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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 or 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 analysis 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 $p < 0.05$.

3. Results and discussion

Although it is well known that trypanosomatids must obtain extracellular heme from their hosts as a nutritional cofactor [24], it remains unclear how heme is catabolized in these parasites. To study the heme degradation products of *T. cruzi*, we adopted a liquid-chromatography-method from Paiva et al. [25] using heme (Fe-protoporphyrin IX) and biliverdin IXα as standards to analyze the production of heme metabolites (Fig. 1A).

T. cruzi epimastigotes were treated as described in the Material and Methods section and analyzed by HPLC. The reverse-phase HPLC analysis of the cell extracts revealed three principal peaks (1, 2 and heme) at 632-nm detection in the control and test samples (Fig. 1A). The heme peak was identified using a heme standard. The culture medium contained heme proteins, which explains the heme degradation intermediates from the control sample in which the cells were not grown with heme supplementation.

Peak 1 from the two samples showed an elution time very close to the biliverdin standard (Fig. 1A) and was identified by light spectroscopy with a λ_{max} at approximately 405 nm (Fig. 1B). This spectrum suggests α-meso-hydroxyheme, the first step of the conversion of heme to BV by HO [26,27]. The ferric α-meso-hydroxyheme-HO-1 complex has a broad Soret band at 405 nm, with a featureless visible region [28–30].

Peak 2 from the control sample displayed a light absorption spectrum with the λmax at 392 and 655 nm (Fig. 1C). The extraction of this product by chloroform gives a spectrum typical of the pyridine hemochrome of verdoheme [31]. This spectrum profile is very similar to that observed by Wilks and Moënne-Loccoz [32] in which the verdoheme molecule was detected by chloroform extraction with 10% pyridine from an HO reaction in *Corynebacterium diphtheriae* (Hmu O).

Peak 2 from test sample exhibited an identical elution time (Fig. 1A) and spectrophotometric profile (1C) to the commercial BV used as a standard. The test sample showed the increased production of the identified metabolites and, therefore, the level of observed BV was altered.

Most organisms, from mammals and plants to bacteria and yeast, cope with heme toxicity by means of degradation via HO [26], and the catalytic conversion of heme proceeds through two known heme derivatives, α-meso-hydroxyheme and verdoheme, until biliverdin is produced [26,27]. However, the mechanism for the oxidative conversion of heme to biliverdin is unknown in *T. cruzi*. These compounds may note alternative mechanisms as part of the adaptation of these parasites to heme fate.

The verdoheme is the intermediate product oxidation of heme to biliverdin by heme oxygenese. Furthermore, a verdoheme-protein is generated in horseradish peroxidase with H_2O_2 or small organic [33–35]. Andreoletti et al. [35] describes an oxidative degradation of a catalase of *Proteus mirabilis* (PMC) which leads to the formation of verdoheme in which a meso-carbon of the porphyrin cycle is replaced by oxygen atom.

Thus, some different mechanisms are involved in verdoheme formation in several organisms. In this paper we are showing the presence of the molecule and the studies of the mechanism are continuing.

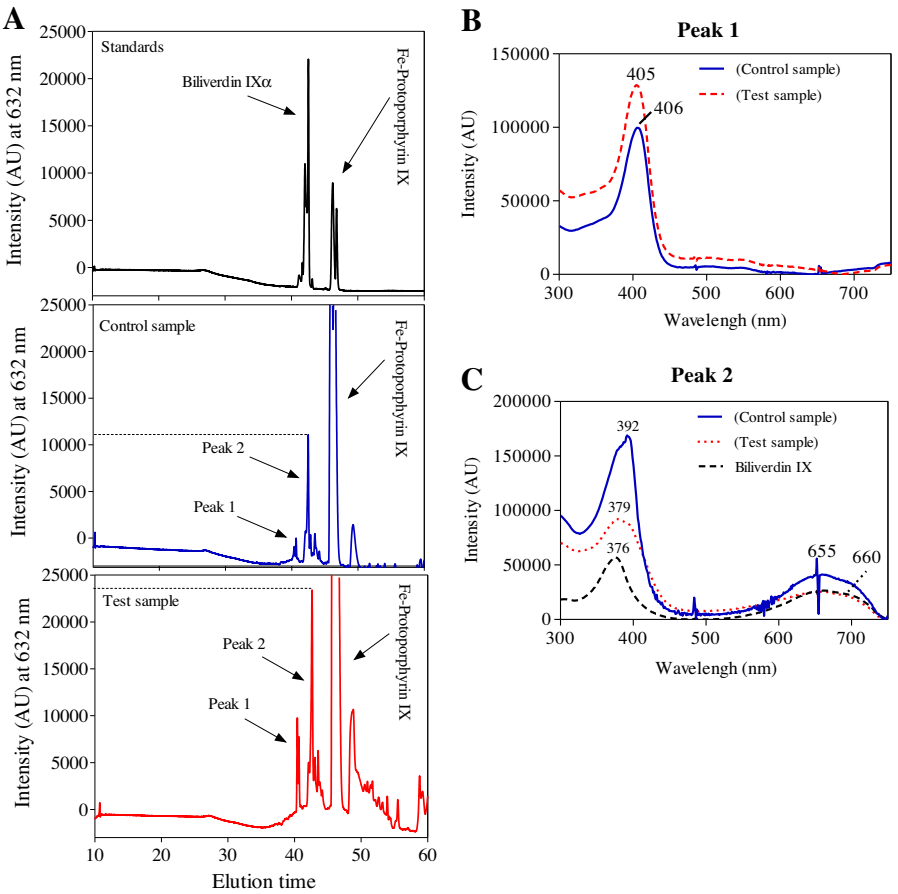

Fig. 1. Heme degradation products. HPLC analysis of epimastigotes extracts. The cell cultures, extractions and chromatography conditions were done as described in Section 2. (A) (black line) Biliverdin and heme (Fe-Protoporphyrin IX) standards, (blue line) extracts of a control cells (without extra heme) and (red line) with extra 30 μM heme in the growth medium for 24 h. The numbers indicate the chromatogram peaks. Light absorbance spectra of peak 1 (B) and peak 2 (C) from chromatograms were identified using the HPLC diode array detector. This result is representative of three different experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The first demonstration of such a system in protozoa was HO in the human malaria parasite *Plasmodium falciparum* (PfHO) [36], with the mechanism of heme catabolism being conserved and producing bilirubin IXα (BR IXα). It is important to note that PfHO is an enzyme with dual activities: heme oxygenase and biliverdin reductase [36]. However, using UV absorbance and HPLC assays, Sigala et al. [37] showed that PfHO parasites did not catalyze heme degradation, suggesting that it represents a functional diversification to serve an alternative role in the parasite. Thus, the answer to the long-standing question regarding heme metabolism in *P. falciparum* remains unresolved.

Another important aspect of our results is related to the hydrophobicity of the observed peaks. All of the metabolites were observed to have shorter elution times compared to heme, which suggests a mechanism already observed in other organisms. For example, in *Rhodnius prolixus*, the vector of *T. cruzi*, the final heme degradation product, dicysteinyl biliverdin IXγ, has two cysteine residues, which support BV solubility in water [25]. This was also observed for BV from *Aedes aegypti*, biglutaminyl biliverdin IXα, which has two glutamine residues [20]. In mammals, BV is converted to bilirubin, which is excreted only after conjugation with glucuronic acid.

To study heme catabolism in *T. cruzi*, we checked the possible role of a heme oxygenase-like enzyme using a monoclonal antibody against mammalian HO-1. *T. cruzi* epimastigotes were incubated in BHI supplemented with 10% FCS with or without 300 μM heme for five days. The parasites were lysed, and immunoprecipitation was performed, followed by immunoblotting using monoclonal anti-HO antibodies. Fig. 2A shows that the antibody had an affinity for a *T. cruzi* protein of an approximate mass of 45 kDa. In addition to the 45-kDa band, two additional bands were observed that were likely the light and heavy chains of the antibody. To investigate whether heme affects the expression of this HO-1-like protein, the epimastigotes were incubated for five days with or without different concentrations of heme. We show the increased HO expression levels in response to heme (Fig. 2B). We also observed a significant increase in the immune complex in cells treated with 300 μM heme at approximately twice the level of the control, which is further evidence that the protein in question is an HO, as these enzymes are responsive to heme, their primary substrate.

The recognition of a *T. cruzi* protein by the anti-HO-1 antibody through immunoblotting and the observation that its expression increased with heme treatment suggests the presence of a heme oxygenase activity with a regulation similar to that in other organisms. Interestingly, the protein recognized by the antibody has a molecular mass (45 kDa) that differs from the classic mammalian HO (32 kDa). The pathogenic bacterium *C. diphtheria* HO (HmuO) exhibits a heme oxygenase activity that is not associated with membranes and has a molecular mass smaller than human HO (24 kDa) [27]. This difference in molecular weight is another indication that the protein is a parasite-specific HO, with its own characteristics and a structure that differs from the mammalian enzyme.

Continuing the investigation of the HO-like activity in *T. cruzi*, we performed immunocytochemistry using monoclonal anti-HO-1 antibodies. The parasites were treated as described for immunoblotting, and the material was prepared as described in Section 2. Through an ultrastructural analysis, we observed the anti-HO-1 antibody in epimastigotes with or without heme, predominantly in the cytoplasm, though it was also found associated with membranes and in vesicles. We also noted that the antibody staining was increased in parasites treated with heme, indicating a greater expression of the enzyme under such conditions (Fig. 3). The ultrastructural immunocytochemistry analysis corroborated the immunoprecipitation and immunoblotting results.

Taken together, this is the first indication of a functional HO in *T. cruzi* epimastigotes. The increased expression of this protein in the presence of heme and the observed heme degradation intermediates α-meso-hydroxyheme and verdoheme and biliverdin by HPLC strongly suggests an active heme catabolism mechanism mediated by an HO-like protein in this parasite. Identifying this protein is extremely important because it is characteristic of the parasite and differs from the classic mammalian enzyme. The search for targets to develop new drugs for the treatment of parasites is typically based on the identification of specific enzymes in the parasite's metabolic pathways. Nevertheless, additional studies are necessary to characterize this particular parasite activity.

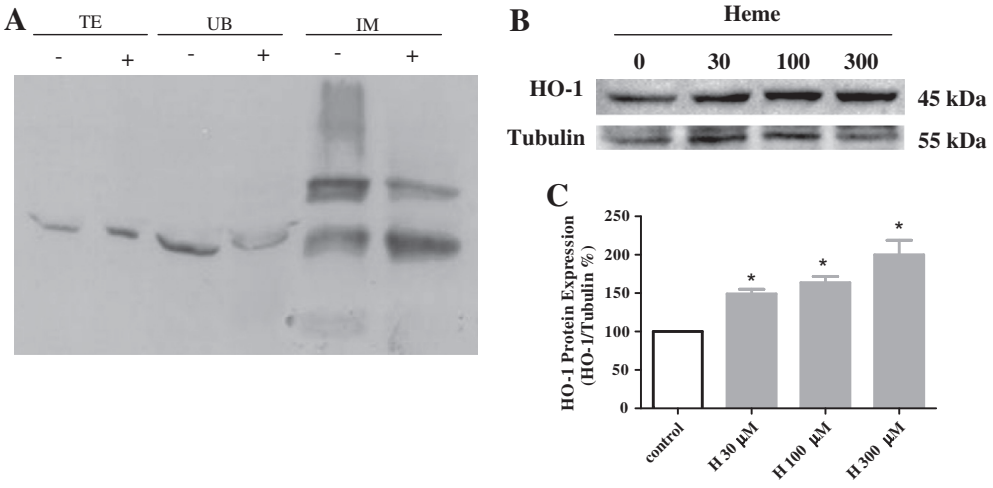

Fig. 2. Immunoprecipitation and immunoblotting analysis of HO expression in epimastigotes treated with heme. (A) Immunoprecipitation of *T. cruzi* epimastigotes performed with monoclonal anti-HO-1 and protein A/G agarose. TE – total extract; UB – unbound; and IMP – immunoprecipitates. "+" means in the presence of heme, and "–" means in the absence of heme. (B) Expression of HO-1 in parasites incubated without (control) or with different concentrations of heme for five days using western blotting with monoclonal anti-HO-1 or anti-tubulin (protein constitutive control) and tested with an ECL kit. (C) Quantification of the HO-1 levels through densitometry. The bands were analyzed using the Adobe Photoshop 5.0 program. The data are presented as the means ± SEM. $^{*}p < 0.05$ for the control group using the Tukey test. Results are representative of four independent experiments.

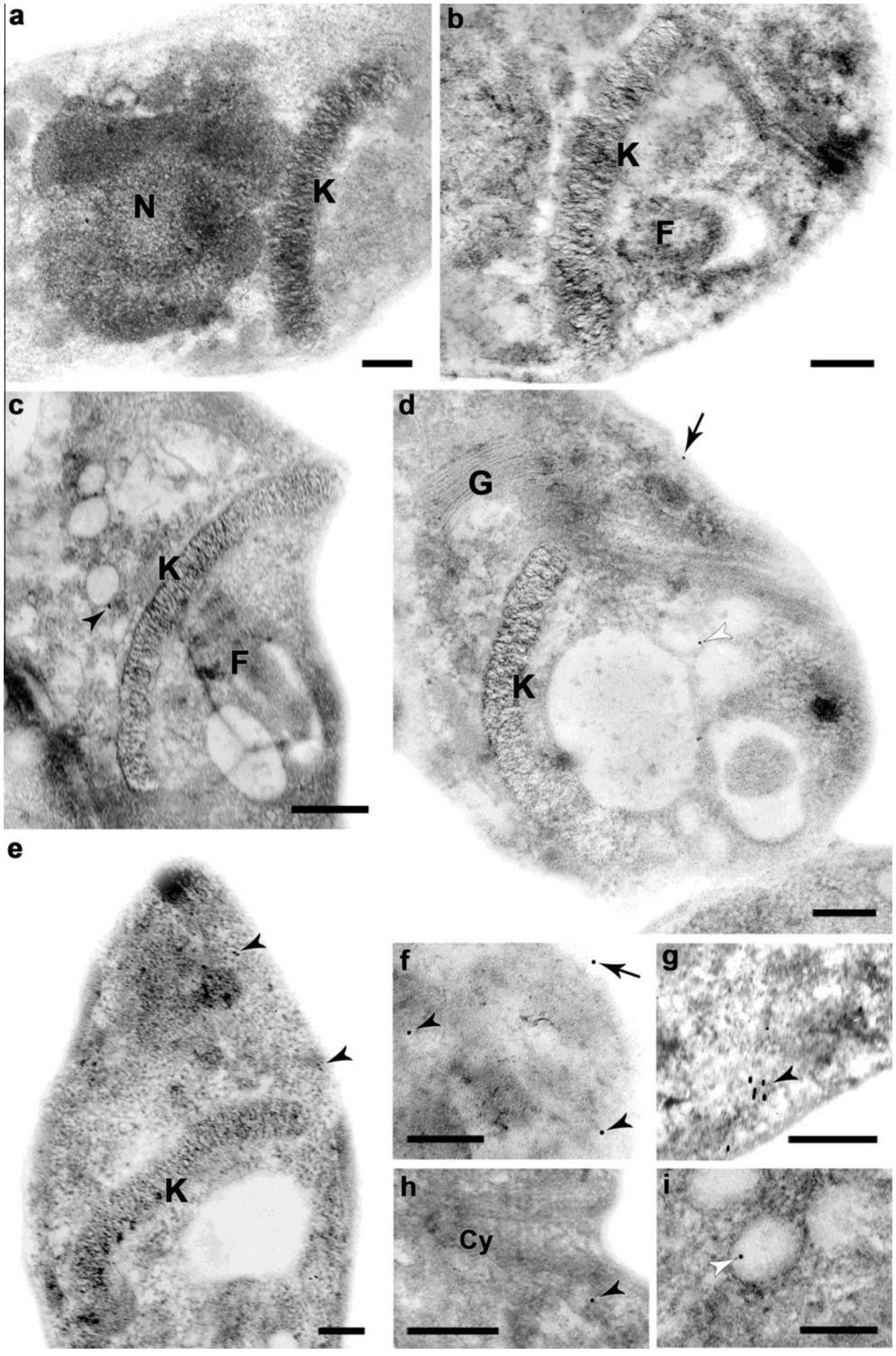

Fig. 3. Ultrastructural Immunocytochemistry for HO-1 in *T. cruzi* epimastigotes. After incubation for five days with or without heme 300 μM, the parasites were fixed, dehydrated and embedded in Lowicryl resin. The sections were incubated with monoclonal anti-HO-1 and then with secondary antibody coupled to colloidal gold particles. Finally, the grids were contrasted and examined using a transmission electron microscope. (A) The secondary control (sample treated only with the secondary antibody) showed no gold particles. (B, C) The control parasites (without heme) showed no immunostaining with HO-1 (B) or rare cytosolic particles (C). (D–I) Epimastigotes treated with heme showed increased labeling in the cytosol (black arrows), plasma membrane (black arrowheads) and in cytosolic vesicles (white arrowheads). N – nucleus; K – kinetoplast; F – flagellum; G – Golgi complex; and Cy – cytosol. Bars = 200 nm.

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