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Eicosanoids up-regulate production of reactive oxygen species by NADPH-dependent oxidase in *Spodoptera exigua* phagocytic hemocytes

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ABSTRACT

Eicosanoids mediate cellular immune responses in insects, including phagocytosis of invading microbes. Phagocytosis entails two major steps, the internalization of microbes and the subsequent killing of them via formation of reactive oxygen species (ROS). Here, we posed the hypothesis that eicosanoids mediate ROS production by activating NADPH-dependent oxidase (NOX) and tested the idea in the model insect, *Spodoptera exigua*. A NOX gene (we named *SeNOX4*) was identified and cloned, yielding a full open reading frame encoding 547 amino acid residues with a predicted molecular weight of 63,410 Da and an isoelectric point at 9.28. A transmembrane domain and a large intracellular domain containing NADPH and FAD-binding sites were predicted. Phylogenetic analysis indicated *SeNOX4* clusters with other *NOX4* genes. *SeNOX4* was expressed in all life stages except eggs, and exclusively in hemocytes. Bacterial challenge and, separately, arachidonic acid (AA, a precursor of eicosanoid biosynthesis) injection increased its expression. The internalization step was assessed by counting hemocytes engulfing fluorescence-labeled bacteria. The phagocytic behavior was inhibited by dsRNA suppression of *SeNOX4* expression and, separately by dexamethasone (DEX, a specific inhibitor of eicosanoid biosynthesis) treatments. However, injecting AA to ds*SeNOX4*-treated larvae did not rescue the phagocytic activity. Hemocytic ROS production increased following bacterial challenge, which was sharply reduced in ds*SeNOX4*-treated, and separately, in DEX-treated larvae. AA partially reversed the suppressed ROS production in ds*SeNOX4*-treated larvae. Treating larvae with either the ROS-suppressing ds*SeNOX4* construct or DEX rendered experimental larvae unable to inhibit bacterial proliferation in their hemocoels. We infer that eicosanoids mediate ROS production during phagocytosis by inducing expression of *SeNOX4*.

1. Introduction

Insect innate immunity is assorted into cellular and humoral defense responses to infection, invasion and wounding (Beckage, 2008). Microbial infections are detected by a surveillance system including pattern recognition receptors that recognize pathogen-associated molecular patterns and stimulate specific immune effectors (Lemaitre and Hoffmann, 2007). The recognition signal propagates to immune tissues, hemocytes and fat body by biochemical mediators including cytokines, biogenic monoamines, and eicosanoids (Gillespie et al., 1997). These mediators lead to cellular immune reactions, including phagocytosis, nodule formation and encapsulation (Stanley and Kim, 2014). Humoral responses include biosynthesis of anti-microbial peptides (AMPs), which appear in hemolymph of challenged insects 6–12 h post-infection (PI), and are thought to clear remaining microbes from hemolymph circulation (Haine et al., 2008).

Eicosanoids are a group of the oxygenated C20 polyunsaturated fatty acids. They are synthesized mostly from arachidonic acid (AA), which is hydrolyzed from cellular phospholipids (PLs) by the catalytic activity of phospholipase A_2 (PLA_2) (Dennis, 1994). They play roles in mediating both cellular and humoral immune responses in insects (Stanley and Kim, 2014), including phagocytosis, microaggregation, nodulation and hemocyte spreading. Eicosanoids mediate AMP production in functional crosstalk with Toll/IMD pathways (Yajima et al., 2003; Shrestha and Kim, 2010). Nodulation is the predominant cellular immune reaction to infection, responsible for clearing most bacteria, fungal spores and some viruses from hemolymph circulation during the first two hours PI (Dunn and Drake, 1983; Miller et al., 1994; Durmuş et al., 2008). In response to relatively large pathogens, such as endoparasitoid eggs, eicosanoids mediate hemocytic encapsulation (Carton et al., 2002). Both nodulation and encapsulation entail a melanizing step,

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in which a final layer of hemocytes are darkened to form melanotic compartments. In this step, quinones are oxidized by phenoloxidase (PO). PO is activated in hemolymph after the zymogen, prophenoloxidase (PPO), is released from a class of hemocytes called oenocytoids in lepidopteran species. The release of PPO from oenocytoids is also mediated by eicosanoids (Shrestha and Kim, 2008).

Hemocyte phagocytosis clears small pathogens from hemolymph circulation. It consists of two steps, engulfment of the microbes into the cells and subsequently killing the pathogens (Lavine and Strand, 2002). Engulfment of pathogens requires cytoskeletal rearrangement to change the cell structure (Metheniti et al., 2001). The intracellular killing mechanism is based on forming reactive oxygen species (ROS) as seen in human neutrophils and invertebrate hemocytes (Conte and Ottaviani, 1995; Bergin et al., 2005; Nappi and Christensen, 2005). Phagocytosis is mediated by eicosanoids in several insect species, including the greater wax moth, *Galleria mellonella* (Mandato et al., 1997) and the beet armyworm, *Spodoptera exigua* (Shrestha and Kim, 2007). However, it is not clear which phagocytic step depends on eicosanoid signaling.

NADPH oxidase (NOX) complex mediates various oxidation reactions against xenobiotic compounds or organisms for defense. In human neutrophils, the NOX system is required for the ROS generation to kill microbial pathogens after phagocytosis (Henderson and Chappell, 1996). Malfunctioning of this oxidase system in neutrophils results in a serious immune disease, chronic granulomatous disease, and patients exhibit high susceptibility to microbial infection (Goldblatt and Thrasher, 2000). NOX is a multicomponent system and works on the plasma membrane (Segal et al., 2012). The main enzyme is called $gp91^{phox}$ (phox stands for phagocytic oxidase) and forms a heterodimer with $p22^{phox}$ to produce superoxide (Wallach and Segal, 1996). Various variants of gp91 have been identified in different taxa: NOX1–5 (Lambeth et al., 2000; Cheng et al., 2001), p138Tox in thyroid, renox in the kidney, and rbohA in plants (Groom et al., 1996). However, to be functional, this heterodimer (gp91 + p22) recruits cytosolic activating factors, such as $p67^{phox}$, $p47^{phox}$, $p40^{phox}$, and Rac2 (Curnutte et al., 1987; Sumimoto et al., 1996). Like human neutrophils, insect hemocytes appear to have a similar NOX system (Bergin et al., 2005). In addition, the production of ROS has been detected in hemocytes of *Drosophila melanogaster* and *G. mellonella* (Slepneva et al., 1999; Nappi and Christensen, 2005).

S. exigua is a model insect used to investigate the roles of eicosanoids in insects among other research channels. The insect is the first known species, in which bacterial pathogens target eicosanoid biosynthesis to suppress host immunity (Park and Kim, 2000). Eicosanoid-mediated cellular and humoral immune responses have been detailed in this species (Shrestha and Kim, 2009). A prostaglandin (PG) receptor and enzymes responsible for PG biosynthesis have been reported for *S. exigua* (Shrestha et al., 2010; Park et al., 2014). The next step in our investigation is to clarify the role of eicosanoids in hemocyte phagocytosis. This motivated our hypothesis that NOX operates in *S. exigua* phagocytosis. In the paper we report on the outcomes of molecular and physiological experiments designed to test our hypothesis.

2. Materials and methods

2.1. Insect and bacterial culture

Beet armyworm larvae were collected from a field population infesting welsh onion in Andong, Korea. The larvae were reared on an artificial diet (Goh et al., 1990) at 25 °C, a 16L:8D photoperiod, and RH 60 ± 5%. The last instar (=fifth instar) was used for immune assessment. Adults were fed 10% sucrose solution. *Escherichia coli* Top10, which was transformed with pCR2.1 vector (Invitrogen, Carlsbad, CA, USA), was cultured at 37 °C for 24 h with 250 rpm in ampicillin-supplemented Luria–Bertani medium (Difco, Sparks, MD, USA).

2.2. In silico gene sequence analysis

A cDNA database was generated from the midgut of *S. exigua* larvae with 454 FLX pyrosequencing and provided a partial sequence of a NADPH oxidase 4 gene, *SeNOX4*. To sequence a full open reading frame (ORF) of *SeNOX4*, partial *SeNOX4* was used as a probe to search the remaining ORF sequence from the deposited-transcriptome data of *S. exigua* using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The matched sequences were put together to get a full ORF by the ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) at the NCBI website. The translated protein sequence was obtained by an ExPASy translation tool (http://web.expasy.org/translate/), and the calculated isoelectric point (pI) and molecular weight (MW) were predicted with an ExPASy proteomics tool Compute pI/MW (http://web.expasy.org/compute_pi/) from the Swiss Institute of Bioinformatics. The N-terminal signal peptide was determined using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). The transmembrane domain and membrane topology was predicted with SMART (http://www.smart.embl-heidelberg.de/smart/). Multiple amino acid sequence alignments were analyzed using ClustalW program (q). Phylogenetic tree was constructed with Neighbor-Joining method (Saitou and Nei, 1987) using MEGA 6 (Tamura et al., 2013).

2.3. Expression profiles of SeNOX4

Total RNA was extracted from whole body of all developmental stages (≈50 eggs, 10 first and second instar larvae, 5 third instar larvae, 3 fourth instar larvae, 2 fifth instar larvae, 1 pupa and 1 adult) and from selected tissues isolated from the fifth instar larvae using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction. The extracted RNAs were digested with RNase-free DNase (Bioneer, Seoul, Korea). The resulting RNAs did not possess genomic DNA, documented by absence of RL32 (a house-keeping gene) product when PCR was performed with the RNA extract as template using RL32 gene-specific primers (Table 1). One microgram of each total RNA was incubated at 70 °C for 3 min and then used for the synthesis of cDNA using RT-mix kit (Intron, Seoul, Korea). The synthesized single-stranded cDNA was used as a template for PCR amplification with gene-specific primer pairs (Table 1) for *SeNOX4* with 38 cycles under the following conditions: 20 s at 94 °C for denaturation, 30 s at 54 °C for annealing, and 30 s at 68 °C for extension.

2.4. Silencing SeNOX4 expression by RNA interference (RNAi)

Template DNA was amplified with primers including a T7 RNA polymerase promoter sequence at the 5′ end of gene-specific *SeNOX4* primers (Table 1). The PCR product was used to prepare double-stranded RNA (dsRNA) using MEGA Script RNAi kit according to the manufacturer's instruction (Ambion, Austin, TX, USA). The dsRNAs were synthesized at 37 °C for 4 h and then left at 70 °C for 5 min to inactivate T7 RNA polymerase. For RNAi, the prepared dsRNA was mixed with Metafectene PRO transfection reagent (Biontex, Plannegg, Germany) in 1:1 volume ratio and incubated for 30 min at 25 °C to form liposomes. For dsRNA injection into larval hemocoel, 4 μL of dsRNA liposome solution (dsNOX4, 250 ng/larva) was injected into each fourth instar larva with a Hamilton syringe equipped with a 26 gauge needle

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