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Effect of L-Arginine Analogs and a Calcium Chelator on Nitric Oxide (NO) Production by *Leishmania* sp.

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***Leishmania amazonensis*, *L. braziliensis* and *L. chagasi* promastigotes were grown in the presence of L-arginine analogs such as N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{G}-nitro-L-arginine (L-NNA) and D-arginine (an inactive L-arginine isomer), besides an intracellular calcium chelator [ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra acetic acid; EGTA] to verify the importance of L-arginine metabolism and the cofactors for these parasites. The parasite's growth curve was followed up and the culture supernatants were used to assay nitric oxide (NO) production by the Griess reaction. The results showed a significant effect of L-arginine analogs on NO production by all *Leishmania* species studied, especially L-NAME, an irreversible inhibitor of the constitutive nitric oxide synthase (cNOS). When *L. amazonensis* promastigotes were pre-incubated with L-NAME, the infection range of the murine macrophages was lowered to 61% in 24 h and 19% after 48 h. These data demonstrated that the parasite NO pathway is important to the establishment of the infection.**

Keywords: *Leishmania* sp; Nitric oxide; Nitric oxide synthase

INTRODUCTION

Cutaneous, mucocutaneous and visceral Leishmaniasis occurs worldwide. The transmission of pathogenic *Leishmania* involves the injection of extracellular promastigotes into the mammalian host by an infected sand fly. Promastigotes rapidly attach and enter monocytes/macrophages and cells of the reticuloendothelial system, where they transform into amastigotes and multiply within phagolysosomes.[1]

Analysis of the interaction of *Leishmania* promastigotes with the target host cell suggests that both parasite and host molecules are involved in cell adhesion. The main function of macrophages is to destroy intracellular pathogens,[2,3] but the manner in which *Leishmania* and other intracellular parasites are able to survive and replicate within this ostensibly hostile intracellular milieu is an important question in cell biochemistry and immunology.[4] It is becoming increasingly evident that *Leishmania* evades host defense mechanisms by disrupting important cell functions. For example, it was reported that *Leishmania* interferes with signal transduction in macrophages.[4,5]

Nitric oxide (NO) a free radical derived from molecular oxygen and the guanidine nitrogen of L-arginine, is involved in a variety of biological functions in different cells,[6] and is an important antimicrobial effector molecule in macrophages against intra- and extracellular pathogens. NO production is catalyzed by the enzyme NO synthase (NOS), which occurs in at least two types. One (with two subtypes) is a Ca^{2+}/calmodulin-dependent enzyme that is constitutively found in the endothelium (eNOS or NOS III) and in neuronal tissue (nNOS or NOS I). The other is inducible (iNOS or NOS II) in vascular tissues, smooth muscle cells, neutrophils, hepatocytes and macrophages and is Ca^{2+}-independent.[7]

NO production during parasitic infections is a common finding.[8] The increase in NO production is mediated by up-regulated expression of the inducible iNOS, in response to secretion of pro-inflammatory cytokines during infections and/or exposure to specific parasite antigens. Parasite-induced cytokines, such as IFN-γ, TNF-α and IL-1β, and pathogen products such glycosylphosphatidylinositol, can stimulate iNOS expression in infected hosts. If NO production fails to be closely regulated and

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is left unchecked, the host may suffer increased morbidity. If the host fails to produce appropriate levels of NO, parasite numbers soar leading to increased host mortality.[9–11]

NO synthesis has been observed in mammals,[12] and also in invertebrates,[13] always through NOS. As part of the studies carried out by our group related to the different signaling pathways in *Leishmania* sp., we described the NO production by promastigotes[14] and further purification and characterization of a cNOS from promastigotes and axenic amastigotes of *L. amazonensis*.[15] Moreover, up to now no studies have been described with respect to NO production by *Leishmania* parasites, and its mode of action on host-parasite interaction. Continuing these studies, the present work provides evidence of the predominance of a constitutive isoform of NOS, through assays carried out with the L-arginine analogs L-NAME (N^{ω}-nitro-L-arginine methyl ester), L-NNA (N-nitro-L-arginine), D-arginine (D-arg) and an intracellular calcium chelator (ethylene glycol-bis (β-amino ethyl ether)-N,N,N′,N′-tetra acetic acid; EGTA) to verify the importance of L-arginine metabolism to macrophage-parasite interaction and of calcium ions as cofactor for the nitric oxide pathway in *Leishmania* sp.

MATERIALS AND METHODS

Reagents

Schneider's Insect Medium, L-NAME, L-NNA, D-arginine, L-arginine, EGTA, RPMI 1640, pyruvate, *N*-1-naphthlethylenediamine, phosphoric acid, sulfanilamide, sodium nitrite, penicillin and streptomycin were from Sigma Chemical Co., St. Louis, MO (USA). Fetal calf serum (FCS) was from Gibco BRL (USA).

Parasites

Leishmania amazonensis (MHOM/BR/77/LTB0016 strain), *L. braziliensis* (MHOM/BR/2000/R616 strain) and *L. chagasi* (MHOD/BR/97/P142 strain) promastigotes were studied comparatively. Parasites were maintained in Schneider's Insect Medium supplemented with 10% of heat-inactivated FCS (20% for *L. braziliensis*) at 26°C and pH 7.2.[16]

Evaluation of the Effect of L-arginine Analogs/EGTA on NO Production by *Leishmania* Promastigotes

To study the effect of L-arginine analogs and EGTA on NO production by *Leishmania* promastigotes, 5×10^5 parasites from the late log phase were incubated with L-arginine analogs (L-NAME, L-NNA, D-arg) 0.5 mmol/L, L-arginine 0.5 mmol/L or EGTA (10, 20 and 40 mmol/L) in 24-well flat bottomed micro-titer plates (final volume = 2 mL) at 26°C for 6, 12, 24, 48, 72, 96 h (*L. chagasi* was analyzed up to 212 h). In order to evaluate the reversibility or irreversibility of the reaction catalyzed by promastigotes- cNOS, an alternative experiment was realized, where parasites were pre-incubated for 40 min with L-NAME, L-NAME/EGTA or EGTA, and then L-arginine was added to promastigote cultures.[17] Supernatant from all cultures were used to assay NO production.

Murine Macrophage Culture

Murine resident peritoneal macrophages obtained from 6–8 week old BALB/c mice were collected in cold serum free RPMI 1640 medium, supplemented with 1 mmol/L L-glutamine, 1 mol/L HEPES [*N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid)], 100 IU penicillin, 100 μg/mL streptomycin and 1 mmol/L pyruvate and seeded on tissue plate wells containing a round coverlid (5×10^5 cells/well) and then incubated for 2 h at 37°C in an atmosphere containing 5% CO_2. Non-adherent cells were removed by washing and RPMI 1640 with 5% of heat-inactivated fetal calf serum (HIFCS) was added. The cells were maintained under the same culture conditions for 24 h before infection.

Evaluation of the Effect of L-NAME on Macrophage-*L. amazonensis* Interaction

L. amazonensis promastigotes were pre-incubated with 0.5 mmol/L L-NAME for 24 h and parasites were harvested from the medium in the late log phase of growth. Parasites were washed in culture medium, resuspended in medium with 1% of HIFCS added and incubated overnight with peritoneal adherent cells (10 parasites/cell, 5×10^6 parasites/well), in a CO_2 incubator at 37°C. After this period, free parasites were removed by washing the monolayers with medium. Infected macrophages were maintained in RPMI 1640 with 5% HIFCS for 24 and 48 h. Coverlids were fixed in methanol and stained with Giemsa's solution. Development of infection was assayed by quantifying the number of infected macrophages and the mean number of parasites per macrophage. Culture supernatants were collected and kept at −20°C until assayed for nitrite.

Nitrite Assay

Nitrite, a stable breakdown product of NO, was measured spectrophotometrically by adding Griess reagent (0.1% N-1-naphthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide) to the same

volume of supernatant from promastigotes and from infected/non-infected murine peritoneal macrophage cultures. After 10–15 min at room temperature, the absorbance was measured at 540 nm. Nitrite concentrations were estimated by comparison with a standard curve prepared with sodium nitrite.[18]

For the experiments with promastigotes, cells were counted in Neubauer's chamber, centrifuged at 1500 × g for 10 min and the supernatants were collected at 6, 12, 24, 48, 72 and 96 h (*L. chagasi* was analyzed up to 212 h, because its late log phase occurred by the sixth to seventh day). For nitrite measurement of macrophage cultures, supernatants were collected at 24 and 48 h after infection.

Data Analysis

Three experiments were performed for all assays to quantify the levels of nitrite in culture supernatants. Data obtained with different treatments were analyzed statistically by 1-way ANOVA and Student *t*-tests and differences were considered to be significant when $p < 0.05$. Statistical analysis was performed using Graph Pad Instat.

RESULTS

Effect of L-arginine Analogs on *Leishmania* sp

Figure 1 shows the effect of L-arginine analogs on NO production by *L. amazonensis*, *L. braziliensis* and *L. chagasi* promastigotes (parasite concentration was adjusted to 1×10^6 cells). The inhibition by L-NAME was the most significant in all strains studied ($p < 0.05$) and at all times analyzed. When parasites were grown with L-arginine, the nitrite measured in the supernatants was not higher compared to the control, also at all times analyzed except for *L. braziliensis,* which showed a NO production very high by 72 and 96 h. Furthermore, NO production by *L. braziliensis* promastigotes was higher than those by *L. amazonensis* and L. *chagasi* promastigotes.

Effect of EGTA on NO Production by *Leishmania* Promastigotes

The results shown in Figure 2 demonstrate that, in general, EGTA in different concentrations was able to affect NO production by *L. amazonensis*, *L. braziliensis* and *L. chagasi* promastigotes through calcium chelation. However, in *L. brasiliensis* promastigotes this inhibition was most significant ($p < 0.05$) and dose-dependent. When parasites were cultured with L-NAME/EGTA, the nitrite measured was similar to those of parasites cultured only with L-NAME 40 mmol/L ($p > 0.05$), and the association L-NAME/EGTA was not reverted by L-arginine addition.

NO Production in L-NAME Test in Macrophage-parasite Interaction

NO production by murine peritoneal macrophages infected by *L. amazonensis* is shown in Table I. Non-infected macrophages used as control produced a basal level of NO, but infected macrophages presented high levels of this radical after 24 and 48 h of culture. Moreover, when promastigotes were pre-incubated with L-NAME for 24 h, NO production was significantly reduced ($p < 0.05$) and a significant decrease of infection after 48 h was observed. In parallel, the infection rate was lowered to 61% by 24 h ($p < 0.05$) and 19% by 48 h ($p < 0.05$).

Microscopy of L-NAME Test in Macrophage-parasite Interaction

Panel 1 demonstrated the influence of L-NAME on parasite-cell host interaction, where comparing non-infected controls (a, d, g), *L. amazonensis* infected macrophages (b,e,i) and L-NAME pre-treated *L. amazonensis* infected macrophages (c,f,j) where it was observed that alterations of the morphology of the parasites, including high level of killed parasites (f,j) and various parasitophorous vacuoles without parasites 24 and 48 h post-infection occurred.

DISCUSSION

Leishmaniasis is characterized by development of lesions where parasites replicate uncontrolled or in the case of visceral leishmaniasis, by the development of a systemic infection, which in extreme cases can be fatal. In the laboratory models of infection and in human studies, the inability to control the disease correlates with defects in INF-γ production by T cells and by their failure to activate macrophages to destroy intracellular amastigotes through TNF-α, oxidative burst and NO.[19,20] The most compelling evidence of a role for NO in parasite killing comes from murine models in which resistance correlated with increased NO levels and was reversed by impaired NO production through treatment with competitive iNOS inhibitors. Moreover, immunohistochemical-staining in resistant mice genetically able to control infection, reveals earlier and significantly higher iNOS expression in the lesion and in the draining lymph nodes, compared to non-healing strains with a susceptible phenotype. In hosts unable to eliminate parasites, the disease becomes chronic and increased expression of NO inhibiting factors such IL-10, TGF-β and IL-4 are observed.[21–23] Li and collaborators[24] recently

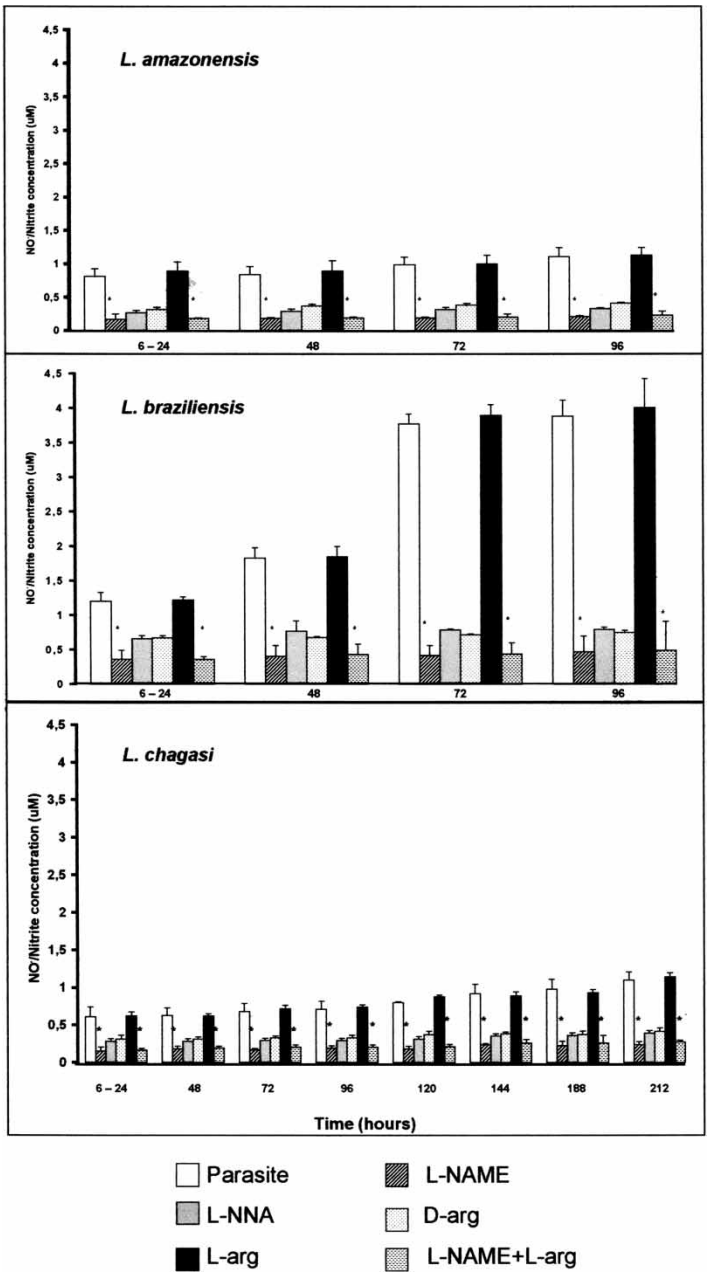


FIGURE 1 Nitric oxide/nitrite production by *Leishmania* species (1×10^6 cell/ml), measured by the Griess reaction in culture supernatants. Concentration of all the L-arginine analogs and L-arginine was 0.5 mmol/L.

observed that anti-TGF-β treatment results in increased NO production within lesions and promotes parasites elimination.

Several L-arginine analogs, exemplified by N^{ω}-nitro-L-arginine-methyl-ester (L-NAME) and N^{G}-nitro-L-arginine (L-NNA), have been routinely used as inhibitors of NOS in order to elucidate the diverse biological roles of NO production. By their nature, L-NNA, N^{G}-methyl-L-arginine (NMMA) and L-NAME are competitive inhibitors of all NOS and have been found to have variable effectiveness against each isoenzyme.[25,26] It has been observed that L-NNA is an irreversible inhibitor of cNOS and a reversible inhibitor of iNOS, although a rigid kinetic analysis was not performed. It has been suggested that the mechanism by which L-NNA inactivates cNOS may involve a direct electrostatic distortion of the active site of the enzyme.[27] Additionally, Kandpal and collaborators[17] showed that L-NAME, L-NNA and the inactive enantiomer D-arginine did not interfere on L-arginine transport in *L. donovani* promastigotes, suggesting a differentiated mechanism of competitive actuation on NOS-promastigotes of *Leishmania* sp.

In this work, the results demonstrated the effect of L-arginine analogs in *L. amazonensis*, *L. braziliensis*

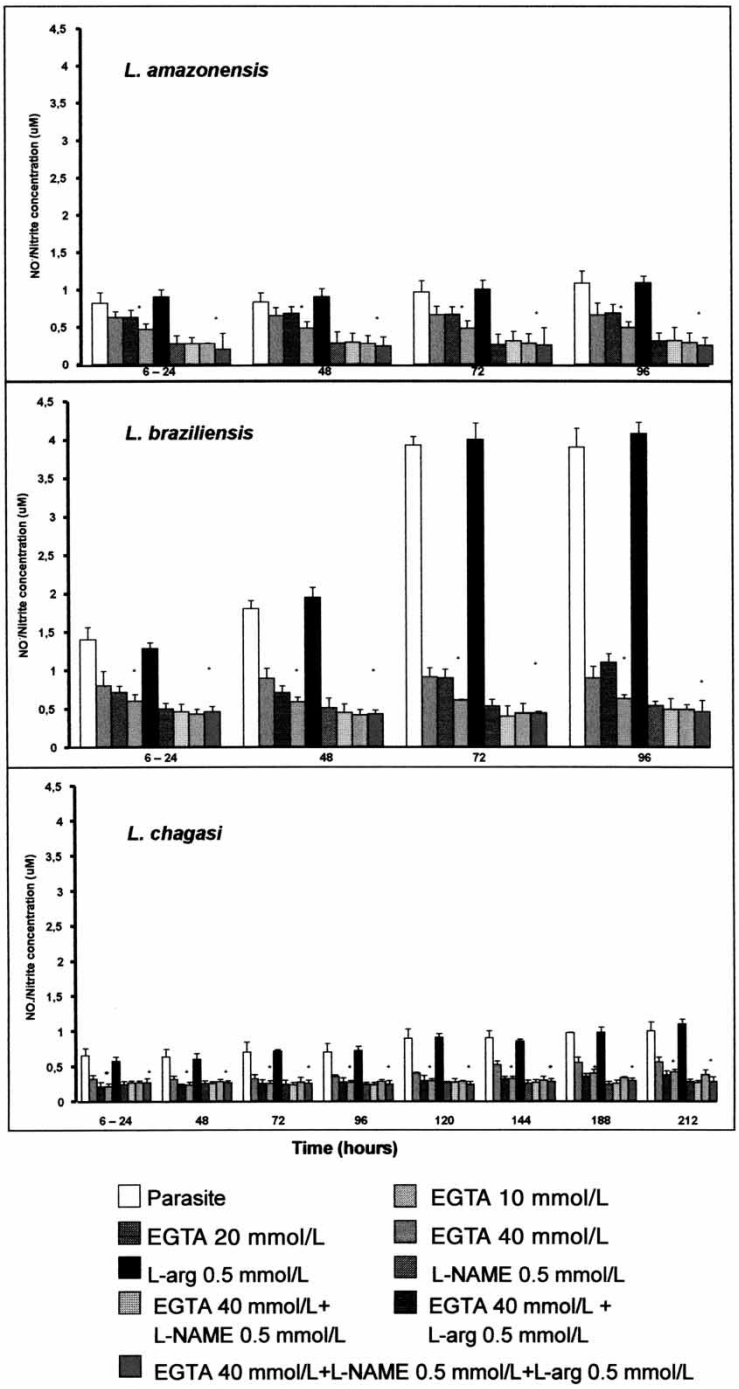


FIGURE 2 Effect of EGTA and EGTA/L-NAME on nitric oxide production by *Leishmania* species (1×10^6 cell/ml).

and *L. chagasi* promastigotes. The three analogs inhibited NO production in all species studied, and at all times analyzed. The inhibition was most evident with L-NAME, and this effect was not reverted by L-arginine addition. These data define the mechanism of action of L-NAME, which is an irreversible inhibitor of the constitutive NOS isoform, the enzyme previously described in *L. donovani* promastigotes. D-arginine and L-NNA showed similar inhibitory effects ($p < 0.05$). The better activity of L-NAME could be explained by its higher solubility compared to those of L-NNA and D-arginine analogs. Kandpall and collaborators[17] demonstrated that no competition occurred by L-NAME, L-NNA and D-arginine on the influx of L-arginine, indicating that it is one of the most specific amino acid transport system characterized so far, showing stereospecificity and recognition of both the guanidino group and the amino acid side chain. This demonstrated that those L-arginine

TABLE I Analysis of percentage infection and NO production (μM) by promastigotes pre-incubated with L-NAME 0.5 mmol/L for 24 h at late log phase of growth

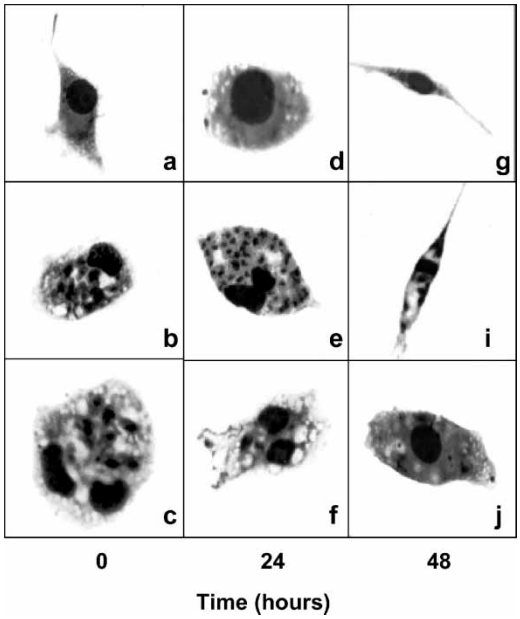
	Infection (%)			Nitrite released in supernatants (μM)[a]	
Macrophages	0 h	24 h	48 h	24 h	48 h
Noninfected	-	-	-	0.17 (±0.02)	0.27 (±0.012)
L. amazonensis	94.6 (±6.6)	91.3 (±2.3)	84 (±6.0)	6.38 (±0.6)	7.15 (±0.36)
L. amazonensis + L-NAME/24 h	92.3 (±1.3)	61 (±1.53)	19 (±4.0)	3.92* (±0.43)	1.63* (±0.048)

[a] Values are means ± standard deviation of three experiments. *$p < 0.05$.

analogs used in our experiments did not interfere with L-arginine transport but in mechanisms of enzymatic intracellular competition.

Ca^{2+} participates in the NO pathway as an important cofactor for NOS activity. The differences between the isoenzymes concern their tissue distribution and regulation. Binding of Ca^{2+}-calmodulin (CaM) to nNOS and eNOS is sensitive to Ca^{2+} influx through the cell membrane, whereas iNOS binds CaM Ca^{2+}-independently and is regulated mainly at the level of gene transcription. Each isoenzyme has a unique N-terminal sequence that is strictly necessary for catalytic activity.[28–30]

In previous work, we demonstrated NO production by *L. amazonensis* promastigotes and axenic amastigotes, and a constitutive NOS was characterized and purified.[14,15,31] Interestingly, an intriguing event in the biology of another trypanosomatid, *Trypanosoma cruzi*, has been the discovery of a Ca^{2+}-dependent NOS, which can be modulated by glutamate, possibly through the *N*-methyl-D-aspartate (NMDA)-activated ion channel. The resultant Ca^{2+} influx leads to a rise of a cyclic guanosine monophosphate (cGMP), ascribable to NO-dependent activation of soluble guanylate cyclase (sGC).[32,33] In *Leishmania* genus, the importance of Ca^{2+} was established in various mechanisms; for example, the presence of low Ca^{2+} in the cytosol, existence of intracellular Ca^{2+} pools and presence of mechanisms to maintain Ca^{2+} homeostasis in the cells, suggests that Ca^{2+} can be an appropriate candidate for a second messenger during the morphogenetic transformation of *L. donovani*[34] and total-reflection X-ray fluorescence has been used to study whether *L. infantum* kinetoplastid membrane protein-11 acts as a Ca^{2+}-binding protein.[35] Thus, with the aim of evaluating the importance of calcium ions as cofactor for NOS-promastigotes, we carried out assays with EGTA, a well known calcium chelator[36] in different concentrations that did not affect the parasite viability. The results showed that, in *L. amazonensis* -promastigotes, EGTA at 10 and 20 mmol/L did not significantly diminish NO production in a time ranging from 6 to 96 h, but when this calcium chelator was used at 40 mmol/L, there was a significant reduction of NO concentration in supernatant cultures, at all the times analyzed. However, when parasites were incubated simultaneously with EGTA 40 mmol/L and L-NAME (0.5 mmol/L), the NO production was seriously affected, and this effect was not reverted by L-arginine addition to *L. amazonensis* promastigote cultures. These data demonstrated that the association EGTA/L-NAME severely diminishes the NOS-promastigotes activity, and the same results were observed in *L. braziliensis* and *L. chagasi* strains. These data also strengthen and corroborate previous results that pointed to the presence of a constitutive isoform of the enzyme in *L. amazonensis* promastigotes and axenic amastigotes. All the experiments were also done with *L. chagasi* (MHCAN/BR/98/R619 strain), a canine isolate, and the results of the tests with L-arginine analogs and the effect of EGTA were similar to *L. chagasi* MHOD/97/P142 strain (data not shown). A relevant



PANEL 1 Infection of murine peritoneal macrophages by *L. amazonensis*, where a,d,g = macrophage control, without parasites; b,e,i = macrophages infected by *L. amazonensis*; c,f,j = macrophages infected by promastigotes pre-incubated with 0.5 mM L-NAME for 24 h and further incubation (overnight) with murine peritoneal adherent cells. Development of infection was assayed by quantifying the mean number of parasites per macrophage.

fact is that Mbati and collaborators[37] verify that EGTA, in a 0.05, 0.1 and 0.2 mg/ml concentration, contributed significantly to a decline in *L. donovani* amastigote parasite-loads within macrophages. Therefore, maybe one of the mechanisms exerted by EGTA is to form a Ca^{2+}-chelate, so decreasing the availability of Ca^{2+} for constitutive NOS-promastigotes. In our assays, we carried out experiments using EDTA (results not shown), another Ca^{2+} chelator, for all strains, but the parasites viability was seriously affected.

In order to evaluate the importance of the NO pathway in the *Leishmania*-host interaction, since the existence of this pathway stands out in the parasite, we investigated the percentage infection of murine macrophages when the parasites were pre-cultured with L-NAME (an inhibitor of the constitutive isoform of NOS) for 24 h. Previous results from our laboratory demonstrated, through immunofluorescence, streptoavidin-biotin system and affinity chromatography, that these isoform of the enzyme is predominant in axenic amastigotes and also in promastigotes cultures containing a high percentage of the metacyclic forms.[14,15,31] It was possible to observe the interference of L-NAME on the metabolic NO pathway of parasites, at the point of interference on macrophage infection. The results of Table I demonstrated that the infection range of the murine macrophages by *L. amazonensis* pre-cultured with L-NAME decreases significantly from 93.3% to 6% in 24 h and was still lower at 48 h post infection. This fact indicated strongly that the promastigotes-NO pathway exerts a fundamental role in establishment of the infection; and this data agreed with studies of Ballestieri and collaborators[38] who stated that the increase of the parasite number on macrophage phagolysosom, at these times evaluated, leads to downregulation of NO production by iNOS.

It was possible to observe that parasite pre-cultures with L-NAME for 24 hours when within phagolissosoms at 24 and 48 h post-infection showed morphological alterations. At 24 h of infection it was observed that several parasitophorous vacuoles contained dead parasite residues.

These data demonstrated that the parasite-NO pathway inhibition modifies important biochemical and structural characteristics of parasites, and deserves further investigations. Amastigotes within macrophages at zero time showed significant morphological alterations when compared with the control (Panel 1b and 1c). 48 hours post infection, it was possible to observe (Panel 1f) an expressive decrease in parasite number per cell. Interestingly, the macrophages presented structural modifications in 24 and 48 h after the infection (Panel 1f and 1j), perhaps due to the adverse effect promoted by *Leishmania*-L-NAME treatment interfering on the parasite-host interaction.

Little is known about the mechanisms that underly the inhibition of NO production by *Leishmania* or their products. Infection with live parasites is not an absolute requirement because parasite-derived molecules such as GIPLs or LPG extracted from *L. major* were also found to inhibit NO production. Balestieri and collaborators[38] demonstrated that heat-killed *L. amazonensis*, but not an antigenic extract of these parasites, is also capable of inhibiting NO production. A LPG-associated molecule of *L. donovani*, the kinetoplastid membrane protein-11 (KMP11) has been described to contain at position 45 a structural analogue of N-monomethyl-L-arginine, a well-known inhibitor of NO production, that acts by competing with L-arginine.[39] This could be a mechanism by which NO synthesis by macrophages is down regulated by these species of *Leishmania*, which have a similar metabolic pathway, since they also possess one of the isoforms of NOS.

Finally, *Leishmania* parasites are small and intriguing organisms, and more and more efforts are being concentrated on the further identification of their constituent molecules and definition of their functional roles. NO is one of them, and new studies will provide important information on the biology and biochemistry of the parasites and probably will identify new potential target for immunological and chemotherapeutical approaches. It is important to stimulate classical studies of the metabolic pathway, now significantly facilitated by new techniques and equipment available. The use of NOS inhibitors has broad therapeutic applications. However, the use of conventional L-arginine analogs is restricted because of lack of isoform selectivity.

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