Signal transduction in *Plasmodium*-Red Blood Cells interactions and in cytoadherence

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ABSTRACT

Malaria is responsible for more than 1.5 million deaths each year, especially among children (Snow et al. 2005). Despite of the severity of malaria situation and great effort to the development of new drug targets (Yuan et al. 2011) there is still a relative low investment toward antimalarial drugs. Briefly there are targets classes of antimalarial drugs currently being tested including: kinases, proteases, ion channel of GPCR, nuclear receptor, among others (Gamo et al. 2010). Here we review malaria signal transduction pathways in Red Blood Cells (RBC) as well as infected RBCs and endothelial cells interactions, namely cytoadherence. The last process is thought to play an important role in the pathogenesis of severe malaria. The molecules displayed on the surface of both infected erythrocytes (IE) and vascular endothelial cells (EC) exert themselves as important mediators in cytoadherence, in that they not only induce structural and metabolic changes on both sides, but also trigger multiple signal transduction processes, leading to alteration of gene expression, with the balance between positive and negative regulation determining endothelial pathology during a malaria infection.

Key words: cytoadherence, erythrocyte, malaria, *Plasmodium*, signal transduction.

INTRODUCTION

Plasmodium parasites are able to sense host signals to modulate their function through a complex cellular machinery and membrane receptors (Hotta et al. 2000, Garcia et al. 2008, Koyama et al. 2009). Moreover Ca^{2+} signalling pathways in *Plasmodium* are crucial for parasite development thus molecules involved in these pathways can be promising antimalarial targets (McCallum-Deighton and Holder 1992, Hotta et al. 2000, Gazarini and Garcia 2004).

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Interestingly, cytoadherence of infected erythrocyte is also regulated by host signalling responses and *Plasmodium falciparum* proteins such as PfEMP1 binding to CD36 or ICAM-1 host receptors can modulate dendridic cells, monoycytes, endothelium and T cells (Urban et al. 1999, Ndungu et al. 2006, Chakravorty et al. 2008, Langhorne et al. 2008) in addition to soluble mediators. Inhibitors of TNF (tumor necrosis factor) also reduced infected erythrocyte cytoadherence (Wassmer et al. 2006b, Chakravorty et al. 2008) and comparison of murine malaria strains indicates that host immunological responses such as TNF

secretion, T cell activation and pro-inflammatory cytokine activation lead to differences in parasite growth or death (Wykes and Good 2008). Based on this we have reviewed the importance of Ca^{2+} modulated targets as antimalarial drugs.

HOST SIGNALS AND MODULATION OF *PLASMODIUM* CELL CYCLE

Signal transduction regulation inside *Plasmodium* has been shown as a major mechanism controlling parasite development. *Plasmodium* is able to sense host signals to modulate its function through a complex cellular machinery and membrane receptors (Garcia et al. 2008, Koyama et al. 2009). Moreover Ca^{2+} signalling pathways in *Plasmodium* are crucial for parasite development. The ion can be released from internal stores or from the extracellular medium (Gazarini and Garcia 2004, Hotta et al. 2000, Mccallum-Deighton and Holder 1992). During the intraerytrocytic development the ion is involved in *P. falciparum* and *P. chabaudi* synchronization through the hormone melatonin and its precursors N-acetylserotonin, tryptamine, serotonin and N(1)-acetyl-N(2)-formyl-5-methoxykynuramine (AFMK) (Beraldo et al. 2005, 2007, Budu et al. 2007, Hotta et al. 2000). Interestingly, the presence of the hormone melatonin induces release of intracellular Ca^{2+} which can be blocked by PLC inhibitor or melatonin antagonist. Recently, it has been shown by caged-IP3 and confocal analysis that melatonin elicited intracellular Ca^{2+} increase though IP3-PLC pathway in *P. falciparum* trophozoite (Alves et al. 2010). Measurements of intracellular cAMP and protein kinase A (PKA) activity also showed that melatonin triggers cAMP production and PKA activation (Gazarini et al. 2011).

Differences among developmental stages within *Plasmodium falciparum* signalling was reported and melatonin modulation was not observed in ring or schizont stages (Alves et al. 2010). Furthermore melatonin did not elicit calcium rise in the rodent

parasites *P. berghei* and *P. yoelii* (Bagnaresi et al. 2009) once those are asynchronous parasites with a distinct profile of proteolysis modulation (Cruz et al. 2011, Farias et al. 2005).

Another important extracellular signal that modulates *Plasmodium*-RBC interaction is ATP which has been described to induce increase of intracellular Ca^{2+} in *P. falciparum*, *P. berghei* and *P. yoelii* thus suggesting the presence of a putative purinoreceptor in the parasite (Levano-Garcia et al. 2011, Cruz et al. in press). Erythrocytes infected by *Plasmodium*, as damaged cells, can increase ATP level of the medium (Akkaya et al. 2009) and *P. falciparum* was unable to infect erythrocytes after ATP depletion (Rangachari et al. 1986). Moreover, the ATP receptor blocker suramin binds to merozoite surface proteins-1 (MSP1) inhibiting its cleavage and erythrocyte invasion by *Plasmodium falciparum* merozoites (Fleck et al. 2003). ATP is also thought to be involved in host induced inflammation which is followed by malaria infection (Clark et al. 2006).

Among the host signalling molecules Xanthurenic acid (XA), produced by the mosquito, triggers differentiation of male/female gametes, although it is not yet identified the parasite receptor for XA (Muhia et al. 2001, Ward et al. 2004). Recently gametocyte exflagellation, DNA synthesis and Ca^{2+} mobilization was shown to be inhibited by U73122 indicating the importance of phosphoinositide-specific phospolipase C pathway during gametocyte activation (Raabe et al. 2011).

Cyclic GMP-dependent protein kinase (PKG) might be involved in gametogenesis by cyclic GMP once exflagellation and rounding up of microgametocytes are induced in the presence of zaprinast (a specific inhibitor of cGMP-PDE). The presence of an upstream Ca^{2+} pathway activated by cyclic GMP-dependent protein kinase (PKG) to initiate gametogenesis (Mcrobert et al. 2008) is suggested. Regarding asexual stages, PKG is involved in the progression of *P. falciparum*

schizogony as in the presence of a PfPKG inhibitor formation of rings is reduced and electron micrographs showed alteration in schizonts morphology (Taylor et al. 2010).

During the *Plasmodium* cell cycle, Ca^{2+} is also involved in invasion of hepatocyte by triggering microneme secretion (Ono et al. 2008, Vaid et al. 2008), phosphorylation of merozoyte motility components via PfPKB induced by calmodulin (Vaid et al. 2008), translocation of microneme proteins (e.g. EBA175), apical membrane antigen-1 (AMA1) to merozoite surface (Singh et al. 2010) and to induce secretion of rhoptry protein to the merozoite surface.

Following merozoite attachment at the erythrocyte membrane there is a decrease in cytosolic Ca^{2+} level, similarly described in *T. gondii* tachyzoites (Singh et al. 2010, Lovett and Sibley 2003). This signalling mechanism might be a feedback to reduce further microneme protein secretion to merozoite surface (Singh et al. 2010).

Ca^{2+} MODULATED ANTIMALARIAL TARGETS

The calcium-dependent protein kinases (CDPKs) are only found in plants and protists (Harper and Harmon 2005) and interestingly the *Plasmodium* has at least 5 CDPKs and gene knock out studies pointed out the importance of these kinases (Doerig et al. 2010, Hall et al. 2005, Ward et al. 2004). Disruption strategies of *P. falciparum* PfCDPK1 were not successful whereas PfCDPK1 inhibitory assays blocked the cell cycle at shizont stage (Kato et al. 2008). Reverse genetics studies carried out in *Plasmodium berghei* CDPK reported that PbCDPK3 is involved in ookinete motility in the mosquito midgut which requires intracellular Ca^{2+} mobilization (Ishino et al. 2006). PbCDPK4 is calcium dependent and is involved in male gametocyte exflagellation induced by XA in the mosquito gut (Billker et al. 2004, Ward et al. 2004). Mutant PbCDPK6 sporozoites showed a reduced

invasion phenotype probably due to contact with the host heparin sulfate proteoglycans (HSPGs) and cAMP-dependent protein kinase (PKA) activation (Coppi et al. 2007).

P. falciparum cAMP-dependent protein kinase (PfPKA) activity is expressed at the sexual and asexual stages of the parasite (Wurtz et al. 2011). PfPKA is involved in Ca^{2+} signalling pathways (Beraldo et al. 2005, Wurtz et al. 2009), motility of sporozoites, apical exocytosis, cell invasion (Kebaier and Vanderberg 2010, Ono et al. 2008) and regulation of mitochondrial protein traffic (Wurtz et al. 2009, De Rasmo et al. 2008).

The protein kinase B-like enzyme (PKB) in mammalian cells, has been shown to be involved in regulation of transcription factors, apoptosis and glycogen pathways (Alessi et al. 1996, Andjelkovic et al. 1997, Vanhaesebroeck and Alessi 2000). Interestingly in *P. falciparum* the protein kinase B-like (PfPKB) does not contain the PH (phosphoinositide interaction) homology domain and its activity is regulated by Ca^{2+}/ CaM autophosphorylation and N-terminal region modulation (Vaid and Sharma 2006, Ward et al. 2004). PfPKB is present at the cytoplasm of schizonts/trophozoites and at the apical end of merozoites (Kumar et al. 2004). Blocking the function of the upstream activators of this pathway resulted in impaired invasion. PfPKB inhibitors dramatically reduced the ability of the parasite to invade erythrocytes and the work of PfPKB was associated with actin-myosin motor and phosphorylates PfGAP45 (glideosome-associated protein 45) (Vaid et al. 2008). PfPI3K, a Phosphatidyl-inositol-3-kinase in *P. falciparum* was reported as a second messenger involved in endocytosis and trafficking of haemoglobin. Inhibition of PfPI3K resulted in attenuated hemoglobin digestion and the inhibition of parasite growth (Vaid et al. 2010).

It is noteworthy that protein kinase C (PKC) activity is not present in the *P. falciparum* genome database (Carlton et al. 2008) although it has been

suggested that in plants CDPK may function as PKC (Satterlee and Sussman 1998). Interestingly, the receptor for activated C kinase 1 (RACK1) is a protein involved in Ca^{2+} signalling by recruiting protein kinase C (PKC) isoforms, InsP3 receptors, proteins G and others to its substrates (Schechtman and Mochly-Rosen 2001, Chen et al. 2004, Patterson et al. 2004). In mammalian cells, RACK1 has been shown to be involved in the circadian clock through interaction with PKC alpha and inhibition of CLOCK-BMAL1 activity (Robles et al. 2010). *Plasmodium falciparum* expresses a PfRACK (Madeira et al. 2003). Moreover functional genomics approaches show that PfRACK modulates Ca^{2+} signalling by inhibiting InsP3 evoked Ca^{2+} release in mammalian cells (Sartorello et al. 2009). PfRACK has also been identified in Maurer´s clefts indicating a role for the protein in signalling pathways within host cells (Garcia et al. 2008, Lanzer et al. 2006).

Another important Ca^{2+} modulated targets are the proteases. During the intraerytrocytic development the parasite alters the RBC plasma membrane and a variety of proteins are proteolyticaly processed (Wu et al. 2003, Garcia et al. 2008, Maier et al. 2009, Cruz et al. 2011, in press). Proteases have been shown to mediate the rupture of merozoites from RBC (Arastu-Kapur et al. 2008, Blackman 2008), as well as protein trafficking by processing of the PEXEL motif in the endoplasmatic reticulum (Boddey et al. 2010, Russo et al. 2010) and degradation of the erythrocyte cytoskeletal proteins through the calcium activated host protein calpain 1 (Chandramohanadas et al. 2009).

Interestingly, calpains are cysteine proteases activated by calcium present in the host and the parasite. Phylogenetic analyses showed that many *Plasmodium* species has a calpain with homology on the N-terminal domain not found in the mammalian homology calpain (Russo et al. 2009, Wu et al. 2003). Interestingly the host calpain is involved in cytoskeletal remodeling (Goll et al.

2003) and *Plasmodium* calpain-1 function has been related to parasite egress from erythrocyte whereas cysteine protease inhibitor blocked erythrocyte membrane disruption (Chandramohanadas et al. 2009, Roiko and Carruthers 2009). Efforts to knockout the *Plasmodium falciparum* calpain was not successful, indicating the importance of this protease (Russo et al. 2009).

To illustrate *Plasmodium* intraerythrocitic calcium signalling a putative model is shown in figure 1.

PARASITE LIGANDS AND ENDOTHELIAL RECEPTORS

The most notable parasite ligand for cytoadherence is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), an antigenically diverse protein family that is expressed on the surface of knob-like structures of mature IEs (Leech et al. 1984). PfEMP-1 varies within and between genetically identical parasite clones resulting in changes in their avidity for endothelial cell receptors. Other potential parasite ligands include Rifin, Stevor, modified Band 3 and Surfin that have been reviewed elsewhere (Rowe et al. 2009). Several endothelial surface receptors have been identified and characterised, including CD36 (Barnwell et al. 1989, Ockenhouse et al. 1989) Thrombospondin (TSP) (Roberts et al. 1985), VCAM, E-selectin (Ockenhouse et al. 1992), P-selectin (Ho et al. 1998), gC1qR (Biswas et al. 2007) and Intercellular adhesion molecule-1 (ICAM-1) (Berendt et al. 1989). Both CD36 and ICAM-1 have been identified as receptors commonly mediating cytoadherence of patient derived Pf-IE (Newbold et al. 1997) and their effects in trigging and regulating signal transduction events have been well characterised; in this section we will review recent advances in cytoadherence and signalling which is illustrated by figure 2 showing the interface of *Plasmodium falciparum* infected erythrocyte (PRBC) and endothelial cell (EC).

ICAM-1 MEDIATED ADHESION AND SIGNALLING

ICAM-1 is constitutively present on ECs but its expression is highly increased by pro-inflammatory cytokines such as interleukin-1 (IL-1), tumour necrosis factor (TNF) (Pober 2002). The stimulation of human vascular endothelial cells with Pf-IE resulted in the non-transient up-regulation of ICAM-1 expression on human ECs. The induction was independent of TNF (Esslinger et al. 1994) indicating that TNF-independent mechanisms (in addition to TNF-dependent) might exist in the pathogenesis of cerebral malaria (CM). The ability of Pf-IE to directly modulate EC depends on the avidity of IE for the receptors on host endothelium and is proportional to the level of activation of the signalling pathways, which usually requires direct physical contact (Chakravorty et al. 2007, Gray et al. 2003, Viebig et al. 2005). ICAM-1 signalling occurs by receptor multimerization that is triggered when ligands bind in an 'outside-in' fashion. The ICAM-1 promoter contains several potential transcription factor binding sites, which have been implicated in induction of ICAM-1 gene transcription in response to various pro-inflammatory stimuli (Muller et al. 1995, Ledebur and Parks 1995). It has been identified that the -187/-178 on the ICAM-1 promoter is a NF-κB site, which is necessary and sufficient for rhinovirus activating ICAM-1 expression (Papi and Johnston 1999). Thrombin-induced ICAM-1 expression is also mediated by NF-κB activation via protease-activated receptor-1 (PAR-1), which induces binding of both p65/RelA and NFATc1 to the NF-κB binding site of ICAM-1 (Xue et al. 2009); the activation of the JNK pathway likely involves $G\alpha_q$, Gβγ, Ras, Rac1 and Src family kinase (Miho et al. 2005). ICAM-1 is a dynamic molecule that is localized in the apical membrane of the endothelium which clusters and aggregates upon binding to leukocyte ligands, such as LFA-1 (Grakoui et al. 1999). A similar process is involved in actin cytoskeleton rearrangement via Rho-like small GTPases family (Burridge and Wennerberg 2004). The cytoplasmic domain of ICAM-1 is composed of 28 amino acids (478-505): RQRKIKKYRLQQAQKGTPMKPNTQATPP (Carpen et al. 1992), this domain does not have intrinsic kinase activity or a Scr homologue domain that can recruit tyrosine-phosphorylated protein, but it does interact with actin-binding proteins, suggesting that ICAM-1 induced signalling may be initiated at the membrane-cytoskeleton interface (Vogetseder and Dierich 1991). ICAM-1 ligation or clustering at the cell surface leads to a range of signalling-mediated activities through cascades involving a number of kinases including p38 MAPK (Mitogen-activated protein kinases)-inducing heat-shock protein 27 phosphorylation in pulmonary microvascular ECs, which in turn modulates cytoskeletal rearrangements and neutrophil migration toward EC junctions (Wang et al. 2002). The tyrosine phosphorylation of cytoskeletal proteins, focal adhesion kinase, paxillin, and p130 in cerebral ECs were mediated by Rho activation (Etienne et al. 1998). Experimental cerebral malaria (ECM) infected with *P. berghei* ANKA (PbA) in C57BL/6J mice (which has virtually no IE sequestration in the brain but shares features of vessel occlusion and inflammation) has revealed the activation of all key elements in the JNK pathway such as p-MKK4, p-JNK and p-c-Jun in mice brains (Anand and Babu 2011). JNK not only caused c-Jun phosphorylation but also affected the activities of multiple proteins such as ATF2, P53, Elk1 c-Myc and bcl-2 family relating to apoptosis (Liu and Lin 2005), indicating that these pathways and components played critical roles in mediating neuronal cell death during ECM.

The regulation of EC due to *P. falciparum* cytoadherence is seen at the transcriptional level, using microarray analysis of HUVEC transcriptome following *in vitro* co-culture of IE and HUVEC. This showed significant differential regulation of genes which defined gene ontologies such as cell

communication, cell adhesion, signal transduction and immune response (Chakravorty et al. 2007). The investigation of global gene responses of human brain microvascular endothelial cells (HBMEC) after the interaction with Pf-IE resulted in the similar results and more specifically, several members of NF-κB signalling cascade was induced with 4 of 5 NF-κB subunits (REL-B, cREL, NF-κB1 and NF-κB2) up-regulated together with significant increase of NF-κB target genes. It was found that one of the NF-κB family members, REL-1A (P65) almost completely translocated into the nuclear compartment within 10 min of IE-EC interaction, suggesting a direct role for parasite factors in NF-κB activation (Tripathi et al. 2009). ICAM-1 was significantly increased upon IE interaction EC and there was a correlation between adherent IEs with this increased level. The interacting IE induced a 730% increase in ICAM-1 on HBMEC; two NF-κB inhibitors, Bay11-7082 and MG132, were able to reduce IE-induced ICAM-1 up-regulation, Bay11-7082 completely abolished IE-induced ICAM-1 expression at a concentration of 40mM (Tripathi et al. 2006). An antioxidant and NF-κB indirect inhibitor, pyrrolidine dithiocarbamate (PDTC), which acts by removing the reactive oxygen species (ROS) intermediates, also had similar functions indicating that ROS may also involved in IE-EC ICAM-1 signalling as IEs can generate a wide variety of ROS (Atamna et al. 1994, Ginsburg and Atamna 1994). How IE are activating NF-κ-B in EC is not clear. Possibly, *P. falciparum*-derived glycosylphosphatidylinositol (PfGPI) toxin activates vascular endothelial cells by tyrosine kinase mediated signal transduction, leading to NFκB/c-rel activation (Schofield et al. 1996). Specific MAPK pathways have been linked to severe malaria through work on the response of macrophages to PfGPI, normally resulting in TNF secretion. A JNK inhibitor alleviated phosphorylation of a number of signalling molecules, and reduced amount of TNF secreted by PfGPI-stimulated macrophages

(Tachado et al. 1996, 1997). More evidence has demonstrated that NF-κB is essential for ICAM-1 expression induced by inflammatory cytokines (Ledebur and Parks 1995) and neutrophil adhesion (Rahman et al. 1999). The mechanism of NF-κB activation via a variety of extracellular stimuli is unique in that it is induced rapidly and does not require de novo protein synthesis, thereby allowing cells to respond quickly to emergent situations, such as bacterial infection.

CD36 MEDIATED CYTOADESION AND SIGNALLING

CD36 is expressed on the EC surface and binds to many ligands such as collagen, thrombospondin, oxidized low density lipoprotein, native lipoproteins, oxidized phospholipids and long-chain fatty acids (Tandon et al. 1989, Silverstein et al. 1992, Endemann et al. 1993, Nicholson et al. 1995, Calvo et al. 1998, Podrez et al. 2002). CD36 has been shown to be a major sequestration receptor on microvascular endothelial cells. In *P. falciparum* malaria, CD36 has long been considered a major contributor to pathogenesis by acting as a vascular receptor for the adhesion of IE (Ho and White 1999). It is thought that CD36 is involved in the PfGPI induced MAPK activation and proinflammatory cytokine secretion, and has been shown to have a role in the innate immune response to malaria in mouse models. Protein kinase C (PKC)–dependent sequence RGPYTYRVRFLA is a target of Thr[92] phosphorylated CD36 in endothelium (Daviet and Mcgregor 1997), and there is minimal binding domain with 179-amino-acid peptide located in exon 1 of PfEMP-1 that anchors CD36 and induces an intracellular signal (Yipp et al. 2003b). In an immunohistochemistry study examining the distribution of sequestration receptors in different organs from fatal cases of *P. falciparum* malaria and non-infected controls, fatal malaria was associated with significantly higher levels of ICAM-1 and E-selectin expression on vessels in the brain, as

well as CD36, but CD36 and thrombospondin staining were thinly scattered in this tissue (Turner et al. 1994). Adhesion receptors have co-operative functions that contribute to cytoadherence. As shown by single-molecule force spectroscopy technique, both CD36 and TSP are involved in specific ligand-receptor interactions, but CD36-mediated signal molecule interactions were more stable than TSP, although the TSP-IE interaction was stronger than the CD36-IE interaction in the high pulling rate regime, indicating that TSP mediated interactions may initiate cytoadhesion whereas CD36 might function to stabilise binding (Li et al. 2011). ICAM-1 and CD36 have been shown in *ex vivo* binding assays to synergize to mediate adherence of Pf-IE to cultured human microvascular EC (Mccormick et al. 1997). It has also been reported that cytoadhesion of CSA-binding IEs on EC can inhibit the cytoadhesion of CD36-binding IEs, which is mediated by a CD44-transduced signal on SBEC-1D (*Saimiri sciureus* brain EC) cells. Two elements from signal transduction pathways Src-kinase and MAP-kinase were identified downstream of CD44 receptor (Jurzynski et al. 2007).

The mechanisms of intracellular signalling events have been studied extensively using parallel plate flow chamber *in vitro* assays or in intact human microvessels in a human/severe combined immunodeficient (SCID) mouse model *in vivo*. These have demonstrated major roles for CD36 in IE cytoadherence on microvascular endothelial cells. A recombinant peptide corresponding to the minimal CD36-binding domain in PfEMP1 can inhibit and even reverse the IE adhesion of clinical isolates, and an anti-CD36 monoclonal antibody can inhibit IE binding both *in vitro* and *in vivo* (Yipp et al. 2003a). ICAM-1 appears to stabilize the IE-CD36 interaction without an increase in the number of IE recruited to the endothelium, while the induction of P-selectin can increase the number of rolling IEs which subsequently adhere to CD36 (Ho et al. 2000). Treatment of HDMECs with a

Src-family kinase-selective inhibitor (PP1) resulted in a significant reduction of IE adhesion by 72% in a flow-chamber assay. The effect of PP1 could be mimicked by a specific alkaline-phosphatase inhibitor levamisole. These results pointed to the use of inhibitors for Src-family kinases and ecto-alkaline phosphatase therapeutically (see below) (Yipp et al. 2003a). Binding of IE to HDMEC also resulted in the downstream activation of the MAPK pathway, but inhibition of the extracellular signal-regulated kinase 1/2 (ERK 1/2) and p38 MAP kinase pathways had no immediate effect on IE adhesion, although the mitogen-activated protein (MAP) kinase pathway activation was Src-family kinase dependent. NIH 3T3 cells transfected with wild-type CD36 or a mutant protein in which Thr^{92} was substituted by Ala were used as a model to support the rolling and adhesion of IEs under flow conditions. It was also shown that the target of the alkaline phosphatase is the ectodomain of CD36 at threonine-92 (Thr^{92}), and this regulated IE adhesion to CD36 under flow conditions in a Src family kinase and alkaline phosphatase dependent manner. A molecular mechanism underlying IE-CD36 binding has been proposed: CD36 is constitutively phosphorylated; upon initial IE adhesion and subsequent Src family kinase activation, CD36 becomes dephosphorylated through the activation of an ecto-alkaline phosphatase, which is expressed on the surface of endothelial cells. Dephosphorylated CD36 binds to IE with higher affinity than to phosphorylated CD36 (Ho et al. 2005).

SIGNALLING INSIDE IEs

Sicard *et al* have shown that *P. falciparum* infection induced modulation of the host PAK1→MEK1 pathway and the pharmacological interference with this pathway using highly specific allosteric inhibitors of human PAK and MEK enzymes caused a block of parasite proliferation in both liver

and blood stages of parasites (Sicard et al. 2011). The role of signalling in Pf-IE has not been studied extensively in relation to cytoadherence except for one study on casein kinase inhibition and its effect on adhesion (Hora et al. 2009).

Extracellular domains of PfEMP1 are variable and bind various host endothelial receptors, whereas their cytoplasmic domains (VARCs) are relatively conserved with binding affinity to *P. falciparum* knob-associated histidine-rich protein (KAHRP). The interaction of VARC and KAHRP was affected by PfEMP1 phosphorylation on VARC and was considered as an important parameter in cytoadherence. By using specific inhibitors and enhancers, an enzyme responsible for VARC phosphorylation has been identified as erythrocytic casein kinase II (CKII) and a set of cell-permeable CKII inhibitors have been tested on cytoadhesion of IEs to soluble EC receptors. These inhibitors possibly block or reverse the interaction between VARC and its binding partners, and so have the ability to reduce cytoadherence and could be considered in the development of therapies against severe malaria.

CYTOADHERENCE OF *P. FALCIPARUM* MEDIATED BY PLATELETS

Increasing evidence shows that platelets may be involved in CM pathogenesis. Platelets have recently been shown to accumulate in brain microvessels of patients with CM and to modulate the binding of *P. falciparum*-infected red cells to human brain endothelium *in vitro* (Wassmer et al. 2006a). Several groups have suggested that platelet-mediated clumping of IEs to form apparent auto-agglutinates is a common adhesive phenotype in severe malaria (Wassmer et al. 2008, Mayor et al. 2011) although others have questioned this (Arman et al. 2007). Using a platelet-endothelial cell co-culture model, Wassmer et al. 2006b have investigated the mechanisms by which platelets modify the function of HBMEC, and have found that platelets have a pro-apoptotic effect

on TNF-activated HBMEC in a contact-dependent manner and the supernatants of thrombin-activated platelets killed TNF-stimulated HBMEC. TGF-1 was found as a major molecule involved in endothelial cell death (Wassmer et al. 2006b), which could be a mechanism of microvascular damage during human CM. The interaction of *P. falciparum* with platelets might also lead to platelet activation and release of inflammatory mediators such as platelet factor 4 (PF4)/CXCL4 by moderating the effects of heparin-like molecules on ECs (Srivastava et al. 2008). However, purified human platelets killed cultured *P. falciparum* IE and platelet-deficient or aspirin-treated mice were more susceptible to death during *P. chabaudi* infection, indicated the protective aspect of platelets in early stage of erythrocytic infection (Mcmorran et al. 2009). Several EC receptors are likely responsible for platelet-mediated IE cytoadhesion including P-selectin (CD62P) (Udomsangpetch et al. 1997); Thrombospondin (TSP) (Roberts et al. 1985); Platelet endothelial cell adhesion molecule 1 (PECAM1 or CD31) (Treutiger et al. 1997, Heddini et al. 2001), globular C1q receptor (gC1qR/ HABP1/p32) (Biswas et al. 2007) and CD36 (Pain et al. 2001). It has also been shown that platelets expressing CD36 serve as a bridge to bind IEs via PfEMP1 to the endothelial cell membrane either directly (Wassmer et al. 2004) or through ultra-large von Willebrand factor strings (Bridges et al. 2010).

The transcriptional changes of HBMEC in response to platelets in the presence or the absence of TNF and IEs have been analyzed by Barbier et al. 2011. The platelets induced the expression of genes involved in inflammation and apoptosis and altered canonical pathways such as chemokines, TGFβ, death-receptors, erythropoietin and TREM1-signalling (Barbier et al. 2011), supporting the hypothesis that platelets play a pathogenic role in CM. The platelet mediated cytoadhesion also involved the activation of Weibel-Palade (WP) bodies and release of von Willebrand factor

(VWF) (Hollestelle et al. 2006). Released VWF is in an activated conformation that could interact with platelet receptor gpIa/V/IV and trigger intravascular platelet aggregation (Groot et al. 2007). Ultra-large VWF multimers are 'unfurled' under flow, presenting a highly adherent platform for platelet recruitment and subsequent IE adhesion via platelet-expressed CD36. Induction of this pathway *in vitro* is very rapid with significant levels of VWF available for platelet adhesion within minutes of WP body activation, making this an attractive mechanism for efficient cytoadherence early in infection. Interestingly, such an IE pro-adhesive state can be removed through the action of the VWF protease ADAMTS-13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) that rapidly clears ultra-large VWF on ECs (Dong et al. 2002), but activity of this enzyme is reduced in severe malaria (De Mast et al. 2009, Larkin et al. 2009, Lowenberg et al. 2010).

Cytoadherence Inhibition Via Signal Transduction Pathways

The importance of signal transduction in *Plasmodium* infection has been also obtained using known inhibitors of the signalling system. Many of these inhibitors are drugs already in clinical use, which makes them more feasible as therapeutic agents in anti-malaria regimes. For example, the *in vitro* anti-plasmodial activity of antioxidant compounds, Flavonoids, from Artemisia *annua L* has recently been reported as protein kinases inhibitor that able to several kinases including Akt/protein kinase B (Akt/PKB), Fyn, Janus kinase 1 (JAK1), mitogen-activated protein kinase kinase 1 (MEK1), phosphoinositide 3-kinase (PI3K), mitogen-activated protein (MAP) kinase, kinase 4 (MKK4), Raf1, and zeta chain-associated 70-kDa protein (ZAP-70) kinase (Hou and Kumamoto 2010). Artemisinin has also been shown to inhibit nuclear translocation of NF-κB complex (Aldieri et al. 2003).

Levamisole is a specific alkaline-phosphatase inhibitor, has been shown *in vitro* to inhibit of sequestration of Pf-IE to endothelium. It has been used in a pilot clinical study for uncomplicated malaria, which resulted in increased peripheral blood parasitaemia of mature *P. falciparum* IE 24 hours after Levamisole administration. The sequestration ratio (between observed and expected peripheral blood parasitaemia) of early trophozoite and midtrophozoite parasites increased after Levamisole treatment, with near complete prevention of early trophozoites sequestration and >65% prevention of mid-trophozoite sequestration. These findings strongly suggest that levamisole decreases iRBC sequestration in *P. falciparum* malaria *in vivo* (Dondorp et al. 2007). Such an inhibitor for ecto-alkaline phosphatase could be exploited therapeutically although it may work through other mechanisms such inhibition of ion channel blockade and platelet aggregation (Mun et al. 1998). Levamisole could enhance immune response towards T helper 1 development through the activation of dendritic cells or T cell aspects (Chen et al. 2008). It has been reported that Levamisole inhibited rat platelet aggregation by releasing of a prostacyclin-like factor *in vivo* (Pinto et al. 1990).

Recently a rho-kinase inhibitor Fasudil, a drug already in clinical use for cardio- and neuro-vascular diseases, was successfully tested on laboratory strains of *P. falciparum* using an *in vitro* co-culture model. It was shown that adhesion of IE to primary human lung endothelial cells (HLECs) activated the Rho kinase signalling pathway, which led to endothelial barrier permeabilization. When Fasudil was added concomitantly with IE, this decreased both NF-κB activation and endothelial cell apoptosis. Fasudil also helped to restore endothelial barrier integrity after IE adhesion. Rho kinase inhibition thus appears to be a promising adjunctive therapeutic agent for severe malaria (Taoufiq et al. 2008). Fasudil was also tested in patient isolates at schizont stage in contact and non-

contact experiments with HLEC. In both conditions, 30 μM Fasudil significantly reduced *P. falciparum*-mediated HLEC apoptosis after 24 hours, but it did not decrease significantly *P. falciparum* cytoadherence (Zang-Edou et al. 2010, Waknine-Grinberg et al. 2010). It has been reported that the anti-inflammatory effect of the Rho kinase inhibitor fasudil was via inhibition of NF-κB activation in rheumatoid arthritis (Okamoto et al. 2010).

Atorvastatin is a cholesterol-lowering medication and is widely used in patients with cardiovascular disorders. It has been shown that atorvastatin protected EC from senescence and apoptosis. The molecular mechanism of this protection is correlated with the activation of the anti-apoptotic inositol triphosphate (PI3)-Akt kinase pathway, resulting in the phosphorylation of endothelial nitric oxide synthase (Kureishi et
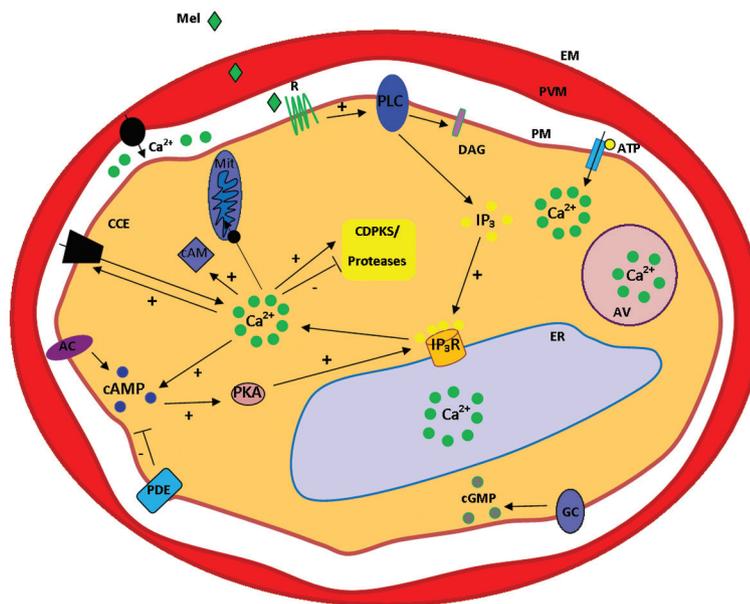

Figure 1. Schematic model for calcium signalling in intraerythrocitic stages of *Plasmodium*. Calcium signalling in intraerythrocitic stages of *Plasmodium*. Melatonin (Mel) binds a putative receptor (R) at the parasite membrane (PM), leading to the activation of a phospholipase C (PLC), which generates the second messengers diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP$_3$). IP$_3$ binds to putative IP$_3$ receptors (IP$_3$R) located at the membrane of the endoplasmic reticulum (ER), leading to the release of calcium (Ca^{2+}). Ca^{2+} rise in the parasite cytosol is responsible for the activation of numerous downstream effectors, as calmodulin (cAM), calcium-dependent protein kinases (CDPKs) and proteases, as well as generating further Ca^{2+} increase in the cytosol via influx from the plasma membrane, a process known as capacitative calcium entry (CCE). Ca^{2+} may also enter the mitochondria (Mit), activating its metabolism. Ca^{2+} is also able to induce an increase in cyclic adenosine monophosphate (cAMP) in the cytosol, whose production is regulated by adenylyl cyclase (AC) and phosphodiesterase (PDE). cAMP is able to promote further calcium increase from the ER. Besides the ER, calcium is also stocked in intracellular acidic pools (AC). The parasitophorous vacuole is a milieu rich in Ca^{2+}. Adenosine triphosphate (ATP) is able to induce calcium influx from the external milieu via putative purinergic-like receptors. Guanylate cyclase (GC) activity, which leads to the production of cyclic guanosine monophosphate (cGMP) is also implicated in cell cycle signalling to initiate gametogenesis and progression of schizogony. EM: erythrocyte membrane, PVM: parasitophorous vacuole membrane.
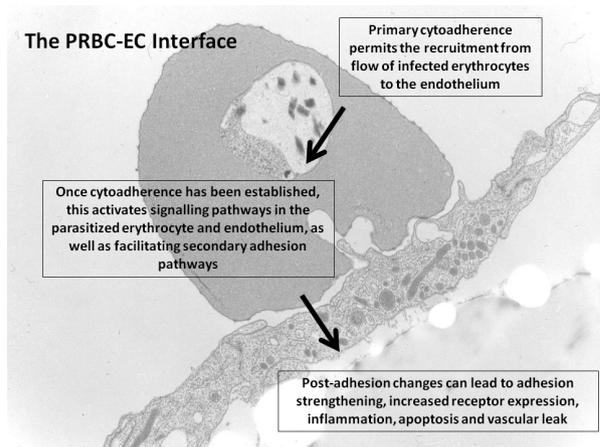
Figure 2. Summary figure for cytoadherence linked signalling showing interface of *Plasmodium falciparum* infected erythrocyte (PRBC) and endothelial cell (EC).

al. 2000). Atorvastatin decreases ICAM-1 expression in stimulated EC and monocytes (Romano et al. 2000). The roles of atorvastatin in the activation of cell survival protein kinase B or Akt signalling pathway is well established (Mason 2003, Wolfrum et al. 2003). The rho-kinase pathway in many aspects is antagonistic to PI3K/Akt/eNOS endothelial cell survival pathway (Zhou and Liao 2009). Recently, co-culture using mature *P. falciparum* 3D7 and primary HLEC IE-induced increased levels of endothelial adhesion molecules, ICAM-1 and P-selectin. Pre-treatment of EC with atorvastatin significantly prevented this up-regulation, protected endothelial cells from IE-induced apoptosis and protected endothelial barrier integrity from IE-induced impairment. The increased Akt expression in HLEC exposed to IE was observed by both Western blot and confocal microscopy techniques (Taoufiq et al. 2011).

CONCLUSIONS

Taken together Ca^{2+} mediated signalling offers potentials new molecular targets for the development of novel antimalarial. Several studies have explored the possibilities of using existing medications as therapeutic modalities to reduce IE sequestration

e.g. Src-family kinases, ecto-alkaline phosphatase, rho-kinase and AKT activator. However, the regulation of signalling pathways is complicated and results are difficult to interpret due to the interconnection of different signalling pathways via protein networks which are controlled by multiple positive and negative feedback mechanisms. Some pathways in certain situation may be redundant, some activation may only be transient and some key components of pathways could often be shared by multiple signalling cascades. More elegant methods and techniques are needed to elucidate the molecular mechanism mediating cytoadhesion and thereby find the key issues in these for the design of new therapies to control disease.

RESUMO

A Malária é responsavel por mais de 1.5 milhões de mortes anualmente, especialmente entre crianças (Snow et al. 2005). Apesar da gravidade da situação e grande esforço para o desenvolvimento de novas drogas (Yuan et al. 2011), os investimentos em drogas antimaláricas ainda é relativamente baixo. Brevemente, os alvos antimaláricos atualmente testados incluem: quinases, proteases, canais iônicos para GPCR, receptores nucleares entre outros (Gamo et al. 2010). No presente trabalho nós revisamos as vias de transdução de sinal em eritrócitos assim como eritrócitos infectados e interações com células endoteliais, denominada citoaderência. Este processo é conhecido por sua importante função na patogenicidade da malária severa. As moléculas expressas na superfície dos

eritrócitos infectados (IE) e células endoteliais vasculares (EC) são importantes mediadores da citoaderência, não apenas induzindo mudanças estruturais e metabólicas nos dois lados, mas também desencadeiam diversos sinais do processo transducional, levando a alteração da expressão gênica, com o balanço entre a regulação positiva e negativa determinando a patologia endotelial durante a infecção malárica.

Palavras-chave: citoaderência, eritrócito, malária, *Plasmodium*, transdução de sinal.

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