Mitochondrial superoxide radicals mediate programmed cell death in *Trypanosoma cruzi*: cytoprotective action of mitochondrial iron superoxide dismutase overexpression

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Trypanosoma cruzi undergo PCD (programmed cell death) under appropriate stimuli, the mechanisms of which remain to be established. In the present study, we show that stimulation of PCD in *T. cruzi* epimastigotes by FHS (fresh human serum) results in rapid (< 1 h) externalization of phosphatidylserine and depletion of the low molecular mass thiols dihydrotrypanothione and glutathione. Concomitantly, enhanced generation of oxidants was established by EPR and immuno-spin trapping of radicals using DMPO (5,5-dimethylpyrroline-*N*-oxide) and augmentation of the glucose flux through the pentose phosphate pathway. In the early period (< 20 min), changes in mitochondrial membrane potential and inhibition of respiration, probably due to the impairment of ADP/ATP exchange with the cytosol, were observed, conditions that favour the generation of O₂^{•−}. Accelerated rates of mitochondrial O₂^{•−} production were detected by the inactivation of the re-

dox-sensitive mitochondrial aconitase and by oxidation of a mitochondrial-targeted probe (MitoSOX). Importantly, parasites overexpressing mitochondrial FeSOD (iron superoxide dismutase) were more resistant to the PCD stimulus, unambiguously indicating the participation of mitochondrial O₂^{•−} in the signalling process. In summary, FHS-induced PCD in *T. cruzi* involves mitochondrial dysfunction that causes enhanced O₂^{•−} formation, which leads to cellular oxidative stress conditions that trigger the initiation of PCD cascades; moreover, overexpression of mitochondrial FeSOD, which is also observed during metacyclogenesis, resulted in cytoprotective effects.

Key words: free radicals, mitochondrial superoxide dismutase, programmed cell death (PCD), reactive oxygen species (ROS), *Trypanosoma cruzi*.

INTRODUCTION

Apoptosis is characterized by a series of morphological changes, including nuclear DNA condensation and fragmentation, cell shrinkage, exposure of PS (phosphatidylserine) and, ultimately, formation of apoptotic bodies that are rapidly eliminated *in vivo* by phagocytes. The biochemical mechanisms leading to apoptosis depend on the cell type and the nature of the death signal triggering either an extrinsic (plasma membrane receptor-mediated) or an intrinsic (mitochondrial-mediated) pathway, which in general operate synergistically [1]. The extrinsic pathway involves the proteolytic activation of a family of cysteine proteinases, known as caspases, thought to represent a major regulatory step in the apoptotic process [1]. The intrinsic pathway involves mitochondrial outer membrane permeabilization (regulated by the balance between the anti- and pro-apoptotic members of the Bcl-2 protein family), leading to the release of mitochondria intermembrane space proteins that activate caspases [e.g. cyt *c* (cytochrome *c*), Smac/Diablo] or proteins that favour the activation of caspase-independent execution pathways [e.g. AIF (apoptosis inducing

factor)] into the cytosol. Different studies have provided evidence for the existence of PCD (programmed cell death) in kinetoplastid parasites of the genera *Trypanosoma* and *Leishmania* with morphological features resembling those of mammalian apoptosis [2].

The biological significance of the existence of PCD in these parasites is unknown. It has been postulated that parasite PCD may serve to control cell proliferation and differentiation, a crucial event for the maintenance and dissemination of the infection during the life cycle of the parasite. Moreover, parasite PCD may function to evade the host immune response through an alternative transforming growth factor β -mediated macrophage activation, which in turn inhibits inflammation and enhances parasite proliferation in the vertebrate host [3–5]. Interestingly, apoptotic-like *Trypanosoma cruzi* [6] and *Leishmania* [7] have been found in mammalian tissues following infection *in vivo*. The molecular mediators and biochemical pathways involved in the trypanosomatid death process are under intensive investigation. The absence of genes encoding caspases and homologues of the mammalian Bcl-2 protein family implies that the

Abbreviations used: $\Delta\Psi_m$, mitochondrial membrane potential; $\Delta\Psi_p$, plasma membrane potential; AIF, apoptosis inducing factor; ANT, adenine nucleotide translocase; AT, atractyloside; cyt *c*, cytochrome *c*; DEVD-CHO, acetyl-Asp-Glu-Val-Asp aldehyde; DiBAC₄, bis-(1,3-dibutylbarbituric acid)trimethine oxonol; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; DMPO, 5,5-dimethylpyrroline-*N*-oxide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FeSOD, iron superoxide dismutase; FHS, fresh human serum; IHS, heat-inactivated human serum; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; KH, Krebs–Henseleit; MitoSOX, 3,8-phenanthridinediamine, 5-(6'-triphenylphosphoniumhexyl)-5,6 dihydro-6-phenyl; oxMitoSOX, oxidized MitoSOX; PCD, programmed cell death; PI, propidium iodide; PPP, pentose phosphate pathway; PS, phosphatidylserine; PTP, permeability transition pore; RA, reverse addition; ROS, reactive oxygen species; SOD, superoxide dismutase; TcSODA, *T. cruzi* mitochondrial SOD; Tet, tetracycline; TR, trypanothione reductase; T(SH)₂, reduced trypanothione; TS₂, oxidized trypanothione; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; VDAC, voltage-dependent anion channel.

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kinetoplastid PCD pathway differs from that of typical mammalian apoptosis, rendering it a promising target for chemotherapy.

T. cruzi, the causative agent of Chagas disease, undergoes complex morphological and biochemical changes during its life cycle. In the gut of the insect vector, proliferating extracellular epimastigotes differentiate into infective metacyclic trypomastigotes which are pre-adapted to survive in the vertebrate host. One of the most striking differences between non-infective epimastigotes and infective metacyclic trypomastigotes is the sensitivity to complement-mediated cell damage. Epimastigotes are killed in less than an hour by complement, whereas metacyclic trypomastigotes are resistant, owing to their ability to inhibit complement activation on their surface [8]. It has been demonstrated previously that epimastigotes exposed to FHS (fresh human serum) as a source of complement died by a process that had morphological and molecular features of apoptosis, instead of necrosis, as was proposed previously [5,9]. Although it is still speculative, the apoptotic response of epimastigotes to complement attack may have evolved in order to benefit the invading trypomastigotes by preventing the triggering of an early inflammatory response [4,5]. FHS treatment of epimastigotes produces a massive and synchronous induction of PCD, a feature that allowed us to study the early biochemical changes produced in response to death stimuli [5,9]. Morphological and biochemical changes associated with FHS-induced PCD in *T. cruzi* epimastigotes included inhibition of parasite proliferation, DNA fragmentation and activation of undefined caspase-like proteases, with maintenance of the plasma membrane integrity during the process [9]. PCD can be prevented by L-arginine (a substrate for nitric oxide synthase) or by chemically generated low fluxes of $\bullet\text{NO}$ [9]. In mammalian cells, anti-apoptotic actions of $\bullet\text{NO}$ can be attributed to cGMP-dependent regulation of gene expression, modulation of the mitochondrial electrochemical gradient, S-nitrosylation of cysteine proteases and inhibition of the cardiolipin oxygenase activity of *cyt c* [10,11]. Mitochondria play a central role during the 'execution' phase of apoptosis, releasing pro-apoptotic factors (e.g. procaspases, *cyt c*, AIF) into the cytosol. Moreover, mitochondrially derived ROS (reactive oxygen species) have also been associated with the initiation phase of apoptosis, acting as mediators for different signal transduction pathways [10]. However, the classification of the precise role and identity of the ROS involved in the initiation process has been hampered by methodological limitations. Increases in ROS levels and oxidative stress have also been implicated in trypanosomatid, yeast and plant PCD, suggesting the existence of an ancestral redox-sensitive death pathway, independent of caspases and Bcl-2 protein members [2,12,13].

In trypanosomatids, redox metabolism relies on the bis-glutathionyl-spermidine conjugate T(SH)_2 (reduced trypanothione) and the flavoenzyme TR (trypanothione reductase), working in concert with different cellular peroxidases [14]. The T(SH)_2 system protects the parasites from oxidative stress and heavy metal toxicity, and delivers reducing equivalents for DNA synthesis [15]. As TS_2 (oxidized trypanothione) is a powerful inhibitor of the trypanothione-mediated deoxyribonucleotide synthesis, changes in the redox status of the cell, based on the $\text{T(SH)}_2/\text{TS}_2$ ratio, are a key regulator of parasite proliferation [16] and probably PCD [4].

One family of metalloproteins that play a major role in maintaining the oxidative balance in cells is the superoxide dismutases (SODs). These catalyse the dismutation of $\text{O}_2^{\bullet-}$ to form H_2O_2 and oxygen. In trypanosomatids, four SOD isoforms have been characterized [17]. These are found at various subcellular locations, including the mitochondrion, and unusually for eukaryotes,

their activities are dependent on iron, a trait normally associated with bacterial enzymes. Notably it has been reported, from proteomic analysis, that transformation of epimastigotes to metacyclic trypomastigotes is associated with an enhanced expression of antioxidant enzymes, including FeSOD (iron superoxide dismutase) [18]. Overexpression of antioxidant defences during metacyclogenesis is consistent with a pre-adaptation of metacyclic forms to counteract the oxidative burst of host phagocytic cells during infection [18].

In the present study, we have investigated the molecular mechanisms that govern PCD in *T. cruzi* epimastigotes, using FHS as the death stimulus. Through the combined use of specific methods for measurement of intracellular and mitochondrial oxidant formation and overexpression of mitochondrial FeSOD, we have assessed whether enhanced ROS production, in particular mitochondrial $\text{O}_2^{\bullet-}$, is implicated in mediating PCD, and we have evaluated the role of the mitochondrion as the key organelle for the oxidant-dependent signalling of the death process in *T. cruzi*.

MATERIALS AND METHODS

Parasites

T. cruzi epimastigotes (Tulahuen-2) were cultured at 28 °C in brain heart infusion medium as described previously [9]. Cells in the exponential phase of growth (5 days) were collected by centrifugation at 800 *g* for 5 min at room temperature (22 °C) and washed in KH (Krebs–Henseleit) buffer (pH 7.3) containing 15 mM NaHCO_3 , 5 mM KCl, 120 mM NaCl, 0.7 mM Na_2HPO_4 and 1.5 mM NaH_2PO_4 . FHS was obtained from healthy volunteers and used as the death stimulus for all experiments. IHS (heat-inactivated human serum) was generated by incubation of FHS at 56 °C for 40 min and used as control for all experiments.

Induction and evaluation of *T. cruzi* PCD

Parasite PCD was triggered by incubating 3×10^8 cells/ml in KH buffer at 28 °C in the presence of 10–80 % (v/v) FHS. Control conditions involved exposing cells to IHS. Cell death was evaluated by assessing cell proliferation by [^3H]-thymidine (American Radiolabeled Chemicals) incorporation, and by DNA fragmentation using the TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; Apoptosis Detection System Fluorescein; Promega) and resolving the samples on agarose gels as described previously [9]. Relative DNA fragmentation was determined by densitometric techniques using Scion Image (Scion). Results are expressed as the percentage of DNA fragmentation with respect to conditions that yield 100 % fragmentation [20 % (v/v) FHS].

PS exposure

A time course (30–120 min) for the detection of PS on the outer plasma membrane of FHS-treated parasites was performed using Annexin V–Alexa Fluor[®] 488 (Molecular Probes), according to the manufacturer's protocol. Fluorescence analysis was performed in a FACSCalibur apparatus (Becton Dickinson). Results are expressed as the percentage of Annexin-V positive cells with respect to the total cell count (1×10^4 cells were considered as 100 %) in each condition.

Low molecular mass thiol content

After incubation of epimastigotes in 0–30 % (v/v) FHS in KH buffer, 20 % (v/v) IHS in KH buffer or KH buffer alone (control)

with 30 μ M DMNQ (2,3-dimethoxy-1,4-naphthoquinone; 3 nM O₂^{•-}/min per 10⁸ cells; estimated from H₂O₂ formation using the *p*-hydroxyphenyl acetic acid–horseradish peroxidase assay [19]), non-protein thiols were derivatized with monobromobimane and analysed by reverse phase HPLC with fluorimetric detection [20]. Quantification and validation were accomplished using authentic standards of T(SH)₂ and GSH. After 1 h of treatment with 20% (v/v) FHS, TS₂ and GSSG in parasite extracts and supernatants were reduced with TR (1 unit/ml) and glutathione reductase (1 unit/ml) in the presence of 1 mM NADPH [20].

EPR studies and immuno-spin trapping of protein radicals

Detection of ROS was performed by EPR studies using DMPO (5,5-dimethylpyrroline-*N*-oxide) as the spin trap [21]. Cells (1 × 10⁸) were incubated for 10 min with KH buffer (control), 20% (v/v) FHS or 20% (v/v) IHS, collected by centrifugation at 800 *g* for 5 min at room temperature, and then resuspended in 300 μ l of KH buffer containing 0.1% Triton X-100 (added to render the cell suspension homogeneous, thereby avoiding cell–cell interactions) and 100 mM DMPO. The DMPO-OH adduct was determined at room temperature in a Bruker EMX EPR spectrometer operating at 9.7 GHz and 100 kHz field modulation using a 200 μ l flat cell. Spectra were recorded at a central field of 3480 G with a sweep width of 100 G. The spectra of FHS-treated parasites were recorded in the presence and absence of Cu/Zn SOD (5000 units). Results represent five accumulated scans (5 min). Immunodetection of DMPO nitron protein adducts on parasite samples was performed using an anti-(DMPO nitron) antiserum, which binds to the one-electron oxidation product of the initial DMPO nitroxyl-protein spin adduct [21]. Parasites (3 × 10⁸ cells/ml) were incubated in KH buffer containing 100 mM DMPO for 2 h in the presence of 10–80% (v/v) FHS or 20% (v/v) IHS. After treatment, parasites were washed in KH buffer and lysed at a concentration of 5 mg/ml by SDS loading buffer [30 mM Tris/HCl, pH 6.6, 1% (w/v) SDS and 5% (v/v) glycerol]. In the RA (reverse addition) condition, parasites were incubated as above, with 100 mM DMPO added at the end of the treatment. Protein extracts (50 μ g) were resolved by SDS/PAGE (12% gels), blotted on to nitrocellulose and probed with rabbit anti-(DMPO nitron) antiserum diluted 1:5000 as described previously [21]. Immunoreactive proteins were detected using the Immun-StarTM Chemiluminescence kit (Bio Rad).

PPP (pentose phosphate pathway) flux

Glucose utilization through the PPP was evaluated in FHS-treated parasites [22]. Cells were exposed to different experimental conditions in the presence of 0.2 μ Ci [¹⁴C¹]- or [¹⁴C⁶]-D-glucose (American Radiolabeled Chemicals). Incubation was performed in 3 ml vials with a 2 M KOH-soaked filter paper in the cap for 1 h at room temperature with constant stirring. After incubation, the filter paper and cell suspension (1 ml) were assessed for radioactivity. DMNQ (30 μ M; 3 nM O₂^{•-}/min per 10⁸ cells) in the presence of 20% (v/v) IHS was used as a control for oxidative stress. Results were calculated as the percentage difference between ¹⁴C¹O₂ and ¹⁴C⁶O₂, and expressed as the fold increase with respect to the IHS treatment. The basal rate of CO₂ production in the IHS condition was 1.8 ± 0.7 nmol/min per mg of protein.

Enzyme activities

Aconitase was measured in the different parasite samples in the early period (15 min) after exposure to FHS [23]. Cells (3 × 10⁸)

were resuspended in hypotonic buffer (5 mM Tris/HCl, pH 7.4, containing 2 μ M fluorocitrate), then subjected to freeze–thaw lysis in liquid nitrogen and centrifuged at 13 000 *g* for 15 min at 4 °C (total extract). Cytosolic fractions and membrane fractions containing mitochondria were obtained by incubating cells in hypotonic buffer for 15 min, followed by differential centrifugation as described previously [24]. The pellet (membrane fraction containing mitochondria) and supernatant (cytosolic fraction) were used to measure aconitase activity (1 unit of aconitase activity corresponds to 1 μ mol NADPH formed/min per mg of protein). TR activity was measured in total parasite extracts (prepared as described above) following the oxidation of NADPH at 340 nm in the presence of T(SH)₂, trypanedoxin, trypanedoxin peroxidase and H₂O₂ [25].

Mitochondria-derived fluorimetric detection of O₂^{•-}

O₂^{•-} production by epimastigotes was assayed fluorimetrically using the O₂^{•-}-sensitive-hydroethidine-analogue mitochondrially targeted probe, MitoSOX {3,8-phenanthridinediamine, 5-(6'-triphenylphosphoniumhexyl)-5,6 dihydro-6-phenyl; Invitrogen-Molecular Probes [26]}. This yields oxMitoSOX (oxidized MitoSOX), which becomes highly fluorescent upon binding to nucleic acids. Cells were loaded with 5 μ M MitoSOX for 10 min and washed with KH buffer before the assay. Loaded parasites (3 × 10⁸ cells/ml) were exposed to 20% (v/v) FHS or 20% (v/v) IHS in the absence or presence of 1 mM NOC-18 (which generates 0.145 μ M •NO/min⁻¹), 1 μ M FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) and 200 μ M DEVD-CHO (acetyl-Asp-Glu-Val-Asp aldehyde) for the indicated times. In the case of DEVD-CHO, parasites were pre-incubated in the presence of the caspase inhibitor for 2 h at 28 °C and then MitoSOX was added for the last 30 min. After incubation, parasites (3 × 10⁷ cells) were centrifuged at 800 *g* for 5 min at room temperature and resuspended in 100 μ l of KH buffer. Detection of oxMitoSOX was performed using black 96-well plates in a fluorescence microplate reader at λ_{ex} 510 nm and λ_{em} 580 nm. Results are expressed as arbitrary units of oxMitoSOX. Mitochondrial localization of oxMitoSOX was confirmed by fluorescence microscopy, which showed intense staining of the organellar genome (results not shown).

Measurement of mitochondrial and plasma transmembrane potential

$\Delta\Psi_m$ (mitochondrial membrane potential) was measured using the J-aggregate, lipophilic cation JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) and the cationic dye Safranin-O (Sigma) [27,28]. For measurements of early $\Delta\Psi_m$ changes (< 1 h), 1 × 10⁹ cells/ml were loaded with 2 μ M JC-1 for 30 min, washed in KH buffer and resuspended at 3 × 10⁸ cells/ml. After exposure to FHS, aliquots (3 × 10⁷ cells) were taken every 10 min, washed and used to measure fluorescence as described above, with filters at λ_{ex} 485 nm and λ_{em} 520 and 590 nm. Results are expressed as the percentage of fluorescence recorded at 520 nm and 590 nm for time point zero of the IHS-treated condition. For the Safranin-O experiments, $\Delta\Psi_m$ in FHS-treated parasites was measured in medium containing 200 mM sucrose, 20 mM Hepes (pH 7.0), 1 mM MgCl₂, 5 mM succinate, 5 μ M Safranin-O and 0.3 mM EGTA. After incubation with FHS, 2.5 × 10⁷ parasites were permeabilized with 40 μ M digitonin and the fluorescence recorded at λ_{ex} 495 nm and λ_{em} 586 nm. Where indicated, 200 μ M ADP, 2 μ M AT (atractyl-oxide) and 1 μ M FCCP were added. Fluorescence was converted into mV by calibration with sequential additions of KCl in the

presence of valinomycin as described previously [28]. The 20 % (v/v) IHS control condition corresponds to a $\Delta\Psi_m$ of approx. 120 mV. $\Delta\Psi_p$ (plasma membrane potential) was measured using DiBAC₄ [bis-(1,3-dibutylbarbituric acid)trimethine oxonol] anionic dye [29]. Briefly, parasites (3×10^8 cells/ml) were incubated with 20 % (v/v) FHS or 20 % (v/v) IHS. After exposure, aliquots (3×10^7 cells) were taken every 10 min, washed and incubated for 10 min in KH buffer containing 5 nM DiBAC₄. Fluorescence was measured as described above with filters at λ_{ex} 492 nm and λ_{em} 515 nm. Results are expressed as the percentage of the fluorescence emitted with respect to the control condition [20 % (v/v) IHS].

Oxygen consumption

Cells (1×10^8 cells/ml) were incubated with 20 % (v/v) IHS or 20 % (v/v) FHS and then, at the indicated times, parasites were washed in KH buffer and analysed at 28 °C in a gas-tight, 1.5 ml chamber equipped with a Clark-type oxygen electrode connected to a computer with the DUO.18 processing program (World Precision Instruments). In some experiments, oxygen consumption was monitored before and after the addition of the uncoupler FCCP (1 μ M). Results show mitochondrial oxygen consumption calculated as the antimycin A-inhibitable fraction, and are expressed as μ M oxygen consumed/min, assuming oxygen solubility at 28 °C in KH buffer to be 280 μ M (taken as 100 %).

Mitochondrial [2,8-³H]-ADP import and cellular ATP levels

After 30 min exposure to FHS, cells (3×10^8) were centrifuged at 800 *g* for 10 min at 22 °C. The cells were resuspended in 1 ml KH buffer containing 40 μ g digitonin and 0.55 mCi of [2,8-³H]-ADP (American Radiolabeled Chemicals), and then incubated for a further 10 min. At the concentration used, digitonin permeabilization is known not to affect mitochondrial function [28]. Cells were centrifuged at 800 *g* for 10 min at 22 °C and the mitochondrial-rich (pellet) and cytosolic (supernatant) fractions were assayed for radioactivity. Results are expressed as the percentage of [2,8-³H]-ADP present in the mitochondrial-rich fraction in comparison with the total radioactivity (pellet and supernatant). ANT (adenine nucleotide translocase)-dependent import of [2,8-³H]-ADP was evaluated in the presence of 25 μ M AT in the incubation medium [30]. ATP levels were determined in total parasite extracts incubated in the presence of 10–40 % (v/v) FHS or 20 % (v/v) IHS and 25 μ M AT at the indicated times using a chemiluminescence ATP-assay kit (Calbiochem).

Cyt *c* release

After exposure to FHS, the parasites (3×10^8 cells) were washed and resuspended in 200 μ l of KH buffer containing 40 μ g digitonin, and incubated on ice for 10 min. The obtained mitochondrial-rich fraction (pellet; resuspended in 50 μ l of KH buffer) and cytosolic fraction (200 μ l) were subjected to Western blotting. Protein extracts (10 μ l and 30 μ l of mitochondrial and cytosolic fractions respectively for each condition) were resolved by SDS/PAGE (15 % gels), blotted on to nitrocellulose and probed with rabbit anti-(*Trypanosoma brucei* cyt *c*) antiserum diluted 1:500 [31]. Immunoreactive proteins were detected using the Immuno-Star™ Chemiluminescence kit. Relative cyt *c* content in the different fractions (cytosolic, mitochondrial-rich and total parasite extracts) was determined by densitometric techniques using Scion Image (Scion). Results are expressed in arbitrary units.

Overexpression of TcSODA (*T. cruzi* mitochondrial SOD)

T. cruzi CL-Brener [pLew13] epimastigotes containing the bacteriophage T7 RNA polymerase and Tet (tetracycline)-repressor genes were maintained as described above except that 5 % (v/v) Tet-free foetal calf serum was used. The *T. cruzi* gene encoding for the mitochondrial FeSOD (TcSODA; XM.807064) was amplified from genomic DNA with the primers 5'-ggggga-tccATGTTGAGACGTGCGGTGAA-3' and 5'-ggttgatcTTTT-ATTGCCTGCGCAT-3'. Restriction enzyme sites (lower case letters) were incorporated into the primers to facilitate DNA cloning. The 699 bp product was ligated into pTcINDEX-9E10, such that the 9E10 epitope derived from the human c-Myc protein was fused to the C-terminus of TcSODA [32]. Expression of the recombinant protein was under the control of a Tet-inducible T7 promoter. Transformed cells were selected in medium containing 100 μ g/ml hygromycin. To induce TcSODA expression, cells were cultured in medium supplemented with 250 ng/ml Tet, which was added to the culture medium every 3 days. Control cells were maintained in Tet-free medium. For SDS/PAGE, Western blotting and SOD assays, cells were collected by centrifugation at 13 000 *g* for 30 min at 4 °C and lysed by freeze-thawing in PBS containing Complete™ proteinase inhibitors (Roche). SDS/PAGE and Western blotting were carried out as described above. Membranes were probed with mouse anti-c-Myc (9E10) antibody (Santa Cruz Biotechnology) diluted 1:2000. SOD activity was determined by the inhibition of cyt *c* reduction using the xanthine/xanthine oxidase system [33]. Localization of the tagged TcSODA was determined by immunofluorescence as described previously [32]. PI (propidium iodide) was used as the DNA stain. Slides were examined on a Zeiss LSM 510 confocal laser-scanning microscope.

RESULTS

Early depletion of trypanothione and GSH, and inhibition of TR during PCD

In mammalian cells, a change in the intracellular redox status of the cell to a more oxidized environment is associated with the activation of factors involved in the execution of PCD [34]. Therefore we evaluated the levels of reduced T(SH)₂ and GSH, the major non-protein thiols in *T. cruzi* epimastigotes, during FHS-induced PCD. When parasites were incubated with increasing concentrations of FHS [10–30 % (v/v)] for 2 h, a dose-dependent depletion of T(SH)₂ and GSH was observed (Table 1A). Time course analysis of the depletion produced by 20 % (v/v) FHS indicated that this is an early event in the death process, with a significant decrease of T(SH)₂ and GSH levels after a 10 min incubation (Table 1B). Thiol depletion was due to intracellular oxidation, and passive leakage due to the increased membrane permeability to low molecular mass components induced by complement activation on the surface of the parasite. Approx. 25 % of the T(SH)₂ and 10 % of the GSH levels were recovered after the treatment of parasite extracts with TR or glutathione reductase respectively (results not shown). In some models of mammalian PCD, it has been observed that GSH is actively extruded from the cell [35], contributing to the generation of intracellular oxidative stress. In addition to intracellular thiol depletion, inhibition of TR was observed in parasites exposed to the death stimuli [34 ± 3 and 13 ± 2 μ M NADPH/min per mg of protein after 1 h of incubation with 20 % (v/v) IHS or 20 % (v/v) FHS respectively]. The treatment of control cells with the O₂^{•-}-forming, redox-cycling compound DMNQ (30 μ M; 3 nM O₂^{•-} min/10⁸ cells) produced similar levels of T(SH)₂

Table 1 T(SH)₂ and GSH content following FHS-induced PCD

Intracellular T(SH)₂ and GSH content are expressed as the percentage observed in IHS-treated cells (4.6 ± 0.8 and 0.9 ± 0.4 nmol per 10^8 cells respectively). (A) Endpoint values after 2 h of incubation under the indicated conditions. DMNQ (30 μ M) was used as an O₂^{•−}-forming compound in KH buffer. Control, parasites in KH buffer alone. (B) Time course under 20 % (v/v) FHS-treatment. Results represent one typical experiment out of four. nd, not determined.

(A)

Condition	T(SH) ₂ content (%)	GSH content (%)
20 % (v/v) IHS	100	100
10 % (v/v) FHS	79	64
20 % (v/v) FHS	32	45
30 % (v/v) FHS	16	20
Control	100	nd
DMNQ (30 μ M)	28	nd

(B)

Time (min)	T(SH) ₂ content (%)	GSH content (%)
0	100	100
10	40	60
20	38	45
60	35	42
120	30	40

depletion (Table 1A) and inhibition of TR activity (70 % inhibition) compared with those caused by 20 % (v/v) FHS. Thus the inhibition of TR [the enzyme responsible for maintaining T(SH)₂ in its reduced state] contributed to the persistent depletion of thiols. In *Leishmania donovani*, similar extents of GSH depletion were observed following induction of PCD by camptothecin, a topoisomerase I inhibitor [36]. This suggests that thiol depletion could be a common feature in trypanosomatids that links the cellular redox status with the initiation of PCD.

ROS production and oxidative stress during PCD

The rapid oxidation of thiols suggests that ROS could be generated in response to PCD, with the establishment of intracellular oxidative stress. Thus we investigated the generation of ROS directly by EPR studies using DMPO as the spin trap and indirectly by the detection of oxidative modifications on proteins by Western blot analysis using an anti-(DMPO nitron) antibody (immuno spin-trapping) [21]. DMPO reaction with O₂^{•−} yields the DMPO-OOH adduct that is rapidly ($t_{1/2} < 1$ min) converted into DMPO-OH in the cellular medium [37]. Figure 1(A) shows that an EPR signal corresponding to the stable DMPO-OH adduct (quartet signal; $a_N = a_H = 14.9$ G) was observed after a 10 min exposure of parasites to 20 % (v/v) FHS. The signal was abolished

Figure 1 Enhanced production of oxidants during FHS-induced PCD

(A) EPR studies. Parasites (1×10^8) were incubated for 10 min with KH buffer as a control (CTL) or with 20 % (v/v) IHS or 20 % (v/v) FHS, then centrifuged and resuspended in 300 μ l KH containing 0.1 % Triton X-100. Immediately after permeabilization, 100 mM DMPO was added and EPR spectra for the spin adduct DMPO-OH (*) were determined for five accumulated scans. CuZnSOD (5000 units) was added to the FHS-treated cells after the incubation period. (B) Detection of protein radicals by immuno-spin trapping. Parasites (3×10^8 cells/ml) were incubated with 100 mM DMPO in the absence or presence of 20 % (v/v) IHS or 10–80 % (v/v) FHS for 2 h. After incubation, the parasites were collected by centrifugation, then washed in KH buffer and lysed at a concentration of 5 mg/ml in SDS loading buffer. In the RA condition, parasites were incubated as described previously and 100 mM DMPO (final concentration) was added at the end of the treatment. MW, molecular mass markers, sizes given in kDa. Western blot analysis was carried out as described in the Materials and methods section. (C) PPP. Cells (3×10^8) were incubated with [¹⁴C]- or [¹⁴C⁶]-D-glucose in the presence of 20 % (v/v) IHS or 20 % (v/v) FHS or 30 μ M DMNQ (3 nM O₂^{•−}/min per 10^8 cells) as an O₂^{•−} donor for 1 h. ¹⁴CO₂ released from the PPP was calculated and results expressed as means $\pm$ S.D. of the fold increase with respect to the IHS condition for at least three independent experiments. The basal rate of CO₂ production in the IHS condition was 1.8 ± 0.7 nmol/min per mg of protein.

Figure 2 Enhanced mitochondrial $O_2^{\bullet-}$ production after FHS-induced PCD

(A) Aconitase activity. Cells (3×10^6) were incubated with 20% (v/v) IHS, or 20 or 40% (v/v) FHS and aconitase was measured in total parasite extracts at the indicated times. The results represent one typical experiment out of three performed. (B) MitoSOX oxidation. MitoSOX pre-loaded parasites were incubated with 20% (v/v) IHS or 20% (v/v) FHS in the absence or presence of 1 mM NOC-18 (0.145 μ M •NO/min) or 1 μ M FCCP. At the indicated times, parasites were washed with KH buffer and 3×10^7 cells were used to fluorimetrically measure oxMitoSOX. Results are expressed in arbitrary units as means $\pm$ S.D. for three independent experiments.

in the presence of Cu/ZnSOD, indicating the generation of $O_2^{\bullet-}$. No adduct was detected when cells were incubated with KH buffer or in the presence of 20% (v/v) IHS (Figure 1A). DMPO–protein adducts were detected immunochemically after a 2 h exposure to different concentrations of FHS (10–80%) (Figure 1B). No immunoreaction was observed in the RA condition, indicating the specificity of the detection (Figure 1B). A major immunoreactive band at approx. 55 kDa was detected after 2 h of incubation with FHS, whereas at longer incubation periods (18 h), other high molecular mass bands were present (results not shown). Finally, as a metabolic response to the generation of oxidative stress, the glucose flux through the PPP was evaluated. A 7-fold increase in glucose utilization through the PPP was observed after the addition of 20% (v/v) FHS (Figure 1C). The $O_2^{\bullet-}$ -forming compound DMNQ also produced a 4-fold increase of the PPP pathway. The increased rates of the PPP reflect an enhanced formation of NADPH, which fuels the trypanothione-dependent antioxidant system in parasites undergoing FHS-induced PCD.

Mitochondrial $O_2^{\bullet-}$ production during PCD

The above data indicate that early in the FHS-induced death programme there is increased production of ROS, although the subcellular site of this generation is unknown. One of the most sensitive targets for $O_2^{\bullet-}$ is the iron–sulphur [4Fe–4S]-containing enzyme aconitase [23]. Upon oxidation by $O_2^{\bullet-}$, the iron–sulphur cluster is disrupted to [3Fe–4S], resulting in enzyme inactivation [23]. In *T. cruzi*, we found that approx. 60% of aconitase activity is localized to the mitochondria (with a total activity of approx. 20 ± 3 milliunits per mg of protein). After induction of PCD by FHS, a dose- and time-dependent inhibition of aconitase activity was observed (Figure 2A). Both mitochondrial and cytosolic aconitase were inhibited after 2 h of treatment with 20% (v/v) FHS (80% and 50% inhibition respectively),

reflecting the early generation of mitochondrial $O_2^{\bullet-}$. This was later confirmed by the mitochondrial overexpression of TcSODA (described in more detail below). In mammalian cells, GSH is involved in the reactivation of aconitase by reassembling the iron–sulphur cluster [38]. Therefore the observed profiles of aconitase inactivation in FHS-treated parasites could be more pronounced owing to the marked depletion of T(SH)₂ and GSH early in the death process (Table 1). Mitochondrial $O_2^{\bullet-}$ production was also evaluated using an $O_2^{\bullet-}$ -sensitive mitochondrially targeted hydroethidine analogue, MitoSOX [26] (see Supplementary Figure 1 at <http://www.BiochemJ.org/bj/403/bj4030323add.htm>). MitoSOX localizes to the mitochondrion due to its hydrophobic nature and its positively charged triphenylphosphonium moiety [26]. MitoSOX oxidation was significantly higher in FHS-treated parasites compared with control conditions [20% (v/v) IHS], reaching a maximum value 20 min after the addition of the death stimulus (Figure 2B). Moreover, addition of the uncoupler FCCP partially inhibited MitoSOX oxidation in FHS-treated cells, indicating that $O_2^{\bullet-}$ production is dependent on $\Delta\Psi_m$ (Figure 2B). DMNQ (30 μ M) caused MitoSOX oxidation comparable with that observed in FHS-treated cells and was not altered by the presence of FCCP (results not shown). The MitoSOX oxidation time course is in agreement with that observed for aconitase inactivation (Figure 2A), further supporting early mitochondrial production of $O_2^{\bullet-}$. Previous results [9] have shown that low •NO fluxes and the presence of the caspase inhibitor DEVD-CHO can inhibit FHS-induced PCD in *T. cruzi*. Addition of the •NO-generating compound NOC-18 to FHS-treated cells reduced MitoSOX oxidation to values similar to those observed with IHS treatment, suggesting that less $O_2^{\bullet-}$ is available. This is in agreement with the observation in $O_2^{\bullet-}$ -producing (antimycin A-treated) isolated mitochondria (Figure 2B and Supplementary Figure 1). Presence of the caspase inhibitor DEVD-CHO did not affect MitoSOX oxidation in FHS-treated parasites, indicating that mitochondrial $O_2^{\bullet-}$ generation occurs earlier than protease activation in the death process.

Figure 3 Mitochondrial function studies during PCD

(A) Mitochondrial respiration. Time course of oxygen consumption by *T. cruzi* epimastigotes (1×10^8 cells) incubated in the presence of 20% (v/v) IHS or 20% (v/v) FHS. Results are expressed as μM oxygen consumed/min for one typical experiment out of three performed. Inset: changes in oxygen consumption by the addition of $1 \mu\text{M}$ FCCP to parasites incubated for 20 min with 20% (v/v) IHS [control (CTL); open bars] or 20% (v/v) FHS (closed bars). Results represent means $\pm$ S.D. for at least ten independent experiments. * $P < 0.05$ in comparison with CTL. (B) Mitochondria and plasma membrane potential. $\Delta\Psi_m$ was measured using JC-1 in entire cells. Pre-loaded parasites (3×10^8 cells/ml) were incubated in the presence of 20% (v/v) FHS or 20% (v/v) IHS. At the indicated times, aliquots (3×10^7 cells) were taken and the fluorescence at λ_{em} 520 nm and λ_{em} 590 nm was measured. Results are expressed as the percentage of the fluorescence at time point zero. The results represent means $\pm$ S.D. for at least three independent experiments. Inset: $\Delta\Psi_p$ was measured using the bis-oxonol anionic dye DiBAC₄. Parasites (3×10^8 cells/ml) were exposed to 20% (v/v) IHS or 20% (v/v) FHS for the indicated times. After incubation, cells were washed with KH buffer and incubated for an additional 10 min with 5 nM DiBAC₄. Aliquots containing 3×10^7 cells were used to measure cellular fluorescence as described in the Materials and methods section. Results are expressed as DiBAC₄ fluorescence (arbitrary units) and represent means $\pm$ S.D. for three independent experiments. (C) Mitochondrial energy metabolism during PCD. After incubation (10–60 min) with 20% (v/v) IHS or 20% (v/v) FHS, 2.5×10^7 cells were permeabilized by 40 μM digitonin in the presence of 5 μM Safranine-O as described in the Materials and methods section. Oxidative phosphorylation was evaluated by the addition of 200 μM ADP following an increase in Safranine-O fluorescence (which correlated with $\Delta\Psi_m$ dissipation). Where indicated, 1 μM AT and 1 μM FCCP were added. In the control condition, $\Delta\Psi_m$ was estimated to be approx. 120 mV. Results show one typical experiment out of three. (D) ADP mitochondrial import. Mitochondrial ADP uptake was assayed in parasites incubated for 30 min with 20% (v/v) IHS or 20% (v/v) FHS in the absence or presence of 25 μM AT, as described in the Materials and methods section. Results are expressed as the percentage of mitochondrial [^3H]-ADP content with respect to total incorporated radioactivity (mitochondria + cytosol). Results are shown as means $\pm$ S.D. for at least three independent experiments. Inset: cellular ATP content was measured at the indicated times with the ATP-assay kit (Calbiochem) in parasites treated with 20% (v/v) FHS and 20% (v/v) IHS in the absence or presence of 25 μM AT. Results show means $\pm$ S.D. for one typical experiment out of three.

Altered mitochondrial function during FHS-induced PCD

The above results indicate that, after the addition of the death stimulus, mitochondrial physiology is altered, with increased generation of O₂^{•-} radicals. Thus we evaluated mitochondrial

function by measuring oxygen consumption and $\Delta\Psi_m$ in response to FHS. Figure 3(A) shows the time-dependent inhibition of mitochondrial respiration in FHS-treated parasites. Inhibition of mitochondrial oxygen consumption in the early phase (< 20 min), following PCD induction, was not due to alterations in the

Figure 4 Cyt c release

After incubation with 20% (v/v) IHS or 20% (v/v) FHS, cyt c was detected, at the indicated times, in 50 µg of cytosolic or mitochondrial-rich fractions of digitonin-treated parasites by immunoblot using an anti-(*T. brucei* cyt c) antibody. Ponceau S staining was performed after protein transfer to ensure equal loading of the gel. Densitometric analysis of the immunoblots are shown and expressed as arbitrary units. The total (mitochondria + cytosol) cyt c content of IHS- (x) and FHS-treated (o) parasites, mitochondrial cyt c content of FHS-treated parasites (●) and cytosolic cyt c content of FHS-treated parasites (■) are shown. Results represent one typical experiment out of three. In all cases, the total amount of detectable cyt c (mitochondrial + cytosol) remained constant.

respiratory chain complexes, since the addition of FCCP brought respiration to the same rate observed under control [20% (v/v) IHS] conditions (Figure 3A, inset). $\Delta\Psi_m$ was evaluated in intact cells using the lipophilic cation JC-1, which exists as a green fluorescent monomer (λ_{em} 520 nm) at low concentrations in the cytosol and forms red fluorescent (λ_{em} 590 nm) J-aggregates at higher concentrations in mitochondria, where it concentrates due to $\Delta\Psi_m$. Also, $\Delta\Psi_m$ was evaluated in digitonin-permeabilized cells using the cationic dye Safranin-O, which accumulates in mitochondria and results in the quenching of its fluorescence [28]. Both methods showed a rapid (< 30 min) but moderate decrease in $\Delta\Psi_m$ by approx. 15% using JC-1 (observed as the loss in fluorescence at 590 nm compared with IHS-treated cells; Figure 3B) and by approx. 20% using Safranin-O (Figure 3C). After the initial drop, $\Delta\Psi_m$ remained constant for up to 1 h after the induction of PCD (Figure 3B). In contrast, depolarisation of the plasma membrane ($\Delta\Psi_p$), which was measured as the loss in JC-1 fluorescence at 520 nm (Figure 3B), or as the accumulation of the bis-oxonol anionic dye DiBAC₄ (Figure 3B, inset), decreased by approx. 35% in the first 30 min, suggesting a relatively large loss of the $\Delta\Psi_p$ after addition of the death stimulus. Overall the results indicate that both inhibition of oxygen consumption and almost total preservation of the mitochondrial electrochemical gradient in FHS-treated parasites (Figure 3A, inset) favour the monovalent reduction of oxygen to $O_2^{\cdot-}$. This

Figure 5 Expression, activity and immunolocalization of inducible TcSODA

(A) Western blots probed with the anti-c-Myc (9E10) antibody, with SOD activity in units per mg of protein shown underneath. The ratio of SOD activity in Tet-induced cells (TcSODA; +Tet) with respect to the control conditions (– Tet) is approx. 15. (B) (a) TcSODA cells stained with anti-c-Myc (9E10) (green) and (b) DNA staining with PI (red); (c) merged image of (a) and (b); (d) phase contrast.

is probably due to a more reduced state of the electron transport chain components [39].

Impairment of mitochondrial energy metabolism

Since oxidative phosphorylation is coupled with mitochondrial respiration, we evaluated the capability of mitochondrial ATP synthesis in FHS-treated parasites. Figure 3(C) shows that, early after the addition of FHS (< 30 min), ATP synthesis (seen as the ADP-dependent increase in Safranin-O fluorescence) by mitochondria was partially inhibited, and it becomes negligible after > 30 min of treatment. Since the components of the electron transport chain function correctly in the early phase (Figure 3A, inset), we tested whether mitochondrial ATP/ADP exchange with the cytosol through ANT, localized to the mitochondrial inner membrane, and the VDAC (voltage-dependent anion channel; localized to the mitochondrial outer membrane) was impaired. ADP import into mitochondria was assayed in the absence or presence of AT, an ANT inhibitor. AT on its own inhibited ATP synthesis (Figure 3C) and ADP import (Figure 3D), decreased cellular ATP levels (Figure 3D, inset) and increased $\Delta\Psi_m$ in IHS-treated control cells (Figure 3C and results not shown), mimicking the response obtained after parasite FHS-challenge. Thus, in our model, inhibition of oxygen consumption and maintenance

Figure 6 Protective effects of TcSODA overexpression on PCD markers

(A) Proliferation assays. CTL and Tet-induced (TcSODA) cells (1×10^6) were pre-incubated with 20% (v/v) IHS or 20% (v/v) FHS and pulsed with 1 μ Ci of [³H]-thymidine for 18 h at 28 °C. After incubation, cells were harvested and assayed for radioactivity. Results are expressed as percentage of [³H]-thymidine incorporation with respect to the IHS-treated cells. *, $P < 0.05$ with respect to IHS treatment; **, $P < 0.05$ with respect to FHS treatment. (B) DNA fragmentation. Cytosolic DNA fragments from control and TcSODA-overexpressing cells were obtained following treatment with 20% (v/v) IHS or 20% (v/v) FHS for 18 h at 28 °C. Samples were resolved on agarose gels and visualized by ethidium bromide. (C) PS exposure was measured using Annexin V–Alexa Fluor[®] 488. Control (CTL) and TcSODA-overexpressing parasites were treated for the indicated time points with 20% (v/v) IHS or 20% (v/v) FHS and the percentage of positive cells was measured. (D) Aconitase activity was measured in control (CTL) and TcSODA-overexpressing cells (3×10^6) after incubation with 20% (v/v) IHS or 20% (v/v) FHS as described in the legend for Figure 2(A). Results are expressed as the percentage of aconitase activity with respect to TcSODA-overexpressing cells in the presence of 20% (v/v) IHS. The results represent one typical experiment out of three. (E) MitoSOX oxidation. Measurements of oxMitoSOX in control (CTL) and TcSODA-overexpressing cells following a 30 min incubation with 20% (v/v) IHS (CTL) or 20% (v/v) FHS were performed as described in the legend for Figure 2(B). Results represent means $\pm$ S.D. for three independent experiments. (F) Cyt c release. After incubation of control (CTL) and TcSODA-overexpressing cells with 20% (v/v) IHS or 20% (v/v) FHS, cyt c was detected in cytosolic extracts of digitonin-treated parasites as described in the legend for Figure 4.

of the mitochondrial electrochemical gradient appear to be due to the impairment of mitochondrial ADP/ATP exchange with the cytosol. Finally, as a central hallmark of mammalian apoptosis, we evaluated cyt c release into the cytosol after treatment of epimastigotes with 20% (v/v) FHS (Figure 4). Cyt c was detected in the parasite cytosol after a 15 min exposure to 20% (v/v) FHS, with a concomitant decrease in the mitochondrial cyt c content. Densitometric analysis of the immunoblots shows that the total

cyt c (mitochondrial cyt c + cyt c released into cytosol) in FHS-treated parasites remains constant and similar to that following IHS treatment (Figure 4), indicating the absence of parasite lysis during the process, as shown previously [9]. In our model, treatment with PTP (permeability transition pore) inhibitors, 10 μ M bongkreikic acid and 5 μ M cyclosporin A, before the addition of FHS did not prevent cyt c release, suggesting that mitochondrial PTP opening is not involved (results not shown).

Inhibition of PCD by overexpression of mitochondrial SOD

In order to confirm the role of mitochondrial $O_2^{\bullet-}$ in FHS-induced PCD in *T. cruzi*, parasites overexpressing TcSODA were produced by genetic manipulation. Briefly, CL-Brener cells were engineered to express the T7 RNA polymerase and Tet repressor genes [32]. Recombinant parasites were obtained that contained Tet-inducible *TcSODA*, a paralogue of the *T. brucei* mitochondrial SOD [17]. Expression of Myc-tagged TcSODA protein was confirmed by Western blotting using the anti-c-Myc (9E10) antibody that strongly recognized the recombinant protein (Figure 5A). Weak protein expression was detected in the non-induced parasites, indicating slight leakiness in the Tet repressor system, which has been described for other Tet-inducible systems (Figure 5A). This overexpression resulted in an approx. 15-fold increase in SOD activity levels (control cells, 2.2 ± 1 units/mg of protein; Tet-induced cells, 32.0 ± 4 units/mg of protein). The TcSODA protein was localized by immunofluorescence to a structure with the lattice-like morphology of the single mitochondrion, with particularly strong staining around the kinetoplast DNA (Figure 5B), which has been described previously and is similar to the staining observed for the *T. brucei* paralogue [17,32]. Importantly, induction of *TcSODA* rendered epimastigotes more resistant to FHS-induced PCD, as shown by the recovery of parasite proliferation rates (Figure 6A), protection from DNA fragmentation (Figure 6B), decrease of TUNEL-positive parasites after 2 h of treatment with 20% (v/v) FHS (83 ± 10 and 14 ± 5 % positive TUNEL staining in control and TcSODA cells respectively; see Supplementary Figure 2 at <http://www.BiochemJ.org/bj/403/bj4030323add.htm>) and PS externalization (Figure 6C). Moreover, a reduction in aconitase inactivation (Figure 6D), an approx. 80% inhibition of MitoSOX oxidation (Figure 6E) and delayed cyt *c* release (Figure 6F) were observed in the TcSODA-overexpressing cells, supporting a key role of mitochondrial $O_2^{\bullet-}$ in the induction of the death process triggered by FHS.

DISCUSSION

The present study aimed to unravel the molecular mechanisms that govern PCD in *T. cruzi*. In our model, PCD is a response to the activation of the complement system on the surface of the parasite [5], which is transduced intracellularly by yet-to-be-established signals that trigger PCD. Even though it was initially described as a necrotic stimulus, it is now well documented that complement activation can induce apoptosis in several models of mammalian cells [40], as well as in *T. cruzi* epimastigotes [5,9], although the precise mechanisms have not been elucidated. In the present study, we demonstrate that the mitochondrion is a key organelle for *T. cruzi* apoptotic signalling and that early changes in mitochondrial physiology lead to increased production of $O_2^{\bullet-}$, a free radical that serves as a key mediator for initiation of the death process. Mechanisms that link extracellular complement activation with mitochondrial changes are still under investigation, but preliminary results point to a massive influx of Ca^{2+} ions into the cytosol and mitochondria of treated parasites (F. Irigoín, L. Piacenza, N. Inada, A. Vercesi and R. Radi, unpublished work). The increase in mitochondrial Ca^{2+} has been associated with loss of $\Delta\Psi_m$, alterations of the ADP-ATP carrier and increased production of $O_2^{\bullet-}$ [41,42], events that are consistent with the present results obtained for FHS-induced PCD.

In our model, mitochondrial respiration and oxidative phosphorylation were impaired soon after exposure to FHS. The inhibition of oxidative phosphorylation could be due either to the reduction in $\Delta\Psi_m$ or to the inhibition of the mitochondrial

ADP transport systems present in mitochondria [42]. In support of the latter, exposure of epimastigotes to AT, an inhibitor of ANT, mimicked the early changes in mitochondrial physiology induced by FHS. In fact, impairment of the mitochondrial ADP/ATP exchange with the cytosol has been described in mammalian models as one of the earliest defects that contribute to the initiation of apoptosis [43]. In FHS-treated epimastigotes, ADP transport impairment could be due to alterations of VDAC and/or by oxidative modifications of ANT, an issue that requires further investigation.

The early changes observed in mitochondrial physiology are compatible with an increased production of ROS. Indeed, soon after FHS exposure, cells underwent oxidative stress, as evidenced by increased ROS production (Figures 1A and 2B), decreased levels of T(SH)₂ and GSH (Table 1), oxidative damage to proteins (Figure 1B) and an increase in the glucose flux through the PPP (Figure 1C). ROS have also been shown to be formed, and involved, in *L. donovani* PCD, although they were triggered by different stimuli [13,36,44]. However, in these studies the methodologies used were ambiguous in terms of the ROS involved or the site of ROS production. Through measuring the activity of the redox-sensitive aconitase, and the oxidation of a new $O_2^{\bullet-}$ -specific hydroethidine-analogue that localizes to mitochondria, we have established that $O_2^{\bullet-}$ is the principal ROS generated by this organelle during the first stages of *T. cruzi* FHS-induced PCD. Most importantly, mitochondrial $O_2^{\bullet-}$ plays a key role in the initiation of the death process. Overexpression of TcSODA renders *T. cruzi* more resistant to FHS-induced PCD. Lower levels of $O_2^{\bullet-}$ were detected in cells expressing elevated levels of TcSODA, either by MitoSOX oxidation or aconitase inhibition, in line with the higher proliferation rates, delayed externalization of PS and decreased DNA fragmentation observed in response to FHS-treatment. Moreover, cyt *c* release was delayed significantly in the recombinant parasites, suggesting the participation of $O_2^{\bullet-}$ radicals in this process. The results of the present study unambiguously indicate the participation of mitochondrial $O_2^{\bullet-}$ in the signalling of the death programme. In this scenario, our results support the concept that the overexpression of anti-oxidant enzymes (including mitochondrial FeSOD) observed during metacyclogenesis [18] may render the parasites more resistant to as-yet-unidentified death stimuli present in the insect vector.

In mammalian cells, as well as in *Leishmania*, the production of ROS has been associated with permeabilization of the mitochondrial outer membrane and the release of pro-apoptotic factors, typically cyt *c* [36]. During mammalian apoptosis, increased levels of oxidants facilitate the cardiolipin oxygenase activity of cyt *c*, which favours its detachment and cytosolic release [11]. It is interesting to note that, in parasites overexpressing TcSODA, cyt *c* release was significantly delayed compared with control cells. It is tempting to speculate that cardiolipin oxidation [45] in *T. cruzi* mitochondria participates in the release of cyt *c*. Thus although we cannot infer directly the mechanism of cyt *c* release, it seems unlikely to involve the PTP opening, as a complete dissipation of $\Delta\Psi_m$ was not observed during the early stages of cyt *c* release. The role of cytosolic cyt *c* in the activation of PCD effectors in trypanosomatids is unknown, owing to the absence of caspases, and the limited characterization of the cytosolic protein partners and proteolytic activities involved. However, cyt *c* release into the cytosol could be involved in the activation of *T. cruzi* metacaspases (homologues of mammalian caspases), which change their subcellular localization during FHS-induced PCD [46]. Cyt *c* could have pro-apoptotic roles mediated by mechanisms involving interactions at the endoplasmic reticulum [47] or translocation to the nucleus [48].

Our work is the first to report a mitochondrial and O₂^{•−}-dependent PCD in *T. cruzi*, and the anti-apoptotic role of mitochondrial FeSOD in trypanosomatids. Moreover, the present study has validated novel experimental tools (MitoSOX and immuno spin-trapping), whose uses can be extended to unravel the mitochondrial formation of O₂^{•−} in other PCD models.

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