Veterinary Immunology and Immunopathology 160 (2014) 1–11

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Ovocalyxin-36 is an effector protein modulating the production of proinflammatory mediators

Jennifer Kovacs-Nolan[b], Cristianne Cordeiro[a], Denise Young[b], Yoshinori Mine[b], Maxwell Hincke[a,*]

[a] Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5
[b] Department of Food Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1

ARTICLE INFO

Article history:
Received 12 December 2013
Received in revised form 29 January 2014
Accepted 3 March 2014

Keywords:
Lipopolysaccharide
Ovocalyxin-36
Gene expression
Innate immunity

ABSTRACT

Sepsis is a systemic inflammatory response syndrome during infection. Therapeutic agents are essential to protect the host from sepsis. Ovocalyxin-36 (OCX-36) is a chicken eggshell membrane protein and shares protein sequence and gene organization homology with bactericidal permeability-increasing protein (BPI), lipopolysaccharide-binding protein (LBP) and palate, lung and nasal epithelium clone (PLUNC) proteins that play a major role in innate immune protection. We recently reported that OCX-36 binds to both lipopolysaccharide (LPS) and lipoteichoic acid (LTA) (Cordeiro et al., 2013, PLoS ONE 8, e84112), which is an important activity to neutralize endotoxins and non-endotoxin pyrogens during an inflammatory response. Here we investigated the immune modulating effects of OCX-36 and enzymatically digested OCX-36 (dOCX-36) *in vitro* and in a mouse model of endotoxemia. OCX-36 alone dose-dependently induced both TNF-α and nitric oxide (NO) production by RAW 264.7 macrophage cells, and this immunostimulatory effect was reduced by enzymatic digestion. In the presence of LPS, dOCX-36 was more effective than intact OCX-36 at reducing LPS-induced secretion of TNF-α from RAW 264.7 cells, but did not reduce NO production. In contrast, OCX-36 increased LPS-induced NO production, both in the presence and absence of FBS, PCR array analysis confirmed that OCX-36 and dOCX-36 differentially regulated genes involved in innate immunity, and dOCX-36 down-regulated the expression of genes involved in LPS signaling and inflammatory responses. *In vivo*, dOCX-36 was more effective at reducing LPS-induced inflammatory symptoms and inhibiting the local production of pro-inflammatory mediators in the small intestine. These results suggest that OCX-36 and OCX-36 derived peptides may differentially modulate innate immune responses, and support our hypothesis that OCX-36 derived peptides have potential therapeutic applications in sepsis.

Abbreviations: ACE, angiotensin-I converting enzyme; BCA, bicinchoninic acid; BPI, bactericidal permeability-increasing protein; BW, body weight; CCL2, MCP-1, monocyte chemotactic protein-1; CXCR4, chemokine receptor 4; Cybβ, cytochrome b-245, beta polypeptide; DMEM, Dulbecco's modified Eagle's medium; dOCX-36, digested ovocalyxin-36; HRP, horseradish peroxidase; HTAB, hexadecyltrimethyl ammonium bromide; iNOS, inducible nitric oxide; LAL, Limulus Amebocyte Lysate; LBP, lipopolysaccharide-binding protein; LTA, lipoteichoic acid; MPO, myeloperoxidase; MWCO, molecular weight cutoff; MyD88, myeloid differentiation primary response gene 88; NO, nitric oxide; OCX-36, ovocalyxin-36; PAMPs, pathogen-associated molecular patterns associated molecular patterns; Pglyrp1, peptidoglycan recognition protein 1; PLUNC, palate, lung and nasal epithelium clone; PMSF, phenylmethanesulfonyl fluoride; PRR, pattern recognition molecule; Proc, protein C; TMB, tetramethylbenzidine; TLR, Toll-like receptor.

* Corresponding author at: Department of Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Canada K1H 8M5. Tel.: +1 613 562 5800x8193; fax: +1 613 562 5687.
E-mail address: mhincke@uottawa.ca (M. Hincke).

http://dx.doi.org/10.1016/j.vetimm.2014.03.005

1. Introduction

Sepsis is a disease characterized by the invasion of bacterial pathogens into the bloodstream that activates an inflammatory response. The uncontrolled immune response leads to septic shock that involves tissue damage and multiple organ dysfunction and failure (Opal, 2007).

LPS is the main component of the Gram-negative bacterial cell wall and the principal activator of the innate immune system which promotes the production of pro-inflammatory mediators during infection (Beutler and Rietschel, 2013). LPS is one of several pathogen-associated molecular patterns (PAMPs) and is recognized by Toll-like receptor (TLR) 4 which is expressed on the surface of macrophages. This stimulates the host cells to secrete a large amount of proinflammatory mediators and cytokines such as nitric oxide (NO), tumor necrosis factor (TNF)-α, and interleukins (ILs) (Kumar et al., 2009). NO and TNF-α are associated with antimicrobial activity, the host innate immune response to pathogens and tumor cell killing (Bogdan, 2001).

The toxic effect of LPS is modulated by a large family of proteins such as the LBP/BPI/PLUNC protein family. These proteins bind LPS and mediate the LPS signal to innate immune receptors (Wiesner and Vilcinskas, 2010). For example, BPI protein suppresses the delivery of LPS to immune receptors and promotes LPS uptake by macrophages *via* the macrophage phagocytic process (Iovine et al., 2002). On the other hand, low concentrations of LBP deliver LPS to CD14 molecules and then boost the inflammatory response induced by LPS; in contrast, high concentrations of LBP reduce LPS activation of macrophages (Lamping et al., 1998). Some studies have reported that LBP/BPI/PLUNC proteins inhibit proinflammatory activities of LPS in macrophages such as induction of cytokines secretion, stimulation of neutrophil oxidase enzymes and NO formation (Schumann, 2001; Lukinskiene et al., 2011).

The current therapy for severe sepsis and septic shock includes treatment of circulatory failure, the administration of antibiotics and the use of activated protein C (Rivers et al., 2001). Newer strategies are the identification and development of improved antimicrobial peptides that also neutralize the LPS functionality that leads to overproduction of proinflammatory mediators (Schuerholz et al., 2012).

OCX-36 is an avian protein enriched in the eggshell membranes of chicken eggs. OCX-36 shares similarity in protein sequence and gene structure with LBP, BPI and PLUNC proteins, which is the origin of our hypothesis that OCX-36 participates in the innate immune protection against pathogens (Gautron et al., 2007). We have recently characterized the biological function of purified OCX-36 extracted from eggshell membranes, demonstrating that it is a pattern recognition molecule (PRR) which has antimicrobial activity against *S. aureus* and the ability to bind to *Escherichia coli* LPS and to *S. aureus* LTA (Cordeiro et al., 2013).

In order to evaluate the potential of OCX-36 for therapeutic and neutraceutical applications, we compared whole and enzymatically digested OCX-36 to determine their immune-stimulating and anti-endotoxin properties *in vivo* and *in vitro*.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), sodium pyruvate and penicillin–streptomycin were purchased from Gibco. FBS was purchased from Cansera. 48-Well tissue culture plates and 96-well medium binding microplates were purchased from Corning Costar. Recombinant mouse TNF-α, IL-6, and IL-1β, anti-mouse TNF-α, IL-6, and IL-1β antibodies, biotinylated anti-mouse TNF-α, IL-6, and IL-1β antibodies, and avidin-conjugated HRP were purchased from BD Biosciences. Mouse TNF-α and IL-6 ELISA Ready-SET-Go® kits were purchased from eBioscience. WST-1 Cell Proliferation Reagent was purchased from Roche Applied Science. Bicinchoninic acid (BCA) protein assay, bovine serum albumin (BSA), cell culture grade water (endotoxin-free, <0.005 EU/mL) and Limulus Amebocyte Lysate (LAL) Chromogenic Endotoxin Quantification Kit were purchased from Thermo Scientific. Pepsin from porcine gastric mucosa, LPS from *E. coli* O111:B4, PMSF, aprotinin, leupeptin, pepstatin A, 3,3′,5,5″-tetramethylbenzidine (TMB) and hexadecyltrimethyl ammonium bromide (HTAB) were purchased from Sigma–Aldrich. The Griess reagent system was purchased from Promega. Aurum™ Total RNA Mini Kit was purchased from Bio-Rad Laboratories. RT^2 First Strand cDNA Kit and Mouse Innate and Adaptive Immune Response RT^2 Profiler PCR Array were purchased from SA Biosciences.

2.2. Ovocalyxin-36

OCX-36 was extracted from eggshell membranes and purified as previously described (Cordeiro et al., 2013). Purified OCX-36 was dissolved in PBS buffer (10 mM sodium phosphate buffer, 0.154 M NaCl, pH 7.4) prepared with endotoxin-free water (<0.005 EU/mL) and the concentrations of OCX-36 for all assays were determined by the BCA protein assay using BSA as standard. Endotoxin levels in OCX-36 samples were measured by the LAL assay (Thermo Scientific).

2.3. Enzymatic digestion of OCX-36

To prepare pepsin-digested OCX-36, freeze-dried OCX-36 (2 mg/mL) was dissolved in 0.15 M HCl, and pepsin was added to the OCX-36 solution at an enzyme to substrate ratio of 1:250 (w/w). Samples were incubated at 37 °C for 0, 30 s; 1.5, 5 and 30 min; and 1.5 h, 5 h and 10 h, followed by heating at 90 °C for 5 min to inactivate the enzyme. The digested samples were dialyzed against water (MWCO 100 Da; Spectrum Laboratories, Inc.) and lyophilized for further use in cell culture and animal studies.

To prepare thermolysin-digested OCX-36, freeze dried OCX-36 was dissolved in endotoxin-free PBS buffer and diluted in thermolysin digestion buffer (50 mM Tris–HCl, 0.5 mM $CaCl_2$, pH 7.4). Thermolysin was dissolved in the