

Changes in Hepatic Phosphoprotein Levels in Mice Infected with *Plasmodium berghei*

(Perubahan Aras Fosfoprotein Hepar dalam Mencit Terinfeksi *Plasmodium berghei*)

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ABSTRACT

Hepatic phosphoprotein levels are altered in mouse liver as a manifestation of bacteria, virus or parasite infection. Identification of signaling pathways mediated by these hepatic proteins contribute to the current understanding of the mechanism of pathogenesis in malarial infection. The present study was undertaken to evaluate the changes in hepatic phosphoprotein levels during *Plasmodium berghei* infection. Our study revealed changes in levels of three hepatic phosphoproteins following *P. berghei* infection compared to non-infected controls. Peptide fragment sequence analysis using tandem mass spectrometry (MS/MS) showed these hepatic proteins to be homologs to haemoglobin beta (HBB), class Pi glutathione S-transferase (GSTPi) and carbonic anhydrase III (CAIII) proteins of *Mus musculus* species respectively from the NCBI nr sequence database. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis predicted the involvement of these proteins in specific pathways in *Mus musculus* species; GSTPi in glutathione and drug metabolism and CAIII in nitrogen metabolism. This shows that *P. berghei* infection affects similar signaling pathways as those reported in other pathogenic infections such as that related to GSTPi and CAIII in response to oxidative stress.

Keywords: Chloroquine; hepatomegaly; malaria; phosphoprotein; *Plasmodium berghei*

ABSTRAK

Aras fosfoprotein hepar mencit berubah semasa manifestasi infeksi oleh bakteria, virus dan parasit. Pengenalpastian tapak jalan pengisyaratan yang diperantara oleh protein hepar boleh menyumbang kepada pemahaman terhadap patogenesis infeksi malaria. Kajian ini dilakukan untuk menentukan perubahan aras fosfoprotein hepar semasa infeksi *Plasmodium berghei*. Dalam kajian ini, perubahan aras tiga fosfoprotein hepar telah diperhatikan sewaktu infeksi *P. berghei* berbanding kawalan tanpa infeksi. Analisis jujukan fragmen peptida menggunakan spektrometri jisim tandem (MS/MS) menunjukkan protein hepar tersebut terdiri daripada homolog kepada hemoglobin beta (HBB), glutathione S-transferase kelas Pi (GSTPi) dan karbonik anhidrase III (CAIII) daripada spesies *Mus musculus* masing-masing dari pangkalan data jujukan NCBI nr. Analisis tapak jalan menggunakan pangkalan data Kyoto Encyclopedia of Genes and Genomes (KEGG) meramalkan penglibatan GSTPi dalam metabolisme glutation dan dadah; dan protein CAIII dalam metabolisme nitrogen, kesemuanya dalam spesies *Mus musculus*. Kajian ini menunjukkan bahawa infeksi *P. berghei* memberi kesan terhadap tapak jalan pengisyaratan yang telah dilaporkan terlibat dalam infeksi patogen lain umpamanya tapak jalan berkaitan GSTPi dan CAIII sebagai respons terhadap tekanan oksidatif.

Kata kunci: Fosfoprotein; hepatomegali; klorokuin; malaria; *Plasmodium berghei*

INTRODUCTION

Malaria is still a deadly disease which causes more than one million deaths each year in the endemic areas of the world. The increasing parasite resistance towards available antimalarial drugs and the unavailability of antimalarial vaccines are amongst the main challenges in curbing this endemic disease (Alkahtani 2010). Understanding the host response induced by malaria is crucial for development of specific antimalarials.

Plasmodium spp., the parasitic protozoan which causes malaria, is carried by the female *Anopheles* mosquito vectors for infection in its vertebrate host. Upon entry into the host, the parasite is carried through the blood stream into the liver where it multiplies (exoerythrocytic cycle)

prior to entering the erythrocytes (erythrocytic cycle). Common symptoms shown by the host during the latter cycle in the erythrocytes include anaemia, hepatomegaly, liver inflammation and tissue injury (Greenwood et al. 2008). These symptoms are however not observed during infection in the exoerythrocytic cycle.

An infection model system frequently adopted for malarial studies is the *Plasmodium berghei* erythrocytic-stage infection system in rodents especially mice (Sherman 2008). *P. berghei* shares common characteristics with *P. falciparum*, the most pathogenic human malarial parasite (Ishino et al. 1994; Sherman 2008).

Erythrocytic-stage *Plasmodium* infection causes liver apoptosis at a significantly higher rate compared to

exoerythrocytic-stage infection (Sand et al. 2005). During exoerythrocytic-stage infection, infected cells containing viable parasites are protected from apoptosis for parasite survival (Sand et al. 2005).

The hepatic pathological changes during malarial infection may lead to liver dysfunction and other systemic complications (Kochar et al. 2003). During malarial infection, the host liver does not only serve as a site for parasite development, but also acts as an important immune effector towards erythrocytic-stage parasite infection (Nobes et al. 2002). Kupffer cells, the resident macrophages in the liver, are able to eliminate parasite-derived haemozoin as well as *Plasmodium*-infected erythrocytes. Localised production of pro-inflammatory cytokines by Kupffer cells is one of the causes of liver injury during erythrocytic-stage *P. berghei* infection (Coban et al. 2007).

Interrupted protein signaling contributes to liver pathogenesis; for example, hepatitis C viral infection causes oxidative stress through the regulation of p38 MAPK and JNK protein signaling (Choi & Ou 2006). Protein phosphorylation has been shown to be an important event in bacteria, virus or parasite infection. For example, infection of peritoneal macrophages with *Salmonella typhimurium* induced extensive phosphorylation in a set of proteins involved during the early phases of interaction between *Salmonella* organisms and macrophages (Saito et al. 1994). Exposure of *Campylobacter jejuni*-infected cells with protein kinase inhibitors was shown to involve tyrosine protein kinase-linked pathways which regulate the internalisation of *C. jejuni* into intestinal epithelial cells (Biswas et al. 2004). Also, infection of HeLa cells by *Yersinia pseudotuberculosis* activates Src-mediated signaling pathway and involves tyrosine phosphorylation of Fak protein (Viboud & Bliska 2005). In the case of hepatitis B viral infection, phosphorylation of an intermediate filament protein keratin 18 in hepatocytes correlates with progression of infection (Shi et al. 2010).

In malarial infection, erythrocyte-stage *Plasmodium* infection activates the MyD88 pathway which activates the immune system, chronic inflammation, apoptosis, and hemozoin clearance from the host liver (Adachi et al. 2001; Thomas et al. 2004). A total of 59 phosphoproteins have been identified in *P. falciparum*-infected red blood cells, including two HSP70 heat shock proteins (Wu et al. 2009). *P. falciparum* infection leads to a dramatic increase in the phosphorylation level of erythrocyte protein 4.1 which forms a tight complex with the mature parasite-infected erythrocyte surface antigens (Wu et al. 2009).

The basis and mechanism of intracellular signaling during liver pathogenesis that causes its dysfunction is still not fully understood. Since the ability of *Plasmodium* to manipulate its host defence system and to cause pathogenesis may be likely to involve phosphorylation of key proteins (Nelson et al. 2008), it is therefore of interest to evaluate the effects of *P. berghei* infection on the liver phosphoprotein profiles.

The present study was undertaken to elucidate the effects of erythrocyte-stage malarial infection towards liver tissue histology and phosphoprotein levels in *P. berghei*-infected mice.

MATERIALS AND METHODS

ETHICS STATEMENT

All animal experiments were performed in accordance with the animal ethics guideline compiled by Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC). Animals were obtained from the Universiti Kebangsaan Malaysia Animal House Facility. The HSD strain ($n=60$) mice were housed in individual cages of five mice per cage and had free access to pelleted protein-enriched diet and drinking water *ad libitum*.

PARASITE

P. berghei NK65 parasites, obtained from the School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor, were maintained by weekly passage of infected blood in HSD mice. A standard inoculum consisting of 10^6 parasitised erythrocytes was prepared from infected blood of stock mice with $>25\%$ parasitemia, and used to infect experimental mice.

MICE

Male HSD mice (4 weeks old) were adapted for two weeks. The mice were randomly divided into four groups of 5 per cage and were inoculated intraperitoneally with 1×10^6 parasitised red blood cells containing *P. berghei* strain NK65 on Day 0.

FOUR-DAY SUPPRESSIVE TEST

The four-day suppressive test was employed as described by Peters et al. (1975). Briefly, mice were administered with daily doses of chloroquine phosphate (Sigma, USA) (10 mg/kg body weight) or normal saline (placebo) intraperitoneally for four consecutive days beginning three hours after *P. berghei* infection on Day 0. Thin films of tail blood were prepared daily beginning from Day 1 and stained with Giemsa to estimate parasitemia levels. Once the blood parasitemia level of infected mice reaches 10%, 30% or 50%, the infected mice, the control non-infected mice and chloroquine-treated infected/non-infected mice were euthanised and liver harvested on the same day.

HISTOLOGICAL STUDIES

Liver organs were harvested from the euthanised mice, washed in 0.9% sodium chloride solution, weighed, cut into 1 cm \times 1 cm \times 1 cm cubes and placed in Bouin solution for fixation. The rest of the organ was stored in -80°C until ready for protein extraction for western blotting.

experiment. The organ was dehydrated in increasing concentrations of alcohol (70–100%) and embedded in paraffin blocks which were then sectioned in 5–10 μm slices on a rotary microtome (American Optical Spencer Rotary Microtome, Model 820, Fischer Scientific, USA). The organs sectioned were stained with hematoxylin and eosin for evaluation of tissue morphology by light microscopy (Kshreerasagar & Kaliwal 2006).

WESTERN BLOTTING

Harvested liver organs were homogenised in five volumes of ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA) containing protease and phosphatase inhibitors (1 mM phenylmethylsulphonylfluoride, 1 mM sodium orthovanadate, 50 $\mu\text{g}/\text{mL}$ leupeptin, 1 mM sodium fluoride) (Lee 2007). Homogenised liver was centrifuged at 12 000 g for 30 min. Protein concentration was determined by the Bradford method (Bradford 1976). Sample buffer (0.25 M Tris HCl pH 6.8, 5% SDS, 10% glycerol, 0.25% bromophenol blue, 2% β -mercaptoethanol) was added to supernatant and samples designated for electrophoresis were boiled in a waterbath for 5 min. Equal amounts of protein samples were loaded into each lane in 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Laemmli 1970). Proteins separated by SDS-PAGE were electro-transferred onto nitrocellulose membrane (Towbin et al. 1979) following which the membrane was blocked with 0.5% gelatine dissolved in phosphate buffered saline containing 0.1% Tween-20 (0.1% PBST), and then incubated with primary mouse monoclonal antibodies; anti-phosphotyrosine (3000x dilution in 0.1% PBST; Sigma, USA), or anti-phosphoserine (1000x dilution in 0.1% PBST; Sigma, USA), or anti-phosphothreonine (500x dilution in 0.1% PBST; Sigma, USA) overnight at 4°C prior to incubation with a corresponding secondary antibody (15 000x dilution in 0.1% PBST; HRP-conjugated anti-mouse IgG; Promega, USA) for 2 h at room temperature. Membranes were stripped (2% SDS, 62.5 mM Tris pH 6.7, 100mM β -mercaptoethanol, 50°C, 30 minutes) and reprobed with anti- β -actin (3000x dilution in 0.1% PBST; Sigma, USA) to ensure equal protein loading (Marlovits et al. 2004). Immunoreactive protein bands were detected using enhanced chemiluminescence (ECL) reagent (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific, USA) following manufacturer's protocol.

The results of western blots were quantified using an image analyser (Alpha Imager 2200, USA). Molecular weights of proteins significantly ($p < 0.05$) affected by *P. berghei* infection (as compared to homologous proteins in control mice) were determined and then subjected to analysis by MALDI-TOF/TOF MS/MS.

MASS SPECTROMETRY

Major liver protein bands which showed significantly altered intensities with infection compared to that of non-

infected control were excised from Coomassie blue-stained polyacrylamide gels and sent to the Protein and Proteomics Centre, Department of Biological Sciences, National University of Singapore for MS/MS protein identification.

MALDI TOF/TOF MS/MS analysis (MALDI TOF/TOF Analyser, Applied Biosystems, USA) was carried out as previously described (Zhang et al. 2005). Briefly, the excised band was digested by in-gel protein digestion. The digest and the matrix solution was spotted on the MALDI target plate and allowed to air dry prior to analysis by the 4800 MALDI-TOF/TOF Analyser. The peptide mass fingerprints obtained were searched against the National Center for Biotechnology Information nonredundant (NCBI nr) Protein Database using the MASCOT search program (version 2.0; Martix Science Ltd., UK). Results were scored using the probability-based Mowse score (the protein score is $-10 \times \log(P)$ where P is the probability that the observed match is a random event). In the conditions of this experiment, a score greater than 81 indicated a significant identification ($p < 0.05$).

IN SILICO ANALYSIS

Proteins identified by MS/MS were assigned to phosphorylation site identification using PhosphoSitePlus database (<http://www.phosphosite.org>), a web-based bioinformatics resource dedicated to physiological sites of protein phosphorylation in humans and mice, as well as the NCBI protein database (Kanehisa et al. 2010). Pathways involving the identified proteins were predicted using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis software (<http://www.genome.jp/kegg/pathway.html>) (Mao et al. 2005).

STATISTICAL ANALYSIS

Statistical analysis of the data was done using the Student's *t*-test. *p* values less than 0.05 were considered significant.

RESULTS

PHOSPHOPROTEIN PROFILE

Figure 1 shows Coomassie-blue stained liver proteins resolved by SDS-PAGE. Densitometric analysis of western blot films revealed a total of three phosphoprotein bands (i.e. ~18 kDa, ~28 kDa and ~50 kDa) which exhibited a 1.5-fold or greater quantitative difference between *P. berghei*-infected and non-infected mice liver samples (Figure 2).

Proteins can be phosphorylated at tyrosine, serine or/and threonine residues. In order to identify the changes in the phosphorylation profile of *P. berghei*-infected mice liver proteins, immunoblotting was carried out using anti-phosphotyrosine, anti-phosphoserine or anti-phosphothreonine antibodies. Immunoblotting using an anti-phosphotyrosine antibody did not show

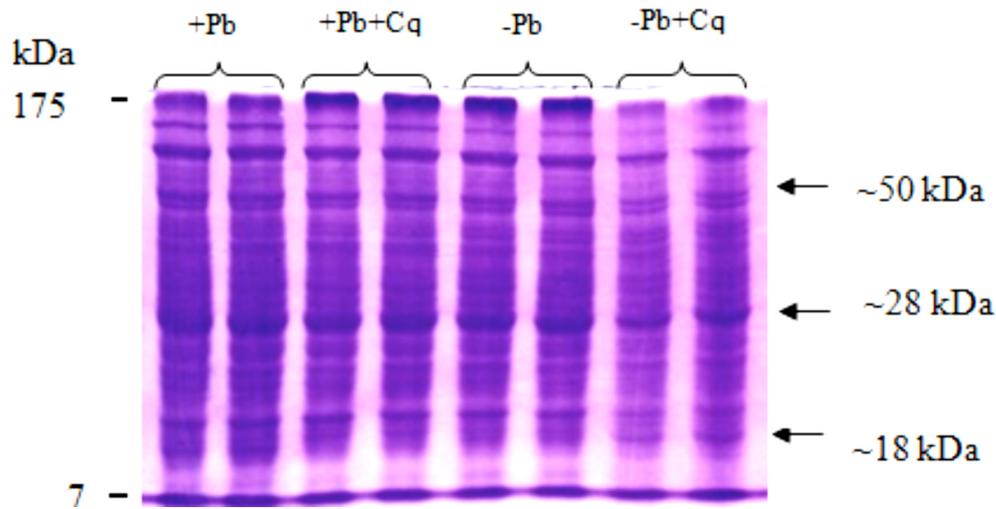


FIGURE 1 Coomassie-stained polyacrylamide gel profile for liver proteins of mice harvested at 50% parasitemia [+Pb: *P. berghei*-infected mouse liver; +Pb+Cq: *P. berghei*-infected + chloroquine treatment; -Pb: non-infected control mouse liver; -Pb+Cq: non-infected + chloroquine treatment]

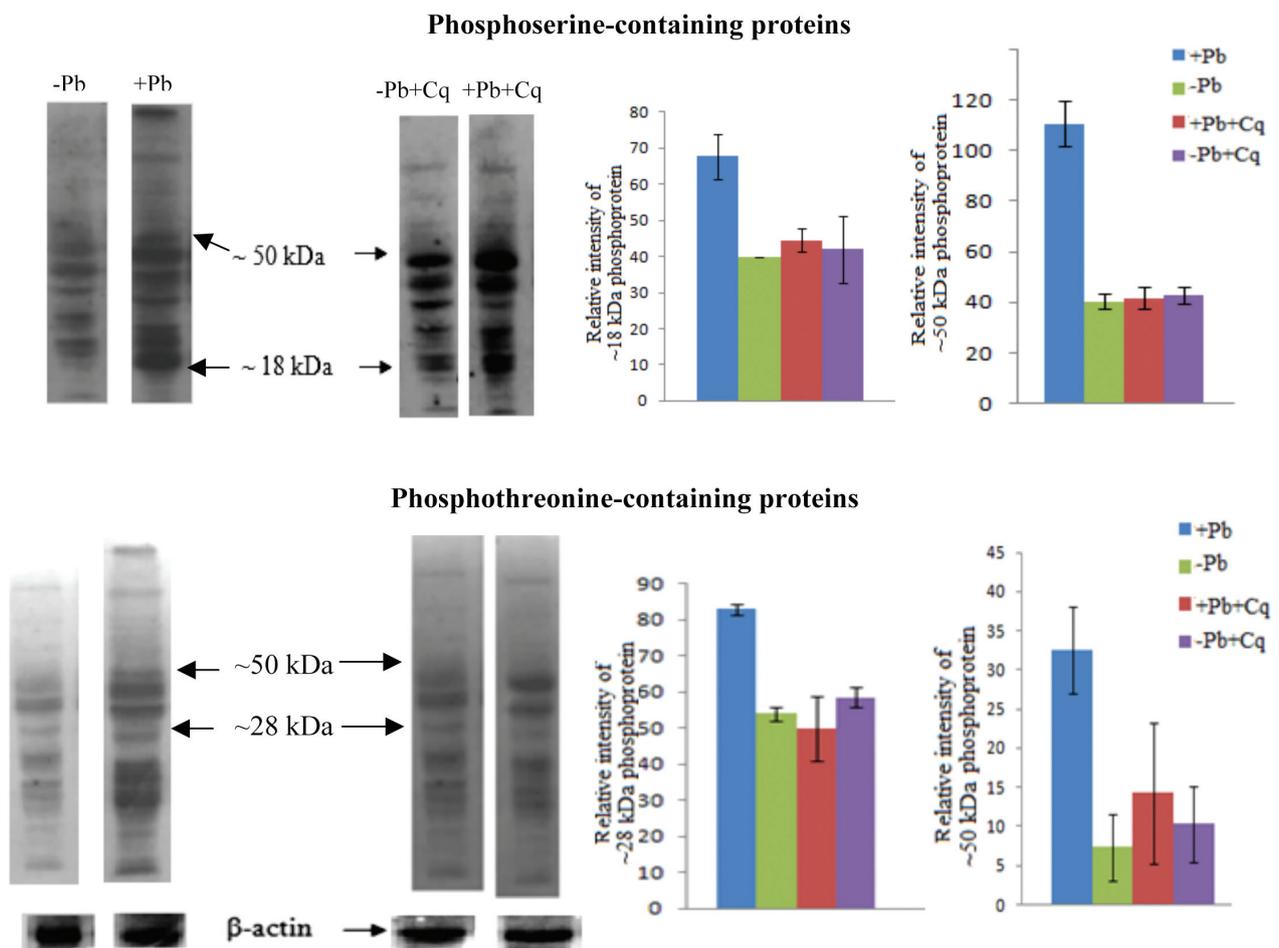


FIGURE 2 Immunoblot analysis of liver samples showing significantly altered levels of phosphoserine- and phosphothreonine-immunoreactive protein bands with its respective densitometry data for *P. berghei*-infected and chloroquine-treated mouse liver samples. β -actin expression level was used as protein loading control. Protein band intensities were expressed in terms of fold control. * Significantly different from control group at $p < 0.05$ by Student's t-test. [~18 kDa protein band= HBB; ~28 kDa= GSTPi; ~50 kDa= CAIII] [+Pb: *P. berghei*-infected mouse liver; +Pb+Cq: *P. berghei*-infected + chloroquine treatment; -Pb: non-infected control mouse liver; -Pb+Cq: non-infected mouse + chloroquine treatment]

TABLE 1 Summary of protein identification by MALDI TOF/TOF MS/MS and data of in silico analysis.

Items	Molecular Weight/ MS/MS details		
	~18 kDa	~28 kDa	~50 kDa
NCBIInr accession no. ¹	gil229301	gil576133	gil31982861
Protein ID ²	Haemoglobin beta (HBB)	Mouse liver class Pi Glutathione S-Transferase (GSTPi)	Carbonic anhydrase 3 (CAIII)
MOWSE score ³	281*	644*	740*
Database/version, sequence, species ⁴	NCBIInr/ 090202, 7777454, <i>Mus musculus</i>	NCBIInr/ 090202, 7777454, <i>Mus musculus</i>	NCBIInr/ 090202, 7777454, <i>Mus musculus</i>
Sequence coverage [%] ⁵	43	44	56
Phosphorylation site ⁶	Ser10, Tyr36, Ser45, Ser50	Tyr63, Tyr108, Thr109	Not identified
Signaling pathway ⁷	Not identified	Glutathione metabolism, metabolism of xenobiotics by cytochrome P450, drug metabolism- cytochrome P450, prostate cancer	Nitrogen metabolism

* Significant score

¹⁻⁴ Peptides homologous with NCBIInr sequence database

⁵ Amino acid sequence coverage determined based on matching peptide sequence

⁶ Phosphorylation site identified through PhosphoSite database

⁷ Signaling pathway identified through KEGG pathway analysis database

any altered protein bands. Use of an anti-phosphoserine antibody (Figure 2) revealed increased levels of hepatic phosphoserine-containing proteins with molecular weights of ~18 kDa (1.7-fold increase) and ~50 kDa (2.78-fold increase) with 50% parasitemia of *P. berghei* infection compared to that in non-infected controls. Use of an anti-phosphothreonine antibody (Figure 2) showed that the intensities of hepatic proteins with molecular weights of ~28 kDa and ~50 kDa were respectively increased by 1.54-fold and 3.45-fold following *P. berghei* infection.

In the presence of chloroquine (10 mg/kg), the levels of the hepatic phosphoprotein bands significantly affected by *P. berghei* infection described above (~18 kDa, ~28 kDa and ~50 kDa) were closer to that in normal non-infected controls (Figure 2). No change in levels of β -actin, which was used as protein loading control, was observed in either group.

MASS SPECTROMETRY ANALYSIS

The hepatic protein bands significantly affected by *P. berghei* infection (~18 kDa, ~28 kDa and ~50 kDa) were subjected to MS/MS protein identification (Table 1). All proteins that matched spectra with a MOWSE score above 81 were considered significant (Zhang et al. 2005). The sequence coverage of the amino acids of the proteins is also shown in Table 1. Protein bands with molecular weights of ~18 kDa, ~28 kDa and ~50 kDa were identified to be homologous to haemoglobin beta (HBB), class Pi glutathione S-transferase (GSTPi) and carbonic anhydrase

III (CAIII) respectively from the NCBIInr database. The percentage of sequence coverage is as low as 43% i.e. for the ~18kDa protein. This low sequence coverage may be associated with loss of peptides during preparation of samples for MS/MS procedure (Fowler et al. 2010). The matching protein taken into account in this study is the one with highest percentage of sequence coverage.

IDENTIFICATION OF PHOSPHORYLATION SITES

Details of the phosphorylation sites of the identified proteins as predicted by PhosphoSitePlus database are summarised in Table 1. Briefly, two of the proteins identified by MS/MS were recognised as phosphorylated proteins in the database [HBB (phosphorylated on Ser10, Tyr36, Ser45 and Ser50) and GSTPi (phosphorylated on Tyr63, Tyr108, Thr109)].

SIGNALING PATHWAY ANALYSIS

Pathway prediction by KEGG pathway analysis database predicted the signaling pathways of GSTPi to glutathione metabolism, metabolism of xenobiotics and drug by cytochrome P450 and prostate cancer; and CAIII to nitrogen metabolism (Table 1).

HISTOLOGY

Sections of liver obtained from the studied animals showed that histological changes occurred during erythrocytic-stage *P. berghei* infection [Figure 3(a)-3(f)].

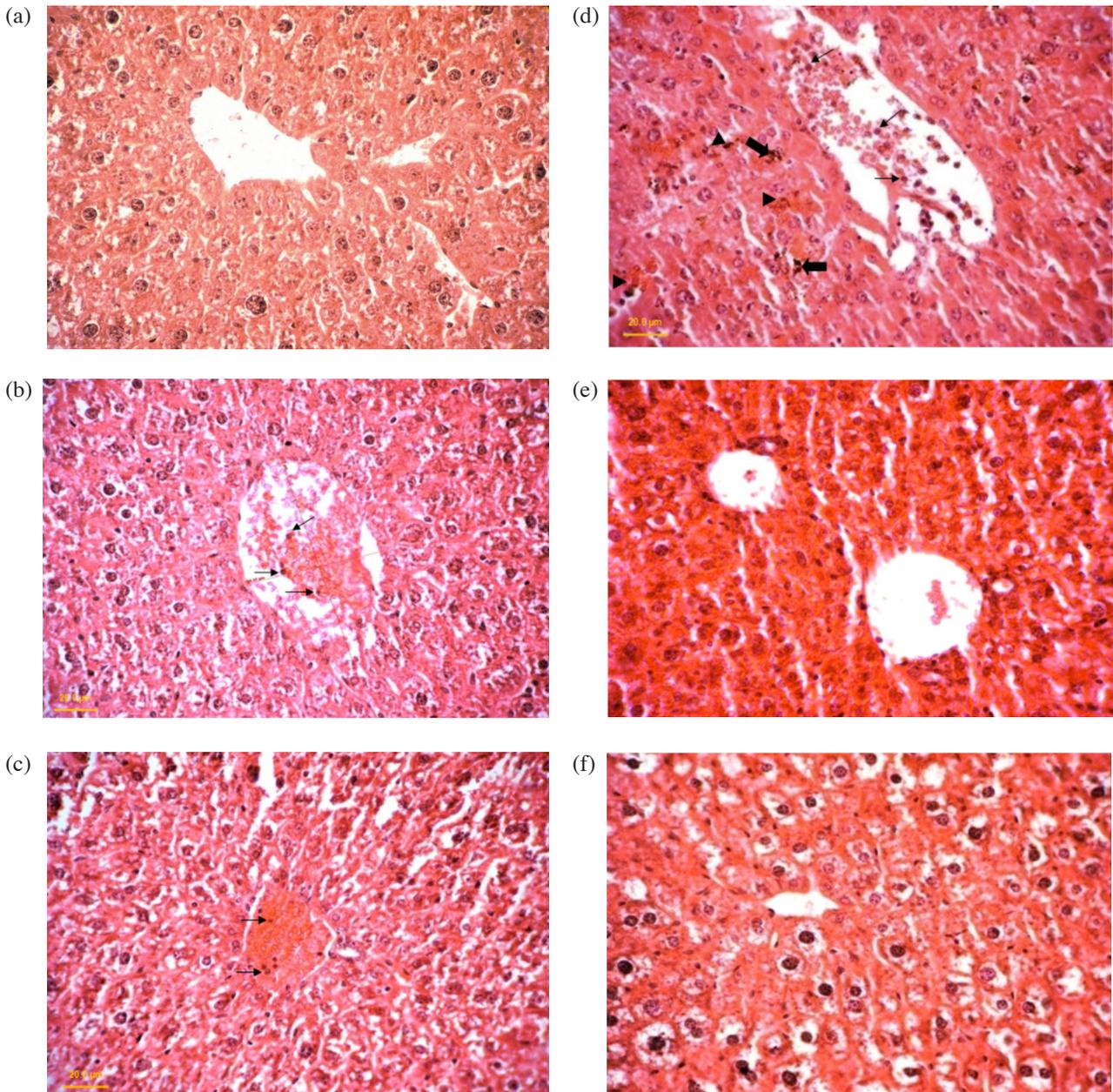


FIGURE 3. Hematoxylin and eosin-stained liver sections (40 \times). Sections were prepared from control non-infected mice (a), *P. berghei*-infected mice (b, c, d; 10%, 30% and 50% parasitemia respectively), and chloroquine-treated non-infected (e) and infected mice (f). Infected liver shows the presence of *P. berghei*-infected erythrocytes (\rightarrow) in central vein, haemozoin deposition in Kupffer cells ($\blacktriangleright\blacktriangleright$), haemozoin granule in sinusoidal lining (\blacktriangleright)

Hematoxylin and eosin-stained liver sections showed accumulating parasitised erythrocytes in infected mice liver sections, which is prominent in mice infected at 50% parasitemia (Figure 3(d)). This was demonstrated by the presence of parasitised erythrocytes in the central vein, presence of parasite-derived haemozoin and haemozoin deposition in Kupffer cells within the liver from *P. berghei* infected animals compared to the normal histology in control non-infected mouse liver sections (Figure 3(a)). Chloroquine-treated mouse liver tissues (Figure 3(e) and 3(f) generally showed tissue structure similar to that in non-infected control (Figure 3(a)).

DISCUSSION

Interrupted signaling pathways associated with metabolism and immune response of the liver is one of the reasons for mortality during *Plasmodium* parasite infection (Krucken et al. 2005). Phosphorylation is one of the mechanisms which alter the activity of certain proteins during pathogenic infection. Phosphorylation at tyrosine, serine or/and threonine residues may activate or inhibit the activity of a protein. This study was undertaken to evaluate alterations in liver phosphoprotein levels during erythrocytic-stage malarial infection.

Generally, pathological changes associated with malaria only occurs during erythrocytic-stage infection, and the host shows no symptoms such as anemia, hepatomegaly, liver inflammation and tissue injury during hepatic exoerythrocytic-stage infection (Greenwood et al. 2008; Prudêncio et al. 2006).

In this study, changes in liver tissue histology and altered levels of specific liver phosphoproteins were observed following erythrocytic-stage *P. berghei* infection of up to 50% parasitemia. The presence of haemozoin granules, Kupffer cell-bound haemozoin and the presence of infected erythrocytes in central vein from infected animals were observed (Figure 3) as evidence of hepatomegaly due to *P. berghei* infection (Wilairatana et al. 2008).

In the present study, we were able to identify (by MALDI TOF/TOF MS/MS) the three mouse liver phosphoproteins significantly affected by *P. berghei* infection. The proteins identified were haemoglobin beta (HBB), glutathione S-transferase class Pi (GSTPi) and carbonic anhydrase III (CAIII).

The intensity of an ~18 kDa liver phosphoserine-containing protein band (identified as HBB) increased upon *P. berghei* infection (Figure 2). During *Plasmodium* infection, haemoglobin digestion by the parasite produces haem, which is toxic to the parasite (Orjih 1997). Polymerisation of haem to non-toxic haemozoin then allows the survival of the parasite. The presence of haemozoin-bound Kupffer cells in liver (Figure 3(d)) suggests phagocytosis of haemozoin by Kupffer cells. The increasing levels of HBB with parasitemia progression in our study could be attributed to accumulation of infected erythrocytes in the central vein or liver sinusoids as observed in our histology results.

The ~28 kDa liver phosphothreonine-containing protein band, the intensity of which was increased with *P. berghei* infection, was further identified as class Pi glutathione S-transferase (GSTPi) (Table 1). PhosphoSitePlus database predicted GSTPi phosphorylation sites at Tyr63, Tyr108 and Thr109 residues (Table 1). KEGG pathway analysis predicted GSTPi to be involved in glutathione metabolism, and drug and xenobiotic metabolism (Table 1). Generally, phosphorylation-dephosphorylation of GSTPi regulates various cellular processes such as stress, growth factor-induced signaling, cell proliferation, immune response, differentiation, cell transformation and apoptosis (Lo et al. 2004; Okamura et al. 2009; Rahman & MacNee 2000). GSTPi has been reported to be one of the proteins upregulated during schistosome parasite infection (Harvie et al. 2007). The enzyme has also been commonly used as a cancer diagnostic marker, which suggests that development of liver cancer and liver injury induced by schistosome infection shared similar pathways and responses (Harvie et al. 2007). However, to our knowledge there have been no reports on the alteration of levels of phosphorylated-GSTPi during infection. In malarial infection, GSTPi mRNA was shown to be upregulated in blood of *P. vivax*-infected

individuals as compared to healthy subjects (Sohail et al. 2010). Also, decreasing activity of GSTPi and elevated activity of lipid peroxidation and catalase activity were shown to upregulate host oxidative defence mechanisms during *P. vivax* infection (Sohail et al. 2010). This suggest the potential of GSTPi for assessing disease progression during clinical malaria (Sohail et al. 2010). In view of the present findings, we suggest that the altered levels of phosphorylated GSTPi may play an important role in host defence mechanisms against *P. berghei* infection.

The ~50 kDa phosphoserine- and phosphothreonine-containing liver protein affected by *P. berghei* infection in this study was identified as carbonic anhydrase III (CAIII). Possible pathways involving CAIII as predicted using the KEGG database showed that CAIII is involved in signaling of nitrogen metabolism. In the current study, CAIII only showed a significant increase ($p < 0.05$) in phosphoprotein levels at 50% parasitemia. The CAIII phosphorylation site is not listed in the PhosphoSitePlus database. However, the phosphorylation site of human CAII, an isoform of CAIII, was identified to be between lysine 64 and arginine 67 at the active site of the enzyme (Paranawithana et al. 1990). Electron microscopy studies on *P. falciparum*-infected erythrocytes showed a higher amount of electron-dense carbonic anhydrase by-products in trophozoite, schizont and merozoite stage of the parasite compared to the infected/non-infected erythrocytes (Sein & Aikawa 1998). Inhibition of carbonic anhydrase causes death to the parasite, which suggests that carbonic anhydrase is crucial for parasite survival (Sein & Aikawa 1998). Kim et al. (2004) suggested that CAIII may be involved in host defence due to its role in reducing reactive oxygen species, a factor which leads to oxidative stress during pathogenic infection. Similarly, the increased level observed in liver ~50 kDa (CAIII) phosphoserine- and phosphothreonine-containing protein in the present study may be associated with oxidative stress occurring as a result of *P. berghei* infection at a high level of parasitemia (50%).

Chloroquine administration for four consecutive days following *P. berghei* infection successfully returned liver histology and phosphoprotein levels to that observed in non-infected control animal liver (Figure 2). Chloroquine interrupts haemoglobin digestion by the parasite (Sharrock et al. 2008). Free haem monomers have been shown to cause oxidative damage to *Plasmodium* parasite (Patel et al. 2005). Oxidative stress in liver cells during malaria parasite infection is reported to return to its normal level following chloroquine administration (Patel et al. 2005).

Non-infected mice administered chloroquine (without *P. berghei* infection) showed relatively loose arrangement of hepatocytes because of lysosomotropic properties of chloroquine (Patel et al. 2005). Administration of chloroquine or other lysosomotropic antimalarials causes activation of lysosome enzymes such as nuclease leading to cell damage and/or necrosis.

The current study provides evidence that erythrocytic-stage *P. berghei* infection altered the levels of phosphorylated

GSTP α and CAIII. *P. berghei* infection seems to affect signaling pathways also reported in other pathogen infections such as GSTP α and CAIII-associated oxidative stress response which can cause apoptosis. Elucidation of the interactions between these proteins (liver GSTP α and CAIII) is important in understanding the mechanism of pathogenesis of *P. berghei* infection.

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