Evaluation of leukotriene biosynthetic capacity in lung tissues from horses with recurrent airway obstruction

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**Objective**—To evaluate leukotriene (LT) biosynthetic capacity in lung tissue from healthy horses and horses with recurrent airway obstruction (RAO).

**Sample Population**—Lung parenchyma and airway specimens from 8 RAO-affected and 5 healthy horses.

**Procedure**—Horses were stabled for ≥72 hours. Blood was drawn before euthanasia, after which lung specimens were collected. Tissue strips from small airways and parenchyma were incubated in organ baths with the precursor LTA₄ or stimulated with calcium ionophore A23187 or the tripeptide N-formyl-Met-Leu-Phe (fMLP), with or without exogenous arachidonic acid.

**Results**—Stabling induced typical signs of airway obstruction in RAO-affected horses but not control horses. When lung parenchyma or airway specimens from both groups of horses were incubated with calcium ionophore, with or without arachidonic acid, they did not form LT. In contrast, addition of LTA₄ to both tissues resulted in conversion to LT, although concentrations of LTC₄ were negligible in airways and parenchymal strips from healthy and RAO-affected horses. Incubation of airway and parenchymal strips with suspensions of autologous neutrophils did not influence formation of LT stimulated by calcium ionophore or fMLP, with or without exogenous arachidonic acid.

**Conclusions and Clinical Relevance**—Results suggest that lung parenchyma and airway tissues themselves are not of substantial importance for LT formation in the lungs, although these tissues possessed some LTA₄ hydrolase activity, enabling LTB₄ formation. It may be speculated that LTB₄ originates primarily from neutrophils and may play a role in the inflammatory events of RAO. (Am J Vet Res 2002; 63:794–798)

Equine recurrent airway obstruction (RAO) is a widespread respiratory disorder characterized by bronchoconstriction, mucus secretion, airway inflammation, and bronchial hyperresponsiveness. The hyperreactivity wanes and disease remission occurs when horses are pastured or when changes in the environment reduce exposure to dust and molds. The predominant inflammatory cell in the airways in RAO is the neutrophil granulocyte.

Leukotrienes (LT) are arachidonic acid-derived compounds that play an important role in asthma. The first step in LT biosynthesis involves release of arachidonic acid from membrane phospholipids, a reaction that can be catalyzed by various phospholipases, in particular cytosolic phospholipase A2. The subsequent metabolism of liberated arachidonic acids to LT is initiated by 5-lipoxygenase, which in cooperation with 5-lipoxygenase-activating protein (FLAP) catalyzes transformation of the substrate to an epoxide, LTA₄. This unstable intermediate can either be converted to LTB₄ by a cytosolic LTA₄ hydrolase or transformed to LTC₄ by a membrane-bound LTC₄ synthase, which specifically conjugates LTA₄ with glutathione. Once formed, LTC₄ is actively transported to the extracellular space, where it is metabolized to LTD₄ and LTE₄ by successive elimination of a γ-glutamyl residue and glycine, respectively. Neutrophils can produce LTB₄, but lack the capacity to convert LTA₄ to LTC₄, whereas eosinophils produce LTC₄. The cysteinyl LT, LTC₄ and LTD₄, are potent bronchoconstrictors and mucus secretagogues, and they provoke vascular leakage. Furthermore, LTA₄ can be exported from neutrophils and further metabolized by surrounding cells to LTC₄ or LTB₄ via transcellular mechanisms. Thus, platelets as well as endothelium and smooth muscle cells convert LTA₄ to LTC₄. However, whereas platelets contain a specific LTC₄-synthase, this activity in endothelial cells is attributable to a microsomal glutathione S-transferase type 2.

Leukotriene B₄ is a potent proinflammatory mediator that induces chemotaxis, cytokine production, and activation of certain gene transcription factors and delays apoptosis. The LTs can provide a positive feedback loop that can amplify the inflammatory response, whereas the cysteinyl LT provoke airway obstruction and edema formation.

The physiologic actions of LTB₄ and cysteinyl LT make them likely candidates in the pathogenesis of RAO; LTB₄ could be involved in neutrophil chemotaxis, and cysteinyl LT could be involved in airway obstruction. Indeed, aerosol administration of LTB₄ causes neutrophil accumulation in horse lungs.
whereas LTD₄ has been reported to cause contraction of airway smooth muscle, and its aerosol administration causes difficult breathing.

Lung tissues from various species produce LT after stimulation with calcium ionophore, antigen, and grain-dust, and production is inhibited by a 5-lipoxygenase inhibitor. The LT-synthesizing capacity in lung tissue increases after allergen challenge and in the presence of inflammatory cells. The key enzymes involved in LT biosynthesis, 5-lipoxygenase and FLAP, are present in leukocytes, and FLAP expression is associated with inflammatory cells within the lung tissue. Furthermore, allergic airway challenge amplifies 5-lipoxygenase expression in lung inflammatory cells.

The purpose of the study reported here was to investigate LT synthesis in lung tissue from RAO-affected and healthy horses and determine whether these tissues could participate in transcellular LT formation in the presence of autologous neutrophils.

**Materials and Methods**

**Horses**—Horses with RAO and healthy control horses were tissue donors for this in vitro study. Horses were brought from a pasture, stabled for various periods (≥72 hours) on straw, and fed hay. The control group consisted of 5 horses (3 mares and 2 geldings) that were 5 to 32 years old (mean ± SD, 16 ± 8.3 years) and had no history of disease or evidence of respiratory tract disease via clinical examination.

The RAO-affected group consisted of 8 horses (3 mares and 5 geldings) that were 7 to 25 years old (mean ± SD, 19 ± 4.8 years) with a history of RAO; these horses developed subclinical respiratory tract disease. 

**Preparation of blood neutrophils**—Before euthanasia, blood (300 ml) was collected into 50-ml syringes via jugular vein puncture and poured into EDTA-containing tubes (final concentration, 4.1 mM). Neutrophil suspensions were prepared by use of a centrifugation gradient according to Fairbairn et al; the leukocyte-rich plasma was collected from blood samples after spontaneous erythrocyte sedimentation and was centrifuged at 200 × g for 15 minutes. The leukocyte pellet was resuspended in 20 ml of the supernatant and layered onto a centrifugation-plasma gradient (15 ml each of 60% and 80% centrifugation gradients and 5 ml of 100% centrifugation gradient) that was centrifuged at 400 × g for 30 minutes. The neutrophils were flushed between the 60% and 80% gradient layers. The pellet, which contained ≥98% neutrophils and 0 to 2% eosinophils, was resuspended in Krebs-Henseleit (K-H) solution (NaCl, 118.4 mM; NaHCO₃, 25.0 mM; dextrose, 11.7 mM; KCl, 4.7 mM; CaCl₂ × 2 H₂O, 2.6 mM; MgSO₄ × 7H₂O, 1.19 mM; and KH₂PO₄, 1.16 mM) to a final concentration of 15 × 10³ cells/ml. Cells were kept on ice until incubation.

**Tissue collection and preparation**—Horses were euthanatized with pentobarbital sodium (100 mg/kg, IV); lung specimens were collected within 20 minutes of administering pentobarbital, placed in K-H solution, and continuously exposed to 95% O₂ and 5% CO₂ (38°C). Tissues were kept in K-H solution during dissection and experimental protocols. Rectangular columns of lung parenchyma (2 × 2 × 5 mm) with no major blood vessels or large airways were prepared, and 5-mm-long strips of small airways (outer diameter, 1 to 2 mm) were isolated from the peripheral parts of the lungs. Specimens (mean ± SD wet weight, 300 ± 30 mg) were placed in a 2-ml tissue bath filled with K-H solution (38°C), which was infused with 95% O₂ and 5% CO₂ and replaced every 15 minutes throughout the experiment. Specimens were tied to a glass tissue holder with surgical silk, which held the tissue covered with K-H solution. Specimens were equilibrated for at least 1 hour before the experimental protocols were conducted.

**Results**

**Leukotriene formation in lung tissue**—Biosynthesis of LT in lung tissue was evaluated by...
stimulation of small airway strips and parenchyma specimens with calcium ionophore (A23187; 1 µM). Concentrations of LTB₄ and LTC₄ were negligible (<17 pmol/g of tissue, 5 pmol/ml tissue bath fluid) in both types of tissues in RAO-affected and control horses. Incubation of both tissue types with supplementation of exogenous arachidonic acid (8 µM) also failed to trigger substantial LT formation.

When both types of lung tissue were incubated with LTA₄, meaningful amounts of LTB₄ were formed. In airway strips as well as parenchymal specimens (Fig 1), concentrations of LTB₄ were significantly greater than when tissues were stimulated with calcium ionophore alone. Concentrations of LTB₄ were similar in both tissue types and in RAO-affected and healthy horses (329 ± 171 and 204 ± 54 pmol/g of tissue, respectively [approx 70 and 51 pmol/ml tissue bath fluid, respectively]). A negligible amount of LTC₄ was formed (significantly less than LTB₄), and no differences between groups or tissue samples were detected.

**Effect of coincubation of lung tissue with neutrophils**—In the absence of supplemental arachidonic acid, incubation of neutrophils with calcium ionophore (either alone or with lung tissues) induced only minor LTB₄ biosynthesis (approx 16 pmol/ml of tissue bath fluid; Fig 2). Formation of LTC₄ was negligible (Table 1). Addition of exogenous arachidonic acid to incubations of neutrophils from RAO-affected and healthy horses (121 ± 38 and 108 ± 7 pmol/ml, respectively; Fig 2). In addition, 2 nonenzymatic degradation products of LTA₄ were detected in neutrophil preparations from RAO-affected and healthy horses. Combined mean values (± SD) from both groups were 59 ± 14 pmol/ml of tissue bath fluid for 6-trans-LTB₄ and 64 ± 27 pmol/ml for 6-trans,12-epi-LTB₄. The presence of these compounds indicated that LTA₄ was formed in excess amounts, exceeding the metabolic capacity of the neutrophils. However, stimulation of neutrophils in the presence of arachidonic acid and small airway or parenchymal strips did not result in generation of significantly greater concentrations of LTB₄, nor did the profile of formed LT change; that is, there was no increased LTC₄ formation (Table 1). Similar findings were observed by use of the physiologic agonist fMLP with exogenous arachidonic acid.
The fluid. Leukotriene B4 is a potent chemotactic agent for inflammatory cells. Concentrations of LTC4 did not differ from those of control incubations. Thus, lung tissue did not participate in LT formation, and neutrophils were the main producers of LTB4.

Discussion

Airway inflammatory cells obtained via bronchoalveolar lavage from horses with RAO as well as healthy horses mainly produce LTB4. This is in agreement with the fact that these cells are mostly neutrophils and macrophages, whereas few are eosinophils and mast cells. Furthermore, the concentration of LTB4 in isolated parenchyma was LTB4, and this was true in both healthy horses and RAO-affected horses. However, in that study, LT formation was measured at fixed intervals up to 48 hours after stabilizing, whereas in the present study, the horses were stabled for varying times (≥ 72 hours).

The interaction between epithelial cells and inflammatory cells has been reported to favor LT biosynthesis by allowing transcellular metabolism of LT to occur. Our experiment evaluated this interaction by examining the capacity of lung tissue to convert LT into other LT metabolites and by examining the interaction between activated neutrophils and lung tissue. It is clear from our data that equine lung tissue can convert LT into other LT metabolites, but the capacity to produce LTC4 is negligible. Thus, our data provide no evidence for increased ability of lung tissue from RAO-affected horses to convert LT into other LT metabolites. Further, our observation of limited capacity to produce cysteiny1 LT, coupled with the failure of an antagonist to improve airway function, leads to the conclusion that cysteiny1 LT do not play an important role in RAO.

Results from other species support our conclusion that most of the increase in LT production in disease is a result of the influx of inflammatory cells rather than changes in the metabolic capacity of lung tissue cells. Increased LT synthesizing capacity develops in murine lungs after allergen challenge, and the increased expression of 5-lipoxygenase is primarily accounted for by the proximity of the different cell types involved. It is impossible to mimic these conditions in vitro. The addition of neutrophils to a metabolism chamber containing lung tissue was a reasonable approximation, but the formation of LT via transcellular mechanisms might require neutrophils that have migrated through the epithelium or that have been obtained from bronchoalveolar lavage fluid.

There was no difference between RAO-affected and healthy horses in the formation of LTB4 in isolated blood neutrophil suspensions. This is in contrast to our earlier results, in which peripheral neutrophils obtained from RAO-affected horses generated less LTB4 than did neutrophils from control horses. However, in that study, LT formation was measured at fixed intervals up to 48 hours after stabilizing, whereas in the present study, the horses were stabled for varying times (≥ 72 hours).

Table 1—Mean ± SEM leukotriene C4 concentrations (pmol/ml) in strips of small airways and lung parenchyma specimens obtained from clinically normal control horses and horses with recurrent airway obstruction (RAO). Specimens were incubated with autologous purified neutrophils and calcium ionophore A23187 (1 µM), calcium ionophore A23187 (1 µM) and arachidonic acid (AA; 5 µM), or N-formyl-Met-Leu-Phe (fMLP; 10⁻⁶ M) and AA (5 µM) and AA (8 µM). Numbers in parentheses indicate sample size.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Control</th>
<th>RAO</th>
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<tbody>
<tr>
<td>Small airways</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils + A23187</td>
<td>6.1 ± 5.4 (5)</td>
<td>2.4 ± 4.1 (5)</td>
</tr>
<tr>
<td>Neutrophils + A23187 + AA</td>
<td>5.5 ± 5.4 (5)</td>
<td>2.9 ± 2.9 (5)</td>
</tr>
<tr>
<td>Neutrophils + fMLP + AA</td>
<td>9.3 ± 9.3 (3)</td>
<td>2.6 ± 3.6 (4)</td>
</tr>
<tr>
<td>Parenchyma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils + A23187</td>
<td>4.7 ± 4.4 (5)</td>
<td>4.3 ± 5.2 (6)</td>
</tr>
<tr>
<td>Neutrophils + A23187 + AA</td>
<td>4.8 ± 3.1 (5)</td>
<td>6.7 ± 7.4 (5)</td>
</tr>
<tr>
<td>Neutrophils + fMLP + AA</td>
<td>2.2 ± 3.0 (3)</td>
<td>3.5 ± 4.3 (4)</td>
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As expected, calcium ionophore-induced generation of LTB4 in neutrophils was greatly enhanced by exogenous arachidonic acid, confirming our earlier observations that equine blood neutrophils require exogenous arachidonic acid to generate LTB4. Stimulation of neutrophils in the presence of arachidonic acid and lung tissue did not induce LTC4 formation or an increase in generation of LTB4, compared with incubations of neutrophils alone.

The capacity of lung tissue from RAO-affected and healthy horses to produce LT was further evaluated in experiments in which the tissue was incubated with autologous neutrophils. The presence of lung tissue did not alter LT biosynthesis, despite the fact that neutrophils produced excess LT, which was confirmed by the presence of nonenzymatic degradation products of LTC4. During migration, activated leukocytes, primarily neutrophils, may exchange LTAs with surrounding cells, including epithelial cells. Thus, transcellular metabolism of LT is favored by adhesion and by the proximity of the different cell types involved. It is impossible to mimic these conditions in vitro. The addition of neutrophils to a metabolism chamber containing lung tissue was a reasonable approximation, but the formation of LT via transcellular mechanisms might require neutrophils that have migrated through the epithelium or that have been obtained from bronchoalveolar lavage fluid.
by invading inflammatory cells.\textsuperscript{44} Furthermore, lung parenchyma from asthmatic patients does not generate more LT than does tissue from nonasthmatic patients.\textsuperscript{33}

The primary LT formed in the lung tissue was LTB\textsubscript{4}, a potent chemotactic agent for neutrophils. Results of a recent study\textsuperscript{2} indicate that inflammatory cells obtained from the airways have a much greater capacity to produce LT than do peripheral neutrophils. In accordance, migration of human neutrophils results in increased LT-forming capacity.\textsuperscript{7} Leukotriene B\textsubscript{4} could therefore be a factor in the neutrophilic inflammation that is characteristic of RAO.

References
29. Tornhamre M, Sjolander M, Lindberg A, et al. Expression of active leukotriene C4 synthase in CD16+ neutrophils from the airways have a much greater capacity to produce LT than do peripheral neutrophils. In accordance, migration of human neutrophils results in increased LT-forming capacity. Leukotriene B4 could therefore be a factor in the neutrophilic inflammation that is characteristic of RAO.