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Sequestration and metabolism of host cell arginine by the intraerythrocytic malaria parasite *Plasmodium falciparum*

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Summary

Human erythrocytes have an active nitric oxide synthase, which converts arginine into citrulline and nitric oxide (NO). NO serves several important functions, including the maintenance of normal erythrocyte deformability, thereby ensuring efficient passage of the red blood cell through narrow microcapillaries. Here, we show that following invasion by the malaria parasite *Plasmodium falciparum* the arginine pool in the host erythrocyte compartment is sequestered and metabolized by the parasite. Arginine from the extracellular medium enters the infected cell via endogenous host cell transporters and is taken up by the intracellular parasite by a high-affinity cationic amino acid transporter at the parasite surface. Within the parasite arginine is metabolized into citrulline and ornithine. The uptake and metabolism of arginine by the parasite deprive the erythrocyte of the substrate required for NO production and may contribute to the decreased deformability of infected erythrocytes.

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Introduction

Malaria poses a significant burden on world health and was responsible for an estimated 198 million clinical cases and approximately 584 000 deaths in 2013 (WHO, 2014). Of the five malaria parasite species that infect humans, *Plasmodium falciparum* accounts for the majority of clinical cases of severe malaria and is the most common cause of lethal malaria. The symptoms of malaria manifest themselves during the intraerythrocytic stage of the parasite life-cycle. As it develops within the human erythrocyte, the parasite 'remodels' the host cell, exporting a multitude of proteins to facilitate nutrient transport, protein trafficking and evasion of the host immune system (Boddey and Cowman, 2013; Kirk and Lehane, 2014).

Following its invasion by the malaria parasite, the host erythrocyte undergoes a decrease in deformability, leading to blockage of microcapillaries and thereby contributing to malaria pathogenesis (Nash *et al.*, 1989; Dondorp *et al.*, 2000). This loss of deformability is mediated in part by parasite-exported proteins binding to the cytoskeleton of the host (Coppel, 1992; Mills *et al.*, 2007; Maier *et al.*, 2008; Glenister *et al.*, 2009) and in part by the presence of the non-deformable parasite itself (Nash *et al.*, 1989; Hosseini and Feng, 2012). The deformability of uninfected erythrocytes is regulated by the short-lived free radical nitric oxide (NO), which is generated by endothelial cells and by the erythrocytes themselves (Heitzer *et al.*, 2001; Kleinbongard *et al.*, 2006; Cortese-Krott *et al.*, 2012). NO is formed by nitric oxide synthase (NOS), which converts the amino acid L-arginine to citrulline and NO. NO is produced constitutively by erythrocytes, maintaining normal erythrocyte membrane fluidity through the S-nitrosylation of cytoskeleton proteins including α- and β-spectrin (Kleinbongard *et al.*, 2006; Horn *et al.*, 2011; Grau *et al.*, 2013).

Whether infection by the malaria parasite perturbs, NO metabolism in the host erythrocyte is unclear. Ghigo *et al.* reported that the intraerythrocytic parasite itself can synthesize NO, based on measurements of citrulline production (Ghigo *et al.*, 1995). More recently, it was reported that there is, in the parasite, a pool of NO localized to the digestive vacuole (Ostera *et al.*, 2008); however, no citrulline production was detected, and it was proposed that the parasite's NO pool is maintained not by a NOS but by the ingestion of host cell cytosol and the subsequent conversion

of host nitrate to NO by an unknown parasite nitrate reductase (Ostera *et al.*, 2008; Ostera *et al.*, 2011). By contrast, Rey and colleagues reported that there to be an NO pool present throughout the parasite, sustained by an arginine-dependent pathway (Rey *et al.*, 2014).

Although the source of NO in the parasite is uncertain, it has been demonstrated that *P. falciparum* parasites in culture deplete arginine from the culture medium over a 48-h period (Olszewski *et al.*, 2009). It was postulated that a 'futile' arginine metabolism may contribute to the development of hypoarginemia in patients with severe malaria (Olszewski *et al.*, 2009); however, the biochemical mechanisms underpinning the depletion of extracellular arginine have not been determined.

In this study, we have investigated the uptake and metabolism of arginine in the asexual-stage *P. falciparum*-infected erythrocyte. We show that, following invasion, the arginine pool within the infected cell is localized exclusively to the parasite; i.e., the erythrocyte cytosol is depleted of arginine. Arginine enters the infected cell via endogenous host cell transporters and is taken up by the intracellular parasite via a high-affinity cationic amino acid transporter that is influenced by the parasite membrane potential. The disruption of NO metabolism in the infected erythrocyte may have significant consequences for the maintenance of erythrocyte deformability, vascular regulation and malaria pathogenesis.

Results

Arginine metabolism in uninfected and Plasmodium falciparum*-infected erythrocytes*

The metabolism of arginine both in uninfected erythrocytes and in erythrocytes infected with mature asexual *P. falciparum* parasites was investigated using stable-isotope labelling and liquid chromatography mass spectrometry (LC-MS) detection. Uninfected red blood cells (uRBCs) and enriched (>95% parasitaemia) trophozoite-stage-infected red blood cells (iRBCs) were each suspended in Roswell Park Memorial Institute (RPMI) containing 6-^{13}C-arginine (i.e. arginine labelled with ^{13}C at all six carbon positions) at the concentration present in RPMI culture medium (1.1 mM noting that this is significantly higher than the normal serum concentration of 75–218 μM (Psychogios *et al.*, 2011)). The samples were incubated for 3 h at 37°C, and the cells were then extracted. And the extracts subjected to LC-MS analysis. The total size of the arginine pool in iRBCs was approximately 1.6 fold than in uRBCs (Fig. 1A). However, whereas in uRBCs, the arginine pool was comprised entirely of the + 6 isotopologue (parental mass plus six atomic mass units; referred to henceforth as M + 6), and in iRBCs, the M + 6 species comprised approximately half of the arginine pool, with the other half comprised of unlabelled arginine (referred to as M0, and likely to be derived from haemoglobin catabolism).

In the urea cycle, the enzyme arginase converts arginine to ornithine with the loss of one carbon, in the form of urea (Fig. 1A). The next reaction in the cycle, catalysed by ornithine carbamoyl transferase, involves the incorporation of a carbon atom (from carbamoyl-phosphate) to form citrulline. Citrulline is converted, via argino-succinate, back to arginine. One turn of the cycle therefore converts M + 6 arginine to M + 5 arginine, via M + 5 citrulline. The fact that there was no M + 5 arginine detected in either uRBCs or iRBCs is consistent with the absence of a functional urea cycle in either the parasite or host cell. The absence of labelling of arginine following the addition of ^{13}C-labelled fumarate to iRBCs (data not shown) and the fact that the urea cycle intermediate argino-succinate was undetectable in iRBC extracts via the methods used here both provide additional support for the absence of a functional urea cycle. Nevertheless, the urea cycle intermediates ornithine and citrulline were detected in both cell types, with significantly lower levels in iRBCs than in uRBCs ($P < 0.05$; Fig. 1A).

Arginine is converted to ornithine (by arginase), which can either be converted to citrulline (via ornithine carbamoyl transferase), utilized for biosynthesis of the polyamine putrescine (via ornithine decarboxylase) or exported out of the infected cell, as has been observed to occur (Olszewski *et al.*, 2009). The ornithine pool in iRBCs was approximately 20% the size of that in uRBCs and had a labelling pattern similar to arginine, with approximately half of the pool composed of the labelled species (in this case M + 5) and half composed of the unlabelled species (Fig. 1A). The citrulline pool in iRBCs was approximately 40% the size of that in uRBCs (Fig. 1A) and was composed primarily of the unlabelled species (M0) and the completely ^{13}C-labelled form (M + 6). The M + 6 species comprised 42 ± 15% of the citrulline pool in iRBCs (compared with only 3 ± 2% in uRBCs), indicating a much higher turnover of the citrulline pool in infected than in uninfected cells. The presence of the M + 6 citrulline species is consistent with the formation of citrulline directly from arginine (Fig. 1A). M + 5 citrulline comprised just 7 ± 4% of the total citrulline pool in infected cells, consistent with ornithine carbamoyltransferase contributing a minor component of citrulline biosynthesis.

The direct conversion of arginine to citrulline may be catalysed either by NOS or via an arginine deiminase. The *P. falciparum* genome does not encode an obvious arginine deiminase but does encode a putative NOS (PF3D7_0923200).

In order to determine *where* in the infected cell arginine was undergoing metabolism, we conducted a compartmental analysis of infected erythrocytes, using rapid saponin-permeabilisation of the erythrocyte membrane to release the contents of the erythrocyte cytosol, while leaving

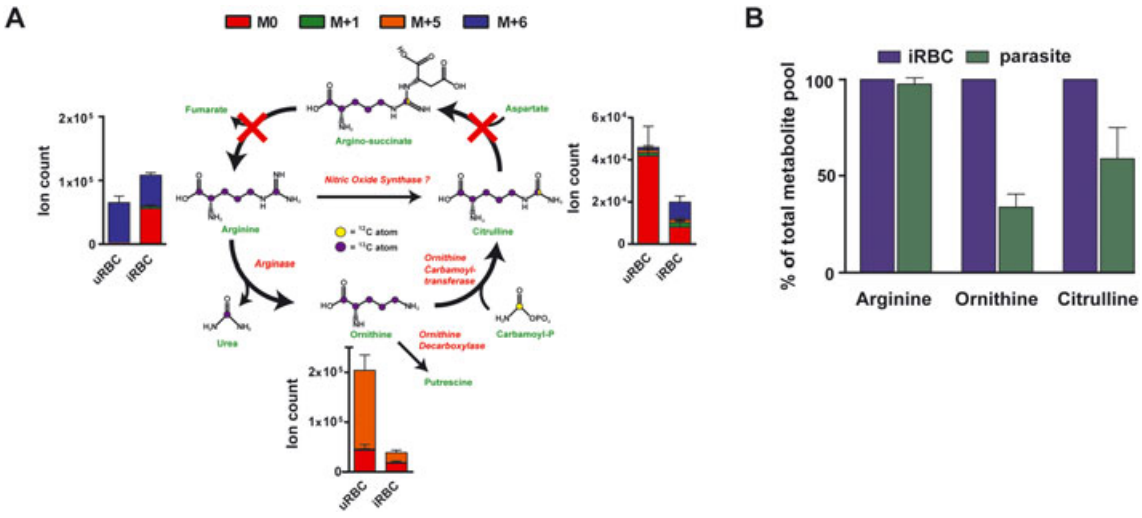

Fig. 1. 6-^{13}C-arginine labelling of uninfected and *Plasmodium falciparum*-infected erythrocytes.
A. *P. falciparum*-infected erythrocytes (> 95% parasitaemia; trophozoite stage) were resuspended in RPMI containing 6-^{13}C-arginine (1.1 mM) and incubated for 3 h. ^{13}C-labelling was determined using the atomic mass shifts arising from incorporation of ^{13}C: M0 = unlabelled, M + 1 = containing one ^{13}C atom, M + 2 = containing two ^{13}C atoms, M + 5 = containing five ^{13}C atoms and M + 6 = containing six ^{13}C atoms. ^{13}C atoms are depicted as purple circles and ^{12}C atoms as yellow circles. Two alternative labelling patterns can be used to discriminate between the possible citrulline-biosynthetic routes. Citrulline formed via ornithine will incorporate an unlabelled carbon, whereas direct formation of citrulline from arginine will maintain complete ^{13}C-labelling at each carbon; half-and-half colouring indicates the two possible labelling patterns. The data are presented as total ion count detected from 10^8 uninfected red blood cell (uRBC) or infected red blood cell (iRBC) erythrocytes. They were corrected for natural abundance and are presented as mean ± standard error of the mean from five independent experiments. The standard urea cycle is shown, with those steps not observed in *P. falciparum*-infected erythrocytes indicated with a red cross.
B. Compartmentalization of arginine-related metabolites in infected erythrocytes. The distribution of arginine, ornithine and citrulline between the parasite and host erythrocyte was determined by comparing the metabolite profile of intact iRBCs with that of parasites isolated from the contents of the host cell compartment by saponin-permeabilisation of the erythrocyte membrane and the release, thereby, of the contents of the erythrocyte cytosol. A suspension of enriched iRBCs (parasitaemia > 95%) was divided into two, with one half treated with saponin before washing in ice-cold PBS to remove the contents of the erythrocyte cytosol and the other simply washed in ice-cold PBS to preserve the entire contents of the infected cells. The relative ion signal between infected erythrocytes and saponin-isolated parasites is presented as mean ± standard error of the mean from $n = 3$.

the parasite intact. As shown in Fig. 1B, the arginine pool of the iRBC was associated wholly with the parasite, i.e., little if any remained in the erythrocyte compartment. By contrast, ornithine and citrulline were more evenly distributed between parasite and host compartments.

Arginine is taken up into the infected erythrocyte via endogenous transporters

It is evident from the LC-MS data that ^{13}C-labelled arginine is taken up from the extracellular solution, into both uninfected and *P. falciparum*-infected erythrocytes and that within the parasitised cell arginine is sequestered by the intracellular parasite, thereby depleting the host cell compartment. The uptake of arginine, both into the infected erythrocyte and into the parasite itself, was investigated using radiolabelled [^{14}C]arginine.

The initial rate of uptake of radiolabelled amino acid into uRBCs was similar to that into iRBCs (Fig. 2A); however, whereas in uRBCs, the uptake of [^{14}C]arginine levelled off within 45–60 min, and in iRBCs, the uptake of the radiolabelled amino acid increased approximately linearly with time for up to 60 min, reflecting the incorporation of radiolabelled arginine into protein by the intraerythrocytic parasite (Supporting Information Fig. 1).

For both neutral (Martin and Kirk, 2007; Cobbold *et al.*, 2011) and acidic (Kirk *et al.*, 1999; Winterberg *et al.*, 2012) amino acids, the new permeability pathways (NPPs) induced by the parasite in the erythrocyte membrane serve as a major route of entry into the infected cell. However, the NPP inhibitor furosemide had no significant effect on the initial rate of [^{14}C]arginine uptake into iRBCs (Fig. 2A), consistent with the NPP not making a significant contribution to the uptake of this basic amino under the conditions tested here.

Arginine is taken up into uninfected erythrocytes via a combination of two transporters, known as system 'y$^+$' and system 'y$^+$L' (Torrents *et al.*, 1998; Pfeiffer *et al.*, 1999; Closs *et al.*, 2004; Rotoli *et al.*, 2009). The relative contributions of these systems to the uptake of arginine into uRBCs and iRBCs under the conditions of our experiments were investigated by measuring the uptake of [^{14}C]arginine in the presence of leucine (a competitive inhibitor of arginine transport via system y$^+$L), lysine (a competitive inhibitor of arginine transport via both system y$^+$ *and* y$^+$L) and/or NEM, an effective inhibitor of system y$^+$. Leucine (2 mM) caused a

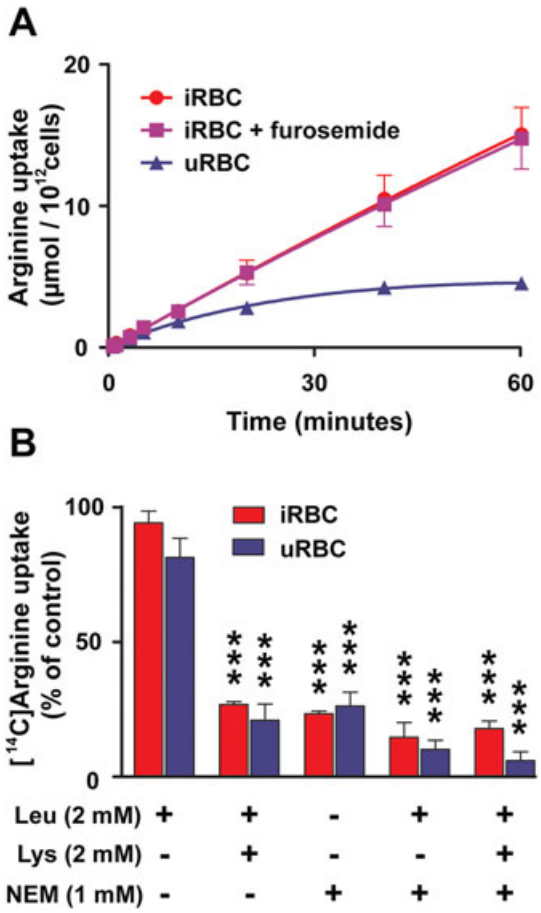

Fig. 2. Uptake of [^{14}C]arginine into uninfected red blood cells (uRBCs) and infected red blood cells (iRBCs).
A. Time courses for [^{14}C]Arginine uptake were measured at 37°C in the presence of physiological concentrations of the naturally occurring amino acids (including 88 μM arginine) and, for iRBCs, in the presence or absence of furosemide (200 μM) an inhibitor of the broad-specificity new permeability pathways induced by the parasite in the erythrocyte plasma membrane. [^{14}C]Arginine uptake is presented in terms of the total incorporation of [^{14}C]radiolabel into infected and uninfected erythrocytes. The data are presented as the mean ± standard error of the mean from three independent experiments. B. The contribution of the endogenous transport systems y^+ and y^+L to arginine uptake in infected erythrocytes. [^{14}C]Arginine uptake was assessed by measuring the uptake of [^{14}C]arginine over 30 min at 37°C into both iRBCs (red bars) and uRBCs (blue bars) in the absence and presence of 2 mM leucine (to inhibit uptake via system y^+L), 2 mM lysine (to inhibit uptake via both systems y^+ and y^+L) and/or 1 mM NEM (to inhibit system y^+ alone). Cell suspensions exposed to NEM were preincubated for 10 min prior to experimentation. Furosemide (200 μM) was present in all samples. [^{14}C]Arginine uptake (mean ± standard error of the mean from three independent experiments) is shown as a percentage of that measured under control conditions. ***denotes $P < 0.001$ for a particular treatment compared with the control (one-way analysis of variance with Dunnett's post-hoc testing).

small (< 5%) decrease in [^{14}C]arginine influx (Fig. 2B), consistent with system y^+L making only a minor contribution to arginine uptake under the conditions of the experiment. By contrast, the system y^+ inhibitor NEM (1 mM) slowed uptake of [^{14}C]arginine in both uninfected and infected erythrocytes by over 70% ($P < 0.001$). The reduction in [^{14}C]arginine uptake seen in NEM-treated cells is similar to that caused, in both uRBCs and iRBCs, by the combination of 2 mM leucine and 2 mM lysine (which, together, inhibit both the systems y^+ and y^+L). The results indicate that the uptake of arginine into iRBCs is via the same mechanism as that into uRBCs, with the NEM-sensitive system y^+ mediating the majority of the arginine uptake in both uRBCs and iRBCs and system y^+L making a much smaller contribution under the conditions tested.

Arginine is taken up into the intracellular parasite via a high-affinity transporter

The uptake of arginine into the parasite itself was investigated using parasites that had been functionally isolated from their host erythrocytes by saponin-permeabilisation of the host erythrocyte membrane. Uptake was measured both into parasites suspended in glucose-containing medium (and therefore ATP-replete) and into parasites preincubated and resuspended in glucose-free medium (and therefore ATP-depleted; (Saliba and Kirk, 1999)). In all such isolated parasite experiments, incorporation of the radiolabel into protein was prevented by the inclusion in the media of protein synthesis inhibitors. In ATP-replete parasites (in glucose-containing medium), the radiolabel accumulated rapidly, reaching a distribution ratio (i.e. the intracellular concentration of radiolabel relative to the extracellular concentration of radiolabel) of approximately 14 within 5 min before undergoing a gradual decline (Fig. 3A). By contrast, in ATP-depleted parasites (in glucose-free medium), the radiolabel equilibrated to a distribution ratio of approximately 1.5 within the first 5 min and remained at this level thereafter. The accumulation of radiolabel by isolated parasites is therefore ATP-dependent.

The effect of the parasite's membrane potential on arginine transport was investigated by measuring the initial rate of [^{14}C]arginine uptake into ATP-replete parasites (measured over 30 s) under conditions in which the membrane potential was manipulated using ionophores or by altering the extracellular K^+ concentration. Depolarisation of the parasite plasma membrane by the addition of the H^+ ionophore CCCP or the addition of the K^+-selective ionophore valinomycin (Allen and Kirk, 2004) significantly reduced [^{14}C]arginine uptake (Fig. 3B; $P < 0.001$ and $P < 0.01$, respectively). Increasing the extracellular K^+ concentration (which causes a more modest depolarization of the parasite plasma membrane) caused a slight apparent decrease in [^{14}C]arginine uptake; however, this was not statistically significant (Fig. 3B; $P > 0.05$). By contrast, under conditions in which the parasite plasma membrane was hyperpolarized, by reducing the extracellular [K^+] to zero, [^{14}C]arginine uptake increased to approximately 140% of that measured

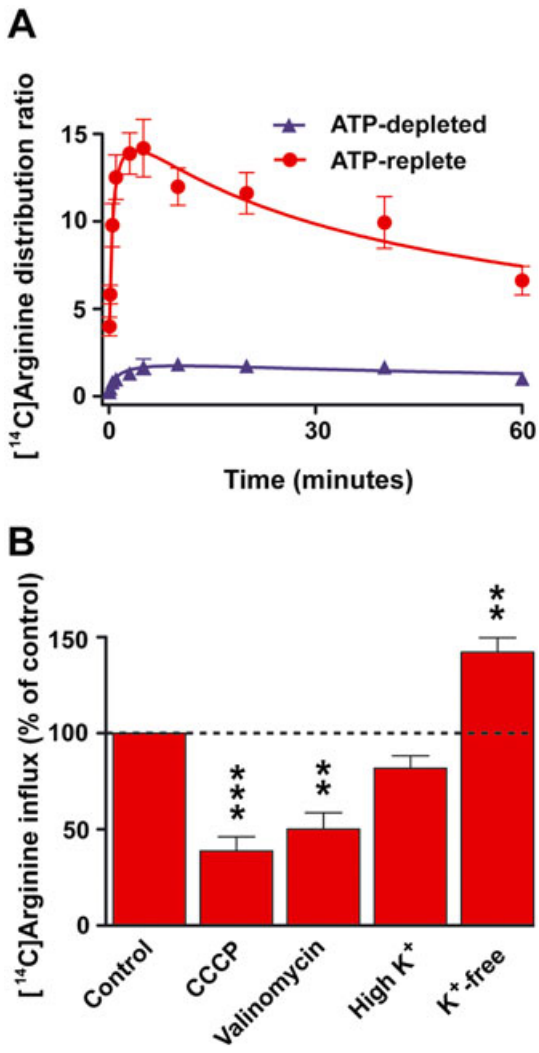

Fig. 3. Uptake of [^{14}C]arginine into saponin-isolated *Plasmodium falciparum* trophozoites. Protein synthesis was inhibited by the inclusion of cyclohexamide (40 μM) and anisomycin (150 μM) in all experiments.

A. Influx of [^{14}C]arginine into ATP-replete or ATP-depleted parasites at 37°C. The extracellular arginine concentration was 10 μM. The distribution ratio is the concentration of (acid-soluble) radiolabel within the parasite relative to that in the extracellular medium.

B. Effect of manoeuvres that influence the membrane potential on the influx of [^{14}C]arginine influx into ATP-replete isolated parasites. Influx is given as a percentage of that measured under control conditions (ATP-replete isolated parasites in standard Hepes-buffered saline solution). For the CCCP experiment, pH_o was 7.35, under which conditions $pH_o \approx pH_i$, and the addition of the protonophore CCCP therefore had little effect on pH_i (Lehane *et al.*, 2004). All other experiments were carried out at $pH_o = 7.1$. CCCP and valinomycin were added at a concentration of 10 μM and 1 μM, respectively. For the high-K^+ condition, the NaCl normally present in the medium (125 mM) was replaced with KCl, resulting in a final K^+ concentration of 130 mM. In the K^+-free medium the 5 mM KCl present under control conditions was replaced with an additional 5 mM NaCl. [^{14}C]Arginine influx was, in each case, measured over 30 s, and the statistical significance between [^{14}C]arginine influx under control conditions and each of the different conditions tested was determined using a one-way analysis of variance with Dunnett's post-hoc testing (**denotes $P < 0.01$ and ***denotes $P < 0.001$). The data are averaged from three to five separate experiments performed on different days, each on cells from different donors, and are shown ± standard error of the mean.

under control conditions ($P < 0.01$). Together, the data are consistent with the uptake of arginine by the parasite being membrane-potential dependent.

Experiments in which arginine uptake into isolated (ATP-depleted) parasites were measured in parasites suspended in media having a range of arginine concentrations revealed Michaelis–Menten kinetics, with a K_m of 40 ± 9 μM and $V_{max} = 227 \pm 11$ μM/(10^{12} cells · h) ($n = 4$; Fig. 4A).

The interaction of other potential substrates with the arginine transport system was investigated in experiments in which the uptake of [^{14}C]arginine was measured into (ATP-depleted) parasites suspended in the presence or absence of a range of unlabelled amino acids, each at a concentration of 5 mM (Fig. 4B). [^{14}C]arginine uptake was significantly reduced in the presence of unlabelled cationic amino acids (ornithine, arginine, lysine and histidine; all $P < 0.001$), whereas the neutral and anionic amino acids were without significant effect ($P > 0.05$).

The reduction in the uptake of [^{14}C]arginine in the presence of ornithine and lysine is consistent with these two amino acids competing with arginine for the transport system(s) involved. To explore further the potential interaction of ornithine and lysine with the putative transporter, we compared the uptake of [^{14}C]arginine into ATP-depleted parasites under conditions in which parasites had been preincubated and resuspended in the presence of a 1 mM concentration of unlabelled arginine, lysine, ornithine or leucine (i.e. the unlabelled amino acids were present in both the intracellular and extracellular solutions, referred to here as 'exchange' conditions) with that into parasites suspended under conditions in which 1 mM of arginine, lysine, ornithine or leucine was present in the extracellular medium alone (nominally 'zero-*trans*' conditions). Pre-loading parasites with unlabelled arginine, lysine or ornithine (i.e. exchange conditions) caused a significant increase in [^{14}C]arginine influx relative to that seen under the corresponding nominally zero-*trans* conditions (Fig. 4C). By contrast, there was no such '*trans*-stimulatory' effect ($P > 0.2$) observed when the parasites were preloaded with the neutral amino acid leucine. The *trans*-stimulation of [^{14}C]arginine influx by intracellular arginine, lysine or ornithine is consistent with the transport system(s) operating more effectively when the radiolabelled amino acid is able to enter the parasite in exchange for intracellular arginine, lysine or ornithine than in the absence of intracellular amino acids with which exchange can occur.

Together, the data are consistent with the parasite having at its surface a basic amino acid transporter, which interacts with arginine, lysine, histidine and ornithine, has a high (micromolar) affinity for arginine and transports it via a membrane potential-dependent mechanism.

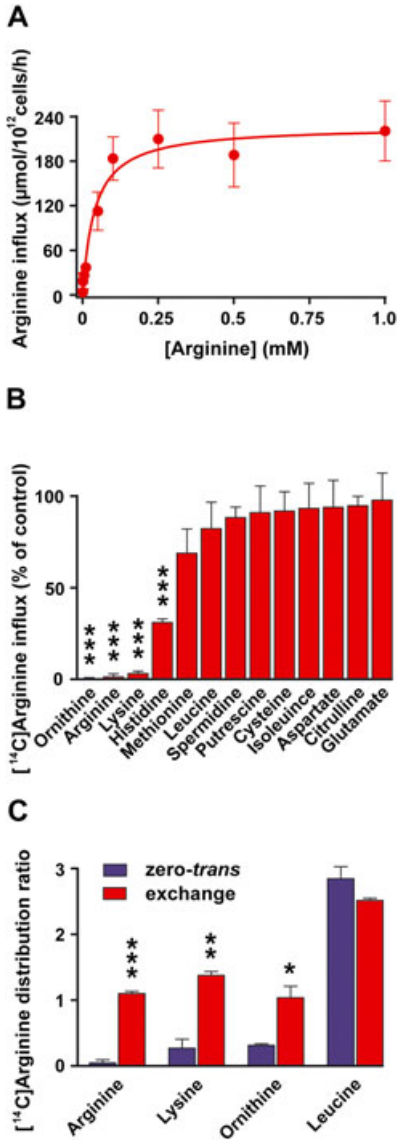

Fig. 4. Kinetics and substrate specificity of [^{14}C]arginine influx into isolated ATP-depleted *Plasmodium falciparum* trophozoites. Protein synthesis was inhibited by the inclusion of cyclohexamide (40 µM) and anisomycin (150 µM) in all experiments.
A. The concentration dependence of arginine influx was determined by measuring the uptake of [^{14}C]arginine over 30 s at 37°C in media in which the concentration of unlabelled arginine ranged from 0.5 µM to 1 mM. Symbols show data averaged from four separate experiments, ± standard error of the mean (SEM). The Michaelis–Menten equation was fitted to the data, yielding $K_m = 40 \pm 9$ µM and $V_{max} = 227 \pm 11$ µM/(10^{12} cells · h).
B. The effect of unlabelled amino acids, each added at a concentration of 5 mM on [^{14}C]arginine influx, was measured over 30 s at 37°C. The data are from $n = 3$ experiments and are presented as mean ± SEM. ***denotes $P < 0.001$ using a one-way analysis of variance (with Dunnett's post-hoc testing).
C. The ability of (unlabeled) arginine, lysine, ornithine and leucine to *trans*-stimulate the influx of [^{14}C]arginine into isolated ATP-depleted *P. falciparum* trophozoites at 37°C was investigated by comparing the uptake of [^{14}C]arginine measured under nominally 'zero-*trans*' conditions with that measured under 'exchange' conditions. Isolated parasites were washed three times in an amino acid-free solution and then preincubated (10 min, 37°C) in either amino acid-free saline (for the nominally zero-*trans* conditions) or saline supplemented with 1 mM unlabelled arginine, lysine, ornithine or leucine (for the exchange conditions). At the end of the 10 min preincubation, [^{14}C]arginine uptake was measured over 30 s, in parasites suspended (at time-zero) in media containing a 1 mM concentration of either arginine, lysine, ornithine or leucine. Under zero-*trans* conditions, unlabelled amino acid was present in the extracellular medium but nominally absent from the parasite cytosol, whereas under exchange conditions, the parasites were preloaded with the unlabelled amino acid, which was therefore present at similarly high concentrations at both the extracellular (*cis*) and intracellular (*trans*) faces of the parasite plasma membrane throughout the uptake period. The data were collected from $n = 3$ experiments and presented as mean ± SEM. *denotes $P < 0.05$, **denotes $P < 0.01$ and ***denotes $P < 0.001$ using a paired Student's *t*-test.

PF3D7_0629500 encodes a putative transporter that shows some similarity to the *Trypanosoma cruzi* and *Leishmania major* high-affinity arginine transporters (Shaked-Mishan *et al.*, 2006; Carrillo *et al.*, 2010). In experiments to investigate the transport capacity of PF3D7_0629500, the protein was expressed in *Xenopus laevis* oocytes. Although the expressed protein was shown to localize to the oocyte surface, there was no detectable increase in the uptake of radiolabelled arginine, nor of any other radiolabelled substrate tested (Supporting Information Fig. 2).

Discussion

As shown here, arginine in the extracellular medium is taken up into parasitised erythrocytes and into the intracellular parasite, where it undergoes metabolism. Under the (approximately physiological) conditions tested here, arginine entered iRBCs primarily via the endogenous erythrocyte transporter system y^+. There was no significant flux of arginine via the parasite-induced NPPs, consistent with results of early haemolysis experiments that indicated that the parasite-induced pathways have a very low permeability to basic amino acids (Ginsburg *et al.*, 1985). Although the NPPs do have a significant permeability to both neutral and acidic amino acids (Ginsburg *et al.*, 1985, Martin and Kirk, 2007; Cobbold *et al.*, 2011) and to monovalent organic (Staines *et al.*, 2000) and inorganic (Staines *et al.*, 2001) cations, they are impermeable to the divalent cation Ca^{2+} (Staines *et al.*, 1999), and the presence of two positive charges on the zwitterionic arginine mole-

cule is likely to be a significant impediment to its passage via the NPP.

The uptake of arginine from the host erythrocyte compartment into the intraerythrocytic parasite is via a high-affinity transporter that interacts with a range of basic amino acids. The uptake of arginine by the parasite leaves the host cell arginine pool largely depleted. Within the parasite arginine is metabolized to produce ornithine and citrulline. Ornithine has been shown previously to be exported from parasitised erythrocytes (Olszewski *et al*., 2009). The finding here that the rate of uptake of arginine by the intraerythrocytic parasite was much higher when the parasite was preloaded with ornithine than in the absence of ornithine (or any of the other candidate substrates) points to a physiological role for the parasite plasma membrane transporter in mediating the import of arginine in exchange for the export of ornithine.

The biphasic nature of the time-course for the uptake of arginine by isolated parasites is somewhat unusual. The accumulation of radiolabel to high levels and the subsequent time-dependent decrease in the amount of radiolabel retained by the parasites are ATP-dependent, consistent with the involvement of metabolic processes and/or the parasite's inwardly-negative membrane potential. However, what proportion of the accumulated radiolabel remains as arginine (as opposed to its metabolites), what becomes of the membrane potential over the time-course of the experiment and in what form the radiolabel effluxes from the cell following the accumulation reaching a maximum value at around 5 min are not known.

The molecular identity of the parasite's arginine transporter, and those of the enzymes responsible for the direct conversion of arginine to citrulline, remains to be established. A putative amino acid/auxin permease (encoded by PF3D7_0629500; (Martin *et al*., 2005)) shows some similarity to the *T. cruzi* and *L. major* high-affinity arginine transporters (Shaked-Mishan *et al*., 2006, Carrillo *et al*., 2010). However, injection of cRNA encoding this protein into *X. laevis* oocytes yielded no significant increase in the uptake of arginine, nor that of any other amino acid, despite the protein being expressed and localized to the oocyte plasma membrane.

As noted in Fig. 1A, the high prevalence of M + 6 citrulline in infected cells is consistent with the conversion of arginine to citrulline being mediated by a nitric oxide synthase. *P. falciparum* encodes a putative NOS (PF3D7_0923200), which contains three of the four domains found in most NOSs characterized to date. However, despite the presence of flavodoxin-like domain and both NADP-binding and FMN-binding domains, the protein lacks an obvious oxygenase domain, which facilitates the NOS protein binding of haem. It is possible that the *P. falciparum* NOS is so divergent that the oxygenase domain lacks significant homology to that of characterized NOS sequences or that the enzyme functions as a heteromer, with a yet-to-be-identified partner (Ostera *et al*., 2008). Alternatively, the conversion of arginine to citrulline in iRBCs may involve a parasite arginine deiminase; however, there is no obvious arginine deiminase homolog encoded in the *P. falciparum* genome.

The depletion of the host erythrocyte arginine pool by the parasite may have significant pathophysiological consequences, preventing the synthesis of NO by the host cell. Disruption of erythrocyte NO metabolism by the parasite could contribute to the reduced deformability of the erythrocyte and thereby could inhibit circulation of infected erythrocytes through capillaries. Rey and colleagues recently demonstrated that the deformability of infected erythrocytes is, in part, dependent on exogenous arginine, and hence NO biosynthesis (Rey *et al*., 2014). The molecular identity of the enzyme(s) involved is yet to be resolved, as is that of the high-affinity transporter, which mediates the uptake of arginine into the parasite, thereby depleting arginine from the host cell compartment. Identifying these proteins and determining their contributions both to parasite development and to the pathophysiology of malaria are the subject of ongoing work.

Experimental procedures

Parasite culture

Human erythrocytes infected with *P. falciparum* (strain 3D7 and NF54) were maintained under standard culturing conditions (Trager and Jensen, 1976; Allen and Kirk, 2010). Experiments were carried out using trophozoite-infected cells (approximately 30–35 h post-invasion). Synchronized parasite cultures were obtained by suspending ring-stage cells in 5% w/v sorbitol as described previously (Lambros and Vanderberg, 1979). For experiments with *P. falciparum*-infected erythrocytes, parasite cultures were concentrated to between 85% and 99% parasitaemia using magnetic enrichment (Cobbold *et al*., 2013).

Stable-isotope labelling and chromatography mass spectrometry detection

Stable-isotope labelling and LC-MS detection were performed as described previously (Olszewski *et al*., 2009; Babbitt *et al*., 2012), with minor modifications. 6-^{13}C-Arginine (Cambridge Isotopes, Tewksbury, MA, USA) was added to arginine-free RPMI at a concentration of 1.1 mM. iRBCs were magnetically enriched to $>$ 95% parasitaemia and allowed to recover for 1 h in complete RPMI 1640 media (haematocrit $<$ 1% at 37°C). iRBCs and uRBCs were then resuspended in the ^{13}C-arginine-containing RPMI and incubated for 3 h (1×10^8 cells). Metabolites were then extracted with 1 ml of 90% methanol, dried under nitrogen and then dissolved in HPLC-grade water. Metabolites were separated via liquid chromatography and detected on a Finnigan triple

quadrupole mass spectrometer (Thermo, Waltham, MA, USA) operating in positive mode with selected reaction monitoring.

In experiments to assess compartmentalization of metabolites within the iRBC, iRBCs were magnetically enriched and 1×10^8 cells transferred to microcentrifuge tubes. Samples were treated with 0.02% w/v saponin for 10 s (at room temperature), centrifuged 12 000 × *g* for 15 s and then washed with 1 ml ice-cold PBS before metabolites were extracted with 1 ml 90% v/v methanol as described earlier. An identical cell suspension was left untreated, washed with ice-cold PBS and performed with metabolite extraction to use as the intact infected erythrocyte control. Giemsa smears and haemocytometer counts were performed to confirm the efficiency of saponin treatment and that the cell number had not changed following isolation.

Influx measurements in infected and uninfected erythrocytes

Influx of [^{14}C]arginine into uRBCs and iRBCs was measured as described previously for other amino acids (Martin and Kirk, 2007; Cobbold *et al.*, 2011). These measurements were carried out in the presence of the naturally occurring amino acids at concentrations typical of those present in human plasma (Psychogios *et al.*, 2011): alanine (356 μM), arginine (88 μM), asparagine (13 μM), aspartate (13 μM), cystine (37 μM), glutamate (57 μM), glutamine (476 μM), glycine (217 μM), histidine (85 μM), hydroxyproline (8 μM), isoleucine (70 μM), leucine (100 μM), lysine (163 μM), methionine (17 μM), phenylalanine (100 μM), proline (165 μM), serine (128 μM), threonine (112 μM), tryptophan (50 μM), tyrosine (62 μM) and valine (190 μM). The inclusion of the full range of amino acids at concentrations present in human plasma allows an assessment of the relative contributions of different transport systems to amino acid uptake under conditions approximating those that prevail *in vivo*.

iRBCs (85–99% parasitaemia) or uRBCs (maintained under culture conditions for 48 h prior to experimentation) were suspended at a concentration of approximately 5×10^7 cells/ml at 37°C in a Hepes-buffered saline solution (130 mM NaCl, 25 mM Hepes, 5 mM KCl, 20 mM glucose, 1 mM $MgCl_2$ and supplemented with RPMI 1640 vitamins and glutathione (Invitrogen, Carlsbad, CA, USA); pH 7.4). An equal volume of [^{14}C]arginine-containing saline solution (identical to the Hepes-buffered saline but supplemented with two times the concentration of amino acids listed earlier and, where appropriate, other compounds) was dispensed into a microcentrifuge tube and warmed to 37°C. Influx commenced with the addition of an equal volume of cell suspension, immediately followed by mixing. At predetermined times, 200 μl aliquots of cell suspension were transferred to microcentrifuge tubes containing 300 μl dibutyl phthalate (density 1.04 g ml^{-1}) layered over 30 μl 30% v/v perchloric acid. The tubes were centrifuged immediately (12 000 × *g*; 1 min) to sediment the cells through the oil and into the acid layer, thereby terminating the flux, lysing the cells and precipitating the protein. The remaining radiolabelled solution was aspirated, and the sides of the tube were washed three times to remove residual radioactivity before the remaining oil was then aspirated. Trichloroacetic acid (1 ml, 5% w/v) was added to the perchloric acid extract, and the sample was then centrifuged (12 000 × *g*; 8 min). The radioactivity in the supernatant solution (approximately 1 ml; referred to here as 'acid-soluble') was measured using a β-scintillation counter. The acid-insoluble protein pellet at the bottom of the microcentrifuge tube was dissolved, and the radioactivity was measured as described previously (Martin and Kirk, 2007).

The volume of extracellular solution (and hence the amount of radiolabel) trapped between the cells as they were centrifuged through the oil layer was estimated by taking replicate samples as quickly as possible after combining the cells and radiolabel, then centrifuging these through an oil layer. The amount of radiolabel in the extracellular space was subtracted from the total radiolabel in the cell pellet to give the amount of radiolabel associated with the cells.

Influx measurements in isolated parasites

Parasites were functionally isolated from their host erythrocytes by the addition to *P. falciparum*-infected erythrocyte cultures of 0.05% w/v saponin. The isolated parasites were washed three times with a minimal saline solution (NaCl 125 mM, KCl 5 mM, Hepes 25 mM, glucose 20 mM, $MgCl_2$ 1 mM at pH 7.1) and then allowed to recover for 15 min prior to experimentation. Influx of [^{14}C]arginine (Perkin-Elmer, Waltham, MA, USA) was measured at 37°C with parasites suspended at a concentration of $0.5–1.0 \times 10^8$ cells ml^{-1} in the presence of the protein synthesis inhibitors cycloheximide (40 μM) and anisomycin (150 μM) (Sigma-Aldrich, St. Louis, MO, USA) (Martin and Kirk, 2007). In experiments in which the parasites were depleted of ATP, the parasites were preincubated for 20 min and resuspended in glucose-free medium (NaCl 135 mM, KCl 5 mM, Hepes 25 mM, $MgCl_2$ 1 mM at pH 7.1).

The prolonged incubation of the isolated parasites in minimal saline (> 20 min in total) prior to measuring the uptake of radiolabel resulted in a depletion of intracellular amino acids (unpublished data), such that amino acid influx measurements were carried out under nominally 'zero-*trans*' condition. The uptake of radiolabel was initiated by combining an aliquot of the suspension of isolated parasites with an equivalent volume of solution containing [^{14}C]arginine. Uptake was terminated at predetermined times by centrifuging the isolated parasites through an oil layer comprising a blend of dibutyl phthalate and dioctyl phthalate (5:4; 1.015 g ml^{-1}), into 30% v/v perchloric acid. Samples pelleted below the oil were collected and processed for β-scintillation counting.

In experiments in which the ability of intracellular (unlabelled) amino acids to stimulate the influx of [^{14}C]-arginine was investigated, isolated ATP-depleted parasites were suspended in Hepes-buffered saline in the presence of protein synthesis inhibitors cycloheximide (40 μM) and anisomycin (150 μM).

Unlabeled amino acids were added individually (at a concentration of 1 mM) and allowed to equilibrate then, at time-zero, an identical suspension (containing the unlabeled amino acid) with [^{14}C]-arginine was mixed 1:1. After 30 s incubations, the parasites were centrifuged through an oil layer, and the pellets were processed for β-scintillation counting. The comparative 'zero-*trans*' condition was equilibrated in an identical solution without any amino acids, and at time-zero, an identical suspension (containing the unlabeled amino acid) with [^{14}C]-arginine was mixed 1:1. The zero-*trans* condition was sampled and processed as described earlier.

As in experiments with intact erythrocytes, the volume of extracellular solution (and hence the amount of radiolabel) trapped between the cells as they were centrifuged through the oil layer was estimated by taking replicate samples as quickly as possible after combining the cells and radiolabel, then centrifuging these through an oil layer. Intracellular concentrations of radiolabelled solute (and hence the 'distribution ratio'; i.e. the concentration of radiolabel inside the cell, relative to that in the extracellular solution) were calculated from the amounts of radiolabel taken up by the cells, in conjunction with previous estimates of the water volume of saponin-isolated trophozoite-stage parasites (28 fL) (Saliba *et al*., 1998).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Distribution of radiolabel between acid-soluble and acid-insoluble pools in iRBCs incubated with [14C]arginine at 37°C. The extracellular medium contained all of the common, naturally occurring amino acids at concentrations that fall within the normal plasma range. The data are averaged from 3 separate experiments performed on different days, each on cells from different donors and are shown ± S.E. Where not shown, error bars fall within the symbols.

Fig. S2. Heterologous expression of PF3D7_0629500 in Xenopus laevis oocytes. Dark field image, bright field image, and an overlay of the two, for (A) oocytes injected with 'Elution Buffer' alone (20 nL), and (B) oocyte injected with the same volume of Elution Buffer in which was dissolved cRNA (40 ng) encoding an C-terminally HA-tagged form of PF3D7_0629500. Using commercially available antibodies specific for the HA epitope, the localisation of each protein was determined from the fluorescence arising from the secondary antibody. Each image is representative of images taken from multiple oocytes, originating from three different frogs. (C) Uptake of radiolabelled forms of candidate substrates into oocytes expressing (untagged) PF3D7_0629500. Oocytes were injected with cRNA encoding PF3D7_0629500 and tested for their ability to take up a range of radiolabelled amino acids and metabolites (grey bars). EAAT3, the mammalian glutamate transport, was used as a positive control (black bar). The uptake of each substrate was measured (5 days post-injection) over 1 or 2 h. For all substrates tested, 10 μ of the unlabelled form was present to reduce binding of the radiolabel to the oocyte surface. The data is presented as the mean ± S.E from different experiments (n = 2–15), utilizing oocytes from different frogs and shown in terms of fold-increase relative to the negative control (i.e. the uptake measured in oocytes expressing the P. falciparum Formate/Nitrate Transporter (PfFNT, PF3D7_0316600)). Statistical significance was determined using a paired Student's t-test; *denotes $P < 0.05$.