Parasitology International xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

# Effect of thioredoxin peroxidase-1 gene disruption on the liver stages of the rodent malaria parasite *Plasmodium berghei*

Miho Usui, Hirono Masuda-Suganuma, Shinya Fukumoto, Jose Ma. M. Angeles, Hassan Hakimi, Noboru Inoue, Shin-ichiro Kawazu *

*National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, 2-13 Inada-cho, Obihiro, Hokkaido 080-8555, Japan*

ARTICLE INFO

Available online xxxx

*Keywords:*
Liver stage
Malaria
Peroxiredoxin
*Plasmodium berghei*
Thioredoxin peroxidase

ABSTRACT

Phenotypic observation of thioredoxin peroxidase-1 (TPx-1) gene-disrupted *Plasmodium berghei* (TPx-1 KO) in the liver-stage was performed with an *in vitro* infection system in order to investigate defective liver-stage development in a mouse infection model. Indirect immunofluorescence microscopy assay with anti-circumsporozoite protein antibody revealed that in the liver schizont stage, TPx-1 KO parasite cells were significantly smaller than cells of the wild-type parent strain (WT). Indirect immunofluorescence microscopy assay with anti-merozoite surface protein-1 antibody, which was used to evaluate late schizont-stage development, indicated that TPx-1 KO schizont development was similar to WT strain development towards the merozoite-forming stage (mature schizont). However, fewer merozoites were produced in the mature TPx-1 KO schizont than in the mature WT schizont. Taken together, the results suggest that TPx-1 may be involved in merozoite formation during liver schizont development.



## 1. Introduction

Malaria remains a major public health threat worldwide and is responsible for high burdens of mortality and morbidity in disease-endemic areas [1]. The invasion of malaria parasites into a patient's body begins in the liver where they replicate and generate thousands of progeny. Thus, inactivating the liver-stage parasite offers clear advantages, which include blocking the developmental phase in liver cells before parasites are able to infect erythrocytes and symptoms begin to develop in the patient. At present, the number of drugs that target the liver-stage parasite is limited due to a lack of studies on the liver-stage parasite, particularly on its metabolism during this stage. Primaquine is the only drug to target the liver-stage parasite that is currently available. However, its use is limited due to the increased risk of hemolysis when it is administered to patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency [2]. A better understanding of the liver-stage parasite's basic biology is needed in order to produce drugs that are effective against it in this stage and/or to formulate a strategy to inactivate liver-stage parasites with minimal side effects. Since malaria parasites are sensitive to oxidative stress [3], their antioxidant defense mechanisms represent a potential target for new strategies against malaria. A number of enzymatic and non-enzymatic antioxidants possessed by the malaria parasite allow it to maintain low intracellular levels of reactive oxygen species (ROS). Among these are the superoxide dismutases (SODs), which act as the first line defense against ROS. SODs are a ubiquitous family of enzymes that efficiently catalyze the dismutation of superoxide ($O^{2-}$) into oxygen and hydrogen peroxide [4]. Hydrogen peroxide ($H_2O_2$) is then reduced into water and oxygen to prevent the oxidation of other cellular components. This reaction is catalyzed by a variety of peroxidases including glutathione (GSH)-dependent peroxidases (GPx) and thioredoxin-dependent peroxidases or peroxiredoxins (Prxs). Genuine GPx does not exist in *Plasmodium*. However, the GSH system is present in *Plasmodium falciparum* and GSH is synthesized *de novo* by γ-glutamyl-cysteine synthetase and glutathione synthetase [5]. GPx and Prx obtain their reducing equivalents from two distinct systems, the GSH and the thioredoxin redox systems, respectively [6–9]. Both comprised a cascade of redox-active proteins which transfer reducing equivalent from NADPH to acceptor molecules, in this case is $H_2O_2$. Malaria parasites possess two SODs [10], but do not encode catalase or Gpx [11], the two major antioxidant enzymes in other organisms, indicating that their cellular redox homeostasis is critically dependent on Prx. In addition, Prx is known as a multifunctional molecule; it reduces peroxynitrite ($ONOO^-$) and also is involved in a $H_2O_2$-mediated signal transduction cascade [12,13].

*Abbreviations:* AOP, antioxidant protein; CSP, circumsporozoite protein; GSH, glutathione; GPx, GSH peroxidase; MSP-1, merozoite surface protein 1; Prx, peroxiredoxin; ROS, reactive oxygen species; SOD, superoxide dismutase; TSA, thiol-specific antioxidant; Trx, thioredoxin; TPx, Trx peroxidase.

* Corresponding author. Tel.: +81 155 495846; fax: +81 155 495643.
*E-mail addresses:* kasiopeia0716@gmail.com (M. Usui), shirono@obihiro.ac.jp (H. Masuda-Suganuma), fukumoto@obihiro.ac.jp (S. Fukumoto), amoj28@gmail.com (J.M.M. Angeles), hassanhakimi@gmail.com (H. Hakimi), ircpmi@obihiro.ac.jp (N. Inoue), skawazu@obihiro.ac.jp (S. Kawazu).

http://dx.doi.org/10.1016/j.parint.2014.09.013
1383-5769/

The Prxs are a family of proteins that are structurally homologous to the thiol-specific antioxidant (TSA) of yeast [14]. Prxs have been identified in all living organisms from bacteria to humans [15,16]. There are three subtypes of Prxs, namely 1-Cys Prx, typical 2-Cys Prx and atypical 2-Cys Prx. 1-Cys and 2-Cys Prxs are distinguished by a number of conserved cysteine residues in their catalytic sites. Although the proposed cellular function and electron donor for 1-Cys Prx are not fully understood [16,17], 2-Cys Prx has been found to act as a terminal peroxidase that reduces hydrogen peroxide and organic hydroperoxides using electrons donated by the thioredoxin (Trx) system [16,14]. During this catalytic action, typical 2-Cys Prx forms a homodimer through an intersub-unit disulfide bond that is reduced by an electron donated by Trx. Atypical 2-Cys Prx forms a monomer with an intramolecular disulfide bond that is also reduced by Trx during the catalytic action. Several findings have been reported on the cellular functions of 2-Cys Prx in mammals, including its involvement in the modulation of cytokine-induced $H_2O_2$ levels, which have been shown to mediate signaling cascades leading to cell proliferation, differentiation and apoptosis [16,18].

*Plasmodium* species possess six peroxidases localized in the cytoplasm, mitochondrion and apicoplast and nucleus [19] (Table 1). These include 1-Cys Prx, two typical 2-Cys Prxs, 1-Cys antioxidant protein (AOP), GSH peroxidase-like thioredoxin peroxidase ($TPx_{Gl}$) [19] and nuclear Prx (nPrx), which was previously known as merozoite capping protein-1 (MCP-1) [20]. The 1-Cys Prx [21] and one of the 2-Cys Prxs (thioredoxin peroxidase-1; TPx-1) [22,23] are expressed in cytosol, whereas the other 2-Cys Prx (TPx-2) is expressed in the mitochondrion [23,24]. The AOP has a signal that targets apicoplast [25]. $TPx_{Gl}$ is suggested to be localized in cytosol and apicoplast [26,27]. nPrx was recently found in the nucleus of the parasite [20].

The phenotype of typical 2-Cys Prx (TPx-1) gene-disrupted parasite population (TPx-1 KO) has previously been studied in *P. berghei* [28]. In a mouse infection model, the parasite population showed defective growth in the liver stage. Thus, in the present study, to further investigate the phenotypes that have thus far been found in mouse infection experiments, we observed the phenotype of the TPx-1 KO population during liver-stage development using an *in vitro* infection system with HepG2 cells.

## 2. Materials and methods

### 2.1. Parasites

The *P. berghei* ANKA strain was obtained from the Armed Forces Research Institute of Medical Sciences, Thailand. The TPx-1 KO population (referred in our previous study as Prx KO) with a targeted disruption of *pbtpx-1* (PlasmoDB, PBANKA_130280) was established by double-crossover homologous recombination [29].

### 2.2. Infection of mosquitoes

Six-week-old ICR mice (Clea Japan) were infected with *P. berghei* by intraperitoneal (i.p.) injection of parasites that had been stored as frozen stock at −80 °C. The parasitemia of the animals was monitored daily by light microscopic observation of Giemsa-stained thin blood smears. *Anopheles stephensi* mosquitoes were maintained on 10% sugar solution at 27 °C and 80% relative humidity under a 12 h light/dark cycle. *A. stephensi* mosquitoes were fed on mice for 2 h at room temperature (RT), or 19 °C, when the number of microgametocytes that could exflagellate *in vitro* had reached 20–30 per $1 \times 10^5$ erythrocytes [30]. The parasite-infected mosquitoes (100–200 mosquitoes in each group) were maintained at 19 °C with 10% sugar solution. The animal experiments in this study were carried out in compliance with the Guide for Animal Experimentation at Obihiro University of Agriculture and Veterinary Medicine (Permission number: 23–43).

### 2.3. Maintenance of hepatoma cells and sporozoite infections

HepG2 cells, which are usually used as host cells in the *P. berghei* liver-stage infection model system [31,32], were maintained in Eagle's minimum essential medium (MEM) (Sigma Aldrich Japan Co., Tokyo, Japan) supplemented with 10% heat inactivated-fetal bovine serum (HI-FBS), 1% MEM nonessential amino acid (Nacalai Tesque Inc., Kyoto, Japan) and 1% penicillin/streptomycin (Invitrogen Japan, Tokyo, Japan). The cells were constantly subcultured until use by trypsinization and kept at 37 °C in a 5% $CO_2$ cell incubator. HepG2 cells ($5 \times 10^4$ per well) were maintained in 8-chamber plastic Lab-Tek slides (Nalge Nunc International, Cergy Pontoise, France). The salivary glands of parasite-infected mosquitoes were excised and sporozoites were released by gentle triturating of the organ. HepG2 cells were inoculated with sporozoites ($1 \times 10^4$ per well) and incubated for 3 h. After washing, the infected cells were cultured at 37 °C in a 5% $CO_2$ cell incubator.

### 2.4. Indirect immunofluorescence microscopy assay

The infected HepG2 cells were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 15 min and permeabilized with 0.1% polyoxyethylene (10) octylphenyl ether (equivalent to Triton®-X 100) (Wako Pure Chemical Industries) for 15 min. The cells were incubated for 30 min at room temperature with phosphate-buffered saline containing 5% skim milk (Wako Pure Chemical Industries) for blocking. The samples were stained with antibody against circumsporozoite protein (CSP) of *P. berghei* at 1:100 dilution. Monoclonal antibody against CSP (MRA-100) [33] was supplied by the Malaria Research and Reference Reagent Resource Center (MR4/ATCC, Manassas, VA, USA). Alexa-Fluor®488 conjugated goat anti-mouse IgG (Invitrogen Japan; 1:1000 dilution) was used as the secondary antibody. Hoechst-33342 (Dojindo, Kumamoto, Japan;

**Table 1**
Peroxidases of *Plasmodium* parasites.

| Gene name | Abbr. | PlasmoDB ID[a] | Localization | Citation |
|---|---|---|---|---|
| Thioredoxin peroxidase-1 | TPx-1 | PF3D7_1438900<br>PBANKA_130280 | Cytoplasm | [22] |
| Thioredoxin peroxidase-2 | TPx-2 | PF3D7_1215000<br>PBANKA_143080 | Mitochondrion | [23] |
| 1-Cys peroxiredoxin | 1-Cys Prx | PF3D7_0802200<br>PBANKA_122800 | Cytoplasm | [21] |
| 1-Cys antioxidant protein | AOP | PF3D7_0729200 | Apicoplast | [25] |
| GSH peroxidase-like thioredoxin peroxidase | $TPx_{Gl}$ | PF3D7_1212000<br>PBANKA_061050 | Apicoplast, Cytoplasm | [26,27] |
| nuclear Prx | nPrx | PF3D7_1027300 | Nuclear | [20] |

[a] Upper ID is for *Plasmodium falciparum* 3D7 strain and lower ID is for *P. berghei* ANKA strain.

Please cite this article as: Usui M, et al, Effect of thioredoxin peroxidase-1 gene disruption on the liver stages of the rodent malaria parasite *Plasmodium berghei*, Parasitology International (2014), http://dx.doi.org/10.1016/j.parint.2014.09.013