Elevated amount of Toll-like receptor 4 mRNA in bronchial epithelial cells is associated with airway inflammation in horses with recurrent airway obstruction

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Berndt A, Derksen FJ, Venta PJ, Ewart S, Yuzbasiyan-Gurkan V, Robinson NE. Elevated amount of Toll-like receptor 4 mRNA in bronchial epithelial cells is associated with airway inflammation in horses with recurrent airway obstruction. *Am J Physiol Lung Cell Mol Physiol* 292: L936–L943, 2007. First published December 8, 2006; doi:10.1152/ajplung.00394.2006.—Recurrent airway obstruction (RAO) is characterized by neutrophilic airway inflammation and obstruction, and stabling of susceptible horses triggers acute disease exacerbations. Stable dust is rich in endotoxin, which is recognized by Toll-like receptor (TLR) 4. In human bronchial epithelium, TLR4 stimulation leads to elevation of interleukin (IL)-8 mRNA expression. The zinc finger protein A20 negatively regulates this pathway. We hypothesized that TLR4 and IL-8 mRNA and neutrophil numbers are elevated and that A20 mRNA is not increased in RAOs during stabling compared with controls and with RAOs on pasture. We measured the maximal change in pleural pressure (ΔPpl_{max}), determined inflammatory cell counts in bronchoalveolar lavage fluid (BAL), and quantified TLR4, IL-8, and A20 mRNA in bronchial epithelium by quantitative RT-PCR. We studied six horse pairs, each pair consisting of one RAO and one control horse. Each pair was studied when the RAO-affected horse had airway obstruction induced by stabling and after 7, 14, and 28 days on pasture. Stabling increased BAL neutrophils, ΔPpl_{max}, and TLR4 (4.14-fold change) significantly in RAOs compared with controls and with RAOs on pasture. TLR4 correlated with IL-8 (R² = 0.75), whereas stabilizing increased IL-8 in all horses. A20 was unaffected. IL-8 was positively correlated with BAL neutrophils (R² = 0.43) and negatively with A20 (R² = 0.44) only in RAO-affected horses. Elevated TLR4 expression and lack of A20 upregulation in bronchial epithelial cells from RAO-affected horses may contribute to elevated IL-8 production, leading to exaggerated neutrophilic airway inflammation in response to inhalation of stable dust.

Although adaptive immune mechanisms were shown to contribute to the pathogenesis of RAO (1, 8), innate immune mechanisms are also important (37). Hay dust is rich in microbial products such as endotoxin (38, 39), and inhalation of endotoxin-depleted hay dust significantly attenuates airway neutrophilia in affected horses (38). The inflammatory response can be reestablished by adding endotoxin back to the endotoxin-depleted hay dust (36). These observations confirm that attenuation of airway inflammation is due specifically to endotoxin. In conventional horse stables, airborne endotoxin concentrations exceed those on pasture (28) and those that can induce airway inflammation in human subjects (46, 47). In our own stables, we observed that the endotoxin concentrations in the breathing zone of stabled horses are at least 10-fold higher than concentrations on pasture (unpublished data).

Microbial-derived products, such as endotoxin, have been shown to play an important role in human lung diseases such as asthma, acute respiratory distress syndrome, and chronic obstructive pulmonary disease (43). Pathogen-associated molecules are recognized by pattern recognition receptors commonly referred to as Toll-like receptors (TLRs); TLR4 is crucial for the recognition of endotoxin, in particular lipopolysaccharide (LPS) (43). TLR4 is expressed in a variety of cell types within the lung, including pulmonary epithelial cells (3, 17, 29), alveolar macrophages (11), endothelial cells (4, 49), and airway smooth muscle cells (30), and its own expression can be stimulated by LPS itself (3, 31, 45). The TLR4 stimulation leads to production of cytokines, such as interleukin (IL)-8 (15, 29).

The TLR4 signaling cascade is under the influence of both positive and negative feedback regulation. A variety of proteins, such as Tollip (50), suppressor of cytokine-signaling-1 (SOCS1) (21), IL-1R-associated kinase M (IRAK-M) (22), and A20 (15), are involved in the reduction of the TLR signal transduction. The gene of the zinc finger protein A20 was originally characterized as TNF-α-inducible (33). Subsequently, it was shown that it is a NF-κB target gene, and such A20 is inducible by a wide variety of stimuli (23). The A20 transcript is rapidly but transiently induced, reaching its highest level within 1 h following stimulation (9). A20-deficient mice develop multiorgan inflammation due to LPS stimulation (24). Therefore, A20 has been suggested to be an endogenous regulator of LPS-induced inflammation. Elevated
A20 expression functions as a negative feedback loop to block NF-κB-dependent gene expression (5). For example, it has been shown that A20 overexpression leads to inhibition of TLR4-mediated IL-8 synthesis in human airway epithelial cells (15). Also, A20-deficient fibroblasts display prolonged NF-κB activity and are unable to terminate NF-κB activation (24). A20 interferes with the TLR signaling pathway at the level of the tumor necrosis factor receptor (TNFR)-associated factor (TRAF)-6 (6, 18, 26), leading to the inhibition of the phosphorylation of IκB kinase (IKK), which is necessary for NF-κB activation.

Little is known about TLR signaling in horses. TLR4 mRNA can be found in the lung tissue of healthy horses (45). The effect of LPS on TLR4 expression in the bronchial epithelial cells of horses is unclear. Whereas, in lung tissue obtained from unaffected horses, LPS exposure increases TLR4 mRNA expression (45), a recent report suggests that hay dust exposure does not change the TLR4 mRNA expression in the bronchial epithelial cells of RAO-affected horses (3). Furthermore, IL-8 mRNA expression and protein concentration measured in bronchoalveolar lavage fluid (BAL) are elevated in RAO-affected horses compared with control animals during stabiling and in RAO-affected horses in remission (2, 3, 14).

In the present study, we hypothesized that the amount of TLR4 mRNA, but not A20, is elevated in RAO-affected horses during stabiling compared with control horses and with RAO-affected horses on pasture. We further hypothesized that in RAO-affected horses, the increased mRNA expression of the receptor would be paralleled by an elevated expression of IL-8 mRNA. Furthermore, we hypothesized that the severity of neutrophilic airway inflammation in RAO-affected horses during stabiling is correlated with an increase in TLR4 but not with A20 expression.

MATERIALS AND METHODS

Animals. RAO-affected horses were selected from a herd maintained by the Pulmonary Laboratory at Michigan State University. These animals have a history and clinical signs compatible with a diagnosis of RAO. To enter the herd, animals fulfilled the following criteria: 1) horses develop airway obstruction and inflammation when stabilized and fed hay; 2) airway obstruction and inflammation are reversed by pasturing, where horses have no exposure to stable dust or hay; and 3) airway obstruction is reversible with atropine. Control animals did not develop airway obstruction when stabilized and fed hay (40).

Study design. Six RAO-affected horses (2 mares, 4 geldings; 18.3 ± 3.3 yr) and six control horses (3 mares, 3 geldings; 16 ± 4.6 yr) were studied in age-matched horse pairs. Before the study, horses were kept on pasture. Acute airway obstruction was initiated by stabiling horses in a barn. Horses were brought into the barn in pairs consisting of one RAO-affected and one control horse and were bedded on straw and fed dusty hay. We defined the beginning of the study as the day the RAO-affected horse of each pair developed acute airway obstruction (day 0). When the RAO-affected horses developed a clinical score of 5 or greater, we measured the total change in pleural pressure during tidal breathing ($\Delta P_{\text{pmax}}$). The clinical criterion to define acute exacerbations in RAO-affected horses was a $\Delta P_{\text{pmax}}$ of 15 cmH$_2$O or greater. Furthermore, we obtained bronchial brushing samples for gene expression analyses and bronchoalveolar lavage samples for total and differential cell count analyses. Horses were then returned to the pasture. Subsequent measurements were obtained on days 7, 14, and 28. The protocol was approved by the All-University-Committee for Animal Use and Care of Michigan State University.

Determination of the total clinical score. A scoring system for the subjective clinical assessment of respiratory effort was used as previously described (42). Nasal flaring and abdominal movement were each scored separately on a scale of 1 (normal) to 4 (severe signs). To determine the total clinical score (TCS), scores for nasal flaring and abdominal movement were summed. Therefore, the TCS could range from 2 (normal) to 8 (severe signs).

Measurement of the maximal change in pleural pressure during tidal breathing. The maximal change in pleural pressure ($\Delta P_{\text{pmax}}$) was used as an indicator of airway obstruction. Measurements were made in unsedated horses by means of an esophageal balloon connected via a 240-cm-long polypropylene catheter and a pressure transducer (Validyne DP-45-22) to a physiograph (Dash 18, Astro-Med). The balloon was passed through the nose and placed into the middle third of the esophagus. Twenty breaths were averaged at each measurement period.

Collection of bronchial brushing samples. Bronchial brushings (BBs) were made between the third and sixth generation bronchi via bronchoscopy. Bronchoscopy was performed with a 3-m-long endoscope (9 mm diameter) using a transnasal approach. The brushing was performed by advancing a cytology brush (CytoSoft Cytology Brush, Medical Packaging) throughout the biopsy channel of the endoscope into the bronchial lumen. The brush was gently stroked against the airway wall 15–20 times. Care was taken to avoid bleeding. The brush was then withdrawn into the biopsy channel and the endoscope was removed from the horse’s airways. The cytology brushes were then flushed in 1-ml phosphate-buffered saline (PBS) and stored on ice until further analysis. The procedure was repeated twice in the same lung. The side of the lung chosen for brushing was alternated between the measurement periods. The beginning lung side was chosen randomly for each horse.

Quantification of cells. Total and differential cell counts in BBs were performed manually using a hemocytometer. Cell preparations were made with a cytocentrifuge and stained with hematoxylin and eosin stain. Differential cell counts were performed by counting 200 cells per slide.

Collection of BAL. BAL was obtained by means of a 3-m-long endoscope that was passed via the nose and wedged in a peripheral bronchus. Three 100-ml aliquots of PBS were infused into the tube.

Table 1. Primer pair sequences used for amplification of TLR4, IL-8, A20, and 18S rRNA transcripts in bronchial brushing samples

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Amplicon Length, bp</th>
<th>NCBI Entrance Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>GCC ATT GCC CAT GAA CG</td>
<td>GCC CTC ACT AAA GCA TCC AA</td>
<td>123 (13)</td>
</tr>
<tr>
<td>TLR4</td>
<td>TCT GGA GAC GAC TCA GGA AAG C</td>
<td>GCA AGA AGC ACC TCA GGA GCT T</td>
<td>91 AY05808</td>
</tr>
<tr>
<td>IL-8</td>
<td>CAG CAT CTC TGC TGA ACA TGA CT</td>
<td>AGA GCT GCA GAA AGC AGG AAG A</td>
<td>73 AY184956</td>
</tr>
<tr>
<td>A20</td>
<td>CCT GCT TGA GGA GTC CAT GCT</td>
<td>TCC ACA CTC ACC CAT CAG TTC</td>
<td>69 Human: NM_006290 Mouse: NM_009397</td>
</tr>
</tbody>
</table>
Fig. 1. Clinical parameters in recurrent airway obstruction (RAO)-affected and control horses during stabling and on pasture. Lung function [ΔPpmax (cmH2O)] (A) and percentage of neutrophils in bronchoalveolar lavage fluid (BAL) (Neu) (B) from control (gray bars) and RAO-affected horses (black bars) during (day 0) and after (days 7, 14, and 28) stabling. Each “a” indicates significant difference (P ≤ 0.05) between the horse groups (control and RAO) within a time point (days 0, 7, 14, and 28). Each “b” indicates significant difference (P ≤ 0.05) from day 0.

Table 2. Cellular composition of bronchoalveolar lavage fluid

<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>RAO</th>
<th>Control</th>
<th>RAO</th>
<th>Control</th>
<th>RAO</th>
<th>Control</th>
<th>RAO</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>6.47 ± 0.21</td>
<td>5.9 ± 0.21</td>
<td>6.61 ± 0.15</td>
<td>6.62 ± 0.33</td>
<td>6.61 ± 0.24</td>
<td>6.34 ± 0.2</td>
<td>6.41 ± 0.32</td>
<td>6.11 ± 0.42</td>
</tr>
<tr>
<td>logTCC</td>
<td>13.08 ± 8.21</td>
<td>53.13 ± 11.79</td>
<td>5.38 ± 0.33</td>
<td>5.09 ± 0.19</td>
<td>4.89 ± 0.2</td>
<td>5.38 ± 0.31</td>
<td>5.02 ± 0.26</td>
<td>5.02 ± 0.31</td>
</tr>
<tr>
<td>%Neu</td>
<td>11.75 ± 7.35</td>
<td>17 ± 8.71</td>
<td>11.75 ± 7.35</td>
<td>17 ± 8.71</td>
<td>11.75 ± 7.35</td>
<td>17 ± 8.71</td>
<td>11.75 ± 7.35</td>
<td>17 ± 8.71</td>
</tr>
<tr>
<td>logMac</td>
<td>6.28 ± 0.26</td>
<td>5.22 ± 0.27</td>
<td>6.44 ± 0.14</td>
<td>6.46 ± 0.3</td>
<td>6.44 ± 0.14</td>
<td>6.46 ± 0.3</td>
<td>6.44 ± 0.14</td>
<td>6.46 ± 0.3</td>
</tr>
<tr>
<td>%Lym</td>
<td>72.17 ± 10.99</td>
<td>26.13 ± 11.31</td>
<td>70.8 ± 9.3</td>
<td>71.6 ± 9.22</td>
<td>70.8 ± 9.3</td>
<td>71.6 ± 9.22</td>
<td>70.8 ± 9.3</td>
<td>71.6 ± 9.22</td>
</tr>
</tbody>
</table>

Values are means ± SE of total (log10 transformed) and differential cell counts in bronchoalveolar lavage fluid (BAL) from recurrent airway obstruction (RAO)-affected and control horses during stabling (day 0) and after stabling (days 7, 14, and 28). Each “a” indicates significant difference (P ≤ 0.05) between the horse groups (control and RAO) within a time point (days 0, 7, 14, and 28). Each “b” indicates significant difference (P ≤ 0.05) from day 0. logTCC, log10-transformed total BAL cell count; logNeu, log10-transformed total BAL neutrophil count; %Neu, percentage of neutrophils in BAL; logMac, log10-transformed total BAL macrophage count; %Mac, percentage of macrophages in BAL; logLym, log10-transformed total BAL lymphocyte count; %Lym, percentage of lymphocytes in BAL.
Pplmax was significantly elevated compared with control horses. Airway function (Pplmax) and after (days 7, 14, and 28) nor between the time points within the control group. Macrophage, eosinophil, and mast cell counts did not differ between groups or time points except for the day 14 measurement period, when the differential macrophage count in BAL of RAO-affected horses was significantly lower than in control horses.

**RESULTS**

**Airway Function (ΔPpl\text{max})**

In RAO-affected horses during acute exacerbation (day 0), ΔPpl\text{max} was significantly elevated compared with control horses and with RAO-affected horses during remission (Fig. 1A). No statistically significant differences in ΔPpl\text{max} were observed between horse groups at days 7, 14, and 28, nor between the time points within the control group.

**Cellular Composition of BAL**

Total and differential cell counts of BAL for the two horse groups at the four different time points are shown in Table 2. In RAO-affected horses at day 0, differential neutrophil numbers were significantly greater (Fig. 1B), and both absolute and differential lymphocyte counts were significantly reduced compared with control horses and with RAO-affected horses during remission. No statistically significant differences in the differential neutrophil and lymphocyte numbers were observed between horse groups at days 7, 14, and 28 nor between the time points within the control group. Macrophage, eosinophil, and mast cell counts did not differ between groups or time points except for the day 14 measurement period, when the differential macrophage count in BAL of RAO-affected horses was significantly lower than in control horses.

**Quantitative mRNA Expression Analyses in Bronchial Brushing Samples**

**TLR4 mRNA expression.** RAO-affected horses during acute exacerbation showed a significantly greater TLR4 mRNA expression compared with control horses (P = 0.05) and with RAO-affected horses at days 7, 14, and 28 (P = 0.01, 0.005, and 0.002, respectively; Fig. 2A). The fold changes in mRNA expression are shown in Table 3. The fold change in TLR4 mRNA expression between RAO-affected and control horses at day 0 was 4.14 (P = 0.05). TLR4 mRNA expression in control horses was significantly lower at day 28 than at day 0 (P = 0.03). No statistically significant differences in TLR4 expression were observed between horse groups at days 7, 14, and 28 nor between other time points within the RAO or the control horse group.

**IL-8 mRNA expression.** RAO-affected horses during acute exacerbation had a significantly higher IL-8 mRNA expression compared with remission values at days 14 and 28 (P = 0.04 for each; Fig. 2B). IL-8 mRNA expression in control horses was significantly lower at days 7, 14, and 28 than at day 0 (P = 0.009, 0.05, and 0.01, respectively). No statistically significant differences in IL-8 expression were observed between horse groups at any time points.

**A20 mRNA expression.** There were no significant group or time effects in A20 mRNA expression (Fig. 2C).

**Table 3. Fold changes for the amount of TLR4, IL-8, and A20 mRNA in bronchial brushing samples**

<table>
<thead>
<tr>
<th>Time</th>
<th>TLR4</th>
<th>IL-8</th>
<th>A20</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAO/Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>4.14*</td>
<td>1.72</td>
<td>1.86</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.98</td>
<td>3.10</td>
<td>1.71</td>
</tr>
<tr>
<td>Day 14</td>
<td>1.49</td>
<td>1.24</td>
<td>1.13</td>
</tr>
<tr>
<td>Day 28</td>
<td>1.85</td>
<td>1.74</td>
<td>1.12</td>
</tr>
<tr>
<td>RAO/RAO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days 0–7</td>
<td>7.10*</td>
<td>4.40</td>
<td>0.80</td>
</tr>
<tr>
<td>Days 0–14</td>
<td>9.29*</td>
<td>6.83*</td>
<td>0.92</td>
</tr>
<tr>
<td>Days 0–28</td>
<td>9.99*</td>
<td>7.10*</td>
<td>0.89</td>
</tr>
<tr>
<td>Days 7–14</td>
<td>1.31</td>
<td>1.55</td>
<td>1.15</td>
</tr>
<tr>
<td>Days 7–28</td>
<td>1.41</td>
<td>1.61</td>
<td>1.11</td>
</tr>
<tr>
<td>Days 14–28</td>
<td>1.07</td>
<td>1.04</td>
<td>0.97</td>
</tr>
<tr>
<td>Control/Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days 0–7</td>
<td>1.68</td>
<td>7.91*</td>
<td>0.73</td>
</tr>
<tr>
<td>Days 0–14</td>
<td>3.33</td>
<td>4.92*</td>
<td>0.56</td>
</tr>
<tr>
<td>Days 0–28</td>
<td>4.46*</td>
<td>7.18*</td>
<td>0.54</td>
</tr>
<tr>
<td>Days 7–14</td>
<td>1.99</td>
<td>0.62</td>
<td>0.76</td>
</tr>
<tr>
<td>Days 7–28</td>
<td>2.66</td>
<td>0.91</td>
<td>0.73</td>
</tr>
<tr>
<td>Days 14–28</td>
<td>1.34</td>
<td>1.46</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Values are means ± SE. Fold changes for the amount of TLR4, IL-8, and A20 mRNA in bronchial brushing samples between RAO-affected and control horses (RAO/Control) during stabling (day 0) and after stabling (days 7, 14, and 28) as well as between time points within the RAO-affected (RAO/RAO) and within the control horse group (Control/Control). *Indicates that the fold change is significantly different (P ≤ 0.05) to 1 (no change).
Associations Among mRNA Expression with Clinical Responses

We observed a highly significant correlation between TLR4 and IL-8 mRNA expression in bronchial brushing samples in both RAO-affected and control horses ($P = 3.95 \times 10^{-6}$ and $6.15 \times 10^{-7}$, respectively; Fig. 3, A and B, respectively). Interestingly, TLR4 and IL-8 mRNA expression were significantly correlated to the percentage of neutrophils within BAL in RAO-affected horses ($P = 0.01$ and $0.002$, respectively; Fig. 3, C and E, respectively), but not in control horses ($P = 0.33$ and $0.31$, respectively; Fig. 3, D and F, respectively). Furthermore, there was a significant negative correlation between IL-8 and A20 mRNA expression in the RAO-affected horses ($P = 0.005$; Fig. 3G), but not in control horses ($P = 0.12$; Fig. 3H).

DISCUSSION

LPS is able to increase expression of TLR4 in inflammatory cells (3, 31) and in lung tissue from healthy horses (45).
data demonstrate that this may also be true in bronchial epithelial cells from horses, as in our control group the amount of TLR4 mRNA in BBs decreased gradually from day 0, when endotoxin exposure was greatest, to days 7, 14, and 28 (Fig. 2A). When horses were on pasture, there was no significant difference in the amount of epithelial-derived TLR4 mRNA between control and RAO-affected horses, but stable dust exposure increased amounts of TLR4 mRNA in BBs from RAO-affected horses significantly more than in controls. This observation could explain the exacerbated neutrophilic airway inflammation of RAO-affected horses to airborne endotoxin (36, 38).

There was no significant difference in the amount of epithelial-derived IL-8 mRNA between RAO-affected and control horses (Fig. 2B). In both horse groups, stable dust exposure was associated with significantly higher amounts of epithelial-derived IL-8. We observed a strong and highly significant correlation between the expressions of TLR4 and IL-8 mRNA in BBs in RAO-affected and control horses (Fig. 3A and B, respectively). This suggests a role for TLR4 in the stimulation of IL-8 production in equine bronchial epithelial cells. Similarly, asthmatic patients also show elevated production of IL-8 in bronchial epithelial cells (27, 48), and, in cell cultures of human airway epithelial cells, stimulation of TLR4 with LPS leads to increased IL-8 production (15, 29). However, in contrast to IL-8 expression, we found TLR4 expression to be significantly higher in RAO-affected horses at day 0 compared with control horses (Fig. 2A). This suggests that the amount of epithelial-derived TLR4 is most likely not the only factor responsible for IL-8 production in horses' airways. Other factors that may stimulate IL-8 expression include bradykinin (41), TNF-α (35), IL-1β (19), or inhaled air pollution particles (7).

IL-8 is a potent neutrophil chemotactic in human inflammatory airway diseases (32), and this has also been demonstrated for RAO-affected horses (12). Indeed, in RAO-affected horses there was a significant correlation between the amounts of epithelial cell-derived IL-8 mRNA and neutrophils in BAL (Fig. 3E). However, whereas there was no significant difference in epithelial-derived IL-8 mRNA between RAO-affected and control horses at day 0, there was a significantly higher number of neutrophils in RAO-affected horses compared with control horses at this time point (Fig. 1C). Therefore, epithelial-derived IL-8 mRNA is unlikely to be the only source for stimulation of neutrophil migration into the airway lumen in horses. Other cells, such as BAL cells (3), airway smooth muscle cells (34), endothelial cells (20), and monocytes (10), also release IL-8 upon stimulation. Also, other neutrophil chemoattractants such as leukotriene B4, TNF-α, granulocyte/macrophage colony-stimulating factor (GM-CSF), complement activation, and reduced apoptosis (16, 25, 44) may contribute to airway neutrophilia.

A20, overexpressed in other animal models, inhibits NF-κB activation, thereby decreasing LPS-induced IL-8 production (15). Our data suggest that this mechanism is functional in RAO-affected horses because we found a strong and significant negative correlation between the epithelial A20 and IL-8 mRNA expressions (Fig. 3G). This suggests that A20 upregulated in RAO-affected horses would reduce IL-8 expression and neutrophilic airway inflammation. Because TNF-α in BAL from RAO-affected horses is greater during stabling than on pasture and compared with control horses (14), and because A20 is a TNF-α response gene, one could assume an upregulation of A20 mRNA in BBs of RAO-affected horses during stabling and compared with controls. Instead, we found that A20 expression was unaffected by stabling and did not differ between RAO-affected and control horses (Fig. 2C). This suggests either that the mechanism responsible for A20 upregulation is defective or that A20 mRNA expression is downregulated in RAO-affected horses and, therefore, may contribute to the exaggerated airway inflammation in RAO-affected horses during stabling. The lack of a significant correlation between IL-8 and A20 in control horses was unexpected. However, this suggests that other regulatory mechanisms are important in the control of airway inflammation in this group of horses. An alternative explanation for the lack of change in A20 mRNA expression is its rapid and transient expression, reaching its highest level within 1 h following stimulation (9). Our horses were studied after several days of stable dust exposure, and we may have missed an early increase in epithelial A20 mRNA.

In the present study, we measured the amount of TLR4, IL-8, and A20 mRNA in bronchial epithelial cells from RAO-affected and control horses following natural challenge induced by stabling. It has been shown previously that stable dust is rich in endotoxin (38, 39) and endotoxin is involved in RAO pathogenesis (36, 38). Because hay dust contains other proinflammatory agents (38, 39) that may interact with endotoxin to cause airway inflammation, we chose this natural challenge model. The disadvantage of this approach is that these other proinflammatory agents may have contributed to airway inflammation in a TLR4-signaling-independent manner.

In conclusion, we showed that exposure to stable dust leads to increased TLR4 mRNA expression in bronchial epithelial cells from RAO-affected horses, that the amount of epithelial TLR4 mRNA correlates with IL-8 mRNA expression as well as airway inflammation, and that, in RAO-affected horses, A20 is negatively correlated with IL-8. These data suggest that an increased TLR4 signaling in combination with a nonsufficient feedback regulation by A20 contributes to the pathogenesis of RAO.

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REFERENCES


