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Plasmodium falciparum avoids change in erythrocytic surface expression of phagocytosis markers during inhibition of nitric oxide synthase activity

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ABSTRACT

Nitric oxide (NO) accumulates in *Plasmodium falciparum*-infected erythrocytes. It may be produced by a parasite NO synthase (NOS) or by nitrate reduction. The parasite's benefit of NO accumulation is not understood. We investigated if inhibiting the *P. falciparum* NOS with specific and unspecific NOS inhibitors led to a decrease in intraerythrocytic NO accumulation and if this was associated with a change in surface expression of the phagocytosis markers CD47 and phosphatidyl serine. The specific inducible NOS inhibitors L-canavanine and GW274150 dose-dependently decreased intraerythrocytic NO while L-NMMA (an unspecific NOS inhibitor) and caveolin-1 scaffolding domain peptide (a specific endothelial NOS inhibitor) did not affect NO levels. Phosphatidyl serine externalization markedly increased upon *P. falciparum* infection. L-canavanine did not modify this whereas caveolin-1 scaffolding domain peptide increased the fraction of phosphatidyl serine exposing cells significantly. The infection did not change the level of expression of neither total CD47 nor its oxidized form. Unrelated to NOS inhibition, incubation with caveolin-1 scaffolding domain peptide lead to a decrease in oxidized CD47. In conclusion, the data imply that NOS inhibitors decrease NO accumulation in *P. falciparum*-infected erythrocytes but this does not correlate with the level of two major erythrocytic phagocytosis markers.

1. Introduction

Nitric oxide (NO) is a potent signalling molecule and has been implicated in several aspects of malaria pathology and pathogenesis. Host-derived NO has been suggested to protect against severe malaria [1]. Also the malaria parasite *Plasmodium falciparum* has been reported to produce NO as well as reactive nitrogen species [2,3]. NO production is catalyzed by either nitric oxide synthase (NOS) [2], nitrate reductase activity [4] or both. Regardless of the pathway, intracellular NO accumulation has been localized to the food-vacuole in *P. falciparum* [3]. NO appears important for the growth of *P. falciparum* as NOS inhibitors reduce parasite growth *in vitro* at a rate that is directly related to the NO reduction [2,3]. The mechanisms by which NO improves parasite growth are unknown.

A possible role of NO-production by the parasite is to cope with the oxidative stress during the intra-erythrocytic part of its life cycle the parasite as NO has anti-oxidant properties [5,6]. In addition to the protective effects of NO and other anti-oxidants on intracellular oxidative stress an increase in intracellular NO could reduce the processes leading to phagocytosis *in vivo* as suggested by previous *in vitro* studies [7]. Two processes that mediate phagocytosis of erythrocytes are externalization of the inner leaflet lipid phosphatidyl serine (PS) [6,8] and a reduction of the total level of CD47 surface expression on erythrocytes as well as changing the conformation of CD47 [9,10]. Although erythrocytes lack nuclei and mitochondria they display features associated with apoptosis including cell shrinkage and membrane blebbing, termed

Abbreviations: Cav-1, caveolin-1 scaffolding domain peptide; DMSO, dimethyl sulfoxide; eNOS, endothelial nictric oxide synthase; HE, hydro ethidine; iNOS, inducible nitric oxide synthase; iRBCs, infected erythrocytes; L-NMMA, L-NG-monomethyl arginine; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; PS, phosphatidyl serine.

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eryptosis [8]. PS exposure in part depends on intracellular NO levels [7].

The discordance of the previous studies on the production of NO in *P. falciparum* [2,3] has raised doubt about the existence of a parasite NOS. Since the original finding by Ghigo et al. [2] suggested that parasite NOS has similarities to inducible NOS (iNOS)-like, we studied the effect of highly selective inhibitors for iNOS on NO accumulation in *P. falciparum*-infected erythrocytes (iRBCs) and compared it with a commonly used NOS inhibitor and one specific for endothelial NOS. Furthermore, we addressed whether NOS inhibition would render the iRBCs susceptible to phagocytosis by measuring the levels of PS exposure and CD47 expression on iRBCs.

2. Materials and methods

2.1. P. falciparum culture and synchronization

P. falciparum (FCR3) was cultured *in vitro* according to standard protocols [11]. Briefly, the parasites were grown in culture flasks at 37 °C at 4% hematocrit in RPMI 1640 medium (Gibco, Life Technologies, Paisley, UK) supplemented with 5 mg/ml Albumax II (Life Technologies), 0.02 mg/ml hypoxanthine (Sigma–Aldrich, MO, US), 0.05 mg/ml gentamycin (Gibco, Life Technologies), 0.18 mg/ml L-glutamine (Sigma–Aldrich) in an atmosphere of 2% O_2, 5.5% CO_2 and 92.5% N_2. Subculture with the addition of blood group O erythrocytes was done throughout the study. Human blood was obtained with verbal informed consent from healthy volunteers; a procedure that did not need ethical approval from the Ethics committee in the capital region of Denmark. After collection, blood was stored cooled and used within one week after venipuncture. Prior to experiments, parasite cultures were synchronized to the schizont stage and enriched with infected RBCs by high gradient magnetic separation [12] or gelatine flotation [13]. Magnetic separation was carried out using a size CS column placed in a VarioMACS magnet (MiltenyiBiothec, Lund, Sweden). Gelatine flotation was carried out for 20 min at 37 °C in sterile filtered 0.75% gelatine (Gibco, Life Technologies) dissolved in RPMI 1640 medium. Parasite stage and parasitemia was determined by microscopy of Giemsa-stained thin blood-smears.

2.2. Detection and quantification of intra-erythrocytic NO

Intracellular NO was detected using 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM, Molecular Probes, Life Technologies), a fluorescent dye that readily crosses the cell membrane, and emits increased fluorescence after reaction with an active intermediate of NO [14]. Synchronized iRBCs were incubated with 40 μM DAF-FM and 5 μg/ml Hoechst 33342 (Life Technologies) for 1 h at 37 °C, and washed twice in PBS before microscopic observation of live cells. Confocal immunofluorescence microscopy photos were taken using a Nikon TE 2000E Eclipse with a 60× numerical aperture 1.4 Apoplan oil immersion objective lens with gain adjusted for each laser (408 nm: 450/35, 488 nm: 515/30). Images were acquired and processed by EZ-C1 gold version 3.90 software (Nikon Corporation).

For quantitative flow cytometric analyses, iRBCs were co-stained with hydro ethidine (HE, Sigma–Aldrich). iRBCs were synchronized as described and resuspended in culture medium at 1.0×10^6 cells/ml. Cells were incubated with DAF-FM (40 μM) and HE (2 μg/ml) for 1 h at 37 °C in the dark and washed twice in PBS prior to flow cytometric detection. NO was quantified using excitation and emission wavelengths of 488 nm and 520 nm, respectively (FL-1 channel), and HE using wavelengths of 488 nm and 620 nm, respectively (FL-3 channel) by using a Beckman Coulter FC500 MPL (CA, US) or a FACSCanto flow cytometer (BD Biosciences, San Jose, CA, US).

2.3. Inhibition of NO production

Four different compounds, known to inhibit one or more NOS isoforms, were tested. L-canavanine (Sigma–Aldrich), a naturally occurring amino acid with a structural resemblance to L-arginine, inhibits both iNOS and endothelial NOS (eNOS), with strongest effect on iNOS [15,16]. L-NG-monomethyl arginine (L-NMMA, Sigma–Aldrich) is a non-selective inhibitor and has been shown to inhibit iNOS, eNOS and neuronal NOS [17]. Caveolin-1 scaffolding domain peptide (cav-1, Alexis Biochemicals, Enzo, NY, US) selectively inhibits eNOS activity [18], while GW274150 (Alexis Biochemicals) is a potent and highly selective inhibitor of iNOS [19]. Synchronized parasites at a concentration of 1.0×10^6 cells/ml were preincubated with either 0.25, 1, or 4 mM L-canavanine in PBS, 0.1, 0.3, 1 or 3 mM L-NMMA or 10 nM, 100 nM, 1 μM or 10 μM cav-1 (Alexis Biochemicals) in DMSO (Sigma–Aldrich) for 15 min. For cav-1 DMSO was used as control, while PBS was used for the other inhibitors. Inhibition assays with GW274150 (Alexis Biochemicals) at 0.01 or 0.04 mM concentration in PBS were carried out for 24 h prior to quantification of intracellular NO production as this is a slowly acting inhibitor of NOS activity [20].

2.4. Quantification of CD47 expression on the RBC surface

Two antibodies for CD47 were used: one conformation-dependent antibody with specificity against the oxidized epitope of CD47; the positive switch for phagocytosis [10,21] (clone 2D3, fluorescein (FITC) conjugated, eBioscience, CA, US) and one detecting total CD47 (clone 472603, phycoerythrin conjugated, R&D Systems, MN, US). RBCs or synchronized iRBCs at a concentration of 1.0×10^6 cells/ml were preincubated for 24 h with or without 0.04 mM GW274150, 1 μM cav-1, 3 mM L-NMMA or 4 mM L-canavanine. For cav-1, DMSO was used as control for other inhibitors PBS was used. Incubation of RBCs in 0.2 mM $CuSO_4$ and 5 mM ascorbic acid (Sigma–Aldrich) in PBS for 60 min at 37 °C was used as a positive control of RBC oxidation/ageing [10]. The cells were washed in PBS supplemented with 0.5% human serum prior to 45 min of incubation with either of the two antibodies (conc. 10 μg/ml). To gate iRBCs, cells were co-stained with 0.5 μl/10^5 cells of the DNA-binding dye Vybrant ruby (Life Technologies) according to the manufacturer's instructions. Flow cytometry was performed using a FACSCanto flow cytometer (BD Biosciences) detecting FITC in FL-1, phycoerythrin in FL-3 and Vybrant ruby in FL-6.

2.5. Quantification of phosphatidylserine exposure on the RBC surface

PS exposure on RBCs and iRBCs were examined using FITC-annexin V (BD biosciences), a highly photostable fluorescent annexin V conjugate. RBCs or synchronized iRBCs at a concentration of 1.0×10^6 cells/ml were preincubated for 24 h with or without 1 μM cav-1, 3 mM L-NMMA or 4.0 mM L-canavanine, washed twice and resuspended in annexin-binding buffer (BD Biosciences), prior to incubation with FITC-annexin V (2 μl) for 20 min at room temperature. Prior to flow cytometry, the cell suspensions were diluted 5 times with annexin binding buffer. As a positive control, an RBC sample was preincubated for 2 h in hyperosmotic sucrose (600 mM), known to induce PS exposure [22]. To gate for iRBCs, cells were co-stained with Vybrant ruby (Life Technologies). Flow cytometry was performed using a FACSCanto flow cytometer (BD Biosciences) detecting FITC in FL-1 and Vybrant ruby in FL-6.