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Mechanism of tachyplesin I injury to bacterial membranes and intracellular enzymes, determined by laser confocal scanning microscopy and flow cytometry

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ABSTRACT

Tachyplesin I is a 17 amino acid, cationic, antimicrobial peptide with a typical cyclic antiparallel β-sheet structure. Interactions of tachyplesin I with living bacteria are not well understood, although models have been used to elucidate how tachyplesin I permeabilizes membranes. There are several questions to be answered, such as (i) how does tachyplesin I kill bacteria after it penetrates the membrane and (ii) does bacterial death result from the inactivation of intracellular esterases as well as cell injury? In this study, the dynamic antibacterial processes of tachyplesin I and its interactions with *Escherichia coli* and *Staphylococcus aureus* were investigated using laser confocal scanning microscopy in combination with electron microscopy. The effects of tachyplesin I on *E. coli* cell membrane integrity, intracellular enzyme activity, and cell injury and death were investigated by flow cytometric analysis of cells following single- or double-staining with carboxyfluorescein diacetate or propidium iodide. The results of microscopy indicated that tachyplesin I kills bacteria by acting on the cell membrane and intracellular contents, with the cell membrane representing the primary target. Microscopy results also revealed that tachyplesin I uses different modes of action against *E. coli* and *S. aureus*. The results of flow cytometry showed that tachyplesin I caused *E. coli* cell death mainly by compromising cell membrane integrity and causing the inactivation of intracellular esterases. Flow cytometry also revealed dynamic changes in the different subpopulations of cells with increase in tachyplesin I concentrations. Bacteria exposed to 5 μg/mL of tachyplesin I did not die instantaneously; instead, they died gradually via a sublethal injury. However, upon exposure to 10–40 μg/mL of tachyplesin I, the bacteria died almost immediately. These results contribute to our understanding of the antibacterial mechanism employed by tachyplesin I.

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Introduction

Antimicrobial peptides (AMPs) have attracted much attention because of their potent and broad-spectrum activities, low cytotoxicity toward normal cells, low levels of acquired resistance in microbes and unique mechanism of action; thus, this mechanism has become an intensely studied topic. Among the studies on bactericidal mechanisms, the mechanism of action of AMPs on bacterial cell membranes has been examined most thoroughly, and several action models of AMPs on target cell membranes have been described (Epand and Vogel, 1999; Hale and Hancock, 2007). However, the antibacterial mechanisms of different AMPs vary (Han et al., 2011). For example, magainin-2 only acts on the cell wall, instead of penetrating the cell membrane (Park et al., 1998). Pexiganan, an analog of magainin, kills bacteria by compromising cell membrane integrity (Gottler and Ramamoorthy, 2009). Moreover, some evidence suggests that most AMPs act on intracellular targets in bacteria by inhibiting the synthesis of DNA, RNA, and protein, as well as enzyme activity, to kill bacteria (Brogden, 2005; Nguyen et al., 2011; Tang et al., 2009). PR-39 inhibits phagocyte NADPH oxidase activity by binding Src homology 3 domains of p47 phox (Shi et al., 1996), and histatins and pyrrhocoricin inhibit

Abbreviations: AMPs, antimicrobial peptides; cF, carboxyfluorescein; cFDA, carboxyfluorescein diacetate; FCM, flow cytometry; FITC, fluorescein isothiocyanate; LCSM, laser confocal scanning microscopy; TEM, transmission electron microscopy; SEM, scanning electron microscopy; MIC, minimum inhibitory concentration; PBS, phosphate-buffered saline; PI, propidium iodide.

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the activity of enzymes produced by bacteria, such as trypsin-like proteinase and heat shock proteins (Andreu and Rivas, 1998; Otvos et al., 2000).

In the present study, tachyplesin I, a cationic AMP originally isolated from the hemocytes of horseshoe crabs (Nakamura et al., 1988), exhibited potent and broad-spectrum activities against both Gram-positive and Gram-negative bacteria. Previous studies have shown that tachyplesin I can kill bacteria by permeabilizing the bacterial membrane and also by binding DNA and RNA, which inhibits the synthesis of macromolecules (Yonezawa et al., 1992; Hirakura et al., 2002; Xie et al., 2008). A previous report showed that tachyplesin I forms a toroidal pore composed of peptides and lipids, which is similar to the actions of magainin-2, MSI-78, and LL-37 (Imura et al., 2007). Taken together, these results suggest that tachyplesin I exploits multiple effects and synergistic antibacterial mechanisms. However, several questions still remain: (i) how is cell death accomplished after tachyplesin I disrupts the cell membrane or changes the membrane permeability, (ii) what is the primary, most important antibacterial target, and (iii) can tachyplesin I cause alterations in intracellular enzyme activity? To date, the mechanisms tachyplesin I employs in its dynamic bactericidal process and intracellular esterase activity and to cause cell death remain unclear.

Fluorescent techniques in combination with flow cytometry (FCM) have been used extensively to study the morphological and physiological characteristics of individual cells. Previous studies have utilized FCM to examine several aspects of microbiology: calcium ion concentrations and pH; bacterial growth and metabolism; bacterial viability (Braga et al., 2003; Wang et al., 2010); membrane potential; membrane integrity; enzymatic activity (Amor et al., 2002; Ananta et al., 2004; Herrero et al., 2006); analysis of the sublethal state of bacteria, which can be caused by various factors; and the physiological status of bacteria (Papadimitriou et al., 2006; Schenk et al., 2011). The results of these studies indicate that it would be appropriate to apply this methodology for determining the mechanism of action of tachyplesin I on *Escherichia coli*.

To explore the mechanism of action of tachyplesin I against bacteria, we investigated the effect of tachyplesin I on the dynamic antibacterial process, membrane integrity, intracellular enzyme activity, and cell injury or death. The results of our study showed that tachyplesin I kills bacteria by acting on the bacterial cell membrane, cytoplasm, and intracellular enzymes, and that the cell membrane is the critical target. Furthermore, tachyplesin I has distinct modes of action against *E. coli* and *Staphylococcus aureus*. We also determined the mechanism of cell injury or death by using increasing tachyplesin I concentrations. Taken together, our results aid in further elucidation of the mechanism of action of tachyplesin I against bacteria.

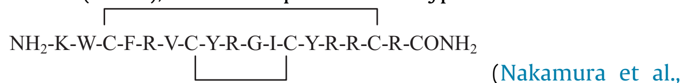
Materials and methods

Microorganisms, media, and growth conditions

E. coli ATCC25922 and *S. aureus* ATCC25923 were provided by the Microbial Culture Collection Center of Guangdong (GIMCC, China). Unless otherwise stated, the bacteria were cultured in nutrient broth (1% peptone, 0.3% beef extract, and 0.5% NaCl) and on nutrient agar plates. A portion of an overnight culture of each strain was transferred into new nutrient broth and cultivated at 37 °C with constant shaking at 180 rpm. Log-phase cultures were diluted to an appropriate concentration with new broth and used for antibacterial assays.

Antibacterial agent

The agent used in this study was the AMP tachyplesin I. Tachyplesin I and fluorescein isothiocyanate (FITC)-labeled tachyplesin I (>95% purity) were both synthesized by Shenzhen HYBIO (China), and the sequence of tachyplesin I was as follows:



(Nakamura et al., 1988). The peptide was solubilized in phosphate-buffered saline (PBS; pH 7.2) to yield a 10 mg/mL stock solution, which was filter-sterilized before use. Peptide solutions were prepared fresh on the day of the assay or stored at −20 °C for a short period.

Probes

Carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) were obtained from Sigma-Aldrich Co. (USA).

Killing assays

Cells from the mid-logarithmic phase were harvested and diluted to a final concentration of 10^6 cells/sample. The bacteria were exposed to a range of tachyplesin I concentrations and incubated at 37 °C for 60 min in *E. coli* and for 30 min in *S. aureus*, and appropriate dilution series of the samples were plated on nutrient agar plates. The samples were incubated at 37 °C for 20 h, and surviving cells were counted subsequently.

Laser confocal scanning microscopy observation

Cells from the mid-logarithmic phase were harvested, centrifuged at 1500 × *g* for 5 min, washed twice with 0.1 mol/L PBS (pH 7.2), and resuspended in the same buffer. The cells were immobilized on a sterile petri dish (BD, USA). Before the sterile petri dish was used, it was coated with 0.01% poly-L-lysine for 15 min; then, it was rinsed with 0.1 mol/L PBS and dried in the air. FITC-labeled tachyplesin was added to the petri dish containing 10^8 CFU/mL *E. coli* and *S. aureus*. Then, laser confocal scanning microscopy (LCSM; Leica TCS-SP5, Germany) was used to examine the interaction between FITC-labeled tachyplesin I and the cells. Fluorescent images were obtained with a 488 nm band-pass filter for excitation of FITC. Images were recorded using a screen-star film recorder (Leica Application Suite, Germany).

Scanning electron microscopy and transmission electron microscopy

E. coli and *S. aureus* cells from the mid-logarithmic phase were harvested after centrifugation; then, they were washed with 0.1 mol/L PBS (pH 7.2) and resuspended in the same buffer to produce a final cell density of approximately 1.5×10^8 CFU/mL. Tachyplesin I was added to 4 mL suspension to achieve the range of final concentrations required, and the mixtures were incubated at 37 °C for different times. After incubating cells for the appropriate times, the mixtures were harvested after centrifugation at 1500 × *g* at room temperature for 5 min. The cells were then washed twice with 0.1 mol/L PBS and centrifuged at 1500 × *g* for 5 min, and the supernatants were then removed. The samples were prepared for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) according to a previously published method (Dai et al., 2009).

FCM observation

This procedure (sample preparation and staining) was performed according to a previously reported method (Amor et al., 2002; Ananta et al., 2004).

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