Title:
Role of Peroxidoxins in *Leishmania chagasi* Survival: Evidence of an Enzymatic Defense Against Nitrosative Stress

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ABSTRACT

The mechanisms by which *Leishmania* parasites survive exposure to highly reactive oxygen (ROS) and nitrogen (RNS) species within phagosomes of macrophages are not well known. Recently it has been shown that RNS alone is sufficient and necessary to control *L. donovani* infection in mice (Ref. 17). No enzymatic defense against RNS has been discovered in *Leishmania* to date. We have previously isolated two peroxidoxins (LcPxn1 and LcPxn2) from *L. chagasi* and showed that recombinant LcPxn1 protein was capable of detoxifying hydrogen peroxide, hydroperoxide and hydroxyl radicals (Ref. 25). In further characterizing the physiological role of peroxidoxins in *Leishmania* survival, we show here that recombinant LcPxn1 protein can detoxify RNS in addition to ROS, whereas recombinant LcPxn2 protein can only detoxify hydrogen peroxide. LcPxn1 and LcPxn2 are localized to the cytoplasm and overexpression of LcPxn1 in *L. chagasi* parasites enhanced survival when exposed to exogenous ROS and RNS and also enhanced survival within U937 macrophage cells. Site-directed mutagenesis studies revealed that the conserved Cys52 residue is essential for detoxifying hydrogen peroxide, t-butyl hydroperoxide and hydroxyl radicals, whereas the conserved Cys173 residue is essential for detoxifying t-butyl hydroperoxide and peroxynitrite. This is the first report of an enzymatic defense against RNS in *Leishmania*.

INTRODUCTION

*Leishmania* is a protozoan parasite that affects over 12 million people worldwide with an estimated 2 million new cases each year. Depending on the species involved, symptoms range from the self-healing cutaneous form (eg. *L. major*) to the fatal visceral form (eg. *L. chagasi*). The parasites are transmitted as promastigotes from the gut of its sandfly vector to mammalian
host macrophages wherein they transform into amastigotes and proliferate. As a macrophage defense mechanism, nitric oxide (\(\ \cdot\ \text{NO}\)), peroxynitrite (\(\ \cdot\ \text{ONOO}^\circ\)), hydroxyl radicals (\(\ \cdot\ \text{OH}\)), hydrogen peroxide (\(\ \text{H}_2\text{O}_2\)), hydroperoxide (\(\ \text{ROOH}\)) and superoxide radicals (\(\ \text{O}_2^\cdot\)) are produced in an attempt to destroy the parasites. These reactive nitrogen species (RNS) and oxygen species (ROS) readily react with proteins, DNA and lipids and have been implicated in a wide variety of cell functions such as signal transduction, redox homeostasis, apoptosis, ageing, activation of T lymphocytes, control of blood pressure, tumor progression, protection of eye tissue and pathogen infection/defense (1-4).

Numerous reports have shown that \textit{Leishmania} parasites are susceptible to ROS-mediated killing (5-9) and RNS-mediated killing (10-17). It has been shown that RNS alone is both necessary and sufficient to control \textit{L. donovani} infection in mice (17) and more recently that both ROS and RNS produced by macrophages act together early to control infection by \textit{L. chagasi} (18) and \textit{L. donovani} (11,17). These studies suggest that further characterization of antioxidant molecules within \textit{Leishmania} and the role that they play in parasite survival in the promastigote and amastigote stages could lead to the development of novel strategies to compromise parasite survival.

Despite the ability of ROS and RNS to control \textit{Leishmania} infection within macrophages, strains causing cutaneous and visceral leishmaniasis persist long enough within macrophages to produce skin lesions or death. The molecular mechanisms by which \textit{Leishmania} circumvent the toxic effects of these reactive species is not fully understood. Some \textit{Leishmania} molecules implicated in antioxidant defense against ROS include intracellular thiols (19), lipophosphoglycan (20,21),
FeSOD (22), HSP70 (23), ovothiol A and trypanothione (24) and peroxidoxins (25-28). The mechanisms by which \textit{Leishmania} withstand the toxic effects of RNS is much less well-defined. Glutathione has recently been implicated in protecting \textit{L. major} from 'NO-induced cytotoxicity (29). To date, an enzymatic defense against RNS has not been identified in \textit{Leishmania}.

Peroxidoxins (or peroxiredoxins) are highly conserved enzymes found in all kingdoms ranging from bacteria to humans. 2-Cys peroxidoxin proteins are characterized by two conserved cysteine residues corresponding to approximately positions 47 and 170 and exist in nature predominantly as head-to-tail dimers, although high molecular weight multimers have been reported (25,30-32). Peroxidoxins were initially characterized as enzymes able to detoxify ROS, namely H$_2$O$_2$ and alkyl hydroperoxides (33), with 'OH recently being added to the substrate list (25). Peroxidoxins have also been implicated in detoxifying RNS in bacteria, yeast and human cells (34-36).

We have previously isolated two peroxidoxin genes from \textit{L. chagasi} that are differentially regulated, where LcPxn1 RNA transcripts are highly abundant in the amastigote stage and LcPxn2 transcripts are highly abundant in the promastigote stage (25). Recombinant LcPxn1 protein was shown to detoxify H$_2$O$_2$, ROOH and 'OH, but the mechanism of its action and the role that \textit{L. chagasi} peroxidoxins play in detoxifying RNS and in parasite survival has not been characterized. In this paper, we demonstrate that recombinant LcPxn1 protein, but not LcPxn2, can detoxify RNS in addition to ROS and show that LcPxn1 protects \textit{L. chagasi} parasites from ROS- and RNS-mediated toxicity \textit{in vitro} and enhances survival within macrophages.
Furthermore, we have identified the key catalytic residues of LcPxn1 involved in detoxifying both ROS and RNS which differs from peroxidoxins isolated from other organisms.

**EXPERIMENTAL PROCEDURES**

*Site-directed Mutagenesis*- LcPxn1 mutants C52A, C173A and C52A/C173A were generated by site directed PCR mutagenesis as previously described (37). All peroxidoxin constructs were amplified using PCR, cloned into the pGEX-2T vector (Amersham Biosciences) and confirmed by sequencing. Transformed *E. coli* DH5α cells were grown shaking at 37°C in Luria-Burtani broth containing 100µg ml⁻¹ ampicillin for 8 hours, after which 0.2mM isopropyl-1-thio-β-D-galactoside (IPTG) was added to the culture and shaken overnight. GST fusion proteins were harvested by sonication and passed over a glutathione-agarose resin column as described by manufacturer (Amersham Biosciences). The fusion proteins were cleaved with Thrombin overnight at 24°C, further purified and protein purity (>95%) was verified on a SDS-PAGE gel. Protein concentrations were determined using the BCA Protein assay kit (Pierce Chemical, Rockford, IL).

*Peroxide Assays*- Peroxide metabolism was measured as previously described (38). Briefly, the reaction mixture contained 50mM Tris-HCl (pH 8.0), 0.2mM dithioerythritol (DTE), 50µM H₂O₂ or 50µM t-butyl hydroperoxide, and 0.125µg/ml of protein (pre-incubated with 0.2mM DTE for 30 mins at 37°C). The reaction was stopped with the addition of 1ml of trichloroacetic acid (10% w/v). 0.2ml of 10mM ferrous ammonium sulfate and 0.1ml of 2.5M potassium thiocyanate were added and the peroxide concentrations were determined spectrophotometrically.
at 480nm using known amounts of peroxide (1-50µM) as a standard. All solutions were made fresh immediately before use.

**Deoxyribose Degradation Assay for •OH Scavenging** - The production of •OH and the •OH-induced damage of 2-deoxy-D-ribose were measured as previously described (39). A 50µl reaction mixture was set up to contain the following components to give the final concentrations as stated: 10mM potassium phosphate buffer (pH 7.4); 63mM NaCl, 0.8mM 2-deoxy-D-ribose; 0.2mM DTE; 0.125µg/µl protein (proteins were pre-incubated in 0.2mM DTE for 30 mins at 37°C). 21µM ferrous ammonium sulfate was added and the tubes were incubated at 37°C for 15 mins. 100µl of thiobarbituric acid (TBA) (1% w/v) and 100µl of trichloroacetic acid (TCA) (2.8% w/v) were then added to the mixture and boiled for 10 mins. Fluorescence was measured in a 96-well plate using a SpectraMax Gemini plate reader (Molecular Devices) with six reads per well (Excitation= 532nm, Emission= 553nm). All solutions were made fresh immediately before use.

**•OH-induced DNA Nicking Assay** - 3µM FeCl₃, 0.1mM EDTA and 10mM DTE were allowed to react for 10 mins at 37°C to generate •OH as previously described (40). 0.5mg/ml protein (pre-incubated with 0.2mM DTE for 30 mins at 37°C) was then added to the mixture and incubated at 37°C for 30 mins. 2µg of pGEM-2 plasmid (Promega) was then added to each tube and incubated at 37°C for 4 hours. The DNA was separated on a 1% agarose gel containing 0.2µg/ml ethidium bromide at 100V constant. All solutions were made fresh immediately before use.
Pyrogallol Red Bleaching Assay for ONOO⁻ Scavenging- Reagent peroxynitrite was generated from acidified hydrogen peroxide and nitrite using the quenched-flow method (41) and passaged over MnO₂ column as previously described (42). The reaction assay was carried out as previously described (43). The reaction mixture contained 100mM phosphate buffer (pH 7.0), 1µM DTE, 50µM Pyrogallol Red ($\varepsilon=2.4\times10^4 \text{ mol}^{-1} \text{ liter}^{-1} \text{ cm}^{-1}$) and 20µM protein at 25°C. 20µM of reagent peroxynitrite was added to the reaction for 5 mins after which the absorbance at 542nm was measured. All solutions were made fresh immediately before use.

ONOO⁻ -induced DNA Nicking Assay- A reaction mixture containing 50mM sodium phosphate (pH 7.0), 10mM NaCl, 0.1mM diethylenetriaminepentaacetic acid (DTPA), 0.5µg intact pGEM-2 plasmid DNA and 20µM protein was prepared as previously described (44). 50µM reagent ONOO⁻ was added to the reaction mixture and incubated at room temperature for 5 mins. The DNA was separated on a 1% agarose gel containing 0.2µg/ml ethidium bromide at 100V constant. All solutions were made fresh immediately before use.

‘NO Detoxification Assay- ’NO levels were measured as previously described (45). 100mM sodium nitroprusside and 0.125µg/ml protein were incubated in phosphate buffered saline (PBS) pH 7.4 for 5 mins. 2,2’-azonbis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was then added to the reaction mixture (5mM final concentration) and the ’NO-induced oxidation of ABTS to ABTS⁺ was measured by monitoring the change in absorbance at 420nm at room temperature. All solutions were made fresh immediately before use.
Construction of Expression Vectors- The *Leishmania*-specific expression vector pX63NEO (kindly provided by Dr S.M. Beverley, Washington University in St. Louis) was used to express LcPxn1, LcPxn1-C52A, LcPxn1-C173A and LcPxn1-C52A/C173A in *L. chagasi*. The coding regions were PCR amplified using a 5’ primer (5’ ACCAGGGATCCATGTCTCGCGTGACGCC 3’) and a 3’ primer (5’ ACATCGGATCCCTACTATTGTGATCGACCTTCAGGCC 3’) with incorporated BamHI sites (underlined). The pX63NEO vector and the PCR products were digested with BamHI, ligated and sequenced for the correct orientation. To express LcPxn1 and LcPxn2 as GFP fusions, the coding region of GFP was PCR amplified using a 5’ primer (5’ GTCGGATCCATG GTGAGCGAGGCGAGG 3’) and a 3’ primer (5’ CCGGAATTCTACTTTGTACAGCTCGTCC 3’). The stop codon of GFP was mutated from TAA to TAC (bold). The PCR fragment was digested with BamHI and EcoRI and purified. The coding region of LcPxn1 was PCR amplified using a 5’ primer (5’ GTGAATTCATGTCCTGCGTGACGCC 3’) and a 3’ primer (5’ ACATCTCTAGATTACTTTATTGTGATCGACCTTCAGGCC 3’). The coding region of LcPxn2 was PCR amplified using a 5’ primer (5’ GTGAATTCATGTCCTGCGTGACGCC 3’) and a 3’ primer (5’ GTCTCTAGATTACTTTATTGTGATCGACCTTCAGGCC 3’). The PCR products of LcPxn1 and LcPxn2 were each digested with EcoRI and XbaI and purified. PCR products of GFP and LcPxn1 or LcPxn2 were mixed and ligated and cloned into the BamHI and XbaI restriction sites of the pXNEO vector (provided by Dr S.M. Beverley). All constructs were re-confirmed by sequencing at the University of Calgary DNA Sequencing Lab.
**Western Blotting**- To detect GFP-LcPxn fusion proteins, $8 \times 10^9$ parasites were pelleted by centrifugation and washed once in ice-cold PBS. Parasites were resuspended in 2.5ml pre-chilled lysis buffer (50mM HEPES pH 7.4, 150mM NaCl, 10mM CaCl$_2$, 5µg/ml leupeptin, 5µg/ml aprotenin, 0.5% Triton X-100) and placed on ice for 30 mins. Lysate was centrifuged at 1000g for 10mins and then 10,000g for a further 10mins at 4°C. 40µl of the supernatant was boiled in sample buffer (2% SDS, 60mM Tris pH 6.8, 2.5% β-mercaptoethanol) for 5mins and resolved on a 10% SDS-PAGE gel and transferred to Hybond-P membrane (Amersham Biosciences). Western blotting was performed using anti-GFP (1:2500) antibodies and detected using an enhanced chemiluminescence method (Amersham Biosciences). To compare the over-expression of LcPxn1 and LcPxn1 mutants (Figure 2), $5 \times 10^6$ parasites of each transfectant were washed in PBS, freeze-thawed and boiled in sample buffer for 10mins before loading onto a 10% SDS-PAGE gel. Samples were subjected to Western blot as described above with anti-LcPxn1 antibodies. Monoclonal antibody (E7) to β-tubulin was purchased from Developmental Studies Hybridoma Bank, University of Iowa.

**3H-uracil Incorporation Assay**- The 3H-uracil incorporation assay was performed as previously described (5,20,46). Briefly, triplicate samples of $2 \times 10^6$ midlog phase promastigotes or stationary phase promastigotes were washed with and resuspended in 100µl Krebs-Ringer Phosphate-Glucose Solution pH 7.4 (0.154M NaCl, 0.154M KCl, 0.11M CaCl$_2$, 0.154M MgSO$_4$$\cdot$7H$_2$O, 0.1M Na$_2$HPO$_4$$\cdot$2H$_2$O pH 7.4, 10mM Glucose). Parasites were exposed for two hours at 26°C to either reagent 100µM H$_2$O$_2$, 100µM t-butyl hydroperoxide (tBOOH), 5mM sodium nitroprusside, 1mM ONOO$^-$ or the •OH-generating system. •OH was generated as previously described (20). The •OH-generating system contained 1.0mM xanthine, 2.4x10$^-$
2 units/ml xanthine oxidase and 30µM Fe²⁺ for 90 mins at 26°C. The parasites were then grown for another 2 hours at 24°C in the presence of 2µCi of ³H-uracil (Amersham Biosciences) for measurement of RNA synthesis. The parasites were solubilized in 2 ml of solubilization solution (0.1% SDS, 0.1% diethyl pyrocarbonate, 25mM HEPES, 10mM EDTA, 100µg/ml of uracil) for 15 mins on ice. After the 15 mins, 200µl of trichloroacetic acid (TCA) (100% w/v) was added to the tubes and then placed on ice for 15 mins. Samples were then applied to Whatman GF-C glass microfibre filters and washed with 10 ml of ice-cold TCA (10% w/v) and then 10 ml of ice-cold ethanol (95%) using a vacuum apparatus. Filters were air-dried, placed in 2 ml of scintillation fluid and monitored for incorporated ³H-uracil for 5 mins.

Macrophage Infection Assay- Infection of U937 cells from human origin (American Type Cell Collection, Rockville, Md.) was carried out as previously described (47). U937 cells were seeded at a concentration of 2.5x10⁵ cells/cm² in 8-chamber slides and differentiated into adherent macrophages by treatment with 7.5ng phorbol myristate acetate (Sigma) per ml of RPMI 1640 with 10% fetal calf serum, 2mM glutamine and 50µg/ml gentamicin (Invitrogen) (RPMI) under 5% CO₂ at 37°C for 72 hours. Non-adherent cells were washed 3-5 times with warm RPMI media followed by incubation with L. chagasi parasites at a parasite to U937 cell ratio of 10:1 for 6 hours. Non-engulfed parasites were washed away 3-5 times with warm RPMI and incubated in fresh RPMI media. The level of infection in 200 U937 cells was determined at 12, 24, and 48 hours by optical microscopy following Diff Quick staining of cell preparations (48). Values are expressed as the average number of parasites per infected U937 cell.

RESULTS
**Recombinant LcPxn1 Protein can detoxify RNS, whereas LcPxn2 cannot**

Assays containing reagent ONOO− or the •NO donor sodium nitroprusside (SNP) and target molecules susceptible to RNS-induced oxidation were used to test whether recombinant LcPxn1 protein can detoxify RNS. Pyrogallol Red (PR) has been previously shown to be bleached by ONOO− but not by decomposed ONOO−, nitrite or nitrate (49). When added to the reaction mixture, recombinant LcPxn1 and the ONOO− scavenger Trolox significantly (P<0.001) protected PR from bleaching compared to boiled LcPxn1, GST and BSA controls (Figure 1A). DNA has also been shown to be a target of ONOO− which converts the supercoiled DNA into a slower migrating nicked DNA (50). Consistent with the results observed above, LcPxn1 protein and Trolox were able to protect supercoiled DNA from ONOO−-induced nicking, whereas boiled LcPxn1, GST and BSA were unable to provide protection (Figure 1A-inset). Known scavengers of H₂O₂, •OH, •NO and O₂•⁻ (catalase, mannitol, PTIO and superoxide dismutase respectively) did not significantly protect the PR from bleaching or the supercoiled DNA from nicking (data not shown). Recombinant LcPxn2 protein did not significantly protect PR (Figure 1A) or the supercoiled DNA from nicking (Figure 1A-inset) compared to boiled LcPxn2, GST or BSA controls.

A colorimetric assay for measuring •NO produced from SNP was used to test the ability of recombinant LcPxn1 protein to protect ABTS from •NO-induced oxidation into the strongly absorbing green ABTS⁺ complex (45). When added to the reaction mixture, LcPxn1 protein and the •NO scavenger PTIO significantly (P<0.0001) protected ABTS from oxidation compared to boiled LcPxn1 and GST controls (Figure 1B). There was no significant difference in protection between LcPxn2 protein and boiled LcPxn2 or GST controls. As further controls, scavengers of
H$_2$O$_2$, $\cdot$OH, ONOO$^-$ and O$_2^-$ (catalase, mannitol, Trolox and superoxide dismutase respectively) did not significantly protect ABTS from oxidation (data not shown). Taken together, the above data suggests that recombinant LcPxnl protein is capable of detoxifying RNS whereas LcPxnl2 does not appear to detoxify RNS.

**Amino acid residues involved in RNS- and ROS-detoxifying activity**

The two cysteine residues corresponding to Cys47 and Cys170 in all 2-Cys peroxidoxins are highly conserved among all organisms. The amino terminus Cys47 has been implicated as the catalytic residue in the detoxification of H$_2$O$_2$ and alkyl hydroperoxides (51,52) and peroxynitrite (36). To identify the catalytic residues in *Leishmania* LcPxnl1, we performed site-directed mutagenesis of the corresponding conserved Cys52 and Cys173 residues and constructed three recombinant LcPxnl1 protein mutants containing Cys to Ala mutations (LcPxnl1-C52A, LcPxnl1-C173A and LcPxnl1-C52A/C173A).

In studying the amino acid residues involved in detoxifying ROS, we found that LcPxnl1-C52A protein failed to detoxify H$_2$O$_2$, t-butyl hydroperoxide (tBOOH) and $\cdot$OH (P<0.001 in each case) compared to wildtype LcPxnl1 protein (Figure 1C-E respectively). In addition, LcPxnl1-C52A protein failed to protect supercoiled DNA from $\cdot$OH-induced nicking (Figure 1E-inset). Since there was no significant ROS-detoxifying activity by LcPxnl1-C52A compared with boiled LcPxnl1 or GST, Cys52 appears to be essential for detoxifying ROS which is consistent with previous findings with other peroxidoxins. There was no significant difference observed in the ability of LcPxnl1-C173A to detoxify H$_2$O$_2$ (Figure 1C) or $\cdot$OH (Figure 1E) compared to wildtype LcPxnl1 and LcPxnl1-C173A protected supercoiled DNA from $\cdot$OH-induced nicking.
(Figure 1E-inset). In contrast, LcPxn1-C173A did not demonstrate significant tBOOH-detoxifying activity compared to boiled LcPxn1 and GST (Figure 1D). Taken together, these results suggest that the Cys173 residue is not essential for detoxifying H$_2$O$_2$ or ¦OH, but is essential in detoxifying tBOOH. LcPxn1-C52A/C173A protein did not demonstrate any significant activity against H$_2$O$_2$, tBOOH or ¦OH compared with boiled LcPxn1 and GST and did not prevent supercoiled DNA from ¦OH-induced nicking. LcPxn2 protein was found to detoxify only H$_2$O$_2$. It did not demonstrate significant activity towards tBOOH or ¦OH (P>0.05) nor did it protect DNA from ¦OH-induced nicking compared to controls (Figure 1C-E).

In studying the amino acid residues involved in detoxifying RNS, we found that LcPxn1-C52A protein exhibited similar levels of activity in detoxifying ONOO$^-$ and protecting supercoiled DNA from ONOO$^-$-induced nicking compared to wildtype LcPxn1 protein (Figure 1A), suggesting that Cys52 is not essential for detoxifying ONOO$^-$. LcPxn1-C173A protein did not demonstrate a significant difference in detoxifying ONOO$^-$ compared to boiled LcPxn1 or GST (P<0.005) and could not protect DNA from nicking (Figure 1A), suggesting that Cys173 is the catalytic cysteine residue and is essential for detoxifying ONOO$^-$. Both LcPxn1-C52A/C173A and LcPxn2 proteins did not detoxify ONOO$^-$ and could not prevent ONOO$^-$-induced nicking of DNA. Interestingly, wild type LcPxn1, LcPxn1-C52A, LcPxn1-C173A and LcPxn1-C52A/C173A proteins were all found to significantly (P<0.002) detoxify ¦NO compared to boiled LcPxn1, LcPxn2 and GST controls (Figure 1B). Remarkably, LcPxn1-C52A/C173A protein provided significantly (P<0.0001) more protection from ¦NO-induced oxidation compared to wildtype LcPxn1 (Figure 1B). Collectively, these results suggest that the Cys52 residue is essential for detoxifying H$_2$O$_2$, tBOOH and ¦OH, Cys173 is essential for detoxifying
Over-expression of LcPxn1 protein in *L. chagasi* enhances survival against ROS and RNS

In order to test whether LcPxn1 protein can protect *L. chagasi* parasites from exposure to an environment enriched in ROS and RNS, we over-expressed LcPxn1 protein in the parasites to see if they exhibited enhanced survival. Parasites were transfected with the pX expression vector containing the various constructs of LcPxn1 and selected at 800µg/ml G418. Southern blot verified the presence of the vectors (data not shown) and Western blot and densitometry analysis showed that each of the early log and stationary phase transfectants had more than a 1.8 fold increase in the level of LcPxn1 protein compared to the control transfectant which contained the pX vector alone (Figure 2).

Both early log phase and stationary phase parasites over-expressing LcPxn1 protein exhibited a significant (P<0.01 in each case) enhanced survival upon exposure to H$_2$O$_2$, tBOOH, *'OH,* ONOO$^-$ and *'NO compared to control parasites containing the expression vector alone (Figure 3A-E). Consistent with previous findings (5), we found that the control (pX) stationary phase parasites were significantly (P<0.01) more resistant to H$_2$O$_2$ toxicity than early log phase parasites (Figure 3A). We also found that the control (pX) stationary phase parasites were significantly more resistant to tBOOH (P<0.001) (Figure 3B) and ONOO$^-$ (P<0.03) (Figure 3D).

In support of our findings that Cys52 is essential in detoxifying H$_2$O$_2$, tBOOH and *'OH and that Cys173 is essential in detoxifying *tBOOH and ONOO$^-$, parasites over-expressing LcPxn1-C52A
did not exhibit an enhanced survival upon exposure to H$_2$O$_2$, tBOOH or 'OH but did exhibit enhanced survival upon exposure to ONOO$^-$ compared to pX control parasites (Figure 3A-D). LcPxn1-C173A exhibited an enhanced survival upon exposure to H$_2$O$_2$ and 'OH but not upon exposure to tBOOH or ONOO$^-$ (Figure 3A-D) which is consistent with our observations with recombinant LcPxn1-C173A protein (Figure 1). Contrary to our findings with recombinant LcPxn1 proteins, parasites over-expressing LcPxn1-C52A and LcPxn1-C173A did not exhibit enhanced survival upon exposure to 'NO (Figure 3E). Parasites over-expressing LcPxn1-C52A/C173A did not exhibit enhanced survival upon exposure to any of the ROS or RNS.

**Cellular localization of LcPxn1 and LcPxn2 proteins**

To further define the functions of LcPxn1 and LcPxn2 in parasite survival, we studied the cellular localization of these proteins within *L. chagasi*. The amino acid sequence of LcPxn1 does not appear to contain a typical organellar-targeting signal sequence which suggests that it may be localized to the cytoplasm. The last three amino acids at the carboxyl terminus of LcPxn2 are SKQ and conspicuously resembles the glycosomal targeting signal sequence SKL. Although mutational analysis of the SKL glycosomal targeting signal in *Trypanosoma brucei* showed that this signal is highly degenerate, mutation of the signal to SKQ was not sufficient to target proteins to the glycosome but rather remained cytosolic (53).

We created GFP-LcPxn1 and GFP-LcPxn2 fusion protein gene constructs and over-expressed them in *L. chagasi* parasites. Parasites were selected at 50µg/ml G418 and Western blot analysis of each parasite extract with anti-GFP revealed the presence of an ~48kDa fusion protein suggesting that both the GFP-LcPxn1 and GFP-LcPxn2 fusion proteins were intact (Figure 4A).
No bands corresponding to the GFP protein alone (27kDa) were observed in either of the extracts isolated from parasites expressing the fusion proteins. Fluorescence microscopy showed that both the GFP-LcPxn1 and GFP-LcPxn2 fusion proteins are localized throughout the parasite including the flagella, similar to the fluorescence pattern observed with the control parasites expressing the GFP protein alone (Figure 4B-D). The fluorescence patterns observed for both LcPxn1 and LcPxn2 are distinct from the pattern of glycosomal localization (data not shown). These results suggest that both LcPxn1 and LcPxn2 are localized to the cytoplasm.

**Over-expression of LcPxn1 protein in *L. chagasi* enhances intracellular survival within macrophages**

During the initial stages of infection with a foreign pathogen, an oxidative burst occurs in human macrophages (9,18,54,55) including U937 cells (56-58) in which ROS is produced in response to phagocytosis. Human macrophages including U937 cells have also been shown to produce RNS once infection is established (18,59,60).

We have previously shown that the level of LcPxn1 mRNA expression increases significantly towards the amastigote phase compared to early log phase parasites (25). In order to gain insight into the role that LcPxn1 plays in intracellular survival within macrophages, we tested the ability of *L. chagasi* parasites over-expressing LcPxn1 to survive within the human macrophage cell-line U937. The average number of amastigotes over-expressing wild type LcPxn1 per infected macrophage at 12, 24 and 48 hours post infection was 8.3 (+/-0.44), 12.1 (+/-0.35) and 14.2 (+/-0.65) respectively (Figure 5). These numbers are significantly (P<0.05) greater than pX control parasites at the corresponding time intervals with 5.9 (+/-0.1), 8.5 (+/-0.76) and 9.4 (+/-0.4)
respectively (Figure 5). At 12 hours post infection, parasites over-expressing LcPxn1-C173A had a significantly (P<0.05) higher average number of amastigotes per infected macrophage of 7.6 (±/0.31) compared to 5.9 (±/0.10) for the pX control parasites. At 24 and 48 hours post infection, there was no significant difference in the average numbers between parasites over-expressing LcPxn1-C173A and the pX control parasites. Parasites over-expressing LcPxn1-C52A or LcPxn1-C52A/C173A did not exhibit a significant increase in parasite load compared to the pX control parasites at any time interval (Figure 5). No significant difference in the percentage of infected U937 cells was observed at any time interval (data not shown).

**DISCUSSION**

Previous reports have shown that both ROS and RNS contribute to the early control of *Leishmania* infection and that RNS alone is necessary and sufficient to control *Leishmania* infection (11,17,18). Clearly, the possession of an antioxidant defence system against ROS and more importantly RNS would provide intracellular pathogens like *Leishmania* a selective advantage for survival. In further characterizing the role of peroxidoxins in *L. chagasi* survival, we have shown that recombinant LcPxn1 protein can detoxify RNS in addition to ROS, whereas recombinant LcPxn2 protein appears to play a more limited role by only being able to detoxify H$_2$O$_2$. Over-expressing LcPxn1 within *L. chagasi* parasites significantly enhanced parasite survival within macrophage cells and upon exposure to exogenously added ROS and RNS. In addition, we have implicated the Cys52 residue as being essential in detoxifying H$_2$O$_2$, tBOOH and •OH and Cys173 as being essential for detoxifying tBOOH and ONOO⁻.
Consistent with previous studies on peroxidoxins from other organisms, the Cys52 residue of LcPxn1 is essential for detoxifying peroxides which fits with the proposed mechanism of action for 2-Cys peroxidoxins which involves the attack of the amino terminus cysteine residue by peroxide to form a sulphenic acid residue intermediate which can then react with the adjacent Cys173 residue and/or a diffusible thiol such as dithioerythritol \textit{in vitro} or trypanothione \textit{in vivo} in \textit{Leishmania} to form a disulphide bond (52,61). The mechanism of action for the detoxification of \textsuperscript{1}OH has not been previously described. Similar to the mechanism of action for detoxifying H\textsubscript{2}O\textsubscript{2}, our studies revealed that the Cys52 residue is also essential for detoxifying \textsuperscript{1}OH which fits with a mechanism of action where a \textsuperscript{1}OH can abstract a hydrogen from the Cys52 R-SH group to form a thyl radical (R-S\textsuperscript{•}) which can subsequently be attacked by another \textsuperscript{1}OH to form a sulphenic acid residue intermediate and react with the adjacent Cys173 residue and/or a diffusible thiol. It is debatable whether \textsuperscript{1}OH can be scavenged \textit{in vivo} since \textsuperscript{1}OH reacts very quickly with almost every type of molecule in living cells and as such a high concentration of peroxidoxins would have to be present to compete with biological targets. However, peroxidoxins have been shown to be highly abundant in cells such as yeast (0.7\% of the total soluble protein) and peroxidoxins are the most abundant protein in erythrocytes after haemoglobin (62,63). Peroxidoxins have also been shown to be able to protect biological targets such as DNA from attack by \textsuperscript{1}OH (25,40) and furthermore we have shown here that LcPxn1 is cytoplasmic which significantly increases its chance of coming into contact with \textsuperscript{1}OH (Figure 4) and whose over-expression can protect parasites from an exogenous source of \textsuperscript{1}OH (Figure 3C).

Numerous studies have identified alkyl hydroperoxides as substrates for peroxidoxins, implicating peroxidoxins as very important enzymes in reducing phospholipid hydroperoxides.
which can arise from oxidation and thereby protecting cells from plasma membrane damage. *Leishmania* parasites lack typical glutathione peroxidases which are well-known protectors of lipid peroxides in eukaryotes. We previously demonstrated that recombinant LcPxn1 protein can detoxify alkyl hydroperoxides and have extended these studies to show that LcPxn1 can also protect *L. chagasi* from tBOOH (Figures 1D and 3B). Interestingly, we found that both the conserved Cys52 and Cys173 residues are essential for detoxifying tBOOH which suggests an alternative mechanism for the detoxification of ROOH compared to the detoxification of H$_2$O$_2$ and •OH. Recent studies have emphasized the importance of the microenvironment surrounding the active site residues of peroxidoxins (52,64). It is therefore possible that the Cys173 residue of LcPxn1 may be essential for coordinating the active site into a more favourable environment for donating a proton to the poor and much more bulky RO$^-$ leaving group. The lack of an available proton donor could cause the sulfenic acid intermediate (R-SOH) that forms on the catalytic cysteine to be further oxidized into R-SOOH which has been found to lead to reduced activity (65). Remarkably, we could not detect alkyl hydroperoxidase activity with recombinant LcPxn2 protein which is 89% identical to LcPxn1 (Figure 1D). There has been a report of a peroxidoxin from *Leishmania major* (Lmf30 TryP) that is highly homologous to LcPxn2 and is also incapable of significantly detoxifying alkyl hydroperoxides (26). Crystallographic studies with the peroxidoxin AhpC from *S. typhimurium* has revealed that structural conformations and the mobility of key residues present in loop structures encompassing the active site cysteines is very important for activity of the protein (64). The main difference between LcPxn1 and LcPxn2 is the presence of a nine amino acid extension at the carboxyl terminus of LcPxn2. It is possible that this extension alters the microenvironment surrounding the active site into one that is not
favorable for activity or is more prone to inactivation as described above. Characterization of the
crystal structures of LcPxn1 and LcPxn2 will provide more insight into this mechanism.

Bacterial and yeast peroxidoxins have been previously shown to protect cells from \textsuperscript{\textasteriskcentered}NO and
ONOO\textsuperscript{-}-mediated toxicity (34-36). Our findings also show that LcPxn1 can detoxify \textsuperscript{\textasteriskcentered}NO and
ONOO\textsuperscript{-} and protect \textit{Leishmania} from RNS- mediated toxicity, however the mechanism by which
this occurs in \textit{Leishmania} differs from the proposed mechanism by which bacterial peroxidoxins
detoxify ONOO\textsuperscript{-}. The conserved amino terminus cysteine (Cys46) residue of the AhpC
peroxidoxin from \textit{S. typhimurium} was found to be essential for activity (36), whereas we
demonstrate that the carboxyl terminus cysteine (Cys173) residue of LcPxn1 is essential for
activity (Figure 1A and 3D). This finding will be important for future drug design studies with
\textit{Leishmania} peroxidoxins that target the ability of peroxidoxins to detoxify RNS. A possible
mechanism of action is one where the Cys173 residue can attack the O-O bond in ONOO\textsuperscript{-}
resulting in the transfer of one oxygen atom to the Cys173 and the release of nitrite, alternatively
ONOO\textsuperscript{-} can oxidize the R-SH group of Cys173 converting it into R-S\textsuperscript{\textasteriskcentered} and releasing \textsuperscript{\textasteriskcentered}NO\textsubscript{2} and \textsuperscript{-}OH. Although peroxidoxins have been shown to protect bacteria, yeast and human cells from the
toxic effects mediated by ONOO\textsuperscript{-} and \textsuperscript{\textasteriskcentered}NO (34,35), no evidence has been presented of a
recombinant peroxidoxin protein capable of detoxifying \textsuperscript{\textasteriskcentered}NO directly. We found that over-
expression of LcPxn1 protein in parasites also protected them from both ONOO\textsuperscript{-} and \textsuperscript{\textasteriskcentered}NO-
mediated toxicity, but we also found evidence that recombinant LcPxn1 protein can detoxify \textsuperscript{\textasteriskcentered}NO
(Figure 1B). Interestingly, site-directed mutagenesis of LcPxn1 protein revealed that neither of
the conserved cysteine residues (Cys52 or Cys173) were essential for detoxifying \textsuperscript{\textasteriskcentered}NO and the
mutation of both cysteines led to increased activity. This result suggests that LcPxn1 possesses a
different mechanism for detoxifying \textsuperscript{\textsuperscript{1}}NO which we are currently investigating. Oddly, the over-expression of wildtype LcPxn1 but not the mutant proteins within the parasites provided significant protection to the parasites when exposed to \textsuperscript{\textsuperscript{1}}NO (Figure 3E). It is possible that formation of heterogeneous multimers between mutant and wildtype LcPxn1 monomers within the parasites led to an inhibitory effect.

Of significant interest is our finding that over-expression of LcPxn1 in \textit{Leishmania} parasites enhanced survival within macrophages (Figure 5). Despite the findings that ROS and RNS act together early to control \textit{Leishmania} infection and that RNS alone is sufficient to control infection, \textit{L. chagasi} persists long enough to establish a potentially fatal infection. Our data shows that \textit{L. chagasi} parasites possess a protective enzymatic defense against ROS and most notably against RNS. Interestingly, over-expression of the Cys173 mutant in the parasites which we found to be active only towards H\textsubscript{2}O\textsubscript{2} and \textsuperscript{\textsuperscript{1}}OH but not RNS enhanced survival early in infection (12 hours post infection) when ROS are produced in abundance during phagocytosis. Later when infection is established (24-48 hours post infection) and when iNOS mRNA and protein are expressed, the same parasites over-expressing Cys173 failed to exhibit enhanced survival. The Cys52 mutant which is active only towards RNS did not provide enhanced survival early in infection and failed to enhance survival at later stages of infection. These findings support previous findings that ROS are important early in controlling \textit{Leishmania} infection and that RNS alone appear to be sufficient to control infection. Taken together, our results strongly suggest that LcPxn1 enhances parasite survival in culture and within macrophages by providing a last line of defense against the most biologically important ROS and RNS. Since LcPxn1 is predominantly expressed in the amastigote stage, it could provide much needed protection from
the ROS and RNS that are produced within the macrophages that are free to diffuse across lipid membranes into the parasites. LcPxn2 is predominantly expressed in the early log phase of the parasites and our finding that LcPxn2 appears to be able to only detoxify H2O2 suggests that LcPxn2 plays a more limited function in parasite survival in the promastigote stage in the gut of the sandfly vector which may not be as enriched in ROS and RNS as within the phagosome of the host’s macrophages.

The discovery of an enzymatic defense against RNS in addition to ROS in *L. chagasi* is very exciting since it identifies a factor present within the parasites that can detoxify molecules produced by the host that have been previously shown to control *Leishmania* infection. It will be interesting to see if a homolog to LcPxn1 is present in *Leishmania* strains that cause the self-healing cutaneous form of leishmaniasis. So far, peroxidoxins isolated from *L. major* (26) and *L. infantum* (27,66) have not been shown to possess the ability to detoxify RNS. It is possible that LcPxn2 is an evolutionary precursor to LcPxn1 which through evolution underwent a deletion in the carboxyl terminus. This deletion could have altered the microenvironment of the active site surrounding the conserved cysteine residues resulting in the acquisition of higher functions such as being able to detoxify phospholipid hydroperoxides, ‘OH, ONOO’ and ‘NO which ultimately led to a selective advantage for *L. chagasi* survival. It will be interesting to see whether the acquisition of the higher functions of LcPxn1 contribute to the pathogenicity of *L. chagasi*. Our findings provide a better understanding of the mechanisms that *Leishmania* utilize for intracellular survival and the role that peroxidoxins play in *Leishmania* survival. We are currently using homologous recombination and anti-sense technology and mouse infection
models in order to gain a better understanding of the role that peroxidoxins play in the pathogenesis of leishmaniasis.

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**Abbreviations**

'NO, nitric oxide; ONOO^−, peroxynitrite; 'OH, hydroxyl radicals; H_2O_2, hydrogen peroxide; ROOH, hydroperoxide; O_2^−, superoxide radicals; tBOOH, tert-butyl hydroperoxide; RNS, reactive nitrogen species; ROS, reactive oxygen species; *L. chagasi*, *Leishmania chagasi*; *L. major*, *Leishmania major*; GST, Glutathione-S-Transferase; GFP, Green Fluorescence Protein.

**FIGURE LEGENDS**

**Figure 1:** Recombinant LcPxn1 protein assays. Recombinant LcPxn1 protein and the various LcPxn1 protein mutants were tested for their ability to detoxify various RNS and ROS. A, activity was assessed by the ability of the LcPxn1 proteins to protect 50µM Pyrogallol Red from ONOO^−-induced bleaching and to protect supercoiled pGEM-2 plasmid (s) from ONOO^− attack into the slower migrating nicked band (n) (A-inset); B, activity towards protecting 5mM ABTS from 'NO-induced oxidation into the highly absorbing green ABTS^+ complex. C, Activity expressed as nmol/min/µg recombinant protein towards 100µM H_2O_2 and 100µM t-butyl hydroperoxide (D); E, activity towards protecting 0.8mM 2-deoxy-D-ribose from 'OH-induced damage and the ability to protect supercoiled (s) pGEM-2 DNA from 'OH-induced nicking (n) (E-inset). The average +/- SE of at least four independent experiments are shown. Insets were representative of three independent experiments.
**Figure 2:** Western blot analysis of *L. chagasi* over-expressers. Lysates from early log phase or stationary phase parasites transfected with pX, pX-LcPxn1, pX-LcPxn1-C52A, pX-LcPxn1-C173A or pX-LcPxn1-C52A/C173A expression vectors were separated on a 10% SDS-PAGE gel and subjected to Western blotting using LcPxn1 antisera (upper panel). The same blots were stripped and incubated with monoclonal β-tubulin antibody to serve as a loading control (lower panel).

**Figure 3:** Parasite protection assays. Early log phase (black bars) or stationary phase (open bars) *L. chagasi* parasites over-expressing LcPxn1 or the various mutant LcPxn1 proteins were re-suspended in 100μl Krebs-Ringer Phosphate-Glucose Solution pH 7.4 and exposed to 100μM H$_2$O$_2$ (A), 100μM t-butyl hydroperoxide (B), *OH*-generating system (C), 1mM ONOO⁻ (D) or 5mM nitroprusside (E) for two hours as described in the Experimental Procedures section. Parasite viability was assessed by determining the percent of $^3$H-uracil incorporation into parasite RNA. The average +/- SE of at least three independent experiments are shown.

**Figure 4:** LcPxn1 and LcPxn2 cellular localization. A, Lysates from wild type *L. chagasi* cells transfected with pX-GFP, pX-GFP-LcPxn1 and pX-GFP-LcPxn2 were separated on a 10% SDS-PAGE gel and subjected to Western blot analysis using anti-GFP antibodies. A ~48kDa band was observed in lanes 2 and 3 which corresponds to the sizes of LcPxn1 (21kDa) and LcPxn2 (22kDa) fusion proteins with GFP (27kDa). GFP fluorescence patterns of *L. chagasi* parasites expressing GFP alone (B), LcPxn1-GFP (C) or LcPxn2-GFP (D).
Figure 5: Survival of *L. chagasi* parasites within U937 cells. U937 cells were incubated with stationary phase parasites transfected with pX (meshed bars), pX-LcPxn1 (black bars), pX-LcPxn1-C52A (slashed bars), pX-LcPxn1-C173A (open bars) or pX-LcPxn1-C52A/C173A (checkered bars) for 6 hours as described in Experimental Procedures. Non-engulfed parasites were washed away and the infected U937 cells were incubated for 12, 24 and 48 hours after which the infected macrophages were stained with Diff Quick and examined using optical microscopy to determine the level of infection which was expressed as the average number of amastigotes per infected macrophage. The average +/- SE of four independent experiments are shown.

TABLE I

| Summary of the antioxidant profiles of recombinant LcPxn1, LcPxn1 mutants and LcPxn2 proteins |
|----------------------------------------------------------|
| LcPxn1 | LcPxn1- C52A | LcPxn1- C173A | LcPxn1- C52A/C173A | LcPxn2 |
| H₂O₂  | +         | −               | +                | −      | +     |
| ROOH  | +         | −               | −                | −      | −     |
| ‘OH   | +         | −               | +                | −      | −     |
| ONOO⁻ | +         | +               | −                | −      | −     |
| ‘NO   | +         | +               | +                | +      | −     |
| O₂⁻   | −         | −               | −                | −      | −     |
Figure 2

LcPxn1

β-tubulin

Early Log Phase

Stationary Phase
Figure 4
Figure 5
Role of peroxidoxins in leishmania chagasi survival: Evidence of an enzymatic defense against nitrosative stress
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