Oxidative stress damage in the protozoan parasite *Trypanosoma cruzi* is inhibited by Cyclosporin A

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SUMMARY

Cyclosporin A (CsA) specifically inhibits the mitochondrial permeability transition pore (mPTP). Opening of the mPTP, which is triggered by high levels of matrix [Ca^{2+}] and/or oxidative stress, leads to mitochondrial dysfunction and thus to cell death by either apoptosis or necrosis. In the present study, we analysed the response of *Trypanosoma cruzi* epimastigote parasites to oxidative stress with 5 mM H_2O_2, by studying several features related to programmed cell death and the effects of pre-incubation with 1 μM of CsA. We evaluated *Tc*PARP cleavage, DNA integrity, cytochrome *c* translocation, Annexin V/propidium iodide staining, reactive oxygen species production. CsA prevented parasite oxidative stress damage as it significantly inhibited DNA degradation, cytochrome *c* translocation to cytosol and *Tc*PARP cleavage. The calcein-AM/$CoCl_2$ assay, used as a selective indicator of mPTP opening in mammals, was also performed in *T. cruzi* parasites. H_2O_2 treatment decreased calcein fluorescence, but this decline was partially inhibited by pre-incubation with CsA. Our results encourage further studies to investigate if there is a mPTP-like pore and a mitochondrial cyclophilin involved in this protozoan parasite.

Key words: *Trypanosoma cruzi*, cyclosporin A, programmed cell death, oxidative stress, cyclophilin.

INTRODUCTION

Mitochondria contain a structure that forms a large and non-specific pore, permeable to molecules smaller than 1·5 kDa, which opens in the inner mitochondrial membrane and is called the mitochondrial permeability transition pore (mPTP). Opening of mPTP, which is greatly enhanced under stressful conditions such as Ca^{2+} overload, nucleotide starvation or oxidative stress, allows the release of apoptosis-related factors such as apoptosis-inducing factor, cytochrome *c* and endonuclease G into the cytosol (Crompton *et al*. 1999; Vaseva *et al*. 2012) and leads to programmed cell death. Although the identity of the pore-forming proteins remains under debate, recent works propose that dimers of the F_1F_0 ATP synthase display the key features of the mPTP. A well-characterized protein regulator of the PTP is cyclophilin D (CyPD), which binds the F_1F_0 ATP synthase (complex V) (Giorgio *et al*. 2013) and is the only component that has been genetically proven to be indispensable for this process (Kroemer *et al*. 2007). CyPD can be displaced from the lateral stalk of F_1F_0 ATP synthase by Cyclosporin A (CsA), which is a mPTP opening inhibitor. It is proposed that when CsA binds CyPD, its displacing from the F_1F_0 ATP synthase results in enzyme reactivation (Giorgio *et al*. 2013).

However, other proteins have been extensively studied and are proposed to have a role in mPTP opening regulation, such as voltage-dependent anion channel (VDAC) and adenine nucleotide translocase (ANT) (Halestrap and Davidson, 1990; Crompton *et al*. 1999; Halestrap, 2014).

Trypanosoma cruzi is the protozoan parasite that causes Chagas disease or American trypanosomiasis. This is a potentially life-threatening illness and it is estimated that about 7 million to 8 million people are infected, mostly in Latin America, where Chagas disease is endemic, but also in other parts of the world through migration from endemic areas (WHO, 2014).

We have previously described the CyP gene family in *T. cruzi* which consists of 15 members (Potenza *et al*. 2006; Bua *et al*. 2008). Analyses of clusters formed by the *T. cruzi* cyclophilins with others encoded in various genomes revealed that 8 of them (*Tc*CyP19, *Tc*CyP21, *Tc*CyP22, *Tc*CyP24, *Tc*CyP35, *Tc*CyP40, *Tc*CyP42 and *Tc*CyP110) have orthologues in many different genome. Besides, 4 CyPs of the family (of 19, 22, 28 and 40 kDa, respectively) have been isolated with a

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CsA-affinity column from parasites lysate. They present PPIase activity, which could be inhibited by CsA *in vitro* (Potenza *et al*. 2006).

Programmed cell death has been described in mammalian cells but has also been suggested for unicellular organisms, including Trypanosomatid parasites affecting humans, such as *Leishmania* spp. (Das *et al*. 2001; Gannavaram *et al*. 2008) and *Trypanosoma* spp. (Figarella *et al*. 2005; Piacenza *et al*. 2007). Some of the main features observed in mammalian cell apoptosis, namely the loss of mitochondrial membrane potential, release of cytochrome *c*, condensation of nuclear chromatin, fragmentation of genomic DNA and surface binding of Annexin V, have been described in these unicellular organisms in response to a variety of stress inducers and parasiticidal drugs (Ameisen *et al*. 1995; Duszenko *et al*. 2006). However, the protozoan cell death pathways and the effector molecules involved in these processes remain to be identified. Several articles have reported these events as apoptotic (Jiménez-Ruiz *et al*. 2010) *et al*. or incidental (Proto *et al*. 2013). Furthermore, the existence of a mPTP-like pore in protozoan parasites has not been previously described.

In the present work, we studied the effects of oxidative stress on *T. cruzi* epimastigotes by analysing several apoptotic features described for mammals and the protective effect exerted by the cyclophilin (CyP) inhibitor CsA on the parasite.

MATERIALS AND METHODS

Chemical compounds

Protease inhibitors, MOPS and EDTA were obtained from Sigma-Aldrich (Sigma Co., USA). The *In Situ* Cell Death detection kit was purchased from Roche Diagnostics (Manheim, Germany). Anti-tubulin, anti-cytochrome *c* were from Santa Cruz Biotechnology, Inc. (TX, USA). The Annexin V: FITC Apoptosis Detection Kit was from BD Biosciences (Becton Dickinson & Co., NJ, USA). The anti-rabbit immunoglobulin G conjugated with horseradish peroxidase was from Jackson Immunoresearch Labs Inc. (PA, USA). The ECL Western Blotting Detection kit was from GE Healthcare (IL, USA). MitoTracker Deep Red, dichloro-dihydro-fluorescein diacetate (DCFH-DA) and Calcein-AM were from Molecular Probes (NY, USA) CsA, used as a CyP inhibitor, was a gift from Novartis (Basel, Switzerland).

Parasite culture

Trypanosoma cruzi CL Brener clone parasites were cultured in Brain Heart Infusion medium (BHI) (Difco Michigan, USA) 33 g L^{-1}, tryptose 3 g L^{-1}, $Na_2HPO_4{\cdot}2H_2O$ 4 g L^{-1}, ClK 0·4 g L^{-1}, glucose 0·3 g L^{-1}, hemin 0·02 g L^{-1}) supplemented with 10% fetal bovine serum-heat inactivated (FBS). Epimastigotes were collected at 5 days of culture (late log phase) at 28 °C (Potenza *et al*. 2006).

Induction of oxidative stress

Oxidative stress was triggered by incubating 1×10^8 epimastigotes in exponential phase of growth with 5 mM H_2O_2 (in some assays 1 mM H_2O_2 incubation was performed) for different periods of time (30 to 180 min, as indicated for each assay) at room temperature. Parasites were centrifuged at 2300 rpm for 10 min and washed once in PBS-3% glucose. Pellets were resuspended in PBS-3% glucose for subsequent studies. To inhibit CyP, parasites were incubated with 1 μM CsA for 1 h at room temperature prior to H_2O_2 incubation.

Detection of Annexin V binding

A common method to detect phosphatidylserine (PS) on a cell surface is the use of Annexin V, a protein which binds PS with high affinity (Van Heerde *et al*. 1994). Oxidative stress was induced with 5 mM H_2O_2 and samples were collected at different timepoints (30, 60 or 180 min). When inhibitory conditions were required, pre-incubation with 1 μM CsA for 1 h was performed. Annexin V binding was detected on the external surface of the plasma membrane of treated parasites using the Annexin V: FITC Apoptosis Detection Kit according to the manufacturer's protocol. Co-staining of the parasites with propidium iodide was performed, to evaluate the integrity of plasma membrane during the treatments. Fluorescence was detected in a FACSCalibur equipment (Becton Dickinson & Co., NJ, USA). Data were analysed using Cyflogic software.

Reactive oxygen species (ROS) production

To evaluate ROS production, the fluorescent dye DCFH-DA was used. DCFH-DA is a nonpolar dye that is converted into the polar derivative non-fluorescent DCFH by cellular esterases, and can be switched into the highly fluorescent DCF when oxidized by intracellular ROS and other peroxides. Epimastigotes (1×10^7 in log phase) were collected, washed once in PBS-3% glucose and resuspended in PBS-3% glucose. Parasites were incubated with 1 μM CsA for 1 h at room temperature prior to H_2O_2 incubation. To induce oxidative stress, two different concentrations of H_2O_2 (1 and 5 mM) were tested for 1 h at room temperature. After treatment, parasites were loaded with 10 μM of the fluorescent dye DCFH-DA for 30 min at 28 °C. Parasites were washed once in PBS-3% glucose and resuspended in PBS for flow cytometry. Fluorescence

was detected in a FACSCalibur equipment (Becton Dickinson & Co., NJ, USA). Data were analysed using Cyflogic software.

Subcellular fractionation

Parasites were fractionated following a described protocol (Gannavaram *et al*. 2008). Briefly, 1×10^8 epimastigotes (in log phase) were collected and washed three times in 15 mL MES buffer (20 mM MOPS, pH 7·0, 250 mM sucrose, 3 mM EDTA). Parasites were resuspended in 0·2 mL MES buffer containing 4 mg mL^{-1} digitonin and protease inhibitors: 1 mM pepstatine A, 1 mM phenylmethylsulfonylfluoride and 0·1 mM Na-ptosyl-L-lysine chloro-methyl ketone. The suspension was incubated at room temperature for 5 min and centrifuged at 10 000 ***g*** for 5 min. The resulting supernatant was collected as the cytosolic fraction and the membrane pellet as the mitochondria-enriched fraction. This fraction was resuspended in phosphate buffer (20 mM sodium phosphate, pH 7·0, 3 mM EDTA) and citrate synthase, a mitochondrial marker enzyme, was assayed as previously described (Cannata and Cazzulo, 1984), to confirm a successful procedure.

Cytochrome c release

To study cytochrome *c* localization after oxidative stress induction, 1×10^8 epimastigotes (in log phase) were collected, washed once in PBS-3% glucose and resuspended in PBS-3% glucose. Parasites were incubated with 1 μM CsA for 1 h at room temperature prior to H_2O_2 incubation. To induce oxidative stress, parasites were incubated with 5 mM H_2O_2 for 3 h at room temperature. After treatment, cytosolic and mitochondria-enriched fractions were obtained as described before. Fractions were separated by SDS-PAGE, carried out as described (Laemmli, 1970). Proteins were electrotransferred from 13·5% polyacrylamide gels to nitrocellulose membranes, which were blocked with a 5% (W/V) non-fat milk suspension for 1 h at room temperature. After incubation for 2 h with a rabbit polyclonal antibody against cytochrome *c* (1:200) and a 1:1500 dilution of the goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidise. The *T. cruzi* cytochrome *c*, size ~12 kDa protein (TriTrypsDB annotated as TcCLB.506949·50) was visualized by chemiluminescence with an ECL Western Blotting Detection kit.

In situ *labelling of DNA fragments*

To evaluate DNA integrity after oxidative stress treatment, 1×10^7 epimastigotes (in log phase) were collected, washed once and resuspended in PBS-3% glucose. Parasites were incubated with 1 μM CsA for 1 h at room temperature prior to H_2O_2 incubation. To induce oxidative stress, parasites were incubated with 5 mM H_2O_2 for 3 h at room temperature.

In situ detection of DNA fragments by TUNEL was performed using the *In Situ* Cell Death detection kit (Roche). Epimastigotes were harvested, fixed in 4% formaldehyde and coated onto poly-(L-lysine) covered slides. Permeabilization was done with 0·1% (v/v) Triton X-100/PBS for 5 min at room temperature, followed by incubation with TdT buffer containing nucleotide mix (50 mM fluorescein-12-dUTP, 100 mM dATP, 10 mM Tris-HCl, 1 mM EDTA, pH 7·6) for 1 h at 37 °C. The samples were counterstained with 10 mg mL^{-1} propidium iodide and visualized under a fluorescence microscope. The percentage of parasites showing a clearly visible nuclear staining was determined in treated and control parasites by counting 100 cells in triplicate (blind-coded samples).

DNA fragmentation assay by agarose gel electrophoresis

DNA integrity after treatments was also evaluated by electrophoresis in agarose gel. Briefly, 1×10^7 epimastigotes (in log phase) were collected, washed once and resuspended in PBS-3% glucose. Parasites were incubated with 1 μM CsA for 1 h at room temperature prior to H_2O_2 incubation. To induce oxidative stress, parasites were incubated with 5 mM H_2O_2 for 3 h at room temperature. Total cellular DNA was isolated by a usual procedure and analysed by agarose gel electrophoresis. Pellets of 10^7 epimastigotes were treated with sarkosyl detergent lysis buffer (50 mM Tris, 10 mM EDTA, 0·5% w/v sodium-N-lauryl sarcosine, pH 7·5) and 7 mM proteinase K, vortexed and allowed to digest overnight at 50 °C. RNase A (10 mM) treatment followed for 1 h at 37 °C. The lysates were then extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged at 16 000 ***g*** for 5 min. The upper phase was treated with 3 M sodium acetate and 100% ethanol overnight at −20 °C. The sample was centrifuged at 16 000 ***g*** for 10 min and the pellet was washed with 0·5 ml of 70% ethanol. DNA was solubilized in Tris/EDTA (10/1 mM) buffer and spectrophotometrically quantitated at 260/280 nm. Total DNA was mixed with tracking dye and loaded on 1% agarose gels containing ethidium bromide. Gels were run for 2·5 h at 50 V.

mPTP-like opening

Calcein is a fluorescent dye of 1 kDa, which enables the study of mPTP. Thus, the calcein quenching method is a highly selective indicator of sustained mPTP opening *in situ* in mammals (Petronilli et al. 1998). In the presence of $CoCl_2$, fluorescence of

calcein is quenched from the cytosol and nuclear compartments, remaining in mitochondria. When the mPTP opens, calcein is lost from mitochondria.

To perform this assay with *T. cruzi* epimastigotes, a protocol adapted from a previous report was followed (Petronilli *et al*. 1998). Briefly, 1×10^6 epimastigotes were loaded with 2 μM of Calcein-AM and 1 mM $CoCl_2$ at 37 °C for 30 min. After staining, parasites were incubated with 1 μM CsA (when CyP inhibition was assayed) or with a combination of the other inhibitors of mPTP components (1 mM ADP and 10 μM Bongkrekic acid) for 1 h at room temperature. Oxidative stress was induced with 5 mM H_2O_2 for 45 min at room temperature and washed with PBS-3% glucose to remove the excess of Calcein-AM and $CoCl_2$. Samples were analysed using a FACSCalibur equipment as mentioned above, with appropriate excitation and emission filters for fluorescein. The decrease in fluorescence intensity indicated mPTP - like opening.

Statistical analysis

Experiments were performed in duplicate or triplicate, as previously indicated, in two to four independent experiments. Statistical analysis of the *in vitro* parasite experiments was performed using Student's *t* test, for paired values. The values presented are the means±S.D. of 2 or more independent experiments. Values of $P < 0{\cdot}05$ were considered statistically significant.

RESULTS

CsA *decreases Annexin V binding induced by* H_2O_2

One of the first phenotypic changes observed during metazoan programmed cell death is the exposure of PS in the external surface of plasma membrane, which occurs after the cell has received the injure signal and will start to develop the cell death program. This event can be tested with the protein Annexin V, which binds PS with high affinity.

To investigate if PS exposure also occurs in *T. cruzi* after oxidative stress induction, parasites were treated with hydrogen peroxide (H_2O_2). CsA was included to evaluate this event in the presence of a CyP inhibitor.

Epimastigotes in exponential growth phase were incubated with 5 mM H_2O_2 for different periods of time. Annexin V binding was detected after 30 min incubation with 5 mM H_2O_2 and increased up to 180 min (Fig. 1A), compared to the Control (untreated) parasites. When incubation with 1 μM CsA for 1 h was done prior to oxidative stress induction, Annexin V binding was significantly decreased after 30 and 60 min of H_2O_2 treatment. The absence of propidium iodide staining in the elapsed time indicated membrane integrity and ensured only the detection of Annexin V binding in the external membrane surface (Fig. 1B).

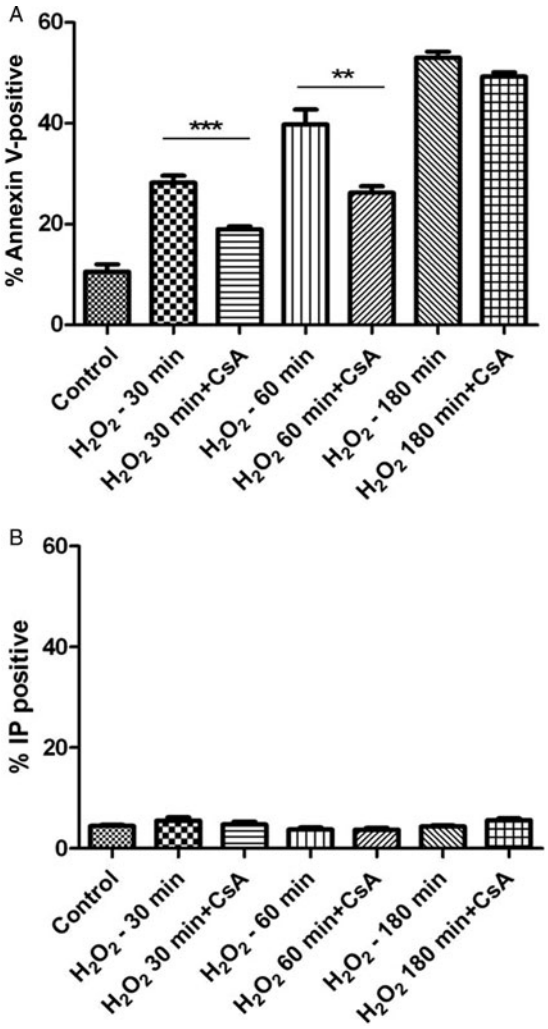

Fig. 1. The CyP inhibitor CsA decreases Annexin V binding in *T. cruzi* epimastigotes after oxidative stress. (A) Untreated epimastigotes (control), parasites treated with 5 mM H_2O_2 for different periods of time or pre-incubated with 1 μM CsA were stained with Annexin V-FITC to detect phosphatidylserine exposure in the external surface of the plasma membrane by flow cytometry. Means±S.D., $n = 4$ are shown, ***(H_2O_2-30 min/H_2O_2-30 min+CsA), **(H_2O_2-60 min/H_2O_2-60 min+CsA), $P < 0{\cdot}05$, Student's *t*-test). (B) Propidium iodide was also measured in parasites from (A). Absence of significant propidium iodide staining during the experiment indicates plasma membrane integrity, meaning that Annexin V-FITC is only binding to the exposed phosphatidylserine. Means±S.D., $n = 4$ are shown, ***(H_2O_2-30 min/H_2O_2-30 min +CsA), **(H_2O_2-60 min/H_2O_2-60 min+CsA), $P < 0{\cdot}05$, Student's *t*-test).

In this oxidative stress condition, *T. cruzi* epimastigotes responded by exposing PS in their external surface of the plasma membrane, as an early indicator of programmed cell death in this protozoan parasite. Pre-incubation with CsA significantly decreased the Annexin V signal, indicating that a CyP may be involved in the cascade of events that

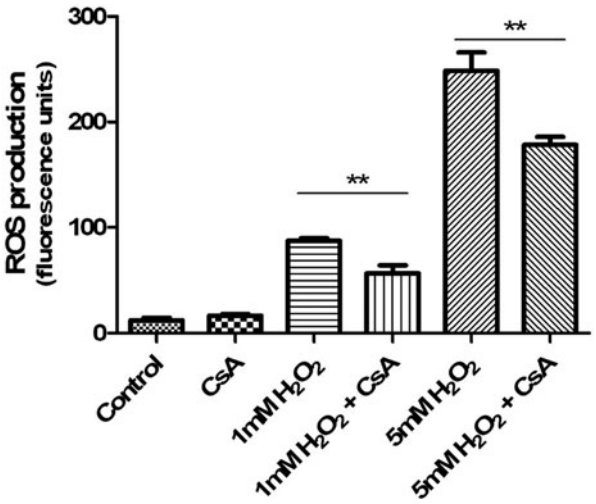

Fig. 2. CsA decreases ROS production in *T. cruzi* epimastigotes after oxidative stress. Parasites were incubated with two different concentrations of H_2O_2 (1 or 5 mM). Pre-incubation with 1 μM CsA was performed when inhibitory conditions were required. After oxidative stress induction, parasites were loaded with the fluorescent dye DCFH-DA. ROS were quantified by flow cytometry (means±S.D., $n = 3$, $P < 0{\cdot}05$, Student's *t*-test).

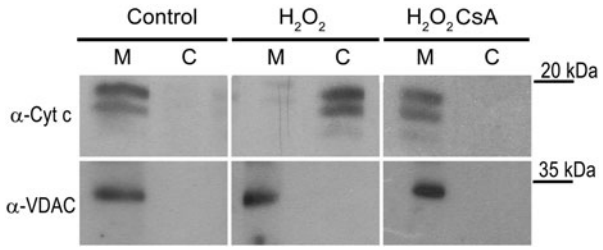

Fig. 3. CsA prevents cytochrome *c* translocation into the cytosol of *T. cruzi* epimastigotes during oxidative stress. Oxidative stress was induced with 5 mM H_2O_2 for 3 h at room temperature. Pre-incubation with 1 μM CsA for 1 h at the same temperature was performed when inhibitory conditions were required. Anti-cytochrome *c* antibodies were used in Western blots with the mitochondria-enriched subcellular fraction (M) and the cytosolic subcellular fraction (C). Membranes were stripped and re-incubated with antibodies against the mitochondrial protein VDAC to confirm the purity of the mitochondria-enriched fractions.

lead to PS exposure. However, after longer exposures with the inducer (180 min), the protective effect of CsA disappeared.

Reactive oxygen species (ROS) production after treatment with H_2O_2

In mammals, mPTP opening is triggered by a synergic combination of increased ROS production together with high levels of Ca^{2+} in the mitochondrial matrix (Figueira *et al*. 2013).

To detect oxidative species in parasites after H_2O_2 treatment and in the presence of CsA, the DCFH-DA dye was used, as detailed in the M&M section. Epimastigotes were treated with two different concentrations of H_2O_2 for 1 h. Incubation with 1 μM CsA for 1 h was performed prior to H_2O_2 treatment. Pre-incubation with CsA significantly decreased ROS production when parasites were incubated with both 1 mM H_2O_2 and 5 mM H_2O_2 (Fig. 2), showing that when the CyP inhibitor is present there is a protection against the damaging effects of H_2O_2. It is important to highlight that the concentration of CsA used in control parasites did not yield a significant amount of ROS *per se*.

Cytochrome c translocation into the cytosol after oxidative stress is prevented by CsA

Translocation of proteins such as cytochrome *c*, apoptosis-inducing factor and endonuclease G from the mitochondrial matrix into the cytoplasm is a critical event during metazoan programmed cell death. This change in localization of apoptosis-related proteins occurs as a consequence of the change in mitochondrial membrane permeability caused by increased ROS and Ca^{2+} overload.

Having established that the induction with H_2O_2 caused an increase in oxidative species in *T. cruzi*, we wanted to investigate if this would also result in translocation of these mitochondrial proteins in the parasite.

To evaluate cytochrome *c* localization after oxidative stress induction, parasites were either treated treated with 5 mM H_2O_2 or not treated (control), or incubated with 1 μM CsA prior to H_2O_2. After treatment, parasites were fractionated using digitonin to obtain a mitochondria-enriched fraction and a cytosolic fraction, and their proteins were separated by SDS-PAGE and immuno-blotted with antibodies against cytochrome *c*. After oxidative stress induction with H_2O_2, cytochrome *c* was released from mitochondria and detected in the cytosolic fraction (Fig. 3, H_2O_2). However, when parasites were pre-treated with CsA, cytochrome *c* remained mostly in the mitochondria-enriched fraction (Fig. 3, H_2O_2 CsA). To confirm the purity of the mitochondria-enriched fractions, these blots were stripped and re-probed for VDAC, protein size ~30 kDa (TriTrypsDB annotated as TcCLB.509141.40) which is located to the outer mitochondrial membrane. These results show that the presence of CsA prevented the release of cytochrome *c* from mitochondria in response to oxidative stress, in agreement with the results obtained for ROS production, suggesting that a CyP is involved in these mitochondria-related events.

CsA *inhibits DNA degradation induced by H_2O_2*

A later step in the cascade of events leading to programmed cell death is marked by changes to the

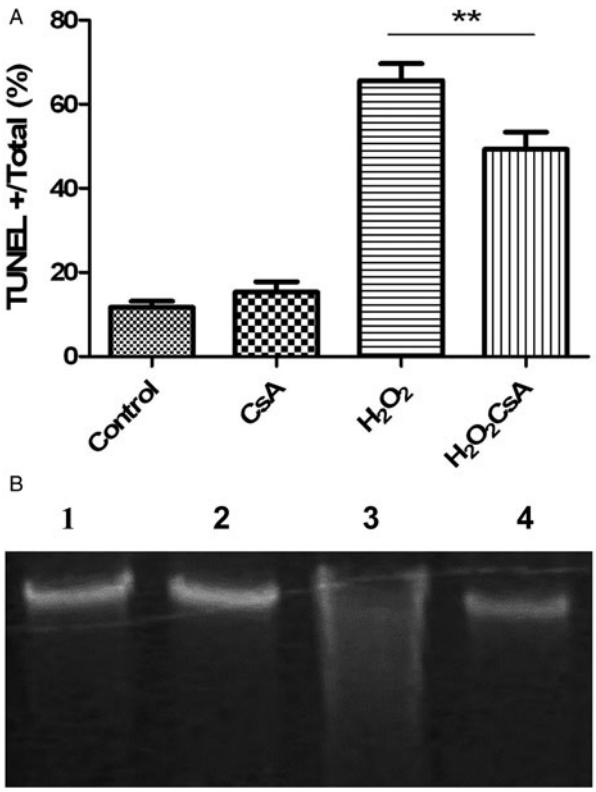

Fig. 4. CsA decreases DNA damage in *T. cruzi* epimastigotes during oxidative stress. (A) *T. cruzi* epimastigotes were subjected to oxidative stress with 5 mM H_2O_2. Parasites with positive nucleus staining were counted as TUNEL positive. The percentage is expressed as TUNEL-positive/total parasites. Significant differences were observed when parasites were treated with CsA prior to incubation with 5 mM H_2O_2 (means±S.D., $n = 3$, $P <$ 0·05, Student's *t*-test). (B) After inducing oxidative stress with 5 mM H_2O_2 genomic DNA was extracted with phenol/chloroform, electrophoresed in 1% agarose gel and visualized by ethidium bromide. DNA degradation observed in 5 mM H_2O_2-treated parasites (lane 3) was prevented by pre-incubation with 1 μM CsA (lane 4), compared to untreated parasites (lane 1) and 1 μM CsA-treated parasites (lane 2).

nucleus. DNA degradation, chromatin condensation and changes in nuclear morphology are often considered the best indicators of an apoptotic process, being DNA degradation probably the most frequent marker of apoptotic death used in metazoans. DNA fragmentation revealed by the presence of strand breaks is considered to be the gold standard for identification of apoptotic cells (Jiménez-Ruiz *et al.* 2010).

To further characterize the changes occurring in H_2O_2-treated *T. cruzi* epimastigotes, *in situ* detection of DNA fragments by TUNEL was performed to detect DNA 3′ free ends after breakage. Figure 4A shows the percentage of cells with positive staining in control (C and CsA) or treatment (H_2O_2 and H_2O_2+CsA) conditions. Epimastigotes treated with 5 mM H_2O_2 showed 67% of TUNEL-positive cells *vs* 42% when parasites were pre-incubated with CsA.

To evaluate genomic DNA degradation, we used electrophoresis in agarose gel of total epimastigotes DNA. A massive degradation of genomic DNA was visualized in 5 mM H_2O_2-treated epimastigotes (Fig. 4B, lane 3). This pattern was not observed when parasites were incubated with CsA prior to oxidative stress induction with H_2O_2 (Fig. 4B, lane 4).

The degraded DNA pattern observed for trypanosomatids showed some degree of smearing and although it is not as clear as the typical expected DNA ladder from metazoan DNA, it is consistent with other reports in protozoan parasites (Jiménez-Ruiz *et al.* 2010).

These results taken together showed that, when CsA is present prior to the addition of the oxidative stress inducer, the genetic material of the parasites can be partially protected from the damaging effect of H_2O_2. Again, this is another feature that can be partially inhibited by CsA, showing indirectly that a CyP is involved in programmed cell death in the parasite.

mPTP-like opening occurs in Trypanosoma cruzi *and responds to mammalian mPTP blockers*

The mPTP is a crucial component of cell death events in mammals. Although its molecular identity remains under debate, it is well established that it is the mPTP opening in mitochondrial membrane which leads to the release of apoptotic-related proteins and in consequence, the completion of cell death program.

To investigate whether the same mechanism occurs in trypanosomatids, we used the highly selective indicator of sustained mPTP opening *in situ*, the fluorescent dye calcein. When Calcein-AM is co-loaded with $CoCl_2$, calcein fluorescence is quenched in both cytosolic and nuclear compartments, allowing the detection of calcein fluorescence in mitochondria. As we found no previous reports about the calcein quenching method in protozoan parasites, we first attempted to establish the correct loading dye in parasites. *T. cruzi* epimastigotes were loaded with calcein and the quencher $CoCl_2$. The proper localization of calcein in the parasite mitochondrion was confirmed by fluorescence microscopy of live cells (data not shown). After finding that epimastigotes could be loaded with the fluorescent probe, we evaluated if calcein was released from parasite mitochondria in response to oxidative stress induction. After 15 min in the presence of 5 mM H_2O_2, almost 50% of the calcein was released from parasite mitochondria, and remained constant up to 90 min in oxidative stress conditions (Fig. 5). Having established the appropriate conditions to study calcein release from parasite mitochondria, we evaluated the effect of CsA and other inhibitors described for mammalian mPTP

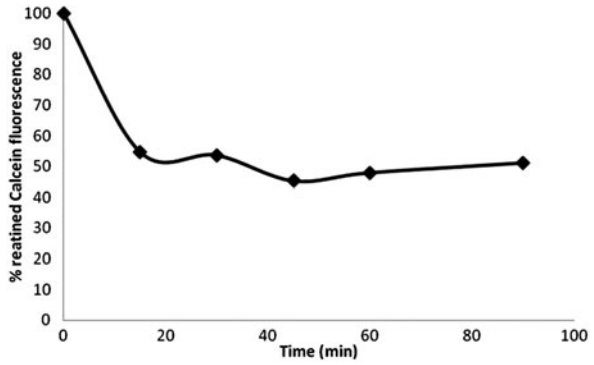

Fig. 5. To evaluate mPTP-like opening in *T. cruzi* epimastigotes, the calcein quenching method was used. Parasites were loaded with 2 μM calcein-AM and 1 mM $CoCl_2$ to detect the fluorescent dye retained within the mitochondria and treated with 5 mM H_2O_2 for 90 min. At the indicated time points, fluorescence was measured by flow cytometry. Data were analysed with Cyflogic software. Results are representative of two independent experiments.

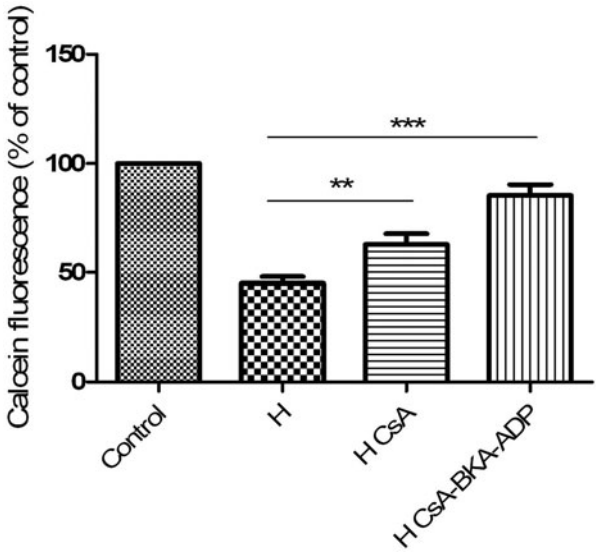

Fig. 6. mPTP-like opening occurs in *T. cruzi* and responds to mammalian mPTP blockers. After having established the proper conditions for the calcein quenching method in our model, the effect of CsA or a combination of known mPTP blockers were evaluated. Parasites were untreated (control) or treated with 5 mM H_2O_2 (H). When inhibition of mPTP was assayed, pre-incubation with CsA (H CsA) or with a combination of the inhibitors CsA+ ADP+ Bongkrekic acid (H CsA–ADP–BKA) was performed. (means±S.D., $n = 3$, **(H_2O_2/H_2O_2 CsA), ***(H_2O_2/H_2O_2 CsA–BKA–ADP), $P < 0{\cdot}05$, Student's *t*-test).

components: Bongkrekic acid and ADP, which inhibit ANT. Figure 6 shows the fluorescent calcein retained from parasites treated with 5 mM H_2O_2 for 45 min (H) or pre-incubated either with CsA (H CsA) or a combination of CsA + Bongkrekic acid + ADP (H CsA-ADP-BKA) compared to untreated parasites (control). When parasites were treated with H_2O_2, ~45% of calcein fluorescence was retained in mitochondria (Fig. 6, column H). However, parasites pre-treated with CsA showed increased calcein retention (60%). When the combination of mPTP inhibitors was assayed, parasites were able to retain a higher amount (86%, Fig. 6).

These results suggest the existence of a mPTP-like pore in *T. cruzi* parasites, which responds to inhibitors described for mammalian mPTP components. In particular, *T. cruzi* mPTP-like appears to respond to CsA, which is one of the best characterized mPTP inhibitors in mammals by binding the mitochondrial CyPD.

DISCUSSION

In this study, we report several programmed cell death features in the protozoan parasite *Trypanosoma cruzi* that were sensitive to the inhibitors of the mammalian mPTP. It has been described that mPTP opening in mammals leads to cell death (Vaseva *et al*. 2012), and whether cell death in protozoan parasites is programmed (Ameisen *et al*. 1995) or incidental (Proto *et al*. 2013) the molecular mechanisms and effectors remain unknown. Several cell death inducers have been tested in different protozoan parasites: chloroquine and staurosporine were assayed in *Plasmodium falciparum* (Meslin *et al*. 2007), prostaglandins in *T. brucei* (Figarella *et al*. 2005, 2006), miltefosine in *Leishmania donovani* (Paris *et al*. 2004) and fresh human serum (Piacenza *et al*. 2001) and starvation (Jimenez *et al*. 2008) in *T. cruzi*.

Oxidative stress with H_2O_2 has also been used by other research groups and, in this work, in the same range of concentrations, from 1 to 5 mM, as an inducer of cell death in *T. cruzi* (Das *et al*. 2001; Fernández Villamil *et al*. 2008; Jiménez-Ruiz *et al*. 2010).

PS exposure, which is an early event in programmed cell death in mammals, had been already reported to occur in *T. cruzi* (Piacenza *et al*. 2007; Jimenez *et al*. 2008) and in *T. brucei* (Figarella *et al*. 2005) as shown by Annexin V binding. Although there may be other PS-containing phospholipids, Annexin V binds PS with high affinity in *T. cruzi*. After oxidative stress induction with 5 mM H_2O_2, we demonstrated that Annexin V binding occurred in *T. cruzi* epimastigotes after 30 min exposure to H_2O_2 and continued up to 180 min. Interestingly, pre-incubation with CsA partially decreased Annexin V binding in H_2O_2-treated parasites.

Regarding mitochondrial function, cytochrome *c* was detected in parasite cytosolic fractions after 30 min treatment with 5 mM H_2O_2, consistent with a

previous report (Piacenza *et al*. 2007). In our model, pre-incubation for 60 min with 1 μM CsA partially prevented the translocation of cytochrome *c* into the parasite cytosolic fraction.

PARP proteolytic processing is another feature of programmed cell death frequently evidenced in metazoans. PARP is a family of abundant nuclear proteins, some of which are involved in the DNA base excision repair system. In metazoans, the specific cleavage of PARP-1 has been used extensively as a biochemical marker of apoptosis (Jiménez-Ruiz *et al*. 2010). In *T. cruzi,* the counterpart *Tc*PARP has been characterized (Fernández Villamil *et al*. 2008). After inducing oxidative stress with 5 mM H_2O_2, we were also able to detect that *Tc*PARP was cleaved. This cleavage was decreased by pre-incubation with CsA (data not shown).

During apoptosis, as repair mechanisms are inhibited, DNA degradation will occur by activated nucleases that migrate to the nucleus to degrade DNA.

In *T. cruzi* epimastigotes, DNA degradation had been reported after cell death induction with 20% (V/V) fresh human serum. We confirmed by both TUNEL and electrophoresis in agarose gel that degradation occurred after treatment with 5 mM H_2O_2. This degradation pattern was not observed when epimastigotes were pre-incubated with 1 μM CsA, probably due to the inhibition of previous steps of the cell death cascade. This phenomenon has also been reported in other protozoan parasites as *Leishmania donovani* under oxidative stress induction with 3 mM H_2O_2 (Das *et al*. 2001). It is important to highlight that, although an internucleosomal ladder pattern is the common feature in metazoan programmed cell death, this is not the case in protozoan parasites (Jiménez-Ruiz *et al*. 2010), where a smear pattern is usually observed.

It is important to point out that, in all the experiments performed in this study, a protective effect was achieved with pre-incubation with 1 μM CsA. When we added some other inhibitors such as ADP and Bongkrekic acid, which block the mammalian mPTP related protein ANT, we observed an even higher protective effect against cell death in the parasite. This potential effect was observed using the calcein/$CoCl_2$ quenching method, which is a highly selective indicator of mPTP opening in mammals (Petronilli *et al*. 1998; Kroemer *et al*. 2007; Vaseva *et al*. 2012). After 15 min of oxidative stress induction in *T. cruzi* epimastigotes, most calcein was released from mitochondria, but pre-incubation with CsA retained up to ~60% calcein inside the parasite. When inhibitors of other mammalian mPTP components were included (1 mM ADP+10 μM Bongkrekic acid), the calcein retained in parasite mitochondria increased up to 86%, evidencing a drug additive effect in inhibiting a probable mPTP-like opening present in *T. cruzi*.

When we searched similarities with proteins that participate in the formation of the mammalian mPTP in the *T. cruzi* genome databases, we found some putative predicted proteins in the parasite with high similarity to mammalian ANT and VDAC, among others. The mPTP protein components have not been fully described in mammals and, to our knowledge, in any protozoan parasite.

Although CsA may exert some inhibitory effect on other Cyclophilins, it is well known that it has a specific cell death protective effect as a mPTP blocker by binding the mitochondrial CyPD (Connern and Halestrap, 1994; Friberg *et al*. 1998). CsA has thus become the usual molecular tool to analyse the role of CyPD in the mPTP in different models (Vaseva *et al*. 2012; Kim *et al*. 2014), while more specific CyPD inhibitors are still under development (Malouitre *et al*. 2009).

We have previously described the CyP gene family in *T. cruzi* which consists of 15 members, while four CyPs of 19, 22, 28 and 40 kDa have been isolated as CsA-binding proteins (Potenza *et al*. 2006; Bua *et al*. 2008).

As *T. cruzi* has a CyPD orthologue in its genome, current efforts are being made to confirm the role of a mitochondrial parasite CyP in these mPTP-related events. Ongoing experiments are expected to produce parasites overexpressing a mitochondrial CyP, which will allow to specifically address its responsibility in the programmed cell death features described in this work.

To our knowledge, the results presented in this article describe for the first time a mPTP-like opening that would be involved in a unicellular parasite cell death. Our findings also encourage further biochemical studies to determine the specific role of a *T. cruzi* mitochondrial CyP homologous to mammalian CyPD, considering that it has not been described in any unicellular parasite yet.

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