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***Plasmodium falciparum*: food vacuole localization of nitric oxide-derived species in intraerythrocytic stages of the malaria parasite**

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Abstract

Nitric oxide (NO) has diverse biological functions. Numerous studies have documented NO's biosynthetic pathway in a wide variety of organisms. Little is known, however, about NO production in intraerythrocytic *Plasmodium falciparum*. Using diaminorhodamine-4-methyl acetoxymethylester (DAR-4M AM), a fluorescent indicator, we obtained direct evidence of NO and NO-derived reactive nitrogen species (RNS) production in intraerythrocytic *P. falciparum* parasites, as well as in isolated food vacuoles from trophozoite stage parasites. We preliminarily identified two gene sequences that might be implicated in NO synthesis in intraerythrocytic *P. falciparum*. We showed localization of the protein product of one of these two genes, a molecule that is structurally similar to a plant nitrate reductase, in trophozoite food vacuole membranes. We confirmed previous reports on the antiproliferative effect of NOS (nitric oxide synthase) inhibitors in *P. falciparum* cultures; however, we did not obtain evidence that NOS inhibitors had the ability to inhibit RNS production or that there is an active NOS in mature forms of the parasite. We concluded that a nitrate reductase activity produce NO and NO-derived RNS in or around the food vacuole in *P. falciparum* parasites. The food vacuole is a critical parasitic compartment involved in hemoglobin degradation, heme detoxification and a target for antimalarial drug action. Characterization of this relatively unexplored synthetic activity could provide important clues into poorly understood metabolic processes of the malaria parasite,

Introduction

The presence of NO, a molecule that control diverse biological functions in higher organisms (Kers, et al., 2004) has also been documented in plants and bacteria (Adak, et al., 2002, Chen and Rosazza, 1995, Guo, et al., 2003). Within the family of protozoan parasites, NO production has been described in promastigote preparations of *Leishmania amazonensis* (Genestra, et al., 2006), *Toxoplasma spp.* (Gutierrez Escobar and Gomez-Marin, 2005) and in *Trypanosoma*

cruzi (Paveto, et al.). Surprisingly, the mechanism of NO production in *P. falciparum* has not been studied in detail and the possible functions of this molecule in the parasite's biology are totally unknown. In 1995, Ghigo and colleagues (Ghigo, et al., 1995) described a NOS activity in *P. falciparum* parasites. This study opened a very relevant area of inquiry in *P. falciparum* biology; however, no other study since then confirmed that intraerythrocytic forms of the parasites produce NO, further characterized the putative *P. falciparum* NOS or localized the area possibly responsible for this activity in the parasite.

Direct detection of NO presents technical difficulties in cellular systems. As a result, a downstream product of NO metabolism, nitrite anion (NO_2^-), is frequently used as a surrogate marker. Nitrite, however, is already present ($\approx 300 \text{ nM}$) in the cytosol of uninfected erythrocytes (Dejam, et al., 2005). Erythrocytic nitrite participates in reactions with oxyhemoglobin, deoxyhemoglobin and methemoglobin as part of a complex heme-nitrite chemistry (Basu, et al., 2007, Kim-Shapiro, et al., 2005). Additional nitrite generated by *P. falciparum* NO metabolism, could potentially be incorporated into this intraerythrocytic nitrite pool and participate in reactions with hemoglobin and methemoglobin, which increases during intracellular *P. falciparum* infection, adding even more complexity to the variety of products generated by these chemical interactions. Understanding these factors, we preferred to use a direct approach to assess RNS generation by *P. falciparum* parasites. DAR-4M AM is a membrane permeable, fluorescent indicator that allows direct visualization of NO and NO^- derived reactive RNS (Balcerczyk, et al., 2005), (Gomes, et al., 2006) and (Lacza, et al., 2005). In the absence of NO, DAR-4M AM is known to have negligible cross-reactivity with other radicals frequently present in the intracellular compartment (Lacza et al., 2005). Therefore, the presence of a fluorescent signal in cells after DAR-4M AM treatment is a good indicator of NO being generated in the system and can provide additional information about the sub-cellular localization of the RNS.

Here, we show images indicating the production of NO-derived RNS in intracellular *P. falciparum* parasites. Our images also show RNS-derived DAR-4M fluorescence signal localized in isolated food vacuoles of *P. falciparum* trophozoites. We confirm the previously reported antiproliferative effect of the NOS inhibitor L-canavanine in *P. falciparum* cultures. However, we did not obtain evidence of the reduction in NO-derived RNS in intraerythrocytic parasites after treatment with NOS inhibitors or about the presence of a putative *P. falciparum* NOS. Finally, we identified a molecule that resembles a plant nitrate reductase that localizes in the food vacuole and that may be responsible for NO-derived RNS in *P. falciparum* parasites.

Materials and Methods

Processing of human blood for culture: O+ blood was processed using leukocyte reduction filter (Sepacell R-500, Baxter, Deerfield, IL). Washed erythrocytes were suspended in RPMI 1640 and stored at 4 °C.

***P. falciparum* cultures:** 3D7 parasites were used in all experiments. Parasites were grown in either 5% or 2.5% hematocrit (leukocyte filtered human O+ RBC) in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 0.5% Albumax II (Invitrogen, Carlsbad, CA), 2 mg/ml sodium bicarbonate (Gibco, Invitrogen, Carlsbad, CA), 0.10 mM hypoxanthine (Sigma-Aldrich, St Louis, MO), 25 mM HEPES pH 7.4 (Calbiochem, EMD Chemicals, Inc., Gibbstown, NJ) and 10 mg/L Gentamicin (Gibco, Invitrogen, Carlsbad, CA) at 37 °C in a 5% O_2 , 5% CO_2 , 90% N_2 atmosphere.

Incubation on M199: mature forms of the parasite were incubated on M199 media supplemented with 0.5% Albumax II and 10 mg/L Gentamicin for up to 48 hr.

Food vacuole isolation: was performed in synchronized parasite cultures at late trophozoite stage following the method published by Goldberg et al (Goldberg, et al., 1990).

Macrophage cultures: murine macrophages (cell line J774) were grown to confluence on RPMI 1640 medium, 10% fetal calf serum and 1% penicillin/streptomycin. J774 murine macrophages were grown in cover slips and incubated overnight with 10 ng/ml LPS and incubated with 1 iM DAR-4M AM.

Detection of NO-derived RNS using DAR-4M AM: asynchronous *P. falciparum* cultures, trophozoites purified by magnetic separation (Miltenyi Biotech, Auburn, CA) or food vacuole preparations were incubated with 1 iM DAR-4M AM (Calbiochem, EMD Chemicals, Inc., Gibbstown, NJ) for 25 min at 37°C and then washed three times with HEPES buffer before microscopic observation. Gametocytes were magnetically separated and stained with 0.2 iM DAR-4M AM. Unstained *P. falciparum* parasites did not exhibit auto-fluorescence. For the detection of superoxide anion using MitoSOX, red mitochondrial superoxide indicator (Molecular Probes, Invitrogen, Carlsbad, CA), *P. falciparum* cultures or food vacuole preparations were incubated with 5 iM MitoSOX for 10 minutes at 37 °C. Fresh mounted cells were observed with a Leica DMI6000 inverted fluorescence light microscope (Leica Microsystems, Inc., Bannockburn, IL) using a 100X objective with n.a. = 1.30, an Omega 303 filter set (Omega Optical, Brattleboro, VT) and an ORCA ER digital camera (Hamamatsu Photonic System, Bridgewater, NJ). Images were acquired and processed by ImagePro 5.1 software (Media Cybernetics, Silver Spring, MD).

Quantification of NO-derived RNS using DAR-4M AM: trophozoites from a synchronized 3D7 parasite culture were purified using magnetic separation columns, washed with HEPES buffer, the parasite concentration adjusted and mixed with 10 µM DAR-4M AM. Cells were washed three times with HEPES after 25 min incubation. Fluorescence was measured in a Gemini XPS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) at ex: 550 nm, em: 590, cut off: 570 nm.

L-canavanine 24 hr for fluorescence measurement: 1 or 3 mM L-canavanine was added to synchronized cultures at ring stage at approximately 4% parasitemia and incubated for 22 to 24 hr. The next day, the number of mature forms developed were compared in L-canavanine treated cultures and in untreated controls in Giemsa smears. Parasites were purified using magnetic separation columns and treated with 1 iM DAR-4M AM as described.

L-canavanine 1 hr for fluorescence measurement: 1 or 3 mM L-canavanine was added to synchronized cultures at trophozoite stage at approximately 4% parasitemia and incubated for 1 hr. Parasites were purified using magnetic separation columns and treated with 1 iM DAR-4M AM, as described above.

NOS inhibitor effect on *P. falciparum* proliferation: synchronized *P. falciparum* cultures at trophozoites stage were grown either under standard conditions or adding to the culture from 1 to 4 mM of the following NOS inhibitors: L-canavanine (Sigma-Aldrich, St Louis, MO), L-NAME (Sigma-Aldrich, St Louis, MO) and L-NIO (Axxora, LLC, San Diego, CA). NOS inhibitors were added to the culture media when the culture media was replaced the next day. Parasite proliferation was assessed as the number of healthy mature forms present in culture after one cell cycle (48 hr).

Flow cytometry: we magnetically purified *P. falciparum* cultures treated for 1 cell cycle with 4 mM L-canavanine, L-NAME or left untreated. Isolated parasites were washed with HEPES buffer 1X and reacted with 2.5 iM DAF-FM diacetate (Molecular Probes, Invitrogen, Carlsbad, CA). Median fluorescence intensity was measured in a FACS Calibur (Becton and Dickinson, BD Biosciences, San Jose, CA).

Investigation of NOS activity using [^3H] L-arginine: NOS activity was measured using the NOSdetect Assay Kit (Stratagene, La Jolla, CA) using either 3D7 or FBC (formerly FCR-3) *P. falciparum*. Whole parasite experiments were performed with 2.6×10^7 magnetically purified trophozoites, obtained from approximately 10 ml of culture. Parasite homogenates were obtained from 50 ml of Percol-sorbitol purified cultures, resuspended in the homogenization buffer supplied in the NOS activity kit and sonicated for 30 seconds on maximum power on crushed ice. Protein concentration used in these assays was adjusted according to kit manufacturer's suggestion. [^3H] L-arginine (1.0 mCi/ml) was purchased from Amersham (GE Healthcare UK Limited, Buckinghamshire, England). Rat cerebellum extract was used as positive control of NOS activity, with and without the addition of the NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) at the concentration suggested by the kit's manufacturer.

RT-PCR: Total RNA was extracted from late trophozoite stage of *P. falciparum* 3D7 by using Trizol reagent following the manufacture's instruction (Invitrogen). The total RNA (2 μg) was treated with DNase I (Invitrogen) to remove potential genomic DNA contamination and used for synthesis of cDNA primed with oligo dT by using Superscript first strand cDNA synthesis kit (Invitrogen). RT-PCR was performed by using Platinum *Taq* polymerase (Invitrogen) with one-hundredth of the cDNA in the mixture and specific primers for each gene. The PCR products were separated in 4% E-gel (Invitrogen) for analysis.

Preparation of antisera by DNA immunization: we followed the procedure for antisera production by DNA immunization of mice published by Oliveira et al., 2006. Briefly, cDNA form PFI1140w and PF13_0353 coding regions were cloned into the vector VR2001-TOPO (Vical, Inc., San Diego, CA). Constructs were sequenced to verify correct direction of the inserted coding sequences. DNA from selected plasmids was amplified, purified, concentrated and sterilized. Plasmid preparations were used to immunize either Swiss Webster or Balb-C mice. Immune antisera were collected after a minimum of three consecutive immunizations, allowing a minimum of 2 weeks between immunizations.

Immunoblot analysis: a 3D7 *P. falciparum* protein extract was prepared using 80 ml of a culture containing 7 % trophozoites and grown at 5% hematocrit. The pellet of this parasite culture was resuspended in 10 ml PBS plus 100 μl of 10% saponin, incubated 10 min at room temperature and washed three times in PBS. This washed pellet was resuspended in 250 μl of Complete Lysis-M (Roche Diagnostics, Mannheim, Germany), incubated at room temperature for 5 min and then sonicated in a series of 15-second pulses in crushed ice, for a total of 2 minutes. After sonication, the sample was spun down at 20,000 rpm for 20 min at 4°C. Total protein concentration in the protein extract was 4,990 $\mu\text{g}/\text{ml}$ by BCA protein assay. A 200- μg aliquot of this protein extract was loaded in a single well, 2-D Tris-Glycin SDS gel (Invitrogen, Carlsbad, CA) under reducing and non-reducing conditions. After protein transfer, the membrane was blocked overnight with TBS/0.05% Tween in milk at 4°C. Individual sera from different mice immunized with either the PFI1140w or the PF13_0353 constructs were incubated at a 1/50 dilution using a Mini-Protean Multi Screen (Bio-Rad, Hercules, CA). The membrane was then washed and incubated with a 1/10,000 dilution of anti-mouse AP-conjugated antibody (Zymed, San Francisco, CA) and developed with Western Blue alkaline phosphatase substrate (Promega, Madison, WI).

Immunofluorescence assay: magnetically purified *P. falciparum* trophozoites were attached to coverslips, fixed and stained with an established procedure published by Tokumasu et al., 2005. Intraerythrocytic parasites were reacted with a 1/500 dilution of immune antisera raised against PFI1140w or PF13_0353 coding sequences in humid chamber at 37 °C for 1 hr. Intraerythrocytic parasites were also reacted with anti-PFI1140w antisera at a 1/50 dilution. Goat anti-mouse IgG conjugated as Alexa 633 (Invitrogen, Carlsbad, CA) was used as secondary antibody at a 1/1000 dilution. Nuclear DNA was identified using Hoechst 33258

(Molecular Probes, Invitrogen, Carlsbad, CA). Images were collected using a TCS-SP2 AOBS confocal microscope (Leica Microsystems GmbH) using a 63 X oil immersion objective.

Results and Discussion

Specificity of DAR-4M AM for the detection of RNS

Direct measurement of NO generation in cellular systems is technically challenging due to its short half-life (≈ 5 sec) and very low concentrations in the intracellular compartments where NO is generated. Recently, NO production has been documented in a variety of cell lines using fluorescent indicators (Manser and Houghton, 2006, Vatsa, et al., 2006). DAR-4M AM is a sensitive, cell-permeable NO fluorescent indicator with excitation maximum at ≈ 560 nm and emission maximum at ≈ 575 nm and high photo-stability, which make it suitable for live-cell imaging. DAR-4M AM releases membrane impermeable DAR-4M intracellularly, by the action of endogenous esterases. NO generated *in situ* is quickly oxidized by oxidants present in the intracellular compartment to NO^+ equivalents, such as N_2O_3 , which actually reacts with DAR-4M to give the fluorescent benzotriazole derivative, DAR-4M T (Kojima, et al., 2001). As a consequence, the generation of N_2O_3 implies that NO, the immediate upstream compound, is present in the system. Since N_2O_3 is the predominant form reacting with DAR-4M, some authors prefer to call this chemical a RNS indicator rather than an NO indicator (Lacza, et al., 2005). Although the formation of N_2O_3 may be increased by the presence of oxidants in the intracellular compartment, it has been documented that in the absence of NO, neither peroxynitrite, superoxide or hydrogen peroxide *alone* can generate fluorescent products with DAR-4M AM (Lacza et al., 2005). Consequently, the presence of a fluorescent signal in cells after DAR-4M AM treatment is a good qualitative indicator of NO-derived RNS being generated in the system.

Detection of RNS in intraerythrocytic *P. falciparum* using DAR-4M AM—We performed the parasite cultures and experiments presented here using leukocytes-free human blood, to avoid potential cross-contamination with mammalian NOS activity.

Using 1 μM DAR-4M AM in *P. falciparum* cultures, we obtained positive fluorescent signals in intraerythrocytic parasites suggesting NO production both at the ring and at the trophozoite stages of development (Fig 1 a, b, e and f). Fluorescence images generated by DAR-4M AM in *P. falciparum* parasites were very consistent throughout multiple microscopy sessions. There was no fluorescence observed in uninfected erythrocytes present in the preparation. The characteristic live-cell hemozoin crystal shimmer, observed by polarized light microscopy, was used to confirm that the parasite was alive after treatment with the fluorescent indicator (Fig 1 c and g). Intraerythrocytic trophozoites generated a much more noticeable fluorescent signal compared to rings. Merging bright field images with fluorescence and polarized light microscopy images, we noticed that the fluorescent signals overlapped with the food vacuole of trophozoites stage parasites (Fig 1 d and h). As a control for DAR-4M AM performance, we incubated murine macrophages (cell line J774) in the presence or absence of 10 $\mu\text{g/ml}$ lipopolysaccharide (LPS) for 24 hr to activate nitric oxide production. When macrophages were stained with 1 μM DAR-4M AM, cells incubated with LPS showed positive fluorescent images while macrophages incubated with no LPS displayed no fluorescence, demonstrating sensitive and specific NO detection (Fig 1 i and j).

To semi-quantify the DAR-4M AM fluorescence observed, we magnetically purified a synchronized culture at trophozoites stage and adjusted their concentration to three different levels of parasite density: 0.34, 0.68 and 1.36×10^7 parasites per experiment. Next, we incubated these parasites with DAR-4M AM following a similar protocol to the one used for the light microscopy experiments, and the fluorescent signal generated by the parasites was quantified by spectrofluorometry. The DAR-4M AM fluorescence detected was proportional

to the number of trophozoites present in each experiment, within the range of parasite numbers used (Fig 2), indicating that a similar amount of RNS was generated by individual trophozoites at a similar developmental stage.

High levels of nitrate salts present in RPMI 1640 (848 iM) were not responsible for the DAR-4M AM fluorescence observed since trophozoites incubated on M199, a culture media that contains very low nitrate concentration (5 iM), also displayed positive DAR-4M AM fluorescence (supplementary material).

Sub-cellular localization of DAR-4M signals—To better assess if DAR-4M AM fluorescent signals observed actually originated in the parasite's food vacuole, we isolated food vacuoles from late trophozoite *P. falciparum* parasites by differential centrifugation of partial parasite lysates and extensive wash of the food vacuole pellets (Goldberg, et al., 1990) (Fig 3 a). We observed that, when reacted with DAR-4M AM, the membranous system associated with food vacuoles still displayed a fluorescent signal very close to the hemozoin crystal (Fig 3 b and c).

Because it is possible that DAR-4M AM fluorescent signals could have been affected by the presence of reactive oxygen species (ROS) (such as superoxide anion), we investigated the reactivity of *P. falciparum* food vacuoles from the previous preparation with MitoSOX, a superoxide fluorescent indicator. MitoSOX exhibits red fluorescence (Ex/Em: 510/580 nm) when oxidized by superoxide, which is a byproduct of mitochondrial oxidative phosphorylation. As expected, when trophozoite stage parasites were stained with the superoxide probe, they displayed positive fluorescent signals within the boundaries of the parasitophorous vacuole, most likely associated with the diffusion of the superoxide anion from their point of origin in the parasite's mitochondria (Fig 3 d, e and f). However, extensively washed food vacuole fractions show no fluorescent signal when reacted with the superoxide indicator (Fig 3 g, h and I), implying that positive RNS signals from DAR-4M AM in food vacuoles did not depend on the simultaneous presence of superoxide anion. Further, when food vacuole preparations were digested with Triton-X100, all fluorescent signals associated to the hemozoin crystal disappeared (Fig 4 a, b and c), showing that the hemozoin crystal itself was not involved in the generation of the fluorescent signal with the probe. The lack of DAR-4M AM reactivity after detergent treatment of hemozoin-rich fractions also reinforced our hypothesis that RNS fluorescence originated in the membrane system surrounding the hemozoin crystal, but not in the hemozoin itself.

Effect of NOS inhibitors on *P. falciparum* proliferation and nitric oxide production—Over a decade ago, researchers observed that nitric oxide inhibitors (NOS) hindered the proliferation of blood stages of the *P. falciparum* parasite *in vitro* to different extents (Ghigo, et al., 1995). The authors of that study proposed the presence of a NOS activity in *P. falciparum* parasites and hypothesized that NO played a crucial role in parasite metabolism. We confirmed that NOS inhibitors have a detrimental effect on parasite proliferation using a slightly different NOS inhibitor panel. We cultured mature forms of the parasite for 48 hr (one cell cycle) in the presence of L-canavanine (iNOS inhibitor), L-NIO (eNOS inhibitor) and L-NAME (non-selective NOS inhibitor) in concentrations from 1 to 4 mM. Similar to what was described by Ghigo and colleagues, we observed that L-canavanine had the strongest negative effect on parasite proliferation. L-canavanine at 1 mM reduced the number of mature forms in culture after 48 hr to approximately 50% compared to untreated control values (normalized to 100%), whereas the addition of 3 mM of L-canavanine caused a drastic reduction in the number of mature forms of the parasite present in culture (2%) compared to untreated controls. In contrast, the number of mature forms present after addition of either 1 or 3 mM of L-NIO to parasite cultures remained very similar to the values of untreated controls (90% in both cases). The addition of 1 or 3 mM L-NAME to our cultures

caused a moderate reduction in the number of mature forms present in culture after one cell cycle (87% and 61% respectively), but far less than the effect observed with L-canavanine. (Fig 5 a).

Since L-canavanine had a considerable antiproliferative effect in *P. falciparum* parasites, we studied L-canavanine's ability to reduce RNS generation in trophozoites *in vitro*. To quantify the possible reduction in fluorescence intensity after the treatment with L-canavanine, we used flow cytometry with 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM) (Itoh, et al., 2000), another NO indicator with excitation/emission maxima of 495/515 nm, compatible with our flow cytometry laser detection system. We observed that synchronized *P. falciparum* cultures treated with 4 mM L-canavanine for 20–24 hr (starting at ring stage and measured at the trophozoite stage) had the number of cells within the R1 gate that was 40% lower compared to untreated *P. falciparum* cultures or cultures treated with the non-selective NOS inhibitor L-NAME (Fig 5 b). In addition, the mean fluorescence intensity for L-canavanine and L-NAME treated parasites in R1 gate was 16% and 6% lower respectively, compared to untreated controls.

After overnight incubation with L-canavanine, microscopic observation of Giemsa smears from the cultures revealed the presence of a lower number of trophozoites than expected, while some rings were still present. Untreated control or cultures treated with L-NAME showed the expected number of trophozoites and no presence of rings (data not shown). The reduced number of trophozoites present in the L-canavanine-treated cultures can explain the lower number of parasites found in the R1 gate. Ring forms, also present in L-canavanine-treated cultures, generate a lower fluorescent signal, as we have observed previously by wide-field fluorescence microscopy (Fig 1 b).

Next, we wanted to determine if the negative effect of L-canavanine on parasite proliferation was related to the inhibition of NO-derived RNS generation under standard conditions used to test the effect of NOS inhibitors. We incubated 10^5 purified *P. falciparum* trophozoites for 1 hr and 24 hr with either 1 or 3 mM of L-canavanine at 37°C, then treated the cell's with DAR-4M AM and quantified the fluorescent signal generated by the parasites by spectrofluorometry (Fig 5c). The fluorescence generated by trophozoites incubated with 1 or 3 mM L-canavanine for 1 hr and untreated parasites was indistinguishable. Incubation for 24 hr (starting with ring and measured at trophozoite stage the next day) with 1 mM L-canavanine caused a slight reduction in the measured DAR-4M AM fluorescence, while 24 hr incubation with 3 mM L-canavanine showed a significant reduction (>50%) in measured fluorescence (P value = 0.0163; <<0.05) when analyzed using a two-tailed, paired t-test at 95% confidence level. These results are in agreement with previously shown data in Fig 5b.

In our opinion, the previous results do not support a role of L-canavanine as a *P. falciparum* NOS inhibitor since L-canavanine did not show any measurable reduction of RNS-related fluorescence when the parasites were exposed for 1 hr to this drug at 1 and 3 mM concentrations. In mammalian cells, it is important to remember that the majority of NOS inhibitors are L-arginine-analogs and exert their NOS inhibitory action by substrate competition at the L-arginine binding site. Since the molecular structure of the system responsible for NO synthesis in *P. falciparum* is not known, we cannot assume that these inhibitors are acting by a similar mechanism in the parasite's putative NOS molecule as they do in mammalian NOSs. NO is produced in many eukaryotic species in a reaction catalyzed by any of the three known isoforms of nitric oxide synthase (NOS): neuronal, inducible and endothelial NOS, all of which contain a heme-oxygenase and a flavin reductase domain. Bacteria also have NOS enzymes, but these are smaller molecules, with similarities to the mammalian oxygenase domain both in sequence and function (Chen and Rosazza, 1995), although lacking the reductase domain present in mammalian NOS. Plants, such as *Arabidopsis thaliana*, contain a NOS-like enzyme that uses

L-arginine as a substrate, but also bears little resemblance to the molecular sequence of NOS enzymes in higher organisms. Instead, *A. thaliana* NOS-like enzyme contains a sequence similar to an NO producing enzyme in the snail *Helix pomatia* (Guo, et al., 2003). Taken together, current evidence from a variety of organisms seems to indicate that even when arginine-dependent NO production seem to be fairly conserved among different organisms, considerable re-arrangement of molecular structures occurred along the evolutionary process (Chen and Rosazza, 1995, Guo, et al., 2003, Huang, et al., 1997).

The *P. falciparum* antiproliferative activity of NOS inhibitors, particularly L-canavanine, first described by Ghigo and colleagues and confirmed in our experiments could be the result of the cytotoxic effect of this drug at the concentrations used. It had been described that L-canavanine's structural similarity to L-arginine causes the accidental incorporation of this drug into proteins since it can be a substrate for arginyl tRNA synthase and it may be erroneously incorporated into various proteins, resulting in functionally inactive products (Bence, et al.). This phenomenon could explain the detrimental effect of L-canavanine on *P. falciparum* cultures.

Investigation of *P. falciparum* NOS activity

Since we did not observe any difference in NO-derived RNS after short-term treatment of trophozoites with 1 or 3 mM L-canavanine, we decided to measure the possible *P. falciparum* NOS activity using a highly sensitive and specific method. We purified 2.6×10^7 3D7 trophozoites from a leukocyte-free culture and incubated them in a reaction buffer mixture containing [3 H] L-arginine, NADPH and calcium in Tris-HCl buffer at pH 7.4. We also prepared parasite homogenates using 5X that amount of parasites to ensure non-limiting amounts of the putative *P. falciparum* NOS in the assay. As expected, positive controls for NOS activity using rat cerebellum extract generated [3 H] L-citrulline in a reaction that was sensitive to the addition of L-NAME. However, neither reaction mixtures using whole parasites nor preparations of parasite homogenates generated [3 H] L-citrulline above the baseline levels obtained with the reaction buffer alone or with 2.6×10^7 unparasitized erythrocytes (Fig 6). Similar results were obtained using whole FCB parasites (data not shown). In another set of experiments, calmodulin was added to parasite homogenates to compensate for its possible depletion during the process of sample preparation, however no increase in the level of [3 H] L-citrulline generated was observed (data not shown). Our results, do not support the presence of an arginine-dependent NOS activity in *P. falciparum* parasites; however, we cannot rule out that such activity might be present but undetectable by this classic test. If a *P. falciparum* NOS exists, we can hypothesize that the parasite's utilization of L-citrulline in an intracellular metabolic pathway could explain the absence of this expected product of NOS activity.

Looking for genes possibly related to NO synthesis in the *P. falciparum* genome

Our observations strongly suggest NO production by intraerythrocytic *P. falciparum* parasites, although the structure of the molecule responsible for that synthetic function is not known. When we searched the Plasmodium genomic database (PlasmoDB: <http://www.plasmodb.org/plasmo/home.jsp>) for coding sequences related to nitric oxide synthesis, we identified two gene candidates. The first one, PlasmoDB ID PFI1140w, was found by BLAST search of PlasmoDB v 5.4 using the protein sequence of human iNOS as query. We identified three hits (PFI1140w, PFF1115w and PF14_0478) with PFI1140w having a high score of 206 and a p-value of 8.2×10^{-23} , while the other genes had scores less than 142 and p-values between 0.036 and 6.3×10^{-8} . PFI1140w and human iNOS have 32% identity and 56% similarity in aminoacid sequence. When we performed an alternative BLAST search using the protein sequence of mouse iNOS as query, we identified the same three hits (PFI1140w, PFF1115w and PF14_0478): PFI1140w had a score of 194 and a p-value of 4.7×10^{-27} , while the other genes had scores less than 160 and p-values between 0.00013 and 6.6×10^{-10} . PFI1140w

and mouse iNOS have 32% identity and 55% similarity in aminoacid sequence. PFI1140w is designated as NADPH-cytochrome p450 reductase. PFI1140w shares the highest similarity in the C-terminal NADPH and flavin-derived binding regions of the mammalian iNOSs. The PFI1140w sequence, however, does not contain the oxygenase homologous domain present in the N-terminus of all mammalian NOSs. The oxygenase domain is responsible for the binding of co-factors tetrahydrobiopterin (BH4) and heme as well as for forming the pocket where the substrate L-arginine binds to the mammalian NOS molecule. The dissimilarity between PFI1140w and typical NOS structures is not surprising considering the evolutionary distance that separates *P. falciparum* from mammalian systems. It is possible, however, that the molecular structure of the putative *P. falciparum* NOS may not resemble the multi-domain structure of a mammalian NOS. NOS-like activity may be achieved by biochemical interaction of separately encoded heme oxygenase and reductase domains, such as it has been described in some bacteria (Adak, et al., 2002). If this were the case in the malaria parasite, the product of the PFI1140w sequence could represent the subunit responsible for the reductase activity in a putative *P. falciparum* NOS complex.

The other gene candidate, PF13_0353, was found by BLAST search of PlasmoDB v 5.4 using the protein sequence from zeamays (corn) nitrate reductase as query. BLAST search identified four hits (PF13_0353, PF14_0266, PFL1555w and PFI0885w) with PF13_0353 having a score of 243 and a p-value of 3.0×10^{-20} , while the other genes had scores less than 132 and p-values between 2.3×10^{-05} and 3.4×10^{-07} . PF13_0353 and zeamays nitrate reductase have 28% identity and 47% similarity in aminoacid sequence. PF13_0353 is a putative NADH-cytochrome b5 reductase and is structurally similar to plant nitrate reductases (NR) that produce NO using nitrite as a substrate. The presence of PF13_0353, a coding sequence structurally similar to plant nitrate reductase in the *P. falciparum* genome is suggestive of this mechanism being active in the parasite and should be further investigated. Interestingly, substantial amounts of nitrite are present in the erythrocyte cytosol under physiological conditions (Dejam, et al., 2005) and could be readily available as a substrate for NO synthesis.

Although no specific function has been described for the product of these two genes, PFI1140w and PF13_0353, we hypothesized that either one or both of these gene products could be associated to NO synthesis in *P. falciparum*. The presence of NO synthesis by dual mechanisms -- a NOS-like enzyme and a nitrate reductase -- has been described in plants (Guo, et al., 2003).

Using reverse transcription, (RT)-PCR, with total RNA from late trophozoite stage *P. falciparum*, we detected mRNA from PFI1140w and PF13_0353 (Fig 7 a). Looking both at the relative (percentiled) and absolute expression levels obtained on intraerythrocytic 3D7 oligo arrays (PlasmoDB), we observed that these two genes are transcribed in asexual stages of *P. falciparum*, as well as at the gametocyte stage (Fig 7 b). It is important to note that, although the y-axis for the absolute expression profiles is given in different scales in the two genes, the peak values expression for PFI1140w and PF13_0353 are similar.

Interestingly, the expression patterns for PFI1140w and PF13_0353 indicate that these gene products should be present in *P. falciparum* parasites at the developmental stages where we observed DAR-4M AM positive signals. For the purposes of verification, we confirmed that *P. falciparum* stage II/III gametocytes also displayed strong fluorescence signals when treated with DAR-4M AM (Fig 8).

Next, we studied expression of these two gene products and their localizations in intraerythrocytic *P. falciparum* parasites. For this purpose, we raised antibodies against these two genes using DNA immunization. Purified plasmid preparations were generated by cloning cDNA from PFI1140w and PF13_0353 coding regions into the DNA plasmid VR2001-TOPO

(Oliveira, et al., 2006). Sequence fragments coding for trans-membrane regions were not included in the constructs. Immunizations were performed in a minimum of five animals, either Swiss Webster or Balb-C mice, and immune sera were obtained after three consecutive immunizations following a protocol described by Oliveira et al., 2006.

Immunoblot analysis using antisera from 5 mice immunized with PFI1140w coding sequence and from 5 different mice immunized with PF13_0353 coding sequence was performed after SDS gel electrophoresis of *P. falciparum* extracts prepared from synchronized trophozoite stage cultures (See Materials and Methods) (Figure 7 c). None of the five anti-PFI1140w antisera tested recognized a protein of ≈ 90 kD, the molecular weight of the predicted protein from the PFI1140w gene, using either reducing or non-reducing conditions. Instead, two high molecular weight bands were recognized at approximately 150 and 250 kD. On the other hand, three independent immunoblot assays showed that, under reducing conditions, one out of five anti-PF13_0353 antisera recognized a single band of approximately 42 kD that matched the molecular weight of the protein predicted by the PF13_0353 gene.

Immunofluorescence microscopy assays (IFA) with antiserum against PFI1140w failed to show reactivity with parasitic structures (Fig 9 a, b, c and d). On the contrary, when anti-PF13_0353 antiserum was tested by IFA in trophozoites, it showed positive fluorescence signals that did not overlap with nuclei detected by Hoescht 33258 (Fig 9 e, f, g and h). In addition, the observed reactivity of anti-PF13_0353 antiserum was localized in the membranous region surrounding the food vacuole area.

The previous experiments could not confirm that the protein product of PFI1140w is present in intracellular *P. falciparum* parasites. It is possible that this protein exists in a multimeric form and that the conformation of the reactive epitope(s) is inaccessible in the parasite. Another explanation for the lack of reactivity with anti-PFI1140w antiserum might be that this protein is present at very low levels in the intraerythrocytic parasite. Alternatively, PFI1140w's product could be a cytosolic protein that was lost during the permeabilization process that is part of the cell preparation for IFA. Finally, the cDNA region cloned in the process of generating the PFI1140w immune antiserum might have not include the antigenic region of this protein. Consequently, at the present time, we cannot rule-out that a protein product of PFI1140w gene might be present in *P. falciparum* trophozoites. On the other hand, anti-PF13_0353 antiserum recognized a band of the expected molecular size in immunoblot analysis, which suggested that a product of this gene might be present in intraerythrocytic *P. falciparum*. Intracellular localization of the protein coded by gene PF13_0353, a putative nitrate reductase, was confirmed by IFA. These observations cannot confirm that the protein coded in PF13_0353 is involved in the synthesis of NO or NO-derived products. Further studies are required to characterize the function of this gene product in *P. falciparum* parasites. It is important to note that possible confirmation of NO synthetic function by PF13_0353 does not preclude the simultaneous presence of an NOS-like complex in which a PFI1140w-derived protein might have a functional role. However, it still remains to be confirmed whether a protein product of PFI1140w is present in intracellular *P. falciparum* parasites.

Conclusions

Our results suggest that NO is produced in intraerythrocytic *P. falciparum* and that this function is localized in or in close proximity to the food vacuole. We showed that the product of PF13_0353, a molecule that is structurally similar to a plant nitrate reductase, localizes in the membranous system that surrounds the food vacuole. In our opinion, future efforts leading to the identification of the molecular nature of a *P. falciparum* NO synthetic activity should focus on this region. The food vacuole, a lysosome-like organelle containing hydrolytic enzymes in an acidic environment, is a critical parasitic compartment involved in hemoglobin degradation,

heme detoxification and a target for antimalarial drug action. Oxidative conditions generated in the food vacuole by NO and NO-derived oxidative radicals might contribute to food vacuole functions, such as hemoglobin degradation and heme polymerization. Understanding the role of NO and molecular nature of the enzymatic system responsible for its synthesis should be a goal for those interested in the biology of this medically relevant organism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. RNS detection using DAR-4M AM

a human erythrocyte infected with two late ring stage *P. falciparum* 3D7 parasites viewed under bright field microscopy, **b**: fluorescence microscopy and **c**: polarized light microscopy, after reaction with 1 iM DAR-4M AM. **d**: merged images of a, b and c. **e**: human erythrocyte infected with a trophozoite stage parasite viewed under bright field, **f**: fluorescence and **g**: polarized light microscopy, after reaction with 1 iM DAR-4M AM. **h**: merged images of e, f and g. **i**: murine macrophages (cell line J774) incubated in the absence (**i**) or presence (**j**) of 10 $\mu\text{g/ml}$ LPS for 24 hr and reacted with 1 μM DAR-4M AM.

Figure 2. Semi-quantification of DAR-4M AM in trophozoites

Magnetically purified *P. falciparum* trophozoite, adjusted to three different concentrations and treated with DAR-4M AM. Fluorescence intensity was measured by spectrofluorometry. Bar graph represents the mean of three different experiments.

Figure 3. DAR-4M AM and MitoSOX treatment of *P. falciparum* food vacuoles

a: image of individual food vacuole reacted with 1 iM DAR-4M AM, viewed under bright field microscopy and **b:** fluorescence microscopy. **c:** merged images of a, b. **d:** human erythrocyte infected with a trophozoite stage parasite reacted with 5 iM MitoSOX viewed under bright field and **e:** fluorescence microscopy. **f:** merged images of d, e. **g:** image of individual food vacuole reacted with 5 iM MitoSOX viewed under bright field and **h:** fluorescence microscopy. **i:** merged images if g, h.

Figure 4. Food vacuoles treated with DAR-4M AM after digestion with Triton X-100
a: food vacuole preparation treated with DAR-4M AM after Triton X-100 digestion viewed under bright field, **b:** polarized light and **c:** fluorescence microscopy.

Figure 5. a: Effect of NOS inhibitors on *P. falciparum* proliferation

*P. falciparum*3D7 parasites cultured for 48 hr in the presence of 1 to 4 mM L-canavanine (v), L-NAME (Δ) and L-NIO (λ). Parasitemia was normalized against that of parasites cultured without the addition of inhibitor. Parasitemia was assessed as the number of healthy mature forms present in culture after one cell cycle. Data points represent the mean $\pm$ SEM of three different experiments. **b: Flow cytometry with DAF-FM after treatment with L-canavanine.** *P. falciparum* cultures incubated with 4 mM L-canavanine for 20–24 hr and treated with 2.5 μ M DAF-FM diacetate. L-canavanine-treated cultures had a 40% lower number of cells in R1 gate compared to untreated parasite cultures or cultures treated with the non-selective NOS inhibitor L-NAME. Bar graph represents the mean of three independent

experiments. **c: DAR-4M AM fluorescence after short or long-term incubation with L-canavanine.** *P. falciparum* trophozoites incubated with 1 or 3 mM L-canavanine at 37°C for either 1 hr or 24 hr and then treated with DAR-4M AM. Bar graph represents the mean of three independent experiments.

Figure 6. NOS activity in *P. falciparum* parasites

2.6×10^7 3D7 trophozoites, parasite homogenates or unparasitized erythrocytes were incubated for 30 minutes at 37°C in a reaction buffer mixture containing [³H] L-arginine, NADPH and calcium in Tris-HCl buffer at pH 7.4. Results are expressed as the ratio between eluted [³H] L-citrulline and unreacted [³H] L-arginine.

Figure 7. a: mRNA detection from genes possibly related for NO synthesis in *P. falciparum*. mRNA from PFI1140w and PF13_0353 was detected in total RNA from late trophozoite stage of *P. falciparum* using (RT)-PCR. **b: Relative and absolute expression levels for PFI1140w and PF13_0353 in intraerythrocytic 3D7** (source: Plasmo DB: <http://www.plasmodb.org/plasmo/home.jsp>). Relative expression level (percentiled) of the genes intensity is compared relative to all other genes for a given experiment; absolute expression is the affymetrix MOID expression value normalized by experiment level. Green bars represent stage synchronization using sorbitol, purple bars represent stage synchronization by temperature and gray bars represent expression level that is close to the background. Abbreviations: ER = early rings, LR = late rings, ET = early trophs, LT = late trophs, ES = early schizonts, LS = late schizonts, M = merozoites, S = sporozoites, G = gametocytes. **c: Immunoblot analysis using anti-PFI1140w and anti-PF13_0353 antibodies.** Predicted MW of proteins coded in PFI1140w and PF13_0353 are 90 and 42 kD respectively. A reactive band at the expected MW, ~42 kD was observed using anti-PF13_0353 antisera (lanes a, b and c). No bands were detected in the expected MW using anti-PFI1140w antisera (lanes d, e and f).

Figure 8. NO and NO-derived RNS detection in gametocytes using DAR-4M AM
a 3D7 stage II-III gametocyte viewed under bright field, **b**: polarized light and **c**: fluorescence microscopy, after reaction with 0.2 iM DAR-4M AM.

Figure 9. Immunofluorescence assay using anti-PFI1140w and anti-PF13_0353 antisera
Purified *P. falciparum* trophozoites were incubated with a 1/500 dilution of antisera recognizing coding sequences of either PFI1140w or PF13_0353. Goat anti-mouse IgG conjugated with Alexa 633 was used as a secondary antibody. Nuclear DNA was labeled with Hoescht 33258. **a:** DIC image of trophozoites treated with anti-PFI1140w antiserum **b:** nuclear DNA stained with Hoescht 33258 and **c:** negative Alexa 633 signal. **d:** merged images of a, b and c. **e:** DIC image of trophozoites treated with anti-PF13_0353 antiserum, **f:** nuclear DNA stained with Hoescht 33258 and **g:** Alexa 633 signal recognizing anti- PF13_0353 antibody. **h:** merged images of e, f and g.