**Plasmodium yoelii**: Correlation of TEP1 with mosquito melanization induced by nitroquine

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**A B S T R A C T**

The antimalarial drug nitroquine is not only an effective antimalarial drug, it is also able to induce the melanization of *Plasmodium* species. However, the molecular mechanisms of the recognition reaction induced by this drug remain unclear. Silencing of thioester-containing protein-1 (TEP1) significantly compromised the ability of *Anopheles gambiae* to melanize the *Plasmodium*, leading to investigation of the involvement of *A. stephensi* TEP1 in melanization induced by nitroquine. This study shows that (1) binding of AsTEP1 to oocysts, especially melanized oocysts, (2) after ingestion of anti-AsTEP1 antibody, the melanization rate in antibody-treated mosquitoes are significantly lower than in control mosquito (*p* < 0.05). The results suggest that nitroquine is able to induce *Plasmodium* recognition by TEP1, possibly triggering the resulting melanotic encapsulation. Further elucidation of the molecular mechanisms of mosquito immunity induced by antimalarial drugs will provide theoretical evidence for the use of antimalarial drugs, and a meaningful pathway for the design of novel antimalarial drugs.

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1. Introduction

Malaria is a severe infectious disease transmitted by mosquito vectors that causes over one million deaths each year. Almost 90% of these deaths occur in sub-Saharan Africa, especially among young children. In spite of intense efforts, the number of malaria cases is on the rise, and it is predicted to double over the next two decades (World Malaria Report, 2005). Although attempts to develop a vaccine for malaria are ongoing, drugs continue to be the only treatment option (Nwaka and Hudson, 2006).

Previous studies on the effects of potential antimalarial drugs have focused on their impact upon the development of *Plasmodium* species (sexual and sexual blood-stage parasites). While the importance of antimalarial drugs in influencing the infectivity of parasites in mosquitoes is generally recognized, i.e., chloroquine ingestion by the host at the time of the blood meal has been associated with an increase in parasites numbers in mosquitoes (Abrantes et al., 2008), nitroquine is known to contribute to a reduction in *Plasmodium* infectivity (Zhang et al., 2008).

In an attempt to further elucidate mechanisms underlying these effects, Abrantes et al. (2005) demonstrated that mosquitoes treated with chloroquine showed a significant down regulation of serine protease and antimicrobial peptide. Recently, they further reported a detailed survey of chloroquine and found that it was able to downregulate important immune-related genes (Abrantes et al., 2008). However, previously, we found that antimalarial nitroquine is not only effective against *Plasmodium* species, it also can induce melanotic encapsulation of *Plasmodium* species in *Anopheles stephensi*. Further, upregulation of the prophenoloxidase (PPO) gene and phenoloxidase (PO) activity might be involved in the process of nitroquine-induced melanotic encapsulation (Zhang et al., 2008). It is well known that melanotic encapsulation is an important defense mechanism of the refractory *Anopheles* against *Plasmodium*.

Melanotic encapsulation, also called melanization, is characterized by the deposition of melanin on the invading pathogen surface. In *Anopheles gambiae*, there is clear evidence that some critical up-stream regulators of the melanization pathway are essential for melanization of abiotic targets, as evidenced by the strongly reduced melanization of Sephadex beads when TEP1 (thioester-containing protein-1) is silenced (Warr et al., 2006). Specific silencing of TEP1 in *Anopheles quadriannulatus*, a non-vector species of mosquito, resulted in complete abolition of melanization of *Plasmodium berghei* and dramatically increased the number of oocysts, thus transforming *A. quadriannulatus* into a highly permissive parasite host (Habtewold et al., 2008). TEP1, a complement-like protein, has been shown to specifically bind to the surface of the ookinete stage of *Plasmodium* parasites in susceptible mosquitoes (Blandin et al., 2004). RNAi-mediated depletion of TEP1 causes a fivefold increase in the number of oocysts developing in the midgut of susceptible mosquitoes, while in the melanizing refractory strain, TEP1 silencing...
resulted in elimination of parasite killing and normal development of oocysts (Blandin et al., 2004).

In this study, the rodent malaria parasite *Plasmodium yoelii*, the vector mosquito *Anopheles stephensi* and the antimalarial drug nitroquine were used as a model to study mosquito melanotic response induced by antimalarial drug. We have found that nitroquine is able to upregulate the expression of the TEP1 gene and the binding of *An. stephensi* TEP1 (AsTEP1) to the melanized oocysts. Further, ingestion of anti-AsTEP1 antibody decreases the melanization rate in mosquitoes, implying a direct correlation between AsTEP1 and melanization induced by nitroquine.

2. Material and methods

2.1. Mosquitoes

*An. stephensi* (Hor strain) mosquitoes were reared at 24 °C and 75% humidity with a 12-h light/dark cycle. Adult mosquitoes were fed on a 5% sucrose solution.

2.2. Experimental procedures

2.2.1. Yoelii infection

*P. yoelii* 17XNL constitutively expressing GFP (PyGFP) was kindly donated by the Malaria Research and Reference Resource Center (MR4). This parasite line can express high levels of GFP during the complete parasite life cycle in the liver, blood, and mosquito stages, and also has normal growth properties. Mice were inoculated intraperitoneally with 1 × 10^7 red blood cells parasitized with PyGFP, and infection was verified with Giemsa staining (Payne, 1988). For infection experiments, female adult mosquitoes (3–5 days old) were starved for 6 h and then allowed to feed for 2 h on mice harboring PyGFP gametocytes.

2.2.2. Cloning and sequencing

Degenerate primers were designed according to the conserved amino acid sequence of the TEP gene from mosquitoes. The degenerate primer sequences were as follows: AsTEP f: 5'-GGITGGYGGIGAG-CAGAATATG-3', AsTEP r: 5'-AGDGCCCAAAYGTRCTCGT-3'. Cycling conditions included 35 cycles of 30 s at 94 °C, 30 s at 45 °C, and 90 s at 72 °C, followed by a final extension at 72 °C for 5 min. The PCR product was separated by 1% agarose gel electrophoresis, and the fragment was purified using the Silverbeads DNA Recovery Kit (Sangon, China) and cloned into the pMD18-T Simple Vector (TakaRa, Japan). A clone of 800 bp was sequenced and analyzed using BLAST programs.

2.2.3. Transcriptional profiling by real-time PCR

Quantitative real-time PCR was performed in order to analyze relative levels of AsTEP1-4 cDNA transcripts at selected time points after feeding on: (1) normal blood, (2) blood infected with PyGFP gametocytes, and (3) infected blood following nitroquine treatment. Total RNA from at least 50 female mosquitoes was extracted with Trizol reagent (Invitrogen) and subjected to reverse transcription. Specific primers were designed and synthesized by Takara, Japan, and were as follows: AsTEP1 f: 5'-CCTCCACAGACCAAAGA-3'; AsTEP1 r: 5'-ATCTGGTCCGGACAAAG-3'; AsTEP2 f: 5'-TGTACCTCTCTATGCCTCT-3'; AsTEP2 r: 5'-TGGATACCTGAAGGCTACAAC-3'; AsTEP3 f: 5'-TTGCCCTCCATGCTGGT-3'; AsTEP3 r: 5'-CATGACCTCTTGTGGT-3'; AsTEP4 f: 5'-ACTTGGTCCGGACAAAG-3'; AsTEP4 r: 5'-TTGCTGCTCGATGGT-3'. SYBR Premix Ex Taq (Perfect Real Time) (Takara) was used in real-time PCR analyses, which were run on a Gene Amp 5700 Sequence Detector (Applied Biosystems). The reaction mix (25 µl total volume) contained 1× reaction buffer, 1 mM MgCl2, 200 µM dNTPs, 0.4 µM primer concentration, 1 U/µl Takara EX Taq enzyme, SYBR green, and 1 µl cDNA template. The ribosomal protein S7 gene obtained from GenBank (accession number: AF539918) was used for data normalization. *A. stephensi* S7 gene (AsS7) primers were as follows: AsS7 f: 5'-CTAACGACAGGACCACAAGA-3'; AsS7 r: 5'-CAACCTGACAGGACAAAA-3'.

2.2.4. Preparation of AsTEP1 antibody

A BamHI-HindIII fragment of AsTEP1 cDNA was cloned into corresponding sites of the pQE-80L expression vector. Fusion proteins were expressed in *Escherichia coli* strain DH-5x and purified according to standard procedures (Pharmacia) on a glutathione Sepharose 4B column. Rabbits were immunized subcutaneously at multiple sites with 250 µg protein using Freund’s complete adjuvants and control rabbits were immunized with PBS and adjuvants, and were boosted every 4 weeks with 250 µg antigen for 6 months. SDS–PAGE, mass spectrometry (MS), ELISA and western blotting were used to determine the expression protein.

2.2.5. Immunostaining

Mosquito midguts were dissected in fixative (polyoxymethylene in 0.05 M Na–phosphate buffer, pH 7.6) at 4 °C. After an 8–12 h fixation at 4 °C, the midguts were washed in 0.05 M Na–phosphate buffer. Briefly, after blocking, the midguts were incubated overnight at 4 °C with AsTEP1 antibody (1:800 dilution), followed by a 1-h incubation with secondary antibodies [Rhodamine (TRITC)-Conjugated AffiniPure Goat Anti-Rabbit IgG, 1:1000]. The samples were analyzed with a Leica TCS-NT 165211 confocal microscope. Pre-immunized serum and serum of rabbit immunized with PBS and adjuvants were used as controls.

2.2.6. Identification correlation of AsTEP1 with melanization induced by nitroquine

Nitroquine was ground and dissolved in Tween-80, and then added to 5% sucrose, with a final concentration of nitroquine of 0.1%. *A. stephensi* mosquitoes were fed anti-AsTEP1 antibody from immunized rabbits combined with a 5% sucrose solution containing 0.1% nitroquine in order to identify the relationship between AsTEP1 and melanization induced by nitroquine. Immune sera were mixed with sucrose solution at 1:100. Five days after infection with *P. yoelii*, mosquitoes were randomly divided into four groups: (1) the untreated group, D1, (2) the nitroquine-treated group, Y, (3) the nitroquine + control sera-treated group, D2, and (4) the nitroquine + antibody-treated group, K. Unfed or partially fed females were removed. Nine days post-infection (3 days after antibody treatment), midguts were collected in order to observe melanization of *Plasmodium* in *A. stephensi*.

2.2.7. Determination of the melanization rate of *P. yoelii* in *An. stephensi* midgut

A minimum of 50 adult *A. stephensi* females were dissected from each group. Nine days post-infection, and the midguts were scored as either bearing only non-melanized oocysts, bearing only melanized oocysts, or bearing both melanized and non-melanized oocysts, with the data representing three independent experiments. The difference of melanization rates between the groups was analyzed by the Chi-square test, with p < 0.05 being considered statistically significant.

3. Results

3.1. Molecular cloning of AsTEP1 cDNAs

Using degenerate primers, we obtained a 0.8 kb cDNA fragment by PCR from hemocytes of *A. stephensi* (Fig. 1). Sequencing of plasmid clones containing the PCR product resulted in four unique clones.
representing four different partial thioester-containing protein cDNAs: AsTEP1 (EF076041), AsTEP2 (EF076042), AsTEP3 (EF076043), and AsTEP4 (EF076044). The translated sequence of cDNA was used to conduct a BLAST search. All four sequences contained the conserved canonical thioester motif GCGEQ, leaving no doubt that all four sequences are members of the thioester-containing protein family.

The predicted amino acid sequence of AsTEP1 was similar to Drosophila melanogaster TEP3 and Aedes aegypti TEP2, with the highest similarity to A. gambiae TEP1 (156/259), suggesting that this cDNA is TEP1 of A. stephensi. The deduced proteins of AsTEP2, AsTEP3, and AsTEP4 were similar to non-annotated TEP sequences of A. gambiae.

3.2. Transcription profiles of AsTEP cDNAs

For the quantitative real-time PCR experiments, the expression level of AsTEP cDNAs were normalized to that of the ribosomal protein AsS7 gene, and results are shown as fold-change relative to the unfed control. The results showed that the antimalarial drug nitroquine can induce upregulation of the AsTEP1 from 9 days post-infection (3 days after nitroquine treatment). The transcript abundance of the AsTEP1 cDNA in the nitroquine-treated group is significantly greater than in the untreated group, by 2.5-fold ($p < 0.05$), as shown in Fig. 2A; oocysts were also shown to be melanized. No upregulation of other AsTEP cDNAs was detected in association with nitroquine induction (Fig. 2B–D).

3.3. Binding of AsTEP1 to P. yoelii oocyst

The dsRNA knockdown of AgTEP1 in adult mosquitoes completely abolished melanotic encapsulation in a genetically selected refractory strain, suggesting an essential role of AgTEP1 in melanization. In our study, P. yoelii is susceptible to the A. stephensi. However, after nitroquine treatment, the Plasmodium was melanized in mosquitoes. To explore whether the AsTEP1 has a similar role in melanization induced by nitroquine, we observed the recognition reaction of AsTEP1 to the Plasmodium in A. stephensi, using rabbit polyclonal antibodies against AsTEP1 (the antibody titer was 1:8000, and SDS–PAGE, western blotting determined the specificity of the antibody, which showed that antigen was exclusively present in the hemolymph). Female A. stephensi mosquitoes were infected with P. yoelii 17XNL GFP, dissected at selected time points and analyzed by confocal microscopy. Light microscopy showed that the melanized oocysts markedly degenerated compared to those of the control (Fig. 3A). The surface of the oocysts was rough.

![Fig. 1. PCR amplification of thioester-containing protein cDNAs from A. Stephensi. Thioester-containing protein PCR fragment was obtained with degenerate primers.](image1)

![Fig. 2. Transcript variation of AsTEP1-4 cDNA induced by the antimalarial drug nitroquine. Quantitative real-time PCR using specific primers for AsTEP1-4 cDNA in mosquitoes fed normal blood (NB), blood infected with PyGFP gametocytes (IB), and infected blood following nitroquine treatment (NA). (A) The transcript variation of AsTEP1 cDNA; (B) the transcript variation of AsTEP2 cDNA; (C) the transcript variation of AsTEP3 cDNA; (D) the transcript variation of AsTEP4 cDNA.](image2)
and uneven, and was coated by some melanin (Fig. 3B). Laser confocal microscopy revealed that 9 days post-infection, binding of AsTEP1 with the oocysts in the drug-treated group was obviously stronger than in the untreated group (Fig. 3C and D). There was no AsTEP1 signal in the control.

3.4. Involvement of AsTEP1 in melanization induced by nitroquine

If AsTEP1 binding is essential for Plasmodium melanization induced by nitroquine, its absence should result in elimination or decrease of melanization. To understand the relationship between

the AsTEP1 and melanotic encapsulation induced by antimalarial drugs, mosquito midguts from four groups were dissected 9 days post-infection (3 days after antibody treatment). Compared with the nitroquine + control sera-treated group (D2) and the nitroquine-treated group (Y), melanization in the nitroquine + antibody treated group (K) was significantly decreased, but no melanized Plasmodium was observed in the untreated group (D1) ($p < 0.05$), shown in Fig. 4A and B and Table 1. The melanization rate in the nitroquine + antibody treated group (K) was 6.5%, but that of the nitroquine + control sera-treated group (D2) and the nitroquine-treated group (Y) was 26.0% and 31.3%, respectively (Table 1).

Fig. 3. Binding of AsTEP1 to P. yoelii 17XNL in A. stephensi. (A) Normal oocysts in A. stephensi midgut, light microscope; (B) melanization of oocyst in A. stephensi midgut, light microscope; (C) binding of AsTEP1 to normal oocysts in A. stephensi midgut, green channel; (D) binding of AsTEP1 to melanized oocysts in A. stephensi midgut, green channel. Scale bars in μm: (A–D), 10 μm.

Fig. 4. Melanized oocysts in A. stephensi. (A) Nitroquine-antibody treated group (K), ×100, light microscope; (B) nitroquine- control sera-treated group (D2), ×100, light microscope.

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4. Discussion

Although knowledge of the mosquito immune response has recently improved, less is known about the impact of antimalarial drugs on mosquito immunity. Abrantes reported that the presence of the antimalarial drug chloroquine in the mosquito has been associated with an increase in Plasmodium infectivity, possibly due to chloroquine leading to reduction of the capacity of the mosquito to defend against the infection. In this context, it is possible that the effects of chloroquine on the mosquito response to infection have contributed to the rapid dispersal of chloroquine resistance observed in the last 40 years. Nevertheless, it is worth noting that nitroquine can induce mosquito melanization and decrease Plasmodium infectivity. In a genetically selected refractory mosquito A. gambiae L35, Plasmodium can be fully blocked by melanotic encapsulation of late ookinetes or early oocysts (Collins et al., 1986; Paskewitz et al., 1988). Further, in infected Anopheles dirus, melanotic encapsulation of oocysts occurs between 7 and 15 days post-infection (Xu et al., 2007). It is unknown whether melanization induced by nitroquine and natural melanization share common mechanisms. This study explored whether A. stephensi TEP1 was related to melanization induced by the antimalarial drug nitroquine.

We obtained CDNA fragments of four TEPs from A. stephensi. TEPs are represented by multi-member families, both in the fruitfly, D. melanogaster, and in the mosquito, A. gambiae. BLAST analysis indicated they were orthologs of A. gambiae TEPs, designated as AsTEP1, AsTEP2, AsTEP3, and AsTEP4. Real-time PCR analysis showed an upregulation of AsTEP1 9 days post-infection (3 days after nitroquine treatment) that coincided with melanization of oocysts induced by nitroquine, suggesting the possible role of AsTEP1 in melanization induced by nitroquine. The transcription of other AsTEP cDNAs implies that they are not implicated in defense of the mosquito against Plasmodium and immune reaction induced by nitroquine, they may be involved in immunity of the mosquito to other pathogens. So far, only mosquito TEP1 has been characterized in detail. Characterization of loss-of-function phenotypes and the development of new tools for further biochemical analysis will be instrumental for uncovering other TEP functions.

To investigate whether AsTEP1 has stronger recognition for P. yoelii in nitroquine-treated mosquitoes compared to the control, we observed binding of AsTEP1 to P. yoelii. The results implied that binding of AsTEP1 to oocysts in the drug-treated group is significantly stronger than in the untreated group, and it is interesting that the AsTEP1 signal appeared on the lodgement of melanin. The binding of AgTEP1 to Plasmodium showed that early and extensive TEP binding to ookinetes correlates with ookinete melanization, and the potential role of AgTEP1 in melanization indicated that the PPO signal was detected only on the surface of AgTEP1-labeled Plasmodium. However, AgTEP1 has been localized between the ookinete surface and the melanin layer of the capsule and PPOs (Blandin et al., 2004), suggesting that AgTEP1 is associated with ookinete melanization but not directly involved in tethering of the PPO complex on the parasite surface. In our study, we found AsTEP1 signal on the lodgement of melanin, implying that AsTEP1 is able to directly tether the components of melanization. In addition, we also found that melanized oocysts were smaller and markedly degenerated compared with those of the control. The surface of the oocysts was rough and uneven and coated by some melanin. This parasite degeneration was consistent with the effects of nitroquine on the erythrocytic and exoerythrocytic forms of Plasmodium and may be related to its mechanism of action. It was presumed that nitroquine degenerated and exposed some ligand on the surface of Plasmodium, leading to involvement of pattern recognition receptors in the melanization process. As a consequence, it is important to identify the correlation of AsTEP1 with melanization induced by nitroquine.

Antibodies against Plasmodium or mosquitoes, which are produced by the vertebrate host, can cross the midgut epithelia and be detected in the hemolymph of Anopheles mosquitoes (Vaughan and Azad, 1988; Kumari et al., 2009; Beier et al., 1989). This property allowed observation of the variation of melanization induced by nitroquine in A. stephensi mosquitoes that ingested anti-AsTEP1 antibody. ELISA was performed in order to determine the specificity of the antibody to TEP1. The results showed that after ingestion of this antibody, the melanization rate would decrease in mosquitoes treated with antibody, suggesting correlation of AsTEP1 with melanotic encapsulation induced by the antimalarial drug nitroquine.

These findings, together with those of previous studies, suggest that nitroquine-induced melanization in A. stephensi requires TEP1. Although we are still not able to dissect the exact mechanism of melanization induced by nitroquine, the extent of the impact of nitroquine on mosquito immune responses is evident from our study, and it suggests correlation of AsTEP1 with melanotic encapsulation induced by the antimalarial drug nitroquine, either directly through participation in capsule formation as proteins cross-linked with melanin, or indirectly through triggering the activation of serine protease cascades that activate PPOs.

5. Conclusions

The impact of antimalarial modulation on murine malaria transmission is evident from the present studies. However, it is unclear whether these results can be extrapolated to human malaria; differences in the transmission of rodent and human malaria cannot be disregarded. Furthermore, future work will serve to provide a better understanding of the impacts of antimalarial drugs on vector and parasite transmission. Understanding of the level of antimalarial interference with the mosquito anti-Plasmodium defense is emerging as an issue of major importance for the future of malaria treatment strategies.

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