

Effect of haemin on growth, protein content and the antioxidant defence system in *Trypanosoma cruzi*

A. CICCARELLI¹, L. ARAUJO^{1,2}, A. BATLLE^{1*} and E. LOMBARDO^{1,2}

¹ Centro de Investigaciones sobre Porfirinas y Porfirias, CONICET-UBA, Argentina

² Departamento de Química Biológica, FCEN-UBA, Argentina

(Received 18 October 2006; revised 18 December 2006; accepted 22 December 2006; first published online 22 February 2007)

SUMMARY

A nutritional characteristic of trypanosomatid protozoa is that *in vitro* they need a haem-compound as a growth factor, which is supplied as haemoglobin, haematin or haemin. Because haemin and related porphyrins are an important source of oxidative stress in biological systems, the effect of haemin on growth, protein content and the antioxidant defence system in *Trypanosoma cruzi* was evaluated. We have observed that, in epimastigotes grown under different haemin concentrations in the culture medium (0–30 mg/l), 5 mg/l was the haemin concentration yielding optimum growth. Above 15 mg/l there was a clear decrease in growth rate, producing the epimastigote to amastigote transformation. Such morphological change was observed together with a marked injury of the enzymatic machinery of the parasite, leading to diminished protein synthesis as well as lower activity of the antioxidant enzymes (superoxide dismutase, ascorbate peroxidase and trypanothione reductase), reduced total thiol content and a marked increase in the HaemOx-1 activity and expression. The current work demonstrates that there is a correlation between higher haemin concentrations in the culture medium and oxidative damage in the cells. Under these conditions induction of HaemOx-1 would indicate the important role of this enzyme as an antioxidant defence response in *Trypanosoma cruzi*.

Key words: *Trypanosoma cruzi*, haemin, antioxidant enzymes, cell transformation, haeme oxygenase.

INTRODUCTION

The protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease, infects nearly 18 million people in Latin America. Haem-compounds are necessary as a growth factor for the parasite. This nutritional requirement would be due to the inability of *T. cruzi* to synthesize haeme (Lombardo *et al.* 2003). Because high levels of haemin and related porphyrins have an important cytotoxic action through the generation of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($OH\cdot$) (Afonso *et al.* 1996; Kumar and Bandyopadhyay, 2005), it was of interest to investigate the effect of haemin on the growth of *T. cruzi*. The potentially deleterious reactions involved are controlled by an antioxidant defence system that eliminates pro-oxidants and scavenges free radicals. Trypanosomatids do not possess catalase or glutathione peroxidase, the antioxidant defence system of these parasites includes the enzymes superoxide dismutase (SOD), ascorbate peroxidase (APx), trypanothione peroxidase (TpxPx) and low molecular weight antioxidants, mainly trypanothione (N^1, N^8 -bisglutathionylspermidine; Try) (Turrens, 2004).

In recent years a non-Se-dependent glutathione peroxidase form that reacts with organic peroxides but not with hydrogen peroxide has been detected in both African and American trypanosomes (Wilkinson *et al.* 2002, 2003). Another enzyme involved is trypanothione reductase (TryR), a NADPH-dependent enzyme, which regenerates reduced trypanothione (Try_{red}). The combined action of APx, TpxPx and TryR would be central to the maintenance of a low steady-state concentration of H_2O_2 .

Independent of its toxic effects, haem plays an essential role in various biological reactions, such as regulation of protein synthesis. Requirement of haem for expression of numerous proteins in mammalian reticulocytes, and also in a number of non-erythroid cells, is well established (Chen and London, 1995). The effect of haemin on protein synthesis and cell proliferation in *Leishmania donovani* has also been reported (Pal and Joshi-Purandare, 2001).

Finally, several detoxification-defence mechanisms exist in the cell to counteract the oxidative damage induced by free haem (Kumar and Bandyopadhyay, 2005). Among them, the most important involves the activity of haeme oxygenase (HaemOx). HaemOx is the rate-limiting enzyme in the degradation of haem. It breaks down the pro-oxidant haem to yield equimolar amounts of carbon monoxide, free iron and the antioxidant

* Corresponding author: Viamonte 1881, 10 "A", CP-1056 Buenos Aires, Argentina. Tel: +5411 4812 3357. Fax: +5411 4811 7447. E-mail: batlle@mail.retina.ar

biliverdin (Maines, 1997; Tomaro and Batlle, 2002). HaemOx is expressed in virtually all forms of life (bacteria, fungi, plants, mammals), to date at least 2 isoforms have been identified: HaemOx-1 and HaemOx-2. It was proposed that the inducible isoform HaemOx-1 could play an effective role to counteract the damage caused by oxidative and nitrosative stress (Maines, 1997; Naughton *et al.* 2002). HaemOx-1 is induced by host oxidative stress stimuli (H_2O_2 , haem-compounds, metal ions, GSH depletion and others) and activation of HaemOx-1 gene expression is considered to be an adaptive cellular response to different types of chemicals and mediators that change the redox status of the cell (Motterlini *et al.* 2002).

Taking into account the different effects of haemin discussed above, our aims have been to investigate the role of haemin on the rate of growth, cellular morphology, total content of proteins, the antioxidant defence system stage and HaemOx activity and expression in *T. cruzi*. The results obtained could potentially be used for the treatment of Chagas' disease.

MATERIALS AND METHODS

Chemicals

Haemin, NADPH, 5,5' dithio-bis(2-nitrobenzoic acid) (DTNB), EDTA, NADH, hydrogen peroxide and ascorbic acid were from Sigma Chem (St Louis, MO, USA). Yeast extract, tryptose, powdered beef liver and brain heart infusion were from Difco. Trypanothione was purchased from Bachem Bioscience Inc. (USA). All other chemicals were of the highest purity commercially available. The following primary polyclonal antibodies, rabbit anti-HaemOx-1 (Stresgen Biotech, Victoria, BC Canada) and goat anti-actin (I-10) (Santa Cruz Biotech, Santa Cruz, CA, USA) were used. The secondary antibodies used for HaemOx-1 Western blot analysis were anti-goat IgG-HRP or anti-rabbit IgG-HRP (Santa Cruz Biotech).

Organism and growth conditions

T. cruzi cells, Tulahuén strain, Tul 2 stock, were grown at 28 °C with constant shaking in a liquid medium containing 0.3% yeast extract, 0.9% tryptose, 0.4% dextrose, 1% disodium phosphate 2-hydrate, 0.36% sodium chloride, 0.04% potassium chloride, 0.15% powdered beef liver, 0.5% brain heart infusion and 0.5 mg/100 ml haemin.

Preparation of extracts from *T. cruzi*

All manipulation steps were performed at 2–4 °C. The cells were harvested by centrifugation at 12 000 g for 10 min and then washed once with either

0.05 M Tris-HCl buffer, pH 7.4, 0.05 M sodium phosphate buffer, pH 7.4, or 0.05 M potassium phosphate buffer, pH 7.2, depending on the measurement under study. The cells from 200 ml of culture were resuspended in 5 ml of (a) 0.05 M Tris-HCl buffer, pH 7.4, for APx activity, (b) 0.05 M sodium phosphate buffer, pH 7.4, for SOD activity and (c) 0.05 M potassium phosphate buffer, pH 7.2, for TryR activity and HaemOx activities and total thiol content. Cells in suspension were disrupted by sonication in a Soniprep 150, MSE Ultrasonic Power for 45 sec. The resulting homogenate was centrifuged at 5000 g for 15 min, the precipitate was discarded and the supernatant was employed as source of enzyme.

SOD activity

Superoxide dismutase activity has been assayed by a spectrophotometric method based on the inhibition of a superoxide-driven NADH oxidation (Paoletti, 1986). One enzyme unit (EU) is defined as the amount of protein required to inhibit 50% NADH oxidation.

APx activity

APx activity was measured at 25 °C following the change in absorbance at 265 nm due to ascorbate oxidation. The method described by Boveris *et al.* (1980) was employed. Usually corrections for oxidation of ascorbate in the absence of hydrogen peroxide were not significant, instead non-enzymatic oxidations of ascorbate by hydrogen peroxide had to be considered. Enzyme activity was calculated by using an ϵ value of 10.36 mM^{-1} . The EU is defined as the amount of enzyme forming 1 nmol of product per sec under the standard incubation conditions.

TryR activity

TryR activity was determined at 25 °C following NADPH oxidation at 340 nm (Ariyanayagan and Fairlamb, 2001). The S supernatant dialysed against 5 mM potassium phosphate buffer, pH 7.2 (5 changes of 160 volumes during 45 min) was employed as enzyme preparation. The activity was calculated using an extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. One EU is defined as the amount of enzyme forming 1 nmol of product per min under the standard incubation conditions.

HaemOx activity

HaemOx activity was determined by measuring bilirubin formation, which was calculated as the difference in absorbance measured at 455 and 520 nm. From the supernatant of *T. cruzi*, both a

microsomal HaemOx fraction and a biliverdin reductase preparation were obtained according to the method of Erario *et al.* (2002). The standard assay mixture and incubation conditions were those reported by Erario *et al.* (2002). Enzyme activity was determined using an ϵ value of $50 \text{ mM}^{-1} \text{ cm}^{-1}$. The EU is defined as the amount of enzyme forming 1 nmol of product per hour under the standard incubation conditions.

Western-blot analysis for HaemOx-1 expression

T. cruzi supernatants prepared in 0.05 M sodium phosphate buffer, pH 7.4, were subjected to 12% SDS-PAGE, transferred to nitrocellulose membranes, and then incubated with specific antibodies, according to the protocol of Foresti *et al.* (1997). Rainbow-coloured protein molecular mass standards obtained from Amersham were used for the estimation of molecular size. For quantification of immunoblots, relative intensities of bands were quantified by densitometry using IMAGE MASTER image analysis software (Amersham Pharmacia Biotech). Control for loading and transfer was obtained by probing with anti- β -actin.

Assay of total thiol groups

The content of thiol groups was determined by measuring the change in light absorbance (410 nm) occurring when SH-groups reduce DTNB. Quantification was carried out in both supernatant and pellet after precipitation of S supernatant with TCA to a final concentration of 5% (Rossi *et al.* 1995).

Protein determination

Protein concentration was determined by a modification of Lowry's method for insoluble proteins. Cells from 10 ml of culture were harvested and washed as indicated above. They were then resuspended in 5 ml of 0.6 M NaOH and disrupted by ultrasonic treatment for 45 sec. The broken cell suspension was employed for protein determination as described by Lowry (1951), except that Na_2CO_3 was dissolved in H_2O instead of 0.1 M NaOH.

Statistical analysis

All data are expressed as means \pm standard deviations of 3 or 4 separate experiments, running in duplicate. The significance of differences was evaluated using Student's *t*-test, taking $P < 0.05$ as significant.

RESULTS

Growth under different haemin concentrations

Epimastigotes of *T. cruzi* were grown in the presence of different haemin concentrations, between 0 and

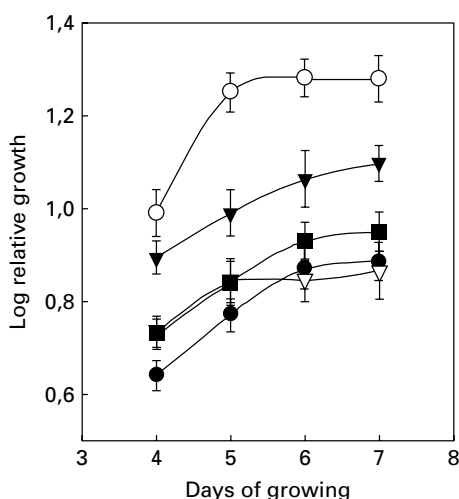


Fig. 1. Relative growth of *Trypanosoma cruzi* under different haemin concentrations. (●) Without added haemin, (○) 5 mg haemin per l of culture, (▼) 15 mg haemin per l of culture, (▽, ■) 30 mg haemin per l of culture. The two lines for 30 mg/l haemin correspond to considerer (■) all living cells independent of the morphology or (▽) only living cells showing an extracellular flagellum. Relative growth is defined as the number of cells per ml of culture at the end of the growth period with respect to the number of cells per ml of culture at the beginning of growth. All other experimental conditions were as indicated in the Materials and Methods section.

30 mg/l, at 28 °C (Fig. 1). The relative growth of the culture was followed as a measure of parasite proliferation. Cells were counted using a Neubauer chamber at the beginning of the experiment and every 24 h during 4–7 days. The indicated haemin concentration corresponds to the concentration of exogenously added haemin. Measurements before 4 days and after 7 days were not recorded because the cellular mass at intervals shorter than 4 days was insufficient for carrying out determinations of enzymes and metabolites and, at longer times, problems inherent to depletion of nutrients or formation of undesired metabolites could arise.

Curves for haemin concentrations of 10 mg/l and 15 mg/l were practically superimposed (data not shown). After 5 days of growing, 2 different curves were observed for 30 mg/l haemin. The upper curve corresponds to all living cells (independent of the morphology). The lower curve only takes into account the living cells showing an extracellular flagellum. Haemin concentrations higher than 15 mg/l clearly decreased growth rate and induced morphological changes. We have therefore found that 6 days of growth and 5 mg/l haemin were the conditions yielding optimum growth and proliferation.

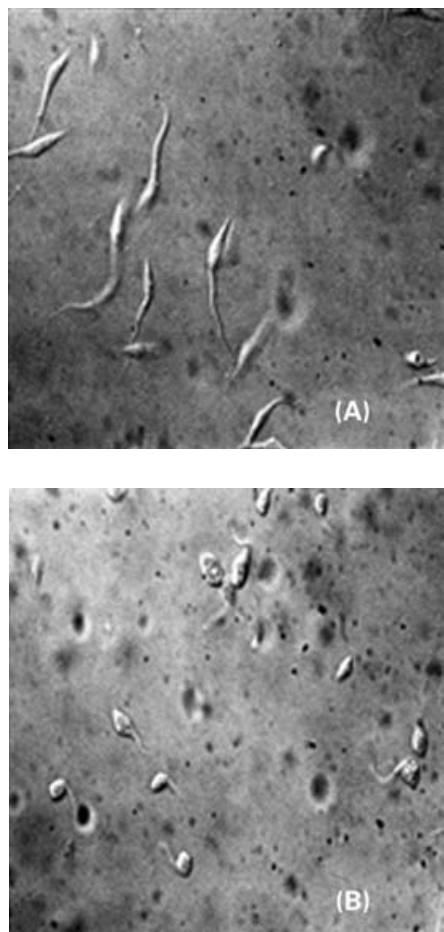


Fig. 2. Morphology of *Trypanosoma cruzi* epimastigotes grown in the absence (A) and presence of haemin (B). Cells were grown without haemin (A) and in the presence of 30 mg/l haemin (B) during 6 days. Then they were examined under light microscopy (ZEISS West Germany AXIOSKOP). Original magnification, $\times 400$.

Effect of haemin on behaviour and morphology of the cells

Optical microscopy observations are presented in Fig. 2. When *T. cruzi* cells were grown in a medium containing a haemin concentration of 30 mg/l, they appeared less elongated (stumpy), with short flagella and therefore with diminished mobility (Fig. 2B). These morphological changes could correspond to the transformation from epimastigote to amastigote. Such differentiation was corroborated employing the methodology of serial cultivation described by Zaidenberg *et al.* (2000). Three consecutive cultures of 5 days each at the 30 mg/l haemin concentration employing transformed cells (like-amastigote) were carried out. Maintenance of the cellular morphology evaluated by optical microscopy would indicate that transformation from epimastigote to amastigote was induced by high haemin concentrations.

For haemin concentrations between 0 and 10 mg/l in the culture medium, the proportion of trypomastigotes increased with time, reaching 25–30% after 7 days.

Effect of haemin on total soluble protein content

We have analysed, the effect of haemin (0–30 mg/l) on total protein content dissolved in 0.6 M NaOH (see Materials and Methods section) in cultures growing between 3 and 7 days (Fig. 3). Considering the results shown in Fig. 3A and B, Fig. 3C can be drawn, which shows total protein content per cell with time. In the absence of haemin, the protein concentration progressively increased from 6.41 ± 0.18 to $8.73 \pm 0.25 \mu\text{g}/10^6$ cells. In the presence of haemin 10 mg/l values were 0–15% higher, showing a maximum level after 5 days of growth, without further changes. For a haemin concentration of 30 mg/l a different response was obtained, protein concentration increased within 3–5 days (from 9.26 ± 0.10 to $10.12 \pm 0.08 \mu\text{g}/10^6$ cells) and then rapidly decreased to $7.24 \pm 0.18 \mu\text{g}/10^6$ cells.

We have seen that between 3 and 6 days of growth, significant differences ($P < 0.05$) among the 3 different haemin concentrations were found.

Effect of haemin on content of thiol groups

Levels of reduced low molecular mass thiols are indicative of the redox state of the cell. Therefore we could expect that if haemin induces the formation of oxidant species it will affect thiol group levels.

In Fig. 4 we can see that SH-content remains constant for haemin concentrations between 0 and 5 mg/l, diminishing between 22% and 37% for haemin 15 and 30 mg/l respectively.

Effect of haemin on antioxidant enzymes

As seen in Table 1, the activities of antioxidant enzymes SOD, TryR and APx showed a maximum value at 5 mg/l of haemin. For higher concentrations of haemin (15 and 30 mg/l) all activities diminished, attaining values very similar to the controls for TryR and APx or slightly lower for SOD.

Diminished activities of these enzymes cannot be attributed to an inhibitory effect of the haemin present in S supernatant (which varies between 0.23 ± 0.08 and $42.46 \pm 7.84 \mu\text{g}/\text{ml}$ for haemin concentrations in the culture medium of 0 to 30 mg/l, respectively) on the enzyme activity evaluated *in vitro*.

Haem oxygenase activity and expression

T. cruzi cells were grown during 6 days in the absence and in the presence of 5, 15 and 30 mg haemin per 1 of culture. The values of HaemOx activity (data not shown) corresponding to 0 and 5 mg/l haemin did not show significant differences (average value: $1.22 \pm 0.15 \text{ EU}/\text{mg}$), for 15 mg/l haemin the activity slightly increased (up to $2.01 \pm 0.23 \text{ EU}/\text{mg}$) whilst for 30 mg/l haemin an activity approximately 3 times

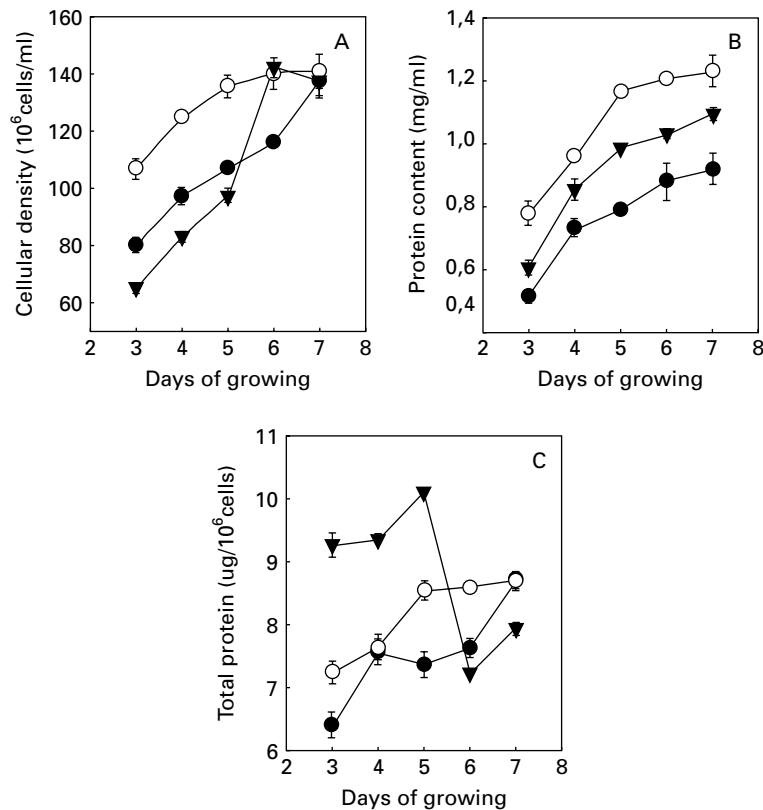


Fig. 3. Effect of different haemin concentrations on cellular density (A) and protein content expressed per ml of culture (B) as well as per cell number (C). (●) Without added haemin, (○) 10 mg haemin per l of culture, (▼) 30 mg haemin per l of culture.

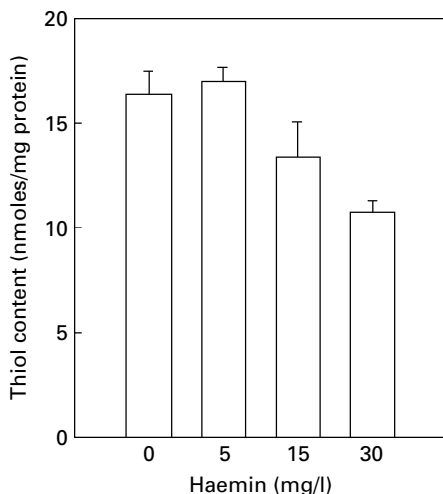


Fig. 4. Effect of haemin on thiol group content. The cells were grown under different haemin concentrations during 6 days. Thiol groups were measured as described in the Materials and Methods section.

higher (3.81 ± 0.12 EU/mg) was observed. Western blot analysis for HaemOx-1 (Fig. 5) showed only a single band with a molecular mass of 32–34 kDa. The maximum expression of the enzyme (9.5 to 10.2-fold) was observed for 30 mg/l haemin, whilst for 5 and 15 mg/ haemin the increase was lower, 1.4

to 1.9-fold and 4.5 to 4.9-fold respectively (0 mg/l haemin was considered as the control value).

DISCUSSION

T. cruzi is the aetiological agent of the Chagas' disease, affecting a significant proportion of the American population. In this parasitic protozoa the effects of haemin on proliferation, differentiation and the redox state of the cells have been investigated. A haemin concentration of 5 mg/l and 6 days of growth at 28 °C with constant shaking, were the optimum conditions to obtain epimastigotes in culture. Haemin concentrations higher than 10 mg/l produced a marked decrease in the rate of growth, together with significant morphological changes, such as shortening of extracellular flagellum, reduction in size and rounding up. Taking into account the changes in cellular morphology and the behaviour observed employing the methodology described by Zaidenberg *et al.* (2000), these changes correspond to cell differentiation towards the amastigote state. These results are in agreement with those obtained in *Leishmania donovani* culture *in vitro* in medium RPMI 1640, in which a haemin concentration of 50 μ M (equivalent to 32.6 mg/l) triggered promastigote-amastigote transformation (Pal and Joshi-Purandare, 2001). Haem regulation of

Table 1. Effect of haemin on antioxidant enzyme activities

(*T. cruzi* cells were grown for 6 days in the presence of different haemin concentrations. Enzymatic activities were assayed and expressed as described in the Materials and Methods section. Activity corresponding to haemin 0 mg/l was considered as the control value.)

Haemin (mg/l)	SOD activity (EU/mg)	TryR activity (EU/mg)	APx activity (EU/mg)
0	3.92 ± 0.10	2.83 ± 0.07	12.77 ± 0.08
5	4.50 ± 0.05	3.72 ± 0.08	15.20 ± 0.06
15	3.90 ± 0.12 ^a	2.70 ± 0.15 ^a	14.22 ± 0.05
30	3.17 ± 0.10	2.95 ± 0.20 ^a	13.58 ± 0.08 ^a

^a No significant differences ($P > 0.05$) when compared to the control (0 mg/l haemin) as assessed by Student's *t*-test.

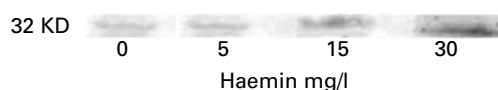


Fig. 5. Effect of haemin on HaemOx-1 expression in *Trypanosoma cruzi*. Western blotting was performed by standard procedures with polyclonal antibody to HaemOx-1. β -actin was used as a loading and transfer control. Other experimental conditions were as described in the Materials and Methods section.

differentiation and proliferation in other cell types, such as a human leukaemic cell line, mouse 3T3 cells and embryonic cells in culture, have also been reported (Kumar and Bandyopadhyay, 2005).

Because high haemin levels stimulate protein translation, we have investigated the effect of haemin on total protein content in cells grown for between 3 and 7 days. We have found that up to day 5 of growth our results are in agreement with the described behaviour, i.e. the higher the haemin concentration, the greater the intracellular total protein content. However, from day 5 onwards this response changes, and a 30% reduction in protein content was found in cells grown in the presence of 30 mg/l haemin. This concentration of haemin not only down regulated protein synthesis but also led to its degradation. Similar results were obtained in other trypanosomatids where high concentrations of haemin (50 μ M) reduced the level of protein synthesis, in general and beta-tubulin in particular (Pal and Joshi-Purandare, 2001). In mammals, the haemin-catalysed degradation of protein to small peptide fragments has also been reported (Kumar and Bandyopadhyay, 2005). In our *T. cruzi* cultures, high haemin concentrations, induced non-synchronized morphological changes, leading to a heterogeneous culture, whereby the process of cellular replication corresponding to the amastigote stage is favored over the synthesis of proteins.

The well-known capacity of haemin to generate ROS, could well support the process of haemin-mediated oxidative degradation of proteins. The increased expression of heat shock protein hsp90 in trypanosomatids and mammalian cells cultured with high haemin concentrations (50 μ M) would be further evidence that haemin does act as a stress-inducing compound (Pal and Joshi-Purandare, 2001). As far as we know this is the first report in *T. cruzi* where a correlation between haemin levels in the culture medium and the state of the antioxidant defence system into the cells has been established. We have shown that the 3 enzymes examined, SOD, APx and TryR showed their maximum activity with haemin at a concentration of 5 mg/l. However, at higher haemin concentrations enzyme activities were lower, in the case of SOD to values even lower than controls. Reduced thiols show the same behaviour profile as the antioxidant enzymes. It is important to consider that evaluation of the antioxidant defence system has been carried out after 6 days of growth, when the deleterious action of higher haemin concentrations is evident. If measurements had been made at earlier time-points we may have detected a stimulation of the antioxidant defence system to prevent or overcome the oxidative deleterious actions (experiments in progress).

HaemOx is a haem-degrading enzyme that also plays a significant role in protecting cells from haeme-induced oxidative stress. HaemOx activity and expression have also been investigated. So far, HaemOx activity in *T. cruzi* cells had not been reported. According to our results, a positive correlation between enzyme activity and expression was observed. If we compare results obtained for HaemOx and those for the antioxidant enzymes defence system we observe a different response. For low concentrations of haemin (5 mg/l), the antioxidant enzymes activity and the reduced thiol content show their maximum levels, instead the activity of HaemOx was not modified and its expression slightly increased. For concentrations of haemin higher than 15 mg/l, the antioxidant defence system was reduced while HaemOx-1 was markedly induced, increasing both its activity and expression. These findings indicate that the mode of action of both antioxidant systems is different.

In conclusion, to obtain *T. cruzi* epimastigotes in culture, the haemin concentration in the medium should not exceed 5–10 mg/l and the time of growth should be 6 days. Higher haemin concentrations produced oxidative stress which induced transformation of the epimastigote to the amastigote form, along with an injury of the enzymatic machinery of the parasite, leading to diminished protein synthesis as well as lower activity of the antioxidant defence system. Under these conditions HaemOx-1 acts as a stress-responsive antioxidant enzyme.

Our results suggest the possibility that HaemOx participates in the catabolism of haeme in *T. cruzi*. At present we are purifying the protein recognized by the anti-HaemOx-1 polyclonal antibody.

Finally, better knowledge of the biochemical systems affording the antioxidant defences of the parasite, would provide a valuable target to be exploited for the development of an effective chemotherapy.

María Elisa Lombardo and Alcira Batlle hold the post of Scientific Researchers at the Argentine National Research Council (CONICET). Alejandra Beatriz Ciccarelli is a Research Fellow of CONICET. Lidia Susana Araujo is a Research Fellow of the University of Buenos Aires (UBA). This work was supported by grants from CONICET, UBA and the Agencia de Promoción Científica y Tecnológica.

REFERENCES

- Afonso, S. G., Polo, C. F., Enriquez de Salamanca, R. and Batlle, A.** (1996). Mechanistic studies on uroporphyrin I-induced photoinactivation of some heme-enzymes. *International Journal Biochemistry and Cell Biology* **28**, 415–420. doi: 1357-2725(95)00159-X.
- Ariyanayagam, M. and Fairlamb, A.** (2001). Ovoidiol and trypanothione as antioxidants in trypanosomatids. *Molecular and Biochemical Parasitology* **115**, 189–198. doi: 10.106/S0166-6851(01)00285-7.
- Boveris, A., Sies, H., Martino, E. E., Docampo, R., Turrens, J. F. and Stoppani, A. O. M.** (1980). Deficient metabolic utilization of hydrogen peroxide in *Trypanosoma cruzi*. *The Biochemical Journal* **188**, 643–648.
- Chen, J. and London, I.** (1995). Regulation of protein synthesis by heme-regulated eIF-2 α kinase. *Trends in Biochemical Science* **20**, 105–108. doi: 10.1016/S0968-0004(00)88975-6.
- Erario, M. A., Gonzales, S., Noriega, G. O. and Tomaro, M. L.** (2002). Bilirubin and ferritin as protectors against hemin-induced oxidative stress in rat liver. *Cellular and Molecular Biology* **48**, 8787–8884.
- Foresti, R., Clark, J. E., Green, C. J. and Motterlini, R.** (1997). Thiol compounds interact with nitric oxide in regulating heme oxygenase-1 induction in endothelial cells. *The Journal of Biological Chemistry* **272**, 18411–18417.
- Kumar, S. and Bandyopadhyay, U.** (2005). Free heme toxicity and its detoxification systems in human. *Toxicology Letters* **157**, 175–188. doi: 10.1016/j.toxlet.2005.03.004.
- Lombardo, M. E., Araujo, L. S. and Batlle, A.** (2003). 5-Aminolevulinic acid synthesis in epimastigotes of *Trypanosoma cruzi*. *The International Journal of Biochemistry and Cell Biology* **35**, 1263–1271. doi: 10.1016/S1357-2725(03)00033-5.
- Lowry, O. H., Roseborough, N. J., Farr, A. L. and Randall, R. J.** (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry* **193**, 265–275.
- Maines, M.** (1997). The heme oxygenase system: a regulator of second messenger gases. *Annual Review of Pharmacology and Toxicology* **37**, 517–554. doi: 10.1146/annurev.pharmtox.37.1.517.
- Motterlini, R., Green, C. and Foresti, R.** (2002). Regulation of heme oxygenase-1 by redox signals involving nitric oxide. *Antioxidants and Redox Signaling* **4**, 615–624.
- Naughton, P., Foresti, R., Bains, S. K., Hoque, M., Green, C. and Motterlini, R.** (2002). Induction of heme oxygenase-1 by nitrosative stress. A role for nitroxyl anion. *The Journal of Biological Chemistry* **277**, 265–275. doi: 10.1074/jbc.M203863200.
- Pal, J. and Joshi-Purandare, M.** (2001). Dose-dependent differential effect of hemin on protein synthesis and cell proliferation in *Leishmania donovani* promastigotes cultured *in vitro*. *Journal of Biosciences* **26**, 225–231.
- Paoletti, F., Aldinucci, D., Mocali, A. and Caparrini A.** (1986). A sensitive spectrophotometric method for the determination of superoxide dismutase activity in tissue extracts. *Analytical Biochemistry* **154**, 536–541.
- Rossi, R., Cardaioli, E., Scaloni, A., Amiconi, G. and Di Simplicio, P.** (1995). Thiol groups in proteins as endogenous reductants to determine glutathione-protein mixed disulphides in biological systems. *Biochimica et Biophysica Acta* **1243**, 230–238. doi: 10.1016/0304-4165(94)00133-I.
- Tomaro, M. L. and Batlle, A. M.** (2002). Bilirubin: its role in cytoprotection against oxidative stress. *The International Journal of Biochemistry and Cell Biology* **34**, 216–220. doi: 10.1016/S1357-2725(01)00130-3.
- Turrens, J. F.** (2004). Oxidative stress and antioxidant defenses: a target for the treatment of diseases caused by parasitic protozoa. *Molecular Aspects of Medicine* **25**, 211–220. doi: 10.1016/j.mam.2004.02.021.
- Wilkinson, S. R., Obado, S. O., Mauricio, E. L. and Kelly, J. M.** (2002). *Trypanosoma cruzi* expresses a plant-like ascorbate-dependent hemoperoxidase localized to the endoplasmic reticulum. *Proceedings of the National Academy of Sciences, USA* **99**, 13453–13458. doi: 10.1073/pnas.202422899.
- Wilkinson, S. R., Hom, D., Prathalingam, S. R. and Kelly, J. M.** (2003). RNA interference identifies two hydroperoxide metabolizing enzymes that are essential to the bloodstream form of the African trypanosome. *The Journal of Biological Chemistry* **278**, 31640–31646. doi: 10.1074/jbc.M303035200.
- Zaidenberg, A., Tournier, H. A., Schinella, G. R. and Buschiazzo, H. O.** (2000). *Trypanosoma cruzi*: Obtención de amastigotes extracelulares y estudio de crecimiento en diferentes condiciones de cultivo. *Revista Latinoamericana de Microbiología* **42**, 21–26.