Nitric oxide participates in the toxicity of *Bacillus thuringiensis* Cry1Ab toxin to kill *Manduca sexta* larvae

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ABSTRACT

Nitric oxide (NO) produced by the nitric oxide synthase (NOS) enzyme is a reactive oxygen molecule widely considered as important participant in the immune system of different organisms to confront microbial infections. In insects the NO molecule has also been implicated in immune response against microbial pathogens. *Bacillus thuringiensis* (Bt) is an insect-pathogenic bacterium that produces insecticidal proteins such as Cry toxins. These proteins kill insects because they form pores in the larval-midgut cells. Here we show that intoxication of *Manduca sexta* larvae with Cry1Ab activates expression of NOS with a corresponding increase in NO. This effect is not observed with a non-toxic mutant toxin Cry1Ab-E129K that is affected in pore formation. The increased production of NO triggered by intoxication with LC_{50} dose of Cry1Ab toxin is not associated with higher expression of antimicrobial peptides. NO participates in Cry1Ab toxicity since inhibition of NOS by selective L-NAME inhibitor prevented NO production and resulted in reduced mortality of the larvae. The fact that mortality was not completely abolished by L-NAME indicates that other processes participate in toxin action and induction of NO production upon Cry1Ab toxin administration accounts only for a part of the toxicity of this protein to *M. sexta* larvae.

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Introduction

The reactive oxygen molecules participate in the immune system defense of different organisms against microbial infections [15,20]. For example, in mammalian hosts, nitric oxide (NO) and superoxide anion (O_2^-) have been identified as intermediates in the cell immunity response to microbial pathogens [15]. NO is a diffusible, transient and highly reactive molecule that can induce different effects at low concentrations ranging from pico- to micromolar [3,15]. This compound may affect the cardiovascular, nervous and immunological systems, playing an important role as a defense mechanism against virus, bacteria and parasites infections in both vertebrates and invertebrates [15,20].

NO molecule is produced by the nitric oxide synthase (NOS) enzyme, during oxidation of L-arginine to L-citrulline [3]. In mammalian organisms, NO is produced by two different NOS enzymes, either constitutive or inducible (cNOS or iNOS). cNOS activity is part of the basal metabolism of neurons (nNOS) or endothelial (eNOS) cells [3,12]. Intracellular calcium levels modulate the activity of cNOS and eNOS. In contrast, iNOS is absent in resting cells but its expression can be induced as an immune response in many cells and tissues during infectious diseases [3,15]. iNOS can produce 100–1000 fold higher concentrations of NO than cNOS [3]. High concentrations of NO are toxic to many pathogens but could also be detrimental to the host or even trigger host cell death [3,12].

In invertebrates a NOS activity that synthesize low concentrations of NO was found in the brain of several insects, participating in the processing of chemosensory and visual information and in long-term memory [16]. Inducible synthesis of NO in insects was also reported and shown to participate in immune responses [7,20]. However, it is not clear whether two different enzymes exist and what are the cellular signals involved in activation of inducible NOS synthesis in invertebrates. In anophelines mosquitoes the

induced expression of NOS was observed after infection with different microorganism such as the yeast *Saccharomyces cerevisiae*, the Gram-positive bacteria *Micrococcus luteus* and ookinetes of *Plasmodium* parasites [8,14]. The role of NO in insect immune response was demonstrated by the fact that NOS expression was decreased if insect-midguts were treated with a mixture of antibiotics and antimycotics [8]. In relation to the immune response induced by NO, it was reported that the increased levels of NO in *Anopheles albimanus* mosquito after infection with *Plasmodium berghei* correlated with a higher expression of antimicrobial peptides such as attacin, cecropin and gambicin [9]. Similarly, increase of NO production in *Drosophila* larvae after infections with Gram-negative bacteria induced the synthesis of anti-microbial peptides [7].

The innate immune mechanisms in insects against microbial pathogens could be activated by microbial surface molecules such as peptidoglycans and lipopolysaccharides, resulting in activation of different responses such as increased NO and superoxide anion, phagocytosis, encapsulation, melanization and synthesis of antimicrobial peptides [13]. NOS (accession number (AN): AF062749) is induced in the midgut of *Manduca sexta* larvae fed with *Photorhabdus luminescens*. If the expression of NOS is knocked-down the survival of orally infected insects is affected and a significant increase in the number of bacteria crossing into the haemolymph was observed [6]. In *Bombyx mori* NOS is induced in the fat body by lipopolysaccharides [11]. The NOS enzyme from *M. sexta* (AN: AF062749) has 85% identity to NOS enzyme from *B. mori* (AN: NM_001043498), 55% identity with the NOS from *D. melanogaster* (AN: AAF25682) and 56% with the NOS from *Aedes aegypti* (AN: XM_001660278). It was proposed that NOS is inducible in *M. sexta* and *B. mori* and that it may play diverse physiological roles in different tissues [6,11]. However, little is known about the immune responses to other bacterial factors such as bacterial pore-forming toxins that would also affect insect viability. In this work we analyzed the activation of inducible NOS (AN: AF062749) [6] and NO production in *M. sexta* larvae after intoxication with insecticidal toxin Cry1Ab produced by the bacterium *Bacillus thuringiensis* (Bt). Bt is an insect pathogen that produces different types of insecticidal proteins, such as Cry and Cyt toxins, which are pore-forming toxins [17]. The Cry toxins are highly specific against some insects and are biodegradable, so they are considered an environmental-compatible insecticide. These toxins kill insects because they are capable to specifically bind certain proteins found in the insect gut and induce the formation of pores in the apical membrane of these cells. As a consequence of pore formation, the midgut tissue is damaged resulting in larval death [17]. We found that intoxication of *M. sexta* larvae with Cry1Ab activates expression of NOS with a corresponding increase in NO. Our data indicate that this effect is associated with the pore-forming activity of the toxin. Also that induction of NO production by Cry1Ab toxin is toxic to *M. sexta* suggesting that NO is participating as a part of the death response induced by Cry1Ab in *M. sexta* larvae.

Materials and methods

Cry1Ab toxin purification

Bt strains expressing Cry1Ab or Cry1Ab-E127K [21] were grown for 3 days at 30 °C in nutrient broth sporulation medium supplemented with erythromycin at $10 \mu g \, ml^{-1}$. After complete sporulation, the harvested products were washed twice in 300 mM NaCl, 10 mM EDTA and crystal inclusions were purified using discontinuous sucrose gradients [23]. Protoxins were produced by solubilization of crystal inclusions in alkaline buffer, composed of 50 mM Na_2CO_3, 0.2% β-mercaptoethanol, and pH 10.5 for 2 h. The soluble protoxins were recovered after centrifugation (20 min at

16,873 × *g*). Protein concentration was determined by the method of Bradford, using bovine serum albumin as standard.

Toxicity assays against M. sexta larvae

Bioassays were performed with *M. sexta* second instar larvae using different doses of protoxin solutions that were poured on the diet surface in a 24 well polystyrene plates and allowed to dry. Twenty-four *M. sexta* larvae per plate and one plate per dose were analyzed in triplicate (five different doses were assayed, corresponding to a total of 360 larvae per bioassay). Mortality was monitored after 7 days and the 50% lethal concentration (LC_{50}) was analyzed with Probit LeOra software. To analyze the mortality of *M. sexta* larvae when fed with other compounds, 24 second instar larvae were fed with diet contaminated with $26 \mu g/cm^2$ sodium nitroprusside (SNP) or 2 mg/ml L-NAME incorporated into the diet, with or without addition of the Cry1Ab toxin at a dose corresponding to the LC_{50} value in the surface of the diet. Mortality in these assays was recorded after 7 days. These experiments were performed in triplicate and significance of the data was analyzed by chi-squared test.

Determination of NO production in M. sexta larvae after feeding with Cry1Ab, L-NAME, sodium nitroprusside and a mixture of Cry1Ab plus L-NAME

Twenty-four *M. sexta* larvae in the second instar were fed with diet contaminated with Cry1Ab, with SNP, with L-NAME, or with L-NAME plus Cry1Ab toxin incorporated into the surface of the diet. After 24 h incubation time the larvae were washed with 70% ethanol followed by one washing step with PBS. The haemolymph was obtained after cutting the prominent horn on the rear of the larvae and collecting it with a micropipette. Midguts were dissected also from 24 larvae subjected to the same treatments, the food bolus was removed and the midgut tissue was suspended in $200 \mu l$ PBS. All samples were stored immediately at −70 °C until analyzed. NO concentration in the samples was determined by quantifying nitrites and nitrates (NO_2^-/NO_3^-) using the Griess reaction [5]. The midgut tissue samples in PBS were disrupted with an automatic homogenizer rotor at 4 °C. Cellular debris were discarded by centrifugation at 15,000 × *g* for 10 min at 4 °C. The concentration of protein in the midgut supernatant and haemolymph was determined by the method of Bradford, using bovine serum albumin as standard. One hundred μg of protein from midgut supernatant or haemolymph in PBS buffer (30 μl total volume) of each sample were used to perform the Griess reaction using the nitrate/nitrite colorimetric assay kit (Sigma-Aldrich). The nitrates were reduced to nitrites by treatment with nitrate reductase and NADPH according to the manufacturer's instructions and nitrite concentration was determined by Griess reaction using a standard curve of nitrates from a solution of $NaNO_3$ as specified in the manufacturer's instructions. After the addition of the Griess reagents, the samples were immediately centrifuged at 15,000 × *g* for 5 min to eliminate precipitated proteins and the supernatant was measured at 540 nm after 5 min of incubation at room temperature. The concentration of nitrites was adjusted to the total protein in the samples. These experiments were performed in triplicate and significance of the data was analyzed by ANOVA and Tukey's tests.

Reverse transcription-PCR and quantitative real time-PCR

For the reverse transcription-PCR (RT-PCR) total RNA was isolated from midgut tissue of 24 larvae using the TRIzol Reagent (Invitrogen). Two μg of total mRNA and 500 μg of oligo(dT25)VN were used to synthesize cDNA with 1 μl (200 Units/μl) of Moloney Murine Virus Retro transcriptase (MoMLVRT) (Invitrogen, Carlsbad,