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Proteome changes associated with *Leishmania donovani* promastigote adaptation to oxidative and nitrosative stresses☆

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ABSTRACT

Phagocytic cells produce reactive oxygen and nitrogen species (ROS & RNS) as the most common arsenal to kill intracellular pathogens. *Leishmania*, an obligate intracellular pathogen also confronts this antimicrobial assault during the early phase of infection but nevertheless is able to survive these attacks and proliferate in macrophage. Adaptation of *Leishmania* to the toxic effects of ROS and RNS, involves a rapid change in the parasite proteome to combat the host defense response that macrophage mount in combating pathogen. To understand the events associated with combating ROS and RNS species, we performed a proteomic analysis of *L. donovani* promastigotes treated with sub-lethal doses of menadione (ROS), S-nitroso-N-acetylpenicillamine (RNS) or combination of both compounds. Proteomic changes triggered by these reagents were evaluated by iTRAQ labeling and subsequent LC–MALDI-TOF/TOF-MS analysis. Across the 3 stress conditions, the quantitative analysis identified changes in the proteins which encompass ~20% of the parasite proteome. Major changes were observed in enzymatic machinery of pathways involved in maintaining redox homeostasis, trypanothione metabolism, oxidative phosphorylation, superoxide metabolism, mitochondrial respiration process and other essential metabolic pathways. These observations shed light on how *Leishmania* promastigotes counter ROS and RNS effects during the initial stage of infection. Q4
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1. Introduction

Leishmania sp. is an obligate intracellular parasite that infects and replicates within mammalian macrophages. It has two principal life cycle stages: the flagellated mobile promastigote that is adapted to live in the gut of the sand-fly vector and an aflagellated amastigote form that residing within phagolysosomal vesicles of the host macrophage. Promastigotes introduced to the host during the taking of blood meal by the sand-fly are phagocytized in a receptor mediated process by macrophages

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and in the phagolysosome, they lose their flagella and transform into amastigotes and replicate. Intracellular amastigotes are the infectious stage that cause the clinical manifestations associated with leishmaniasis, a disease characterized by various symptoms ranging from self-healing cutaneous ulcer to a potentially fatal visceral disease resulting in high parasite burdens in the liver and spleen. Visceral leishmaniasis is generally caused by the species; *L. chagasi*, *L. donovani* and *L. infantum* [1].

The various life-cycle stages have different sensitivities to reactive oxygen species (ROS) and provoke different oxidative responses in the macrophage. After recognition of *Leishmania* spp., macrophages are activated and become so-called "effector cells" that can phagocytose and destroy the unwanted guest. Various cellular processes start after macrophage activation, including production of phagolysosomal degradation enzymes (e.g., proteases, nucleases, phosphatases, lipases, and esterases), oxidative burst generation, and nitric oxide (.NO) production [2]. The initial response that macrophages marshal in combating phagocytosed microbe is the production of superoxide reactive oxygen species that is part of human and murine macrophage's respiratory burst mechanism [3,4]. Menadione produces the superoxide radical in aqueous solution which is a predominant ROS species [5]. Superoxide production is catalyzed by the NADPH oxidase, a heme-containing cytochrome complex. *In vitro* studies showed a direct toxic effect of $O_2.^-$ on *L. chagasi* promastigotes (within 2 h of treatment with 2–14 μM menadione as $O_2.^-$ donor) [6]. The susceptibility was dependent on the parasite stage: metacyclic promastigotes were more resistant when compared to procyclic promastigotes. *Leishmania* promastigotes have been shown to be susceptible to killing by exposure to superoxide and hydroxyl radicals generated from H_2O_2 [7]. A second anti-leishmanial oxidant produced by macrophages is nitrous oxide (NO) [8–10]. Unlike reactive oxygen species (ROS) which are generated during phagocytosis, NO is generated after macrophage activation by IFN-γ and TNF-α, cytokines those have been linked to the killing of intracellular amastigotes [11]. SNAP produces NO radical as RNS [12]. In combination of both compounds the peroxynitrile is produced [13]. NO has also been reported to participate in the killing of *Leishmania major* by human macrophages stimulated through the low affinity Fcε receptor, CD23 and IFN-γ [14].

In spite of all the anti-microbial oxidative species and particularly nitrosative responses produced by activated macrophages a subset of metacyclic promastigotes are able to transform into obligate intracellular amastigotes, and eventually lead to disease symptoms [15–17].

The exact mechanisms that *Leishmania* use to resists the toxic effects of ROS is not known, however, several parasite molecules that include the surface molecules, lipophosphoglycan (LPG) and glycoprotein GP63 have been found to be important in protecting parasites by scavenging toxic oxygen products that inhibit macrophage responses [18,19]. Trypanosomes and *Leishmania* also possess trypanothione (TSH), a unique redox-cycling glutathione-spermidine conjugate which, in concert with trypanothione reductase, maintains the intracellular reducing environment in the parasites [20,21]. Disruption of the trypanothione reductase gene or transfection of parasite with *trans*-dominant negative form of trypanothione reductase renders these parasites more susceptible to intracellular killing by macrophages [21,22]. Earlier reports suggest that *Salmonella* sp. has redundant systems to detoxify oxidative stress, however the mechanisms by which ROS damage bacteria in the phagosome is unclear [23].

Finally, like other organisms, *Leishmania* up-regulates expression of heat shock proteins in response to a temperature increase or other environmental stresses. Cellular damage associated with protein denaturation and aggregation [24].

Quantitative proteomics has become the dominant strategy to investigate disease-specific targets and biomarkers. Previous investigations examining alteration in the *Leishmania* proteome have focused principally on events associated with promastigote to amastigote differentiation [25–34 and reviewed in 35]. Surprisingly no analyses of the proteomic changes that occur in *Leishmania* during early and late stage of infection have been performed to examine parasite response that are important to counter the stress inflicted by ROS and RNS. To better understand the molecular events necessary for the adaption to this oxidative and nitrosative stresses at early and late stage of infection, we performed an *in-vitro* proteomic analysis of *Leishmania* promastigotes treated with sub-lethal dose of ROS, RNS or a combination of both to mimic the physiological conditions found during early and late stages of infection that occur in the macrophage phagolysosome.

To examine the changes in the *Leishmania* promastigote proteome triggered by the harsh oxidative environment we employed an iTRAQ approach which provides a relative and absolute quantification of protein expression levels [36]. This method utilizes amine-reactive isobaric tags to label peptides in tryptic digest generated from whole cell proteome. An advantage of this method is that parasites exposed to four different protein conditions can be directly compared in a single experiment by combining samples prior to LC–MALDI-TOF/TOF mass spectrometry, where, on fragmentation, every fragmented peptide tag produces distinct signature ions differing by an m/z value of 114–117. The relative intensities of these signals represent the relative abundance of the analyzed peptide in each sample. Relative abundance values of all peptides attributed to each specific protein are averaged to represent the relative abundance of the entire protein.

Across the 3 stress conditions, the quantitative analysis identified changes in the proteins which encompass ~20% of the entire theoretical parasite proteome. We observed major changes in the enzymatic machinery of redox homeostasis, superoxide metabolism, and mitochondrial respiration pathways. These proteomic observations shed light on how *Leishmania* promastigotes respond and counter ROS and RNS during the initial stage of infection.

2. Materials and methods

2.1. *Leishmania* cell culture

Promastigotes of *Leishmania donovani* clones, AG83 (MHOM/IN/1983/AG83) were used in all experiments. The promastigotes were grown at 25 °C in 25 cm^2 flasks (Nunc) in fresh RPMI-1640 media (Sigma) supplemented with 10% FBS (Gibco). Cultures were allowed to reach stationary phase (5–6 days post inoculation), as determined by growth curve analysis (data not shown),

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