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Comparing bacterial membrane interactions and antimicrobial activity of porcine lactoferricin-derived peptides

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ABSTRACT

Antibiotic treatment for microbial infections is under scrutiny due to increasing resistance to conventional antibiotics, warranting discovery of new classes of antibiotic agents. Antimicrobial peptides are part of the innate defense system found in nearly all organisms and possess bactericidal mechanisms that make it more difficult for bacteria to develop resistance. Porcine lactoferricin (LFP-20) is an antimicrobial peptide located in the N terminus of lactoferrin (LF). To develop novel cell-selective antimicrobial peptides with improved antimicrobial specificity compared with LFP-20, analogs LF2A LF-2, LF-4, and LF-6 were substituted with Ala, Ser, or Trp residues at different positions in the molecule. Analogs displayed a 2- to 16-fold higher antimicrobial activity than LFP-20, but were hemolytic at 64 μg/mL. Additionally, LFP-20, LF2A, LF-2, and LF-4 exhibited lower cytotoxicity against human peripheral blood mononuclear cells than LF-6 at concentrations of 25 to 100 μg/mL. To better understand the antibacterial mechanisms of LFP-20 and its analogs we examined their effect on the cytoplasmic membrane of *Escherichia coli*. The LFP-20 was not effective in depolarizing cytoplasmic membranes, whereas the other 3 analogs gradually dissipated the membrane potential of *E. coli*. Membrane potential increased with minimal inhibitory concentrations changes, demonstrating a correlation between bactericidal activity and membrane depolarization. Analogs were more efficient than LFP-20 in displacing lipopolysaccharide-bound dansyl-polymyxin B, which also rapidly increased 1-N-phenylnaphthylamine uptake and release of cytoplasmic β-galactosidase by increasing the permeability of the outer and inner membranes of *E. coli*. The 3 analogs caused an increased potential for calcein leakage from negatively charged lipid vesicles at high concentrations. Collectively, these results suggest that the first targets of LF-2, LF-4, and LF-6 in *E. coli* are cytoplasmic membranes. The 3 analogs exhibited lethal effects based on their abilities to disrupt membranes and permit transit of large intracellular components, such as calcein.
Key words: porcine lactoferricin, antimicrobial activity, mechanism, cytoplasmic membrane

INTRODUCTION

Antibiotics represent some of the major scientific and medical advances of the 20th century. The discovery and development of conventional antibiotics, which are primarily based on bacteria- or fungi-generated antimicrobial compounds, has led to dramatic improvements in the ability to treat infectious diseases and significant increases in animal production. Although antibiotic therapy remains the first choice for treating microbial infections in humans and animals, increasing rates of antibiotic resistance is a growing public health concern (Gordon et al., 2005; Sang and Blecha, 2008). This has driven the search for new antimicrobials that are broadly effective and less likely to induce antimicrobial resistance. Thus, one potentially useful but underdeveloped class of antibiotic agents is the antimicrobial peptides (**AMP**; Rathinakumar et al., 2009).

Antimicrobial peptides generally contain 12 to 100 AA residues, have a net positive charge, and have an amphipathic structure that facilitates interaction with negatively charged microbial membranes or other cellular targets (Yeaman et al., 2007; Sang and Blecha, 2008). Compared with conventional antibiotics, AMP often exert broad spectrum activity against microorganisms, including bacteria, fungi, parasites, enveloped viruses, and even some cancer cells. Additionally, conventional antibiotics generally target a metabolic enzyme and may selectively induce resistance in microorganisms, whereas AMP kill microbes primarily through membrane-targeting pore-forming mechanisms, which are inherently more difficult for microbes to circumvent by developing resistance (Hancock and Sahl, 2006; Sang and Blecha, 2008; Fan et al., 2011).

Porcine lactoferricin (**LFP-20**) is one of the 20 amino acid AMP identified in the N terminus of the lactoferrin (**LF**), a member of the porcine LF family. It

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exhibits moderate antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* through a mechanism directly leading to cell wall disruption and outer membrane breakdown (Chen et al., 2006a). A limitation of AMP for use in therapeutics is that many possess cytotoxic and hemolytic properties, whereas others have relatively weak antimicrobial activity. Therefore, several studies have focused on designing cell-selective peptides with strong antimicrobial activity but no toxicity against mammalian cells for use as antibiotic agents (Oren et al., 1997; Chen et al., 2005; Wang et al., 2009). In our previous study, we found that LFP-20 does not exhibit hemolysis and has very low cytotoxicity to eukaryotic cells (unpublished data). This study focuses on identifying key AA in the LFP-20 sequence and enhancing the activity of various LFP-20 peptide analogs using AA substitutions and activity assays. We also developed a plausible bactericidal mechanism by measuring depolarization of the cytoplasmic membrane potential of intact *E. coli* cells and their ability to cause leakage of a fluorescent dye from lipid vesicles to mimic bacterial membranes.

MATERIALS AND METHODS

Peptide Synthesis

Peptides were synthesized by standard solid-phase procedures with 9-fluorenyl- methoxycarbonyl using an Apex 396 peptide synthesizer (Aapptec, Louisville, KY). Ninety-five percent purity of synthetic peptides was achieved and verified using Agilent 1200 Series HPLC (Agilent Technologies, Santa Clara, CA) and a Thermo Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). A concentrated stock solution of each test compound was prepared in endotoxin-free water and stored at −80°C until use.

Bacterial Strains and Reagents

Escherichia coli ATCC25922, *E. coli* K88, *E. coli* AG1, *E. coli* JM109, *E. coli* DH 5α, *E. coli* UB1005, *E. coli* ML-35, *Staphylococcus epidermidis* C621, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosa* H103, *P. aeruginosa* PA14, *Salmonella choleraesuis* CMCC50020, and *Salmonella typhimurium* CMCC50013 were included in this study. *Escherichia coli* AG1, *E. coli* UB1005, *E. coli* ML-35, *Staph. epidermidis* C621, *P. aeruginosa* H103, and *P. aeruginosa* PA14 were donated by R. E. W. Hancock (Centre for Microbial Diseases and Immunity Research, The University of British Columbia, Vancouver, Canada). All strains were grown in Mueller Hinton broth (**MHB**; Difco Laboratories, Detroit, MI) at 37°C unless otherwise noted. The fluorescent dyes, $diSC_35$ and dansyl-polymyxin B, were purchased from Molecular Probes (Eugene, OR); O-nitrophenyl β-D-galactoside (**ONPG**), N-phenyl-1-naphthylamine (**NPN**), and calcein were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). Zwitterionic phospholipid, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (**POPC**); anionic phospholipid, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-(1-glycerol) (**POPG**), 1-oleoyl-2-hydroxy-sn-glycero-3-phospho-(1'-rac-glycerol), cardiolipin (**CL**), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (**PE**) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). All other reagents were of the highest grade commercially available.

Cell Isolation

Fresh human venous blood from healthy volunteers was collected in vacutainer collection tubes containing sodium heparin as an anticoagulant (BD Biosciences, Franklin Lakes, NJ) in accordance with Zhejiang University ethical approval and guidelines. The blood was diluted in an equal volume of complete RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 2 m*M* L-glutamine, and 1 m*M* sodium pyruvate (Invitrogen, Carlsbad, CA), and separated using centrifugation over a Ficoll-Paque Premium (GE Healthcare Bio-sciences Corp., Uppsala, Sweden) density gradient. Mononuclear cells and erythrocytes were collected and washed twice in RPMI 1640 medium. The number of peripheral blood mononuclear cells (**PBMC**) was determined using trypan blue exclusion. The PBMC were seeded into 96-well tissue culture plates (BD Biosciences) at 1×10^6 cells/mL at 37°C in 5% CO_2. All experiments using human PBMC or erythrocytes involved at least 4 biological replicates.

MIC Determination

Minimal inhibitory concentrations of the peptides were measured using a modified broth microdilution method in MHB (Wiegand et al., 2008). Briefly, cells were grown overnight at 37°C in MHB medium and were diluted in the same medium to give 1×10^6 cfu/mL. The assay was performed in sterile 96-well polypropylene microtiter plates (Costar, Cambridge, MA). Serial dilutions of the peptides at 10-fold of the desired final concentration were added to each well of the microtiter plates in a volume of 10 μL, followed by addition of 90 μL of diluted bacteria. Plates were incubated at 37°C for 18 h, and MIC were determined as the lowest peptide concentration at which no bacterial growth was observed.