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Research brief

Plasmodium berghei: Effect of protease inhibitors during gametogenesis and early zygote development

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Abstract

Plasmodium berghei: The effect of five protease inhibitors, TPCK, TLCK, PMSF, leupeptin, and 1,10-phenanthroline on in vitro gametogenesis and early zygote development of *P. berghei* was investigated. PMSF and leupeptin showed no effect. Cysteine/serine protease inhibitors TPCK/TLCK at concentrations of 75 and 100 μM were effective on inhibiting exflagellation center formation, and this effect was reversible with the addition of L-cysteine. Exflagellation center formation was most effectively blocked by 1,10-phenanthroline (1 mM), and exflagellation center numbers were restored by the addition of Zn^{2+}. A reduction of ookinete production was observed when TPCK/TLCK (100 μM) was added at 2 h after gametogenesis, but no effect was observed with 1,10-phenanthroline (1 mM). Our results suggest that proteolysis is important in both gametocyte activation and sexual development of *P. berghei*.

Index Descriptors and Abbreviations: *Plasmodium berghei*; Malaria; Parasite; Gametogenesis; Gametes; Zygotes; Exflagellation centers; Leupeptin; PMSF, phenylmethylsulfonyl fluoride; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosylamide-2-phenylethyl-chloromethyl ketone; 1,10-phenanthroline; Protease; Protease inhibitor

The sexual development of malaria parasites within the midgut of mosquitoes is an essential step of the parasite life cycle. Shortly after an infected bloodmeal, male and female gametes are formed from gametocytes (0–60 min post-feeding [pf]), and their fertilization (30–60 min pf) results first in zygotes and then in motile ookinetes (9–24 h pf), that escape to form oocysts on the hemolymph side of the midgut epithelium (Sinden, 1999). A decrease in temperature and xanthurenic acid, a waste product derived from mosquito metabolism, are the main factors that trigger gametogenesis (Kawamoto et al., 1991; Billker et al., 1998).

The participation of proteolysis in parasite development and midgut invasion has been documented at various levels: midgut digestive enzymes have a deleterious effect on *Plasmodium* ookinetes (Gass and Yeates, 1979; Ramasamy et al., 1997; Yeates and Steiger, 1981); chitinase and subtilisin-like serine proteases are used by ookinetes to penetrate the peritrophic matrix (Langer and Vinetz, 2001; Shahabuddin and Kaslow, 1993), and invade the midgut epithelium (Han et al., 2000). However, little is known about the effect of midgut and parasite proteases on the earlier sexual parasite processes (gametogenesis, fertilization, and zygote formation). Understanding these mechanisms may provide insight into malaria parasite biology and vector–parasite interactions. Data supporting a possible role of proteolytic activity on *Plasmodium berghei* gametogenesis and zygote production is presented here.

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Plasmodium berghei, ANKA strain, gametocyte-producing clone 2.34- (kindly provided by R. E. Sinden, Imperial College, UK) infected blood was obtained from blood-passage-infected BALB/c mice treated with 6 mg/ml of phenylhydrazine (Sigma, St. Louis Mo. USA) previous to the infection to induce reticulocytosis. Sexual *P. berghei* cultures were obtained as described (Rodríguez et al., 2002) using complete ookinete medium (RPMI 1640 medium, 20% heat-inactivated fetal bovine serum [Invitrogen Life Technologies, Carlsbad CA], 50 μg/ml hypoxanthine, 25 mM Hepes, 24 mM $NaHCO_3$, 5 μg/ml penicillin, 5 μg/ml streptomycin, and 1 μg/ml neomycin, pH 8.3). Gametogenesis in vitro took 10–15 min after which exflagellation centers were observed.

To analyze the effect of protease inhibitors on gametocyte activation (number of exflagellation centers), freshly prepared protease inhibitors were added to five replicate gametocyte-infected blood cultures for each compound at concentrations of 25 μM to 1 mM (Leupeptin [stock solution of 10 mM in 10 mM sodium phosphate, pH 7.4], phenylmethylsulfonyl fluoride, PMSF [stock solution of 200 mM in methanol], *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, TLCK [stock solution 10 mM in methanol], L-1-tosylamide-2-phenylethyl-chloromethyl ketone, TPCK, [stock solution of 10 mM in methanol] [Sigma], and 1,10-phenanthroline [stock solution of 200 mM in methanol] [Merck, Darmstadt, Germany]). Control cultures received only medium added with the same amount of solvent of the corresponding protease inhibitor. Cultures were incubated at 19–21 °C, and the number of exflagellation centers in a total of 40 microscopic fields (400×) were counted after 15 min of culture. In these and the following experiments, the mean ± SD of the percentage inhibitions were compared between experimental against control groups using one-way ANOVA, Tukey–Krammer test ($P < 0.05$) with one degree of freedom (Fry, 1996).

An inhibitory effect of 99.2 and 100% ($P < 0.05$) on exflagellation center formation was observed when TPCK was added at 75 μM and 100 μM, respectively, to gametocyte-infected blood cultures (Fig. 1). TLCK added at concentration of 50 μM induced 83.7% inhibition and complete inhibition was obtained at 100 μM ($P < 0.05$). The addition of 1 mM 1,10-phenanthroline also resulted in a significant reduction ($P < 0.05$) in exflagellation centers, but all concentrations (up to 1 mM) of PMSF and leupeptin had no detectable effect (Fig. 1). These results suggest the possible participation of cysteine proteases and metalloproteases in gametocyte activation.

To investigate the effect of protease inhibitors on the sporogonic cycle of *P. berghei*, from gametocyte activation to ookinete development, gametocyte-infected blood cultures were prepared as above, but 100 μM TPCK and 100 μM TLCK, 1 mM leupeptin, 1 mM PMSF, and 1 mM 1,10-phenanthroline were added at 0, 10, 20, 30, 60, and 120 min and exposed at 21 °C during 10 min, after which the proteases inhibitors were eliminated by washing twice with complete ookinete medium. Washed parasites were suspended in fresh ookinete medium at a 1:5 dilution and incubated at 21 °C for 24 h to allow ookinete development. Ookinetes/10,000 erythrocytes were recorded in Giemsa-stained smears of pelleted cultures. Control cultures were set by adding the corresponding proteases inhibitors solvents at the corresponding times, and statistical comparisons were performed as above. Leupeptin and PMSF had no effect on ookinete development no matter which parasite stage was exposed to them (Fig. 2). A significant reduction ($P < 0.05$) was observed when TLCK and TPCK were added to cultures at all experimental times (inhibitions of 82.35 ± 3.16, 92.4 ± 0.65, 95.31 ± 0.81, 89.07 ± 2.15, and 90.2 ± 5.62 with the addition of TLCK at 0, 10, 20, 30, and 60 min in culture, respectively, and inhibitions of 87.47 ± 1.92, 91.1 ± 0.56, 79.59 ± 3.1,

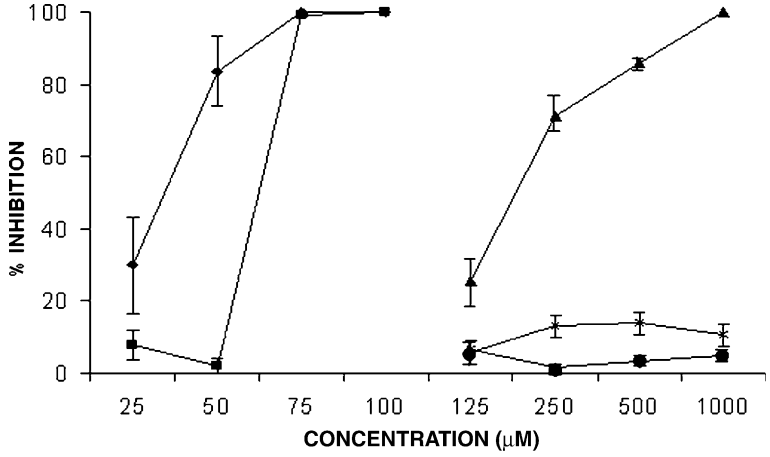

Fig. 1. Effect of protease inhibitors on *P. berghei* gametogenesis. *P. berghei* gametocyte-infected mouse blood cultures ($n = 5$, each) were treated with protease inhibitors (final concentrations are indicated) and the numbers of exflagellation centers were determined in 40 fields using a phase contrast microscope. The percentage ± SD inhibition of exflagellation centers was calculated by comparison to those of control cultures added with same amount of solvent of the corresponding protease inhibitor. (■) TPCK, (♦) TLCK, (●) leupeptin, (×) PMSF, and (▲) 1,10-phenanthroline.