Effects of buthionine sulfoximine nifurtimox and benznidazole upon trypanothione and metallothionein proteins in *Trypanosoma cruzi*.

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ABSTRACT

Proteins rich in sulfhydryl groups, such as metallothionein, are present in several strains of the parasite *Trypanosoma cruzi*, the etiological agent of Chagas’ disease. Metallothionein-like protein concentrations ranged from 5.1 to 13.2 pmol/mg protein depending on the parasite strain and growth phase. Nifurtimox and benznidazole, used in the treatment of Chagas’ disease, decreased metallothionein activity by approximately 70%. *T. cruzi* metallothionein was induced by ZnCl₂. Metallothionein from *T. cruzi* was partially purified and its monobromobimane derivative showed a molecular weight of approximately 10,000 Da by SDS-PAGE analysis. The concentration of trypanothione, the major glutathione conjugate in *T. cruzi*, ranged from 3.8 to 10.8 nmol/mg protein, depending on the culture phase. The addition of buthionine sulfoximine to the protozoal culture considerably reduced the concentration of trypanothione and had no effect upon the metallothionein concentration. The possible contribution of metallothionein-like proteins to drug resistance in *T. cruzi* is discussed.

Key terms: *Trypanosoma cruzi*, metallothionein, glutathione, trypanothione, benznidazole, nifurtimox, buthionine sulfoximine.

INTRODUCTION

Several thiol-containing molecules play an important role in trypanosomatid defense against free radicals and electrophilic agents. The most important thiol molecules are trypanothione (bis-glutathionyl spermidine, T(SH)₂), glutathione (GSH), ovothiols (Fairlamb and Cerami, 1985; Steenkamp and Spies, 1994; Repetto et al., 1996; Maya et al., 1997; Maya et al., 1999; Maya et al., 2001a; Ariyanayagam and Fairlamb, 2001; Steenkamp, 2002), and metallothionein-like proteins (MTs), as shown in this paper. All these compounds act as free radical scavengers by virtue of their thiol groups.

Metallothioneins (MTs) are low molecular weight, soluble proteins found in vertebrates, invertebrates and microorganisms. In addition, MTs have a high content of cysteinyl residues; in this respect, they are very similar to T(SH)₂. MTs appear to be mainly cytoplasmic but several studies have shown that these proteins are also present in nuclei (Jeremias and Kägi, 1991; Sato and Bremner, 1993). The term metallothionein may be applied to proteins with the following properties: a) low molecular weight (6-14 kDa); b) high content of heavy metals (6-12 metal atoms per molecule); c) amino acid composition containing approximately one third of cysteinyl residues and without disulfide...
bonds, and no aromatic amino acids or histidine; d) amino acid sequence with conserved distribution of cysteinyl residues; e) single polypeptide chain; f) heat stability; and g) the presence of isoforms (Sato and Bremner, 1993).

MT has multiple functions that include the detoxification of drugs and heavy metals, the hepatic metabolism of zinc and copper, and the scavenging of electrophilic compounds and free radicals (Lazo et al., 1998; Klaassen et al., 1999; Hanada, 1998; He et al., 2000; Coyle et al., 2002; Sato and Kondoh, 2002). Many agents producing oxidative or physical stress induce these proteins. Hepatic MT content may be increased 20-30 fold by several chemical agents, such as heavy metals (Dunn et al., 1987; Jeremias and Kägi, 1991; Sato and Bremner, 1993).

Nifurtimox and benznidazole are nitroheterocyclic compounds used as anti-*T. cruzi* drugs in the treatment of Chagas’ disease (Sanchez et al., 2002; Contreras et al., 2002). The susceptibility of several *T. cruzi* strains and forms to nifurtimox and benznidazole correlate negatively with the intracellular free thiol (GSH and T(SH)2) levels (Maya et al., 1997; Maya et al., 2001a). Buthionine sulfoximine (BSO), an inhibitor of glutathione (GSH) synthesis, renders *Trypanosoma cruzi* parasites more susceptible to nifurtimox and benznidazole (Moncada et al., 1989; Repetto et al., 1996; Maya et al., 1997). The significant decrease in the GSH and T(SH)2 concentration caused by conjugation with nifurtimox and benznidazole nitroreductive metabolites could explain the toxic effects of both drugs (Maya et al., 1997). Because of their high content of cysteinyl residues, MTs may play a similar or complementary role to GSH and T(SH)2 in detoxification and drug metabolism in *T. cruzi* parasites.

MTs have been isolated from many sources including protozoa such as *Oxytrichia* and *Tetrahymena* (Piccini et al., 1994; Irato et al., 1995). However, in this paper we describe for the first time evidence of the presence of an MT-like protein in a protozoon of medical importance and its possible role in its resistance to drugs.

**MATERIALS AND METHODS**

**Parasites**

*T. cruzi* epimastigotes (Tulahuén, LQ and MF strains and CL-Brener and Dm28c clones) were grown at 28°C in modified Diamond’s medium as described previously (Maya et al., 1997). 80x10^6 parasites are equivalent to 1 mg of protein or 12 mg of wet weight.

**Metallothionein analysis**

MT concentration was determined by the radioactive cadmium method (Eaton and Toal, 1982). Basically, the parasites were suspended in an antioxidant and antiproteolytic cocktail (1 mM phenyl methyl sulfonyl fluoride (PMSF), 0.1 mM Nα-p-Tosyl-L-lysine chloro methyl ketone (TLCK), 0.05 mM trans-epoxysuccinyl L-leucylamido (4-guanidino)-butane (E64), 2 mM orthophenanthroline, 5 mM mercaptoethanol, Tris-HCl 10 mM, pH 7.4), sonicated twice for 30 seconds, and heated for two minutes at 100°C. The sample was centrifuged at 10,000 x g for 5 minutes. An aliquot of 200 µL of the supernatant was mixed with 100 µL of a Tris-HCl 10 mM, pH 7.4 ^109^CdCl2 solution containing 2 µg of CdCl2 per mL and one µCi per mL. The mix was incubated for ten minutes at room temperature. Then, 100 µL of fresh solution of 2% bovine hemoglobin was added and heated for 2 minutes at 100°C followed by centrifugation at 10,000 x g for 10 minutes. The supernatant was treated twice with hemoglobin. Finally the resultant supernatant was counted in a gamma counter. The parasite’s GSH and T(SH)2 and mercaptoethanol did not interfere with the MT determination. The concentration of MT was calculated assuming that six atoms of cadmium bind to a molecule of MT (Eaton and Toal, 1982), thus one picomole of MT corresponds to approximately 1080 cpm.

**Analysis of thiols**

Reduced GSH, glutathionyl-spermidine and T(SH)2 in control and drug-treated parasites
were determined by derivatization with monobromobimane (Thiolyte) and then separated by HPLC as described previously (Fairlamb et al., 1987; Repetto et al., 1996).

Effect of nifurtimox and benznidazole upon MT concentration

On the second day of the exponential growth phase, 400x10^6 parasites/ml of the Tulahuén strain were incubated at 28°C for 2 hours with nifurtimox and benznidazole, at 2.6 mM final concentration. The control without drugs was also incubated at 28°C for two hours. The nifurtimox and benznidazole growth inhibition IC_{50} is approximately 10 µM for 3x10^6 parasites/ml (Maya et al., 2003). The concentrations employed correspond to twice the IC_{50} multiplied by a factor of 260 to correct for the difference in parasite concentration. No lysis of the parasites was observed at these concentrations. MT concentration was determined as described above.

Chromatography of T. cruzi MTs

Approximately 20 grams wet weight of Dm28c clone epimastigotes were suspended in the antioxidant and antiproteolytic cocktail, sonicated, and centrifuged at 100,000 x g for 1 hour. The supernatant was heated for 2 minutes at 100ºC. The pellet was discharged, the resulting supernatant was eluted through a Sephadex G-75 column (1.5 x 25 cm) with Tris-HCl 0.1 M, pH 7.4, and one mL fractions were collected. The MTs were determined by the radioactive cadmium method. Tubes with positive radioactivity were pooled and concentrated by freeze-drying and then suspended in the antiproteolytic and antioxidant buffer and divided in two aliquots. One aliquot was derivatized with monobromobimane (Thiolyte®) producing a fluorescent derivative due to the high concentration of thiol groups in MTs (Kosower et al., 1983). The other aliquot was used as such. Both aliquots were eluted through a Sephadex G-75 column (1.5 x 40 cm) with Tris-HCl 0.1 M, pH 7.4 buffer. One mL fractions were collected, and fluorescence at 385 nm excitation and 480 emission (RF-540 Spectrofluorophotometer, Shimadzu) or radioactivity in a gamma counter was measured.

SDS-Polyacrilamide Gel Electrophoresis of MTs

Control and MT samples from epimastigotes treated with nifurtimox and benznidazole as described in section 2.4 were derivatized with monobromobimane. Then the parasites were subjected to MT derivatization with monobromobimane as described in section 2.5. The supernatants were dialyzed overnight against distilled water, concentrated by lyophilization and separated by 15% SDS-PAGE. The fluorescent bands corresponding to MT were visualized by UV-transillumination. Monobromobimane-derivatized horse kidney MT standard and prestained molecular weight standards (SigmaMarkerTM Low range [M.W. 6500-66000]) were used.

Chemicals

^{109}CdCl_2 was obtained from NEN Life Science products, Boston, Massachusetts; tryptose, tryptone, yeast extract and fetal calf serum were obtained from Difco, Co.; dimethylsulfoxide was obtained from Merck, Co.; monobromobimane (Thiolyte®) was obtained from Calbiochem Corp. Horse kidney MT, TLCK, PMFS, and all other chemicals were obtained from Sigma Chemical Co.

Statistical Analysis

Student’s T, ANOVA and Tuckey’s or Dunnett’s multiple comparison tests were performed when necessary using Prism GraphPad 2.01 software from GraphPad Software Inc.

Values are expressed as mean ± SD for three independent experiments, each one performed in triplicate.
RESULTS

Metallothionein concentration in several strains of Trypanosoma cruzi epimastigotes

Table I shows the concentrations of MTs in several strains of *T. cruzi*. The LQ strain and the clone Dm28c show the highest concentration at the fourth day of growth, equivalent to the exponential phase. In all strains studied, except the CL-Brener clone, the MT content tends to decline in the stationary phase of growth.

The differences between the exponential and stationary phase are significant (p<0.0001) except for the CL-Brener clone. No significant differences between clones or strains, except for the Dm28c clone (p=0.0006), were observed.

Effect of buthionine sulfoximine upon total thiol content of Trypanosoma cruzi epimastigotes

Figure 1 shows the change in thiols concentration with culture time. The concentration increased up to 10.8 nmol/mg protein during the exponential phase of growth, to decline to 3.8 nmol/mg protein during the stationary phase. These results agree with those reported recently by Ariyanayagam and Fairlamb (2001). Figure 1 also shows that buthionine sulfoximine (BSO) decreased thiols concentration to less than 1 nmol/mg protein after three days without affecting parasite growth. The main burden of the thiols decrease corresponded to T(SH)₂ (data not shown). No effect of BSO upon MT concentration was observed (Table II).

Effect of nifurtimox, benznidazole and zinc chloride upon metallothionein content in Trypanosoma cruzi

Table II shows the effect of nifurtimox and benznidazole upon MT concentration in the Tulahuén epimastigotes. MT content diminished significantly by 73% in relation to controls with both drugs (p<0.001). Because of the presence of mercaptoethanol, no oxidized MTs were present and, accordingly, the decrease in the amount of MTs is most probably due to conjugation of the protein with nifurtimox or benznidazole nitro reduced metabolites,

### Table I

<table>
<thead>
<tr>
<th>Strain</th>
<th>Exponential MTs (pmol /mg protein)</th>
<th>Stationary MTs (pmol /mg protein)</th>
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<tbody>
<tr>
<td>CL-Brener Clone</td>
<td>7.2 ± 1.8</td>
<td>7.5 ± 0.3</td>
</tr>
<tr>
<td>Dm28c Clone</td>
<td>18.0 ± 1.8</td>
<td>6.9 ± 0.7</td>
</tr>
<tr>
<td>LQ</td>
<td>13.2 ± 1.3</td>
<td>6.3 ± 0.9</td>
</tr>
<tr>
<td>MF</td>
<td>10.2 ± 1.6</td>
<td>5.4 ± 0.6</td>
</tr>
<tr>
<td>Tulahuén</td>
<td>10.0 ± 1.7</td>
<td>6.3 ± 0.5</td>
</tr>
</tbody>
</table>

*T. cruzi* epimastigotes were grown at 28°C in a modified Diamond’s medium and harvested on the fourth day of culture (exponential phase) or on the eighth day (stationary phase).

* MTs (Metallothioneins) were determined by the radioactive cadmium method. See Materials and Methods section.
Figure 1. Effect of buthionine sulfoximine upon total thiol content of Trypanosoma cruzi epimastigotes. Continuous line: control thiol content. Hatched line: BSO 0.5 mM added at 0 hours. Total Thiols correspond to the sum of GSH, T(SH)₂ and the intermediate glutathionyl-spermidine and are expressed as GSH. Dotted line: number of parasites treated with BSO 0.5 mM. The \textit{T. cruzi} MF strain was used in these experiments. See Materials and Methods section.

TABLE II

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metallothionein content % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0</td>
</tr>
<tr>
<td>Nifurtimox</td>
<td>26.3 ± 8.0</td>
</tr>
<tr>
<td>Benznidazole</td>
<td>27.5 ± 12.8</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>140.0 ± 10.0</td>
</tr>
<tr>
<td>Buthionine sulfoximine</td>
<td>100.0</td>
</tr>
</tbody>
</table>

\textit{T. cruzi} epimastigotes of the Tulahuén strain were treated with nifurtimox and benznidazole at 2.6 mM concentration, respectively, for two hours and then determined as described in the Materials and Methods section. ZnCl₂ 0.4 mM or buthionine sulfoximine 0.5 mM was added, and after 24 or 48 hours, respectively, the content of MT was determined. The MT content was determined by the radioactive cadmium method as described in the Materials and Methods section.

Table II also shows that ZnCl₂ increased the MT concentration by 40 percent.

Chromatographic analysis of metallothioneins of Trypanosoma cruzi

Figure 2 shows the elution pattern of \textit{T. cruzi} MTs. Monobromobimane derivatized MT from \textit{T. cruzi} eluted in a bimodal pattern at fractions 52-66 and 64-74. Similarly, the native MT detected with the \textsuperscript{109}Cd method also showed a bimodal pattern. Standard monobromobimane-derivatized MT from horse kidney eluted with one peak at 50-58 fractions. Both peaks of native MT showed no measurable which react with free thiol groups (Repetto \textit{et al.}, 1996; Maya \textit{et al.}, 1997). MTs cannot bind cadmium or other metals when their thiol groups are oxidized or conjugated with reactive electrophiles.
absorbance at 280 nm and negligible reaction in the Lowry protein determination indicating a low concentration or absence of aromatic amino acids.

When *T. cruzi* homogenates or the fractions obtained from the Sephadex columns (Fig. 2) were prepared without mercaptoethanol, the amount of MTs analyzed by the radioactive cadmium method decreased to almost zero. The addition of mercaptoethanol recovered the original value for MTs, due to the reduction of disulfide bonds formed. Figure 2 also shows the peak obtained from standard horse kidney MT (6800 KDa, Kojima et al., 1976) derivatized with monobromobimane. This standard demonstrates that *T. cruzi* MTs are in the molecular weight range for MTs.

The proteolytic degradation of MT during the homogenization process is inhibited by the addition of antiproteases, excluding this possibility as an explanation for the two peaks observed in Figure 2 when determined by the radioactive cadmium method or by monobromobimane derivatization.

**Effect of nifurtimox and benznidazole upon MT-like proteins of Trypanosoma cruzi epimastigotes**

Figure 3 shows the 15% SDS-PAGE of nifurtimox- and benznidazole-treated epimastigotes. Both panels, A and B, correspond to the same gel. Panel A was stained with Coomasie blue, and Panel B depicts UV-transillumination visualization. Line 1 is the underivatized, standard horse kidney metallothionein. Line 2 is the same standard but monobromobimane-derivatized. Lines 3 and 4 correspond to 1.3 mM and 2.6 mM benznidazole treatment, respectively, and lines 5 and 6 correspond to 1.3 and 2.6 mM nifurtimox treatments, respectively (Table II). Line 7 is the *T. cruzi* monobromobimane control. There are no apparent differences between control
and treatment lines in the Coomasie blue gel. Figure 3B, lines 2B and 7B, show that *T. cruzi* and horse kidney MT range between 6000 and 14000 KDa. Lines 3B to 6B show that treatment of the parasites with both nifurtimox and benznidazole produces disappearance of this fluorescent band corresponding to MT. When the two peaks of *T. cruzi* monobromobimane-derivatized MT (Fig. 2) were subjected to SDS-PAGE, no difference in their migration was observed. This indicates that both peaks have a very similar molecular weight. Nevertheless, very low-intensity, small fluorescence bands in the high molecular weight region persist throughout the treatment and control lines.

**DISCUSSION**

Metallothionein content in all *T. cruzi* strains studied was higher during exponential phase of growth, except in the CL-Brener clone (Table I). In the Cl-Brenner clone the MT content did not change significantly between the exponential and the stationary phase. At present we have no explanation for this observation. However, we might speculate that in the CL-Brenner clone the MT protein regulatory processes (synthesis and degradation) do not differ in the two growth phases. Nevertheless, other explanations are possible.

T(SH)$_2$ is the major thiol-containing compound in *T. cruzi* and may play a significant role in the parasite’s resistance to nifurtimox and benznidazole (Meister, 1983; Repetto et al., 1996; Maya et al., 1997).

Nifurtimox and benznidazole decrease T(SH)$_2$ and GSH concentration producing oxidative stress (Repetto et al., 1996; Maya et al., 1997). In this report, both drugs also decreased MT levels (Table II and Fig. 3). The reaction of the nifurtimox or benznidazole’s electrophilic metabolites with the MT’s thiol groups most probably causes this effect. Such a reaction is similar to the conjugation of the nifurtimox or benznidazole’s electrophilic metabolites.
with the thiol groups of T(SH)₂ and GSH (Repetto et al., 1996; Maya et al., 1997).

MTs are inducible by several factors including oxidative and physical stress. The results with ZnCl₂ (Table II) indicate that T. cruzi MTs are inducible.

The radioactive cadmium method only detects free reduced MTs and consequently, does not detect MTs conjugated with drug metabolites (Table II). Also, MT conjugated with drug metabolites does not react with monobromobimane and thus does not produce fluorescence (Fig. 3). MTs are better radical scavengers than GSH. The rate constant for the reaction of MT with hydroxyl radicals and electrophilic agents is about three hundred times higher than with GSH (Sato and Bremner, 1993).

Nitroheterocyclic agents, such as nifurtimox, produce hydroxyl radicals (Docampo and Moreno, 1984), which may react with thiol groups. In this respect, we may see in Figure 3 that the parasites’ treatment with both nifurtimox and benznidazole produces disappearance of the fluorescence bands indicating reaction between MT and drug metabolites. Also, Figure 3 shows that low-intensity fluorescence bands in the high molecular weight range of the gel do not disappear, indicating the presence of proteins with less reactive thiol groups.

In previous reports we showed evidence that correlates T(SH)₂ and GSH concentration with the susceptibility to nifurtimox and benznidazole of several T. cruzi strains (Moncada et al., 1989; Repetto et al., 1996; Maya et al., 1997). In this report, we found no direct evidence for the same role of MTs in T. cruzi (tables I and II). Nevertheless, in other organisms, MTs have been shown to have this property (Perego et al., 1998; Lazo et al., 1998; He et al., 2000).

It is probable that MTs and other thiol-containing compounds, such as ovothiols (Steenkamp and Spies, 1994; Ariyanayagam and Fairlamb, 2001), may contribute to protection of the parasite against oxidative stress.

We conclude that MT-like proteins are present in T. cruzi because: i) they can bind cadmium; ii) are heat-stable; iii) they have a molecular weight in the same range as other MTs (Figs. 2 and 3); iv) two possible isoforms elute from the chromatographic columns (as seen in Fig. 2); v) the low O.D. at 280 nm indicates the absence of aromatic amino acids; and vi) they are induced by zinc (Table II). In addition, these proteins may contribute to the resistance of drugs that act producing free radicals or electrophilic metabolites. As stated in the introduction section of this paper, MTs have been isolated from other protozoa. However, this is the first time that MTs are identified in parasites of medical importance.

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