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Research Article

Uptake and kinetic properties of choline and ethanolamine in *Plasmodium falciparum*

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Abstract

Purpose: *The asexual proliferation of Plasmodium, inside the erythrocyte, is accompanied by the synthesis of huge quantities of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn). These needful phospholipids for the cytoplasmic membrane of the merozoites are provided by the precursors choline and ethanolamine. PtdCho and PtdEtn are synthesized by the parasite because the erythrocyte is unable to do it. In order to assess the dynamism of the phospholipid pathways, we aimed to investigate the respective shape of the uptake of choline and ethanolamine by Plasmodium falciparum.*

Method: *Time-course experiments and kinetic assays were performed respectively with fixed and ranged concentrations of radioactively-labelled choline and ethanolamine. The labelled-precursors were added in the culture of P. falciparum infected-erythrocytes and the incorporated molecules in phospholipids were measured with a scintigraph counter.*

Result: *The results showed that the incorporation of precursors in the infected-erythrocyte occurred with a Michaelis-Menten's kinetic shape. According to the maximum rate (V_{max}), the pathway of ethanolamine incorporation was faster than that of choline. Similarly, affinity for ethanolamine was greater than that of choline.*

Conclusion: *Although PtdCho is the major phospholipid in the membrane, this study rules out that the influx of ethanolamine in the infected-erythrocyte, in vivo conditions, is more dynamic than choline.*

Keywords: *Plasmodium,* phospholipids, kinetics, metabolism, choline, ethanolamine

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INTRODUCTION

Like many other animal cells, the cytoplasmic membrane of the *Plasmodium* is made of bulk phospholipid molecules organized in a double layer. The major ones are phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn). After the invasion of the erythrocyte, the parasite starts a schizogonic cycle marked by the production of several merozoites. This asexual proliferation of the parasite inside the erythrocyte is accompanied by the synthesis of huge quantities of needful phospholipids for the membrane of the merozoites. It means a biosynthesis of PtdCho and PtdEtn by the parasite because the erythrocyte is unable to do it on its own. In order to assess the needs of phospholipids for the parasite, we evaluated the uptake of its precursors, choline and ethanolamine. Many studies reveal metabolic differences within the species of *Plasmodium* [1-3]. The uptake of choline and ethanolamine by the *Plasmodium* seem to vary with the species, the stage of the life cycle and the experimental conditions[4].

In this study, we determined the uptake and apparent kinetic properties of choline and ethanolamine in *P. falciparum* infested-erythrocyte, the most widespread human malarial parasite in West Africa.

EXPERIMENTAL

In vitro culture of *P. falciparum*

The 3D7 strain of *P. falciparum* in infected erythrocytes was incubated in modified RPMI 1640 medium supplemented with 25 mM Hepes (pH 7.6). The RPMI 1640 was free of choline and ethanolamine. The parasitemia was 7 - 12 % with a hematocrit of 2.5 % in a total amount of 3.5 $x10^6$ – 6 $x10^6$ infected cells. The cell suspension was distributed in microwell plate and incubated at 37°C in a 5 % carbon dioxide atmosphere at time 0.

Labelling for time-course studies

To assess the uptake of the phospholipid precursors by the infected-erythrocyte, radioactive isotopes of [1-^{3}H]ethan-1-ol-2-amine and [methyl-^{3}H]choline, purchased with Amersham™ corp were used They were added, in the respective microwells, at time 60, 120, 180 and 210 min (final concentration of 2.5 µM; specific activity of 2 Ci/mmol). The final volume of the suspension in each microwell was 200 µl. At time 240 min, reactions were stopped at 4°C. Radioactive incorporation in bulk molecules was measured after haemolysis, filtration and transfer onto glass-fiber filter plate using a cell harvester. Radioactivity was measured on a scintigraph counter from Beckman™. Incorporation of the labelled precursors into the infected-erythrocyte was expressed in nmol/10^{10} cells.

Labelling for kinetic assay

To assess the kinetic features, different specific activity of radioactive choline and ethanolamine were added at indicated concentrations in the suspension of the infected-erythrocytes. The suspension was incubated for 2 hours at 37 °C in a 5 % CO_2 incubator. Reactions were stopped at 4 °C and radioactive incorporation was measured after filtration and transfer on fiber glass plate using a cell harvester. Radioactivity was measured as described above.

The blank test was performed with an uninfected-erythrocyte suspension provided by an uninfected human to assess non-specific uptake by the erythrocyte.

Data analysis

Experiments were performed twice. Blank test was deduced from each result and all of them were means of triplicate assays. Regression curve was drawn and the Michaelis constant (K_m) and V_{max} were calculated with the software GraphPad Prism® version 4.

RESULTS

Time course of choline and ethanolamine uptake

P. falciparum incorporates choline and ethanolamine in its bulk molecules retained by the cell harvester. For the two precursors, the uptake is constant and linear at least from 60 to 240 minutes. The rate of choline uptake is 0.91 ± 0.03 nmol.10^{-10} parasites.h^{-1} and 5.01 ± 0.24 nmol.10^{-10} parasites.h^{-1} for ethanolamine uptake (Figs.1 and.2). The *in vitro* conditions

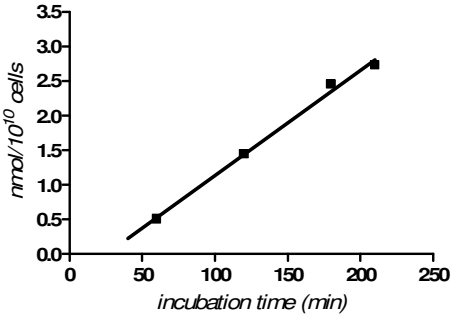

Figure 1: Choline incorporation rate in *P. falciparum.*

P. falciparum-infected erythrocytes (3D7 strain) (12 % parasitemia, 2.5 % hematocrit in 6.10^6 total infected cells) were incubated in a final volume of 200 µl of modified RPMI 1640 supplemented with 25 mM Hepes (pH 7.6). Suspensions were incubated at 37 °C in a 5 % CO_2 incubator at time 0. Radioactive choline (2.5 µM, 2 Ci/mmol) was added at time 60, 120, 180 and 210 min. At time 240 min, reaction was stopped at 4 °C and radioactive incorporation in phosphatidylcholine was measured after filtration using a cell harvester. Incorporation of the labelled precursors into phosphatidylcholine is expressed in nmol/10^{10} cells and is mean of triplicate values ± SEM.

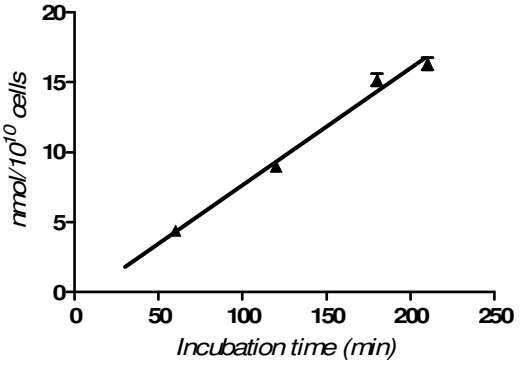

Figure 2: Ethanolamine incorporation rate in *P. falciparum*

P. falciparum-infected erythrocytes (3D7 strain) (12 % parasitemia, 2.5 % hematocrit in 6.10^6 total infected cells) were incubated in a final volume of 200 µl of modified RPMI 1640 supplemented with 25 mM Hepes (pH 7.6). Suspensions were incubated at 37 °C in a 5 % CO_2 incubator at time 0. Radioactive ethanolamine (2.5 µM, 2 Ci/mmol) was added at time 60, 120, 180 and 210 min. At time 240 min, reaction was stopped at 4 °C and radioactive incorporation in phospholipids was measured after filtration using a cell harvester. Incorporations of the labelled precursors into phospholipids are expressed nmol/10^{10} cells and are means of triplicate values ± SEM.

show that the uptake of ethanolamine is greater than that of choline.

Kinetics of uptake

This assay was performed assuming an enzymatic process for the uptake of precursor

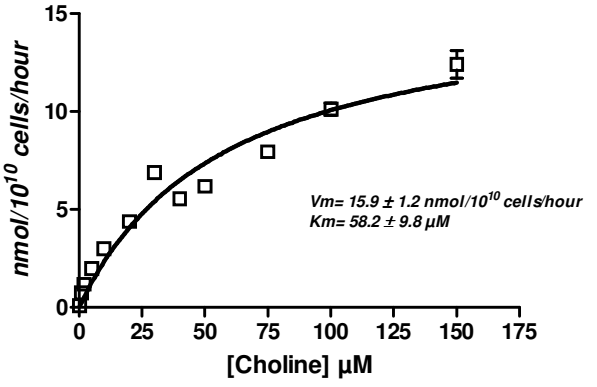

Figure 3: Kinetics of choline incorporation in *P. falciparum*

P. falciparum-infected erythrocytes (3D7 strain) (7 % parasitemia, 2.5 % hematocrit in 3.5x10^6 total infected cells) were incubated in a final volume of 200 µl of modified RPMI 1640 supplemented with 25 mM Hepes (pH 7.6) and choline at indicated concentration (0,066 - 84 Ci/mmol). Suspension was incubated for 2 hours at 37 °C in a 5 % CO_2 incubator. Reaction was stopped at 4 °C and radioactive incorporation was measured after filtration using a cell harvester. Results are means of triplicate values ± SEM.

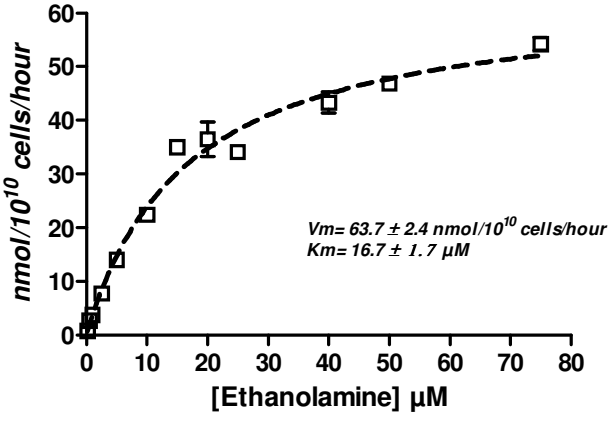

Figure 4: Kinetics of ethanolamine incorporation in *P. falciparum*

P. falciparum-infected erythrocytes (3D7 strain) (7 % parasitemia, 2.5 % hematocrit in 3.5x106 total infected cells) were incubated in a final volume of 200 µl of modified RPMI 1640 supplemented with 25 mM Hepes (pH 7.6) and ethanolamine at indicated concentration (0,066 - 28 Ci/mmol). Suspension was incubated for 2 hours at 37 °C in a 5 % CO2 incubator. Reaction was stopped at 4 °C and radioactive incorporation was measured after filtration using a cell harvester. Results are means of triplicate values ± SEM.

and their incorporation in bulk molecules of the parasite. The uptake of choline and ethanolamine in *P. falciparum* showed an apparent *Michaelis-Menten* kinetics with the following features: choline uptake had a V_{max} of 15.9 ± 1.2 nmol.10^{-10} cells.h^{-1} with an apparent K_m of 58.2 ± 9.8 µM and ethanolamine uptake had a V_{max} of 63.7 ± 2.4 nmol.10^{-10} cells.h^{-1} with an apparent K_m of 16.7 ± 1.7 µM (Figs 3 and 4). The metabolic

pathway of precursor incorporation in the phospholipids of *P. falciparum* seems to have a greater affinity for ethanolamine than for choline. Beside this, the incorporation of ethanolamine was faster than that of choline.

DISCUSSION

In human infection, signs of clinical morbidity, associated with malaria may occur at a parasitaemia range of about 100 – 720,000 per µl (i.e., 0.02 to 15%)[5, 6]. Our experimental conditions were chosen to get an optimal growth of the parasite. It means not too low parasitaemia to get a sufficient measurable metabolic reaction through the radioactivity signal. But not too high parasitaemia to get a dynamic and optimal growth of the parasite and avoid a high amount of lactic acid or other biological wastes that a high parasitaemia can release. It is the same caution that led to a relatively short incubation time (less than 240 min).

In human blood, the choline concentration is about 10 - 12 µM and ethanolamine is about 2.5 µM[7]. In order to assess the match of choline and the ethanolamine pathways in *in vitro* conditions, we chose an identical final concentration of precursors (2.5 µM for each).

The uninfected-erythrocyte is not expected to incorporate choline or ethanolamine in any metabolic pathways. However, Staines and Kirk showed that in an infected mammalian, the choline enters in uninfected erythrocyte with the same rate than in infected erythrocyte[4]. Therefore we performed a blank test with uninfected-erythrocytes from healthy humans to deduce the non-specific adsorption of the precursors on any bulk molecules of the erythrocytes.

Several pathways from choline or ethanolamine are known to lead to phosphatidyl choline and phosphatidyl ethanolamine. The one known as the Kennedy pathway, consists in the phosphorylation of choline in phosphocholine (PC), catalyzed by a choline kinase (EC 2.7.1.32). Then a cytidylmonophosphate (CMP) is transferred on the PC leading to CDP-Choline. This reaction is catalyzed by a cholinephosphate cytidyltransferase (EC 2.7.7.15). And finally, the CDP-Choline is transferred on a diacylglycerol (DAG) to get the PtdCho. This reaction is catalyzed by CDP-Choline:1,2 diacylglycerol choline phosphotransferase (EC 2.7.8.2). Another pathway goes through the tri-methylation of the phosphoethanolamine (PE) into the PC and then leads to PtdCho via CDP-Cho [8]. The last one, known as occurring in mammalian cells and yeast, is the tri-methylation of PtdEtn into PtdCho[9]. The involvement of each pathway depends on the type of cell.

The respective biosynthetic and catabolic pathways of phosphatidylcholine and phosphatidylethanolamine involve many kinds of molecules : choline is known to be mainly taken up and incorporated in phosphatidylcholine (PtdCho) without betaine or acetylcholine biosynthesis[10]. Ethanolamine is retrieved from PtdCho and Phosphatidylethanolamine (PtdEtn). But nothing is known about the kinetics of their catabolism.

Our experiments show that *P. falciparum* incorporates choline in a constant way within 60 – 240 minutes while *P. knowlesi* optimal incorporation of choline into PtdCho is less than 180 min. But the rate of choline uptake, supposed to be mainly incorporated in PtdCho, is about 100 fold lesser for *P. falciparum* than for *P. knowlesi*[11]. In the same way, Staines and Kirk showed that the rate of choline uptake by *P. vinckei* is greater than that of *P. falciparum* one[4]. This correlates with the length of life cycle of *P. knowlesi* or *P. vinckei* which is 24 hours while *P. falciparum* is much longer, 48 hours.

Infestation of the erythrocyte by *Plasmodium* entails a huge change of phospholipid composition in the erythrocyte membrane and in the whole complex parasite-erythrocyte relationship. At the schizonte stage phospholipid composition increases to about 500 – 700% and is mainly composed of PtdCho and PtdEtn (75 – 85%) with predominantly PtdCho[12-14]. However in our condition of experiments, we noticed that the rate of ethanolamine uptake was superior to the rate of choline uptake. The normal

mammalian plasma choline concentration is in the range 10 – 40 µM[15]. Therefore, we expected the rate of choline uptake to increase and be superior to that of ethanolamine. But on the one hand V_{max} of choline uptake was inferior to V_{max} of ethanolamine uptake. On the other hand, the apparent K_m of ethanolamine uptake showed a higher affinity of the parasite for ethanolamine rather than choline. These observations contribute to the hypothesis that PtdCho is mainly issued from ethanolamine according the three steps of N-methylation of phosphoethanolamine to phosphocholine and catalyzed by phosphoethanolamine N-methyltransferase (EC 2.1.1.103)[8, 16]. Otherwise, in *in vivo* conditions, the ethanolamine pathway, is inhibited by a transcriptional repression of the phosphoethanolamine methyltransferase as recently shown by Witola and Ben Mamoun[17]. This apparent feature of Mickaelis-Menten kinetics for the choline and ethanolamine uptakes hide the possibility that these pathways possess any key-steps regulated by a possible allosteric enzyme.

CONCLUSION

Choline and ethanolamine are incorporated in bulk molecules of *P. falciparum*. The uptake of these precursors by infected-erythrocyte increased when compared with uninfected erythrocyte. Such differences in the apparent kinetic properties of the choline and ethanolamine uptake are easily understandable if we are aware that ethanolamine has to provide both PtdCho and PtdEtn metabolic pathways. On the contrary, choline provides only the PtdCho pathway. These results are in favor for a most dynamic influx of ethanolamine in the *P. falciparum* phospholipids but may not be the case in *in vivo* conditions.

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