J Vet Intern Med 2013;27:164–170

PI3K and MAPKs Regulate Neutrophil Migration Toward the Airways in Heaves

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Background: Neutrophils accumulate in the airways of horses with heaves. They likely play an important role in the disease pathogenesis. Understanding the pathways regulating their migration may help identifying new therapeutic targets.

Hypothesis: MAPK and PI3K pathways are involved in neutrophil migration toward the airway lumen in heaves.

Animals: Twelve heaves-affected horses and 4 healthy horses.

Methods: Migratory activity of bronchoalveolar lavage fluids (BALF) from horses with heaves and healthy horses was compared by means of a Boyden chamber. Involvement of MAPK and PI3K pathways in neutrophil migration was investigated by pretreating neutrophils with inhibitors of p38 MAPK, JNK, MEK1/2, and PI3K. The capacity of a p38 MAPK inhibitor at decreasing neutrophil chemotaxis toward the airways was also evaluated in vivo.

Results: BALF from symptomatic heaves-affected horses induced a greater degree of chemokinesis ($P = .0004$) than BALF from healthy horses. Although all pathways tested were involved in neutrophil migration, inhibition of PI3K was most potent in vitro. An inhibitor of p38 MAPK administered before challenge in horses with heaves did not alter BALF chemokinetic properties. BALF neutrophil percentage and BALF migratory activity were positively correlated after 14 and 35 days of antigen challenge in healthy ($P = .05$; $R^2 = 0.82$) and heaves-affected horses ($P = .03$; $R^2 = 0.76$), respectively.

Conclusions and Clinical Importance: MAPK and PI3K pathways regulate neutrophil migration induced by BALF of horses with heaves. Inhibition of multiple pathways might be required to completely abolish BALF-induced neutrophil migratory activity and possibly inflammation in heaves.

Key words: Horses; Inflammation; Lungs; RAO.

Neutrophils migrate toward sites of inflammation and infection where they act as early responder cells toward the external insults. They are key actors in host defense and also mediate tissue damage of various noninfectious inflammatory processes. Neutrophils accumulate in the airways of horses affected by heaves starting few hours after their exposure to antigen-rich environments.[1] Within the airways, they likely contribute to bronchoconstriction, mucus hypersecretion, and pulmonary remodeling by releasing proinflammatory mediators, including TNF-α, MMPs, elastase, and reactive oxygen species.[2,3]

Peripheral neutrophils adhere to endothelial cells and migrate toward a gradient of stimuli (chemotaxis) in a dose-dependent or independent manner in both humans and horses. Mechanisms regulating their recruitment in the tissues are complex and incompletely understood.[4] Bronchoalveolar fluids (BALF) of heaves-affected horses have increased chemotactic activity when compared with that of healthy horses, although this is not a constant finding.[5,6] Indeed, several chemoattractants including CXCL8, MIP-2, and TNF-α[7–9] are present in greater concentrations in the airways of horses with heaves compared with normal subjects during antigen challenge. However, it is not yet clear which cells are mainly involved in synthesis and release of these chemokines.

Abbreviations:

BALF	bronchoalveolar lavage fluid
ERK	extracellular signal-regulated kinase
fMLP	Nformyl-Met-Leu-Phe
JNK	c-Jun NH_2-terminal kinases
MAPK	mitogen-activated protein kinase
MIP-2	macrophage inflammatory protein-2
MMP	matrix metalloproteinase
PAF	platelet-activating factor
PI3K	phosphoinositide-3 kinase.
PKB	protein kinase B

The ability of peripheral neutrophils to respond to chemoattractants by migration is mediated by activation of several complex intracellular signaling pathways.[10,11] Among those, mitogen-activated protein kinases (MAPKs) and phosphoinositide-3 kinase (PI3K) have been shown to be of primary importance in neutrophil migratory activity.[12] Expression of chemotaxis-related pathways is usually studied in the presence of a single stimulus, which is clearly not what occurs in vivo, where complex regulation of several cell functions in response to multiple simultaneous mediators is required to maintain homeostasis. Furthermore, the use of chemoattractants alone or in combination might induce different effects on chemotaxis and its regulatory mechanisms.[13]

The presence of neutrophils in the airways coincides with exacerbation of the disease and possibly contributes to tissue damage and repair. Glucocorticoids are anti-inflammatory drugs believed to inhibit neutrophil

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Submitted April 30, 2012; Revised July 9, 2012; Accepted September 25, 2012.

10.1111/jvim.12008

recruitment into the airways in heaves. Recently, our group has shown that these drugs do not reverse luminal neutrophilic inflammation, unless the antigenic stimulus is withdrawn.[14–16] Because of the poorly defined role of neutrophils in pathogenesis of heaves, understanding the mechanisms underlying their migration and accumulation in the airways could highlight important therapeutic targets.

In this study, we explored several pathways possibly involved in the recruitment of neutrophils toward the airway lumen. We hypothesized that MAPKs and PI3K contribute to the regulation of neutrophil migration in heaves. The aims of this study were to I) investigate differences in chemokinetic activity of BALFs from heaves-affected and normal horses; II) examine the effects of inhibitors of MAPKs and PI3K on neutrophil chemokinesis induced by BALFs from heaves-affected horses in vitro; and III) study the effects of a p38 MAPK inhibitor on equine neutrophil airway-directed chemotaxis.

Materials and Methods

Animals

Sixteen mixed-breed healthy and heaves-affected horses from a research herd were studied. All horses were stabled together and regularly dewormed and vaccinated. During the 4 months preceding the beginning of the study, horses had been pastured together to ensure complete remission of the disease in the affected animals. Horses undergoing antigen challenge were stabled, bedded on straw, and fed hay twice a day.

Three additional horses (mean±SD, 501 ± 38 kg; 13 ± 2 year old) from a teaching herd considered healthy based on clinical examination and hematology findings were used as donors of neutrophils. They were regularly vaccinated and dewormed, and had no history of drug administration within the last month.

The experimental protocol was performed in accordance with the guidelines for the Canadian Council on Animal Care and was approved by the Animal Care Committee of the Faculty of Veterinary Medicine of the Université de Montréal.

Respiratory Function Test

Respiratory mechanics were performed on unsedated horses as previously described.[17] Respiratory flow rates were measured with a heated pneumotachograph. Transpulmonary pressures (ΔP_L) were measured with a balloon sealed over the end of a polyethylene catheter placed in the distal third of the esophagus. All input signals were analyzed by a dedicated data acquisition and analysis software.[a] Values of pulmonary resistance (R_L) and elastance (E_L) were also obtained for each breath by applying the multiple regression equation for the single compartment model of the lung.

Bronchoalveolar Lavage

BALs were performed on horses sedated with xylazine[b] and butorphanol[c] (IV dose) as previously described.[18] Briefly, a fiberoptic endoscope was introduced into a nostril and advanced until it wedged in a bronchus. Two boluses (250 mL) of sterile prewarmed (37°C) isotonic saline solution were sequentially infused and aspirated with a suction pump. BAL fluids were filtered through sterile gauze, centrifuged at 4°C and supernatant stored at −80°C until chemotactic assays were performed. The total cell count was determined with a hemacytometer and differential cell counts were performed on cytospin preparations[d] following Wright Giemsa staining. A minimum of 400 cells were counted for each sample.

Neutrophil Isolation

Blood was collected in heparinized tubes[e] by jugular venipuncture from healthy horses. Neutrophils were isolated from peripheral blood by lympholyte-poly density gradient centrifugation.[f] Neutrophils were resuspended in PBS containing Ca^{2+} (1 mM), Mg^{2+} (1 mM), and 0.5% BSA to obtain a final concentration of 5×10^6 cells/mL). The total neutrophil count was determined with a hemacytometer, and cell viability was assessed by a Trypan blue dye exclusion test. Neutrophils were used for chemotactic assays if they demonstrated greater than 95% purity and 98% viability.

Neutrophil Pretreatment with Pharmacological Inhibitors

MAPKs, PI3K, and c-Jun N-terminal kinase (JNK) inhibitors were used to study the signaling pathways involved in neutrophil migration induced by BALFs of heaves-infected horses. Neutrophils isolated from healthy horses were pretreated with SB203580[g] (3 μM), SP600125[h] (15 μM), and PD98059[i] (50 μM) to block p38 MAPK, JNK, and MEK1/2, respectively. Wortmannin[j] (100 nM) was used to block PI3K. Neutrophils were pretreated with all inhibitors for 1 hour at 37°C. Effective concentrations of all inhibitors were selected according to data obtained in preliminary experiments performed on equine neutrophils, to validate concentrations used in previous studies (data available online).

Chemokinetic Assays

Chemokinetic activity of BALFs on neutrophils was measured with 48-well microchemotaxis chambers.[k,19] The lower wells were filled with 30 μL of the chosen chemoattractant. They were then overlaid with Sartorius cellulose nitrate filters[l] (8-μm pore size, 140-μm thick) premoistened with PBS containing Ca^{2+} (1 mM), Mg^{2+} (1 mM), and 0.5% BSA. The same solution was used in the lower wells as negative control in preliminary tests (data not shown). Aliquots of 55 μL of pretreated or untreated neutrophils suspension ($\gg 1.5 \times 10^5$ cells in the comparative experiment between healthy and heaves horses, $\gg 2.75 \times 10^5$ cells in all other experiments) were then added over each well. The chambers were incubated for 1 hour at 37°C in 5% CO_2 and humidified air. The filters were harvested, fixed with mercuric chloride, and stained with hematoxylin[m] and Chromotrope 2R.[n,20] Then, they were dehydrated, cleared, and counted as described elsewhere.[21] Chemokinetic activity of BALFs was determined by counting the number of neutrophils on the lower side of the filter, with a light microscope and an eyepiece graticule to facilitate the counting. Five distinct fields were counted at high magnification (400 ×). All chemotactic assays were performed as triplicates. Mean values of triplicates were reported and used for statistical examinations.

Experimental Protocol

Part 1. Five heaves-affected horses (460 ± 15 kg, 15 ± 4 year old) and 4 control horses (491 ± 85 kg, 12 ± 3 year old) were exposed to antigen challenge for 35 days. Migratory activities of BALFs obtained from the 2 groups at T_{35} were measured in vitro.

BALFs collected at T_{35} from the 5 heaves-affected horses were employed in chemokinetic assays to assess the involvement of selected kinase inhibitors in regulating equine neutrophil migration.

Part 2. Ten asymptomatic heaves-affected horses (500 ± 38 kg; 13 ± 2 year old) were exposed to antigen challenge for 14 days. Three horses had participated in study 1; a minimum of 8-week washout period was observed between the studies. During the 14 days of antigen exposure, 5 horses were treated with a p38 MAPK inhibitor as previously reported by Lavoie,[22] whereas 5 horses were used as untreated controls. Briefly, the MRL-EQ1 compound was administered IV twice a day at decreasing dosages (1.5 mg/kg for 3 days; 1 mg/kg for 6 days; and 0.75 mg/kg for 5 days). BALs were performed before and after the treatment period (T_0 and T_{14}). Percentage of neutrophils in BALF was measured and chemokinetic assays were performed to assess migratory activity of BALFs collected at the different time points.

Statistical Analysis

The effects of antigen challenge and group (heaves versus control) on transpulmonary pressure and neutrophil percentage in BALF, and the in vitro inhibition of neutrophil migrations with pharmacological agents, were analyzed with repeated-measure mixed linear models with a priori contrasts and sequential Bonferroni correction with SAS v.9.1.[o] All other data were analyzed by GraphPad Prism 5 Software.[p] An unpaired *t*-test was used to analyze data from the comparative studies of control and heaves-affected horses or in vivo p38 MAPK inhibitor-treated and untreated horses. One-tailed Pearson tests were used to correlate neutrophil percentage in BALF and BALF-induced neutrophil migratory activity as higher chemotactic activity was expected in BALF with greater percentage of neutrophils. Graphs show raw data, expressed as mean ± standard deviation [SD]. *P* values less than .05 were considered as statistically significant.

Results

PI3K Pathway

Horses from both groups had normal lung function (ΔP_L<10 cmH_2O) and no airway inflammation (neutrophils <5%, mast cells <2%, eosinophils <1%) while at pasture. Horses with heaves developed airway obstruction and a greater bronchial luminal neutrophilia ($P = .005$) compared with controls when challenged (Fig 1). BALF from heaves-affected horses induced a significantly greater (approximately 3-fold) migration of neutrophils compared with BALF from healthy horses ($P = .0004$, Fig 2). The percentages of neutrophils in BALF correlated positively with BALF migratory activity at T_{35} in healthy horses ($P = .05$, $R^2 = 0.82$), but not in horses with heaves ($P = .18$; $R^2 = 0.27$). All inhibitors studied significantly decreased the chemokinesis of equine neutrophils triggered by BALF collected from heaves-affected horses (Fig 3). Wortmannin (100 nM), a PI3K inhibitor, was most potent at decreasing neutrophil migration ($P \leq .002$) compared with other inhibitors tested. Blocking both p38 MAPK and MEK1/2 had stronger inhibitory effects than blocking p38 MAPK ($P = .0004$) alone, but not MEK1/2. Therefore, the combination of p38 MAPK and MEK1/2 inhibitors showed at most an additive effect on BALF-induced neutrophil chemokinesis.

MAPK Pathway

BALF-induced migration of neutrophils was not significantly different in the 2 groups of heaves-affected horses at T_0 (data not shown). Administration of a p38 MAPK inhibitor did not reduce BALF migratory activity at T_{14} when compared with BALF migratory activity of untreated heaves-affected horses at the same time (Fig 4). The percentage of neutrophils in BALF and BALF migratory activity was positively correlated in untreated horses ($P = .03$, $R^2 = 0.76$), but not in p38 MAPK inhibitor-treated horses ($P = .35$, $R^2 = 0.03$).

Discussion

Using BALF supernatants from antigen challenged heaves-affected horses as a chemokinetic compound in our experiments, we demonstrated that both MAPK and PI3K pathways are involved in regulating airway neutrophil migratory activity in vitro, with the latter being most potent. The methodology we employed measures generalized increase in the migratory activity of neutrophils (chemokinesis), but did not allow to differentiate between chemotaxis and chemokinesis. The previously reported reduction in neutrophil percentages in BALF with the administration of a p38 MAPK inhibitor to horses with heaves[22] was likely caused by a reduction of peripheral blood neutrophil ability to migrate, given the unchanged BALF migratory activity observed in this study. Taken together, these results suggest that multiple signaling pathways contribute to airway neutrophilia in this disease, but that PI3K might represent an effective target for the control of inflammation in heaves.

The accumulation of neutrophils within the airway lumen likely plays a central role in the clinical and pathological features of equine heaves. Drugs preventing neutrophil chemotaxis toward the airways could thus represent novel tools for the control of this disease. Studies using both human and animal neutrophils have attempted to clarify which pathways regulate their migration toward the airway lumen.[23–27] In vitro studies generally use single chemoattractants to evaluate the possible contribution of several pathways to cell migration. Interestingly, the stimuli used to stimulate neutrophil chemotaxis in vitro elicit different responses when applied separately or simultaneously, as a consequence of the ability of several enzymatic pathways to regulate each other once activated.[13,28] Thus, a chemotactic stimulus as similar as possible to that present in vivo is more likely to yield results leading to effective treatment.

Several molecules, including chemokines, cytokines, and eicosanoids, have been identified in greater concentrations in BALF of heaves-affected horses compared with normal horses. CXCL8, LTB_4, and MIP-2 are three of the most potent chemoattractants synthesized by neutrophils and other cells that have been

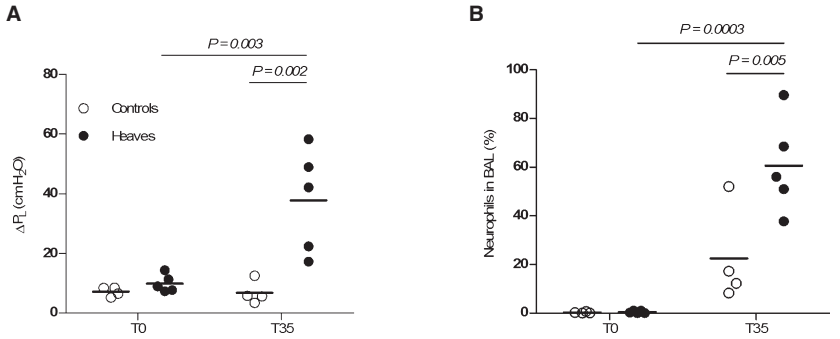

Fig 1. Effects of antigen challenge on lung function (A) and inflammation (B) in controls (n = 4) and heaves-affected horses (n = 5). A 35-day period (T_{35}) of antigen challenge induced a significant increase in percentage of BALF neutrophils and maximal change in transpulmonary pressure (ΔP_L) only in heaves-affected horses.

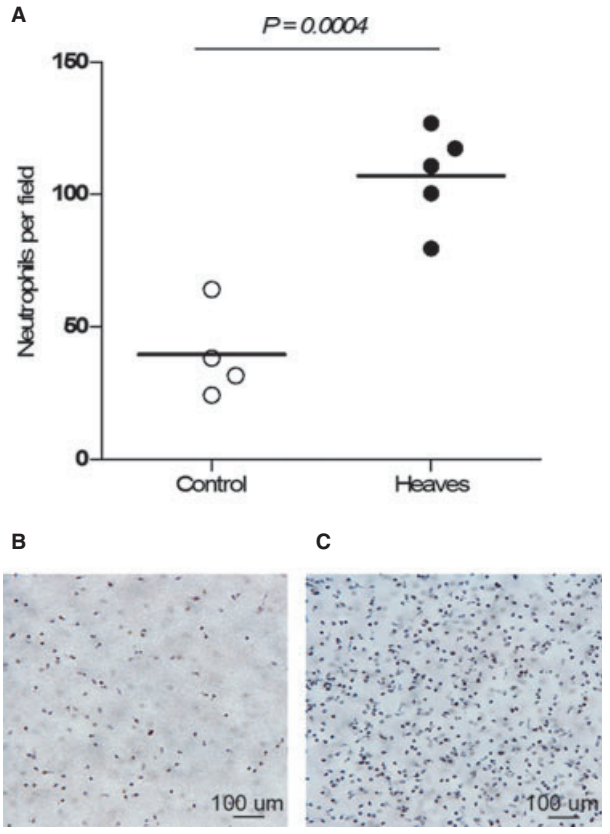

Fig 2. Migratory properties of BALF from heaves-affected (n = 5) and control (n = 4) horses. BALFs collected from horses with heaves after 35 days of antigen challenge induced a significantly greater neutrophil chemokinesis in vitro, compared with BALF from healthy horses kept in the same environment (A). Representative microphotographs of neutrophil migration induced by BALF of healthy (B) and heaves-affected horses (C). Hematoxylin and Chromotrope 2R, 40x.

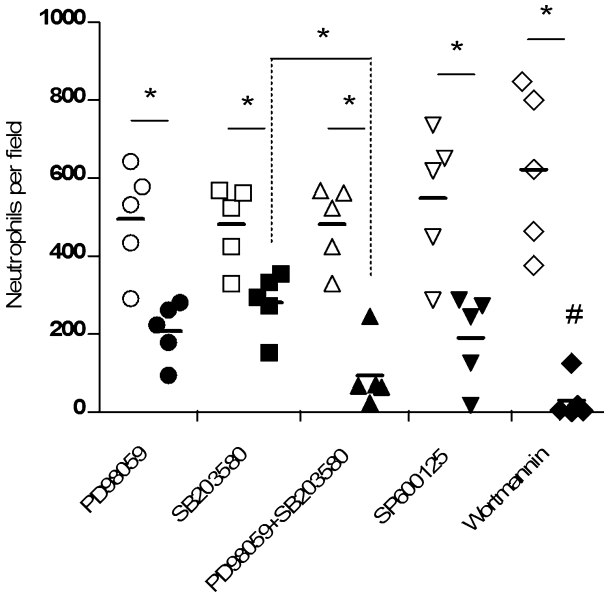

Fig 3. Inhibition of neutrophil migration by kinase inhibitors in vitro. BALF were collected from horses with heaves after 35 days of antigen challenge. White: untreated; black: treated. *$P < .001$; #: significantly greater than all other inhibitors tested ($P \leq .002$).

associated with heaves.[29–34] IL-4 and TNF-α also contribute to recruitment and retention of neutrophils into inflammatory sites.[35,36] TNF-α induces an increase in the expression of the adhesion molecule ICAM-1 in both human endothelial and bronchial epithelial cells in vitro, which may foster local neutrophil influx in vivo.[37–39] Given the nature and concentration of the molecules detected in BALF from heaves-affected horses, it is not surprising that our experiments, in agreement with previous reports,[6,40,41] showed an increased migration of neutrophils toward BALF from heaves-affected horses compared with healthy horses.

MAPKs are serine/threonine-specific protein kinases activated by different extracellular stimuli following a cascade mechanism. MEK1/2 is one of the kinases that activate MAPKs. The MAPKs subfamilies include the extracellular signal-regulated kinases (ERK), further subclassified as ERK1/2, ERK3/4, ERK5 and ERK6/7, the c-Jun NH_2-terminal kinases (JNK), and the p38 MAPK, of which ERK1/2, JNK, and p38 MAPK are expressed in neutrophils.[12,42] The p38 MAPK pathway has been shown to regulate proinflammatory angiogenesis, cytokine and chemokine expression, and synthesis

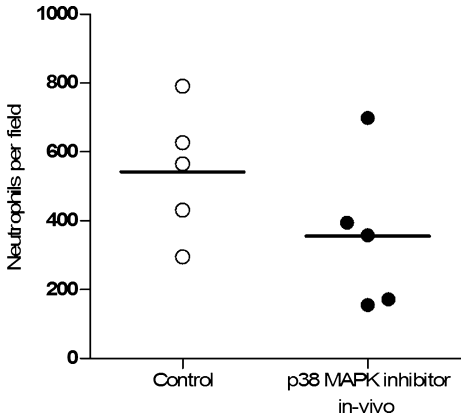

Fig 4. BALF migratory activity after 14 days of antigen challenge and simultaneous in vivo treatment with p38 MAPK inhibitor. BALF collected from heaves-affected horses receiving systemic treatment (n = 5) had a chemokinetic activity not significantly different compared with BALF from untreated heaves-affected horses (n = 5; $P = .111$).

by local cells and neutrophils in the lung.[36,43–46] PI3Ks are a family of intracellular kinases activated by a number of molecules and stressing events for the cell. They are involved in regulation of cellular proliferation, cell cycle, differentiation, apoptosis, and gene expression.[2,42,47] Recent findings suggest that the nature of the stimuli determines which of the above pathways is primarily involved in chemotaxis activation. Indeed, PI3K pathway activation has been linked to "intermediary chemoattractant"-induced migration, whereas the p38 MAPK pathway is activated by "end-target chemoattractants". Based on this theory, LTB4, CXCL8, and PAF have been classified as intermediary, whereas other molecules like TNF-α, fMLP, and C5a are end-target chemoattractants. Interestingly, all chemoattractants separately can activate PI3K in vitro, while when they are present simultaneously, p38 MAPK activation predominates, suggesting that the latter can suppresses PI3K activity.[28] However, in our experiments, although all MAPK pathways were activated (given the significant decrease in migratory activity that we observed after MAPKs inhibition), PI3K was the major pathway involved in BALF-induced equine neutrophil chemokinesis in vitro. In support of our findings, horses exposed to antigen challenge and simultaneously treated with a p38 MAPK inhibitor were reported to have lower percentage of neutrophils in BALF compared with untreated horses,[22] although airway neutrophilia persisted in treated horses. Taken together, these findings suggest that p38 MAPK contributes to neutrophil migration both in vitro and in vivo, but may not be the primary pathway involved in heaves. The pivotal role of class I PI3Ks in regulation of neutrophil migration is well known, although its inhibition cannot completely avoid neutrophil motile activity.[48,49] Accordingly, our data show a large, yet not complete, reduction in neutrophil migration toward the migratory stimulus when cells were pretreated with the PI3K-inhibitory compound Wortmannin. These observations support Wortmannin or other PI3K inhibitors as new potential treatments to control bronchial inflammation in heaves.

In vitro neutrophil chemotaxis toward a gradient of stimuli is mainly dependent on the motile machinery, in which actin filament activation and polarization represent the central events. In vivo, neutrophil chemotaxis from the vessels to the tissues starts with rolling and subsequent adhesion of the cells to the endothelium, followed by extravasation and migration toward the stimuli. Our results support an effect of p38 MAPK inhibition mainly on intrinsic migratory capacity of neutrophils or on their adhesion process to the endothelium.[11,25] Indeed, after blocking p38 MAPK activity, neutrophil chemokinesis was decreased in vitro. Neutrophil recruitment into the airway lumen was decreased as well, and the lack of a concomitant decrease in BALF migratory activity suggests that the concentration of chemoattractants in BALF was not significantly affected by the treatment administered. Alternatively, the overall contribution of this pathway may be small and drug effects could have been masked by other more potent chemotactic factors.[22] In support of this hypothesis, inhibition of p38 MAPK significantly decreased equine neutrophil polarization and adhesion in response to LTB_4 and PAF, and TNF-α, respectively.[11]

Neutrophils synthesize chemoattractant molecules and they are found in great quantity in airways of horses with heaves. Correlation between neutrophil percentage in BALF and BALF migratory activity varied in the different groups of horses and at the different time points evaluated. Indeed, a positive correlation between these 2 parameters was present in healthy horses after stabling, whereas a positive correlation was detected at T_{14} in horses with heaves, but not at T_{35}. The lack of correlation at T_{35} might be attributable in part to the great variability of data collected in this group of horses, or to the fact that different subsets of cytokines and chemokines could be synthesized and present into the airway lumen at the 2 time points studied, which might elicit a different migratory response by neutrophils. Alternatively, the neutrophil migration rate may be maximum after 14 days of antigen challenge and a further increase in chemotactic signaling may not be accompanied by an augmented neutrophil influx into the bronchial lumen.

In conclusion, our results indicate that neutrophil influx toward the airways in heaves is regulated by both MAPK and PI3K pathways, which can potentially control different cell functions related to chemotaxis other than those regulating the migratory processes per se (ie, production of chemoattractants). A p38 MAPK modulation of cytoskeleton processes, as previously suggested,[11] could possibly explain the combined reduced percentages of neutrophils in BALF and unaltered BALF chemotactic activity observed with the administration of a p38 MAPK inhibitor in horses with heaves. Blocking PI3K had a profound inhibitory effect on neutrophil chemotaxis in vitro;

inhibition of this pathway might represent a new target for the control of airway neutrophilia in heaves.

Footnotes

[a] Anadat, RHT-Infodat, Montreal, QC, Canada
[b] Rompun 100 mg/mL, Bayer Healthcare, Toronto, ON, Canada
[c] Torbugesic, Pfizer Animal Health, Kirkland, QC, Canada
[d] Cytospin model II, Shandon Southern Instruments, Sewickley, PA
[e] BD Vacutainer Tubes, BD, Franklin Lakes, NJ
[f] Lympholyte, Cedarlane Laboratory Limited, Burlington, ON, Canada
[g] SB203580, Cedarlane Laboratory Limited
[h] SP600125, Cedarlane Laboratory Limited
[i] PD98059, Cedarlane Laboratory Limited
[j] Wortmannin, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada
[k] AP48 Chemotactic Chamber, Neuro Probe Inc, Gaithersburg, MD
[l] SCB8 Cellulose Nitrate Filters, Neuro Probe Inc
[m] Hematoxylin, VWR International, Ville Mont-Royal, QC, Canada
[n] Chromotrope 2R, Sigma-Aldrich Canada Ltd
[o] SAS v.9.1, Cary, NC
[p] GraphPad Software Inc, La Jolla, CA

Acknowledgments

The authors thank Dr William S. Powell and Sylvie Gravel for their help in setting up the chemotactic assay. This study was supported by a discovery grant (JPL) and by an undergraduate student (KM) research award from the Natural Sciences and Engineering Research Council of Canada.

Conflict of Interest: Authors disclose no conflict of interest.

References

1. Fairbairn SM, Page CP, Lees P, Cunningham FM. Early neutrophil but not eosinophil or platelet recruitment to the lungs of allergic horses following antigen exposure. Clin Exp Allergy 1993;23:821–828.

2. Leclere M, Lavoie-Lamoureux A, Lavoie JP. Heaves, an asthma-like disease of horses. Respirology 2011;16:1027–1046.

3. Monteseirin J. Neutrophils and asthma. J Investig Allergol Clin Immunol 2009;19:340–354.

4. Wang F. The signaling mechanisms underlying cell polarity and chemotaxis. Cold Spring Harb Perspect Biol 2009;1:a002980.

5. Grunig G, Witschi U, Winder C, et al. Neutrophil migration induced by equine respiratory secretions, bronchoalveolar lavage fluids and culture supernatants of pulmonary lavage cells. Vet Immunol Immunopathol 1991;29:313–328.

6. Franchini M, Gill U, von Fellenberg R, Bracher VD. Interleukin-8 concentration and neutrophil chemotactic activity in bronchoalveolar lavage fluid of horses with chronic obstructive pulmonary disease following exposure to hay. Am J Vet Res 2000;61:1369–1374.

7. Giguere S, Viel L, Lee E, et al. Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate. Vet Immunol Immunopathol 2002;85:147–158.

8. Joubert P, Cordeau ME, Lavoie JP. Cytokine mRNA expression of pulmonary macrophages varies with challenge but not with disease state in horses with heaves or in controls. Vet Immunol Immunopathol 2011;142:236–242.

9. Ainsworth DM, Grunig G, Matychak MB, et al. Recurrent airway obstruction (RAO) in horses is characterized by IFN-gamma and IL-8 production in bronchoalveolar lavage cells. Vet Immunol Immunopathol 2003;96:83–91.

10. Arndt PG, Young SK, Lieber JG, et al. Inhibition of c-Jun N-terminal kinase limits lipopolysaccharide-induced pulmonary neutrophil influx. Am J Respir Crit Care Med 2005;171:978–986.

11. Eckert RE, Sharief Y, Jones SL. p38 mitogen-activated kinase (MAPK) is essential for equine neutrophil migration. Vet Immunol Immunopathol 2009;129:181–191.

12. Niggli V. Signaling to migration in neutrophils: Importance of localized pathways. Int J Biochem Cell Biol 2003;35:1619–1638.

13. Brooks AC, Rickards KJ, Cunningham FM. CXCL8 attenuates chemoattractant-induced equine neutrophil migration. Vet Immunol Immunopathol 2011;139(141–147):13.

14. Leclere M, Lavoie-Lamoureux A, Martin J, et al. Inhaled corticosteroids accelerate the reversal of airway smooth muscle remodeling in an equine model of chronic asthma. ATS International Conference, New Orleans May 14–19, 2010;A2302.

15. Lavoie JP, Leguillette R, Pasloske K, et al. Comparison of effects of dexamethasone and the leukotriene D4 receptor antagonist L-708,738 on lung function and airway cytologic findings in horses with recurrent airway obstruction. Am J Vet Res 2002;63:579–585.

16. Lavoie JP, Pasloske K, Joubert P, et al. Lack of clinical efficacy of a phosphodiesterase-4 inhibitor for treatment of heaves in horses. J Vet Intern Med 2006;20:175–181.

17. Jean D, Vrins A, Lavoie JP. Monthly, daily, and circadian variations of measurements of pulmonary mechanics in horses with chronic obstructive pulmonary disease. Am J Vet Res 1999;60:1341–1346.

18. Jean D, Vrins A, Beauchamp G, Lavoie JP. Evaluation of variations in bronchoalveolar lavage fluid in horses with recurrent airway obstruction. Am J Vet Res 2011;72:838–842.

19. Falk W, Goodwin RH Jr, Leonard EJ. A 48-well micro chemotaxis assembly for rapid and accurate measurement of leukocyte migration. J Immunol Methods 1980;33:239–247.

20. Kay AB. Studies on eosinophil leucocyte migration. II. Factors specifically chemotactic for eosinophils and neutrophils generated from guinea-pig serum by antigen-antibody complexes. Clin Exp Immunol 1970;7:723–737.

21. Hiester AA, Metcalf DR, Campbell PA. Interleukin-4 is chemotactic for mouse macrophages. Cell Immunol 1992;139:72–80.

22. Lavoie JP, Thompson D, Hamilton E, et al. Effects of a MAPK p38 inhibitor on lung function and airway inflammation in equine recurrent airway obstruction. Equine Vet J 2008;40:577–583.

23. Yum HK, Arcaroli J, Kupfner J, et al. Involvement of phosphoinositide 3-kinases in neutrophil activation and the development of acute lung injury. J Immunol 2001;167:6601–6608.

24. Reutershan J, Saprito MS, Wu D, et al. Phosphoinositide 3-kinase gamma required for lipopolysaccharide-induced transepithelial neutrophil trafficking in the lung. Eur Respir J 2010;35:1137–1147.

25. Nick JA, Young SK, Arndt PG, et al. Selective suppression of neutrophil accumulation in ongoing pulmonary inflammation by systemic inhibition of p38 mitogen-activated protein kinase. J Immunol 2002;169:5260–5269.

26. Roussel L, Houle F, Chan C, et al. IL-17 promotes p38 MAPK-dependent endothelial activation enhancing neutrophil recruitment to sites of inflammation. J Immunol 2010;184:4531–4537.

27. Underwood DC, Osborn RR, Bochnowicz S, et al. SB 239063, a p38 MAPK inhibitor, reduces neutrophilia, inflamma-

tory cytokines, MMP-9, and fibrosis in lung. Am J Physiol Lung Cell Mol Physiol 2000;279:L895–L902.

28. Williams MR, Azcutia V, Newton G, et al. Emerging mechanisms of neutrophil recruitment across endothelium. Trends Immunol 2011;32:461–469.

29. Joubert P, Silversides DW, Lavoie JP. Equine neutrophils express mRNA for tumour necrosis factor-alpha, interleukin (IL)-1beta, IL-6, IL-8, macrophage-inflammatory-protein-2 but not for IL-4, IL-5 and interferon-gamma. Equine Vet J 2001;33:730–733.

30. Ainsworth DM, Wagner B, Erb HN, et al. Effects of in vitro exposure to hay dust on expression of interleukin-17, -23, -8, and -1beta and chemokine (C-X-C motif) ligand 2 by pulmonary mononuclear cells isolated from horses chronically affected with recurrent airway disease. Am J Vet Res 2007;68:1361–1369.

31. Ainsworth DM, Matychak M, Reyner CL, et al. Effects of in vitro exposure to hay dust on the gene expression of chemokines and cell-surface receptors in primary bronchial epithelial cell cultures established from horses with chronic recurrent airway obstruction. Am J Vet Res 2009;70:365–372.

32. Lindberg A, Nasman-Glaser B, Lindgren JA, Robinson NE. Evaluation of leukotriene biosynthetic capacity in lung tissues from horses with recurrent airway obstruction. Am J Vet Res 2002;63:794–798.

33. Marr KA, Lees P, Page CP, Cunningham FM. Inhaled leukotrienes cause bronchoconstriction and neutrophil accumulation in horses. Res Vet Sci 1998;64:219–224.

34. Lee E, Lindo T, Jackson N, et al. Reversal of human neutrophil survival by leukotriene B(4) receptor blockade and 5-lipoxygenase and 5-lipoxygenase activating protein inhibitors. Am J Respir Crit Care Med 1999;160:2079–2085.

35. Lokuta MA, Huttenlocher A. TNF-alpha promotes a stop signal that inhibits neutrophil polarization and migration via a p38 MAPK pathway. J Leuk Biol 2005;78:210–219.

36. Lavoie-Lamoureux A, Moran K, Beauchamp G, et al. IL-4 activates equine neutrophils and induces a mixed inflammatory cytokine expression profile with enhanced neutrophil chemotactic mediator release ex vivo. Am J Physiol Lung Cell Mol Physiol 2010;299:L472–482.

37. Look DC, Rapp SR, Keller BT, Holtzman MJ. Selective induction of intercellular adhesion molecule-1 by interferon-gamma in human airway epithelial cells. Am J Physiol 1992;263:L79–L87.

38. Rider P, Carmi Y, Guttman O, et al. IL-1alpha and IL-1beta recruit different myeloid cells and promote different stages of sterile inflammation. J Immunol 2011;187:4835–4843.

39. Krunkosky TM, Fischer BM, Martin LD, et al. Effects of TNF-alpha on expression of ICAM-1 in human airway epithelial cells in vitro. Signaling pathways controlling surface and gene expression. Am J Respir Cell Mol Biol 2000;22:685–692.

40. Hall JA, Hoyt D, Zuver C, et al. Rapid, multiwell colorimetric assay for measuring neutrophil chemoattractant activity in bronchoalveolar lavage fluid of horses with recurrent airway obstruction. J Vet Diagn Invest 2006;18:257–263.

41. Franchini M, Gilli U, Akens MK, et al. The role of neutrophil chemotactic cytokines in the pathogenesis of equine chronic obstructive pulmonary disease (COPD). Vet Immunol Immunopathol 1998;66:53–65.

42. Chen LW, Lin MW, Hsu CM. Different pathways leading to activation of extracellular signal-regulated kinase and p38 MAP kinase by formyl-methionyl-leucyl-phenylalanine or platelet activating factor in human neutrophils. J Biomed Sci 2005;12:311–319.

43. Hashimoto S, Gon Y, Matsumoto K, et al. N-acetylcysteine attenuates TNF-alpha-induced p38 MAP kinase activation and p38 MAP kinase-mediated IL-8 production by human pulmonary vascular endothelial cells. Br J Pharmacol 2001;132:270–276.

44. Kim SR, Lee KS, Park SJ, et al. Inhibition of p38 MAPK reduces expression of vascular endothelial growth factor in allergic airway disease. J Clin Immunol 2012;32(3):574–586.

45. Shan L, Redhu NS, Saleh A, et al. Thymic stromal lymphopoietin receptor-mediated IL-6 and CC/CXC chemokines expression in human airway smooth muscle cells: Role of MAPKs (ERK1/2, p38, and JNK) and STAT3 pathways. J Immunol 2010;184:7134–7143.

46. Rajashekhar G, Kamocka M, Marin A, et al. Pro-inflammatory angiogenesis is mediated by p38 MAP kinase. J Cell Physiol 2011;226:800–808.

47. Huang G, Shi LZ, Chi H. Regulation of JNK and p38 MAPK in the immune system: Signal integration, propagation and termination. Cytokine 2009;48:161–169.

48. Pinho V, Russo RC, Amaral FA, et al. Tissue- and stimulus-dependent role of phosphatidylinositol 3-kinase isoforms for neutrophil recruitment induced by chemoattractants in vivo. J Immunol 2007;179:7891–7898.

49. Thomas MJ, Smith A, Head DH, et al. Airway inflammation: chemokine-induced neutrophilia and the class I phosphoinositide 3-kinases. Eur J Immunol 2005;35:1283–1291.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Preliminary dose titration studies to determine the concentrations of the kinase inhibitors to be used.