Original article

Expression of Toll-like receptor 4 and 2 in horse lungs

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Abstract – Toll-like receptor (TLR) is a key component in launching innate immune response to microbial challenge. TLR4 and TLR2 are recognized as specific receptors for components of Gram-negative and Gram-positive bacteria, respectively. Horses are extremely sensitive to endotoxin-induced cardiopulmonary distress and mortality which causes significant economic losses. To date, there are no data on the expression of TLR4 and TLR2 in horse lungs. Therefore, we examined the expression of TLR4 and TLR2 in lungs from normal or *Escherichia coli* lipopolysaccharide (*E. coli* LPS; 50 ng/kg; iv) treated horses. We also studied the impact of the depletion of pulmonary intravascular macrophages (PIM) on TLR4 and TLR2 expression in normal or LPS-treated horses. RT-PCR showed TLR4 mRNA but not TLR2, in normal horse lungs. PIM depletion reduced TLR4 mRNA expression without affecting TLR2. The LPS treatment increased the expression of TLR4 and TLR2 mRNA in normal and PIM-depleted horses compared to normal saline-treated horses. Light and electron microscopic immunocytochemistry showed TLR4 protein in PIM, alveolar macrophages and septal endothelium in lungs from normal or LPS-treated horses. Immuno-gold electron microscopy showed TLR4 in PIM and dual-label immuno-electron microscopy co-localized TLR4 and LPS in the cytoplasm and nucleus of PIM of LPS-treated horses. The present manuscript is the first report on the expression of TLR4 and TLR2 in normal and LPS-treated horses and direct co-localization of TLR4 with LPS molecules in PIM. These data provide evidence that PIM are equipped with TLR4 to handle and rapidly respond to circulating endotoxins.

immunohistochemistry / lungs / immuno-gold EM / pulmonary intravascular macrophages / LPS

1. INTRODUCTION

Lipopolysaccharide (LPS) is a principal pro-inflammatory component of the Gram-negative bacterial cell wall. Bacterial LPS can induce inflammation in many organs including the lungs. Toll-like receptors (TLR) are mammalian homologues of the toll receptors identified in *Drosophila* and to date 11 mammalian TLR have been iden-

tified [15, 20]. TLR4 and TLR2 recognize conserved molecular patterns associated with a wide range of microbial pathogens [1, 2] to initiate transcription of proinflammatory cytokines such as IL-1 β , TNF- α , IL-6 and IL-8 [3, 6, 10]. Mammalian TLR4 regulates innate immune response and is the most critical sensor for the recognition of LPS and triggering signaling events associated with it [9]. TLR2 is involved in the

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recognition of lipoteichoic acid from Gram positive bacteria to generate inflammatory response [21]. LPS, apart from regulating expression of TLR4, can also influence expression of TLR2 in human leukocytes and mouse lungs [11, 16]. Therefore, it is critical to understand the expression of these proteins for a better comprehension of the host response to pathogens.

Compared to other animal species, horses are extremely sensitive to LPS-induced cardiopulmonary distress and mortality which causes significant economic damage to the equine industry [22]. Recently, we reported that pulmonary intravascular macrophages (PIM) in equine lungs produce inflammatory mediators and their depletion partially inhibits LPS-induced lung inflammation including increases in pulmonary arterial pressures [14]. However, the precise mechanisms of increased susceptibility of horses for LPS-induced pulmonary inflammation remain incompletely understood. Although TLR4 expression has been well reported in various tissues including lungs in humans and mice [6, 19], there are no data on the expression of TLR4 and TLR2 in horse lungs. Therefore, we investigated TLR4 and TLR2 expression in lungs from normal and LPS-treated horses. Because PIM appear to regulate LPS-induced lung inflammation in horses [14], we also evaluated TLR4 and TLR2 expression in the lungs of horses with or without challenge with LPS following depletion of PIM.

2. MATERIALS AND METHODS

2.1. Animals

The experimental protocols for this study were approved by the Animal Care Assurance Committee of the University of Saskatchewan in accordance with the national guidelines for the use of animals in research. Male horses ($n = 20$) of 1–2 years of age were purchased from the Saskatoon Auction Market and were acclimatized in animal care housing for a period of one

week. The horses were housed in a barn and isolated from other horses or animals. The horses were examined regularly for clinical signs of any respiratory or other systemic diseases. The examination included recording of temperature, heart and respiration rates and blood examination. The horses were used for experiments after they were found to be clinically normal and acclimatized.

2.2. Treatments

The treatment groups have been reported previously [13, 14]. The horses ($n = 8$) were administered gadolinium chloride (GC; Sigma Co., St. Louis, USA) dissolved in endotoxin-free saline (10 mg/mL intravenously) to deplete PIM followed by euthanasia at 24 h and 48 h of the treatment ($n = 4$ each). The GC treatment induced significant depletion of PIM at 48 h after the treatment [13, 14]. Another group of horses ($n = 8$) was treated with either the saline ($n = 4$) or GC ($n = 4$) 48 h prior to administration of *E. coli* LPS (50 ng/kg body weight; B0128:12; Sigma Co., St. Louis, USA). The LPS was dissolved in physiological saline and infused over 20 min. The control horses ($n = 4$) were administered 500 mL of endotoxin-free physiological saline (vehicle) intravenously twice 48 h apart. The horses were euthanized 2 h after the LPS or saline treatments (Euthenol; 10 mL/50 kg body weight intravenously; Biomed-MTS Animal Health, Mississauga, Canada).

2.3. Tissue collection and processing

The horses were euthanized and the lungs were collected as described previously [13]. Briefly, the lungs were fixed *in situ* for 30 min by pouring 5 L of 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) into the trachea. The lungs were removed *en bloc* and tissue pieces were collected from 7 pre-determined areas of each lung and fixed in 4% paraformaldehyde for 24 h at

4 °C. The necropsy was conducted to determine any gross abnormalities in the viscera. The lungs were dehydrated, cleared in xylene and embedded in paraffin. For immuno-electron microscopy, lung tissues of approximately 1 mm³ were fixed in 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 3 h, dehydrated and polymerized in LR-White resin (Electron Microscopy Sciences, Hatfield, USA) under ultraviolet (UV) light at -8 °C for 48 h.

2.4. RNA isolation and reverse transcription – PCR

Total RNA was isolated from the lungs of horses by sequential extraction with TRIzol reagent (Invitrogen, Ontario, Canada) followed by treatment with RNase-free DNase (Qiagen, Ontario, Canada) purification on RNeasy mini columns (Qiagen) according to the manufacturer's instructions. RNA integrity was confirmed by agarose gel electrophoresis, and RNA was quantified by spectrophotometric analysis. The SuperScript III one-step RT-PCR system with Platinum *Taq* DNA polymerase (Invitrogen) was used for semi-quantitative analysis of the expression of TLR4 (GenBank Accession No. AY005808), TLR2 (GenBank Accession No. AY429602) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank Accession No. AF157626). The reactions were performed using the primer pairs; 5'-GTGGCCGTGGAGACAA-3' and 5'-AGTCCGCGCAGGGCA-3' for TLR4, 5'-CATGCTTTGTGGACGGT-3' and 5'-GTATTGGTTTCAGGGTCA-3' for TLR2 and 5'-TCACCATCTTCCAGGAG-3' and 5'-GTCTTCTGGGTGGCAG-3' for GAPDH. Each reaction was performed using 10 ng of total RNA and reverse-transcribed at 55 °C for 30 min. After initial denaturation of the cDNA at 94 °C for 2 min, 30 amplification cycles (94 °C for 15 s, 57 °C for 30 s, and 68 °C for 1 min) were performed, followed by a final extension at 68 °C for 5 min. To ensure the lack

of DNA contamination, 2 units of Platinum *Taq* DNA polymerase were substituted for the SuperScript III RT/Platinum *Taq* mixture in the reaction. A negative control reaction consisted of all the components of the reaction mixture except RNA. Reactions were amplified and PCR products were electrophoresed on a 1% TAE-agarose gel, stained with ethidium bromide and photographed under UV light. The intensity of the amplified bands was analyzed by densitometry and all results were normalized to GAPDH.

2.5. Immunohistology with anti-TLR4 antibody

Immunohistology with anti-TLR4 antibody (M16; SC-12511, Santa Cruz Biotechnology Inc., USA) was done on one lung section from each of saline and LPS treated horses. Briefly, following dewaxing, rehydration and quenching of endogenous tissue peroxidase, the sections were incubated with pepsin (2 mg/mL 0.01 N hydrochloric acid) to unmask the antigens. After blocking nonspecific sites with 1% bovine serum albumin in PBS buffer for 30 min, the sections were treated with anti-TLR4 antibody (4 µg/mL) and with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 60 and 30 min, respectively. Previously, we showed the specificity of the TLR4 antibody using a blocking peptide against which the antibody was raised [23]. The reaction was visualized using a color development kit (Vector Laboratories, Ontario, Canada). Slides were also counterstained with methyl green (Vector Laboratories). Immunohistochemical controls included incubation with normal goat IgG or omission of primary antibody or both primary and secondary antibodies.

2.6. Sequence alignment and immuno-precipitation

Information on the amino acid sequence of the peptide used to raise the antibody

against TLR antibody (M16; SC-12511) was obtained from the manufacturer and compared with the C-terminus of horse TLR4 sequences using the Clustal W program to ensure its specificity. Furthermore, the specificity of this antibody to recognize horse TLR4 protein was tested by immunoprecipitation. Lungs from control animals were grounded in liquid nitrogen and lysed in 1 mL of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, phenylmethylsulfonyl fluoride, protease inhibitors). After 30 min of incubation on ice, cell-free lysate was pre-cleaned on protein G bead slurry and stored at -80°C . The protein was quantified by using the Bradford method and for immunoprecipitation 1 mg of the protein was mixed with 10 μg of anti-TLR4 antibody. After overnight incubation at 4°C , the immune complexes were washed with lysis buffer and solubilized in 50 μL of SDS sample buffer (1.25% SDS, 2.5% glycerol, 62.5 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol). Immuno-precipitated proteins were separated on 12% tris-glycine polyacrylamide gel and blotted onto polyvinylidene difluoride membranes. The membranes were blocked with 5% dry milk-0.1% Tween-20 in Tris-saline buffer for one hour and incubated overnight with anti-TLR4 antibody followed by one hour exposure to horseradish peroxidase-labeled rabbit anti-goat antibody. Staining was detected by chemiluminescence and photographs were taken using a gel documentation unit.

2.7. Single and dual label immuno-electron microscopy

Thin sections were placed on nickel grids and floated on a blocking buffer (1% BSA and 0.1% Tween 20 in Tris-buffered saline, pH 7.9) for 30 min followed by incubation with primary antibody against TLR4 (1:25) for 1 h. Tissue sections were rinsed three times for 5 min each in Tris-buffered saline and incubated with 15 nm gold-conjugated anti-goat secondary antibody

(1:100; British Biocell International, USA) for 1 h. Some of the sections labeled with anti-TLR4 antibody were further stained with anti-LPS antibody and 10 nm gold conjugated anti-rabbit antibody. This was followed by staining with 2% aqueous uranyl acetate and lead citrate. The controls for immuno-electron microscopy included omission of primary antibody and incubation with isotype-matched immunoglobulins.

2.8. Statistical analyses

Data were analyzed using SigmaStat version 3.1. Groups were compared by one-way analysis of variance and differences were considered significant at $P < 0.05$.

3. RESULTS

3.1. TLR2 and TLR4 mRNA expression

PCR on lung samples treated with DNase as well as RT-PCR on pure water (without RNA) did not yield any bands indicating the absence of non-specific amplification (data not shown). The total amount of RNA in each sample appeared to be uniform as evident from GAPDH amplification which yielded a 340 bp band (Fig. 1A). TLR4 and TLR2 values were normalized to GAPDH values. The data show expression of TLR4 mRNA of expected 450 bp size but not TLR2 mRNA in the lungs of normal horses (Fig. 1A). We found a significant reduction in expression of TLR4 mRNA at 24 h followed by a further decline at 48 h of GC treatment (Fig. 1B). However, GC treatment had no effect on TLR2 mRNA expression (Fig. 1C). There were no differences in levels of TLR4 and TLR2 mRNA (740 bp) in normal or PIM-depleted horses treated with LPS (Figs. 1A–1C). However, both TLR4 and TLR2 mRNA levels were higher in LPS-treated normal or PIM-depleted horses compared to those administered the saline (Figs. 1A–1C).

Figure 1. RT-PCR for the expression of TLR4 and TLR2. Total RNA extracted from the lungs of horses with different treatments were subjected to RT-PCR for TLR4 (A, upper panel), TLR2 (A, middle panel). PCR products separated on 1% agarose gel (A), showed differential expression of TLR4 and TLR2 transcripts. The amount of total RNA was the same in all reactions as supported by the GAPDH transcripts (A, lower panel). The graphs represent integrated density values (IDV) of TLR4 and TLR2 (mean $\pm$ SE) normalized to GAPDH. Densitometric analysis showed a significant ($P = 0.006$) increase in the TLR4 mRNA transcripts in LPS challenged horses compared to control animals (B). The level of TLR2 in control animals was negligible and horses challenged with LPS resulted in a significant ($P = 0.001$) increase in the expression of TLR2 transcripts (C). Groups with different letter superscripts for example "a" and "b" are different ($P < 0.05$) while those bearing the same letter superscripts are similar ($P > 0.05$).

3.2. Immunohistochemistry

The specificity of the anti-TLR4 antibody was indicated by 80% homology in C-terminus amino acid sequences of the peptide used to raise the antibody and C-terminus sequence of horse TLR4 (Fig. 2A). The specificity of anti-TLR4 antibody to horse TLR4 antigen was confirmed by detection of a single band of 85 kDa by the

antibody from an immuno-precipitated fraction (Fig. 2B) but not from the supernatant. These two experiments confirm that anti-mouse TLR4 antibody used in our experiments does recognise horse TLR4.

Substitution of primary antibody with isotype-matched immunoglobulins in the protocol showed no staining in tissue sections (Fig. 3A). Sections incubated with only secondary antibody did not reveal any

Figure 2. Amino acid sequence alignment of the C-terminal region of mouse TLR4 (GenBank Accession No. Q9QUK6) and horse TLR4 (GenBank Accession No. AY005808). Anti-TLR4 antibody used was raised against the 16 amino acid sequence of mouse TLR4 in this region and the region is 80% similar (Box) to that in the horse (A). Anti-TLR4 antibody raised against the C-terminus of mouse TLR4 also specifically recognizes TLR4 proteins in the horse (B). A single 85 kDa band was observed in the total extracted protein (TP), and immuno-precipitate (IP) but not in the supernatant (S).

tissue reactivity (data not shown). Staining without primary and secondary antibodies to determine neutralization of endogenous peroxidase resulted in the lack of a reaction (data not shown). In the lungs from the control horses, TLR4 staining was observed in the septa (Fig. 3B), endothelium of large blood vessels, alveolar macrophages and bronchiolar epithelial basement membrane (Fig. 3C), and the PIM (Fig. 3E). No staining was observed in the bronchiolar epithelium. In the lungs from LPS-treated horses, the pattern of TLR4 staining was similar to that observed in control lungs with a possible increase in intensity of staining in the septum and PIM (Figs. 3D, 3F).

3.3. Immunoelectron microscopy

First, we labeled lung sections from normal horses with anti-TLR4 antibody and found TLR4 localization in the cytoplasm and nucleus of PIM (Fig. 4A) and septal endothelial cells (Fig. 4B) in normal and inflamed lungs. Lungs from LPS-treated horses showed disruption of alveolar septa and localization of TLR4 in neutrophils (Fig. 5A). To directly address the issue of the interaction of TLR4 with the LPS, we performed dual label immuno-electron microscopy to localize TLR4 as well as the

LPS in lung sections. The data show co-localization of TLR4 with the LPS in the cytoplasm and the nucleus of PIM to establish a direct spatial relationship between the two (Fig. 5B).

4. DISCUSSION

Although it is well established that horses are extremely sensitive to endotoxin-induced lung inflammation and cardio-pulmonary shock, the underlying physiological mechanisms have remained a mystery. To further expand on our recently reported observations that PIM play a critical role in LPS-induced lung inflammation in horses [14], we examined the expression of TLR4 and TLR2 in lungs from normal or LPS-treated horses. We focused on TLR4 and TLR2 due to their established roles in bacterial diseases [20]. First, we report that TLR4 but not TLR2 is expressed in normal horse lungs. Second, PIM depletion reduced the expression of TLR4 without affecting the absence of TLR2 in the lungs of normal horses. Lastly, LPS increased lung expression of both TLR4 and TLR2 in normal as well as PIM-depleted horses.

In normal horses, we show TLR4 mRNA expression using RT-PCR and protein

Figure 3. Immunohistochemistry for TLR4: Staining with normal goat IgG served as a negative control and showed no staining which ruled out non-specific reactions (A) whereas staining control horse (B, C, E) lungs with anti-TLR4 antibody showed the presence of TLR4 in the septum, alveolar macrophages (thick arrows) and pulmonary intravascular macrophages (arrowheads). No staining was present in the bronchiolar epithelium (arrows). However, peribronchial blood vessels (arrowheads in C) and epithelial basement membranes (double arrow) showed TLR4 expression. In LPS treated horse lungs (D and F), the expression was similar to controls except for increased expression in the septum and pulmonary intravascular macrophages (arrowheads). Original magnification: Scale bar: A-D: 50 μ m; E and F: 25 μ m. (A color version of this figure is available at www.edp-sciences.org.)

expression in PIM and microvascular endothelium with immunocytochemistry. Lung tissue immunochemistry was done with a commercially available mouse anti-TLR4 antibody. We found 80% similarity in the C-terminus amino acid sequence between horse TLR4 and the epitope that was used to raise the mouse antibody. Secondly, the mouse anti-TLR4 antibody immuno-precipitated a single band of 85 kDa, which is the expected molecular weight of TLR4, from the horse lung

homogenates. These two experiments confirmed that the antibody used in our experiments does indeed recognize horse TLR4. PIM have been recognized as central to phagocytosis of blood-borne endotoxins and bacteria and production of inflammatory mediators such as $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ in various species including the horse [14, 18]. Our data provide the first evidence that horse PIM express TLR4 and that LPS-treated horses show co-localization of TLR4 and LPS in PIM. TLR4 is a critical

Figure 4. Immunoelectron microscopy for TLR4: TLR4 was localized in the cytoplasm and nucleus of PIM (A; arrows), septal endothelial cells (B; arrows). AS: Alveolar space; Ep: alveolar epithelium; RBC: red blood cell; En: endothelial cell; PIM: pulmonary intravascular macrophage. Scale bar: 1 μ m.

LPS sensor to induce cell signaling and production of inflammatory mediators [3, 6, 10]. Based on co-localization of TLR4 and LPS in PIM in our studies, it is possible that TLR4 may be the reason for the ability of PIM for rapid phagocytosis of blood-borne endotoxins, rapid activation, and production of inflammatory mediators [14, 17, 18]. The TLR4 and LPS colocalization in horse lungs is interesting because we recently reported similar data from a rat model of LPS-induced lung inflammation [7]. There have been previous ultrastructural data that LPS micelles disrupt endosomal membranes and enter into the nucleus of monocytes in vitro and PIM in vivo [8, 17]. However, the mechanisms that facilitate the entry of LPS into the nucleus have not been clear. Our new data showing

co-localization of TLR4 and LPS does not indicate whether the complex was formed on the membrane or following entry of the LPS into the cytoplasm and nucleus. Therefore, further studies are needed to elucidate the molecular interactions of TLR4 that might facilitate the movement of LPS inside the target cells. We also need to probe nuclear extracts from lung homogenates from LPS-treated horses to further confirm the co-localization of TLR4 and the LPS. Our data does show that PIM are the major contributors or regulators of TLR4 expression in horse lungs because their depletion caused significant suppression of TLR4 expression in normal horse lungs. This could also be one of the reasons for the reduced LPS-induced lung inflammation and cytokine expression which we recently

Figure 5. Lungs from LPS-treated horses showed disruption of alveolar septa (Se) and localization of TLR4 (A: arrows) in neutrophils (N). Dual label immuno-EM localized (B; circles) TLR4 (large gold particles) and the LPS (small gold particles) in cytoplasm and the nucleus of a pulmonary intra-vascular macrophage. AS: Alveolar space; Ep: alveolar epithelium; Se: septum; RBC: red blood cell; En: endothelial cell. Scale bar: 1 μ m.

observed in PIM-depleted horses [14]. These data on TLR4 expression in lungs provide a molecular framework to explain the susceptibility of horses for endotoxin-induced pulmonary dysfunction.

In lungs from LPS-treated horses, increased expression of TLR4 and TLR2 mRNA was observed within 2 h of LPS treatment of both normal as well as PIM depleted horses. The increase in expression of TLR4 and TLR2 was in agreement with

previously published reports in other species [12, 16]. Because TLR2 was absent in the lungs of the normal or PIM-depleted horses, the increase in the expression of TLR2 following LPS treatment is more striking than that of TLR4, which is normally present in the lungs. It is possible that increased mRNA expression for TLR2 is a consequence of TLR4 signaling, as shown in mice [5] and it appears to be a part of broader up-regulation of immune functions

in lung cells. The LPS-induced increase in TLR4 expression in PIM-depleted horses is even more striking because PIM depletion suppressed TLR4 expression in normal horses. It is possible that LPS administration, in the absence of PIM, induced vigorous expression of TLR4 in other lung cells such as the endothelium, alveolar epithelium and inflammatory cells. The immediate effects of increased expression of TLR4 and TLR2 on lung inflammation in horses are not understood. However, there are some possibilities. Early up-regulation of TLR4 and TLR2 plays a role in the pro-inflammatory response and pathophysiology of polymicrobial sepsis [24]. Increased expression of TLR4, for example in PIM, may impart them the additional capability to scavenge circulating LPS and respond to it. However, our data differed from recently reported suppression of TLR4 and TLR2 expression in LPS challenge of mice following depletion of alveolar macrophages [16]. These difference may be species-specific because horse lungs are inherently more sensitive compared to mice to LPS-induced lung inflammation. Furthermore, a comparison of porcine PIM with alveolar macrophages revealed PIM to be more inflammatory [4]. Therefore, depletion of PIM versus alveolar macrophages may result in different effects on TLR4 expression in lungs.

In conclusion, we show that normal horse lungs express TLR4 but not TLR2. We also show that LPS administration increases TLR4 and TLR2 expression. Abundance of TLR4 expressing PIM and the presence of a large number of these macrophages in the horse lungs highlight their central role in lung inflammation and them as one of the reasons for higher sensitivity of horses to endotoxin-induced pulmonary dysfunction. We are aware that our data do not directly address the functions of TLR4 and TLR2 in cardiopulmonary complications associated with endotoxemia in horses. Therefore, further studies are needed to clarify and establish the roles of TLR4 and TLR2 in such clinical conditions.

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