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Lysinibacillus sphaericus binary toxin induces apoptosis in susceptible *Culex quinquefasciatus* larvae

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ABSTRACT

During sporulation, a Gram-positive bacterium *Lysinibacillus sphaericus* (*Ls*) produces the mosquito larvicidal binary toxin composed of 2 subunits, BinA and BinB. Full toxicity against *Culex* and *Anopheles* mosquito larvae is achieved when both subunits are administered together at equimolar amounts. Although cellular responses to Bin toxin have been reported in previous studies, it remains essential to extensively examine the cytopathic effects *in vivo* to define the underlying mechanism of larval death. In this study, 4th instar *Culex quinquefasciatus* larvae fed with different doses of Bin toxin were analyzed both for ultrastructural as well as biochemical effects. Typical morphological changes consistent with apoptosis were observed in mosquito larvae exposed to Bin toxin, including mitochondrial swelling, chromatin condensation, cytoplasmic vacuolization and apoptotic cell formation. Bin toxin also induced the activation of caspase-9 and caspase-3 in larval midgut cells. Our current observations thus suggest that Bin toxin triggers apoptosis via an intrinsic or mitochondrial pathway *in vivo*, possibly contributing to larval death.

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1. Introduction

Highly toxic strains of *Lysinibacillus sphaericus* (*Ls*) such as 1593 M, 1691, 2362, 2297 and IAB59 produce the binary toxin (Bin), accumulated as crystalline protein during the sporulation stage (Baumann et al., 1991; Charles et al., 1996). The Bin toxin is highly active against different species of mosquito larvae such as *Culex* and *Anopheles* species, whereas it is rather inactive towards *Aedes* species (Berry et al., 1993). The high specificity and potency of Bin toxin has made possible its use as a biopesticide to control mosquito populations, and also to prevent transmission of mosquito-borne diseases such as West Nile fever, encephalitis, or malaria (Sweeney, 1999). Both BinA and BinB proteins are produced as inactive protoxins. Upon Bin toxin ingestion, protoxin inclusions are solubilized under alkaline gut conditions and activated by larval gut proteases to form active cores of about 40 kDa and 45 kDa, for BinA and BinB, respectively (Charles et al., 1996; Silva-Filha et al., 1999). Specificity is enabled by binding of the BinB subunit to receptors present on the midgut epithelial membranes of mosquito larvae (Charles et al., 1997; Nielsen-Leroux and Charles, 1992). In *Culex pipiens*, this receptor is GPI-anchored Cpm1 (*C. pipiens* maltase 1), whereas in *Anopheles gambiae*, the receptor is a GPI-anchored Agm3 (*A. gambiae* maltase 3) (Darboux et al., 2001; Opota et al., 2008; Silva-Filha et al., 1999). The role of BinA is still unclear, although it has been proposed to have a toxicity role (Charles et al., 1997; Nicolas et al., 1993). Recently, we have elucidated the crystal structure of BinB (Srisucharitpanit et al., 2014), which consists of lectin-like antiparallel β-rich domains, similar to the architecture found in aerolysin-type β-pore forming toxins (Parker et al., 1994). Consistent with this similarity, BinB has been reported to insert into artificial lipid membranes, to perturb membrane integrity and increase permeability (Boonserm et al., 2006; Schwartz et al., 2001; Singkhamanan et al., 2010), supporting a cytolytic role via pore formation.

However, the mechanism of action of Bin toxin at a cellular level is still poorly understood. Some reports have demonstrated cytopathological alterations of Bin-treated *Culex* larvae. These are shown by the presence of mitochondrial swelling, endoplasmic reticulum breakdown, enlargement of cytoplasmic vacuoles, and microvillar disruption. These alterations were observed in midgut cells, specifically those of the gastric caecum and posterior stomach (de Melo et al., 2008; Silva-Filha and Peixoto, 2003). Additionally, Bin-induced autophagic response was observed in mammalian cells (MDCK) expressing Cpm1 receptor (Opota et al.,

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2011). Nevertheless, the precise role of Bin toxin in activation of apoptotic pathways has not been established.

Apoptosis is induced through the activation of caspases, via two principal signaling pathways, the extrinsic and the intrinsic – or mitochondrial – pathways (Li and Yuan, 1999; Schulze-Osthoff et al., 1998). The extrinsic pathway involves the oligomerization of death receptors (CD95/Fas/Apo-1; TNF receptor-1) that leads to the activation of initiator caspase-8 (Ashkenazi and Dixit, 1998; Nunez et al., 1998; Schulze-Osthoff et al., 1998). The intrinsic pathway is a consequence of disruption of mitochondrial integrity, resulting in cytochrome c release, leading to caspase-9 activation. Both pathways converge activating downstream executioner caspases such as caspase-3, and caspase-6. The activation of the executioner caspases results in cleavage of cytoskeleton proteins, activation of nucleases, and subsequent DNA fragmentation (Desagher and Martinou, 2000; Green and Reed, 1998; Zimmermann et al., 2001).

Until now, no direct evidence has been reported of *in vivo* apoptosis after Bin intoxication in mosquito larvae. In the present study, we have investigated the mechanism of cell death in Bin toxin-treated *Culex quinquefasciatus* larvae using ultrastructural and biochemical assays. This study is the first to demonstrate caspase activation and apoptosis mediated by the mitochondrial pathway in susceptible mosquito larvae as a result of Bin intoxication.

2. Materials and methods

2.1. Mosquito larvae intoxication

Larvicidal toxicity tests were performed against 2nd-instar *C. quinquefasciatus* larvae as described previously (Promdonkoy et al., 2008). Before doing the experiment, the larvae were fasted at least for 3 h. Purified soluble BinA and BinB protoxins were prepared as reported earlier (Srisucharitpanit et al., 2012). The mixtures of BinA and BinB proteins at 1:1 (w/w) ratio were diluted in distilled water with 2-fold serial dilutions, ranging from 1 μg/ml to 0.25 ng/ml. Then 1 ml of each dilution was added into each well of 24-wells tissue culture plate containing 10 larvae in 1 ml distilled water. After 48 h incubation at room temperature, mortality was recorded. These assays were performed in duplicate at each protein concentration with at least three independent experiments before calculating LC_{50} and LC_{90} by GWbasic Probit analysis (Finney, 1971).

2.2. Transmission electron microscopy

The 4th-instar *C. quinquefasciatus* larvae were treated with mixtures of BinA and BinB proteins at concentration 20-fold higher than the LC_{90} dose (2 μg) for 6, 12 and 18 h, or without Bin toxin (negative control). Larvae from untreated and treated groups were dissected in 1% glutaraldehyde in Millonig's phosphate buffer on ice to discard the food particles inside tubular peritrophic membranes and malpighian tubules. Specimens were fixed with 4% glutaraldehyde at 4 °C for 2 h, and post-fixed with 4% osmium tetraoxide for 30 min, followed by being stained with 2% uranyl acetate for 20 min. Then, they were dehydrated in ethanol (70%, 80%, 90%, 95%, 100%) followed by propylene oxide treatment three times. The dehydrated specimens were incubated in mixtures of propylene oxide and Spurr's epoxy resin (volume ratio 1:1) at 37 °C for 30 min and followed by 100% Spurr's epoxy resin (Electron Microscopy Sciences; EMS) at 37 °C for 2 h. Following polymerization, they were incubated at 70 °C overnight. The ultra-thin sections of 50–70 nm thickness in the grids were stained with 4% uranyl acetate and Reynold's lead citrate. Finally, the sections of the gastric caeca, anterior stomach and posterior stomach were examined under the transmission electron microscope (Jeol-1230).

2.3. TUNEL assay

The TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay was used to detect DNA fragmentation, a hallmark of apoptosis. The 4th-instar *C. quinquefasciatus* larvae were treated with Bin toxin at the LC_{90} dose for 6 and 12 h before fixing in 4% paraformaldehyde. Paraffin-embedded sections of larval gut and immunohistochemical detection were prepared according to standard histological method. Tissue sections were pre-treated with 20 μg/ml proteinase K, and the endogenous peroxidase was blocked by incubating with 0.1% TritonX-100 and 3% H_2O_2. Sections were analyzed with In Situ Cell Death Detection Kit, POD (Roche Applied Science). The signal was developed by incubating with DAB (3,3′-diaminobenzidine) chromogenic substrate for 2 min and the reaction was stopped by rinsing with distilled water. The brown stained TUNEL positive cells were observed under a light microscope. The intensity of TUNEL staining in the region of interest (ROI) was quantified by using ImageJ analysis software. The corrected integrated density was calculated as: integrated density – (area of selected cell × mean gray value of background). Controls included the sections treated with DNaseI as a positive control and those incubated with a reaction mixture without terminal deoxynucleotidyl transferase (TdT) as a negative control.

2.4. Quantitation of caspase-3 and caspase-9 activities

Larvae were treated with Bin toxin at either the LC_{90} dose (100 ng) or at high doses (2 μg and 10 μg) for 6, 12 and 18 h. Following treatment, 50 larvae were lysed in the cell lysis buffer (Molecular Probes) for measuring caspase-3 activity while they were lysed in cell lysis buffer (Biovision) for measuring caspase-9 activity by four cycles of freeze-thawing. Cell debris was separated by centrifugation at 6000×g for 5 min at 4 °C. The supernatant fraction was collected and assayed for caspase activity. Protein concentration was estimated using a Bradford assay (Bio-Rad).

Caspase-3 activity was measured by using a fluorometric caspase-3 assay kit (Molecular Probes), whereas caspase-9 activity was measured by using a fluorometric caspase-9 assay kit (Biovision) following the manufacturer's instructions. The supernatants were incubated with Z-DEVD-R110 substrate used for caspase-3 at room temperature for 30 min, while LEHD-AFC substrate was used for caspase-9 by incubating at 37 °C for 1 h. In addition, the supernatants were incubated with Ac-DEVD-CHO, a caspase-3 inhibitor, and LEHD-FMK, a caspase-9 inhibitor, to confirm that the observed fluorescence signals were due to those caspase activities. Rhodamine 110 (R 110 λ_{ex}, λ_{em} of 496 nm and 520 nm), and 7-amino-4-trifluoromethyl-coumarin (AFC λ_{ex}, λ_{em} of 400 nm and 505 nm) were used as reference standards for caspase-3 and caspase-9, respectively. The fluorescence signal was quantified by using a DTX880 Multimode detector (Beckman Coulter, CA, USA) and caspase activity was calculated as nmole/min/μg protein. Three independent experiments were performed and analyzed by using student's unpaired *t*-test.

3. Results and discussion

3.1. Ultrastructural changes after larval intoxication

Prior to studying the ultrastructural changes of the susceptible midgut cells upon intoxication by Bin toxin, purified BinA and BinB proteins were tested for mosquito-larvicidal activity against *C.*

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