Redox Report
Communications in Free Radical Research

ISSN: 1351-0002 (Print) 1743-2928 (Online) Journal homepage: https://www.tandfonline.com/loi/yrer20

Inactivation of *Trypanosoma cruzi* and *Crithidia fasciculata* topoisomerase I by Fenton systems

Dolores Podestá, Andrés Stoppani & Silvia Fernández Villamil

To cite this article: Dolores Podestá, Andrés Stoppani & Silvia Fernández Villamil (2003) Inactivation of *Trypanosoma cruzi* and *Crithidia fasciculata* topoisomerase I by Fenton systems, Redox Report, 8:6, 357-363, DOI: 10.1179/135100003225003366

To link to this article: https://doi.org/10.1179/135100003225003366

Published online: 19 Jul 2013.

Research article

Inactivation of *Trypanosoma cruzi* and *Crithidia fasciculata* topoisomerase I by Fenton systems

Dolores Podestá, Andrés Stoppani, Silvia Fernández Villamil

Bioenergetics Research Centre (CONICET) and School of Medicine, University of Buenos Aires, Buenos Aires, Argentina

Fenton systems (H_2O_2/Fe(II) or H_2O_2/Cu(II)) inhibited *Trypanosoma cruzi* and *Crithidia fasciculata* topoisomerase I activity. About 61–71% inactivation was produced by 25 μM Fe(II) or Cu(II) with 3.0 mM H_2O_2. Thiol compounds and free radical scavengers prevented Fenton system effects, depending on the topoisomerase assayed. With the *T. cruzi* enzyme, reduced glutathione (GSH), dithiothreitol (DTT), cysteine and N-acetyl-L-cysteine (NAC) entirely prevented the effect of the H_2O_2/Fe(II) system; mannitol protected 37%, whereas histidine and ethanol were ineffective. With *C. fasciculata* topoisomerase, GSH, DTT and NAC protected 100%, cysteine, histidine and mannitol protected 28%, 34% and 48%, respectively, whereas ethanol was ineffective. With the H_2O_2/Cu(II) system and *T. cruzi* topoisomerase, DTT and histidine protected 100% and 60%, respectively, but the other assayed protectors were less effective. Similar results were obtained with the *C. fasciculata* enzyme. Topoisomerase inactivation by the H_2O_2/Fe(II) or H_2O_2/Cu(II) systems proved to be irreversible since it was not reversed by the more effective enzyme protectors. It is suggested that topoisomerases could act either as targets of 'reactive oxygen species' (ROS) generated by Fenton systems or bind the corresponding metal ions, whose redox cycling would generate reactive oxygen species *in situ*.

INTRODUCTION

Topoisomerases catalyse the breakage of DNA phosphodiester bonds and then rejoin them, mediating changes in DNA topological status. Topoisomerase I transiently cleaves and then rejoins a single DNA chain while topoisomerase II does so with both chains and requires ATP for its activity. These enzymes are involved in DNA replication, transcription and recombination as well as in chromosome condensation and decondensation.[1–6] The topoisomerases of several kinestoplastid protozoa have been purified and cloned, including those of *Trypanosoma cruzi* (the agent of Chagas' disease, American trypanosomiasis) and *Crithidia fasciculata*.[7–10] Trypanosomes differ from mammalian cells in many aspects and are characterized by a considerable amount of mitochondrial DNA called kinetoplast DNA (kDNA), which is a network consisting of thousands of intercatenated circular DNAs known as maxicircles and minicircles. Network replication requires topoisomerase activity.[11,12] Parasite topoisomerases have been the focus of many investigations due to their intrinsic interest and because of their role as potential targets for antiparasite drugs.[12–14]

Transition group metals including Fe and Cu play a major role in the oxidation and denaturation of proteins, both properties attributed to their capacity to form 'reactive oxygen species' (ROS).[15–19] Such reactive species would be closely involved with the action of certain drugs on *T. cruzi* (such as nitrofurans),[20] given the weakness of parasite antioxidant defenses.[21,22]

To obtain information on the response of *T. cruzi* topoisomerase I activity to ROS, we studied the action of

Received 20 March 2003
Revised 10 July 2003
Accepted 25 August 2003

Correspondence to: Silvia Fernández Villamil, Bioenergetics Research Centre (CONICET) and School of Medicine, University of Buenos Aires, Paraguay 2155, 1121-Buenos Aires, Argentina
Tel/Fax: +54 11 4508 3680; E-mail: villamil@mail.retina.ar

Abbreviations: ROS, reactive oxygen species; superoxide anion radical ($O_2^{\bullet}$); hydroxyl radical ($HO^{\bullet}$); GSH, reduced L-glutathione; DTT, DL-dithiothreitol; NAC, N-acetyl-L-cysteine; BSA, bovine serum albumin; NEM, N-ethylmaleimide

DOI 10.1179/135100003225003366

Fenton systems (H_2O_2/Fe(II) or H_2O_2/Cu(II)) on the activity of this enzyme. Complementary experiments were carried out with the topoisomerase of *C. fasciculata*, which may be successfully used to support trypanosomatid studies, because of its relatively fast growth rate and lack of pathogenicity.[23] Results achieved show that the unwinding activity of topoisomerases present in both *T. cruzi* and *C. fasciculata* was sensitive to ROS.

MATERIALS AND METHODS

Materials

Phenylmethylsulphonyl fluoride, DTT, cysteine, GSH, NAC, Trizma Base, mannitol, histidine, HEPES, EDTA, BSA, NP-40 (Igepal CA-630) non-ionic detergent, dimethyl sulphoxide, $CuSO_4.5H_2O$, φX 174 phage DNA, ethidium bromide, and proteinase K were purchased from Sigma Chemical Co. (St Louis, MO, USA). H_2O_2 was obtained from Merck (Darmstadt, Germany), and sample concentration was determined spectrophotometrically at 240 nm ($\varepsilon = 0.04\ mM^{-1}cm^{-1}$). $FeSO_4.7H_2O$ was purchased from ANALAR (UK). H_2O_2 and $FeSO_4$ solutions were prepared immediately before use. Other reagents were of analytical grade.

Organisms

C. fasciculata (ATCC 11745) was provided by Dr S. Hutner from Haskins Laboratory, Pace University, New York, USA. Cells were cultured in a medium containing (g/l): NaCl (5.0); KCl (2.0); NaH_2PO_4 (0.6); $MgSO_4$ (0.2); sucrose (2.5); sodium ascorbate (0.2); MOPS (0.5); powdered dried liver (5.0); pluripeptone (7.5); brain-heart infusion (7.5); and hemin (0.02; dissolved in triethanolamine:H_2O 1:1 [v/v]). Cells were cultured at 28°C for 48 h in a New Brunswick Gyratory Shaker model G-25 at 100 rpm. Cells were harvested by centrifugation at 750 *g* at 4°C. *T. cruzi* epimastigotes in their logarithmic growth phase were kindly provided by Dr González Cappa of the Microbiology Department at the School of Medicine, University of Buenos Aires.

Cell-free extract preparations

Cell-free extracts for topoisomerase I assays were prepared as follows. *T. cruzi* or *C. fasciculata* cells (~0.2 g wet weight) were washed with 0.15 M NaCl and the cell suspension centrifuged at 750 *g*. The pellet was resuspended in 0.8 ml of lysis buffer (20 mM Tris-HCl pH 8.8, 50 mM NaCl, 1 mM EDTA pH 8.0, 0.1 mM DTT, 5% [v/v] glycerol, 10 mM $NaHSO_3$, 1 mM phenylmethylsulphonyl fluoride) and sonicated in a sonifier cell disruptor (Model W 185, Heat System-Ultrasonic, Inc.) at 45 W for 10 s. Extracts were kept in a 1:1 mixture of lysis buffer and dilution buffer (40 mM HEPES pH 8.0, 0.2 mg/ml BSA, 0.5 mM EDTA pH 8.0, 40% [v/v] glycerol, 6% [w/v] polyethyleneglycol) at –20°C until used.

Topoisomerase I activity assay

Cell-free extracts were used to assay the unwinding of a supercoiled plasmid φX 174 to its relaxed circular form,[24] as follows. Extracts were diluted in reaction buffer (50 mM Tris-HCl pH 7.5, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA pH 8.0, 30 µg/ml BSA) and then 10 µl of each preparation, containing two enzyme units approximately, were pre-incubated in reaction buffer for 12 min at 30°C with the drug systems indicated under 'Results' (final volume 20 µl). After adding 0.2 µg plasmid (RF I) (SC), the incubation mixture was left for 20 min at 30°C. The reaction was stopped by adding 5 µl of 5% (w/v) sodium dodecyl sulphate and 2 µl of 0.5 µg/ml proteinase K solution. After 60 min at 37°C, 3 µl of stop buffer (0.4% [w/v] bromophenol blue, 50% [v/v] glycerol) were added. The supercoiled (RF I) and relaxed plasmid (RF IV) (OC) topoisomers were separated by electrophoresis in a 1% (w/v) agarose gel in TAE buffer (40 mM Tris-HCl pH 7.6, 20 mM acetic acid, 1 mM EDTA) for 2.5 h. Gels were then stained with 0.7 µg/ml ethidium bromide for 30 min and destained for 30 min with distilled water. DNA bands were visualised by UV light in a transilluminator, quantitated by scanning and analysed using the Gel-Pro Analyzer 3.1 (Media Cybernetics) program.

Assay with Fenton systems

Cell-free extracts were pre-incubated for 12 min at 30°C in 50 mM Tris-HCl pH 7.5, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA pH 8.0, 30 µg/ml BSA with 3 mM H_2O_2 and 25 or 100 µM Fe(II) or Cu(II). Aliquots were then taken and activity measured as described above. Protectors were added prior to the Fenton system as described in 'Results'.

Expression of results

Enzyme activity is expressed by the percentage value of SC plasmid converted into the OC form. Control unwinding activity was taken as 100%. Percentage protective effect of the antioxidants assayed, is represented by the equation: P (%) = 100 (FS – FSP)/FS), where P (%) represents

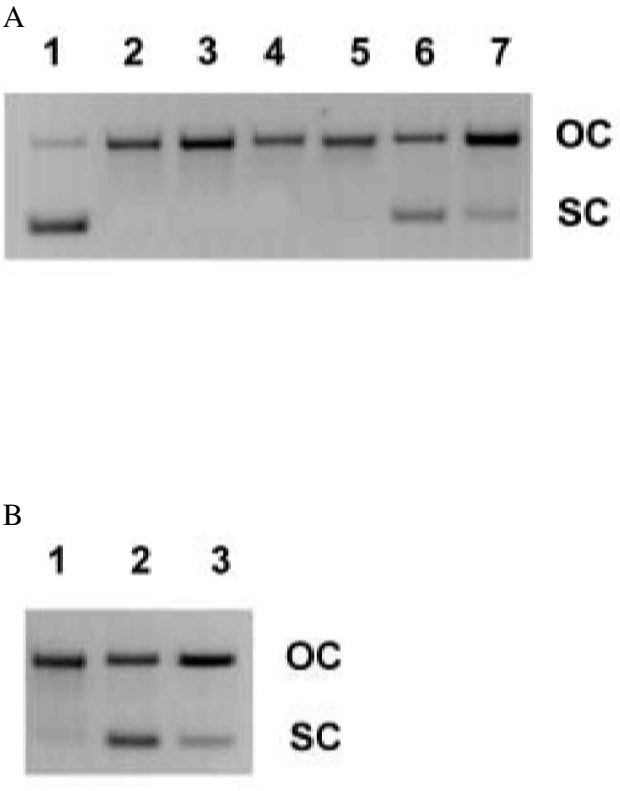

Fig. 1. Effect of Fenton systems on *T. cruzi* topoisomerase I activity. The reaction mixture contained 50 mM Tris-HCl pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 30 μg/ml BSA and the compounds as stated above. The order of addition of reagents to the reaction mixture containing 10 μl of *T. cruzi* cell-free extract (0.2 mg protein/ml) was as follows: (i) the Fenton system; (ii) after 12 min pre-incubation, the reaction was started by adding 0.2 μg of the plasmid φX 174. SC, supercoiled circular form DNA (RF I); OC, relaxed circular form DNA (RF IV). (A): *T. cruzi* cell-free extract in the presence of the H_2O_2/Fe(II) system. Lane 1, control DNA φX 174; lane 2, topoisomerase control activity; lane 3, 3 mM H_2O_2; lane 4, 100 μM Fe(II); lane 5, 25 μM Fe(II); lane 6, H_2O_2 + 100 μM Fe(II); lane 7, H_2O_2 + 25 μM Fe(II). A typical experiment is shown from at least three independent determinations. (B) *T. cruzi* cell-free extract in the presence of the H_2O_2/Cu(II) system. Lane 1, topoisomerase control activity; lane 2, H_2O_2 + 100 μM Cu(II); lane 3, H_2O_2 + 25 μM Cu(II). A typical experiment is shown from at least three independent determinations.

the protective effect; FS, enzyme inactivation by the Fenton system; and FSP, enzyme inactivation by the Fenton system in the presence of the protector. All determinations were carried out in at least duplicate samples. Figures illustrate typical experiments.

RESULTS

Inhibition of Trypanosoma cruzi *and* Crithidia fasciculata *topoisomerase I activity by Fenton systems*

Incubation of φX 174 plasmid supercoiled DNA with *T. cruzi* or *C. fasciculata* cell-free extract, under conditions as described in this study, demonstrated the presence of topoisomerase I unwinding activity in the assayed extract. This activity was inhibited by heating (100%), by 5 mM NEM (70%), and in the presence of the trypanocide Berenil (100 μM), reaching 78% and 82% for *T. cruzi* and *C. fasciculata* enzymes, respectively. These latter results agree with those documented by Riou *et al*. for *T. cruzi*.[12] Camptothecine, a well-known inhibitor of mammalian topoisomerase I,[13,25] inhibited the *T. cruzi* enzyme *in vitro* (data not shown), in close agreement with its effect on intact parasites.[13]

When an extract from *T. cruzi* was pre-incubated with H_2O_2 plus a metal cation system (Fe[II] or Cu[II]), the relaxing activity diminished significantly (Fig. 1A,B). Inhibition was dependent on the concentration of the metal used, as illustrated by comparison of lanes 6 and 7 in Figure 1A for the H_2O_2/Fe(II) system (100 μM or 25 μM), respectively, and of lanes 2 and 3 in Figure 1B for the H_2O_2/Cu(II) system (100 μM or 25 μM), respectively. H_2O_2 or Fe(II) alone failed to affect enzyme activity as depicted in Figure 1A, lanes 3–5, thus confirming the role of the Fenton reaction in topoisomerase inactivation. Similar results were obtained with *C. fasciculata* extracts (data not shown). For subsequent Fenton systems assays, the lower metal concentration (25 μM) was used.

Effect of thiol compounds and scavengers against Fenton systems

Figure 2A,B allows comparison of the effects of several free radical scavengers on the inhibition of *T. cruzi* topoisomerase I activity by the H_2O_2/Fe(II) system. GSH, DTT, cysteine or NAC (1 mM) completely prevented the inactivation induced by this system, whereas histidine failed to do so (Fig. 2A). None of the assayed compounds had any effect on DNA unwinding activity in the absence of the Fenton system (Fig. 2A). DTT was assayed at different concentrations with the H_2O_2/Fe(II) system and showed its protective effect starting from 0.5 mM DTT (Table 1). Inhibition of the unwinding activity by the H_2O_2/Fe(II) (or Cu[II]) system proved to be irreversible, since incubation with 1 mM GSH, DTT, cysteine or NAC after pre-incubation with such systems, failed to re-activate topoisomerase activity either in *T. cruzi* or in *C. fasciculata* extracts (data not shown), despite the protective effect exerted by the same antioxidants. Figure 2B shows that 200 mM mannitol protected *T. cruzi* topoisomerase against the deleterious effect of the H_2O_2/Fe(II) system by 37%, whereas ethanol was ineffective (Fig. 2B and Table 1). Benzoate, a well-known scavenger of free radicals,[15,26] exerted a direct inhibitory effect (100%) on topoisomerase activity (Fig. 2B) and, therefore, it could not be used as topoisomerase I protector. Neither ethanol nor mannitol had any effect on topoisomerase activity in the absence of the Fenton system (Fig. 2B).

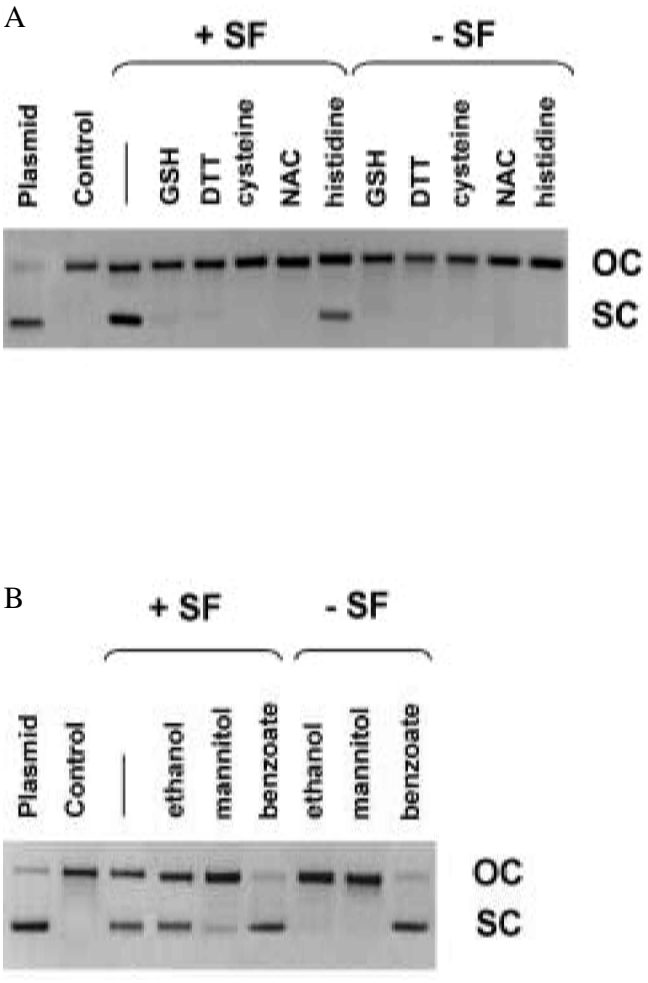

Fig. 2. Effect of thiols and scavengers on the inactivation of *T. cruzi* topoisomerase I activity by the H_2O_2/Fe(II) system. Experimental conditions were as described in the caption to Figure 1 and Materials and Methods. The order of addition of reagents to the reaction mixture containing 10 µl of *T. cruzi* cell-free extract (4.5 mg protein/ml) was as follows: (i) the protector; (ii) the Fenton system (3 mM H_2O_2/25 µM Fe(II)), 3) after 12 min incubation the reaction was started by adding the plasmid. (A) Plasmid, control DNA φX 174; Control, topoisomerase control activity; GSH, DTT, cysteine, NAC or histidine (1 mM) effect on topoisomerase activity in the presence (+SF) or absence (–SF) of Fenton system. A typical experiment from at least three independent determinations. (B) Plasmid, control DNA φX 174; Control, topoisomerase control activity; ethanol (4 mM), mannitol (200 mM) or benzoate (200 mM) effect on topoisomerase activity in the presence (+SF) or absence (–SF) of Fenton system. A typical experiment from at least three independent determinations.

With *C. fasciculata* and the H_2O_2/Fe(II) system, results were as follows: GSH, DTT and NAC (1 mM) totally protected the unwinding activity; mannitol (200 mM), histidine and cysteine (1 mM) protected the enzyme 48%, 34% and 28%, respectively, whereas ethanol (4 mM) failed to protect (Table 2).

Table 1. Effect of thiols and scavengers on *T. cruzi* topoisomerase I inactivation by Fenton systems

Addition	Topoisomerase I inactivation (%)	
	H_2O_2 + Fe(II)	H_2O_2 + Cu(II)
None	71 (0)	62
GSH (1 mM)	0 (100)	64 (0)
Cysteine (1 mM)	0 (100)	56 (10)
NAC (1 mM)	0 (100)	76 (0)
Histidine (1 mM)	75 (0)	25 (60)
DTT (0.05 mM)	77 (0)	–
DTT (0.1 mM)	74 (0)	–
DTT (0.5 mM)	45 (37)	–
DTT (1 mM)	0 (100)	0 (100)
Ethanol (4 mM)	80 (0)	54 (13)
Mannitol (200 mM)	45 (37)	66 (0)

Experimental conditions were as described in the captions to Figures 1–3 and in Materials and Methods. Control unwinding activity was taken as 100%. In parenthesis, thiol or scavenger protective effect (%):P(%).

When in the Fenton system Fe(II) was replaced by Cu(II), inhibition was 62% and 61%, respectively, for *T. cruzi* and *C. fasciculata* topoisomerase I (Tables 1 and 2 and Fig. 1). DTT and histidine (1 mM), efficiently prevented the loss of *T. cruzi* topoisomerase activity (100% and 60%, respectively; Fig. 3 and Table 1). GSH, NAC, cysteine (1 mM), ethanol (4 mM) and mannitol (200 mM) failed to protect enzyme activity against topoisomerase Fenton system inactivation (Fig. 3 and Table 1). Similar results were obtained with *C. fasciculata* enzyme (Table 2). However, ethanol and mannitol prevented the effect of H_2O_2/Cu(II) to a limited degree (46% and 54%, respectively; Table 2).

Table 2. Effect of thiols and scavengers on *C. fasciculata* topoisomerase I inactivation by Fenton systems.

Addition	Topoisomerase I inactivation (%)	
	H_2O_2 + Fe(II)	H_2O_2 + Cu(II)
None	65	61
GSH (1 mM)	0 (100)	61 (0)
Cysteine (1 mM)	47 (28)	54 (11)
NAC (1 mM)	0 (100)	53 (13)
Histidine (1 mM)	43 (34)	0 (100)
DTT (1 mM)	0 (100)	0 (100)
Ethanol (4 mM)	66 (0)	33 (46)
Mannitol (200 mM)	34 (48)	28 (54)

Experimental conditions were as described in the captions to Figures 2 and 3, except for *C. fasciculata* cell-free extract (0.15 mg protein/ml), and in Materials and Methods. Control unwinding activity was taken as 100%. In parenthesis, thiol or scavenger protective effect (%):P(%).

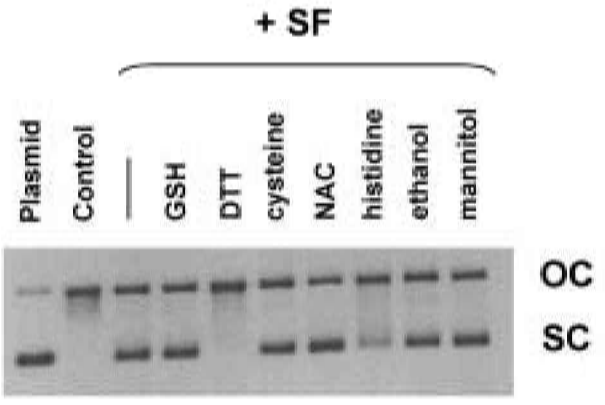

Fig. 3. Effect of thiols and scavengers on the inactivation of *T. cruzi* topoisomerase I activity by the H_2O_2/Cu(II) system. Experimental conditions were as in the captions to Figures 1 and 2. The order of addition of reagents to the reaction mixture containing 10 µl of *T. cruzi* cell-free extract (4.5 mg protein/ml) was as follows: (i) the protector; (ii) the Fenton system (3 mM H_2O_2/25 µM Cu(II); (iii) after 12 min incubation, the reaction was started by adding the plasmid. Plasmid, control DNA φX 174; Control, topoisomerase control activity; GSH, DTT, cysteine, NAC, histidine (1 mM), ethanol (4 mM) or mannitol (200 mM) effect on topoisomerase activity in the presence (+SF) of Fenton system. A typical experiment from at least three independent determinations.

Discussion

Fenton chemistry is a prime example of damaging free-radical reactions catalysed by transition metals. A mixture of H_2O_2 with a Fe(II) salt oxidises a broad spectrum of organic molecules. The H_2O_2/Fe(II) system probably involves several oxidising species, the best characterised being the hydroxyl radical.

$$\begin{aligned} Fe(II) + H_2O_2 &\rightarrow \text{Intermediate complex(es)} \\ &\rightarrow Fe(III) + HO^{\bullet} + HO^{-} \end{aligned} \qquad \text{Eq. 1}$$

The chemical identity of the oxidising species additional to $HO^{\bullet}$ formed during Fenton chemistry is uncertain. Fe(II) chelators may prevent the Fenton reaction by: (i) altering the oxidation potential of iron; (ii) blocking available sites on the iron to which the H_2O_2 might attach; (iii) promoting oxidation of Fe(II) to Fe(III); or (iv) scavenging $HO^{\bullet}$. Hydroxyl radicals react quickly with a wide variety of molecules.[15] These reactions may involve hydrogen abstractions, addition and electron transfer. Amino acids such as arginine, cysteine, glutamic acid, histidine, phenylalanine and tryptophan react with second order rate constant in the range of 10^9–10^{10} $M^{-1}s^{-1}$, which means that the probability of multiple sites of attack for topoisomerase I by the assayed Fenton systems is very high. In this connection, it should be recalled that *T. cruzi* F_0F_1-ATPase and *C. fasciculata* PARP are also inactivated by the H_2O_2/Fe(II) system.[27,28]

Copper-induced oxidative damage by H_2O_2 is also generally attributed to the formation of the highly reactive hydroxyl radical by a mechanism analogous to the iron-catalysed Haber-Weiss cycle.[15,17]

$$Cu(II) + H_2O_2 \rightarrow Cu(I) + HO_2^{\bullet} + H^{+} \qquad \text{Eq. 2}$$

$$2\,HO_2^{\bullet} \rightarrow 2\,O_2^{\bullet} + 2\,H^{+} \rightarrow H_2O_2 + O_2 \qquad \text{Eq. 3}$$

$$Cu(II) + O_2^{\bullet} \rightarrow Cu(I) + O_2 \qquad \text{Eq. 4}$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + OH^{\bullet} + OH^{-} \qquad \text{Eq. 5}$$

Evidence for the formation of the Cu(I) intermediate from Cu(II) in the presence of H_2O_2 was provided by inhibiting DNA damage from this reaction with a Cu(I)-specific chelator. However, the reaction between Cu(I) and H_2O_2 seems not to be a simple one-electron oxidation of Cu(I) to Cu(II) with concomitant hydroxyl radical formation as represented in Equation 5. There are many reports where addition of a hydroxyl radical scavenger had a less than expected or insignificant effect in preventing oxidative damage in various systems.[29]

Treatment of *T. cruzi* and *C. fasciculata* extracts with H_2O_2/Fe(II) or H_2O_2/Cu(II) systems decreased topoisomerase I unwinding activity, an inhibition that proved to be irreversible. Metals were effective only in the presence of H_2O_2, thus demonstrating the role of ROS produced by the Fenton reaction as inactivating agents (Fig. 1). To confirm the role of the metal in such enzyme inactivation, several compounds with complexing capacity were used. The effects described herein depended on the metal used and on the origin of the enzyme. DTT was a strong protector of topoisomerase I unwinding activity against the oxidising effect of either Fenton system (Figs 2 and 3; Tables 1 and 2). Concentrations required to achieve the protective effect were similar to those used for the mammalian lipoamide dehydrogenase enzyme; however, inactivation due to such Fenton systems could not be reversed, as described for lipoamide dehydrogenase and H_2O_2/Fe(II).[26] The irreversibility of inactivation by thiols as DTT suggests that, besides –SH, other amino acid residues (histidyl, prolyl and tyrosyl) could be affected by ROS generated by the Fenton system, as described for other proteins.[17,26,30–34] Both in *T. cruzi* and in *C. fasciculata,* GSH protected topoisomerase activity against the H_2O_2/Fe(II) system, as reported for lipoamide dehydrogenase after a –SH/Fe(II) molar ratio of about 1:1, which led to complete protection of the latter enzyme. The antioxidant action of 1 mM GSH would result from GSH oxidation by the $OH^{\bullet}$ radical.[35]

The H_2O_2/Cu(II) system differed somewhat from the H_2O_2/Fe(II) system (Tables 1 and 2). Besides DTT, compounds such as GSH, cysteine and NAC effectively protected both *T. cruzi* and *C. fasciculata* topoisomerases against the H_2O_2/Fe(II) system, whereas with the

H_2O_2/Cu(II) system only DTT and histidine were effective (Fig. 3; Tables 1 and 2). The effect of histidine seems attributable to a decrease in metal concentration, since in the presence of Cu(II) histidine forms a complex. Thus, $OH^{\bullet}$ radicals generated by the redox cycle of the metal are taken up and inactivated by histidine itself, which is destroyed, so that they cannot react with essential amino acid residues.[36,37] The H_2O_2/Cu(II) system yielded $OH^{\bullet}$ radicals, though the kinetics differed from that of H_2O_2/Fe(II). Accordingly, a combination of $OH^{\bullet}$ and other ROS, possibly $O_2^{\bullet}$ and/or singlet oxygen, would explain the dissimilar action of scavengers on topoisomerase inactivation, as compared with that of the H_2O_2/Fe(II) system. Likewise, evidence for the formation of Cu(III) intermediates during the reaction of Cu(I) with H_2O_2 has been reported from kinetic data[38,39] and is in agreement with the observation that oxidative damage from copper is not easily quenched by hydroxyl radical scavengers. Results with mannitol and ethanol support the hypothesis that oxyradicals generated by Fenton systems act at a short distance from their formation site. They would mainly arise on the surface of the enzyme, rather than in the aqueous phase where they may be taken up by scavengers. Therefore, the generation of 'site-specific' radicals would facilitate their action on the enzyme.[31,40–42] While it is possible to explain the latter results solely by a 'site-specific' mechanism, whereby hydroxyl radicals react immediately upon formation close to their site of generation, formation of Cu(III) or metal-bound oxygen intermediates as the oxidising species has been described.[38,39] Highly oxidising Fe(IV) intermediates have also been proposed in reactions between Fe(II) or Fe(II) complexes and H_2O_2.[15] These higher oxidation state metal intermediates can oxidise organic reagents such as alcohols commonly used for scavenging hydroxyl radicals.[29,40]

Site-specificity of metal-mediated protein damage depends on the co-ordination of transition metal ions by proteins and peptides predominantly through the sulphydryl, imidazole, and the deprotonated peptide bond and side chain nitrogens, followed by generation of metal-associated oxidising species at these particular sites. Therefore, tryptophan, tyrosine, phenylalanine and methionine, which are also sensitive to oxygen radicals generated by ionising radiation, but fail to bind to metal cations under physiological conditions, are less likely to be targeted by metal-catalysed oxidation, depending on the specific folding of the protein molecule.[41] Metal ion-catalysed oxidation of amino acids is most likely a 'caged' process, and 'site-specific' generation of $OH^{\bullet}$ is very difficult to protect against by adding most $OH^{\bullet}$ scavengers, since they cannot be present at the site of metal ion binding at concentrations sufficient to protect the target, to which the metal ion is bound, against immediate attack by $OH^{\bullet}$.

Owing to dissimilar affinities of various metal cations for various side-chain donors in proteins and diverse co-ordination modes of resulting complexes, damage extent and scavenger behaviour may strongly depend on the metal. The co-ordination mode would also be the likely cause of a difference in redox activity between the two complexes.[33]

Although it is quite probable, it remains uncertain whether the hydroxyl radical is the sole species responsible for the oxidation of the protein. An alternative explanation would be that other reactive oxygen species promoted through the linkage of transition metals to the protein, such as the perferryl, ferryl ion or singlet oxygen which attacks side chains of amino acid residues at the metal-binding site, would behave as active intermediates in the loss of enzymatic activity.[40,43]

Conclusions

These observations demonstrate that both *T. cruzi* and *C. fasciculata* topoisomerase I may constitute a suitable target for free-radical generating systems. More relevantly, with the former parasite it may contribute to chemotherapy for Chagas' disease. The inactivation of topoisomerase I from *T. cruzi* and *C. fasciculata* by Fe(II) and Cu(II) Fenton systems suggests that the molecular structure of the protein could act either as a target for oxyradicals or else to link metals as Cu(II) or Fe(II), facilitating the generation of 'site-specific' radicals, both mechanisms leading to the inactivation of enzyme activity.

Acknowledgements

This work was supported by grants from the University of Buenos Aires and the Ministry of Public Health of Argentina. We are grateful to M. I. Garcia-Herreros for her useful collaboration. M. A. Veron and S. del Valle lent able technical assistance. D. Podestá is a doctoral Research Fellow of the University of Buenos Aires (UBA). S. Fernández Villamil is a Career Investigator of CONICET, Argentina. Dr Andrés Stoppani died on March 18, 2003.

References

1. Wang JC. DNA topoisomerases. *Annu Rev Biochem* 1985; **54**: 665–697.
2. Wang JC. DNA topoisomerases: why so many? *J Biol Chem* 1991; **266**: 6659–-6662.
3. Giaccone G. DNA topoisomerases and topoisomerase inhibitors. *Pathol Biol (Paris)* 1994; **42**: 346–352.
4. Wigley DB. Structure and mechanism of DNA topoisomerase. *Annu Rev Biophys Biomol Struct* 1995; **24**: 185–208.

5. Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. *Mol Cell Biol* 2002; **3**: 430–440.
6. Zhu Q, Pongpech P, Di Gate RJ. Type I topoisomerase activity is required for proper chromosomal segregation in *Escherichia coli*. *Proc Natl Acad Sci USA* 2001; **98**: 9766–9771.
7. Melendy T, Ray DS. Purification and nuclear localization of a type I topoisomerase from *Crithidia fasciculata*. *Mol Biochem Parasitol* 1987; **24**: 215–225.
8. Melendy T, Ray DS. Novobiocin affinity purification of a mitochondrial type II topoisomerase from the trypanosomatid C*rithidia fasciculata*. *J Biol Chem* 1989; **264**: 1870–1876.
9. Fragoso SP, Mattei D, Hines JC, Ray D, Goldenberg S. Expression and cellular localization of *Trypanosoma cruzi* type II DNA topoisomerase. *Mol Biochem Parasitol* 1998; **94**: 197–204.
10. Fragoso SP, Goldenberg S. Cloning and characterization of the gene encoding *Trypanosoma cruzi* DNA topoisomerase II. *Mol Biochem Parasitol* 1992; **55**: 127–134.
11. Burri C, Bodley AL, Shapiro TA. Topoisomerases in kinetoplastids. *Parasitol Today* 1996; **12**: 226–231.
12. Riou GF, Gabillot M, Douc-Rasy S, Kayser A. DNA topoisomerase(s) of trypanosomes: inhibitory effects of some chemicals. *Molecular Biology of Host-Parasite Interactions* 1984; 279–289.
13. Bodley AL, Shapiro TA. Molecular and cytotoxic effects of camptothecin, a topoisomerase I inhibitor, on trypanosomes and *Leishmania*. *Proc Natl Acad Sci USA* 1995; **92**: 3726–3730.
14. Nenortas EC, Bodley AL, Shapiro TA. DNA topoisomerases: a new twist for antiparasitic chemotherapy? *Biochim Biophys Acta* 1998; **1400**: 349–354.
15. Halliwell B, Gutteridge JMC. *Free Radicals in Biology and Medicine*, 3rd edn. Oxford: Oxford University Press, 1999.
16. Stadtman ER, Berlett BS. Fenton chemistry. Amino acid oxidation. *J Biol Chem* 1991; **266**: 17201–17211.
17. Liochev SI. The mechanism of 'Fenton-like' reactions and their importance for biological systems. A biologist's view. In: Sigel A, Sigel H. (eds) *Metal Ions in Biological Systems. Interrelations between free radicals and metal ions in life processes*, vol 36. New York: Marcel Dekker, 1999; 1–31.
18. Goldstein S, Meyerstein D, Czapski G. The Fenton reagents. *Free Radic Biol Med* 1993; **15**: 435–445.
19. Stadtman ER, Berlett BS. Reactive oxygen mediated protein oxidation in aging and disease. *Drug Metab Rev* 1998; **30**: 225–243.
20. Docampo R, Stoppani AOM. Generation of superoxide anion and hydrogen peroxide induced by nifurtimox in *Trypanosoma cruzi*. *Arch Biochem Biophys* 1979; **197**: 317–321.
21. Boveris A, Stoppani AOM. Hydrogen peroxide generation in *Trypanosoma cruzi*. *Experientia* 1977; **33**: 1306–1308.
22. Boveris A, Sies H, Martino EE, Docampo J, Turrens JF, Stoppani AOM. Deficient metabolic utilization hydrogen peroxide in *Trypanosoma cruzi*. *Biochem J* 1980; **188**: 643–648.
23. Stoppani AOM, Goijman S, Fernández Villamil SH, Molina Portela MP, Biscardi AM, Paulino M. Cytotoxicity of lipophilic *o*-naphthoquinones: structure-activity relationship. *Trends Comp Biochem Physiol* 2000; **7**: 1–16.
24. Riou JF, Gabillot M, Philippe M, Riou G. Purification and characterization of *Plasmodium berghei* DNA topoisomerases I and II: drug action, inhibition of decatenation and relaxation, and stimulation of DNA cleavage. *Biochemistry* 1986; **25**: 1471–1479.
25. Liu LF, Desai SD, Li T-K, Mao Y, Sun M, Sim S-P. Mechanism of action of camptothecin. *Ann NY Acad Sci* 2000; **922**: 11–46.
26. Gutierrez Correa J, Stoppani AOM. Inactivation of lipoamide dehydrogenase by cobalt (II) and iron (II) Fenton systems: effect of metal chelators, thiol compounds and adenine nucleotides. *Free Radic Res Commun* 1993; **19**: 303–314.
27. Cataldi de Flombaum MA, Stoppani AOM. Inactivation of mitochondrial adenosine triphosphatase from *Trypanosoma cruzi* by oxygen radicals. *Biochem Int* 1986; **12**: 785–793.
28. Fernández Villamil S, Podestá D, Molina Portela MD, Stoppani A. Characterization of poly(ADP-ribose)polymerase from *Crithidia fasciculata*: enzyme inhibition by β-lapachone. *Mol Biochem Parasitol* 2001; **115**: 248–256.
29. Hanna PM, Mason RP. Direct evidence for inhibition of free radical formation from Cu(I) and hydrogen peroxide by glutathione and other potential ligands using the EPR spin-trapping technique. *Arch Biochem Biophys* 1992; **295**: 205–213.
30. Hong J, Schöneich C. The metal-catalyzed oxidation of methionine in peptides by Fenton system involves two consecutive one-electron oxidation processes. *Free Radic Biol Med* 2001; **31**: 1432–1441.
31. Li S, Schöneich C, Borchardt RT. Chemical pathways of peptide degradation. VIII. Oxidation of methionine in small model peptides by prooxidant/transition metal ion systems: influence of selective scavengers for reactive oxygen intermediates. *Pharm Res* 1995; **12**: 348–355.
32. Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J* 1997; **324**: 1–18.
33. Kasprzak KS. Oxidative DNA and protein damage in metal-induced toxicity and carcinogenesis. *Free Radic Biol Med* 2002; **32**: 958–967.
34. Akagawa M, Suyama K. Oxidative deamination by hydrogen peroxide in the presence of metals. *Free Radic Res* 2002; **36**: 13–21.
35. Gutierrez Correa J, Stoppani AOM. Inactivation of heart lipoamide dehydrogenase by copper Fenton systems. Effect of thiol compounds and metal chelators. *Free Radic Res* 1995; **22**: 239–250.
36. Tachon P. DNA single strand breakage by H_2O_2 and ferric or cupric ions: its modulation by histidine. *Free Radic Res Commun* 1990; **9**: 39–47.
37. Shinar E, Navok T, Chevion M. The analogous mechanisms of enzymatic inactivation induced by ascorbate and superoxide in the presence of copper. *J Biol Chem* 1983; **258**: 14778–14783.
38. Johnson GR, Nazhat NB, Saadala-Nazhat RA. Reaction of the aquocopper (I) ion with hydrogen peroxide evidence against hydroxyl free radical formation. *J Chem Soc Chem Commun* 1985; 407–408.
39. Sutton HC, Winterbourn C. On the participation of higher oxidation states of iron and copper in Fenton reactions. *Free Radic Biol Med* 1989; **6**: 53–60.
40. Kremer ML. Is $OH^{\bullet}$ the active Fenton intermediate in the oxidation of ethanol? *J Inorg Biochem* 2000; **78**: 255–257.
41. Stadtman ER. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu Rev Biochem* 1993; **62**: 797–821.
42. Welch KD, Davis TZ, Van Eden ME, Aust SD. Deleterious iron-mediated oxidation of biomolecules. *Free Radic Biol Med* 2002; **32**: 577–583.
43. Qian SY, Buettner GR. Iron and dioxygen chemistry is an important route to initiation of biological free radical oxidations: an electron paramagnetic resonance spin trapping study. *Free Radic Biol Med* 1999; **26**: 1447–1456.