Assessment of leukotriene B_4 production in leukocytes from horses with recurrent airway obstruction

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Objective—To determine the ex vivo leukotriene (LT) biosynthesis in peripheral blood neutrophils (PBNs) and inflammatory cells in bronchoalveolar lavage fluid (BALF) obtained from horses affected with recurrent airway obstruction (RAO).

Animals—6 RAO-affected and 6 control horses.

Procedures—Before and 6, 24, and 48 hours after stabling, disease severity was determined subjectively by clinical and mucus scores and measurement of the maximal change in pleural pressure (ΔPpl_{max}); PBNs were isolated and BALF samples were examined cytologically. The PBN and BALF cells were activated with a calcium ionophore in the presence of arachidonic acid, and production of LTC_4 and LTB_4 was measured per 10^6 cells.

Results—Clinical and mucus scores and ΔPpl_{max} increased during stabling in RAO-affected horses, but not in control horses. In neutrophils and BALF cells from both groups, production of LTB_4 exceeded that of LTC_4. At all times, LTB_4 production by PBNs was less in RAO-affected horses than it was in control horses. Before stabling, LTB_4 production by cells in BALF was low in RAO-affected horses, but increased considerably after 6 hours of stabling. This increase coincided with the migration of neutrophils into the airways. In control horses, production of LTB_4 did not change during stabling.

Conclusions and Clinical Relevance—Results suggested increased production of LTB_4 in airways of RAO-affected horses, compared with control horses, that may contribute to the infiltration of neutrophils into the lungs and the sustained inflammation associated with RAO. (*Am J Vet Res* 2004;65:289–295)

Recurrent airway obstruction (**RAO**; also known as heaves) in horses is a widespread respiratory tract disorder characterized by neutrophil influx and mucus secretion into the airways, bronchoconstriction, airway inflammation, and bronchial hyperresponsiveness.[1] The bronchial hyperresponsiveness wanes and disease remission occurs when horses are pastured or when changes in the environment reduce exposure to dust and moulds. Confinement in stables and inhalation of hay dust result in development of airway obstruction and migration of neutrophils into the lung within 4 to 6 hours, at which time large numbers of inflammatory cells can be recovered from samples of **bronchoalveolar lavage fluid (BALF)**.[2]

In inflammatory respiratory conditions associated with neutrophilia such as RAO, neutrophils provide a potential target in the treatment of the condition because the accumulation and activation of those cells at an inflamed site result in release of proteases and oxygen radicals that are responsible for tissue damage. Furthermore, neutrophils produce large amounts of **leukotriene (LT)** B_4 and can also secrete LTA_4 (the precursor of LTB_4); LTA_4 can be converted into the potent bronchoconstrictor LTC_4 by certain surrounding cells.[3] The unstable epoxide LTA_4 is synthesized from arachidonic acid by the action of **5-lipoxygenase (5-LO)**.[4] In neutrophils and various other cell types, LTA_4 can be converted to LTB_4 by the enzyme LTA_4-hydrolase, whereas eosinophils contain LTC_4-synthase, which converts LTA_4 to LTC_4.[5] For LTB_4 production, equine neutrophils require exogenous arachidonic acid during stimulation with calcium ionophore A23187; for LTC_4 production, equine eosinophils do not require exogenous arachidonic acid during stimulation with calcium ionophore.[6] In the experience of 1 of the authors (AL), cells in BALF obtained from healthy and RAO-affected horses also need exogenous arachidonic acid for leukotriene formation.

Leukotriene B_4 is a potent proinflammatory mediator that primarily affects leukocyte functions.[7,8] In neutrophils, it induces chemotaxis, upregulation of adhesion molecules, and extravascular migration to the site of inflammation. In horses, aerosol administration of LTB_4 is followed by migration of neutrophils into the lung.[9] In humans, LTB_4 stimulates cytokine production and activates the transcription factor, **nuclear factor-κB (NF-κB)**, thereby promoting tran-

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scription of inflammatory genes and cytokine production.[10] High levels of NF-κB activity develop in bronchial cells during acute airway obstruction in RAO-affected horses.[11] There is also evidence that LTB_4 maintains survival of neutrophils in humans by delaying apoptosis.[12] Increased numbers of neutrophils and LTB_4 formation have been detected in humans with chronic bronchitis and experimentally induced bronchial hyperresponsiveness.[13,14]

Because LTB_4 can initiate so many of the changes observed in RAO-affected horses, the purpose of the study reported here was to determine the ex vivo LTB_4 biosynthesis in **peripheral blood neutrophils (PBNs)** and inflammatory cells in BALF obtained from horses affected with RAO, compared with that of control horses. In vitro stimulation of these cells for LT biosynthesis was performed to test the hypothesis that the formation of LTs in purified PBNs and BALF leukocytes was greater in RAO-affected horses than it was in control horses. Furthermore, we tested the hypothesis that the leukotriene production was correlated with the infiltration of neutrophils into lung airways and with the onset of airway obstruction.

Materials and Methods

Horses—Twelve horses from the Pulmonary Laboratory's herd were used in the study. Six RAO-affected horses of various breeds (4 mares and 2 geldings) were selected; the criteria for inclusion of these horses fulfilled those defined by the International Workshop on Heaves (ie, they developed signs of RAO when exposed to the organic dust found in hay).[1] The RAO-affected horses were 14 to 25 years old (mean ± SEM age, 21.0 ± 2.7 years). Six control horses of various breeds (3 mares and 3 geldings) were selected on the basis that they did not develop airway obstruction when exposed to hay. The control horses were 12 to 27 years old (17.7 ± 6.3 years). The All-University Committee for Animal Use and Care of Michigan State University approved the study.

Experimental protocol—Horses were studied in randomly selected pairs (1 RAO-affected horse and 1 control). All horses had been at pasture for at least 1 month and had no clinical signs of airway obstruction before the start of the study. Horses were transported from pasture and kept in the same stable; they were bedded on straw and fed from the same batch of poor quality hay, which induced the onset of airway obstruction in the RAO-affected horses. Clinical and mucus scoring, lung function testing, and withdrawal of peripheral blood and BALF samples were performed just before (0 hours) and 6, 24, and 48 hours after stabling.

To determine whether repeated bronchoalveolar lavage affected cytologic findings in BALF samples, lavage was repeated in 5 of the control horses kept on pasture. The schedule was the same as that used in the main study (ie, at baseline [0 hours], 6, 24, and 48 hours).

Clinical scoring—One observer (AL) conducted clinical scoring in all horses. For each horse, the extents of nostril flaring and abdominal effort of breathing were each scored on a scale[15] of 1 to 4 and the scores were summed. This scoring system correlates well with the severity of airway obstruction.[15]

Measurement of lung function—Changes in pleural pressure were measured in unsedated horses by use of an esophageal balloon (length, 10 cm; circumference, 3.5 cm; wall thickness, 0.06 cm) that was sealed over the distal end of a polypropylene catheter (length, 2.4 m; internal diameter, 3 mm; external diameter, 4.4 mm). The balloon was passed into the distal third portion of the esophagus and connected to a pressure transducer,[a] which was calibrated before each study by means of a water manometer. The position of the esophageal balloon was adjusted to obtain the **maximal change in pleural pressure during tidal breathing (ΔPpl_{max})**. At least 30 breaths were recorded on a physiograph,[b] and the mean value was calculated for each measurement period.

Mucus scoring and bronchoalveolar lavage—Horses were sedated with xylazine hydrochloride[c] (0.3 to 0.5 mg/kg, IV), and a 3-m-long endoscope[d] was passed into the trachea via 1 of the nares. Mucus accumulation in the trachea was scored by 1 individual (AL) as follows: 1, no visible mucus accumulation; 2, a few drops; 3, coalescent drops; and 4, large ventral pool of mucus.[16] The endoscope was then advanced and wedged into a peripheral bronchus. A sample of BALF was collected by infusing and manually aspirating three 100-mL aliquots of PBS solution that was warmed to room temperature (20°C). The lavage fluids retrieved were pooled, and samples were placed on ice. Because the protocols required repeated bronchoalveolar lavage during 48 hours, different regions of the lung were lavaged at each time period.

An aliquot of BALF was used for cytologic evaluation and the remainder for investigation of biosynthesis of leukotrienes. To obtain a total cell count, each BALF sample was evaluated manually by use of a hemacytometer. Smears of BALF were prepared after cytocentrifugation and stained with Geimsa; differential cell counts (including percentages of lymphocytes, monocytes and macrophages, neutrophils, eosinophils, mast cells, and epithelial cells) were performed by counting 2 × 200 cells per smear sample via light microscopy.

Preparation of purified PBNs—Blood (100 mL) was collected into 50-mL syringes by jugular venipuncture and transferred to EDTA-containing test tubes (final concentration of EDTA was 4.1mM). Neutrophil suspensions were prepared by centrifugation on a Percoll[e] density gradient.[2] After spontaneous erythrocyte sedimentation, the leukocyte-rich plasma was centrifuged at 200 × *g* for 15 minutes. The leukocyte pellet was resuspended in 20 mL of the supernatant and gently layered on a Percoll-plasma gradient (15 mL each of 60% and 80% Percoll solutions and 5 mL of 100% Percoll solution) and centrifuged at 400 × *g* for 30 minutes. The neutrophils were found on the gradient between the layers of 60% and 80% Percoll. The pellet (containing ≥ 98% neutrophils and 0% to 2% eosinophils) was resuspended in PBS solution containing 0.9mM calcium chloride (pH, 7.4) to a final concentration of 15×10^6 cells/mL.

Biosynthesis of leukotrienes—Each BALF sample was centrifuged (200 × *g* for 10 minutes at 4°C) and resuspended at a concentration of 10^7 cells/mL before the incubation of the cells. A 0.5-mL aliquot of BALF cells was incubated with calcium ionophore A23187[c] (1μM) and arachidonic acid[f] (8μM) for 5 minutes. One milliliter of purified PBN suspension was incubated with calcium ionophore A23187 (1μM) or **N-formyl-methionyl-leucyl-phenylalanine**[e] (**fMLP**; 10^{-7}M) in the presence of arachidonic acid (8μM) for 5 or 10 minutes, respectively. All incubations were terminated by addition of ethanol containing prostaglandin B_2[f] as an internal standard. Samples were stored at –20°C until analyzed for LT concentrations.

Leukotriene assays—After centrifugation and evaporation, the samples for LT assay were dissolved in mobile **high performance liquid chromatography (HPLC)** phase and

recentrifuged. Leukotriene analysis was performed by use of reversed-phase HPLC with a C18 column[g] (3.9 × 150 mm) eluted with mixture of acetonitrile, methanol, water, and acetic acid (vol:vol basis, 27:18:54:0.8, respectively; apparent pH, 5.6) at a flow rate of 1.0 mL/min. A variable wavelength UV-detector[h] connected to an integrator[i] was used for UV-detection at 280 nm. Quantitative determination was performed by peak area integration. The compounds were identified by cochromatography with synthetic standards. Limits of detection were 10 pmol/mL with a sample size of 1 mL. Data were collected as LT production per 10^6 cells.

Statistical analyses—Data were categorized according to the groups of horses (ie, RAO-affected or control horses) and sampling times (ie, 0, 6, 24, and 48 hours after stabling). Values are given as mean ± SEM. Analysis of data was performed by repeated measures ANOVA with group (RAO vs control) and time as main effects. Before analysis, data were checked for equal variance and, if necessary, transformed (log10). Student-Newman-Keul's test was used for post-hoc analysis. The relationship between LT biosynthesis and BALF cell percentages was evaluated by determination of Pearson's correlation coefficient. Values of $P < 0.05$ were considered significant for all tests.

Results

Pulmonary function and BALF cell counts—Before horses were stabled, there were no differences in clinical score, mucus score, or ΔPpl_{max} between the 2 groups. In control horses, none of these variables was affected by stabling (**Table 1**). In RAO-affected horses, stabling significantly increased clinical and mucus scores and the ΔPpl_{max} , compared with values in control horses.

The recovered BALF volume was significantly less in RAO-affected horses (75 ± 10 mL) than in control horses (155 ± 10 mL), but there was no effect of stabling. Before stabling, there were no differences in total or differential cell counts in BALF samples between the 2 groups (**Table 2**). However, from 6 to 48 hours after stabling, significant differences between the groups were detected. In the RAO-affected horses, a large increase in the total number of cells in BALF samples was noted because of an increase in the number of neutrophils. In control horses, the percentage and total number of those cells also increased, but the differences from baseline (0 hours) were not significant. Interestingly, there was a decrease in differential counts of lymphocytes, and monocytes and macrophages in RAO-affected horses after stabling, but the total number of these cells increased, compared with values in control horses. In RAO-affected horses, the differential percentage of mast cells was less than that of control horses. There were no differences in total or differential percentage of eosinophils or epithelial cells between RAO-affected and control horses. Repeated lavage of 5 of the control horses kept on pasture was associated with no adverse effects and revealed no significant changes in either total or differential cell counts in BALF samples (data not shown). However, 3 of the 5 horses had a transient increase in differential percentage of neutrophils at 24 hours.

Production of LTB_4 in purified PBNs—After stimulation of PBNs with calcium ionophore A23187, there was significantly less production of LTB_4 in purified PBN suspensions from RAO-affected horses, compared with PBN suspensions from control horses; however, there was no significant effect of duration of stabling. The formation of LTB_4 per 10^6 cells at 0, 6, 24, and 48 hours was 18.3 ± 3.8, 19.6 ± 3.1, 18.8 ± 5.7, and 22.8 ± 7.7 pmol, respectively, in control horses, whereas at those time points, LTB_4 formation was 11.7 ± 5.8, 10.3 ± 3.5, 11.4 ± 5.1, and 10.4 ± 2.1 pmol, respectively, in RAO-affected horses. The mean value of LTB_4 production over all time points was 10.8 ± 4.2 and 19.9 ± 2.5 pmol/10^6 cells in RAO-affected and control horses, respectively.

After stimulation of PBNs with the more physiologic agonist fMLP, there was significantly less production of LTB_4 in purified PBN suspensions from RAO-affected horses, compared with PBN suspensions from control horses; similar to findings with A23187 stimulation, there was no significant effect of duration of stabling. The mean value of LTB_4 biosynthesis over all time points was 4.3 ± 1.5 and 6.6 ± 1.9 pmol/10^6 cells in RAO-affected and control horses, respectively. Overall, the LTB_4 production by PBNs was less after stimulation with fMLP than it was after stimulation with the calcium ionophore.

Biosynthesis of leukotrienes in BALF leukocytes—Incubation of BALF cells with calcium ionophore A23187 and exogenous arachidonic acid resulted in formation of LTB_4. There was 10 times as much LTB_4 produced as there was LTC_4 (**Table 3**). In

Table 1—Mean ± SEM clinical and mucus scores and maximal change in pleural pressure during tidal breathing (ΔPpl_{max}) in 6 horses with recurrent airway obstruction (RAO) and 6 control horses before (0 hours) and 6, 24, and 48 hours after stabling

	Duration of stabling (h)			
Variable	**0**	**6**	**24**	**48**
Clinical score				
RAO-affected horses*	3.0 ± 1.0	4.5 ± 0.5[a,b]	6.5 ± 1.2[a,b]	7.0 ± 0.7[a,b]
Control horses	2.0 ± 0	2.0 ± 0	2.3 ± 0.4	2.7 ± 0.7
Mucus score				
RAO-affected horses*	2.2 ± 0.8	2.2 ± 0.9	3.0 ± 0.7[a]	3.7 ± 0.4[a,b]
Control horses	2.0 ± 1.0	1.3 ± 0.4	1.3 ± 0.4	1.5 ± 0.5
ΔPpl_{max} (cm of water)				
RAO-affected horses*	16.6 ± 13.4	20.9 ± 7.5	35.8 ± 14.6[a,b]	37.2 ± 3.8[a,b]
Control horses	5.8 ± 1.1	5.5 ± 1.0	6.9 ± 2.8	5.2 ± 1.5

*N = 5 at 0 hours. [a]Value significantly ($P < 0.05$) different from that of control horses. [b]Value significantly ($P < 0.05$) different from time 0 within group.

Table 2—Total and differential cell counts (mean values ± SEM) in samples of bronchoalveolar lavage fluid (BALF) obtained before (0 hours) and 6, 24, and 48 hours after stabling of 6 RAO-affected horses and 6 control horses

	Duration of stabling (h)							
	0		6		24		48	
Variable	RAO-affected horses	Control horses	RAO-affected horses	Control horses	RAO-affected horses	Control horses	RAO-affected horses	Control horses
Differential percentage								
Neutrophils	15.0 ± 10.0	6.3 ± 6.2	62.6 ± 26.7[a,b]	14.5 ± 6.5	82.6 ± 6.6[a,b]	21.8 ± 10.5	74.8 ± 15.1[a,b]	25.3 ± 11.5
Lymphocytes[c]	40.2 ± 9.8	55.7 ± 14.6	18.1 ± 13.4	52.0 ± 11.2	9.8 ± 3.7	49.1 ± 12.5	12.2 ± 8.2	44.3 ± 10.2
Monocytes and macrophages	39.8 ± 10.0	32.1 ± 9.5	16.3 ± 11.0[b]	27.9 ± 6.4	6.8 ± 2.8[b]	24.7 ± 11.0	10.4 ± 6.6[b]	25.1 ± 9.3
Mast cells[d]	4.0 ± 0.3	4.2 ± 2.1	1.6 ± 1.3	4.7 ± 1.1	0.8 ± 0.4	3.7 ± 2.9	1.6 ± 1.9	4.8 ± 3.5
Eosinophils	0.2 ± 0.1	0.4 ± 0.5	0.1 ± 0.2	0.3 ± 0.3	0	0.5 ± 0.5	0.1 ± 0.1	0.1 ± 0.2
Epithelial cells	0.9 ± 0.8	1.7 ± 0.6	1.2 ± 1.2	0.5 ± 0.3	0.1 ± 0.1	0.4 ± 0.3	1.0 ± 0.8	0.4 ± 0.3
No. of cells (×10^3/mL)								
All cells[c]	46 ± 21	23 ± 8.4	209 ± 159[a,b]	27 ± 14	1,080 ± 1,030[a,b]	105 ± 103	906 ± 918[a,b]	119 ± 109
Neutrophils	7.8 ± 7.8	1.6 ± 1.4	160 ± 159	3.2 ± 1.7	913 ± 879	13.3 ± 10.2	693 ± 707	23.1 ± 21.1
Lymphocytes	17.2 ± 7.5	9.8 ± 6.2	23.6 ± 15.8[a,b]	14.4 ± 7.0	88.0 ± 77.5[a,b]	50.7 ± 49.4	83.3 ± 67.3[a,b]	44.6 ± 36.4
Monocytes and macrophages	19.2 ± 10.9	6.1 ± 4.0	21.9 ± 9.2[b]	8.1 ± 5.4	67.2 ± 66.6[a,b]	35.5 ± 42.3	106 ± 127[a,b]	45.2 ± 53.2

[a]Values significantly ($P < 0.05$) different between groups. [b]Values significantly ($P < 0.05$) different from baseline (0 hours) within groups. [c]Significant time and group differences but no interaction detected. [d]Significant effect of group detected.

Table 3—Mean ± SEM values of leukotriene (LT) B_4 and C_4 production (per 10^6 cells) and capacity (per milliliter) in samples of BALF stimulated with the calcium ionophore A23187 (1μM) in the presence of arachidonic acid (8μM) in 6 horses with RAO and 6 control horses before (0 hours) and 6, 24, and 48 hours after stabling

	Duration of stabling (h)			
Variable	0	6	24	48
LTB_4 production (pmol/10^6 cells)				
RAO-affected horses	5.5 ± 0.1[a]	34.0 ± 6.2[b]	42.2 ± 14.3[b]	40.0 ± 17.0[b]
Control horses	15.9 ± 4.4	2 0.5 ± 10.6	23.0 ± 13.3	34.3 ± 16.0
LTC_4 production (pmol/10^6 cells)				
RAO-affected horses	1.2 ± 0.2	2.0 ± 1.3	1.8 ± 0.4[b]	2.4 ± 1.2
Control horses	2.6 ± 1.0	2.2 ± 1.3	3.0 ± 1.4	3.2 ± 1.4
LTB_4 capacity (pmol)				
RAO-affected horses	0.2 ± 0.1	9.9 ± 7.2[a,b]	34.0 ± 27.2[a,b]	38.2 ± 43.0[a,b]
Control horses	0.4 ± 0.2	0.4 ± 0.2	2.6 ± 2.9	4.3 ± 4.4[b]
LTC_4 capacity (pmol)				
RAO-affected horses	0.1 ± 0.1	0.8 ± 0.8	2.1 ± 2.1[b]	1.9 ± 1.7[b]
Control horses	0.1 ± 0.1	0.1 ± 0.1	0.4 ± 0.5	0.5 ± 0.5

*N = 5 at 0 hours. [a]Value significantly ($P < 0.05$) different from that of control horses. [b]Value significantly ($P < 0.05$) different from time 0 within group.

BALF cells obtained before stabling (0 hours), the stimulation resulted in significantly less production of LTB_4/10^6 cells in RAO-affected horses (5.5 ± 2.2 pmol/10^6 cells), compared with that produced in control horses (15.9 ± 4.4 pmol/10^6 cells); however, there were no differences in BALF cell counts between the 2 groups (Table 2 and 3). In RAO-affected horses, there was a significant increase in LTB_4 formation by 6 hours (34.6 ± 6.3 pmol/10^6 cells) that persisted through 24 and 48 hours. Production of LTB_4 was not significantly increased in control horses during stabling. At no point after 0 hours was there a significant difference in LTB_4 production between groups. In all BALF samples, a positive correlation ($P < 0.05$) was detected between LTB_4 production and the number of neutrophils (results not shown); there was no correlation between the number of monocytes and macrophages and LTB_4 formation.

The formation of LTC_4 after stimulation with calcium ionophore A23187 in the presence of arachidonic acid did not differ between the 2 groups or over time. The amounts of LTC_4 formed by both groups were small (ie, < 3 pmol/10^6 cells).

To evaluate the potential capacity for LT production in both groups of horses, the LT production per milliliter of BALF was calculated as the product of LT formed per 10^6 cells and the number of cells per milliliter of BALF. At 48 hours, the LTB_4 capacity per milliliter of BALF in RAO-affected horses was 150 times as great as that calculated at 0 hours; in control horses, LTB_4 capacity per milliliter of BALF was only 10 times as great at 48 hours as it was at 0 hours (Table 3). The capacity to produce LTC_4 increased significantly with duration of stabling, but the magnitude of the increase was much less than that calculated for LTB_4, and the difference between groups did not achieve significance ($P < 0.057$).

Discussion

The neutrophil is the predominant cell that invades the airways of RAO-affected horses after a hay or straw challenge. For this reason, neutrophil func-

tion in RAO-affected horses has been a major focus of investigation. Results of studies[17-20] to investigate the production of reactive oxygen species and neutrophil adhesion in horses have indicated that the type of stimulus and type of functional response measured appear to be important in determining whether differences between RAO-affected and control horses will be detected. The study reported here was intended to investigate production of LTB_4 (an important chemotactic agent for neutrophils) by neutrophils in peripheral blood and BALF samples from RAO-affected and control horses before and during exposure to hay or straw. The study was designed to measure the production capacity of neutrophils, and therefore we did not measure LTB_4 concentration in BALF of the horses.

Results of our study indicated that LTB_4 production in purified PBNs from RAO-affected horses was less than that in PBNs from controls; furthermore, there was an increase in ex vivo LTB_4 formation in BALF cell suspensions obtained from RAO-affected horses in the first few hours after stabling, compared with that detected in control horses. In the RAO-affected horses, the increased LTB_4 formation per million BALF cells occurred concurrently with changes in clinical score, lung function (ΔPpl_{max}), and cellular composition of BALF. The increases in clinical score, ΔPpl_{max}, and BALF neutrophils soon after stabling illustrated that the affected study horses fulfilled the criteria for RAO; moreover, the absence of such changes in control horses indicated that they did not have the disease.[1] The clinical score used in the study of this report correlates well with changes in pulmonary function that are indicative of airway obstruction.[15] Furthermore, the increase in ΔPpl_{max} in RAO-affected horses is a result of airway obstruction. In addition, an increase in mucus score in RAO-affected horses was detected during stabling, but there was no similar increase in controls.

Infiltration of airways by large numbers of neutrophils derived from the peripheral blood circulation is a characteristic feature of acute exacerbations of RAO[21,22]; the small increase in the differential percentage of neutrophils in BALF samples from control horses, compared with that detected in RAO-affected horses, has also been reported previously.[23] Although we cannot rule out that repeated bronchoalveolar lavage contributed somewhat to the increased numbers of neutrophils in the airways, the changes observed in RAO-affected horses were characteristic of the disease. As expected, there was a correlation between neutrophil counts in BALF samples and LTB_4 formation, demonstrating the importance of neutrophils for LTB_4 production in the airways. For this reason, it was important to understand the production of leukotrienes by purified PBNs so that changes observed in the production of leukotrienes by BALF cells could be evaluated. Our results indicated that there was less LTB_4 formation after ex vivo stimulation with either calcium ionophore A23187 or fMLP in the presence of arachidonic acid in equine purified PBNs from RAO-affected than there was in those cells from control horses. Compared with neutrophils from humans, equine neutrophils require low concentrations of exogenous arachidonic acid for the production of LTB_4.[6] In humans, that LT-producing capacity of neutrophils is dependent on cellular maturation[24]; segmented neutrophils in the bone marrow have a lower capacity, compared with those in the peripheral blood circulation. It is possible that the PBN population in RAO horses is somewhat immature, resulting in low LTB_4-producing capacity. Similarly, reduced capacity of purified PBNs for production of reactive oxygen species and for phagocytosis in horses with chronic airway disease has been detected.[25] However, in RAO-affected horses, Rickards et al[20] reported increased production of reactive oxygen species after stimulation of PBNs with C5a in the presence of cytochalasin B but no change in production after stimulation of PBNs with serum-treated zymosan. Thus, it may be important in the future to examine the effects of different stimuli on LTB_4 production by neutrophils.

The production of reactive oxygen species by neutrophils in response to fMLP requires priming of the neutrophils (eg, by endotoxin) that results in functional coupling of fMLP receptors.[17] In the absence of arachidonic acid, equine neutrophils produce little LTB_4 when activated with fMLP; however, in the experience of 1 of the authors (AL), the addition of arachidonic acid (which enhances translocation of 5-LO to the nucleus of equine neutrophils) has resulted in the production measurable amounts of LTB_4 in the presence of 10^{-7}M fMLP.

In both groups of horses, the main metabolite of cells in BALF samples was LTB_4; formation of LTB_4 was approximately 10 times as great as that of LTC_4. In cells in BALF samples from healthy humans, the ratio of LTB_4:LTC_4 is similar to that detected in horses (approx 12:1).[26] On stimulation ex vivo, cells in BALF samples obtained from RAO-affected horses before stabling (0 hours) produced significantly less LTB_4 than did cells in BALF samples from control horses, despite there being no differences in cell counts in BALF samples, clinical score, or ΔPpl_{max} between the groups at this time. Similarly, cells in BALF samples from humans with asthma but without clinical signs are reported to produce less LTB_4 than cells from nonasthmatic individuals, whereas the concentrations of LTC_4 are similar between the groups (as detected in the horses of our study).[26]

Before horses were stabled, the production of LTB_4 was similar in PBNs and BALF leukocytes, but there was significantly less production in RAO-affected horses than there was in control horses. After stabling of RAO-affected horses, there was an influx of neutrophils into the airways (such that the BALF samples contained primarily neutrophils), but the production of LTB_4 per 10^6 cells was 3 to 4 times as great as that of the purified PBNs at the same time period. This increased LT-synthesizing potential could be a result of translocation of 5-LO, but could also be associated with an increase in expression of activity of 5-LO or LTA_4-hydrolase after recruitment of neutrophils to the airways.[27] A similar up-regulation of phagocytosis and respiratory burst has been reported[25] in leukocytes from airways of horses with chronic airway disease, which provides further evidence of the activation of leukocytes after entering the lung.

During stabling, there was a large increase in LTB_4 formation per 10^6 cells in BALF samples from RAO-affected horses, but the increase in the differential percentage of neutrophils was even greater than the increase in LTB_4 formation in these cells; this suggests that there was down-regulation of the LTB_4 formation in each neutrophil or that significant amounts of LTB_4 originated from lymphocytes and macrophages. This observation was particularly noticeable after 48 hours of stabling when the LTB_4 formation per million cells was similar in both groups of horses, yet the differential percentages of neutrophils in BALF samples from RAO-affected and control horses were 75% and 25%, respectively. We speculate that there may be decreased ability of neutrophils in BALF from RAO-affected horses to produce LTB_4 ex vivo because of high synthetic activity in vivo, which initiates regulation by inhibitory feedback loops. The enzyme that is critical in LT formation is 5-LO, and it is inactivated by its end product LTA_4.[28] Furthermore, in humans, generation of PGE_2 and 15-hydroxyeicosatetraenoic acid (HETE) by airway epithelial cells suppresses the synthesis of LTB_4 by neutrophils.[29-31] The level of production of both of these mediators in horses with chronic airway disease has been reported to be high.[32,33] In those horses, the persistently high level of production of 15-HETE during clinical remission may suppress LTB_4 production. Such a phenomenon may explain the much lower LTB_4 production by cells in BALF samples from RAO-affected horses obtained before stabling (after at least 1 month on pasture), compared with that detected in control horses.[29,32,33] Thus, similar mechanisms may attenuate the formation of LTB_4 in both horses and humans.

The results of our study indicated that there was formation of small amounts of cysteinyl LTs per million cells in BALF samples obtained from RAO-affected and control horses, with no differences between those groups or during stabling. This is in agreement with the lack of eosinophils and small numbers of mast cells in BALF samples detected in RAO-affected horses. However, when the capacity of cells in BALF to produce LTC_4 was calculated for samples from RAO-affected horses, there was only a slight increase after stabling, compared with the value obtained before stabling. These observations suggest that cysteinyl LTs may play a small role in RAO. Although LTD_4 causes contraction of equine small airways in vitro and bronchoconstriction in vivo, that effect is less in horses with chronic airway disease than it is in healthy horses.[9,34,35] Furthermore, an LTD_4-receptor antagonist failed to improve airway function in RAO-affected horses.[36] Overall, these results do not support a role for the cysteinyl-LTs in RAO.

By comparison, LTB_4 has the potential to play an important role in the pathogenesis of RAO. Although RAO-affected and control horses produced similar amounts of LTB_4 per million cells after 48 hours of stabling, there were vastly more millions of cells in the airways of RAO-affected horses. At 48 hours, the LTB_4 capacity per milliliter of BALF in RAO-affected horses was 150 times as great as that calculated at 0 hours. In control horses, the LTB_4 production capacity also increased over time, but the value at 48 hours was only 10 times as great as that at 0 hours, which was a much smaller increase than that detected in RAO-affected horses. Because there was a large increase in the LTB_4 producing capacity in cells in BALF samples from RAO-affected horses during stabling, it is tempting to speculate that LTB_4 is of importance for the maintenance of the inflammation and recruitment of neutrophils in RAO. Furthermore, it is noteworthy that, although less potent than the cysteinyl LTs, LTB_4 is a bronchoconstrictor, at least in guinea pigs.[37] Our enthusiasm for suggesting a role of LTB_4 in RAO is tempered somewhat by the observation that the 5-lipoxygenase inhibitor fenleuton did not diminish the accumulation of neutrophils in the lungs of horses with chronic obstructive pulmonary disease.[38] Despite the latter observation, it would be interesting to evaluate the effect of specific LTB_4-antagonists in RAO.

[a]Model DP45-34, Validyne, Northridge, Calif.
[b]Dash model II, Astromed, West Warwick, RI.
[c]Vedco Inc, St Joseph, Mo.
[d]Olympus Corporation of America, New Hyde Park, NY.
[e]Sigma Chemical Co, St Louis, Mo.
[f]Biomol Research Laboratories, Plymouth Meeting, Pa.
[g]Waters Assoc, Milford, Mass.
[h]LDC spectromonitor III, Riviera Beach, Fla.
[i]LDC/Milton Roy CI-4000, Riviera Beach, Fla.

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