Activation of Insect Phenoloxidase after Injury: Endogenous versus Foreign Elicitors

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Key Words

Coagulation • Comparative immunology • *Drosophila melanogaster* • Hemostasis • Innate immunity • Insects • Pattern recognition • Sepsis • Wound healing

Abstract

The enzyme phenoloxidase (PO) is one of the first immune molecules that was identified in invertebrates. Recently, the immune function of PO has been challenged. We tested how PO is activated following injury in 2 insects, i.e. the fruit fly *Drosophila melanogaster* and the wax moth *Galleria mellonella*. Rapid PO activation in *Drosophila* was limited to discrete areas of the hemolymph clot which forms after injury. Surprisingly, unlike systemic PO activation during bacterial sepsis, clot melanization was not sensitive to microbial elicitors in our assay. Instead, *Drosophila* clot melanization was activated by endogenous signals such as apoptotic cells and was superinduced by phosphatidylserine, a negatively charged phospholipid normally found on the inner surface of the plasma membrane and exposed during apoptosis. In contrast, melanization in *G. mellonella* hemolymph was stronger and more uniform and was sensitive to peptidoglycan. This shows that both exogenous and endogenous signals can trigger the same immune mechanism in species- and context-dependent ways. Our findings have implications for the evolutionary dynamics of immune mechanisms and are in agreement with recent comparisons of insect immune transcriptomes.

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Introduction

One of the key immune reactions in invertebrates is the activation of tyrosinase-type phenoloxidases (POs; EC 1.10.3.1), which catalyze several reactions leading to the crosslinking of proteins, the production of reactive intermediates with potential cytotoxic activity [1] and ultimately to the production of melanin [2, 3]. The proenzyme prophenoloxidase (proPO) can be activated upon contact with microbes as well as during the formation of capsules that surround larger foreign objects such as parasitoid eggs. In *Drosophila melanogaster*, full systemic activation of proPO during septic infections has been shown to depend on the activation of a proteolytic cascade and breakdown of the inhibitor serpin 27A (Spn27A) [4–8]. Both these events are induced after recognition of microbial products through pattern recognition receptors [in particular peptidoglycan (PGN) recognition proteins], leading to transcription of genes including regulators of proPO activation. This activation of PO during the insect immune response is consistent with the idea that microbial signals are key activators of immune reactions [9]. Nevertheless, the importance of proPO activation for insect immunity is controversial [2, 7, 8, 10–12].

PO is also involved in coagulation in insects. POs were identified in the clot in *Galleria mellonella* [13], in the *Anopheles gambiae* mosquito [14] and in *Drosophila* [15], where it was shown to contribute to clot hardening [10, 16]. While tyrosinase-type PO activity in the clot has a

physiological aspect similar to sclerotization of the cuticle, it is noted that sclerotization of the insect cuticle appears to require laccase-type POs (EC 1.10.3.2), rather than tyrosinases [17].

To identify activators of proPO and to learn more about the mode of proPO activation, we compared melanization in *D. melanogaster* and the wax moth *G. mellonella*, two established insect models for wound healing [15, 18, 19]. In both these insects, proPO is released from specialized hemocytes, called crystal cells and oenocytoids, respectively, within minutes after bleeding. We observed marked differences in the pattern of melanization. In *Drosophila* in vivo and ex vivo preparations, melanization was highly localized even in the presence of a bacterial elicitor (PGN). In contrast, *Galleria* larvae injected with PGN melanized systemically, and ex vivo clot preparations darkened more uniformly. We also found that *Drosophila* proPO could be superactivated by phosphatidylserine (PS), an endogenous elicitor, while PGN was more effective in *Galleria*. When infection-dependent breakdown of *Drosophila* Spn27A was mimicked using *Spn27A* mutants, the pattern of melanization resembled that in uninfected *Galleria*, suggesting that differences in the inhibition of proPO activation might explain some of our observations. We propose a model for proPO activation incorporating these findings.

Materials and Methods

Insect Culture

Flies were kept at 25°C on a 12-hour light/dark cycle on potato sucrose medium. Washed 3rd-instar larvae of *Drosophila* were used. The *Drosophila* strains used were: *w¹¹¹⁸* (wild type), *spn27A¹/CyO actin-GFP* [4], *Hemese-Gal4 UAS-GFP.nls* [20] and *UAS-grim* [21]. Gal4 >> UAS experiments were performed at 25°C. *Galleria* larvae were reared on a 12-hour light/dark cycle at 25°C on an artificial diet.

Histology

The preparation of clot samples was essentially performed as previously described in the hanging drop assay [10]. Hemolymph from 5 *Drosophila* larvae and a comparable amount for *Galleria* were each incubated upside down for 30 min in a humid chamber at 25°C. The formed clot was then caught on a coverslip, and images were taken with a Coolpix 4500 digital camera (Nikon) adapted to a Leitz Orthoplan microscope. The clot preparation shown in figures 4F and G was reconstituted by incubating the clot on the coverslip for another 30 min with 20 mM DL-3,4-dihydroxyphenylalanine (DOPA) in phosphate buffer (pH 6.6).

PO Activation

Photometric measurement of hemolymph PO activity has recently been described [16]. L- α -Phosphatidylethanolamine (PE)

(P8068), L- α -phosphatidylinositol sodium (P0639), L- α -phosphatidylcholine (PC) (P3556) and L- α -phosphatidyl-L-serine (P0474) were purchased from Sigma. Each lipid was dissolved in buffer A (50 mM Tris, 0.1 M NaCl, pH 7.5), which has been described elsewhere [22], and used at a final concentration of 10 μ g/ml. Lipopolysaccharide (LPS; *Escherichia coli* O55:B5, Sigma) and PGN (*Bacillus megaterium* Bm11, kindly provided by H. Steiner, Stockholm, Sweden) were similarly suspended.

By quickly bleeding 12 larvae of *Drosophila* each time into a well of a 15-well slide and withdrawing 7 μ l, this volume of hemolymph was pooled on ice in 50 μ l of anticoagulant Ringer's solution containing 20 mM EDTA instead of CaCl₂. After pipette mixing, 5 μ l of pooled hemolymph was aliquoted in triplicate or quadruplicate to 1 μ l of each phospholipid, PGN, LPS and buffer A (control) on ice. For *Galleria*, 4 drops of hemolymph from 4 4th-instar larvae were pooled on ice in 2 ml of *Galleria* Ringer's anticoagulant saline by amputating a pro-leg. *Galleria* hemolymph was then pipette-mixed, and triplicate 140- μ l aliquots were transferred to 10 μ l of different elicitors on ice.

After samples were allowed to activate at 25°C for 10 min, 45 and 150 μ l of DOPA (20 mM) were added to *Drosophila* and *Galleria* samples, respectively, and the optical density (OD) was read at 490 nm with a Vmax™ Kinetic Microplate Reader (Molecular Devices). The OD was measured again after incubating the samples for 30 min at 37°C. Activation of PO was measured as the change in OD. Photometric measurements were repeated at least 3 times, basically with the same result.

PGN Injection

Drosophila and *Galleria* last-instar larvae were injected with PGN from *B. megaterium* Bm11 (kindly provided by H. Steiner) suspended in distilled sterile water at a concentration of approximately 2.5 mg/ml. Larvae were injected in the abdominal region using glass-capillary-drawn needles connected to a custom-made injector. Melanization was monitored over a period of 2 h in both *Drosophila* and *Galleria*. As a control, distilled sterile water was injected instead of PGN.

Statistical Analysis

Samples from the photometric measurements were compared using ANOVA (available at <http://faculty.vassar.edu/lowry//anova1u.html>) or a Student t test (STT; Microsoft Excel). The samples shown in figure 3 were triplicates and differed significantly from the control at <0.05 (STT for *Drosophila*, fig. 3A) or <0.01 (STT for *Galleria*, fig. 3B, and *Drosophila* when analyzing quadruplicates).

Results

Melanization Patterns in Bacterial Elicitor-Challenged *Galleria* and *Drosophila* Larvae Are Different

To compare proPO activation between *Galleria* and *Drosophila*, we injected PGN into larvae from both species. Injection of PGN in *Galleria* led to melanization throughout the hemocoel (fig. 1A, B), while injected

Fig. 1. Melanization in PGN-challenged larvae. Healthy *Galleria* (A, B) and *Drosophila* (C, D) last-instar larvae were injected with PGN and photographed 5 min (A, C) and 90 min (B, D) after injection. The arrow in D indicates the injection site.

Fig. 2. Activation of PO in *Galleria* and *Drosophila* hemolymph clots. Hemolymph from *Galleria* (A, B) and *Drosophila* (C, D) were bled onto multiwell slides (a single well has a diameter of 4 mm), allowed to melanize for 30 min and either photographed directly (insets in A and C) or prepared using the hanging drop method and analyzed microscopically (A–D) using phase contrast (A, C) or transmission light (B, D). The arrows in A and B indicate a *Galleria* oenocytoid that has released PO. Scale bar = 10 μ m.

Drosophila larvae showed melanization only at the injection site (fig. 1C, D). To study the activation of proPO, larvae were bled and the clots that formed were examined. Melanization of hemolymph became visible with the naked eye after a few minutes. *Galleria* hemolymph

melanized extensively (fig. 2A, inset), while comparable samples from *Drosophila* hemolymph remained almost transparent except for a few darker spots and strands (fig. 2C, inset). Microscopically, melanization of *Galleria* clot preparations was diffusely distributed over the en-

tire sample (fig. 2A, B). In contrast, as expected from its macroscopic appearance, melanization in the *Drosophila* clot was highly localized and most intense along clot fibers and folds, as well as on cellular fragments derived from ruptured crystal cells or other hemocytes (fig. 2C, D) [16].

Drosophila proPO Is Activated after Injury by Endogenous Elicitors

Our previous data suggested that in *Drosophila*, proPO activation after injury involves signals released from ruptured crystal cells and other dying hemocytes [16]. This led us to speculate that inner-membrane lipids exposed during crystal cell rupture might activate proPO, similar to what happens during platelet activation in vertebrate blood clotting [23, 24]. Supporting this idea, we found we could significantly superinduce melanization in ex vivo hemolymph preparations from noninfected an-

imals using inner-membrane lipids [PS and phosphatidylinositol (PI); fig. 3A, B], which are hallmarks of damaged cells. These in vitro data are consistent with the melanization we observed after inducing apoptosis in hemocytes in vivo, which also leads to cell surface exposure of PS and subsequent formation of melanotic aggregates [16] (fig. 3D). Thus, proPO activation can also be induced in vivo in a restricted manner. Conversely, neither of the outer-membrane lipids (PC and PE) led to any significant increase in PO activity in a photometric assay (fig. 3A, B). The bacterial elicitors increased melanization in some cases (for example, for LPS in fig. 3A, and for PGN in some samples in fig. 3B), but in photometric assays, this effect was too variable to be significant. Melanization was completely inhibited when Spn27A was ubiquitously overexpressed (fig. 3B, 27A/OE), supporting our previous data from histological clot preparations [16].

Fig. 3. PO activity is induced by endogenous signals. **A** The outer-membrane lipid PC, bacterial LPS and the inner-membrane lipid PS were mixed with *Drosophila* hemolymph, and melanization was determined using a dot blot assay [36]. ctrl = Control sample with solvent alone. The experiment was repeated twice with the same result. **B, C** Hemolymph from *Drosophila* (**B**) and *Galleria* (**C**) was mixed with the same lipids as in **A** and in addition with bacterial PGN, PE and PI. Change of OD was measured as described (see Materials and Methods). Samples that differ significantly from the control are indicated by an asterisk. p values are: PS: 0.014; PI: 0.038; LPS: 0.0019; PGN: 0.00095. The *Drosophila* set was repeated in quadruplicate (see Materials and Methods for further details). When Spn27A was overexpressed ectopically using a tubulin driver line (27A-OE [16]), melanization was inhibited completely and was not detectable (ND). **D** Expression of the apoptotic inducer Grim in hemocytes (*heGal4* crossed to *UAS-grim*) leads to the formation of melanotic aggregates (some of which are indicated by arrows [see also 16]).

Fig. 4. proPO is not activated in clots from Spn27A mutants. Direct bleeds (**A**) and hanging drop preparations (**B–I**) from *spn27A* mutants (left) and control larvae (right) showing strong systemic activation in *spn27A* as described before [4, 5] (the insets in **D** and **E** show PO activity detected with the dot blot assay [36]). **F–I** Clot preparations from *w¹¹¹⁸* (**F, G**) and *spn27A* (**H, I**) larvae were reconstituted with DOPA. All microscopic figures are shown in both phase-contrast (**B, C, F, H**) and transmission microscopy (**D, E, G, I**).

Fig. 5. Model of proPO activation in the clot. After release from crystal cells, PO is activated in a complex containing activating proteases and inner-membrane lipids released during crystal cell rupture. Inside the clot, proPO-activating complexes have limited contact with Spn27A, due to restricted diffusion of Spn27A into the clot matrix, while outside the clot, the proPO-activating proteases are inhibited. nu = Nucleus.

4

5

The superactivation of proPO was different in *Galleria*. Here, both LPS and PGN significantly superinduced PO activity, while PS showed only marginal, nonsignificant effects, and neither PC nor PI had any effect (fig. 3C). This indicates that during activation of proPO after bleeding, endogenous signals such as inner-membrane lipids can act as activators in some species and not in others. In summary, proPO activation after injury appears to differ between *Drosophila* and *Galleria*; in *Drosophila*, activation is more locally restricted and can be enhanced by endogenous signals, while in *Galleria*, activation is more systemic and can be superinduced by microbial elicitors but not by endogenous lipids.

proPO in the Clot Is Not Activated in Drosophila Spn27A Loss-of-Function Mutants

Our results showed that in noninfected *Drosophila* larvae, PO activity is limited to the clot matrix, where it contributes to clot hardening and sequestration of bacteria [10]. We wished to relate these findings to previous studies on proPO activation in *Drosophila* that had been performed after septic injury. Following infection, *Drosophila* Spn27A is transiently degraded, allowing systemic activation of proPO (see Introduction). Spn27A loss-of-function mutants thus resemble infected animals and show high PO activity in their hemolymph [4, 5]. Surprisingly, in spite of the absence of Spn27A and subsequent high systemic PO activity (fig. 4A, inset in fig. 4D), the clot from *spn27A* mutants showed no signs of melanization (fig. 4D). Similarly, clots from mutants expressing a dominant active form of Toll (Tl⁸) mimicking immune activation during septic injury show no sign of melanization (not shown), in spite of the presence of melanotic masses in Tl⁸ hemolymph [20, 25]. The lack of melanization in *spn27A* mutants appeared to be due to limited availability of PO substrate in the clot, since exogenous addition of DOPA restored clot melanization (fig. 4H, I) to a level which was comparable to or even stronger than that in normal animals treated identically (fig. 4F, G). Hemolymph preparations from *spn27A* mutants looked similar to those from *Galleria*, with high systemic PO activity and weak PO activity in the clot (compare fig. 2 and 4).

Discussion

We have incorporated our findings into a model for proPO activation in *Drosophila* (fig. 5). Based on our finding that melanization in the clot can be inhibited by

spn27A (fig. 3B) [16], we propose that clot proPO is activated proteolytically. Following wounding, crystal cells are activated and rupture [16] and proPO is released and forms complexes with activating proteases and aminophospholipids such as PS that are also released during cell rupture. Association with activating proteases may be facilitated by the fact that some of them are expressed in crystal cells, for example CG3066 (or MP2 [6, 8]). Association with lipids may facilitate proteolytic cleavage, in agreement with previous findings that slightly denaturing conditions can activate proPO [summarized in 26]. We propose that normally proPO activation is limited to the clot matrix by Spn27A, which has limited access to the clot but inhibits any proPO activation outside the clot. In many cases, the formation of a clot matrix will be sufficient to sequester any microbes that have entered via the wound, thus preventing sepsis. In case bacterial entrapment by the clot is insufficient and results in bacteremia, microbial products trigger the transcriptional activation of immune genes and activate a humoral response, including breakdown of Spn27A and systemic activation of proPO [4, 5].

Thus, in *Drosophila*, one and the same immune reaction, i.e. melanization, is activated differently at different times and places following injury. Endogenous triggers such as lipids (this report) and basement membrane components released during injury [summarized in 27, 28] activate proPO at the clot soon after wounding, contributing to crosslinking and melanization independent of bacterial elicitors. This is somewhat unexpected since microbial breakdown products are likely present in high concentrations in decaying fruits, where *Drosophila* larvae usually feed. In the case of a septic infection, however, it has been shown that microbial products do cause systemic gene transcription-dependent proPO activation [4, 5].

The situation appears to be different in *Galleria*, where proPO activation at the pre-septic stage is more systemic and sensitive to microbial elicitors before the onset of any transcriptional activation (fig. 1A, B, 2A, B, 3C). A possible molecular mechanism to explain the systemic melanization is suggested by the similarity of clots from *Galleria* and *Drosophila spn27A* mutants. According to our model (fig. 5), the different mode of activation of *Galleria* proPO might be due to a lower concentration of inhibitory activities such as Spn27A allowing activation of proPO even outside the clot matrix. In addition, the availability of PO substrates may influence its activity [29] (fig. 4).

At this stage, we can only speculate on the environmental and evolutionary factors that determine how proPO is activated in different animals. It may be that more localized and endogenously activated melanization ensures fast and efficient crosslinking of clot fibers in small animals like *Drosophila* and mosquitoes, where clot melanization appears similar [10, 14, Hauling et al., unpubl. results]. Reliance on endogenous signals may on the other hand enhance the risk of autoimmune reactions. This appears to happen in *spn27A* mutants, where the prevalence of spontaneous melanization increases, leading to a reduction in viability [5].

Our results add new findings to the ongoing discussion regarding the role of PO in innate immunity. It is interesting to note that most of the recent reports arguing against an immune function involve *Drosophila* or mosquitoes, where our data suggest PO is regulated by endogenous signals and contributes primarily to hemostasis – at least at the wound. In contrast, we found that microbial elicitors were more effective at activating proPO in

Galleria, a lepidopteran. Here, the interference with proPO activation caused by virulence factors isolated from parasitoid wasps or bacterial commensals from parasitic nematodes of lepidopterans [30–32] is strong evidence for an immune function of PO.

Taken together, our data suggest that PO activity is used in different ways depending on physiological and ecological requirements. This may caution against generalizations based on studies performed in one particular insect species or with one particular pathogen, and is in complete agreement with recent studies based on genomic comparisons which show faster evolution for factors that regulate PO activity [2, 33–35].

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