Airway collagen and elastic fiber content correlates with lung function in equine heaves

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Setlakwe EL, Lemos KR, Lavoie-Lamoureux A, Duguay JD, Lavoie JP. Airway collagen and elastic fiber content correlates with lung function in equine heaves. Am J Physiol Lung Cell Mol Physiol 307: L252–L260, 2014. First published May 30, 2014; doi:10.1152/ajplung.00019.2014.—The consequences on lung function and inflammation of alterations in the extracellular matrix affecting the peripheral airway wall in asthma are largely unknown. We hypothesized that remodeling of collagen and elastic fibers in the peripheral airway wall leads to airway obstruction and contributes to neutrophilic airway inflammation. Animals used were six heaves-affected horses and five controls. Large peripheral lung biopsies were obtained from horses with heaves in clinical remission (Baseline) and during disease exacerbation and from age-matched controls. The area of collagen and elastic fiber content in the lamina propria was measured by histological staining techniques and corrected for airway size. Collagen type 1 and type 3 content was further assessed from additional horses after postmortem lung samples by immunohistochemistry. The collagen breakdown products proline-glycine-proline (PGP) and N-acetylated-PGP (Nα-PGP) were also measured in bronchoalveolar lavage fluids (BALF) by mass spectrometry. Compared with controls, heaves-affected horses had an increase in collagen (P = 0.05) and elastic fiber contents (P = 0.04) at baseline. Collagen types 1 and 3 content was also significantly increased in diseased horses (P = 0.015) when both collagen types were combined. No further change in collagen content was observed after a 30-day antigenic challenge. Airway collagen at baseline was positively correlated with pulmonary resistance in asthmatic horses (r² = 0.78, P = 0.03) and elastic fiber content was positively associated with pulmonary elastance in controls (r² = 0.95, P = 0.02). No difference between groups was appreciated in PGP and Nα-PGP peptides in BALF. Increased airway wall collagen and elastic fiber content may contribute to residual obstruction in the asthmatic airways.

CLINICAL SIGNS OF ASTHMA result from airflow obstruction due to bronchospasms and, possibly, to the structural changes affecting the airway wall. This remodeling is thought to arise as a consequence of repeated cycles of inflammation and repair to the airway epithelial-mesenchymal trophic unit (15). Given its contractile properties, much attention has been paid to the contributions of the extracellular matrix (ECM) to lung disease. The ECM is a dynamic three-dimensional fibrous network that is essential to the mechanical properties of the airways. Collagen gives tensile strength, whereas elastin is responsible for the extensibility and elastic recoil of the airways (5, 24). They are thus likely to modulate both bronchoconstriction and airway reopening. In asthmatic patients, ECM in the ASM has been positively correlated with changes in the dynamics of airway function (47) possibly as a result of alterations of the type and spatial organization of the collagen fibers. However, others have failed to link mechanical properties and airway collagen content in asthma (4, 14). Furthermore, it has also been suggested that increased airway collagen rather has a protective effect in asthma by stiffening the airways (23, 26, 36). Elastic fiber content in the airways of asthmatic patients has been described as increased, unaltered, or decreased and its architecture unaltered, disorganized or fragmented in the few studies where it has been measured (reviewed in Ref. 30). Thus, not surprisingly, at present little is known of the functional consequences of altered collagen and elastin content within the airway wall.

The contribution of the ECM to lung disease may not be limited to its mechanical properties because it may modulate airway inflammation and remodeling through its cross talk with other cells and the secretion of bioactive molecules. Proline-glycine-proline (PGP) and N-acetylated-PGP (Nα-PGP) are small peptides produced by the degradation of collagen by matrix metalloproteases and neutrophil proteases and endopeptidases (28). These potent neutrophil chemoattractants are increased in human neutrophilic lung diseases such as cystic fibrosis, chronic obstructive pulmonary disease, and bronchiolitis obliterans syndrome (10, 27, 33). The finding that Nα-PGP directly activates human neutrophils and induces the release of matrix metalloprotease (MMP)-9, suggests that this pathway may contribute to the development of unrelenting chronic neutrophilic pulmonary inflammation (46).

We thus hypothesized that remodeling of collagen and elastic fibers in the peripheral airway wall leads to airway obstruction and that the increased turnover of ECM contributes to the recruitment of neutrophils within the small airways. Our objective was to quantify collagen and elastic fiber remodeling in the lamina propria of the peripheral airways of horses with heaves, a spontaneously occurring asthmalike disease (20), and explore its relationship to lung function and airway neutrophilic inflammation.

METHODS

Animals and experimental protocol. In a prospective study of lung function, bronchoalveolar lavage fluid (BALF) cytology and lung biopsy procedures were performed on five healthy control horses and six horses with heaves. Testing was performed when the horses had been pastured for 3 mo (Baseline) to induce clinical remission of the disease, and after a natural 30-day antigenic challenge (Antigen challenge) consisting of stabling and hay feeding. Thoracoscopic guided lung biopsies specimens were obtained from the 12th intercostal space as previously described (31). Horses with heaves, but not controls, developed airway neutrophilia (BALF neutrophils; means ± SE
32 ± 22.6% in horses with heaves, 3.1 ± 2.5% in control horses) and obstruction [pulmonary resistance (R<sub>t</sub>) 4.86 ± 3.52 cmH<sub>2</sub>O·l<sup>-1</sup>·s<sup>-1</sup> in horses with heaves; R<sub>t</sub> 0.51 ± 0.23 cmH<sub>2</sub>O·l<sup>-1</sup>·s<sup>-1</sup> in control horses] with stabilizing and hay feeding. These horses were part of a larger study evaluating asthmatic remodeling and inflammation (19). Lung autopsy specimens from well-characterized horses with heaves (n = 7) and aged-matched control horses (n = 6) were also studied.

All experimental procedures were conducted in accordance with the guidelines of the Canadian Council for Animal Care and were approved by the Animal Care Committee of the Faculty of Veterinary Medicine of the Université de Montréal.

**Histomorphometric analysis: collagen.** Formalin-fixed peripheral lung tissue biopsies were embedded in paraffin, cut to a 5-μm thickness, and stained with picrosirius red (41), a collagen-specific histological stain. Coded slides were evaluated under bright-field microscopy. Digitized peripheral airway images were transferred to a computer software program (Image J, National Institutes Health). Collagen in the lamina propria (stained red, Fig. 1A) was subtracted from the rest of the image (Fig. 1B) using image-processing steps described above. Lung tissue autopsy specimens from the cranial and caudal lobes of each separate cohort of well-characterized horses with heaves in exacerbation of the disease (n = 6) and controls (n = 6) were processed and analyzed as described above but immunostained for collagen type 1 and type 3.

**Immunohistochemistry.** Lung tissue containing airways of different sizes and embedded in paraffin were cut into 5-μm-thick sections. Steps performed for the image analysis of peripheral airways (horses with heaves and controls) stained with picrosirius red. A: digitized peripheral airway stained with picrosirius red. B: background correction factor applied and lamina propria collagen selected with a black-colored tracing tool. C: lamina propria collagen is extracted from the image and area can be measured. D: internal airway epithelium perimeter is hand traced with a black-colored tracing tool and measured.

**Histomorphometric analysis-elastic fibers.** The surface area of elastic fibers located in the lamina propria was quantified by using the Russell modification of the Movat Pentachrome stain (34) and the image-processing steps described above.

**PGP and N-α-PGP assessment in BALs by mass spectrometry (LC-MS/MS).** Bronchoalveolar lavages (BAL) were performed prior to, 24 h after, and 30 days after the start of the antigenic challenge in standing sedated horses by using a videoscope and two boluses of 250 ml of isotonic saline, as previously described (19). BALF was kept on ice, filtered, and frozen at −80°C until assayed.

LC-MS/MS was performed by the Proteomics core facility of the Institute for Research in Immunology and Cancer associated to Université de Montréal. The samples were separated with an Agilent (Santa Clara, CA) nano-LC (1100 Series) system by using a C18 analytical column (150 mm ID x 1 mm) packed with a C18 (5 μm, 300 A) Jupiter (Phenomenex, Torrance, CA) stationary phase. The injection volume was 20 μl and sample loading was performed at a flow rate of 50 μl/min for 4 min. Sample elution on the analytical column was achieved with a gradient going from 100% of formic acid 0.2% to 40% acetonitrile with formic acid 0.2% at a flow rate of 50 μl/min. For the calibration curve, PGP (customized synthesis, >90% purity; Feldan) and N-acetyl-PGP (ac-PGP trifluoroacetate salt hydrate, ≥98% purity; Sigma) standards ranging from 0.001 to 100 ng were injected on column. The R<sup>2</sup> values for the calibration curves were 0.989 and 0.991 for PGP and N-α-PGP, respectively, and the
detection limit was 1 pg. liquid chromatography-multiple reaction monitoring (LC-MRM) analyses were performed on the Q-trap 4000 mass spectrometer under the following conditions: source temperature 160°C; curtain gas 10; nebulizer gas 5; source voltage 5,400 V; and declustering potential 120 V. Positive electrospray mass transitions were at 270–70 and 270–116 (PGP) and 312–140 and 312–112 (N-α-PGP) (0.2 s each). Optimal sensitivity was obtained with the following conditions: collision energy 25 eV, collision gas 10, and declustering potential 120 eV.

Statistical analysis. Values of collagen or elastic fiber contents of individual airways from each horse were averaged, and mean values were compared between groups (heaves and controls) with two-sided unpaired *t*-test with Welch’s correction. Associations between lung function, BALF neutrophilia, and mean collagen or elastic fiber contents were evaluated by a least squares linear regression test. BALF concentrations of PGP and N-α-PGP were logarithmic transformed before performing two-way repeated-measures ANOVA followed by Bonferroni’s post hoc comparisons to evaluate the effect of group and time. Pearson correlation analysis between PGP and N-α-PGP was performed on log-transformed data. *P* ≤ 0.05 was considered significant.

RESULTS

Collagen content in the lamina propria of peripheral airways of heaves-affected horses compared with controls. Fifty-six (range 6–15 airways per control horses) and 58 (range 4–15 airways per horses with heaves) peripheral airways from large peripheral lung biopsy samples were assessed. Lamina propria collagen distribution was heterogeneous, and no specific pattern or fiber orientation was visualized among the digitized picrosirius red-stained airways in both groups of horses. Some airways had a very dense collagen fiber network (Fig. 1), taking up most of the lamina propria, whereas others had an uncondensed collagen network, with large areas of empty space between fibers, likely due to a processing artifact.

The airways evaluated were of similar size (perimeters) in both groups of horses (Baseline: Heaves 1,062 ± 169 μm, Controls 1,278 ± 166 μm, *P* = 0.33; Antigen exposure: Heaves 1,259 ± 93 μm, Controls 1,112 ± 129 μm, *P* = 0.66). Compared with controls, horses with heaves have a significantly (*P* = 0.05 at baseline) increased lamina propria collagen content in the airways (Fig. 2A). The increase in collagen content in heaves-affected horses compared with controls was more marked in airways of smaller diameter (Fig. 2B). Lamina propria collagen content in the peripheral airways was positively correlated with pulmonary resistance (*r*² = 0.78, *P* = 0.03) in heaves-affected horses at baseline (Fig. 2, C and D). No other association was present with pulmonary function or BAL neutrophilia (%) (data not shown).

Collagen type 1 and type 3 content was assessed in the lamina propria of 113 and 158 airways from autopsy samples of heaves-affected horses (7–21 airways per horse), and 83 and 81 airways from control animals (7–16 airways per horse), respectively. Collagen types 1 and 3 were present in the lamina propria and the adventitia of the airways, but not in the basement membrane (Fig. 3, A and B). The increase in collagen type 1 (*P* = 0.1; Fig. 3C) and type 3 (*P* = 0.1; Fig. 3D) content in horses with heaves compared with control

![Fig. 2. Lamina propria collagen content in peripheral airways of horses with heaves and controls. A: lamina propria collagen content corrected for internal perimeter squared in peripheral airways of horses with heaves (*n* = 6) and controls (*n* = 5) before (Baseline) and after (Antigen exposure) a 30-day antigenic challenge (means ± SD). B: collagen content area in function of basement membrane length in heaves-affected horses (58 airways) and in controls (56 airways). The increase in collagen content is more marked in airways of smaller diameter. C and D: lamina propria collagen content in the peripheral airways was positively correlated with pulmonary resistance (*r*² = 0.78, *P* = 0.03) in heaves-affected horses at baseline but not in control horses (*r*² = 0.21).]
horses was significant ($P = 0.015$) only when values of the two collagen types for each horse were combined (Fig. 3E).

**Elastic fiber content in the lamina propria of peripheral airways of heaves-affected horses compared with controls.** Twenty-four airways ($n = 3$ per horse) were evaluated for elastic fiber content in horses with heaves ($n = 4$) and controls ($n = 4$) during clinical remission of the disease. Tissue availability prevented evaluation after antigen challenge. Most elastic fibers were localized at the interface between the ASM and the lamina propria ECM in both control and diseased animals (Fig. 4). The elastic fibers varied according to the orientation of the airway myocytes. For instance, they were generally oriented radially when the ASM fibers were sectioned longitudinally (Fig. 4, C and D), whereas they appeared as single long fibers when the ASM was cross-sectioned (Fig. 4E). In some airways, the elastic fibers were diffusely present within the ECM (Fig. 4, A and B). Large bundles of elastic fibers were also observed in some airways, suggesting abnormal deposition (Fig. 4, F and G).

Elastic fiber content in the lamina propria was significantly greater in the airways of horses with heaves compared with controls ($P = 0.04$; Fig. 5A). It was positively correlated to pulmonary elastance (Ei) in controls ($r^2 = 0.95$, $P = 0.02$; Fig. 5B) but not in heaves-affected horses ($r^2 = 0.06$, $P = 0.75$; Fig. 5C). Elastic fiber content was not correlated with other lung function measurements, the airway neutrophilia, or the collagen content within the airways (data not shown).

**PGP and N-α-PGP contents in BALF in horses with heaves and controls before and after antigen challenge.** Both forms of PGPs were detected in BALF samples from most horses. For PGP, 10 BAL samples showed peak areas lower than the peak area of the most diluted standard (Fig. 6, A and C). There was no significant difference between groups or time points in the other BAL samples, with mean $\pm$ SE PGP concentrations of $488 \pm 125$ pg/ml in BALs from controls and $367 \pm 88$ pg/ml in BALs from heaves-affected horses (Fig. 6C). For N-α-PGP, 11 BAL samples showed peak areas under the lowest standard (Fig. 6, B and D). The concentration of N-α-PGP in the other BAL samples was $710 \pm 201$ pg/ml for controls and $423 \pm 119$ pg/ml for heaves-affected horses. There was no significant group or time effect, although the group and time interaction was significant ($P = 0.025$). However, Bonferroni’s post hoc tests failed to reveal any significant differences between groups at each time or between time points within groups. Similarly, no
differences between groups were observed when PGP and N-α-PGP concentrations were corrected for dilution effect by using urea and total protein in blood and BALF at 30 days (data not shown). Furthermore, neutrophil percentages in BALF were not correlated with PGP and N-α-PGP concentrations corrected for dilution effect using urea and total protein (data not shown). The concentrations of PGP and N-α-PGP were strongly correlated (Fig. 6E; $r^2 = 0.89$, $P < 0.001$), when both were detectable. The ratio of PGP/N-α-PGP in BAL samples in control and heaves-affected horses revealed stable levels of the two forms at each time point (Fig. 6F).

**DISCUSSION**

It is generally accepted that increased ECM in the airway wall leads to airflow obstruction. Indeed, several studies have linked increased subepithelial collagen content in endobronchial biopsies with airway hyperreactivity or asthma severity (1, 6). However, it has also been argued that fibrosis stiffens the airways and rather has a protective role by opposing forces to ASM contraction (17, 26). Tissue availability, especially when studying the peripheral airways of humans, has prevented the prospective evaluation of collagen and elastic fiber content in the same individuals during different phases of the disease (remission/exacerbation). In the present study, we found that, in a naturally occurring asthma-like disease of horses, an increase in collagen and elastic fiber content in the peripheral airways correlates with alterations in airway function. Thus the increased collagen content possibly contributed to the residual airway obstruction, whereas the association between elastic fiber content and the elastance (compliance) of...
the lung observed in control animals was lost in diseased horses. We found no association between collagen content and lung function during exacerbation of the disease, suggesting that collagen is not an important contributor (protection or exacerbation) to active airway obstruction in these animals or that a more complex relationship between the various components of the ECM and ASM may be implicated. Furthermore, bioactive peptides resulting from increased ECM turnover do not appear to contribute to the sustained airway neutrophilia in this condition. Collectively, the results suggest that chronic airway ECM remodeling alters airway function in diseased lungs, but its contribution to disease exacerbation and inflammation remains to be better defined.

Heaves is a common disease of horses that, similarly to asthma, is characterized by airflow obstruction, bronchial hyperresponsiveness, and airway inflammation (20). Clinical signs are triggered in susceptible horses by inhalation of antigens present in the stables, especially those associated with hay feeding. Airway neutrophilia is a characteristic finding and it has recently been shown that increased airway smooth muscle mass is present in affected horses (11, 19). It is perhaps the only naturally occurring animal disease developing throughout multiple cycles of inflammation and repair over a period of many years that captures the clinical features of asthma. A study performed by our research group has recently shown, using suppression subtractive hybridization techniques, that collagen type I alpha 2 (COL1A2) and collagen type III alpha 1 (COL3A1) are overexpressed in the lungs of heaves-affected horses (18). Furthermore, the collagenase MMP-9 (16) and neutrophil elastase (7) are increased in the airway secretions of heaves-affected horses, suggesting that collagen and elastin turnover is increased in the lungs of affected horses. Together, these findings suggested that remodeling of airway collagen also occurs in this natural disease and lead us to undertake this study.

**Collagen.** Because collagen provides tissues with stiffness, it is hypothesized to assume most of the load bearing in the expanded lungs (25, 39). Although the progression of fibrosis typically leads to organ failure, it has been suggested that fibrosis in the inner airway wall may be beneficial, since it opposes forces against contraction of the airway smooth muscle, thus limiting occlusion of the airway lumen during bronchospasm (36, 44). The positive correlation we observed between collagen content in the lamina propria of the peripheral airways of heaves-affected horses and pulmonary resistance herein, rather suggests a detrimental effect of fibrosis on the resting airways. In agreement with our findings, and although there is little information on the ECM remodeling affecting the peripheral airways of asthmatic patients, thickening of the epithelial reticular basement membrane in asthmatic central airways was also correlated to lung function deficits or airway hyperresponsiveness in some studies (6, 12, 37). Nevertheless, such an association is not a universal finding in asthma (4, 14), indicating that differences in the site evaluated (central vs. peripheral airways, basement membrane, or lamina propria), the airway phenotype, and the severity of disease may influence the biomechanical properties of the airways. Our results also extend the findings of a recent study reporting a variable and nonsignificant increase in collagen type 1 and type 3 in equine asthmatic airways (9), by showing that collagen remodeling is most marked in the smallest airways, where the largest increase in ASM is also observed in these animals, and that
both collagen types are involved in the remodeling process (11, 19).

The lack of association between collagen content and lung function after a 30-day antigenic exposure could be explained by the overriding influence of bronchospasm on lung function in diseased animals. However, the simple linear function we used to evaluate the relationship between the mechanical properties of the lungs and collagen and elastic fiber content likely underscores the complexity of the contribution of the various ECM molecules to airway function (40). Furthermore, our analysis does not take into account alterations occurring within the lung parenchyma, which also likely influence lung function during bronchospasm.

Elastic fiber content. The elastic fiber network present throughout the lung parenchyma and in most layers of the airway wall is responsible for elastic recoil of the lungs. In the bronchi of normal human subjects, distinct layers of elastic fibers are found beneath the basement membrane and close to the airway smooth muscle cells (2). As observed in horses, they appear as longitudinal bundles usually perpendicular to the orientation of the human airway smooth muscle cells (23).

Elastic fibers are also present within the smooth muscle layer and are connected to the network of adventitial fibers (22).

In the present study, elastic fiber content in the airways from control animals was positively correlated with EL. This is in agreement with findings suggesting that at low strain, most of the stress in tissue is borne by elastin fibers (39). There is great variability in the reported elastic fiber content within the various airway layers in asthma (30). However, and similar to our findings, elastin content is usually reported to be increased in the deeper layer of the submucosa (30). The functional effects of these changes are largely unknown and difficult to predict, since elastin produced in adulthood often fails to polymerize or form functional three-dimensional fibers (30, 42). Values of EL in the asthmatic horses in clinical remission (in absence of disease related bronchoconstriction) were similar to those of control horses, but it was not correlated with the elastic fiber content in their airways. This uncoupling of elastic fiber content and elastic recoil in diseased animals supports impaired elastic properties of newly deposited fibers. This finding is in agreement with the mathematical modeling suggesting that collagen, rather than elastin content, provides most...
to the overall viscoelastic properties of the lung tissue when both are increased in diseases (39). It would also suggest that the dysfunctional elastic fiber network may contribute to airway obstruction in asthma, as elastin is believed to limit ASM constriction.

**PGP and N-α-PGP.** In addition to their structural role, the ECM macromolecules are bioactive and play an important role in mechanotransduction. They influence cellular behaviors including growth, migration, adhesion, and differentiation and interact with cellular receptors to regulate key signaling pathways (5, 29, 35). It has been suggested that endogenous proteases secreted by inflammatory and structural cells contribute to tissue remodeling in various lung diseases. PGP and N-α-PGP are small fragments generated by the degradation of collagen by MMPs and by neutrophil proteases and endopeptidases (28). They are potent neutrophil chemoattractants, as they share homology with ELR-positive CXC chemokines, and activate CXC chemokine receptors CXCR1 and CXCR2 present on neutrophils and various other cell-types (46).

Given that heaves is associated with ECM remodeling and airway neutrophilia, we hypothesized that PGP and N-α-PGP would be increased in airway secretions in this disease. However, although they were detectable in BALF samples of most animals, there was no difference between groups for either peptides. This is somewhat surprising considering that MMP-9 and neutrophil elastase, two potent collagenases, are present at increased concentrations in the airway secretions of horses with heaves (3, 16), suggesting increased collagen turnover in the airways of these horses. Similar observations were made in severely asthmatic patients (28), which are known to have increased collagen content and turnover in their airways but do not have increased PGP or N-α-PGP in their airway secretions. In a mice model of influenza infection, PGP was not increased in BALFs despite the upregulation of PGP generating enzymes. This was shown to be due to the rapid degradation of PGP by leukotriene A4 hydrolase (LT₄H) produced by bronchial epithelial cells and neutrophils (38). Although LT₄H has long been known to generate leukotriene B₄ (LTB₄), it has also been recently shown to possess aminopeptidase activity for PGP (38). While the presence of LT₄H in BALF of human asthmatic patients and horses with heaves has not, to our knowledge, been investigated to date, equine and human lungs have endogenous LT₄H activity (8, 21), and we have recently reported its increased expression in the lung tissue of diseased horses compared with controls (18). The lack of differences between groups in PGP or N-α-PGP may also have resulted by the difficulty to correct for possible dilution effects of epithelial lining fluids when studying BALFs (43).

In conclusion, findings from this study suggest that ECM remodeling in asthmatic disease may result in residual lung function deficits at rest (clinical remission). These results support the need to determine specific contributions of ECM components on airway function and their possible reversibility and significance in overall disease progression.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


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