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# The effect of nitric oxide and hydrogen peroxide in the activation of the systemic immune response of *Anopheles albimanus* infected with *Plasmodium berghei*

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ABSTRACT

The expression of genes encoding the antimicrobial peptides (AMPs) attacin, cecropin and gambicin, as well as the effects of NO and $H_2O_2$ on their expression was investigated in midguts and fat bodies of *Anopheles albimanus* during the midgut infection with *Plasmodium berghei*. Midgut infection induced an increase in the expression of the three AMPs in both tissues; while NO and $H_2O_2$ were present in haemolymph. Treatment with L-NAME and vitamin C reduced the effect of *P. berghei* infection on the AMP's expression, and exogenous NO and $H_2O_2$ induced their expression in the mosquito fat body. The induction of AMPs in abdominal tissues, while the malaria parasites are in the mosquito midgut, suggests communication between the midgut epithelial cells and the abdominal tissue which has not yet had direct contact with the parasites. Free radical production in mosquito midgut and haemolymph during *Plasmodium* infection and their inductive effect on AMPs in abdominal tissues indicates the possible participation of these radicals in mediating a systemic immune response in this mosquito.



## 1. Introduction

Malaria parasites are transmitted by *Anopheles* mosquitoes. *Plasmodium* male and female gametocytes are ingested during blood feeding and form extracellular gametes within the insect midgut. After fertilization, the resulting zygotes differentiate into ookinetes. These motile forms cross the peritrophic membrane and the intestinal epithelium and establish themselves between the epithelial cells and the basal membrane where they develop into oocysts. After 12–15 days post-infected blood ingestion, sporozoites emerge from mature oocysts into the haemocele. Sporozoites migrate to the salivary glands and accumulate in the salivary ducts until they are injected into a vertebrate host during blood feeding (Beier, 1998; Shahabuddin and Kaslow, 1994).

During the infection, the mosquito mounts a very strong immune response and parasites abundance experiences a bottle neck, resulting in large losses during its development in the mosquito (Sinden and Billingsley, 2001). A small percentage of ingested gametocytes develop into ookinetes and of these only a portion reach the oocysts stage. At the later phases of infection, more than 80% of the haemocele sporozoites do not succeed to reach the salivary glands and they are rapidly cleared from the haemolymph (Sinden and Billingsley, 2001; Dimopoulos, 2003). These losses occur at the moment of mosquito immune responses peak and these responses have been linked to parasite elimination.

The mosquito's immune response is complex involving melanization, phagocytosis, production of antimicrobial peptides (AMPs), and the participation of several organs such as the midgut, fat body, dorsal vessel and haemocytes (reviewed in Dimopoulos, 2003). The exact mechanisms for the induction and regulation of these responses have not yet been fully characterized (reviewed by Yassine and Osta, 2010).

In *A. gambiae*, hydrogen peroxide ($H_2O_2$) levels increase dramatically after a blood meal, this is probably due to an increase in the metabolic processes associated with blood digestion and oogenesis (DeJong et al., 2007), as well as the defense mechanism induced by bacterial growth in the midgut after blood feeding.

It has been suggested that $H_2O_2$ participates in mosquito defenses; levels of $H_2O_2$ significantly increases in an *A. gambiae* malaria refractory strain (compared to a susceptible strain) after an infected bloodmeal (Kumar et al., 2003). Also, in the presence of L-DOPA, the haemolymph and midgut of anopheline mosquitoes, including *A. albimanus*, generate superoxide anion that is toxic to *P. berghei* ookinetes (Lanz-Mendoza et al., 2002; Kumar and Barillas-Mury, 2005). The involvement of $H_2O_2$ in the activation of the acute phase of the immune response via NF-κB is well documented (Gloire et al., 2006). However, its participation in the activation of the mosquito systemic immune response remains uncharacterized.

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Nitric oxide (NO) is produced in *Anopheles* midguts during *Plasmodium* infection (Luckhart et al., 1998; Dimopoulos et al., 1998; Herrera-Ortíz et al., 2004), and this molecule and its metabolites limit the parasite development (Luckhart et al., 1998; Peterson et al., 2007). In *Drosophila*, infection with Gram-negative bacteria induces the expression of nitric oxide synthase (NOS) and the inhibition of NOS activity decreased larval survival to Gram-negative bacterial infection. Also, NO produced during infection is involved in signal transduction for the expression of immune response genes as NOS activity inhibition prevents the induction of diptericin (Nappi et al., 2000; Foley and O'Farell, 2003), exogenous NO induces the production of this antimicrobial peptide in uninfected *Drosophila* larvae (Foley and O'Farell, 2003), and the injection of a NO donor induced the expression of the cecropin B gene in *Bombix mori* (Imamura et al., 2002).

In this paper, we report the induction and increase of the transcript abundance of the AMPs attacin, gambicin and cecropin genes and their induction by exogenous NO and $H_2O_2$ in the midgut and abdominal tissues of *A. albimanus* (a major malaria vector in Mexico and Central America) infected with *P. berghei*. These findings are indicative that NO and $H_2O_2$, produced in mosquito midgut during infection, function as signals for the activation of the mosquito systemic immune response.

## 2. Material and methods

### 2.1. Insects

*Anopheles albimanus* adult mosquitoes, age range 3–7 days post-emergence, from the insectary at the Centro de Investigaciones sobre Enfermedades Infecciosas (INSP), were used in all experiments. They were maintained at 27 °C and 75% relative humidity with 12 h light/dark cycle and fed on a 5% sucrose solution.

### 2.2. Infection of mosquitoes with P. berghei

Mosquitoes were treated with a 5% sucrose solution supplemented with antibiotic–antimycotic (GIBCO) (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin) for two days. Mosquitoes were fed with a BALB/c mouse infected (10% parasitemia) with a recombinant *Plasmodium berghei*, $PbGFP_{CON}$ (Franke-Fayard et al., 2004) (provided by R. E. Sinden, Imperial College UK), during 30 min, this point was considered time 0 h for the analysis of the parasite development kinetics over 48 h. Mosquitoes' infection was determined after 48 h by the presence of oocysts in their midguts under a fluorescent microscope (Leica DM 1000, Germany).

In some cases, mosquitoes were inoculated by enema into the midgut lumen, with approximately 1000 *P. berghei* ookinetes in RPMI, using a fine glass needle (Herrera-Ortíz et al., 2004). One hour after inoculation, midguts were removed and cultured. RPMI-inoculated and non-inoculated midguts were included as control. Cultured *P. berghei* ookinetes were obtained as described by Rodríguez et al. (2002).

### 2.3. Mosquito tissue culture

Midguts were collected from female adult mosquitoes. Prior to dissection, mosquitoes were cold-anesthetized and abdomens were washed with 70% ethanol and air-dried. Midguts were dissected in 20 μl of PBS (2.6 mM KCl, 1.5 mM $KH_2PO_4$, 140 mM NaCl and 8.2 mM $NaH_2PO_4$) containing protease inhibitors (2 mM phenylmethylsulfonyl-fluoride [PMSF], 0.1 mM Na-p-tosyl-l-lysine chloro-methyl ketone [TLCK], 1 mM EDTA and 0.1 mg/ml leupeptin [Sigma, St. Louis, MO]). To obtain abdominal tissues, the abdomen was separated from the mosquito body and cut longitudinally with the bevel edge of a needle through the pleural membrane; the abdomen wall was placed in culture with the cuticle side up and the fat body in contact with Schneider culture medium (Invitrogen) supplemented with 10% fetal bovine serum (SFB) and antibiotic–antimycotic mixture (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin). Tissues were cultured at 22–24 °C for 60 min and their viability was assessed by observing their motility and by superoxide anion generation using 3-(4,5-dimetil-2-thiazolil)-2,5-difenyl-2H tetrazolium bromide (MTT, Sigma Chem.) reduction assay, as described by Lanz-Mendoza et al. (2002).

### 2.4. Identification of antimicrobial peptides expressed in A. albimanus

Antimicrobial peptides were identified in an *A. albimanus* EST database (Martínez-Barnetche, manuscript in preparation) available at the National Institute of Public Health website http://funcgen.vectorbase.org/ESTs/Anopheles_albimanus/INSP/v1 and NCBI. The sequences of individual genes of interest (gambicin, cecropin and attacin) were analyzed by BlastX against *A. gambiae* (Agam P3.46) and *D. melanogaster* (BDGP4.3.46).

The complete sequence of the *A. albimanus* homologue of gambicin was obtained from the corresponding clone. Incomplete sequence clones of attacin and cecropin were completed by RACE-5′ technique (SMART RACE II, Clontech) using the oligonucleotides 5 Atta-5R′-GGTCCCGTAGCCGTCCTCATGGGTGG-3′ and C3_5R5′-GCCGCCTTGAACACATTCCGACCCAGC-3′ for attacin and cecropin, respectively. The sequences were assembled and annotated in InterPro using Vector NTI (Invitrogen). Access numbers corresponding to GenBank are Aa-Gambicin (accession EF686019), Aa-Cecropin (EF686020) and Aa-Attacin (EF686018). Once gene sequences were completed, specific oligonucleotides were designed to amplify each gene by RT-PCR using the Vector NTI (Invitrogen).

### 2.5. Reverse transcription real-time PCR

Total RNA from 10 whole female mosquitoes or their tissues (20 midguts and 20 abdominal tissues) was obtained by Trizol method (Invitrogen) and then re-purified using RNA Clean-Up Kit (Zimo Research). cDNA was synthesized by reverse transcription using 1 μg of RNA, 100 ng of oligonucleotide dT, and 200 U of the enzyme reverse transcriptase RNase H-SuperScript II (Gibco BRL). For real-time PCR, 2.5 μl of (cDNA from the previous reaction) was used in Syber Green I Kit (Applied Biosystems) following the kit instructions. The primers used were for gambicin (AGAP008645) RT_Gam_F (CGTGCGATGGTCAGACGAT) and RT_Gam_R (CGCCGCGTTCACAAGAA); for attacin (AGAP005620) Atta_F (CGC TAC AAA GGC AAG ATG AAC) and Atta_R (TGT TTC CGC TCG CAC TCT TC); and for cecropin (AGAP000694) Cec3_F (GAAATTGGCAAACGACGTGAA) and Cec3_R (GCGATGCTAAAAGACTAAGGGC). As an internal control, a fragment of actin was amplified using primers RT_ActU_R (CGA TCC ACT TGC AGA GCC AGT) and RT_Act3.2_F (TAC GCC AAC ATT GTC ATG TCC). The amplification and detection of specific products was performed on ABI Prism 7900 HT real-time PCR system (Applied Biosystems, USA), using the following conditions: 1 cycle at 48 °C for 10 min, 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The fold changes in expression were calculated using the comparative "delta delta Ct" ($\Delta\Delta$Ct) method against the bloodfed control (Pfaffl, 2006) using three replicates per sample. Two independent experiments were done. The data represents the average fold-change relative to the control group. The amplification efficiency was similar between the test and control genes.