Studies on Antioxidant Defense System in the Liver Stage of Rodent Malaria Parasite

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USUI Miho

Doctoral Program in Animal and Food Hygiene

Graduate School of Obihiro University of Agriculture and Veterinary Medicine

ローデントマラリア原虫肝臓型
における
抗酸化機構の研究

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帯広畜産大学大学院畜産学研究科
博士後期課程　畜産衛生学専攻
薄井　　美帆

Contents

Abbreviations

CSP: circumsporozoite protein

Cys: cysteine

GFP: green fluorescent protein

GPx: glutathione-dependent peroxidases

GS: glutathione synthetase

GSH: glutathione

GR: glutathione reductase

ICR: imprinting control region

KO: knock-out

MCP-1: merozoite capping protein-1

MSP-1: merozoite surface protein-1

P. berghei: *Plasmodium berghei*

Prx: peroxiredoxin

PV: parasitophorous vacuole

ROS: reactive oxygen species

RNS: reactive nitrogen species

RT: room temperature

SOD: superoxide dismutase

TPx-1: thioredoxin peroxidase-1

TPx-2: thioredoxin peroxidase-2

Trx: thioredoxin

TrxR: thioredoxin reductase

TSA: thiol-specific antioxidant

WHO: World Health Organization

WT: wild type

General Introduction

I. Overview of malaria

Malaria is endemic in 106 countries and territories, which include large parts of Africa and Asia, Central and South America, Haiti and the Dominican Republic, some Pacific islands, such as Papua New Guinea and some parts of the Middle East. Around 3.3 billion people living in these areas are at risk of malaria transmission. The signs and symptoms of malaria vary from being non-specific such as fever, nausea, vomiting and diarrhea; to severe, such as anemia, jaundice and systemic organ failure, which can result in death if not treated promptly. According to the World Health Organization, in 2010, there were 216 million episodes of malaria and 655,000 deaths worldwide. Of these deaths, around 91% occurred in Africa, followed by Southeast Asia (6%), and the Eastern Mediterranean (3%). About 86% of deaths globally were seen in children.

Malaria is caused by apicomplexan parasites of the *Plasmodium* genus, five species of which are known to infect humans, namely *Plasmodium falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi*. Among these species, *P. falciparum*

accounts for most malaria-associated mortality. At present, public health concern in relation to *P. vivax* infection is increasing (WHO, 2012).

The life cycle of the *Plasmodium* parasite requires two important hosts: mosquitoes and mammals (Fig. 1). *Plasmodium* sporozoites residing at the mosquito salivary gland are injected into the skin of their mammalian host during blood meal. The sporozoites then use their capacity to migrate through the host cells (Mota et al., 2001), to reach a blood vessel (Amino et al., 2008), and possibly to cross the liver sinusoids (Ishino et al., 2004), as well as several hepatocytes, before infecting a hepatocyte in a process called productive invasion, which occurs with the formation of a parasitophorous vacuole (PV). Inside the PV, the parasites multiply, generating liver-stage parasites that grow in size as *Plasmodium* replicates to generate thousands of new parasites. The liver stage of infection is an obligatory step in the life cycle of the parasite and culminates in the release of parasite-filled vesicles known as merosomes, into the blood stream (Sturm et al., 2009). The merosomes eventually burst, presumably in the microvasculature of the lungs (Baer et al., 2007), releasing erythrocyte-infective parasites called merozoites (Prudencio et al., 2006; Aly et al., 2009). The merozoite release marks the beginning of the

blood stage of infection, when *Plasmodium* cyclically invades and ruptures erythrocytes, giving rise to disease symptoms. During this phase, some parasites differentiate into male and female gametocytes, which can be ingested by mosquitoes. The sexual phase of the life cycle of the parasite takes place in the mosquito midgut and eventually results in the formation of sporozoites that will reach the salivary glands responsible for another transmission cycle.

The life cycle of the parasite has been intensively studied in the search for an effective method to control the disease and possibly eliminate the parasite. At present, however, there is no effective vaccine available and key interventions rely on prompt and effective treatment with artemisinin-based combination therapies, use of insecticidal nets; and indoor residual spraying of insecticide to control vector mosquitoes. With the emergence of drug-resistant parasites and insecticide-resistant mosquitoes in many parts of the world, there is an increased need to develop novel effective interventions for malaria control.

II. The redox system of *Plasmodium* spp.

Reactive oxygen species (ROS) are inevitably produced in the cell during normal aerobic metabolism under oxygen-containing environments (Finkel, 2003; Georgiou et al., 2003). The *Plasmodium*-infected erythrocyte is under constant oxidative stress caused by exogenous ROS and reactive nitrogen species (RNS) produced by the immune system of the host, and by the parasite's endogenous production of ROS, which is generated during the digestion of host hemoglobin and by concomitant biochemical reactions. It has also been proposed that hemozoin, the mammalian host heme metabolite produced by the parasite, induces the gene expression of nitric oxide synthase i*n vivo* in *A. stephensi* and that it affects the parasite's development in the insect vector (Akman-Anderson et al., 2007). Furthermore, ROS can be produced by the mitochondrial electron transport chain and by various metabolic processes. The actions of most anti-malarial drugs are attributed to the production and effect of ROS/RNS against the parasite.

In order to maintain low intracellular levels of ROS, the malaria parasite possesses a number of enzymatic and non-enzymatic antioxidants. These include 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 (Fridovich et al., 1995). 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) (Fig. 2). Genuine glutathione peroxidase does not exist in *Plasmodium*. However, the GSH system is present in *P. falciparum* and GSH is synthesized *de novo* by γ-glutamyl-cysteine synthetase (γGCS) and glutathione synthetase (GS) (Jortzik and Becker, 2012). GPx and Prx obtain their reducing equivalents from two distinct systems, the GSH and the thioredoxin redox systems, respectively (Arner and Holmgren, 2000; Becker et al., 2000; Yodoi et al., 2001; Filomeni et al., 2002). Both comprised a cascade of redox-active proteins which transfer reducing equivalent from NADPH to acceptor molecules, which in this case is from hydrogen peroxides. Malaria parasites possess two SODs (Sienkiewicz et al., 2004), but do not encode catalase or glutathione peroxidase (Sztajer et al., 2001; Clarebout et al., 1998), the two major antioxidant enzymes in other organisms, indicating that their cellular redox homeostasis is critically dependant 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 (Rhee and Woo, 2011; Klomsiri et al., 2011).

III. Peroxiredoxin

The Prxs are a family of proteins that are structurally homologous to the thiol-specific antioxidant (TSA) of yeast (Chae et al., 1994). Prxs have been identified in all living organisms from bacteria to humans (Chae et al., 1999; Wood et al., 2003). 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 (Wood et al., 2003; Manevich et al., 2004), 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 (Chae et al., 1994; Wood et al., 2003). 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. Different findings have been reported on the cellular functions of 2-Cys Prx in mammals, including its involvement in the modulation of cytokine-induced hydrogen peroxide levels, which have been shown to mediate signaling cascades leading to cell proliferation, differentiation and apoptosis (Georgiou et al., 2003; Wood et al., 2003).

Plasmodium species possess six peroxidases localized in the cytoplasm, mitochondria and apicoplast and nucleus (Nickel et al., 2006; Richard et al., 2011). These include one 1-Cys Prx, two typical 2-Cys Prxs, one 1-Cys antioxidant protein (AOP), one GSH peroxidase-like thioredoxin peroxidase (TPx_{Gl}) (Rahlfs et al., 2002; Nickel et al., 2006) and one nuclear Prx (nPrx), which was previously known as merozoite capping protein-1 (MCP-1) (Richard et al., 2011). The 1-Cys Prx (Krnajski et al., 2001) and one of the 2-Cys Prxs (TPx-1) (Krnajski et al., 2001; Kawazu et al., 2001; Rahlfs et al., 2001) are expressed in cytosol, whereas the other 2-Cys Prx (TPx-2) is expressed in the mitochondria (Rahlfs et al., 2001; Boucher et al., 2006). The AOP has a signal that targets apicoplasts (Sarma et al., 2005). TPx_{Gl}

is suggested to be localized in cytosol, apicoplasts and mitochondria (Kehr et al., 2010). nPrx was recently found in the nucleus of the parasite (Richard et al., 2011).

IV. *Plasmodium berghei*

P. berghei is one of the many species of malaria parasites that infect mammals other than humans and one of the four species that have been described in murine rodents of West Africa. These parasites and their infection in mice provided models for malaria parasites and malarias of humans and other primates that are applicable to a number of aspects, including biochemistry, cell biology, molecular biology immunology and pathology (Carter and Nijhout, 1977).

V. Objectives of the present study

The objectives of the present study were: (1) to examine protein and mRNA expression profiles for three Prxs [thioredoxin peroxidase-1 (TPx-1), 1-cystein peroxiredoxin (1-Cys Prx) and thioredoxin peroxidase-2 (TPx-2)] of liver-stage *P. berghei*; (2) to examine mRNA expression profiles of TPx-1, 1-Cys Prx and TPx-2

during the liver stage of *P. berghei*, in TPx-1 knock-out (KO) and TPx-2 KO parasite populations; and (3) to examine and compare the phenotypes of three types of *P. berghei* [TPx-1 KO, TPx-2 KO and wild type (WT)] in the liver stage.

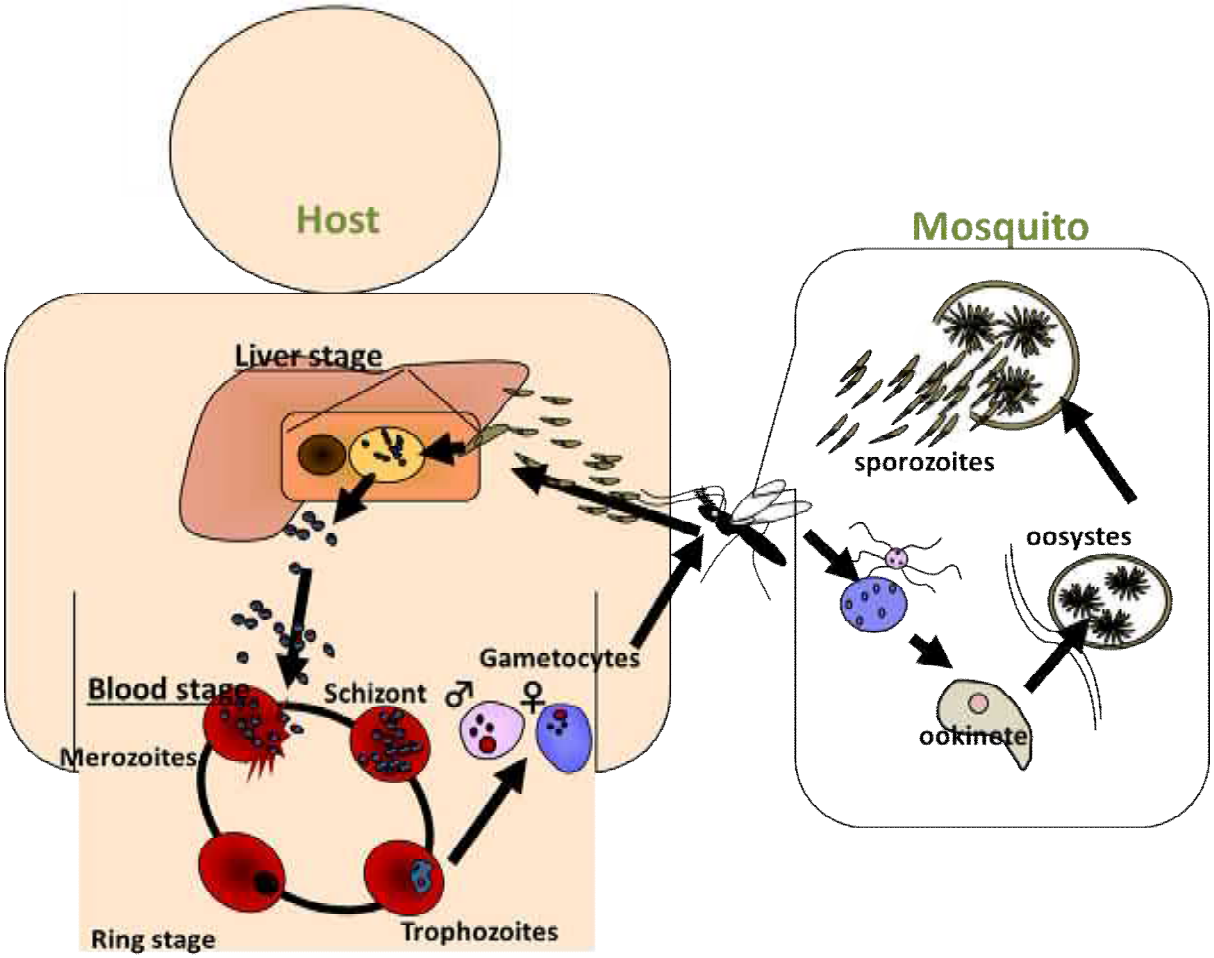


Figure 1. The life cycle of the malaria parasite.

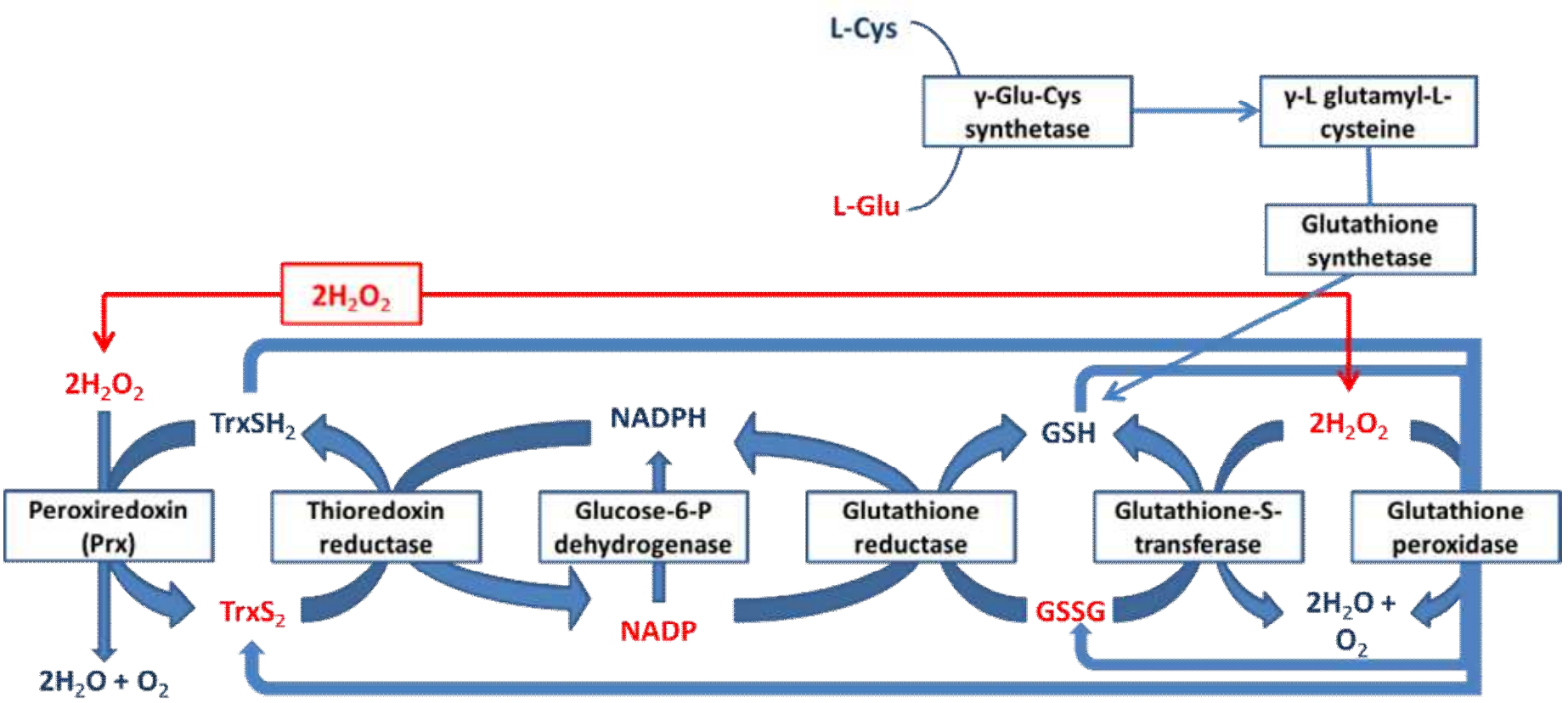


Figure 2. The endogenous antioxidant defense system in *Plasmodium*.

Chapter 1

Expression profiles of peroxiredoxins in the liver stage of the rodent malaria parasite *Plasmodium berghei*

1-1 Introduction

As *Plasmodium* species actively proliferate in the erythrocytes of their vertebrate hosts, the parasites are subjected to the toxic effects of ROS throughout their asexual development (Becker et al., 2004; Müller et al., 2004). In the *Anopheles* mosquito, *Plasmodium* has to defend against high concentrations of ROS produced in response to their invasion into midgut epithelial cells (Molina-Cruz et al., 2008). In these environments, the parasites are also likely to be subjected to oxidative stress (Han et al., 2000; Radyuk et al., 2001). However, despite multiple entries of SOD (Bécuwe et al., 1996; Sienkiewicz et al., 2004), which dismute superoxide into hydrogen peroxide, malaria parasites do not express two of the major peroxidases, catalase and genuine GPx (Becker et al., 2004; Müller et al., 2004). To reduce peroxides, which are produced in each cellular

compartment by metabolism and parasitism, the parasites are equipped with peroxidases. The expression of Prxs has been studied both in the blood stage and the mosquito stage. In the liver stage, however, the expression of Prxs remains to be studied.

The liver stage of infection is an obligatory step in the life cycle of the parasite. During growth and proliferation in the liver, the parasite is subjected to the toxic effects of ROS that originate from the host cell as well as those from its own metabolism (Guha et al., 2006).

Because *Plasmodium* is sensitive to oxidative stresses, its antioxidant defenses are considered to play significant roles in survival and thus represent a promising target for novel chemotherapies (Becker et al., 2004; Müller et al., 2004; Jaeger et al., 2006; Krauth-Siegel et al., 2005).

In this study, the author examined mRNA and protein expression profiles for three Prxs (TPx-1, 1-Cys Prx and TPx-2) of liver-stage *P. berghei*, which have not previously been reported in malaria parasites.

1-2 Materials and Methods

Parasites

Two strains of *P. berghei,* (ANKA, and ANKA Mie), were used. The ANKA strain was obtained from the Armed Forces Research Institute of Medical Science, Thailand (Yano et al., 2008). The ANKA Mie strain, which expresses green fluorescent protein (GFP) (Ishino et al., 2006) was provided by Dr. Masao Yuda from Mie University School of Medicine, Japan. The parasite was maintained by mosquito transmission in *A. stephensi,* interspersed by a maximum of two serial passages in ICR imprinting control region (ICR) mice (Clea Japan, Tokyo, Japan).

Cells

HepG2 cells, which are usually used as host cells in the *P. berghei* liver-stage infection model system (Sturm et al., 2006; Rankin et al., 2010), 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 37ºC in a 5% CO_2 cell incubator.

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. *A. stephensi* was provided by Dr. Shinya Fukumoto from the author's research center. The mosquitoes were maintained on 10% sugar solution at 27ºC 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 19ºC, when the number of microgametocytes that could exflagellate *in vitro* (Cao et al., 1998) had reached 20-30 per 1 x 10^5 erythrocytes. The parasite-infected mosquitoes (100-200 mosquitoes in each group) were maintained at 19ºC 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).

Sporozoite infections

HepG2 cells (5×10^4 per well) were maintained in 8-chamber plastic Lab-Tek slides (Nalge Nunc International, Cergy Pontoise, France). *P. berghei* sporozoites were collected from the infected *A. stephensi* mosquitoes through dissection. HepG2 cells were inoculated with sporozoites (5×10^4 per well) and incubated for 3 h. After washing, the infected cells were cultured at 37ºC in a 5% CO_2 cell incubator.

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 RT with

phosphate-buffered saline containing 5% skim milk (Wako Pure Chemical Industries) for blocking. The following antibodies were used: anti-circumsporozoite protein (CSP) of *P. berghei*, anti-recombinant TPx-1 of *P. falciparum* (rPfTPx-1), anti-recombinant 1-Cys Prx of *P. falciparum* (rPf1-Cys Prx), and anti-recombinant TPx-2 of *P. berghei* (rPbTPx-2). Monoclonal antibody against CSP (MRA-100) (Yoshida et al., 1980) was supplied by the Malaria Research and Reference Reagent Resource Center (MR4, ATCC; Manassas, VA, USA). This antibody stains the plasma membrane and cytoplasm of the liver stage parasite during its early developmental stage (Dr. T. Ishino of Ehime University, personal communication). Interspecific cross-reactivity between the proteins of the rodent malaria parasite and the rabbit antisera against rPfTPx-1 and rPf1-Cys Prx has been confirmed (Kawazu et al., 2003). Rabbit antiserum against rPbTPx-2 was prepared in this study as previously described (Yano et al., 2005). The specificity of the rabbit serum against rPbTPx-2 has also been confirmed in the experiment with asexual stage parasite (Masuda-Suganuma et al., 2012). Rabbit antisera to rPfTPx-1, rPf1-Cys Prx and rPbTPx-2 were each used at 1:200 dilution. Mouse monoclonal antibody to CSP was used at 1:100 dilution. Alexa-Fluor®488 conjugated goat anti-mouse IgG

(Invitrogen Japan; 1:1000 dilution) and Alexa-Fluor®546 conjugated goat anti-rabbit IgG (Invitrogen Japan; 1:1000 dilution) were used as secondary antibodies. Hoechst 33342 (Dojindo, Kumamoto, Japan; 1:500) staining was performed with the secondary antibody reaction to visualize the parasite nuclei. The slides were mounted with Dako Fluorescent Mounting Medium (Dako Cytomation, Denmark) and observed under a confocal laser-scanning microscope (TCSSP5, Leica Microsystem, Wetzlar, Germany).

Real-time quantitative reverse transcription-PCR

Total RNA was extracted with Trizol (Invitrogen) according to the manufacturer's instructions. It was then treated with DNase I (QIAGEN, Hilden, Germany), and purified using RNeasy MinElute Cleanup columns (QIAGEN). cDNA was synthesized using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). mRNA expression profiles were examined by quantitative reverse transcription-PCR (RT-qPCR) using an ABI 7300 Real Time PCR System (Applied Biosystems Japan). The primers used were designed based on the available sequences in the *P. berghei* genome database provided by

PlasmoDB Version 8.2 (Table 1). The cycling conditions used were as follows: 2 min at 50°C, 10 min at 95°C and 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 68°C for 1 min. Real time detection of cDNA amplification was performed using an SYBR Green PCR Master Mix Kit (Applied Biosystem). Post-amplification analysis by agarose gel electrophoresis indicated a single PCR product and confirmed the absence of non-specific amplicons and primer dimers. Standard curve graphs were prepared using serial dilutions of blood-stage genomic DNA. To compare the relative quantity of PCR products, the fluorescence intensity was recorded by cycles for each amplification and analyzed using the ABI PRISM Sequence Detection software Version 1.2.2 (Applied Biosystems Japan). The 18S rRNA gene served as the reference to standardize the target gene expressions in each sample.

1-3 Results & Discussion

HepG2 cells infected with the GFP-expressing parasite were observed by confocal microscopy, affirming the transformation of the sporozoites into liver-stage parasites and the development of the parasites into merozoites in the cell

culture as previously described (Rankin et al., 2010; Graewe et al., 2012) (Fig. 3). RT-qPCR experiments revealed that mRNA expression for TPx-1 was detected shortly after (5 h) sporozoite infection, with expression maintained until the schizont stage (40 h). Expression decreased at the cytomere stage (50 h) (Fig. 4). In contrast, mRNA expression for 1-Cys Prx began increasing when the parasite developed into the schizont stage and became elevated toward the cytomere stage (Fig. 4). This finding suggests the existence of developmental stage-specific expression of these cytosolic enzymes in the liver-stage parasite. In contrast, mRNA expression for TPx-2 began increasing at trophozoite stage (17 h) after sporozoite infection and peaked at the schizont stage (Fig. 4). Western blotting that could at least semi-quantify the expression of these proteins and support the RT-qPCR results was not performed because of the high expectation of cross-reaction of the antibody between the enzymes of both the parasite and the host. Indirect immunofluorescence microscopy assay (IFA) with antibody against CSP of *P. berghei* also confirmed the transformation of sporozoites into liver-stage parasites and the development of the parasites in the cell culture (Fig. 5). IFA with the Prxs-specific rabbit antisera confirmed the cytosolic localization of TPx-1 and

1-Cys Prx in the liver-stage parasites (Fig. 5). The branched staining pattern with TPx-2-specific serum in IFA suggested that the enzyme was localized in the mitochondria in liver-stage parasites (Graewe et al., 2012) (Fig. 5). Double staining of parasite cells with anti-TPx-2 serum and Mito Tracker® revealed the presence of TPx-2 in the mitochondria (Fig. 6).

Prompt and cytosolic expression of TPx-1 suggests a possible role of this enzyme to protect the parasite from the intrahepatocyte environment. This finding on liver-stage expression, together with the previous studies showing constitutive expression of TPx-1 in the intraerythrocytic and insect stages (Kawazu et al., 2008; Yano et al., 2006) indicates that the enzyme is expressed throughout the entire life cycle of the parasite. TPx-1 might therefore be a housekeeping gene, which protects the parasite from the oxidative stresses produced under the host environment. In the case of yeast, TPx-1 is known to act as molecular chaperone, which prevents the denaturation of protein substrates during heat shock or oxidative stresses (Jang et al., 2004). Due to the prompt and constitutive expression profiles of TPx-1, it might also be possible that the enzyme acts as a molecular chaperone in the parasite, protecting its cytosolic protein substrates from the sharp cellular environmental

changes that occur due to host cell invasion and subsequent rapid multiplication. The oligomeric structure of the enzyme, which is the key structure in the mediation of the chaperone function has previously been observed in TPx-1 of *P. falciparum* (Akerman and Müller, 2003).

The present findings on the liver-stage expression of 1-Cys Prx and TPx-2 revealed that the enzymes are expressed throughout all of the mammalian stages but not in all of the insect stages, in which only the ookinete expresses the enzymes (Hall et al., 2005). In the liver stage, 1-Cys Prx was expressed during the schizont to cytomere stage when the parasite develops exponentially to produce ~30,000 of merozoites per schizont (Graewe et al., 2012). TPx-2 was also expressed in this time period but the expression peaked at the schizont stage. The rapid expansion of membranous organelles, such as mitochondria at the schizont stage also coincides with a marked increase in the expression of TPx-2. Furthermore, the ATP production required for the extraordinary development of the parasite also increases ROS in mitochondria and may induce TPx-2 expression at this stage. The cytosolic enzymes may also act on the ROS that leaks out from the organelle, and this might explain the increase in 1-Cys Prx expression at the schizont stage. The

phospholipase A_2 activity, which has been reported in mammalian Prx 6 (1-Cys Prx) for its function in the repair of oxidized membranes (Nevalainen et al., 2010; Chen et al., 2000), may also assist in protecting the rapidly expanding membrane from oxidative stresses at this stage. This function of 1-Cys Prx in the parasite should be the subject of future study.

Here, the author has described the localization and expression profile of the TPx-1, 1-Cys Prx and TPx-2 in the liver stages of the rodent malaria parasite *P. berghei*. The results suggest that the parasite expresses these proteins in separate cellular compartments with different profiles to manage intracellular oxidative stresses during liver-stage development. How the parasite orchestrates the antioxidant network in the parasite's cells during infection in the host liver cells would be an interesting matter for future study. Investigations on the transcriptional regulation of these antioxidant proteins in the parasite cell should provide further insight into the response of the malaria parasites to ROS at the cellular level in the liver stage. Further studies to elucidate the role and expression mechanism of Prxs would therefore be interesting and could provide new insight on the redox regulation mechanisms in malaria parasites. In addition, it will be important to

compare the observations in the liver stage with those in the blood and insect stages, in order to investigate the specific and/or conserved functions of thiol-specific antioxidant molecules in the parasite life cycle.

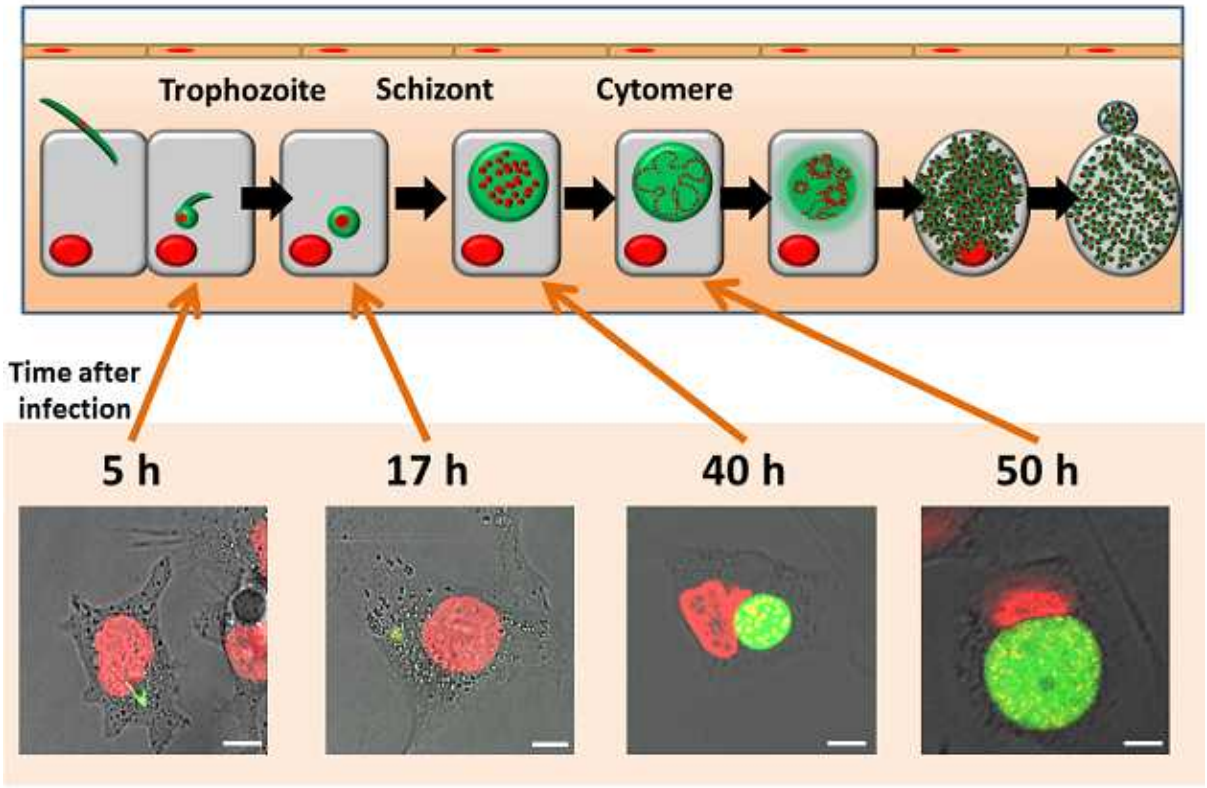


Figure 3. Development of the GFP-expressing parasite in HepG2 cell culture.

Development of the GFP-expressing parasite (green color) under the cell culture conditions was observed using laser-scanning microscope (TCSSP5, Leica Microsystem). Hoechst 33342 solution was used for nucleus staining (red color). The observation confirmed the transformation of the sporozoites into liver-stage parasites and the development of the parasites into merozoites in the *in vitro* cell culture. Briefly, after infecting the cell, the sporozoite underwent initial

morphological change (5 h), became a round-shaped trophozoite (17 h), and matured into a multinucleated schizont (40 h). The schizont developed into the cytomere stage. At this stage, the plasma membrane of the parasite invaginated and surrounded the parasite nuclei, which were aligned and clustered along the membrane. In some parts, the membrane surrounded a single parasite nucleus (50 h). Repeated membrane invagination resulted in meroblast formation to produce merozoites (55-60 h). Merosomes eventually budded off from the host cell (60-65 h). Scale bar indicates 10 μm.

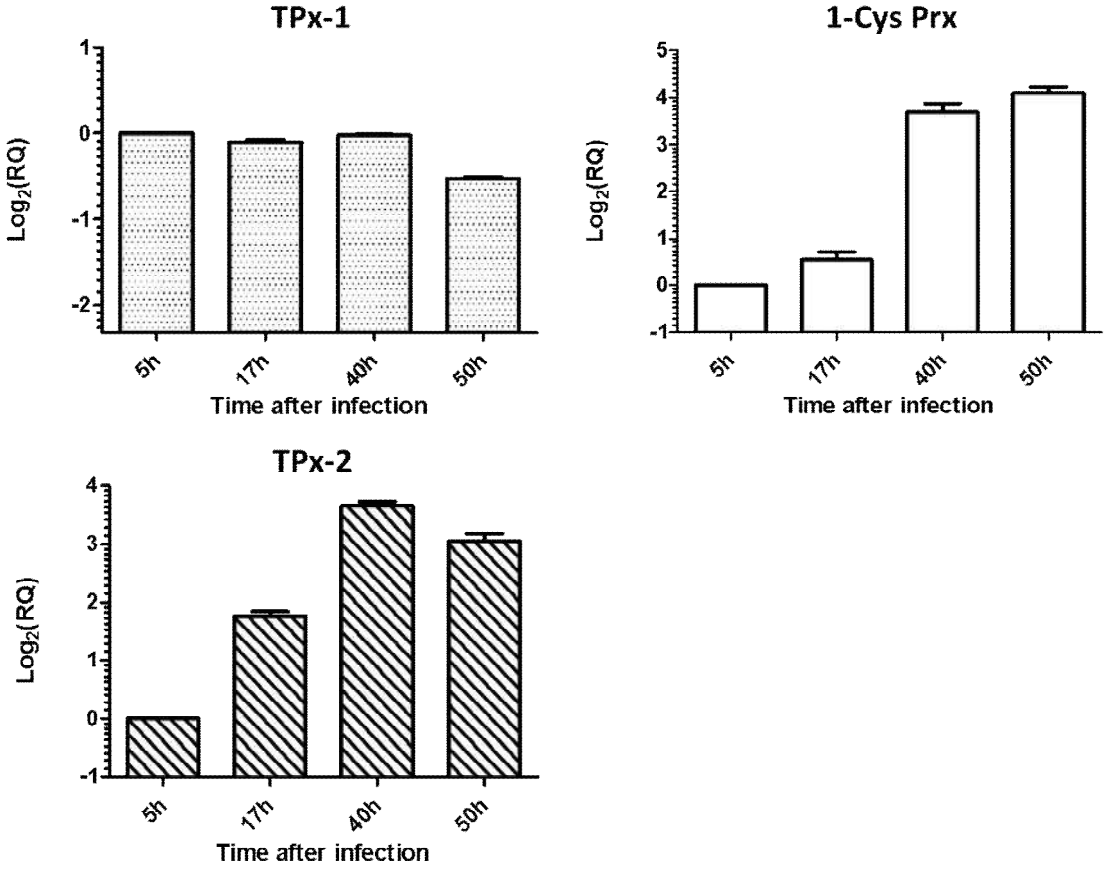


Figure 4. Patterns of expression of TPx-1, 1-Cys Prx and TPx-2 mRNAs in *P. berghei* cells during the liver stage.

Histogram representation of real-time RT-qPCR analysis of relative gene expression in *P. berghei* cells shortly after sporozoite infection (5 h), and at the trophozoite (17 h), schizont (40 h) and cytomere (50 h) stages in the WT parasite. 18S rRNA gene served as the reference to standardize the target gene expressions in each sample. The data are expressed as $\log_2$ of (the relative quantity of PCR

product: RQ). The figures show the representative results of two similar experiments. Data are mean + S.D. of triplicate assays.

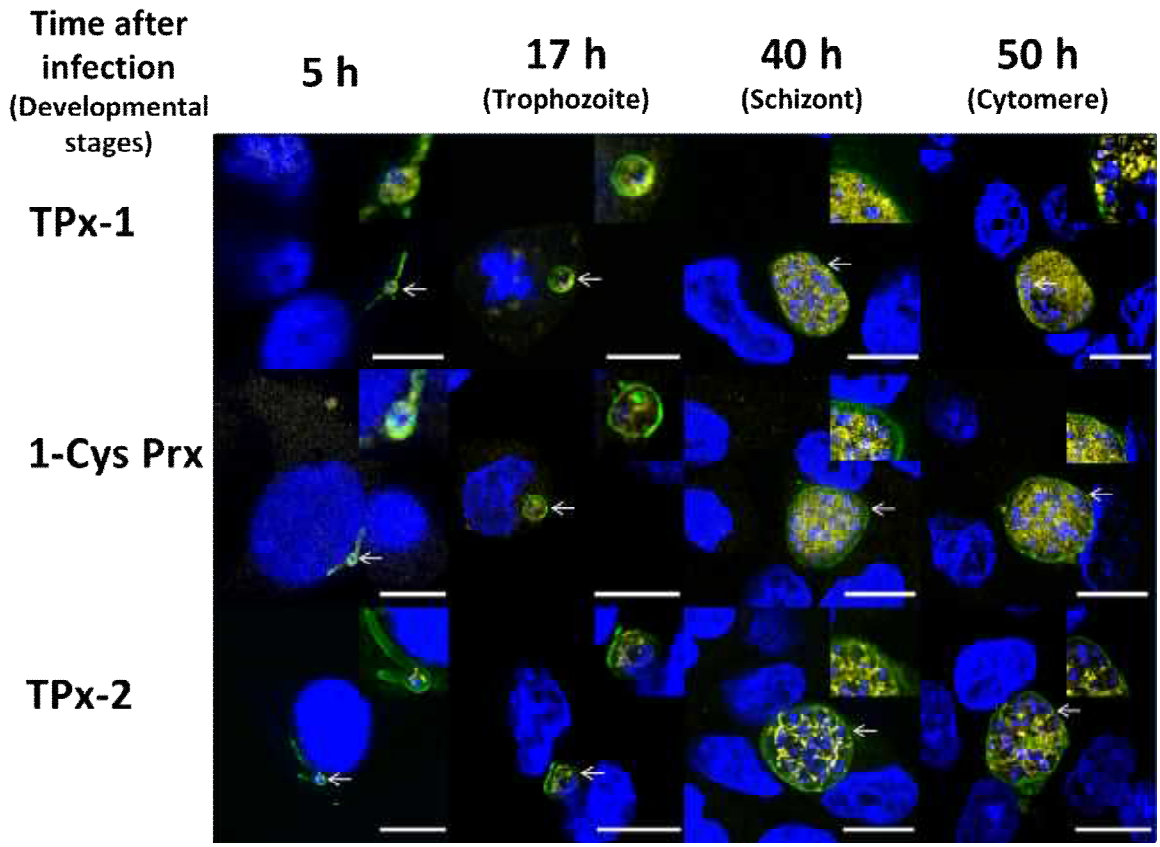


Figure 5. The expression of TPx-1, 1-Cys Prx and TPx-2 proteins in *P. berghei* cells during the liver stage.

The localization of each of the Prxs was observed by IFA. Figures show triple-stained images with the monoclonal antibody to CSP (green), rabbit antiserum to Prx (yellow; pseudo color) and Hoechst to nuclei (blue). Insets show magnified images (×1.5 – ×3) of the part indicated with an arrow. Scale bar indicates 10 μm. All three Prxs were detected shortly after sporozoite infection (5 h), and at the trophozoite (17 h), schizont (40 h) and cytomere (50 h) stages of the

parasite. The IFA confirmed the cytosolic localization of TPx-1 and 1-Cys Prx. The branched staining pattern with TPx-2-specific serum in the IFA suggested the mitochondrial localization of the enzyme in the liver-stage parasites.

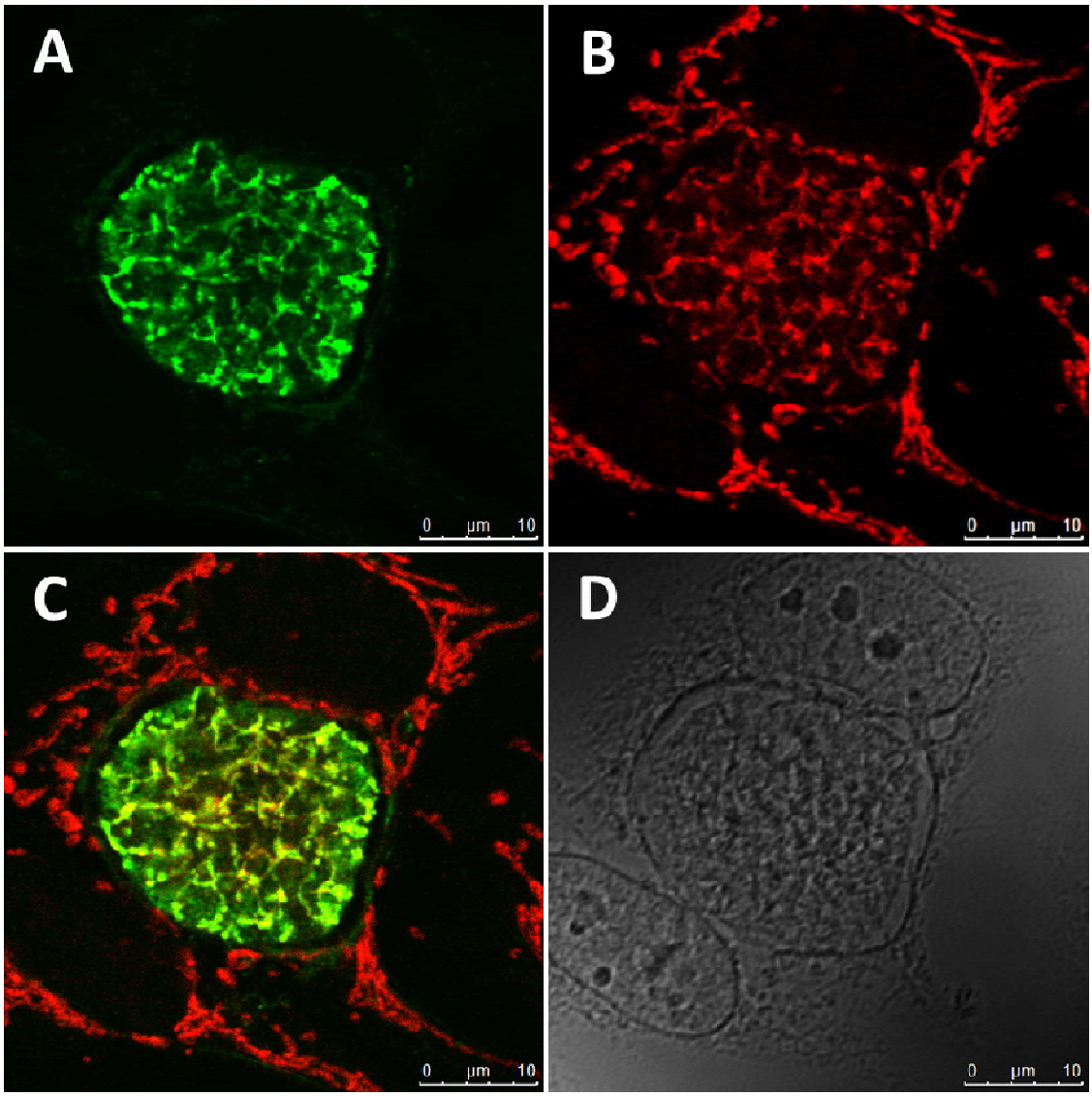


Figure 6. Mitochondrial localization of TPx-2 protein in the liver stage of *P. berghei.*

TPx-2 protein was double stained (C; yellow color) with Mito Tracker® (B; red color) and antiserum (A; green color), indicating the mitochondrial localization of the protein. Panel D shows the brightfield image. Scale bar indicates 10 µm.

Table 1

Primer sequences used in the RT-qPCR assay.

Gene name and ID*	Primer pair sequences
PbTPx-1 (PBANKA_130280)	F: 5’-GTGTAGCATTAAGAGCGTTTGTTCTC -3’ R: 5’-GGTGTTGCAAAGCATCAATTAGTC -3’
Pb1-Cys-Prx (PBANKA_122800)	F: 5’-CAACTGGCAGAAATGCTCAAG -3’ R: 5’-CATCATCCTGAAGAGTTGGAATG -3
PbTPx-2 (PBANKA_143080)	F: 5’-AACGATTTATCAATAGGCAGAAACG -3’ R: 5’-TGGGTCTAAACGATTTGTCTCC -3’
Pb18S rRNA (berg07_18S)	F: 5’-GAATCTTGGCTCCGCCTCG -3’ R:5’-GGGCTCTCAAAGGGTCTGTAATTAAAAGAAC -3’

* Gene accession numbers were derived from PlasmoDB version 8.2.

Chapter 2

Expression profiles of peroxiredoxins in the liver stage of the peroxidase gene-disrupted rodent malaria parasite *Plasmodium berghei*

2-1 Introduction

Plasmodium parasites are transmitted by Anopheline mosquitoes. During mosquito's blood meal, sporozoites are injected into the final host. In the case of mammalian infection, sporozoites are rapidly transported to the liver, where they invade the hepatocytes. In the liver, the sporozoite multiplies and develops into a liver-stage parasite. This process is called the liver stage of infection and is an obligatory step in the life cycle of the parasites. The Intercellular environment of the hepatocyte generally provides optimal conditions for transformation and maturation of the liver-stage parasite, however, at the same time, the parasite is under several stresses, which include the emergence of ROS (Guha et al., 2006).

Cells are generally exposed to ROS and RNS from various sources and therefore possess protective mechanisms involving enzymatic and non-enzymatic antioxidant molecules. Two major interdependent antioxidant systems have been described in *Plasmodium*, namely the thioredoxin (Trx) and glutathione (GSH) systems. The GSH system consists of a small ubiquitous redox active tripeptide GSH and its corresponding reducing enzyme, GSH reductase (GR). In contrast, Trx is central to the Trx system and mainly functions as an electron donor to diverse target proteins, including key antioxidant enzymes, such as the Prxs. Trx is maintained in its reduced, active state by Trx reductase (TrxR). Recently, *trxr* (−) *P. berghei* sporozoites were found to produce a lower number of mature liver-stage parasites than those of WT parasites (Buchholz et al., 2010). This finding supports the idea that the Trx system plays an important role in the liver stage. As for its downstream, the disruption of gene coding for cytosolic 2-Cys Prx, TPx-1, in *P. falciparum* results in an increased sensitivity of the parasite to ROS and RNS in the blood stage (Komaki-Yasuda et al., 2003). Disruption of the gene in *P. berghei* resulted in multiple phenotypes, including a reduced number of sporozoites in the mosquito stage and defective growth of the parasite in the liver stage (Yano et al.,

2006; Yano et al., 2008). On the other hand, the disruption of gene coding for mitochondrial 2-Cys Prx, TPx-2, suggested that this Prx family might not be essential for the blood and insect stages of *P. berghei* development (Masuda-Suganuma et al., 2012). Its liver-stage phenotype, however, remains unknown. Another cytosolic Prx, 1-Cys Prx, has been suggested to have an antioxidant activity (Kawazu et al., 2000) and the function of scavenging endogenous ROS in the blood stage (Kawazu et al., 2005), however, its liver-stage function also remains unknown.

In the present study, the author examines the expression profiles of three Prxs (TPx-1, 1-Cys Prx and TPx-2) of the rodent malaria parasite, *P berghei*, in TPx-1 KO and TPx-2 KO parasite populations, and compares the profiles with those in the WT strain.

2-2 Materials and Methods

Parasites

The *P. berghei* ANKA strain was obtained from the Armed Forces Research Institute of Medical Sciences, Thailand. The TPx-1 KO population, with the targeted disruption of *pbtpx-1* (PlasmoDB, PBANKA_130280), and the TPx-2 KO population, which carries a targeted disruption of *pbtpx-2* (PlasmoDB, PBANKA_143080) were established by double-crossover homologous recombination (Yano et al., 2006; Masuda-Suganuma et al., 2012). The parasite populations with the disruption of *pbtpx-1* and *pbtpx-2* were established separately using the dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene with a pyrimethamine-resistance mutation (*dhfr-ts/mt*) as a selectable marker. Parasite populations with pyrimethamine resistance (*dhfr-ts/mt* at the DHFR-TS locus) were named Prx-WT, and served as control. Prx-WT develops normally (in comparison to WT) in the blood and mosquito stages (Yano et al., 2006; Yano et al., 2008; H. Masuda-Suganuma et al., 2012).

The parasites were maintained by mosquito transmission in *A. stephensi* interspersed with a maximum of two serial passages in ICR mice (Clea Japan). The animal experiments in this study were carried out in compliance with the Guide for

Animal Experimentation at the Obihiro University of Agriculture and Veterinary Medicine (Permission number: 24-39).

Cells

HepG2 cells were maintained as described in Chapter 1.

Infection of mosquitoes

Six-week-old ICR mice (Clea Japan) were infected with *P. berghei* by i.p. injection of the parasite-infected mouse blood, which had been stored as frozen stock at -80 °C. Parasitemia of the animals was monitored daily by light microscopic observation of Giemsa-stained thin blood smears. The mosquitoes were maintained and fed on *P. berghei*-infected mice in the same conditions as described in Chapter 1.

Sporozoite infections

HepG2 cells (5×10^4 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 (5×10^4 per well) and incubated for 3 h. After washing, the infected cells were cultured at 37ºC in a 5% CO_2 cell incubator.

Real-time quantitative reverse transcription-PCR

RT-qPCR was conducted with SYBR® Green system in same conditions as described in Chapter 1. The post-amplification analysis by agarose gel electrophoresis indicated a single PCR product and confirmed the absence of non-specific amplicons or primer dimers.

Determination of pre-patent period for the erythrocytic stage

P. berghei sporozoites, were collected from the infected *A. stephensi* mosquitoes. Three mice were each inoculated with 1×10^4 sporozoites by

intravenous injection. Parasitemia of the animals was monitored every 12 h by observing Giemsa-stained blood smears, and the number of days between sporozoite inoculation and detection of 0.5% parasitemia of the blood stage (pre-patent period of the infection) was determined.

Statistical analysis

Differences were evaluated by Student's *t*-test. $p < 0.05$ was considered statistically significant.

2-3 Results & Discussion

RT-qPCR experiments on the TPx-1 KO population revealed that mRNA expression of 1-Cys Prx was detected shortly after sporozoite infection (5 h). The expression decreased once in the trophozoite stage (17 h) and increased again when the parasite developed into the schizont stage (40 h). The expression then decreased toward the cytomere stage (50 h) (Fig. 7). At 17 h and 40 h post-infection, the expression levels of 1-Cys Prx mRNA in the TPx-1 KO population were

significantly higher than in WT parasites. They were approximately 3.8 (17 h) times and 2.5 times higher (40 h) in TPx-1 KO than in WT parasites. In contrast, the mRNA expression of TPx-2 began increasing at the trophozoite stage, 17 h after sporozoite infection, and peaked at the schizont stage (40 h), which was the same pattern as was observed in WT parasites. Interestingly, the expression level of TPx-2 mRNA in the TPx-1 KO population was three times higher than that of schizont-stage WT (40 h) (Fig. 7). In WT parasites, TPx-1 is constantly expressed at 5, 17, 40 and 50 h of development.

In the TPx-2 KO population, TPx-1 mRNA was detected shortly after sporozoite infection (5 h). The expression decreased once in the trophozoite stage (17 h) and again when the parasite developed into the schizont stage (40 h). The expression then decreased toward the cytomere stage (50 h) (Fig. 8). Despite the similar expression pattern, the expression level of TPx-1 mRNA in the TPx-2 KO population shortly after sporozoite infection (5 h) was approximately two times higher than in WT. Similarly, 1-Cys Prx was highly expressed shortly after sporozoite infection (5 h) and decreased in the trophozoite stage (17 h). The expression increased again when the parasite developed into the schizont stage (40

h) and decreased in the cytomere stage (50 h) (Fig. 8). Despite similar expression patterns seen between TPx-2 KO and WT until 40 h, the expression levels of 1-Cys Prx mRNA at 5 h, 17 h and 40 h were approximately four times higher in TPx-2 KO than in WT.

The mean pre-patent period for the blood-stage infection in animals (n=3) inoculated with 1 x 10^4 sporozoites of WT and TPx-2 KO parasites was 4.6 ± 0.14 and 4.4 ± 0.14 days, respectively (Table 2). These data indicate that the pre-patent period of TPx-2 KO in mice was equivalent to that of WT. Taking account the fact that the blood-stage development of TPx-2 KO is similar to that of WT, the TPx-2 KO parasite would seem to grow normally, in comparison to WT, in the liver stage.

Here, the author described the expression profiles of TPx-1, 1-Cys Prx and TPx-2 mRNAs in two Prx-KO parasite populations (TPx-1 KO and TPx-2 KO) of *P. berghei* in the liver stage. Previous studies showed that the disruption of the TPx-1 gene was not lethal in either *P. falciparum* (Komaki-Yasuda et al., 2003) or *P. berghei* (Yano et al., 2006; Yano et al., 2008). Moreover, the TPx-2 gene was not essential for the blood and insect developmental stages of *P. berghei* (Masuda-Suganuma et al., 2012). There is a defect in the parasite development of

the TPx-1 KO population in the liver stage (Yano et al., 2008). However, the results from the sporozoite inoculation experiment for the TPx-2 KO population in this study suggest that *pbtpx-2* disruption does not affect liver-stage parasite development. It should be noted, however, that redox homeostasis in these phenotypes is not yet fully understood. There are several reports from different groups suggesting that such non-lethal phenotypes found in the KO of antioxidant proteins may occur due to compensation by other antioxidant proteins (Cao et al., 1998; Masuda-Suganuma et al., 2012; Buchholz et al., 2008). A recent report showed that *Plasmodium* ookinete regulated antioxidant protein expression in response to environmental changes in order to maximize the probability of survival, maturation and escape (Benjamin et al., 2013). In the present study, the author also observed that mRNAs coding for other Prx family members in the both TPx-1 KO and TPx-2 KO parasites were upregulated during their liver-stage development. These results suggest that the upregulation might compensate for the lack of Prx in liver-stage development. Interestingly, significant increases in the expressions of 1-Cys Prx and TPx-2 mRNA were seen in TPx-1 KO at the schizont stage (40 h), when karyokinesis takes place in the liver-stage parasite. The ATP production

required for an extraordinary development turn in ROS, may induce the upregulation of other Prx family members. The upregulation of the mRNA of other Prxs in the Prx-KO at specific times may depend on the environmental conditions of the host liver cell. The upregulation of the mRNA of other Prxs was observed *in vitro* in both TPx-1 KO and TPx-2 KO during liver-stage development. However, the TPx-1 KO parasite showed defective growth in the liver stage. The upregulation should be examined in a mouse infection model *in vivo*. The ROS emerged because the lack of TPx-1 might exceed the level which could be compensated by the upregulation of other Prxs. The ROS level in the liver-stage parasite should be evaluated to test this hypothesis. The parasite population that expresses HyPer (Belousov et al., 2006), the H_2O_2 sensor protein, could be used in the experiment. Another hypothesis is that RNS, but not ROS, emerged due to lack of TPx-1 that could not be compensated by the upregulation of other Prxs, since TPx-1 is the only member of the Prx family to have peroxynitrite ($ONOO^-$) reductase activity (Nickel et al., 2005). The RNS level in the liver-stage parasite should also be evaluated to test this hypothesis. A bioimaging experiment with NO probes (Kojima et al., 2001) may be performed. At present, work is underway in the author's laboratory to

investigate in detail the correlation between the upregulation of the Prx family and the TPx-1 KO phenotype during the liver stage.

The present study suggested the possibility that liver-stage *Plasmodium* utilizes an inducible antioxidant response during adaptation to changing conditions in the host liver cell. These findings support the hypothesis that TPx-1, 1-Cys Prx and TPx-2 may play overlapping roles, and that these enzymes may orchestrate the antioxidant network in parasite cells. The updated knowledge yielded from further study will facilitate research into the development of novel antimalarial drugs and vaccination strategies.

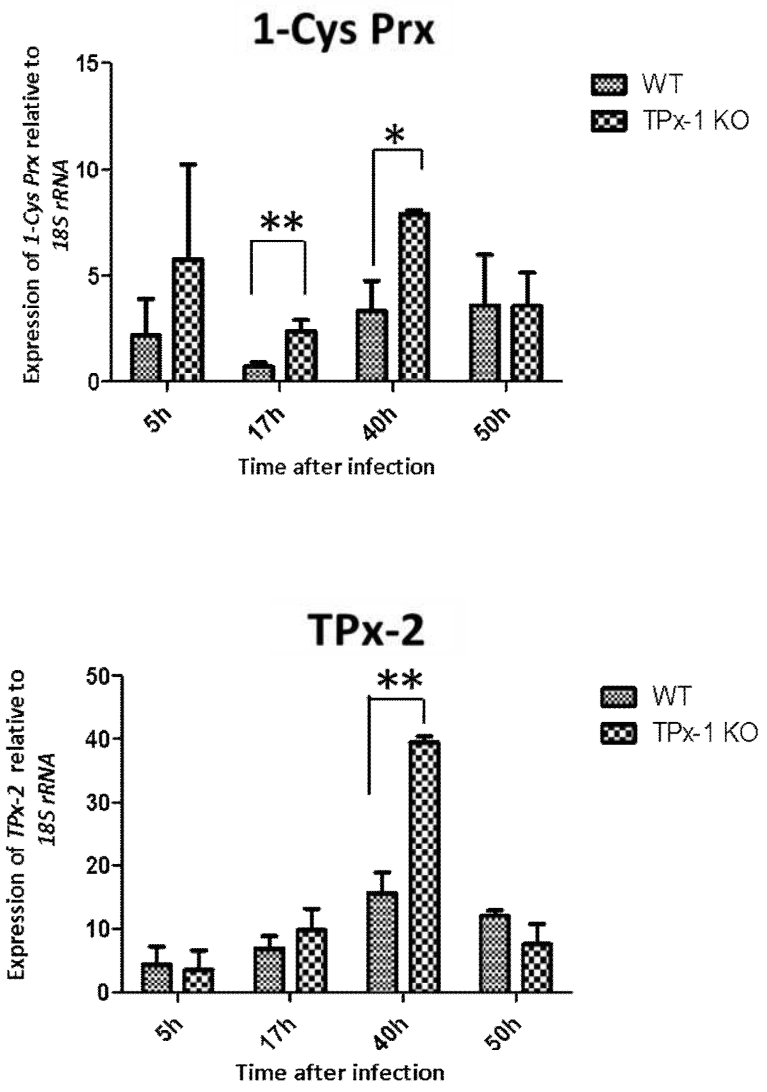


Figure 7. Patterns of expression of *pb1-cysprx* and *pbtpx-2* in WT and TPx-1 KO populations during the liver stage.

A histogram representation of real-time RT-qPCR analysis of relative gene expression in *P. berghei* cells during the liver stage at 5, 17, 40 and 50 h post-infection in WT and the TPx-1 KO population. The 18S rRNA gene served as

the reference to standardize the target gene expressions in each sample. The average of three independent experiments is shown. Error bars represent standard deviation. Student's *t*-test was performed to compare the expression level of each Prx in WT and TPx-1 KO populations. Asterisks (*) indicate statistical significance.

*: $p < 0.05$; ** $p < 0.001$

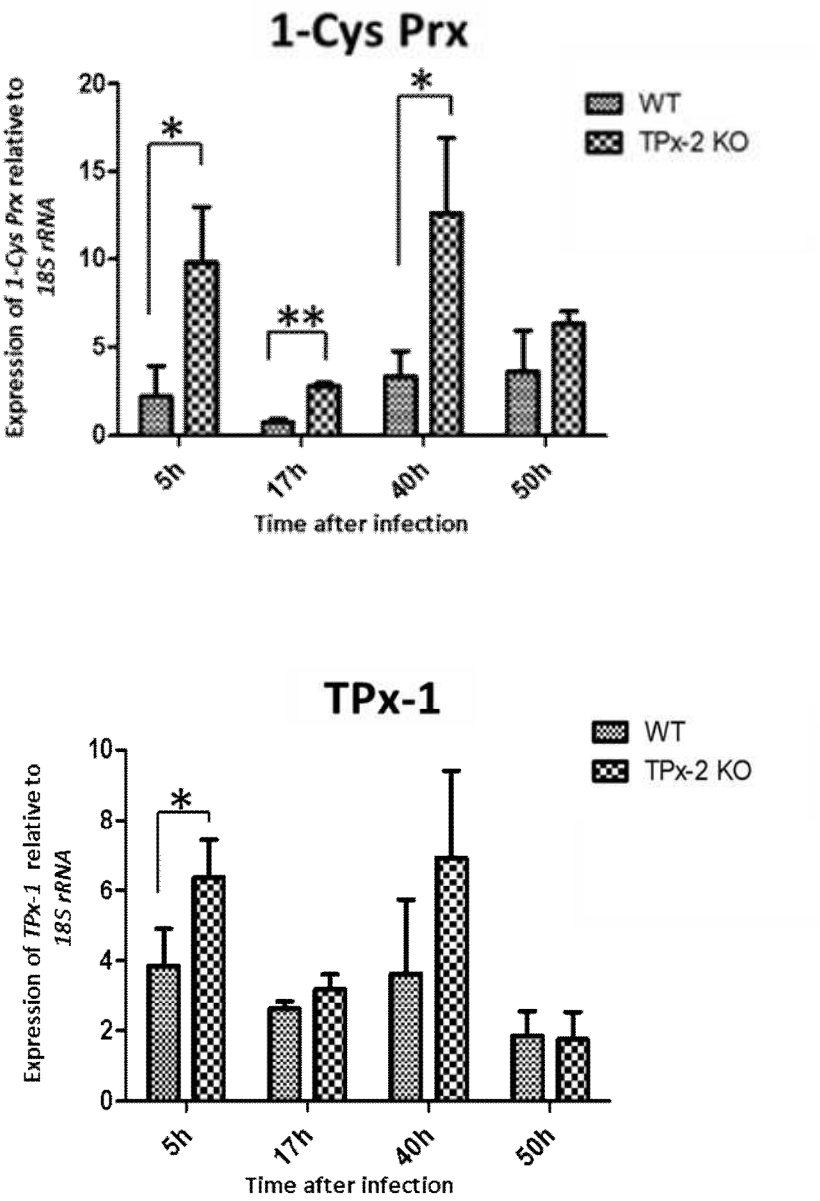


Figure 8. Patterns of expression of *pb1-cysprx* and *pbtpx-1* in WT and TPx-2 KO populations during the liver stage. Histogram representation of RT-qPCR analysis of relative gene expression in *P. berghei* cells during the liver stage at 5, 17, 40 and 50 h post-infection in WT and TPx-2 KO populations. The 18S rRNA gene

served as the reference to standardize the target gene expressions in each sample. The average of three independent experiments is shown. Error bars represent standard deviation. Student's *t*-test was performed to compare the expression level of each Prx in WT and TPx-2 KO parasites. Asterisks (*) indicate statistical significance.

*: $p < 0.05$; ** $p < 0.001$

Table 2

Parasite	No. of injected sporozoites	Sporozoite infection rate[a]	Prepatent of period[b] infection (days)
WT	1x 10^4	3/3	4.6 ± 0.14
TPx-2 KO	1x 10^4	3/3	4.6 ± 0.14

[a]Number of infected mice/number of mice injected with salivary gland sporozoites.
[b]Number of days required for the parasite to develop 0.5% parasitemia at blood stage.

Chapter 3

Phenotypic analysis of peroxidase gene-disrupted rodent malaria parasite *Plasmodium berghei* in the liver stage

3-1 Introduction

Malaria remains a major public health threat worldwide, and is responsible for high burdens of mortality and morbidity in malaria-endemic areas (WHO, 2012). The invasion of malaria parasites into the patient 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. In *P. ovale* and *P. vivax*, a subset of the sporozoites that have invaded the hepatocyte do not develop beyond the early hepatic trophozoite stage, but remain as latent or dormant forms called hypnozoites.

At present, there are limited numbers of drugs that target the liver-stage parasite. This is due to a lack of studies on the liver-stage parasite, particularly on the metabolism of the liver-stage parasite. Primaquine is the only currently available drug that targets the liver-stage parasite, but its use is limited due to increased risk of hemolysis when administered to patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency (Baird et al., 2001). To produce effective drug against liver-stage parasites or to formulate a strategy to inactivate liver-stage parasites with minimal side effects, a better understanding of their basic biology is needed.

Since malaria parasites are sensitive to oxidative stress (Müller et al., 2001), their antioxidant defense mechanisms represent a potential target for new strategies against malaria. For this reason, the author focused on the antioxidant defense system of malaria parasites and conducted studies on Prxs, which are one of the main antioxidant enzymes of the parasites, using Prx-KO populations. The phenotype of the TPx-1 KO population has previously been studied in liver-stage *P. berghei* (Yano et al., 2008). In a mouse model, the population showed defective growth in the liver stage . As described in Chapter 2, the TPx-2 KO population in *P.*

berghei seems to develop similarly to WT parasites in mice during the liver stage. However, these phenotypes of liver-stage Prx-KO populations have not yet been investigated in detail.

Thus, in the present study, the author observed the phenotypes of the TPx-1 KO and TPx-2 KO populations in their liver-stage development using an *in vitro* infection system with HepG2 cells to investigate mechanisms under the phenotypes that have thus far been found in the liver stage using mouse infection experiments.

3-2 Materials and Methods

Parasites

The *P. berghei* ANKA strain was obtained from the Armed Forces Research Institute of Medical Sciences, Thailand. The TPx-1 KO population with a targeted disruption of *pbtpx-1* (PlasmoDB, PBANKA_130280) and the TPx-2 KO population, which carries a targeted disruption of *pbtpx-2* (PlasmoDB, PBANKA_143080) were established by double-crossover homologous

recombination (Yano et al., 2006, Masuda-Suganuma et al., 2012) as described in Chapter 2.

The parasite populations were maintained by mosquito transmission in *A. stephensi* under the same conditions as described in Chapter 1. The animal experiments in the present study were carried out in compliance with the Guide for Animal Experimentation at the Obihiro University of Agriculture and Veterinary Medicine (Permission number: 24-39).

Infection of mosquitoes

Mosquitoes were maintained and fed on *P. berghei* infected mice as described in Chapter 2.

Sporozoite infections

Sporozoites were obtained by dissecting the salivary glands of parasite-infected mosquitoes, and inoculated to HepG2 cells. The cells were

incubated as described in Chapter 2 for infection and development of liver-stage parasites.

Indirect immunofluorescence microscopy assay

IFA was performed under the same conditions as described in Chapter 1. At 40-50 h post-infection (schizont stage to cytomere stage), the samples were stained with antibody against CSP of *P. berghei* (provided by MR-4) at 1:100 dilution with Hoechst 33342. The slides were mounted with Prolong® Gold antifade reagent (Invitrogen Japan, Tokyo, Japan) and observed under a confocal laser-scanning microscope (TCSSP5, Leica Microsystem).

At 40 h and 50 h post-infection, the area of the liver-stage parasite was measured using TSCCP5 software (Leica Microsystem) to evaluate its size. At 50-60 h post-infection (cytomere stage to merozoite-forming stage), the samples were stained with antibodies against merozoite surface protein-1 (MSP-1) of *P. berghei* (provided by T. Holder, National Institute for Medical Research, London, UK) at 1:500 dilution. At 50 h post-infection, each of the developmental stages

towards merozoite formation was counted. At 60 h post-infection, the number of merozoites in each schizont was evaluated.

Statistical analysis

Differences were evaluated with Student's *t*-test. $p < 0.05$ was considered statistically significant.

3-3 Results & Discussion

To evaluate the liver-stage development of the TPx-1 KO population *in vitro*, IFA was performed with anti-CSP antibody. The area of TPx-1 KO was significantly smaller than that of WT at the schizont and cytomere stages (40 h and 50 h post-infection). The mean area values calculated for TPx-1 KO were approximately 3/4 of WT at the schizont stage (40 h) and 1/2 of WT at the cytomere stage (50 h) (Fig. 9). These results suggest that TPx-1 KO parasites were smaller in size in the schizont and cytomere stages than WT parasites.

To observe liver schizont development in detail, anti-MSP-1 antibody, which serves as a marker of MSP-1 expression during merozoite formation in mature liver-stage parasites, was used. MSP-1 is primarily located on the rim of the developing schizont. Afterwards, its expression widens within the parasite. Its final location is the cell membrane of the merozoite (Fig. 10). The staining patterns of TPx-1 KO and WT were therefore evaluated at 50 h post-infection according to three criteria as follows: (a) no staining of MSP-1, (b) rim staining of MSP-1, and (c) intracellular merozoite staining of MSP-1 (Fig. 11). In WT parasites, the percentages of each staining pattern were 25.4 %, 42.45 % and 32.15 % for (a), (b) and (c), respectively. On the other hand, in TPx-1 KO, the percentage of each staining pattern was 21.65 %, 42.95 % and 35.4 % for (a), (b) and (c), respectively. There was no statistical significance between TPx-1 KO and WT found in percentage of any of the staining patterns. This result indicates that the development of TPx-1 KO liver schizonts could be comparable to WT towards the merozoite-forming stage.

The author next evaluated merozoite formation in TPx-1 KO liver schizonts. To assess the number of merozoites formed in each schizont, the sizes of

merozoites in TPx-1 KO and WT were compared. The result showed that the mean merozoite size in TPx-1 KO was 1.44 um^2, while the mean size of WT was 1.36 um^2 ($P>0.05$) (Fig. 12). Taken together with the finding that the TPx-1 KO liver schizont and cytomere stages are smaller in size than those of WT, it is suggested that TPx-1 KO liver schizont produces fewer merozoites than WT. It has been reported that the sizes of *P. yoelli* liver schizont and cytomere stages correlate with the number of merozoites formed within the parasite (Baer et al., 2007).

The area values of TPx-2 KO at the schizont stage (40 h) and cytomere stage (50 h) were equivalent to WT (Fig. 13).

In this chapter the phenotypes of two Prx-KO populations in the liver stage were investigated using an *in vitro* infection system with HepG2 cells. IFA using anti-CSP mouse antisera confirmed that TPx-1 KO schizonts and cytomeres were smaller in size than those of WT. The TPx-1 KO population has a defect in the liver stage in the mouse (Yano et al., 2008). The present finding, together with the previous result, suggested that the developmental disorders in the liver stage of TPx-1 KO took place in or after the liver schizont stage.

In the schizont stage, intracellular ROS density may be high due to ATP production during extensive karyokinesis. The highest expression level of TPx-2, which localizes mitochondrion, has also been observed in WT parasites at the liver schizont stage. TPx-1 KO parasites may be more susceptible to ROS than WT, since disruption of gene coding for TPx-1 in *P. falciparum* rendered the parasite more susceptible to paraquat-induced ROS than WT in the blood stage (Komaki-Yasuda et al., 2003).

There was no difference between TPx-1 KO and WT in liver-stage parasite development during the schizont to cytomere stage. However, the author's results suggest that fewer merozoites were produced in TPx-1 KO liver schizonts than in WT. In the mosquito stage, the numbers of TPx-1 KO and WT oocysts were comparable; however, the fewer sporozoites were produced by TPx-1 KO oocysts than by WT oocysts (Yano et al., 2008).

TPx-1 may protect the parasite from oxidative stress in the developmental stage when the parasites exponentially multiply. Several reports state that TPx-1 possesses multiple functions other than its function as peroxidase, including the functions of molecular chaperone and sensor protein (Veal et al., 2004; Jara et al.,

2007). Recently, the function of TPx-1 as a molecular chaperone has been reported in *P. falciparum* (Kimura et al., 2013). The mechanisms by which TPx-1 supports sporozoite formation in oocysts at the mosquito stage and merozoite formation in schizonts at the liver stage would be important and interesting avenues for future study.

There was no significant difference in size between TPx-2 KO and WT in liver-stage parasites *in vitro*. This result supports the previous finding that TPx-2 KO development in the liver stage is comparable to that of WT (as discussed in Chapter 2). Comprehensive gene expression profiling in liver stage parasites at each developmental stage by RNA sequencing may provide information to answer why the TPx-1 KO population, but not the TPx-2 KO population, had defective liver-stage development.

The biological functions of Prxs in malaria parasites will be interesting topics for future study, especially with regard to their functions in mosquito and liver developmental stages. Our results indicated an import role for Prx in the liver-stage development of the malaria parasite. Environmental conditions should be taken into

account into future investigations into the host–parasite relationship of this developmental stage.

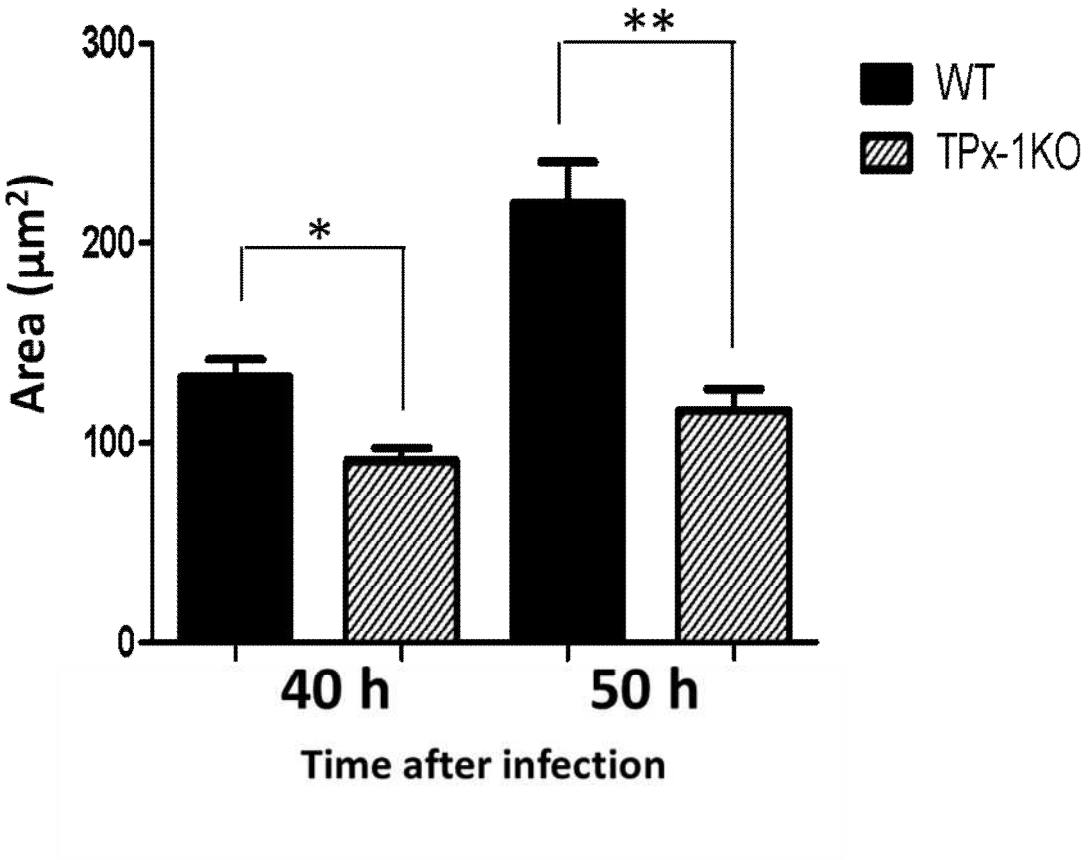


Figure 9. Liver-stage development of *P. berghei* after sporozoite infection of HepG2 cell. The area of the parasite was measured based on IFA at 40 h (schizont stage) and 50 h (cytomere stage) post-infection in WT and TPx-1 KO liver-stage parasites using TSCCP5 software (Leica Microsystem). The average of three independent experiments is shown. Error bars represent standard deviation. Student's *t*-test was performed to compare the values of WT and TPx-1 KO parasites. Asterisks (*) indicate statistical significance. *:$p < 0.05$; ** $p < 0.001$

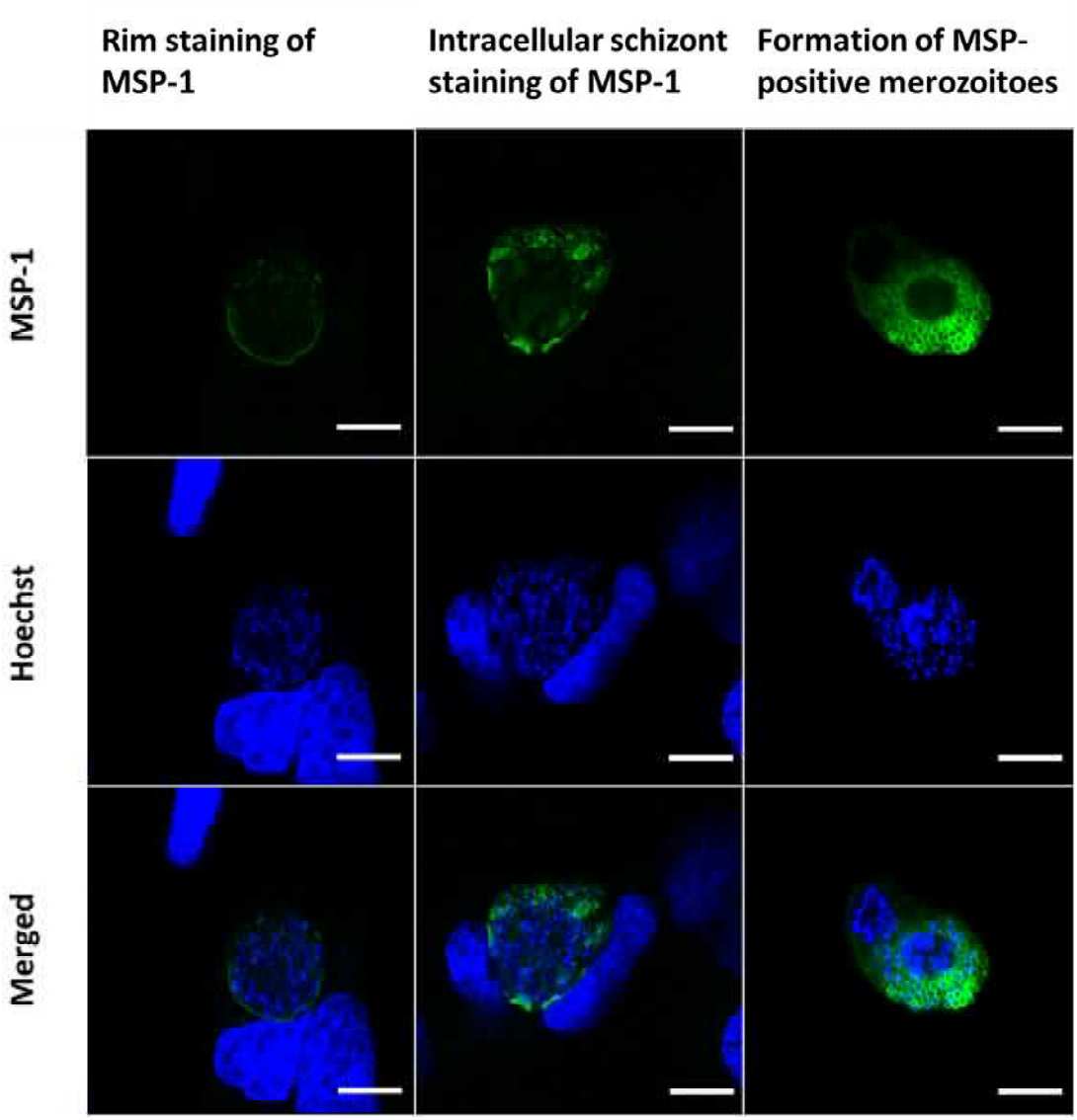
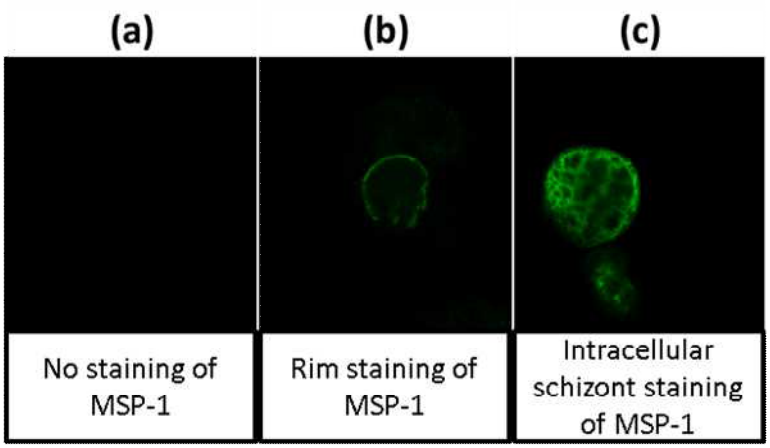


Figure 10. *in vitro* liver-stage development of the *P. berghei* WT parasite. Development of liver-stage *P. berghei* was observed by IFA. Images show staining patterns with anti-MSP-1-antibodies at liver cytomere stage (left and center panels) and merozoite-forming schizont stage (right panels) (50-55 h post-infection in the *in vitro* assay). Anti-MSP-1 antibody recognizes the parasite cytoplasm (green).

Anti-MSP-1 antibody is used as a marker of MSP-1 expression during merozoite formation in mature liver-stage parasites (Annoura et al., 2012). Nuclei are stained with Hoechst-33342 (blue). Scale bar indicates 10 µm.


(a)
(b)
(c)
No staining of MSP-1
Rim staining of MSP-1
Intracellular schizont staining of MSP-1

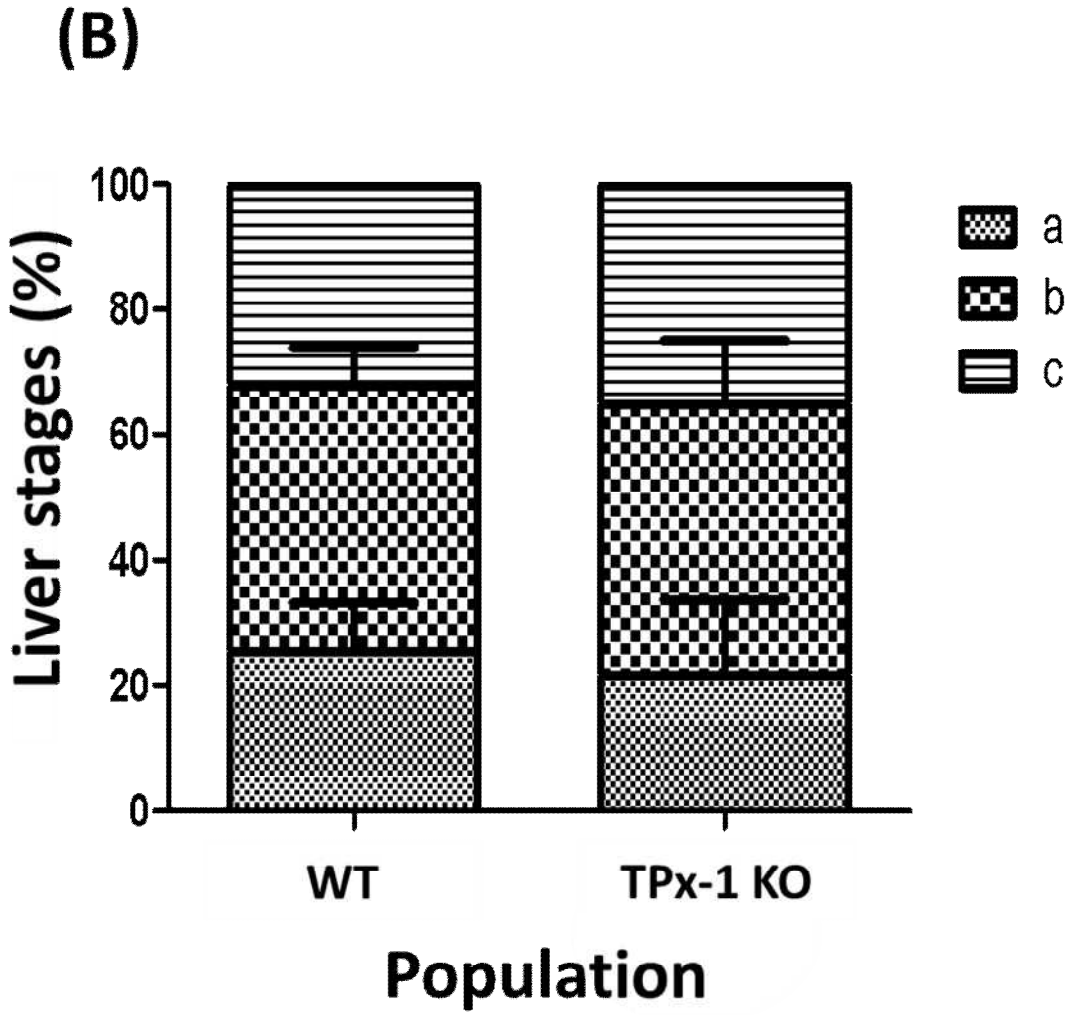

(B)
Liver stages (%)
100
80
60
40
20
0
a
b
c
WT
TPx-1 KO
Population

Figure 11. Quantitative evaluation of liver merozoite formation.

The liver-stage parasite was stained with anti-MSP-1 antibody. The developmental stages of parasites in WT and TPx-1 KO were evaluated at 50 h post-infection by counting the parasites with different staining patterns according to the criteria indicated by the representative images in panel A: (a) no MSP-1, (b) rim staining of MSP-1, (c) intracellular merozoite staining of MSP-1. The percentage of each staining pattern in WT and TPx-1 KO parasites are shown in panel B. The average of two independent experiments is shown. Error bars represent standard deviation.

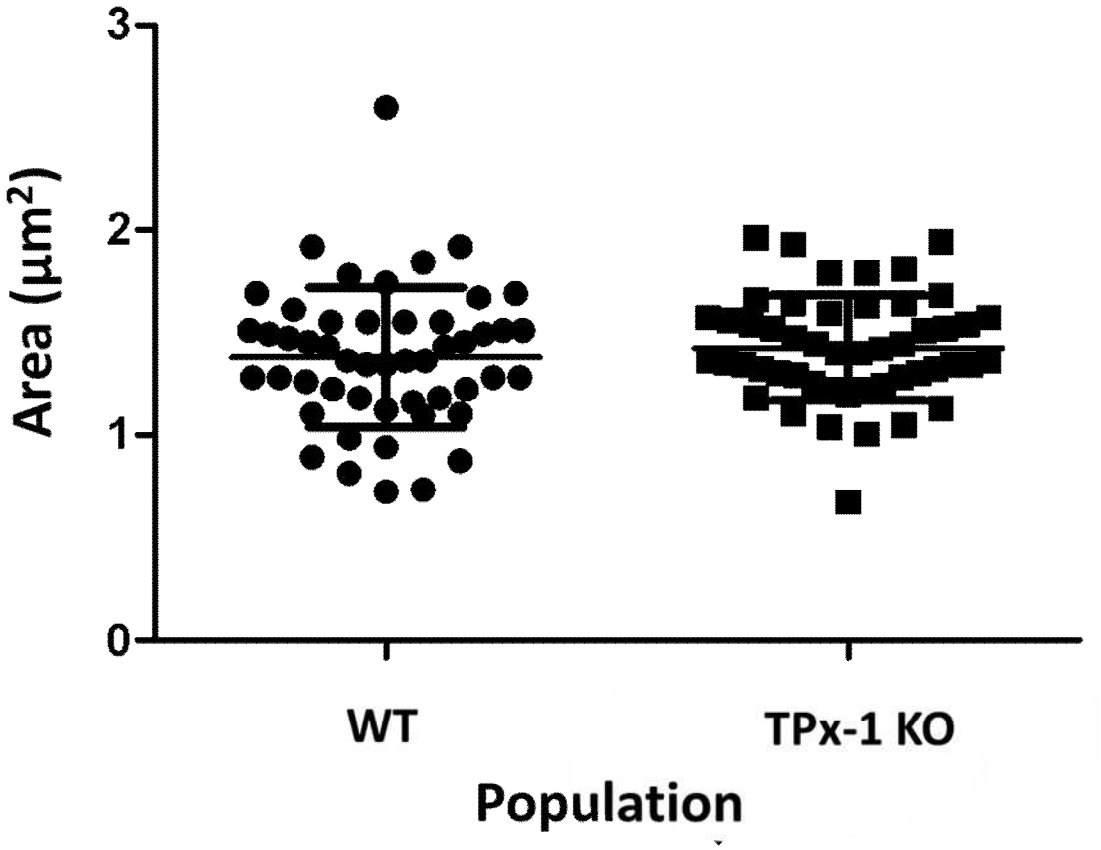


Figure 12. Evaluation of merozoite size in WT and TPx-1 KO liver-stage parasites. The area of liver merozoites were measured based on the IFA assay with anti-MSP-1 antibody using TSCCP5 software (Leica Microsystem) at 60 h post-infection. Error bars represent standard deviation.

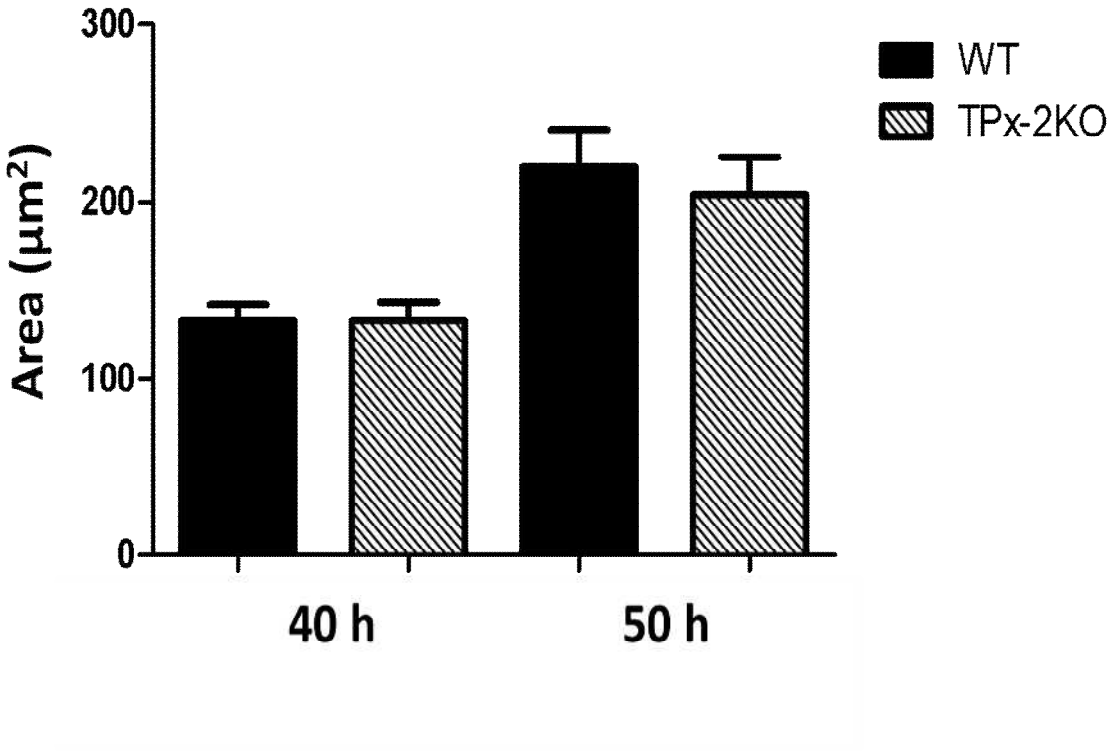


Figure 13. Liver stage development of *P. berghei* after sporozoite infection of HepG2 cells. The area of the parasite was measured based on IFA at 40 h (schizont stage) and 50 h (cytomere stage) in WT and TPx-2 KO parasites using TSCCP5 software (Leica Microsystem). The average of three independent experiments is shown. Error bars represent standard deviation. Student's *t*-test was performed to compare the values of TPx-2 KO and WT. No significant difference was found at 40 h and 50 h.

General Discussion

This study aims to examinethe antioxidant defense system of *Plasmodium* in the liver stage, and consists of three chapters: 1) analysis of the localization and expression profile of three members of Prxs namely, TPx-1, 1-Cys Prx and TPx-2 in WT *Plasmodium berghei* during the liver stage; 2) examination of the expression profiles of TPx-1, 1-Cys Prx and TPx-2 in TPx-1 KO and TPx-2 KO *P. berghei* during the liver stage, and comparison with WT; and 3) examination and comparison of the phenotype of three types of TPx-1 KO, TPx-2 KO and WT in the liver stage. In a series of experiments, the author observed expression of Prxs in the liver stage, and discussed how these enzymes are engaged with host-parasite adaptation and the growth of the liver-stage parasite.

With regards to expression of Prxs in *Plasmodium*, TPx-1 is expressed throughout the whole life cycle of the parasite. 1-Cys Prx and TPx-2 are expressed in the blood stage, but in the insect stage they are only expressed in the ookinetes (Hall et al., 2005). These enzymes are expressed again after parasite invasion in the host liver cell. *P. falciparum* TPx-1 is abundantly and constitutively expressed in the cytoplasm of the blood-stage parasite, suggesting that this enzyme performs a

housekeeping function (Yano et al., 2005). High expression of 1-Cys Prx during trophozoite (heme digesting) stage in the cytoplasm suggests that this enzyme act as an antioxidant to cope with the oxidative burden of heme (ferriprotoporphyrin IX; FP) (Kawazu et al., 2005). However, the roles of these cytoplasmic Prxs in the liver stage remain unknown at the time of writing. TPx-2 KO *P. berghei* are able to grow normally in the blood and the insect stages, suggesting this Prx might not be essential for parasite development in these stages (Masuda-Suganuma et al., 2012), however, the high expression of TPx-2 in liver stage suggests that the enzyme is still important in this stage. It is interesting to investigate the role of each enzyme in the development of the parasite in host liver cells.

The comparison of mRNA expression profiles of Prxs in TPx-1 KO and TPx-2 KO to WT revealed that the expression of mRNAs coding for other Prxs in the KO populations were upregulated during liver-stage development. These results suggested that the stage specific expression of Prxs may be affected and regulated by environmental changes. A recent report suggested that the *P. berghei* ookinete regulates antioxidant protein expression in response to environmental changes in order to maximize the probability of survival, maturation and escape (Benjamin et

al., 2013). In order to analyze the biological function of the Prxs in detail, it is very important to reveal the relationship of each Prx with the other Prx family members. In this study, Prx expression is observed in an *in vitro* model system using human HepG2 hepatoma cells, however, if considering the host-parasite interaction, a mouse infection model should be utilized. Besides, considering the upstream of Prxs is essential for revealing the whole picture of the antioxidant defense mechanism in malaria parasites. Therefore, comprehensive gene expression profiling in the liver stage parasite at each developmental stage by RNA sequencing should be performed in future.

The TPx-1KO population has a defect in liver stage development *in vivo* (Yano et al., 2008). The observation of WT and two KO populations in the liver stage *in vitro*, suggested that the TPx-1KO defect might be due to its smaller cell size rather than developmental delay. Therefore, it is postulated that the level of ROS exceeded the amount that could be compensated for by the overexpression of other Prx in the absence of TPx-1, and that these stresses somehow resulted in the cell size being smaller than usual. The ROS level in the liver stage parasite and the

mechanism by which the stresses make the cell size smaller should be evaluated in order to confirm this hypothesis.

The liver stage is the least researched stage in *Plasmodium* development, mainly due to the relative inaccessibility and low abundance of these stages for detailed cellular and molecular studies (Kappe and Duffy 2006). However, this stage of *Plasmodium* infection bears enormous potential for anti-malarial intervention. Investigation of such redox systems in the malaria parasite for the purpose of developing novel antimalarial drugs and vaccination strategies would be interesting topics for a future study.

Conclusion

In this study, mRNA and protein expression profiles of three peroxiredoxins (TPx-1, 1-Cys Prx and TPx-2) in the liver stage *P. berghei* were examined through IFA and RT-qPCR. The IFA results indicated the cytosolic localization of TPx-1 and 1-Cys Prx, and mitochondrial localization of TPx-2 in the liver-stage parasite. The findings suggested that the parasite expresses these proteins in different cellular compartments with different profiles to manage intracellular oxidative stress.

Comparison of the expression profiles of Prxs in the TPx-1 KO and TPx-2 KO populations with expression profiles of Prxs in WT suggested a compensatory mechanism of these antioxidant enzymes under conditions of oxidative stress during liver-stage development.

The phenotypic observation of KO populations in the liver stage suggested that the defect in liver stage development seen in TPx-1 KO *in vivo* might be due to there being a lower number of merozoites formed than in WT, as opposed to developmental delay. On the other hand, TPx-2 KO developed normally, in comparison to WT, in liver cell *in vivo* and *in vitro* experiments. It is the author's

hope that these findings can facilitate a better understanding of the antioxidant defense mechanisms in malaria parasite for development of novel and potential therapeutic strategies for this parasitic disease.

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要約

マラリア原虫は生活環の中で絶えず酸化ストレスに曝されている。そのためマラリア原虫抗酸化機構は、その寄生適応の成否を左右する重要なメカニズムと考える。本研究ではローデントマラリア原虫肝臓型における抗酸化機構が宿主寄生適応に果たす役割を考察するため、原虫の主要抗酸化酵素ペルオキシレドキシンを対象として以下の研究を行った。

第一章ではローデントマラリア原虫 *Plasmodium berghei*（ANKA 株）野生型（WT）肝臓型におけるペルオキシレドキシン（TPx-1、 1-Cys Prx および TPx-2）の発現解析をおこなった。

WT 感染蚊唾液腺から調製したスポロゾイトを、*in vitro* で培養した HepG2 細胞へ接種した。接種後 5 時間後（スポロゾイト感染初期）、17 時間後（肝臓型トロホゾイト期）、40 時間後（肝臓型シゾント期）、50 時間後（肝臓型サイトメア期）に TPx-1、1-Cys Prx および TPx-2 の局在を、各抗酸化酵素タンパク質に特異的な抗体を用いた間接蛍光抗体法（IFA）により確認し、また mRNA 発現量を各ペルオキシレドキシン遺伝子に特異的なプライマーを用いたリアルタイム RT-PCR（RT-qPCR）にて相対定量した。

IFA の結果、TPx-1 と 1-Cys Prx は原虫細胞質に、TPx-2 は原虫ミトコンドリアに局在することが確認された。また RT-qPCR の結果、TPx-1 mRNA は構成的な発現を示した。1-Cys Prx mRNA の発現はシゾント期から亢進しサイトメア期でピークとなった。一方 TPx-2 の mRNA 発現はトロホゾイト期から亢進しシゾント期でピークとなった。以上の成績から、マラリア原虫肝臓型において、これらの抗酸化タンパク質はそれぞれ異なった細胞分画に異なったプロファイルで発現し、原虫を酸化ストレスから保護していることが示唆された。

第二章では、*P. berghei* で作製した TPx-1 遺伝子欠損株（TPx-1 KO）および TPx-2 遺伝子欠損株（TPx-2 KO）肝臓型でのペルオキシレドキシン（TPx-1、1-Cys Prx および TPx-2）の発現プロファイルを WT のそれと比較した。

各原虫株感染蚊唾液腺から調製したスポロゾイトを、*in vitro* で培養した HepG2 細胞へ接種した。接種後 5 時間後、17 時間後、40 時間後、50 時間後に TPx-1、1-Cys Prx および TPx-2 の mRNA 発現量を RT-qPCR にて相対定量した。

TPx-1 KO では、トロホゾイト期およびシゾント期での 1-Cys Prx mRNA の発現量が WT のそれと比較して有意に増加した。TPx-2 mRNA の発現量もシゾント期で WT のそれに比較して有意に増加した。TPx-2 KO でも、スポロゾイト感染初期、トロホゾイト期およびシゾント期での 1-Cys Prx mRNA の発現量が WT のそれと比

較して有意に増加した。一方 TPx-1 mRNA の発現量は、スポロゾイト感染初期で WT のそれと比較して有意に増加した。以上の成績から、マラリア原虫肝臓型でも酸化ストレス応答メカニズムの存在が示唆された。

第三章では、TPx-1 KO と TPx-2 KO について肝臓型での表現型を観察した。

WT、TPx-1 KO および TPx-2 KO 各原虫株感染蚊唾液腺から調製したスポロゾイトを、*in vitro* で培養した HepG2 細胞へ接種した。接種後 5 時間後、17 時間後、40 時間後及び 50 時間後に、肝臓型原虫の発育状態を抗 CSP (circumsporozoite protein) 抗体と抗 MSP (merozoite surface protein)-1 抗体 による IFA で観察した。

TPx-1 KO での抗 CSP 抗体を用いた IFA の成績から、この原虫株では肝臓型シゾント期以降で、原虫細胞の大きさが WT に比較して有意に小さいことが解った。TPx-1 KO では、スポロゾイトをマウスに接種すると、赤血球型出現の潜伏期が WT に比較して遅延するが、今回の成績から、肝臓型シゾント期以降に発育障害が起きていることが示唆された。また、この時期の表現型について更に詳しく調べる目的で抗 MSP-1 抗体を用いた IFA をおこなったところ、TPx-1 KO では WT と同様にメロゾイトの形成進行することが解った。また形成されるメロゾイトの大きさも WT と同等であった。このことから、TPx-1 KO で観られた赤血球型出現の潜伏期

遅延は、肝臓型シゾントの小型化すなわち形成メロゾイト数の減少によるものと考察された。一方、スポロゾイトのマウス接種試験における TPx-2 KO での赤血球型出現の潜伏期は WT と同等であった。また、TPx-2 KO は、HepG2 細胞接種試験においても WT と同等の発育と増殖を示した。

以上の成績から、これまで不明であったマラリア原虫肝臓型での抗酸化機構の役割の一端が明らかとなった。マラリア原虫の肝臓型ステージにおける抗酸化機構の意義の更なる解明によって、このステージを標的とする新規の創薬やワクチン開発研究に有用な知見が供給されることが期待される。