The CysLTs are potent proinflammatory agents that play an important role in the pathogenesis of many diseases. Leukotrienes have been implicated as mediators of acute synovial inflammation, cardiovascular disease, gastrointestinal tract disorders, allergic dermatologic conditions, and certain cancers. The best characterization of the effects is for those within the respiratory tract, where they mediate airway hyperresponsiveness, mucus production, bronchoconstriction, increases in vascular permeability, and inflammation.5 The importance of CysLTs in human respiratory medicine is supported by the clinical efficacy of the CysLT1 receptor antagonists, montelukast and zafirlukast, in asthmatic human patients.5 The role of LTs in pulmonary disease of horses has not been definitively established. Administration of an experimental phosphodiesterase-4 enzyme inhibitor attenuated LT production in equine whole blood exposed to lipopolysaccharide, but treatment of horses with RAO failed to result in improved lung mechanics.3 Montelukast also had no significant effect on pulmonary mechanics in horses with RAO but did reduce the change in pleural pressure in 2 of the most severely affected horses.6 Failure to detect significant improvement may have been attributable to poor bioavailability and a low plasma concentration. However, results

Effects of leukotriene C4 on the bioelectric properties and ion transport of equine tracheal epithelium

Guy D. Lester, BVMS, PhD, and Brett L. Rice, BS

Objective—To determine effects of leukotriene (LT) C4 on ion transport across equine tracheal epithelium.

Sample—Tracheal epithelium from cadavers of 24 horses considered free of respiratory tract disease.

Procedures—Mucosae were mounted into Ussing chambers, and short-circuit current (Isc) was monitored over time. Effects of LTC4 were examined for various conditions, including addition of amiloride (10µM) to the mucosal bath solution, addition of bumetanide (10µM) to the serosal bath solution, addition of barium (1mM) to the serosal bath solution, and substitution of gluconate for chloride and HEPES for bicarbonate in bath solutions. Electrolyte transport was assessed via 22Na and 36Cl isotope fluxes.

Results—Addition of LTC4 (50nM) to the serosal bath solution caused an increase in Isc for basal conditions and a larger increase after pretreatment with amiloride. The increase was negated in part by the addition of bumetanide to the serosal bath solution and further reduced by substitution of HEPES for bicarbonate in bath solutions. Remaining current was reduced to values less than those before treatment with LTC4 by the addition of barium to the serosal solution. There was a small increase in Isc after the addition of amiloride and substitution of gluconate for chloride. Radioisotope flux indicated that addition of LTC4 to the serosal bath solution increased chloride secretion and reduced sodium absorption.

Conclusions and Clinical Relevance—LTC4 stimulated chloride secretion through a predominantly bumetanide-sensitive pathway, with a smaller contribution from a bicarbonate-dependent pathway. Thus, LTC4 appears to be a potential mediator of airway hypersecretion in horses. (Am J Vet Res 2012;73:2007–2012)

ABBREVIATIONS

CysLT Cysteinyl leukotriene
Isc Short-circuit current
LT Leukotriene
PD Potential difference
RAO Recurrent airway obstruction

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hydrolyase or LTC4 and its downstream metabolites (LTD4 and LTE4) by LTC4 synthase. Leukotriene C4, LTD4, and LTE4 are released by a range of inflammatory cells, including macrophages, eosinophils, and mast cells. Bronchial mast cells are also an important source of LTC4 synthase in humans with asthma.6

The role of LTs in pulmonary disease of horses has not been definitively established. Administration of an experimental phosphodiesterase-4 enzyme inhibitor attenuated LT production in equine whole blood exposed to lipopolysaccharide, but treatment of horses with RAO failed to result in improved lung mechanics.3 Montelukast also had no significant effect on pulmonary mechanics in horses with RAO but did reduce the change in pleural pressure in 2 of the most severely affected horses.6 Failure to detect significant improvement may have been attributable to poor bioavailability and a low plasma concentration. However, results
of a few studies support a role of CysLTs in RAO. Inhalation of LTD₄ at concentrations ≥ 5 μg/mL in clinically normal horses and horses with RAO resulted in bronchoconstriction that persisted for up to 20 minutes. The magnitude of the response indicated a potency 305 to 970 times as great as that for methacholine on an equivalent molar basis. The CysLTs also induce contraction of isolated equine lung parenchymal strips, and this response is inhibited by specific receptor antagonists. Production of LTB₄ is increased in RAO strips, and this response is inhibited by specific receptor antagonists. The CysLTs are also likely involved in the acute phases of airway inflammation in RAO, where they increase cholinergic tone and probably facilitate airway hypersecretion. There may be an even more important role of CysLTs in the pathogenesis of inflammatory airway disease, particularly in mast cell– or eosinophil-associated inflammatory airway disease. Leukotrienes can induce chloride secretion across a number of membranes. Ion transport across respiratory epithelium is responsible for fluid movement and maintenance of mucociliary clearance. Impaired ion transport could potentially lead to excessive mucus accumulation within the airways, which is a prominent clinical feature of RAO and inflammatory airway disease. The effect of other mediators of airway hypersecretion on ion transport across equine respiratory epithelia has been examined in excised equine respiratory epithelium. In that study, the addition of histamine to the mucosal bath solution enhanced chloride secretion and impaired sodium absorption across equine tracheal epithelium mounted in Ussing chambers. The objective of the study reported here was to examine the effect of LTC₄ on the bioelectric properties of and electrolyte transport across equine tracheal epithelium.

Materials and Methods

Sample—Tracheas were obtained from the cadavers of 24 adult horses. The horses had been donated to the Veterinary Medical Teaching Hospital at the University of Florida. Overt respiratory tract disease in the horses was excluded on the basis of the medical history, results of physical examination, and results of gross examination of the respiratory tract after horses were euthanized. None of the horses were receiving medications at the time of euthanasia. The project was approved by a university institutional animal care and use committee.

Horses were sedated with an α₁-adrenergic agonist and then were euthanized by administration of a commercial solution containing 390 mg of sodium pentobarbital/mL and 50 mg of phenytoin/mL. Immediately after each horse was euthanized, the trachea was removed and transported to our laboratory in a cold (5ºC) solution that contained 146 mmol Na, 5 mmol K, 140 mmol Cl, 0.7 mmol Mg, 3 mmol Ca, 20 mmol HCO₃⁻, 1.3 mmol HPO₄²⁻, 0.3 mmol H₂PO₄⁻, and 10 mmol dextrose (pH 7.4).

At the laboratory, the mucosa was stripped from the underlying tissues and mounted in Ussing chambers with an aperture area of 1.327 cm². The tissues were maintained at 38.0°C, and a mixture of 95% O₂ and 5% CO₂ was continuously bubbled through the bath solution. The bath solution was identical to the solution used during transport of tissues to our laboratory. Samples from 17 horses were used to investigate baseline characteristics of tissues and responses to LTC₄, and samples from 7 horses were used for electrolyte flux experiments.

Electrical measurements—The transepithelial PD and the current required to nullify the spontaneous PD (ie, Iₛ) were measured with an automatic voltage clamp. Tissue resistance and conductance were calculated by use of Ohm’s Law as follows:

\[ I = gV, \text{ with } g = 1/R \]

where I is current, g is conductance, V is the PD measured across the tissue, and R is resistance. Electrical convention dictated that the PD was measured with the serosal surface being the ground and a positive current representing a flow of positive charge from the serosa to mucosa.

Bath solutions and treatments—The aforementioned basal bath solution was modified in chloride and bicarbonate substitution experiments. Chloride was replaced with gluconate, and bicarbonate was replaced with 20 mM HEPES. The sodium channel blocker amiloride (10 μM) was added to the mucosal bath solution. Bumetanide (10 μM) and BaCl₂ (1 mM), which is a nonselective potassium channel blocker, were added to the serosal bath solution. All these chemicals were purchased from a single supplier. Leukotriene C₄, which is (5S,6R,7E,9E,11Z,14Z)-6-[(2R)-2-[(4S)-4-amino-4-carboxybutanoyl]amino]-3-(carboxymethylamino)-3-oxopropyl)sulfanyl-5-hydroxyicos-7,9,11,14-tetraenoic acid, was purchased from another supplier.

Baseline characterization of tissues and effects of LTC₄—The bioelectric properties of excised equine tracheal mucosa were assessed for baseline conditions and after the addition of the sodium channel blocker amiloride. Chloride secretion was examined via the addition of bumetanide, an inhibitor of the Na⁺-,K⁺-2Cl⁻ cotransport system, and through substitution of HEPES for bicarbonate in the bath solution, which thereby inhibited parallel Na⁺-,H⁺-,Cl⁻,HCO₃⁻ exchange. Basolateral transport of potassium was inhibited by the addition of BaCl₂.

Preliminary experiments were conducted to examine the differential changes in Iₛ in response to when LTC₄ was added to the mucosal or serosal bath solution and when various concentrations of LTC₄ were added to the serosal bath solution. Effects of LTC₄ on bioelectric properties were examined for baseline conditions, after pretreatment with amiloride, and after pretreatment with bumetanide or substitution of HEPES for bicarbonate (or both pretreatment with bumetanide and substitution of HEPES for bicarbonate).

Transmepithelial flux measurements—Effects of LTC₄ on sodium and chloride transport were assessed with ⁵¹Na or ⁴⁰Cl isotopes via short-circuit conditions. Methods were as described elsewhere. Tissues were
allowed to stabilize in the Ussing chambers for 30 minutes. Chambers then were matched in pairs on the basis of similar conductance (within 20%), and 3 µCi of $^{22}$Na or 6 µCi of $^{36}$Cl was added to the mucosal or serosal bath solution, respectively; tissues were allowed to equilibrate for 60 minutes. Flux was measured during a 30-minute (control) period, which was followed by the addition of LTC$_4$ (50nM) to the serosal bath solution, and a second 30-minute period of flux measurement. Finally, bumetanide (10µM) was added to the serosal bath solution, and flux was measured during a third 30-minute period. A liquid scintillation counter was used to measure $^{36}$Cl activity, and a gammacounter was used to measure $^{22}$Na activity.

**Statistical analysis**—Statistical analyses were performed with a commercial software package.

For baseline characterization of tissues and investigation of the effect of LTC$_4$ on Isc for various pretreatment conditions, a related-samples Wilcoxon signed rank test was used. Replicate observations on tissues collected from a single horse were used to calculate a mean value for inclusion in the statistical analyses. Values of $P < 0.05$ were considered significant. A series of paired $t$ tests were used to investigate the effects of LTC$_4$ and the addition of bumetanide to the serosal bath solution on sodium and chloride measurements. Significance for this portion of the study was based on an adjusted value of $P < 0.025$ via the Benjamini-Hochberg algorithm.

Results

**Baseline characterization of tissues**—Mean ± SEM baseline $I_{sc}$ for tissues obtained from the 17 horses was 84.6 ± 5.8 µA•cm$^{-2}$ (range, 41 to 173 µA•cm$^{-2}$). The spontaneous PD was serosal-positive and ranged from 5 to 24 mV. The addition of amiloride to the mucosal bath solution significantly ($P = 0.001$) decreased the $I_{sc}$ by 54.7% (from 93.4 to 42.3 µA•cm$^{-2}$; tissues were allowed to stabilize in the Ussing chambers for 30 minutes. Chambers then were matched in pairs on the basis of similar conductance (within 20%), and 3 µCi of $^{22}$Na or 6 µCi of $^{36}$Cl was added to the mucosal or serosal bath solution, respectively; tissues were allowed to equilibrate for 60 minutes. Flux was measured during a 30-minute (control) period, which was followed by the addition of LTC$_4$ (50nM) to the serosal bath solution, and a second 30-minute period of flux measurement. Finally, bumetanide (10µM) was added to the serosal bath solution, and flux was measured during a third 30-minute period. A liquid scintillation counter was used to measure $^{36}$Cl activity, and a gammacounter was used to measure $^{22}$Na activity.

**Statistical analysis**—Statistical analyses were performed with a commercial software package. For baseline characterization of tissues and investigation of the effect of LTC$_4$ on $I_{sc}$ for various pretreatment conditions, a related-samples Wilcoxon signed rank test was used. Replicate observations on tissues collected from a single horse were used to calculate a mean value for inclusion in the statistical analyses. Values of $P < 0.05$ were considered significant. A series of paired $t$ tests were used to investigate the effects of LTC$_4$ and the addition of bumetanide to the serosal bath solution on sodium and chloride measurements. Significance for this portion of the study was based on an adjusted value of $P < 0.025$ via the Benjamini-Hochberg algorithm.

Results were reported as mean ± SEM.

**Concentration of LTC$_4$**

The effect of different concentrations of LTC$_4$ was evaluated in amiloride-pretreated tissues from 4 horses. The mean increase in $I_{sc}$ after various concentrations of LTC$_4$ were added to the serosal bath solution was as follows: 1nM, 4.61 µA•cm$^{-2}$; 10nM, 11.75 µA•cm$^{-2}$; 25nM, 13.67 µA•cm$^{-2}$; 50nM, 15.30 µA•cm$^{-2}$; 100nM, 18.44 µA•cm$^{-2}$; and 200nM, 17.32 µA•cm$^{-2}$. The increase in $I_{sc}$ did not differ among concentrations, except for the value for the lowest concentration, which differed from the values for all other concentrations. A concentration of 50nM was used for subsequent experiments.

**Mucosal effects versus serosal effects**

To differentiate mucosal from serosal effects, LTC$_4$ at a final concentration of 50nM was added to the appropriate bath solution for baseline (10 tracheal tissues from 4 horses) or amiloride-pretreated (12 tracheal tissues from 4 horses) conditions. The mean ± SEM increase in $I_{sc}$ was consistently greater when LTC$_4$ was added to the serosal bath solution (baseline to serosal, 84.4 ± 10.9 µA•cm$^{-2}$; amiloride pretreated to mucosal, 77.9 ± 11.9 µA•cm$^{-2}$; baseline to amiloride pretreated to serosal, 35.6 ± 7.7 µA•cm$^{-2}$; and amiloride pretreated to mucosal, 28.6 ± 12.1 µA•cm$^{-2}$). All subsequent LTC$_4$ treatments were added to the serosal bath solution.

Consistent with the dose-response experiment, 50nM LTC$_4$ added to the serosal bath solution resulted in a significant ($P < 0.001$) increase of 13% in the mean

![Figure 1](image-url)
tissues were pretreated with the addition of amiloride to the mucosal bath solution \((42.3 \pm 4.2 \mu A/cm^2)\) to 57.1 \(\pm 4.5 \mu A/cm^2\) \((n = 16 \text{ horses})\). Addition of LTC\(_4\) to tissues after addition of amiloride to the mucosal bath solution and bumetanide to the serosal bath solution resulted in a small but significant \((P = 0.003)\) increase of 16\% in \(I_c\) \((32.5 \pm 3.2 \mu A/cm^2)\) to 38.8 \(\pm 3.4 \mu A/cm^2\) \((n = 12 \text{ horses})\). Much of this increase was negated when HEPES was substituted for bicarbonate \((38.8 \pm 3.4 \mu A/cm^2)\) to 33.8 \(\pm 3.2 \mu A/cm^2\) \((n = 12 \text{ horses})\); Figure 1). Substitution of HEPES for bicarbonate prior to the addition of LTC\(_4\) resulted in a smaller change in \(I_c\) than when bicarbonate was present. The residual current after the addition of amiloride, substitution for bicarbonate, addition of bumetanide, and addition of LTC\(_4\) was further reduced by the addition of barium \((1mM)\) to the serosal bath solution, although the small number of samples precluded a statistical analysis (Figure 2). The addition of LTC\(_4\) to the serosal bath solution after pretreatment with amiloride and substitution for chloride, with or without the addition of bumetanide, resulted in a small increase in \(I_c\) (Figure 3).

**Transepithelial flux**—Transepithelial flux data were summarized (Table 1). For the control conditions, equine tracheal epithelial secreted chloride and absorbed sodium. The addition of LTC\(_4\) inhibited net sodium absorption by approximately 41\% and increased net chloride secretion by 104\%. The addition of bumetanide significantly decreased both serosal-to-mucosal transport of chloride and net chloride flux. The addition of bumetanide did not significantly alter net sodium flux but did reduce both mucosal-to-serosal and serosal-to-mucosal transport of sodium.

**Discussion**

The bioelectric properties of equine tracheal epithelium reported here were similar to those described previously.\(^{13,17,18}\) Equine tracheal epithelium is unique in that 2 independent transport processes are involved in chloride secretion. Basolateral chloride transport is mediated by a Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport system and a parallel Na\(^+\)-H\(^+\)-Cl\(^-\)-HCO\(_3\)^\(^-\) exchange. Apical chloride transport is through special channels, such as the cystic fibrosis transmembrane conductance regulator chloride channel.\(^{19}\) Transport through apical chloride channels is also dependent on basolateral potassium channels. Inhibition of basolateral potassium channels with barium salts effectively blocks apical chloride transport.\(^{19}\)

### Table 1—Mean ± SEM values for electrolyte flux in tracheal tissues obtained from the cadavers of 7 horses.

<table>
<thead>
<tr>
<th>Chloride flux (μmol/cm²·h⁻¹)</th>
<th>Sodium flux (μmol/cm²·h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Mucosal to serosal</td>
</tr>
<tr>
<td>Control</td>
<td>6.56 ± 0.56</td>
</tr>
<tr>
<td>Bumetanide</td>
<td>4.52 ± 0.46*</td>
</tr>
</tbody>
</table>

| Flux was measured for a 30-minute (control) period, which was followed by the addition of LTC\(_4\) \((50mM)\) to the serosal bath solution, and a second 30-minute period of flux measurement. Bumetanide \((10μM)\) then was added to the serosal bath solution, and flux was measured for a third 30-minute period. |

*Within a column, the value differs significantly \((P < 0.025)\) from the value for the control treatment. **Within a column, the value differs significantly \((P < 0.025)\) significantly from the value for the LTC\(_4\) treatment.
A further barium-induced reduction in $I_\text{sc}$ after pretreatment with amiloride, substitution for bicarbonate, and the addition of bumetanide suggests an additional mechanism of action of barium on the serosal surface. Inactivation of basolateral potassium channels may also further limit sodium absorption across the apical membrane.10

Parallel $\text{Na}^+\text{-H}^+\text{-Cl}^-\text{-HCO}_3^-$ exchange was proposed as the major mechanism of chloride transport for basal conditions.18 In the present study, the effect of substitution of HEPES for bicarbonate was not examined for baseline conditions, but substitution for bicarbonate in the bath solution after the addition of amiloride had a small effect, which suggested a minimal role for bicarbonate in an unstimulated environment. A more pronounced effect on $I_\text{sc}$ was seen when bumetanide, an inhibitor of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransport system, was added to the serosal bath solution for both baseline and amiloride-pretreated conditions. The response to bumetanide, although evident in most tissues, was not consistent, which is a similar finding to that reported in another study.18 Another group of authors concluded that equine tracheal tissue was bumetanide-insensitive for basal conditions, although they did detect reductions in $I_\text{sc}$ in some tissues that were attributed to poor tissue viability.17 Findings of the present study suggested that most basal chloride transport was mediated by a basolateral parallel $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransport system, with a smaller contribution from a bicarbonate-dependent $\text{Na}^+\text{-H}^+\text{-Cl}^-\text{-HCO}_3^-$ exchange.

Electrogenic sodium transport is a feature of most mammalian epithelia, including equine tracheal epithelium.17,21 Sodium uptake is dependent on amiloride-sensitive sodium channels in the apical membrane.19 The large and consistent decrease in $I_\text{sc}$ after the addition of amiloride to the mucosal bath solution in the present study was consistent with this finding. This concentration of amiloride has no effect on chloride transport.19 The absorption of sodium across equine tracheal epithelium is through both amiloride-sensitive and -insensitive pathways. Amiloride-sensitive absorption predominates for neutral conditions.17 Efflux of sodium through the basolateral membrane is mediated by $\text{Na}^+\text{-K}^+\text{-ATPase}$.

Analysis of the bioelectric data for the present study suggested that physiologic concentrations of LTC$_4$ are capable of inducing net chloride secretion. This is consistent with results of experiments in other species.3 Investigators for a study22 that involved cultured rabbit tracheal epithelial monolayers concluded that exogenous LTC$_4$ also enhanced chloride secretion but enhanced net sodium absorption, which is in contrast to the findings of the present study. In the present study, the increase in $I_\text{sc}$ was largely ameliorated by the addition of bumetanide to the serosal bath solution, which suggested a predominant role of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransport system in chloride secretion. There was a small LTC$_4$-induced increase in $I_\text{sc}$ after pretreatment of the mucosal bath solution with amiloride and the addition of bumetanide to the serosal bath solution that was reduced, in part, through substitution for bicarbonate. Therefore, LTC$_4$ stimulated chloride secretion through a predominantly bumetanide-sensitive pathway, with a smaller contribution from a bicarbonate-dependent pathway.

Limited data are available on the effect of other inflammatory mediators on epithelial transport in horses. The effect of histamine on bioelectric properties and ion transport across equine tracheal epithelium was evaluated in a similar Ussing chamber system.15 Addition of histamine to the mucosal bath solution enhanced chloride secretion through the bumetanide-sensitive electroneutral $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransport system with no evidence of stimulated bicarbonate-dependent chloride transport.15 Additionally, histamine did not increase $I_\text{sc}$ in chloride-free conditions, whereas LTC$_4$ caused a small but significant increase in $I_\text{sc}$. This suggests alternative ionic pathways may be stimulated by LTC$_4$, which results in anion secretion or cation absorption. Further studies are needed to investigate this response.

Results of the present study confirm that LTC$_4$ is capable of inducing fluid hypersecretion in equine airways. Several studies9–12 have found that CysLTs are capable of inducing bronchoconstriction and hypersecretion of fluid in clinically normal horses and horses with RAO. This is consistent with responses in other species, including humans.4 Definitive evidence requires detection of increased concentrations of CysLTs in naturally occurring or experimentally induced pulmonary diseases of horses. On the basis of the predominant cell types, mast cell– or eosinophil-associated inflammatory airway disease is more likely than RAO to involve CysLTs.

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