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MAP kinases mediate phagocytosis and melanization via prophenoloxidase activation in medfly hemocytes

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Abstract

E. coli phagocytosis by medfly hemocytes, in contrast to mammalian macrophages, associates with *E. coli*-challenged hemocyte secretion by mitogen activating protein (MAP) kinases. In the present work, we examined whether this system links with the proteolytic activation of prophenoloxidase (proPO). ProPO and prophenoloxidase-activating proteinases (PAPs) were initially identified within freshly isolated medfly hemocytes. Moreover, flow cytometry and immunocytochemical analysis revealed the constitutive expression of proPO and its stable association with hemocyte surface. The expression level of hemocyte surface proPO is not affected by *E. coli* infection. In addition, flow cytometry analysis in freshly isolated hemocytes showed that *E. coli* phagocytosis is markedly blocked by antibodies against proPO or PAPs, as well as by several serine protease inhibitors, strongly supporting the involvement of proPO cascade in the phagocytosis process. Similarly, it was shown that melanization process depends on proPO activation. MAP kinases appeared to control both phagocytosis and melanization, since they regulate PAPs secretion, a prerequisite for the conversion of proPO to active PO. From this and previous studies, hemocytes appear to be central to immune response in medfly.

Keywords: Prophenoloxidase; Mitogen activating protein kinase; Signalling; Phagocytosis; Prophenoloxidase-activating proteinase; Insect immunity

1. Introduction

Insects respond to invading pathogens and parasites by several innate immune mechanisms, such as phagocytosis, encapsulation, nodule formation, melanization and synthesis of anti-bacterial peptides and proteins [1,2]. Some of these processes appear to be controlled by a serine protease cascade that leads to the proteolytic activation of prophenoloxidase (proPO). ProPO is converted to active phenoloxidase (PO) by proPO-activating proteinase (PAP), also known as proPO-activating enzyme (PPAE), and its cofactor [3–5]. The cofactor consists of two serine protease homologues (SPHs) that associate with immulectin-2 (IML-2) [6,7]. The active proPO catalyzes the early steps of the pathway, leading to melanin formation. It has been assumed that through this pathway, highly reactive and toxic quinone intermediates are produced, which are probably lethal to invading pathogens [8–10]. Although much information has been accumulated concerning the components of proPO cascade and their molecular interaction, the signal transduction pathways leading to the activation of this cascade is still largely unknown.

Hemocytes are the primary mediators of cell-mediated immunity in insects. Their response to pathogen invasion consists of either regulated release of biologically active molecules or endo-phagocytosis. The signalling pathway of these processes is largely unknown. Recently, it has been demonstrated that medfly hemocytes respond to LPS/*E. coli* by changing their morphology and inducing the activation of several signalling pathways, such as MAP kinases,

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phosphatidylinositol-3 kinase (PI-3K) and Rho pathways [11,12]. Furthermore, it has been observed that extracellular signal-regulated kinase (ERK) and p38 pathways, but not c-*jun* N-terminal kinase (JNK), are involved in LPS-dependent hemocyte secretion, whereas all MAP subfamilies participate in *E. coli*-dependent secretion. In addition, flow cytometry analysis in hemocytes has shown that the LPS/*E. coli*-induced release is a prerequisite for LPS/*E. coli* uptake [11]. Similar data has also been demonstrated in a mosquito cell line (C6/36), where it was observed that phagocytosis involves the JNK but not the ERK or p38 pathway [13]. *E. coli* phagocytosis by medfly hemocytes also depends on integrins, via the activation of the focal adhesion kinase (FAK)–Src complex [14,15].

As stated above, LPS/*E. coli*-challenged hemocytes induce MAP kinase activation and secretion. The latter appears to be a critical downstream target of MAP kinases, required for endo- and phagocytosis. On the other hand, hemocytes appear to synthesize and secrete proPO at all times, with the expression level not affected by infection [1]. In addition, PAPs are part of an extracellular serine protease cascade required for proPO activation [1,16]. The concentration in hemocytes and their release in larval plasma increases on bacterial challenge [3–5]. The above data prompted us to study any possible relationship among the MAP kinase subfamilies ERK, p38, JNK signalling pathways, proPO cascade activation and phagocytosis/melanization, the major defense mechanisms in insects.

2. Materials and methods

2.1. Antibodies, inhibitors and activators

Polyclonal antibodies against *Manduca sexta* prophenoloxidase (proPO) were kindly offered by Prof. Michael R. Kanost (Department of Biochemistry, Kansas State University, Manhattan, KS 66506, USA). Polyclonal antibodies against *M. sexta* prophenoloxidase-activating proteinases (PAP1, PAP2 and PAP3) were kindly offered by Dr. Haobo Jiang (Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK 74078, USA). Goat anti-rabbit IgG-HRP (horseradish peroxidase conjugated) was purchased from Cell Signaling Technology (Beverly, MA, USA), while goat anti-rabbit IgG-FITC (fluososcein isothiocyanate conjugated) was from Santa Cruz Biotechnology. U0126 and PD98059, MEK1/2 inhibitors, were obtained from Cell Signaling Technology. The cell secretion inhibitors brefeldin A (from *Penicillium brefeldianum*) and SITS (4-acetamido-4′-isothiocyanatostilbene-2, 2′-disulfonic acid disodium salt hydrate), as well as SB203580 and SB202190, inhibitors of p38 and the LPS–FITC conjugate, were obtained from Sigma (St. Louis, MO, USA). SP600125, an inhibitor of JNK, was obtained from Calbiochem (USA). Serine protease inhibitors aprotinin, leupeptin, pepstatin and PMSF were obtained from Sigma. Amerlex-M second antibody reagent was purchased from Amersham Life Science (UK). Other reagents were obtained as indicated.

2.2. FITC-labelling of E. coli

Fluoroscein isothiocyanate (FITC)-labeled *E. coli* (DH10B) was prepared after the incubation of 10^8 heat-killed bacteria with 1 mg FITC, in 0.5 ml 0.5 M Na_2CO_3/0.5 M $NaHCO_3$, pH 9.5, for 30 min in the dark. FITC-conjugated *E. coli* was rinsed three times with PBS, resuspended in Grace's medium and stored in aliquots at −20 °C.

2.3. Collection of hemocytes and cell-free hemolymph

C. capitata was reared as described previously [17]. The isolation and homogenization of 3rd instar larvae hemocytes were performed according to Charalambidis et al. [18]. In brief, hemolymph was collected and centrifuged at 2000×*g* for 10 min at 4 °C. Sedimented hemocytes were washed 3 times with Ringer's solution (128 mM NaCl, 18 mM $CaCl_2$, 1.3 mM KCl and 2.3 mM $NaHCO_3$, pH 7.0). To collect hemolymph from *B. mori*, 5th instar, 4- to 6-day larvae were immobilized on ice, rinsed with 70% ethanol and bled by proleg puncture. Hemolymph was collected in an eppendorf tube and centrifuged at 2000×*g* for 10 min at 4 °C to separate hemocytes from the plasma. Sedimented hemocytes were washed 3 times with Ringer's solution. The viability of hemocytes was assessed by the exclusion of trypan blue dye (Sigma, USA) under a microscope.

2.4. Immunoprecipitation

Hemocytes were lysed in lysis a buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 5 mM NaF, 1 mM PMSF, 10 μg/ml leupeptin and 10 U/ml aprotinin) at 4 °C. Insoluble material was removed by centrifugation (16,000×*g* for 15 min at 4 °C) and the supernatant was collected. For immunoprecipitation, 400 μg of crude extract protein was incubated with 2 μg anti-proPO, -PAP1, -PAP2 or -PAP3 polyclonal antibody for 2 h at 4 °C and then for an additional hour at 25 °C with an Amerlex-M secondary antibody reagent (Amersham Life Science, UK). The immune complexes were washed 4 times with TBS. Proteins were eluted from the beads by boiling the samples for 3 min in 50 μl of electrophoresis sample buffer. The samples were electrophoresed and immunoblotted with anti-proPO, -PAP1, -PAP2 or -PAP3 polyclonal antibodies.

2.5. Protein determination

Protein concentration was determined according to Bradford [19], with a modified solution containing 10%

(w/v) Coomassie G250 (Merck, Germany) in 5% (v/v) ethanol, 10% (v/v) H_3PO_4. OD was recorded at 595 nm.

2.6. SDS-PAGE and immunoblot analysis

SDS-PAGE was performed on 10% acrylamide and 0.10% bisacrylamide slab gels, according to Laemli [20]. Immunoprecipitants were electrophoretically analysed and electroblotted onto Immobilon P polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA, USA). Membranes were incubated in a SuperBlock™ Blocking Buffer (Pierce Rockford, IL, USA) for 1 h at room temperature. Subsequently, membranes were incubated overnight at 4 °C with the primary antibody (anti-proPO, -PAP1, -PAP2 or -PAP3) diluted 1:2000 in TBS (10 mM Tris–HCl, pH 7.5, 100 mM NaCl) containing 10% (v/v) SuperBlock™ Blocking Buffer and 0.05% (v/v) Tween 20. Membranes were washed with TBS containing 0.05 (v/v) Tween 20, followed by incubation with horseradish peroxidase-linked secondary antibody (Cell Signaling Technology, USA) for 1 h at room temperature. Immunoreactive proteins were visualized on X-ray film by the enhanced chemiluminescence (ECL) methodology (Amersham, UK). The stripping of membranes after ECL detection was performed according to the manufacturer's instructions (Amersham Pharmacia Biotech). Non-immune rabbit serum was used as a negative control in immunoblot analysis of immunoprecipitants, which did not show any bands (Fig. 1b). Prestained Protein Markers, broad range, were use to indicate the size of the protein bands (Cell Signaling Technology, USA).

2.7. Immunocytochemistry

Isolated hemocytes were suspended in 200 μl Grace's medium (5×10^5 cell/sample) and allowed to attach on glass slides for 10 min at 25 °C. The slides were washed with Ringer's solution to remove non-adherent hemocytes. The resulting monolayers were fixed with 4% formaldehyde solution for 10 min. Hemocytes were treated with a protein blocking agent for 10 min to reduce non-specific binding. Slides were incubated with anti-proPO or anti-PAPs (1:100) for 1 h at 37 °C in a humid atmosphere. Following antibody treatment, the slides were washed with TBS and further incubated with a polyvalent biotinylated secondary antibody (Kwik kit, IMMUNON, USA) for 10 min. Cells were washed with TBS and incubated with a streptavidin–biotin alkaline phosphatase reagent for 10 min and washed again with TBS. The visualization of the

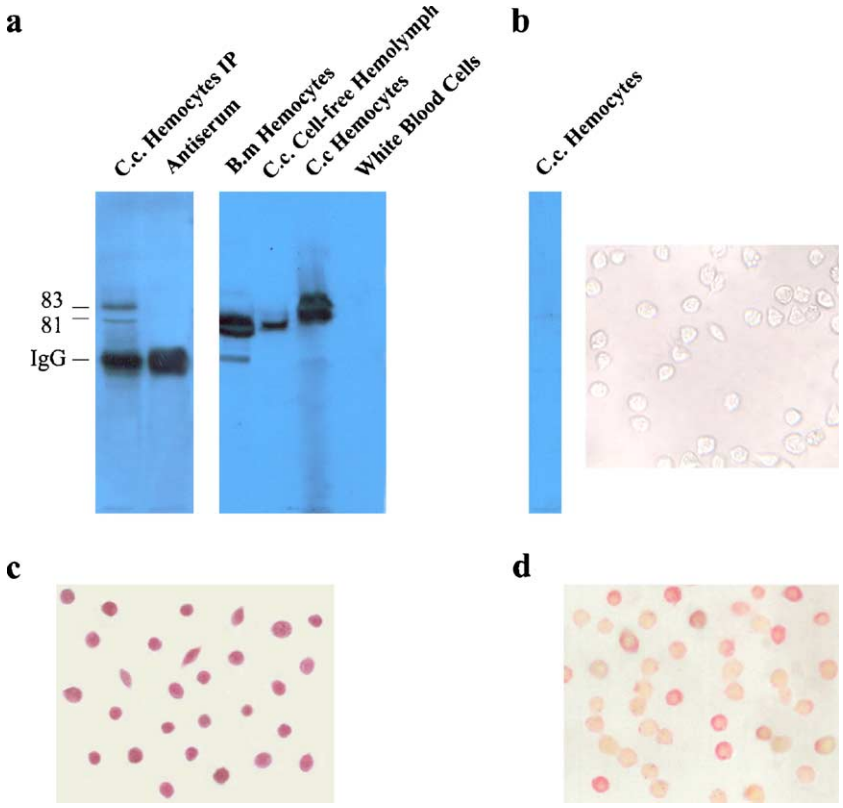

Fig. 1. Identification of prophenoloxidase in medfly hemocytes. Prophenoloxidase was immunoprecipitated from *C. capitata* larvae freshly isolated hemocyte crude extracts. The immune precipitant (IP) and the total hemocyte crude extract showed a doublet of bands at 81 and 83 kDA. Anti-proPO antiserum was analysed as well, showing the IgG band. Immunoblot analyses of *B. mori* hemocyte crude extract and cell-free hemolymph were used as positive controls. Human white blood cell protein extract was also used as a negative control (a). Control experiments were performed for immunoblot analysis and immunocytochemical staining using non-immune rabbit serum (b). Prophenoloxidase was identified immunocytochemically in the cytoplasm (c) and on the surface (d) of hemocytes.

antigen–antibody complex was achieved by incubating the slides with fast red chromogen solution for 20–30 min, until a satisfactory colour developed. Finally, the slides were washed with water and mounted. All steps, except that of primary antibody, were performed at room temperature. Non-immune rabbit serum was used as a negative control (Fig. 1b).

2.8. Flow cytometry analysis

To determine surface proPO, larval hemocytes (5×10^5 cells) were incubated in 100 μl Grace's insect medium with anti-proPO (1:100) for 30 min at 25 °C. Subsequently, FITC-labeled anti-rabbit IgG (Santa Cruz Biotechnology) was added and incubated for 30 min at 25 °C. To study *E. coli* phagocytosis, larval hemocytes (5×10^5 cells) were incubated in 100 μl Grace's insect medium containing *E. coli*-FITC (10 bacteria per hemocyte) for 60 min at 30 °C, in the presence or absence of anti-proPO (1:30), anti-PAPs (1:30), serine proteases (20 U/ml aprotinin, 20 U/ml leupeptin, 1 mg/ml pepstatin and 100 μM PMSF) or cell secretion (20 μM SITS and 35 μM brefeldin A) inhibitors. Internalized *E. coli*-FITC was measured by quenching the attached *E. coli*-FITC with trypan blue 4% in Ringer's solution. Hemocyte suspensions were placed in ice and 400 μl of Ringer's solution was added. Approximately 20,000 cells from each sample were analysed by flow cytometry using a Coulter EPICS-XL-MCL cytometer (Coulter, Miami, FL, USA), and the data was processed using the XL-2 software.

2.9. Immunoenzymic quantization of PAPs secretion

Hemocytes (5×10^5/100 μl) were preincubated in Grace's medium with specific MAP kinase inhibitors (0.4 μM SB203580 or SB202190 for p38, 4 μM PD098059 or 8 μM U0126 for MEK1/2 and 4 μM SP600125 for JNK) or with secretion inhibitors (20 μM SITS and 35 μM brefeldin A) for 30 min. Cells were then stimulated with *E. coli* (10 bacteria/hemocyte) for 30 min at 25 °C. Cell-free incubation media were collected and total protein was determined. Samples were diluted with 150 mM Na_2CO_3-buffered solution, pH 9.0, to a final concentration of 50 mM Na_2CO_3 and 2–4 μg protein/ml. Aliquots of 100 μl from each sample were added in respective wells of 8-well strips (Costar, USA) and left overnight at 4 °C. The strips were emptied and washed four times with TBS containing 0.05% (v/v) Tween 20. To avoid non-specific interactions,

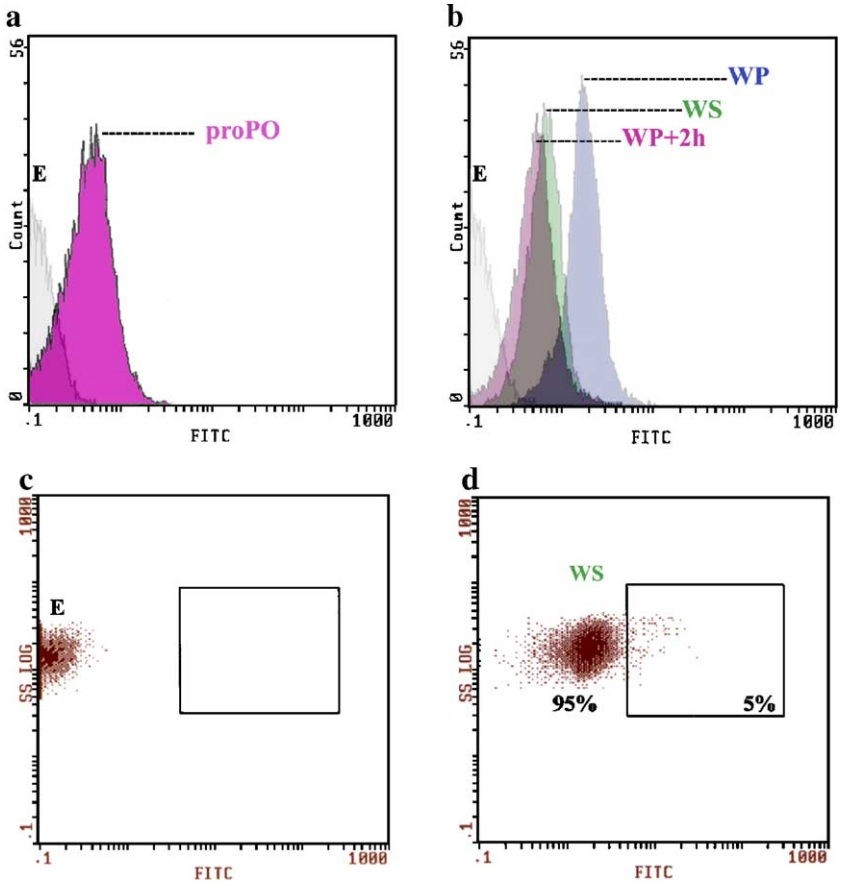

Fig. 2. Identification of prophenoloxidase on the surface of medfly hemocytes by flow cytometry. Indirectly, FITC-labeled proPO is present on the surface of all hemocytes of the 3rd instar larvae (a), with a peak at the white pupa (WP), while seems to be the same at the wandering stage (WS) and 2 h after the white pupa (b). About 5% of the hemocyte population appears to carry 10 times more proPO than does the majority of hemocytes (d). Endogenous fluorescence (E) was presented by analysing hemocyte suspension in the plain medium (a, c).

250 μl SuperBlock™ (Pierce Rockford, IL, USA) was added to all wells, and the strips were allowed to incubate overnight at 4 °C. Secreted PAPs bound onto the wells were detected with a mixture of all three anti-PAPs polyclonal antibodies (1:2000) in TBS 10% (v/v) SuperBlock™ and 0.05% (v/v) Tween 20, for 1 h at room temperature. Wells were washed and incubated with 100 μl goat anti-rabbit IgG antibody labeled with horseradish peroxidase (Cell Signaling Technology, USA) for 1 h at room temperature. After washing, peroxidase activity was determined using tetramethylbenzidine (TMB) as a substrate. The enzymic reaction was stopped with 1 N H_2SO_4, and colour/optical densities were measured at 450 nm with an ELISA reader.

3. Results

3.1. Hemocytes constitutively express proPO and proPO-activating proteinases (PAPs)

Since the serine protease cascade has already been characterized in the hemocytes of several insect species, we were interested to see whether it was present in medfly hemocytes. Therefore, we explored the presence of proPO and the proPO-activating proteinases (PAP1, PAP2 and PAP3) in freshly isolated medfly hemocytes, using antibodies against the lepidopteran *M. sexta* proPO and PAPs, respectively [3–5,21,22]. The immunoprecipitation experiments and immunoblot analysis of hemocyte crude extract with anti-proPO revealed a doublet of bands with a corresponding molecular weight 81 and 83 kDa (Fig. 1a). To show the specificity of anti-proPO, *B. mori* (lepidoptera) hemocyte and cell-free hemolymph were used, instead of *M. sexta*, as positive controls and human white blood cell protein extract as a negative control (Fig. 1). These results and the fact that the sequence of *M. sexta* proPO shows a high degree of identity with other arthropod proPO sequences, including *Drosophila melanogaster* (48% identity) [23], suggest the presence of a homologue proPO in medfly hemocytes.

Immunocytochemical experiments were performed to localize proPO in freshly isolated medfly hemocytes. Immunocytostaining revealed the presence of proPO in the plasmatocytes of 3rd instar larvae, the major type of the

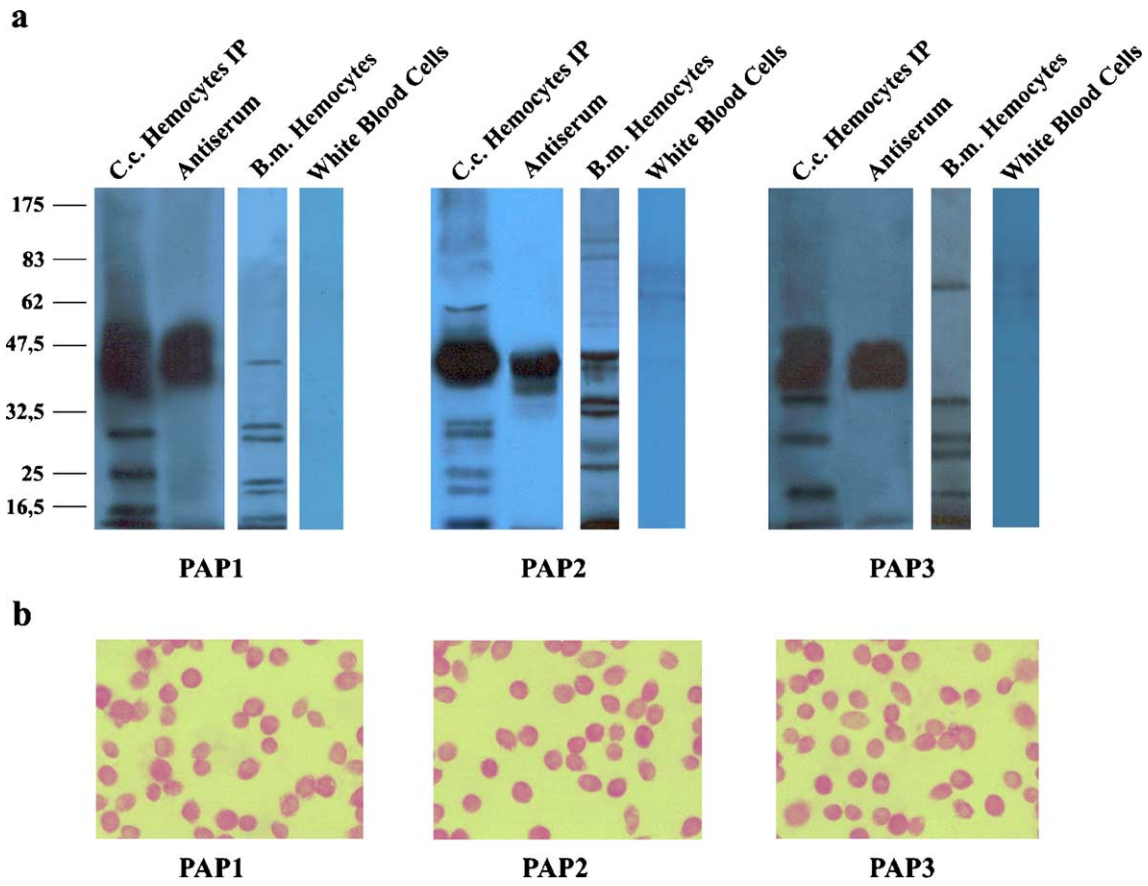

Fig. 3. Identification of prophenoloxidase-activating proteinases in medfly hemocytes. PAPs were immunoprecipitated from *C. capitata* larvae hemocyte crude extracts. The immune precipitants (IP) were immunoblotted with anti-PAP1, -PAP2 and -PAP3. Anti-PAPs antisera were analysed as well, to show the IgG band. The immunoblot analysis of *B. mori* hemocyte crude extract was used as a positive control. Human white blood cell protein extract was also used as a negative control (a). The numbers indicate molecular weights in kDa. PAP1 antiserum slightly cross-reacted with PAP2, and PAP2 antiserum cross-reacted with PAP3. PAPs were identified immunocytochemically in the cytoplasm of the hemocytes (b).

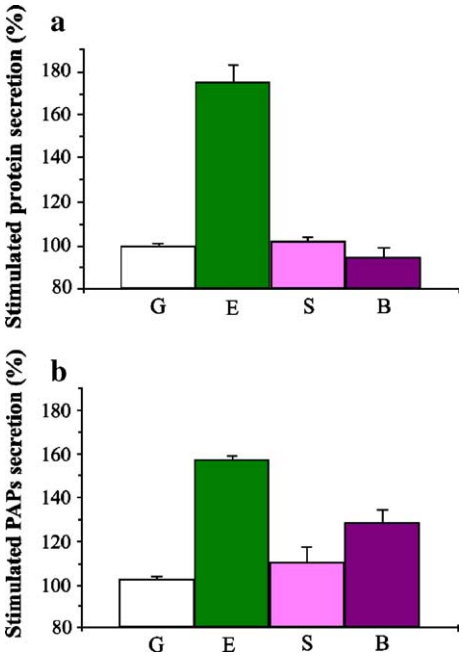

Fig. 4. *E. coli* stimulates the secretion of prophenoloxidase-activating proteinases from cultured hemocytes. Hemocytes were incubated in Grace's medium in the presence of *E. coli* (E), with or without preincubation of the secretion inhibitor SITS (S) and brefeldin A (B). The secreted total protein was determined by the Bradford method (a) and PAPs were determined by ELISA (b) in the cell-free incubation medium. The stimulated secretion (%) was calculated over basal secretion in plain Grace's medium (G).

circulating hemocytes. proPO was identified in the cytoplasm as well as at the surface of the hemocytes (Fig. 1c, d). For more convincing evidence, the presence of proPO on the hemocyte surface was monitored by flow cytometry (Fig. 2a). The results clearly showed that proPO was present on the surface of all hemocytes (Fig. 2c, d). It was interesting that about 5% of the hemocytes appeared to have about 10 times more fluorochrome intensity than the rest of the cells did (Fig. 2d). However, changes in the level of hemocyte-surface proPO were observed during development, with a peak at the white pupae (Fig. 2b). These results demonstrate, for the first time, the constitutive presence of proPO on hemocyte surface, strongly supporting a role for proPO in mediating immune and inflammatory responses.

PAPs are part of an extracellular serine protease cascade required for proPO activation [1,3–5]. Their concentration in hemocytes and their release in larval plasma increase due to bacterial challenge [3–5]. Immunoprecipitation experiments, using antibodies against *M. sexta* PAPs, indicated the presence of PAP1, PAP2 and PAP3 homologues in freshly isolated medfly hemocyte extracts. To show the specificity of the anti-PAPs, *B. mori* (lepidoptera) hemocytes were used, instead of *M. sexta*, as a positive control of anti-PAPs and human white blood cell protein extract as a negative control (Fig. 3). It must be underlined that PAP1 antiserum slightly cross-reacts with PAP2, and PAP2 antiserum cross-reacts with PAP3. PAP3 antiserum appears to be very specific [3–5]. The presence of PAPs in the plasmatocytes of 3rd instar larvae was further confirmed immunocytochemically (Fig. 3b). In addition, it was shown by ELISA that the release of PAPs significantly increased in the incubation medium in hemocyte cultures after *E. coli* challenge, as is the case for PAPs in *M. sexta* (Fig. 4b) [1,3–5]. Secretion inhibitors, SITS and brefeldin A, were used in the control experiments to prevent the stimulated secretion.

3.2. *E. coli* does not affect the expression level of hemocyte-surface proPO

Hemocytes synthesize and secrete proPO at all times [1]. To elucidate the role of the proPO on the surface of hemocytes, we treated hemocytes with *E. coli*, and the level of hemocyte-surface proPO was evaluated by flow cytometry. Fig. 5a clearly shows that both treated and untreated hemocytes with *E. coli* showed similar expression levels of hemocyte-surface proPO. Furthermore, these results indicate that the site of *E. coli* binding on hemocyte surface is independent of surface proPO.

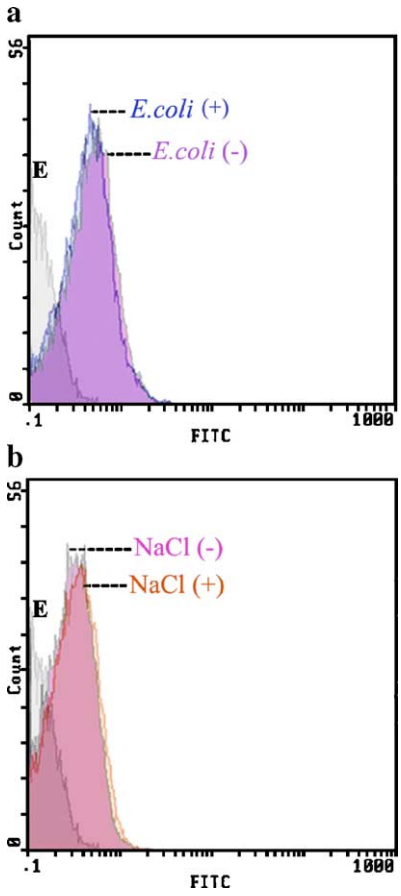

Fig. 5. Surface proPO on medfly hemocytes. Indirectly, FITC-labeled proPO remains the same on the surface of all hemocytes of the 3rd instar larvae after preincubation with *E. coli* (a) or NaCl (b). Endogenous fluorescence (E) was presented by analysing hemocyte suspension in plain medium.

However, these results do not clarify whether hemocyte-surface proPO is an integral part of hemocyte membrane or whether cell-free proPO binds to hemocyte surface. What is known is that the association of proPO with hemocyte membrane is stable, since the treatment of hemocytes with 0.6 M NaCl did not affect the binding of proPO to the hemocyte membrane (Fig. 5b). Consequently, hemocyte-surface proPO is not transient, but rather, a constitutively stable component of plasma membrane.

3.3. proPO cascade, phagocytosis and melanization

Hemocytes respond to infections by phagocytosis, nodule or capsule formation and melanization. They also exhibit a change in their morphology and behavior [11,12,24,25]. The proPO cascade mediates some of these responses. Since proPO and PAPs are present in our system, we focused our interest on whether *E. coli*-challenged hemocyte phagocytosis/melanization is mediated and coordinated by proPO cascade.

Flow cytometry analysis showed that *E. coli* phagocytosis was efficiently blocked by the presence of either anti-proPO or a mixture of all three anti-PAPs (Fig. 6a, b). It must be noted that anti-PAP1, -PAP2 and -PAP3, separately, also blocked *E. coli* phagocytosis (data not shown). The blockage of phagocytosis was also evident when hemocytes were incubated in the presence of several serine protease inhibitors that block, among others, the activation of PAPs (Fig. 6c–g). SITS and brefeldin were used as controls, to prevent the stimulated secretion, as it is clear that *E. coli* phagocytosis by insect hemocytes depends on *E. coli*-challenged secretion [11,15] (Fig. 6h, i). These results strongly support that the activation of proPO via PAPs secretion is a prerequisite for phagocytosis process.

Furthermore, we examined whether *E. coli*-challenged hemocyte melanization is mediated and coordinated by the proPO cascade. Phenoloxidase-based melanization and melanotic encapsulation are well-known immune responses in several insects [26,27]. In *E. coli*-challenged hemocytes, 10 min after infection, a partial melanization response towards cell-free, cell-attached or phagocytosed *E. coli* was

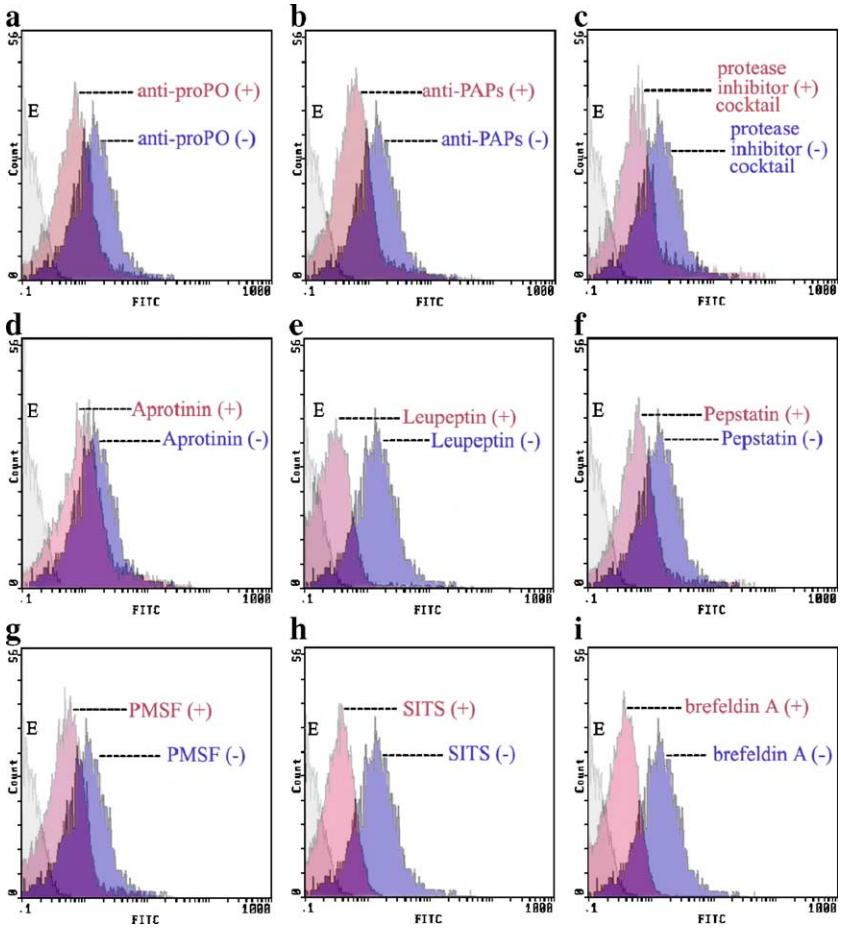

Fig. 6. Prophenoloxidase and its activating proteinases are involved in the phagocytosis of *E. coli* by medfly hemocytes. Hemocytes from the 3rd instar larvae were incubated at 30 °C for 60 min, with FITC-labeled *E. coli* alone or with preincubation with anti-proPO (a), anti-PAPs (b), protease inhibitor cocktail (c), aprotinin (d), leupeptin (e), pepstatin (f), PMSF (g), SITS (h) or brefeldin (i). Endogenous fluorescence (E) was presented by analysing hemocyte suspension in the plain medium.

observed (Fig. 7a). Under the experimental conditions used, the majority of bacteria was attached on hemocytes rather than being phagocytosed by them (data not shown). Melanization appeared under the microscope as a darkening of the *E. coli*. The melanized bacteria were counted on a hemocytometer and were found to be about 40%. Hemocyte viability was tested by trypan blue. One hour later, well-developed hemocyte–*E. coli* aggregates with melanized areas were formed (Fig. 7b). On the contrary, in hemocyte suspensions preincubated with anti-proPO, anti-PAPs or

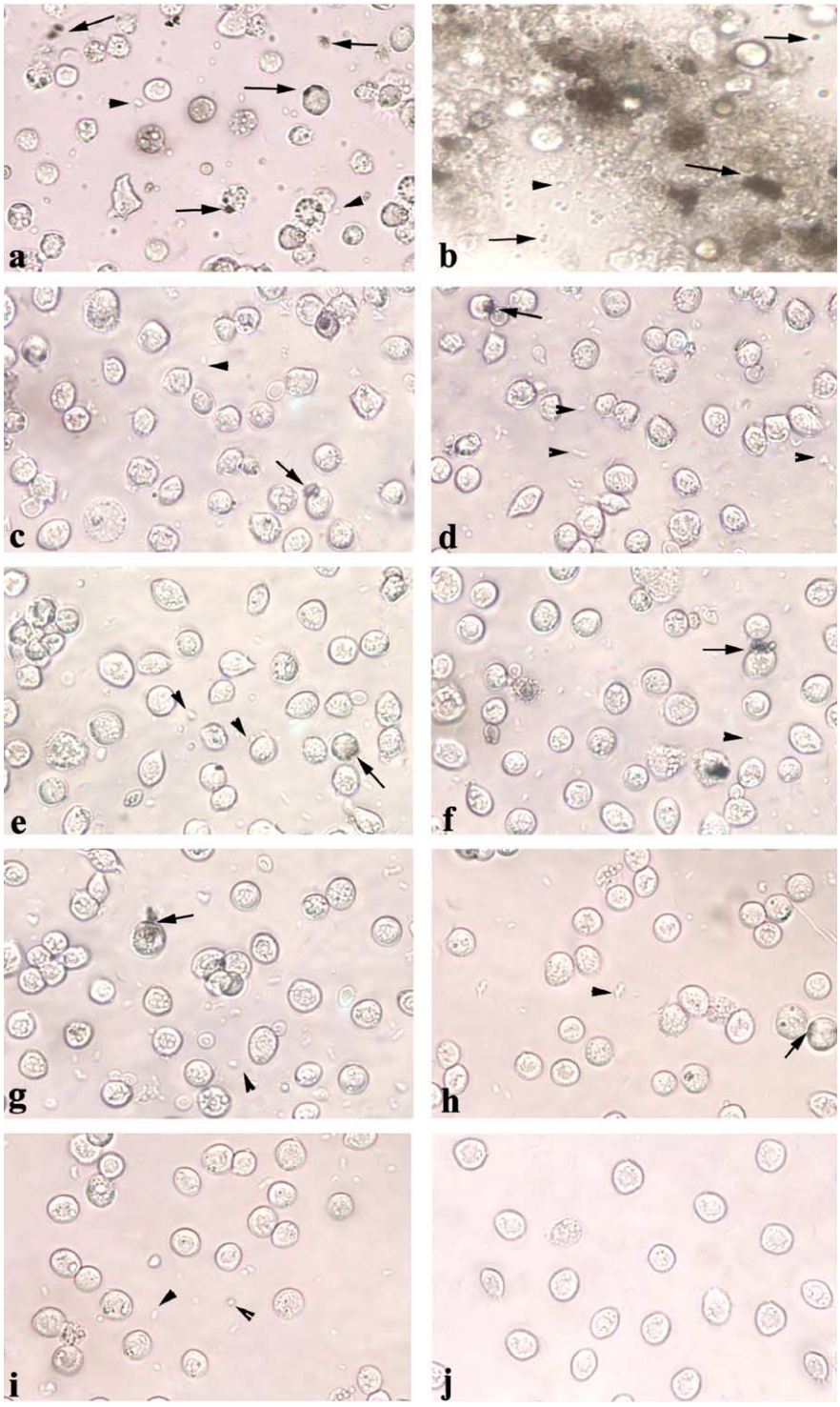

Fig. 7. Prophenoloxidase and its activating proteinases are involved in melanization. Hemocytes were incubated in Grace's medium with *E. coli* alone for 10 min (a) and for 1 h (b) or in the presence of anti-proPO (c), anti-PAPs (d), as well as serine protease inhibitors aprotinin (e), leupeptin (f) and pepstatin (g), SITS (h), and brefeldin A (i). Hemocytes in the plain medium were used as a negative control (j). Arrows indicate melanized bacteria, and arrowheads indicate non-melanized bacteria.

serine protease inhibitors, the cell-free, cell-attached or internalized bacteria remained unmelanized. The melanized bacteria were also counted on a hemocytometer and were found to be less than 10%, although in some cases, small hemocyte aggregates were formed (Fig. 7c–i). These results clearly show that (a) melanization and aggregation are distinct events and (b) proPO cascade participates in hemocyte melanization.

3.4. MAP kinase phosphorylation regulates PAPs secretion

The activation of MAP kinases appears to regulate the LPS/*E. coli*-dependent secretion of TNF-α and IL-1b in macrophages [28]. MAP kinases also regulate LPS/*E. coli*-dependent secretion in medfly hemocytes [11]. These results prompted us to examine whether MAP kinase phosphorylation regulates PAPs secretion also. For this purpose, hemocytes were treated with several inhibitors for ERK, p38 and JNK, and incubation media were subjected to ELISA to determine the secreted PAPs (Fig. 8b). Pretreatment of hemocytes with PD 098059 and U0126, inhibitors of MEK1/2, followed by a 30-min incubation with *E. coli* at room temperature, efficiently suppressed the hemocyte secretion of all PAPs. Similarly, pretreatment of hemocytes with SB203580 and SB202190, inhibitors of p38 kinase, and SP600125, an inhibitor of JNK, also suppressed hemocyte PAPs secretion. Therefore, it is evident that MAP kinases regulate PAPs secretion in response to *E. coli* stimuli and, hence, phagocytosis process.

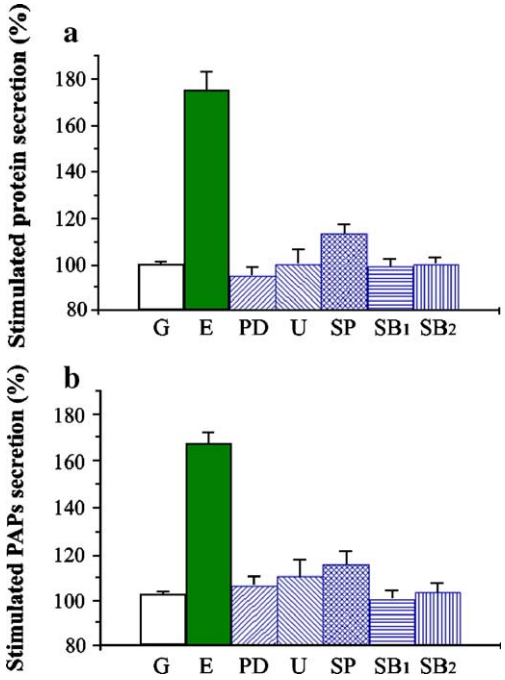

Fig. 8. Prophenoloxidase-activating proteinase secretion is regulated by MAP kinases in *E. coli*-challenged hemocytes. Hemocytes were incubated in Grace's medium in the presence of *E. coli* (E), with or without preincubation of the MAP kinase inhibitors PD98059 (PD), U0126 (U), SP600125 (SP), SB203580 (SB1) and SB202190 (SB2). The secreted total protein was determined by the Bradford method (a), and PAPs were determined by ELISA (b), in the cell-free incubation medium. The stimulated secretion (%) was calculated over basal secretion in plain Grace's medium (G).

To explore the involvement of the MAPK pathway in the melanization process, the ability of several MAPK inhibitors to affect melanization was tested. For this purpose, hemocytes were pretreated with either PD 098059 or U0126, inhibitors of MEK1/2, or SP600125, an inhibitor of JNK, or SB203580 or SB202190, inhibitors of p38, followed by the addition of *E. coli*. The results clearly show that the presence of MAP kinase inhibitors blocked melanization, since the percentage of melanized bacteria reduced from about 40% to less than 8% (Fig. 9). The blockage of *E. coli* phagocytosis in the presence of MAP kinase inhibitors has already been demonstrated [11]. Consequently, MAP kinases are required for both phagocytosis and melanization, since they regulate PAPs secretion, a prerequisite for proPO activation.

4. Discussion

The main aim of this study was to elucidate the possible involvement and regulation of proPO cascade in hemocyte-mediated phagocytosis/melanization processes in *E. coli*-challenged hemocytes. Here, we show that proPO and PAPs are constitutively present in the cytoplasm of freshly isolated medfly hemocytes (Figs. 1 and 3). In addition, flow cytometry analysis demonstrated the presence of proPO on hemocyte surface and its stable association with the membrane, since 0.6M NaCl did not affect the binding of proPO to the hemocytes (Figs. 2a and 5b). Thus, a question arises concerning the proPO function due to its constitutive expression on insect plasmatocyte surface.

ProPO activation is a type of insect immune response. It is synthesized in various hemocyte types, depending upon insect species [1,2]. ProPO expression level is not affected by infection ([1], present results). A current model for the proPO activation system, based on available data, suggests that cell-free proPO is activated by proteolytic cleavage that is stimulated upon infection. This response is analogous to the complement system and the coagulation pathway in mammals [29–31]. The reactions mediated by proPO activation then result in wound healing and melanization [1,32]. Active PO also participates in sclerotization and pigmentation processes in many insect tissues [1,16,32].

The signalling pathways involved in vertebrate immunity are well documented. The signal transduction pathways involved in immune responses, such as phagocytosis and melanization by insect hemocytes, have not been largely characterized. Recently, we showed that insect hemocytes, key mediators of cell-mediated immunity in insects, respond to LPS/*E. coli* challenge by the activation of ERK, p38 and JNK in a Ras/Rho-dependent manner, similarly to mammalian monocytes/macrophages [11,12,33].

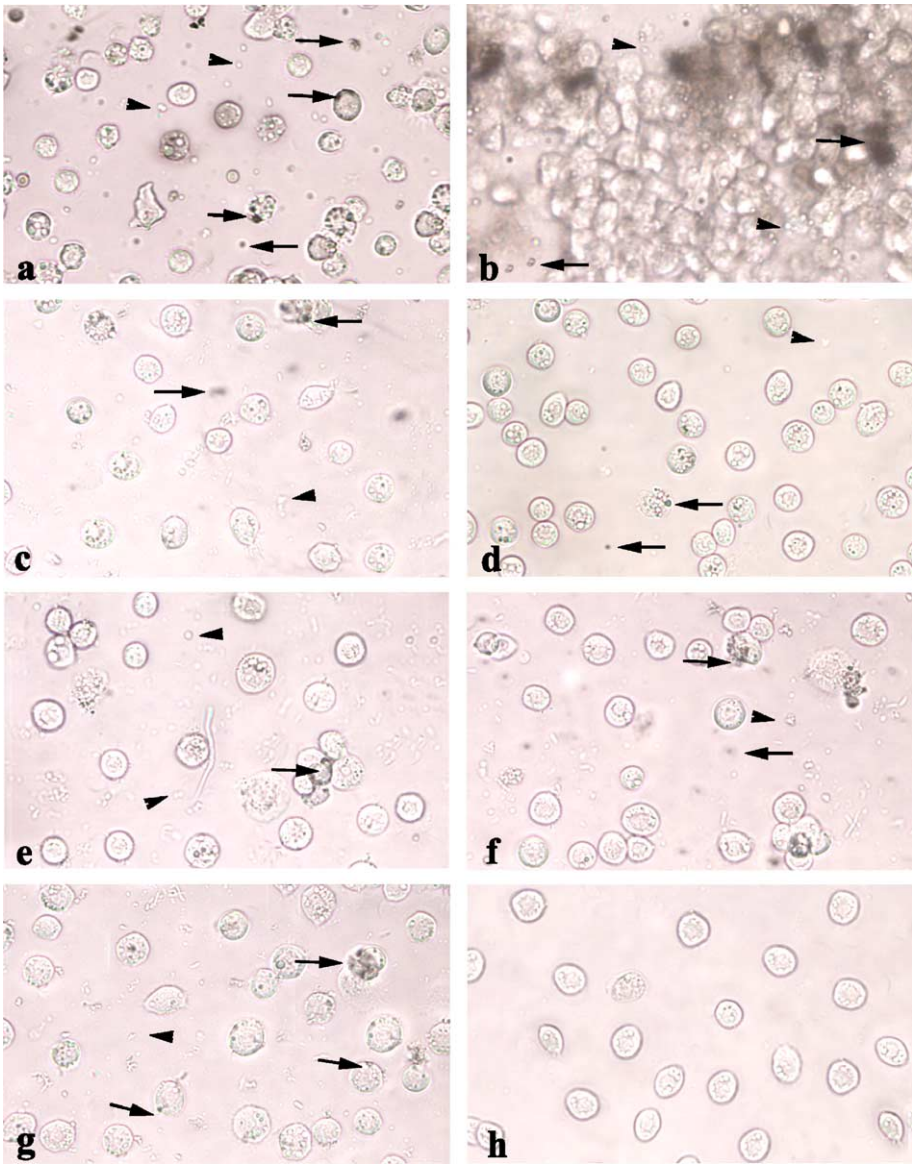

Fig. 9. MAP kinases regulate melanization in *E. coli*-challenged hemocytes. Hemocytes were incubated in Grace's medium with *E. coli* alone for 10 min (a) and for 1 h (b) or in the presence of the MAP kinase inhibitors SB203580 (c), SB202190 (d), SP600125 (e), PD98059 (f) and U0126 (g). Hemocytes in the plain medium were used as a negative control (h). Arrows indicate melanized bacteria, and arrowheads indicate non-melanized bacteria.

The MAP kinase family is a well-characterized intracellular evolutionary conserved phosphorylation cascade and is implicated in the regulation of differentiation control, cell proliferation, development, inflammatory response and apoptosis [34–36]. Three major subfamilies have been characterized, the extracellular signal-regulated kinases (ERKs), the c-*jun* N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs), and the p38 mitogen activated protein.

The comparable responses of the MAP kinase pathways to the LPS/*E. coli* challenge between insects and mammals suggest that MAP kinase signalling in innate immunity may have been conserved through evolution. This activation regulates, among others, the LPS/*E. coli*-dependent secretion, as well as phagocytosis, both in mammalian cell systems and insect hemocytes [11,12,28,37]. However, only in insect hemocytes is the regulated secretion in response to LPS/*E. coli* a prerequisite for LPS/*E. coli* uptake [11,12,15]. Evidently, this means that one or more of the secreted bioactive molecules from hemocytes, in response to LPS/*E. coli*, are somehow actively involved in the internalization of LPS/*E. coli*.

To investigate this hypothesis, we studied the secreted bioactive molecules in response to LPS/*E. coli* and their possible involvement in the phagocytosis and melanization processes. In this paper, we conclusively demonstrated that, in response to LPS/*E. coli*, hemocytes secrete PAPs (Fig. 4), the enzymes that convert proPO to the active PO, which catalyzes the early steps of the pathway leading to melanin formation. In addition, as stated above, we observed that proPO is present on the surface of all freshly isolated hemocytes (Figs. 1 and 2).

In insects, melanization is a critical process in defense against bacteria. A recent report demonstrates that the bacteria tested were melanized, and most importantly, this process appeared to facilitate phagocytosis [26,27]. Moreover, studies in other invertebrates also have linked components of the melanization cascade with phagocytosis [38]. In the current study, we observed that phenoloxidase-based melanization, an innate immune mechanism in insects, works very well in our system (Fig. 7). The mechanism appeared to be dependent, among others, on the secretion of PAPs in response to LPS/*E. coli* via MAP kinase activation (Figs. 8 and 9). In parallel, we observed by flow cytometry analysis that the blockage of the secreted PAPs with their corresponding antibodies largely inhibited the phagocytosis of *E. coli* (Fig. 6). Similar results were obtained using several serine protease inhibitors that blocked PAPs activity. The use of anti-proPO antibodies inhibited phagocytosis as well (Fig. 6). Consequently, the blockage of proPO activation markedly inhibits both melanization and phagocytosis — strongly supporting the involvement of proPO cascade in these processes. It is not clear whether phenoloxidase-based melanization facilitates phagocytosis; however, these unrelated procedures appear to use a common enzyme, phenoloxidase. As is well known, PO creates protein cross-links via quinone intermediates, as in cuticle sclerotization. Therefore, in phagocytosis, PO might be necessary for the stable connection of proteins on the cell surface with pathogens. On the other hand, in melanization, PO is the enzyme for the first step of the conversion of phenols to quinones, which takes place in the serum. [26,27,38].

This study documents the involvement of proPO cascade in medfly hemocyte immune responses against bacteria and conclusively shows that phagocytosis and melanization responses are involved. Both processes appear to be controlled by MAP kinases, since they regulate PAPs secretion, a prerequisite for the conversion of proPO to active PO. This data also provides strong evidence that hemocytes are central to insect immune responses against bacteria.

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