Glycolysis in *Plasmodium falciparum* results in modulation of host enzyme activities

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**Abstract**

**Background & objectives:** *Plasmodium falciparum*, the causative agent of the most serious form of malaria, infects about 5–10% of the world’s population per year. It is well established that the erythrocytic stages of the malaria parasite rely mainly on glycolysis for their energy supply. In the present study, the glucose utilisation of erythrocyte population with parasitaemia levels similar to that of malaria patients was measured. The results allowed us to assess the effect of the parasites on the glucose utilisation of the vast majority of uninfected erythrocytes.

**Methods:** Using \([2-^{13}\text{C}]\)glucose and nuclear magnetic resonance (NMR) technique, the glucose utilisation in normal red blood cell (RBC) and *P. falciparum* infected red blood cell (IRBC) populations was measured. The IRBC population consisted of > 96% RBC and < 4% of parasite infected red blood cells (PRBC). The glycolytic enzymes were assayed to assess the effect of infected red cells on the enzymatic activities of uninfected ones.

**Results:** The rate of glucose utilisation by IRBC was considerably higher than that of RBC. Upon addition of 25% v/v conditioned culture medium (CM) of IRBC, RBCs exhibited a significant decrease in glucose utilisation. The CM could directly inhibit the activities of RBC glycolytic enzymes—phosphofructokinase (PFK) and pyruvate kinase (PK), without interfering with the activity of the pentose phosphate pathway enzyme—glucose-6-phosphate dehydrogenase (G-6-PD).

**Interpretation & conclusion:** The present study showed that the clinical level of *P. falciparum* infected RBCs (< 4% parasitaemia) significantly enhance the glycolytic flux as well as down-regulate the glucose utilisation rate in the majority of uninfected RBC population. The mechanism of inhibition seems to be direct inhibition of the regulatory glycolytic enzymes—PFK and PK.

**Key words** Erythrocytic stages – glucose-6-phosphate dehydrogenase – glycolysis – malaria – phosphofructokinase – *Plasmodium falciparum* – pyruvate kinase

**Introduction**

*Plasmodium falciparum*, the causative agent of malaria, infects about 5–10% of world’s population and kills about 2 million children every year. The parasite goes through a complex life-cycle and the erythrocytic stages of *P. falciparum* are responsible for the pathology in humans. Hypoglycemia and lactic...
Acidosis are often associated with severe malaria, and one of the reasons for hyperlactatemia or acidosis is assumed to be the increased anaerobic glycolysis by the infected erythrocytes\textsuperscript{1,2}. During its intra-erythrocytic growth phase, the malarial parasite relies mainly on glycolysis for its energy requirements\textsuperscript{3,4}. Activation of several enzymes of the glycolytic pathway has also been reported in studies performed with parasite-enriched population and with red cell-free parasites. It has been documented that the parasite-infected red blood cells (PRBCs) utilise glucose at a rate much higher than that of the normal red blood cells (RBCs)\textsuperscript{5-7}. The emphasis of these studies has been to assess the glucose utilisation capabilities of the parasite or parasite-infected red cells.

In a malaria patient the percent of parasite infected RBC rarely exceeds 3–4\%, and is generally around 0.1–1\% (4,000–40,000/µl)\textsuperscript{8}. Thus in a patient blood, which contains largely uninfected RBCs, is there any influence of the small fraction of PRBC on the glucose utilisation of the vast majority of uninfected RBCs? We have attempted to address this question by studying the metabolites produced by a red cell population (IRBC) containing 0.5–4\% PRBC using NMR spectroscopy. In this report we present observations which demonstrate the remarkable ability of a small parasitised RBC cohort (IRBC) to down-modulate glucose utilisation in normal uninfected red blood cells, possibly through some secreted product(s).

**Material & Methods**

**Red cells:** Human blood was collected from 25–27 year old healthy individuals with A\textsuperscript{+} blood group in ACD (38 mM citric acid, 75 mM sodium citrate, 136 mM glucose) as the anticoagulant. Informed and free consent was obtained from the donors. The cells were pelleted, buffy coat was discarded to remove the leucocytes, and the erythrocytes were washed thrice with RPMI (RPMI 1640 supplemented with 27 mM NaHCO\textsubscript{3}, 25 mM HEPES, 0.35 mM hypoxanthine). The washed RBCs were then resuspended in complete RPMI (RPMI with 0.5\% albumax). Asexual stages of *P. falciparum* (3D7 strain) were cultured *in vitro* and synchronised by sorbitol treatment as described earlier\textsuperscript{9}. Briefly, the cells were harvested when maximum IRBCs were predominantly rings, washed and treated with 5\% sorbitol (in double distilled water) at 37\°C for 10 min, washed repeatedly with RPMI, and subcultured with RBCs prepared as described above. Parasites were maintained at 5\% hematocrit in complete RPMI at 37\°C in a humidified chamber containing 5\% CO\textsubscript{2}. RBCs were harvested, washed and resuspended at 50–70\% hematocrit in complete RPMI containing D\textsubscript{2}O and used for NMR spectroscopy. The experiments were started by adding solid [2-\textsuperscript{13}C]glucose (Isotech Inc.) to 11 mM final concentration.

**Mouse malarial parasites:** The *P. yoelii* 17XL strain was maintained by blood passage of parasitised erythrocytes through BALB/c mice by intraperitoneal injections. The maintenance and care of the experimental animals was carried out as per the guidelines for use of laboratory animals in research specified by the Animal Ethics Committee of the Institute. Parasitaemia was monitored using Giemsa stained blood smears made periodically from infected mice. Male siblings were used for each set of experiments.

**Various treatments of the RBCs:** For obtaining the conditioned culture medium (CM) the parasite cultures were synchronised, subcultured with fresh RBCs at a final concentration of 0.5–4\% PRBC and incubated at standard culture conditions for 17–20 h. Supernatant from such cultures, filtered through a 0.22 µm filter, was used as the CM at a final concentration of 25% v/v in the NMR experiments. For the *P. yoelii* experiments, the blood plasma from infected mouse was used as the condition medium after filtration.

**NMR experiments:** \textsuperscript{13}C-NMR spectra on the RBCs
were recorded at 125.78 MHz on a Bruker AVANCE AV500 NMR spectrometer. The acquisition parameters were 225 ppm spectral width, excitation pulse of 30° (5 µs), with a 1.2 s delay between pulses. The transients (408 or 1280) were stored in 8192 data points resulting in acquisition time of 0.15 s and a digital resolution of 3.4 Hz/point. Gated decoupling of the protons was achieved by applying a power of 18 dB only during acquisition to minimise the Nuclear Overhauser Effect from the connected protons. The time domain spectra were subjected to exponential multiplication leading to an additional line broadening of 5 Hz before Fourier transformation. Chemical shifts are in ppm with respect to sodium 3-trimethylsilyl propionate, which served as an external reference. For quantification, integrals of the various resonances were determined using the Bruker software and corrected as reported earlier. Glucose utilisation was determined essentially by the method reported for human RBCs.

The effect of CM on invasion of erythrocytes by P. falciparum merozoites: Conditioned medium, taken from P. falciparum culture (Pf CM) or from normal uninfected RBCs (UI CM), was added (25% v/v) to synchronised parasite culture containing mature trophozoites. After allowing one cycle of invasion (24 h after CM addition) the resultant parasitaemia was estimated as mentioned above.

Preparation of cell lysates and enzyme activity assays: RBC lysates were prepared by a modification of the method described by Beutler. The enzyme activity in the cell lysates was followed spectrophotometrically as the oxidation of NADH to NAD (for PFK and PK) or as the reduction of NADP to NADPH (for G-6-PDH), by linking it to the formation of various products in the presence of an excess of auxiliary enzymes. After adding the cell lysate (10 µl), the mixture was incubated at 37°C for 10 min and the reaction was started by adding 2 mM ATP for PFK, and 5 mM PEP for PK, and 10 mM glucose-6-phosphate for G-6-PDH. The decrease in NADH (PFK and PK) or the increase in NADPH (G-6-PDH) was measured at 340 nm for 5 min with a Perkin-Elmer spectrophotometer and the slopes of the curves were determined. The effects of CM on the enzyme activities were assessed using RBC cell lysate to which 25% v/v of the CM, from P. falciparum IRBC culture (Pf CM), or from normal uninfected RBCs (UI CM), was added.

Results & Discussion

Utilisation of [2-13C]glucose by P. falciparum infected RBCs: The earlier studies on glycolysis in Plasmodium infected red cells have been based on either red cell-free parasites or enriched IRBC. In our experiments, the level of parasitaemia (< 4%) was similar to that reported in patients with clinical symptoms of malaria. Thus, 13C-NMR spectra were recorded for infected red blood cell population (IRBC) under physiological conditions (0.5–4% parasitaemia, 50–70% hematocrit, 10–12 mM glucose, pH 7.4 and 37°C). The quantitative estimates of metabolites and flux routed through various metabolic intermediates were determined as described in Methods section. The IRBC cultures consisted mainly of trophozoites (about 17–20 h post-synchronisation). A typical NMR data profile is shown for the utilisation of [2-13C]glucose for both RBC and IRBC (3% parasitaemia) (Fig. 1 a and b), and the data plotted to show the rates of utilisation of glucose, and production of [2-13C]lactate (Fig. 1 c and d). The rate of glucose utilisation of the IRBC was found to be significantly higher as compared to that of uninfected RBC. Concomitantly the rate of [2-13C]lactate, which originates through glycolysis, was found to increase in the IRBC.

The intraerythrocytic asexual growth phase of P. falciparum has a single acrstate mitochondrion and is devoid of TCA cycle, relying mainly on anaerobic glycolysis for its energy requirement. Genes for certain enzymes of TCA cycle are found in the Plasmodium genome but these appear to be involved in
mitochondrial redox control rather than energy metabolism of the parasites\textsuperscript{14,15}. The \textsuperscript{13}C-NMR spectra showed no resonance(s) corresponding to labelled glutamate carbon(s), and therefore, the signatures of aerobic metabolism were not observed. The stoichiometric production and accumulation of lactate confirmed the earlier observations that neither the RBC nor the IRBC possess a functional TCA cycle.

**Parasitaemia and sub-stage dependence of the glucose flux in IRBCs:** Fig. 2 shows the glucose utilisation in IRBC over several experiments. The increase in glucose utilisation was directly proportional to the percent parasitaemia of IRBC, and was consistent with rates observed earlier for *P. falciparum* IRBC determined through other methods\textsuperscript{4}. The glucose flux clearly depended on the sub-stage of the infected red cells (Fig. 3). The glucose utilisation was only marginally higher 2 h after synchronisation (Fig. 3a), and the maximum rate of utilisation was observed for the trophozoite stage, at about 24 h post-synchronisation (Fig. 3a and b).

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*Fig. 1:* Utilisation of [2-\textsuperscript{13}C]glucose by human RBCs infected with malarial parasite *P. falciparum*. Stacked plot of the time-lapse \textsuperscript{13}C-NMR spectra of RBC (a) and *P. falciparum* infected RBCs (IRBC) containing 3% parasitaemia (b) after incubation with [2-\textsuperscript{13}C]glucose. \textsuperscript{13}C-NMR spectra were recorded and the data processed as mentioned in the methods section. Panels (c) and (d) show the concentration profiles of [2-\textsuperscript{13}C]glucose and C2-lactate of uninfected and infected red blood cells. The lines are linear fits for C2-glucose and C2-lactate obtained from the data shown in (a) and (b) respectively.
The infected red cells showed enhanced utilisation of glucose, and extrapolated to a value of ~100-fold higher glucose utilisation in parasite-infected red cells (PRBC), in agreement with values obtained earlier. The rate of glucose utilisation was proportional to the percent parasitaemia and was highest for the trophozoite stage (24 h post-synchronisation), coinciding with the period of maximum metabolic activity of the erythrocytic parasite.

**Glucose utilisation in red cells isolated from mice infected with murine malarial parasite, P. yoelii.** In addition to *P. falciparum*, glucose utilisation was also measured in the murine model of *P. yoelii*. Results qualitatively similar to *P. falciparum* were observed, such as enhancement of glucose utilisation and lactate production rates by IRBC (Fig. 4 a and b). We have shown earlier the effect of conditioned culture supernatant medium (CM) obtained from IRBC on the glucose utilisation of RBCs. A similar inhibition of glucose utilisation by normal mouse RBCs in the presence of the blood plasma isolated from infected mouse was observed (Fig. 4). However, the parasitaemia was high (28%), and the glucose utilisation rate was only 1.4-fold higher than that of RBCs from uninfected mice. Typically in murine malaria samples, the parasitaemia values were higher, and the glucose utilisation did not extrapolate to the 100-fold levels, but was considerably lower. Since the glucose utilisation was not that high, the higher inhibitory effect of blood-plasma indicates that either there is inhibition at high parasite value, or that there may be additional physiological factors present in the blood-plasma. It is possible that various cytokines, released during immune/stress responses to the parasite infection, may be playing roles in such cases. To address the effect of the conditioned medium, therefore, we restricted ourselves to *P. falciparum* cultures.

**The effect of conditioned medium (CM) on invasion of erythrocytes by P. falciparum merozoites and on host-enzyme activities:** To obtain the conditioned medium (CM) from a *P. falciparum* culture, the cultures were synchronised, subcultured with fresh red
cells at a final concentration of 0.5–4% infected RBC and incubated at standard culture conditions for 17–20 h. Supernatant from such cultures, filtered through a 0.22 µm filter, was used as the CM. To rule out the possibility of the CM compromising the infection of RBCs by the parasite, the effect of CM (25%) on the merozoite invasion of RBCs was evaluated (Fig. 5). However, no significant effect of CM on the efficiency of RBC invasion by merozoites, was observed (Fig. 5).

To investigate the mechanism of this inhibition by the CM, it was decided to check if the CM had any direct inhibitory effect on the glycolytic enzymes of the RBC. Three RBC enzymes involved in the glucose metabolism, phosphofructokinase (PFK), pyruvate kinase (PK) and glucose-6-phosphate dehydrogenase (G-6-PDH) were assayed after incubation with culture supernatant from parasite-infected culture (CM-Pf) and from uninfected culture (CM-Uninf) (Fig. 6). The CM from the parasite-infected culture inhibited both the PFK and PK activities of the RBC extract, while the G-6-PDH activity (required for the pentose phosphate pathway) remained unaffected. The CM from uninfected culture (CM-Uninf) had no effect on the activities of any of these enzymes, showing that the CM from IRBCs specifically inhibited the regulatory PFK and PK enzyme activities. Thus the CM from parasite infected cultures can directly inhibit important host-glycolytic
enzymes (PFK and PK) and possibly modulate glucose utilisation of RBCs through these lowered enzymatic activities. We have recently demonstrated that a drop in pH (occurring during glycolysis due to massive lactic acid production) is selectively inhibitory to host PFK, but not for PK enzyme activity. As opposed to pH, CM seems to affect both PFK and PK enzyme activities. Currently we are assessing the factor(s) present in the CM which may be responsible for the inhibition of the enzyme activities.

In addition to differential effects on glycolysis, infection by the parasite is also known to alter the membrane of erythrocytes considerably. It is believed that the period of maximum metabolic activity of the parasite coincides with an increase in the permeability of the infected erythrocyte membrane to a range of small solutes and ions. This enhanced permeability of the infected erythrocytes has been ascribed to (a) activation of endogenous quiescent channels or (b) the induction of new permeation pathways having characteristics similar to anion selective channels. It follows that the membrane of the infected erythrocytes is significantly different from that of uninfected. Several parasite proteins are transported to the red cell membrane. The hexose transporter Glut1 of RBC is the major glucose transporter of RBC. The single hexose transporter present on the parasite membrane shows certain differences from Glut1. Thus the glucose uptake and utilisation pathway of the parasite-infected erythrocytes is likely to be different from uninfected erythrocytes.

The pathological events that contribute to falciparum malaria are the cerebral syndrome, anaemia, hypoglycemia and lactic acidosis. In these assessments, hypoglycemia is determined by the measurement of the actual blood glucose levels. Our results suggest a possibility of ‘functional’ pseudo-hypoglycemia despite the presence of substantial glucose level in the medium. The consequence of such pseudo-hypoglycemia in malaria patients is significant. At a micro-level, where the percent parasite-infected erythrocytes may be higher through sequestration or rosetting, the effect may be even higher. The glucose utilisation rate of other host tissues exposed to the parasite-infected erythrocytes (e.g. vascular endothelial cells) remains to be assessed.

Fig. 6: Effect of conditioned medium (CM) from parasite-infected and uninfected cultures on the enzymes of RBC. Three RBC enzymes involved in the glucose metabolism, phosphofructokinase (a), pyruvate kinase (b), and glucose 6-phosphate dehydrogenase (c) were assayed after incubation with conditioned medium obtained from parasite-infected culture (CM-Pf) and from uninfected culture (CM-Uninf). Enzyme activity is expressed as % of activity (of the same preparation) in the absence of any CM.
Conclusion

In conclusion we showed that the low clinical level of *P. falciparum* infected RBCs (< 4% parasitaemia) utilise glucose at about 100-fold higher levels as compared to the normal uninfected cells. Product(s) secreted by the parasite into the external medium down-regulate the glucose flux of the uninfected RBC population. The mechanism of inhibition of glucose utilisation in uninfected RBCs seems to involve inhibition of the regulatory glycolytic enzymes—PFK and PK.

References


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