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Expression analysis of reactive oxygen species detoxifying enzyme genes in *Anopheles stephensi* during *Plasmodium berghei* midgut invasion

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ABSTRACT

Objective: To investigate the involvement of reactive oxygen species detoxification system in *Anopheles stephensi* during *Plasmodium berghei* midgut invasion. **Methods:** Eight key reactive oxygen species metabolizing enzymes were cloned and characterized, and their expression was monitored in parasite-infected mosquitoes. **Results:** Superoxide anion detoxifying superoxide dismutases (Fe/Mn SOD, Cu/Zn SOD 2, Cu/Zn SOD 3A, and Cu/Zn SOD 3B) depicted varied expression patterns. Fe/Mn SOD expression declined, whereas Cu/Zn SOD expression was elevated in the infected mosquitoes. Peroxidases, catalase and glutathione peroxidase showed lack of induction in expression during the *Plasmodium berghei* infection. Further, expression of thioredoxin reductase increased in the infected mosquitoes, whereas gluthathione S-transferase levels decreased markedly. **Conclusions:** Detoxification enzymes may play a role in modulating host immunity and parasite transmission.

1. Introduction

Global efforts to curb malaria caused by parasitic protozoa have been impeded by anti-malarial drug resistance, insecticide resistant mosquitoes, poor surveillance and socioeconomic obstacles. According to the latest estimates, released in December 2013, there were about 207 million cases of malaria in 2012 and an estimated 627 000 deaths[1]. Second only to Africa, Asian-Pacific region suffers with maximum global malaria burden due to diversity of vector species. *Anopheles stephensi (A. stephensi)*, an important urban vector of human malaria and a competent laboratory host for the murine parasite, *Plasmodium berghei (P. berghei)*, is predominant in south-east Asia[2,3].

For natural transmission of *Plasmodium* to vertebrate host, the parasite undergoes a series of obligatory developmental processes inside the mosquito vector. Male and female gametocytes, taken up during the blood meal, undergo gametogenesis within the lumen of the midgut. After fertilization, the resulting zygote develops into the motile ookinete which breaches through the peritrophic matrix and laminar surface of the midgut enhanced by extensive microvillae of the brush border membrane. Invaded epithelium cells undergo apoptosis and are extruded from the midgut wall. Ookinete differentiates at the basal lamina and loses its elongated shape to form an oocyst. Within each oocyst, asexual multiplications produce thousands of sporozoites that are released into the hemocoel. Sporozoites invade salivary gland epithelial cells, migrate into the ducts and are delivered to a new vertebrate host during subsequent blood meal[4-6].

The midgut is a critical site for parasite development[7]. The major bottleneck for *Plasmodium*'s development occurs during the ookinete invasion of the midgut epithelium in the complex environment of a blood meal undergoing digestion, prior to the development of oocysts on the basal lamina[8]. Ookinete development, midgut invasion and parasite transmission events are inevitably associated with blood meal digestion. The blood meal itself brings metabolic changes and induces a state of oxidative stress[9]. This is further exacerbated by the *Plasmodium* infection in *Anopheles gambiae* (*A. gambiae*)[10]. During mosquito response to infection, reactive oxygen species (ROS) are generated to limit *Plasmodium* infection. Inspite of these

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active intermediates being beneficial for parasite clearance, they are potentially toxic to the host itself. Therefore, it is important that they are generated transiently and kept well localized, and this task is accomplished through the action of antioxidant enzymes and other proteins that participate in redox metabolism. Mosquito host cells are protected from oxidative damage by numerous detoxifying enzymes in a precise manner including superoxide dismutases (SODs), *eg*., Fe/Mn SOD, Cu/Zn SOD 2, Cu/Zn SOD 3A and Cu/Zn SOD 3B, which transform superoxide anions into hydrogen peroxide[11]; peroxidases, *eg*., catalase (CAT) & glutathione peroxidases (Gpx), detoxify hydrogen peroxide into water molecule[12], and redox enzymes, *eg*., thioredoxin reductase (TrxR) and glutathione S-transferase (GST), maintain cellular redox homeostasis. Thioredoxin reduction is catalysed to maintain a reducing intracellular redox state by the flavin-dependent oxidoreductase (TrxR)[13]. GSTs are involved in cellular detoxification by catalyzing the conjugation of glutathione with a wide range of endogenous and xenobiotic agents and products of oxidative stress[14]. In addition, GSTs have peroxidase activity and can be induced by ROS[15].

Molina-Cruz *et al* first described the induced expression of several detoxifying enzymes in *A. gambiae* after *Plasmodium* infected blood meal[10]. Altered antioxidative enzymatic activities against blood meal induced oxidative stress were reported in *Aedes aegypti* (*A. aegypti*)[16]. Recently, Bahia *et al* revealed detoxification enzymes' expression modulation in the Brazilian natural malaria vector, *Anopheles aquasalis (A. aquasalis)* in response to *Plasmodium vivax* infection[17].

Following evidences for a role of several ROS detoxifying enzymes in response to oxidative stress during infection of human disease vectors, we investigated the recruitment of ROS and its role in immune defense of an important malaria vector, *A. stephensi* against *P. berghei*. Our results revealed modulation of expression of prominent ROS detoxifying enzymes in the infected midgut.

2. Materials and methods

2.1. Ethics statement

All the animal experiments were performed in accordance with the recommendations in the Committee for the Purpose of Control and Supervision on Experiments in Animals (CPCSEA, India) as well as approval by the Institutional Animal Ethics Committee (School of Life Sciences, University of Hyderabad, India).

2.2. A. stephensi and malaria parasite

A. stephensi were reared at 27 ℃ and 70% humidity on a 12-h light-dark cycle on 100 g/L sucrose solution under standard laboratory conditions. *P. berghei*-ANKA strain was maintained by serial passage in 3-4 week-old female BALB/c mice or as frozen stocks. The mice having parasitemia between 4% and 8% as checked by Giemsa staining and 2-3 exflagellations/field under 400× magnification were used to infect mosquitoes.

2.3. P. berghei infection of mosquitoes

Starved (for 2 h) female mosquitoes (3-5 days old) were infected with *P. berghei* by feeding on anesthetized infected BALB/c mice for 15 min. Infected and control blood-fed mosquitoes were kept in an environmental chamber maintained at 21 ℃ and 70% humidity. *P. berghei* midgut infection was confirmed by monitoring chitinase mRNA expression using quantitative real-time PCR (qRT-PCR).

2.4. Mosquito midgut isolation

Twenty mosquitoes from control and infected group were washed with 70% (v/v) ethanol and midguts were dissected under a stereozoom microscope. The dissected midgut obtained from different time points (8, 14, 20, 26, 32 & 38 h) were collected in minimal volume of TRI® reagent (Sigma-Aldrich), snap frozen using liquid nitrogen and stored at -80 ℃ till further use.

2.5. PCR based cloning of antioxidant enzyme genes

Total RNA from the isolated midgut tissue was isolated using PURELink® RNA mini kit (Life Technologies) following manufacturer's protocol. Following DNase Ⅰ treatment, the concentration of total RNA was assessed using NanoDrop-1000 spectrophotometer (NanoDrop Technologies), and the quality was analyzed by formaldehyde agarose gel electrophoresis. First-strand cDNA synthesis was performed using Superscript Ⅲ® (Life Technologies) following manufacturer's protocol. Primers were designed based on conserved regions of *SODs*, *CAT*, *Gpx*, *TrxR* and *GST* of *A. gambiae*, *A. aquasalis*, *A. aegypti* and *Drosophila melanogaster*. Amplicons generated were cloned in pTZ57R/T vector (Thermo Scientific) and plasmids containing inserts were sequenced bidirectionally. The list of primers used to clone the partial cDNA fragments of antioxidant genes are illustrated in Table 1. The cloned sequences were submitted to GenBank under the following accession numbers (Fe/Mn SOD: KJ160261; Cu/Zn SOD 2: KJ160262; Cu/Zn SOD 3A: KJ160263; Cu/Zn SOD 3B: KJ160264; CAT: KJ160265; Glutathione peroxidise: KJ160266; GST: KJ160267; TrxR: KJ739802).

2.6. Relative quantification of antioxidant gene expression

Control and infected midguts collected at different time points as mentioned above were dissected in ice-cold TRI® reagent and the total RNA isolation was carried out immediately. All total RNA samples were treated with DNase Ⅰ prior to first strand cDNA synthesis. Reverse transcription was carried out with 0.5 μg total RNA using random hexamer primers and Superscript® Ⅲ reverse transcriptase according to manufacturer's protocol. Based on the partial cDNA fragments, the primer sets for qRT-PCR were carefully designed in the conserved motifs of the antioxidant genes using Primer Express software (Applied Biosystems). The list of primers is given in Table 1. Standard curve for each gene was plotted with serial dilutions of respective primers and cDNA. Gene expression was assessed by SYBR green qRT-PCR (Applied Biosystems) in ABI-7500 fast real-time PCR system (Applied Biosystems). A 40-cycle two-step PCR was carried out in triplicates with 10.0 μL reaction volume containing the following components: 1.0 μL of cDNA template, 1.0 μL of forward and reverse primers each, and 5.0 μL of 2× master mix. Dissociation or melting curve analysis was performed for all the genes to check for specific amplification. The

amplification efficiency was 95%-99% with slope of the curve ranging between -3.0 to -3.3. During each cycle of the PCR, fluorescence accumulation resulting from DNA amplification was analyzed and converted into cycle threshold (Ct) by the sequence detection system software (Applied Biosystems). Relative quantification results were normalized with conserved *Anopheles* ribosomal protein S7 as endogenous control. Ct values were obtained from the exponential phase of PCR amplification. All the results are represented as change in the transcript levels relative to the reference values obtained for the control (sugar-fed) and were normalized to that of endogenous control gene (S7) Ct values using the $2^{-\Delta\Delta Ct}$ method as described by the reference[18].

2.7. Statistical analysis

Data are expressed as mean±SEM of three independent experiments (n=3). Differences between groups were analyzed for statistical significance by One-Way ANOVA followed by Students-Newman-Keuls' post hoc test using Sigma Plot 11.0 software (Systat Software Inc., USA). A probability of P<0.05 was considered statistically significant.

3. Results

3.1. Identification and characterization of antioxidant enzyme genes from A. stephensi midgut

Initially, partial cDNAs for detoxifying enzymes were obtained using degenerate PCR based approach (data not shown). Expected cDNA fragments of 201-, 532-, 369- and 296-bp for *Fe/Mn SOD*, *Cu/Zn SOD 2*, *Cu/Zn SOD 3A* and *Cu/Zn SOD 3B*, respectively were obtained using specific primers. Amino acid sequence alignment of *A. stephensi* Fe/Mn SOD with its counterparts showed the presence of a highly conserved superoxide dismutase alpha hairpin domain. The deduced *A. stephensi* Cu/Zn SOD 2, Cu/Zn SOD 3A and Cu/Zn SOD 3B proteins have conserved Cu^{2+} and Zn^{2+} binding domains typically found in Cu/Zn superoxide dismutases. The putative *Cu/Zn SOD 2*, *Cu/Zn SOD 3A* and *Cu/Zn SOD 3B* orthologous genes of *A. stephensi* showed high homology (89%, 79% and 81% respectively) with *A. gambiae*. *Cu/Zn SOD 3A* and *Cu/Zn SOD 3B* also showed 82% and 77% similarity with the recently reported counterparts of *A. aquasalis*. The C-terminal region of *Cu/Zn SOD 3B* greatly differs from that of *Cu/Zn SOD 3A*, which facilitated apparent identification of these otherwise highly similar sequences.

The partial cDNAs of the two peroxidases obtained, i.e., CAT and Gpx, were 534- and 204-bp, respectively. *A. stephensi* CAT amino acid sequence is very similar to that of other insect catalases, all showing a long stretch of CAT domain comprising of heme binding pocket and NADPH binding site. The Gpx amino acid sequence obtained from its cDNA fragment harbors a putative glutathione peroxidise domain that matches with Gpxs reported in *A. gambiae*, *Anopheles darlingi*, *A. aegypti*, *Culex quinquefasciatus*, and *Drosophila melanogaster*.

A 136-bp cDNA of *TrxR* belonging to the pyridine nucleotide-disulphide oxidoreductase family was cloned from *A. stephensi*. Its amino acid sequence analysis showed the presence of a small conserved NADH binding domain within a larger flavin adenine dinucleotide binding domain. This region is conserved in all known insect TrxRs. Similarly, deduced amino acid sequence from *A. stephensi GST* cDNA of length 665-bp consists of an N-terminal thioredoxin-fold domain and a C-terminal alpha helical domain that groups its amino acid sequence with other insect GST sequences.

3.2. Relative expression of ROS detoxification enzymes in P. berghei infected A. stephensi

In general, both the control and infected blood meals induced the midgut mRNA expression of most detoxification enzymes. However, detoxification enzyme genes post infection showed varied expression patterns when compared

Table 1
List of primers used for cloning and real-time analysis of *A. stephensi* antioxidative genes.

Gene	Primer	Sequence for cloning (5′→3′)	Sequence for cloning real-time analysis (5′→3′)
Fe/MnSOD	Forward	GGAGCACTCTTTTCCACTGCAAAG	TCCACTGCAAAGAACTGCAGCGC
	Reverse	CACCGCATCCTGCAGCTG	GGTGGTGAAGCTCCATGATTTCGC
Cu/Zn SOD2	Forward	GTGCTAATTGCTCTATCGACCGTGCTGTGC	GTGCTAATTGCTCTATCGACCGTGCTG
	Reverse	CACTGTCCGGCTCATCGAAGGGCTCGA	GCACGATGGTTGCGAGATGGTCAC
Cu/Zn SOD3A	Forward	ATGCCGCTGAAAGCCGTGTGTGTTC	ATGCCGCTGAAAGCCGTGTGTGTTC
	Reverse	GTCCGGATCGGCATGAACGAC	CCGTGCGGGTTGAAGTGAGCG
Cu/Zn SOD3B	Forward	AACGATCACGGGGCTCCGGATGACGCGAAT	ACGATCACGGGGCTCCGGATGACGCG
	Reverse	TCGTCCGTGGTCAGATGCAATCGTTCGGC	TCGTCCGTGGTCAGATGCAATCGTTC
Cat	Forward	GATAAGGTGACGGCTACGATGAGC	ACGATGGCGTGTGGGATATGGTG
	Reverse	GAACAGGAACATCGTCTGGTGCG	GAACAGGAACATCGTCTGGTGCG
Gpx	Forward	GAGAAGTACCGTGGCAAGGTGCT	CAAGGTGCTGCTAATCGTCA
	Reverse	GGCCGAGCGAAGACGACAC	CCTTGTCCGCGTACTTTTGC
TrxR	Forward	TATCCGGACATTCCCGGCGCAGC	ACGAGCGACGACATCTTCAG
	Reverse	ACCAGGGTGGCCATCTGCTGATCG	GAATCGACCGCACCATCAC
GST	Forward	ATGCCTAAGCTAGTTCTGTACACG	CGCGTTGAGTATGTGCAGAAATCGT
	Reverse	TAAGCTTTAGCATTCTCTTCC	CACCCGTAGATCCGAGGATGTTCC
S7	Forward		GTGCACCTGGATAAGAACCAGGA
	Reverse		CAGGTAGTTCTCTGGGAATTCGAA

to the control. Upon *P. berghei* infection, the transcript levels of *Fe/Mn SOD* gradually decreased from 8 h and reached to significant levels at 14 h ($P<0.05$). Thereafter, the expression levels were induced similarly in both the control and infected blood fed *A. stephensi*. Conversely, transcript levels of cytosolic *SODs*, *Cu/Zn SOD 2* and *Cu/Zn SOD 3A* showed a marked increase from 8-26 h after the infected blood meal; however, the increase in expression was more prominent in case of *Cu/Zn SOD 3A* ($P<0.05$). On the other hand, the mRNA levels of *Cu/Zn SOD 3B* were unaltered when compared to the control. For peroxidases, *CAT* and *Gpx*, transcript levels were induced after normal blood meal. However, no significant change was observed in *Plasmodium* infected mosquitoes. In fact, there was a complete lack of induction of *CAT* and *Gpx* mRNAs in the midgut of *P. berghei* infected mosquitoes. The redox enzyme, *TrxR* mRNA levels were up regulated at 20 h in the infected midgut and reverted to normal levels towards the end of the time regimen. The mRNA levels of the other redox enzyme *GST* declined significantly at 14 h post infection ($P<0.05$; Figure 1).

4. Discussion

Blood meal digestion is a vital physiological event in the midgut of female *Anopheles* mosquito. Amino acids obtained from the blood meal are utilized for fat body vitellogenesis required for oocyte development[19]. These metabolic changes lead to ROS generation resulting in oxidative stress in the mosquito[20]. *Plasmodium* infection exacerbates the oxidative stress induced by feeding, as mosquitoes respond by increasing the systemic expression of ROS detoxification enzymes[10]. This role played by ROS detoxification enzymes during *Plasmodium* transmission is so far reported in *A. gambiae* comprehensively[10].

Here we described the role of ROS in the *A. stephensi* in response to *P. berghei* infection. We characterized three key groups of ROS detoxifying enzymes: enzymes that detoxify superoxide anions (Fe/Mn SOD, Cu/Zn SOD2, Cu/Zn SOD 3A and Cu/Zn SOD 3B), hydrogen peroxide detoxifying enzymes (CAT and Gpx) and enzymes that maintain redox homeostasis (TrxR and GST).

Direct experimental evidences indicate that ROS derived from mitochondria can modulate mosquito epithelial responses to *Plasmodium* infection[21]. In our study, Fe/Mn SOD expression decreased significantly post infected blood meal, which corroborates with an earlier report in *A. gambiae*[10] and is also supported by the fact that insulin from human blood administered to *A. stephensi* decreased the mitochondrial SOD activity of midgut[22]. The expression patterns of *Cu/Zn SOD 2* and *Cu/Zn SOD 3A* are also similar to that observed in *A. gambiae*[10]. The dramatic high levels of these two SOD mRNAs upon infection can be explained as an attempt of the mosquito to return cellular superoxide free radicals to normal levels in the midgut. On the other hand, *Cu/Zn SOD 3B* mRNA levels in *A. stephensi* remain unaltered as observed in the case of *A. gambiae*[10] and *A. aquasalis*[17]. However, in *A. aquasalis*, post 24 and 48 h infection, whole body SOD 3B levels increased significantly in relation to control blood-fed insects, suggesting its possible role in fat body and/or hemocytes during parasite exposure to the insect hemolymph[17].

In hematophagous insects, CAT has been detected among transcripts that are up-regulated after ingestion of blood, indicating its possible role in response against the oxidative stress caused by a blood meal[20,23]. Further, silencing of *A. gambiae* CAT increased mosquito mortality after a blood meal[24]. Our study too indicated an induction of the peroxidases, CAT and Gpx post blood meal. However, the suppression of expression of both these midgut peroxidases post infection is likely a part of a defense response triggered by parasite invasion. During midgut traversal, ookinetes trigger a two-step cell nitration process in which nitric oxide is rapidly converted to nitrite. An inducible epithelial peroxidise uses nitrite and hydrogen peroxide as substrates and catalyzes nitration. A reduction in CAT accelerates the peroxidase-mediated nitration reaction and prevents further

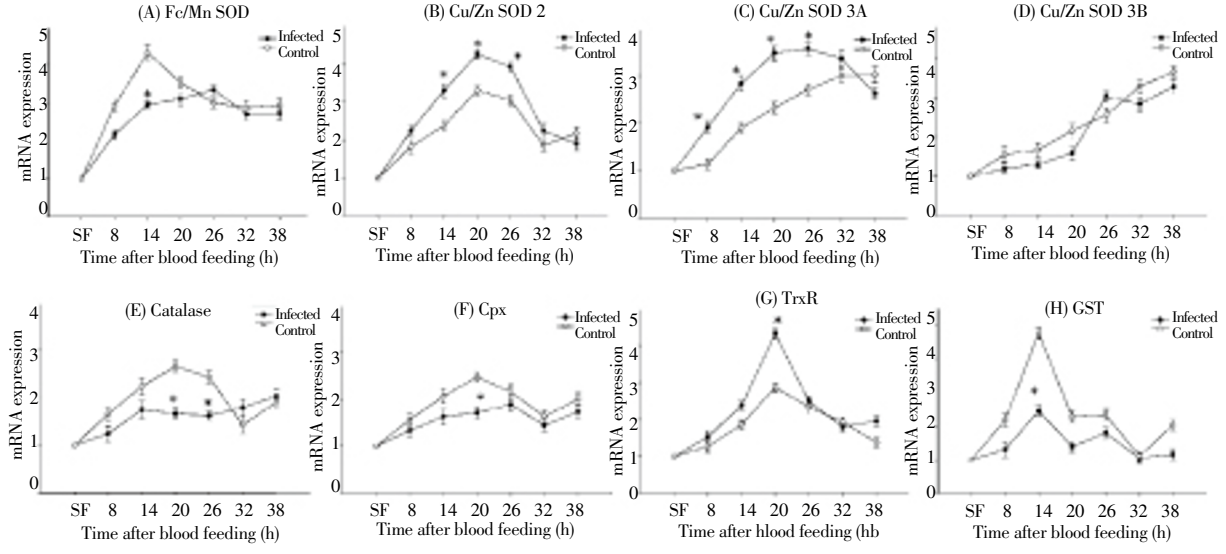

Figure 1. Relative expression of ROS detoxifying enzymes in *A. stephensi* mosquitoes after *P. berghei* infection.
Induction of ROS detoxification enzymes mRNA in midgut of adult females fed with uninfected (○) or *P. berghei* infected blood (●). *, significantly different measurements between control and infected samples ($P\leqslant0.05$) at a given time point.

parasite transmission[9,25,26].

In dipteran insects including mosquitoes, glutathione reductase is functionally substituted by TrxR[27]. The activation of TrxR reported in *A. gambiae*[13,28] supports our result showing a marked increase of TrxR post blood meal. The insect specific GSTs class delta and epsilon play a major role in insecticide resistance[14]. Our study corroborates with an earlier report in *A. gambiae* where *Plasmodium* infection down regulated various epsilon and delta class GSTs, suggesting an antioxidant role of this redox enzyme[15].

The present study contributes to our understanding of the molecular aspects of *A. stephensi* midgut antioxidant defenses to *Plasmodium* during parasite midgut invasion. Clearly, more studies of antioxidant responses of diversified anopheline species to *Plasmodium* parasite, are required to develop potential molecules involved in antioxidant response and redox metabolism. These molecules may serve as targets for novel anti-mosquito strategies and offer a screening methodology for finding targetable mosquitoes to curb parasite transmission.

Conflict of interest statement

We declare that we have no conflict of interest.

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