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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