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Role of *Trypanosoma cruzi* peroxiredoxins in mitochondrial bioenergetics

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Abstract *Trypanosoma cruzi* cytosolic (TcCPx) and mitochondrial tryparedoxin peroxidase (TcMPx) play a fundamental role in H_2O_2 detoxification. Herein, mitochondrial bioenergetics was evaluated in cells that overexpressed TcCPx (CPx) and TcMPx (MPx) and in pTEX. In MPx, a higher expression was observed for TcCPx, and the same correlation was true for CPx. Differences in H_2O_2 release among the overexpressing cells were detected when the mitochondrial respiratory chain was inhibited using antimycin A or thenoyltrifluoroacetone. MPx had higher O_2 consumption rates than pTEX and CPx, especially in the presence of oligomycin. In all of the cells, the mitochondrial membrane potential and the ATP levels were similar. Because of the mild uncoupling that was observed in MPx, the presence or induction of a proton transporter in the mitochondrial membrane is suggested when TcMPx is expressed at higher levels. Our results show a possible interplay between the cytosolic and mitochondrial antioxidant systems in a trypanosomatid.

Keywords *Trypanosoma cruzi* · Tryparedoxin peroxidase · Mitochondria

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Introduction

Trypanosoma cruzi is the etiologic agent of Chagas disease, which is an infection that affects 18–20 million people in Latin America. There is no available vaccine, and chemotherapeutics have not improved over the years. Unfortunately, the currently available treatment is associated with a high frequency of serious side effects and has a low efficacy during the chronic phase of the disease (Wilkinson et al. 2008).

In the search for targets for new chemotherapeutic approaches, the parasite's antioxidant defense system has attracted attention. This system is unique to the trypanosomatids. Although the antioxidant defense system was previously considered deficient (Boveris et al. 1980), research has shown that it has a wide range of substrate specificities and intracellular locations (Wilkinson et al. 2002; Wilkinson et al. 2003). In this sense, the antioxidant defense system enables the parasite to defend itself from the reactive oxygen species (ROS) that are generated by the host immune system (Piacenza et al. 2009) and from its own metabolism (Boveris and Stoppani 1977). The capacity of *T. cruzi* to evade the host-oxidative response is directly correlated to the success of the invasion process and the establishment of the disease (Piacenza et al. 2009; Piñeyro et al. 2008).

The mitochondrion plays a special role in cell viability because it is the main intracellular site of ATP generation and a source of signaling molecules that control cell cycle, proliferation and apoptosis (Cadenas 2004). In mammalian cells during oxidative metabolism, 0.1%–0.5% of the oxygen that is consumed by the mitochondria is partially reduced, leading to the formation of superoxide anions (Vercesi et al. 2006), which are converted to H_2O_2 nonenzymatically or through the action of superoxide

dismutase. In this sense, if this generation takes place inside the mitochondrion and exceeds the antioxidant capacity of this organelle, an oxidative stress state could be created, and mitochondrial activity would be impaired (Monteiro et al. 2004).

In *T. cruzi*, mitochondrial H_2O_2 and small-chain organic hydroperoxide detoxification occurs through mitochondrial tryparedoxin peroxidase (TcMPx) (Wilkinson et al. 2000), which also detoxifies peroxynitrite (Piacenza et al. 2008). TcMPx is found near the kinetoplast and it is suggested to be involved in the protection of mitochondrial DNA from peroxide-mediated damage (Wilkinson et al. 2000). TcMPx is homologous to another peroxiredoxin that is present in the cytoplasm called the cytosolic tryparedoxin peroxidase (TcCPx), which also detoxifies H_2O_2 and peroxynitrite (Piacenza et al. 2008).

In the cytosolic trypanothione-dependent pathway, the reducing substrate for tryparedoxin peroxidase is tryparedoxin (TXN), which in turn is reduced by dihydrotrypanothione ($T(SH)_2$) that is reduced via trypanothione reductase (TR), which uses NADPH as the source of reducing equivalents (Wilkinson et al. 2003). Experimental evidence showing the presence of the trypanothione-dependent pathway in mitochondria is scarce. Intracellular compartmentalization of $T(SH)_2$ has not previously been investigated (Castro et al. 2004), and there is no consensus regarding the presence of TR inside the mitochondrion (Wilkinson et al. 2002; Meziane-Cherif et al. 1994). Two mitochondrial TXN have been identified in *Leishmania infantum*: *Li*TXN2 (Castro et al. 2004) and *Li*TXN3 (Castro et al. 2010). *Li*TXN2 is not essential for parasite survival, and *Li*TXN3 is not a fully active TXN (Castro et al. 2010). Interestingly, phylogenetic analysis suggests that the non-requirement for TXN activity within the mitochondria is a common feature of the Trypanosoma genera (Castro et al. 2010). Therefore, the mechanism that is used by the mitochondrial antioxidant system and is based on TcMPx activity remains unclear.

The relevance of TcCPx and TcMPx in the survival of *T. cruzi*, especially during the invasion process, is now clear. The levels of TcCPx, TcMPx and trypanothione synthetase increase during the differentiation of epimastigotes to metacyclic trypomastigotes (Piacenza et al. 2009), which reflects a preadaptation process of infective parasites to cope with the host immune cell–derived oxidants upon macrophage invasion (Piacenza et al. 2008). Moreover, parasites overexpressing either TcCPx or TcMPx display a higher infectivity in phagocytic and nonphagocytic cells (Piñeyro et al. 2008).

Data from the literature have been focused on understanding how the parasite copes with ROS that have been generated from exogenous sources, i.e., generated during the invasion process (Piacenza et al. 2009) or from H_2O_2 treatment (Wilkinson et al. 2000; Finzi et al. 2004) rather than by its own metabolism. In addition, *T. cruzi* has only one mitochondrion. The study of parasitic bioenergetics and its molecules may be useful for the development of new strategies for chemotherapy. In the current study, the relative importance of TcCPx and TcMPx in the maintenance of mitochondrial integrity and function as well as the resistance to the oxidative challenge that was generated inside the mitochondrion is addressed.

Material and methods

Cell cultures

pTEX (control), TcCPx and TcMPx overexpressing *T. cruzi* epimastigotes, denominated CPx and MPx respectively were grown in LIT medium containing 20 mg/L of hemin and 10% fetal calf serum (Castellani et al. 1967) at 28 °C in the presence of 200 μg/mL of G418 (Finzi et al. 2004). MPx cells were transfected using the pTEX-TcMPx vector, as described for pTEX and CPx cells (Finzi et al. 2004). At the early stationary phase, cells were harvested by centrifugation (1000 × *g* at 4 °C), washed in phosphate-buffered saline (PBS) at a pH 7.3. The number of cells was determined using a Neubauer chamber.

Western blotting analysis

Protein extracts (20 μg) were separated by 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane using the XCell™ II Mini-Cell System (Novex™). Membranes were blocked by incubating with 5% instant nonfat dried milk in 0.05% PBS Tween 20 (PBS-T) for 1 h, washed and incubated in the presence of polyclonal antibodies raised against *T. cruzi* TcMPx (1:1000) for 2 h. After three 15-min washes with PBS-T, the membranes were incubated with HRP-linked anti-rabbit IgG (Cell Signaling Technology®, 1:6000 dilution) for 1 h at room temperature and washed three times with PBS (Nogueira et al. 2006). Bands were revealed using the Super Signal® Detection Kit (Pierce). Data were analyzed using the Scion Imaging® Program and normalized using a loading control (α-GAPDH).

Determination of H_2O_2 IC_{50}

H_2O_2 susceptibility assays to MPx cells were performed as described previously (Finzi et al. 2004).

Determination of H_2O_2 release

10^8 cells/mL were incubated in PBS with 1 mM $MgCl_2$ in the presence of 5 mM succinate, 60 μM digitonin, 1 U/mL of horseradish peroxidase (HRP) and 25 μM Amplex Red

(Molecular Probes®), antimycin A (AA) (2 μg/mL) or thenoyltrifluoroacetone (TTFA) (500 μM). The fluorescence was monitored at excitation and emission wavelengths of 563 nm and 587 nm, respectively, using a Hitachi F2500 fluorescence spectrophotometer with continuous stirring. The quantitative correlation between the fluorescence and the H_2O_2 released by the cells was determined as previously described (Barros et al. 2004).

NADPH production by the pentose phosphate pathway (PPP)

NADPH production in 10^8 cells/mL was determined as previously described (Mielniczki-Pereira et al. 2007).

Oxygen uptake measurements

O_2 consumption was monitored using a computer-interfaced Clark-type oxygen electrode with continuous stirring at 28 °C (Hansatech® Systems Inc., Norfolk, England). Cells (10^8 cells/mL) were incubated in PBS with 1 mM $MgCl_2$ in the absence or presence of 60 μM digitonin and 5 mM succinate. Respiratory control ratios (RCR) (State 3/State 4) were determined after the addition of 400 μM ADP (State 3) and 2 μg/mL of oligomycin (State 4).

Mitochondrial membrane potential ($\Delta\Psi$)

The quantitative determination of $\Delta\Psi$ was performed as previously described (Vercesi et al. 1991).

Determination of intracellular ATP

Experiments were performed according to the manufacturer's instructions using the Enzylight™ ATP Assay Kit (EATP-100). Briefly, cells were harvested by centrifugation (1000 × *g* at 4 °C) and washed in PBS. The luminescence that was obtained by the reaction between the D-luciferin, luciferase and ATP that was released after cell lysis was determined using a luminometer (Packard Bioscience Company®) coupled to the Instrument Control MFC Application, version 3.02. The quantitative luminescence was correlated to the ATP concentration using a standard ATP curve.

Statistical analysis

Data were represented as the averages ± standard errors of at least four independent experiments that were performed in duplicate. Comparisons were conducted using Student's *t*-test in Origin 6.0 software, and $p<0.05$ was considered significant.

Results and discussion

The mitochondrion is the most relevant site for ATP production in eukaryotic cells and is an important intracellular ROS-generating site. Therefore, an efficient antioxidant defense system is required to prevent the effects of oxidative stress. In *T. cruzi*, protection was conferred by the overexpression of the mitochondrial superoxide dismutase against programmed cell death, which was induced by fresh human serum (Piacenza et al. 2007) and nifurtimox or benznidazole treatment (Prathalingham et al. 2007). These results support the relevance of the ROS detoxification pathways in parasitic survival.

To identify the contributions of TcCPx and TcMPx in mitochondrial bioenergetics, stable transformants that overexpressed TcMPx (MPx) were generated according to the protocol that was established for pTEX and CPx cells (Finzi et al. 2004). Transfection did not alter the mobility and the morphological characteristics of the cells (data not shown). As expected, MPx cells showed a 138% increase in TcMPx expression. Interestingly, CPx cells also showed higher levels of TcMPx (46%) compared with those in the control (pTEX) (Fig. 1c). In addition, MPx cells displayed an increase of 24% in TcCPx expression (Fig. 1f). These results suggest the interplay between the antioxidant defense systems that are present in the cytosol and mitochondria.

The growth index (GI) of MPx cells (15.0±0.1) was significantly different from that of pTEX cells but not that of CPx cells (Finzi et al. 2004). Low concentrations of H_2O_2 stimulate cell proliferation in *T. cruzi* (Finzi et al. 2004) and in mammalian (Wiese et al. 1995) and yeast cells (Davies et al. 1995). Therefore, cells with a higher TcMPx or TcCPx content maintain lower H_2O_2 levels than control cells. The decrease in H_2O_2 allows for the stimulation of growth. Regarding the resistance to the oxidative stress that is generated by H_2O_2, other studies have reported a 2-fold increase (Wilkinson et al. 2002) and a 1.4-fold increase (Piacenza et al. 2008) in the resistance to the H_2O_2-mediated oxidative stress in CPx and MPx, respectively. Under our experimental conditions, the resistance to H_2O_2-mediated effects was increased in MPx cells (IC_{50}=142.64±0.6), which is similar to the values reported for CPx cells (Finzi et al. 2004).

In mammalian cells, the bulk of mitochondrial ROS production is derived from the leaking of electrons from the mitochondrial respiratory chain, mainly at complex I, II and III (Kowaltowski et al. 2009). Therefore, mitochondrial H_2O_2 release in digitonin-permeabilized epimastigotes using succinate to support oxygen consumption was determined (Fig. 2). Because of the lack of an inhibitor of trypanosomatid complex I (Hernandez and Turrens 1998), we could not establish the contribution of trypanosomatid

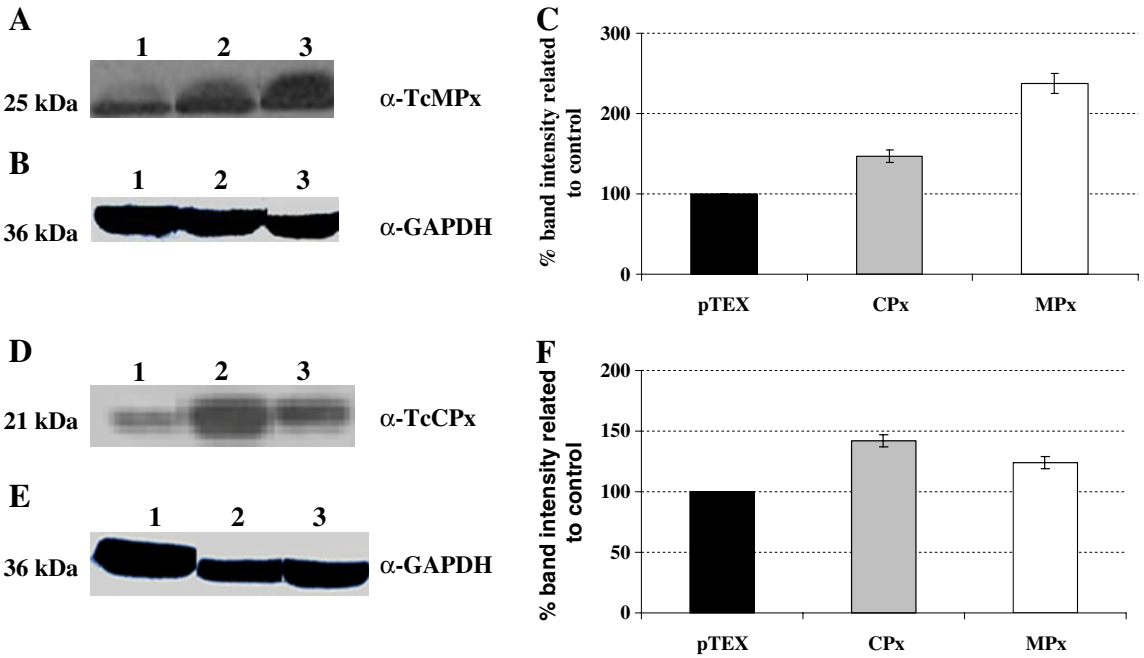

Fig. 1 Overexpression of a tryparedoxin peroxidase increases the expression of its homolog. *T. cruzi* lysates from pTEX, CPx and MPx cells were prepared and resolved by SDS-PAGE (20 μg protein/lane). (**a**) Western blotting analysis of α-TcMPx; lane 1: pTEX, lane 2: CPx, lane 3: MPx. (**b**) Western blotting analysis of α-GAPDH. (**c**) The percentage of the band intensities of (**a**) were normalized to the band in lane 1, which was set to 100%. (**d**) Western blotting analysis of α-TcCPx; lane 1: pTEX, lane 2: CPx, lane 3: MPx. (**e**) Western blotting analysis of α-GAPDH. (**f**) The percentage of the band intensities of (**d**) were normalized to the band in lane 1, which was set to 100%. The best representatives of three independent experiments are shown. *Statistical analysis*: *t*-test: $p<0.05$

complex I to the release of ROS. Under basal conditions, a low level of H_2O_2 was released in pTEX cells, and no H_2O_2 released was detected in overexpressing cells. Significant differences were only observed when the mitochondrial respiratory chain was inhibited using AA or TTFA, which are inhibitors of complex III or II, respectively. Upon mitochondrial respiratory chain impairment by these inhibitors it was clear the protection conferred by TcMPx once lowers levels of H_20_2 than the one in CPx cells were released. Under our experimental conditions, TTFA inhibition led to a higher increase in released H_2O_2 compared with AA treatment. This result confirms the previous findings in *Leishmania donovani* (Mehta and Shaha 2004). H_2O_2 is relatively stable and it is a membrane-

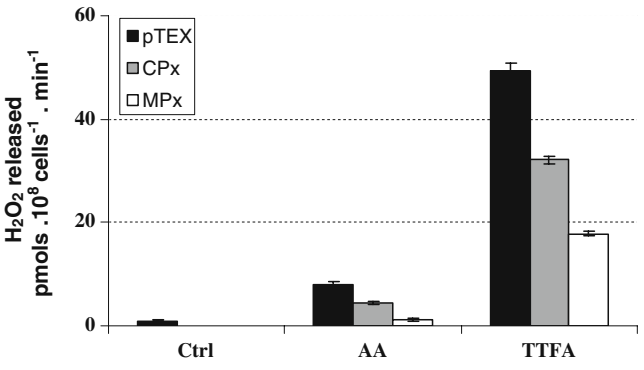

Fig. 2 The levels of H_2O_2 release in CPx and MPx cells increase after inhibition of the mitochondrial respiratory chain. 10^8 cells/mL were incubated in PBS with 1 mM $MgCl_2$ in the presence of 60 μM digitonin, 5 mM succinate, 1 U/mL of HRP, 25 μM Amplex Red (Ctrl) and 2 μg/mL AA or 500 μM TTFA. The data are shown as the average ± standard errors of five independent experiments performed in duplicate. *Statistical analysis*: *t*-test: $p<0.05$ for all, but CPx and MPx under control conditions

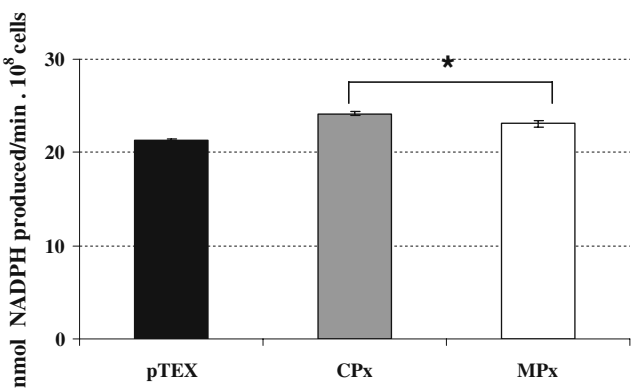

Fig. 3 NADPH production is higher in CPx and MPx cells than in control cells. 10^8 cells/mL were incubated in 50 mM Tris-HCl, pH 7.6, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 250 μM $NADP^+$, 1 mM glucose-6-phosphate and 1 mM 6-phosphogluconate. NADPH production was measured using a spectrophotometer. The values are averages ± standard errors from three independent experiments performed in duplicate. *Statistical analysis*: *t*-test: * $p>0.05$

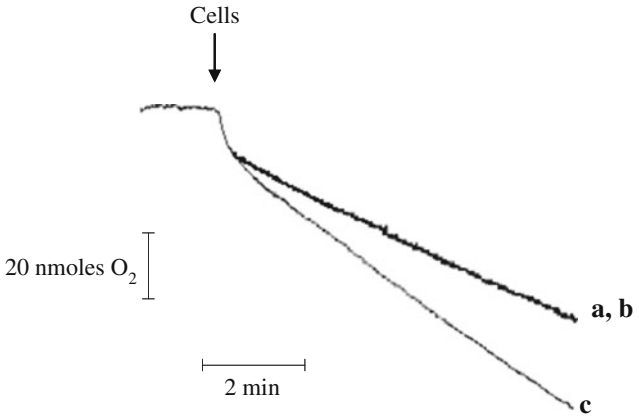

Fig. 4 MPx cells have higher respiratory rates than pTEX and CPx cells. 10^8 cells/mL were added to PBS with 1 mM $MgCl_2$, and the oxygen consumption rates were determined in (**a**) pTEX, (**b**) CPx and (**c**) MPx cells. The best representative of three independent experiments that were performed in duplicate is shown

permeable molecule that is transported by aquaporins that are present in the mitochondrial inner membrane (Kowaltowski et al. 2009). Therefore, H_2O_2 diffuses from the mitochondria into the cytosol and is removed by cytosolic peroxidases, such as TcCPx, which explains the lower levels of H_2O_2 in CPx cells compared with that in control cells. The higher levels of TcMPx that were observed in CPx cells (Fig. 1) may also contribute to the lower levels of released H_2O_2 compared with those of control cells (Fig. 2).

The antioxidants pathways that have been described thus far in *T. cruzi* use trypanothione, which is reduced by TR using NADPH as an electron source (Wilkinson et al. 2003). Therefore, the hydroperoxide detoxification cascade will only function properly if NADPH is being supplied. As shown in Fig. 3, MPx cells showed significantly higher levels of NADPH production compared with pTEX cells. However, MPx cells displayed similar levels of NADPH

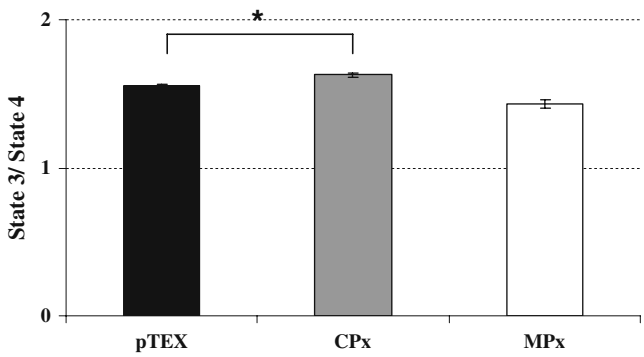

Fig. 5 Respiratory control in MPx cells is lower than that in control and CPx cells. 10^8 cells/mL were added to PBS with 1 mM $MgCl_2$, containing 60 μM digitonin and 5 mM succinate. Respiratory rates were determined in the presence of 400 μM ADP and 2 μg/mL of oligomycin. The data are presented as the average ± standard error of six independent experiments that were performed in duplicate. *Statistical analysis*: *t*-test: * $p>0.05$

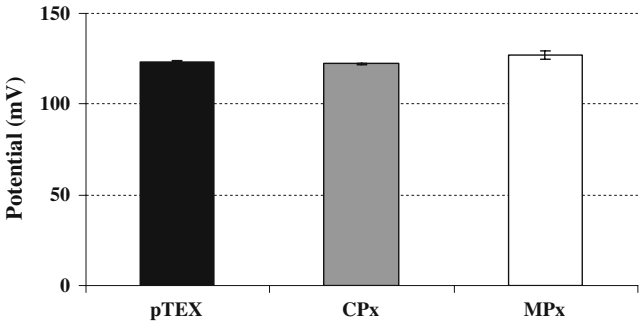

Fig. 6 The mitochondrial membrane potential is similar in pTEX, CPx and MPx cells. 10^8 cells/mL were added to a medium containing 200 mM sucrose, 20 mM Na-HEPES, pH 7.0, 1 mM $MgCl_2$, 0.33 mM EGTA, 60 μM digitonin, 5 mM succinate and 5 μM safranine. The ΔΨ was determined as described. *Statistical analysis*: *t*-test: $p>0.05$

production compared with CPx cells. Until now, the mechanism of the mitochondrial antioxidant system is not well understood. If an interaction between this antioxidant defense system and the cytosolic system exists (Fig. 1), one of the mitochondrial sources for this coenzyme may be the PPP that is present in the cytosol. In addition, the increase in GI in overexpressing cells may be a consequence of an increased G6PD activity in these parasites. In *T. cruzi*, the Tulahuen 2 strain has increased GI and G6PD activity compared with that of the Y strain (Mielniczki-Pereira et al. 2007). In addition, an increase in GI was reported in mammalian cells that overexpressed G6PD (Tian et al. 1998).

The oxygen consumption rates in nonpermeabilized cells are shown in Fig. 4. MPx cells have a higher respiratory rate (10.5 nmoles O_2/min) than pTEX and CPx cells (approximately 7.2 nmoles O_2/min). To further examine this phenomenon, the respiratory control ratio (RCR), which is used to indicate the tight correlation between the mitochondrial respiratory chain and oxidative phosphorylation, was determined. Because of the difficulty in isolating the mitochondrion of *T. cruzi*, the oxygen consumption rates were analyzed in cells that were permeabilized with digitonin (Vercesi et al. 1991; Vercesi et al. 1993). As shown in Fig. 5, MPx cells displayed a 10% lower RCR than the

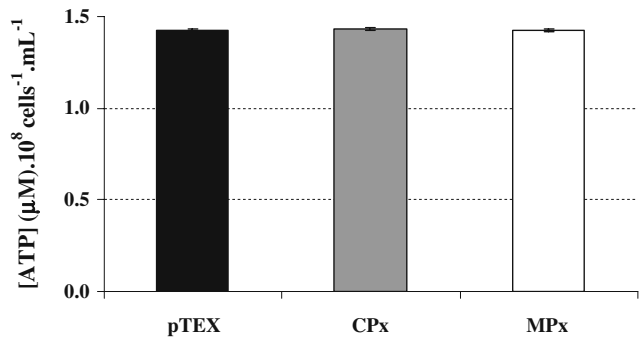

Fig. 7 The intracellular ATP content is similar in pTEX, CPx and MPx cells. ATP levels were determined in 10^8 cells/mL using the manufacturer's protocol. *Statistical analysis*: *t*-test: $p>0.05$

other cells. The increased oxygen consumption rates in the presence of oligomycin (State 4) brings respiration to a nonphosphorylating state (Vercesi et al. 1991) and reflects a mild uncoupling of the respiratory chain. Interestingly, this partial uncoupling and lower RCR did not significantly alter the $\Delta\Psi$ (Fig. 6) or ATP production (Fig. 7).

The activation of mild uncoupling pathways, such as uncoupling proteins (Kowaltowski et al. 1998) or ATP-sensitive K^+ channels (Facundo et al. 2007), which are key regulators of mitochondrial ROS generation (Kowaltowski et al. 2009), decreases the efficiency of oxidative phosphorylation and increases electron transport (Kowaltowski et al. 2009). Therefore, the partial uncoupling of the respiratory chain in MPx cells may occur by a similar process. Although there is no evidence for the presence of the trypanothione-dependent system in the mitochondrion of *T. cruzi*, the mitochondrial detoxification system may be linked to another reduction system, which was proposed by Wilkinson et al. (Wilkinson et al. 2000). This reduction system could be the cytosolic one, where a protein that is anchored in the mitochondrial membrane may link the two systems. This would create another path for the electrons to reenter the matrix, which causes the mild uncoupling and the lower RCR. MPx cells increased their respiratory rates (Fig. 4) to maintain $\Delta\Psi$ at the desired levels for ATP production (Figs. 6 and 7).

Another possibility is that at very low levels, all of the components of the trypanothione-dependent pathway may be present in the mitochondrion. $NADP^+$-dependent isocitrate dehydrogenase (Jo et al. 2001) or NADH-transhydrogenase, which is found in mammalian cells, is the source for NADPH (Kowaltowski et al. 2009). The NADH- transhydrogenase functions as a proton pump using the respiration-generated electrochemical H^+ gradient to displace the reaction toward NADPH formation (Kowaltowski et al. 2009). This feature links the mitochondrial coupling and the membrane potential to the redox potential (Kowaltowski et al. 2009). However, NADH- transhydrogenase has not been identified in trypanosomatids.

Although further research should be conducted to test the above hypothesis, our results indicate for the first time a possible interconnection between the antioxidant defense systems present in the mitochondrion and in the cytosol in a trypanosomatid. In addition, the results suggest the presence or induction of an unidentified H^+ transporter in the mitochondrial membrane when TcMPx is expressed at high levels.

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