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Original Contribution

Glutathione-deficient *Plasmodium berghei* parasites exhibit growth delay and nuclear DNA damage

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ABSTRACT

Plasmodium parasites are exposed to endogenous and exogenous oxidative stress during their complex life cycle. To minimize oxidative damage, the parasites use glutathione (GSH) and thioredoxin (Trx) as primary antioxidants. We previously showed that disruption of the *Plasmodium berghei* gamma-glutamylcysteine synthetase (*pbggcs-ko*) or the glutathione reductase (*pbgr-ko*) genes resulted in a significant reduction of GSH in intraerythrocytic stages, and a defect in growth in the *pbggcs-ko* parasites. In this report, time course experiments of parasite intraerythrocytic development and morphological studies showed a growth delay during the ring to schizont progression. Morphological analysis shows a significant reduction in size (diameter) of trophozoites and schizonts with increased number of cytoplasmic vacuoles in the *pbggcs-ko* parasites in comparison to the wild type (WT). Furthermore, the *pbggcs-ko* mutants exhibited an impaired response to oxidative stress and increased levels of nuclear DNA (nDNA) damage. Reduced GSH levels did not result in mitochondrial DNA (mtDNA) damage or protein carbonylations in neither *pbggcs-ko* nor *pbgr-ko* parasites. In addition, the *pbggcs-ko* mutant parasites showed an increase in mRNA expression of genes involved in oxidative stress detoxification and DNA synthesis, suggesting a potential compensatory mechanism to allow for parasite proliferation. These results reveal that low GSH levels affect parasite development through the impairment of oxidative stress reduction systems and damage to the nDNA. Our studies provide new insights into the role of the GSH antioxidant system in the intraerythrocytic development of *Plasmodium* parasites, with potential translation into novel pharmacological interventions.

1. Introduction

Malaria remains the most deadly parasitic disease resulting in approximately 584,000 deaths from 198 million clinical cases reported in 2013 and one-third of the world population at risk of infection [1]. This disease is caused by the apicomplexan parasites of the genus *Plasmodium*, which are transmitted to humans by the bite of infected *Anopheles* mosquitoes.

Plasmodium parasites are exposed to multiple sources of oxidative stress throughout their complex life cycle. During the intraerythrocytic development, endogenous oxidative stress results from reactive oxygen species (ROS) produced during hemoglobin (Hb) digestion in the parasite's food vacuole [2,3]. In addition, the parasite is exposed to exogenous oxidative stress when merozoites egress from the red blood cells (RBC), inducing the production of nitric oxide and ROS by the host's immune system [2,4]. *Plasmodium* parasites depend on two major NADPH-dependent redox

Abbreviations: ART, artemisinin; DCF, dichlorofluorescein; DCFH, 2′,7′-dichlorodihydrofluorescein; DHOD, dihydroorotate dehydrogenase; DNPH, dinitrophenylhydrazine; FP IX, ferritoprotoporphyrin IX; FTC, fluorescein 5-thiosemicarbazide; GR, glutathione reductase; Grx, Glutaredoxin; GSH, glutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; Hb, hemoglobin; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; *pbggcs-ko*, *P. berghei* gamma-glutamylcysteine synthetase knockout; *pbgr-ko*, *P. berghei* glutathione reductase knockout; PBS, phosphate-buffered saline; PPP, Pentose phosphate pathway; QPCR, quantitative polymerase chain reaction; RBC, red blood cells; RNR, ribonucleotide reductase; ROS, reactive oxygen species; SOD, superoxide dismutase; Trx, thioredoxin; WT, wild type; γGCS, gamma glutamylcysteine synthetase

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networks, the GSH and the Trx antioxidant systems, to detoxify ROS and prevent oxidative damage [5].

Plasmodium synthesizes GSH by the sequential action of the rate limiting enzyme γ-glutamylcysteine synthetase (γ-GCS), and the glutathione synthetase [6]. The reduced GSH is oxidized to GSH disulfide (GSSG) by the reduction of glutaredoxin or glutathione-S-transferase (GST) [3,6,7]. The oxidized GSSG is reduced back to GSH by the glutathione reductase (GR), maintaining the GSH/GSSG ratio in the cell [8]. Glutathione plays an important role in a wide range of cellular processes including the detoxification of xenobiotics and protection against ROS [7,9,10]. In *Plasmodium* parasites, GSH is also involved in the degradation of the toxic ferritoprotoporphyrin IX (FP IX), escaped from hemozoin formation [11]. Additionally, GSH functions as an electron donor for the enzyme ribonucleotide reductase (RNR), [12,13], crucial for DNA synthesis and cellular proliferation [2,3,10].

Previous reports from our laboratory demonstrated that disruption of the *pbggcs* gene in *Plasmodium berghei* resulted in mutant parasites displaying reduced GSH levels and growth impairment during intraerythrocytic development [14]. Moreover, disruption of the *pbggcs* gene inhibited oocyst development and the production of sporozoites in the mosquito, indicating that GSH biosynthesis is critical to complete parasite transmission [14]. Similarly, *P. berghei* parasites with a disrupted glutathione reductase (*pbgr-ko*) gene displayed significantly reduced GSH levels and interruption of parasite development in the mosquito, with parasites arrested at the oocyst stage [15]. Although the relevance of GSH for parasite development has been shown, the underlying mechanisms of the delayed parasite development as a consequence of reduced GSH levels as well as the oxidative status of the mutant parasites deserves further investigation.

In this study, we further characterized the effects of reduced GSH levels in *P. berghei* and demonstrate that parasites with significantly low levels of GSH show a growth delay during the ring to schizont transition, have significantly smaller trophozoites and schizonts and a vacuolated cytoplasm. These changes occur concomitantly with an impairment capacity to handle oxidative stress and increased levels of nDNA damage. These results show for the first time a causal interplay between low GSH levels, impaired response to oxidative stress, oxidative DNA damage, and delayed parasite growth during the ring to schizont transition. The findings presented herein provide a better understanding of the need for maintaining GSH homeostasis during *P. berghei* development. Because the redox balance plays a vital role for parasite survival, our results have important applications for antimalarial treatments. Strategies aiming to promote oxidative stress and/or inhibiting the parasite antioxidant system will lead to a redox imbalance affecting parasite growth, and therefore a potential improvement in treatment response.

2. Materials and methods

2.1. Mice and P. berghei lines

Random-bred Swiss albino CD-1 female mice (Charles River Laboratories, Wilmington, MA, USA), 6–8 weeks old, weighing 20–35 g were used for the study. All mice procedures were conducted at the AAALAC accredited University of Puerto Rico Medical Sciences Campus (UPR-MSC) Animal Resources Center and approved by the Institutional Animal Care and Use Committee (IACUC). All work was done in strict accordance with the "Guide for the Care and Use of Laboratory Animals" (National-Research-Council, Current Edition) and regulations of the PHS Policy on Humane Care and Use of Laboratory Animals. Mice were maintained and housed according to NIH and AAALAC regulations and guidelines. Mice were allowed to acclimatize for 1 week prior to the beginning of the studies. The *P. berghei* ANKA WT, reference line (507cl1), which expresses green fluorescent protein under the control of the constitutive *eukaryotic elongation factor 1A* promoter, was used as a control in all experiments [16]. The following *P. berghei* lines (mutants) were included in the study: *pbggcs-ko1* and *pbggcs-ko2* [14], and *pbgr-ko1* and *pbgr-ko2* [15].

2.2. Intraerythrocytic growth assay

To investigate the delayed parasitemia of *pbggcs-ko* parasites in mice [14], WT and *pbggcs-ko* parasites were synchronized in *in vitro* cultures as described by Janse and Waters [17]. Briefly, *P. berghei* infected blood was harvested from mice with 5–15% parasitemia, diluted in complete medium [RPMI 1640 (Gibco®), 25% heat inactivated fetal bovine serum (Gibco®), 50 IU/ml of neomycin (Sigma)] and cultured for 24–26 h at 37 °C in an atmosphere of 10% O_2, 5% CO_2 and 85% N_2 gas mixture. The schizonts were purified by 55% Nycodenz gradient [17] and injected intravenously into mice [18]. The synchronized ring infected blood was collected from mice 4–6 h post infection and cultured *in vitro* for a total of 28 h. The parasite intraerythrocytic development was analyzed by light microscopy of Diff-Quick stained thin smears in samples collected every 2 h, beginning after the initial 16 h of culture. Parasite intraerythrocytic developmental stages (rings, trophozoites and schizonts) and morphology were determined by counting at least 100 parasites per slide. Images were acquired with a microscope (BX51, Olympus) at 100 × magnification using a digital camera (DP72, Olympus). The size of trophozoites and schizonts stages was assessed at 16, 24 and 28 h and vacuoles at 16 h in WT, *pbggcs-ko1* and *pbggcs-ko2* parasites using a calibrated ocular micrometer at 100 × magnification ($n=20$).

2.3. Determination of intraparasitic response to oxidative stress levels by measuring the oxidation of 2′,7′-dichlorodihydrofluorescein (DCFH).

To determine the levels of intraparasitic response to oxidative stress, the fluorescent intensity of dichlorofluorescein (DCF), the green fluorescent oxidation product of DFCH, was determined in WT and *pbggcs-ko* parasites cultured *in vitro*. The DCF fluorescent intensity was measured in the basal (untreated) or hydrogen peroxide (H_2O_2)-treated cultures as described by Trivedi et al. [19]. Briefly, *P. berghei* infected blood was cultured, as previously described, in the presence or absence of 2 mM H_2O_2 for 1 h. The cultures were then incubated with 10 μM of 2′,7′-dichlorofluorescein diacetate for 30 min at 37 °C, washed with 0.01 M, potassium phosphate buffer (PBS) (pH 7.4), centrifuged and subsequently lysed with saponin (0.15%) for 10 min at 4 °C. Parasites were isolated by centrifugation at 1300*g* for 5 min at 10 °C and lysed by sonication (30 s pulse, bath-type sonicator) at 4 °C. Fluorescent intensities were recorded from the lysates in a spectrofluorometer (SpectraMax® M3, Molecular Devices©) at 502 nm and 523 nm excitation and emission wavelengths, respectively. Protein concentrations were determined in each well using the Pierce™ Modified Lowry Protein Assay Kit (Thermo Scientific) and data was expressed as fluorescence intensity/milligram of parasite lysate protein.

2.4. DNA isolation and quantification

High molecular weight genomic DNA was isolated from WT, *pbggcs-ko* and *pbgr-ko* parasites using a Genomic DNA extraction Kit (Qiagen©) following the manufacturer's instructions. Quantification of DNA was performed using the PicoGreen® dsDNA Quantitation Reagent (Molecular Probes®) with modifications as