Acta Tropica 120 (2011) 211–218

Contents lists available at SciVerse ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

The heme uptake process in *Trypanosoma cruzi* epimastigotes is inhibited by heme analogues and by inhibitors of ABC transporters

Mauricio Peixoto Cupello[a], Cíntia Fernandes de Souza[a], Celeste Buchensky[b], Juliana Baptista Rocha Corrêa Soares[c], Gustavo Augusto Travassos Laranja[a], Marsen Garcia Pinto Coelho[a], Julia A. Cricco[b,*], Marcia Cristina Paes[a,d,**]

[a] *Laboratório de Interação Tripanossomatídeos e Vetores, Departamento de Bioquímica, Instituto de Biologia Roberto Alcântara Gomes, IBRAG, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil*
[b] *Departamento de Química Biológica and Instituto de Biología Molecular y Celular de Rosario (IBR, CONICET-UNR), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina*
[c] *Laboratório de Bioquímica Redox, Programa de Biologia Molecular e Biotecnologia, Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil*
[d] *Instituto Nacional de Ciência e Tecnologia - Entomologia Molecular (INCT-EM), Brazil*

ARTICLE INFO

Article history:
Received 27 April 2011
Received in revised form 18 July 2011
Accepted 20 August 2011
Available online 26 August 2011

Keywords:
Heme
Trypanosoma cruzi
Porphyrins
Heme transport

ABSTRACT

Heme (iron protoporphyrin IX) is an important molecule involved in many biological reactions, including oxygen transport, respiration, photosynthesis and drug detoxification. *Trypanosoma cruzi* parasites, the etiological agent of Chagas' disease, take up heme from the environment to supply their nutritional needs because they do not synthesize this cofactor. However, the mechanisms involved in heme transport across biological membranes are poorly understood. Indeed, in *T. cruzi*, no heme transporter has yet been characterized. In the present work, we evaluate the heme uptake processes by *T. cruzi* epimastigotes using fluorescent heme-analogues. Heme uptake decreased significantly when cells were pretreated with different concentrations of SnPPIX, PdMPIX or ZnMPIX, this observed competition suggests that they are taken up by the same transport system. We studied the growth behavior of epimastigotes using the same heme-analogues and the treatments with SnPPIX or PdMPIX impaired cell growth but when heme was added to the culture medium the observed inhibition was partially reversed. In addition, we tested how the heme uptake processes are affected by the presence of different transporter inhibitors. When the cells were treated with inhibitors and then incubated with heme, heme uptake decreased significantly for all treatments. These results constitute a strong indication for the existence of a protein associated with porphyrin transport in *T. cruzi*, possibly ATP-binding cassette transporters (ABC-transporter).

1. Introduction

Trypanosoma cruzi is a protozoan parasite that resides in the blood and other tissues and causes American Trypanosomiasis, or Chagas' disease, which is recognized by the World Health Organization (WHO) as one of thirteen major neglected diseases throughout the world. Chagas' disease is a significant problem in Latin America (Hotez et al., 2007), where it is endemic, but other countries (considered non-endemic) are considerably affected as well because of the migration movements of the Latin American population (Coura and Viñas, 2010). Currently, an estimated 10 million people are infected worldwide, primarily in Latin America, and more than 25 million people are at risk of being infected (WHO, 2010). The treatment is not very effective; the available medications have serious side effects and little efficacy during the chronic stage of the disease. As there are currently no prospects for vaccines or satisfactory medical treatments, the search for new therapies is a priority. This disease is transmitted by triatomine vector insects while they feed on a vertebrate host. These bloodsucking insects ingest 6–12 times their original weight in blood (Wigglesworth, 1943). This blood is continuously digested through a variety of proteinases, releasing amino acids, peptides and heme. Usually, approximately 10 mM of heme bound to hemoglobin is obtained in a single ingest (Graça-Souza et al., 2006).

The heme molecule is an important cofactor that is involved in several essential biological processes in aerobic organisms, such as oxygen transport (hemoglobin and myoglobin), cellular respiration

* Corresponding author at: Departamento de Química Biológica and Instituto de Biología Molecular y Celular de Rosario (IBR, CONICET-UNR), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, S2002LRK Rosario, Argentina. Tel.: +54 341 435 1235x109; fax: +54 341 439 0465.
** Corresponding author at: Boulevard 28 de setembro, 87 fundos IBRAG 4o andar Bioquímica, Rio de Janeiro, Brazil. Tel.: +55 21 28688043; fax: +55 21 28688036.
E-mail addresses: cricco@ibr.gov.ar (J.A. Cricco), mcpaes@uerj.br, marcia.paes@gmail.com (M.C. Paes).

0001-706X/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.actatropica.2011.08.011

(cytochrome *c*, cytochrome *c* oxidase, and cytochrome *c* reductase), drug detoxification (enzyme P450) and enzymes involved in antioxidant defense (catalases and peroxidases) (Ponka, 1999). Heme is a highly toxic molecule due to its pro-oxidant properties (Ryter and Tyrrel, 2000; Deterding et al., 2004), its biosynthesis and degradation are tightly regulated in accordance with cellular requirements. The heme biosynthetic pathway is conserved throughout evolution, in non-photosynthetic eukaryotic cells, it starts and finishes in the mitochondria, while still involving several cytosolic steps (Severance and Hamza, 2009). There are some organisms that depend on essential heme–proteins but lack in part or in total a heme biosynthetic pathway. Trypanosomatids are included in this group (revisited by Koreny et al., 2010; Tripodi et al., 2011). Biochemical studies have demonstrated the void of a complete heme biosynthesis pathway in *T. cruzi* (Lombardo et al., 2003), and this was later corroborated by the absence of conserved enzymes in its genomic sequence (El-Sayed et al., 2005).As heme is an essential cofactor for trypanosomatids, the processes of heme binding, transport and intracellular distribution must involve critical pathways in these organisms. Heme uptake in trypanosomatids is a topic of actual debate and, specifically for *T. cruzi*, there is limited information concerning transporters or carriers and there is no biochemical characterization of the process itself. Our group has demonstrated that in *T. cruzi* epimastigotes, heme and hemoglobin internalization proceed through different routes and/or mechanisms (Lara et al., 2007). We have observed by fluorescence microscopy using fluorescent heme analogues that heme uptake might involve the activity of a P-glycoprotein (Pgp) homologue, an ABC transporter. Latter, the protein TbHpHbR was described as a receptor for the complex haptohemoglobin-hemoglobin (Hp–Hb) (Vanhollebeke et al., 2008). The authors demonstrated that TbHpHbR is a bloodstream stage-specific protein, but it is not expressed in the procyclic form. TbHpHbR is present in *Trypanosoma brucei subsp. gambiense* and *Trypanosoma brucei subsp. rhodesiense* but absent from the related kinetoplastids *T. cruzi* and *Leishmania*. This protein directs the internalization of heme carried by the Hp–Hb complex into hemoproteins in order to optimize growth of bloodstream forms. Recently, it was demonstrated that an ATP-binding cassette protein was involved in intracellular heme trafficking in *Leishmania* (Campos-Salinas et al., 2011). The protein LABCG5 was associated with the internal distribution of hemoglobin-bound heme to the mitochondria, but LABCG5 was not involved in the internal trafficking of free heme. Currently, the mechanisms by which heme is transported through the plasma membrane and targeted to heme proteins in the intracellular medium remain unknown for all the trypanosomatids.

Considering the implications of new discoveries in the transport of porphyrins in *T. cruzi*, it becomes great importance, especially from the standpoint of chemotherapy, to elucidate the mechanisms for heme transport. In this study, we investigate the effects of heme transport blockage by heme analogues and specific transporters inhibitors and, consequently, the disruption of the life-cycle of *T. cruzi*.

2. Materials and methods

2.1. Reagents

BHI (brain–heart infusion medium) was obtained from DIFCO (Sparks, MD, USA). Cyclosporin A (CsA) was purchased from Novartis Pharma S.A.S. (Huningue, France). Fetal calf serum (FCS) was purchased from CULTLAB (São Paulo, Brazil). Heme (Fe–protoporphyrin IX), Sn–protoporphyrin IX (SnPPIX), Pd–mesoporphyrin IX (PdMPIX) and Zn–mesoporphyrin IX (ZnMPIX) were obtained from Frontier Scientific (Logan, UT, USA). Indomethacin (1-[p-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid) was obtained from SIGMA–ALDRICH CHEME (Steinheim, Germany). Pyridine was purchased from MERCK (Darmstadt, Germany). Verapamil (5-[N-(3,4-dimethoxyphenylethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile hydrochloride) was also purchased from SIGMA–ALDRICH CHEME (Steinheim, Germany). All other reagents used were of analytical grade.

2.2. Parasites

T. cruzi epimastigotes, strain Dm28c, were grown at 28 °C for seven days in BHI supplemented with 10% FCS and in the absence or presence of 30 μM heme. The growth was monitored by cell counting in a Neubauer chamber.

2.3. Porphyrins

Fe–protoporphyrin IX stock (20 mM) was prepared by dissolving in 0.1 N NaOH, and after it was buffered using PBS (100 mM sodium phosphate buffer and 150 mM NaCl at pH 7.4). The stock was diluted immediately before use to 10 mM in the same PBS buffer. Stocks of Sn–protoporphyrin IX, Pd–mesoporphyrin IX and Zn–mesoporphyrin IX (heme analogues) were prepared in 0.1 N NaOH and buffered in PBS in the same way as the heme and then used in the experiments.

2.4. ABC transporter inhibitors (cyclosporin A, indomethacin and verapamil)

The inhibitor stock solutions were prepared as follows: the 5 mM cyclosporin A and 10 mM verapamil stock solutions were prepared in water, and the 10 mM indomethacin stock solution was prepared in 0.1 N NaOH and PBS.

2.5. The effect of SnPPIX, PdMPIX, and ZnMPIX on T. cruzi epimastigote heme uptake

The parasites were maintained in BHI supplemented with 10% FCS for 7 days. After 7 days, the cells were washed twice with PBS. After washing, the cells were suspended with PBS and incubated for 10 min with different concentrations of the heme analogues (SnPPIX, PdMPIX, and ZnMPIX). After incubation, they were washed twice with PBS, suspended with PBS and then incubated with 10 μM heme for 10 min. After this incubation, the cells were collected, washed twice with PBS and lysed through repeated freezing and thawing. Heme uptake was measured in epimastigote lysates with the alkaline pyridine method (Falk, 1964) using a GBC UV/VIS 920 absorption spectrometer with the following volume modifications: 500 μL of sample was combined in a 1 mL cuvette with 500 μL of stock solution containing 0.2 N NaOH and 48% pyridine. The oxidized and reduced (via the addition of DTH) spectra were recorded between 500 nm and 600 nm. The 557 and 541 nm peaks were identified in the differential reduced minus oxidized spectra. The heme concentration was estimated using the molar extinction coefficient 20.7 mM^{-1} cm^{-1}. As controls we recorded the reduced minus oxidized spectra of heme alone and heme plus different amounts of SnPPIX and we did not observe any difference in the reduced minus oxidized spectra, indicating that these analogues did not show interference in this test.

2.6. Analysis of ZnMPIX uptake by T. cruzi epimastigotes measured via direct fluorescence from total cell extracts

The *T. cruzi* epimastigotes were maintained in BHI supplemented with 10% FCS and 30 μM hemin at 28 °C. Parasites in the

stationary phase were used to study the ZnMPIX-uptake processes. The cells were collected by centrifugation and washed twice with PBS. The cells were then suspended in PBS, and the assays were performed in PBS buffer at 28 °C using 2×10^7 parasites per assay, volume of 100 μL. The epimastigotes were incubated for 5 min with 10 μM ZnMPIX at different concentrations of hemin (0–100 μM) added to epismatigotes to complete a volume of 200 μL. After incubation, the reaction was stopped by the addition of 800 μL of cold PBS with 1 mM hemin. Then the cells were collected and washed 3 times with cold PBS (we tested that the last washing buffer does not contain hemin nor ZnMPIX). The cells were then suspended in lysis buffer (0.5% Triton X-100 in PBS) and disrupted by repeated freezing and thawing. The lysates were clarified by centrifugation, and the supernatant was used to measure the ZnMPIX fluorescence intensity in the total cell extracts using a Varian Eclipse fluorometer (λ_{ex} = 405 nm and λ_{em} = 578 nm). The fluorescence intensity measured is directly proportional to the ZnMPIX concentration. Every data point is the average of 3 independent assays, and the uptake experiments were reproduced at least 3 times. The same experiments were carried out in stationary phase and we observed the same effects on ZnMPIX uptake.

2.7. The effects of SnPPIX, PdMPIX, and ZnMPIX on T. cruzi epimastigote growth

Parasites were maintained at 28 °C for a week in BHI containing both 10% FCS and 30 μM heme, and then epimastigotes in the stationary phase were collected by centrifugation and washed three times with BHI without FCS. The cells were suspended in BHI with 10% FCS. The heme analogues (SnPPIX, PdMPIX, and ZnMPIX) were added to cells suspensions at different concentrations and were incubated for 10 min. Then 30 μM heme was added to the medium. The cells were then maintained for 12 days in culture. Cell growth was monitored by cell counting in a Neubauer chamber.

2.8. The effect of cyclosporin A, indomethacin and verapamil on epimastigotes heme uptake

Parasites were maintained in BHI supplemented with 10% FCS. The cells were washed twice with PBS and then treated with different ABC-transporter modulators (300 μM indomethacin, 10 μM cyclosporin A or 2 μM verapamil) for 30 min. Then 10 μM heme was added to the medium, and the samples were incubated for another 10 min. After this incubation, the cells were lysed through a freeze/thaw process. The heme uptake was measured from the *T. cruzi* lysates as previously described in Section 2.5.

2.9. Evaluation of the effect of cyclosporin A on the proliferation of T. cruzi epimastigotes

The cells were maintained at 28° for a week in BHI containing both 10% FCS and 30 μM heme. During the stationary phase, they were collected by centrifugation and washed three times with BHI without FCS. The cells were suspended in BHI with 10% of FCS. The epimastigotes were pre-treated with increasing doses of cyclosporin A (1–20 μM) for 30 min, and then 10 μM heme was added to the cells. The cells were maintained for 7 days in this culture. Cell growth was monitored by cell counting in a Neubauer chamber.

2.10. Evaluation of the effect of cyclosporin A on the heme uptake in T. cruzi epimastigotes

Parasites were maintained in BHI supplemented with 10% FCS and 30 μM heme at 28° for one week. Then, the cells in the stationary phase were collected by centrifugation and washed three times with BHI without FCS. After washing, the epimastigotes were suspended in BHI with 10% FCS and pre-treated with 10 μM of cyclosporin A for 30 min. After 30 min, 300 μM heme was added to the medium, and the cells were maintained for 7 days in culture. After 7 days, the cells were collected, washed twice with PBS, lysed as described above and the heme uptake was measure as described previously in Section 2.5.

2.11. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). The data are presented as the mean ± SD. The experimental data were analyzed by one-way analysis of variance (ANOVA), and differences between groups were assessed using Tukey's post-test. The level of significance was set at $p < 0.05$, and all experiments were repeated at least three times.

3. Results

3.1. The effect of the heme analogues (SnPPIX, PdMPIX and ZnMPIX) on heme uptake and cell proliferation in T. cruzi epimastigotes

The heme analogues used in this work have a similar chemical structure to heme. The mesoporphyrin derivatives have replaced the two vinylene groups of the original structure of protoporphyrin IX by two ethyl groups besides, they contain Pd(II) and Zn(II) as central ion (PdMPIX and ZnMPIX, respectively). The SnPPIX analogue have replaced only central metal ion but conserve the protoporphyrin ring structure. All three molecules are good fluorophores, and several have been used to study heme uptake by fluorescence microscopy and heme trafficking in different tissue types and organisms (Worthington et al., 2001; Lara et al., 2005; Rao et al., 2005; Sartonello et al., 2010), including our previous work where PdMPIX was used to follow by fluorescence microscopy heme internalization in *T. cruzi* epimastigotes (Lara et al., 2007).

As a first approach to try to elucidate the process of heme transport in *T. cruzi* epimastigotes, we designed and optimized conditions to evaluate heme uptake in the presence of the fluorescent heme analogues SnPPIX, PdMPIX and ZnMPIX. The intracellular heme concentration was determined using hemochrome pyridine methods (Falk, 1964), as described in Section 2. We observed that epimastigotes in the stationary phase can accumulate approximately 10–15 nmol of heme per 10^8 cells when they are incubated with 10 μM heme for 10 min after being depleted of heme, as shown in Fig. 1 (panels A, B and C; the concentration 0 represent cells without treatment with heme analogues). When the cells were treated with different concentrations of heme analogues, we observed a decrease in the intracellular heme concentration. This reduction is proportional to the analogue concentration, as shown in Fig. 1(A–C). In particular, we followed ZnMPIX uptake by spectrofluorometry, as described in Section 2. The fluorescence intensity (IF) of ZnMPIX, which was measured from total cell extracts treated with different heme concentrations, is presented in Fig. 2. We observed lower ZnMPIX IF values as the heme concentration increased. This reduction in ZnMP uptake is in agreement with the previous results presented in Fig. 1, when we followed the intracellular heme uptake in parasites treated with different concentrations of analogues. A similar effect was observed when different *T. cruzi* strains were subject of the same treatment, such as CL-Brener and CL 14 (data not shown). These results indicate that heme analogues (PPIX or MPIX) might be imported into cells through the same system used to uptake heme.

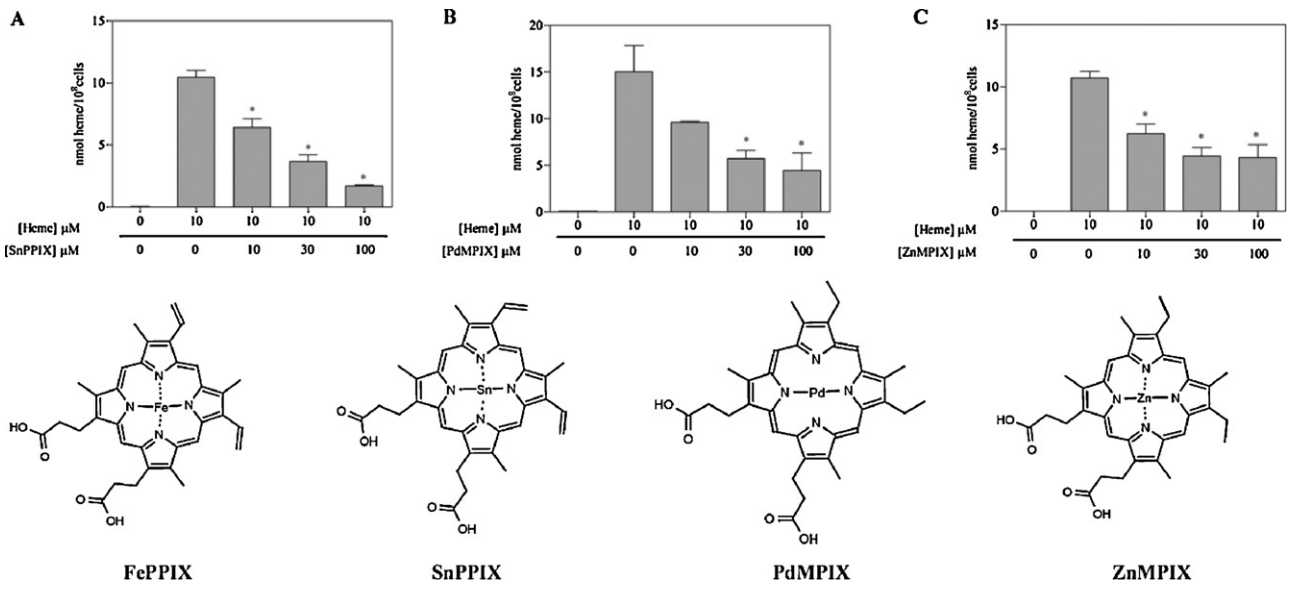

Fig. 1. The heme analogues (SnPPIX, PdMPIX and ZnMPIX) prevents heme uptake by *T. cruzi* epimastigotes. *Trypanosoma cruzi* Dm28c were maintained in BHI supplemented with 10% FBS at 28 °C for 7 days. The epimastigotes were washed with PBS and treated with different concentrations of SnPPIX (A), PdMPIX (B) and ZnMPIX (C) for 10 min and then incubated with 10 μM heme for 10 min. The negative control (0) did not receive any treatment, whereas the positive control (0 heme analogue) was treated with 10 μM heme for 10 min. Heme uptake in *T. cruzi* was measured in lysed epimastigotes using the alkaline pyridine method. All data are presented as the means ± SD, $^{*}p < 0.05$ in relation to the heme treatment, as determined using Tukey's test. This result is representative of 3 experiments.

We also evaluated the effect of these heme analogues on the proliferation of *T. cruzi* epimastigotes, as described in Section 2. Briefly, we treated the epimastigotes with heme and various concentrations of SnPPIX, PdMPIX or ZnMPIX. The parasites were kept in culture for 12 days, and cell growth was monitored by counting in a Neubauer chamber. We observed a marked inhibition in parasite growth when the parasites were treated with heme analogues (Fig. 3). However, the addition of heme after treatment with SnPPIX and PdMPIX partially alleviated this inhibitory effect (Fig. 3A and B). Interestingly, the inhibitory effect observed after treatment with ZnMPIX did not revert in the presence of heme (Fig. 3C), suggesting that under these experimental conditions the effect of this compound might be more deleterious or that ZnMPIX cannot be displaced by heme after being imported by the cells. However, we cannot exclude other factors related with the experimental conditions and/or related to any specific strain. We performed several controls to evaluated ZnMPIX toxicity in CL-14 and Cl Brener epimastigotes and these assays did not show the deleterious effect of ZnMPIX. In those cases the cells were treated during 10 min with (or without) 50 μM ZnMPIX in PBS buffer, then they were washed and cultured in BHI with 10% FCS (plus 30 μM heme) and we did not observe differences between the treated and non-treated cells (data not show). More experiments are necessary to distinguish between these hypotheses about ZnMPIX behavior compared to the others heme analogues. Besides, all the heme analogues showed a deleterious effect when they replaced heme in the culture media. This result confirms that heme is an essential cofactor for *T. cruzi* epimastigotes and these analogues may compete with heme for the same carrier in the parasite cell.

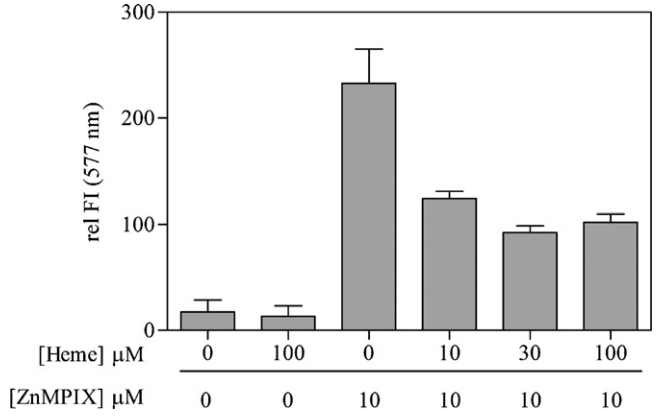

Fig. 2. The presence of increased heme concentration affects the ZnMPIX uptake in *T. cruzi* epimastigotes. *T. cruzi* Dm28c epimastigotes were maintained in BHI supplemented with 10% FCS and 30 μM heme at 28 °C for 7 days. The cells were washed twice with PBS and suspended in the same buffer at 2×10^8 cells/ml. The cells were incubated with ZnMPIX and different concentrations of heme for 5 min before the reaction was stopped by the addition of a cold PBS solution containing 1 mM heme. The amount of uptake was measured in lysed epimastigotes, after several steps of centrifugation and pellet suspension in cold PBS buffer, by determining fluorescence intensity in a spectrofluorometer (λ_{ex} = 405 nm and λ_{EMmax} = 577 nm). All data are presented as the means ± SD. This result is representative of 3 experiments.

3.2. The effect of ABC transporter inhibitors on heme uptake and cell proliferation in T. cruzi epimastigotes

Previously, we observed that the internalization of PdMPIX by epimastigotes, as measured by fluorescence microscopy, decreased in the presence of ABC transporter inhibitors (Lara et al., 2007). In addition, preliminary results obtained by Cricco and colleagues show that heme uptake by epimastigotes are dependent on ATP (personal communication, data not shown). These results suggest that heme uptake is mediated by an active transport system and that an ABC transporter is involved in this process. To elucidate what types of transporters or carriers are associated with heme transport in epimastigotes, we evaluated the changes in intracellular heme levels when parasites were treated with different transporter inhibitors. We measured the intracellular heme level spectrophotometrically, as described before, to evaluate the effect produced by the ABC transporter inhibitors cyclosporin A, indomethacin and verapamil at concentrations of 10 μM, 300 μM and 2 μM, respectively. The chosen inhibitors and concentrations were based on our previous results (Lara et al., 2007). We observed that when parasites are treated with the inhibitors and then incubated with heme, less heme was incorporated than in the untreated cells. The heme level decreased approximately 50% in all cases,

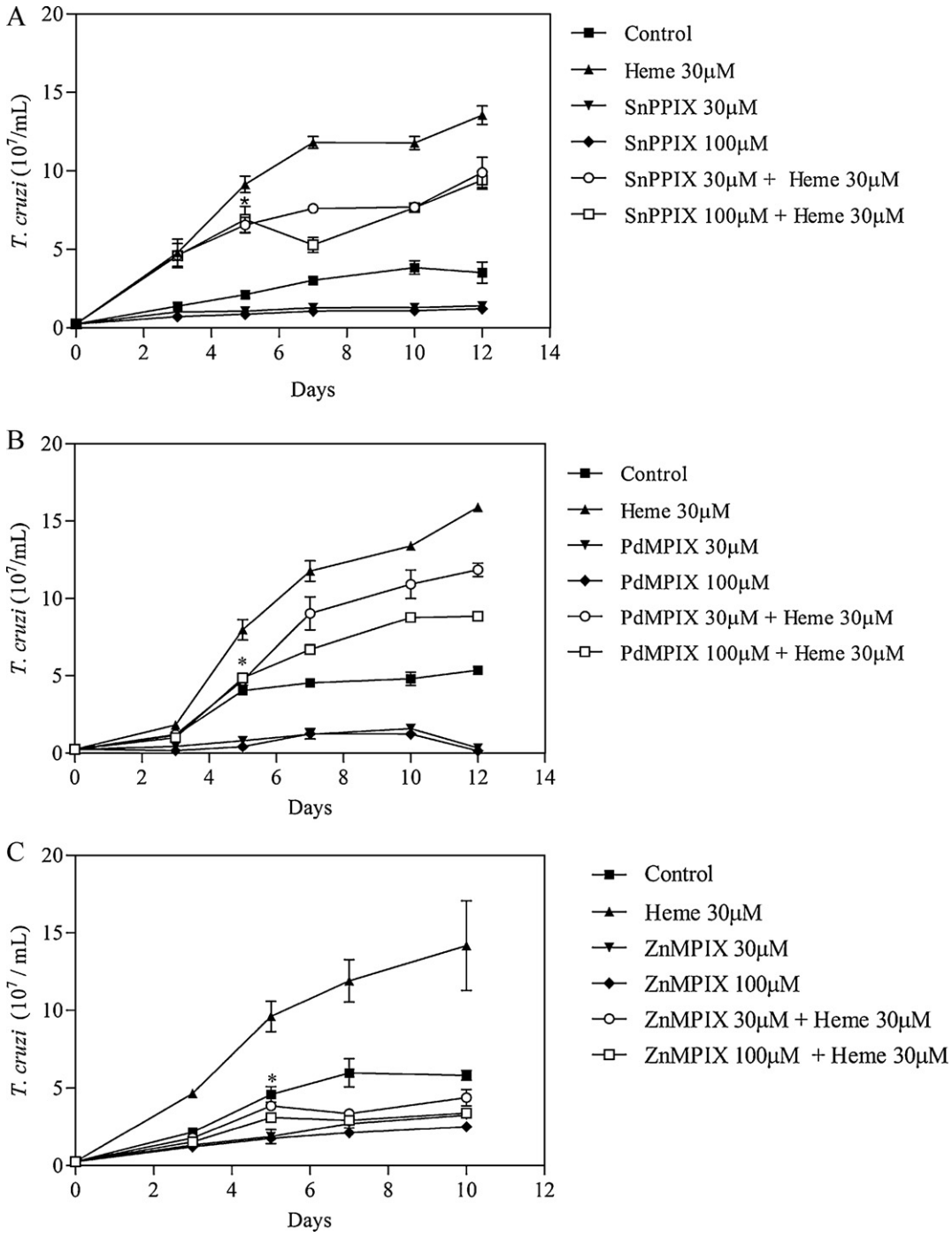

Fig. 3. The heme analogues (SnPPIX, PdMPIX and ZnMPIX) negatively affect the growth kinetics of *T. cruzi* epimastigotes. *Trypanosoma cruzi* Dm28c strain were maintained at 28 °C in BHI supplemented with 10% FBS and 30 μM heme. Epimastigotes in the stationary phase were washed with PBS and incubated in BHI supplemented with 10% FBS. The epimastigotes were then treated with different concentrations of SnPPIX (A), PdMPIX (B) and ZnMPIX (C) for 10 min before incubation with 30 μM heme. All data are presented as the means ± SD, $^{*}p < 0.05$ in relation to the heme treatment by Tukey's test. The results are representative of three independent experiments.

shown in Fig. 4. This result supports our hypothesis that an ABC transporter could be involved in the translocation of heme through the lipid bilayer in *T. cruzi*. To evaluate the effect of these inhibitors on the parasite proliferation, we treated them with increasing doses of cyclosporin A (1–20 μM). It was observed that doses smaller than 5 μM do not interfere with proliferation. At concentration of 10 μM CsA we observed a small reduction on parasite proliferation. However, at cyclosporin A concentrations greater than 10 μM, the proliferation induced by heme incubation is prevented, as shown in Fig. 5. Finally, we decided to evaluated how increasing doses of heme (10–300 μM) modulate the effect of 10 μM cyclosporin A treatment as shown in Fig. 6, our results demonstrate that heme does not reverse the inhibitory effects of cyclosporin A, suggesting that CsA, at this concentration, is affecting other processes in addition to heme uptake. To further investigate this point, we evaluated the intracellular heme level in the presence of CsA.

Parasites were treated with 10 μM cyclosporin A for 30 min and then incubated with or without heme in culture for 7 days, as detailed in Section 2. These results are shown in Fig. 7. We observed that CsA treatment prevents heme uptake when the parasites are

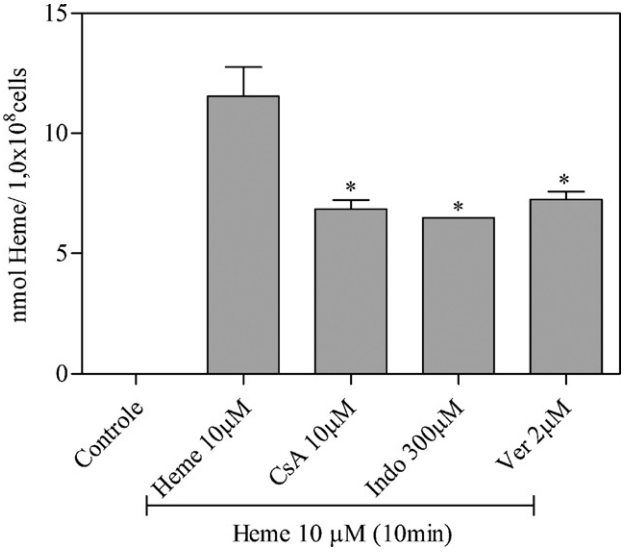

Fig. 4. The treatment with ABC transporter inhibitor affects the heme uptake in *T. cruzi* epimastigotes. *Trypanosoma cruzi* Dm28c were maintained in BHI supplemented with 10% FBS at 28 °C for 7 days. The epimastigotes were washed with PBS and treated with 10 μM CsA, 300 μM indomethacin or 2 μM verapamil for 30 min and then incubated with 10 μM heme for an additional 10 min. The control did not receive any treatment. Heme uptake in *T. cruzi* was measured in lysed epimastigotes using the alkaline pyridine method. All data are presented as the means ± SD of three independent experiments, $^{*}p < 0.05$ in relation to the heme treatment, as measured by Tukey's test.

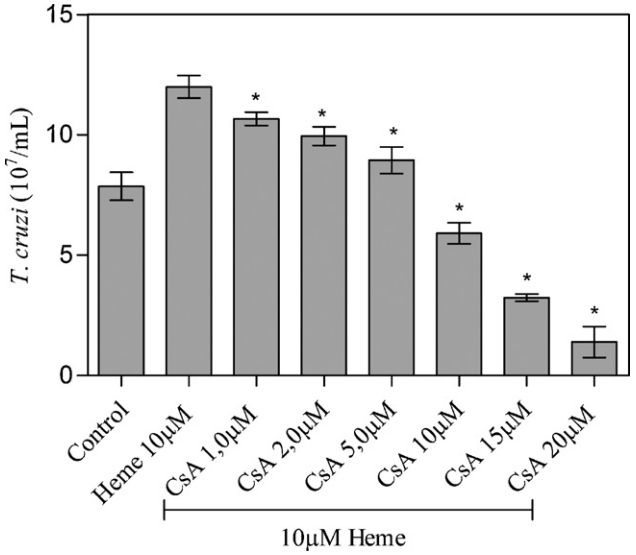

Fig. 5. The dose-effect of CsA on the proliferation of *T. cruzi* epimastigotes grown in the presence of heme. *Trypanosoma cruzi* Dm28c were maintained at 28 °C in BHI supplemented with 10% FBS and 30 μM heme. Epimastigotes in the stationary phase were washed with PBS and treated with different concentrations of cyclosporin A (CsA) for 30 min. The parasites were then incubated with 10 μM heme for 7 days. All data are presented as the means ± SD of two independent experiments, $^{*}p < 0.05$ in relation to the heme treatment, as determined using Tukey's test.

incubated with 300 μM heme. However, a basal level of heme was maintained in the cells, this amount was presumably sufficient to allow the parasite regular proliferation. In addition, the results presented in Figs. 6 and 7 allowed us consider that at 10 μM CsA the parasite proliferation might be affected because extra heme import is prevented, inhibiting at least in part, the heme uptake processes

4. Discussion

The study of heme acquisition by *T. cruzi* is critical for understanding the following aspects of *T. cruzi* infection: (i) the biology of the parasite, (ii) heme uptake in *T. cruzi*, and (iii) the interaction between the parasite and the vector. Advancing knowledge regarding the transporters used by parasites is necessary because blocking heme transport may interrupt the relationship between *T. cruzi* and the vector or host. In this work, we developed a method to evaluate the heme uptake processes in *T. cruzi* epimastigotes. We used the fluorescent heme analogues SnPPIX, ZnMPIX and PdMPIX to evaluate heme uptake. We observed that the presence of these compounds decreased intracellular heme levels and affected parasite proliferation, suggesting a competition between the porphyrins and heme for the same transport machinery. The presence of common porphyrin-transporter in *T. cruzi* agrees with the results presented by Campos-Salinas et al. (2011) where they study the heme transport by *Leishmania donovani* promastigotes using MgPPIX as heme analogue by flow citometry. Our results indicate that *T. cruzi* proliferation is mediated almost exclusively by heme through unknown mechanisms. We observed that treatment with SnPPIX, ZnMPIX and PdMPIX inhibited parasite proliferation, and this inhibition is reverted by the presence of heme, although the case of

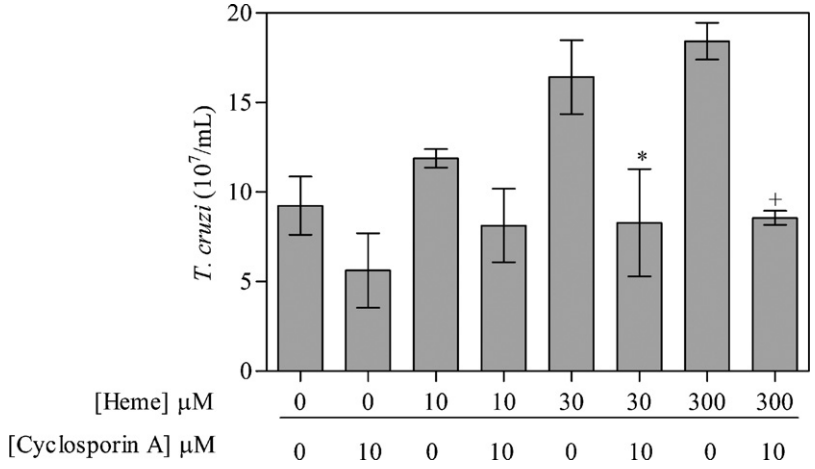

Fig. 6. The dose-effect of heme on the proliferation of *T. cruzi* epimastigotes grown in the presence of CsA. *Trypanosoma cruzi* Dm28c were maintained in BHI supplemented with 10% FBS and 30 μM heme at 28 °C. Epimastigotes in the stationary phase were washed with PBS and treated with 10 μM cyclosporin A (CsA) for 30 min. The cells were then incubated with different concentrations of heme for 7 days. All data are presented as the means ± SD of two independent experiments. $^{*}p < 0.05$ in relation to the cells grown with 30 μM heme, and $^{+}p < 0.05$ in relation to the cells grown with 300 μM heme, as determined using Tukey's test.

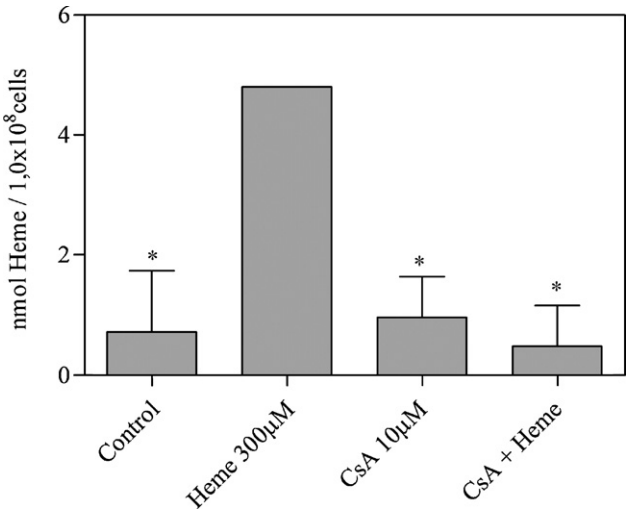

Fig. 7. The effect of cyclosporin A on heme uptake in *T. cruzi* epimastigotes. *Trypanosoma cruzi* Dm28c were maintained at 28 °C in BHI supplemented with 10% FBS and 30 μM heme. Epimastigotes in the stationary phase were washed with PBS and treated with 10 μM cyclosporin A (CsA) for 30 min. The cells were then incubated with 300 μM heme for 7 days. Heme uptake in *T. cruzi* was measured in lysed epimastigotes using the alkaline pyridine method. All data are presented as the means ± SD with $^*p < 0.05$ in relation to the heme treatment, as determined using Tukey's test. This result is representative of two independent experiments.

ZnMPIX is controversial and more studies will be necessary to address it behavior.

ABC transporters are responsible for moving a variety of molecules through the lipid membrane of cells and organelles, mostly against a concentration gradient at the expense of ATP hydrolysis. These carriers are remarkably variable with regards to their physiological functions, their substrates and the direction of transport, alternating between import and export (Higgins, 1992). Klokouzas et al. (2003) identified approximately 30 members of the ABC family in the main protozoan parasites. Sauvage et al. (2009) presented a review on ABC proteins that have been identified in the main protozoan parasites, including *Leishmania*, *Trypanosoma*, *Plasmodium*, *Toxoplasma*, *Cryptosporidium* and *Entamoeba*. It has also been observed that the activity of ABC transporters is a basic biological strategy to defend living cells against cytotoxic attack by xenobiotics, but ABC transporters also play an important role in other cellular functions that have yet to be characterized in protozoan parasites. In the present research work, we studied the effects of specific ABC transporter inhibitors (cyclosporin A, verapamil and indomethacin) to investigate the possibility for the involvement of a protein of this family in the translocation of heme across the plasma membrane of *T. cruzi* epimastigotes. We showed that heme uptake by the parasite decrease approximately 50% in the presence of these inhibitors. In subsequent analyses, we evaluated the effect of cyclosporin A on parasite proliferation and heme accumulation. It was possible to observe a reduction in cell proliferation concomitantly to a reduction on heme accumulation. These results allow us to hypothesize that interference with heme transport will interrupt the trypanosome life cycle. The inhibition of *T. cruzi* cell proliferation by cyclosporin A was also observed by Búa et al. (2008). The authors reported inhibitory effects of cyclosporin A on the proliferation of epimastigotes, the internalization of trypomastigotes, the development of intracellular amastigotes, and infection by *T. cruzi in vivo*. They associate the inhibitory effect of CsA with a role of a Pgp protein, but this was unrelated to the transport of heme and/or porphyrins. The involvement of ABC transporters in heme transport has been observed in other studies. Krishnamurthy et al. (2006) published a study demonstrating the involvement of an ABC transporter in the translocation of coproporphyrinogen III (CPIII), a precursor of heme, from the cytoplasm into the mitochondria in mammalian cells by linking the expression of this transporter with heme synthesis. Other studies in mammals have shown that ABCG2 (Krishnamurthy et al., 2004) has a potential heme export activity during the development of erythroid cells. Yet another example can be seen in the recent study by Desuzinges-Mandon et al. (2010), which demonstrates the ability of the membrane protein ABCG2 to transfer the heme molecule to human serum albumin. Recently, an ABC protein was shown to be involved in internal heme trafficking in *L. donovani* (Campos-Salinas et al., 2011). This protein is utilized for hemoglobin-bound heme trafficking to the mitochondria but not in the movement of free heme. This protein is conserved in other trypanosomatids. Other studies presented by Vanhollebeke et al. (2008) demonstrated that TbHpHbR is a bloodstream stage-specific protein and directs the internalization of heme carried by the Hp–Hb complex into hemoproteins in order to optimize growth of bloodstream forms. TbHpHbR is present in *T. brucei subsp. gambiense* and *T. brucei subsp. rhodesiense* but absent from the related kinetoplastids *T. cruzi* and *Leishmania*. It is important to note that although only 3 ABC genes have been characterized in *T. cruzi*, 28 have been identified in the genome of this parasite; the functions of the remaining 25 are still unknown (Sauvage et al., 2009).

In summary, our results indicate that a common porphyrin transporter may be involved in the entry of amphipathic porphyrins, as suggested by the observation that different heme analogues compete with heme for cellular uptake. We present evidence indicating that heme/porphyrins translocation in *T. cruzi* epimsatigote is mediated by a transporter protein and possibly a member of the ABC transporter family; however, a molecular approach for identifying the transporter is required. The answer to this question in *T. cruzi* will be of great importance because heme transport is essential for parasite survival. The identification of this essential transporter might allow for the development of chemotherapy targets for the treatment of Chagas' disease.

Acknowledgement

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Instituto Nacional de Ciência e Tecnologia - Entomologia Molecular (INCT-EM) – Brasil to MCP. PIP 2010-0685 Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) Argentina and Subsidio de la Fundación Bunge y Born 2010/2012, para Investigación de la Enfermedad de Chagas (FBB27/10) to JAC.

References

Búa, J., Fichera, L.E., Fuchs, A.G., Potenza, M., Dubin, M., Wenger, R.O., Moretti, G., Scabone, C.M., Ruiz, A.M., 2008. Anti-*Trypanosoma cruzi* effects of cyclosporin A derivatives: possible role of a P-glycoprotein and parasite cyclophilins. Parasitology 135, 217–228.

Campos-Salinas, J., Cabello-Donayre, M., García-Hernández, R., Pérez-Victoria, I., Castanys, S., Gamarro, F., Pérez-Victoria, J.M., 2011. A new ATP-binding cassette protein is involved in intracellular heme trafficking in *Leishmania*. Mol. Microbiol. 79, 1430–1444.

Coura, J.R., Viñas, P.A., 2010. Chagas disease: a new worldwide challenge. Nature 465, S6–S7.

Desuzinges-Mandon, E., Arnaud, O., Martinez, L., Huche, F., Di Pietro, A., Falson, P., 2010. Abcg2 transports and transfers heme to albumin through its large extracellular loop. J. Biol. Chem. 285, 33123–33133.

Deterding, L.J., Ramirez, D.C., Dubin, J.R., Mason, R.P., Tomer, K.B., 2004. Identification of free radicals on hemoglobin from its self-peroxidation using mass spectrometry and immuno-spin trapping: observation of a histidinyl radical. J. Biol. Chem. 279, 11600–11607.

El-Sayed, N.M., Myler, P.J., Bartholomeu, D.C., Nilsson, D., Aggarwal, G., Tran, A.N., et al., 2005. The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. Science 309, 409–415.

Falk, J.E., 1964. Porphyrins and Metalloporphyrins. Amsterdam, Elsevier Publishing Company.

Graça-Souza, A.V., Maya-Monteiro, C., Paiva-Silva, G.O., Braz, G.R.C., Paes, M.C., et al., 2006. Adaptations against heme toxicity in blood-feeding arthropods. Insect Biochem. Mol. Biol. 36, 322–335.

Higgins, C.F., 1992. ABC transporters: from microorganisms to man. Annu. Rev. Cell. Biol. 8, 67–113.

Hotez, P.J., Molyneux, D.H., Fenwick, A., Kumaresan, J., Sachs, S.E., Sachs, J.D., Saviolli, L., 2007. Control of neglected tropical diseases. N. Engl. J. Med. 357, 1018–1027.

Klokouzas, A., Shahi, S., Hladky, S.B., Barrand, M.A., Van Veen, H.W., 2003. ABC transporters and drug resistance in parasitic protozoa. Int. J. Antimicrob. Agents 22, 301–317.

Koreny, L., Lukes, J., Oborník, M., 2010. Evolution of the heme synthetic pathway in kinetoplastid flagellates: an essential pathway that is not essential after all? Int. J. Parasitol. 40, 149–156.

Krishnamurthy, P.C., Du, G., Fukuda, Y., Sun, D., Sampath, J., Mercer, K.E., Wang, J., Sosa-Pineda, B., Murti, K.G., Schuetz, J.D., 2006. Identification of a mammalian mitochondrial porphyrin transporter. Nature 443, 586–589.

Krishnamurthy, P., Ross, D.D., Nakanishi, T., Bailey-Dell, K., Zhou, S., Mercer, K.E., Sarkadi, B., Sorrentino, B.P., Schuetz, J.D., 2004. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. J. Biol. Chem. 279, 24218–24225.

Lara, F.A., Lins, U., Bechara, G.H., Olivera, P.L., 2005. Tracing heme in a living cell: hemoglobin degradation and heme traffic in digest cells of the cattle tick *Boophilus microplus*. J. Exp. Biol. 208, 3093–3101.

Lara, F.A., Sant'Anna, C., Lemos, D., Laranja, G.A.T., Coelho, M.G.P., Reis Salles, I., Michel, A., Oliveira, P.L., Cunha-e-Silva, N., Salmon, D., Paes, M.C., 2007. Heme requirement and intracellular trafficking in *Trypanosoma cruzi* epimastigotes. Biochem. Biophys. Res. Commun. 355, 16–22.

Lombardo, M.E., Araújo, L.S., Batlle, A., 2003. 5-Aminolevulinic acid synthesis in epimastigotes of *Trypanosoma cruzi*. Int. J. Biochem. Cell Biol. 35, 1263–1271.

Ponka, P., 1999. Cell biology of heme. Am. J. Med. Sci. 318, 241–256.

Rao, A.U., Carta, L.K., Lesuisse, E., Hamza, I., 2005. Lack of heme synthesis in a free-living eukaryote. Proc. Natl. Acad. Sci. 102, 4270–4275.

Ryter, S.W., Tyrrel, R.M., 2000. The heme synthesis and degradation pathways: role in oxidant sensitivity: heme oxygenase has both pro and antioxidant properties. Free Radic. Biol. Med. 28, 289–309.

Sartonello, R., Budu, A., Bagnaresi, P., Fernandes, C.A.H., Sato, P.M., Bueno, V.B., Fontes, M.R.M., Oliveira, P.L., Paiva-Silva, G.O., Alves, S.V., Netto, L.E.S., Catalani, L.H., Garcia, C.R.S., 2010. In vivo uptake of a heme-analogue Zn–protoporphyrin IX by the human malaria parasite *P. falciparum*-infected red blood cells. Cell Biol. Int. 34, 859–865.

Sauvage, V., Aubert, D., Escotte-Binet, S., Villena, I., 2009. The role of ATP-binding cassette (ABC) proteins in protozoan parasites. Mol. Biochem. Parasitol. 167, 81–94.

Severance, S., Hamza, I., 2009. Trafficking of heme and porphyrins in metazoa. Chem. Rev. 109, 4596–4616.

Tripodi, K.E.J., Menendez Bravo, S.M., Cricco, J.A., 2011. Role of heme and heme–proteins trypanosomatid essential metabolic pathways. Enzyme Res. 87323 (April) (Epub 2011).

Vanhollebeke, B., De Muylder, G., Nielsen, M.J., Pays, A., Tebabi, P., Dieu, M., Raes, M., Moestrup, S.K., Pays, E., 2008. A haptoglobin–hemoglobin receptor conveys innate immunity to *Trypanosoma brucei* in humans. Science 320, 677–681.

Wigglesworth, V.B., 1943. The fate of haemoglobin in *Rhodnius prolixus* (Hemiptera) and other blood-sucking arthropods. Proc. R. Soc. Lond. 131, 313–339.

World Health Organization Fact sheet n° 340. June 2010 from http://www.who.int/mediacentre/factsheets/fs340/en/print.html.

Worthington, M.T., Cohn, S.M., Miller, S.K., Luo, R.Q., Berg, C.L., 2001. Characterization of a human plasma membrane heme transporter in intestinal and hepatocyte cell lines. Am. J. Physiol. Gastrointest. Liver Physiol. 280, G1172–G1177.