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***Trypanosoma cruzi* infection disturbs mitochondrial membrane potential and ROS production rate in cardiomyocytes**

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Abstract

In this study, we investigated the role of *Trypanosoma cruzi* invasion and inflammatory processes in reactive oxygen species (ROS) production in mouse atrial cardiomyocyte line (HL-1) and primary adult rat ventricular cardiomyocytes. Cardiomyocytes were incubated with *T. cruzi* (Tc) trypomastigotes, Tc lysate (TcTL) or Tc secreted proteins (TcSP) for 0-72 h, and ROS measured by amplex red assay. Cardiomyocytes infected by *T. cruzi* (but not those incubated with TcTL or TcSP) exhibited a linear increase in ROS production during 2-48 h post-infection (max.18-fold increase) which was further enhanced by recombinant cytokines (IL-1 β , TNF- α and IFN- γ). We observed no increase in NADPH oxidase, xanthine oxidase, and myeloperoxidase activities, and specific inhibitor of these enzymes did not block the increased rate of ROS production in infected cardiomyocytes. Instead, the mitochondrial membrane potential was perturbed, and resulted in inefficient electron transport chain (ETC) activity, and enhanced electron leakage and ROS formation in infected cardiomyocytes. HL-1 rho (p) cardiomyocytes lacked a functional ETC, and exhibited no increase in ROS formation in response to *T. cruzi*. Together, these results demonstrate that invasion by *T. cruzi* and inflammatory milieu affect mitochondrial integrity and contribute to electron transport chain inefficiency and ROS production in cardiomyocytes.

Keywords

Trypanosoma cruzi; Chagas disease; primary cardiomyocytes; mitochondria; reactive oxygen species; inflammatory cytokines

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Introduction

Chagasic cardiomyopathy, initiated by *Trypanosoma cruzi*, accounts for >50,000 deaths and the loss of 2.74 million disability-adjusted life years per year in Latin America. Approximately 30% of infected individuals develop dilated cardiomyopathy that may ultimately lead to heart failure and patients' death [1].

Several investigators have suggested that Chagas disease is an inflammatory pathology. This idea is supported by the detection of extensive inflammatory infiltrate in the heart, and enhanced expression of interleukin 1 β (IL-1 β), tumor necrosis factor α (TNF- α) and IL-6 cytokines and a relatively low level of interferon γ (IFN- γ) in the myocardium of *T. cruzi*-infected experimental models [2][3]. The macrophages and neutrophils infiltrating the heart are the main source of IL-1 β and TNF- α cytokines [4,5] while CD4⁺ and CD8⁺ T cells contribute to IFN- γ production [6]. Oxidative burst of activated phagocytes result in the release of reactive oxygen species (ROS), e.g., superoxide (O₂^{•-}), hydrogen peroxide (H₂O₂), and hydroxyl radical ([•]OH) via activation of NADPH oxidase (NOX) and/or myeloperoxidase (MPO) enzymes [7]. The inflammatory cytokines and ROS are important for the control of *T. cruzi*, and may be cytotoxic to the host cellular components.

Many of the ROS are highly reactive and diffusible, and may be released in extracellular milieu. While intracellular ROS serve mainly for host defense against infectious agents, the extracellular release of ROS, when present in abundance, directly damage the surrounding tissues or promote inflammatory processes [8]. We have shown an antioxidant/oxidant imbalance pursue in the heart of *T. cruzi*-infected mice, and was associated with enhanced oxidative modification of cellular and mitochondrial proteins and lipids [9][10]. Subsequently, mitochondria isolated from infected murine hearts were shown to produce enhanced ROS [11], indicating that cardiomyocytes contribute to oxidative stress during *T. cruzi* infection.

Overall, the published literature has demonstrated that inflammatory and oxidative pathology pursue in the heart during *T. cruzi* infection. No studies have been conducted to demonstrate whether invasion by *T. cruzi* or inflammatory processes induce ROS production in cardiomyocytes and whether it is a mitochondrial process. In this study, we have used HL-1 cardiomyocyte cell line and primary adult rat cardiomyocytes to examine whether cardiomyocytes produce ROS in response to *T. cruzi* infection, or if it is a bystander effect in response to *T. cruzi* antigens. We treated cardiomyocytes with a variety of inhibitors and specific fluorescent probes to identify the source of ROS release. Since cardiomyocytes *in vivo* encounter a complex and varying mix of cytokines, we studied the additive and/or synergistic effect of these cytokines on *T. cruzi*-induced ROS production.

Material and Methods

Cell culture, primary cardiomyocytes and infection

HL-1 cardiomyocytes were cultured in Claycomb media supplemented with 10% FBS, as described [12]. HL-1 rho (p) cells were generated by culturing in presence of ethidium bromide (1 μ g/ml) and uridine (50 μ g/ml) for 59 passages. HL-1 p clones were screened for loss of mtDNA by Southern blot analysis. Briefly, total DNA (3- μ g) was digested with *Bam*HI, resolved on 10% agarose gels, and transferred to Zeta probe (Bio-Rad). Membranes were hybridized with [α ³²P]-labeled *COX II* probe (mtDNA-encoded) and *18S rDNA* (nuclear DNA-encoded), and the images captured using a phosphorimager (Molecular Devices) [13]. The expression level of mtDNA-encoded transcripts was determined by Northern blot analysis. For this, total RNA was isolated by guanidinium thiocyanate-phenol-chloroform method, and treated with DNase (Ambion) to remove contaminating DNA. Samples (2.5- μ g total RNA) were denatured in 50% formamide/2 M formaldehyde, resolved on 1% agarose gel containing

2 M formaldehyde in MOPS buffer and transferred. Membranes were hybridized with [$\alpha^{32}\text{P}$]-labeled cDNA probes and images captured as above [14].

Primary cardiomyocytes were isolated from adult rats using the Langendorff apparatus for heart perfusion [15]. Sprague Dawley rats (6-8 weeks old) were purchased from Harlan (Indianapolis, IN). After intra-peritoneal injection of heparin (1000 IU/kg) and administration of anesthesia, heart was removed in ice cold Tyrode's buffer, and sequentially perfused with Ca^{2+} free Tyrode solution for 6 min at 37° C (flow rate of 12 ml/min), and 1 mg/ml collagenase (Type II, Worthington Biochem) until the heart became flaccid (20-30 min). Finally, heart was minced, cell suspension rinsed in Tyrode's buffer with a gradual increase in Ca^{2+} concentration (up to 1 mM), and cells were plated on petri dishes (60 mm) previously coated with laminin (10 $\mu\text{g}/\text{ml}$). Cultures consisting of $\geq 95\%$ viable cardiomyocytes, validated by trypan blue exclusion method, were used for all experiments. Animal experiments were performed according to the National Institutes of Health Guide for Care and Use of Experimental Animals and approved by the UTMB Animal Care and Use Committee.

T. cruzi trypomastigotes (SylvioX10/4) were propagated in C2C12 cells. *T. cruzi* trypomastigotes lysate (TcTL) was prepared by repeated freeze thaw method (1×10^9 trypomastigotes/ml PBS). *T. cruzi* secreted proteins (TcSP) were prepared as described [16]. HL-1 and primary cardiomyocytes were seeded in 6-well ($5 \times 10^4/\text{well}$), or 24-well ($1 \times 10^4/\text{well}$) plates and infected with *T. cruzi* trypomastigote (cell: parasite ratio, 1:5) for 3 h. Plates were rinsed with RPMI to remove free parasites, and then incubated in complete medium at 37°C, 5% CO_2 for 0-72 h. In some experiments, cells were incubated with TcTL or TcSP (equivalent to 1×10^6 trypomastigotes/ml), and treated with recombinant human cytokines IL-1 β , IFN- γ , and TNF- α (100 ng/ml each cytokine, from BD Pharmingen). Cell viability, determined by Trypan blue exclusion and MTT cell growth assay (Millipore), was decreased by 10% in *T. cruzi*-infected cardiomyocytes compared to controls during the course of incubation (up to 48 h).

ROS level

We measured H_2O_2 release by cardiomyocytes using amplex red assay. Briefly, supernatants (50- μl), collected at various time-points post-infection (pi), were added in triplicate to 96 well, black flat-bottomed plates, and reaction was started with addition of 100 μM 10-acetyl-3,7-dihydroxyphenoxazine (amplex red, Invitrogen) and 0.3 U/ml horseradish peroxidase (HRP). HRP-catalyzed amplex red oxidation by H_2O_2 , resulting in fluorescent resorufin formation, was monitored at $\text{Ex}_{563\text{nm}}/\text{Em}_{587\text{nm}}$ using a Spectra Max^R M2 microplate reader (Molecular Devices) [11]. In some experiments, cardiomyocytes were incubated with 10- μM diphenyliodonium (DPI), 100 nM salicylhydroxamic acid (SHX), and 100 nM allopurinol, the specific inhibitors of NADPH oxidase (NOX), myeloperoxidase (MPO) and xanthine oxidase (XOD), respectively. To measure the mitochondrial ROS production, mitochondria were isolated by differential centrifugation, incubated with 5 mM pyruvate/5 mM malate (pyr/mal, provide electrons to complex I) or 5 mM succinate (provides electron to complex II), and substrate-stimulated H_2O_2 generation was monitored by amplex red assay. Standard curve was prepared with H_2O_2 (50 nM - 5 μM) [11].

Fluorescence microscopy

HL-1 or primary cardiomyocytes ($10^4/\text{well}$) were cultured in Nunc^R Lab Tek II chamber slides, infected with *T. cruzi* (cell: parasite ratio, 1:5), and incubated for 48 h ($\pm$ recombinant cytokines, as above). Cardiomyocytes were washed thrice, and labeled for 30 min with following fluorescent probes: 200 nM MitoTracker red (mitochondria-specific, $\text{Ex}_{498\text{nm}}/\text{Em}_{598\text{nm}}$), 200 nM MitoTracker green (mitochondria specific, $\text{Ex}_{498\text{nm}}/\text{Em}_{598\text{nm}}$), 5 μM MitoSOX red (specifically detects mitochondrial ROS, $\text{Ex}_{498\text{nm}}/\text{Em}_{598\text{nm}}$), or 10 μM $\text{H}_2\text{DCF-DA}$ (detects

cellular ROS, Ex_{498nm}/Em_{598nm}). Fluorescence was detected on an Olympus BX-15 microscope and images captured using a mounted digital camera (magnification 40× and 60×).

Mitochondrial membrane potential (Ψ_m) in normal and infected cardiomyocytes was determined using 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). Briefly, cells were incubated with 10 μ M JC-1 for 30 min. Cells were washed, and green monomers (Ex_{485nm}/Em_{535nm}) and red J-aggregates (Ex_{560nm}/Em_{595nm}, a sensitive marker of $\Delta\Psi_m$) were monitored by fluorescence microscopy. The fluorescent intensity of J-monomers (green) and J-aggregates (red) was determined using a Spectra Max^R M2 microplate reader. All fluorescent probes were purchased from Invitrogen/Molecular Probes.

Respiratory complex activities

Cardiomyocytes were cultured in Lab Tek II chamber slides and infected with *T. cruzi* (cell: parasite ratio, 1:5). At 24 h and 48 h pi, cells were permeabilized with 50 μ g/ml saponin for 5 min, and washed thrice. For complex I catalytic staining, slides were immersed in 1 mM Tris buffer (pH 7.4) containing 0.5 mg/ml NADH substrate and 12.5 mg/ml nitroblue tetrazolium (NBT) electron acceptor. Staining for complex II activity was performed in 1.5 mM phosphate pH 7.4, 5 mM EDTA, 10 mM KCN reaction buffer containing 84 mM succinate substrate, and 0.2 mM phenazine methosulfate and 10 mM NBT electron acceptors. For complex IV staining, permeabilized cells were incubated in reaction buffer (50 mM phosphate pH 7.4, 24 units/ml catalase, 75 mg/ml sucrose) containing 1 mg/ml reduced cytochrome c substrate and 1.0 mg/ml 3,3'-diaminobenzidine electron acceptor. The specificity of the reaction for complex activity was determined using specific inhibitors, i.e., 6.5 μ M rotenone (complex I), 10 mM sodium malonate (complex II), 1 μ M antimycin (complex III), and 1 mM KCN (complex IV). Spectrophotometric evaluation of respiratory complex activities was performed in isolated mitochondria from infected and normal cardiomyocytes [10].

Statistical analysis

Data are expressed as means $\pm$ SD, and were derived from at least triplicate observations per experiment. All experiments were repeated at least twice. Results were analyzed for significant differences using ANOVA procedures and Student's t-tests. The level of significance was accepted at $p < 0.01$.

Results

We employed amplex red fluorescent probe (detects H₂O₂) to monitor *T. cruzi*-induced ROS production in cardiomyocytes. HL-1 cells infected with *T. cruzi* exhibited an increase in ROS production beginning at 2 h pi (Fig. 1A). The ROS level in infected HL-1 cells was increased by 8- and 15-fold at 24 h and 48 h pi, respectively, as compared to that detected in normal controls (Fig. 1A). Likewise, primary cardiomyocytes infected by *T. cruzi* exhibited a 12-fold and 18-fold increase in ROS level at 24 h and 48 h pi, respectively (Fig. 1B). HL-1 cells (Fig 1C) and primary cardiomyocytes (data not shown) exhibited a marginal increase in H₂O₂ release when incubated with trypomastigote lysate (TcTL) or secreted (TcSP) proteins. The specificity of amplex red was confirmed by >80% decline in fluorescence when catalase (CAT, scavenges H₂O₂) was added to cardiomyocytes infected by *T. cruzi* or incubated with TcTL or TcSP (Fig. 1C). Together, these results suggest that active invasion by *T. cruzi* elicits ROS production in cardiomyocytes.

In an *in vivo* state, cardiomyocytes are exposed to proinflammatory cytokines (e.g., IL-1 β , TNF- α) and IFN- γ produced by macrophages and T cells, respectively. The cytokine treatment of HL-1 cells infected by *T. cruzi* resulted in a 76.4% and 86.2% increase in H₂O₂ level at 24 h (data not shown) and 48 h pi (Fig. 2A), respectively, compared to that detected in infected/

untreated cardiomyocytes. Likewise, primary adult rat cardiomyocytes treated with cytokines exhibited a 2-fold increase in *T. cruzi*-induced H₂O₂ release at 24 h and 48 h pi (Fig. 2B). Cytokine treatment did not enhance ROS release in HL-1 only or HL-1 cells incubated with TcTL or TcSP (Fig. 2A). These results suggest that proinflammatory cytokines augment the ROS release in cardiomyocytes infected by *T. cruzi*.

ROS are elicited as a host defense response against infectious agents through the activation of NOX, MPO, and XOD. To examine whether these enzymes are activated and contributed to increased ROS in infected cardiomyocytes, we first determined the effect of specific inhibitors on ROS level in infected cardiomyocytes. The H₂O₂ levels in *T. cruzi*-infected HL-1 and primary cardiomyocytes, measured at 24 h and 48 h pi, was not altered by DPI (NOX inhibitor), SHX (MPO inhibitor) and allopurinol (XOD inhibitor) (data not shown). Likewise, the inhibitors of NOX, XOD, and MPO had no effect on the cytokine-stimulated enhanced ROS production in *T. cruzi*-infected HL-1 and primary cardiomyocytes (data not shown). Further, HL-1 and primary cardiomyocytes infected by *T. cruzi* and incubated with or without recombinant cytokines exhibited no detectable level of NOX, MPO, and XOD activities at 24 and 48 h pi (data not shown). These results suggested that ROS release by cardiomyocytes is not a defense response against *T. cruzi*, and other processes contribute to ROS release in infected cardiomyocytes.

T. cruzi attachment to and invasion of host cells stimulates Ca⁺² flux [17]. Changes in Ca⁺² may promote opening of mitochondrial membrane permeability transition pores (MPTP) and affect the ψ_m and electrochemical gradient, leading to electron leakage to O₂ and O₂^{•-} generation. We determined mitochondrial $\Delta\psi_m$ in infected cardiomyocytes using JC-1 dye. JC-1 accumulates in coupled, healthy mitochondria as red aggregates. Upon collapse of $\Delta\psi_m$, JC-1 remains in a monomeric form and fluoresces green. Normal HL-1 cells exhibited a high red fluorescence of J-aggregates (Fig. 3A, panels a, c) and very low green fluorescence of monomers (Fig. 3A, panel b). In comparison, *T. cruzi*-infected HL-1 cells exhibited a marked increase in green fluorescence (Fig. 3A, panels e, f) with a corresponding decline in red fluorescence (Fig. 3A, panel d). Likewise, primary adult rat cardiomyocytes exhibited a *T. cruzi*-induced decline in red J-aggregates and a substantial increase in green monomers (Fig. 3B, compare panels a, b with panels d, e, respectively). Fluorometric analysis showed that JC-1 ratio (green monomers/red aggregates) was increased by ≥ 4 -fold in infected HL-1 and primary cardiomyocytes at 24 h and 48 h pi (Fig. 3C, data not shown). Incubation of cardiomyocytes with TcTL or TcSP had no effect on JC-1 ratio (data not shown). HL-1 cardiomyocytes incubated with H₂O₂ (20 μ M) exhibited a similar increase in JC-1 ratio as was noted in *T. cruzi*-infected cardiomyocytes (Fig. 3C). Treatment of *T. cruzi*-infected or H₂O₂-treated cardiomyocytes with catalase prevented the increase in green fluorescence, and preserved the J-red aggregates and JC-1 ratio to control level (Figs. 3C). Together, these results demonstrate that ROS altered the mitochondrial $\Delta\psi_m$ in cardiomyocytes infected by *T. cruzi*.

We determined the mitochondrial ROS production in infected cardiomyocytes using specific fluorescence probes. Representative images of HL-1 cardiomyocytes stained with MitoTracker red (localizes to mitochondria) and H₂DCF-DA (detects intracellular ROS, green fluorescence) at 48 h pi are shown in figure 4A. HL-1 cells, irrespective of infection status, exhibited punctuated mitochondrial staining with MitoTracker red (Fig. 4A, panels a, d). Normal HL-1 exhibited minimal-to-no DCF fluorescence (Fig. 4A, panel b). In comparison, *T. cruzi*-infected HL-1 cardiomyocytes exhibited bright, punctuated, ROS-specific DCF fluorescence (Fig. 4A, panel e). Overlay images (yellow color) showed that DCF fluorescence (green) co-localized with MitoTracker red signal (Fig. 4A, panel f), thus, suggesting that mitochondrial ROS level was increased in *T. cruzi*-infected HL-1 cardiomyocytes. To validate in primary cardiomyocytes, we used MitoTracker green (mitochondrial probe) and MitoSOX red (detects O₂^{•-} in mitochondria of live cells). In absence of mitochondrial ROS, MitoSOX accumulates

in nucleus. Primary adult cardiomyocytes exhibited substantial level of MitoTracker green signal (Fig. 4B, panels a, d). MitoSOX red fluorescence was detected in the nucleus of normal primary cardiomyocytes (Fig. 4B, panel b), also evident in overlay image (Fig. 4B, panel c). *T. cruzi*-infected primary cardiomyocytes exhibited a punctuated mitochondrial localization of MitoSOX red fluorescence (Fig. 4, panel e), also evidenced by overlay image (yellow) showing co-localization of MitoSOX red and MitoTracker green signal in infected cardiomyocytes (Fig. 4, panel f). These results demonstrated that mitochondrial rate of ROS production is increased in HL-1 and primary cardiomyocytes infected by *T. cruzi*.

The effect of the disturbance in $\Delta\psi_m$ on electron transport chain efficiency was determined by monitoring the a) activity of the respiratory complexes, and b) the rate of electron leakage to O_2 resulting in $O_2^{\bullet-}$ formation. Histochemical staining showed a substantial decline in complex I activity in HL-1 (Fig. 5A, panels a, c) and primary cardiomyocytes (data not shown) at 48 pi. Catalytic staining of complex II was not significantly decreased (Fig. 5A, panels d, f) while complex IV staining was decreased in >30% of HL-1 (Fig. 5A, panels g, i) and primary cardiomyocytes at 48 h pi. Specificity of the reaction was confirmed by a loss in staining when normal cardiomyocytes were incubated with rotenone (inhibits complex I, panel b), malonate (inhibits complex II, panel e), and KCN (inhibits complex IV, panel h). Spectrophotometric assays validated the histochemical results. Isolated mitochondria from infected HL-1 cardiomyocytes, as compared to normal controls, exhibited 63.6% and 92% decline in complex I activity, and 51% and 46.3% decline in complex III activity at 24 h and 48 h pi, respectively (Fig. 5B, data not shown).

Inefficient transport of electrons due to altered $\Delta\psi$ and a loss in complex activities can result in increased electron leakage and ROS formation. To validate, that enhanced ROS formation in infected cardiomyocytes is due to impaired mitochondrial ETC efficiency, we generated HL-1 ρ cardiomyocytes by ethidium bromide treatment that selectively inhibits mtDNA replication and transcription. Selected HL-1 ρ cultures, examined by Southern blot analysis using *COX II* probe, showed 50-63% decline in the 16-kb mtDNA compared to HL-1 wt cells (Fig. 6A & B). Northern blot analysis exhibited the mRNA level for mtDNA-encoded *COX II* was decreased by 65-84% in HL-1 ρ cells compared to wt controls (Fig. 6C & D). Similar level of signal for *18S rDNA* in HL-1 wt and ρ cardiomyocytes demonstrated that ρ cells were not compromised for nuclear DNA (Fig. 6A & B). The clone 3 (ρ 3), consisting maximal repression of mtDNA-encoded transcripts, exhibited 95% loss of complex I activity and >52% loss in complex III activity as compared to HL-1 wt cardiomyocytes (Fig. 6E). These data showed that HL-1 ρ cells contain partial activity of respiratory complexes.

Upon infection with *T. cruzi*, HL-1 ρ 3 cardiomyocytes exhibited no significant difference in the number of parasites/cell than that noted in wild-type HL-1 cells at 24 h and 48 h pi. We isolated mitochondria from infected cardiomyocytes at 24 h and 48 h pi by differential centrifugation. The rate of pyr/mal- and succinate-supported ROS production in isolated mitochondria from *T. cruzi*-infected HL-1 cardiomyocytes was increased up to 121% and 157%, respectively, when compared to that noted in normal controls (Fig. 7). HL-1 ρ cells, due to a partial ETC activity, exhibited a substantially higher level of substrate-stimulated ROS production than the HL-1 wt, and did not respond by further increase in ROS when infected with *T. cruzi* (Fig. 7). A substantial increase in pyr/mal- and succinate-supported ROS generation in mitochondria of HL-1 wt in response to *T. cruzi* infection, but not in the HL-1 ρ , confirms that respiratory chain inefficiency contributes to increased ROS production in cardiomyocytes infected by *T. cruzi*.

Discussion

In this study, we demonstrate that HL-1 and primary adult rat cardiomyocytes elicit a strong response to invading *T. cruzi* by substantial ROS production that was augmented by proinflammatory cytokines. The increase in ROS production in cardiomyocytes was not a bystander effect caused by parasite antigens and was not an outcome of activation of classical ROS producers (NOX, MPO, and XOD). Instead, invasion by *T. cruzi* altered the mitochondrial ψ_m , and subsequently, the activities of respiratory complexes were compromised leading to increased electron leakage and superoxide production in cardiomyocytes. HL-1 ρ cardiomyocytes exhibited no ROS response to *T. cruzi* infection. This, to the best of our knowledge, is the first study documenting that invasion by *T. cruzi* initiates ROS production and mitochondrial dysfunction in cardiomyocytes and the presence of inflammatory milieu augments the *T. cruzi*-induced ROS production.

In pathologic conditions, diverse pathways result in excessive ROS production that if not efficiently scavenged by the antioxidant system, leads to oxidative stress. ROS can rapidly oxidize proteins, lipids and DNA, and thereby result in dysfunction of physiological processes, oxidative damage and cell/tissue death [18]. Several studies have shown that oxidant/antioxidant imbalance and oxidative damage are increased in the myocardium of experimental animals infected by *T. cruzi* [9] [19] and in peripheral blood of human chagasic patients [20, 21]. The detection of inflammatory infiltrate constituted of neutrophils and macrophages in cardiac biopsies from infected mice [22] and humans [23] has led to the suggestion that oxidative burst of immune cells might be the primary source of oxidative stress during Chagas disease. We have demonstrated that mitochondrial electron transport chain is compromised in chagasic hearts, and the resultant electron leakage to molecular oxygen contributes to oxidative stress during the course of *T. cruzi* infection and disease development [10,11]. Because of the presence of multiple cell types in the heart, it was not clear whether cardiomyocytes produced ROS in response to *T. cruzi* infection or infiltrating immune cells were the only source of ROS in the heart. Our data in this study clarify this dilemma, and demonstrate that HL-1 and primary cardiomyocytes respond to *T. cruzi* by elicitation of ROS within 2 h pi, and ROS level increased exponentially up to 48 h pi (Fig. 1). ROS response was not a bystander effect caused by the presence of parasite antigens, and active invasion by *T. cruzi* was essential to elicit ROS production in cardiomyocytes. This is the first observation of *T. cruzi*-induced ROS production in isolated primary cardiomyocytes.

The participation of cytokines, peptide hormones, and immune regulators in cellular pathways is modulated by redox status [24][25]. Conversely, cytokine themselves are mediators of oxidative stress, and have the potential to alter redox equilibrium in cells [25]. Some reports have shown ROS generation in cardiomyocytes directly exposed to TNF- α [26][27], and cytotoxicity of TNF- α was enhanced by IL-1 β and IFN- γ . In this study, we found no detectable production of ROS in normal cardiomyocytes exposed to recombinant cytokines (IL-1 β , TNF- α and IFN- γ , individually or in combination). The recombinant cytokines did not enhance the low level of ROS produced in cardiomyocytes treated with parasite antigens (membrane or secreted). Instead, when cardiomyocytes were infected with *T. cruzi*, exposure to IL-1 β , TNF- α , and IFN- γ cytokines together, not individually, resulted in 2-fold increase in parasite-induced ROS production (Fig. 2). Whether an interaction between TNF- α , IFN- γ and IL-1 β enhanced the modulating activity of each cytokine or all three cytokines played an essential role in signaling increased ROS formation remain to be investigated in future studies. Yet, our data demonstrate that infection by *T. cruzi* and the inflammatory milieu crosstalk, and enhance the oxidative stress in cardiomyocytes.

The oxidases and oxygenases expressed in different cell types and organelles contribute to ROS formation and oxidative stress. For example, monoamine oxidase, present in

mitochondrial outer membrane, converts O_2 to H_2O_2 on the cytoplasmic face [28]. Xanthine dehydrogenase and XOD produce H_2O_2 and $O_2^{\bullet-}$ during purine hypoxanthine degradation to uric acid [29]. The NOX and MPO enzymes are the primary source of ROS in macrophages, endothelial cells, and neutrophils. Activation of NOX, MPO, and XOD is documented in the heart of *T. cruzi*-infected mice [30]. Increased activity of XOD is also noted in the heart during acute ischemia-reperfusion injury [31]. Yet, these oxidases and peroxidases were not the source of ROS in cardiomyocytes infected by *T. cruzi*. We detected no change in NOX, MPO and XOD activities in infected cardiomyocytes as compared to controls. Treatment of infected cardiomyocytes with DPI, an inhibitor of NOX and other mixed function oxidases; SHX, an inhibitor of MPO and other ROS-generating peroxidases; and allopurinol, a specific inhibitor of XOD; did not attenuate ROS generation in infected cardiomyocytes. We surmise that processes other than the classical ROS producers contributed to redox stress in the infected cardiomyocytes.

Several observations in this study indicate that mitochondrial electron transport chain was the primary source of enhanced ROS release in cardiomyocytes infected by *T. cruzi*. One, our data showed co-localization of mitochondria-specific MitoTracker probes with ROS-specific H_2DCF -DA and MitoSOX red fluorescence in infected cardiomyocytes (Fig. 4). Second, isolated mitochondria from infected cardiomyocytes exhibited a significant increase in ROS generation when energized with complex I (pyr/mal) or complex II (succinate) substrates (Fig. 7). Third, HL-1 p cardiomyocytes that harbored a dysfunctional electron transport chain (Fig. 6) did not respond to *T. cruzi* infection and cytokine treatment by an increase in ROS production (Fig. 7). Our data provide validation to the reports demonstrating the defects of mitochondrial respiratory complex III resulted in an increased electron leakage to molecular O_2 and $O_2^{\bullet-}$ formation in the heart of chagasic mice [10,11], and suggest that these defects are primarily accrued in cardiomyocytes of the infected host.

The question then arises, how mitochondrial ROS is initiated in infected cardiomyocytes. Our observation that ROS was elicited in cardiomyocytes only when infected by live *T. cruzi* and incubation with parasite lysates or secreted proteins had marginal-to-no effect on ROS release (Fig. 1 & 2) provides clues to this query. It is documented that invasion by *T. cruzi* trypomastigotes elicit transient elevations in intracellular Ca^{+2} (reviewed in [32]). In mammalian cells, Ca^{+2} overload is known to induce the opening of MPTPs [33]. An increase in MPTP opening affects several mitochondrial functions. One of these is the dissipation of protonmotive force that in itself serves as a negative modulator of respiratory chain activity [34]. It is, thus, likely that *T. cruzi*-induced Ca^{+2} overload might be the primary signal in mitochondrial dysfunction. We have noted a substantial decline in JC-1 fluorescence during *T. cruzi* infection (Fig. 3), indicating that significant fall in mitochondrial ψ_m must have occurred, and subsequently, respiratory chain activity was decreased (Fig. 5). Our notion is supported by others indicating the deleterious effects of Ca^{+2} flux on mitochondrial respiratory complex activities in cardiac and retinal cells [35,36]. Amplification of cellular damage due to Ca^{+2} signaling of ROS generating pathways in mitochondria is reported in numerous *in vitro* and *in vivo* cellular systems (reviewed in [37]). We, thus, propose that *T. cruzi* invasion-dependent Ca^{+2} flux is the initial event that results in mitochondrial depolarization, ETC inefficiency and ROS production in cardiomyocytes. Further, we found that immunofluorescence staining with antibody to 4-hydroxynonenal (HNE, lipid peroxidation marker) co-localized with MitoTracker (specific for mitochondria) and H_2DCF -DA (specific for H_2O_2) fluorescent probes in infected cardiomyocytes (unpublished observation). A substantial increase in malonyldialdehyde and protein carbonyl contents in cardiac mitochondria of mice infected by *T. cruzi* is also documented [10]. Treatment of infected cardiomyocytes with catalase preserved the mitochondrial ψ_m , while treatment of normal cardiomyocytes with H_2O_2 resulted in a loss in JC1-red aggregates staining (Fig. 3). Thus,

ROS-induced oxidation of mitochondrial membranes may constitute a secondary signal affecting $\Delta\psi_m$ and respiratory chain efficiency.

The extent of mitochondrial ROS production and membrane alterations promotes cell death [38]. Mild-to-moderate level of ROS resulting in mitochondrial depolarization and $\Delta\psi_m$ results in cytochrome c release that is an important catalytic activator of apoptotic pathway [39]. Excessive ROS production switches the mode of cell death from apoptosis to oncosis due to severe oxidative damage and downstream effects [40]. Cardiomyocytes undergoing apoptotic and necrotic death are reported in chagasic hearts [41,42]. In our study, infected cardiomyocytes exhibited a 10% reduction in viability and a corresponding increase in Annexin V staining that is an indicator of early apoptotic events (data not shown). *In vivo* studies are required to delineate further the role of mtROS in cardiomyocyte death and progression of Chagas disease.

In summary, we have demonstrated that cardiomyocytes respond to *T. cruzi* infection by an exponential increase in ROS production that is augmented by the proinflammatory cytokines. The ROS production by cardiomyocytes was not a defense response against *T. cruzi*. Instead, invasion by *T. cruzi* induced cellular events that affected the mitochondrial ψ_m , and initiated a feedback cycle of electron transport chain inefficiency and increased electron leakage and ROS production in the cardiomyocytes. The ROS-induced signaling and injurious processes may contribute to cardiomyocyte death and chronic heart failure in chagasic patients, to be investigated in future studies.

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Abbreviations

Amplex red	10-acetyl-3,7-dihydroxyphenoxazine
CI	NADH ubiquinone oxidoreductase
CII	succinate decylubiquinone 2, 6 dichlorophenolindophenolreductase
CIII	ubiquinol cytochrome c oxidoreductase
CIV	cytochrome c oxidase
cyt c	cytochrome c
ETC	electron transport chain

H₂DCF-DA	dichlorodihydrofluorecein diacetate
HNE	4-hydroxynonenal
JC-1	5 5' 6 6'-tetrachloro-11' 3 3'-tetraethylbenzimidazolylcarbocyanine iodide
IL-1β	interleukin 1 β
IL-6	interleukin 6
$\Delta\psi_m$	membrane potential
MDA	malonyldialdehyde
MPO	myeloperoxidase
MPTP	membrane permeability transition pores
Pyr/mal	pyruvate/malate
rho or ρ	cells depleted of mitochondrial DNA
ROS	reactive oxygen species
O₂^{•-}	superoxide
TNF-α	tumor necrosis factor α <i>T. cruzi</i> or Tc, <i>Trypanosoma cruzi</i>
TcTL	Tc trypomastigote lysate
TcSP	Tc secreted proteins
XOD	xanthine oxidase

Figure 1. *T. cruzi* infection elicits ROS generation in cardiomyocytes

(A) HL-1 cardiomyocytes were infected with *T. cruzi* (cell: parasite ratio, 1:5), and ROS level in supernatant measured using amplex red fluorescent probe. (B) ROS generation in primary adult rat cardiomyocytes at 24 h and 48 h pi with *T. cruzi*. (C) HL-1 cardiomyocytes were infected with *T. cruzi*, or incubated with trypomastigote lysate (TcTL) or secreted proteins (TcSP) in presence or absence of catalase (CAT), and ROS level measured at 48 h pi. Data (mean $\pm$ SD) are representative of three independent experiments (** p < 0.01, *** p < 0.001, normal (N) versus *T. cruzi*-infected).

Figure 2. Proinflammatory cytokines augment the *T. cruzi*-induced ROS production in cardiomyocytes

(A) HL-1 cells were infected with *T. cruzi*, or incubated with TcTL or TcSP in presence or absence of 100 ng/ml each of IL-1 β , TNF- α , and IFN- γ cytokines for 48 h and ROS level measured (as above). (B) Shown are H_2O_2 levels in primary cardiomyocytes infected with *T. cruzi* $\pm$ recombinant cytokines. Data (mean $\pm$ SD) are representative of triplicate observations ($n = 3$ experiments, ** $p < 0.01$, normal versus infected). Significance ($p < 0.05$) of cytokine treatment is shown by dot-head.

Figure 3. Mitochondrial membrane potential ($\Delta\psi$) is disturbed in cardiomyocytes infected by *T. cruzi*
(A & B) HL-1 and primary cardiomyocytes (PrC) were infected with *T. cruzi*, and at 48 h pi, loaded with JC-1 probe. Shown are the representative fluorescence micrographs of JC-1-stained HL-1 **(A)** and primary **(B)** cardiomyocytes. Note the accumulation of red J-aggregates in normal cardiomyocytes (panels a) and an accumulation of green monomers in infected cardiomyocytes (panels e). Overlay images show that a majority of mitochondria fluoresced red in normal cardiomyocytes (panels c) while mitochondria in infected cardiomyocytes fluoresced green (panels f). **(C) ROS contributes to mitochondrial depolarization in *T. cruzi*-infected cardiomyocytes.** HL-1 cardiomyocytes were infected with *T. cruzi* or incubated with 20 μ M H₂O₂ in presence or absence of catalase (CAT). Shown are the ratios of fluorescence intensity of J-monomers (green fluorescence) to J-aggregates (red fluorescence). Data (mean $\pm$ SD) are representative of triplicate observations (n = 3 experiments, ** p < 0.01, normal versus H₂O₂-treated or *T. cruzi*-infected). Significance of catalase treatment (p < 0.05) is shown by dot-head.

Figure 4. Mitochondrial ROS release is increased in cardiomyocytes infected by *T. cruzi*
 (A) Shown are the representative fluorescence micrographs of HL-1 normal (panels a, b, c) and *T. cruzi*-infected (panels d, e, f) cardiomyocytes at 48 h pi, stained with MitoTracker red (localizes to mitochondria, panels a, d) and H₂DCFDA (detects intracellular ROS, green fluorescence, panels b, e). (B) Normal (panels a, b, c) and *T. cruzi*-infected (panels d, e, f) primary cardiomyocytes were stained at 48 h pi with MitoTracker green (localizes to mitochondria, panels a, d) and MitoSOX red (detects mitochondrial O₂^{•−}, panels b, e). Overlay images demonstrate co-localization of MitoTracker and ROS-specific probes in infected cardiomyocytes (panel f).

Figure 5. Electron transport chain activity is compromised in mitochondria of *T. cruzi*-infected cardiomyocytes

(A) Shown are representative histochemical micrographs for complex I (panels a, b & c), complex II (panels d, e & f) and complex IV (panels g, h & i) in normal HL-1 (panels a, d & g), HL-1 treated with specific inhibitors (b: rotenone, e: malonate, h: KCN), and infected HL-1 at 48 h pi (panels c, f & i). (B) Spectrophotometric evaluation of activities of complex I and complex III in mitochondria isolated from HL-1 cardiomyocytes at 48 h pi. Data (mean ± SD) are representative of triplicate observations (n = 3 experiments, (**p < 0.01, ***p < 0.001, normal versus infected).

Figure 6. HL-1 rho (ρ) cardiomyocytes are compromised in mitochondrial function

(A & B) Southern blotting. Total DNA from HL-1 wt and ρ cultures ($\rho 1$, $\rho 2$, $\rho 3$) was digested with BamHI, and hybridization was performed with [32 P]-labeled *COX II* or *18S* rRNA probes. Densitometric analysis of *COX II* signal (indicative of mtDNA content), normalized with *18S* signal (indicative of nuclear DNA) is shown in panel B. **(C & D) Northern blot analysis.** Total RNA was resolved on gel, and hybridized with [32 P]-labeled cDNA probes for *COX II* or *18S* rRNA. Densitometric analysis of *COX II* mRNA signal, normalized with *18S* rRNA signal, is shown in panel D. **(E)** Mitochondria were isolated from HL-1 wt and ρ cardiomyocytes, and activities of complex I and complex III determined by spectrophotometry.

Figure 7. *T. cruzi* infection does not elicit ROS production in HL-1 p cardiomyocytes
 HL-1 (wt and p3) cardiomyocytes were infected with *T. cruzi*. At 48 h pi, freshly isolated mitochondria were energized with pyruvate/malate (pyr/mal) (**A**) or succinate (**B**) substrates, and the rate of H₂O₂ production monitored using amplex red. Data (mean $\pm$ SD) are representative of triplicate observations (n = 3 experiments, ***p* < 0.01, ****p* < 0.001, normal versus infected).