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

Annerose Berndt, Frederik J. Derksen, Patrick J. Venta, Susan Ewart, Vilma Yuzbasiyan-Gurkan, and N. Edward Robinson

Pulmonary Laboratory, Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan

Submitted 5 October 2006; accepted in final form 8 December 2006

Recurrent airway obstruction (RAO), also known as heaves, is a common pulmonary disorder in horses. Acute exacerbations are characterized by neutrophilic airway inflammation, mucus accumulation, and bronchospasm, which are triggered by exposure of susceptible horses to stable and hay dust. RAO is a diffuse airway disease involving large conducting airways down to the terminal bronchioles. Periods of acute exacerbation are interspersed by periods of remission, which can be achieved by returning RAO-affected horses from stables to pasture (40).

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concentrations in the breathing zone of stabled horses are at least 10-fold higher than concentrations on pasture (unpublished data).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
Table 1. Primer pairs sequences used for amplification of TLR4, IL-8, A20, and 18S rRNA transcripts in bronchial brushing samples

<table>
<thead>
<tr>
<th>Primer</th>
<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>GCA ATT ATT CCC CAT GAA CG</td>
<td>GCC CTC AGT AAA GCA TGC AA</td>
<td>123</td>
<td>(13)</td>
</tr>
<tr>
<td>TLR4</td>
<td>TCT GGA GAC GAC TCA GGA AAG C</td>
<td>GCA AGA AGC ACC TCA GGA GTT T</td>
<td>91</td>
<td>AY058908</td>
</tr>
<tr>
<td>IL-8</td>
<td>CAC CAT CTC GTC TGA ACA TGA CT</td>
<td>AGA GCT GCA GAA AGC AGG AAG A</td>
<td>73</td>
<td>AY184956</td>
</tr>
<tr>
<td>A20</td>
<td>CCT GAT TGA GGA GTC CAT GCT</td>
<td>TCC ACA CTC ACC CAC CAG TTC</td>
<td>69</td>
<td>Human: NM_006290</td>
</tr>
</tbody>
</table>

The oligonucleotide primer pairs sequences used for amplification of TLR4, IL-8, A20, and 18S rRNA transcripts in bronchial brushing samples. Amplicon length of the primer pairs and National Center for Biotechnology Information (NCBI) entrance code for sequences used during primer design or sequence source are shown. TLR4, Toll-like receptor 4; A20, zinc finger protein.
and recovered by suction. The lavaged fluids were pooled and the volume was determined.

**Quantification of inflammatory cells.** Total and differential cell counts in BAL were performed using a hemocytometer. Cell smears were made by use of a cytocentrifuge and stained with Wright-Giemsa stain. Differential cell counts were performed by counting 200 cells per slide.

**RNA isolation and quantitative reverse transcriptase-polymerase chain reaction.** Total RNA was isolated from BBs using a phenol/guanidine isothiocyanate mixture (TRI reagent, Sigma) and 1-bromo-3-chloro-propane (Sigma-Aldrich, St. Louis, MO). Total RNA was treated with DNA-free kit (Ambion). Yields of total RNA were determined using NanoDrop technology (ND-1000 Spectrophotometer) and the integrated software v3.1.2. The quality of total RNA was assessed using the Agilent 2100 Bioanalyzer, and the integrity of the 18S and 28S rRNA was determined visually and by the 18S-to-28S ratio. Depending on the total RNA yield of each sample, 40–200 ng total RNA were used as template for the RT reaction using the Omniscript RT kit 200 (Qiagen) according to the manufacturer’s protocols. The RT reaction contained 10× RT buffer, 0.5 mM of each deoxynucleotide triphosphate (dNTP), 10 μM random hexamer primers (Applied Biosystems), 10 U/μl of RNase inhibitor, and 4 units of Omniscript RT. The conditions for the RT reaction were 37°C for 60 min, followed by 93°C for 5 min using an Eppendorf Mastercycler. Quantitative PCR (qPCR) was performed using the ABI 7900 Sequence Detection System (Applied Biosystems). The qPCR reaction contained 20 ng of cDNA as template, QuantiTect SYBR Green PCR kit (Qiagen), and oligonucleotide primer pairs specific to each mRNA of interest (Table 1). Thermal cycling conditions in the ABI 7900 were: 95°C for 15 min, followed by 40 cycles; 95°C for 15 s; and 60°C for 60 s. A dissociation curve for each amplicon in each sample was generated to verify specificity of primer pairs. In addition, each RNA sample was analyzed for genomic contamination by testing RT-negative samples (use of RNase-free H₂O instead of Omniscript RT in RT reaction) for the reference gene. The oligonucleotide primer pair sequences used for amplification of TLR4, IL-8, and A20, and 18S rRNA transcripts, their amplicon length, and their National Center for Biotechnology Information (NCBI) entrance code or the source for the sequence used in the study are listed in Table 1. The 18S rRNA served as the reference gene. Primer pairs were designed with Primer Express software v2.0 (Applied Biosystems). The primer pair for A20 was designed by choosing regions of high sequence identity between the human and mouse A20 mRNA sequences.

**Statistical analysis.** The relative amount of mRNA was calculated using the relative standard curve method. Quantitative means and their standard deviations (SD) for TLR4, IL-8, and A20 of each BB sample were standardized to the reference gene (18S rRNA). If standardized quantitative mRNA expression data were not normally distributed (Kolmogorov-Smirnov P ≤ 0.05), they were log₁₀ transformed. Only BB samples with more than 80% epithelial cells were included in the statistical analysis. Standardized data were analyzed by repeated-measure ANOVA. Fold changes in mRNA expression were determined by calculating the ratio between RAO-affected and control horses at the different time points or between different time points within each group. Data were analyzed by use of SAS v9.1 (SAS Institute, Cary, NC). Associations between single variables were determined using Spearman correlation and regression analysis. Associations were determined within a treatment group (RAO or control group) by taking the measurements of all time points (days 0, 7, 14, and 28) into account. Differences were considered significant for P values ≤ 0.05.

**Table 2. Cellular composition of bronchoalveolar lavage fluid**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>RAO</th>
</tr>
</thead>
<tbody>
<tr>
<td>logTCC</td>
<td>6.47 ± 0.21</td>
<td>5.9 ± 0.21</td>
</tr>
<tr>
<td>logNeu</td>
<td>5.31 ± 0.24</td>
<td>5.59 ± 0.19</td>
</tr>
<tr>
<td>%Neu</td>
<td>13.08 ± 8.21</td>
<td>53.13 ± 11.79</td>
</tr>
<tr>
<td>logMac</td>
<td>5.38 ± 0.33</td>
<td>5 ± 0.36</td>
</tr>
<tr>
<td>%Mac</td>
<td>11.75 ± 3.75</td>
<td>17 ± 8.71</td>
</tr>
<tr>
<td>logLym</td>
<td>6.28 ± 0.26</td>
<td>5.22 ± 0.27</td>
</tr>
<tr>
<td>%Lym</td>
<td>72.17 ± 10.99</td>
<td>26.13 ± 11.31</td>
</tr>
</tbody>
</table>

Values are means ± SD of (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.
RESULTS

Airway Function (ΔPplmax)

In RAO-affected horses during acute exacerbation (day 0), ΔPplmax was significantly elevated compared with control horses and with RAO-affected horses during remission (Fig. 1A). No statistically significant differences in ΔPplmax 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>Day 0</td>
<td>4.14*</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>0.98</td>
<td>3.10</td>
</tr>
<tr>
<td></td>
<td>Day 14</td>
<td>1.49</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>Day 28</td>
<td>1.85</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>Days 0–7</td>
<td>7.10*</td>
<td>4.40</td>
</tr>
<tr>
<td></td>
<td>Days 0–14</td>
<td>9.29*</td>
<td>6.83*</td>
</tr>
<tr>
<td></td>
<td>Days 0–28</td>
<td>9.99*</td>
<td>7.10*</td>
</tr>
<tr>
<td></td>
<td>Days 7–14</td>
<td>1.31</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td>Days 7–28</td>
<td>1.41</td>
<td>1.61</td>
</tr>
<tr>
<td></td>
<td>Days 14–28</td>
<td>1.07</td>
<td>1.04</td>
</tr>
<tr>
<td>RAO/RAO</td>
<td>Day 0</td>
<td>1.68</td>
<td>7.91*</td>
</tr>
<tr>
<td></td>
<td>Day 0–14</td>
<td>3.33</td>
<td>4.92*</td>
</tr>
<tr>
<td></td>
<td>Days 0–28</td>
<td>4.46*</td>
<td>7.18*</td>
</tr>
<tr>
<td></td>
<td>Days 7–14</td>
<td>1.99</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>Days 7–28</td>
<td>2.66</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>Days 14–28</td>
<td>1.34</td>
<td>1.46</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 ($P = 3.95 \times 10^{-06}$ and $6.15 \times 10^{-07}$, 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. 3, A 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 chemoattractant 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 chemotactic agents 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 down-regulated 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 stimulus (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.

ACKNOWLEDGMENTS

We gratefully acknowledge Annette Thelen from the Research Technology Support Facility at Michigan State University for guidance in quantitative RT-PCR analysis and Sue Eberhart and Cathy Berney from the Pulmonary Laboratory for skillful assistance in sample collection.

REFERENCES

TLR4 IN EQUINE BRONCHIAL EPITHELIUM

L942


