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Antiviral, anti-parasitic, and cytotoxic effects of 5,6-dihydroxyindole (DHI), a reactive compound generated by phenoloxidase during insect immune response

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ABSTRACT

Phenoloxidase (PO) and its activation system are implicated in several defense responses of insects. Upon wounding or infection, inactive prophenoloxidase (proPO) is converted to active PO through a cascade of serine proteases and their homologs. PO generates reactive compounds such as 5,6-dihydroxyindole (DHI), which have a broad-spectrum antibacterial and antifungal activity. Here we report that DHI and its spontaneous oxidation products are also active against viruses and parasitic wasps. Preincubation of a baculovirus stock with 1.25 mM DHI for 3 h near fully disabled recombinant protein production. The LC_{50} for lambda bacteriophage and eggs of the wasp *Microplitis demolitor* were 5.6 ± 2.2 and 111.0 ± 1.6 μM, respectively. The toxicity of DHI and related compounds also extended to cells derived from insects that serve as hosts for several of the aforementioned pathogens. Pretreatment of *Sf*9 cells with 1.0 mM DHI for 4 h resulted in 97% mortality, and LC_{50} values of 20.3 ± 1.2 μM in buffer and 131.8 ± 1.1 μM in a culture medium. Symptoms of DHI toxicity in *Sf*9 cells included DNA polymerization, protein crosslinking, and lysis. Taken together, these data showed that proPO activation and DHI production is strongly toxic against various pathogens but can also damage host tissues and cells if not properly controlled.

1. Introduction

Phenoloxidase (PO) and its activation system is a conserved component of the immune system of insects and other arthropods (Gillespie et al., 1997; Cerenius et al., 2008; Ragan et al., 2009). Active phenoloxidase (PO), which has a broad substrate specificity on monophenols and diphenols, catalyzes the formation of quinones which spontaneously cyclize and oxidize to yield other reactive intermediates (Sugumaran, 2002; Nappi and Christensen, 2005). Among them, semiquinones may produce free radicals while quinones polymerize to form eumelanin. Due to the high reactivity and assumed toxicity of quinolic compounds, protease-mediated proPO activation has always been considered as a mechanism for pathogen immobilization and killing.

Do proPO activation and PO-generated compounds play a role in insect immune defense? Several lines of evidence suggest yes. In response to bacteria, for example, our previous study shows that PO-catalyzed oxidation of dopamine generates reactive compounds that immobilize, aggregate, and kill *Escherichia coli* and *Bacillus subtilis* (Zhao et al., 2007). Against viruses, Trudeau et al. (2001) reported that *Helicoverpa zea*, a semipermissive host of *Autographa californica* multi-capsid nucleopolyherdrovirus (AcMNPV), melanizes infection foci, whereas this response was not observed in the fully permissive host *Heliothis virescens* due to AcMNPV reducing proPO levels in the hemolymph (Li et al., 2008). Shelby and Popham (2006) elaborated on these results by showing that PO inhibitors abolish the virucidal activity of *H. virescens* plasma against a baculovirus, while Clarke and Clem (2002) report that hemocytes also affect spreading of AcMNPV in *Trichoplusia ni* and *Spodoptera frugiperda*. Melanization and other immune mechanisms also play essential roles in resistance against endoparasitoids (Beckage, 1998; Schmidt et al., 2001). Oxidation of eumelanin precursors (*e.g.* dopamine and DHI) affects parasite survival and growth in *Drosophila melanogaster* (Kohler et al., 2007), while several parasitoids suppress melanization and other host defense responses to prevent the immune system from killing offspring (Nappi et al., 2009). For example, a venom protein from the parasitic wasp *Cotesia rubecula* encodes a clip-domain serine protease homolog that blocks melanization of host hemolymph by likely interfering with proPO activation (Zhang et al., 2004). The bracovirus (MdBV) carried by the wasp *Microplitis demolitor* encodes a 26 kDa protein named Egf1.0 that suppresses melanization by

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inhibiting proPO-activating proteases (PAPs) (Beck and Strand, 2007; Lu et al., 2008). A related protein, encoded by MdBV Egf1.5, also suppresses melanization (Lu et al., 2010), while functional assays show that suppresssion of the host proPO activation system by Egf proteins greatly enhances the survival of both *M. demolitor* and MdBV (Beck and Strand, 2007).

These results collectively suggest a critical role for PO-mediated melanization in host defense, yet it remains unclear which PO-generated compounds are responsible for killing pathogens and parasites (Kanost and Gorman, 2008). Prior studies with vertebrates indicate that the melanin precursors 5,6-dihydroxyindole (DHI) and DHI carboxylic acid are cytotoxic to human cells (Pawelek and Lerner, 1978; Urabe et al., 1994), while our own data indicate that DHI kills selected bacteria and fungi (Zhao et al., 2007). In this study, we extend our survey of DHI antibiotic activity to two kinds of viruses, a parasitic wasp, and an insect cell line. Our results provide new insights on the effects of DHI and its oxidation products against different targets while also providing evidence that improper regulation of the PO cascade can cause serious damage to hosts.

2. Materials and methods

2.1. Effect of DHI on a baculovirus

A recombinant stock of *A. californica* nucleopolydrosis virus (AcNPV) (30 μl, 1–2 × 10^8 pfu/ml) expressing *Schizaphis graminum* acetylcholinesterase-1 (AChE1) (Zhao et al., 2010) was treated with 50 μl, 1.25 mM DHI in PB (50 mM sodium phosphate, pH 6.5) or PB alone at room temperature for 3 h. The treated and control samples (80 μl) were separately added to *S. frugiperda Sf*9 cells (10^5 cells/ml, 2.0 ml) in fresh Sf900–III serum-free medium (Life Technologies, Inc) in a 6-well plate. After incubation at 27 °C for 72 h, the AChE activity in the conditioned culture medium (20 μl) was measured in triplicate using the modified Ellman method. *Sf*9 cells infected with DHI- or buffer-treated virus were examined by fluorescence microscopy using a monoclonal antibody against a hexahistidine tag at the carboxyl-terminus of *S. graminum* AChE1. The cells were collected, added to a microscope slide, fixed in cold methanol for 5 min, and permeabilized with cold acetone for 5 min. After washing with ethanol once and phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) three times, 1:1000 diluted anti-$(His)_5$ antibody (Qiagen) was added to the treated cells and incubated at 37 °C for 1 h. Following washing with PBS three times, 1:1000 fluorescein-labeled goat-anti-mouse secondary antibody (Sigma–Aldrich) was added to the slide. After incubation and washing, the cells were observed under a fluorescence microscope, Olympus BX-51.

2.2. Effect of DHI on bacteriophage lambda

Aliquots (10 μl) of 0, 0.2, 2, 20, 200, and 2000 μM DHI in sterile SM buffer (0.0001% gelatin, 0.1 M NaCl, 7 mM $MgSO_4$, 50 mM Tris–HCl, pH 7.5) were individually incubated with 10 μl, 1:100 diluted lambda bacteriophage stock (10^9 pfu/ml) for 4 h at room temperature. After being diluted 1:10^2–10^6 in SM buffer, 10 μl of treated samples were separately incubated at 37 °C for 15 min with 200 μl fresh *E. coli* Xl1-Blue cells suspended in 10 mM $MgSO_4$ (OD_{600} = 1.0). Each reaction mixture was mixed with 4.0 ml, 55 °C NZY broth containing 0.75% agarose, poured onto a pre-warmed NZY agar plate, and incubated at 37 °C. Numbers of plaques formed on plates were counted to calculate viral titers: pfu/ml = plaque number × dilution factor × 100. An LC_{50} was determined by plotting bacteriophage titer against final DHI concentration (0–1.0 mM) using Prism 3.0 (GraphPad Software).

2.3. Effect of DHI on the eggs of a parasitoid wasp

Newly laid (<1 h old) eggs from the wasp *M. demolitor* were collected from host larvae as described (Beck and Strand, 2007), and incubated with 0, 2, 20, 200 and 2000 μM DHI in TC-100 insect cell medium (Sigma–Aldrich) at room temperature for 1 h. After washing with the medium three times, the embryos were cultured in TC-100 supplemented with 30% heat-treated *Pseudoplusia includens* plasma. An egg was scored as alive if it hatched to form a first instar parasitoid after 30 h. An LC_{50} value was determined by plotting viability of the wasp embryos against DHI concentration (0–2 mM) as described in Section 2.2.

2.4. Cytotoxicity of DHI to insect cells

*Sf*9 cells (1 × 10^5 cells/ml, 200 μl) in a 96-well plate were incubated with 0, 1, 10, 20, 50, 100 μM DHI in PB at 27 °C for 4 h. After the cells were washed with PB, 200 μl Sf900–III medium and 20 μl, 5 μg/μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added to each well. Following incubation at 27 °C for 4 h, the medium-dye mixture was removed and the cells in each well were washed with PB. Aliquots of DMSO (150 μl) were added to wells to dissolve reduced MTT taken up by live cells. A_{492nm} readings, which correlate with cell viability, were taken using a VersaMax microplate reader (Molecular Devices) and plotted against DHI concentration for determination of LC_{50} values as described in Section 2.2.

Viability of DHI-treated *Sf*9 cells was further examined by flow cytometry: 1 × 10^5 *Sf*9 cells were first treated with 0, 31, 63, 94, 125, 188, 250, 375 and 500 μM DHI in Sf900–III medium at 27 °C for 5 h in a shaker-incubator (100 rpm). The cells were then harvested by centrifugation at 500 × *g* for 5 min and resuspended in 50 μl propidium iodide (PI) and fluorescein-labeled Annexin V for 15 min in the staining buffer (Annexin V-FITC Apoptosis Detection Kit, BD Biosciences). After addition of 400 μl buffer, cell suspensions were analyzed on a BD FACS Calibur flow cytometer. Data analysis and LC_{50} calculation were performed as described in Section 2.2.

2.5. Morphological changes of DHI-treated Sf9 cells

*Sf*9 cells (2 × 10^4) in a 6-well plate were treated with 200 μl PB or 0.5 mM DHI in PB at 27 °C for 6 h, stained with 300 nM 4′,6-diamidino-2-phenylindole (DAPI) for 15 min, and observed by fluorescence microscopy. Additionally, *Sf*9 cells (2 × 10^4) were incubated with Sf900–III SFM or 0.5 mM DHI in the medium at 27 °C for 5 h, washed with 0.85% NaCl twice and staining buffer once, and stained with Evans Blue, a membrane-impermeable dye. The control and treated cells were observed under bright field to detect differences in staining patterns.

2.6. Staining of phosphatidylserine

*Sf*9 cells (2 × 10^4) were treated with Sf900–III or 0.5 mM DHI in the medium at 27 °C for 6 h and washed with 0.85% NaCl and staining buffer. Fluorescein-labeled Annexin V was added to stain phosphatidylserine, a phospholipid exposed on the surface of cells undergoing early-stage apoptosis. PI was also included to stain dead cells for 15 min. Stained cells were washed with buffer and observed under the fluorescence microscope. The images were processed by adjusting brightness and contrast using Auto Levels in Photoshop 6.0 (Adobe Systems Inc).

2.7. Detection of fragmented genomic DNA

*Sf*9 cells (2 × 10^5) were treated with Sf900–III or 0.25 mM DHI in the medium at 27 °C for 5 h, washed with PBS once, and