Toxicon 58 (2011) 46–53

Contents lists available at ScienceDirect

Toxicon

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

Membrane-damaging activity of Taiwan cobra cardiotoxin 3 is responsible for its bactericidal activity

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ARTICLE INFO

Article history:
Received 7 March 2011
Received in revised form 22 April 2011
Accepted 26 April 2011
Available online 7 May 2011

Keywords:
Snake venom
Cardiotoxin
Bactericidal effect
Membrane-damaging activity

ABSTRACT

This study investigates the causal relationship between membrane-damaging activity and bactericidal activity of *Naja naja atra* (Taiwan cobra) cardiotoxin 3 (CTX3). CTX3 showed greater inhibitory activity for the growth of *Staphylococcus aureus* (Gram-positive bacteria) relative to that of *Escherichia coli* (Gram-negative bacteria). The CTX3 antibacterial activity is positively correlated with the increase in membrane permeability of bacterial cells. Morphological examination showed that CTX3 disrupted bacterial membrane integrity.CTX3 showed similar binding capability with lipopolysaccharide (LPS) and lipoteichoic acid (LTA), and destabilization of LPS layer and inhibition of LTA biosynthesis on cell wall increased the CTX3 bactericidal effect on *E. coli*. and *S. aureus*, respectively. Compared with that of *E. coli*, CTX3 notably permeabilized model membrane of *S. aureus*. CTX3 membrane-damaging activity was inhibited by LPS and LTA, while increasing the CTX3 concentration counteracted the inhibitory action of LPS and LTA. Oxidation of Met residues on loop II of CTX3 simultaneously reduced the membrane-permeabilizing activity and bactericidal effect of CTX3. Taken together, our data indicate that CTX3 bactericidal activity depends highly on its ability to induce membrane permeability.

1. Introduction

Cardiotoxins (CTXs), a group of major venom polypeptides of around 60 amino acid residues present abundantly in the elapid family of snakes, show pharmacological functions including hemolysis, cytotoxicity and depolarization of muscles (Dufton and Hider, 1991). All three-dimensional structures of CTXs adopt a three-fingered loop-folding topology dominated by β-sheet (Bilwes et al., 1994; Jayaraman et al., 2000), but differ in details including the extent of secondary structure and the positions of invariant side-chains. Wu and his colleagues proposed that CTXs may damage cells by their ability to interact with anionic lipids or negatively charged oligosaccharides on the cell membrane and subsequently form an oligomeric toxin complex (Patel et al., 1997; Sue et al., 2002; Forouhar et al., 2003; Wang et al., 2006).These studies imply that positively charged residues of CTXs should critically contribute to their binding with phospholipids and membrane-damaging effect.

Several lines of evidence show that snake venom proteins exhibited antimicrobial activities (San et al., 2010). Among some of the common antimicrobial components that have been isolated from snake venom are L-amino acid oxidase (LAAO) and phospholipase A_2 (PLA_2) (Stiles et al., 1991; Santamaria et al., 2005; Perumal Samy et al., 2007, 2008; Costa Torres et al., 2010; Lee et al., 2011). The LAAO antibacterial action appears to result from hydrogen peroxide generated by the oxidative action of the enzymes, as the effect is abolished in the presence of hydrogen peroxide scavengers such as catalase (Lee et al., 2011). Membrane-permeabilizing and damage mechanism are believed to be responsible for bactericidal effect of PLA_2 enzymes (Perumal

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0041-0101/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2011.04.021

Samy et al., 2008). Apart from enzymatic proteins, antimicrobial peptides including cathelicidins, vgf-1 and omwaprin have been isolated from the venom of *Bungarus fasciatus*, *Naja atra* and *Oxyuranus microlepodotus*, respectively (Xie et al., 2003; Nair et al., 2007; Wang et al., 2008). Although antimicrobial proteins and peptides may kill bacteria by macro-molecular biosynthesis inhibition and/or interacting with specific vital components inside the bacteria, most antimicrobial proteins and peptides are cationic in nature and exert their bactericidal effect by permeabilizing the bacterial membrane, thinning the membrane or destabilizing the membrane structure (Brogden, 2005). Treating infection is becoming more difficult as microorganisms become resistant to therapeutic agents. The growth inhibition on various types of bacteria by snake venom or isolated substances from these venoms is being studied for developing prototype of antibacterial agents (Stiles et al., 1991; Santamaria et al., 2005; Perumal Samy et al., 2007, 2008; Costa Torres et al., 2010; Lee et al., 2011; Xie et al., 2003; Nair et al., 2007; Wang et al., 2008). Given that CTXs are suggested to damage phospholipid bilayers via the formation of a membrane pore structure (Forouhar et al., 2003), it is likely that CTXs may potentially show bactericidal action. The antibacterial action of *Naja naja atra* CTX3 was investigated in this study to address that question.

2. Materials and methods

CTX3 was isolated from the venom of *Naja naja atra* (Taiwan cobra) according to the procedure described by Lin et al. (2002). Met residues of CTX3 were oxidized with chloramine-T according to the procedure described in Kao et al. (2009). Antisera against CTX3 were prepared in our laboratory (Chang et al., 1998), and CTX3 antibodies were further purified by CTX3-Sepharose column essentially according to the procedure for affinity purification of anticobrotoxin antibodies (Chang et al., 2003). Calcein, chloramine-T, rifampin, propidium iodide (PI), cardiolipin, egg yolk phosphatidylglycerol (EYPG), egg yolk phosphatidylethanolamine (EYPE), lipopolysaccharide (LPS) 0111:B4 from *Escherichia coli* and lipoteichoic acid (LTA) from *Staphylococcus aureus* were purchased from Sigma–Aldrich Inc., and 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) was obtained from Invitrogen. Peroxidase conjugated anti-rabbit IgG was obtained from Bio-Rad, and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was from Boehringer Mannheim GmbH. Unless otherwise specified, all other reagents were analytical grade.

2.1. Bacterial strains

E. coli (JM109) and *S. aureus* (ATCC 25923) were used in this study. *E. coli* were maintained on Luria-Bertani (LB) agar plate at 37 °C, and *S. aureus* were maintained on tryptic soy agar plate at 37 °C.

2.2. Antimicrobial assay

Antimicrobial assay was performed using the colony-forming unit (CFU) assay. *E. coli* (JM109) were grown in LB medium and *S. aureus* were grown in tryptic soy broth (TSB) from a single colony with agitation at 37 °C. Bacterial number was then evaluated by measuring the optical density (OD) at 550 nm ($OD_{550\ nm} \sim 0.3$ for *E. coli* and $OD_{550\ nm} \sim 0.5$ for *S. aureus* corresponding to approximately to 10^8 CFU/ml). The bacteria were pelleted, washed with PBS, and then concentrated to 2×10^8 CFU/ml in PBS.One hundred microliters of the bacteria suspension were incubated with CTX3 or Met-modified CTX3 at 37 °C for indicated time periods. Then the cells were diluted 10^4-fold in PBS and pour in solid LB agar for *E. coli* or tryptic soy agar for *S. aureus*. Bacterial colonies were counted after incubation overnight at 37 °C. Antimicrobial activity of CTX3 was calculated as (number of clonies after CTX3 treatment)/(number of clonies without CTX3 treatment) × 100%.

2.3. Bacterial membrane permeability assessment

Bacterial suspensions (10^8 CFU/ml) were treated with CTX3 or PBS for 1 h in a total volume of 100 μl under the same conditions used in antimicrobial assay. Subsequently, the bacterial cells (*E. coli* or *S. aureus*) were incubated with propidium iodide (PI) for 15 min in the dark at room temperature. Then the bacterial cells were washed with PBS, and resuspended in 1 ml PBS. A total of 10,000 bacterial cells were analyzed in each sample by Beckman Coulter Epics XL flow cytometer.

2.4. Bacterial cytoplasmic membrane depolarization assay

Membrane depolarization was monitored using the $DiSC_3(5)$ lipophilic dye. *S. aureus* cells were grown to mid-exponential phase ($OD_{550} = 0.5–0.6$). Cells were collected by centrifugation, washed with buffer (5 mM glucose, 5 mM HEPES, pH 7.2), and resuspended to an OD_{550} of 0.05 in 5 mM HEPES (pH 7.2) containing 5 mM glucose and 100 mM KCl. Subsequently, cells were incubated with 0.4 μM $DiSC_3(5)$ until $DiSC_3(5)$ uptake was maximal (as indicated by a stable reduction in fluorescence due to quenching of accumulated dye in the membrane interior). The fluorescence of the cytoplasmic membrane potential were continuously monitored at 30 °C by fluorescence emission at 670 nm and excitation at 650 nm. CTX3-induced membrane depolarization of *S. aureus* in a concentration-dependent manner, and maximal activity was noted when CTX3 concentration was higher than 9.5 μM (data not shown). Thus, 9.5 μM CTX3 was used to examine the effect of LTA on the interaction of CTX3 with membrane. After the addition of 9.5 μM CTX3, changes in fluorescence signal with time were recorded.

2.5. Competitive replacement of LPS-bound or LTA-bound rhodamine-labeled CTX3 (Rh-CTX3) by unlabeled CTX3

Rh-CTX3 were prepared according to the procedure described in Kao et al. (2010). Rh-CTX3 (1 μM) was titrated with increasing concentrations of LPS or LTA until maximal changes in fluorescence intensity of Rh-CTX3 was achieved. Then increasing concentrations of unlabeled CTX3 were added to compete for binding of Rh-CTX3 with LPS or LTA. Competitive binding was monitored at excitation