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Silencing an *Anopheles gambiae* Catalase and Sulfhydryl Oxidase Increases Mosquito Mortality After a Blood Meal

T. Magalhaes^{1,2,*}, D.E. Brackney², J.C. Beier¹, and B.D. Foy²

¹Department of Epidemiology and Public Health, University of Miami, Miami, Florida

²Department of Microbiology, Immunology and Pathology, Arthropod-borne and Infectious Diseases Laboratory, Colorado State University, Fort Collins, Colorado

Abstract

Catalase is a potent antioxidant, likely involved in post-blood meal homeostasis in mosquitoes. This enzyme breaks down H₂O₂, preventing the formation of the hydroxyl radical (HO[•]). Quiescins are newly classified sulfhydryl oxidases that bear a thioredoxin motif at the N-terminal and an ERV1-like portion at the C-terminal. These proteins have a major role in generating disulfides in intra- or extracellular environments, and thus participate in redox reactions. In the search for molecules to serve as targets for novel anti-mosquito strategies, we have silenced a catalase and a putative quiescin/ sulfhydryl oxidase (QSOX), from the African malaria vector *Anopheles gambiae*, through RNA interference (RNAi) experiments. We observed that the survival of catalase- and QSOX-silenced insects was reduced over controls following blood digestion, most likely due to the compromised ability of mosquitoes to scavenge and/or prevent damage caused by blood meal-derived oxidative stress. The higher mortality effect was more accentuated in catalase-silenced mosquitoes, where catalase activity was reduced to low levels. Lipid peroxidation was higher in QSOX-silenced mosquitoes suggesting the involvement of this protein in redox homeostasis following a blood meal. This study points to the potential of molecules involved in antioxidant response and redox metabolism to serve as targets of novel anti-mosquito strategies and offers a screening methodology for finding targetable mosquito molecules.

Keywords

malaria; mosquito; RNAi; antioxidant; redox metabolism; survival

INTRODUCTION

Blood meals are used by anautogenous female mosquitoes to produce eggs; however, ingested blood leads to oxidative stress for the insect (Dansa-Petretski et al., 1995; Kumar et al., 2003). Certain mosquito proteins are thought to help reduce oxidative stress and maintain the post-blood meal homeostasis. These may include antioxidant enzymes and other proteins that participate in redox metabolism. Some examples are catalase, superoxide dismutase (SOD), thioredoxins, and glutathione (Halliwell, 1981; Missirlis et al., 2001; Storz et al., 1990). Several publications have documented up-regulated transcription of putative mosquito proteins involved in antioxidant responses and redox metabolism that occur after ingestion of a blood meal (Holt et al., 2002; Marinotti et al., 2005; Ribeiro, 2003; Sanders et al., 2003).

*Correspondence to: T. Magalhaes (current address): Department of Entomology, Centro de Pesquisas Aggeu Magalhaes (CPqAM)/ FIOCRUZ, Av. Prof. Moraes Rego s/n, Campus UFPE, Cidade Universitaria, Recife-PE 50670-420, Brazil. E-mail: E-mail: teca@cpqam.fiocruz.br.

Catalase is one of the key antioxidant enzymes and is part of the SOD/catalase system that prevents the formation of the hydroxyl radical ($\text{HO}^\bullet$) (Halliwell, 1981). SOD is responsible for O_2^- dismutation into H_2O_2 and oxygen, while catalase converts H_2O_2 to water and molecular oxygen (Halliwell, 1981; Mackay and Bewley, 1989). In hematophagous insects, catalase 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 (Kumar et al., 2003; Munks et al., 2005; Sanders et al., 2003).

A new member of the sulfhydryl oxidase family has been identified and named quiescin, which possesses a thioredoxin domain at the N-terminal and a FAD-binding domain similar to the ERV1 protein in yeast, at the C-terminal (Coppock et al., 1998). These enzymes insert disulfide bonds into reduced proteins by reducing molecular oxygen to H_2O_2 , or may act as secreted oxidases to counteract reductants in the cellular milieu (Chakravarthi et al., 2007; Thorpe and Coppock, 2007). Quiescin has been found in all multicellular organisms of which the genome has been sequenced (Chakravarthi et al., 2007; Thorpe and Coppock, 2007). However, more studies are needed to better elucidate their functions in vivo. In insects, no studies of this protein have been documented.

In the present study, we report the influence of silencing transcription of a catalase and a putative quiescin/sulfhydryl oxidase (QSOX) in *Anopheles gambiae* adult females, using RNA interference (RNAi).

MATERIALS AND METHODS

Mosquito Rearing

The *Anopheles gambiae* (G3) colony was maintained in a 28–30°C and 75% humidity insectary on a 14-h light/10-h dark cycle at the Arthropod-borne and Infectious Diseases Laboratory (AIDL) at Colorado State University. Larvae were fed with ground fish food, and adults were kept with 10% sucrose and water ad libitum. Adult females at 3–5 days post-emergence were used in all experiments.

Anopheles gambiae Catalase and QSOX

An. gambiae catalase and QSOX, identified as AGAP004904 and AGAP007491 at Ensembl (<http://www.ensembl.org>), were cloned from a cDNA library made with 24-h post-blood fed *An. gambiae*. Primers were designed according to the transcript open reading frame (ORF); restriction enzymes sites were added on the flanking ends to allow cloning into a plasmid vector.

Primers for catalase were:

Forward: *Hind*III—5'-
ATTATCAAGCTTATGTCGCGCAATCCGGCCGAAAAC-3'

Reverse: *Xba*I—5'-ATTATCTCTAGATTACAGATTGGCCGTGCGGCGCAG-3'

Primers for QSOX were:

Forward: *Xho*I—5'-ATTATCCTCGAGATGGTGGTGGTGCGT-3'

Reverse: *Hind*III—5'-ATTATCAAGCTTTTACACTTTGCCCAG-3'

PCR was performed using the *An. gambiae* cDNA library as the template, under the following conditions: 94°C for 2 min followed by 25 cycles of 94°C for 1 min, 58°C for 2 min, and 72°C for 1 min, and a last step at 72°C for 10 min. PCR fragments were purified using the QIAGEN PCR purification kit (QIAGEN, Valencia, CA) and cloned into the pcDNA3.1+ plasmid vector (Invitrogen, Carlsbad, CA). Catalase and QSOX had ORFs of 1.6 kb and 2.7 kb, respectively.

Mosquito Injections With dsRNA

For double-stranded RNA (dsRNA) preparation, sense and anti-sense primers containing a T7 promoter site were designed to amplify the last 500 bp of the catalase and QSOX ORFs.

Primers for catalase were:

Forward: 5'-TAATACGACTCACTATAGGTCGAGCCGTCGCCCCGAC-3'

Reverse: 5'-TAATACGACTCACTATAGGTTACAGATTGGCCGTGC-3'

Primers for QSOX were:

Forward: 5'-TAATACGACTCACTATAGGACGAGGCCGTGCTGTGG-3'

Reverse: 5'-TAATACGACTCACTATAGGTTACACTTTGCCAGTA-3'

For control, β -galactosidase (β -gal) dsRNA was produced from a 500-bp amplicon of the β -gal gene from the commercial plasmid pcDNA3.1/His/LacZ (Invitrogen).

Primers for β gal were:

Forward: 5'-

TAATACGACTCACTATAGGGGGTCGCCAGCGGCACCGCGCCTTC-3'

Reverse: 5'-

TAATACGACTCACTATAGGGGGCCGGTAGCCAGCGCGGATCATCGG-3'

The amplicons were purified using the QIAGEN PCR purification kit and used as a template to make dsRNA in the following reaction: 5 μ g of the 500-bp DNA amplicon was incubated with rNTPs and T7 Enzyme Mix (Ambion, Austin, TX) for 4 h at 37°C, allowing transcription to occur and then incubated with DNase I and RNase Cocktail (Ambion) for 1 h at 37°C for degradation of template DNA and ssRNA. The dsRNA was purified according to manufacturer's instructions. The resulting dsRNA pellet was resolubilized in sterile PBS and diluted to 1 μ g/ μ l. *An. gambiae* adult females were injected intrathoracically with 1–2 μ l of either β -gal dsRNA (ds β gal, control), dsRNA catalase (dsCat), or dsRNA QSOX (dsQSOX), by using pulled capillary needles. Two days (48 h) post-injection, mosquitoes were blood fed on naive BALB/c mice and used for the experiments described below.

qRT-PCR

At 24 h post-blood feeding, pools of five bloodfed midguts from dsRNA-injected mosquitoes were dissected and pooled for each group. Total RNA was extracted using the QIAGEN RNeasy Kit. After quantification, RNA was normalized at 30 ng/ μ l and 2 μ l (60 ng) were used for quantitative reverse-transcriptase Real-Time PCR (qRT-PCR). The QuantiTect SYBR Green RT-PCR QIAGEN Kit was used, and optimal primers were designed according to the manufacturer's instructions. These primers were designed to be within the gene's ORF but excluded the 500 bp used for making the dsRNAs.

For catalase, primers for qRT-PCR amplified a fragment of 162 bp and were the following:

Forward: 5'-AGGTTACCCACGACATCA-3'

Reverse: 5'-TCGTCCGTGTAGAACTTGA-3'

For QSOX, primers amplified a fragment of 121 bp and were:

Forward: 5'-CCAGTGAAAGAGCACGATGA-3'

Reverse: 5'-ACTTATCGTCCGTCCGATTG-3'

Primers that amplified a fragment of the S7 mosquito ribosomal gene were used for all templates as a normalization control. Data were analyzed using the comparative C_T method (Livak and Schmittgen, 2001). RNAi experiments for qRT-PCR analyses were repeated 3 times in independent experiments. S7 transcripts vary ≤ 1.6 -fold over the course of a blood meal (<http://www.angagepuci.bio.uci.edu>). More importantly, this normalization target gave consistent C_T measurements between both experimental and control treatments and between experimental replicates (18.00–19.00), thus verifying that off-target treatment effects were unlikely.

Catalase Activity

The activity of catalase was measured at 24 h post-blood feeding in dsRNA-injected mosquitoes by using the Catalase Assay Kit (Sigma, St. Louis, MO). Pools of 5 mosquitoes per group were each ground in 400 μ l of buffer (50 mM potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100) and centrifuged for 10 min at 14,000 rpm. The supernatant was transferred to a new tube and kept on ice for the remainder of the experiment. An aliquot (30 μ l) of the supernatant was diluted into 470 μ l of cold buffer (50 mM potassium phosphate, pH 7.0) and 500 μ l of cold 20 mM H_2O_2 was added, thus the 1-ml final reaction contained 10 mM H_2O_2 . The tube was inverted and immediately placed in a quartz cuvette where change in A_{240} was read in six 5-s intervals over a 30-s time span in a spectrophotometer at room temperature. Catalase units/ml were calculated according to the following: 1 unit of catalase will decompose 1.0 μ mol of H_2O_2 to oxygen and water per minute at pH 7.0 at 25°C at a substrate concentration of 10 mM H_2O_2 . RNAi experiments for catalase activity assay were repeated 3 times independently.

TBARS Assay

Lipid peroxidation was measured as one marker for elevated redox activity in mosquitoes. For that, dsRNA-treated/bloodfed whole mosquitoes were used for quantitation of malondialdehyde-thiobarbituric acid (MDA-TBA) chromophore complexes through the thiobarbituric acid reactive substances (TBARS) assay (Valenzuela, 1991). The assay performed here was adapted from an experiment described previously by Dansa-Petretski et al. (1995). Twenty-four hour post-bloodfed mosquitoes were put at 4°C for 5 min, transferred to a 1.5-ml tube, and kept at -80°C until use. Each group had 5 independent replicates with 3 mosquitoes per tube, which were then homogenized with 100 μ l of PBS with a pestle. Samples were centrifuged at 14,000 rpm for 5 min and the supernatant transferred to a new tube, where 10 μ l of 20% of trichloroacetic acid (TCA) was added for protein precipitation. Tubes were incubated on ice for 10 min and centrifuged as before. Supernatants (100 μ l) were transferred to a new tube, and 20 μ l of 1 mg/ml of 2,6-di-ter-butyl-4-methylphenol (BHT) was added as an antioxidant. For the formation of MDA-TBA complexes, 100 μ l of 8 mg/ml of TBA was added and samples were incubated for 1 h at 4°C and then for 15 min at 98°C. After incubation, 300 μ l of 1-butanol was added; tubes were vortexed and centrifuged as before. Samples (100 μ l) were read at A_{532nm} in a Beckman spectrophotometer. The μ M concentrations of MDA-TBA were calculated on the basis of a regression plot of a MDA curve. RNAi experiments for TBARS assay were repeated 3 times independently.

Mosquito Survival Analysis

Mosquitoes were bloodfed on naive mice at 48 h post-dsRNA injections and fully engorged females were transferred to gallon-size cages for mortality assessment. For each group, there were 40–50 females. Dead mosquitoes were counted for 3 days and the total number of surviving insects recorded at the end of the 3rd day post-bloodfeeding. Dead mosquitoes were counted for only 3 days post-blood meal for two reasons: (1) we wanted to ensure that the observed mortality effects were well within the window of effective RNAi silencing via

injection of dsRNA (Blandin et al., 2002); and (2) we wanted to focus on mortality effects that were due to blood meal processing and not aging. RNAi experiments for survival analyses were repeated independently 4 times, life tables were pooled for each group, and survival curves were analyzed by Kaplan—Meier log-rank analyses.

Statistical Analysis

Statistical significance between results from the control and experimental groups for the catalase activity assay, TBARS assay, and mortality curves was obtained through the Student's *t*-test. Differences were considered significant when $P < 0.05$.

Analysis of *Anopheles gambiae* QSOX

The similarity of the *An. gambiae* QSOX with quiescins from *Aedes aegypti*, *Drosophila melanogaster*, and *Homo sapiens* was analyzed through the program MegAlign/DNASTAR by the ClustalW method.

RESULTS

qRT-PCR

Among the 3 experiments, the reduction in catalase transcript in dsCat-injected mosquitoes varied from 88% to 93%, while the reduction in QSOX transcript in dsQSOX-injected mosquitoes varied from 72% to 88%, compared with dsβgal-injected mosquitoes. Figure 1 shows the average (SD) catalase and QSOX transcripts reduction on dsCat and dsQSOX-injected mosquitoes, respectively, from 3 separate experiments.

Catalase Activity

The activity of catalase was significantly lower in dsCat-injected mosquitoes compared to dsβgal and dsQSOX-injected groups (Fig. 2). Mean units of catalase activity decomposing 1 μM of H₂O₂ per minute (at pH 7.0 at 25°C at a substrate concentration of 10 mM H₂O₂) were 72.7, 72.6, and 13.8 for dsβgal, dsQSOX, and dsCat, respectively.

TBARS Assay

Figure 3 shows the differences in MDA-TBA μM among mosquitoes injected with dsβgal, dsCat or dsQSOX. The mean MDA-TBA concentration from 3 experiments was 0.85 μM in dsβgal-injected mosquitoes compared with 0.87 μM in dsCat-injected mosquitoes, with no significant difference. However, MDA-TBA concentration was 0.78 μM in dsQSOX-injected mosquitoes compared with 0.73 μM in dsβgal-injected mosquitoes, being significantly different.

Mosquito Survival Analysis

In the pooled survival curves, at the end of the 3rd day after a blood meal, the percentage of surviving dsβgal-injected mosquitoes ($n = 188$) was 86% compared with 66% for dsCat-injected mosquitoes ($n = 179$) and 77% for dsQSOX-injected mosquitoes ($n = 200$) (Fig. 4). The difference in survival between dsβgal and dsCat-injected mosquitoes was highly significant. There was also a significant difference in survival between dsβgal and dsQSOX-injected mosquitoes, however it was not as pronounced as that from the dsCat-injected mosquitoes. Figure 5 shows the individual survival curves of the 4 sets of replicate mortality experiments.

Analysis of *Anopheles gambiae* QSOX

The *An. gambiae* QSOX has a thioredoxin (Thio) and an ERV1-like domain, as shown in Figure 6, adapted from Ensembl (http://jun2007.archive.ensembl.org/Anopheles_gambiae/geneview?gene=AGAP007491). Comparison of this newly cloned *An. gambiae* QSOX with quiescins from *Ae. aegypti* (Ensembl ID: AAEL012054), *D. melanogaster* (Fly-Base ID: CG4670), and *H. sapiens* (Ensembl ID: ENSG00000116260) showed high similarity of the Thio and ERV1 motifs. Analysis was performed the same way as described in Thorpe et al. (2002), where alignment was carried out for the 25-aa Thio domain, and the 3 regions of the ERV1 motif, consisting of: CxxC motif (Region 1), representing the active site of quiescins; HN×VN motif (Region 2); and another CxxC motif (Region 3), unique of quiescins. Region 3 of the ERV1 domain was the most variable among the analyzed species. As is the case of other invertebrate quiescins, the *An. gambiae* QSOX does not have a 48-aa conserved region downstream the ERV1 domain that is found in vertebrate quiescins and named the QSOX homology zone 10 (QHZ10) (Thorpe et al., 2002). The highest sequence homology was observed between *An. gambiae* and *Ae. aegypti* and the least homology between *An. gambiae* and *H. sapiens*. The Thio domain and Regions 1 and 2 of the ERV domain of *D. melanogaster* was slightly more related to the respective regions of *An. gambiae*. Alignments are shown in Figure 7.

DISCUSSION

Catalase activity in dsCat-injected mosquitoes was reduced to low levels, possibly indicating a lack of redundancy of catalase enzymes in *An. gambiae*. Indeed, there is only one catalase gene annotated in the *An. gambiae* genome database (Ensembl), which corresponds to the one used in our study (AGAP004904). Since this enzyme is the sole H₂O₂ scavenger in insects (Yamamoto et al., 2005), the increased mortality observed in these insects was likely caused by H₂O₂ damage after a blood meal. The inactivation or silencing of catalase in *Drosophila*, *Rhodnius prolixus*, and *Musca domestica* has led to increased susceptibility of the insects to H₂O₂ challenge and to higher levels of endogenous molecular H₂O₂ (Allen et al., 1983; Mackay and Bewley, 1989; Paes et al., 2001). In acatalasemic *Drosophila*, viability and longevity of mutants were reduced (Griswold et al., 1993; Mackay and Bewley, 1989), under normal conditions, but only when catalase activity was close or equal to zero. H₂O₂ can diffuse through cell membranes and is involved in cell death and mutagenic effects (Lee et al., 2006; Mackay and Bewley, 1989). Moreover, in the presence of reduced transition metals, H₂O₂ can be converted through the Fenton reaction to the hydroxyl radical (HO•), the most reactive free radical, at innumerable cellular sites (Griswold et al., 1993; Halliwell, 1981). Because ingestion of mammalian blood results in a large dietary dose of iron for the insect (Dansa-Petretski et al., 1995), a H₂O₂ scavenger after a blood meal is likely important to prevent formation of HO•. The low amount of catalase activity in catalase-silenced mosquitoes was probably due to the few transcripts that RNAi failed to degrade and/or to low amounts of protein present before the blood meal (because RNAi will interfere in protein translation but not destroy mature proteins). The residual level of catalase in dsCat-injected mosquitoes possibly accounted for the percentage of mosquitoes surviving. The lack of a significant difference in lipid peroxidation in catalase-silenced mosquitoes may be because lipids are only one of the many biomolecules that can be damaged by ROS (Halliwell, 1981); thus it serves as one among other indicators of oxidative stress damage. Moreover, insects are generally very low in polyunsaturated fatty acids, which would reduce the potential for oxidative damage. Nucleic acid or protein molecules might have been damaged instead of lipids in these insects, but the specific death-causing mechanism is beyond the scope of this paper.

The gene encoding the *An. gambiae* QSOX was initially identified as a thioredoxin (Ribeiro, 2003). However, the protein used here is a member of the quiescin/sulfhydryl oxidases family,

or QSOX, bearing a thioredoxin and an ERV (essential for respiration and vegetative growth) 1 domain (Wang et al., 2007). Analysis of *An. gambiae*, *Ae. aegypti*, *D. melanogaster*, and human quiescins show that both thioredoxin and ERV1 domain are similar among these species. This family of proteins participates in disulfide bond formation in eukaryotes and may be involved on extracellular matrix formation, assembly of Fe/S center in proteins, and growth factors (Lange et al., 2001; Thorpe et al., 2002). Substrates for QSOX that have been identified in vitro include thiol molecules such as dithiothreitol, reduced glutathione, and thioredoxins (Thorpe et al., 2002). It has also been suggested that QSOX acts as an oxidant that opposes cellular thiol reductants (Chakravarthi et al., 2007; Thorpe and Coppock, 2007). In hematophagous insects, reductants such as thioredoxins are up-regulated after a blood meal (Munks et al., 2005; Ribeiro, 2003; Sanders et al., 2003). Here, the higher lipid peroxidation and mortality observed in QSOX-silenced mosquitoes may be an indication that the redox balance was altered in these insects after a blood meal.

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Fig. 1.

Percentage reduction of *Anopheles gambiae* catalase and sulfhydryl oxidase transcripts in dsRNA-injected mosquitoes, measured by quantitative reverse-transcriptase Real-Time PCR (qRT-PCR). *An. gambiae* females were injected with ds β gal, dsCat or dsQSOX and blood fed 48 h later on naive mice. At 24 h post-blood feeding, midguts were dissected and RNA was extracted and used for qRT-PCR. Bars show the mean (SD) of transcript reduction of *An. gambiae* catalase and thioredoxin over control treatment (ds β gal) obtained from 3 separate experiments (a pool of 5 midguts per group was assayed per experiment).

Fig. 2.

Catalase activity in dsRNA-injected mosquitoes. *Anopheles gambiae* females were injected with dsβgal, dsCat, or dsQSOX, blood-fed 48 h later and assayed for catalase activity at 24 h post-blood feeding. Bars show the mean and standard deviation of catalase units/ml from 3 independent RNAi experiments (a pool of 5 midguts per group was assayed per experiment). Catalase activity was based on the following: 1 unit of catalase will decompose 1.0 μmol of H₂O₂ to oxygen and water per minute at pH 7.0 at 25°C at a substrate concentration of 10 mM H₂O₂.

Fig. 3. Thiobarbituric acid reactive substances (TBARS) assay performed on 24 h post-blood fed *Anopheles gambiae* females that had been injected with dsβgal, dsCat or dsQSOX. Bars show the mean in μM concentration of MDA-TBA chromophore complexes from 3 separate experiments. Scales are different in the two graphs due to different standard curves of MDA obtained in separate experiments.

Fig. 4. Survival curves of *Anopheles gambiae* mosquitoes injected with dsβgal, dsCat, or dsQSOX and blood fed on naive mice. Life tables were pooled for each group from 4 experiments and survival curves compared by Kaplan—Meier log-rank analyses.

Fig. 5.

Survival curves of *Anopheles gambiae* mosquitoes injected with dsβgal, dsCat, or dsQSOX and blood fed on naive mice. Life tables were constructed for each group and survival curves compared by Kaplan—Meier log-rank analyses. Four separate experiments are shown.

Fig. 6. Structure of *Anopheles gambiae* quiescin/sulfhydryl oxidase (QSOX)/quiescin protein, adapted from Ensembl. The 700-aa protein has a thioredoxin domain at the N-terminal and a ERV1-like (or Alr) domain at the C-terminal. Note that a thioredoxin reductase motif was also recognized within the thioredoxin domain.

Fig. 7.

Alignment of *Anopheles gambiae* (Ensembl ID: AGAP007491), *Aedes aegypti* (Ensembl ID: AAEL012054), *Drosophila melanogaster* (Fly-Base ID: CG4670), and *Homo sapiens* (Ensembl ID: ENSG-00000116260) quiescin regions. These regions were determined by Thorpe et al. (2002) and comprise a thioredoxin domain and 3 regions of the ERV1-like domain, which are: CxxC motif (Region 1), representing the active site of quiescins; H×VN motif (Region 2); and another CxxC motif (Region 3), unique of quiescins.