Inducible Resistance to Oxidant Stress in the Protozoan
Leishmania chagasi*

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Leishmania sp. protozoa are introduced into a mammalian skin by a sandfly vector, whereupon they encounter increased temperature and toxic oxidants generated during phagocytosis. We studied the effects of 37 °C “heat shock” or sublethal menadione, which generates superoxide and hydrogen peroxide, on Leishmania chagasi virulence. Both heat and menadione caused parasites to become more resistant to H$_2$O$_2$-mediated toxicity. Peroxide resistance was also induced as promastigotes developed in culture from logarithmic to their virulent stationary phase form. Peroxide resistance was not associated with an increase in reduced thiols (trypanothione and glutathione) or increased activity of ornithine decarboxylase, which is rate-limiting in trypanothione synthesis. Membrane lipophosphoglycan can increased in size as parasites developed to stationary phase but not after environmental exposures. Instead, parasites underwent a heat shock response upon exposure to heat or sublethal menadione, detected by increased levels of HSP70. Transfection of promastigotes with L. chagasi HSP70 caused a heat-inducible increase in resistance to peroxide, implying it is involved in antioxidant defense. We conclude that leishmania have redundant mechanisms for resisting toxic oxidants. Some are induced during developmental change and others are induced in response to environmental stress.

Mammalian phagocytes generate superoxide, hydrogen peroxide, and nitric oxide as a part of their antimicrobial armamentarium. These in turn rapidly react to form toxic effectors such as the hydroxyl radical (·OH) and peroxynitrite (ONOO$^-$). Intracellular microbes residing in macrophages in particular must rise to this challenge by preventing oxidant formation, resisting damage from toxic oxygen products, or metabolizing oxidants. Microorganisms have developed a diverse array of mechanisms allowing them to evade oxidant-mediated killing (1).

The Leishmania sp. are obligate intracellular protozoa residing in macrophages of their mammalian hosts. The promastigote form of the parasite is inoculated into mammalian skin by a sand fly vector, subjecting it to a sudden change in ambient temperature. Parasites undergo phagocytosis by dendritic cells and macrophages in the skin, exposing them to antimicrobial oxidants generated during phagocytosis (2, 3). In the face of these exposures a subset of parasites survive, convert to obligate intracellular amastigotes, and can eventually lead to disease symptoms (2, 4, 5).

Mechanisms through which the Leishmania sp. resist the toxic effects of oxidant exposure include the promastigote surface glycolipid lipophosphoglycan (LPG) which scavenges toxic oxygen products and inhibits macrophage responses (6, 7). Several Leishmania antioxidant enzymes have been identified and cloned (8–11). Trypanosomes and Leishmania sp. also possess trypanothione (TSH), a unique redox-cycling glutathione-stermine conjugate which, in concert with trypanothione reductase, maintains the intracellular reducing environment (11–13). Disruption of the trypanothione reductase gene or transfection with trans-dominant inactive trypanothione reductase renders parasites more susceptible to intracellular killing in macrophages capable of generating reactive oxygen intermediates (13, 14). Finally, similar to other organisms, Leishmania sp. augment their expression of heat shock proteins in response to elevated temperature or other environmental stress. The heat shock proteins are involved in the prevention and repair of damage caused by denatured and aggregated proteins. Their expression can result in a cell with increased resistance to thermally induced injury and cross-resistance to other toxic environmental exposures (15).

We previously found that Leishmania chagasi promastigotes undergo a stress response after exposure to environmental conditions they encounter in their mammalian hosts, including heat or sublethal concentrations of oxidants. This response results in cross-resistance to the toxic effects of either superoxide or hydrogen peroxide and increased virulence (16, 17). Promastigotes also undergo a developmental change as they progress from logarithmic to stationary phase in culture medium, which is accompanied by an increase in resistance to...
H$_2$O$_2$-induced injury and an increase in virulence. Our investigations suggested that different mechanisms account for the inducible oxidant resistance encountered during development as opposed to environmental stress (17). The purpose of the present study was to investigate mechanisms that might be responsible for inducible resistance to oxidant-mediated toxicity in *L. chagasi* promastigotes. Such mechanisms are likely essential for survival of the parasite in a mammalian host.

**EXPERIMENTAL PROCEDURES**

**Parasites**—A Brazilian isolate of *L. chagasi* (MHOM/BR/00/1669) was maintained in hamsters by serial intracardiac injection of amastigotes. Parasites were grown as promastigotes at 26 °C in liquid hemoflagellate-modified minimal essential medium (HOMEM) (18) and used within 3 weeks of isolation. Some parasites were transferred to serum-free medium (SFM) (16). Promastigote cultures were seeded at 1 × 10$^6$/ml and harvested during logarithmic or stationary phase of growth, defined according to concentration and morphology as we have described previously (19).

**Stress Exposures and Viability Assays**—Promastigotes in HOMEM were exposed to heat shock by incubation in a 37 °C water bath. Menden is dissolved in Me$_2$SO (1:50) and added at 2.5 μM to cells adapted to SFM, a concentration that we previously determined was sublethal but generated detectable superoxide (16). Control parasites were suspended in the equivalent volume of Me$_2$SO alone.

2 × 10$^8$ stress-exposed or control promastigotes in 100 μl of HBSS were exposed in triplicate to varying concentrations of H$_2$O$_2$ (Sigma) in 96-well plates at 26 °C. After 1 h the reaction was stopped by addition of 10% heat-inactivated fetal calf serum and 500 units/ml catalase. Parasite viability was measured by incubation in 0.5 mg/ml (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) for 3 h followed by addition of 100 μl of 0.04% n-heptanol in isopropl alcohol. Living mitochondria convert MTT to dark blue formazan that is soluble in acid isopropl alcohol. Formazan was detected on a microplate reader at 570 nm (19). The percent viability was calculated from the ratio of OD readings in wells rendered void of H$_2$O$_2$ versus wells without H$_2$O$_2$ × 100.

**Orotate Decarboxylase Activity**—Promastigote pellets were suspended in 50 volumes of PSDEP buffer (60 mM sodium phosphate, pH 7.8, 44 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.05 mM pyridoxal phosphate). They were lysed by six rounds of freeze-thaw and clarified by 15 min of microcentrifugation. ODC activity in recombinant ODC or lysates was measured by the amount of [1-14C]ornithine produced per mg of protein lysate over 1 h.

**Polyamines and Reduced Thiols**—Putrescine, spermidine, and spermine were quantified as the o-phthalaldehyde derivatized from acid extracts by reverse phase HPLC on a C-18 Percosil 10-μm column as described previously (21). To measure reduced thiols, 10$^8$ promastigotes in 100 μl of HOMEM were metabolically labeled by overnight incubation in 300 μCi of [3H]mannose (60 Ci/mmol, Amersham Pharmacia Biotech). LPG was extracted sequentially with CHCl$_3$/CH$_3$OH (3:2) and other solvents as described (22). After electrophoresis on an 8% SDS Tris-Tricine gel (30), radioactive LPG was detected by autoradiography.

**Plasmid Constructs and Parasite Transfection**—The HSP70 coding region was amplified from a full-length HSP70 cDNA previously cloned from an L. chagasi promastigote cDNA expression library (31) using the polymerase chain reaction. Primers introduced BamHI sites (underlined) at both ends of the coding region (top primer 5'-CGGCCATTCG-GACAGATGACGGTTGCAG-3'; bottom primer 5'-CGCCGGATCCT-GCTCGGGCAGTATCG-3'). The reaction product was cloned into the BamHI site of p63XNeo, a *Leishmania* plasmid shuttle vector kindly provided by Steven Beverly, Washington University, St. Louis. The insert sequence was fully sequenced. Logarithmically growing L. chagasi promastigotes were transfected with 20 μg of purified plasmid DNA without insert or p63XNeo containing the HSP70 gene. Single colonies were selected on medium 199-agar plates as described (32). Clones were gradually adapted in liquid medium to 1000 μg/ml G418.

**RESULTS**

**Inducible Resistance to Hydrogen Peroxide-mediated Toxicity**—At the onset of mammalian infection, *Leishmania* sp. promastigotes are transferred from ambient temperature in an insect vector to 37 °C in a mammalian host. Upon phagocytosis by a macrophage, they are exposed to products of the NADPH oxidase (2, 33). In order to reproduce these conditions, promastigotes were exposed *in vitro* to 37 °C heat or sublethal concentrations of menadione, a compound that generates both superoxide and H$_2$O$_2$. These exposures each elicited a response that rendered promastigotes more resistant to the toxic effects of H$_2$O$_2$ than unexposed parasites (Fig. 1) (16). We previously showed that stationary phase promastigotes are more resistant to oxidant toxicity than logarithmically growing promastigotes (17).

**ODC Expression after Environmental Stress**—Trypanothione is a conjugate of glutathione and spermidine that cycles between oxidized and reduced thiol forms. Similar to GSH in other eukaryotic cells, trypanothione maintains the intracellular reducing environment and contributes to antioxidant defense (12, 13). The rate-limiting enzyme in trypanothione biosynthesis is ODC (12). We queried whether inducible oxidant resistance in promastigotes is associated with increased ODC activity and/or increased levels of reduced trypanothione.

Both sublethal menadione and growth to stationary phase caused an increase in the steady state level of ODC RNA (Fig. 2). By using light exposures of Northern blots, mRNA abundance was quantified by densitometry as a ratio with 18 S RNA. ODC RNA increased 4.7-fold after 20 h of menadione exposure and 9.2-fold during growth to stationary phase. Changes in HSP70 and HSP90 RNAs were less dramatic. HSP70 RNA increased 1.3- or 2.1-fold, respectively, after menadione exposure or stationary growth. The ratios of HSP90 RNAs were 1.6 and 3.7, respectively. We previously documented increased amounts of HSP70 and HSP90 proteins after menadione treatment and decreased HSP70 levels with growth to stationary phase (16, 34). Despite the increase in ODC mRNA, ODC enzyme activity decreased significantly during growth to stationary phase, and there was a trend toward decreased ODC activity after exposure to sublethal menadione (Fig. 3). There is only one copy of the ODC gene in *L. donovani* (27). It therefore seems unlikely that the band on ODC North-
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Fig. 1. Stress-induced resistance of promastigotes to H$_2$O$_2$-mediated toxicity. A, promastigotes were pre-exposed to 37 °C heat shock overnight or to a sublethal concentration of menadione (2.5 μM) for 2 h 1 day prior to the assay. They were suspended in HBSS and exposed to the indicated concentrations of H$_2$O$_2$ for 1 h. Promastigote viability was measured according to their conversion of the dye MTT to formazan, a function that depends on mitochondrial activity. The mean viability in triplicate wells compared with wells with no H$_2$O$_2$ is shown in a representative experiment. B, mean H$_2$O$_2$ resistance of promastigotes exposed to heat or sublethal menadione. Promastigotes were pre-exposed to heat (n = 16 assays) or menadione (n = 5 assays) as in A. Controls for menadione exposure were suspended in the equivalent volume of Me$_2$SO as used for the menadione solvent. Viability was determined after a 1-h exposure to 200 μM H$_2$O$_2$. Shown are the mean ± S.E. percent of control viability in untreated (open bars) versus pre-exposed (hatched bars) promastigotes in replicate MTT assays, each with triplicate conditions. Statistical analyses were done by t test.

Fig. 2. Induction of ornithine decarboxylase mRNA. Equivalent flasks of promastigotes in logarithmic phase of growth (Log) were suspended in buffer alone (Control, Cont) or a sublethal concentration of menadione (2.5 μM) (md) for 2 h. 6 or 20 h later, RNA was extracted. Another flask (Sta) was allowed to develop to stationary phase. Northern blots of total RNA were probed with 32P-labeled DNA sequences for L. donovani ODC, L. chagasi hsp70, or L. chagasi hsp90. The final panel shows a control probe in which the ODC blot was stripped and reprobed with 18 S rRNA to verify equal loading of lanes. The addition of an equal volume of Me$_2$SO as used for the menadione solvent did not alter expression of any genes compared with controls (not shown).

Fig. 3. Assay of ODC activity. Promastigotes in logarithmic growth were exposed for 2 h to the ODC inhibitor DFMO (1 mM) versus control (C). Other promastigotes were harvested in (Log) or stationary (Sta) phases of growth. Logarithmic promastigotes were exposed to 2.5 μM menadione (MD) for 2 h or to 37 °C heat shock (HS) for 12 h, whereas controls were maintained in control medium without (HS) or with (MD) Me$_2$SO at 26 °C. Cytosolic fractions of all promastigotes were assayed for ODC activity. One unit of ODC represents the nanomoles of [$^{14}$C]CO$_2$ produced from L-[1-14C]ornithine per mg parasite protein/h. Data represent means ± S.E. of 4 assays, each with triplicate conditions.

Levels of Reduced Thiols after Environmental Stress—Promastigotes can become resistant to heavy metals by overproducing trypanothione (37). To determine whether inducible oxidant resistance was associated with a similar increase, we quantified reduced thiols in promastigotes harvested under different conditions. Reduced thiols were derivatized with monobromobimane and separated by reverse phase HPLC (22). Reduced glutathione (GSH), trypanothione (TSH), and glutathionyl-spermidine intermediates (GSH-Spd) were detected as peaks of fluorescence eluted from the column (Fig. 4). To our surprise, the amounts of total reduced trypanothione and glutathione were similar (Fig. 5, A and B). The level of reduced trypanothione in stationary phase promastigotes was paradoxically lower than in logarithmic organisms (n = 4) despite their increased resistance to H$_2$O$_2$, paralleling the activity of ODC (see Fig. 3). Exposure to environmental stress (heat or menadione) did not significantly alter the level of either TSH or GSH (heat, n = 4; sublethal menadione, n = 7).

ODC activity was decreased by 98 ± 1% in the presence of the specific irreversible inhibitor DFMO (see Fig. 3; n = 9) (38, 39). Incubation in DFMO was ultimately lethal for promastigote cultures (data not shown). Four or seven days of incubation in 1 μM DFMO decreased the enzyme product putrescine by 82 and 97%, respectively (Table I). DFMO exposure also caused a significant decrease in the amount of reduced trypanothione (Figs. 4 and 5A; n = 5). However, DFMO caused a paradoxical increase in resistance of promastigotes to H$_2$O$_2$ (Fig. 5, C and D), investigated further below. We conclude that neither developmental nor environmentally induced resistance to oxidant toxicity is accounted for by an increase in ODC activity or reduced trypanothione.

Expression of Proteins and HSP70 after Environmental Stress—To determine whether conditions that augment promastigote resistance to H$_2$O$_2$ induce a stress response, we measured the rate of protein synthesis. Promastigotes were exposed for 2 h to 37 °C heat shock, a sublethal concentration of menadione (2.5 μM), buffer alone, or buffer with Me$_2$SO to control for the menadione solvent. Both heat shock and menadione caused an increase in total protein synthesis compared with control conditions. 37 °C heat shock increased [$^{14}$C]leucine incorporation from 4606 ± 231 to 8393 ± 962 cpm in a representative experiment (3 repeats with duplicate conditions; p < 0.05). Menadione increased leucine incorporation from 5015 ± 214 to 6380 ± 52 cpm in a representative experiment (3 repeats with duplicate conditions; p = 0.06).

We performed immunoblots to determine whether heat shock proteins were among those induced by environmental stress. Logarithmically growing promastigotes were exposed to 37 °C heat shock, sublethal menadione (2.5, 5.0 μM), or 1 mM DFMO to inhibit ODC activity. Other parasites were harvested during logarithmic versus stationary growth phases (Fig. 6).
HSP70 and β-tubulin immunoblots of proteins from these promastigotes showed the following. First, both heat exposure and sublethal menadione caused an increase in the amount of HSP70 protein. Second, growth to stationary phase was associated with a decrease rather than an increase in HSP70 protein expression. Third, DFMO exposure also increased HSP70 protein expression, in addition to its effects on ODC and trypanothione (see Figs. 3 and 5). Thus, the paradoxical increase in H$_2$O$_2$ resistance after DFMO exposure could be due to induction of a stress response, similar to other environmental exposures. These blots make it apparent that different responses are induced upon exposure of parasites to environmental stress as opposed to the growth to stationary phase, even though each produces a state of increased oxidant resistance.

**TABLE I**

| Conditions | Putrescine | Spermidine | Spermine |
|------------|------------|------------|----------|
| Logarithmic | 1422*      | 3456       | 330      |
| Stationary | 354        | 1687       | 33       |
| DFMO*      | 9.0        | 131        | 6.1      |
| Menadione control† | 1760 | 1080 | 82 |
| Menadione† | 1942       | 2230       | 215      |

* Polyamine levels are mean pmol/10$^7$ cells in four replicate HPLC runs.
† Data for DFMO exposure are shown in stationary phase promastigotes.
‡ Menadione exposure was performed on logarithmic promastigotes. Controls were incubated in the same concentration of the solvent Me$_2$SO as menadione-exposed promastigotes. Slight differences in growth phase account for differences in levels.

**FIG. 6.** HSP70 and β-tubulin immunoblots of promastigotes exposed to environmental stress. Left panels, promastigotes were pre-exposed at 37 °C overnight (hs) or sublethal (2.5 μM) menadione (md) for 2 h. Parasites in control lanes were maintained in growth medium alone (c1) or incubated with the solvent Me$_2$SO (c2; control for menadione). Total proteins were extracted and analyzed on identical immunoblots probed with either rabbit polyclonal antibody to recombinant L. chagasi HSP70 or monoclonal antibody to β-tubulin. Gels of lanes contain proteins from 5 × 10$^7$ promastigotes. Middle panels, promastigotes were harvested during growth from logarithmic (days 1 and 2) to stationary phase (day 6). Total parasite proteins were analyzed for HSP70 and β-tubulin as above. Right panels, promastigotes were grown in 1 mM DFMO (D) without or with 300 μg/ml putrescine (DP) for 3 days prior to harvesting and analysis of proteins on HSP70 immunoblots. Controls were cultivated with no additive (C) or putrescine alone (P).

**FIG. 5.** Levels of reduced trypanothione (A) and glutathione (B) are shown in control promastigotes (open bars) or promastigotes exposed to 1 mM DFMO, 2.5 μM menadione (MD), or 37 °C heat shock (hatched bars). The solvent Me$_2$SO was added to controls (Cont) for menadione exposure. Levels are also shown for promastigotes in logarithmic (open bar) versus stationary (hatched bar) phases of growth. Data represent the mean ± S.E. values for 5 assays, each with triplicate conditions. C and D, promastigotes were exposed to the indicated concentrations of DFMO for 3 days. Susceptibility to H$_2$O$_2$ toxicity was assessed by pre-exposing parasites to varying concentrations of H$_2$O$_2$ followed by measuring viability according to the uptake of MTT. C shows a representative toxicity curve. D shows the mean ± S.E. viability after exposure to 0 versus 200 μM H$_2$O$_2$ in 9 assays, each with triplicate conditions.
with rabbit polyclonal antiserum to p63XNeo vector (−) or vector containing an L. chagasi HSP70 gene (+). Transfected promastigotes were incubated either at 26 or 37 °C for 2 h prior to harvesting proteins. Two parallel immunoblots were probed for β-tubulin and hsp70. A, transfected promastigotes were incubated either at 26 or 37 °C for 2 h to 26 °C in control HOMEM growth medium (C1), 37 °C heat shock (hs), HOMEM containing the solvent Me2SO (C2), or two sublethal concentrations of menadione (2.5 and 5.0 μM MD). In a separate experiment, virulent promastigotes were harvested during logarithmic (L) or stationary (S) phase growth. Parasites were then metabolically labeled in [3H]mannose, and LPG was extracted. After separating on a 7.5% SDS-polyacrylamide gel, LPG appeared as a smear on autoradiograms.

**Fig. 7.** A, HSP70 and β-tubulin immunoblots of transfected promastigotes. L. chagasi promastigotes were transfected with the empty pX63Neo vector (−) or vector containing an L. chagasi HSP70 gene (+). Transfected promastigotes were incubated either at 26 or 37 °C for 2 h prior to harvesting proteins. Two parallel immunoblots were probed for β-tubulin and hsp70. B, promastigote LPG. L. chagasi promastigotes were pre-exposed for 2 h to 26 °C in control HOMEM growth medium (C1), 37 °C heat shock (hs), HOMEM containing the solvent Me2SO (C2), or two sublethal concentrations of menadione (2.5 and 5.0 μM MD). In a separate experiment, virulent promastigotes were harvested during logarithmic (L) or stationary (S) phase growth. Parasites were then metabolically labeled in [3H]mannose, and LPG was extracted. After separating on a 7.5% SDS-polyacrylamide gel, LPG appeared as a smear on autoradiograms.

**Fig. 8.** H2O2 resistance of transfected promastigotes. A, stably transfected promastigotes containing either the pX63Neo vector without insert (vector) or pX63Neo with the L. chagasi HSP70 gene (hsp70) were incubated 26 or at 37 °C heat shock for 2 h. After exposure to varying H2O2 concentrations in triplicate wells, viability was measured by the uptake of MTT. A representative assay is shown. B, mean resistance of transfected promastigotes to H2O2. Stable transfectants containing pX63Neo without (vector) or containing the L. chagasi HSP70 gene (hsp70) were exposed to 150 μM H2O2, and viability was measured by the uptake of MTT. The mean ± S.E. viability is shown (n = 12 for the 2-h heat shock; n = 7 for the 12-h heat shock, each with triplicate conditions).

**DISCUSSION**

Antioxidant defenses are critical for the survival of microorganisms such as Leishmania that reside intracellularly in mammalian macrophages. Not only must they contend with oxidants generated as a result of their own aerobic metabolism, but they must also survive exposure to oxidants generated during phagocytosis by and activation of host macrophages. Our prior studies showed that L. chagasi promastigotes possess inducible means for resisting the toxic effects of oxidant exposure (16, 17). Environmental factors that induced oxidant resistance included exposure to temperatures encountered in the mammalian host (37 °C "heat shock") and sublethal concentrations of H2O2 or superoxide. Promastigotes also developed to an oxidant-resistant state during their growth from logarithmic to stationary phase in vitro. At least some aspects of the log-stationary transition mirror developmental changes that occur as promastigotes develop to their virulent form in the gut of the sand fly vector (41). Heat shock or superoxide exposure did not alter the amount of hydroxyl radical formed after exposure to H2O2, whereas stationary phase parasites generated significantly less hydroxyl radical than logarithmic promastigotes (16, 17). These data suggested to us that different mechanisms accounted for developmentally versus environmentally induced changes in oxidant resistance.

The purpose of the current study was to investigate mechanisms accounting for these inducible states of oxidant resistance. Induced resistance was not due to increased levels of reduced thiols (trypanothione or glutathione) or to increased activity of the enzyme ornithine decarboxylase, which is rate-limiting in trypanothione synthesis. Reduced thiols may therefore be more important for constitutive rather than inducible oxidant resistance. However, exposure to environmental stress (heat, menadione-derived oxidants H2O2 and superoxide) resulted in a stress response causing a burst of protein synthesis and increased levels of the heat shock protein HSP70. HSP70 itself must contribute to oxidant resistance, since overexpression of HSP70 in transfected promastigotes caused a heat-inducible increase in resistance to the toxic effect of H2O2 exposure. In contrast, development to stationary phase did not induce a stress response as we have defined it, since stationary phase organisms had decreased levels of HSP70 compared with logarithmic promastigotes. Instead stationary phase organisms possessed larger LPG molecules than their logarithmic counterparts, similar to changes that occur in L. major LPG during metacyclogenesis (40). These results are consistent with the existence of two distinct mechanisms for inducible peroxide resistance that are differentially invoked in response to environmental or developmentally programmed stimuli.

Antioxidant mechanisms that protect other microbes against reactive oxygen intermediates can be divided into enzymatic and non-enzymatic defenses. Enzymes that detoxify toxic oxygen-containing intermediates include the superoxide dismutases, catalase, peroxidoxins, flavohemoglobins, and glutathione S-transferase/glutathione peroxidase coupled to glutathione reductase (42, 43). Non-enzymatic microbial defenses include membrane-associated oxygen radical scavengers, small molecules that detoxify oxygen radicals (glutathione, ascorbate, α-tocopherols, retinoids, ascorbic acid, and spermine), and proteins that sequester the transition metals catalyzing free radical generation (iron and copper) (1, 6, 44–46). In addition, proteins that limit or repair oxidative damage include DNA repair systems (47) and the heat shock proteins (48).

The current study documented two non-enzymatic mechanisms, the heat shock response and developmental changes in LPG, that correlate with inducible resistance of Leishmania sp. to oxidant-mediated toxicity. LPG contributes to antioxidant defenses by scavenging O2− radicals (6), inhibiting protein kinase C (7, 49), and promoting intracellular Leishmania survival in macrophages (50, 51). A developmental increase in the size of LPG due to the addition of increasing numbers of phosphosaccharide residues is a defining characteristic of the highly...
infectious metacyclic form of \textit{L. major} promastigotes (40, 52, 53). Our observation that the LPG of \textit{L. chagasi} also increases during development establishes the following points. First, stationary growth is the \textit{L. chagasi} equivalent of metacyclogenesis as defined in \textit{L. major}. Second, the modulation of LPG provides an explanation for increased oxidant resistance of stationary phase \textit{L. chagasi}. Furthermore, our prior observation that lesser amounts of ‘OH are formed after the addition of H$_2$O$_2$ to stationary as opposed to logarithmic \textit{L. chagasi} promastigotes (17) is likely due to the more efficient oxidant scavenging by the larger stationary LPG.

In contrast to developmental changes, stress-induced oxidant resistance of \textit{L. chagasi} is not explained by an increase in the scavenging of H$_2$O$_2$/‘OH (17) or an increased size of LPG. Instead the increased synthesis of stress-inducible proteins, including but undoubtedly not limited to HSP70, likely limits or sequesters the oxidant-induced damage. The HSP70 family of heat shock proteins includes members localized in the cytosol, mitochondrion, and endoplasmic reticulum of eukaryotic cells (54). These proteins reversibly bind hydrophobic domains of polypeptide chains in a cyclical manner requiring ATP hydrolysis and additional heat shock proteins (HSP40; DNAJ in \textit{Escherichia coli}) (55). HSP70 proteins assist in protein translocation across membranes, and they suppress aggregation of damaged proteins and reactivate denatured proteins. Cytoplasmic HSP70 homologues are induced in response to environmental stress. HSP70-mediated protection from toxic environmental conditions may occur via two mechanisms. First, it is likely that HSP70 cooperates with other stress-induced proteins to prevent heat-induced denaturation prior to aggregation of proteins in vitro (55, 56). Second, HSP70 family proteins can suppress programmed cell death by preventing the activation of critical kinases leading to heat-induced apoptosis (57).

It has been shown in several mammalian cell lines that the overexpression of HSP70 protects cells from the toxic effects of heat, H$_2$O$_2$, monocyte-induced cytotoxicity, or superoxide generated by several methods (58–62). In addition, transgenic expression of either rat or human HSP70 in mice protects them from myocardial ischemic injury (63, 64). To our knowledge the current study contains the first evidence that overexpression of heat-inducible HSP70 renders a microbial pathogen more resistant to the toxic effects of oxidant exposure.

The above findings lead to a model in which programmed developmental changes modulate the form of LPG and generate an oxidant-resistant promastigote ready for inoculation into a mammal. The increased temperature in a mammalian host in conjunction with oxidants generated during phagocytosis trigger the synthesis of proteins required for continued oxidant resistance of the intracellular amastigote form of the parasite. HSP70 is among the first proteins induced, but others including some inducible antioxidant enzymes are likely of equal importance (10). It has been hypothesized that transiently increased expression of heat shock proteins precedes transformation between the life stages of protozoan parasites (65). In \textit{Leishmania} promastigotes these could serve the dual purposes of protecting parasites from phagocyte-induced toxicity and preparing for the extensive protein remodeling that must accompany the morphologic stage change. We conclude that \textit{Leishmania} sp. promastigotes possess both constitutive and inducible mechanisms for defending themselves against oxidant-induced damage. These mechanisms undoubtedly ensure the successful adaptation of the parasite to the mammalian host environment.

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