merino-cross sheep were sensitized with HDM extract (*Dermatophagoides pteronyssins*; CSL, Melbourne, Australia) in alum via three subcutaneous injections as previously described [16]. Atopic sheep with high HDM-specific serum IgE levels (>2 fold increase) were selected for lung challenges with HDM as previously described [16]. The remaining sheep were used as non-allergic controls. A fibre-optic bronchoscope was used for localized delivery of solubilized HDM (1 mg in 5 mL phosphate buffered saline; PBS) into spatially separate lung segments. Previous studies have shown that both functional changes and inflammatory cell infiltration following a bolus HDM-challenge are localized to the specifically treated lung segment [17]. All experimental animal procedures and the collection of tissues were approved by the Animal Experimentation Ethics Committee of the University of Melbourne.

Segmental Challenge Regimes

Progressive vascular remodeling

To investigate progressive stages of vascular remodeling, we studied archived airway tissue collected from the right apical, right medial, right caudal, and left caudal lung segments that were challenged weekly with HDM for different durations [17]. The commencement of challenges in each segment was staggered so that the right apical, right medial, right caudal, and left caudal segments received 0, 8, 16, or 24 weekly challenges respectively ($n = 7$). To ensure that there were no natural differences in vascular indices between segments a separate group of control sheep that did not receive HDM-treatment to any of their lung segments was used ($n = 7$).

Lung function correlations

Segmental lung function indices were taken from a separate study performed on these sheep [17] and used to ascertain whether the vascular remodeling found in this model was associated with lung function decline.

Reversibility of vascular remodeling

The reversibility of allergen-induced vascular remodeling was investigated in a separate group of sheep, which were sensitized to allergen as described above. The right and left caudal segments each received 24 weeks of HDM-challenges and then either 0 or 12 weeks without HDM respectively ($n = 8$; Figure S1). The challenges in each segment were staggered so that they concluded together.

Lung Tissue Collection, Immunohistochemistry and Morphometric Analysis

Sheep were euthanized 7 days after the final HDM challenge. This time-point was chosen to avoid the acute effects of HDM challenge on blood vessel size. Lung segments were inflated with optimal cutting temperature compound diluted in PBS (1:1). Blood vessels were identified on frozen sections of lung tissue immunostained with an antibody against type IV collagen. Sections were fixed with 95% ethanol for 10 minutes were blocked for endogenous peroxidase with 3% H_2O_2 . After washing sections were incubated for 2 hours with mouse anti-human type IV collagen (1:100; Dako, Kingstree, Australia). After washing, sections were incubated with rabbit anti-mouse HRP-conjugated secondary antibody (1:100; Dako) for 1 hour. Sections were then washed and a peroxidase-based detection system was used for visualization. Specificity for type IV collagen was determined by omission of the primary antibody.

The number and area of blood vessels within the airway wall was determined by digital image analysis of photographs using Image-Pro Plus (Media Cybernetics, version 4.1.0.0). The area of the airway wall was defined as the area enclosed by the outer perimeter of the basement membrane and the outer perimeter of the airway wall, as previously described [18]. Two bronchi from the same position in the tracheo-bronchial tree, approximately 1–2 mm diameter and free from branching, were analyzed for each segment and the results averaged for each sheep. All measurements were made by a single observer who was blind to the experimental group and lung segment.

Data Analysis

Vessel density, % vascularity and vessel size values are reported as the mean $\pm$ standard error. Normality of variables was assessed using a Shapiro–Wilk's test. A Friedman test with a Dunn's multiple comparison post-test were used to compare between segments in one group. Differences between the HDM-challenged and control groups were assessed using a

student's t test for data with a normal distribution and a Mann–Whitney test for data with a nonparametric distribution. Correlations were assessed by the Spearman correlation coefficient (r_s). A P value of $< .05$ was taken as significant.

RESULTS

Progressive Vascular Remodeling

It was established by challenging spatially separate lung segments with HDM for sequentially longer periods of time that it took between 16 weeks and 24 weeks to significantly increase blood vessel density. The number of vessels per mm^2 of airway wall in the HDM-challenged sheep group was significantly increased in the segment that received 24 weekly challenges compared to the unchallenged control segment (295 ± 29 versus 217 ± 22 vessels per mm^2 ; $P < .05$, $n = 7$; Figure 1A). Vessel density in segment challenged for 24 weeks was also significantly increased compared to the corresponding segment in the naïve control sheep group (295 ± 29 versus 201 ± 29 vessels per mm^2 ; $P < .05$, $n = 7$). This increase in blood vessel number was observed throughout both the lamina propria and the outer airway wall of bronchi, as shown in Figure 2. There were no differences in the number of vessels per mm^2 of airway wall between the unchallenged control segment and the segments that received 8 HDM or 16 HDM challenges (Figure 1A). Nor were there any differences in blood vessel density between any of the segments in the control sheep group.

The percent area occupied by blood vessels (percent airway vascularity) in the HDM challenged group was significantly increased in the segment that was challenged for 24 weeks compared with the unchallenged control segment (8.3 ± 0.5 versus $5.8 \pm 0.5\%$, $P < .05$, $n = 7$; Figure 1B) and the corresponding control segment in the control sheep group (8.3 ± 0.5 versus $5.5 \pm 0.6\%$; $P < .005$, $n = 7$). There were no other differences in the percent vascularity between the unchallenged control segment and the segments that received 8 HDM or 16 HDM challenges. Nor were there any significant differences in the percent airway vascularity between any of the segments in the control sheep group (Figure 1B).

An examination of the mean size of vessels revealed that there were no significant differences in mean vessel size between the lung segments that received 0, 8, 16, or 24 weeks of HDM challenge (Figure 3). Nor were there any differences between the mean size of vessels in the lung segments of the untreated control group (Figure 3). There was no significant

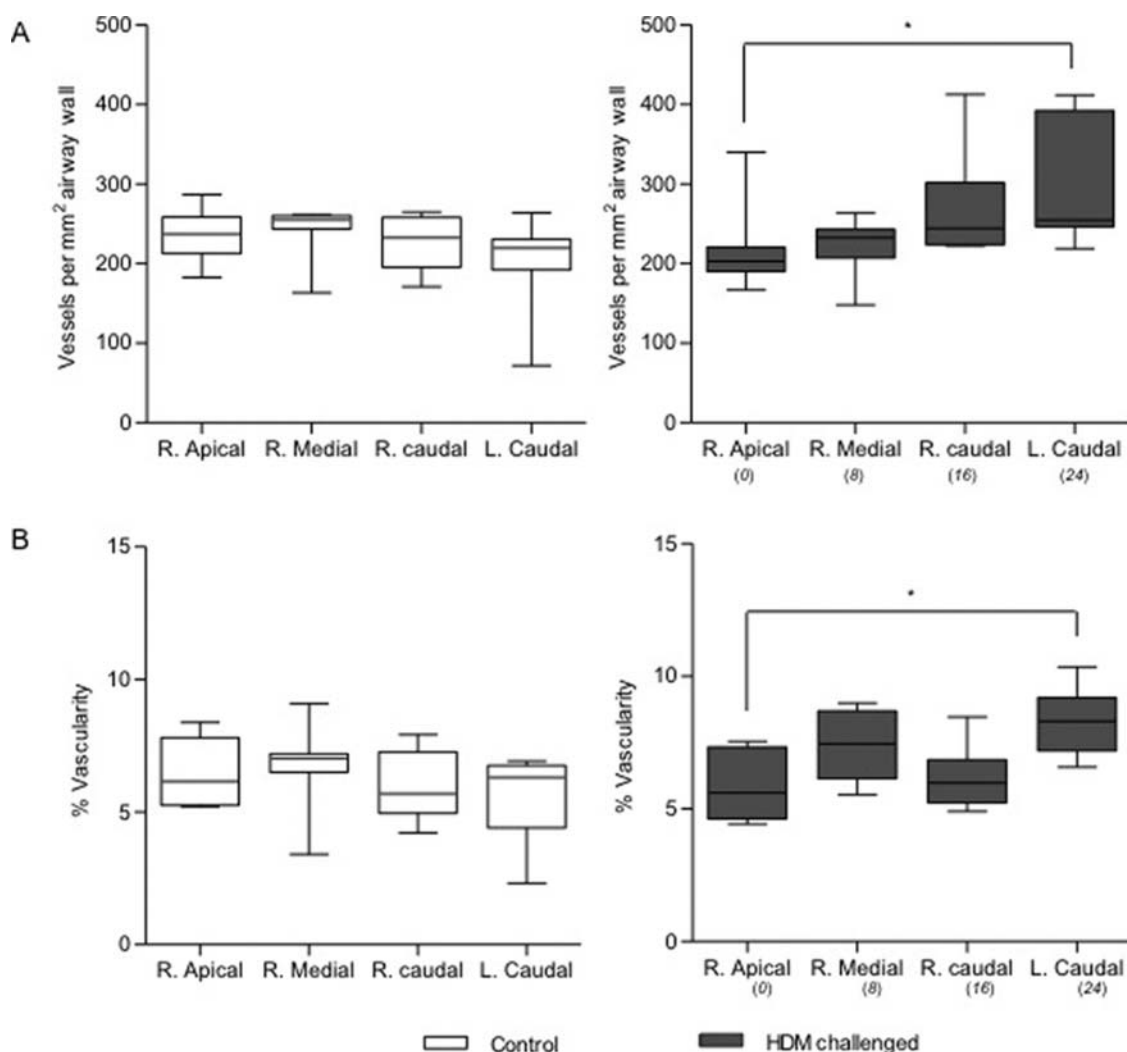


FIGURE 1 Bronchial blood vessel density and percent vascularity increase following chronic allergen challenge. Graphs show (a) blood vessels per mm² airway wall in untreated control and HDM challenged sheep, and (b) vascularity, expressed as the percent area occupied by blood vessels in the bronchial walls in untreated control and HDM challenged sheep. HDM-challenged sheep received 0, 8, 16, or 24 weeks of HDM challenges to four spatially separate lung segments. Matched lung segments in an untreated sheep group were used as controls. Line shows the mean, boxes show 25th and 75th percentile, whiskers represent 5th–95th percentile; * $P < .05$, $n = 7$.

difference in the distribution frequency of blood vessel sizes between the segment that received 24 weekly HDM challenges and the corresponding segment in the control sheep group (Figure S2).

We correlated vascular density and percent vascularity collected in the present study with indices of peripheral airway function collected from a previous study performed on these sheep. There was no significant correlation between increased airway vascularity after 24 challenges and measurements of allergen-induced bronchoconstriction or airway responsiveness (i.e., the percent dose of methacholine required for PC₁₀₀) ($r_s = -0.363$ and $r_s = 0.414$ respectively, $n = 7$; Figure 4). Nor was there any relationship between increased vessel density and

percent change in resistance post-HDM challenge or airway responsiveness after 24 challenges ($r_s = 0.250$ and $r_s = -0.252$ respectively, $n = 7$; results not shown). There was also no correlation between allergen-induced airway responses and vascular indices after 0, 8, and 16 weeks of HDM challenges. Similarly, resting R_p did not correlate with percent vascularity or vascular density at any of the time-points assessed.

Reversibility of Vascular Remodeling

Next we examined whether the increases in blood vessel density and vascularity induced by 24 weeks of allergen challenge returned to prechallenge levels

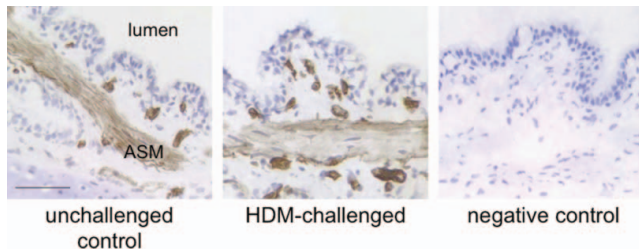


FIGURE 2 Airway sections stained with type IV collagen staining. Representative photomicrographs showing blood vessels in the bronchial walls from an untreated control lung segment and a lung segment which has been challenged with HDM for 24 weeks. The sections have been stained with antibodies to type IV Collagen. Negative control shows the omission of the primary antibody. Arrows indicate examples of blood vessels. Scale bar – 50 μm .

following a 12-week period without allergen. There was a significant increase in blood vessels per mm^2 airway wall in the segment which received 24 weeks of HDM challenges and no withdrawal period relative to the control group (375 ± 25 versus 292 ± 10 vessels per mm^2 , $P < .05$, $n = 8$; Figure 5A), there was also a significant increase in airway vascularity compared with controls ($10.3 \pm 0.7\%$ versus $7.9 \pm 0.6\%$, $P < .05$, $n = 8$; Figure 5B). Blood vessel density in the rested segment was still significantly increased relative to controls (344 ± 15 versus 266 ± 26 vessels per mm^2 respectively, $P < .05$, $n = 8$; Figure 5A). Percent vascularity also remained high in the rested segment following 12 weeks without allergen compared with matched segments of HDM naïve control sheep,

although this was not statistically significant ($8.3 \pm 0.7\%$ versus $6.7 \pm 0.8\%$, $P = .058$, $n = 8$; Figure 5B). Consistent with our previous results, there was no difference in the mean size of blood vessels in any segments of the HDM-treated group irrespective of whether they had no recovery time, or 12 weeks rest (271 ± 13 versus $262 \pm 16 \mu\text{m}^2$ and 262 ± 13 versus $258 \pm 18 \mu\text{m}^2$ respectively, $n = 8$; Figure 5C).

DISCUSSION

To our knowledge, this is the first study to attempt to describe the temporal process of vascular changes and their effects on lung function in small airways. In the present study, our key findings are that blood vessel density and percent airway vascularity increase significantly following 24 weeks of chronic allergen exposure and that these changes persist for up to 12 weeks following the cessation of allergen challenge. Until now, primates have been the only large animal species where an increase in the airway vasculature in response to allergen exposure has been documented [19]. The changes to the vascular compartment of sheep airways after allergen challenge has revealed many similarities to the changes observed in the airway walls of asthmatic patients. In particular, the vessel density and vascularity ranges reported here (219–412 vessels/ mm^2 and 5.5%–9.3%) are similar to ranges previously published from the clinic [3]. The experiments described in the present study add to our

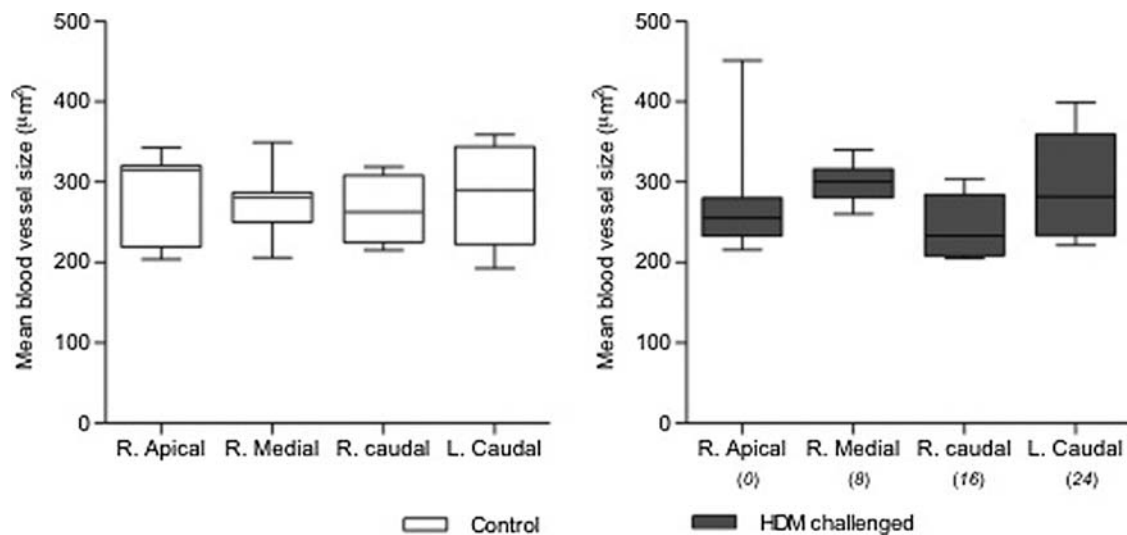


FIGURE 3 Mean vessel size. Mean blood vessel size (μm^2) in bronchial walls from control and house dust mite (HDM) challenged sheep in four spatially separate lung segments. HDM-challenged sheep received 0, 8, 16, or 24 weeks of HDM challenges in four spatially separate lung segments. Matched lung segments in untreated control sheep were used as controls for each segment in the HDM-challenged sheep. Line shows the mean, boxes show 25th and 75th percentile, whiskers represent 5th–95th percentile; $n = 7$.

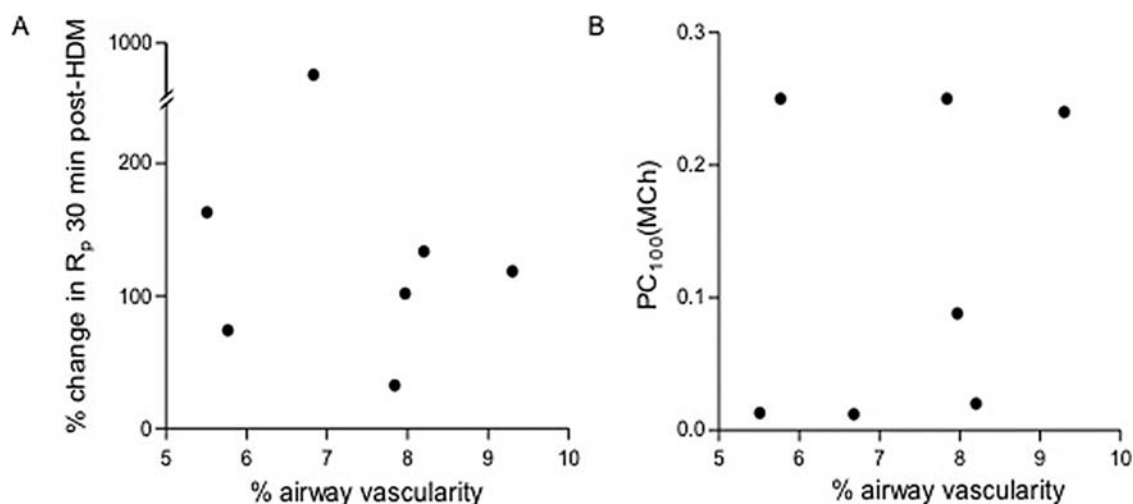


FIGURE 4 Correlation between airway vascularity and airway responses to allergic and nonallergic stimuli. Correlation between percent airway vascularity and lung function taken between 21 weeks and 23 weeks of chronic HDM challenges. The percent airways vascularity versus (a) the percent change in peripheral airways resistance (R_p) 30 minutes after HDM challenge ($r_s = -0.363$) (b) Airway hyperresponsiveness represented by the percent dose of methacholine (MCh) needed to increase the R_p to 100% above the resting R_p (PC_{100}) ($r_s = 0.414$). $n = 7$.

understanding of the temporal dynamics of blood vessel growth over long periods of allergic inflammation.

Our findings from two separate experiments indicate that 24 weeks of HDM challenges were needed to significantly increase blood vessel density and vascular area. This suggests that a prolonged, rather than an acute, allergic stimulus is required for vascular

remodeling to occur. In a previous study of asthmatic airways, there was an increased in blood vessel number relative to healthy controls when standardized against the basement membrane length, but not when standardized against airway wall area [11]. This finding suggests that increases in airway vascularity are directly proportional to increases in airway wall

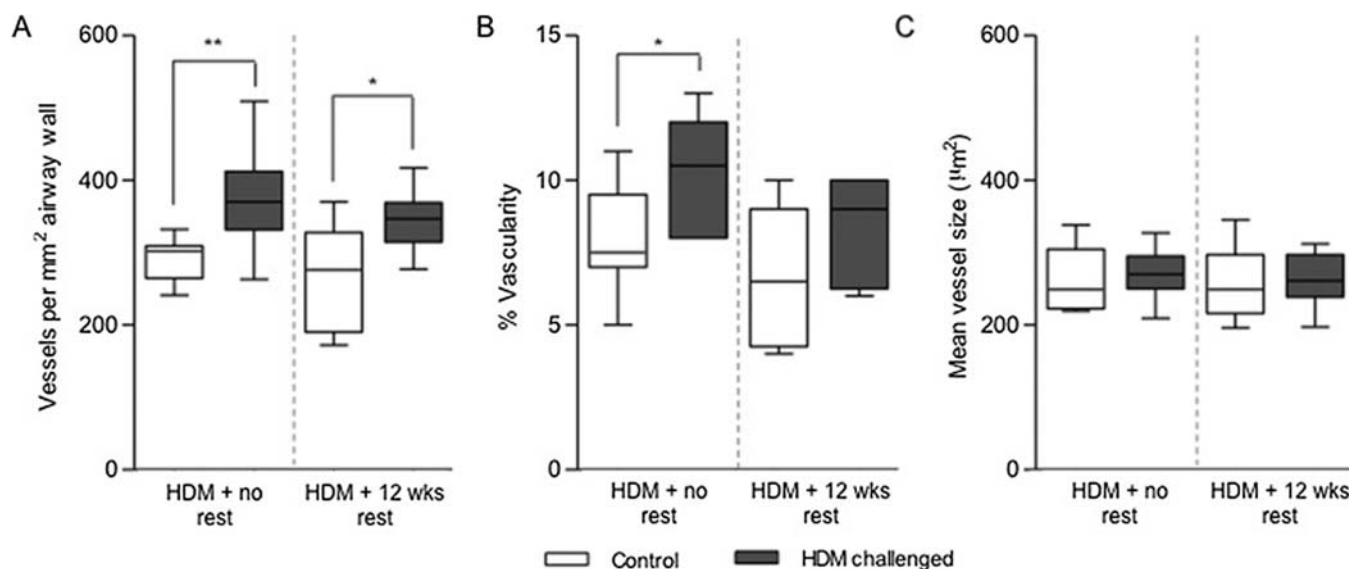


FIGURE 5 Bronchial vascularity following the cessation of allergen challenges. Sheep received 24 weeks of HDM challenges and then either 0 week or 12 weeks rest from HDM exposure to spatially separate lung segments (labeled “HDM + no rest” and “HDM + 12 wks rest” respectively). Matched lung segments in an untreated sheep group were used as controls. (a) Blood vessel density in control and HDM-challenged. (b) Percent airway vascularity in control and HDM-challenged and rested segments. (c) Mean blood vessel size in control sheep and HDM-challenged. * $P < .05$, ** $P < .005$, $n = 8$.

thickness that occurs as a part of airway remodeling. However, this was not the case in the present study as vascular indices were expressed relative to airway wall area.

The molecular and cellular mechanisms whereby vascularization is stimulated by repeated immunologic challenge are not completely understood. Previous studies have shown that mast cells and eosinophils are elevated in the airway walls of HDM-challenged sheep compared to control animals following a similar duration of allergen exposure [16, 20]. These cell types are known sources of angiogenic factors, such as VEGF, angiogenin, and basic fibroblast growth factor (bFGF) [21–23] and thus may be important cells linked to the changes to the microvasculature in asthma. Supporting evidence for this notion is that chymase-positive mast cells which express VEGF have been specifically associated with the microvascular changes in airway remodeling in a small group of asthmatics [24]. In cell culture, VEGF is known to stimulate primitive endothelial tube growth, which supports its role in stimulating increased vascularization in asthma [25, 26].

Remodeling of airway vasculature is known to be a persistent feature of several other nonallergic lung diseases, including chronic obstructive pulmonary disease (COPD) [27], pulmonary arterial hypertension [28] and systemic sclerosis [28, 29]. In the present study, we found that continual exposure to allergen was not required to maintain the increase in vascular density induced by earlier repeated allergen challenges. This suggests that increased vascular density in the airways maybe a persistent feature of asthma, and moreover, that once the blood vessels were formed they were likely to persist even when allergic inflammation subsides. Our finding is supportive of data derived from a mouse model of asthma, where increased vascular density and area persist for at least 4 weeks following antigenic withdrawal despite inflammatory cell numbers returning to baseline in the withdrawal period [10]. The stability of blood vessel number observed in the present study, given our longer recovery period of 12 weeks, compared with 4 weeks in the mouse study, suggests that this aspect of vascular remodeling is a long-term feature of asthma. While the number of vessels observed in the present study did not decrease following cessation of allergen challenges, the percentage area occupied by blood vessels in the airway wall, i.e., percent vascularity, was no longer increased above controls. This suggests the possibility that this aspect of vascular remodeling may be at least partially reversible over time. Consistent with this idea is the finding that airway vascularity, but not vessel density, is de-

creased in bronchial biopsies taken from asthmatics treated with inhaled corticosteroids compared to asthmatics that received no treatment [30]. This clinical study data and our data suggest that allergic inflammation is required to maintain increased airway vascularity.

Our study showed no apparent correlation between vascular remodeling and lung function indices as assessed by the wedged bronchoscope technique. The present study is consistent with the report by Li and Wilson [3] who found no correlation between forced expiratory volume in one-second (FEV_1) or PC_{20} and the percent vascularity or number of vessels in asthmatic biopsies. However, our work and that of Li and Wilson contrasts with Hashimoto et al. [2] and Hoshino et al. [22] who found inverse correlations between percent airway vascularity and the percentage of FEV_1 predicted or airway responsiveness. It is noteworthy that in the studies by Hashimoto et al. and Hoshino et al. vessel density and area from bronchial biopsies was increased by more than 100% in asthmatic patients compared to controls, while in the present study and the study by Li and Wilson, vessel density and percent vascularity was increased by less than 70% [3]. While it is important to acknowledge that increased vascularity may not directly contribute to altered airway function in asthma, it is also possible that the observed increases in airway vascularity reported by the Japanese groups were above a necessary threshold to negatively impact on lung function. It is therefore possible that sheep challenged with allergen for considerably longer than 24 weeks would display a greater increases in airway vascularity, which could impact negatively on lung function.

While the precise mechanism underlying the impact of changes to the airway microcirculation on lung function in asthma remains unknown, it has been suggested that vascular expansion contributes to airway wall thickening, and as a consequence airway reactivity [31, 32] and airway resistance are increased [33]. It is also possible, that increases in airway vascularity increase local blood flow to the airways and hence increase the trafficking of inflammatory cytokines and mediators to the airways compounding the effects of local inflammation [5].

CONCLUSIONS

The results from the present study show for the first time that the airways of sheep chronically exposed to HDM allergen undergo vascular remodeling. Our data gives important information on the temporal

aspects of vascular remodeling in airways that have been chronically exposed to allergen.

ABBREVIATIONS

HDM	house dust mite
MCh	methacholine
PBS	phosphate buffered saline
R_p	peripheral airway resistance
VEGF	vascular endothelial growth factor

Declaration of interest: The authors declare that they have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary Material

Supplementary Figures S1 and S2 are available with the online edition of this paper.

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