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Eicosanoids mediate melanotic nodulation reactions to viral infection in larvae of the parasitic wasp, *Pimpla turionellae*

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Abstract

Nodulation is the quantitatively predominant insect cellular immune function activated in response to bacterial, fungal and some viral infections. We posed the hypothesis that parasitoid insects express melanotic nodulation reactions to viral challenge and that eicosanoids mediate these reactions. Treating fifth-instar larvae of the ichneumonid endoparasitoid *Pimpla turionellae* with Bovine Herpes Simplex *Virus-1* (BHSV-1) induced nodulation reactions in a challenge dose-dependent manner. Experimental larvae treated with the cyclooxygenase inhibitor, indomethacin, the lipoxygenase inhibitor, esculetin, and the phospholipase A_2 inhibitor, dexamethasone, resulted in severely impaired nodulation reactions to our standard BHSV-1 challenge dose. The immunoinhibitory influence of dexamethasone was reversed in larvae reared on culture medium amended with arachidonic acid, the fatty acid precursor of eicosanoid biosynthesis. Larvae that had been reared on media amended with indomethacin, esculetin, or dexamethasone were also compromised in their nodulation reactions to viral challenge. The influence of the orally administered pharmaceutical was expressed in a dose-dependent manner. Finally, wasp larvae reared in the presence of indomethacin and dexamethasone expressed significantly decreased levels of phenoloxidase activity in response to viral challenge. These findings draw attention to the idea that endoparasitoid insects express cellular immune reactions to viral challenge; they also support our hypothesis that eicosanoids mediate nodulation reactions to viral challenge in these highly specialized insects.

Keywords: Parasitoid wasp; *Pımpla turıonellae*; Cellular immunity; Nodulation; Phenoloxidase; Eicosanoids

1. Introduction

Eicosanoids are produced by enzymatic oxygenation of arachidonic acid (AA) and two other C20 polyunsaturated fatty acids. The two major groups of eicosanoids are prostaglandins and the many lipoxygenase products. Virtually all animals are thought to biosynthesize a wide range of eicosanoids, which serve in a large (but unknown) number of molecular, physiological and ecological actions (Stanley, 2000; Curtis-Prior, 2004). Among their important biological actions, eicosanoids act in several aspects of insect immunity. Two categories of insect innate immune reactions are appreciated. Humoral reactions involve induced biosynthesis of various anti-microbial peptides and proteins (Hoffmann, 2003). Cellular reactions include nodulation, encapsulation and phagocytosis (Stanley, 2006; Stanley and Miller, 2006).

Miller et al. (1994) proposed that nodulation reactions to bacterial infection are mediated by eicosanoids. This eicosanoid hypothesis has been supported by reports, on over 20 insect species, from several laboratories (Stanley and Miller, 2006). Looking in more detail than nodulation, eicosanoids also mediate particular steps that lead to observable cell actions. Mandato et al. (1997) reported that

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cell spreading, a distinct phase of nodulation, and phagocytosis are mediated by eicosanoids in larval wax moths, *Galleria mellonella*. Miller (2005) found that eicosanoids mediate hemocyte elongation in hemocytes isolated from tobacco hornworms, *Manduca sexta*. Eicosanoids also mediate microaggregation reactions (another step in nodulation) to bacterial challenge (Miller and Stanley, 2001, 2004; Phelps et al., 2003). Aside from influencing cellular defense actions, Bundey et al. (2003) reported that eicosanoids mediate behavioral fever responses to infection in the locust *Schistocerca gregaria*. The eicosanoids also act in insect humoral immunity. Morishima et al. (1997) found that biosynthesis of antibacterial proteins also depends on eicosanoids in the silkworm, *Bombyx mori*. And Yajima et al. (2003) reported a functional coupling between the immune deficiency pathway and eicosanoid biosynthesis in *Drosophila*. We surmise eicosanoids are crucial elements in the mediation of in insect immunity.

With respect to the nature of infecting organisms, eicosanoids act in insect defense reactions to several species of bacteria, two fungal species and eggs of a parasitoid (Stanley, 2006; Stanley and Miller, 2006). Most recently, Büyükgüzel et al. (2007) suggested that eicosanoids mediate melanotic nodulation reactions to Bovine Herpes Simplex Virus-1 (BHSV-1) challenge in larvae of the greater wax moth, *G. mellonella*. At a higher level of biological organization, parasitoid insects can become exposed to viral infection via interactions with their hosts. We posed the hypothesis that parasitoids also express melanotic nodulation reactions to viral challenge and that eicosanoids mediate these reactions. In this paper, we report on experiments with the ichneumonid endoparasitoid *Pimpla turionellae* designed to test our hypothesis.

2. Materials and methods

2.1. Organisms

A colony of *P. turionellae* was maintained in the laboratory at $23 \pm 1\,^{\circ}C$, $75 \pm 5\%$ RH, and a photoperiod of 16-h light:8-h dark. Wasp larvae were reared on the pupae of the greater wax moth, *G. mellonella*. Adults were fed daily with a 50% filtered honey solution and *G. mellonella* pupal hemolymph every other day. Adult wasps were allowed to oviposit in wax moth pupae. Fifth-instar *P. turionellae* (20–30 mg) larvae were used in the nodulation reaction and PO activity experiments.

Stock BHSV-1 was kindly donated by Dr. Aykut Özkul (Veterinary Faculty, Ankara University). The virus stock was kept at $-70\,^{\circ}C$ until used. The concentrations of virus solutions (4×10^6, 4×10^5, 4×10^4 plaque-forming units (PFU)/ml) were prepared by serially diluting the original liquid suspension with distilled water. Dilutions of the stock solution were made immediately before injection.

2.2. Reagents

The cyclooxygenase (COX) inhibitor, indomethacin (1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-3-indolyl-acetic acid), the 5- and 12-lipoxygenase (LOX) inhibitor esculetin (6,7-dihydroxycoumarin), the PLA_2 inhibitor dexamethasone [(11β,16α)-9-fluoro-11-17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione], arachidonic acid (AA), dopamine, bovine serum albumin (BSA), and Folin-Ciolcalteu Reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Injections and nodulation assay

Before injection, larvae were anesthetized by chilling them on ice for 5 min and surface-sterilized by swabbing their cuticles with 95% ethanol (EtOH). Injections were carried out with a 10 μl Hamilton micro-syringe (Hamilton, Reno, NV). Injections were performed dorso-laterally in the abdominal segments with the pharmaceutical treatments and BHSV-1 challenge doses on opposite sides. The abdomen was palpated gently after injection to mix the contents of the hemocoel. Eicosanoid biosynthesis inhibitors (EBIs), indomethacin, esculetin and dexamethasone were dissolved in EtOH at 1 mg/ml.

Control insects were injected with 70% EtOH (5 μl) and experimental wasps with EBIs (5 μg/5 μl EtOH). All larvae were immediately challenged with BHSV-1 in a standard dosage of 2×10^4 PFU in 5 μl of culture medium, except in dose–response experiments, following the injection protocols of Miller and Stanley (1998). We assessed nodulation at selected times after injections. The larvae were anesthetized by chilling them on ice, then their hemocoels were exposed to count melanized, brownish-black nodules under a stereomicroscope at $45\times$. After initial counting, the alimentary canals were removed and nodules in the remaining internal tissues were then counted. The nodules were distinct and direct counting reliably reflected the extent of the nodulation response to infections (Miller and Stanley, 1998).

2.4. Control experiments

Several control experiments were conducted to determine the level of background nodulation in the wasp larvae. Because the larvae were reared in non-sterile conditions, control experiments (no treatment) were performed to register the background number of nodules in the larvae. Nodulation in unchallenged larvae was assessed by randomly taking 10 fifth-instar larvae from artificial diet, chilling them on ice, and counting the nodules, as described above. The effect of wounding on nodulation was determined by wounding 10 larvae with the needle of the micro-syringe. Nodulation was assessed 1 h post-injection (PI). The effect on nodulation of the COX inhibitor indomethacin, the LOX inhibitor esculetin, and the phospholipase A_2 inhibitor dexamethasone, BHSV-1