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The Role of Eicosanoids on Nodulation Reactions to Bacterium *Serratia marcescens* in Larvae of *Ostrinia nubilalis*

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Abstract: Nodulation is the first step of the cellular defense reactions to bacterial infections in insects. In the present study, injecting larvae of the European corn borer, *Ostrinia nubilalis*, with eicosanoid biosynthesis inhibitors, immediately prior to intrahemocoelic injections of the bacterium, *Serratia marcescens*, sharply reduced the nodulation response to bacterial challenges. Separate treatments with specific inhibitors including dexamethasone (a phospholipase A_2 inhibitor), indomethacin (a cyclooxygenase inhibitor) and phenidone (a dual cyclooxgenase/lipoxygenase inhibitor) also impaired the ability of European corn borers to form nodules in reaction to bacterial challenges. The inhibitory influence of phenidone was apparent within 30 mins after infection, and nodulation was significantly reduced, relative to the control insects, over following 4 h. These findings support the hypothesis that eicosanoids act in nodulation reactions to bacterial infections in European corn borers.

Key Words: Insect cellular immunity; nodulation; *Ostrinia nubilalis*

Ostrinia nubilalis'in Larvalarında *Serratia marcescens* Bakteriyal İnfeksiyonuna Karşı Oluşan Şırınga ile Nodülasyon Reaksiyonunda Eikosanoidlerin Rolü

Özet: Nodülasyon reaksiyonu bakteri infeksiyonlarına karşı böcekleri koruyan ilk hücresel reaksiyondur. Yapılan bu çalışmada mısır kurdu larvalarına şırınga ile eicosanoid biyosentezini engelleyen kimyasallar injekte edildikten sonra aynı larvalara şırınga ile bakteri, *Serratia marcescens*, verilmesi bu böceklerde nodülasyon oluşumunu önemli derecede azaltmıştır. Bunun yanı sıra yine eicosanoid biyosentezi oluşması aşamasındaki enzimleri (phospholipase A_2, cyclooxygenase, ve lipoxygenase) engelleyen kimyasallar (dexamethasone, indomethacin ve phenidone) larvalara injekte edildiğinde, bu böceklerde nodülasyon oluşumu önemli ölçüde azalmıştır. Eicosanoid biyosentezini engelleyen kimyasallardan phenidone'nun eicosanoid biyosentezini engelleme etkisinin 30 dakika içerisinde gerçekleştiği ve nodulasyon sayısının, kontroldeki böceklere göre önemli ölçüde azaldığı ve bu etkinin yaklaşık 4 saat devam ettiği saptanmıştır. Bu bulgular mısır kurdunda bakteriyal infeksiyonlara karşı oluşturulan hücresel bağışıklardan nodülasyon reaksiyonunda, eicosanoidlerin önemli bir yer aldığı hipotezini doğrulamıştır.

Anahtar Sözcükler: Böcek hücresel bağışıklığı; Nodülasyon; *Ostrinia nubilalis*

Introduction

Insects produce 2 categories of defense responses to bacterial infections, humoral and hemocytic (Dunn, 1986; Gupta, 1986, 1991). Humoral reactions take several hours for full expression, and they involve the induced synthesis of antibacterial proteins, such as cecropins, attacins, diptericins and defensins (Dunn, 1986; Cociancich et al., 1994). In the presence of these proteins, bacteria lose their cellular integrity because of the detergent properties of peptides. Insects also synthesize lysozymes, which enzymatically attack bacteria by hydrolyzing their peptidoglycan cell walls (Dunn, 1986; Russell and Dunn, 1996).

Hemocytic reactions involve direct cellular interactions between circulating hemocytes and bacteria. In contrast to humoral defense reactions, hemocytic responses are very quick, typically occurring within minutes of an infection cycle. Specific cellular defense mechanisms include phagocytosis, nodulation and encapsulation (Gupta, 1986; 1991).

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While humoral and hemocytic immune reactions to bacterial infections are well documented, until recently there was virtually no information on the biochemical events responsible for mediating insect immune reactions. Drawing on the background of signal transduction systems in mammalian immunity, Stanley-Samuelson et al. (1991) suggested that insect cellular immune reactions are mediated by eicosanoids. Eicosanoids are oxygenated metabolites of arachidonic acid and two other polyunsaturated fatty acids, the structures and biosynthesis of which are outlined elsewhere (Stanley, 2000). Eicosanoids are very well understood in the contexts of human and animal medicine, where they mediate many pathophysiological events, such as inflammation. Eicosanoids are also important to many actions in invertebrates, as reviewed recently (Stanley and Howard, 1998; Stanley, 2000).

Stanley-Samuelson et al. (1991) first showed that eicosanoids mediate one or more cellular reactions responsible for clearing bacterial infections from hemolymph circulation. This suggestion prompted more detailed research to determine which of the several cellular defense reactions depends on eicosanoid biosynthesis. Nodulation is an early cellular reaction responsible for clearing large numbers of bacterial cells from circulation during the first two hours of an infection (Horohov and Dunn, 1983). Since nodulation is the predominant cellular immune reaction to bacterial infections, it has been hypothesized that eicosanoids mediate nodulation reactions to bacterial infections (Miller et al., 1994). The idea was tested by injecting larvae of hornworms, *Manduca sexta* with an eicosanoid biosynthesis inhibitor, and then infecting them with *Serratia marcescens*. Compared to ethanol-treated controls, the experimental larvae produced significantly fewer nodules in response to similar bacterial challenges. This finding supported the idea that nodulation is one of the cellular immune responses to bacterial infections that are mediated by eicosanoids (Miller et al., 1994).

On the basis of these findings, Stanley-Samuelson and his colleagues developed the hypothesis that eicosanoids mediate nodulation reactions to bacterial infections in most, if not all, insect species, now known as the eicosanoid hypothesis (Stanley and Howard, 1998; Stanley, 2000). They tested the eicosanoid hypothesis in several additional insect species. Using similar experimental protocols, they have shown that nodulation reactions to bacterial infections depend on eicosanoid biosynthesis in the tenebrionid beetle, *Zophabos atratus* (Miller et al., 1996), in the silkworm, *Bombyx mori* (Stanley-Samuelson et al., 1997), in two other moths, black cutworms, *Agrostis ipsilon* and true armyworms, *Pseudaletia unipuncta* (Jurenka et al., 1997), in adults of the cockroach, *Periplaneta americana* (Tunaz and Stanley, 1999), the 17-year periodical cicadas, *Magicicada septendecim* and *M. cassini* (Tunaz et al., 1999) and honey bees, *Apis mellifera* (Bedick et al., 2001). In related research, Mandato et al. (1997) found that cell spreading, a distinct phase of nodulation, and phagocytosis are mediated by eicosanoids in wax moths, *Galleria mellonella*. The eicosanoid hypothesis is also supported by another line of research on humoral immunity. Morishima et al. (1997) found that the biosynthesis of anti-bacterial proteins also depends on eicosanoids in the silkworm, *B. mori*. These findings uniformly support the eicosanoid hypothesis.

It may therefore be asked whether all insects are competent to produce cellular defense reactions. In this paper we tested the hypothesis in larvae of the European corn borer, *Ostrinia nubilalis*. We observed nodulation reactions to bacterial infections in these larvae and found that these reactions depend on eicosanoid biosynthesis. This finding supports the idea that eicosanoids mediate nodulation in most insect species.

Materials and methods

Organisms

Late-stage larvae of the European corn borer were collected from corn fields in Türkoğlu/Kahramanmaraş in Turkey. The larvae were maintained in a container at room temperature on a laboratory bench.

The pathogen used was a non-pigmented strain of an entomopathogenic bacterium, *S. marcescens* (Miller and Stanley, 1998). The bacteria were grown in 50 ml of nutrient broth in an environmental horizontal shaker at 37 °C and 100 rev./min. The bacteria were freeze-dried, and then when needed the material was taken up into sterile distilled water for injection into larvae of the European corn borer.

Injections and assays for nodulation

The protocols formalized by Miller and Stanley (1998) were followed. Larvae of the European corn borer were

injected with either the phospholipase A_2 (PLA_2) inhibitor dexamethasone {(11β, 16α)-9- fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-dione}, the cyclooxygenase inhibitor indomethacin {1-P-(chlorobenzyl)-5-methoxy-2-methyl-3-indolyl-acetic acid} or the dual cyclooxygenase and lipoxygenase inhibitor phenidone {1-pheny-3-pryazolidinone}. Control insects were injected with 85% ethanol. Drugs and control substances were injected into the opposite side of the abdomen using a 50 µl Hamilton 701 micro-syringe. All injections of pharmaceuticals were in a standard dosage of 52 µg in 4 µl of ethanol, except in dose-response experiments. Before being injected, the larvae were surface sterilized by swabbing their cuticles with 85 % ethanol.

Immediately after the drug injections, the larvae were infected by injecting 50 µg of freeze-dried bacterial preparation, made up in 500 µl sterile distilled water, into each larva, following the injection protocols of Miller and Stanley (1998). Bacteria were injected in 5 µl aliquots, using an insulin syringe.

We assessed nodulation at selected times after injections. The larvae were anesthetized by chilling them on ice, then their hemocoels were exposed. Melanized, brownish black nodules were counted under a stereo microscope at 45x. The nodules were distinct, and direct counting reliably reflected the extent of the nodulation response to infections (Miller and Stanley, 1998; Miller et al., 1999). After the first counting, the alimentary canal was removed. Nodules in the previously unexposed areas and remaining internal tissues were then counted.

Control experiments

To determine the level of the background nodulation in larvae of the European corn borer, several control experiments were conducted. To record the nodulation in unchallenged larvae, 10 larvae were taken from culture at various times. We anesthetized the larvae on ice for 5 minutes and then assessed nodulation. To determine the influence of the drug vehicle, ethanol, on nodule formation, 5 larvae were injected with 4 µl of ethanol. Nodulation was assessed 4 h post injection (hpi), following the same protocol. To assess the effect of phenidone on nodulation in unchallenged larvae of the European corn borer, a standard dosage (52 µg) of phenidone in 4 µl ethanol was injected into 5 larvae. Nodulation was assessed by standard methods at 4 hpi. Injection of 5 µl sterile distilled water into 5 larvae of the European corn borer was used as control for nodulation by following the standard protocol.

Dose-response curve for freeze-dried bacteria

The freeze-dried bacterial preparations were made up in 500 µl sterile distilled water in three concentrations, 5, 25 and 50 µg in 5 µl aliquots per injection for each larva. The larvae were anesthetized, sterilized and injected as described. Nodulation was assessed at 4 hpi.

Time course of nodulation: Influence of phenidone

Individuals in 2 groups of larvae of the European corn borer were injected with ethanol or with 52 µg of phenidone. The larvae were immediately injected with bacteria as described. At 0.5, 1, 2 and 4 hpi, sub-groups of control and experimental insects were anesthetized, and nodulation was assessed.

Influence of other eicosanoid biosynthesis inhibitors on nodulation

We divided the larvae into two groups and injected individuals in each group with either the cyclooxygenase inhibitor indomethacin or the dual cyclooxygenase and lipoxygenase inhibitor phenidone, all in standard dosages of 52 µg in 4 µl of ethanol. Control insects were injected with 4 µl of ethanol. Following injection, the larvae were infected with a standard dosage of bacteria as described. At 4 hpi, the larvae were anesthetized and nodulation was assessed.

Statistical Analysis

Data were analyzed using the general linear models procedure, and mean comparisons were made using the least significant difference (LSD) test ($P \leq 0.01$) (SAS Institute Inc., 1989).

Results

Control experiments

Table 1 displays the results of several control experiments. We recorded approximately 24 nodules per larva (n=10 larvae) in untreated insects taken directly

Table 1. Outcomes of background control experiments. Insects were treated as specified in the left column, and nodulation was assessed at 4 hpi as described in the materials and method section.

Treatment	Nodules per larva (Mean ± SEM)
None (untreated)	24.6 ± 3.48
Vehicle (ethanol only)	57.6 ± 7.10
Phenidone only	46.2 ± 9.19
Distilled water only	43.8 ± 4.74
Bacterial challenge (50 mg)	201 ± 48

from the culture. Similarly, we observed approximately 57 nodules per larva of the European corn borer injected with ethanol, and 46 nodules per larva injected with phenidone. Injections with sterile distilled water resulted in about 43 nodules per larva. By comparison, infections with the highest dosage of freeze-dried *S. marcescens* resulted in about 201 nodules per larva.

Dose-response curve for *S. marcescens*

We recorded increased nodulation with increased doses of the freeze-dried bacterial preparation, from approximately 112 nodules per insect at the lowest dose to approximately 201 nodules per insect at the highest dose (Figure 1) ($y = 2.762x + 74.911$; $R^2 = 0.8521$).

Time course of nodulation

Figure 2 shows the time course of visible nodule formation in the two groups of larvae of the European corn borer, experimentals and controls. Phenidone-treated larvae formed about 32 nodules per insect at 0.5 hpi, which increased to 40 at 4 hpi, whereas the ethanol-treated control larvae produced significantly more nodules at each time, from 63 nodules at 0.5 hpi to 159 at 4 hpi (LSD, $P \leq 0.01$).

Influence of other eicosanoid biosynthesis inhibitors on nodulation

We considered the effects of three pharmaceutical inhibitors of eicosanoid biosynthesis on nodulation in response to bacterial infections. Figure 3 shows that, compared to the control (EtOH) larvae, all inhibitor-treated larvae of the European corn borer exhibited significantly reduced nodulation in response to bacterial infections (LSD, $P \leq 0.01$). We did not obtain any significant differences among the effects of individual inhibitors on nodulation (LSD, $P \leq 0.01$).

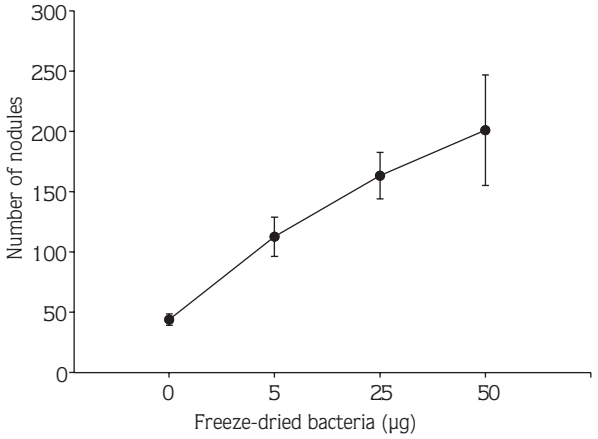

Figure 1. The influence of different bacterial concentrations on nodulation reactions in larvae of *O. nubilalis*. The larvae were challenged with injection of the indicated dosages of bacterial solutions, then nodulation was recorded at 4 hpi. Each point indicates the mean number of nodules found in each insect (n = 5 individuals), and the error bars represent ± S.E.M.

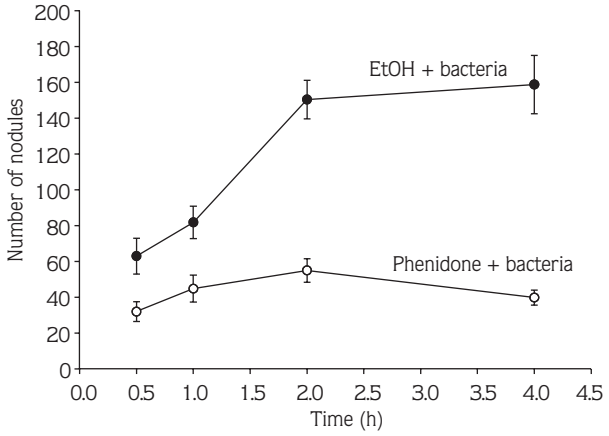

Figure 2. Time course of nodulation in larval *O. nubilalis*, in response to infections with *S. marcescens*. Each point indicates the mean number of nodules found in each insect (n = 5 individuals), and the error bars represent ± S.E.M.

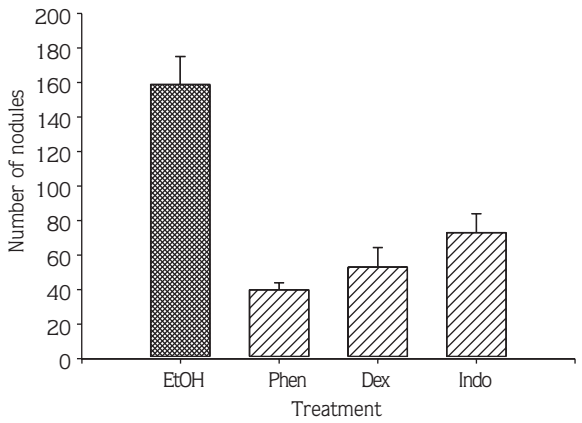

Figure 3. Effect of treating *O. nubilalis* larvae with individual eicosanoid biosynthesis inhibitors on nodule formation in response to infections with *S. marcescens*. Each point indicates the mean number of nodules found in each insect, and the error bars represent ± SEM. Histogram bars with the same fill pattern are not significantly different from each other (LSD, $P \leq 0.01$).

Discussion

In this paper, we obtained results that support the hypothesis that eicosanoids mediate nodule formation in response to bacterial infections in the European corn borer, *O. nubilalis*. The results of all the experiments support this hypothesis. Treating experimental European corn borers with phenidone prior to infecting them with freeze-dried bacteria significantly reduced nodulation at all points in the time course experiments. Moreover, three different eicosanoid biosynthesis inhibitors significantly reduced nodulation when compared to control treatments. When taken together, these two separate lines of evidence strongly support the overall hypothesis.

The results of the time course experiment showed that treating experimental European corn borers with phenidone produced significantly fewer nodules than the control group at all time points in the experiment. We infer from this finding that inhibition of eicosanoid biosynthesis influences the cellular events involved in nodulation early in the infection process, and continues to exert a negative influence for a few hpi. The time course experiment also indicates the upper limits of nodulation reactions to the bacterial infections in European corn borers. In our experiments, European corn borers produced a maximum of about 160-200 nodules per individual within 4 hpi, which is in line with the outcomes of similar experiments with other insect species (Miller et al., 1994; Bedick et al., 2001). The larvae of several Lepidoptera produced approximately 80-120 nodules per individual by 6 h after infection with the same number of bacterial cells. Tobacco hornworms, *M. sexta* produced approximately 120 nodules per individual (Miller et al., 1994), and silkworms, *B. mori*, produced approximately 80 nodules per individual (Stanley-Samuelson et al., 1997). Similarly, honey bees, *Apis mellifera*, produced approximately 130 nodules per individual by 4 hpi. (Bedick et al., 2001). With hemimetabolous insect species, Miller et al. (1999) recorded approximately 45 nodules in identical experiments with crickets, *Gryllus assimilis*.

Howard et al. (1998) suggested that differences in nodulation intensity might be due to differences in circulating hemocyte populations. In their work with tobacco hornworms and larvae of *Z. atratus*, they recorded numbers of nodules formed in reaction to similar bacterial challenges as a function of insect size, weight and age. These experiments showed that nodulation was not influenced by these three parameters. Noting that insects tend to maintain fairly similar concentrations of circulating hemocytes (about 4 to 6 x 10^6 cells/ml hemolymph), those insect species with copious amounts of hemolymph, such as tobacco hornworms, would have far larger absolute numbers of circulating hemocytes (Howard et al. 1998). They speculated that if circulating hemocyte population sizes account for differences in nodulation capacity, then it should not be surprising to record considerable differences among insect species in nodulation responses to similar infection challenges.

Due to their importance in human medicine, many different inhibitors of eicosanoid biosynthesis are available. Some, such as aspirin and ibuprofen, are available as analgesic drugs for relief of minor pains, while many others are available to researchers, and are not approved for use in humans (Needleman et al., 1986; Capdevila et al., 2000). These compounds, which we refer to with the general term "eicosanoid biosynthesis inhibitors", perform different actions in cellular eicosanoid biosynthesis (Smith, 1989). For example, dexamethasone inhibits PLA_2. Dexamethasone performs other actions, as well, including influence on gene expression. Several compounds specifically inhibit cyclooxygenase, the first step in prostaglandin

biosynthesis (Schnitzer, 2001). Our experiments with different inhibitors showed that all three of the compounds we tested resulted in similar reductions in nodulation. The observation that separate experiments with different inhibitors of eicosanoid biosynthetic pathways similarly retarded nodulation in larvae of the European corn borer indicates that eicosanoids act in nodule formation. This makes sense because nodulation results from complex cellular physiology, which involves many separate cellular actions. Inhibiting one or more of the eicosanoid-mediated steps may impact the overall nodulation process. We interpret the results of our experiments to suggest that prostaglandins and various lipoxygenase products mediate various, still unknown, steps in nodulation.

The results with three different inhibitors are subject to a slightly more subtle interpretation. Indomethacin is a cyclooxygenase inhibitor. However, it inhibits mammalian cyclooxygenases in slightly different ways (Vree et al., 1993). Moreover, phenidone is a dual lipoxygenase/cyclooxygenase inhibitor. Again, this compound acts in a slightly different way from the other cyclooxygenase inhibitors (Stanley-Samuelson, 1994). Hence, our observation with these compounds yielded similar results indicates that their actions are exerted through a common mechanism, specifically, inhibition of cyclooxygenase. Our background control experiments indicate that the nodules we recorded were due to the experimental treatments, and not to adventitious infections. European corn borers taken directly from the field had a low background of nodulation. The inject treatments, similarly, did not influence the low background of nodulation. We note that control experiments with ethanol yielded higher numbers of nodules than those recorded with other insect species (Miller et al., 1994, 1996). However, the key point is that the drug vehicle did not, in itself, diminish the European corn borers' ability to form nodules. Hence, the experimental protocols allow a physiological interpretation of the data; inhibition of eicosanoid biosynthesis impairs European corn borer immunity.

Although it has not yet been possible to use eicosanoid biosynthesis inhibitors with entomopathogenic microorganisms for insect pest control, the possible use in the future could help to improve the effectiveness of microbial control agents against insect pests. The potential discovery of insect-specific prostaglandin (PG) biosynthesis inhibitors opens a new frontier for the application of basic research into insect PGs and other eicosanoids to real-world problems in agricultural and medical insect pest management in the future.

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