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Experimental TLR4 inhibition improves intestinal microcirculation in endotoxemic rats

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ABSTRACT

Introduction: Toll like receptor 4 (TLR4) represents a critical cellular link for endotoxin-induced pathology. The aim of this study was to evaluate the potential role of TLR4 inhibition on the intestinal microcirculation during experimental endotoxemia.
Materials and methods: The intestinal microcirculation was studied by intravital microscopy in four groups of Lewis rats (n = 10 per group): healthy controls (CON group), endotoxemic animals (15 mg/kg lipopolysaccharide, LPS group), endotoxemic animals treated with a TLR4 antagonist (1 mg/kg CRX-526, LPS + CRX526 group), and controls treated with CRX-526 (C-CRX526 group). Plasma samples were obtained for cytokine measurements at the end of the experiments.
Results: Endotoxemia significantly increased leukocyte adhesion in intestinal submucosal venules (e.g., V1 venules: CON 20.4 ± 6.5 n/mm^2, LPS 237.5 ± 36.2 n/mm^2, $p < 0.05$) and reduced capillary perfusion of the intestinal wall (e.g., longitudinal muscular layer: CON 112.5 ± 5.9 cm/cm^2, LPS 71.3 ± 11.0 cm/cm^2, $p < 0.05$) at 2 h. TLR4 inhibition significantly reduced endotoxemia-associated leukocyte adhesion (V1 venules: 104.3 ± 7.8 n/mm^2) and improved capillary perfusion (longitudinal muscular layer: 111.0 ± 12.3 cm/cm^2). Cytokine release was not significantly affected.
Conclusions: The TLR4 pathway may be a target in clinical Gram-negative sepsis since administration of the TLR4 antagonist CRX-526 improved intestinal microcirculation parameters in experimental endotoxemia.

Introduction

Sepsis is a systemic inflammatory response to infection (Matot and Sprung, 2001). During the early stages of sepsis, large quantities of inflammatory cytokines and mediators are released into the circulation by a dysregulated immune response. If left untreated, immune system dysregulation leads to impairment of circulatory function, resulting in tissue hypoperfusion and organ dysfunction or failure (Cohen, 2002; Rittirsch et al., 2008). It has been suggested that altered perfusion of the intestinal microcirculation plays an important role in the development of ongoing sepsis and in the pathogenesis of septic multiple organ dysfunction syndrome (Frey and Kesel, 2000). Underlying factors for the disturbances in the microcirculation include changes in plasma viscosity, leukocyte adhesion and red blood cell aggregation (Sallisalmi et al., 2014; Santos et al., 2011; Yang et al., 2008). Hypoperfusion-induced ischemia during endotoxemia may also compromise the gut barrier, resulting in translocation of bacteria and toxins into the systemic circulation and subsequent enhancement of sepsis (Savva and Roger, 2013; Theuer et al., 1993).

Lipopolysaccharide (LPS) or endotoxin is the major component of the outer membrane of Gram-negative bacteria. LPS generally consists of a hydrophobic lipid A and the outmost O-antigen polysaccharide. Lipid A provokes immune cell activation through the pattern recognition receptor, Toll-like receptor 4 (TLR4) (Ramachandran, 2014). Endotoxin binds to CD14 on immune cells and subsequently interacts with the adaptor protein MD2 and TLR4 to form a heterodimeric cell-surface signaling complex (Park and Lee, 2013; Deng et al., 2013; Beutler, 2000; Tidswell and LaRosa, 2011). TLR4 recruits the Toll-adaptor protein and myeloid differentiation factor 88 (MyD88) to induce NF-kB activation and the up-regulation of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), interleukin 6 (IL-6) and interferon γ (IFN-γ) (Park and Lee, 2013; Płóciennikowska et al., 2015). Increased levels of TNF-α, IL-1, IL-6 and IFN-γ have been reported in LPS induced acute lung injury (Wei and Huang, 2014), experimental endotoxemia (Patel et al., 2010; Zhou et al., 2012; Yeh et al., 2012; Copeland et al., 2005) and inflammatory

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bowel disease (Fort et al., 2005). LPS activation of immune cells triggers enhanced phagocytosis of bacteria and the release of cytokines, promoting recruitment of other immune cells to the site of infection. Thus, signaling through the TLR4R complex actively contributes to the development of inflammation and is useful for limiting the spread of infection. However, if the immune system is activated systemically (hyper-immune response), destructive activities not only target pathogens but also healthy tissues (Hotchkiss and Karl, 2003).

CRX-526 is a synthetic lipid A mimetic molecule that belongs to the class of aminoalkyl-glucosaminide-phosphates (AGP) (Bazin et al., 2008). CRX-526 has been used as an antagonist for the TLR4 complex to block the pro-inflammatory actions of LPS in vitro and in vivo (Fort et al., 2005), to reduce acute inflammation in lung ischemia-reperfusion injury (Zanotti et al., 2009) and to prevent diabetic nephropathy (Lin et al., 2013). The aim of this study was to evaluate the impact of CRX-526 on the intestinal microcirculation during endotoxemia. Intravital microscopy was used to examine leukocyte recruitment and capillary perfusion in vivo. In addition, since TLR4 activation is associated with pro-inflammatory cytokine production, we examined the effects of CRX-526 on plasma levels of TNF-α, IL-1, IL-6 and IFN-γ.

Materials and methods

Animals

Male Lewis rats (250–300 g) were purchased from Charles River (Wilmington, MA, USA) and maintained in the Carlton Animal Care Facility at Dalhousie University. The animals were provided with water and rodent chow ad libitum under standard 12 h light/dark cycles. All procedures were undertaken in compliance with the guidelines of the Canadian Council on Animal Care and approved by the University Committee on Laboratory Animals at Dalhousie University.

Anesthesia and preparation

The animals were initially anesthetized intraperitoneally (i.p.) with 60 mg/kg pentobarbital (Ceva Sante Animale, Montreal, QC, Canada) and were supplemented intravenously (i.v.) with 20 mg/kg pentobarbital during the experiment. The animals were placed in the supine position on a heating pad that maintained rectal body temperature at 37.5 ± 0.5 °C. A tracheostomy was performed to maintain airway patency, and the animals breathed room air spontaneously. The left jugular vein and carotid artery were cannulated with polyethylene tubing (PE 50, Clay Adams, Sparks, MD, USA) for continuous fluid supplementation and monitoring of arterial blood pressure and heart rate (Hewlett Packard monitor, Model 66S, Saronno, Italy), respectively.

Four groups of animals (n = 10 per group) were studied: 1) healthy control animals (CON group), received normal saline solution vehicle (0.9% Sodium Chloride, Hospira, Montreal, QC, Canada); 2) endotoxemic animals received lipopolysaccharide (15 mg/kg, LPS group); 3) endotoxemic animals treated with TLR4 antagonist (1 mg/kg CRX-526, LPS + CRX526 group), and 4) CRX-526 treated controls (C-CRX526 group). LPS was administered i.v. at time 0 and CRX-526 was given i.v. 15 min after LPS or saline injection. Two hours after LPS challenge, intravital microscopy was performed.

Intravital microscopy (IVM)

5 min before IVM, the animals were given 0.05% rhodamine 6G (1.5 ml/kg, Sigma-Aldrich, Oakville, ON, Canada) and 5% FITC albumin (1 ml/kg, Sigma-Aldrich) solution i.v. for leukocyte staining and capillary flow visualization, respectively. After a midline laparotomy, a section of the small intestine proximal from the ileocoecal valve was placed on custom stage (Pavlovic et al., 2006) and viewed using an epifluorescent microscope (Leica DMLM, Wetzlar, Germany). The images were captured by a CCD video camera (SIT 68, DAGE MTI, Michigan City, IN, USA), transferred to a black and white monitor (Speco Technologies, Amityville, NY, USA) and recorded by a videotape recorder (DSR-25 DVCAM, Sony, Japan) for off-line evaluation. During the entire in vivo microscopic procedure the intestine was superfused with temperature-controlled (37.5 °C) saline solution to avoid drying and exposure to ambient air. At the end of each experiment, blood samples were taken and the animals were sacrificed by potassium chloride (EDM Chemicals, Gibbstown, NJ, USA).

Evaluation of video sequences of the intestinal microcirculation was carried out off-line using a video monitor. Leukocyte adhesion was defined as the number of leukocytes that remained immobile for at least 30 s relative to an oblique, cylindrical endothelial surface and reported as cells per mm^2 (n/mm^2). Leukocyte adhesion was analyzed in collecting (V1) and postcapillary (V3) venules of the intestinal submucosal layer. Leukocytes that interacted with but did not adhere to the endothelia were defined as rolling leukocytes. The number of rolling leukocytes passing through the observed vessel segment within a 30 s window was reported as leukocyte rolling (cells per minute, n/min). Functional capillary density (FCD), defined as the length of capillaries with erythrocyte perfusion in relation to a predetermined rectangular field, cm/cm^2, was measured in mucosal and muscular layers. The analysis of the video sequences was performed in a blinded manner by the investigator.

Cytokine analysis

Plasma was obtained from blood samples and stored at −80 °C. Cytokine levels were measured according to the manufacturer's instructions using a rat FlowCytomix multiplex assay (eBioscience, San Diego, CA, USA) for IL-1, IL-6, TNF-α, and IFN-γ.

Statistical analysis

Prism 5 (GraphPad Software, La Jolla, CA, USA) was used to analyze the results. All data are expressed as means ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni corrected Student's t-test. Significance was set at $p < 0.05$.

Results

Intravital microscopy

Leukocyte adhesion

Fig. 1 shows the number of adherent leukocytes in the submucosal collecting (V1) and postcapillary (V3) venules of the intestinal microvasculature. Following LPS challenge (15 mg/kg), there was a significant ($p < 0.01$) increase in the number of adherent leukocytes in the V1 venules (Fig. 1a) and V3 venules (Fig. 1b) in comparison to healthy control animals. There was an 8-fold increase in the V1 venules and a 10-fold increase in the V3 venules. Administration of the TLR-4 antagonist CRX-526 significantly reduced the LPS-induced leukocyte adhesion in both V1 and V3 venules (Fig. 1a & b, $p < 0.01$). Administration of CRX-526 in normal healthy animals had no significant effect on leukocyte adhesion (Fig. 1a & b, $p > 0.05$).

Leukocyte rolling

Fig. 2 shows the number of leukocytes rolling along the endothelium in the intestinal submucosal V1 and V3 venules. In the V1 venules, there were no significant differences in the number of rolling leukocytes among the control, LPS and CRX-526 treated groups (Fig. 2a, $p > 0.05$). However, in the V3 venules, there was a significantly decreased number of rolling leukocytes in LPS challenged animals in comparison to healthy control animals (Fig. 2b, $p < 0.05$). Treatment with CRX-526 in LPS challenged animals did not significantly increased the number of rolling leukocytes in V3 venules (Fig. 2b, $p > 0.05$ vs LPS). Administration of