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Characterization of choline uptake in *Trypanosoma brucei* procyclic and bloodstream forms

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ABSTRACT

Choline is an essential nutrient for eukaryotic cells, where it is used as precursor for the synthesis of choline-containing phospholipids, such as phosphatidylcholine (PC). According to published data, *Trypanosoma brucei* parasites are unable to take up choline from the environment but instead use *lyso*-phosphatidylcholine as precursor for choline lipid synthesis. We now show that *T. brucei* procyclic forms in culture readily incorporate [^{3}H]-labeled choline into PC, indicating that trypanosomes express a transporter for choline at the plasma membrane. Characterization of the transport system in *T. brucei* procyclic and bloodstream forms shows that uptake of choline is independent of sodium and potassium ions and occurs with a K_m in the low micromolar range. In addition, we demonstrate that choline uptake can be blocked by the known choline transport inhibitor, hemicholinium-3, and by synthetic choline analogs that have been established as anti-malarials. Together, our results show that *T. brucei* parasites express an uptake system for choline and that exogenous choline is used for PC synthesis.

1. Introduction

By definition, endoparasites scavenge nutrients from their hosts. Among the essential nutrients for kinetoplastids and apicomplexans are precursors like choline, ethanolamine and *myo*-inositol for the synthesis of phospholipids, the major structural components of membranes in protozoan parasites [1].

In *Trypanosoma brucei*, the causative agent of human African sleeping sickness and the related animal disease, Nagana, phosphatidylcholine (PC) represents the most abundant phospholipid class, representing 45–60% of total phospholipid [2]. The formation of PC can be initiated by uptake of *lyso*-phosphatidylcholine (*lyso*-PC) from the environment, followed by CoA-dependent acylation to PC [3]. This pathway has been shown to contribute most of the PC in *T. brucei* bloodstream forms [4]. Alternatively, PC can be synthesized de novo via the CDP-choline branch of the Kennedy pathway, using choline as precursor [4]. The enzymes of the CDP-choline pathway have been identified and experimentally verified in *T. brucei* [5,6]. In addition, RNAi studies revealed that the pathway is essential in both *T. brucei* bloodstream [1] and procyclic [6] forms. In contrast, little and conflicting data are available on the acquisition of the precursor molecule, choline, for de novo PC synthesis via the CDP-choline pathway. Marginal uptake of choline has been reported in *T. brucei* bloodstream forms isolated from rats [4], however, this finding was not confirmed in more recent studies [7,8].

Choline uptake in mammals is mediated via several distinct transport systems. Based on ion dependence and susceptibility to the specific inhibitor, hemicholinium-3 (HC-3) [9], choline transport can occur via (i) low-affinity facilitated diffusion, mediated by polyspecific organic cation transporters, (ii) intermediate-affinity, sodium-independent transport, using choline transporter-like (CTL) proteins, and (iii) high-affinity, sodium-dependent transport, catalyzed by high-affinity choline transporters [10]. In humans, organic cation transporters and CTL proteins are ubiquitously distributed. In contrast, high-affinity choline transporters primarily occur in cholinergic neurons, where they mediate uptake of choline for acetylcholine synthesis [11,12].

Choline transport systems have also been identified and characterized in *Plasmodium* and *Leishmania*. Uptake of choline in these

Abbreviations: PC, phosphatidylcholine; lyso-PC, lyso-phosphatidylcholine; HC-3, hemicholinium-3; CTL, choline transporter-like; TLC, thin layer chromatography.

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parasites is sodium-independent, can be inhibited by HC-3, and occurs with affinities in the low micromolar range (2.5–25 μM), suggesting that it is mediated by intermediate-affinity CTL proteins [13,14]. Interestingly, a group of synthetic choline analogs was shown to effectively inhibit choline uptake in both groups of parasites [7,14]. It has been suggested that in *Plasmodium* the drugs may act via interference with phospholipid biosynthesis [14–18]. Some of these choline analogs have been used as anti-malarials [15].

In the present study, we show for the first time that choline is readily taken up by *T. brucei* bloodstream and procyclic forms in culture. We characterize the transport system, which is independent of Na^+ and K^+ ions, and show that it is inhibited by HC-3 and synthetic choline analogs. Our analyses also demonstrate that exogenous choline is used by trypanosomes for PC synthesis.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade and purchased from Merck (Darmstadt, Germany), Sigma–Aldrich (Buchs, Switzerland) or ICN Biomedicals (Tägerig, Switzerland). Antibiotics and fetal bovine serum (FBS) were obtained from Invitrogen (Basel, Switzerland). DNA polymerases were purchased from Promega (Madison, USA). Primers and sequencing services were from Microsynth AG (Balgach, Switzerland). All restriction enzymes were purchased from Fermentas (Nunningen, Switzerland). methyl-[^{3}H]choline chloride (60 Ci/mmol; [^{3}H]choline), *myo*-[2-^{3}H(N)]inositol (15–20 Ci/mmol; [^{3}H]inositol) and [1-^{3}H]ethanolamine hydrochloride (40–60 Ci/mmol; [^{3}H]ethanolamine) were from American Radiolabeled Chemicals (St. Louis, USA), and [α-^{32}P]-dCTP (3000 Ci/mmol) from PerkinElmer Life Sciences (Schwerzenbach, Switzerland). Kodak MXB and BioMax MS films were from Kodak SA (Lausanne, Switzerland).

2.2. Trypanosomes and culture conditions

T. brucei strain Lister 427 procyclic forms were cultured at 27 °C in SDM-79 containing 5% (v/v) heat-inactivated FBS. *T. brucei* bloodstream and procyclic forms expressing T7 RNA polymerase and a tetracycline repressor [19] were cultured at 37 °C in HMI-9 containing 10% (v/v) heat-inactivated FBS and 27 °C in SDM-79 containing 15% (v/v) heat-inactivated FBS, respectively, in the presence of 25 μg/ml hygromycin, and 15 μg/ml G418. The derived clones containing different double-stranded RNA constructs against putative choline transporter genes were cultured in the presence of an additional 2 μg/ml puromycin or 1 μg/ml phleomycin. Generation of double-stranded RNA was induced by the addition of 1 μg/ml tetracycline.

2.3. Choline uptake in T. brucei

T. brucei procyclic and bloodstream forms were grown to a density of 1.0–1.5 x 10^7 and 1.0–1.5 x 10^6 cells/ml, respectively, washed once in 25 ml of assay buffer (130 mM NaCl, 10 mM HEPES, 5 mM KCl, 1 mM $CaCl_2$, 3 mM $MgCl_2$, 5 mM NaH_2PO_4 and 5 mM glucose, pH 7.0) by centrifugation (1250 x *g*, 10 min, 24 °C) and resuspended in the same buffer, pre-warmed at 27 °C and 37 °C, respectively, at a density of 5 x 10^7 cells/ml. Parasites were then incubated for 3, 6 and 9 min in 96-well plates at room temperature (for procyclic forms) or 37 °C (for bloodstream forms) in the presence of 65 nM (0.8 μCi) [^{3}H]choline and 120 nM to 62.5 μM (final concentrations) non-labeled choline per well in a total volume of 200 μl. Uptake of [^{3}H]choline was terminated by rapidly transferring the samples to 96-well microplates containing 1.2 μM pore size GF/C glass fiber filters (UniFilter-96 GF/C, Perkin Elmer Life Sciences), immediately followed by filtration. After washing the pellets four times with 100 μl assay buffer, the samples were air-dried, resuspended in 40 μl scintillation fluid and counted in a 1450 MicroBeta Trilux top counter (Perkin Elmer Life Sciences). Uptake of [^{3}H]choline was linear over 9 min for all concentrations tested. The results from 4–5 independent experiments were used for calculation of K_m values, using Graphpad Prism 5 software and Lineweaver–Burk transformation. Choline uptake inhibition experiments were performed using 65 nM [^{3}H]choline and 1 μM non-labeled choline in the absence or presence of different concentrations of inhibitors. To study the ion dependence of choline uptake, trypanosomes were incubated in the absence of Na^+, K^+, or both ions, by replacing Na^+ for K^+, or vice versa (135 mM, final concentrations), or using 135 mM *N*-methylglucamine instead of Na^+ and K^+, respectively, in the assay buffer. No NaH_2PO_4 was used in these experiments.

2.4. Incorporation of [^{3}H]choline into T. brucei lipids

Metabolic labeling of trypanosomes with [^{3}H]choline and [^{3}H]ethanolamine (to control for cell viability and phospholipid synthesis) was done essentially as described before [20]. Briefly, [^{3}H]choline (1 μCi/ml) or a mixture of [^{3}H]choline and [^{3}H]ethanolamine (2 μCi/ml and 0.25 μCi/ml, respectively) was added to *T. brucei* procyclic forms at a density of 0.8–1.2 x 10^7 cells/ml and incubated in the absence or presence of G25 or MS1 (5 μM or 10 μM, respectively, final concentrations) for 2 h at 27 °C. Subsequently, parasites were spun down, washed with ice-cold buffer (10 mM Tris, 144 mM NaCl, pH 7.4) to remove unincorporated label, and phospholipids were extracted with 2 x 10 ml chloroform:methanol (2:1, by volume). Lipids were then separated by one-dimensional thin-layer chromatography (TLC) using Silica Gel 60 plates in a solvent system composed of chloroform:methanol:acetic acid:water (25:15:4:2, by volume). On each plate, appropriate phospholipid standards were run alongside the samples to be analyzed. Lipid spots were visualized by exposing the plates to iodine vapor. Radioactivity was detected by scanning the air-dried plates with a radioisotope detector (Berthold Technologies, Regensdorf, Switzerland) and quantified using the Rita Star® software provided by the manufacturer. Alternatively, plates were sprayed with EN^{3}HANCE (PerkinElmer Life Sciences) and exposed to MXB film at −70 °C.

2.5. Drug sensitivity assays

Susceptibility of *T. brucei* bloodstream forms to G25 was assessed by Alamar blue assays [21]. Briefly, serial dilutions of G25 starting at 10 μM (using a 10 mM stock solution) were prepared in HMI-9 medium containing 10% (v/v) FBS in 96-well plates (100 μl total volume). Parasites were added to a final density of 1 x 10^4 cells/ml. After incubation for 70 h at 37 °C, 10 μl of Alamar blue solution (12.5 mg resazurin/100 ml buffer) was added to all wells and incubation was continued for another 2 h at 37 °C. Fluorescence was measured using a spectromax GEMINI plate reader at 544 nm excitation, 590 nm emission and 570 nm cutoff.

2.6. In silico identification of genes encoding candidate choline transporters

All *T. brucei* proteins with two or more predicted [22] transmembrane domains were hierarchically clustered into similarity groups based on all pairwise global alignments [23]. For each cluster a hidden Markov model profile was made with ClustalW [24] and *hmmbuild* of the HMMer package [25]. The same procedure was carried out with the well-annotated proteomes of *Homo sapiens*, *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. Finally, the