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Translocation of proteins homologous to human neutrophil p47^{phox} and p67^{phox} to the cell membrane in activated hemocytes of *Galleria mellonella*

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Abstract

Activation of the superoxide forming respiratory burst oxidase of human neutrophils, crucial in host defence, requires the cytosolic proteins p47^{phox} and p67^{phox} which translocate to the plasma membrane upon cell stimulation and activate flavocytochrome b_{558}, the redox centre of this enzyme system. We have previously demonstrated the presence of proteins (67 and 47 kDa) in hemocytes of the insect *Galleria mellonella* homologous to proteins of the superoxide-forming NADPH oxidase complex of neutrophils. The work presented here illustrates for the first time translocation of homologous hemocyte proteins, 67 and 47 kDa from the cytosol to the plasma membrane upon phorbol 12-myristate 13 acetate (PMA) activation. In hemocytes, gliotoxin (GT), the fungal secondary metabolite significantly suppressed PMA-induced superoxide generation in a concentration dependent manner and reduced translocation to basel nonstimulated levels. Primarily these results correlate translocation of hemocyte 47 and 67 kDa proteins with PMA induced oxidase activity. Collectively results presented here, demonstrate further cellular and functional similarities between phagocytes of insects and mammals and further justify the use of insects in place of mammals for modelling the innate immune response to microbial pathogens.

Keywords: Human neutrophils; Insect hemocytes; NADPH oxidase; Host defence; Gliotoxin; Phox proteins

1. Introduction

Insects rely upon both cellular and humoral mechanisms to mount a potent antimicrobial defence. Microbial infection results in a range of responses including changes in the hemocyte population [1] and density [2], changes in performance of the hemocytes (i.e. spreading, phagocytosis and nodule/capsule formation) [3], activation of the prophenoloxidase cascade and release of antimicrobial peptides and proteins (i.e. lysozyme, metalloproteinase and defensins) [4].

Given the role of the innate immune response in protecting mammals from microbial infection and the high degree of similarity that exists between the

Abbreviations: ROS, Reactive oxygen species; SOD, Superoxide dismutase; PMA, Phorbol 12-myristate 13-acetate; DPI, Diphenyleneiodonium; GT, Gliotoxin

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mammalian and insect innate immune response, insect models have been developed for the study of microbial virulence [5,6]. Advantages of the use of insects include low cost, ease of rearing in the laboratory, genetic manipulability and fewer ethical considerations than the use of mammalian models [4]. We are interested in developing the use of larvae of the Greater Wax Moth *Galleria mellonella* (*G. mellonella*) which is attracting ever-increasing attention as a model organism for the study of a range of pathogenic bacteria (*Pseudomonas aeruginosa* [5], *Proteus mirabilis* [7], *Escherichia coli, Bacillus cereus,* [8] and *Staphylococcus aureus* [6]) and fungi (*Cryptococcus neoformans* [9], *Aspergillus* and *Candida* species [6,10]). The demonstration of a positive correlation between the virulence of *Candida* mutants in BalbC mice and *G. mellonella* larvae augments the use of *G. mellonella* as a model for evaluating microbial pathogenicity [11].

Immune related proteins and mechanisms that are similar between insects and mammals have been identified. These include the remarkable structural and functional similarities between the systems mediating *Drosophilia* Toll and mammalian IL-1 receptor-mediated signalling [12]. Pattern recognition molecules such as apolipophorin III (apoLp-III) has been identified in insects and found homologous to mammalian apolipoprotein E (apoE) involved in LPS detoxification and phagocytosis [13]. Further lines of defence where direct comparisons can be drawn is in the synthesis of a broad range of antimicrobial peptides [14], which are synthesised by the fat body, released into the open circulatory system and play a crucial role in combating infection [15,16].

Neutrophils play a central role in the innate immune response of mammals and function in a similar manner to phagocytic insect cells (plasmatocytes and granulocytes) by phagocytosing and destroying invading microorganisms [4,15]. The burst in oxidative metabolism associated with activation of either human neutrophils or insect hemocytes results in the manufacture of reactive oxygen species (ROS) as detected by electron spin resonance spectroscopy [17] and more recently by cytochrome c reduction, with evidence of increased oxygen consumption resulting in superoxide (O_2^-) production ($0.25\,\mu M/min/10^6$) by hemocytes of *G. mellonella* [6].

The significance of the oxidase in host defense is evident by the life threatening infections that occur in patients with chronic granulomatous disease (CGD), whose phagocytes are defective in oxidase activity and O_2^- production [18]. The O_2^- generating NADPH oxidase is a multicomponent system consisting of a membrane-bound flavocytochrome b_{558} (composed of two subunits, $p22^{phox}$ and $gp91^{phox}$) [19] and four cytosolic factors, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the small G protein, rac 2 [20]. These cytosolic proteins interact with each other [21,22], with rac [23,24] and with the flavocytochrome [25–27] through a number of *Src* homology 3 (SH3), proline-rich, tetratricopeptide repeat, and PC motifs. Using immunological and matrix-assisted laser desorption ionisation-time of flight analysis (MALDI-TOF), the presence of homologous proteins to $p67^{phox}$ and $p47^{phox}$ were found in insect hemocytes [6] further strengthening the similarities between the oxidative burst pathways in the two cell types.

The cytochrome b_{558} comprises the electron transporting system and forms the membrane-docking site for the translocated cytosolic components. In CGD neutrophils lacking cytochrome b_{558}, neither $p47^{phox}$ nor $p67^{phox}$ can be recruited to the membrane upon cell stimulation [28]. In $p47^{phox}$ deficient phagocytes, membrane targeting of $p67^{phox}$ does not occur whereas $p47^{phox}$ is independently targeted to the membrane in $p67^{phox}$ deficient cells [28,29]. Phosphorylation induced conformational changes in $p47^{phox}$ [30] targets interactions between its SH3 domain and the proline-rich region of $p22^{phox}$ [31,32] an essential step in attaching the translocated $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ complex to the flavocytochrome. The P156Q substitution in $p22^{phox}$, a mutation that has occurred in a case of CGD [27] results in not only impaired interaction between $p22^{phox}$ and $p47^{phox}$ in vitro but also defective translocation of $p47^{phox}$ to the membrane in vivo [33]. Concomitantly rac 2 translocates to the membrane autonomously, with interactions by way of the flavocytochrome and $p67^{phox}$ reported [23,24]. Once activated, the cytochrome takes electrons from NADPH and passes them, via FAD and haem, to O_2 with kinetics of cytochrome reduction correlating with the observed rate of O_2^- generation [34].

Common infectious organisms affecting CGD patients include *S. aureus, Klebsiella, E. coli, Pseudomonas, Serratia marcescens* and also fungi, especially *Aspergillus fumigatus*. Gliotoxin (GT), one of the major metabolites produced by *A. fumigatus* and an inhibitor employed in this study, has received particular attention because it

has potent immunosuppressive [35], genotoxic, cytotoxic [36] and apoptotic effects [37]. In addition this toxin has been associated with cases of clinical aspergillosis [38,39]. Reports have suggested that *A. fumigatus* may retard the action of neutrophils by inhibiting protein kinase C βII-related responses: $p47^{phox}$ phosphorylation, its incorporation to the cytoskeleton, and the subsequent membrane translocation of $p67^{phox}$ and $p47^{phox}$ [40]. In a recent study it was shown that GT directly harms sites crucial for electron transport in flavocytochrome b_{558} [41].

Fundamental experiments are required to further validate the use of insects as an alternative to mammalian models for evaluating microbial virulence. We have previously demonstrated at the cellular level the respiratory burst of insect hemocytes and the presence of proteins (47 and 67 kDa) homologous to neutrophil $p47^{phox}$ and $p67^{phox}$ [6]. However, it remained unresolved whether activation of the NADPH oxidase of hemocytes resulted in translocation of these homologous phox components to the plasma membrane of activated hemocytes. The current study found extensive protein rearrangements accompanying hemocyte activation including translocation of proteins homologous to neutrophil $p67^{phox}$ and $p47^{phox}$ from the cellular cytosolic compartment to a membrane fraction. The effect of GT on oxidase activation and translocation of cytosolic oxidase components was studied with findings further strengthening the similarities between the oxidative burst pathways of neutrophils and insect hemocytes.

2. Material and methods

2.1. Chemicals

All chemicals were of the highest purity and were purchased from Sigma Aldrich Chemical Co. Ltd., Dorset, United Kingdom, unless otherwise indicated.

2.2. Preparation of human neutrophils and insect hemocytes

Blood was obtained from healthy donors and neutrophils were isolated by dextran sedimentation and Ficoll-Hypaque (Axis-ShieldPoC AS, Oslo, Norway) centrifugation [6]. Neutrophils were resuspended in PBS (pH 7.4) containing 5mM glucose (PBS-G) and used immediately.

Storage, handling and inoculation of *G. mellonella* larvae, was as previously described [6]. Insect hemocytes were harvested from healthy, sixth instar, *G. mellonella* larvae (Mealworm Company, Sheffield, United Kingdom) [6]. The cells were washed once in insect physiological saline (IPS) [42] and finally resuspended in PBS-G.

2.3. Effect of GT on superoxide production by PMA stimulated hemocytes

Production of superoxide by hemocytes (1×10^7) following stimulation with Phorbol 12-Myristate 13-Acetate (PMA) (1 μg/ml) was measured by determining the superoxide dismutase (SOD) (50 μg/ml)-inhibitable reduction of cytochrome *c* [43]. Experiments were performed in the presence or absence of diphenyleneiodonium chloride (DPI) (5 μM) [44] or GT (0.01, 0.025, 0.03 or 0.06 μg/ml). Absorbance at 550 nm was recorded with a Synergy HT multi-plate reader (Bio Tek) over 30 min.

2.4. Cellular fractionation of hemocytes and neutrophils

Hemocytes and neutrophils at a concentration of 1×10^8/ml were isolated and stimulated with PMA (1 μg/ml) in the presence or absence of DPI (5 μM) or GT (0.06 μg/ml for hemocytes and 0.25 μg/ml for neutrophils). Following treatment cells were pelleted and Di-isopropyl fluorophosphate (1 mM) added on ice for 10 min. The cells were resuspended in 200 μl Break buffer (10 mM KCl, 3 mM NaCl, 4 mM $MgCl_2$, 10 mM piperazine-N, N′-bis(2-ethanesulfonic acid) (PIPES); pH 7.2) [45] containing protease inhibitors (10 μg/ml leupeptin, pepstatin A, aprotinin, and N-α-p-tosyl-L-lysine chloromethylketone hydrochloride [TLCK]), sonicated (Bandelin Sonopuls; Bandelin Electronics, Germany) three times for 5 s, and centrifuged at 200 × g for 10 min at 4 °C. The postnuclear supernatant (PNS) was layered on top of a continuous sucrose gradient from 10–60% (wt/wt in Break buffer) and centrifuged at 40,000 × g for 3 h at 4 °C in a Beckman SW40 TI head. The percentage sucrose in each fraction was measured using a refractometer (Miltor Roy). Bradford/Protein Assay reagent (Biorad Laboratories) was used to assay the amount of protein in each fraction, using BSA as a standard. Sub-cellular fractionation was also performed on a discontinuous gradient of 10%, 17.5% and 34% sucrose (wt/wt). Membranes at the 17.5%/34%

interface were diluted 1:1 with ice-cold Break buffer and pelleted at 22,000 × g for 30 min.

2.5. Electrophoresis and immunoblotting

Samples from the continuous and discontinuous sucrose gradients were run on SDS-polyacrylamide gel electrophoresis (PAGE) minigels (12.5% acrylamide), and protein profiles visualized by Coomassie blue staining. For Western blotting, the protein was transferred to a nitrocellulose membrane using a semidry blotter for 1 h at 1.4 mA/cm^2. Goat polyclonal antisera raised against human p47^{phox} and p67^{phox} [46] were used at a dilution of 1/1,000 for 1 h. Rabbit polyclonal antibodies against Toll-like receptor 1 (TLR1) were purchased from Abcam, (Cambridge, United Kingdom) and were used at a dilution of 1/1000 for 1 h. Horseradish peroxidase-conjugated rabbit anti-goat antibody (1/5000; Calbiochem, Merck Biosciences Ltd., Nottingham, United Kingdom) was used to detect reactive bands by enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, I11, USA). In certain experiments these ECL bands were quantitated by densitometry using a Syngene Gene Genius, Bio Imaging system.

2.6. Confocal immunofluorescence microscopy

Samples for confocal microscopy were prepared as described previously [47] and viewed with the use of a Olympus Fluoview 1000 confocal microscope. Cells were fixed with 4% (v/v) paraformaldehyde, permeabilized with 0.2% (v/v) Triton X-100 and blocked with 10mM $NaBH_4$. The cells were incubated with rabbit primary antibody against p47^{phox} and p67^{phox} [46] overnight at 4 °C. The slides were washed in PBS and incubated with tetramethyl rodamine iso-thiocyanate (TRITC) goat anti-rabbit IgG secondary antibody (The Jackson Laboratory, Bar Harbor, Maine, USA) for 1 h. The controls for this experiment included cells alone and cells exposed to secondary antibody.

2.7. Statistical analysis

Statistical comparisons were made with student's *t* test using the Sigma Stat statistical analysis package, version 1.00 (SPSS Inc., Chicago, I11). A P value of <0.05 was considered significant.

3. Results

3.1. Effect of GT on PMA activation of NADPH oxidase in hemocytes

Reports of O_2^- production by PMA activated hemocytes [6] and the ability of GT to inhibit O_2^- production by neutrophils [40] prompted an investigation into the effects of GT on O_2^- production by insect hemocytes. O_2^- was measured by determining the superoxide dismutase (SOD) (50 μg/ml)-inhibitable reduction of cytochrome *c* [43] and the characteristics of O_2^- production and cytochrome *c* reduction by hemocytes are summarized in Fig. 1. Basel levels of cytochrome c reduction occurred upon incubation of unstimulated hemocytes (control) within the assay (1.2 ± 0.015 μM/min for a cell population of 1×10^7). Incubation of hemocytes with PMA (1 μg/ml) resulted in increased reduction of the cytochrome, and the O_2^- production was 4.16 ± 0.04 μM/min for 1×10^7 cells. The O_2^- production by hemocytes was reduced to almost control levels when the NADPH oxidase inhibitor DPI (5 μM) [44] was added 5 min prior to the stimulus or 50 μg/ml SOD was added to the cell suspension. When hemocytes were pretreated with

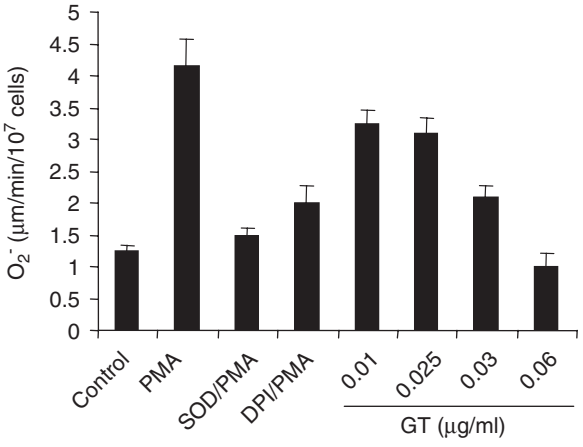


Fig. 1. Effect of DPI or GT on in vitro PMA-stimulated generation of O_2^- by insect hemocytes. The production of O_2^- by unstimulated hemocytes (1×10^7 cells/ml) (Control) and PMA (1 μg/ml)-activated cells was measured using the reduced cytochrome *c* assay. Cellular production of O_2^- was inhibited in the presence of the oxidase inhibitor DPI (5 μM) and scavenged by SOD (50 μg/ml). The inhibitory effect of GT was observed by preincubating cells with increasing concentrations of GT (0.01, 0.025, 0.03 or 0.06μg/ml) for 20 min at 37 °C, prior to the O_2^- generation assay in the presence of PMA (1 μg/ml). Data are means ± S.E. of duplicate assays and representative of three experiments.

GT for 20 min at 37 °C, the O_2^- generation was reduced in a dose-dependent manner. Hemocytes treated with 0.01 μg/ml GT prior to the addition of PMA (1 μg/ml) showed a 22.1% ± 3.3% reduction in O_2^- production to 3.24 ± 0.02 μM/min/10^7 cells. As the dose of GT was increased to 0.06 μg/ml cytochrome *c* reduction was reduced by 76% ± 6.6% with measurements of 1.0 ± 0.04 μM/min/10^7 cells recorded. These decreases in hemocyte O_2^- generation were statistically significant (for DPI, $P < 0.004$; SOD, $P = 0.004$; GT, 0.01 & 0.06 μg/ml, $P < 0.05$) compared to the O_2^- generation of PMA activated cells. The inhibition by 0.06 μg of GT/ml on O_2^- generation by hemocytes was not associated with cell death under conditions employed, as tested by trypan blue dye exclusion.

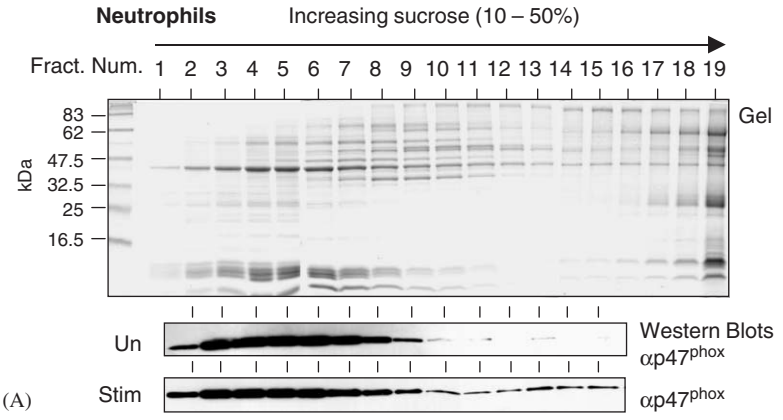
3.2. Membrane translocation of cytosolic protein homologous to $p47^{phox}$ in PMA-stimulated hemocytes

The activation of NADPH oxidase requires the assembly of membrane bound flavocytochrome b_{558} with the cytosolic components $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and rac 2 [20]. Having identified proteins homologous to $p47^{phox}$ and $p67^{phox}$ in insect hemocytes by MALDI-TOF [6], experiments were undertaken to investigate whether these homologous proteins also transferred from the cytosolic compartment to the membrane fraction as a result of cellular activation. Upon subcellular fractionation of neutrophils, it is well established that the membrane fraction is successfully isolated at a 34% (wt/wt) sucrose interface [47]. Fig. 2 shows the result of a study to determine the percentage sucrose at which hemocyte membranes can be isolated. Coomassie stained gels illustrate typical protein profiles of neutrophils and hemocytes fractionated by density-dependent sucrose gradients (10%–50% wt/wt). Fractions were collected and the subcellular distribution of membranes and $p47^{phox}$ located by immunoblotting. In unactivated neutrophils, the cytosolic phox component $p47^{phox}$ was located high up on the density gradient (Fig. 2A, fract. 2–8). As previously described, neutrophil stimulation with PMA (1 μg/ml) caused the translocation of this phox protein to fractions lower in the gradient (Fig. 2A, fract. 12–15) consisting of 34% (wt/wt) sucrose, characteristic of the neutrophil membrane fraction. Antibodies to insect TLR1 (plasma membrane marker, Toll-like receptor 1 [48]) were employed to locate hemocyte membranes within the continuous sucrose gradient. TLR1 reactivity occurred at approximately 34% (wt/wt) sucrose (Fig. 2B, fract. 12–15), indicating that hemocyte membranes located to an equivalent sucrose concentration as neutrophil membranes. Upon PMA activation of hemocytes, polyclonal antibodies against $p47^{phox}$ showed a clear shift of the homologous 47 kDa protein of hemocytes from the cytosolic region of the gradient (approx. 12.5% wt/wt, fract. 6–9) to fractions containing the plasma membrane marker TLR1 (Fig. 2B, fract. 12–15). The sucrose concentration of these fractions (34% wt/wt), was similar to the sucrose concentration to which neutrophil $p47^{phox}$ was shown to migrate (Fig. 2A). This result suggests that the 47 kDa protein of hemocytes was indeed migrating from the cytosol to the plasma membrane upon PMA stimulation and behaves in a similar manner to neutrophil $p47^{phox}$ upon activation of the NADPH oxidase. As previously reported [49] the amount of 47^{phox} that became membrane-associated was a relatively small proportion of that present in the cytosol, as evident with the majority remaining in the higher cytosolic fractions (Fig. 2A, fract 2–8), a phenomenon also observed for the homologous hemocyte 47 kDa protein (Fig. 2B, fract. 6–9).

To further substantiate movement of cellular protein in response to oxidase activation the migration of protein from the cytosolic fraction to the particular cellular compartments that were separated on continuous sucrose gradients was performed employing Bradford/Protein assay (Fig 2C). The protein content of 1×10^8 hemocytes was 12 mg, of which 3.4 mg ± 0.23 mg was in the cytosolic fraction (Fig. 2B, fract. 6–9). Of this 0.45 mg ± 0.12 mg of total cytosolic protein relocated from the cytosolic fractions to the membrane fractions (Fig. 2B, fract. 12–15). This result shows that extensive protein rearrangements accompany hemocyte activation including translocation of the protein homologous to $p47^{phox}$ in insect hemocytes.

3.3. The effect of GT on membrane translocation of cytosolic components in PMA stimulated cells

The NADPH oxidase cytosolic components of neutrophils form an activation complex, which translocates to the membrane to associate with flavocytochrome b_{558} [49]. It has recently been shown that GT inhibits the assembly of these cytosolic phox components [40] by targeting the flavocytochrome b_{558} [41]. Having demonstrated that the 47 kDa hemocyte protein, homologous to


Neutrophils
Increasing sucrose (10 – 50%)
Fract. Num. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
kDa
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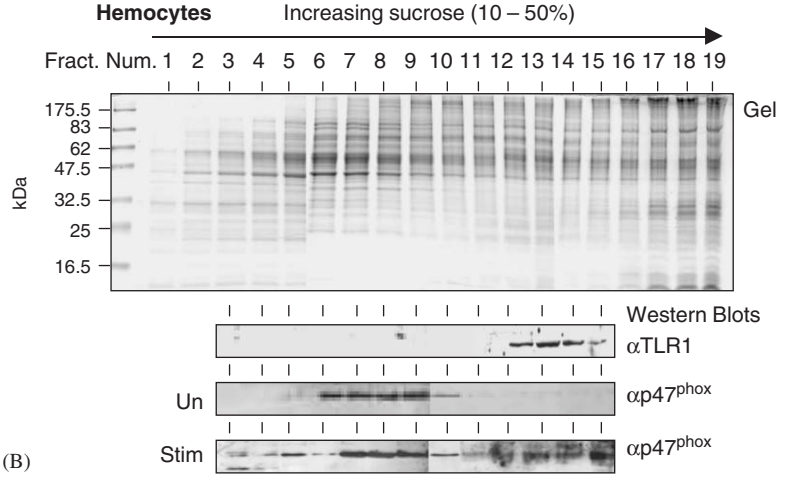
62
47.5
32.5
25
16.5
Gel
Un
Stim
Western Blots
αp47phox
αp47phox

(A)


Hemocytes
Increasing sucrose (10 – 50%)
Fract. Num. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
kDa
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Gel
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Un
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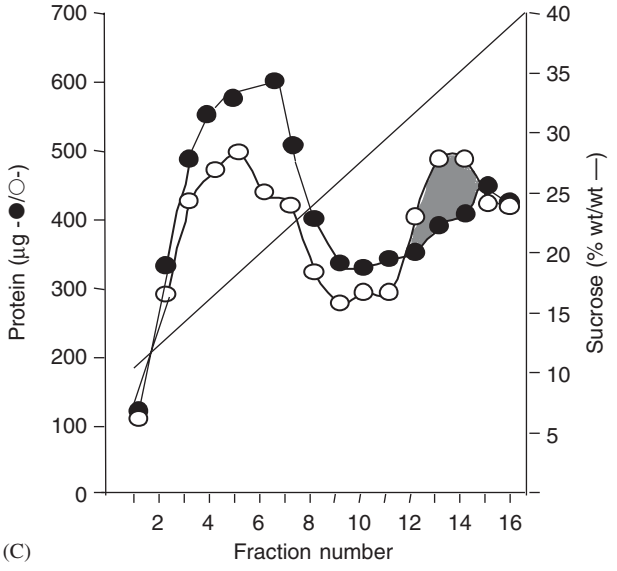
Stim
αp47phox

(B)


Protein (µg -●/○-)
Sucrose (% wt/wt —)
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0
100
200
300
400
500
600
700
5
10
15
20
25
30
35
40
2
4
6
8
10
12
14
16

(C)

p47^{phox} translocated to a membrane fraction upon PMA activation, the ability of this protein and the 67 kDa protein homologous to p67^{phox} [6] to assemble at the membrane in the presence or absence of GT was determined. Discontinuous sucrose gradients were run, with sucrose layers of 17.5% (wt/wt) and 34% (wt/wt) employed to isolate cytosol and plasma membranes respectively (Fig. 3A and B). By Western blotting, confirmation of membrane fractions of both cell types was made employing antibodies against the flavocytochrome gp91^{phox} of neutrophils and TLR1 of hemocytes (Fig. 3C and D). Polyclonal antibodies against human p47^{phox} and p67^{phox} proteins were used to identify the relevant proteins in hemocyte fractions. In unactivated (Un) neutrophils and hemocytes, the phox and homologous 47 kDa and 67 kDa proteins are located in the cytosol (Fig. 3E–H). Subsequent stimulation with PMA (1 μg/ml) caused increased membrane association of these components (Fig. 3E–H). Pre-treatment of hemocytes with GT (0.06 μg/ml) prior to PMA stimulation caused a decrease in oxidase activity (Fig. 1), which correlated with the reduced membrane translocation levels of 47 and 67 kDa proteins (Fig. 3F and H). In Fig. 3H polyclonal antibodies against neutrophil p67^{phox} reacted with the insect hemocyte homologous protein present in the membrane fraction. This immunoreactive band appeared as a doublet possibly due to proteolytic breakdown of the immunologically related protein during experimental procedure.

To extend this study the percentage of untranslocated and translocated proteins present in the cytosol and membranes fractions was determined (Fig. 4). Following PMA stimulation of neutrophils, p47^{phox} and p67^{phox} were distributed between the cytosol and membrane fractions (Fig. 4A and C). The presence of p47^{phox} and p67^{phox} in the membrane fraction increased from 30.5% ± 5.6% to 47.97% ± 5.49% ($P < 0.05$) and 5.58% ± 0.97% to 23.68% ± 2.9% ($P < 0.04$) respectively. Thus

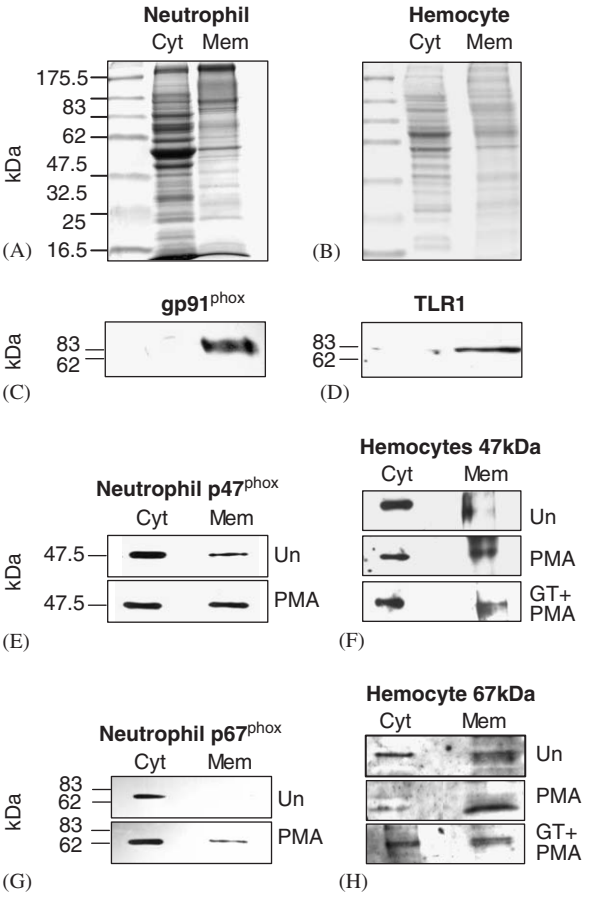

Fig. 3. Distribution of p47^{phox} and p67^{phox} homologues in unstimulated and PMA activated cytosol and membrane fractions. Hemocytes (1×10^8 cell/ml) were pre-treated with GT (0.06 μg/ml) and then PMA (1 μg/ml for 6 min) activated. Coomassie stained gels of neutrophil and hemocyte cytosol (Cyt) and membrane (Mem) fractions isolated on 17.5% and 34% (wt/wt) sucrose are illustrated in A and B. To check membrane purity, antisera to gp91^{phox} (C) was used as a marker for neutrophil membranes and TLR1 for insect hemocyte membranes (D). Aliquots of the unstimulated (Un) or PMA stimulated cytosol (Cyt) and membrane fractions (Mem) of neutrophils (E & G) or hemocytes (F & H) were subjected to immunoblotting with goat antisera to p47^{phox} and p67^{phox}. The results revealed GT (0.06 μg/ml) inhibition of membrane translocation of homologous cytosolic phox components in hemocytes (F & H). The data are representative of three experiments.

Fig. 2. Translocation of the cytosolic components of NADPH oxidase. Neutrophils (A) and hemocytes (B) were either unstimulated (Un) or PMA (1 μg/ml) activated (Stim), disrupted and cellular components separated on continuous sucrose gradient (10–50%). (A) Coomassie blue-stained SDS-PAGE gel (12.5% polyacrylamide) of fractionated PMA activated neutrophil or hemocyte (B) lysate with molecular weight markers indicated on the left. Electrophoretically separated proteins were transferred to nitrocellulose and probed with rabbit antiserum to p47^{phox} or TLR1 as a hemocyte membrane marker. The results revealed the immunologically related hemocyte protein 47 kDa, translocating upon PMA activation to sucrose concentrations of approximately 34%, also containing the membrane TLR1 marker. Data shown are representative of three independent experiments. (C) Separation of cellular lysates was performed on a continuous gradient, and the distribution of total cellular protein in PMA activated hemocytes (-○-) was compared to resting hemocytes (-●-), where shaded/highlighted area indicates movement of protein to fractions containing approximately 34% (wt/wt) sucrose.

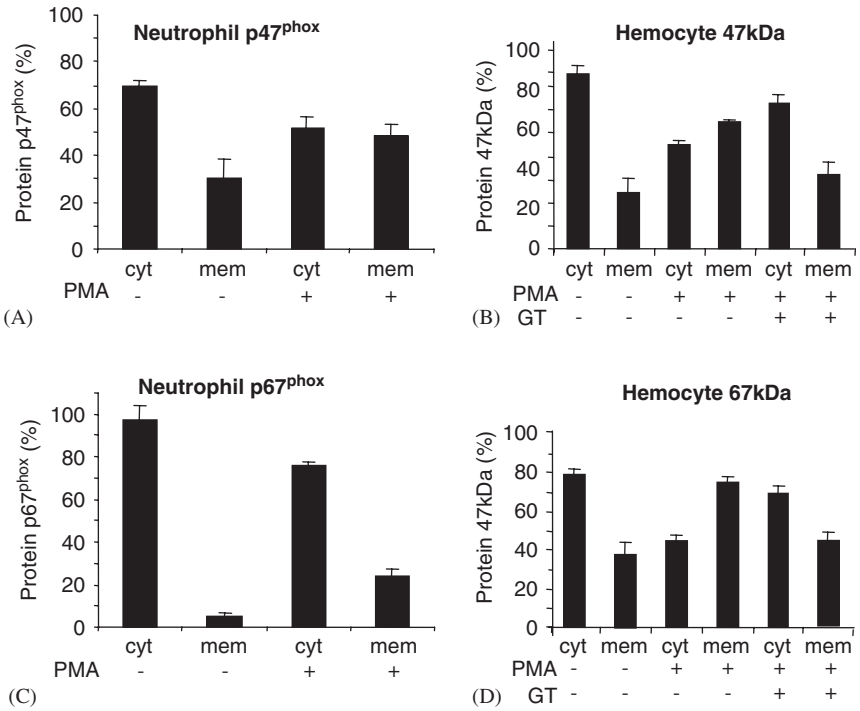

Fig. 4. Effect of GT on membrane assembly of cytosolic components. Subcellular fractions were prepared from unstimulated or GT (0.06 μg/ml) treated cells in the same way as for Fig. 3. Analysis of neutrophil $p47^{phox}$ (A), $p67^{phox}$ (C) and hemocyte 47 kDa (B) and 67 kDa (D) association with the plasma membrane upon PMA (1 μg/ml) stimulation was evaluated by immunoblotting with anti-$p47^{phox}$ and $p67^{phox}$ sera. Immunoreactive bands were detected with an ECL-Plus reaction and quantitated by densitometry. The data are representative of three independent experiments.

approximately 17% of both $p47^{phox}$ and $p67^{phox}$ translocated to the membrane upon PMA stimulation. In insect hemocytes slightly higher levels of translocation were observed with $22.02\% \pm 2.99\%$ ($P < 0.05$) and $21.36\% \pm 3.37\%$ ($P < 0.05$) of proteins homologous to $p47^{phox}$ and $p67^{phox}$ (47 and 67 kDa) translocating from the cytosol to the membranes (Fig. 4B and D). Hemocytes exhibited markedly reduced levels of oxidase activity on the addition of GT (0.06 μg/ml) prior to PMA stimulation (Fig. 1), which corresponded directly to reduced levels of translocation (Fig. 4B & D). In hemocytes where the oxidase had been inhibited by inclusion of GT, translocation was reduced to basal nonstimulated levels.

To further strengthen the evidence that O_2^- production in insect hemocytes is associated with the GT inhibitory translocation of homologous components of the NADPH oxidase complex of human neutrophils, confocal fluorescence microscopy was used to determine the distribution of 47 and 67 kDa in insect hemocytes. TRITC-labelled antibodies to $p47^{phox}$ and $p67^{phox}$ were employed to follow the distribution of these proteins in resting and PMA stimulated cells and to determine the effects of GT. Cells that had attached to a glass coverslip and spread were examined. Results revealed neutrophil *phox* proteins and homologous insect equivalents (47 and 67 kDa) to be predominantly located throughout the cytosol (Figs. 5C–F). After PMA stimulation there was redistribution of a proportion of the *phox* proteins and homologous 47 and 67 kDa to the membrane margins of the cells (Fig. 5G–J) ($p47^{phox}$ and 47 kDa are shown in G and I and had an analogous appearance to $p67^{phox}$ and 67 kDa in H and J). Because of the demonstrated association between the lack of translocation and GT, we examined by confocal microscopy the influence of GT on protein translocation. The striking anomaly observed in cells treated with GT (Fig. K, L, M and N) was the failure of the redistribution of the phox proteins and the complementary hemocyte 47 and 67 kDa proteins after stimulation with PMA.

These results correlate translocation of hemocyte 47 and 67 kDa proteins with PMA induced oxidase activity and secondly indicate that in a similar manner identified in human neutrophils [40], the

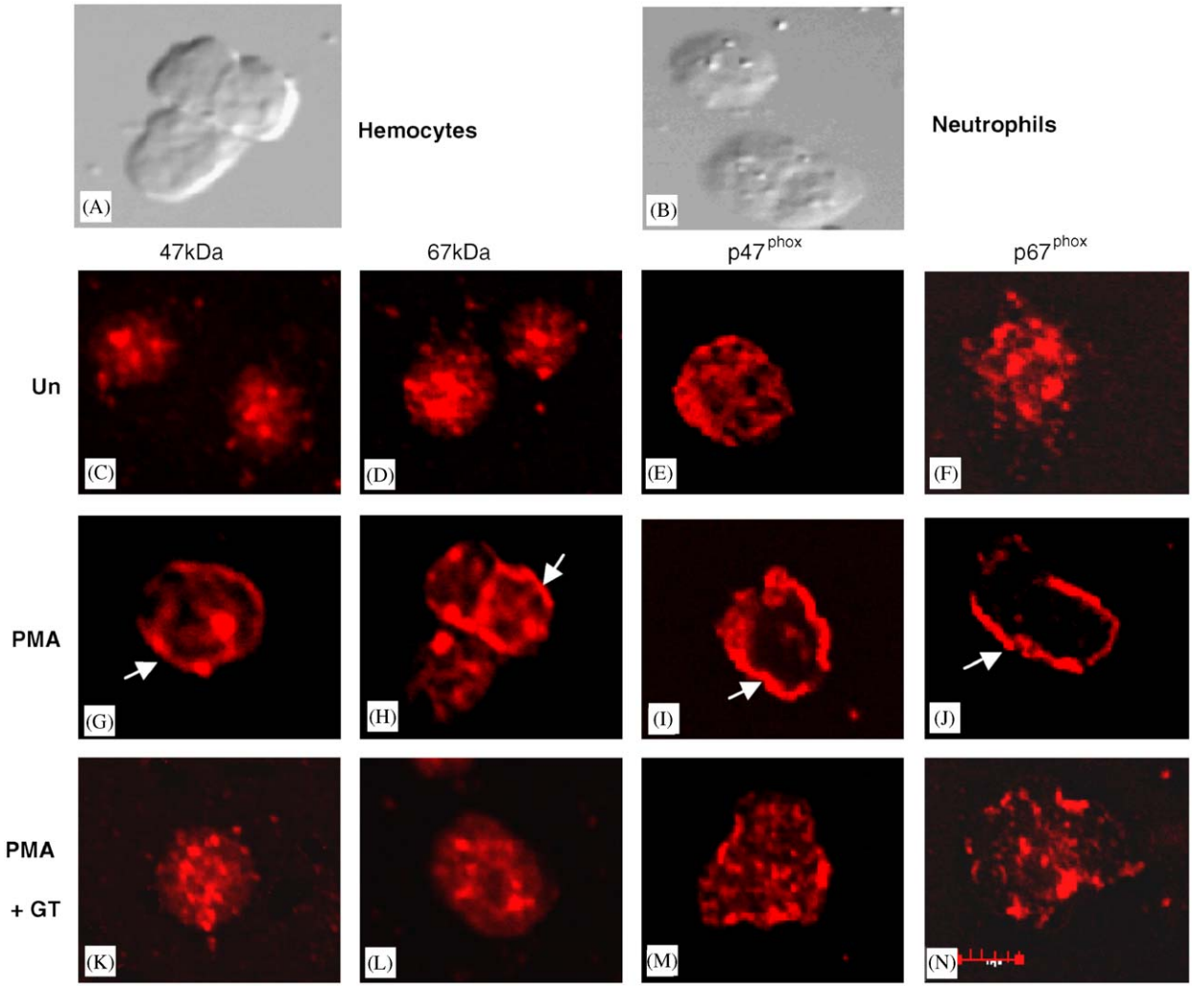

Fig. 5. The distribution of $p47^{phox}$ and $p67^{phox}$ homologous in unstimulated and PMA activated cytosol and membranes by confocal fluorescence microscopy. Light microscope images of hemocytes and neutrophils are depicted in A and B respectively. The distribution of $p67^{phox}$, $p47^{phox}$ and homologous insect proteins, 47 and 67 kDa before (C, D, E and F) and after stimulation with PMA (G, H, I and J). The distribution of the proteins was predominantly throughout the cytosol of unstimulated cells and after stimulation condensed around the membrane (indicated by arrows). Translocation of proteins following PMA stimulation was inhibited in the presence of GT (K, L, M and N). The scale bar indicated in panel N = 10 μM.

membrane translocation of homologous phox components (47 and 67 kDa) in insect hemocytes is involved in the inhibitory process of GT. Such comparative results further strengthen the similarities between the oxidase pathways in insect hemocytes and mammalian neutrophils.

4. Discussion

As the immune response of insects is similar to the innate immune response of mammals, insects have been recognized as valid alternatives for in vivo testing of microbial mutants. Indeed, the *G. mellonella* system has recently been used to detect up-regulation of *Metarhizium anisophilae*-derived *Pr1* (a subtilisin-like protease) [50] and the virulence of the *flhA* mutant of *Bacillus thuringiensis* [51]. Insects have also been used to quantify the role of the *rel A* gene in contributing to the virulence of *P. aeruginosa* [52], and the virulence of mutants of *A. fumigatus* lacking the nucleolar protein Cgr A [53].

The *Drosophilia* and human Toll cascades highlight the similarities of the insect and vertebrate immune response. Mammalian immune cells express

several Toll-like receptors that are considered cellular pattern recognition receptors, because they directly recognise LPS and other microbial products. Immunity-related proteins and mechanism that are similar in insects are under tight regulation, with transcription of insect genes encoding antimicrobial peptides controlled by two separate pathways. Bacterial (gram-positive) or fungal infection results in activation of the Toll pathway, which controls the expression of drosomycin, an antifungal peptide, and other genes via the NF-κB-family member DIF (dorsal-related immune factor) [54]. The second described pathway, referred to as the IMD (immune deficiency) is mainly triggered after gram-negative infection and regulates via the NF-κB protein Relish, the synthesis of additional antibacterial peptides [14].

Adding to this list of comparisons between insect hemocytes and mammalian immune cells was the identification of immunologically related proteins of all the essential components (gp91^{phox} , p67^{phox}, p47^{phox}, and rac) that are required in the cell-free oxidase system of neutrophils and which may play a role in the activation of the respiratory burst oxidase of hemocytes of *G. mellonella* [6]. The critical importance of these phox proteins in the role of the neutrophil in host defence is demonstrated by patients who suffer from CGD who inherit, through an X-linked or autosomal recessive mechanism, the absence or abnormality in one of the essential phox proteins (gp91^{phox}, p22^{phox}, p47^{phox} and p67^{phox}) [18]. It was demonstrated that significant regions of domain structures including the PX domain of p47^{phox} and SH3 and PB1 domains of p67^{phox} are present within the homologous hemocyte 47 and 67 kDa proteins [6].

Activation of the cytochrome b_{558} requires membrane translocation of the cytosolic proteins, including the two SH3 domain-containing oxidase proteins p47^{phox} and p67^{phox}. To further elucidate the similarity between the insect hemocyte 47 and 67 kDa proteins and human neutrophil p47^{phox} and p67^{phox} it was essential to establish whether activation of the NADPH oxidase of hemocytes resulted in translocation of these homologous phox components to the plasma membrane. To address this, we determined the location of the homologous hemocyte 47 and 67 kDa proteins in resting unstimulated hemocytes and in hemocytes stimulated with PMA. For this purpose we employed continuous and discontinuous sucrose gradient systems to obtain subcellular fractions after cellular disruption and the location of neutrophil phox and homologous hemocyte proteins established by Western blot analysis. For identification of hemocyte plasma membranes, antisera raised against membrane bound TLR1 [48] was employed. Studies clearly show that both p47^{phox} and the 47 kDa homologous protein in insect phagocytes, are cytosolic in resting cells and become associated with the plasma membrane after stimulation with PMA (Figs. 3 and 4). As previously shown in neutrophils the association with the plasma membrane does not result in complete disappearance from the cytosol. Clark et al. (1990) [49] carried out quantitative analysis and suggested that approximately 10% of total cellular p47^{phox} became membrane-associated during neutrophil PMA activation and results of this study indicated a 22% decrease in cytosolic hemocyte 47 kDa protein upon PMA activation.

PMA activation of the NADPH oxidase is intimately linked with a series of phosphorylation events. Translocation of p47^{phox} to the membrane requires phosphorylation [55] of a number of serine sites located in its C-terminus, resulting in exposure of its SH3 domain, which binds directly to the p22^{phox} subunit of the flavocytochrome [56]. Neutrophils contain five of the eleven isoforms of PKC and there are several lines of evidence supporting a role for protein kinase C (PKC) α, βII, δ and ζ in the phosphorylation of p47^{phox} [57] with a direct interaction between this substrate and the kinase been observed [58]. PKC is also capable of phosphorylating p47^{phox} at various sites, which are absolutely essential for activation and translocation of the protein [59]. PKC δ has been successfully identified in insect hemocyte lysates [6,60] and as this study revealed the translocation of the insect 47 kDa protein from the cytosol to the membrane, it is not inconceivable that phosphorylation of this immunologically similar protein by PKC may occur upon stimulation of insect phagocytes.

The interactions between p67^{phox} and the other components of NADPH oxidase have been widely studied. p67^{phox} has particular affinity for p40^{phox} [46] and its activation-induced translocation to the membrane depends on the presence of p47^{phox} [28]. Translocation of the insect 67 kDa protein in response to PMA was confirmed by immunoblotting (Fig. 3), which demonstrated that as in neutrophils [49] only a relatively small proportion, about 20%, of this protein translocates when the cells were activated.

p67^{phox} phosphorylation is known to occur during neutrophil activation by agonists such as PMA, fMLP and serum-opsonized zymosan. A relationship between p67^{phox} phosphorylation and translocation has been suggested, with both phosphoserine and phosphothreonine residues identified [61]. Candidate kinases include ERK2 and p38MAPK, with results indicating that phosphorylation of the C-terminal portion of p67^{phox} could trigger protein conformational changes to an active configuration [62]. Further studies are required to examine the association between the insect 67 kDa protein and possible phosphorylation by MAPK, previously identified in hemocytes.

The similarities between hemocyte 67 and 47 kDa proteins and the phox proteins of neutrophils prompted us to repeat the translocation experiments in the presence of GT. GT is an epipolythiodioxopiperazine metabolite of pathogenic fungi which is capable of altering function and inducing apoptosis in macrophages [63]. GT has been shown to inhibit assembly of the NADPH oxidase of neutrophils via inhibition of PKC and the subsequent membrane translocation of p67^{phox} and p47^{phox} [40]. In this study we investigated the effect of GT on insect hemocytes and showed that GT inhibits both translocation of the hemocyte 67 and 47 kDa proteins and O_2^- generation upon PMA activation. A more recent study by Nishida et al. (2005) [41] employed a cell free activation assay to specify the cellular target of GT in human neutrophils and showed that the membrane component (i.e. flavocytochrome b_{558}), was directly affected in the GT treated neutrophils, compromising the flavocytochrome b_{558} function for electron flow. This is of tremendous interest as an immunologically related protein was detected in insect hemocytes [6], possibly suggesting that this antibody recognizes a region of gp91^{phox} that has remained conserved, consisting of cysteine residues against which GT exerts its effect. Compared to other investigators [40], throughout this investigation low concentrations of GT were employed to inhibit membrane translocation (0.06–0.25 μg/ml compared to 1–3 μg/ml). To address this, it has been shown that gliotoxin is actively concentrated in cells in a glutathione-dependent manner and intracellular levels of the toxin can be up to 1500-fold greater than the applied concentration [64].

At the cellular level, the studies presented here further illustrate the strong structural and functional similarities between the innate immune response of mammals and insects. From an evolutionary perspective the success of the NADPH oxidase system is clear, as it is present in immune cells of insects and vertebrates that diverged approximately 500 million years ago. We are beginning to realise just how useful insect models could be for studying mammalian innate immunity and the results presented provide a stringent test of the homology between the insect and mammal oxidase complexes. Given the increased use of insects for evaluating microbial virulence and the need to reduce the number of vertebrates used in such testing, this study further demonstrates the validity of insects as an alternative to the use of mammals.

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