

Effect of Antigenic Exposure on Airway Smooth Muscle Remodeling in an Equine Model of Chronic Asthma

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Recent studies suggest that airway smooth muscle remodeling is an early event in asthma, but whether it remains a dynamic process late in the course of the disease is unknown. Moreover, little is known about the effects of an antigenic exposure on chronically established smooth muscle remodeling. We measured the effects of antigenic exposure on airway smooth muscle in the central and peripheral airways of horses with heaves, a naturally occurring airway disease that shares similarities with chronic asthma. Heaves-affected horses ($n = 6$) and age-matched control horses ($n = 5$) were kept on pasture before being exposed to indoor antigens for 30 days to induce airway inflammation and bronchoconstriction. Peripheral lung and endobronchial biopsies were collected before and after antigenic exposure by thoracoscopy and bronchoscopy, respectively. Immunohistochemistry and enzymatic labeling were used for morphometric analyses of airway smooth muscle mass and proliferative and apoptotic myocytes. In the peripheral airways, heaves-affected horses had twice as much smooth muscle as control horses. Remodeling was associated with smooth muscle hyperplasia and *in situ* proliferation, without reduced apoptosis. Further antigenic exposure had no effect on the morphometric data. In central airways, proliferating myocytes were increased compared with control horses only after antigenic exposure. Peripheral airway smooth muscle mass is stable in chronically affected animals subjected to antigenic exposure. This increased mass is maintained in a dynamic equilibrium by an elevated cellular turnover, suggesting that targeting smooth muscle proliferation could be effective at decreasing chronic remodeling.

Keywords: asthma; animal model; peripheral airways; heaves

Increased airway smooth muscle (ASM) mass is a prominent feature of patients with asthma, and may play a central role in allergen-induced bronchospasms and airway hyperresponsiveness to nonspecific agonists (1). ASM remodeling was demonstrated in the airways of patients with asthma, whether samples were obtained from lung resections or autopsy specimens (2–5), or from endobronchial biopsies (6–9). Although ASM remodeling is considered a target for novel therapies (10, 11), the processes leading to and, to a greater extent, maintaining ASM thickening in chronic disease are unknown. The question is not trivial, because therapeutic approaches targeting established

CLINICAL RELEVANCE

Recent studies suggest that airway smooth muscle remodeling is an early event in asthma, but whether it remains a dynamic process late in the course of the disease, and how antigenic exposure affects established remodeling, are unknown. We showed that a 30-day antigenic exposure had little effect on established remodeling in diseased animals, despite the development of inflammation and bronchoconstriction. In peripheral airways, airway smooth muscle remodeling appears to be maintained in a dynamic equilibrium by an elevated turnover with *in situ* proliferation, suggesting that targeting airway smooth muscle proliferation may be effective at decreasing its mass.

remodeling would differ if the remodeling was the result of ongoing proliferation or else a decrease in cellular death. This is of particular interest in small airways, where direct therapeutic interventions such as bronchial thermoplasty are not possible (12). Recent studies also highlighted the important contributions of the small airways in asthma, while recognizing the difficulty of sampling and imaging those airways (13, 14). Among the advantages of using large animal models is the possibility of repeated sampling of peripheral airways, along with the possibility of controlling their environment and treatment, but without the genetic homogeneity of inbred colonies.

Heaves is a naturally occurring disease of horses, associated with domestication and hay feeding, that shares similarities with asthma, including reversible antigen-induced bronchoconstriction, the accumulation of mucus, and airway inflammation. Ten to fifteen percent of adult horses are affected by this condition, characterized by episodes of coughing, wheezing, and exercise intolerance that can be controlled by environmental management (i.e., avoiding the offending indoor antigens, usually organic dust from poorly conserved hay), or with corticosteroids and bronchodilators (15). Similar to certain categories of asthma, intraluminal inflammation is predominantly neutrophilic, although it was linked to Th2 cytokines in some studies (16, 17). The features of airway remodeling resemble those of asthma, and include epithelial detachment and regeneration, goblet-cell hyperplasia, and increased bronchial and bronchiolar smooth muscle (18, 19). Although heaves is caused by antigens in moldy hay, it does not resemble extrinsic allergic alveolitis, because interstitial fibrosis, alveolitis, lymphocytic bronchoalveolar lavage inflammation, and restrictive pulmonary dysfunction are not characteristic of the disease (20, 21). As in humans, the reversibility of established ASM remodeling in adult horses has yet to be demonstrated.

In this study, we hypothesized that (1) heaves-affected horses have greater ASM mass than age-matched control horses kept in the same environment; (2) a month-long antigenic exposure would further increase ASM mass in heaves-affected horses; (3) this greater ASM mass would be at least partly attributable to hyperplasia; and (4) similar changes would be

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evident in the peripheral airways (full-thickness biopsies) and in endobronchial biopsies (partial sampling of airway walls). To test these hypotheses, we exposed heaves-affected horses and control horses to poorly cured hay, and examined ASM in peripheral and endobronchial biopsies, harvested before and after exposure, via morphometric analyses of ASM mass and markers of proliferation and apoptosis.

MATERIALS AND METHODS

Experimental Design

Data were collected after horses had been on pasture for more than 3 months (baseline) and after 1 day (pulmonary function and bronchoalveolar lavage only) and 30 days of stabling and exposure to poorly cured hay (antigenic exposure).

Animals

Six heaves-affected horses and five age-matched control horses were studied. Heaves-affected horses had a well-documented 3–10-year history of reversible airway obstruction and inflammation upon exposure to hay. Horses were deemed otherwise healthy, based on physical examination, blood count, and biochemistry. Animal manipulations were performed in accordance with the guidelines of the Canadian Council for Animal Care.

Pulmonary Function

In unsedated animals, pulmonary resistance and elastance were calculated from the flow rates obtained from a heated pneumotachograph attached to a mask, and transpulmonary pressure was derived from an esophageal catheter (22).

Bronchoalveolar Lavage

Two 250-ml boluses of isotonic saline were instilled in the main bronchi through a 2.5-m bronchoscope (Olympus Medical Systems Corp., Tokyo, Japan), as previously described (16). Cytospins were stained with Wright-Giemsa and Toluidine blue. Additional information is available in the online supplement.

Endobronchial Biopsies

Biopsies were performed in the contralateral lung after bronchoalveolar lavage (BAL), using disposable forceps (Olympus Medical Systems Corp.). Biopsies (median, $n = 5$; range, $n = 3$ –8) were taken from different branching sites, starting approximately 30 cm distal to the carina and moving cranially.

Lung Biopsies via Thoracoscopy

Peripheral lung tissue (8–12 cm³) was harvested in the caudo-dorsal region of the lung from standing, sedated animals (23). Samples were fixed for 24 hours in 4% formaldehyde and embedded in paraffin.

Immunostaining and Enzymatic Labeling

Immunohistochemical staining was performed for the colocalization of proliferating cell nuclear antigen (PCNA) with smooth muscle-specific α -actin (18). Apoptosis was detected using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. Additional information is available in the online supplement.

Morphometric Analysis

In peripheral lung biopsies, the ASM area of airways in cross section was measured using Image-Pro Plus software (MediaCybernetics, Carlsbad, CA). The ASM area (median number of airways per animal, 10; range, 5–17), ASM nuclei (median number per animal, 8; range, 4–17), PCNA⁺ myocytes (median number per animal, 8; range, 1–17), and TUNEL⁺ myocytes (median number per animal, 7; range, 4–7) were corrected by the internal perimeter squared to account for variations in airway size (24). In endobronchial biopsies, the ASM area was measured as a ratio of the biopsy area (5; 3–8), and ASM cells (median number for PCNA⁺, 4; range, 1–5; median number for

TUNEL⁺, 3; range, 1–4) were counted in random fields over $1\text{--}2 \times 10^4 \mu\text{m}^2$. Measurements were performed by one investigator (M.L.) blinded to group and the time point of sample collection.

Statistical Analysis

Group characteristics (age, weight, and gender) were analyzed according to the Mann-Whitney test, and physiologic data (function and BAL) were analyzed according to repeated-measures ANOVA with *a priori* contrasts. Morphometric data were analyzed according to paired and unpaired two-tailed *t* tests, using an average value for each animal at each time point. Normality was assessed according to the Kolmogorov-Smirnov test and visual inspection of the data. BAL cell counts and mast-cell percentages were transformed (log and arcsine square root, respectively) before analyses. The software SAS version 9.1 (SAS Institute, Cary, NC) was used, and $P < 0.05$ was considered significant.

RESULTS

Animals

Ages, weights, and proportions of mares in heaves-affected horses were not statistically different from those of control horses (ages for heaves-affected horses: median, 16 years; range, 15–20 years; ages for control horses: median, 14 years; range, 11–17 years; weights of heaves-affected horses: median, 467 kg; range, 444–515 kg; weights of control horses: median, 504 kg; range, 450–555 kg; proportions of mares: four heaves-affected horses, and five control horses).

Lung Function

Under conditions of low antigenic exposure, the lung function of heaves-affected horses was not different from that of control horses. Antigenic exposure induced significant airway obstruction only in diseased animals. Figure 1 depicts pulmonary resistance and elastance after 3 months of antigen avoidance (baseline) and after 1 and 30 days of exposure to indoor antigens.

BAL

Antigenic exposure induced a significant increase in the percentage of neutrophils and mast cells in heaves-affected horses after 1 and 30 days, along with a decrease in the percentage of lymphocytes. In control horses, a transient increase in neutrophils was also present on Day 1 (Figure 2). In absolute cell counts, no significant change was evident over time in the number of total cells, lymphocytes, macrophages, or eosinophils recovered from BAL in either group. An increase from baseline occurred in neutrophils and mast cells in both groups after 1 day and in mast cells after 30 days in heaves-affected horses (Table 1).

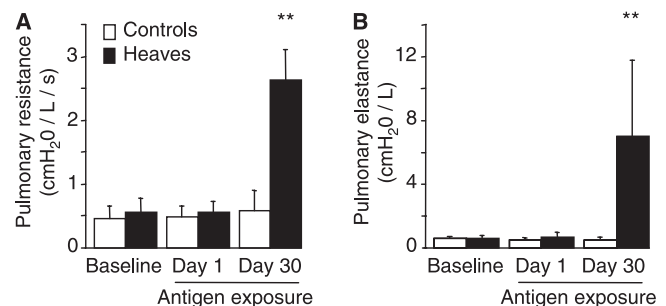


Figure 1. Pulmonary resistance (A) and elastance (B) after 3 months of antigen avoidance (Baseline) and after 1 and 30 days of antigenic exposure. Heaves-affected horses, $n = 6$; control horses, $n = 5$. Mean $\pm$ SD. **Different from Baseline and Day 1 within the same group, and different from controls at the same time point, $P < 0.01$.

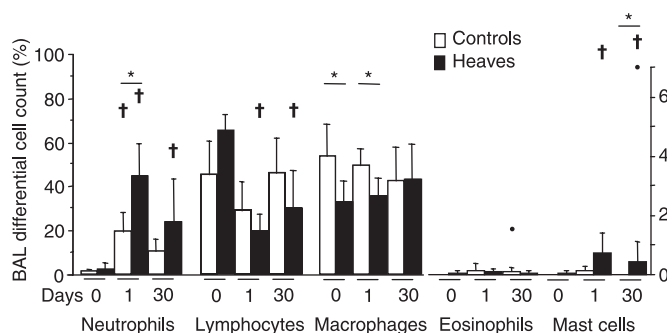


Figure 2. Differential cell count of bronchoalveolar lavage (BAL) after 3 months of antigen avoidance (baseline = Day 0), and after 1 and 30 days of antigenic exposure. Heaves-affected horses, $n = 6$; control horses, $n = 5$. Mean $\pm$ SD. *Different between groups at one time point. †Different from baseline within the same group, $P < 0.05$. •Outliers.

Airway Smooth Muscle in Peripheral Airways

Figure 3A illustrates ASM stained by immunohistochemistry in a peripheral airway. Individual airways of heaves-affected horses exhibited increased ASM mass, which was more pronounced in the most peripheral airways (Figure 4). In horses with heaves, mean ASM mass (Figure 5A) and myocyte nuclei per perimeter length (Figure 5B) were approximately 2-fold greater than in control horses, and were unaffected by a 30-day antigenic exposure. The greater ASM mass observed was not associated with a higher cell density (ASM nuclei per ASM area), but with a significantly lower density (Figure 5C). These findings suggest contributions by both hyperplasia and hypertrophy to ASM remodeling in peripheral airways, with a greater contribution of hyperplasia.

Proliferation and Apoptosis of Airway Smooth Muscle in Peripheral Airways

At baseline, both PCNA⁺ myocytes and TUNEL⁺ myocytes (Figures 3C and 3D) were more numerous in heaves-affected horses than in control horses, which suggests that chronic ASM remodeling is associated with an increased cellular turnover, because both proliferation and apoptosis were increased (Figures 6A and 6B, baseline). This high turnover was unaffected by the 30-day exposure. In contrast, control animals showed an increase in both proliferative and apoptotic cells after antigenic

exposure, compared with baseline (PCNA⁺, 2.1-fold increase, $P = 0.01$; TUNEL⁺, 2.4-fold increase, $P = 0.06$) (Figure 6). This increase remained below the level observed in heaves-affected horses, and was not associated with a change in ASM mass (Figure 5A).

Airway Smooth Muscle Proliferation and Apoptosis in Endobronchial Biopsies

At baseline, no difference was evident between groups in terms of ASM area percentage (Figures 3B and 7A), proliferative density (PCNA⁺ cells/ASM area) (Figure 7C), and percentage of proliferating airway myocytes (Figure 7D), or in terms of apoptotic density (TUNEL⁺ cells/ASM area) (Figure 7E) and percentage of apoptotic myocytes (Figure 7F). Only a modest increase in myocyte density (myocyte nuclei/ASM area) was evident in heaves-affected animals (Figure 7B). After antigenic exposure, the ASM area percentage decreased in heaves-affected horses (i.e., biopsies performed during ongoing bronchospasm), but remained stable in control animals. The proliferative density and percentage of proliferating airway myocytes were significantly greater in heaves-affected horses after antigenic exposure, compared with control horses (Figures 7C and 7D). No significant difference was evident between groups and time in terms of TUNEL⁺ myocytes (Figures 7E and 7F). Interestingly, approximately 50% of PCNA⁺ ASM cells were found in clusters of three or more cells (data not shown).

DISCUSSION

In this study, we examined the effects of antigenic exposure on ASM mass, proliferation, and apoptosis in the peripheral and central airways of mature animals with preexisting ASM remodeling that had been through multiple cycles of antigenic exposure, bronchospasm, and inflammation throughout their lives. After this natural challenge, only heaves-affected horses developed airway obstruction and sustained BAL inflammation. In peripheral airways, ASM remodeling appears to have reached a new dynamic equilibrium, characterized by a high cellular turnover where ASM mass, myocyte number, and markers of proliferation and apoptosis were increased compared with control horses, but were unaffected by an antigenic challenge. Antigenic exposure increased markers of proliferation and apoptosis in control horses without affecting their ASM mass. In the central airways, proliferative myocytes were increased in diseased animals only after challenge, and antigenic exposure

TABLE 1. CELL COUNTS IN BRONCHOALVEOLAR FLUID

			Baseline	Antigenic Exposure (1 Day)	Antigenic Exposure (30 Days)
Total cell count ($\times 10^7$)	C		4.17 (1.45)	7.13 (2.69)	7.97 (0.73)
	H		3.45 (3.59)	8.82 (6.91)	5.91 (13.08)
Neutrophils ($\times 10^7$)	C		0.04 (0.02)	1.53 (1.16)*	0.80 (0.43)
	H		0.03 (0.06)	4.28 (4.29)*	0.90 (1.80)
Lymphocytes ($\times 10^7$)	C		2.02 (1.16)	1.95 (0.97)	3.69 (1.40)
	H		2.20 (2.09)	1.48 (0.97)	2.00 (4.50)
Macrophages ($\times 10^7$)	C		2.11 (0.51)	3.50 (1.32)	3.36 (1.28)
	H		1.21 (1.53)	2.96 (2.00)	3.03 (6.78)
Eosinophils ($\times 10^5$)	C		0.00 (0.00)	1.09 (1.49)	3.42 (5.56)
	H		0.09 (0.21)	0.95 (1.58)	0.02 (0.06)
Mast cells ($\times 10^5$)	C		0.00 (0.00)	1.08 (1.17)*	0.00 (0.00)
	H		0.01 (0.01)	5.65 (6.61)*	0.46 (0.73)**

Definition of abbreviations: C, control horses; H, horses with heaves.

Values are presented as mean (SD).

* Different from baseline within the same group, $P < 0.05$.

† Different between groups at one time point, $P < 0.05$.

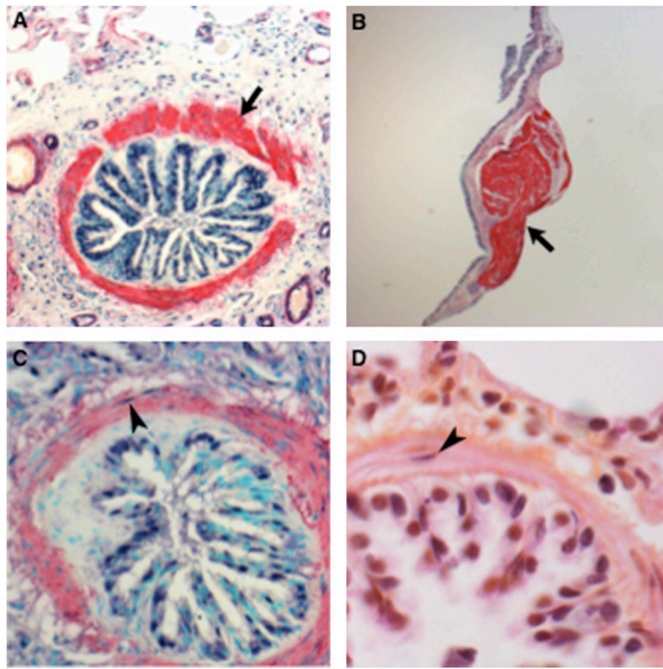


Figure 3. Airway smooth muscle (arrows) (smooth muscle α -actin stained by immunohistochemistry) in peripheral lung biopsies (A) and endobronchial biopsies (B). Airway myocytes were positive for proliferating cell nuclear antigen (PCNA) (C) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) (D) (arrowheads). Original magnifications: $\times 10$, $\times 4$, $\times 20$, and $\times 40$ for A, B, C, and D, respectively.

exerted no measurable effect on control horses. We confirmed our hypothesis, which stated that heaves-affected horses have greater ASM mass than age-matched control horses, and that this remodeling is maintained in part by hyperplasia. However, a month-long antigenic exposure did not further increase ASM mass, and we could not correlate the changes seen in peripheral airways with the changes in endobronchial biopsies.

Inflammation, Bronchoconstriction, and Remodeling

Exposure to indoor antigens induced marked and persistent airway neutrophilia in heaves-affected horses. The neutrophil percentage in BAL, more than in absolute cell counts, has proven useful in monitoring environment-induced airway inflammation in horses (15), but healthy animals can also develop transient inflammation under similar conditions (25). That was the case here, although to a lesser extent than in heaves-affected animals, and without the development of concomitant airflow limitation or increased ASM mass. The present study indicates that the transient inflammation resulting from antigen exposure in healthy animals also leads to an up-regulation of ASM turnover (seen in peripheral airways), without an association with airway obstruction or thickening of the ASM. The inflammation-induced up-regulation of ASM turnover therefore appears to be part of a normal response in healthy subjects. However, we cannot conclude whether ASM remodeling in diseased animals is only the result of a greater and more persistent inflammation, or is the result of factors intrinsic to ASM, as suggested by *in vitro* studies (26, 27).

ASM Remodeling in Peripheral Airways and Lung Function

Using postmortem lung samples, Herszberg and colleagues (18) showed that horses with heaves manifest more ASM in their medium and small airways than horses without respiratory

disease. In the present study, we confirmed and extended these findings by housing age-matched control horses in the same environment and by examining the effects of antigenic exposure on parameters of remodeling. Although all the heaves-affected horses had a greater mean peripheral ASM mass than did the control horses, this mass was not correlated with pulmonary resistance or elastance when horses were symptomatic (data not shown). This finding is in agreement with the findings of James and colleagues (5), in which small airway smooth muscle thickness was not correlated with severity of asthma, despite an increase in thickness compared with control samples. In that study of human asthma using postmortem specimens, only ASM in medium and large airways was associated with disease severity. This result is unlikely to mean that ASM remodeling in small airways is irrelevant, but more likely that clinical signs and criteria of severity based on need for therapy (as in James and colleagues [5]) or conventional assessments of pulmonary function correlate better with larger airway bronchospasm. Moreover, when horses were asymptomatic (at baseline), no difference in lung function was detectable, despite the presence of ASM thickening. This finding is in agreement with a mathematical model predicting that ASM thickening without concurrent bronchoconstriction exerts only a mild effect on airway caliber and lung function (28). More refined techniques to assess function, and especially peripheral airway obstruction, may have shown low-grade persistent airflow obstruction.

ASM Remodeling in Peripheral Airways: Contribution of Hyperplasia to ASM Remodeling

Horses with heaves have more than twice as much ASM mass and approximately twice as many airway myocytes in their peripheral airways as age-matched control animals. This alteration in ASM is associated with an increase in proliferating airway myocytes, rather than a decrease in apoptosis. These results are consistent with a previous study on postmortem equine lung tissue (18). The relatively greater increase in proliferating myocytes than in ASM mass (4.2-fold versus 2.3-fold at baseline, respectively) suggests that *in situ* proliferation accounts for some of the increase in ASM, even if the percentage of proliferating airway myocytes was not signifi-

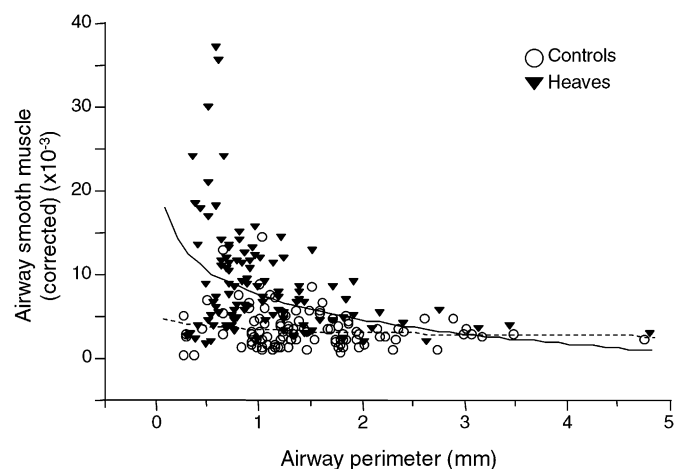


Figure 4. Airway smooth muscle area corrected for internal perimeter squared in peripheral airways at both time points. The internal perimeter of the airways in cross sections ranged from 0.2–4.8 mm (x-axis). Logarithmic regression lines illustrate trends for diseased animals (solid line) and control horses (dashed line). Control horses, $n = 108$ airways. Horses with heaves, $n = 109$ airways.

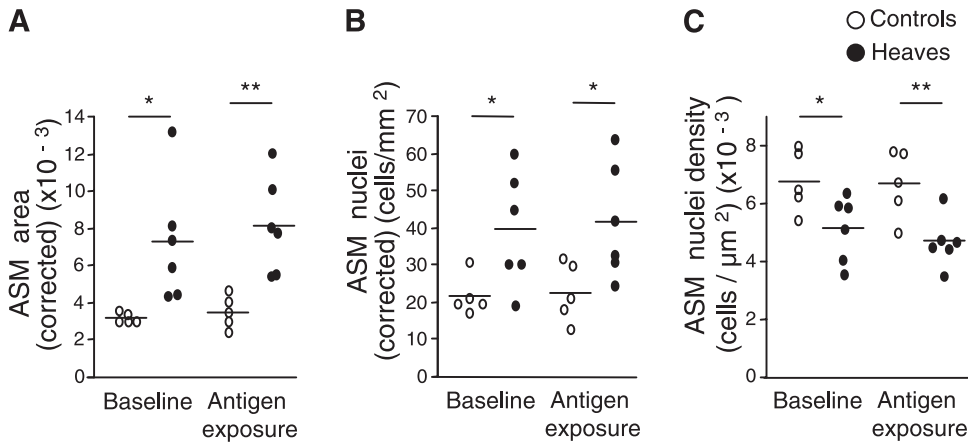


Figure 5. Airway smooth muscle (ASM) area (A) and ASM myocyte nuclei (B) corrected for internal perimeter squared, and (C) myocyte density (ASM nuclei/measured ASM area) after 3 months of antigen avoidance (Baseline) and after 30 days of antigenic exposure. Heaves-affected horses, $n = 6$; control horses, $n = 5$. Horizontal bars represent the mean. * $P < 0.05$, ** $P \leq 0.01$.

cantly different in the peripheral airways (data not shown). The differences in myocytes per airway perimeter squared and per measured surface suggest a contribution of both hyperplasia (i.e., increased mass resulting from increased cell number) and cellular hypertrophy. The contribution of cellular hypertrophy is consistent with the increased cell size described in patients with asthma (3, 9, 29), but hypertrophy was not demonstrated in all patients (7, 30). The decrease in myocyte nuclei per unit area could also be attributable to an increase in extracellular matrix deposition within the smooth muscle bundle, because we did not measure cell size directly. Nevertheless, these phenomena are not mutually exclusive, and in asthma, evidence exists of both hypertrophy and hyperplasia (3, 9, 29, 31, 32), along with increased extracellular matrix (33, 34), and with possible regional differences within the bronchial tree (3). Finally, evidence that ASM hyperplasia in chronic ASM remodeling is at least partly attributable to *in situ* proliferation suggests that limiting the proliferative capacity of airway myocytes may be of therapeutic value, even without directly inducing cell death.

ASM Remodeling in Peripheral Airways: Limited Effect of Antigenic Exposure in Heaves-Affected Horses

The parameters of ASM remodeling were not affected by a month-long antigenic exposure in heaves-affected horses. The lack of further increase in ASM mass or cell number suggests that ASM remodeling may reach a plateau after a certain mass is attained. By showing that ASM remodeling can occur early in the natural progression of asthma (35) and that ASM thickening correlates better with severity than with duration, James and

colleagues (5) findings indirectly support this concept of plateau or equilibrium in ASM remodeling. This plateau, or dynamic equilibrium, may prevent complete airway obstruction by ASM (a phenomenon not known to occur in heaves or in fatal asthma). Interestingly, antigenic exposure was associated with a modest but significant rise in proliferative and apoptotic cells only in control animals, which developed transient inflammation without airflow limitation. Taken together, these results suggest that the normal response to a transient inflammatory event is an increase in myocyte proliferation and an appropriate compensatory increase in apoptosis, whereas more pronounced or persistent inflammation leads to an increase in ASM mass in diseased animals. We postulate that after the ASM mass reaches a new dynamic equilibrium, as in the peripheral airways of chronically affected horses, myocytes show an elevated baseline turnover that is no longer affected by antigenic exposure. Ongoing tissue inflammation may also persist in asymptomatic heaves-affected horses, and may contribute to the ongoing proliferation and apoptosis observed at baseline. However, it is difficult to predict whether a longer exposure could have led to an alteration in this dynamic equilibrium in heaves-affected horses. Because the control horses have spent most of their lives exposed to hay, longer exposure would not likely have led to an increase in their ASM mass.

ASM Remodeling in Central Airways: Bronchospasm Affects ASM Quantification in Equine Endobronchial Biopsies

At baseline, ASM area percentage in endobronchial biopsies was not significantly increased in heaves-affected horses. Most

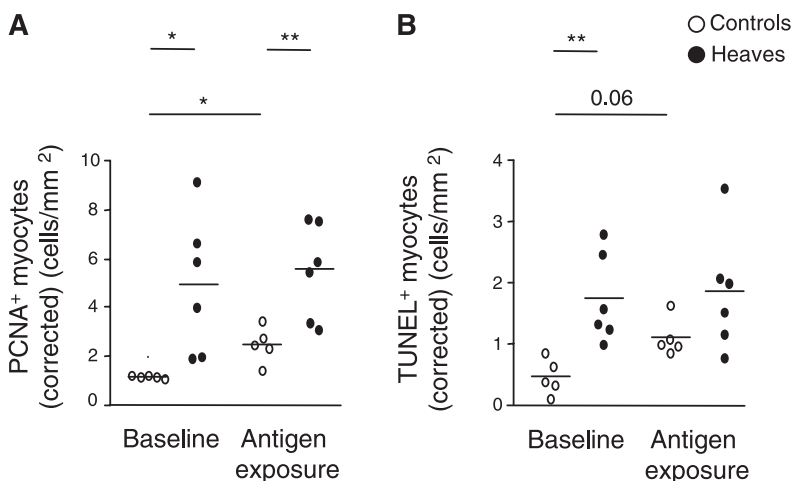


Figure 6. Proliferation and apoptosis in peripheral airways. PCNA⁺ (A) and TUNEL⁺ (B) myocytes were corrected for internal perimeter squared after 3 months of antigen avoidance (Baseline) and after 30 days of antigenic exposure. Heaves-affected horses, $n = 6$; control horses, $n = 5$. Horizontal bars represent the mean. * $P < 0.05$, ** $P \leq 0.01$.

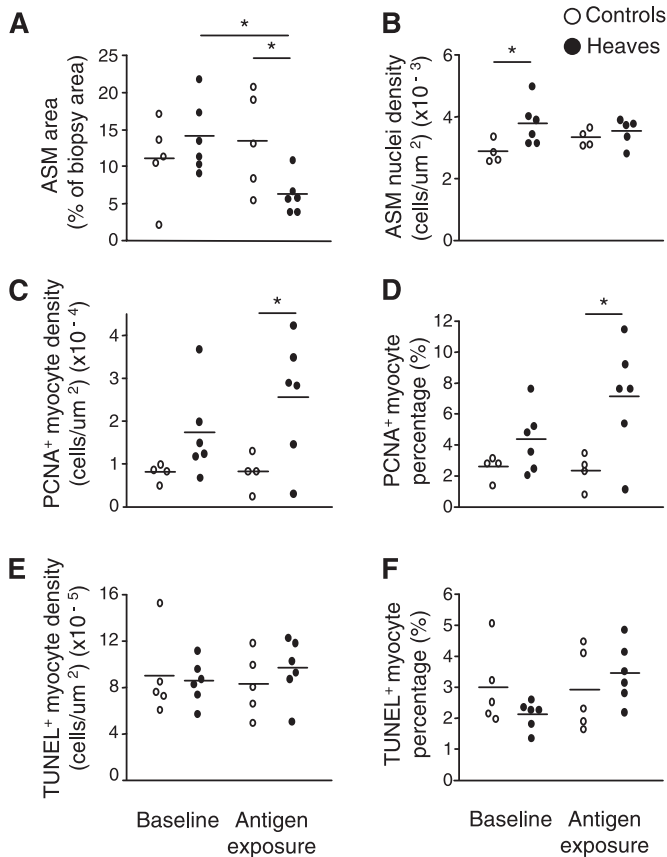


Figure 7. Airway smooth muscle in endobronchial biopsies. (A) Percentage of ASM area (ASM area/biopsy area). (B) Myocyte density (myocyte nuclei/ASM area). (C) PCNA⁺ myocyte density (PCNA⁺ myocytes/ASM area). (D) Percentage of PCNA⁺ airway myocytes. (E) TUNEL⁺ myocytes density (TUNEL⁺ myocytes/ASM area). (F) Percentage of TUNEL⁺ airway myocytes. Biopsies were performed after 3 months of antigen avoidance (Baseline) and after 30 days of antigenic exposure. Heaves-affected horses, $n = 6$ (except for B, exposure $n = 5$); control horses, $n = 5$ (except for B–D, $n = 4$). Horizontal bars represent the mean. * $P < 0.05$.

studies of human patients found an increase in ASM area (total or percentage) (6, 8, 9, 35), although a few could not demonstrate a difference between groups (36), or else the difference was evident only in patients with severe but not intermittent asthma (9). In the present study, a significant decrease in ASM area percentage was observed after antigenic exposure. Kelly and colleagues (37) noted a similar decrease 24 hours after an allergen challenge in patients with asthma, which they attributed to a dedifferentiation to myofibroblasts. The decrease of ASM ratio after exposure could indeed represent an increase in other subepithelial components, but we also found that sampling during ongoing bronchospasm made the positioning of the biopsy forceps more difficult and the sampling more likely to be superficial, because the carinae thicken with constriction. Alternatively, the lengthening of the airways that may occur during hyperinflation (38) could increase the distance of the ASM from the carinae. Furthermore, two-dimensional morphometric analysis may not be appropriate for the quantification of equine ASM using endobronchial biopsies, regardless of clinical stage, because of the smaller size of the forceps relative to the bronchial carina. Because these results suggest that bronchoconstriction may affect morphometric measurements, a bronchodilator could be used at the time of sampling to avoid this possible confounding factor in the future.

ASM Remodeling in Central Airways: Evidence of ASM Cell Hyperplasia in Endobronchial Biopsies

Despite the difficulties in quantifying ASM in endobronchial biopsies, data on proliferating and apoptotic myocytes were still obtained. The greater proliferation density and percentage of proliferating airway myocytes in heaves-affected horses after antigenic exposure, without a concurrent increase in apoptosis, are suggestive of *in situ* proliferation, even if no increase in ASM mass was demonstrated in these biopsies. The observation that approximately 50% of proliferating myocytes were found in clusters also supports the presence of *in situ* proliferation, possibly by the autocrine feed-forward mechanism described by Johnson and colleagues (39), or because of a localized source of growth factors. ASM proliferation was only recently shown to play a role in ASM remodeling in patients with severe, long-standing asthma (40). Previously, James and colleagues (41) found a percentage of PCNA⁺ myocytes in a range similar to the one detected here (5–8%), but with no difference between patients with asthma and control subjects, whereas others failed to detect proliferation markers (PCNA, Ki67, or cyclin D1) in ASM bundles (9, 42, 43). However, in our study, the difference was significant only after antigenic exposure, and after subjects became symptomatic and had gone untreated for a prolonged period, which is less likely to occur in patients with asthma. The severity of disease (7) or the use of corticosteroids (42) can also account for some variations, but the processes leading to ASM hyperplasia may also differ among species (44).

This equine model of chronic asthma produced evidence of hyperplasia associated with *in situ* proliferation, as well as of possible hypertrophy in remodeled ASM. An antigenic exposure had no effect on morphometric measurements in peripheral airways and little effect on the percentage of proliferating myocytes in endobronchial biopsies in these chronically affected animals. These findings are in agreement with the concept that ASM remodeling is an early event in asthma and remains stable in terms of mass later in life (5, 45). We conclude that in peripheral airways at least, ASM remodeling reaches a new dynamic equilibrium in which the increased mass is maintained with an elevated turnover. This finding also suggests that limiting the proliferative capacities of airway myocytes may have a therapeutic value, even without directly inducing cell death.

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