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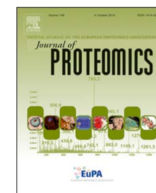

Dynamic molecular events associated to *Plasmodium berghei* gametogenesis through proteomic approach

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ABSTRACT

Plasmodium mature sexual cycle occurs in the vector mosquitoes and ensures the transmission to a new host. Gametogenesis takes place within minutes in the vector midgut. Gametocytes have to complete a deep nuclear reorganization, quick differentiation, and in the case of male gametocytes, intracytoplasmic flagellum assembly that results in free-swimming microgametes required for macrogamete fertilization.

In efforts to improve our knowledge of molecular mechanisms involved in gamete morphogenesis, we carried out a nanoLC/MSMS based quantitative proteomic analysis throughout the xanthurenic acid-induced gametogenesis of the rodent parasite *P. berghei*. Time-course analyses were performed 7 and 15 min after gametogenesis induction. From 2617 iTRAQ-labelled peptides, 49 proteins were found differentially abundant. Proteins related to RNA translation, DNA, and protein biosynthesis were most prominent and strongly regulated. The energetic metabolic pathway, glycolysis, environmental stress response, RNA/protein biosynthesis, mitosis and axoneme formation, both related to tubulin-associated cytoskeleton dynamic, were predominant regulated cell processes at protein level during the differentiation. Our results also include 26 phosphoproteins in gametocytes/gametes. This first iTRAQ-based proteomic time course analysis of *Plasmodium* gamete development sheds light on the biological protein orchestration within gametogenesis.

Significance: Malaria is one of the most serious and widespread parasitic diseases that affected humans in medicine history. The increasing emergence of resistance of parasites from *Plasmodium* genus to the available antimalarial drugs and the absence of efficient vaccines require an urgent need of development of new therapeutic strategies to fight against that disease. The sexual reproduction is a key step of *Plasmodium* life cycle and constitutes an attractive target for the development of new therapeutic approaches since it would control malaria based on an inhibition of the parasite transmission to *Anopheles*, and then to humans. Male and female gamete formation (gametogenesis) is thus a biological event that is determinant for the perpetuation of the parasite in which drastic morphological and metabolic changes occur in a short time interval, resulting in the production of 8 male gametes from a male gametocyte, and fertilization of the female gamete. Development of such transmission-blocking strategies required in deep understanding of the molecular and cellular events associated to gametogenesis. Despite several studies, our understanding on gametogenesis is still incomplete and requires further investigations. This work is the first large-scale quantitative proteomic insight into the *P. berghei* gamete morphogenesis providing valuable time course data. *Plasmodium* gametogenesis clearly requires regulation of expression and phosphorylation of proteins belonging to different metabolic pathways and functions, in order to produce mature male and female gametes.

1. Introduction

Despite the decline of malaria deaths by 48% (438,000 in 2015) in the last 15 years among populations at risk, malaria remains a major cause of death for children, particularly in sub-Saharan Africa, taking the life of a child every 2 min. Moreover, there are no licensed vaccines

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against malaria and in recent years, parasite resistance to the best available treatment, artemisinin-based combination therapy (known as ACT), has emerged [1].

Malaria is due to the development in vertebrates of a protozoan parasite belonging to the *Plasmodium* genus. Uptake of sexual precursor cells, the male and female gametocytes, from vertebrate blood during the *Anopheles* vector meal leads to the mosquito infection and to the genesis of sporozoites that accumulate into the salivary glands. Vertebrate infection occurs by injection of sporozoites from salivary glands to vertebrates during the mosquito bite. Establishment of mosquito infection requires a differentiation of the haploid gametocytes into male and female gametes, a process called gametogenesis, for fertilization and formation of a diploid zygote in the gut lumen. The zygote differentiates into a mobile ookinete that crosses the midgut epithelium and settles to differentiate, after meiosis, into oocyst. The later, after multiple mitosis, produces sporozoites that migrate to the salivary glands.

Gametocyte maturation is blocked in G0 phase in vertebrate blood. Gametogenesis induction requires several changes of the parasite environmental conditions that are met in mosquito gut *viz.*: drop of temperature from 37 °C to around 20 °C, increase of pH from 7.4 to 7.8–8.0, O_2 change, and the presence of the inducer xanthurenic acid, a mosquito intermediate metabolite of tryptophan metabolism [2]. Gametogenesis of *Plasmodium berghei*, a rodent parasite, is a quick process occurring within 15 to 20 min and involving 1) xanthurenic acid-triggered calcium signalling to activate Ca^{2+}-dependent protein kinases; 2) egress of the parasite from the red blood cells (RBCs) by lysis of the parasitophorous vacuole and the erythrocyte membrane; 3) in male gametocyte, completion of three rounds of rapid mitotic DNA replication associated to the intracytoplasmic axoneme assembly that will produce 8 highly motile male gametes by a process called exflagellation [3,4].

Reversible protein phosphorylation, rapid mitosis, and translational repression are major cell processes that have been highlighted to orchestrate the *Plasmodium* sexual development [3,5,6]. Although several proteomic studies of *Plasmodium* gametocytes and gametes have been described [7–12], molecular and cellular events associated to gametogenesis are still incompletely characterized. To provide new insights on molecular processes involved in gametogenesis, we here disclose the first label-based quantitative proteomic time-course analysis of the xanthurenic acid-induced gamete development in *P. berghei*. Front to the quick transformation, the necessity to obtain high quantity of proteins from a reproducible triplicate and to minimize extensive manipulation period, we performed protein relative quantitative comparisons over 7 and 15 min of gametogenesis induction using iTRAQ labelled peptides.

2. Materials and methods

2.1. Ethical compliance

Experiments using rodents were carried out according to European regulations in compliance with the French guidelines and regulations, and approved by the Ethic Committee CUVIER (authorization no. 68-007).

2.2. In vivo P. berghei gametocytes production and in vitro gamete exflagellation process

A pyrimethamine-sensitive clone of *Plasmodium berghei* NK65 strain (kindly provided by R. Ménard) was used throughout the study. *P. berghei* was maintained by cyclic passage in 4–6 weeks old female Swiss OF1 (Janvier, Le Genest Saint Isle, France). Enrichment and purification of gametocytes were achieved using a modified protocol from Beetsma et al. [13], as described in Guttery et al. [14]. Briefly, mice were treated intraperitoneally (ip) with 100 μL of 25 mg/mL phenylhydrazine in saline solution to induce hyper-reticulocytosis [15]. Two to three days later, mice were infected ip with 10^7 infected RBCs from a donor mouse. At day 3 post-infection, mice were treated with sulfadiazine (Sigma) at 15 mg/L in drinking water for two days to kill asexual blood stages. On day five after infection, parasites were mainly constituted of gametocyte stages. Mice were euthanized; blood harvested with heparin and kept on ice. Gametocyte-infected RBCs were separated from uninfected erythrocytes by a 48% NycoDenz gradient (27.6% w/v Nycodenz in 5 mM Tris-HCl, pH 7.2, 3 mM KCl, 0.3 mM EDTA) in coelenterazine loading buffer (CLB: containing PBS, 20 mM HEPES, 20 mM glucose, 4 mM sodium bicarbonate, 1 mM EDTA, 0.1% w/v bovine serum albumin, pH 7.25) during 10 min at 1000 × *g*. Gametocyte-infected RBCs were collected at the interphase, transferred into CLB-containing tubes, centrifuged for 1 min at 800 × *g* and the pellet resuspended in CLB, and Giemsa-stained smears realized. One third of the sample was immediately frozen in liquid nitrogen (Time 0) and xanthurenic acid (100 μM final concentration) was immediately added to the remaining two thirds to induce gametogenesis. A small amount of cell suspension was mounted onto a Malassez chamber to count the RBC number and to follow the exflagellation process under microscope. Half of the sample was frozen in liquid nitrogen after 7 min of incubation at room temperature (RT) and the remainder after 15 min of incubation. Samples were stored at − 80 °C.

In our hands, no free male gametes or gametocytes under process of exflagellation were observed at time 0. Fifteen minutes after xanthurenic acid induction, budding male gametes from gametocytes with vigorous flagellar beating, associated with formation of characteristic nucleation centres with the surrounding cells, as well as free male gametes, were reproducibly observed. Three independent biological replicates were performed. Each enrichment had about 3×10^8 gametocyte-infected RBCs. Purity was checked on Giemsa-stained smears (Fig. 1).

2.3. Sample preparation for LC-MS/MS

Each sample was lysed in 10 volumes of 8 M urea containing a complete mixture of protease and phosphatase inhibitor, 2 × Halt Protease and Phosphatase Inhibitor Cocktail 100 × (Thermo Scientific), through three rapid cycles of freezing in liquid nitrogen and thawing on ice. Lysates were lyophilized and stored at − 80 °C until use. Lyophilized samples were resuspended in 100 mM TEAB (triethylammonium bicarbonate, Fluka), vigorously vortexed and proteins were precipitated by ethanol/acetone. Briefly, each sample was vigorously vortexed and incubated overnight at − 20 °C for protein precipitation. Samples were then centrifuged at 13,000 × *g*, 15 min, at 4 °C and the supernatant was discarded. The pellet was washed three times with ice-cold ethanol/acetone/water solution (4/4/2). Proteins were resolubilized in 8 M urea in 100 mM TEAB, centrifuged at 13,000 × *g*, 15 min, at 4 °C and the supernatant transferred into a new tube. Proteins were reduced by 20 mM DTT at RT for 1 h and alkylated with 40 mM iodoacetamide for another hour. Proteins were first digested at RT by endoproteinase Lys-C (Sigma-Aldrich) for 12 h with 0.01 AU for 100 μg of proteins. The solution was then diluted with 100 mM TEAB and 10% acetonitrile to a final urea concentration of 1 M, supplemented with 1 μg of trypsin per 100 μg of proteins, Sequencing Grade Modified Trypsin (Promega), for 4 h, at RT. The peptide sample was acidified with TFA 0.1% (v/v) final concentration and desalted using homemade 2 cm long microcolumns of Poros 20 R2 and Oligo R3 resins (Applied Biosystems) packed in p200 tips [16]. Peptides were eluted with 70% acetonitrile/0.1% TFA. Dried up peptides were solubilized in 300 mM TEAB and the concentration of the peptide solution was determined by Qubit® quantification assay (Thermo Fischer).

2.4. iTRAQ labelling

Peptides were labelled using 4-plex iTRAQ Reagent Kit (Applied Biosystems) according to the manufacturer's instructions. Briefly,

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