The International Journal of Biochemistry & Cell Biology 45 (2013) 2622–2631

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

Lipopolysaccharide neutralization by a novel peptide derived from phosvitin

Lili Hu [a], Chen Sun [a], Shengnan Wang [a], Feng Su [b], Shicui Zhang [a,*]

[a] Laboratory for Evolution & Development, Institute of Evolution & Marine Biodiversity and Department of Marine Biology, Ocean University of China, China
[b] Institute of Chemical Engineering, Qingdao University of Science and Technology, Qingdao 266042, China

ARTICLE INFO

Article history:
Received 29 May 2013
Received in revised form 27 August 2013
Accepted 2 September 2013
Available online xxx

Keywords:
Antimicrobial peptide
Endotoxin
Sepsis
Lipopolysaccharide (LPS)
Tumor necrosis factor (TNF)-α

ABSTRACT

Lipopolysaccharide (LPS), also known as endotoxin, is the primary trigger of sepsis, which is associated with high mortality in patients. No therapeutic agents are currently efficacious enough to protect patients from sepsis characterized by LPS-mediated tissue damage and organ failure. Previously, a phosvitin-derived peptide, Pt5, which consists of the C-terminal 55 residues of zebrafish phosvitin, has been shown to function as an antibacterial agent. In this study, we have generated six mutants by site-directed mutagenesis based on the sequence of Pt5, and found that one of the six mutants, Pt5e, showed the strongest bactericidal activities against *Escherichia coli* and *Staphylococcus aureus*. We then demonstrated that Pt5e was able to bind to LPS and lipoteichoic acid (LTA). More importantly, we showed that Pt5e significantly inhibited LPS-induced tumor-necrosis factor (TNF)-α and interleukin (IL)-1β release from murine RAW264.7 cells and considerably reduced serum TNF-α and IL-1β levels in mice. Additionally, Pt5e protected the liver from damage by LPS, and remarkably promoted the survival rate of the endotoxemia mice. Furthermore, Pt5e displayed no cytotoxicity to murine RAW264.7 macrophages and no hemolytic activity toward human red blood cells. These data together indicate that Pt5e is an endotoxin-neutralizing agent with a therapeutic potential in clinical treatment of LPS-induced sepsis.

1. Introduction

Sepsis is a serious disease which is characterized by a whole body inflammatory state, called a systemic inflammatory response syndrome (SIRS), that frequently leads to tissue damage and fatal multiple organ dysfunction syndrome (MODS). The primary trigger of sepsis is thought to be lipopolysaccharide (LPS) or endotoxin, a major constituent of the outer membranes of all Gram-negative bacteria, which is released from the cell wall of growing bacteria or when antibiotics or the complement system destroy bacteria. LPS is one of the highly conserved pathogen-associated molecular patterns (PAMPs), and upon entering the blood circulation, it interacts with the Toll-like receptor 4 (TLR4), which is prominently expressed on monocytes and macrophages, stimulating the host cells to produce a large amount of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, by activating several types of transcription factors including the nuclear factor (NF)-κB (Hack et al., 1997; Hernandez-Rodriguez et al., 2004; Zhang and Ghosh, 2000). This TLR-mediated innate immunity is responsible for the host defense against invading pathogenic microbes. However, an unbalanced overproduction of such cytokines may result in sepsis which ranks as the leading cause of mortality in intensive care units (Parrillo, 1993; Warren, 1997).

Sepsis is a common and serious clinical problem. The therapy of sepsis rests on antibiotics, surgical drainage of infected fluid collections, fluid replacement and appropriate support for organ dysfunction. However, no therapeutic agents to date are efficacious enough to protect patients from LPS-mediated tissue damage and organ failure (Russell et al., 2006). To block the recognition of LPS by TLR4, both anti-LPS antibody and TLR4 antagonistic peptides have been developed, yet their trials on patients have not been satisfactory in reducing mortality significantly (Bone et al., 1995; Leaver et al., 2007; Ziegler et al., 1991, 1982). Therefore, search for novel therapeutics that are able to halt the LPS stimulation on the host immunity remains urgent and pressing.

Recently, antimicrobial peptides (AMPs) have emerged as an ideal candidate for sepsis therapeutics because they do not only have a broad-spectrum activity against bacteria but also possess the potential to bind LPS and block LPS-stimulated cytokine release (Mookherjee and Hancock, 2007; Rosenfeld et al., 2006). For example, LL-37, the C-terminal part of cathelicidin and only AMP identified so far in humans, has been demonstrated to be effective in binding and thus neutralizing LPS and consequently has a considerable potential in the treatment of sepsis associated with bacterial infections (Parrillo, 1993; Pirofski and Casadevall, 2006;

* Corresponding author at: Room 205, Ke Xue Guan, Ocean University of China, Qingdao 266003, China. Tel.: +86 532 82032787.
E-mail address: sczhang@ouc.edu.cn (S. Zhang).

1357-2725/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biocel.2013.09.002

Vandamme et al., 2012; Wang et al., 2011). AMPs are crucial humoral components of the innate immunity of virtually all organisms, that are produced in large quantities at the site of infection and/or inflammation and act rapidly to eliminate bacteria (Ren et al., 2010). Our previous studies show that hen egg yolk protein phosvitin (Pv) has an LPS-neutralizing capacity (Ma et al., 2013). We also show that zebrafish Pv is not only directly bactericidal but also binds the PAMPs such as LPS, lipoteichoic acid (LTA) and peptidoglycan (PGN) (Wang et al., 2011). Moreover, Pv-derived peptide Pt5 (consisting of the C-terminal 55 residues of Pv) in zebrafish exhibits antimicrobial-immunomodulatory activities, capable of protecting fish against the pathogenic bacterium *Aeromonas hydrophila* via its antibacterial activity as well as preventing fish from the detrimental effects of an excessive inflammatory response via modulating immune functions (Ding et al., 2012). In an attempt to obtain a peptide with a therapeutic potential for treatment of sepsis, we have generated a series of new mutants by site-directed mutagenesis based on the sequence of Pt5, and found that one of the mutants, Pt5e, showed the strongest antibacterial activity. The aims of this study were thus to explore if Pt5e displays any LPS-neutralizing activity, and if so, to evaluate its application potential as an LPS-neutralizer in patients with sepsis.

2. Materials and methods

2.1. Site-directed mutation, expression and purification

The distribution of positively charged and hydrophobic residues have been suggested to be the major factors of Pt5 to mediate specific electrostatic surface and amphipathicity (Wang et al., 2011), accordingly 6 mutants with modified net charge and hydrophobicity were generated by a single or double mutagenesis, on the basis of Pt5 sequence (NH_2-SRMSKTATIIEPFRKFHKDRYLAHHSATKDTSSG-SAAASFEQMQKQNRFLGNDIP-COOH), using the specific primers (Application number: 201310152263.X; State Intellectual Property Office, PRC) and Takara MutanBEST kit (Takara, Dalian, China). The mutations were confirmed by DNA sequencing, and the mutated recombinant peptides were expressed in *Escherichia coli* and purified as described by Wang et al. (2011). Purified recombinant peptides were run on 16% SDS-PAGE gel, electroblotted onto PVDF membrane (Amersham) and immunostained using the mouse anti-His antibody (Tiangen, China) as described by Liu and Zhang (2009).

2.2. Preparation of bacteria

The Gram-negative bacteria *E. coli* and *Vibrio anguillarum* and the Gram-positive bacteria *Staphylococcus aureus*, *Bacillus subtilis* and *Micrococcus luteus* were incubated in Luria–Bertani (LB) medium at 37 °C for 16 h. They were harvested by centrifugation at 3000 × *g* at 4 °C for 15 min. The bacterial pellets were resuspended in 10 mM phosphate-buffered saline (PBS; pH 7.4) and used for the following experiments.

2.3. Antibacterial activity assay

Initially, microdilution assay was performed as described by Yang et al. (2006) as pilot experiments to test the antibacterial activity of the recombinant peptides using *E. coli* and *S. aureus* as target bacteria. The experiments revealed that Pt5e out of the six mutants showed the strongest antibacterial activity (see below), therefore it was selected and used for the following experiments. The antibacterial activities of Pt5e against the Gram-negative bacteria *E. coli* and *V. anguillarum* and the Gram-positive bacteria *S. aureus*, *B. subtilis* and *M. luteus* were assayed as described by Yang et al. (2006). Briefly, all five strains of the bacteria were cultured in LB medium to mid-logarithmic phase, and then collected by centrifugation at 6000 × *g* at room temperature for 10 min. After washing three times with PBS, they were suspended in fresh LB medium and adjusted to a density of 3×10^6 cells/ml. Totally, 510 μl of each bacterial suspensions was mixed with 90 μl of Pt5e solution (final concentrations from 0.1 to 20.0 μM). Aliquots of 200 μl of the mixtures were sampled, added to each well of a 96-well microplate and incubated at 37 °C for 8 h. In parallel, both recombinant Pt5 and synthetic LL-37 (purity > 99%; synthesized by Sangon Biotech, Shanghai, China) were similarly processed for positive controls, and PBS alone was used for negative control. Inhibition of bacterial growth in each well was determined at each time point by measuring absorbance at 630 nm with a Multiskan MK3 microplate reader (Thermo, Shanghai, China). The minimum inhibitory concentration (MIC) was defined as the lowest peptide concentration at which the growth of the respective bacterium was completely inhibited.

To test if heating affects antibacterial activity, Pt5e was heated at 50 and 100 °C, respectively, for 10 min, and centrifuged at 10,000 × *g* for 5 min. The supernatants were collected, electrophoresed on SDS-PAGE gel, and used for antibacterial activity assay as above.

2.4. Flow cytometry

E. coli and *S. aureus* were used as representative bacteria for flow cytometry. They were grown to mid-logarithmic phase in LB medium, and harvested by centrifugation at 6000 × *g* for 10 min. After washing three times with PBS, the bacterial pellets were suspended in PBS and adjusted to a density of 1×10^6 cells/ml. Pt5e was added to the bacterial suspension to give final concentrations of 0.5 and 1 μM, respectively. In parallel, LL-37 and BSA were used instead of Pt5e for positive and negative controls, individually. The mixtures were incubated at 37 °C for 1 h, and fixed with 10 μM propidium iodide (PI) solution under dark at 4 °C for 15 min. Bacterial cell staining by PI was examined using a FC500 MPL flow cytometer (Beckman). Data were analyzed using WinMDI v.2.9 software (Scripps Research Institute, San Diego, CA).

2.5. Transmission electron microscopy

Aliquots of 700 μl of *E. coli* and *S. aureus* suspensions containing 5×10^7 cells/ml were mixed with 300 μl of Pt5e in 10 mM PBS, giving final concentrations of 1 × MICs (see below), or with 300 μl of PBS alone as control. The mixtures were incubated at 25 °C for 1 h, fixed in 2.5% glutaraldehyde in 100 mM PBS (pH 7.4), and then dropped onto 24 mm × 24 mm cover glasses. The samples were post-fixed in 1% osmium tetroxide, dehydrated with graded ethanol, and dried by the critical point method. Observation was made under a JEOL JSM-840 transmission electron microscope.

2.6. Assay for binding of Pt5e to LPS and LTA

As Pt5e showed antibacterial activities against *E. coli* and *S. aureus* (see below), we thus tested if it could bind LPS and LTA, the major conserved components on the surfaces of Gram-negative and positive bacteria, respectively. The labeling of Pt5e, Pt5c (which has the same molecular size and the similar charge with Pt5e, but shows little antibacterial activity; see below) and BSA with digoxigenin (DIG; Roche, Germany) was performed according to the instruction of DIG protein labeling kit (Roche, Germany). Briefly, Pt5e, Pt5c and BSA (100 μg) were mixed with 16 μl of 20 mg/ml DIG-NHS solution dissolved in DMSO and incubated at 25 °C for 2 h. The free DIG-NHS was removed by gel filtration on a Sephadex G-25 column. Both LPS from *E. coli* and LTA from *S. aureus* (all from Sigma, USA) were individually dissolved in double distilled water, yielding a concentration of 40 μg/ml. A volume of 50 μl (containing 2 μg