Malaria Parasite-Synthesized Heme Is Essential in the Mosquito and Liver Stages and Complements Host Heme in the Blood Stages of Infection

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Abstract

Heme metabolism is central to malaria parasite biology. The parasite acquires heme from host hemoglobin in the intraerythrocytic stages and stores it as hemozoin to prevent free heme toxicity. The parasite can also synthesize heme de novo, and all the enzymes in the pathway are characterized. To study the role of the dual heme sources in malaria parasite growth and development, we knocked out the first enzyme, δ-aminolevulinate synthase (ALAS), and the last enzyme, ferrochelatase (FC), in the heme-biosynthetic pathway of Plasmodium berghei (Pb). The wild-type and knockout (KO) parasites had similar intraerythrocytic growth patterns in mice. We carried out in vitro radiolabeling of heme in Pb-infected mouse reticulocytes and Plasmodium falciparum-infected human RBCs using [4-14C] aminolevulinic acid (ALA). We found that the parasites incorporated both host hemoglobin-heme and parasite-synthesized heme into hemozoin and mitochondrially synthesized cytochromes. The similar fates of the two heme sources suggest that they may serve as backup mechanisms to provide heme in the intraerythrocytic stages. Nevertheless, the de novo pathway is absolutely essential for parasite development in the mosquito and liver stages. PbKO parasites formed drastically reduced oocysts and did not form sporozoites in the salivary glands. Oocyst production in PbALASKO parasites recovered when mosquitoes received an ALA supplement. PbALASKO sporozoites could infect mice only when the mice received an ALA supplement. Our results indicate the potential for new therapeutic interventions targeting the heme-biosynthetic pathway in the parasite during the mosquito and liver stages.

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Introduction

Plasmodium falciparum (Pf) and Plasmodium vivax account for more than 95% of human malaria. P. falciparum is widely resistant to the antimalarial drugs chloroquine (CQ) and antifolates. Sporadic resistance is also seen in P. vivax [1]. Emerging resistance to the artemisinin-based combination therapies [2] and the absence of an effective vaccine highlight an urgent need to develop new drug targets and vaccine candidates [3], [4]. The de novo heme-biosynthetic pathway of the malaria parasite offers potential drug targets and new vaccine candidates. The malaria parasite is capable of de novo heme biosynthesis despite its ability to acquire heme from red blood cell (RBC) hemoglobin. During the intraerythrocytic stages, the parasite detoxifies hemoglobin-heme by converting it into hemozoin [5], [6]. The source of the heme used in the parasite mitochondrial cytochromes and the parasite heme requirements during the mosquito and liver stages are yet unknown. Hence, the role of the de novo heme-biosynthetic pathway throughout the entire parasite life cycle is a subject of considerable interest [7].

Detailed studies in our laboratory and elsewhere have completely characterized all the enzymes in P. falciparum heme-biosynthetic pathway. The parasite enzymes are unique in terms of their localization and catalytic efficiencies. The first enzyme, δ-aminolevulinate synthase (PfALAS) [8], [9], and the last two enzymes, Protoporphyrinogen IX oxidase (PfPPO) and Ferrochelatase (PfFC) [10], [11] localize to the mitochondrion. The enzymes that catalyze the intermediate steps: ALA dehydratase (PfALAD) [12], [13], Porphobilinogen deaminase (PfPBGD) [9], [14], and Uroporphyrinogen III decarboxylase (PfUROD) [15] localize to the apicoplast (a chloroplast relic), whereas, the next enzyme Coproporphyrinogen III oxidase (PfCPO) is cytosolic [16]. Figure 1 depicts the pathway. The enzymes that localize to the apicoplast have very low catalytic efficiency compared with...
RBC counterparts [17], [18]. Earlier studies showed that host ALAD and FC are imported into the parasites in the intraerythrocytic stages, suggesting that the host machinery may augment parasite heme synthesis [6], [19].

The apicoplast is involved in the synthesis of heme, fatty acids, iron-sulfur proteins, and isoprenoids [20]. Yeh and Risi [21] showed that a chemical knockout of apicoplast function could be rescued by isopentenyl pyrophosphate supplement to *P. falciparum* cultures *in vitro*. This suggests that during the intraerythrocytic stages, the parasite requires apicoplast function for isoprenoid synthesis but not for heme or fatty acid synthesis. However, heme as such is essential for parasite survival in the intraerythrocytic stages, minimally constituting the cytochrome component of the Electron Transport Chain (ETC). The ETC is used as a sink for electrons generated in the pyrimidine pathway [22]. Atovaquone inhibits parasite growth by inhibiting cytochrome bc1 activity of the ETC, most likely by competitively inhibiting the cytochrome b quinone oxidation site [23], [24]. Previously, we showed that *PfPPO* requires the ETC and is likewise inhibited by atovaquone [10]. Heme can also serve as a source of iron for the iron-sulfur proteins involved in isopentenyl pyrophosphate synthesis [20].

The question arises whether the parasite depends on *de novo* heme biosynthesis or heme from hemoglobin or a combination of both to make mitochondrial cytochromes. The steps involved in the acquisition of heme from RBC hemoglobin and the storage of heme as hemozoin in the food vacuole of the parasite are reasonably well understood [7], [25]. In addition to the possibility of acquiring heme from hemoglobin to make cytochromes in the blood stages, there is also a suggestion that *Plasmodium* may be able to scavenge heme in the liver stages as well, as is the case with organisms infecting nucleated cells such as *T. cruzi*, *Leishmania* and *M. tuberculosis* [7]. A direct approach to examine the role of the

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**Figure 1. De novo heme-biosynthetic pathway of *P. falciparum*.** The enzymes are localized in three different cellular compartments - mitochondrion, apicoplast and cytosol. The transporters involved in the shuttling of intermediates are yet to be identified. Red bars represent the knockouts generated in *P. berghei* for the first (ALAS) and last (FC) enzymes of this pathway. doi:10.1371/journal.ppat.1003522.g001

**Author Summary**

We demonstrated about two decades ago that the malaria parasite could make heme on its own, although it imports heme from red blood cell hemoglobin during the blood stages of infection. We investigated the role of parasite-synthesized heme in all stages of parasite growth by knocking out two genes in the heme-biosynthetic pathway of *Plasmodium berghei* that infects mice. We found that the parasite-synthesized heme complements the function of hemoglobin-heme during the blood stages. The parasite-synthesized heme appears to be a backup mechanism. The parasite incorporates both sources of heme into hemozoin, a detoxification product, and into mitochondrial cytochromes. The parasite-synthesized heme is, however, absolutely essential for parasite growth during the mosquito and liver stages. We restored the sporozoite formation and liver-stage development of the knockout parasites by providing the missing metabolite. Thus, the heme-biosynthetic pathway could be a target for antimalarial therapies in the mosquito and liver stages of infection. The knockout parasite could also be tested for its potential as a genetically attenuated sporozoite vaccine.
heme-biosynthetic pathway throughout the *Plasmodium* life cycle, including the sexual stages in the mosquitoes and liver stages in the animal host, is to knockout genes in the pathway and determine the effect of the knockouts (KOs) using the *P. berghei* (Pb)-infected mouse model.

Results

The role of parasite-synthesized heme during the intraerythrocytic stages of *P. berghei*

We used an *in vivo* animal model of parasite infection to determine the role of heme biosynthesis during all the stages of parasite development. Figure 2A depicts the double crossover recombination strategy followed to obtain *Pb*ALAS and *Pb*FC KOs. Table S1 shows the primers used to amplify the 5’ upstream and 3’ downstream regions of *Pb*ALAS and *Pb*FC. Figure 2B-M shows the detailed characterization of the KOs based on RT-PCR, Southern, Northern, and Western analyses. We bypassed the liver stage of the infection cycle by injecting 10^5 intraerythrocytic-stage parasites intraperitoneally into mice. There was no significant difference in the growth of the PbKO parasites compared with the PbWT parasites (Figure 3). These results indicate that the parasite may be acquiring host heme during the intraerythrocytic stages.

Radiolabeling of hemoglobin-heme and tracing its path in the parasite

The potential of human RBCs and mouse reticulocytes to synthesize heme was explored in this study. We detected the ALAS and FC proteins by Western analysis in mouse reticulocytes but not in human RBCs (Figure S1Aa and Bb). Unlike in human RBCs, it was possible to radiolabel the total heme and hemoglobin-heme in short-term mouse reticulocyte cultures incubated with [4-14C]ALA (Figure S1C-G). Because *P. berghei* prefers reticulocytes, the experimental system made it feasible to study the availability of hemoglobin-heme not only for hemozoin formation but also for parasite cytochromes. Furthermore, we were able to block heme labeling in the mouse reticulocyte cultures using succinyl acetone (SA), a specific inhibitor of ALAD (Figure S2A-C). Since *P. berghei* can only grow but poorly infect fresh reticulocytes *in vitro*, reticulocytes infected *in vivo* with PbWT and PbKO parasites were used to perform short-term radiolabeling experiments in the presence of [4-14C]ALA. We found [4-14C]ALA incorporation into total heme and hemozoin-heme of PbWT parasites and both of the PbKO parasites. SA inhibited the radiolabeling (Figure 4A and B). Radiolabeled heme appearing in the PbWT and PbALASKO parasites could come from host hemoglobin as well as from parasite heme biosynthesis. But, we would not expect to find [4-14C]ALA incorporated into the heme synthesized by the PbFCKO parasites. The ethyl acetate:acetic acid mixture used to extract heme did not extract hemozoin. Therefore, we extracted hemozoin using acetic-acetone solvent.

We analyzed the labeling of mitochondrial proteins by non-denaturing PAGE and observed a sharp band at the top of the gel after silver staining. The band was radiolabeled in PbWT parasites and in both of the PbKO parasites. The radiolabeling was almost completely inhibited by SA (Figure 4C-E). SDS-PAGE analysis of the band excised and eluted from non-denaturing PAGE showed five separate protein bands and MALDI analysis revealed the presence of two cytochrome oxidase subunits. The sharp silver-stained band in non-denaturing PAGE thus appeared to represent a complex of proteins and needs to be further characterized in detail (Figure S3). For now, it is clear that the PbWT parasites and both of the PbKO parasites incorporated hemoglobin-heme into mitochondrial hemoproteins and into hemozoin.

Next, we examined whether the parasite could use hemozoin-heme to make mitochondrial cytochromes. We tested the effect of CQ, which is known to block hemozoin formation [26], on *P. berghei*-infected short-term reticulocyte cultures. PbFCKO parasite was used to avoid any contribution from parasite-synthesized heme. CQ was injected into PbFCKO-infected mice as described in the Materials and Methods. After 7 h, the infected reticulocytes were incubated in short-term cultures and the incorporation of [4-14C]ALA into hemozoin and mitochondrial cytochromes over a period of 9 h was measured. We resorted to *in vivo* treatment of the animals with the drug, since we found that direct addition of the drug to reticulocyte culture failed to inhibit hemozoin formation under the conditions used, even at high concentrations. Figures 4F and G show that the CQ treatment inhibited hemozoin labeling by 70% but did not affect the labeling of mitochondrial cytochromes. These results suggest that host hemoglobin may provide heme to mitochondrial cytochromes and hemozoin through independent pathways.

The role of parasite-synthesized heme in *P. falciparum* cultures

The radiolabeling of hemoglobin-heme made it impossible to assess the contribution of parasite-synthesized heme using [4-14C]ALA in *P. berghei*-infected reticulocytes. We could, however, assess the contribution of parasite-synthesized heme in *P. falciparum* cultures. In those cultures, all of the radiolabeled heme was synthesized by the parasite. The hemoglobin-heme was not radiolabeled in the *P. falciparum* cultures because the human RBCs used in the *in vitro* cultures lacked the mitochondrial enzymes required to synthesize heme (Figure S1). Although not radiolabeled, the preformed hemoglobin in the RBCs could act as a heme source for the parasite. We found [4-14C]ALA incorporation into the total heme, hemozoin-heme, and mitochondrial hemoproteins in the *P. falciparum* cultures. SA (50 μM) inhibited the radiolabeling (Figure 4H-J). Earlier studies used SA at a fixed concentration ranging from 1 to 2 mM to inhibit heme synthesis and parasite growth [5]. The present study showed that while the 50% growth inhibitory concentration was around 1 to 2 mM (Figure S4A), concentrations as low as 50 μM inhibited heme synthesis (Figure 4H-J). We observed similar results in short-term *P. berghei* cultures (Figure S4B). The *P. falciparum* mitochondrial cytochromes also formed a complex in non-denaturing PAGE and need to be further characterized in detail.

Thus, we showed that both hemoglobin-heme and parasite-synthesized heme could be incorporated into hemozoin in the food vacuole and into mitochondrial cytochromes. Hemozoin formation from host hemoglobin in *P. falciparum* is well characterized [25]. Hemozoin formation from heme synthesized in the parasite mitochondrion, however, needs to be studied further. The relative contributions of hemoglobin-heme and parasite-synthesized heme to parasite cytochrome biosynthesis during the intraerythrocytic stages need to be assessed under different environmental conditions.

The role of parasite-synthesized heme in the mosquito stages

To examine the role of parasite-synthesized heme in the mosquito stages, we allowed *Anopheles* mosquitoes to feed on mice infected with PbWT and PbKO parasites. Figure 5 shows that both PbWT and PbKO parasites formed ookinetes. We found no difference between the WT and KO ookinetes *in vivo* using...
Role of \textit{de novo} Heme in Malaria Parasite Biology

\textbf{A}

\textit{P. berghei ANKA ALAS} Chromosome 14 (PBANKA\_145920)/
\textit{P. berghei ANKA FC} Chromosome 11 (PBANKA\_114070)

$pL0006$ Plasmid

\begin{itemize}
  \item 5' UTR
  \item 3' UTR
\end{itemize}

\begin{itemize}
  \item 670-740 bp
  \item 700-710 bp
\end{itemize}

\begin{enumerate}
  \item Pyrimethamine selection
\end{enumerate}

\textit{P. berghei ANKA ALAS/FC KOs}

\textbf{B}

\begin{tabular}{llllll}
\textbf{B} & \textbf{C} & \textbf{D} & \textbf{E} & \\
P\textit{WT} & P\textit{bALASKO} & P\textit{bWT} & P\textit{bFCKO} & P\textit{bWT} & P\textit{bALASKO} & P\textit{bFCKO} & P\textit{bWT} & P\textit{bFCKO} \\
\end{tabular}

\begin{itemize}
  \item 2.1 Kb
  \item 1.5 Kb
  \item 1.9 Kb
  \item 1.0 Kb
\end{itemize}

\textbf{F}

\begin{tabular}{llllll}
\textbf{F} & \textbf{G} & \textbf{H} & \textbf{I} & \\
P\textit{bWT} & P\textit{bALASKO} & P\textit{bWT} & P\textit{bFCKO} & P\textit{bWT} & P\textit{bALASKO} & P\textit{bFCKO} & P\textit{bWT} & P\textit{bFCKO} \\
\end{tabular}

\begin{itemize}
  \item 5.7 Kb
  \item 5.9 Kb
  \item 1.8 Kb
  \item 1.0 Kb
\end{itemize}

\textbf{J}

\begin{tabular}{llllll}
\textbf{J} & \textbf{K} & \textbf{L} & \textbf{M} & \\
P\textit{WT} & P\textit{bALASKO} & P\textit{bFCKO} & P\textit{bWT} & P\textit{bALASKO} & P\textit{bFCKO} & P\textit{bWT} & P\textit{bALASKO} & P\textit{bFCKO} \\
\end{tabular}

\begin{itemize}
  \item 1.3 Kb
  \item 70 kDa
  \item 40 kDa
  \item 60 kDa
\end{itemize}
gametocyte cultures or in vivo using midgut preparations. In contrast, Figure 6 shows a drastic decrease in PbKO oocysts formation in the midgut and absence of PbKO sporozoites in the salivary glands. We examined whether ALA supplement could overcome the block in PbALASKO parasites for which 0.1% ALA was supplemented in feeding solution (PbALASKO (Mq^ALA)). The results obtained indicate that the formation of oocysts and sporozoites were restored (Figure 6). Our results reveal that parasite heme synthesis was required for oocyst and sporozoite development in the mosquitoes. In the case of PbFKCO parasites, we attempted to supplement heme through blood feeding on mice, but we were not able to rescue the defect. This suggests that the parasite could not acquire heme from the mouse hemoglobin in the mosquito blood meal or from any other mosquito source during the sexual stages of its development.

The role of parasite-synthesized heme in liver stage development

We examined the ability of PbALASKO (Mq^ALA) sporozoites to reinfect mice by measuring the parasitemia in the mice on subsequent days with and without ALA supplement (0.1% in drinking water). We did not detect any parasites in the mice infected with PbALASKO (Mq^ALA) sporozoites that did not receive ALA supplement (PbALASKO (Mq^ALA, Mi^ALA)). We did, however, detect parasites in the mice infected with PbALASKO (Mq^ALA) sporozoites that received ALA supplement (PbALASKO (Mq^ALA, Mi^ALA)). The infected animals died after 14–16 days, when the parasitemia levels reached around 60% (Figure 7). Mosquitoes infected with PbALASKO parasites (without ALA supplement) failed to give rise to blood-stage parasites in mice when we allowed them to feed. This is an additional proof to suggest that the PbALASKO parasites did not form sporozoites in the mosquito salivary glands. We reproduced all the mosquito transmission experiments by intravenously injecting the sporozoites obtained from mosquito salivary gland extracts into mice. Thus, our results suggest that parasite heme synthesis is absolutely essential for liver-stage development. Our results discount the suggestion [7] that the parasite may import host-synthesized heme during the liver stage.

Discussion

In this study, we assessed the role of parasite-synthesized heme in all stages of malaria parasite growth. We generated ALAS and FC KOs in P. berghei. We used the KOs to track parasite-synthesized heme and host hemoglobin-heme during the intraerythrocytic stages of the parasite. The KOs did not affect parasite growth in mice when the parasites were injected intraperitoneally. All infected animals died within 10 to 12 days, when parasitemia reached around 60%. The synthesis of mitochondrial cytochromes is essential for parasite survival, so our results mean that the PbFKO parasites used hemoglobin-heme to synthesize cytochromes during the intraerythrocytic stages. We demonstrated this by radiolabeling hemoglobin-heme with [4-14C]ALA in short-term mouse reticulocyte cultures.

In the short-term in vitro P. berghei cultures, we found radiolabeled hemozoin and mitochondrial cytochromes in reticulocytes infected with PbWT, PbALASKO, and PbFKCO parasites. We could not, however, distinguish between the contributions of hemoglobin-heme and parasite-synthesized heme in those cultures, because the use of [4-14C]ALA to radiolabel heme would bypass the potential ALASKO block. At the same time, the PbFKCO parasites would not be able to incorporate [4-14C]ALA into heme. We showed in a prior study that P. berghei imports host ALAD as well as host FC [6]. Therefore, we cannot rule out the possibility that the parasite used FC imported from the host to synthesize heme. We addressed this possibility using P. falciparum in human RBC culture. Western analysis indicated that the human RBCs used to culture P. falciparum did not contain detectable levels of ALAS and FC. Again, the RBCs did not incorporate [4-14C]ALA into heme (Figure S1). Thus, all of the radiolabeled heme in P. falciparum was synthesized de novo by the parasite. We found that 50 μM SA completely inhibited heme synthesis in P. falciparum (Figure 4H–J), but did not affect parasite growth (Figure S4A). This means that P. falciparum can use hemoglobin-heme to sustain growth under these conditions.

Earlier studies used a fixed, high concentration of SA (1–2 mM) [5], which inhibited both heme synthesis and parasite growth. In this study, SA was found to inhibit heme synthesis at a much lower concentration than that required to inhibit parasite growth, indicating that de novo heme synthesis is not essential for P.
*P. berghei* growth in culture. This is likely to be true of *P. berghei* as well, because 50 μM SA completely inhibited heme synthesis in *P. berghei*-infected reticulocytes (Figure 4A–E) but did not affect *P. berghei* growth in short-term cultures (Figure S4B). The earlier studies correlating the growth of the parasite with inhibition of heme synthesis or host enzyme import [5], [6], [17] have now been re-evaluated with the use of specific gene KOs in the pathway.

Because the parasite can survive in the absence of de novo heme synthesis, it may appear that the parasite heme-biosynthetic pathway has no role in the intraerythrocytic stages. However, our results show for the first time that *P. falciparum* growing in human reticulocytes can use radiolabeled heme to synthesize hemozoin and mitochondrial cytochrome complex, which is consistent with the observation that *P. falciparum* can survive in the absence of de novo heme synthesis.[8]

**Figure 4. Acquisition of radiolabeled heme by *P. berghei* and *P. falciparum* in short-term cultures.** *P. berghei*-infected reticulocytes were isolated from mice infected with WT and KO parasites. Infected reticulocytes were also isolated after CQ treatment. Radiolabeling of *P. berghei* and *P. falciparum* with [4,14C]ALA in short-term cultures was carried out as described in Materials and Methods. Radiolabeling of total parasite heme, hemozoin and mitochondrial cytochrome complex were assessed with (+) and without (−) succinyl acetone (SA) treatment. (A) Radiolabeling of total parasite heme. (B) Radiolabeling of hemozoin-heme. (C–E) Radiolabeling of parasite mitochondrial cytochrome complex. (F,G) Radiolabeling of hemozoin-heme and mitochondrial cytochrome complex after chloroquine (CQ) treatment. Equal numbers of infected reticulocytes were used to perform the radiolabeling of *PbFCKO* parasites and the data obtained for CQ treatment were compared with untreated control. (H–J) Radiolabeling of total heme, hemozoin-heme and mitochondrial cytochrome complex in *P.falciparum*. *Pb*, *P. berghei*; *Pf*, *P. falciparum*. doi:10.1371/journal.ppat.1003522.g004
RBCs incorporated parasite-synthesized heme radiolabeled with [4-\(^{14}\)C]ALA into hemozoin as well as into mitochondrial cytochromes. Hemoglobin-heme in the RBCs was not radiolabeled; so the heme in the parasite hemozoin and mitochondrial cytochromes was synthesized \textit{de novo} by the parasite. It has long been assumed that only hemoglobin-heme is converted into hemozoin in the parasite food vacuole. We found that parasite-synthesized heme can also give rise to hemozoin in the food vacuole. Since hemoglobin transport into the food vacuole involves cytostomes and other vesicle-mediated transformations [25], it is not clear at this stage how the parasite-synthesized heme made in the mitochondrion finds its way to the food vacuole.

Our results also emphasize the fact that hemozoin is, perhaps, the only mechanism for heme detoxification in the parasite. A recent study showed that the malaria parasite lacks the canonical heme oxygenase pathway for heme degradation and relies on hemozoin formation to detoxify heme [27], although an earlier study suggested the possible presence of heme oxygenase in the apicoplast [7], [28]. It appears that the parasite mitochondrion would need a two-way transporter for heme: one to incorporate hemoglobin-heme into the mitochondrion and another to transport mitochondrial heme into the pathway leading to hemozoin formation in the food vacuole. Free heme was also detected in the erythrocyte at a concentration around 1 \(\mu\text{M}\) [29] and the parasite may be able to scavenge this heme directly [7]. It was also suggested that ferririboporphyrin could leach from the food vacuole into the parasite cytosol [30]. We found that SA inhibited the radiolabeling of hemozoin and of mitochondrial cytochromes in \(Pb\) FCKO parasites. But, CQ inhibited the radiolabeling of hemozoin but not of mitochondrial cytochromes (Figure 4F and G). These results suggest that hemoglobin-heme may be incorporated into mitochondrial cytochromes and into hemozoin through independent processes. Figure 8 gives some of the pathways that may be involved.

It needs to be established whether hemoglobin-heme and parasite-synthesized heme are functionally equivalent. The parasite-synthesized heme may be a backup mechanism that could be of significance only if hemoglobin-heme is not available, as may be the case with sickle cell and other hematological disorders. It has been proposed that low levels of free heme in the plasma induce heme oxygenase-1 to generate carbon monoxide that binds with sickle hemoglobin-heme. This could prevent the release of the
Heme, and thus suppress the heme-mediated pathogenesis of cerebral malaria, without affecting the parasite load [31]. In another scenario, it was suggested that human hemoglobin variants offer protection by interfering with host actin remodeling in *P. falciparum*-infected erythrocytes. These variant hemoglobins are unstable and undergo oxidation, leading to the denaturation and release of heme and oxidized forms of iron that can affect host actin dynamics and thus affect parasite virulence. However, malaria parasites develop normally in such erythrocytes, both in culture and in vivo [32]. Therefore, parasite-synthesized heme may sustain parasite survival when hemoglobin-heme is unavailable, although pathogenesis is ameliorated. It is also possible that the parasite-synthesized heme has a function that is presently unknown.

The growth pattern of the KO parasites in the mosquito stages was striking. While ookinetes formed, oocysts formation decreased substantially, and no sporozoites appeared in the salivary glands. Furthermore, when these mosquitoes fed on mice, we found no intraerythrocytic-stage parasites in the blood of the mice. ALA supplement to the mosquitoes enabled PbALASKO to form oocysts and sporozoites. This is clear proof that *de novo* parasite heme synthesis is required for parasite development in mosquitoes [33]. Equally striking was the growth pattern of *PbALASKO* parasites in the liver stage. The sporozoites formed in the mosquitoes with ALA supplement could infect mice only when the mice received ALA supplement. This again shows that parasite *de novo* heme synthesis is required for development in the liver stage. The liver stage is a major focus of malaria interventions and the role of parasite heme synthesis in liver-stage development needs to be investigated in more detail.

Inhibitors of parasite heme synthesis offer newer drug candidates for blocking infection and transmission, since the parasite enzymes involved have unique properties [10], [11], [14]–[18]. Irradiated sporozoites serve as a malaria vaccine candidate [4]. There are several current efforts to design and stabilize irradiated sporozoites for large-scale clinical trials [34]–[36]. Our results with *PbALASKO*(Mq<sup>ALA</sup>) sporozoite infections in mice offer some additional options for a genetically attenuated sporozoite vaccine that can be tested in the animal model.

The biology of parasite heme synthesis may change drastically between the intraerythrocytic stages and the mosquito and liver stages. The malaria parasite essentially depends on glycolysis to generate ATP in the intraerythrocytic stages. Hemoglobin is available as a heme source in addition to parasite-synthesized heme. It is thus possible that the parasite survives when hemoglobin-heme is unavailable, although pathogenesis is ameliorated. It is also possible that the parasite-synthesized heme has a function that is presently unknown.

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possible that the de novo heme-biosynthetic pathway of the parasite is augmented during the mosquito and liver stages. The ATP synthesized by the ETC may be necessary to provide the energy needed for ookinetes in the mosquito midgut to develop into sporozoites in the mosquito salivary glands. The energy provided by the ETC may also be necessary for the sporozoites to explore the mammalian host from the skin to liver and give rise to merozoites in the hepatocytes.

Materials and Methods

Ethics statement

Animal experiments were carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (Registration No: 48/1999/CPCSEA). The guidelines of National Institute of Malaria Research, New Delhi, were followed for all the mosquito infection studies. All the experiments were carried out as approved by the Institutional Animal Ethics Committee of the Indian Institute of Science, Bangalore (CAF/Ethics/102/2007-455 and CAF/Ethics/192/2010).

Parasite maintenance and isolation

In vitro cultures for P. falciparum 3D7 isolate were maintained continuously on human O+ red cells of 3% hematocrit supplemented with 10% O+ serum or 0.5% Albumax II in RPMI 1640 medium containing L-glutamine (GIBCO) by the candle jar method [37] or in a CO2 incubator. Synchronization was carried out by sorbitol treatment [38] and parasites at the late trophozoite and schizont stages were freed from infected erythrocytes by treatment with an equal volume of 0.15% (w/v) saponin in PBS [39]. The released parasites were centrifuged at 10,000 g for 10 minutes and the pellet obtained was washed four times with ice cold PBS to remove any detectable hemoglobin. The routine propagation of P. berghei ANKA strain (MRA-311, MR4, ATCC Manassas Virginia) was carried out in 6–8 weeks old Swiss mice. In brief, mice were injected intraperitoneally with 105 P. berghei infected-RBCs/reticulocytes and the parasite growth was routinely monitored by assessing the percentage of parasitemia in Giemsa stained thin smears prepared from tail vein blood. On day 8–10 post-infection, mice were anesthetized with ketamine/xylazine and the infected blood was collected through cardiac puncture. The blood obtained was diluted with PBS to initiate fresh infections in mice [40], [41]. Parasite isolation was carried out as described earlier [39].

Generation of P. berghei ALAS and FC knockouts

To generate the knockout parasites, primers were designed and PCR was carried out with P. berghei genomic DNA to amplify the 670–740 bp fragments that correspond to the 5’-UTR and 3’-UTR regions of PbALAS/FC genes. The resultant fragments were cloned into the appropriate restriction sites flanking the human
DHFR selection cassette of pL0006 replacement plasmid (MRA-735, MR4, ATCC Manassas Virginia). The plasmid constructs were then digested with ApaI and NotI, and transfected into P. berghei schizonts that were purified from intraerythrocytic stage infections initiated by sporozoite injections [42]. In brief, P. berghei schizonts were purified and subjected to nucleofection with the appropriate constructs, followed by pyrimethamine selection. Limiting dilution was carried out for pyrimethamine-resistant parasites [43] and the targeted deletion of PbALAS and PbFC genes in the respective knockout parasites were confirmed by PCR, Southern, Northern and Western analyses. The details of the primers and restriction sites are provided in Table S1.

Rearing of A. stephensi mosquitoes

A. stephensi mosquitoes were reared under standard insectary conditions maintained at 27°C and 75–80% humidity with a 12 h light and dark photo-period as described [44], [45]. Larvae were reared on yeast tablets at a fixed density of one larva per ml. Upon maturation, the pupae were segregated for adult emergence. The emerged adult mosquitoes were fed on filter-sterilized 10% glucose solution containing 0.05% paraaminobenzoic acid. For egg production, adult female mosquitoes were allowed to take blood feeding on mice anesthetized with ketamine/xylazine.

P. berghei infection studies in A. stephensi

P. berghei infection studies in A. stephensi mosquitoes were carried out as described elsewhere [46]–[48]. In brief, antibiotic-treated adult female mosquitoes of 5–7 days old, starved for 12 h, were allowed to feed on anesthetized-P. berghei infected mice with 8–12% parasitemia showing 2–4 ecdalgeations per field. The fully engorged mosquitoes were then separated and maintained at 19°C. At 20 h post feeding, the mosquito midguts were dissected to remove the blood bolus and ookinete numbers were quantified as described [49]. On day 10 post feeding, mercurochrome staining was carried out for the dissected midguts to determine the number of oocysts formed [50], followed by the dissection of salivary glands on day 19 to examine and count the number of sporozoites present [51]. To supplement the PbALASKO-infected mosquitoes, routine feeding was carried out with sugar solution containing 0.1% ALA from 20 h post feeding until the dissection of salivary glands on day 19. To supplement PbFCKO-infected mosquitoes, blood feeding was given to the mosquitoes in six intervals from the day of infection till the sporozoite analysis, besides the routine feeding with sugar solution.

Sporozoite infections in mice

The ability of the sporozoites to develop asexual stage infections was studied by allowing the mosquitoes infected with P. berghei wild-type and knockout parasites to feed for 15–20 min on 6–8 weeks old Swiss mice (30 mosquitoes/mouse) anesthetized with ketamine/xylazine. The development of asexual stage parasites was monitored by examining the Giemsa stained blood smears from day 5 post infection. To inject 10⁵ sporozoites intravenously in mice, salivary gland extracts of the infected mosquitoes were prepared and sporozoites were counted as described [51]. ALA supplement in mice was carried out immediately after sporozoite infection and continued for 7 days by including 0.1% ALA in drinking water.

In vitro radiolabeling experiments

In vitro radiolabeling of heme in mouse reticulocytes was carried out at 37°C in a CO₂ incubator, for a period of 9 h in RPMI-1640 medium containing 10% FBS, by adding 1 μCi of [4-¹⁴C]ALA to a total volume of 5 ml containing 10⁹ reticulocytes. In brief, reticulocytosis was induced in mice by injecting a single dose of phenylhydrazine (2.5 mg in saline/mouse) intraperitoneally. Two days later, reticulocytes from the mice blood were separated by performing the density-gradient centrifugation on isotonic percoll [52], washed thrice with the medium and used for labeling. Labeling studies in human RBCs were also carried out in a similar fashion with 10⁹ human RBCs in RPMI-medium containing 10% human serum. To perform in vitro labeling for the intraerythrocytic stages of P. berghei wild-type and knockout parasites, the respective blood-stage infections were initiated in phenylhydrazine-treated mice by intraperitoneal injection of 10⁷ infected erythrocytes. The blood was collected when the parasitemia reached around 5–8% with parasites predominantly in early trophozoites. After washing thrice with RPMI-1640 containing FBS, the cells were resuspended in 10 ml of the medium to a final hematocrit of 5% and labeling was carried out for 9 h as described for reticulocytes by adding 3 μCi of [4-¹⁴C]ALA. To study the in vitro effect of SA on heme labeling, cultures were treated for 3 h with 50 μM SA prior to the addition of [4-¹⁴C]ALA, and the labeling was carried out for 9 h in the presence of SA. For CQ treatment, PbFCKO-infected mice were injected intraintraperitoneally with two doses of 0.5 mg CQ dissolved in water at 6 h time interval, when the blood stage parasites were predominantly in early rings. The blood was collected 1 h after the second dosage and the cells were washed with medium, followed by in vitro labeling for 9 h with 3 μCi of [4-¹⁴C]ALA. In vitro labeling for P. falciparum in the presence and absence of SA was carried out with synchronized cultures harbouring 5–8% early trophozoites maintained in RPMI-1640 containing 10% O₂ serum or 0.5% Albumax II.

Preparation of parasite mitochondria and food vacuole

Mitochondria isolation was carried out as described [53] by homogenizing the parasite pellet in 10 volumes of buffer pH 7.4 containing 5 mM Heps-KOH, 75 mM sucrose, 225 mM mannitol, 5 mM MgCl₂, 5 mM KH₂PO₄ and 1 mM EGTA with protease inhibitors. The homogenate was then centrifuged at 4500 xg for 5 min at 4°C and the supernatant obtained was subjected to 44,700 xg for 7 min at 4°C to pellet mitochondria. Labeling of hemoproteins in the parasite mitochondria was examined by solubilizing the pellet in 20 mM Tris buffer pH 7.5 containing 5% Triton X-100 and protease inhibitors, and centrifuging at 20,000 xg to remove membrane debris, followed by loading the supernatant on to a 5% Native-PAGE. The radiolabeled sharp band seen at the top of the gel in silver staining was subjected to MALDI analysis. To measure the intensity of radiolabeling, the gel was dried and exposed to phosphorimager screen for 24 h. For food vacuole preparation, 4500 xg pellet was processed as described [54], [55]. After lysis in ice cold water pH 4.2 and DNaseI treatment in uptake buffer (25 mM HEPES, 25 mM NaHCO₃, 100 mM KCl, 10 mM NaCl, 2 mM MgSO₄, and 5 mM sodium phosphate, pH 7.4), food vacuoles were purified by titratting the pellet in 42% percoll containing 0.25 M sucrose and 1.5 mM MgSO₄, and centrifuging at 16,000 g for 10 min at 4°C. The food vacuole pellet obtained was washed with 1 ml of uptake buffer to remove percoll.

Extraction of total heme and hemoozoin-heme

Extraction of free and protein-bound heme (total heme) was carried out as described earlier [10]. Briefly, the parasite pellet was extracted with 10 volumes of ethyl acetate/glacial acetic acid (4:1) for 30 min at 4°C and centrifuged at 16,000 xg for 10 min. The organic phase containing heme and porphyrins was separated and washed thrice with 1.5 N HCl of one-third total volume, and twice
with water to remove porphyrins and any ALA present. The extracted organic phase containing heme was dried under a stream of nitrogen and dissolved in methanol, followed by thin-layer chromatography (TLC) on silica gel using the mobile phase 2,6-lutidine and water (5:3) in ammonia atmosphere [56]. The intensity of radiolabeling was quantified by exposing the TLC sheets to phosphorimager screen for 8 h. To extract heme from hemozoin, food vacuole pellet was resuspended in 10 volumes of cold acetone containing 0.1 N HCl, vortexed for 30 min at 4°C and centrifuged at 16,000×g for 10 min. The supernatant obtained was dried, dissolved in methanol and analyzed by TLC as described for total heme. The complete extraction of heme from hemozoin can be easily visualized by the color change of the pellet from dark brown to pale and if necessary, the extraction was carried out twice.

Other procedures
Parasite genomic DNA was isolated by SDS/proteinase K method [57]. Total RNA from the parasite was prepared using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. PCR, Western, Southern and Northern analyses were carried out using standard procedures. Polyclonal antibodies for ALAS and FC, cross-reacting with the proteins of both human and mouse origin, were procured from Santa Cruz Biotechnology, Inc. To detect P. berghei ALAS and FC, polyclonal antibodies raised against P. falciparum ALAS and FC, cross-reacting with P. berghei proteins were used. All these antibodies were used in 1:1000 dilution for Western blotting. In vitro ookinete formation in P. berghei wild-type and knockout parasites was analyzed by injecting 2×10⁶ parasites in phenylhydrazine-treated mice, followed by sulfadiazine treatment for two days to remove asexual stages. After removing the leukocytes using CF-11 cellulose columns, the gametocyte-infected blood was loaded on to a UNOsphereQ column (Bio-Rad). After washing the column with 10 mM NaCl, haemoglobin was eluted with lysis buffer containing 20 mM Tris pH 7.5 and centrifuged at 20,000×g for 30 min. The supernatant obtained was dried, dissolved in methanol and analyzed by TLC for total heme and hemoglobin-heme from mouse reticulocyte loaded on TLC with [4-14C]ALA for 9 h in short-term cultures. (E) Total heme and hemoglobin-heme from human RBC loaded on TLC. (F) Radiolabeling of the bands depicted in E. (G) Quantification of radioactivity in total and hemoglobin-heme from mouse reticulocytes and human RBC. The data represent the radioactive counts obtained from three independent experiments. MRet, mouse reticulocytes; HRBC, human RBCs; TH, total heme; HbH, hemoglobin-heme.

Supporting Information
Figure S1 Evidence for the presence and absence of heme synthesis in the mouse reticulocytes and human RBCs, respectively. (A, B) Western analysis for ALAS and FC. 1, mouse reticulocyte lysate; 2, human RBC lysate. (C) Total heme and hemoglobin-heme from mouse reticulocyte loaded on TLC (D) Radiolabeling of bands depicted in C. Labeling was carried out with [4-14C]ALA for 9 h in short-term cultures. (E) Total heme and hemoglobin-heme from human RBC loaded on TLC. (F) Radiolabeling of the bands depicted in E. (G) Quantification of radioactivity in total and hemoglobin-heme from mouse reticulocytes and human RBC. The data represent the radioactive counts obtained from three independent experiments. MRet, mouse reticulocytes; HRBC, human RBCs; TH, total heme; HbH, hemoglobin-heme.

Table S1 Primers used to generate the knockout parasites and PCR analysis. Restriction sites are underlined.

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Author Contributions
Conceived and designed the experiments: VAN GP. Performed the experiments: VAN BS NMV PAS DMK. Analyzed the data: VAN SKG GP. Wrote the paper: VAN GP.

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