Parasite-induced ER stress response in hepatocytes facilitates Plasmodium liver stage infection

Patricia Inácio, Vanessa Zuzarte-Luís, Margarida TG Ruivo, Brie Falkard, Nagarjuna Nagaraj, Koos Rooijers, Matthias Mann, Gunnar Mair, David A Fidock & Maria M Mota

Abstract

Upon infection of a mammalian host, Plasmodium parasites first replicate inside hepatocytes, generating thousands of new parasites. Although Plasmodium intra-hepatic development represents a substantial metabolic challenge to the host hepatocyte, how infected cells respond to and integrate this stress remains poorly understood. Here, we present proteomic and transcriptomic analyses, revealing that the endoplasmic reticulum (ER)-resident unfolded protein response (UPR) is activated in host hepatocytes upon Plasmodium berghei infection. The expression of XBP1s—the active form of the UPR mediator XBP1—and the liver-specific UPR mediator CREBH is induced by P. berghei infection in vivo. Furthermore, this UPR induction increases parasite liver burden. Altogether, our data suggest that ER stress is a central feature of P. berghei intra-hepatic development, contributing to the success of infection.

Keywords CREBH; liver; Plasmodium; UPR; XBP1

Introduction

Plasmodium spp., the causative agents of malaria, are obligate intra-cellular parasites with a complex life cycle involving both a mosquito and a mammalian host. In mammals, Plasmodium life cycle is initiated when a motile sporozoite is injected with the bite of an infected Anopheles mosquito. Sporozoites travel, through the bloodstream, to the liver and infect hepatocytes, where each sporozoite develops and multiplies into thousands of merozoites [1]. Once released into the bloodstream, each merozoite infects a red blood cell (RBC) where a new replication cycle occurs, culminating in the production of new merozoites. The continuous cycle of invasion, intracellular development and proliferation and release of merozoites from RBCs is central to malaria-associated pathology [2]. Plasmodium, as an obligate intracellular parasite, depends on host cell resources to support its development. This feature is particularly important during the liver stage as the Plasmodium replication rate in the liver is exceedingly higher than that in the blood.

Hepatocytes are responsible for a myriad of metabolic processes, including protein synthesis and metabolism of lipids, carbohydrates and other nutrients and micronutrients (such as iron) [3]. The key players of these pathways exist, entirely or partially, in the lumen or membrane domains of the endoplasmic reticulum (ER). As a consequence, hepatocytes are unusually rich in both smooth and rough ER.

In hepatocytes, the ability of the ER to respond to metabolic demands is crucial for cell homeostasis. The unfolded protein response (UPR) of the ER is an elaborate stress signalling pathway activated upon conditions that challenge ER function, in particular the accumulation of unfolded proteins in the lumen owing to an increased demand on its synthesis capacity that the cell cannot cope with, termed ER stress [4]. In eukaryotic cells, three ER transmembrane proteins mediate the canonical UPR: the two kinases, IRE1 (inositol requiring enzyme 1) and PERK (PKR-like eukaryotic initiation factor 2α kinase), and the transcription factor precursor ATF6 (activating transcription factor 6). IRE1, the most conserved signalling branch of the UPR response, functions by activating the transcription factor XBP1 (X-box binding protein 1). XBP1 mRNA is activated by an IRE1-dependent unconventional splicing and generates a mature (spliced) XBP1 transcription factor with a potent transactivation domain [5]. Together, they activate signalling pathways that restore the folding capacity of the ER. Several recent studies have revealed that ER stress and UPR activation regulatory actions are broad-acting and intersect at certain metabolic pathways including lipid and glucose metabolism, particularly in the liver [6]. The metabolic role of the UPR in the liver is further enhanced by the existence of a hepatocyte-specific UPR branch, mediated by the ER transcription factor CREBH (cAMP responsive element-binding
protein, hepatocyte specific). This UPR branch does not activate protein folding transcriptional programmes but rather regulates liver metabolic pathways [7–9]. As such, ER stress and UPR are activated in several metabolic syndromes, including obesity and type II diabetes, as well as in specific liver diseases including fatty liver disease and viral hepatitis [10].

*Plasmodium* infection leads to alterations in the hepatocyte transcriptome, in particular by activating metabolic processes [11]. *Plasmodium* development thus represents a metabolic challenge to the host cell. How hepatocytes cope with a developing parasite and how intracellular host signalling events shape the infection outcome remain unclear. Here, we hypothesized that *Plasmodium* development inside hepatocytes impacts ER function and modulates UPR signalling pathways. We now show that *Plasmodium* infection induces ER stress and that activation of the UPR strongly increases *Plasmodium* liver infection through both the XBP1 and CREBH pathways.

### Results and Discussion

**Plasmodium** hepatocyte infection induces ER stress and UPR activation

Our recent transcriptomic analysis of *P. berghei* sporozoite-infected mouse Hepa 1.6 cells at different time points [11] identified transcriptional upregulation of several key ER stress markers, as early as 6 h after infection. These include *Atf4* and *Chop*, *Atf6* and *Atf3*, *Cebpb*, *Herpud1* and *Trb3* (Fig 1A), all with a well-established role in ER stress [12]. These results were independently validated by analysing the expression of 4 of those transcripts (*Atf4, Chop, Atf3* and *Herpud1*) by quantitative real-time PCR (qRT–PCR) of purified Hepa 1.6 GFP-expressing *P. berghei* ANKA-infected cells infected with GFP-expressing *P. berghei* ANKA sporozoites [13] at 6 and 24 h after infection (Fig 1B). Uninfected cells (Hepa 1.6 GFP-negative cells), from both time points, were used to control for basal mRNA expression. Additionally, comparative quantitative proteomic analysis of infected and uninfected cells (V. Zuzarte-Luís and M. Mota, unpublished data) identified 59 ER proteins that were differentially expressed (DE) within 12 h of Huh7 human hepatoma cells being infected with GFP-expressing *P. berghei* ANKA sporozoites (Fig 1C). Among these, gene ontology (GO) analysis identified an enrichment of proteins involved in protein folding processes, as demonstrated by the induced expression of calnexin (Canx) and calreticulin (Calr). These factors play important roles in the ER quality control apparatus through retention of incorrectly folded proteins [14,15]: the ER resident protein 29 (Erp29) [16] and ERp44 (Erp44) [17], both important to correct protein folding, together with ERp19 (Txdn12) and ERp46 (Txdnc5), members of the thioredoxin family of ER proteins that are highly expressed in the liver [18], and protein-modifying enzymes, including dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunits 1 and 2 (Rpn1/Rpn2) and mannosyl-oligosaccharide glucosidase (Mogs) [19] (Fig 1C). Proteins involved in the ER-associated protein degradation (ERAD) pathway were also overrepresented. These include endoplasmic (Hsp90b1), both components of the ERL1/ERL2 complex, and the UBX domain-containing protein 4 (Ubxn4, also known as Ubxd2) [20] (Fig 1C). The upregulation of the protein folding capacity together with ERAD activation is consistent with the induction of the unfolded protein response (UPR), in response to ER stress. In fact, ERp44 and UBX domain-containing protein 4 are induced, at the protein level, with ER stress [17,21]. Moreover, the major ER stress marker, the 78 kDa glucose-regulated protein also known as BiP (Hspa5) and protein disulphide-isomerase (PDI, P4hb) [22], were clearly upregulated with infection (Fig 1C). Notably, transcriptomic data from hepatocytes infected with a different rodent model, *P. yoelii*, show a similar alteration in ER response [11]. Altogether, our data analysis on both the transcriptional and ER protein responses to infection suggests that *Plasmodium* liver stage parasites induce a clear signature of UPR activation in response to ER stress.

**In vivo** UPR activation strongly increases exoerythrocytic forms (EEFs) in the liver

We next sought to determine how UPR activation impacts *Plasmodium* hepatocyte infection. To that end, we have used a well-described pharmacological ER stress inducer, tunicamycin (TM) [7,12]. We performed a dose–response analysis of TM impact on *P. berghei* ANKA liver stage infection [13]. Mice were intraperitoneally treated with different TM concentrations (or DMSO as control) 8 h prior to intravenous (i.v.) injection of 50,000 *P. berghei* ANKA sporozoites. Liver infection was quantified by qRT–PCR and ER stress induction was confirmed by XBP1 splicing assay. While all tested concentrations induced ER stress response within 8 h after intraperitoneal injection (data not shown), the results show that a sustained ER stress response throughout infection was only observed using 0.75 and 1 mg/kg body weight (bw) of TM. Notably, a clear and significant increase in infection was observed for mice treated with these two concentrations of TM (Fig 2A). To exclude any toxic effect of TM concentrations on mouse livers, we performed histopathological analysis on liver sections of TM-treated and DMSO control mice. No histomorphological differences between control

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**Figure 1.** *Plasmodium* sporozoite infection induces ER stress and activates the UPR in hepatocytes.

A. Heatmap of DE transcripts involved in ER stress/UPR pathways in parasitized hepatocytes at 6, 12, 18 and 24 h after infection. Each row of the plot is a gene and was colour-coded according to the log base 2 of the expression fold changes for each transcript, with red meaning upregulation and blue meaning downregulation. Original data from Albuquerque et al [11].

B. Quantitative real-time PCR (qRT–PCR) analysis of *Atf4, Chop, Atf3* and *Herpud1* mRNA in sorted Hepa 1.6 cells infected with *P. berghei* at 6 and 24 h after infection relative to its GFP-negative control (dashed line), normalized to hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). *P* < 0.05, one-sample t-test. Results are expressed as means ± SEM (n = 3 independent experiments).

C. Heatmap of DE proteins identified as ER proteins by gene ontology (GO) (GO_0044432). Each row of the plot is a protein and was colour-coded according to row-normalized log intensity (z-score) with red meaning upregulation and blue meaning downregulation. Each row is identified with gene name and UniProt accession number. Each column represents a replicate. Highlighted in blue are all the proteins mentioned in the text. Data are available via ProteomExchange with identifier PXD002269.
Figure 1.
Figure 2.
Both XBP1 mice only became apparent 24 h after sporozoite delivery (Fig 2F). The difference in liver infection at 14 h after infection between TM- and TM-treated mice was found (Appendix Fig S1A). Additionally, none of these treatments had an impact on liver weights (Appendix Fig S1B). As such, 1 mg/kg bw was chosen for the subsequent experiments. Confirmation of TM impact on infection was obtained by initiating infection either through i.v. injection of P. berghei ANKA sporozoites or mosquito bite (the natural route of infection) and analysing parasite liver load by both in vivo light emission quantification of luciferase-expressing P. berghei liver stage parasites [23] and qRT–PCR (Fig 2B and C). To exclude a direct effect of TM on parasites, P. berghei ANKA sporozoites were incubated with TM (10 μg/ml) prior to in vitro infection. No differences in sporozoites’ infectivity were found (Appendix Fig S1C).

The observed increase in parasite load can result from either a higher number of parasites that manage to reach and establish a successful liver infection or an increase in parasite replication while the number of infected cells remains constant. To determine the cause of this increase, we isolated liver sections from TM- and DMSO-treated mice and quantified GFP-positive parasite numbers and growth by fluorescence microscopy. Microscopic examination revealed that the increase in infection is due to higher numbers of exoerythrocytic forms (EEFs) as opposed to increased EEF size (Fig 2D and E). Hence, our data suggest that UPR activation potentiates infection by increasing Plasmodium-infected hepatocyte numbers. Importantly, this effect on parasite numbers could not be ascribed to a defect in initial invasion of hepatocytes, as there was no difference in liver infection at 14 h after infection between TM- and DMSO-treated mice (Fig 2F). The increase in infection in TM-treated mice only became apparent 24 h after sporozoite delivery (Fig 2F).

Both XBP1 and CREBH pathways contribute to Plasmodium infection

Our results so far showed that infection led to an UPR activation in the liver and that exogenous induction of ER stress led to an increase in the number of productively infected cells. We thus hypothesized that UPR activation during infection may therefore play a role in the maintenance of a successful infection. Owing to the complexity of UPR signalling, the study of its individual pathways is crucial to understand how liver UPR pathways and Plasmodium EEFs crosstalk to determine in vivo liver infection. In the liver, the XBP1 [24–26] and CREBH [7–9] pathways have been shown to regulate aspects of hepatic metabolism. We first sought to determine whether Plasmodium infection activated splicing of Xbp1 mRNA. We detected maturation of Xbp1 to its active form, Xbp1s (Fig 3A and B), together with an increased expression of its target gene Dnajb9, in P. berghei-infected cells (Fig 3A). We next investigated the relevance of our findings to Plasmodium liver infection. We infected mice with an inducible, conditional disruption of the Xbp1 gene in the liver (Xbp1Δ), as previously described [24] (Fig 3C). We observed a significant decrease in parasite liver load in Xbp1Δ mice compared to their wild-type (WT) littermates (Fig 3D). In agreement with our finding of UPR activation by TM, we observed that the effect in P. berghei liver load is due to an alteration in numbers of EEFs rather than in their development. Our results show that Xbp1Δ livers exhibit a reduction in the number of infected cells (Fig 3E), suggesting a direct effect of XBP1 regulated pathways on P. berghei liver stage infection. During this stage, a bulk of lipids is required to support the generation of thousands of merozoites. Plasmodium possesses a type II fatty acid biosynthetic pathway [27]; however, several studies have shown that in the liver the parasite relies on its own synthesis pathway (in addition to scavenging host fatty acids) only at the transitional stage from the liver to the blood [28–30]. Therefore, during the majority of the liver stage, we hypothesize that the host must fulfil the bulk of the parasite’s lipid needs. The ER has a central role in the regulation of lipid metabolism, and in the liver, XBP1s was shown to regulate the expression of genes involved in fatty acid synthesis [24]. Thus, it is tempting to hypothesize a functional link between XBP1-mediated regulation of de novo hepatic fatty acid synthesis and infection. On the other hand, XBP1s overexpression was shown to induce the synthesis of phospholipids, mainly phosphatidylcholine (PC), the primary phospholipid of eukaryotic membranes in general, and the ER membrane in particular [31]. Importantly, we have now shown that Plasmodium relies on the abundance of PC within hepatocytes to support infection [32]. Whether XBP1 role in infection is mediated by its impact in PC levels remains to be explored.

CREBH is an ER-bound transcription factor that is specifically and highly expressed in the liver and small intestine [9,33] and is activated upon ER stress [33]. We tested whether Crebh mRNA is induced during Plasmodium infection. Results showed that Crebh mRNA expression was highly up-regulated at 6 and 24 h after infection in infected Hepa 1.6 cells (Fig 4A). To understand the functional significance of Crebh upregulation, we infected mouse primary hepatocytes with adenovirus expressing a small hairpin RNA for
Crebh (Ad-CREBH) prior to sporozoite infection. A significant decrease in infection was observed upon Crebh knock-down (Fig 4B and C), without affecting cell viability (Appendix Fig S1D). Additionally, administration of Ad-CREBH in vivo, which led to a significant decrease in expression of both mRNA and protein, resulted in a marked decrease in liver infection (Fig 4D–F). Crebh has been shown to regulate the expression of the major regulator of mammalian iron homeostasis, hepcidin (encoded by Hamp) [7], which we have shown to influence liver stage infection [34].

Iron is an essential nutrient that has been shown to be required for Plasmodium liver infection [34–36] and infected hepatoma cells significantly increase the iron importer divalent metal transporter-1 (DMT1), while ferroportin expression is highly reduced [11]. It is thus tempting to postulate that CREBH role in infection might be mediated by modulation of iron levels and availability. Future research will establish whether that is the case or not.

Altogether, our data show that Plasmodium infection induces ER stress in hepatocytes via both XBP1 and CREBH pathways.
activated and contributing significantly to infection. This raises the question of what is the nature of disrupted ER homeostasis mechanisms in the context of malaria liver infection? The liver, a highly metabolically active organ, has a protein synthesis rate of ~13 million secretory proteins per minute [37]. Thus, in hepatocytes, even subtle perturbations to protein synthesis can lead to ER stress. Moreover, alterations to ER fatty acid/lipid composition can also induce ER stress and activate the UPR [6,38]. We have recently reported a global analysis of the total lipid repertoire of Plasmodium-infected hepatocytes, which revealed an enrichment of neutral lipids as well as the major membrane phospholipid, PC [32]. Whether these alterations are the underlying cause of the ER stress during Plasmodium liver stage infection remains to be elucidated. Many pathogens induce ER stress and use diverse strategies to activate or modulate it. Viral replication, for example, co-opts ER functions to produce viral glycoproteins, which leads to induction of the UPR [39]. On the other hand, several bacteria seem to induce this response via the secretion of virulence factors into the host cell [40–43]. Interestingly, an active...
Plasmodium export machinery may be operating in infected hepatocytes and at least one parasite protein—the circumsporozoite (CS) protein—has been reported to be exported to the hepatocyte cytoplasm where it can impact host inflammatory responses [44]. Whether other parasite factors are exported to interfere with host pathways in hepatocytes, such as the UPR, represents an exciting and yet to be explored field. Our work sheds light on a crucial and unknown aspect of the cell biology of yet to be explored field. Our work sheds light on a crucial and pathways in hepatocytes, such as the UPR, represents an exciting and better understand the implications of the newly revealed ER–Plasmodium interaction. Although several pathogens (including viruses, bacteria and parasites) can induce the UPR during infection, it is not clear in each case whether this response benefits the host or the pathogen. Modulation of ER function by these pathogens may promote infection by providing a replicative niche, but at the same time, it has been shown that the resulting disruption of the secretory pathway can aid the innate immune system in recognizing intracellular infection and in mounting an appropriate defence (reviewed in [45]). Indeed, it has been claimed that some forms of ER stress may use innate immune pathogen-sensing pathways to augment IRF3-regulated type I IFN response [46], which we have recently shown to play an important role during Plasmodium liver stage infection [47]. Furthermore, the UPR is a complex pathway mediated, in the liver, by four different ER sensors. Therefore, the role of PERK-eIF2α and ATF6 pathways in malaria liver infection is still to be determined. Finally, the ER is increasingly being considered an attractive potential therapeutic target, under the premise that maintaining ER function and reducing ER stress may be able to prevent metabolic diseases. In the light of our results, we postulate that resolving ER stress in the liver can mitigate malaria liver infection.

Materials and Methods

Ethics statement

All in vivo protocols were approved by the Animal Care Committee of the Instituto de Medicina Molecular (AEC_2010_024_MM_RDT_ General_IMM) and were performed according to the regulations of the European guidelines 86/609/EEG.

Parasite strains and mice

Sporozoites from GFP-expressing P. berghei (parasite line 259cl2) [13] or luciferase-expressing P. berghei (parasite line 676m1cl1) [23] were dissected from infected female Anopheles stephensi salivary glands 20–24 days after the infectious blood meal. Sporozoite numbers were determined using a Neubauer chamber. Male C57BL/6 mice were obtained from Charles River (Spain) and all experiments performed with mice aged 6–8 weeks. Experimental groups were set up with mice from the same age range, gender and supplier, to exclude variation within groups.

Mosquito bite infection

Mice were intraperitoneally injected with 200 μl of anaesthesia mixture (ketamine, xylazine) diluted in PBS. Each mouse was exposed to A. stephensi mosquitoes infected with GFP-expressing P. berghei for 30 min.

Tunicamycin treatment

For in vivo treatment, tunicamycin (TM) diluted in 150 mM dextrose at 50 μg/ml was intraperitoneally injected at different doses (0.25, 0.5, 0.75 and 1 mg/kg body weight). For sporozoite treatment, TM was added to freshly dissected sporozoites at a concentration of 10 μg/ml and incubated for 30 min at room temperature. After incubation, sporozoites were centrifuged for 5 min at 10,000 g. 4°C, the medium with TM removed, and sporozoites added to cells previously seeded.

Quantification of parasite liver load

Livers were collected at the mentioned time point of infection and homogenized in denaturing solution (4 M guanidine thiocyanate; 25 mM sodium citrate pH 7, 0.5% N-lauroylsarcosine and 0.7% β-mercaptoethanol in DEPC-treated water). Total RNA was extracted using RNeasy Mini kit (Qiagen). One microgram of total RNA was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche) with the following cycle: 25°C for 10 min, 55°C for 30 min and 85°C for 5 min. Parasite burdens in the liver were determined by qRT–PCR using Pb 18S rRNA primers and normalized against the Hprt housekeeping gene. qRT–PCR was performed in a 7500 Fast Applied Bioscience (AB) machine and ViiA™ 7 Real-Time PCR System (both Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems) as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min; melting stage was done at 95°C for 15 s, 60°C for 1 min and 95°C for 30 s. Primer sequences are listed in Appendix Table S1. Real-time in vivo luminescence measurement of P. berghei liver infection was performed as previously described [23].

Quantification of sporozoite infectivity by luminescence

Sporozoite infectivity was determined 24 h after infection by measuring the luminescence intensity in Hepa 1.6 cells infected with a firefly luciferase-expressing P. berghei line, as previously described [23].

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software with unpaired Student’s t-tests. Significance is indicated by * and its value is identified in every graph.

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Author contributions
PI performed the majority of the experimental work. PI and VZ-L performed the collection of sorted infected cells for proteomics screen. VZ-L, NN and MM performed sample processing and proteomics screen. MR and VZ-L performed the in vivo TM dose dependency and sporozoite TM pre-incubation experiments. PI and BF performed the experiments on Xbp1Δ mice. KR performed the analysis of ER proteins from proteomics screen. GM and DF contributed with reagents. PI and BF performed the experiments on Xbp1Δ mice. KR performed the analysis of ER proteins from proteomics screen. GM and DF contributed with reagents. PI and MMM conceived the study and designed the experiments. PI and BF performed the experiments on Xbp1Δ mice. KR performed the analysis of ER proteins from proteomics screen. GM and DF contributed with reagents. PI and MMM conceived the study and designed the experimental procedures. MMO supervised the study. PI and MMM wrote the manuscript. GM and DF provided insightful comments. All authors read and approved the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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activity is only essential for the parasite's progression from liver infection to blood infection. *Mol Microbiol* 75: 957 – 971


