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Involvement of prophenoloxidases in the suppression of *Plasmodium yoelii* development by *Anopheles dirus*

Ying Wang, Hongxing Hao, Zong-wen Qiu, Wen-yue Xu, Jian Zhang, Tao-li Zhou, Xi-lin Zhang *, Fu-Sheng Huang *

Department of Pathogenic Biology, College of Basic Medical Sciences, Third Military Medical University, 30 Gaotanyan Street, Shapingba District, Chongqing 400038, PR China

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ABSTRACT

Anopheles dirus is refractory to a rodent malaria parasite, *Plasmodium yoelii*, and melanized oocysts are manifested in infected mosquitoes. Prophenoloxidase (PPO) is a zymogen whose active form mediates melanotic encapsulation of invading pathogens in mosquitoes. In this study, we cloned cDNA fragments of four *An. dirus* PPOs, that are orthologs of *Anopheles gambiae* PPO2, PPO4, PPO5 and PPO6. *AdPPO4* expression in hemocytes was induced in response to *P. yoelii* infection. RNA interference using double stranded RNA of *AdPPO4* led to depletion of its mRNA and other PPO transcripts. This depletion increased *P. yoelii* infection prevalence and oocyst intensity, and abolished the melanization of oocysts as well. Therefore, *An. dirus* PPOs may play a role in the refractoriness to *P. yoelii*.

1. Introduction

Malaria is a major health problem, causing 200–300 million human infections and over one million deaths annually. It is responsible for 20% of all deaths among children under 5 years old in Africa (WHO: World Malaria Report, 2005). The burden of malaria is increasing despite of ongoing efforts for control directed at mosquito vectors (environmental management, insecticides, and bednets) or anti-malarial medicines. The resurgence of malaria may be attributed mainly to the emergence of drug and insecticide resistance in parasite and vector populations, respectively. New intervention strategies are needed urgently.

The *Plasmodium* parasites undergo a dynamic sporogony within the vector mosquitoes (Vaughan, 2007). Generally the parasites experience numerical losses in abundance during two life-stage transitions, i.e. gametocyte-to-ookinete and ookinete-to-oocyst, in the early sporogony (Alavi et al., 2003; Poudel et al., 2008; Sinden et al., 2007; Vaughan et al., 1992). Mosquito immune attack is believed to be one of the factors that contribute to the parasite reduction. Invading parasites are eliminated by lysis or melanization in the midgut (Blandin et al., 2008; Chen et al., 2008; Vlachou and Kafatos, 2005). Melanotic encapsulation of parasites as a visible trait of defense has become a focus of research since the establishment of a genetic strain of *Anopheles gambiae* that is capable of melanizing several *Plasmodium* species (Collins et al., 1986).

We have been using rodent malaria parasite *Plasmodium yoelii* and *Anopheles dirus* as a model to study mosquito melanotic response against *Plasmodium*. *Plasmodium yoelii* was originally isolated from shiny thicket rats in central Africa. The natural vector of *P. yoelii* is not known. The susceptibility of colonized mosquito species to *P. yoelii* varies. For example, *P. yoelii* can complete its development to the sporozoites in the salivary gland in *An. freeborni* and *An. stephensi*, but it stops development before oocyst stage in *An. albinmanus*. In *An. gambiae* and *An. dirus* the oocysts of *P. yoelii* can be melanized. It is intriguing to note that the patterns of melanotic response are different in these two vector mosquitoes. In *An. gambaie* the parasites are melanized at early oocyst stage and the melanized oocysts are small (10 μm diameter), whereas in *An. dirus* the melanized oocysts are larger (60–70 μm diameter) (Vaughan et al., 1994). We have shown that 5% oocysts began to be melanzied at day 7, and the rate of melanized oocyts increased to 58.3% at day 15 post infection. The hemocytes with granules were found near or attached to the melanized oocysts (Huang and Wang, 1995; Xu et al., 2007). These findings suggest that melanization

* Corresponding authors. Fax: +86 23 68753841.
E-mail addresses: zhxljsc120@163.com (X.-l. Zhang), fushenghster@gmail.com (F.-S. Huang).

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contribute to the refractoriness to *P. yoelii* in *An. dirus*. In insect, melanization response is initiated by an enzymatic cascade that leads to the prophenoloxidase (PPO) activation in response to invaded pathogens (Cerenius and Soderhall, 2004; Christensen et al., 2005). In this study we obtained cDNA fragments of four PPOs from *An. dirus*. The transcriptional analysis suggests that PPOs play an important role in the melanization of *P. yoelii*. RNA interference (RNAi) caused depletion of multiple PPO transcripts in *An. dirus*, which increased the susceptibility to *P. yoelii* infection.

2. Materials and methods

2.1. Mosquitoes rearing and infection with P. yoelii

Anopheles dirus were reared at 27 °C with a relative humidity of 70–80% with supply of 5% sucrose. The mosquitoes were fed on mouse blood for gonotrophic cycle. Larvae were kept in water and fed on yeast powder, and pupae emerge in cage. Adult mosquitoes were infected with *P. yoelii* (BY 265 strain) by feeding on *P. yoelii* infected mice (Kunming strain) after a 10-h starvation, and the infected mosquitoes were kept at 24 °C, an ideal temperature for *P. yoelii* development. Prevalence of infection and oocyst melanization were checked by midgut dissection at 11 days after the blood feeding.

2.2. Cloning of An. dirus PPOs

Haemocytes were collected from 200 female adults infected with *P. yoelii* 24 h before, using a method previously described by Paskewitz and Shi (Paskewitz and Shi, 2005). In brief, after the tip of proboscis was removed with scissors, haemolymph was expelled in a droplet at the wounded proboscis upon pressure to the thorax. Only clear droplets were collected to avoid contamination with fat body. Hemolymph droplets were immediately added into 1 ml of Trizol reagent (Invitrogen). Total RNA was isolated from haemocytes according to the manufacturer's instruction. Approximately 0.6 μg total RNA was used to synthesize the first strand cDNA with MMLV (Promega). Then PCR was carried out with degenerate primers (Table 1), which were designed using Primer 5.0 (PREMIER Biosoft International) according to conserved amino acid sequence of PPOs from nine arthropods, including *An. gambiae, An. stephensi, An. culicifacies, Bombix mori, Tenebrio molitor, Sarcophaga bullata, Hyphantria cunea, Pacifastacus leniusculus, Penaeus monodon.* Products of RT-PCR at the expected size were cloned into the pMD18-T vector (Takara). Plasmid samples were transformed into *Escherichia coli* DH5α. Then the randomly picked clones were sent to Invitrogen company for sequencing. And the nucleotide sequences were analyzed by the DNASIS software (Hitachi Software Engineering Company).

2.3. Semiquantitative RT-PCR

Semiquantitative RT-PCR was used to examine relative abundance of *AdPPO* transcripts in three groups of mosquitoes that were fed on sugar, normal, or infectious blood meal. Total RNA was isolated using Trizol at 24 h after feeding. First-strand cDNA was synthesized using total RNA (1 μg), oligo$(dT)_{15}$ (10 pmol), and MMLV reverse transcriptase at 42 °C for 1 h. Ribosomal protein S7 gene *AdS7* was used as an internal loading control to normalize the cDNA templates in the PCR. The primer sequences were listed in Table 1. Thermal cycling conditions were 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min. The appropriate cycle numbers were chosen to produce comparable band intensities while avoiding saturation. After separation of the PCR products on 1% agarose gel electrophoresis, band intensity was quantified and compared using Quantity One Gel Analysis Software (Bio-rad).

Table 1
Primer sequences. Sense primer sequences (Ps) and antisense primer sequences (Pa) were shown in this table.

	Primer sequence
Degenerate primers to clone PPO	Ps: 5′-AACCTGCA(CT)CA(CT)TGGCA(CT)TGGCA-3′
	Pa: 5′-CCAGCGGTAGAA(ACG) (AG) (ATC) (ATCG) GG(AG) TC-3′
Primers to extend AdPPO4	Ps: 5′-CGCTATGCGAACGGGTGGCCGAAGG-3′
	Pa: 5′-C(GT)(AG)TCGAA(GC)GG(AG)(AT)A(GT) CCCAT-3′
Specific primers of AdPPOs used in semiquantitative RT-PCR	Ps (AdPPO2): 5′-GCGTACTTCCCGAAGATGATCCG-3′
	Pa (AdPPO2): 5′-ATGTTGTGCAGGCTGCCGTACAG-3′
	Ps (AdPPO4): 5′-CGCTATGCGAACGGGTTGCCGAAGG-3′
	Pa (AdPPO4): 5′-CAACGCCGAAGCCCTCCAGGAACGA-3′
	Ps (AdPPO5): 5′-CTACTCCTGTCGCTATCCC-3′
	Pa (AdPPO5): 5′-ATCGTCGTGGTCAAATCTC-3′
	Ps (AdPPO6): 5′-TGCGTCCGCTCACGAGTCTG-3′
	Pa (AdPPO6): 5′-CCCACCACACCGTATCCCTCC-3′
Specific primers of AdS7 used in semiquantitative RT-PCR	Ps (AdS7): 5′-GATCATCATCTACGTGCCGCTG-3′
	Pa (AdS7): 5′-TGGTGGTCTGCTGGTTCTTGTC-3′
Specific primers of GFP used in RNAi	Ps (GFP): 5′-CACAAGTTCAGCGTGTCCG-3′
	Pa (GFP): 5′-AGTTCACCTTGATGCCGTTC-3′

2.4. RNA interference

Gene silencing: An *AdPPO4* cDNA fragment was used as template to make double-stranded RNA (dsRNA) for silencing PPO4 gene. PCR was carried out using the AdPPO4-specific primers mentioned before, each of which has a 5′ extension of T7 promoter sequence (5′-TAATACGACTCACTATAGGG-3′). The control dsRNA was made from a fragment of GFP gene (Table 1). The PCR products were purified and used as templates for in vitro transcription reactions with an Ambion MEGASCRIPT RNAi kit (Ambion) by following the manufacturer's instructions. The resulting dsRNA was analyzed by agarose gel electrophoresis and concentrations were determined using a spectrophotometer ND-1000 (American MET). About 69 nl dsRNA (0.3 mg/ml) was introduced into the hemocoel through thorax of ether-anesthetized 3-day-old female mosquitoes by using a Nanoinject (Drummond). The mortality caused by injection was about 10% with no significant difference among groups.

Challenge with Plasmodium: After being untreated or treated with *AdPPO4* or GFP dsRNA for four days, mosquitoes were fed on the same mouse infected with *P. yoelii*. Mosquito midguts were dissected on day 11 post infection, and oocyst numbers were counted under microscope. Each assay was done with at least 50 mosquitoes, and the data represent three independent experiments. Difference of infection prevalence between groups was analyzed by Chi-square test. Kruskal–Wallis one-way analysis of variance (ANOVA on ranks) was used to test difference of the infection intensity (*i.e.* oocyst load) in infected mosquitoes among groups. The uninfected mosquitoes were not included when infection intensity was analyzed.

3. Results

3.1. cDNA cloning of PPO genes from An. dirus

Using degenerate PCR we amplified fragments with approximately 600 bp from the cDNA of haemocytes of *An. dirus*. The