ORIGINAL RESEARCH

Peripheral Airway Smooth Muscle, but Not the Trachealis, Is Hypercontractile in an Equine Model of Asthma

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Abstract

Heaves is a naturally occurring equine disease that shares many similarities with human asthma, including reversible antigen-induced bronchoconstriction, airway inflammation, and remodeling. The purpose of this study was to determine whether the trachealis muscle is mechanically representative of the peripheral airway smooth muscle (ASM) in an equine model of asthma. Tracheal and peripheral ASM of heaves-affected horses under exacerbation, or under clinical remission of the disease, and control horses were dissected and freed of epithelium to measure unloaded shortening velocity (V_{max}), stress (force/cross-sectional area), methacholine effective concentration at which 50% of the maximum response is obtained, and stiffness. Myofibrillar Mg^{2+}-ATPase activity, actomyosin *in vitro* motility, and contractile protein expression were also measured. Horses with heaves had significantly greater V_{max} and Mg^{2+}-ATPase activity in peripheral airway but not in tracheal smooth muscle. In addition, a significant correlation was found between V_{max} and the time elapsed since the end of the corticosteroid treatment for the peripheral airways in horses with heaves. Maximal stress and stiffness were greater in the peripheral airways of the horses under remission compared with controls and the horses under exacerbation, potentially due to remodeling. Actomyosin *in vitro* motility was not different between controls and horses with heaves. These data demonstrate that peripheral ASM is mechanically and biochemically altered in heaves, whereas the trachealis behaves as in control horses. It is therefore conceivable that the trachealis muscle may not be representative of the peripheral ASM in human asthma either, but this will require further investigation.

Keywords: asthma; airway hyperresponsiveness; airway smooth muscle; smooth muscle mechanics

Asthma is characterized by airway inflammation and airway hyperresponsiveness (AHR), an exaggerated bronchoconstrictive response to various stimuli (1). Because airway smooth muscle (ASM) is the direct effector of bronchoconstriction, it is believed to be hypercontractile in asthma. Indeed, several studies, performed in animals with experimentally induced or innate AHR, have shown that tracheal and bronchial smooth muscle (SM) is hypercontractile, exhibiting increases in maximal velocity of shortening (V_{max}) (2–7) or maximal isometric force (4, 6, 8). To the contrary, measurements of these parameters in human tissues were rather inconclusive. For instance, in human trachealis and main bronchi muscle strips, isometric force (9–11) or V_{max} (9, 10) were not different between subjects with asthma and control subjects. These results were corroborated by electron microscopy measurements showing that there were no structural

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Clinical Relevance

Our data show that the trachealis of horses with heaves behaves similarly to those of control horses, whereas the peripheral airway smooth muscle, which is more readily exposed to inflammation, is hypercontractile (increased unloaded shortening velocity). Moreover, we found a correlation between the muscle hypercontractility and the time elapsed since the end of the corticosteroid treatment.

differences between asthmatic and control trachealis (12). In contrast, stress and maximal extent of shortening was increased in bronchial strips of two patients with asthma (13, 14), although one of the subjects also suffered of lung carcinoma (14). In addition, in one study performed with fatal asthmatic trachealis samples, an increased isometric force and impaired relaxation were reported (11). Finally, bronchial spirals from fatal (11, 15) and nonfatal asthmatic lungs (16) showed hypocontractile responses.

There are several reasons why we may not clearly detect enhanced contractility in asthmatic ASM. The most obvious possibility is that the ASM is mechanically normal in asthma and that alterations occur at different levels. For example, the neural control could be altered in asthma (17) or airway–parenchymal interdependence could be reduced (18, 19), and these effects would not be seen in excised SM. Furthermore, whereas numerous studies point to an increased ASM mass (20), muscle force is always studied with normalization to cross-sectional area (CSA) so that this contribution is overseen. Another possibility is that the trachealis muscle, which is the muscle of choice to study ASM mechanics because of its ease of dissection, is not mechanically representative of the SM of more peripheral airways, which are likely to be involved in asthma. It is also possible that inflammatory mediators, present in greater amounts in the peripheral airways (21, 22), alter the ASM mechanical properties. Indeed, we recently showed that incubating Brown Norway rat trachealis muscle with $CD4^+$ T cells for 24 hours increases its V_{max} along with alterations of the contractile protein expression (7).

Thus, the purpose of this study was to compare the mechanical, biochemical, and structural properties of the trachealis and more peripheral ASM in horses with heaves, a naturally occurring model of asthma (23). This animal model offers a unique opportunity to dissect and study the intrapulmonary ASM mechanics. We report that the trachealis muscle is not mechanically different between heaves-affected horses and controls, whereas the SM of the peripheral airways is hypercontractile in heaves. Some of the results of this study have been previously reported in the form of an abstract (24).

Materials and Methods

Animals

Horses from the Faculty of Veterinary Medicine, University of Montreal (St.-Hyacinthe, PQ, Canada), were studied (*see* clinical details in Table 1). Animals were pooled into three groups according to their symptomatic history: (*1*) the control group contained horses with no history of respiratory diseases; (*2*) the clinical remission group contained heaves-affected horses with inflammation and reversible airway obstruction upon hay exposure; the remission state was obtained using antigen avoidance strategies (none of the horses had received corticosteroids for at least 4 months); and (*3*) the heaves group contained horses in clinical exacerbation of the disease (all of these horses except for one had also received some corticosteroid treatments) (*see* Table 1). All procedures were approved by the Animal Care Committee of the University of Montreal (Protocol Rech-1324) and complied with the guidelines of the Canadian Council on Animal Care.

Harvesting SM

The lower part of trachea (10–15 rings) and intrapulmonary airways (3–7 mm inner diameter from degassed lungs) were placed in Hank's balanced salt solution and dissected from connective tissues and parenchyma. Fine dissection (removal of the epithelium, cartilage, and connective tissues) was performed on ice in Ca^{2+}-free Krebs-Henseleit solution. The buffers composition is detailed in the online supplement.

Muscle Strip Mechanics

The muscle strip was mounted horizontally with foil clips to a length controller (no. 322C-I; Aurora, ON, Canada) at one end and a force transducer (Aurora no. 400A) at the other, controlled by Aurora 600A software in a temperature controlled chamber (37°C) as described previously (7, 25). Reference length (L_{ref}; the *in situ* length under relaxed conditions) (9) and width at L_{ref} were measured using a Hitachi camera (KP-D20A/B; Hitachi, Toronto, ON, Canada) at 10–12 × magnification in Ca^{2+}-free Krebs-Henseleit solution and analyzed with ImageJ software (IJ1.46r; National Institute of Health, Bethesda, MD). Muscle strips from tracheal and intrapulmonary airways SM were used to study the methacholine (MCh) dose response, stress, stiffness, and shortening velocity. The details of the equilibration and mechanical measurements are included in the online supplement.

Smooth Muscle CSA

The total muscle strip CSA in square millimeters was calculated as previously described (26), with the details given in the online supplement. To evaluate the SM CSA, the muscle strips were processed for histology and examined by light microscopy, as detailed in the online supplement.

In Vitro Motility Assay

The *in vitro* motility assay was performed as previously described (27), and detailed in the online supplement.

ATPase Assay

The ATPase assay was performed as previously described (28), with modifications (29), and as detailed in the online supplement.

Western Blot Analysis

The Western blot was performed as previously described (7), and as detailed in the online supplement.

Statistical Analysis

Data are presented as mean (±SEM). The *n* values refer to the number of animals from which two or three repeats were averaged. All parameters were analyzed using GraphPad Prism 5 (GraphPad

Table 1. Clinical Information

Group	Age (years)	Weight (kg)	Sex	Clinical Condition	Medication	Resistance	Date Killed
1. Control	15	NA	Mare	No history of respiratory diseases	No corticosteroid treatment		15-Aug-2013
2. Control	9	550	Mare	No history of respiratory diseases	No corticosteroid treatment		27-Aug-2013
3. Control	20	443	Mare	No history of respiratory diseases	No corticosteroid treatment		05-Mar-2013
4. Control	18	437	Mare	No history of respiratory diseases	No corticosteroid treatment		19-Mar-2013
5. Control	20	184	Mare	No history of respiratory diseases	No corticosteroid treatment		12-Nov-2014
6. Heaves exacerbation	25	250	Mare	Exacerbation (time unknown)	Unknown	Unknown	29-May-13
7. Heaves exacerbation	22	708	Mare	Exacerbation since August 12, 2013	Corticosteroid treatment for 3 months (20 April–22 July, 2013); 3 months before being killed	1.49 cmH2O/L/s	16-Oct-2013
8. Heaves exacerbation	17	450	Mare	Exacerbation since April 30, 2013	Corticosteroid treatment for 2 weeks (23 September–6 October, 2013); 1 months before being killed	1.43 cmH2O/L/s	06-Nov-2013
9. Heaves exacerbation	23	520	Mare	Exacerbation since July 2013	Corticosteroids treatment for 2 weeks (19 November–4 December, 2013); 2 weeks before being killed	0.93 cmH2O/L/s	17-Dec-2013
10. Heaves exacerbation	15	400	Mare	Exacerbation since December 2013	No treatment with corticosteroids since March 2013; 10 months before being killed	1.75 cmH2O/L/s	22-Jan-2014
11. Heaves remission	28	430	Gelding	Remission since March 2013	No treatment with corticosteroids since January 2013	0.664 cmH2O/L/s	22-Jul-2013
12. Heaves remission	22	800	Mare	Remission since March 2013	Treated with corticosteroids a few days in late May	0.74 cmH2O/L/s	09-Oct-2013
13. Heaves remission	15	480	Gelding	In remission for 3 months	No corticosteroids treatment for 1 year	0.96 cmH2O/L/s	31-Jul-2014

Definition of abbreviation: NA, not applicable.
The animals were pooled into three main groups according to their symptomatic history: (*1*) the control group contained horses with no history of respiratory diseases ($n = 5$); (*2*) the clinical remission group contained heaves-affected horses with inflammation and reversible airway obstruction upon hay exposure ($n = 3$); the remission state was obtained using antigen-avoidance strategies (none of the horses had received corticosteroids for at least 4 months); and (*3*) the heaves group contained horses in clinical exacerbation of the disease ($n = 5$). Most of these horses had also received some corticosteroid treatments as described.

Software Inc., La Jolla, CA) and MatLab (Natick, MA), as described previously (7). A two-way ANOVA (linear mixed model with airway location and disease stage as categorical variables) were performed, followed by a Bonferroni *post hoc* test. Correlation analysis was performed by GraphPad InStat version 3.10 using Pearson's test. Differences were considered significant at a P value less than 0.05.

Results

Mechanics of Tracheal and Peripheral ASM

To assess whether the trachealis muscle is mechanically representative of the intrapulmonary ASM in heaves, we measured their mechanics in control and in heaves-affected horses during both clinical exacerbation and remission. There was a significant difference between groups with airway location ($P = 0.01$) and an interaction between airway location and stages of the disease ($P = 0.018$). No differences were observed between groups in V_{max} of the trachealis muscle (Figure 1A). In contrast, V_{max} was significantly greater in the peripheral ASM of heaves-affected horses in exacerbation (0.26 ± 0.05 L_{ref}/s) compared with controls (0.12 ± 0.01 L_{ref}/s; $P = 0.036$), but not compared with the peripheral ASM of horses with heaves under remission (0.17 ± 0.02 L_{ref}/s; $P = 0.21$; Figure 1A). Moreover, V_{max} of the peripheral ASM of heaves-affected horses in exacerbation was significantly greater than that of their own trachealis (0.12 ± 0.01 L_{ref}/s; $P = 0.036$), control trachealis (0.11 ± 0.01 L_{ref}/s; $P = 0.036$), and the trachealis of horses under remission (0.12 ± 0.03 L_{ref}/s; $P = 0.05$; Figure 1A). Importantly, in the heaves group, a linear correlation was observed between V_{max} and the time elapsed since their last corticosteroid treatment for the peripheral airways ($r^2 = 0.598$; $P = 0.009$), but not for the tracheal SM ($r^2 = 0.147$, $P = 0.27$; Figure 1A, *inset*). Figure 1B shows representative force–velocity curves.

MCh dose–response curves were performed for tracheal and peripheral ASM and are expressed in terms of stress (Figures 2A–2C) by normalizing by SM CSA (Figure 2F). For the maximal stress, there was no significant difference between airway location ($P = 0.31$), nor interactions with stages of the disease ($P = 0.12$).

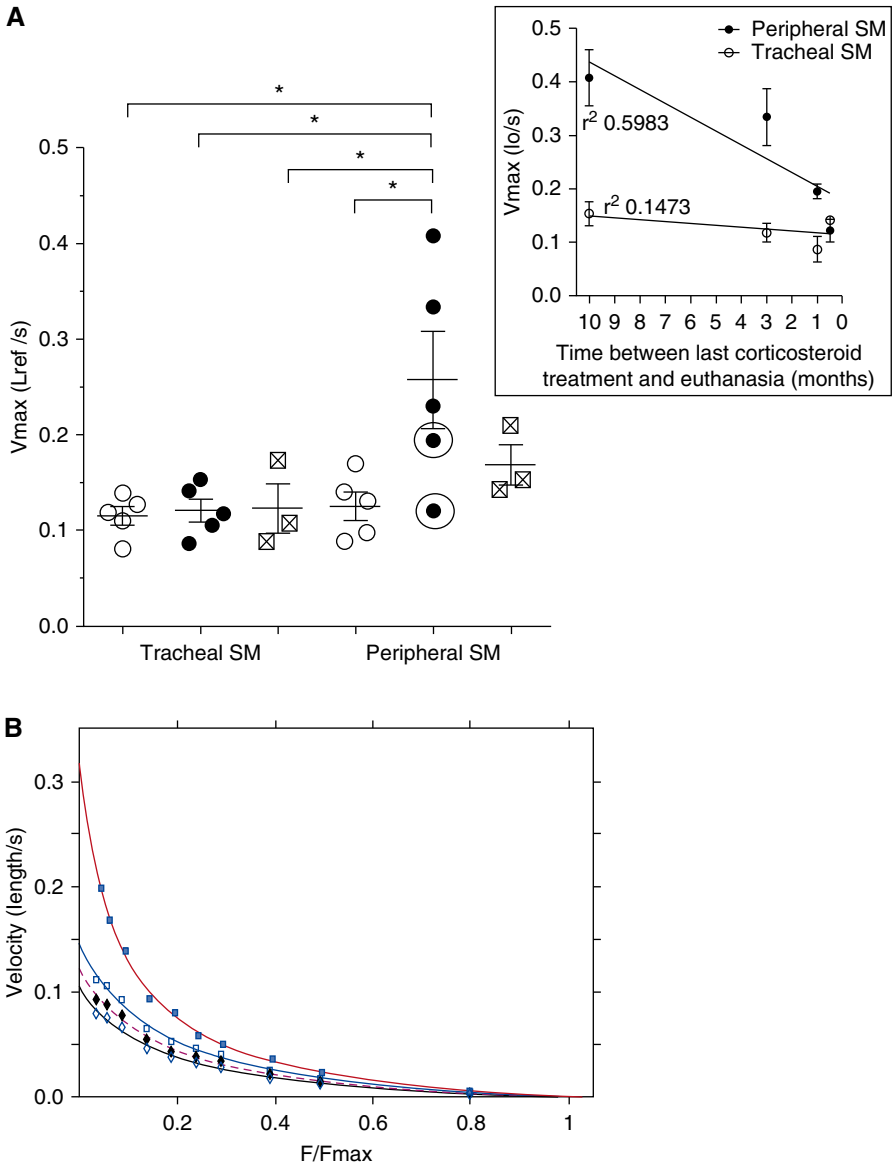

Figure 1. (*A*) Unloaded shortening velocity (V_{max}) of tracheal and peripheral airway smooth muscle (ASM) from controls (*open circles*; $n = 5$ horses), horses with heaves during exacerbation (*solid circles*; $n = 5$ horses), and horses experiencing remission (*crossed squares*; $n = 3$ horses) of the disease. The two *circled values* are from horses affected with heaves but recently treated with corticosteroids (horses 8 and 9 in Table 1). *$P < 0.05$. *Inset*, V_{max} of tracheal (*open circles*) and peripheral (*solid circles*) ASM as a function of time between the last corticosteroid treatments and killing, measured in the horses with heaves. Lo and L_{ref}, reference length. (*B*) Representative force–velocity curves for tracheal control (*open diamonds*) or peripheral control (*open squares*) smooth muscle (SM) and tracheal heaves–affected (*solid diamonds*) or peripheral heaves–affected (*solid squares*) SM. F/Fmax, force normalized to maximal force. The force–velocity relationships were accurately fitted by the Hill hyperbolic model ($r^2 > 0.98$). Data are presented as mean (±SEM).

However, significant differences were observed between peripheral airways of the remission group and the control ($P = 0.039$) or heaves ($P = 0.049$) groups (Figures 2C). Similarly, the active stiffness was not different between airway location ($P = 0.74$), and there was no interaction with stages of disease ($P = 0.10$), but a significant difference was observed between peripheral airways of the remission group and control ($P = 0.049$) or heaves-affected ($P = 0.049$) animals (Figure 2E). The MCh dose at which 50% of the maximum stress is generated (EC_{50}), was not different between groups; no significant differences were found between airway location ($P = 0.88$) and stages of the disease ($P = 0.83$) (Figure 2D). Representative cumulative MCh dose–response traces of tracheal and peripheral ASM are shown in Figures 2G and 2H.

Mg^{2+}-ATPase Activity of Tracheal and Peripheral ASM Myofibrils

To study whether the biochemical properties were altered in tracheal and peripheral ASM of control and heaves-affected horses, measurements of myofibril Mg^{2+}-ATPase activity were performed. Isolated myofibrils showed significant differences in Mg^{2+}-ATPase between airway location ($P = 0.015$) and the interaction between airway location and stages of the disease ($P = 0.013$). Myofibrils isolated from peripheral ASM of heaves-affected horses showed significantly higher ATPase activity (117 ± 24.8 nmol inorganic phosphate (Pi)/mg actomyosin (AM)/min) than myofibrils isolated from control peripheral ASM (28.5 ± 14.9; $P = 0.019$), control trachealis (30 ± 14.1; $P = 0.02$), and heaves-affected horses trachealis (33.9 ± 12.8; $P = 0.026$) (Figure 3).

Tracheal and Peripheral ASM Myosin *In Vitro* Motility

To verify whether or not the molecular mechanics of the myosin motor is also altered in the peripheral ASM of the heaves-affected horses, we purified myosin from tracheal and peripheral ASM and measured the velocity of actin filament propulsion (v_{max}) in the *in vitro* motility assay. There was a significant difference between airway location ($P = 0.0002$), but not at the interaction between airway location and stages of the disease ($P = 0.66$). No differences in v_{max} were observed between control (0.48 ± 0.05 μm/s) and heaves under exacerbation (0.49 ± 0.04; $P = 0.84$) of tracheal SM or between control (0.3 ± 0.03 μm/s) and heaves under exacerbation (0.29 ± 0.01; $P = 0.72$; Figure 4) of peripheral ASM. Note that v_{max} of myosin purified from control tracheal SM was significantly greater than that of peripheral ASM of controls ($P = 0.002$) and heaves-affected horses ($P = 0.003$), and that v_{max} of myosin from tracheal SM of heaves-affected horses was significantly greater than that of peripheral ASM of control ($P = 0.001$) and heaves-affected horses ($P = 0.001$).

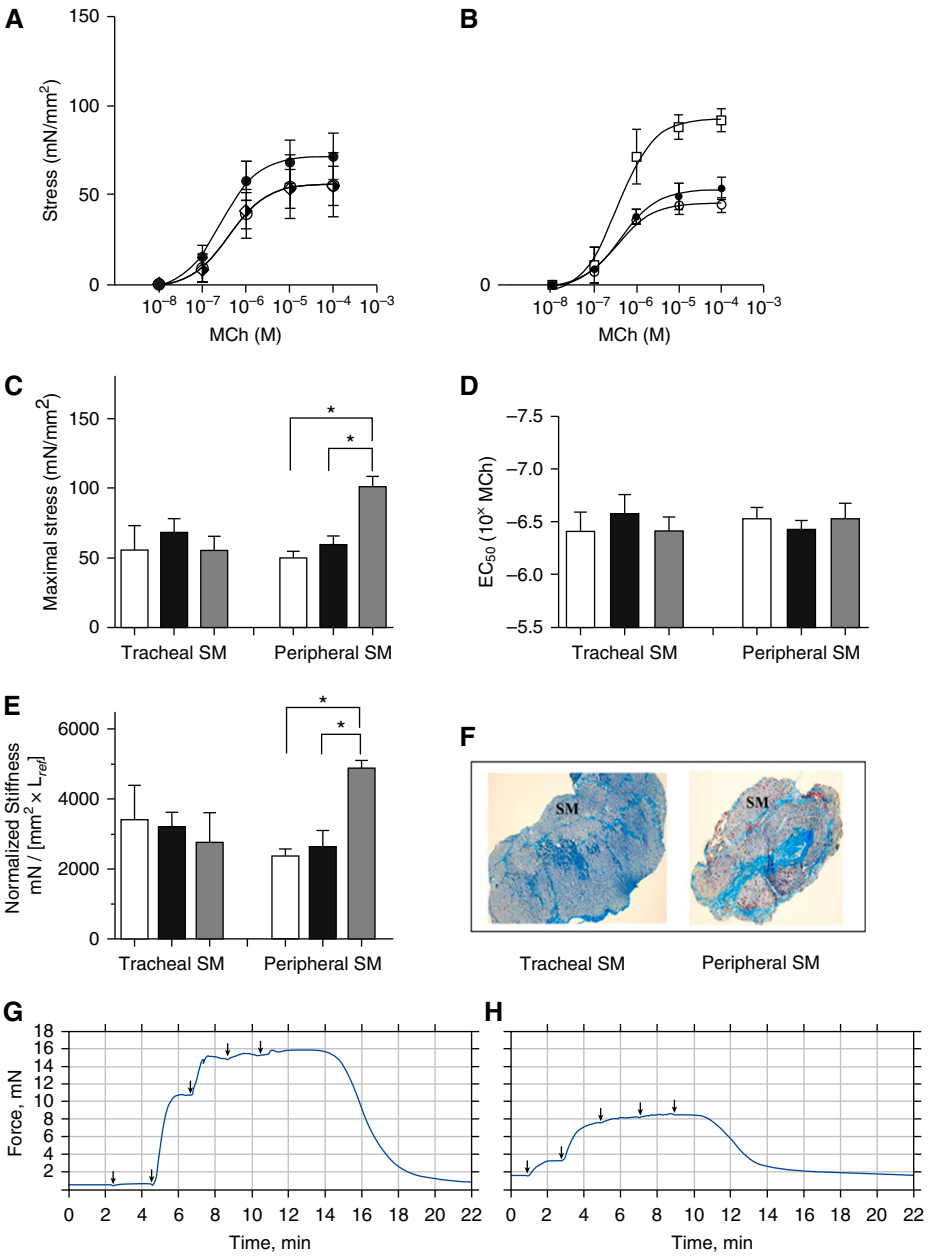

Figure 2. Mean cumulative methacholine (MCh) dose–response curves expressed as SM stress for (*A*) tracheal SM and (*B*) peripheral ASM from control horses (*open circles*; $n = 4$ horses), heaves-affected horses (*solid circles*; $n = 5$ horses), and horses under remission (*open squares*; $n = 3$ horses). (*C*) Maximal stress generated by tracheal and peripheral ASM at 10^{-4} M MCh (maximal force normalized to SM cross-sectional area [CSA]) in control (*white bars*), heaves (*black bars*), and heaves under remission (*gray bars*). *$P < 0.05$. (*D*) Effective concentration at which 50% of the maximum stress (EC_{50}) is generated for the three groups of animals. (*E*) Active stiffness (force normalized to SM CSA and divided by L_{ref}) measured in tracheal and peripheral ASM of control horses (*white bars*), horses with heaves (*black bars*), and horses under clinical remission (*gray bars*). *$P < 0.05$. (*F*) Cross-section of tracheal and peripheral ASM strips used for CSA quantification. (*G* and *H*) Representative cumulative MCh dose–response traces of tracheal (*G*) and peripheral airway (*H*) SM. *Arrows* denote time points of MCh injection. Data are presented as mean (±SEM).

Western Blot Analysis of Contractile Proteins

To determine whether the differences in peripheral ASM mechanics were due to alterations in contractile protein expression, the level of the (+)insert SM myosin heavy chain (SMMHC) isoform (or SMB), calponin (CaP), total SMMHC, myosin light chain kinase (MLCK), and transgelin (SM22) was quantified (Figure 5). No differences between airway location or stage of disease were seen in the expression of SMB ($P = 0.34$; $P = 0.077$; Figure 5A), total SMMHC ($P = 0.75$; $P = 0.93$; Figure 5C), and SM22 ($P = 0.1$; $P = 0.18$; Figure 5E). Significant differences were measured for CaP (Figure 5B) and MLCK (Figure 5D) between airway location ($P = 0.008$ and $P = 0.0003$), but not between stages of disease ($P = 0.2$ and $P = 0.66$, respectively). CaP in the tracheal SM of heaves-affected horses was significantly greater (2.15 ± 0.57 arbitrary units) than in peripheral ASM of controls (0.37 ± 0.14; $P = 0.033$) and heaves-affected horses (0.4 ± 0.16; $P = 0.026$; Figure 5B). The expression of MLCK in control trachealis muscle (0.84 ± 0.13) was greater than that of control peripheral ASM (0.05 ± 0.003; $P = 0.03$). Similarly, the expression of MLCK in heaves-affected horses trachealis muscle (0.83 ± 0.24) was greater than that of control peripheral ASM (0.05 ± 0.003; $P = 0.024$; Figure 5D).

Discussion

In this study, we compared the contractile and biochemical properties of the tracheal and peripheral ASM in heaves, a spontaneously occurring asthma-like disease in horses.

Very few studies have investigated the force generation and velocity of shortening of human asthmatic ASM because of the difficulty in obtaining such human samples. Those few studies, however, did not reveal the expected enhancement in contractility. To the contrary, the results were rather inconclusive. Some studies reported increased contractility in human asthmatic trachealis and bronchi strips or spirals (11, 13,14) or in sensitized bronchi (30), whereas others reported no mechanical differences between human asthmatic and control tracheal and main bronchi muscle strips (9, 10) or bronchial spirals (11, 15, 16). One of the problems of the early studies is that the tissues were often obtained several hours *postmortem*. Conversely, the more recent studies have used transplant-grade lungs, but have addressed only the mechanical properties of the trachealis and main bronchi SM (9, 10), and not those of peripheral ASM because of the difficulty of dissection. If the ASM is not intrinsically altered in asthma, but its

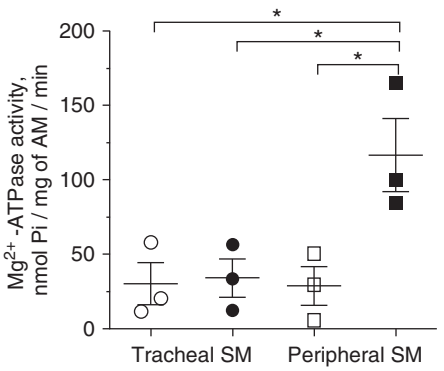

Figure 3. Mg^{2+}-ATPase activity of myofibrils isolated from tracheal and peripheral ASM of control (*open circles* and *open squares*, respectively; $n = 3$ horses for both) and heaves-affected horses during exacerbation (*solid circles* and *solid squares*; $n = 3$ horses for both). $^*P < 0.05$. Data are presented as mean (±SEM).

contractility is only enhanced when exposed to inflammatory cells and mediators, then the peripheral airways, which are more exposed to inflammation (21, 22), are more likely to have hypercontractile SM. Indeed, we recently demonstrated in the Brown Norway rat model of asthma that *ex vivo* exposure of the trachealis muscle to inflammatory cells for 24 hours enhances its mechanical properties (7). Thus, in the current study, we addressed whether or not the trachealis SM behaves differently from the peripheral ASM in a species in which we can easily access and dissect the peripheral ASM.

Horses with heaves (23) develop moderate to severe airway obstruction when kept in an antigen-rich environment characterized clinically by labored breathing at rest (dyspnea). Treatment with corticosteroids, or prolonged antigen avoidance, lead to marked improvement of clinical signs and lung function (clinical remission of the disease) (31). Antigen avoidance was the strategy used in the current study to create clinical remission. Note that, because of the limitations in tissue availability, the muscle mechanics measurements were performed on all tissues, whereas the *in vitro* motility, ATPase activity measurements, and Western blots were performed only on subgroups (the *n* is indicated in the figure legends). Furthermore, the group under remission contained only three animals. This is a limitation of our study and, while it prevents us from drawing definitive conclusions regarding peripheral ASM mechanics in heaves, it generates hypotheses to be tested in human asthma.

V_{max} measured in the trachealis muscle was not different between controls, horses with heaves, and horses with heaves under remission (Figure 1). This is in agreement with the recent data obtained in human trachealis (9, 10). However, one of the most prominent results of our study was the roughly twofold-greater V_{max} measured in the peripheral ASM of the horses with heaves compared with the control peripheral ASM and with all other trachealis SM (Figure 1). In addition, V_{max} of the peripheral airways of the horses with heaves under remission tended to decrease back toward baseline values, but this did not reach significance most probably because of the large variability in the heaves group. Two of the horses with heaves (the two *circled values* in Figure 1) had recently received corticosteroids and showed a somewhat lower V_{max}. Horse no. 8 developed very pronounced heaves symptoms and had to be treated until the scheduled killing for ethical reasons. Although its symptoms improved, it still had a high pulmonary resistance (R_L) (*see* Table 1) and was clearly still under exacerbation. The second horse (#9) that needed treatment with corticosteroids had developed lameness. This horse did not have very pronounced exacerbations and after the treatment transiently had a R_L considered normal. Treatments with corticosteroids improve heaves symptoms, but this is only transient under continued antigen exposure. Thus, although it is obvious that the treatments received improved lung function and potentially contributed to decreasing their V_{max}, the treated horses were not in a controlled state to fit the remission group. Furthermore, all of the horses with heaves except for one had received corticosteroids at given points in time. Conversely, horses with heaves under remission had not been antigen exposed and had not exhibited airway obstruction for at least 3 months, and had not received corticosteroids for at least 4 months.

A strong correlation between the time elapsed since the last corticosteroid treatment and V_{max} of peripheral ASM was found in the horses with heaves under exacerbation (Figure 1A, *inset*). The mechanism behind this effect might be related to a decrease in specific inflammatory cells or mediators in peripheral airways of the heaves-affected horses during treatment. However, it has been shown previously, in a similar cohort of horses, that inhaled corticosteroids do not significantly decrease the number of neutrophils, lymphocytes, mast cells, and macrophages during the first month of treatment (31). Thus, either the observed effect occurred via another type of cell/mediator combination or it is a direct effect of the corticosteroids on the muscle (32).

There are no differences in dose responses between controls and the animals with heaves, either in terms of maximum stress or effective concentration at which 50% of the maximum response is obtained for both the trachealis and the peripheral airways (Figure 2). This is in agreement with the recent stress data obtained in human trachealis in response to electrical field stimulation (EFS) (9) or MCh (10) stimulation. However, we observed significant differences in stress and stiffness of peripheral ASM between the remission and the control groups or remission and heaves under exacerbation (Figure 2E), potentially due to airway remodeling. Several studies have addressed how ASM changes its stiffness and contractility after mechanical oscillation and stretch, because this is believed to be an important contributor to airway normoresponsiveness

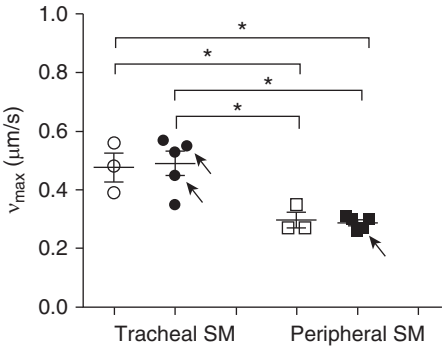

Figure 4. Velocity of actin filament (v_{max}) when propelled by thiophosphorylated myosin purified from tracheal and peripheral ASM of controls (*open circles* and *open squares*, respectively; $n = 3$ horses for both) or heaves-affected horses (*solid circles* and *solid squares*; $n = 5$ horses for both), as measured in the *in vitro* motility assay. *Arrows* show v_{max} for myosin purified from tracheal or peripheral ASM of horses affected by heaves recently treated with corticosteroids. $^*P < 0.0001$. Data are presented as mean (±SEM).

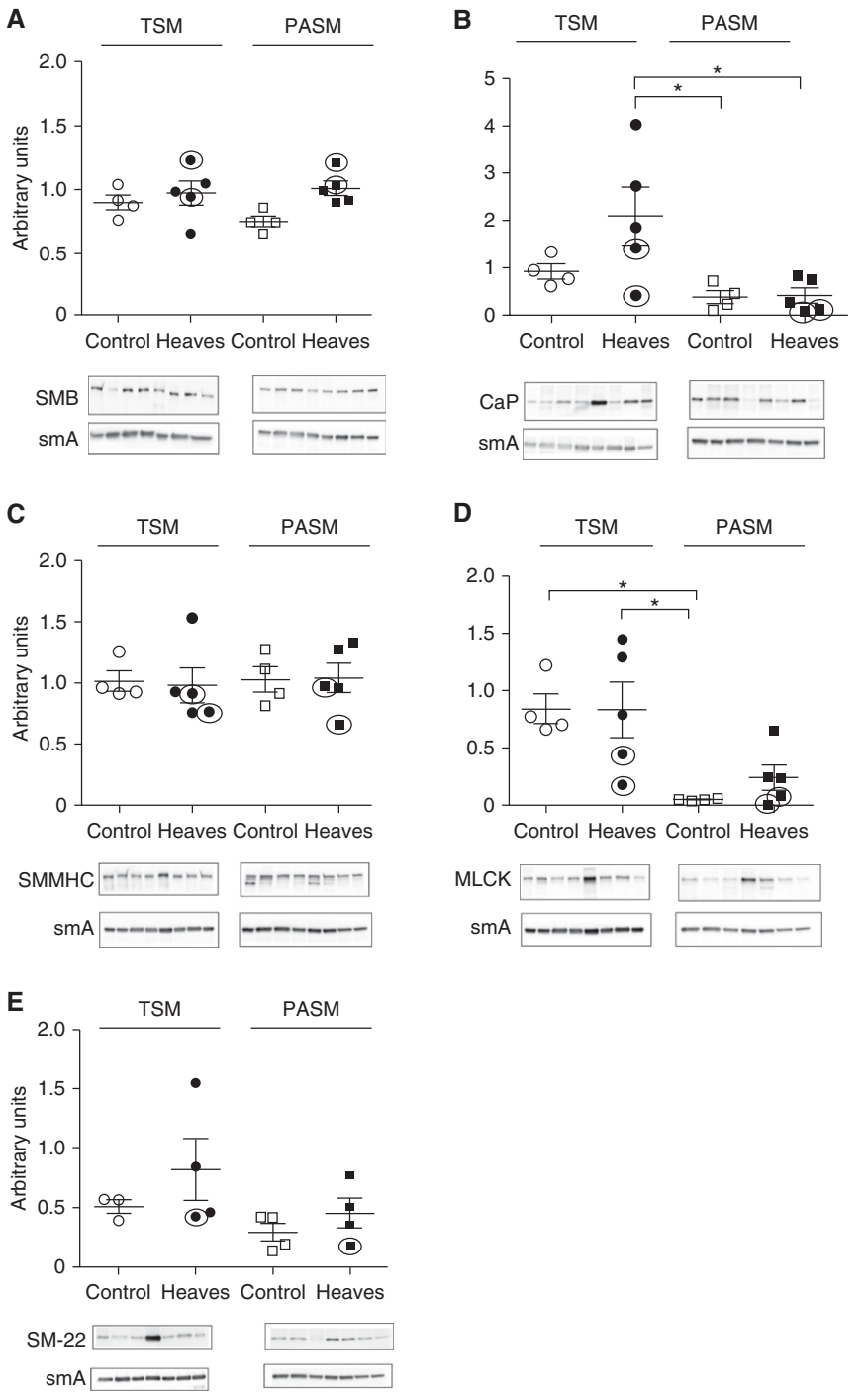

Figure 5. Western blot analysis: (*A*) (+) insert SM myosin heavy chain (SMMHC) isoform B (SM-B); (*B*) calponin (CaP); (*C*) total SMMHC; (*D*) myosin light chain kinase (MLCK); and (*E*) transgelin (SM22) of tracheal SM (TSM) of control (*open circles*) or heaves-affected horses (*solid circles*) and peripheral ASM (PASM) of control (*open squares*) or heaves-affected horses (*solid squares*); $n = 4$ for TSM and PASM of control horses (except for SM22, where $n = 3$ horses); $n = 5$ for TSM and PASM of heaves-affected horses (except for SM22, where $n = 4$ horses). Smooth muscle actin (smA) was used as a loading control. The *circled values* are from horses affected with heaves that were recently treated with corticosteroids (horses 8 and 9 in Table 1). Representative Western blots are shown. $^*P < 0.05$. Data are presented as mean (±SEM).

(9, 33), but we did not test for such effects on muscle contractility.

Intuitively, one would expect that ASM force generation would be increased in AHR and asthma/heaves, but not the velocity of shortening. However, a greater V_{max} was also reported in the canine ragweed allergic model of AHR (3) and in the Brown Norway rat ASM exposed to $CD4^+$ T cells (7). A stronger ASM would lead to a more pronounced bronchoconstriction upon simulation, but this has yet to be demonstrated convincingly in asthmatic tissues (9, 13). Conversely, a faster ASM could lead to greater airway resistance by counteracting the relaxing effect of tidal breathing (34). Extensive studies have shown that tidal or deep breaths stretch ASM and decrease airway resistance in normal lungs, but not in patients with asthma (35). Furthermore, using imaging techniques, Brown and coworkers (36) have shown that asthmatic airways dilate upon stretching, but that this is transient, because they rapidly narrow back to their initial diameter. This suggests that asthmatic ASM can shorten faster than normal ASM after a stretch. Thus, this "fast" muscle might be responsible for maintaining asthmatic airways in a more constricted state, because the ASM has time to shorten significantly between each breath, thus counteracting the relaxing effect of tidal and deep breaths.

To further dissect the mechanisms that led to the increased V_{max} in the airways of heaves-affected horses, we investigated the biochemical activity of these SMs at the myofibrillar level. Previous studies have shown that V_{max} is proportional to the rate of ATP hydrolysis in skeletal and SM (37). Indeed, supporting our muscle mechanics results (Figure 1), the myofibrillar Mg^{2+}-ATPase activity was roughly threefold greater in the peripheral airways of the heaves-affected horses compared with controls and to the trachealis muscle of heaves-affected horses (Figure 3). Similar results were obtained in other animal models; however, in those studies, V_{max} (2, 3) and ATPase activity (3, 38) were increased in both the tracheal and peripheral ASM. These differences may be attributable to the mode of sensitization or to different asthma animal models. The Fisher and Lewis rat model of innate AHR (8) also showed a greater isometric tension for the trachealis of the hyperresponsive Fisher rats, which

suggests that there might be cases of intrinsic alterations of the ASM. Thus, the similarities in tracheal V_{max} and stress between controls and horses with heaves suggest that the equine model is mechanically more representative of human asthma.

To investigate whether the difference in V_{max} and ATPase activity can be attributed, at least partially, to the mechanics of the myosin molecules, we purified the ASM myosin, the molecular motor of muscle contraction, and measured its ν_{max} of actin propulsion in the *in vitro* motility assay. No differences were observed in ν_{max} between control and heaves-affected horses (Figure 4). Similar results were obtained with tracheal SM myosin purified from the ragweed-sensitized dog model (39), despite an increased V_{max} (3) and ATPase activity (38) in that model. This suggests that myosin alone is not sufficient to alter V_{max}, but that other proteins, or post-translational alterations that might have been eliminated during the myosin purification procedure, are required. Finally, it is noteworthy that ν_{max} for the peripheral airways was lower than that of the trachea, both in controls and in horses with heaves, most probably because less SM is available for the purification in the peripheral airways, which might affect the quality of the resulting myosin.

In the current study, the Western blot (WB) analysis did not show major differences between controls and the heaves groups (Figure 5). The choice of the contractile proteins analyzed by WB was based on our previous study (40) that examined the messenger RNA expression in endobronchial biopsies from normal subjects and patients with asthma in which we observed a greater expression of the (+)insert SMMHC isoform, MLCK, and SM22 in the patients with asthma. More (+)insert SMMHC and MLCK pointed toward a more contractile phenotype, whereas the function of SM22 remains unknown. We did not observe any significant differences in the fast (+)insert SMMHC isoform expression in tracheal and peripheral ASM of control and heaves-affected horses. Accordingly, no differences were observed in ν_{max} in the motility assay (Figure 4). Note that, in a previous study, we reported a greater expression of the fast (+)insert SMHHC isoform in the peripheral airways of the heaves-affected horses compared with peripheral airways of controls and horses with heaves under remission (41), but variability between animals and airway location may account for the differences between studies along with the different technology used to quantify protein content (Western blot versus mass spectrometry). The expression of MLCK and CaP showed significant differences between airway locations, but not between disease stages. These results are difficult to interpret, because expression of these proteins may also have been influenced by the corticosteroid treatments (Figures 5B and 5D). It is interesting to note that a lower MLCK content in airways was observed in the ragweed-sensitized dog model, where the activity of MLCK, but not its content was elevated (42).

One surprising result from WB analysis was the significantly greater CaP expression in trachea of heaves-affected horses compared with peripheral ASM of control and heaves animals (Figure 5). It is unclear why CaP should be expressed differently in the trachealis and not in the peripheral airways of the heaves-affected horses. CaP inhibits myosin cycling through its interaction with F-actin. Indeed, it has been shown at the tissue level that V_{max} of CaP knockout mice is higher than that of wild-type mice, and that adding back exogenous CaP inhibits the unloaded V_{max} (43, 44). We obtained additional data comparing the contractile protein profile of the myofibrils described previously here, and our results suggest that filamin and α-actinin are also differently expressed between the trachea and the peripheral airways and potentially differently expressed between heaves and control trachealis (on line supplement). These proteins are known inhibitors of the Mg^{2+}-ATPase activity, ν_{max} and V_{max} (45, 46), and this could have contributed to the decreased V_{max} and Mg^{2+}-ATPase activity that we observed in tracheal SM relative to peripheral SM from horses with heaves under exacerbation.

It is conceivable that several factors are required for a muscle to become hypercontractile. For example, the presence of inflammatory cells and mediators, or the possibility to repetitively shorten excessively upon stimulation, might be necessary for a muscle to become hypercontractile. Mechanical adaptation and plasticity have indeed been studied extensively and are believed to contribute significantly to AHR (47, 48). Thus, the trachealis of the heaves-affected horse might be exposed, although to a smaller extent than the peripheral airways, to inflammation, but is restrained from excessive contraction due to the cartilage rings; this may lead to a different muscle phenotype in which contractile proteins that favor muscle relaxation are up-regulated. This will obviously require further investigations.

The principal site of airway obstruction in asthma has been a topic of debate for several years. Whereas the peripheral airways have initially been termed "silent," because their small size was compensated by the large total CSA of the peripheral airways (49), more recent studies have revealed several reasons why they may play an important role in asthma (50, 51). Our study did not address precisely which airway generation is involved in asthma, but assessed the function of extra- versus intrapulmonary airways. Thus, our observations could be interpreted in terms of the effects of intrapulmonary inflammation (21, 22). Nevertheless, even if it is well accepted that the trachealis and main bronchi SM are not involved in asthma, because of the restriction of their contraction due to the cartilage rings, they have been used extensively in animal models and in human studies to address AHR. Using the SM of those extrapulmonary airways to study asthma assumes that intrinsic mechanical properties of the ASM are altered. Our data demonstrate that this is not the case in the heaves model of asthma. Indeed, the trachealis of the heaves-affected animals behaves similarly to that of the controls, whereas the peripheral ASM exhibits hypercontractility. Thus, the peripheral ASM potentially acquires these abnormal mechanical properties in the presence of the inflammatory mediators (7) or, as suggested by others, because of repeated contractile challenges and adaptation of the SM to shorter lengths (47, 48, 52). The increased enzymatic activity that leads to an increase in V_{max} appears to be the primary response of the ASM in this horse model of asthma; however, the precise pathway involved will await further studies.

Conclusions

Our data show that the trachealis of the horses with heaves behaves similarly to that of control horses, whereas the peripheral ASM, which is more

readily exposed to inflammation, is hypercontractile (increased V_{max}). Having previously demonstrated that incubation of ASM with inflammatory cells enhances their mechanics (7), we conclude that the alterations in peripheral ASM mechanics of the horse with heaves must be induced by the inflammatory cells present in the peripheral airways. ■

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