

Evaluation of lipid markers in surfactant obtained from asthmatic horses exposed to hay

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OBJECTIVE

To evaluate the lipidomic profile of surfactant obtained from horses with asthma at various clinical stages and to compare results with findings for healthy horses exposed to the same conditions.

SAMPLE

Surfactant samples obtained from 6 horses with severe asthma and 7 healthy horses.

PROCEDURES

Clinical evaluation of horses and surfactant analysis were performed. Samples obtained from horses with severe asthma and healthy horses before (baseline), during, and after exposure to hay were analyzed. Crude surfactant pellets were dried prior to dissolution in a solution of isopropanol:methanol:chloroform (4:2:1) containing 7.5mM ammonium acetate. Shotgun lipidomics were performed by use of high-resolution data acquisition on an ion-trap mass spectrometer. Findings were analyzed by use of an ANOVA with a Tukey-Kramer post hoc test.

RESULTS

Results of lipidomic analysis were evaluated to detect significant differences between groups of horses and among exposure statuses within groups of horses. Significantly increased amounts of cyclic phosphatidic acid (cPA) and diacylglycerol (DAG) were detected in surfactant from severely asthmatic horses during exposure to hay, compared with baseline and postexposure concentrations. Concentrations of cPA and DAG did not change significantly in healthy horses regardless of exposure status.

CONCLUSIONS AND CLINICAL RELEVANCE

cPA 16:0 and DAG 36:2 were 2 novel lipid mediators identified in surfactant obtained from asthmatic horses with clinical disease. These molecules were likely biomarkers of sustained inflammation. Further studies are needed to evaluate a possible correlation with disease severity and potential alterations in the plasma lipidomic profile of horses with asthma. (*Am J Vet Res* 2019;80:300–305)

A number of studies performed over the past decade have involved lipidomic analysis of lung secretions or pulmonary tissues to characterize physiologic and pathological changes in the lungs. For nondiseased lungs, lipidomic analysis was used to explore developmental changes in lung surfactant,^{1–4} evaluate surfactant metabolism by the use of stable isotopes,⁵ characterize and localize phospholipids within the lungs,⁶ and assess alterations induced by exposure to inhaled irritants or pathogens.⁷ A variety of pulmonary diseases have been evaluated with lipidomic techniques, including chronic obstructive pulmonary disease,⁸ asthma,^{9,10} pulmonary arterial hypertension,¹¹ interstitial lung disease in children,¹²

acute lung injury,¹³ and lung cancer.^{14,15} Lipidomic analysis has helped investigators characterize specific forms of disease, elucidate inflammatory and signaling pathways, and identify potential biomarkers of pulmonary disease.^{9,12} Lysophosphatidic acid, a lipid mediator identified in BALF collected from asthmatic patients, has been recognized as a potential biomarker for asthma.¹⁶ It is possible LPA could be a useful marker for examination of specific asthma phenotypes and assessment of disease severity and progression.

Only a few studies^{17–22,a} have been conducted that used lipidomic techniques to analyze samples obtained from horses. The lipidomic composition of equine spermatozoa and seminal plasma was recently characterized.¹⁸ Targeted lipidomic analysis was used to identify eicosanoid release in joints of horses with induced synovitis.²² Lipidomic biomarkers of immune activation have been described in horses with leptospirosis and horses vaccinated against leptospirosis.²¹ Lipidomic analysis has also been performed on equine

ABBREVIATIONS

BALF	Bronchoalveolar lavage fluid
cPA	Cyclic phosphatidic acid
DAG	Diacylglycerol
ΔPpl	Maximal change in pleural pressure
LPA	Lysophosphatidic acid
PMN	Polymorphonuclear neutrophilic leukocyte

amniotic fluid.²⁰ To the authors' knowledge, lipidomic analysis of equine surfactant has not been performed. In a study²³ conducted by our research group, significant differences in lung surfactant composition and function were detected between healthy horses and horses with severe asthma. In another study²⁴ conducted by our research group, it was found that horses with severe asthma had significantly lower phospholipid concentrations in surfactant at all clinical stages, compared with concentrations in surfactant from age-matched healthy horses exposed to the same environmental conditions. In that study,²⁴ phospholipid concentrations in surfactant were low during remission when asthmatic horses had no clinical signs of disease, no signs of airway inflammation, and no changes in results of lung function tests. Phospholipid concentrations in surfactant decreased further when asthmatic horses were moved into an environment and exposed to dusty hay and straw. Surfactant is composed of phospholipids (80% to 85%), proteins (5% to 10%), and other lipids (5% to 10%).²⁵ Phospholipids can be subdivided into 5 major classes: phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, and sphingomyelin. Despite the reduction in overall surfactant phospholipid concentration in asthmatic horses, significant alterations were not detected in the percentages of the phospholipid classes typically contained in surfactant.²⁴ However, each phospholipid class is composed of a range of molecular species that share the same head group but contain fatty acid chains of various lengths and degrees of saturation.²⁵ The purpose of the study reported here was to evaluate the lipid composition of surfactant and to identify alterations in specific lipid markers in horses with severe asthma.

Materials and Methods

Sample

The study included surfactant samples that had been obtained from 6 horses with asthma and 7 healthy horses. Detailed clinical characteristics of these horses have been described elsewhere.²⁴ All samples used in the study reported here were collected at the Virginia-Maryland College of Veterinary Medicine in 2006 and 2007; results of surfactant analysis have been reported elsewhere.²⁴ Samples were stored at -80°C until used in the present study. Samples were transferred on dry ice to the Lincoln Memorial University College of Veterinary Medicine in 2016 for lipidomic analysis.

Healthy horses originated from the Virginia-Maryland College of Veterinary Medicine teaching herd, whereas horses with severe asthma were from a pre-existing herd of asthmatic horses maintained at the Virginia-Maryland College of Veterinary Medicine. Healthy horses were housed on pasture and did not develop airway obstruction when housed in a barn and exposed to hay. Horses with asthma were maintained on pasture but developed airway inflammation and obstruction when housed in a barn and exposed

to hay; asthmatic horses had clinical remission when returned to pasture. The initial study was approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee; additional approval was not required for the present study because it only involved sample analysis and did not require additional animal involvement.

Experimental procedures

Experimental procedures, sample collection, and surfactant analysis for the study reported here have been described elsewhere.²⁴ Briefly, horses of both groups were subjected to the same environmental conditions and evaluations. Sample collections were determined by the clinical stage reached by asthmatic horses as assessed on the basis of results of a physical examination and determination of a clinical score (clinical score was the sum of nasal flare and an abdominal component to breathing).²⁶ Samples were collected before (baseline), during, and after exposure to hay. During the baseline period, horses were housed on pasture, and severe asthmatic horses did not have clinical signs (clinical score < 4). Exposure consisted of housing horses in a barn environment with dusty hay and straw. Samples were obtained from severe asthmatic horses after obvious clinical signs of airway obstruction developed (clinical score ≥ 5) or the horses had been housed in the barn for > 3 days. After exposure, horses were returned to pasture, and samples were obtained once severe asthmatic horses had a decrease in clinical signs (decrease in clinical score of ≥ 2 points) or the horses had been housed on pasture for > 7 days.

Evaluation of horses included assessment of the clinical score and results of lung function tests, airway endoscopy, and cytologic examination of BALF. These results have been reported elsewhere.²⁴ Briefly, clinical score, mucus score for endoscopy, ΔPpl during lung function testing, and percentage of PMNs detected during BALF cytologic examination for healthy horses were all within accepted limits and did not change significantly among time periods (baseline, during exposure, and after exposure). In comparison, horses with severe asthma had a low clinical score (clinical score < 4), mucus score for endoscopy, ΔPpl during lung function testing ($\Delta\text{Ppl} \leq 8 \text{ cm H}_2\text{O}$), and percentage of PMNs $< 10\%$ at baseline (ie, while in remission), but they had substantial increases for these variables during exposure (clinical score > 5 , $\Delta\text{Ppl} \geq 20 \text{ cm H}_2\text{O}$, percentage of PMNs $> 15\%$) and intermediate values for these variables during the period after exposure (clinical score > 3 , ΔPpl between 8 and 13 $\text{cm H}_2\text{O}$, and percentage of PMNs $< 10\%$).²⁴

Isolation and analysis of surfactant

Cell-free BALF was separated into crude surfactant pellets and supernatant by ultracentrifugation (40,000 $\times g$ for 1 hour). Organic extraction was performed,²⁷ lipid phosphorus concentration was measured,²⁸ and phospholipid concentration was determined as previously reported.^{23,24,29,30}

Lipidomic analysis of samples

Lipidomic analysis was performed on crude surfactant pellets, which constituted the lipid-rich fraction of cell-free BALF. Crude surfactant pellets were dried by centrifugal vacuum evaporation and dissolved in a solution of isopropanol:methanol:chloroform (4:2:1) containing 7.5mM ammonium acetate. Internal standards included [$^2\text{H}_5$]docosahexaenoic acid, [$^2\text{H}_4$]hexacosanoic acid, [$^2\text{H}_{31}$]phosphatidylethanolamine 34:1, [$^2\text{H}_{54}$]phosphatidylethanolamine 28:0, [$^2\text{H}_{31}$]phosphatidylcholine 34:1, [$^2\text{H}_{54}$]phosphatidylcholine 28:0, [$^2\text{H}_{31}$]sphingomyelin 16:0, [$^2\text{H}_{31}$]phosphatidic acid 34:1, [$^2\text{H}_{62}$]phosphatidyl glycerol 32:0, and bromocriptine. Direct infusion lipidomics were used with high-resolution (mass error, 0.2 to 3 ppm) data acquisition on an ion-trap mass spectrometer.^b Anions of cPAs, ethanolamine plasmalogens, phosphatidylethanolamines, fatty acids, lysophosphoethanolamines, phosphatidylglycerols, phosphatidylinositols, and phosphatidylserines were monitored in negative-ion electrospray ionization. Cations of choline plasmalogens and phosphatidylcholines, sphingomyelins, and oxidized glycerophosphocholines were quantitated in positive-ion electrospray ionization, and lipid identities were validated by use of mass spectrometry. Cations and anions of bromocriptine were used to monitor potential mass axis drift. Between injections, the transfer line was washed with successive 500- μL washes of a solution of methanol and hexane:ethyl acetate:chloroform (3:2:1).

Statistical analysis

Semiquantitative lipidomics data were reported as mean $\pm$ SEM ratio values, which represented the ratio of the endogenous lipid peak area to the peak area of an appropriate internal standard. A *t* test was used to determine differences in metabolite concentrations of crude surfactant pellets between asthmatic and healthy horses. Values were considered significant at $P < 0.05$. In the case of multiple comparisons between groups, significant differences were evaluated with an ANOVA and Tukey-Kramer post hoc analysis.

Results

Lipidomic analysis of crude surfactant pellets was performed on surfactant samples obtained from 7 healthy horses and 6 asthmatic horses before (baseline), during, and after exposure to hay. Results from the lipidomic analysis were evaluated to determine significant differences between groups of horses (healthy vs asthmatic) and among exposure statuses (baseline, during exposure, and after exposure) within groups of horses.

The cPA concentration in surfactant was significantly increased in asth-

matic horses during exposure to hay, compared with concentrations at baseline and after exposure when horses were returned to pasture (**Figure 1**). The cPA concentration increased by $> 50\%$ during exposure to hay in asthmatic horses and then reached values between those at baseline and those during hay exposure. No significant changes were observed in cPA concentration of healthy horses, regardless of exposure status. Tandem mass spectrometry was used as

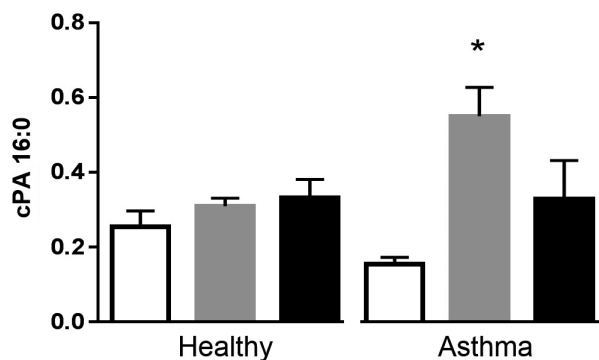


Figure 1—Concentration of cPA 16:0 in crude surfactant pellets of 7 healthy horses and 6 horses with asthma for samples obtained from the horses before (baseline; white bars), during (gray bars), and after (black bars) exposure to hay. Data represent the mean $\pm$ SEM of the ratio of the peak area of cPA 16:0 to the peak area of an internal standard. *Within the horses with asthma, value differs significantly ($P < 0.05$) from the values before and after exposure to hay.

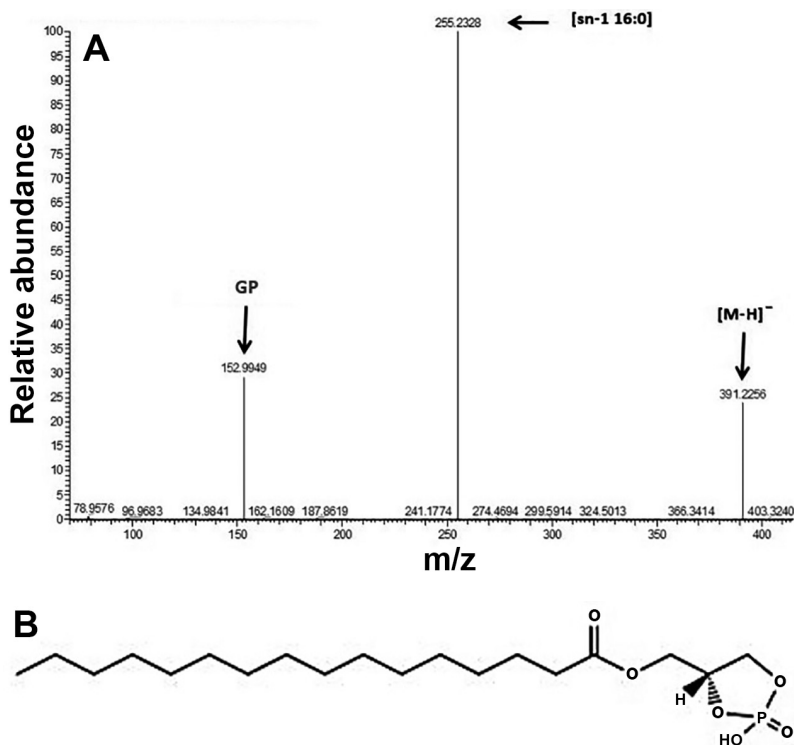


Figure 2—Negative-ion tandem mass spectroscopy spectrum of negative-ion mode ions of cPA 16:0 (A) and the chemical structure of cPA 16:0 (B). GP = Glycerophosphate. M-H⁻ = Molecular anion. sn-1 = Chemical nomenclature for the first carbon of a glycerol backbone.

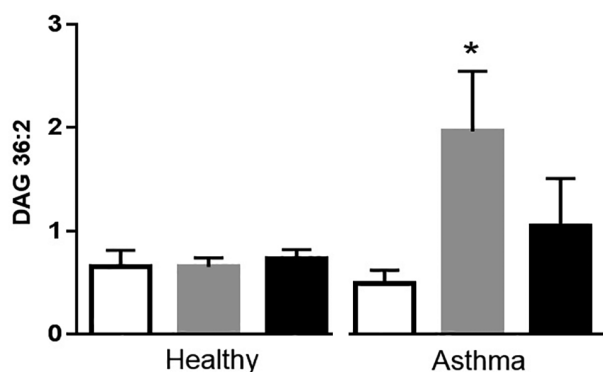


Figure 3—Concentration of DAG 36:2 in crude surfactant pellets of 7 healthy horses and 6 horses with asthma for samples obtained from the horses before (white bars), during (gray bars), and after (black bars) exposure to hay. Data represent the mean $\pm$ SEM of the ratio of the peak area of DAG 36:2 to the peak area of an internal standard. See Figure 1 for remainder of key.

reported elsewhere²¹ to validate the structure of cPA 16:0 (**Figure 2**).

The concentration of DAG 36:2 was significantly elevated in surfactant from asthmatic horses during exposure to hay, compared with concentrations at baseline and after exposure (**Figure 3**). The DAG concentration was more than double in asthmatic horses during exposure to hay, compared with baseline values, and it decreased to intermediate values after exposure. The DAG concentration in healthy horses was not affected by exposure status.

Discussion

The present study focused on lipidomic analysis of the phospholipid-rich fraction (crude surfactant pellets) isolated from BALF of healthy horses and horses with severe asthma before, during, and after exposure to hay. Significant compositional changes included an elevation in cPA 16:0 and DAG 36:2 concentrations in asthmatic horses during exposure to hay once clinical signs, airway inflammation, and altered pulmonary function developed. These changes were not seen while asthmatic horses were in remission (baseline) and had no clinical signs, airway inflammation, or pulmonary function changes. The decrease in surfactant concentration in asthmatic horses in remission appeared to precede the increase in cPA 16:0 and DAG 36:2 concentrations associated with clinical disease.²⁴

The source of cPA and DAG in lung surfactant may be surfactant phospholipid structures (ie, tubular myelin or lamellar bodies), cell membranes, or plasma transudate. Lung surfactant phospholipids consist primarily (60%) of dipalmitoylphosphatidylcholine 16:0/16:0, which may release cPA 16:0 as a result of hydrolysis by phospholipases activated during inflammation.³¹ The DAGs are intermediate molecules in the generation and breakdown of phospholipids.³² The cellular fraction of BALF was removed from the samples before they were processed for surfactant analysis;

therefore, a cellular origin for cPA and DAG was unlikely. Plasma transudation secondary to increased alveolocapillary membrane permeability may occur in asthmatic horses as a result of airway inflammation.³³ Cyclic phosphatidic acid is found in serum and plasma at a tenth of the concentration of its analog molecule LPA.^{34–36} Tissue concentrations of cPA are highest in the brain, followed by the liver, kidneys, lungs, heart, and spleen.³⁶ The concentration of cPA 16:0 is usually the highest, compared with concentrations of other molecular species (ie, cPA 18:0, cPA 18:1, or cPA 18:2). To our knowledge, the present study provided the first evidence of cPA in lung surfactant.

Although cPA and LPA have closely related molecular structures, they have opposing biological actions. Phospholipid synthesis and hydrolysis may lead to the generation of cPA, LPA, and DAG. These molecules play an important role in lipid signaling and modulate a number of cellular events.^{31,37,38} Both cPA and LPA are produced through the action of phospholipases such as phospholipase D and lysophospholipase D (autotaxin) or secretory phospholipases A₁ and A₂.³⁹ Both LPA and cPA share some of the same cell surface receptors (LPA₁ through LPA₇), but they have differing affinities for these receptors. Cyclic phosphatidic acid is a potent inhibitor of autotaxin activation and LPA production, and it has been suggested that cPA may serve as an antimetastatic drug in cancer treatment.^{39,40} The biological activity of cPA includes antimitogenic action, elevation of cAMP concentrations, platelet inhibition, neurotrophic effects, inhibition of tumor cell invasion, and antimetastatic actions.³⁹ The DAGs perform a variety of functions. They serve as building blocks for glycerophospholipids, influence the physical properties of lipid layers, and act as lipid second messengers.³² Elevated DAG concentrations in asthmatic horses may reflect increased phospholipid synthesis as a compensatory response to decreased phospholipid concentrations or may indicate increased phospholipid breakdown through phospholipases.

The role of cPA in respiratory tract disease is poorly understood, and the role of DAG is likely multifaceted.^{32,39} It is known that cPA stimulates respiration in rats through both centrally and peripherally mediated mechanisms.⁴¹ In humans, LPA is considered a potential biomarker of asthma.¹⁶ It has been identified in BALF from asthmatic patients, and a significant increase in concentrations of polyunsaturated LPA species (22:5 and 22:6) has been observed following subsegmental allergen challenge.^{10,42} The autotaxin LPA pathway plays a critical role in asthma, and LPA agonists can inhibit airway inflammation in mice with experimentally induced asthma.^{16,42,43} In contrast to these findings in human medicine, cPA concentrations, but not LPA concentrations, were increased in asthmatic horses in the present study. Because cPA and LPA share some of the same molecular and functional characteristics, it is possible that cPA played a role in these asthmatic horses similar to that of LPA

in asthmatic humans. However, increased concentrations of cPA and DAG have been detected in plasma from horses with leptospirosis and horses vaccinated against leptospirosis.²¹ Similarly, in the experience of the authors, high plasma cPA concentrations can be found in plasma of *Rhodococcus equi*-infected foals (unpublished data). These findings suggest that cPA is a biomarker of sustained inflammation or infection, rather than a specific biomarker for asthma in horses.

The study reported here did not include lipidomic analysis of plasma from asthmatic horses. Future studies should include lipidomic profiling of BALF and plasma obtained from asthmatic horses at different clinical stages. In human asthmatic patients, the increase in polyunsaturated LPA concentrations is limited to the lungs and is not found in the plasma of challenged subjects.^{42,c} A correlation between LPA concentration and disease severity has not been established. Similarly, the role of LPA in the pathophysiology of the lungs needs to be investigated further. It is likely that LPA contributes to epithelial barrier integrity but also has proinflammatory properties in airway inflammation. The same observations apply to cPA and DAG in horses with asthma.

In the present study, cPA and DAG concentrations were significantly increased in surfactant from horses with asthma that had obvious clinical disease. However, concentrations of cPA and DAG did not differ significantly between healthy horses and horses with asthma that were in remission. Therefore, cPA and DAG are not adequate markers for asthmatic horses that are in remission or recovering from clinical signs. In addition, because we induced clinical signs in asthmatic horses through exposure to hay and allowed horses to recover on pasture shortly thereafter, it is not possible to predict the effects of prolonged clinical conditions, airway inflammation, and obstruction on the lipidomic profile of surfactant from asthmatic horses.

The present study had several limitations. It was performed on a small number of samples, the samples were stored for a number of years prior to lipidomic analysis, and a second method was not used to identify cPA. In the initial study,²⁴ surfactant composition was analyzed in samples obtained from 7 healthy horses and 7 horses with asthma at different clinical stages. Samples from only 6 asthmatic horses were included in the present study because the sample quantity for the other asthmatic horse was insufficient to allow lipidomic analysis. Lipidomic analysis was performed almost 10 years after initial sample collection. However, samples were stored at -80°C and were not subjected to repeated freeze-thaw cycles. In addition, it has been reported that lipidomic composition is not significantly altered by prolonged frozen storage.⁴⁴ Finally, the identity of cPA 16:0 was confirmed by use of tandem mass spectrometry.

Both cPA 16:0 and DAG 36:2 were novel lipid mediators identified in surfactant obtained from asthmatic horses with clinical disease. These mol-

ecules likely represented nonspecific biomarkers of sustained inflammation. Further studies are needed to evaluate possible correlations with disease severity and potential alterations in the plasma lipidomic profile of horses with asthma.

Acknowledgments

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Footnotes

- a. Christmann U, Buechner-Maxwell V, Witonsky SG, et al. Levels of cyclic phosphatidic acid are increased in surfactant from asthmatic horses exposed to hay (oral presentation). 34th Annu Symp Vet Comp Respir Soc, East Lansing, MI, September 2016.
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