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Trypanosoma cruzi Antioxidant Enzymes As Virulence Factors in Chagas Disease

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Abstract

Significance: Chagas disease (CD) affects several million people in Latin America and is spreading beyond its classical boundaries due to the migration of infected host and insect vectors, HIV co-infection, and blood transfusion. The current therapy is not adequate for treatment of the chronic phase of CD, and new drugs are warranted. **Recent Advances:** *Trypanosoma cruzi* is equipped with a specialized and complex network of antioxidant enzymes that are located at different subcellular compartments which defend the parasite against host oxidative assaults. Recently, strong evidence has emerged which indicates that enzyme components of the *T. cruzi* antioxidant network (cytosolic and mitochondrial peroxiredoxins and trypanothione synthetase) in naturally occurring strains act as a virulence factor for CD. This precept is recapitulated with the observed increased resistance of *T. cruzi* peroxiredoxins overexpressers to *in vivo* or *in vitro* nitroxidative stress conditions. In addition, the modulation of mitochondrial superoxide radical levels by iron superoxide dismutase (FeSODA) influences parasite programmed cell death, underscoring the role of this enzyme in parasite survival. **Critical Issues:** The unraveling of the biological significance of FeSODs in *T. cruzi* programmed cell death in the context of chronic infection in CD is still under examination. **Future Directions:** The role of the antioxidant enzymes in the pathogenesis of CD, including parasite virulence and persistence, and their feasibility as pharmacological targets justifies further investigation. *Antioxid. Redox Signal.* 19, 723–734.

Introduction

CHAGAS DISEASE (CD), caused by the parasitic protozoan *Trypanosoma cruzi*, remains a major public health concern in Latin America, with an estimated total of 8 million people infected and 28 million at risk (http://apps.who.int/tdr/publications/tdr-research/publications/reporte-enfermedad-chagas/pdf/swg_chagas.pdf). Moreover, the disease is spreading worldwide as a result of migration (mammalian host and insect vectors); HIV co-infection; blood transfusion; and organ transplantation; evidenced by the fact that 1 in every 4700 blood donors test positive for *T. cruzi*. An estimate of 300,000 infected people is found in the United States as of 2010 (www.cdc.gov/chagas/factsheet.html).

Life Cycle of *T. cruzi* and Progression of CD

The life cycle of *T. cruzi* involves the invertebrate host (triatomid hematophagous arthropod), where the noninfective epimastigotes replicate and differentiate to the nonreplicative infective stage (metacyclic trypomastigotes) at the rectum of the insect vector. During the differentiation process from

noninfective epimastigotes to infective metacyclic trypomastigotes (a process called metacyclogenesis), the parasite undergoes complex morphological and biochemical changes in order to effectively infect and survive in the hostile environment of the vertebrate host. Metacyclic trypomastigotes should gain access to the internal medium of the vertebrate host via the mucosal and/or skin wounds invading different cell types, including macrophages, cardiomyocytes, fibroblasts, and smooth and striated muscle cells (4). The acute phase of the disease lasts between 4–8 weeks, generally with mild flu-like self-limiting symptoms, although 10% of symptomatic cases develop severe myocarditis and/or meningoencephalitis. Organ and tissue damage during acute *T. cruzi* infection is caused by the parasite itself and by the host's acute inflammatory response, elicited by the presence of the pathogen. Between 30% and 40% of patients will develop chronic CD either in its cardiac, digestive, or cardio-digestive manifestation between 10 and 30 years after the initial infection. The pathological findings observed in chronic chagasic cardiomyopathy include widespread destruction and dysfunction of mitochondrial myocardial cells (68, 69), myocardium

inflammation, fibrosis, and hypertrophy along with scarce parasite numbers. Exacerbation of the chronic stage with increased blood parasitemia and intracellular parasite replication is found in individuals with immunosuppressive treatment, indicating the pivotal role of the host-immune mediators in the control of parasite proliferation and persistence (57). The pathogenesis of chronic CD myocarditis is complex and involves parasite immune evasion strategies, genetically determined host-immune homeostasis defects, and autoreactive phenomena in the presence of auto-antibodies. Damaged tissue in the chronic phase is also accompanied by genetic material from *T. cruzi*, indicating an active role of parasite persistence in pathology (7, 57). Indeed, in spite of a robust immune response, the host fails to eliminate parasites from tissues, and the pathogen is able to chronically persist.

Host-Derived Nitroxidative Stress During *T. cruzi* Infection

In the acute infection, resident macrophages at the site of parasite invasion are among the first professional phagocytes that are invaded by *T. cruzi* (29, 39). On invasion, infective insect-borne metacyclic trypomastigotes should survive and evade the highly oxidative environment found inside the phagosome in order to establish the infection (1, 46, 50). We have recently shown that during the phagocytosis of *T. cruzi* metacyclic trypomastigotes, macrophage membrane-associated NADPH oxidase is activated, resulting in a sustained (60–90 min) superoxide radical ($O_2^{\bullet-}$) production toward the internalized parasite (1). $O_2^{\bullet-}$ either dismutates to H_2O_2 or reacts with nitric oxide ($\cdot NO$) derived from the inducible nitric oxide synthase (iNOS) in a diffusion-controlled

FIG. 1. Reactive oxygen and nitrogen species cytotoxicity against *Trypanosoma cruzi*. Peroxynitrite anion ($ONOO^-$), formed from the diffusion-controlled reaction between $\cdot NO$ and superoxide radical ($O_2^{\bullet-}$), is in equilibrium with peroxynitrous acid ($ONOOH$). Peroxynitrite cytotoxicity can arise by the direct reaction via two-electron oxidation as well as by secondary-derived radicals via one-electron oxidation processes. Two-electron oxidations include the reaction with low-molecular-weight thiols [RSH , $T(SH)_2$], leading to the corresponding sulfenic ($R-SOH$) and disulfide ($R-SS-R$, TS_2) derivatives. Radical pathways depends on (i) the homolysis of $ONOOH$ to nitrogen dioxide ($\cdot NO_2$) and hydroxyl radicals ($\cdot OH$); (ii) the reaction of $ONOO^-$ with carbon dioxide (CO_2) that yields carbonate ($CO_3^{\bullet-}$) and $\cdot NO_2$ radicals; and (iii) the $ONOO^-$ reaction with transition metal centers (Me) yielding $\cdot NO_2$ and the corresponding oxo-metal complex ($Me=O$). The quantitative relevance of the different reaction pathways in specific cells/tissue compartments has been analyzed elsewhere (21). $\cdot NO_2$, $CO_3^{\bullet-}$, $\cdot OH$, and $Me=O$ can oxidize protein tyrosine to the tyrosyl radical ($\cdot Tyr$) and cysteine to thiyl radicals ($R-S\cdot$). Tyrosyl radicals either dimerize to form 3,3'-di-tyrosine or in the presence of $\cdot NO_2$ react to form protein-3-nitrotyrosine. Thiyl radicals may form mixed disulfides or react with O_2 to yield higher oxidation states of the sulfur ($R-SO_2H$, $R-SO_3H$ sulfenic, and sulfonic acids, respectively). Different parasite targets for $\cdot NO$, $O_2^{\bullet-}$, $ONOO^-$, and its derived secondary radicals have been identified in *T. cruzi*, including the inhibition of cytochrome c oxidase (this work), aconitase (47), enzymes of metabolic pathways [succinate dehydrogenase and NADH-fumarate reductase (60)], mitochondrial iron superoxide dismutase (FeSODA) (this work, unpublished results), and Ca^{2+} -ATPase (65).

reaction, to yield peroxynitrite (ONOO^- ; $k=1 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$), a strong oxidant and potent cytotoxic effector molecule against *T. cruzi* (1, 2, 16). The control of intraphagosomal parasite survival, before the replicative amastigotes reach the "safe" cytoplasmic environment, largely depends on the macrophage production of ONOO^- (1, 46). In this scenario, the levels of parasite antioxidant defenses at the onset of macrophage invasion may tilt the balance toward pathogen survival (44, 46, 48). Some parasites manage to evade the initial assault of the immune system and infect other organs such as the heart and digestive tract, disseminating the infection and favoring the progression to the chronic stage of CD. Chronic chagasic cardiomyopathy is characterized by the presence of pseudo cysts of amastigote nests in the cardiac fiber. *T. cruzi* invasion to cardiomyocytes triggers the production of inflammatory mediators ($\text{TNF-}\alpha$, $\text{IL-1}\beta$) and the induction of cardiomyocyte iNOS with the subsequent generation of sustained amounts of $\cdot\text{NO}$ (13, 32). *T. cruzi* invasion and proinflammatory cytokines along with $\cdot\text{NO}$ generation lead to cardiomyocyte mitochondrial dysfunction with an increase in reactive oxygen species that may contribute to cardiomyocyte death and chronic heart failure in chagasic patients (7, 24).

The $\cdot\text{NO}$ pathway is indicated as a parasite control mechanism, as revealed by experiments performed on infected cardiomyocytes cultures. In addition, the role of the $\text{IL-12}/\text{INF-}\gamma/\text{iNOS}$ axis in the control of *T. cruzi* infection and its implication for the outcome in CD has been well documented in key experiments using knockout mice for $\text{INF-}\gamma$, IL-12 , and NOS isoforms (20, 37). $\cdot\text{NO}$ oxidative damage largely depends on its reaction with superoxide (Fig. 1). Peroxynitrite can cause damage by direct reactions via one or two electron

oxidations mechanisms to several molecules such as thiols and metal centers (21), also yielding secondary reactive species, including hydroxyl radical ($\cdot\text{OH}$), nitrogen dioxide ($\cdot\text{NO}_2$), and carbonate ($\text{CO}_3^{\cdot-}$) radicals that can oxidize lipids, DNA, and participate in protein oxidation and nitration, stable indicators of nitroxidative stress (9, 21, 40) (Fig. 1). Host-tyrosine nitrated proteins have been identified in heart lesions in the mouse model of CD (17, 40). Moreover, nitrated and oxidized plasma proteins as well as circulating myeloperoxidase [a heme-peroxidase released by PMNs that also participates in protein tyrosine nitration (54)] have been found in the plasma of patients with CD (17, 18). $\cdot\text{NO}$ is not a strong oxidant or reactive species per se and by itself is unlikely to account for direct damage to the parasite (21). The inhibition of the respiratory chain by $\cdot\text{NO}$ in mammalian mitochondria via interactions with cytochrome c oxidase is accompanied by a larger steady-state level of reduced respiratory complexes, which, in turn, favors intramitochondrial $\text{O}_2^{\cdot-}$ formation (11, 55). After the acute infection, host-derived $\cdot\text{NO}$ may diffuse and reach intracellular amastigotes, leading to the generation of intramitochondrial $\text{O}_2^{\cdot-}$ and ONOO^- formation. Results from our laboratory showed that *T. cruzi* mitochondrial oxygen consumption was reversibly inhibited by $\cdot\text{NO}$ (Fig. 2A, B), and mitochondrial $\text{O}_2^{\cdot-}$ production under these conditions was detected using the mitochondria-targeted ethidium-derived probe [MitoSOXTM, live-cell permeable probe selectively targeted to mitochondria (59)] (Fig. 3A, B). This result makes feasible the concept of $\cdot\text{NO}$ -mediated control of parasite proliferation in CD via intra-parasitic ONOO^- formation. In this scenario where host-derived oxidant mediators are actively generated, the antioxidant armamentarium of *T. cruzi* becomes crucial for parasite survival and persistence.

FIG. 2. Nitric oxide ($\cdot\text{NO}$) interactions with *T. cruzi*. (A) The reversible $\cdot\text{NO}$ -dependent inhibition of *T. cruzi* mitochondrial respiration was evaluated in cells (1×10^8 epimastigotes) with endogenous respiratory substrates using a gas-tight chamber equipped with a Clark-type oxygen electrode connected to a computer with the DUO.18 processing program (World Precision Instruments). As indicated in the figure, authentic $\cdot\text{NO}$ ($1.25 \mu\text{M}$) was added to the chamber followed by reoxygenation by air bubbling. The transient inhibition of *T. cruzi* mitochondrial respiration in the presence of $\cdot\text{NO}$ was fully displaced by O_2 , demonstrating the reversible nature of the process. (B) Representation of *T. cruzi* mitochondria showing the $\cdot\text{NO}$ -dependent interaction with the terminal cytochrome c oxidase (complex IV). The inhibition of parasite mitochondria respiration by host-derived $\cdot\text{NO}$ leads to $\text{O}_2^{\cdot-}$ production (mainly by ubiquinol-cytochrome c reductase, complex III) with the subsequent intramitochondrial ONOO^- formation. OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 3. Intramitochondrial superoxide radical detection in •NO-challenged parasites. (A) Schematic representation of the reactions involved in Mito-HE (MitoSOX) oxidation. Mito-HE with a triphenylphosphonium moiety is targeted to mitochondria in response to the electrochemical membrane potential. Mito-HE can be oxidized by O₂^{•-} to yield the hydroxylated product (2-OH-Mito-E⁺) or by other oxidants to yield the two-electron oxidation product (Mito-E⁺) (59). Both products have distinctive fluorescence spectra and can be separated by HPLC techniques, providing a specific O₂^{•-} assay (80). (B) Detection of mitochondrial O₂^{•-} production by •NO-challenged epimastigotes. Preloaded (MitoSOX, 5 μM for 30 min) epimastigotes (1 × 10⁸) were exposed to an •NO flux (NOC-18; 1 μM min⁻¹ during 2 h) and MitoSOX oxidation analyzed by flow cytometry. The redox-cycling agent 2,3-dimethoxy-1,4-naphthoquinone (DMNQ, 30 μM, 3 nM O₂^{•-} min⁻¹/10⁸ parasites) was used as a positive control for O₂^{•-} production (48). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

T. cruzi Antioxidant Network

Antioxidant defenses in *T. cruzi* rely on a sophisticated system of linked pathways in which reducing equivalents from NADPH (derived from the pentose phosphate pathway) are delivered to a variety of enzymatic detoxification systems through the dithiol trypanothione [T(SH)₂, N¹,N⁸-bisglutathionylspermidine] and the thioredoxin homologue tryparedoxin (TXN; Fig. 3) (26). Trypanothione is synthesized in two sequential steps in which two molecules of glutathione (GSH) are covalently bound to the terminal NH₂ groups of spermidine by a single cytosolic enzyme called *T. cruzi* trypanothione synthetase (TcTS) (42). Five distinct peroxidases have been identified in *T. cruzi*, differing in their subcellular location and substrate specificity. Glutathione peroxidase-I (TcGPXI, located at the cytosol and glycosome) and TcGPXII (located at the endoplasmic reticulum) confer resistance against hydro- and lipid-hydroperoxides, respectively, and use GSH and/or TXN as reducing substrates (72, 76). The *T. cruzi* cytosolic tryparedoxin peroxidase (TcCPX) and *T. cruzi* mitochondrial tryparedoxin peroxidase (TcMPX, typical two-cysteine peroxiredoxins belonging to the AhpC/Prx sub-

family) have the capacity to detoxify ONOO⁻, H₂O₂ and small-chain organic hydroperoxides using TXN (Fig. 4) (48, 51, 66, 77). Parasite resistance against ONOO⁻ toxicity is afforded by CPX overexpression (Fig. 4A), while the mutation in the *peroxidatic* Cys52 (Cys52Ala) of CPX fails to confer resistance, indicating the catalytic nature of the process (Fig. 5A, inset). Moreover, TcMPX overexpressers are more resistant to •NO fluxes, which is in line with the predicted •NO-mediated intramitochondrial ONOO⁻ formation and detoxification by this peroxiredoxin (Fig. 5B). Whether the complete system of TXN, T(SH)₂, and TR is found in the mitochondria is still a matter of debate. Finally, an ascorbate-dependent heme-peroxidase (TcAPX), located at the endoplasmic reticulum, confers resistance against H₂O₂ challenge using ascorbate as the reducing substrate (48, 73). The complete detoxification capacities of the different peroxidases of *T. cruzi* are still under investigation. In particular, further studies are required to find out whether thiol-dependent GPX-I and GPX-II can catalyze ONOO⁻ reduction and detoxification in *T. cruzi*.

T. cruzi contains four iron superoxide dismutases (FeSODs) that detoxify O₂^{•-} generated in the cytosol (TcSODB1), glycosomes (TcSODB1-2), and mitochondria (TcSODA and C)

FIG. 4. Subcellular distribution of the antioxidant network in *T. cruzi*. The antioxidant network of *T. cruzi* is formed by a number of enzymes and nonenzymatic redox-active molecules distributed in the endoplasmic reticulum, glycosomes, mitochondrion, and cytosol. The final electron donor for all the enzymatic systems is the NADPH derived from the pentose phosphate pathway (PPP). The reducing equivalents are funneled through the trypanothione [T(SH)₂], glutathione (GSH), ascorbate (ASC), and/or trypanredoxin (TXN) redox systems. Endoplasmic reticulum: Hydrogen peroxide (H₂O₂) is metabolized by an ascorbate-dependent hemoperoxidase (APX) using ASC as the electron donor. Dehydroascorbate (DHA) is thought to be reduced by a direct reaction with T(SH)₂. H₂O₂ in the presence of redox-active metals can initiate lipoperoxidation reactions that generate organic hydroperoxides (LOOH), which are substrates of GSH-dependent-peroxidase II (GPX-II) that uses GSH. T(SH)₂ reduces oxidized glutathione (GSSG) to GSH, while trypanothione reductase (TR) reduces oxidized trypanothione (TS₂). Mitochondrion: the electron transport chain (Complex II: succinate dehydrogenase, CII; Complex III: ubiquinol-cytochrome c reductase, CIII and Complex IV: cytochrome c oxidase, CIV) is a principal site for superoxide (O₂^{•-}, blue sphere) formation mainly at CIII. The mitochondrial isoform of FeSODA catalyzes the dismutation of O₂^{•-} to H₂O₂. When host-derived [•]NO (gray sphere) reaches the mitochondrion, it inhibits mitochondrial respiration at CIV (with an enhanced production of O₂^{•-} by CIII) by outcompeting FeSODA for O₂^{•-} to form ONOO⁻ (green sphere). Mitochondrial peroxiredoxin (MPX) catalytically decomposes H₂O₂ and/or ONOO⁻. Figure 6 probably uses reduced TXN (TXN_R) and T(SH)₂ as the reducing substrate (dashed arrows). Cytosol: The antioxidant enzymes in the cytosol include cytosolic peroxiredoxin (CPX), FeSODB, and GPX-I. T(SH)₂ is synthesized from two molecules of GSH and one spermidine in a reaction catalyzed by the enzyme trypanothione synthetase (TS). The oxidative branch of the PPP is induced in the metacyclic trypomastigote, ensuring the provision of reducing equivalents from NADPH (25). The size of the spheres for [•]NO, ONOO⁻, and O₂^{•-} illustrates the diffusional capabilities of the oxidants. Glucose-6-phosphate dehydrogenase (G6PDH); nitrite (NO₂⁻). Adapted from Piacenza *et al.* (46). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(33). The biochemical and cell biology characterization of the *T. cruzi* FeSODs repertoire in terms of free radical events has been minimally explored (28, 47, 64, 74). The role of mitochondrial *Tc*FeSODA could be particularly relevant, as this organelle is a key source of O₂^{•-} and presumably ONOO⁻. The preliminary data of our laboratory indicate that, although fluxes of O₂^{•-} and [•]NO (SIN-1, 0–10 mM) cause a very modest enzyme inactivation, *Tc*FeSODA activity is inhibited by biologically relevant ONOO⁻ concentrations, which is accompanied by nitration on tyrosine residues (Martinez, Piacenza, *et al.*, unpublished results). The nitration of a critical tyrosine near the active site can account for the observed inhibition, as previously shown for Tyr-34 in MnSOD (38, 53, 78). These

data support the concept that *Tc*FeSODA play an important role in parasite oxidant detoxification pathways by preventing ONOO⁻ formation in this organelle through O₂^{•-} dismutation, all of which contribute to neutralize cytotoxicity. Due to its unique characteristics when compared with the mammalian counterparts, components of the *T. cruzi* antioxidant system have been considered good targets for chemotherapy (23, 26, 30).

Since the end of the 1960s and the beginning of the 1970s, two drugs have been used for the treatment of CD: a 5-nitrofur, nifurtimox [NFX, (4[5-nitrofurfurylidene)amino]-3-methylthiomorpholine-1,1-dioxide] and a 2-nitroimidazole, benznidazole (BZ, N-benzyl-2-nitroimidazole-1-acetamide)

FIG. 5. Resistance of *T. cruzi* CPX and MPX overexpressors to peroxynitrite challenge. (A) *T. cruzi* epimastigotes from wild-type (CL-Brener, control) or CPX overexpressors (3×10^8 cells·ml⁻¹) were exposed to ONOO⁻ (0–700 μM), and parasite survival was assayed by [³H]thymidine incorporation (48). Results are expressed as percentage [³H]thymidine incorporation compared with control conditions (no peroxynitrite addition) for each cell line. *Inset*: *T. cruzi* epimastigotes (CL-Brener; *T. cruzi* cytosolic trypanedoxin peroxidase [TcCPX] overexpressing cells, and TcCPX C52A mutants; 3×10^8 cells·ml⁻¹) were exposed to a single dose of 300 μM peroxynitrite. Cell viability was evaluated as in (A). ***p* < 0.05 compared with *. The results show the efficiency of TcCPX in ONOO⁻ detoxification. (B) Protection against intramitochondrial peroxynitrite formation is achieved by *T. cruzi* MPX overexpressors. Wild-type (CL-Brener) and *T. cruzi* mitochondrial trypanedoxin peroxidase (TcMPX) overexpressors were exposed to ·NO fluxes generated by the ·NO donor NOC-18 (NOC-18, 0–2 mM; *t*_{1/2} ~ 18 h, Alexis) for 1 h. After treatment, parasite viability was evaluated as in (A). (C) 6-carboxy-fluorescein di-acetate (6-CFDA, 10 μM) preloaded wild-type (CL-Brener) and TcCPX parasites (3×10^8 cells ml⁻¹) were exposed to a single dose of 250 μM ONOO⁻, and cell morphology was evaluated by epifluorescence microscopy (×60). Adapted from (48). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(62). Both are prodrugs that undergo intracellular activation by nitroreductases (NTRs), leading to cytotoxicity within the parasite, although the precise mechanism of action and parasite targets remain to be established (70, 71, 75). Since a long time, it has been proposed that NFX trypanocidal activity involves the generation of nitroanion radicals through NTR type II activity (one-electron reduction), which, in turn, generates O₂·⁻ radicals (19, 34). In addition, NFX-derived metabolites can lead to adduct formation with GSH and TSH, contributing to parasite oxidative stress (58). However, the direct evidence between drug-induced oxidative stress and trypanocidal activity is limited and arises from functional studies where (i) *T. brucei* parasites lacking cytosolic SODB are more sensitive to NFX and BZ (52); and (ii) the reported overexpression of mitochondrial TcFeSODA in an *in vitro*-derived BZ-resistant *T. cruzi* strain (41). It is now known that BZ and NFX trypanocidal activity depends on NTR type I activity, which is absent in mammalian cells and is the base for parasite selectivity (70). NTR type I activity catalyzes the two-electron reduction of nitroheterocyclic compounds producing

toxic hydroxylamine derivatives and other metabolites that can induce biomolecule modifications and DNA strand breaks, leading to parasite death (8, 36, 75).

Moreover, it was recently found that in the BZ-resistant *T. cruzi* population, the parasite phenotype is associated with the loss of a NTR gene copy with changes in neither parasite SOD expression nor activity (36). The unambiguous determination of BZ-NFX toxicity mechanisms as well as the participation of antioxidant enzymes, and especially that of FeSOD, in resistant *T. cruzi* phenotypes needs further investigation. SODs readily eliminates O₂·⁻ and may contribute to *T. cruzi* survival in the vertebrate host by immune evasion mechanisms, such as (i) protection from direct cytotoxic effects of O₂·⁻, mainly generated by parasite mitochondria; (ii) inhibiting the formation of ONOO⁻ in ·NO-challenged parasites; and (iii) participating in the modulation of redox signaling processes. While the protein fold and structure of FeSOD are considered similar to mammalian MnSOD, efforts are being made to unravel the structural characteristics to allow the generation of specific inhibitors (6).

Redox Signaling of *T. cruzi* Programmed Cell Death

T. cruzi can undergo a programmed cell death process that is triggered by a variety of stimuli and involving alterations of cellular homeostasis (3, 27, 49). The intracellular misbalance of the parasite redox status can disengage programmed cell death in epimastigotes of *T. cruzi* (27, 44, 47). After appropriate challenges (fresh human serum [FHS]), a rapid loss of low-molecular thiols (*i.e.*, T(SH)₂, glutathionyl-spermidine, and GSH) occurs accompanied by mitochondrial dysfunction; disruption of mitochondrial homeostasis in the parasite is characterized by mitochondrial Ca²⁺ influx, an increase in mitochondrial O₂^{•−} production, impaired oxidative phosphorylation, oxygen consumption, and the release of cytochrome c to the cytosol (47). FHS-dependent mitochondrial O₂^{•−} generation is evidenced by aconitase inhibition due to the disruption of the 4Fe4S cluster (Fig. 6A). The *T. cruzi* cell death phenotype is accompanied by phosphatidylserine exposure to the outer leaflet of the plasma membrane and by nuclear DNA condensation and fragmentation, all common morphological features of metazoan apoptosis (27, 47, 49). Notably, the overexpression of FeSODA prevented FHS-triggered processes, including aconitase inhibition, MitoSOX oxidation, and programmed cell death, underscoring the role of mitochondrial O₂^{•−} in the redox signaling of this process (Fig. 6A, B).

Intracellular apoptotic amastigotes have been found in experimentally infected cardiomyocytes as well as *in vivo* in the heart tissue of infected mice (14, 15). Interactions of apoptotic parasites with immune cells may locally down regulate the host-inflammatory response with the concomitant production of anti-inflammatory cytokines (TGF-β), thus favoring parasite proliferation and transition to the chronic stage (22, 44). It has also been proposed that apoptosis enables the regulation of parasite densities in distinct host compartments, facilitating a sustained infection in the vertebrate host (67). Thus, interfering with the parasite apoptotic machinery may reveal new targets for drug design. The presence in *T. cruzi* of four FeSODs localized in different subcellular compartments argues in favor of a pivotal role of O₂^{•−} in parasite redox signaling.

Antioxidant Enzymes As Virulence Factors

T. cruzi consists of a mixed population of strains classified in two discrete typing units recently designated as *T. cruzi* I-VI (81), which circulate in the sylvatic and domestic cycles, respectively (63). The biochemical and genetic heterogeneity between strains is, in part, responsible for the diverse clinical manifestations of the disease, ranging from asymptomatic to severe cardiac and digestive presentations (31). The pathogenesis of CD depends on parasite and host factors that control virulence, tissue tropism, tissue damage, and ability to maintain long-term infections in the vertebrate host. A number of virulence factors have been identified for *T. cruzi* and include complement C2 receptor inhibitor trispanning (CRIT) protein (12), calreticulin (*TcCRT*) (56), gp35/50 (79), gp82 (61), cruzipain (35), oligopeptidase B (10), and transialidases (45), among others. Taking into account the establishment of a nitroxidative stress during CD (acute or chronic stages), the antioxidant armamentarium of *T. cruzi* becomes decisive. Several proteomic analyses have suggested the up-regulation of members of the *T. cruzi* antioxidant network (*TcTS*; *TcMPX*;

FIG. 6. Enhanced mitochondrial O₂^{•−} production during *T. cruzi* programmed cell death. (A) Mitochondrial aconitase inhibition during programmed cell death. Wild-type and mitochondrial FeSODA overexpressers were incubated with 20% (v/v) inactivated human serum (IHS, control), or 20% or 40% (v/v) fresh human serum (FHS), and aconitase was measured in total parasite extracts at the indicated times. The results show that after the death stimuli, mitochondrial (but not cytosolic) aconitase is inactivated in wild-type parasites and partially protected in mitochondrial FeSODA overexpressers, indicating the participation of O₂^{•−} in the death process. **(B)** Preloaded MitoSOX parasites were exposed to the death stimuli (20 min), and this was fluorimetrically followed by MitoSOX oxidation (λ_{ex} = 10 nm and λ_{em} = 580 nm). *TcSODA* overexpressers inhibited MitoSOX oxidation unambiguously indicating mitochondrial O₂^{•−} dependent MitoSOX oxidation. Adapted from (47).

TXN; FeSODA and *TcAPX*) in the infective metacyclic trypomastigote compared with the noninfective epimastigote stage (5, 43). Due to the fact that *T. cruzi* is not a clonal population, we search for the up-regulation of different components of the antioxidant network in several strains belonging to the major phylogenetic groups during the differentiation process to the infective metacyclic trypomastigotes. Our results show for all the analyzed strains an up-regulation of *TcCPX*, *TcMPX*, and *TcTS* protein content during metacyclogenesis, making this a general preadaptation process that allows *T. cruzi* to deal with the nitroxidative environment found in the vertebrate host (50). Most important, a positive

TABLE 1. CORRELATION BETWEEN *TRYPANOSOMA CRUZI* ENZYME CONTENT IN THE INFECTIVE METACYCLIC STAGE AND PARASITEMIA ELICITED IN MICE

	TcMPX	TcCPX	TcTS
Pearson's coefficient	0.771 ^a	0.617 ^a	0.499 ^a
Determinations (n)	59	105	75

^aCorrelation is significant at the 0.001 level (two tailed).

TcMPX, *T. cruzi* mitochondrial trypanodioxidase; TcCPX, *T. cruzi* cytosolic trypanodioxidase; TcTS, *T. cruzi* trypanothione synthetase.

Adapted from Piacenza *et al.* (46)

association was found between virulence and the protein levels of antioxidant enzymes (TcCPX, TcMPX, and TcTS) in several *T. cruzi* strains, highlighting the interplay between the antioxidant enzyme network and host oxidative defense mechanisms (Table 1) (50). In accordance, strains of higher virulence showed an important heart inflammatory infiltrate with high parasitemias in the acute phase of CD. (Fig. 7A). At

the cellular level, parasites that overexpressed TcCPX were resistant to macrophage killing due to peroxynitrite detoxification (Table 2) (1). Moreover, the overexpression of TcCPX in trypomastigotes augments virulence, as evidenced by a threefold increase in parasitemia with the presence of higher inflammatory infiltrates in the heart and skeletal muscle when compared with the wild strain (Fig. 7B). In this line, the experiments performed with genetically modified parasites fully recapitulated the association observed between the virulence and antioxidant enzyme content of *T. cruzi* wild strains (compare Fig. 7A and B) (1). These results reinforce the concept that the success of infection in the acute phase depends on parasite antioxidant enzyme levels. Whether FeSOD constitute a virulence factor for *T. cruzi* infection remains to be established and is currently under investigation in our laboratory. *T. cruzi* cytosolic FeSOD is particularly resistant to peroxynitrite inactivation, suggesting its participation mainly as an antioxidant defense enzyme, while mitochondrial FeSOD may act as an oxidative stress sensor participating in O₂^{•-} mediated redox processes of cell signaling (Martinez, Piacenza, *et al.*, unpublished results). Interestingly, highly

FIG. 7. Elevated parasitemia and inflammatory infiltrates in experimentally infected animals elicited by natural and genetically modified *T. cruzi* strains with increased antioxidant enzyme levels. The behavior of natural attenuated (TCC) and virulent (CL-WT) *T. cruzi* strain (A) and that obtained by genetically modified CL_Brener (wild type, control) and TcCPX overexpressers (B) in experimental infections. Two-month-old Swiss mice were infected by an intraperitoneal inoculation with 1×10^3 complement-resistant metacyclic trypomastigote forms. Parasitemia was followed by parasite count number in blood-tail samples and results presented as trypanomastigotes/100 fields (natural strains TCC and CL-WT) or trypanomastigotes/50 fields (CL-Brener, TcCPX), and values are given as means $\pm$ standard error of the mean. Histological H&E-stained heart tissue sections from mice inoculated with the attenuated and virulent strain (TCC and CL-WT; 25 $\times$ magnification) and skeletal muscle tissue sections of mice inoculated with the genetically modified *T. cruzi* strain (CL-Brener, control, and TcCPX overexpressers; 40 $\times$ magnification) were analyzed for the presence of immune infiltrates (shown with arrows), as previously adapted from Piacenza *et al.* and Alvarez *et al.* (1, 50).

TABLE 2. INTRAPHAGOSOMAL PEROXYNITRITE DETOXIFICATION BY *TRYPANOSOMA CRUZI* TcCPX OVEREXPRESSERS

	T. cruzi amastigotes/100 macrophages ^a	
	CL-Brener	TcCPX
Unelicited	37 ± 13	38 ± 19
INF-γ/LPS ^b	14 ± 3 ^c	36 ± 9

^aThe load of *T. cruzi* amastigotes in the macrophage (J-774 line) culture was evaluated 24 h after infection with metacyclic trypomastigotes (parasite:macrophage ratio for infection 5:1). Data are mean ± standard error of three independent experiments.

^bINF-γ/LPS was added 5 h before the infection.

^c*p* < 0.01.

LPS, lipopolysaccharide.

Adapted from Alvarez *et al.* (1)

virulent strains of *T. cruzi* showed less amastigote apoptotic death and higher proliferation rates than less virulent strains in cardiomyocyte infections (14). Taking into account that mitochondrial O₂^{•−} mediates programmed cell death in *T. cruzi*, we propose that FeSOD may also play a role in the pathogenesis of the disease.

Concluding Remarks

T. cruzi antioxidant enzymes acting synergistically represent the first line of defense to cope with O₂^{•−}, H₂O₂, and ONOO[−] generated while infecting mammalian cells either in the acute and presumably in the chronic stage of the disease. Particularly interesting is the emerging evidence that the single *T. cruzi* mitochondrion can play a central role as a “powerhouse” of the reactive oxygen and nitrogen species, including O₂^{•−} and ONOO[−] (the latter arising from the combination of O₂^{•−} with mammalian host cell-derived •NO), when infecting non-phagocytic cells such as cardiomyocytes; these alterations in *T. cruzi* mitochondrion redox homeostasis and its modulation by mitochondrial antioxidant systems may facilitate the progression of a “low grade” infection to a chronic phase. Deciphering the relative role of different arms of the enzymatic antioxidant network in the various cellular compartments should be a part of future investigations. In summary, the assessment and definition of the contribution of the parasite antioxidant systems toward virulence and persistence could further define them as relevant targets for the development of new pharmacological strategies that treat CD, especially in the chronic stage.

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Abbreviations Used

BZ = benznidazole
 6-CFDA = 6-carboxy-fluorescein di-acetate
 CD = Chagas disease
 $\text{CO}_3 \cdot^-$ = carbonate radical
 DMNQ = 2,3-dimethoxy-1,4-naphthoquinone
 FHS = fresh human serum
 GSH = glutathione
 HIS = inactivated human serum
 IMM = inner mitochondrial membrane
 iNOS = inducible nitric oxide synthase
 LPS = lipopolysaccharide
 NFX = nifurtimox
 $\cdot\text{NO}$ = nitric oxide
 $\cdot\text{NO}_2$ = nitrogen dioxide
 NTRs = nitroreductases
 $\text{O}_2 \cdot^-$ = superoxide radical
 $\cdot\text{OH}$ = hydroxyl radical
 OMM = outer mitochondrial membrane
 ONOO $^-$ = peroxynitrite
 PPP = pentose phosphate pathway
 RSH-RSSR = reduced and oxidized low molecular or protein thiols
 TcCPX = *T. cruzi* cytosolic trypanedoxin peroxidase
 TcFeSOD = *T. cruzi* iron superoxide dismutase
 TcMPX = *T. cruzi* mitochondrial trypanedoxin peroxidase
 TcTS = *T. cruzi* trypanothione synthetase
 T(SH)2/TS2 = reduced and oxidized trypanothione
 TXN = trypanedoxin