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Trypanosomatid essential metabolic pathway: New approaches about heme fate in *Trypanosoma cruzi*

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ABSTRACT

Trypanosoma cruzi, the causal agent of Chagas disease, has a complex life cycle and depends on hosts for its nutritional needs. Our group has investigated heme (Fe-protoporphyrin IX) internalization and the effects on parasite growth, following the fate of this porphyrin in the parasite. Here, we show that epimastigotes cultivated with heme yielded the compounds α-meso-hydroxyheme, verdoheme and biliverdin (as determined by HPLC), suggesting an active heme degradation pathway in this parasite. Furthermore, through immunoprecipitation and immunoblotting assays of epimastigote extracts, we observed recognition by an antibody against mammalian HO-1. We also detected the localization of the HO-1-like protein in the parasite using immunocytochemistry, with antibody staining primarily in the cytoplasm. Although HO has not been described in the parasite's genome, our results offer new insights into heme metabolism in *T. cruzi*, revealing potential future therapeutic targets.

1. Introduction

Chagas disease or American trypanosomiasis is caused by the parasite *Trypanosoma cruzi*, which is transmitted to a vertebrate host by triatomine insects during feeding [1]. This disease is recognized by the World Health Organization as one of the neglected global diseases that infects an estimated 10 million people, primarily in Latin America, where the disease is endemic, and the migration of populations between countries has created new epidemiological, economic, social and political challenges [2]. More than 100 years after its discovery, Chagas disease does not have an efficient chemotherapy and new drugs and therapies should be sought for the treatment of Chagas' disease.

T. cruzi has a complex life cycle within different hosts, including humans, which supply the nutritional requirements lacking in the parasite [3]. Among these nutrients, heme is crucial to the proliferation of parasite epimastigotes [4,5] and is an essential supplement for the culture of these cells [6,7]. Heme catalyzes many of the oxidation processes in biological systems and is important in cellular function and organismal homeostasis. Furthermore it is also a regulatory molecule, and the absolute intracellular concentration must be tightly regulated [8,9].

Although it has been reported that *T. cruzi* lacks a complete pathway for heme biosynthesis [10,11], these species contains hemeprotein-like cytochromes involved in essential metabolic pathways. Buchensky et al. [12] published work showing the first functional characterization of *T. cruzi* ORFs that encode enzymes involved in heme A biosynthesis (TcCox10 and TcCox15), the prosthetic group of the mitochondrial cytochrome c oxidase and several bacterial terminal oxidases. Moreover, the heme porphyrin is important in *T. cruzi* epimastigote biology and must therefore be scavenged from the host. Heme uptake may occur via a specific porphyrin transporter, possibly a member of the ABC-transporter family [5,13].

However, free heme in solution is a potent free radical producer and is extremely harmful to biological systems [8,14,15], therefore its control is essential to the survival of the organism. One heme detoxification pathway occurs via the enzyme heme oxygenase (HO), an enzyme that is physiologically important, in part,

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because of the biological properties of its organic reaction products. In mammals, HO catalyzes heme degradation in an oxygen-dependent reaction and produces biliverdin (BV), carbon monoxide (CO) and iron (Fe^{+2}). BV is reduced by biliverdin reductase to bilirubin, which is excreted as a glucuronic acid conjugate [16,17]. Three HO isoforms have been characterized in several mammals, HO-1, HO-2 and HO-3, products of three different genes [18].

As the system for the enzymatic degradation of heme in *T. cruzi* is unknown, the object of this work was to study heme catabolism in this parasite. We identified heme degradation products and HO expression, a result that suggested the action of a heme oxygenase-like enzyme involved in *T. cruzi* heme homeostasis, underscoring its high potential as a chemotherapeutic target.

2. Materials and methods

2.1. Parasites

The *T. cruzi* strain Dm 28c (CT-IOC-010) was obtained from the Trypanosomatid Collection at the Oswaldo Cruz Institute, FIOCRUZ, Brazil. The epimastigotes were grown at 28 °C for 7 days in brain–heart infusion medium (BHI) and supplemented with 30 μM heme and 10% fetal calf serum (FCS). The cultures were maintained in 100-mL bottles with an initial density of 20–30 × 10^6 cells/mL in 30 mL of medium. Growth was monitored by counting the cells in a Neubauer chamber.

2.2. Extraction of heme and metabolites from T. cruzi epimastigotes

The epimastigote cells were maintained in 500 mL BHI supplemented with 10% FCS for 7 days in an Erlenmeyer flask (1000 mL) at 28 °C in an orbital shaker (Excella E24R, 100 rpm). The cells were then incubated with (test) or without (control) 30 μM heme for 24 h. The parasites were collected by centrifugation at 2000*g* at room temperature and then washed twice with PBS. The method previously described by Braz et al. [19] was used for heme extraction, with the modifications described below. The cells were lysed via three cycles of freezing in liquid nitrogen and thawing in a water bath. The material was acidified with equal volumes of 5 N HCl and conc. acetic acid and then extracted with 2 volumes of chloroform. After centrifugation, the chloroform layer was washed with distilled water and dried under nitrogen. The samples were dissolved in DMSO, and 100-μL aliquots were analyzed using a Shimadzu HPLC system.

2.3. HPLC fractionation and light absorption spectra

HPLC was performed using a Shimadzu CLC-ODS C18 column (15 mm × 22 cm) and a Shimadzu LC-10AT instrument (Tokyo, Japan) equipped with a diode array detector (SPD-M20A UV). The method for chromatography analysis was described by Pereira et al. [20] using 5% acetonitrile with 0.05% trifluoroacetic acid (TFA) as the solvent at a 0.4 mL/min flow rate. Before injection, the cell extracts were dissolved in DMSO and centrifuged (16800*g* for 15 min). At 10 min after sample injection, a 40-min linear acetonitrile gradient (5–80%) was applied, followed by 20 min of 80% acetonitrile. The light absorption spectra were recorded during the chromatography by the HPLC diode array detector. The compounds were identified by comparing their retention time and UV–Vis spectral data to previously injected standards.

2.4. Samples for immunoblotting

T. cruzi epimastigotes were treated for 5 days with different concentrations of heme (30 μM, 100 μM and 300 μM), and the treated samples were prepared as previously described [21]. For the immunoprecipitation, the parasites were resuspended in a different lysis buffer (20 mM Tris–HCl [pH 8.0], containing 150 mM NaCl, 5 mM EDTA and 10 mM NaF) with protease inhibitors and incubated for 30 min at 4 °C for complete lysis. After lysis, the parasites were centrifuged for 10 min at 9400*g* at 4 °C. The sediment was discarded, and the supernatant was collected to determine the protein concentration.

2.5. Immunoprecipitation

After lysis, the supernatant was incubated with a monoclonal antibody against HO-1 at a 1:200 dilution for 2 h at 4 °C with stirring. Protein A/G agarose (20 μL/500 μL sample) was then added for approximately 18 h at 4 °C. After this period, the samples were centrifuged (590*g* for 5 min at 4 °C) and rinsed twice with PBS. The sediment (resin) was eluted by adding sample buffer [22] and boiling for 3 min. The sample was then centrifuged (9400*g* for 5 min, room temperature) to collect the supernatant (eluate). After the first centrifugation, the supernatant (unbound) was collected for later analysis.

2.6. Immunoblotting

Lysates from the parasite cells (50 μg) were used, and the protein concentrations were determined by the method of Lowry et al. [23]. The proteins were separated on a 12% SDS–polyacrylamide gel [22] and blotted onto nitrocellulose membranes. The membranes were blocked in Tris-buffered saline (TBS) supplemented with Tween 0.1% and non-fat milk 5% for 1 h before an overnight incubation with the primary monoclonal antibody against HO-1 (Stressgen®) diluted 1:1000. After the primary antibody was removed, the membranes were washed 5 times in TBS supplemented with Tween 0.1% (TT). The membranes were then incubated with the secondary antibody conjugated to mouse peroxidase for 1 h. The washed blots were developed using a chemiluminescence ECL kit (Amersham). The above procedure was also used for the immunoblotting with the immunoprecipitation samples, but the dilution of the primary antibody was 1:250. As a protein loading control, polyclonal anti-tubulin was used at a 1:1000 dilution.

2.7. Immunocytochemistry

T. cruzi epimastigotes were treated for five days with and without heme (300 μM). After the incubation period, these parasites (10^7 cells/mL) were centrifuged, washed twice with PBS and fixed with 0.1% glutaraldehyde and 4% paraformaldehyde in sodium cacodylate buffer 0.1 M (pH 7.2) at 25 °C for 1 h. The cells were dehydrated in a sequence of solutions with increasing concentrations of methanol and then embedded in Lowicryl resin. The ultrathin sections were incubated with a monoclonal antibody against HO-1 (dilution 1:10) and then with a secondary anti-mouse antibody coupled to 10-nm colloidal gold particles (dilution 1:10). Lastly, the grids were contrasted using uranyl acetate and lead citrate and examined under a transmission electron microscope, Jeol JEM1011 (Tokyo, Japan).

2.8. Statistical analysis

The statistical analyses were performed using GraphPad Prism 3 software (GraphPad Software, Inc., San Diego, CA). The data are presented as the means ± SD. The data were analyzed using a one-way analysis of variance (ANOVA), and differences between the groups were assessed using the Tukey post-test. The level of significance was set at $p < 0.05$.

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