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Eicosanoids Mediate *Manduca sexta* Cellular Response to the Fungal Pathogen *Beauveria bassiana*: A Role for the Lipoxygenase Pathway

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Eicosanoids Mediate *Manduca sexta* Cellular Response to the Fungal Pathogen *Beauveria bassiana*: A Role for the Lipoxygenase Pathway

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Many studies have documented the involvement of eicosanoids in insect cellular immune responses to bacteria. The use of the fungal pathogen *Beauveria bassiana* as a nodulation elicitor, with inhibition of phospholipase A_2 by dexamethasone, extends the principle to fungi. This study also provides the first evidence of involvement of the lipoxygenase (LOX) pathway rather than the cyclooxygenase (COX) pathway in synthesis of the nodulation mediating eicosanoid(s). The LOX product, 5(S)-hydroperoxyeicosa-6E,8Z,11Z,14Z-tetraenoic acid (5-HPETE), substantially reversed nodulation inhibition caused by dexamethasone and the LOX inhibitors, caffeic acid and esculetin. The COX product, prostaglandin H_2 (PGH_2), did not reverse the nodulation inhibition by dexamethasone or the COX inhibitor, ibuprofen. None of the inhibitors tested had a significant effect on the phagocytosis of *B. bassiana* blastospores in vitro. Hemocyte phenoloxidase activity was reduced by dexamethasone, esculetin, and the COX inhibitor, indomethacin. The rescue candidates 5-HPETE and PGH_2 did not reverse the inhibition. Arch. Insect Biochem. Physiol. 51:46–54, 2002. Published 2002 Wiley-Liss, Inc.†

KEYWORDS: eicosanoid; lipoxygenase; *Manduca sexta; Beauveria bassiana*; nodulation; insect immunity; fungus

INTRODUCTION

Eicosanoids are oxygenated metabolites of arachidonic acid with a broad array of informational functions in a diversity of organisms. Among the roles ascribed to eicosanoids in insects are release of cricket oviposition behavior (Destephano and Brady, 1977; Loher et al., 1981), regulation of fluid secretion rates in Malpighian tubules in mosquitoes and ants (Petzel and Stanley-Samuelson, 1992; Van Kerkhove et al., 1995), regulation of temperature set points in cicadas (Toolson et al., 1994), and modulation of the cellular immune response to bacteria (Stanley-Samuelson et al., 1991). Eicosanoid actions in cellular defense mechanisms have been most intensely investigated (see reviews by Stanley-Samuelson, 1994; Stanley and Howard, 1998; Howard and Stanley, 1999; Stanley, 2000).

Insect cellular immune responses to microbial assault are divided into multicellular nodulation and encapsulation involving both granular cells and plasmatocytes, and phagocytosis by individual hemocytes. Several studies have demonstrated a role in various insects for eicosanoids in the hemocyte aggregation and nodulation response to blood-borne bacteria (Jurenka et al., 1997; Miller et al., 1996; 1999; Tunaz et al., 1999). Mandato et al. (1997) reported that eicosanoids mediate phagocytosis of carboxylated latex beads, prophenoloxidase activation, and cell spreading in *Galleria mellonella*. With the tobacco hornworm, *Manduca sexta*, and the tenebrionid beetle, *Zophobas atratus*,

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as models, Howard et al. (1998) demonstrated that the influence of eicosanoids on nodulation applies across bacterial genera.

In this study, we report that the role eicosanoids serve in signal transduction for nodulation applies to the presence of fungi as well as bacteria, as suggested by Dean et al. (2002). We also provide the first evidence that products of the lipoxygenase (LOX) biosynthetic pathway rather than the cyclooxygenase (COX) pathway are involved in the nodulation events.

MATERIALS AND METHODS

Organisms

Fifth instar *M. sexta* larvae weighing 4–6 g were used for all experiments. The larvae were reared at 26°C and 75% RH in 15:9 photoperiod from eggs that were purchased from Carolina Biological Supply Company (Burlington, NC) or North Carolina State University. They were cultured on an artificial diet modified from Baumhover (1985). The diet contained sorbic acid, methyl-p-hydroxybenzoate, and streptomycin sulfate as preservatives.

Beauveria bassiana isolate GHA was obtained from Mycotech Corp. (Butte, MT). It was maintained in Sabouraud dextrose broth throughout the experiments by repeated mycelium-blastospore transfers (Rombach et al., 1988). All experiments were conducted with blastospores that were separated from mycelium by passage through glass wool, washed twice by low speed centrifugation in distilled, deionized water, and adjusted to 4×10^7/ml for injections.

Reagents

The phospholipase A_2 (PLA_2) inhibitor, dexamethasone [(11β,16α)-9-fluoro-11,17, 21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione]; the cyclooxygenase (COX) inhibitor, ibuprofen [α-methyl-4-(isobutyl)phenylacetic acid]; L-DOPA (L-3,4-dihydroxyphenylalanine); and arachidonic acid [5,8,11,14-eicosatetraenoic acid] were purchased from Sigma Chemical Company (St. Louis, MO). The lipoxygenase (LOX) inhibitors, caffeic acid [3,4-dihydroxycinnamic acid] and esculetin [6,7-dihydroxycoumarin]; the COX inhibitor, indomethacin [l-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid]; the LOX product 5-HPETE (5(S)-hydroperoxyeicosa-6E,8Z,11Z,14Z-tetraenoic acid) and the COX product, PGH_2 (prostaglandin H_2) were purchased from Biomol (Plymouth Meeting, PA). Other reagents were obtained from Sigma.

Nodulation Assay

Insects were injected in the third and fourth prolegs with 26 G Hamilton syringes. Inhibitors were dissolved in ethanol at concentrations of 11–14 mM. Each injection was in 5 μl volume. The insects were chill-anesthetized and surface disinfected with 70% ethanol. Drug treatments were injected separately; then fungal blastospores were injected within 2 min. In some experiments, the influence of the pharmaceuticals was potentially rescued by injecting candidate rescue compounds within 2 min of the fungal challenge. After injections, the insects were incubated for 2 h at room temperature. They were then bled through a clipped proleg into iced test tubes, and 250 μl of hemolymph were combined with 100 μl of anticoagulant saline solution containing 98 mM NaOH, 146 mM NaCl, 17 mM EDTA, and 41 mM citric acid (Mead et al., 1986). The hemolymph was placed in CoverWell imaging chambers (Grace Bio-Labs, Bend, OR) and all nodules in 250 ml were counted with an inverted microscope. The hemolymph of five insects per treatment was handled separately in four trials for all treatments except for three trials with esculetin and esculetin with 5-HPETE.

Phenoloxidase Assay

Hemolymph from 12 chilled fifth instar larvae was collected into individual test tubes containing anticoagulant saline. Hemocytes were washed in anticoagulant saline twice by centrifugation at 100*g* for 5 min and resuspended in *Manduca* buffered

saline (MBS) (Willot et al., 1994). Ten microliters of 10 mM test compound in ethanol were added to 1 ml of hemocyte suspension and incubated at room temperature for 1 h. The samples were then placed in ice and sonicated for 15 sec at 30% power with a VirSonic 475 probe sonicator (Virtis, Gardiner, NY). Cellular debris was removed by centrifugation at 13,300*g* for 20 min at 4°C. The supernatant protein was determined by bicinchonic acid assay (Pierce, Rockford, IL). The hemocyte lysate supernatants were distributed in 100-µl aliquots in a 96-well plate with 50 µl of 10^7 *B. bassiana* blastospores/ml and incubated for 30 min at 37°C. Fifty microliters of 3 mg/ml L-DOPA was added to each well and the absorbance was read at 490 nm for 60 min. Units of PO activity were calculated as 0.001 Δ absorbance/mg protein/min and all results were analyzed as proportions of untreated controls. The experiment was carried out three times.

Phagocytosis Assay

Hemocytes from 12 larvae were collected and washed as in the PO assay. The suspensions were adjusted to 10^6 hemocytes/ml and dispensed in 170 µl aliquots into the wells of chamber slides (Nalge Nunc, Naperville, IL). Test compounds were dissolved in ethanol and added in a total of 10 µl for each well. The hemocytes were incubated for 30 min at 30°C with test materials at concentrations of 0.3 mM for dexamethasone, indomethacin, and caffeic acid, and 3.6 µM for PGH_2 and 5-HPETE. After incubation with the eicosanoid synthesis inhibitors and intermediates, 50 µl of fluorescein isothiocyanate-stained blastospores were added to each well to achieve a final 10:1 blastospore: hemocyte ratio, and the cells were incubated for 2 h at 30°C. The hemocytes and blastospores in wells were washed twice with MBS, incubated for 15 min in 2 mg/ml trypan blue solution to quench fluorescence from uningested blastospores (Rohloff et al., 1994), and washed two more times with MBS. The cells were then fixed in 4% formaldehyde for 30 min and washed two more times with MBS. The MBS was replaced by glycerol, the chambers were removed from the slides, and coverslips were added and sealed. Internalization of blastospores was scored by fluorescence microscopy on 100 hemocytes per well. Each treatment was applied to three wells, and the experiment was carried out three times.

Statistical Analyses

Significant treatment effects were identified by one-way analysis of variance at $\alpha = 0.05$ using GraphPad InStat (GraphPad Software, San Diego). The nodulation data of individual assays were not significantly different and were pooled for analysis. The phagocytosis and phenoloxidase (PO) data varied significantly among assays, and arcsine transformed trial means were used in the analysis. Differences among means were determined by the Student-Newman-Keuls procedure.

RESULTS

Nodulation

Dexamethasone significantly reduced the nodulation response to *B. bassiana*, from about 450 nodules/ml hemolymph in control to about 200 nodules/ml hemolymph in dexamethasone-treated experimental hornworms ($F = 28.0$; d.f. = 7,130; $P < 0.01$, Fig. 1). Arachidonic acid injection did not significantly restore the nodulation response ($P > 0.05$). The number of nodules in the hemolymph of larvae injected with water, ethanol, or arachidonic acid did not differ significantly from the number in the hemolymph of unchallenged larvae ($P > 0.05$). The number of nodules in insects injected with fungal blastospores and ethanol carrier was not significantly different from the number injected with the fungal blastospores in water ($P > 0.05$).

In the experiments in which *M. sexta* larvae were injected with specific eicosanoid synthesis inhibitors, nodulation was significantly lower in all of the inhibitor treatments compared to blastospore-challenged controls ($F = 7.94$; d.f. = 9,118; $P < 0.01$, Fig. 2). Injection of the 5-LOX product, 5-HPETE

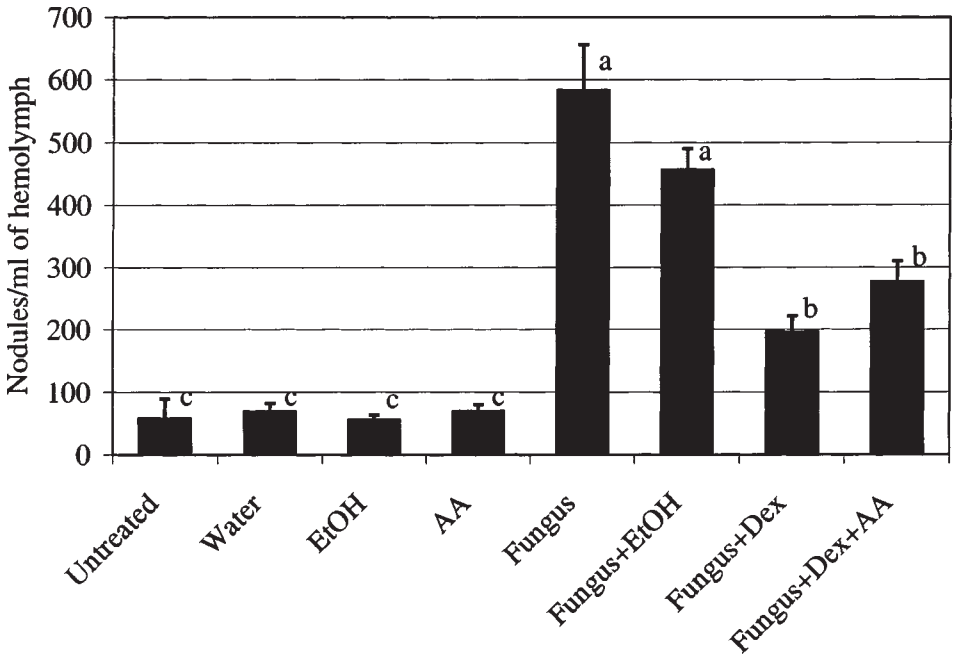

Fig. 1. Numbers of nodules in *Manduca sexta* hemolymph after injection of drug vehicle; *Beauveria bassiana* blastospores; the phospholipase A_2 inhibitor, dexamethasone (Dex); and the eicosanoid substrate, arachidonic acid (AA). The histogram bars = the mean number of nodules, and the error bars = 1 SEM. Histogram bars with the same letter are not significantly different from the mean nodulation in inhibitor-free controls (α = 0.05, Student-Newman-Keuls).

after dexamethasone, caffeic acid, and esculetin injections resulted in significantly greater nodulation relative to hornworms injected with the inhibitors alone, except for the case of esculetin. Injection of the COX pathway intermediate, PGH_2, after dexamethasone or ibuprofen did not increase the mean number of nodules formed ($P > 0.05$).

Phenoloxidase

Several approaches to measuring eicosanoid effects on PO activity resulted in erratic and equivocal responses. The use of washed, intact hemocytes gave the most consistent results. Hemocytes treated with dexamethasone, indomethacin, and esculetin

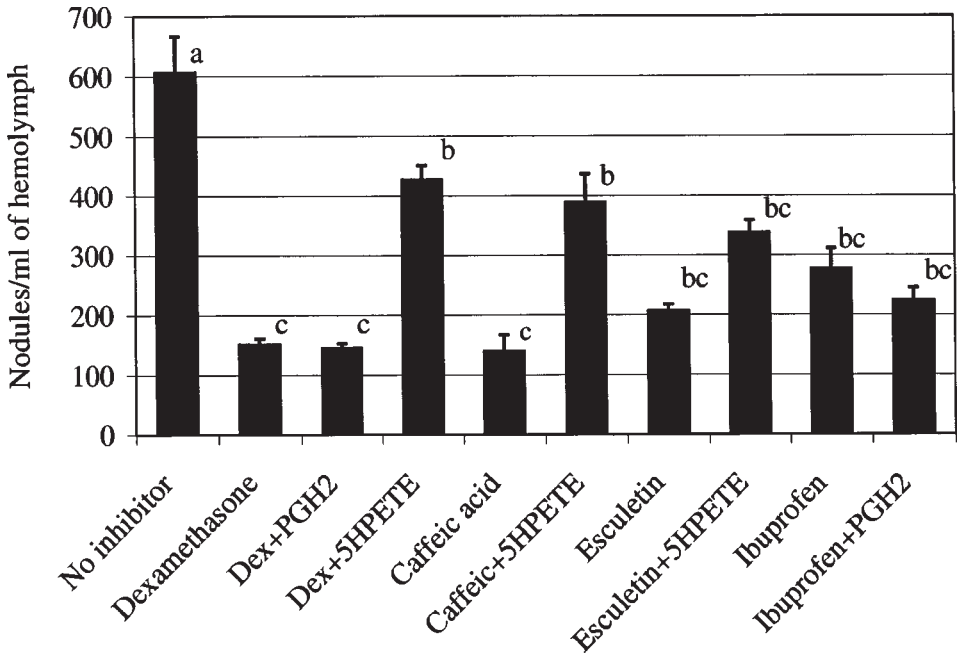

Fig. 2. Nodulation of *Beauveria bassiana* blastospores in the hemolymph of *Manduca sexta* larvae treated with eicosanoid synthesis inhibitors and upstream intermediates in LOX (5-HPETE) and COX (PGH_2) pathways. The histogram bars = mean number of nodules, and the error bars = 1 SEM. Histogram bars with the same letter are not significantly different (α = 0.05, Student-Newman-Keuls).

all showed less apparent activity than the carrier controls, but the differences were not significant. Application of PGH_2 or 5-HPETE did not restore melanization, resulting in PO activities that were significantly less than controls ($F = 5.09$; d.f. = 7,16; $P < 0.01$, Fig. 3).

Phagocytosis

In our assays for effects of eicosanoid biosynthesis inhibitors and intermediate products on phagocytosis of *B. bassiana* blastospores, there were no significant differences in the proportions of hemocytes with internalized fungus cells ($F = 0.19$; d.f. = 8,18; $P > 0.05$, Table 1).

DISCUSSION

There is now considerable evidence for the involvement of eicosanoids in signal transduction in insect antimicrobial responses. We have found that eicosanoids play a similar role in *M. sexta*'s nodulation response to *B. bassiana*, a ubiquitous fungus with infectivity for a broad range of arthropods. This report supports the work of Dean et al. (2002), who suggested that eicosanoids mediate insect cellular defense reactions to infection by the fungus *Metarhizium anisopliae*. Our findings extend the inquiry into the roles of eicosanoids in insect reactions to fungal infection by showing that products of the LOX, but not COX, pathway are crucial mediators of nodulation reactions to fungal infection.

Phospholipase A_2 is responsible for the release of membrane-bound eicosanoid precursor, arachidonic acid from cellular phospholipids. Workers in several laboratories have reversed the effects of dexamethasone on insect cellular immunity by administration of arachidonic acid (Stanley-Samuelson et al., 1991; Mandato et al. 1997; Park and Kim, 2000; Dean et al., 2002). The inhibition by dexamethasone was not as pronounced as has been observed for its inhibition of bacteria-induced nodulation (Stanley-Samuelson et al., 1991; Howard et al., 1998; Miller and Stanley, 2001) nor for infection of the fungus *M. anisopliae* (Dean et al., 2002). Moreover, injection of arachindonic acid did not rescue the dexamethasone influence as strongly as has been observed with other challenges, including bacteria (Miller et al., 1994) and fungi (Dean et al., 2002). Bacteria and fungi differ in their signal molecules for non-self pattern recognition. Bacteria carry surface lipopolysaccharide (LPS) and glucoproteins. Fungal cell walls bear (β1,3-glucans. In *M. sexta*, there are specialized pattern recognition receptors

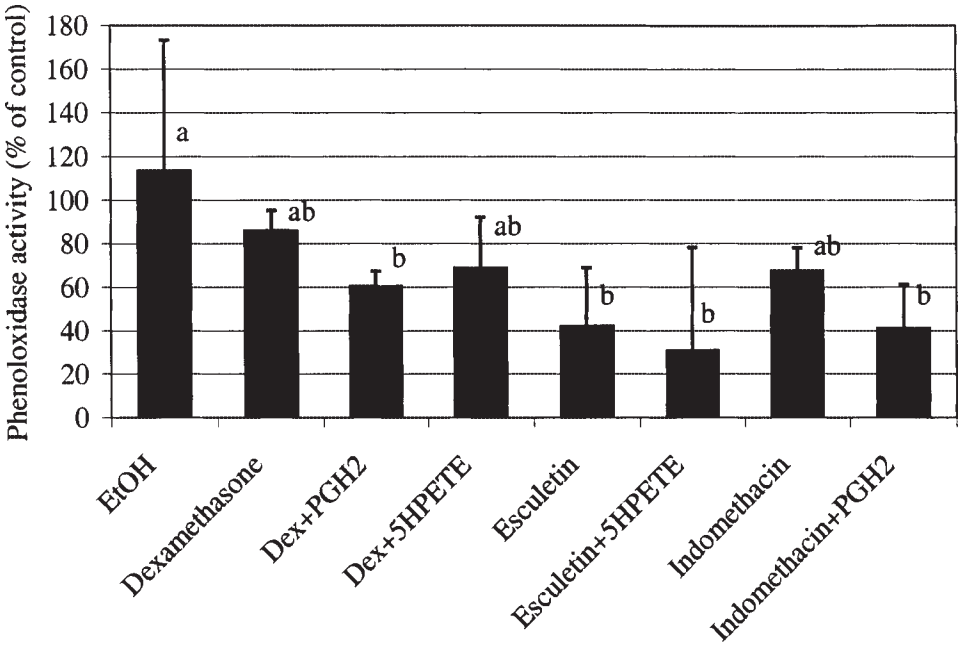

Fig. 3. Phenoloxidase activity in *Manduca sexta* hemocytes treated with eicosanoid synthesis inhibitors and intermediates. The bars = percent of controls with neither treatment chemicals nor ethanol carrier. Error bars = SEM. Histogram bars with the same letter are not significantly different (α = 0.05, Student-Newman-Keuls).

TABLE 1. Influence of Eicosanoid Synthesis Inhibitors and Rescue Agents on Phagocytosis of *Beauveria bassiana* Blastospores*

Treatment	Percent of blastospores within hemocytes ± SEM
Saline	45.1 ± 3.03
Ethanol	51.3 ± 14.84
Dexamethasone	40.0 ± 9.82
Dexamethasone + PGH_2	48.0 ± 7.51
Dexamethasone + 5-HPETE	43.1 ± 4.04
Ibuprofen	49.1 ± 4.16
Ibuprofen + PGH_2	43.0 ± 5.81
Caffeic acid	47.0 ± 10.69
Caffeic acid + 5-HPETE	43.0 ± 12.66

*There are no statistically significant differences among the means ($n = 9$, $P < 0.05$).

for bacterial and fungal components. Immulectin-1 binds both bacteria and yeast, while immulectin-2 is LPS-specific (Yu et al., 1999; Yu and Kanost, 2000). A lectin in *M. sexta* that binds the (β1,3-glucans recognizes fungal infections (Ma and Kanost 2000). It is likely that *M. sexta* also has pattern recognition receptor for peptidoglycan, as does *Bombyx mori* (Ochiai and Ashida, 1999). Eicosanoids have been implicated in initiation of nodule formation in *M. sexta*'s response to purified LPS (Bedick et al. 2000). Our results indicate that eicosanoids play a similar role in the *M. sexta* nodulation response to blastospores of the fungus, *B. bassiana*.

In earlier reports, nodulation rescue experiments consisted of injecting arachidonic acid to provide substrate for eicosanoid synthesis when PLA_2 activity had been inhibited by dexamethasone. While implicating the eicosanoids in the chain of events that result in nodulation, this approach does not identify the operative class or classes of eicosanoids in cellular immunity. Arachidonic acid is converted into three major groups of eicosanoids via separate oxygenation pathways (Stanley, 2000). Cyclooxygenase introduces two dioxygen molecules onto arachidonic acid to form the cyclic endoperoxide prostaglandin G_2. PGG_2 is subsequently reduced to the slightly more stable PGH_2 from which other prostaglandins, as well as thromboxanes and prostacyclins, are formed by various shunts. Accordingly, PGH_2, which is commercially available, is a suitable rescue candidate for the reversal of inhibition of upstream events and the implication of COX products in nodulation, phagocytosis, and PO cascades.

Lipoxygenases catalyze the dioxygenation of 1,4-pentadienyl units in polyunsaturated fatty acids to their 1-hydroperoxy derivatives. Mammalian LOXs produce six HPETEs that differ in the carbon that is oxygenated. The products downstream from HPETEs in the LOX pathways are leukotrienes, hydroxy fatty acids, lipoxins, and hepoxilins. We selected 5-HPETE over the other commercially available HPETEs because of caffeic acid's reported specificity for 5-LOX (Koshihara et al., 1984). The cytochrome P-450 products are hydroxy fatty acids and fatty acid epoxides (Kühn and Borngräber, 1998). These compounds have not yet been considered in insect physiology.

In our nodulation assays, 5-HPETE significantly reversed the effects of the PLA_2 inhibitor, dexamethasone, and the LOX inhibitor, caffeic acid. It also partially reversed the effects of another LOX inhibitor, esculetin, although the reversal was not statistically significant. The COX inhibitor, ibuprofen, was less inhibitory than dexamethasone and the LOX inhibitors, and its effect was not reversed by PGH_2. This could be taken as evidence for the involvement of the LOX products, but not the COX products, in mediation of the *M. sexta* nodulation response to *B. bassiana* blastospores. We note, however, that ibuprofen exerted a significant inhibitory effect on nodulation. Although not reversed by treatment with PGH_2, this would indicate that COX products are involved in the nodulation reaction to fungal blastospores to some extent.

In support of the idea that LOX products mediate cellular reactions to fungal blastospores, biochemical studies show that *M. sexta* fat body and hemocytes express LOX activity. Stanley-Samuelson and Ogg (1994) detected *M. sexta* fat body products that co-chromatographed with LOX products. These products were not seen in eicosanoid biosynthesis reactions unless the reactions were conducted in the presence of a COX inhibitor. Moreover, Gadelhak et al. (1995) reported strong LOX activity in *M. sexta* hemocytes. In reactions conducted in the absence of COX inhibitors, the hemocyte LOX activity yielded substantially more product than the hemocyte COX activity. Prior to this report, however, no physiological function has been assigned

to the LOX products. In light of the findings in this report, one biological role of the LOX products would be their actions in cellular defense reactions to fungal blastospores.

In our system, dexamethasone or indomethacin alone reduced cellular PO activity only slightly and not significantly (Fig. 3). Adding PGH_2 to the reaction mixture did not reverse the influence of these drugs. Esculetin significantly reduced PO activity, and 5-HPETE did not reverse the reduction. Without reversal of the PO inhibition, we are unable to draw conclusions on the possible role of eicosanoids in activation of PO activity. There are several possible explanations of our results. Among them, it may be that signaling for PO activation is independent of other nodulation activities, or it may be that all of the employed compounds are toxic to the hemocytes at the concentrations we used (although they were not toxic to hemocytes at three times this concentration in the phagocytosis experiments). Mandato et al. (1997) reported that injection of *G. mellonella* with dexamethasone depressed PO activity and this was reversed by exogenous arachidonic acid. Furthermore, they reported that indomethacin inhibited PO activity by injection, but not when added to hemocyte lysates. We added the inhibitors to intact hemocytes in vitro, then lysed them. In our hands, PO assays by injection did not give reproducible results, and we were unable to determine eicosanoid effects on PO activity in vivo.

Our phagocytosis assays did not provide indication of an effect of PLA_2, COX, and LOX inhibitors. Nor did PGH_2 or 5-HPETE affect phagocytosis. This is in contrast to the results of Mandato et al. (1997) with *G. mellonella* hemocytes and latex beads, in spite of our use of the same inhibitors at much higher doses.

While we were unable to implicate specific eicosanoid synthesis pathways in the activation of prophenoloxidase or phagocytosis, we believe that the data presented here, in addition to implicating eicosanoids in the *M. sexta* nodulation response to fungi, support the hypothesis that the LOX pathway provides specific signal transducers for the response. This introduces a function of a new class of compounds into insect physiology.

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