Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 355 (2007) 16–22
BBRC
www.elsevier.com/locate/ybbrc

Heme requirement and intracellular trafficking in *Trypanosoma cruzi* epimastigotes ☆

F.A. Lara [b,1,2], C. Sant'Anna [c,2], D. Lemos [a], G.A.T. Laranja [a], M.G.P. Coelho [f], I. Reis Salles [d], A. Michel [e], P.L. Oliveira [b], N. Cunha-e-Silva [c], D. Salmon [d], M.C. Paes [a,*]

[a] *Lab. de Interação de Tripanossomatídeos e Vetores, Departamento de Bioquímica, Instituto de Biologia Roberto Alcântara Gomes, Universidade do Estado do Rio de Janeiro, Brazil*
[b] *Lab. de Bioquímica de Artrópodes Hematófagos, Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*
[c] *Lab. de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*
[d] *Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*
[e] *Biological Chemistry Laboratory, Peptides, University of Mons, Mons, Belgium*
[f] *Lab. de Imunologia Aplicada e Bioquímica de Proteínas e Produtos Naturais (LIA-BPPN), Departamento de Bioquímica, Instituto de Biologia Roberto Alcântara Gomes, Universidade do Estado do Rio de Janeiro, Brazil*

Received 7 December 2006
Available online 23 January 2007

Abstract

Epimastigotes multiplies in the insect midgut by taking up nutrients present in the blood meal including heme bound to hemoglobin of red blood cell. During blood meal digestion by vector proteases in the posterior midgut, hemoglobin is clipped off into amino acids, peptides, and free heme. In this paper, we compared the heme and hemoglobin uptake kinetics and followed their intracellular trafficking. Addition of heme to culture medium increased epimastigote proliferation in a dose-dependent manner, while medium supplemented with hemoglobin enhanced growth after 3-day lag phase. Medium supplemented with globin-derived peptides stimulated cell proliferation in a dose-independent way. Using Palladium mesoporphyrin IX (Pd-mP) as a fluorescent heme-analog, we observed that heme internalization proceeded much faster than that observed by hemoglobin-rhodamine. Binding experiments showed that parasites accumulated the Pd-mP into the posterior region of the cell whereas hemoglobin-rhodamine stained the anterior region. Finally, using different specific inhibitors of ABC transporters we conclude that a P-glycoprotein homologue transporter is probably involved in heme transport through the plasma membrane.

Keywords: Heme; *T. cruzi*; Endocytosis; ABC transporter

Trypanosoma cruzi, the etiologic agent of Chagas's disease [1], is transmitted through the feces of the triatomine insect vectors during the blood meal on a vertebrate host. Bloodstream trypomastigotes are ingested by the vector and transform into replicative epimastigotes in the lumen of the gut where they proliferate, an environment rich in hemoglobin and heme. The subsequent transformation of epimastigotes into metacyclic trypomastigotes occurs mostly in the rectum, and the differentiated parasite leaves the insect together with feces and urine [1–4].

Triatomine insects feed large amounts of blood compared to their body size, which is continuously digested by the action of proteases releasing aminoacids, peptides, and heme. Similarly to other trypanosomatids, *T. cruzi* needs heme to its metabolism. In nature, *T. cruzi* must acquire extracellular heme from their hosts as a nutritional cofactor and incorporate it in various essential hemopro-

☆ This work was supported by FAPERJ, PADCT, CNPq, Howard Hughes Medical Institute (to P.L. Oliveira) and PRONEX (to P.L. Oliveira).
* Corresponding author. Fax: +55 21 25876136.
E-mail address: mcpaes@uerj.br (M.C. Paes).
[1] Present address: Lab. de Microbiologia Celular, Departamento de Micobacterioses, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Brazil.
[2] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.12.238

teins. Biochemical studies have pointed out the absence of a complete heme biosynthetic pathway in *T. cruzi* [5,6] and this is corroborated by the recent sequencing of the *T. cruzi* genome that revealed that the eight enzymes of the classical glycine pathway of heme biosynthesis are lacking in these organisms [7]. This defect in tetrapyrrole biosynthesis is shared by other trypanosomatids, like *Leishmania* which is deficient in at least five enzymes of the porphyrin pathway, as demonstrated by genetic rescue of *Leishmania* deficiency in heme biosynthesis following the construction of mutants overexpressing the mammalian genes encoding δ-aminolevulinate dehydratase and porphobilinogen deaminase, the second and third enzyme of this pathway [8].

Ultrastructural studies using a variety of proteins (BSA, transferrin, LDL, and ferritin) revealed that epimastigotes take up macromolar nutrients by flagellar pocket and cytostome. From the entry sites, endocytic vesicles bud off and fuse with a branched tubulo-vesicular early endosome. At last, cargo-containing vesicles delivery nutrients to the *T. cruzi* storage organelles, the reservosomes [9–11]. A stereological study showed that reservosomes occupy about 6% of the total cell volume of epimastigotes, gradually disappearing during the metacyclogenesis process [12].

In the present work, we demonstrated that heme constitutes an important molecule for the optimal proliferation of *T. cruzi* epimastigotes. Taking advantage of Palladium mesoporphyrin IX (Pd-mP), a fluorescent metalloporphyrin, employed here as a heme analog, we showed that similarly to hemoglobin-rhodamine, Pd-mP concentrated into the posterior end of the cell region that corresponds to reservosomes, but through a mechanism distinct from the endocytic pathway operating for hemoglobin. Furthermore, our data suggest that heme is transported by an ABC transporter.

Materials and methods

Parasites. *Trypanosoma cruzi* strains Y (CT-IOC-106) and Dm28c (CT-IOC-010) strains were provided by the Trypanosomatid Collection of the Oswaldo Cruz Institute, Fiocruz, Brazil. The protozoa were grown at 28 °C for 7 days in brain–heart infusion medium (BHI), and supplemented with 30 μM hemin (heme-Cl) and 10% fetal calf serum (FCS). Parasite growth was monitored by cell counting in a Neubauer chamber.

Heme, hemoglobin, and globin-derived peptides effects on T. cruzi epimastigotes growth. To evaluate the heme requirements of *T. cruzi*, the cells maintained in BHI containing 10% FCS were collected by centrifugation and washed three times with 0.15 M NaCl, 10 mM sodium phosphate, and pH 7.2 (PBS). The cells were maintained for 8 days in culture containing different concentrations of hemin, hemoglobin or globin-derived peptides as indicated. Cell growth was monitored by cell counting in a Neubauer chamber.

Peptide synthesis. Synthetic peptides R 791 (TRMFTTYPQT KT, 12 aa), R 797 (TRMFTTYPQT KTYFPHFDLS, 20 aa) correspond to sequences {30–41} and {30–49}, respectively, of the chicken α^{D}-globin chain. Peptide R 815 (used as control: ISQAVHAAHA EINEAGR, 17 aa) corresponds to sequence {323–339} of ovalbumine (D011.10/OT II TCR epitope).

Peptides were synthesized by the solid-phase method performed an ABI automated synthesizer Model 431A using Fmoc chemistry [13]. Peptides were cleaved from the resin, extracted, and purified by reverse phase HPLC on octadecyl-silica. They were characterized by mass spectrometry on a Micromass Q-Tof II.

Palladium mesoporphyrin IX (Pd-mP). Palladium mesoporphyrin IX (Frontier Scientific, Logan, UT) stock solution of 20 mM was prepared in DMSO and then diluted immediately before use to 10 mM in 0.1 N NaOH. The final concentration in all experiments was 100 μM.

Hemoglobin conjugation to rhodamine. Hemoglobin was labeled with rhodamine isothiocyanate (Molecular Probes, Oregon, USA) in 0.1 M sodium bicarbonate buffer, pH 9.0, during 1 h. The reaction was stopped by adding 1.5 M hydroxylamine, pH 8.5. Unbound probe was removed by sequential dialysis against PBS.

Pd-mesoporphyrin IX (Pd-mP) and hemoglobin-rhodamine uptake by epimastigotes. Parasites were collected by centrifugation at 1500*g* for 10 min, washed twice in PBS, and 10^7 cells was resuspended in modified DMEM medium without serum, containing 9.2 mM Hepes, pH 7.0, and 100 μM Pd-mP or 100 μg/ml hemoglobin-rhodamine at 28 °C. At different times parasites were immediately fixed in 4% paraformaldehyde and unbound tracers were washed out by centrifugation in cold PBS. Cells were mounted between slides and coverslips, and observed by differential interference contrast (DIC) and fluorescence in an Axioplan 2 Zeiss fluorescence microscope using a filtered 100 W mercury lamp as the excitation light source. Pd-mP images were obtained using the following set of filters: BP400-410 nm/FT 500 nm/LP 520 nm. Rhodamine images were taken using Zeiss-15 filter set: BP546-58 nm/FT 580 nm/LP 590 nm. All images obtained are equally processed by Adobe PhotoShop.

Binding assay. Parasites were collected by centrifugation at 1500*g* for 10 min and washed twice in modified DMEM medium without serum, containing 9.2 mM Hepes, pH 7.0. They were incubated for 30 min at 28 °C in the same medium with 0.01% sodium azide and 50 mM 2-deoxyglucose. Temperature was subsequently lowered to 4 °C and, after 10 min, 100 μg/ml hemoglobin-rhodamine or 100 μM Pd-mP were added to the medium and incubated for 30 min at 4 °C. Cells were then centrifuged at 1500*g* for 10 min at 4 °C and washed twice in the same medium containing the endocitose inhibitors. After that, parasites were fixed in 4% paraformaldehyde and observed in a Zeiss Axioplan microscope.

ABC transporter inhibitors on Pd-mesoporphyrin IX uptake by epimastigotes. Parasites were pre-incubated during 30 min in PBS containing different ATP binding cassette (ABC) transporters modulator (300 μM indomethacin and 10 μM cyclosporin) at 28 °C and then incubated for 30 min with 100 μM Pd-mP. The unbound tracer was washed out by centrifugation in ice-cold PBS, and cells were fixed in 4% paraformaldehyde and observed in a Zeiss Axioplan microscope.

Statistical analysis. Data will be presented as means ± SE and all experiments were repeated at least three times. Statistics treatment used analysis of the Student's *t* test with 95% confidence interval.

Results and discussion

Effects of supplementary hemin on different strains of T. cruzi epimastigotes

We investigated the effects of hemin on the *in vitro* growth of two strains (Y, Dm28c) of *T. cruzi*. By varying the hemin concentration from 0 to 1 mM, we observed an increased parasite proliferation in a dose-dependent manner (Fig. 1A and B). Strain Y and clone Dm28c cultivated in BHI medium or BHI medium supplemented with 3, 30, 100, and 1000 μM of heme exhibited respective median generation times of 5.33, 4.07, 3.38, 3.06, 2.66 days ($n = 4$) and 5.40, 4.61, 4.40, 3.59, 3.30 days ($n = 5$), respectively. The generation time of both strains with 1 mM hemin was close to the optimal generation time previously described for axenic growth of epimastigotes in defined medium (1.66–1.87 days) [14]. These growth rate differences

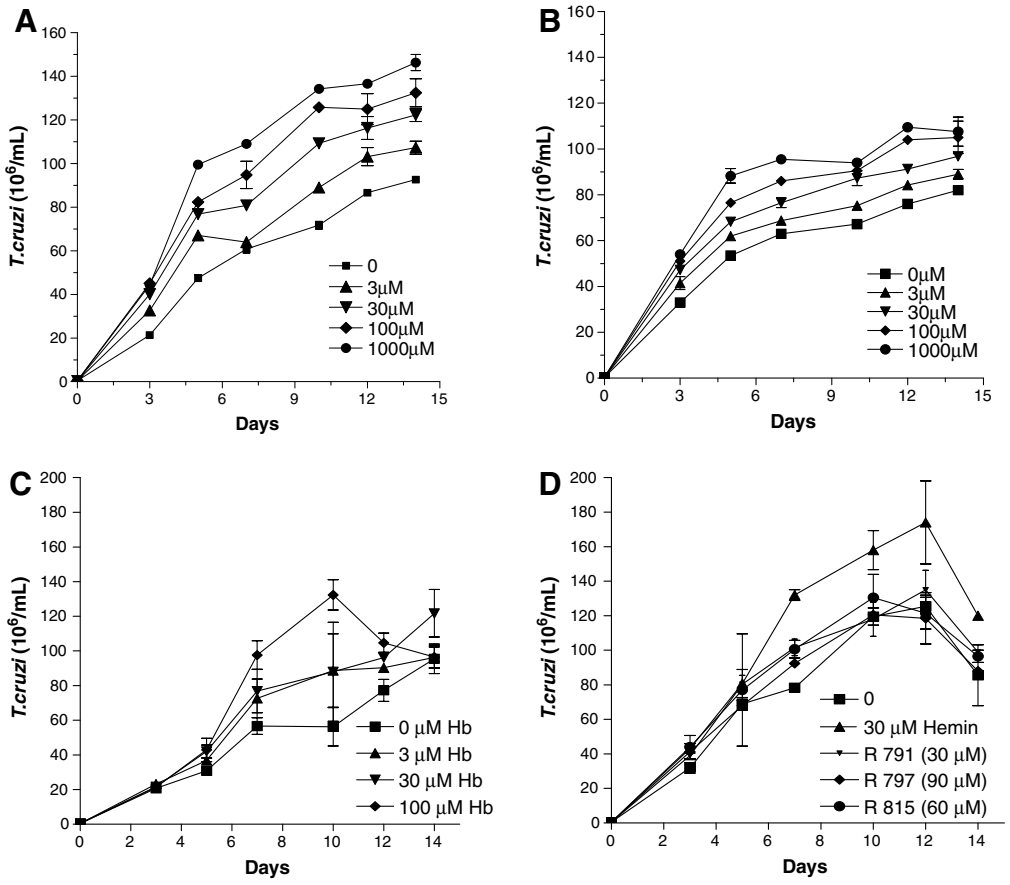

Fig. 1. (A,B) Effects of heme on the growth of *Trypanosoma cruzi* epimastigotes. The curves show effects of supplementary heme concentration (μM) on the Y (A) and Dm28c (B) population density during 14 days of culture in BHI (brain–heart infusion) medium plus 10% fetal bovine serum (FBS). (C,D) Effects of hemoglobin and globin-derived peptides on the growth of *Trypanosoma cruzi* epimastigotes. The curves show effects of supplementary (C) hemoglobin concentration (μM) or (D) globin-derived peptides: R 791 and R 797 and ovalbumin-derived peptide: R 815 on Dm28c clone. Population density during 10 days of culture in BHI (brain–heart infusion) medium plus 10% fetal bovine serum (FBS). Results are means ± SD of three different experiments performed in triplicate.

observed between cells cultivated in BHI medium and BHI medium supplemented with 3, 30, 100, and 1000 μM of heme were considered to be statistically significant, respectively, in four different experiments performed in duplicate for the Y strain (two-tailed unpaired *t* test, $P < 0.0003$, $n = 8$). The growth differences observed for the Dm28c clone performed in the same conditions were less pronounced than the Y strain but still statistically significant (two-tailed unpaired *t* test, $P < 0.0003$, $n = 10$ for medium supplemented with 100 and 1000 μM heme versus complete BHI medium).

Effects of hemoglobin and globin-derived peptides on T. cruzi growth

In order to compare the growth effect of heme alone versus heme bound to hemoglobin protein moiety, we analyzed the *in vitro* cell growth of the Dm28c clone in the presence of different amounts of hemoglobin or globin-derived peptides. Hemoglobin also increased significantly the parasite proliferation in a dose-dependent manner but with a lag phase of 3 days (Fig. 1C). When two different globin-derived peptides (R 791 and R 797) were added to the medium, a very slight dose-independent stimulating effect on growth was observed (Fig. 1D), which was similar to that of an unrelated peptide derived from ovalbumin (R 815) (median generation times of 4.19, 3.37, 3.73, 3.39 days, for BHI free-heme medium or supplemented with R 791 (30 μM), R 797 (90 μM), R 815 (60 μM), respectively; $n = 3$). No statistically differential growth was observed between both globin and ovalbumin derived peptides. In contrast to the data reported by [15] showing that various synthetic peptides of α^D-globin ({1–40}, {30–49}) stimulated the *in vitro* differentiation, the two synthetic peptides (R 791 {30–41}, R 797 {30–49}) of the α^D-globin chain were without effect on the metacyclogenesis process. These differences could be due to the different experimental conditions used in these works.

In conclusion, various peptides only produced a marginal effect on growth of *T. cruzi* epimastigotes. In contrast, both heme and hemoglobin significantly stimulated the growth.

Internalization of fluorescent Pd-mesoporphyrin IX (Pd-mP) in epimastigotes

In order to follow the fate of heme and hemoprotein during the endocytotic process in *T. cruzi,* the time course of internalization of Pd-mesoporphyrin IX (Pd-mP), a fluorescent analogue of Fe-protoporphyrin IX [16], was followed with parasites harvested in log phase. As shown in Fig. 2, after only 30 s of incubation, fluorescent signal could be detected in the anterior end of the parasite (cytostome region, arrow). In addition, some Pd-mP fluorescence was already observed in the posterior region, even in such short incubations. Longer incubation periods (5, 15, and 30 min), revealed increased accumulation of Pd-mP in the posterior region of the trypanosoma accumulated the tracer (reservosomes region).

The time course of internalization of Pd-mP differed significantly from that of hemoglobin coupled to rhodamine. Cells incubated for 30 s with hemoglobin-rhodamine were not labeled, whereas after 2 min of incubation, fluorescence appeared inside and close to cytostome, in the anterior part of the cell. After 5 min it was possible to observe fluorescent tracer on the perinuclear region. Later, the fluorescence signal was concentrated in the posterior region,

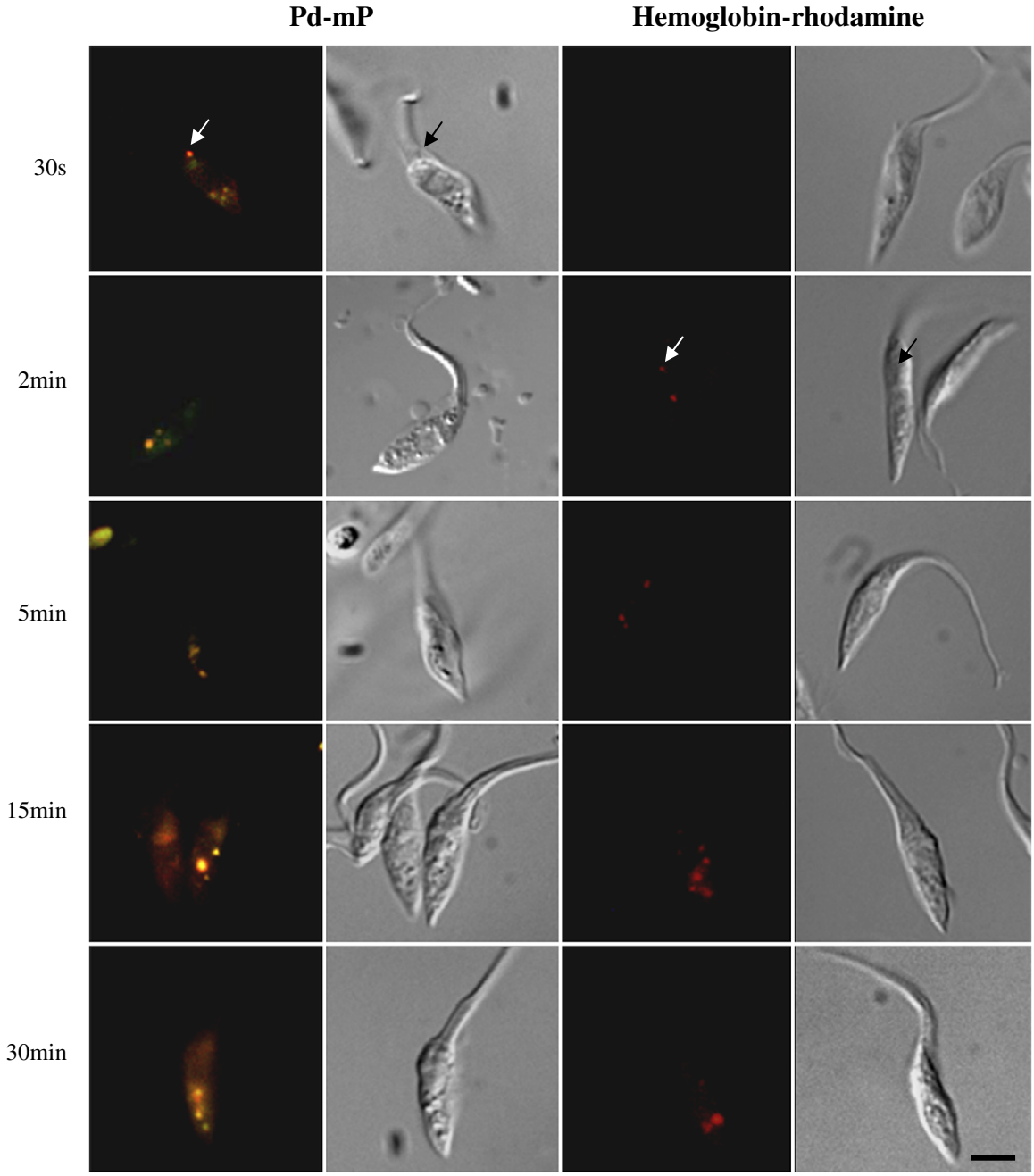

Fig. 2. Time course of Pd-mP or hemoglobin-rhodamine internalization. Cells were incubated with tracers and fixed at the times indicated. Correspondent differential interferential contrast image is presented at right of each fluorescence image. Scale bar represents 10 μm.

probably inside reservosomes, as these organelles are the final destination of several proteins taken up by endocytotic pathways.

It is well known that reservosomes accumulate proteins like cruzipain, transferrin, albumin, and lipoproteins such as low density lipoprotein (LDL) [11]. The marked difference between the distribution of the fluorescence of hemoglobin-rhodamine and Pd-mP (Fig. 2) suggests the existence of two different pathways used to target molecules to the reservosomes. An endocytotical route, used to incorporate Hb as described here and for other proteins such as transferrin and BSA [10], that operates in the time range of some minutes to reach its destination and a second mechanism that transports heme in a few seconds, which is independent of endocytosis.

Binding experiments

In order to exclude the Pd-mP uptake by endocytosis, the cells were maintained at low temperature in the presence of sodium azide and 2-deoxyglucose, with the aim of slow down ATP synthesis. In such conditions, cells are able to maintain its movement by flagellum activity and osmotic pressure by membrane ATPases, but endocytosis processes are inhibited [10]. Hemoglobin-rhodamine, used as endocytic positive control, stained a specific punctuated area at the anterior region of the cell (Fig. 3A) while Pd-mP stained the posterior region, corresponding to the reservosomes (Fig. 3C). According to [10], in similar conditions of binding, both transferrin-FITC and albumin-FITC bind to a punctuate region corresponding to the cell surface at the entry of the cytostome. Therefore, we conclude that even in cells at low temperature, under conditions that prevent endocytosis, the heme analog Pd-mP was still able to concentrate in reservosomes, confirming the existence of a non-endocytotic mechanism involved in transport of heme to the reservosome. These observations indicate that endocytosis is not the only pathway used by molecules to reach the reservosomes and suggest the existence of a specific transmembrane transport mechanism of heme mediated by a transporter located in the membrane of the parasite.

The effect of ABC transporter inhibitors on Pd-mesoporphyrin IX transport in epimastigotes

This led us to investigate the involvement of ABC transporters, which were well known to transport heme in bacteria [17] and have recently been implicated in heme transport in mammalian cells [18]. So, we examined the Pd-mP transport using cyclosporin A, an inhibitor of PgP (P-glycoproteins) [19], and indomethacin, an inhibitor of MRP (multidrug resistance proteins) [20]. Heme-analog accumulation in the surface of *T. cruzi* was observed using the cyclosporin A, whereas no remarkable effect was observed using indomethacin (Fig. 4). As described by Lara et al. [16], Pd-mP has a characteristic shift emission to green (540 nm) when present in a hydrophobic environment, such as the phospholipids moiety of membranes.

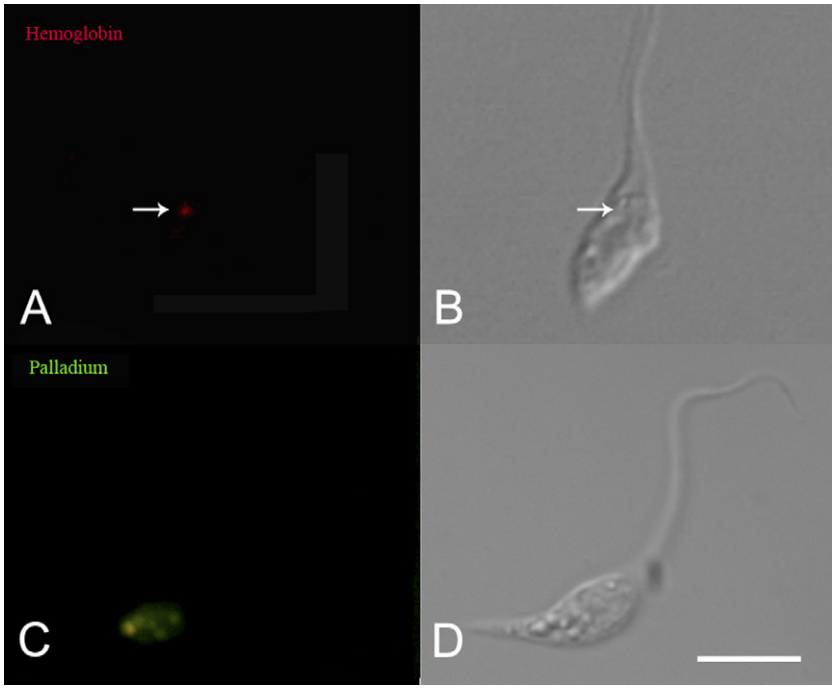

Fig. 3. Binding of Pd-mP hemoglobin-rhodamine to *T. cruzi* epimastigotes. In order to impair the endocytic activity cells were previously incubated with ATP synthesis inhibitors. Subsequently the tracers were added to the incubation medium at 4 °C. After the same incubation time (30 min) hemoglobin-rhodamine was observed at the cytostome region (A,B), while Pd-mP was found concentrated in the reservosomes (C,D). Correspondent differential interferential contrast image is presented at right of each fluorescence image. Scale bar represents 10 μm.

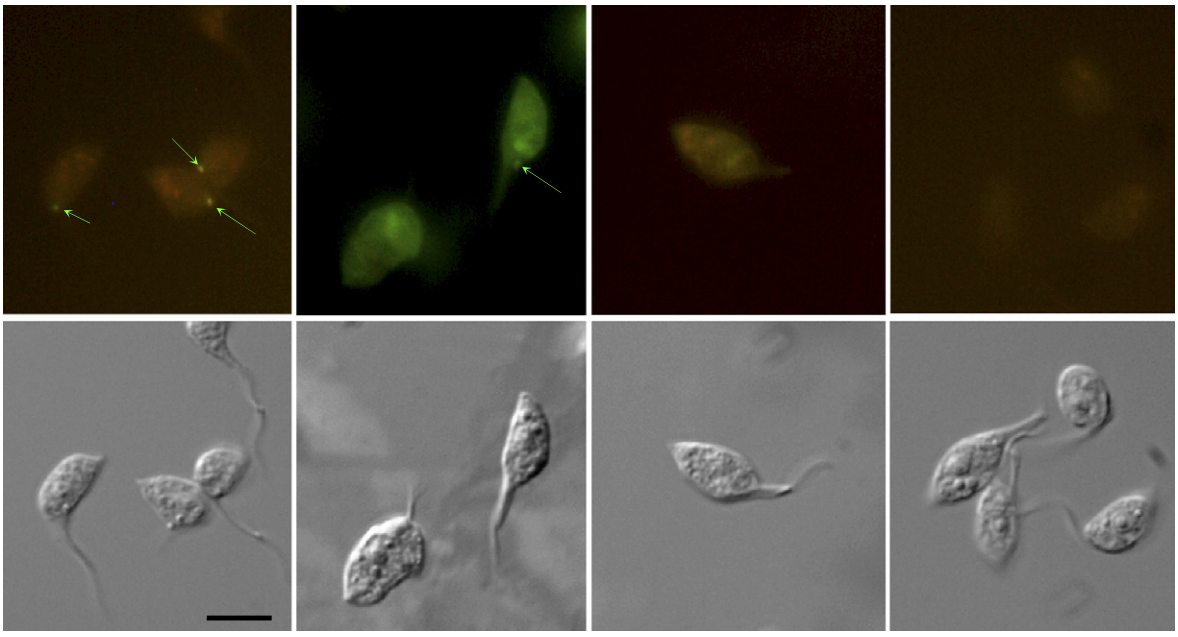

Fig. 4. Palladium mesoporphyrin uptake by *T. cruzi* can be modulated by cyclosporin. Cells were incubated with different drugs for 30 min in BHI, and then exposed to 100 μM of palladium mesoporphyrin IX for 30 min. Green arrows point to the cytostome regions. Drug concentrations were as follows: 10 μM cyclosporin and 300 μM Indomethacin. Scale bar represents 20 μm. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

Inhibition of Pd-mP transport by cyclosporin A, resulted in a strong green emission at the surface of the parasite, which could be explained by accumulation of this amphipathic probe within phospholipid bilayer. On the other hand, all internalized Pd-mP presents a weak red emission, indicating protein-bound form of the probe [16].

In contrast to *Leishmania donovani* promastigotes [21], no obvious toxic effect was detected, even when using a high heme concentration (1 mM), in the range known to disrupt phospholipid membranes [22]. This indicates that *T. cruzi* has an important mechanism to counteract heme toxicity that might allow the parasite to resist oxidative stress generated during the initial days of blood meal digestion, before the heme detoxification due to hemozoin [23]. A heme transporter located in epimastigote membrane could be one of the adaptations of this parasite for dealing with harsh environments containing huge amounts of heme, such as the triatomine midgut. This transporter could be involved in the clearance of free amphipathic porphyrins located in the lipid membrane. It could be speculated that the portion of heme that escapes from this mechanism is captured and stocked into reservosomes by a second transporter. This observation is in agreement with the fact that *T. cruzi* is unable to synthesize heme, which is not easily available in the other environments of its life cycle.

Taken altogether, our data suggest that heme is necessary for the proliferation of *T. cruzi* parasites and points to the existence of a specific transmembrane transport of heme. Uptake of heme could be modulated by a PgP homologue associated to the plasma membrane of the parasite. Unraveling this mechanism should further allow the development of new drugs able to inhibit this class of transporter, compromising the survival of the parasite and thus useful in Chagas's disease treatment. A detailed description of these transporters, and their application in parasite control, is currently underway in our laboratories.

Acknowledgments

We express our gratitude to Dr. Ulysses Lins and Dr. Thais Souto Padron, from Instituto de Microbiologia Prof Paulo de Góes, UFRJ, to allow the mounting of Pd-mP filter on their epifluorescence microscope Zeiss Axioplan 2. We thank Dr. E. Pays and Dr. C. Felu (Brussels) for critical reading of the manuscript.

References

[1] C. Chagas, Nova tripanomíase humana. Estudos sobre a morfologia e o ciclo evolutivo do *Schizotrypanum cruzi*, n. gen., agente etiológico de nova entidade mórbida do homem, Mem. Inst. Oswaldo Cruz 1 (1909) 159–218.

[2] Z. Brener, Biology of *Trypanosoma cruzi*, Annu. Rev. Microbiol. 27 (1973) 347–382.

[3] R. Zeledon, Epidemiology, modes of transmission and reservoir hosts of Chagas disease, Ciba Found. Symp. 20 (1974) 51–77.
[4] E.S. Garcia, P. Azambuja, Development and interactions of *Trypanosoma cruzi* within the insect vector, Parasitol. Today 7 (1991) 240–244.
[5] T.A. Salzman, A.M. Stella, W. Xifra, A.M. Batlle, R. Docampo, A.O. Stoppani, Porphyrin biosynthesis in parasitic hemoflagellates: functional and defective enzymes in *Trypanosoma cruzi*, Comp. Biochem. Physiol. B 72 (1982) 663–667.
[6] M.E. Lombardo, L.S. Araujo, A. Batlle, 5-Aminolevulinic acid synthesis in epimastigotes of *Trypanosoma cruzi*, Int. J. Biochem. Cell Biol. 35 (2003) 1263–1271.
[7] N.M. El-Sayed et al., The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease, Science 309 (2005) 409–415.
[8] J.F. Sah, H. Ito, B.K. Kolli, D.A. Peterson, S. Sassa, K.P. Chang, Genetic rescue of Leishmania deficiency in porphyrin biosynthesis creates mutants suitable for analysis of cellular events in uroporphyria and for photodynamic therapy, J. Biol. Chem. 277 (2002) 14902–14909.
[9] W. De Souza, T.U. Carvalho, M. Benchimol, *Trypanosoma cruzi*: ultraestructural, cytochemical and freeze-fracture studies of protein uptake, Exp. Parasitol. 45 (1978) 101–115.
[10] I. Porto-Carreiro, M.A. Attias, K. Miranda, W. De Souza, N. Cunha-e-Silva, *Trypanosoma cruzi* epimastigote endocytic pathway: cargo enters the cytostome and passes through an early endosomal network before storage in reservosomes, Eur. J. Cell Biol. 70 (2000) 858–869.
[11] N. Cunha-e-Silva, C. Sant'anna, M.G. Pereira, I. Porto-Carreiro, A.L. Jeovanio, W. de Souza, Reservosomes: multipurpose organelles? Parasitol. Res. 99 (2006) 325–327.
[12] M.J. Soares, The reservossome of *Trypanosoma cruzi* epimastigotes: an organelle of the endocytic pathway with a role on metacyclogenesis, Mem. Ins. Oswaldo Cruz 94s 1 (1999).
[13] G.A. Grant, Synthetic Peptides. A User's Guide, Freeman and co, New York, 1992.
[14] J.A. O'Daly, M.B. Rodriquez, G. Garlin, *Trypanosoma cruzi*: growth requirements at different temperatures in fetal bovine serum or peptide supplemented media, Exp. Parasitol. 64 (1987) 78–87.
[15] D. Fraidenraich, C. Pena, E.L. Isola, E.M. Lammel, O. Coso, A.D. Anel, S. Pongor, F. Baralle, H.N. Torres, M.M. Flawia, Stimulation of *Trypanosoma cruzi* adenylyl cyclase by an alpha D-globin fragment from Triatoma hindgut: effect on differentiation of epimastigote to trypomastigote forms, Proc. Natl. Acad. Sci. USA 90 (1993) 10140–10144.
[16] F.A. Lara, U. Lins, G.H. Bechara, P.L. Oliveira, Tracing heme in a living cell: hemoglobin degradation and heme traffic in digest cells of the cattle tick, *Boophilus microplus*, J. Exp. Biol. 208 (2005) 3093–3101.
[17] C. Wandersman, P. Delepelaire, Bacterial iron sources: from siderophores to hemophores, Annu. Rev. Microbiol. 58 (2004) 611–647.
[18] P.C. Krishnamurthy, G. Du1, Y. Fukuda, D. Sun, J. Sampath, K.E. Mercer, J. Wang, B. Sosa-Pineda, K.G. Murti, J.D. Schuetz, Identification of a mammalian mitochondria porphyrin transporter, Nature 443 (2006) 586–589.
[19] G. Liminga, P. Nygren, R. Larsson, Microfluorometric evaluation of calcein acetoxymethyl ester as a probe for P-glycoprotein-mediated resistance: effects of cyclosporin A and its nonimmunosuppressive analogue SDZ PSC 833, Exp. Cell Res. 212 (1994) 291–296.
[20] M.P. Draper, R.L. Martell, S.B. Levy, Indomethacin-mediated reversal of multidrug resistance and drug efflux in human and murine cell lines overexpressing MRP, but not P-glycoprotein, Br. J. Cancer 75 (1997) 810–815.
[21] J.K. Pal, M. Joshi-Purandare, Dose-dependent differential effect of hemin on protein synthesis and cell proliferation in *Leishmania donovani* promastigotes cultured in vitro, J. Biosci. 26 (2001) 225–231.
[22] T.H. Schmitt, W.A. Frezzatti Jr., S. Schreier, Hemin-induced lipid membrane disorder and increased permeability: a molecular model for the mechanism of cell lysis, Arch. Biochem. Biophys. 307 (1993) 103.
[23] M.F. Oliveira, J.R. Silva, M. Dansa-Petretski, et al., Heme detoxification by insect, Nature 400 (1999) 517–518.