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Expression of glyceraldehyde 3-phosphate dehydrogenase is enhanced in *Leishmania* spp naturally resistant to nitric oxide

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ABSTRACT. *Leishmania* spp are the causative agents of a spectrum of diseases termed leishmaniasis that affect mammals, including humans and dogs. Although reactive nitrogen species are employed in the control of parasitism by the immune system, it is known that *Leishmania* can withstand this oxidative stress. As the mechanism by which these species are resistant to nitric oxide (NO) is poorly understood, the main objective of this study was to evaluate the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in *Leishmania amazonensis* and *Leishmania chagasi* promastigotes

showing natural resistance to NO. *GAPDH* transcript levels were quantified by real-time polymerase chain reaction amplification, and GAPDH activity (assessed by levels of NADH oxidation) was measured by spectrophotometry. The level of nitration in total protein was assessed by immunoblotting. The results demonstrated an increase in *GAPDH* expression in resistant isolates of both species compared to susceptible isolates. The increase in *GAPDH* expression led to an increase in the activity of GAPDH in *L. amazonensis* human isolates resistant to NO. The pattern of protein nitration did not differ between sensitive and resistant isolates. Our results suggest that changes in expression of GAPDH may be responsible, at least in part, to natural resistance to NO found in human and canine *Leishmania* spp.

Key words: *Leishmania amazonensis*; *Leishmania chagasi*; Nitric oxide resistance; Glyceraldehyde 3-phosphate dehydrogenase

INTRODUCTION

Leishmania is a protozoan parasite that is responsible for a spectrum of diseases whose clinical manifestations include cutaneous, mucocutaneous, and visceral leishmaniasis, which affect over 12 million people worldwide (WHO, 2010). The flagellate promastigote form of the parasite lives in the midgut of the insect vector. After having been transmitted to a vertebrate host, the promastigote is converted into the amastigote form, which infects mononuclear phagocytes, including macrophages. Because these cells are specialized for the identification and destruction of invading pathogens either directly or by eliciting an immune response, *Leishmania* have developed several mechanisms to suppress a variety of critical macrophage activities, thus allowing them to proliferate and survive in a harsh environment. One of these evasion strategies is the control of nitric oxide (NO) production by inhibiting inducible NO synthase (iNOS) activity (Linares et al., 2001; Balestieri et al., 2002). Thus, the parasite prevents the initiation of an NO-stimulated immune response in addition to other leishmanicidal actions.

The biochemical basis for the microbicidal action of NO is its reactivity with various chemical groups in the biomolecular components of metabolic pathways involved in cellular energy production (James, 1995). Such interaction may lead to an inhibition of the catalytic action of enzymes that are essential for microorganism survival, including cis-aconitase (Lemesre et al., 1997) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mauel et al., 1991). It was previously demonstrated that these two enzymes had higher activity levels in strains of *Leishmania infantum* with induced resistance to NO (Holzmuller et al., 2006).

Clinical isolates of *Leishmania braziliensis* and *Leishmania amazonensis* showing natural resistance to NO have been recently identified. In addition to producing larger cutaneous ulcers in infected patients, the resistant isolates multiplied in human macrophages significantly more efficiently than did susceptible isolates (Giudice et al., 2007).

Recently, our group has reported natural resistance of *Leishmania chagasi* promastigotes to NO. These parasites were isolated from humans and dogs with visceral leishmaniasis and its resistance profile was associated with a greater survival capacity and a greater parasite

burden in murine macrophages (Santos et al., 2012).

Since the molecular basis for the natural resistance of *Leishmania* to NO has not been clarified and based on evidence of the GAPDH involvement in induced NO-resistance, the expression of *GAPDH* was investigated in *L. amazonensis* and *L. chagasi* isolates naturally resistant to NO.

MATERIAL AND METHODS

Parasite and culture conditions

Two *L. amazonensis* and three *L. chagasi* isolates were used in this study. LTCP 10432, an *L. amazonensis* isolate that shows natural resistance to NO, and LTCP 9667, an NO-sensitive isolate, were previously obtained by Giudice et al. (2007) by needle aspiration of lesions from human patients from Corte da Pedra, Ba, Brazil, and were cryopreserved. The NO-resistant *L. chagasi* isolates LVCSE24 and LVCSE30 and the NO-sensitive LVCSE19 isolate were obtained from bone marrow aspirates from canine reservoirs with positive serology for visceral leishmaniasis, recruited at the Center for Zoonosis Control (CCZ) in Aracaju-SE (96/2011, Comitê de Ética em Pesquisa Animal/UFS). Parasites were speciated in our laboratory by polymerase chain reaction (PCR) with primers specific for *L. infantum* (le Fichoux et al., 1999) and by isoenzyme electrophoresis and monoclonal antibodies by Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil (Cupolillo et al., 1994). The parasites selected were expanded in Schneider's insect medium (Sigma Chemical Co., St. Louis, MO, USA), pH 7.2, supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical Co.) and 2% male human urine (for *L. amazonensis* cultures) at 25°-26°C. For each experiment, the parasites were grown until their exponential growth phase was reached (5 x 10^7 parasites/mL). Before RNA and protein extractions, cultures were exposed to 16 mM $NaNO_2$ (Sigma Chemical Co.), pH 5.0 (NO donor, freshly prepared) in Hanks' Balanced Saline (HBSS) (Sigma Chemical Co.), pH 5.0, at 25°-26°C for 2 h. The control cultures were incubated in HBSS pH 5.0 without $NaNO_2$, under the same conditions.

NO sensitivity assay

To confirm the NO sensitivity of the isolates, the cultures were adjusted to 5 x 10^7 promastigotes/mL in HBSS, and 100-µL aliquots were distributed on 96-well U-shaped plates and incubated with dilutions of $NaNO_2$ from 0 to 16 mM in HBSS, pH 5.0, for 4 h at 25°-26°C. Plates were then centrifuged (2500 *g* for 10 min), and parasites were resuspended with 200 µL complete Schneider medium and incubated for an additional 20 h at 25°-26°C. The parasites were incubated with 0.5 mg/mL 3-(4.5-dimetthythiazol-2-yl)-2.5-diphenyltetiazolium bromide (MTT) (Sigma Chemical Co.) in HBSS, pH 7.0, at 25°C for 4 h. The dark blue formazan generated by conversion of MTT by mitochondrial dehydrogenases was solubilized by the addition of 0.04 N HCl in isopropanol. The formazan was read by a spectrophotometer at 540 nm. The percentage of viability was calculated using the optical density ratio of untreated versus NO-treated parasites x 100. Parasites with a survival rate above 70% at 16 mM $NaNO_2$ concentration were considered to be resistant to NO.

Total protein extraction and quantification

Approximately 5 x 10^8 promastigotes were either exposed to 16 mM $NaNO_2$, or left untreated, and then pelleted and washed three times in phosphate-buffered saline by centrifugation for 10 min at 425 *g*. The cells were then suspended in 20 µg/mL protease inhibitor cocktail (Sigma Chemical Co., CAT: P2714) and submitted to 3 freeze-thaw cycles. To improve the degree of lysis, cells were subjected to three water bath ultrasonic treatments for 15 s, with 1 min between each treatment. Lysed cells were pelleted by centrifugation at 23,000 *g* for 10 min at 4°C. Water-soluble proteins (supernatant) were quantified by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

GAPDH enzymatic assay

For GAPDH activity determination, 50 µg total protein was incubated with 0.1 M triethanolamine/HCl buffer, pH 7.6, 1.0 mM dithiothreitol, 1.0 mM ATP, 300 mM NADH, 5.0 mM $MgSO_4$, and 1.0 mM ethylenediaminetetraacetic acid in a final volume of 1.0 mL (Misset and Opperdoes, 1984). The reaction was initiated by adding the enzyme substrate 3-phosphoglycerate (5.6 mM) to the total assay mixture. The GAPDH activity was determined following a change in absorbance at 340 nm over 10 min due to NADH oxidation. Enzyme activity was calculated using the molar extinction coefficient of NADH (6.2 cm^2/10 min due to NADH oxidation). Enzyme activity is expressed as $\mu mol \cdot min^{-1} \cdot mg$ total $protein^{-1}$.

RNA extraction and cDNA synthesis

Total RNA was extracted from 5 x 10^8 promastigotes that were or were not exposed to 16 mM $NaNO_2$ using Trizol reagent (Life Technologies, Grand Island, NY, USA), following the manufacturer protocol. The quantitative analysis of total RNA was determined by measuring absorbance at 260 nm in a Nanodrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was obtained by reverse transcription using products from Fermentas Life Science (Pittsburgh, PA, USA). Briefly, 3 µg total RNA from each sample was added to an RT-mix containing 1 µL RiboLock™ Ribonuclease Inhibitor, 1 µL Dnase I, 1 µL 10 mM dNTPs, 1 µL 100 µM oligodT, and 4 µL 5X First-Strand Buffer in a volume of 19 µL. The mix was incubated for 10 min at 37°C, 5 min at 75°C, and then 2 min at 37°C to inactivate RNAses and DNAses. The reaction was initiated with the addition of 1 µL RevertAid™ M-MuLV Reverse Transcriptase followed by incubation for 1 h at 37°C, 15 min at 75°C, and 2 min at 37°C.

Gene expression by real-time quantitative polymerase chain reaction (qPCR)

qPCR was performed using an Mx3005P qPCR System (Stratagene, Santa Clara, CA, USA). The reactions were performed as biological triplicates and technical duplicates using a SYBR Green kit (SYBR Green ROX Mix, LGC Biotechnology, Teddington, Middlesex, UK). The final concentration of oligonucleotides in the reaction was 0.6 µM. Fluorescence readings were performed under the following conditions: preincubation at 95°C for 10 min, 40 cycles of amplification at 95°C for 30 s, and 60°C for 60 s. The melting or dissociation curve was determined immediately after amplification with the acquisition of continuous readings of fluorescence at 55° to 95°C at a linear temperature transition rate of 0.1°C. The glucose

6-phosphate dehydrogenase (*G6PDH*) gene was used as reference (constitutive expression) for assays performed in *L. amazonensis* and the α-tubulin gene was used for assays in *L. chagasi*. The sequences of primers used in the qPCR assays were: *GAPDH* Forward 5'-TCA AGG GTG GTG CGA AGA AG-3'; *GAPDH* Reverse 5'-TCG CCG TGT AGG AGT GGA TG-3'; *G6PDH* Forward 5'-ACC GCA TTG ACC ACT ACC TC-3', *G6PDH* Reverse 5'-GAT GTT GTT CGA GTT CCA C-3', α-tubulin Forward 5'-AGC ACA CCG ATG TTG CGA CGA T-3' and α-tubulin Reverse 5'-GAT CAG GCG GTT CAC GTT CGT GT-3'. To evaluate the efficiency of the amplification reaction of the target gene, a standard curve was generated using 10-fold dilutions of cDNA. *GAPDH* cycle threshold (C_t) values were first normalized to *G6PDH* ($\Delta C_t = C_{t(GAPDH\ gene)} - C_{t(G6PDH\ gene)}$). Fold change was determined by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = \Delta C_{t(GAPDH\ resistant\ isolate)} - \Delta C_{t(GAPDH\ susceptible\ isolate)}$.

Total proteins nitration

The presence of proteins containing 3-nitrotyrosine (NT) residues, an index of oxidative stress and protein nitration, was analyzed by immunodetection. First, 2.5 µg total protein from *L. amazonensis* was transferred to a nitrocellulose membrane using a vacuum system through a slot blot manifold (BioRad, Hercules, CA, USA). After blocking with 0.2% casein solution, the membrane was incubated for 12 h with rabbit polyclonal 3-nitrotyrosine antibodies (0.5 µg/mL; Upstate Biotechnologies, Waltham, MA, USA). After six washes with TBS-T (200 mM Tris, 1.37 M NaCl, 0.2% Tween-20), the membrane was incubated for 2 h with goat anti rabbit horseradish peroxidase-conjugated secondary antibodies (1:4000; BioRad). Immunoreactive bands were detected by chemiluminescence (Thermo Fischer Scientific) and their intensities were estimated by densitometric analysis (Chemi imager® 5500 system, Alpha Innotech Corporation, Santa Clara, CA, USA). Results were normalized by the band intensity values obtained after staining with Ponceau red.

RESULTS

NO viability

The susceptibility/resistance of *L. amazonensis* and *L. chagasi* isolates was assayed by the colorimetric MTT assay. After exposure for four hours to 16 mM $NaNO_2$, the rate of viability obtained confirmed the NO-resistant profile of the isolates LTCP 10432, LVCSE 24, and LVCSE 30; and the susceptibility of LTCP 9667 and LVCSE 19, as shown in Table 1.

Table 1. Nitric oxide viability of human *Leishmania amazonensis* (LTCP) and canine *Leishmania chagasi* (LVCSE) promastigotes.

	Isolate	$NaNO_2$ viability (%)*
Resistant	LVCSE 24	89.0 ± 26.9
	LVCSE 30	85.0 ± 14.1
	LTCP 10432	92.5 ± 11.3
Susceptible	LVCSE 19	42.0 ± 8.5
	LTCP 9967	57.5 ± 7.3

*Promastigotes were exposed to16 mM $NaNO_2$ for 4 h and the viability was evaluated by MTT assay. Viability is reported as means ± SD of two independent experiments.

GAPDH is upregulated in *Leishmania* isolates resistant to NO

The relative expression of *GAPDH* was evaluated in both species. As shown in Figure 1, the level of *GAPDH* transcripts in the human *L. amazonensis* isolate resistant to NO was more than twice that observed in the susceptible isolate, regardless of NO exposure. On the other hand, only a slight increase in *GAPDH* expression was observed in the resistant canine *L. chagasi* isolates with no exposure to NO (NO-), compared to the susceptible isolate. However, a significant increase in the level of *GAPDH* transcripts could be observed when the *L. chagasi* promastigotes were exposed to NO (NO+).

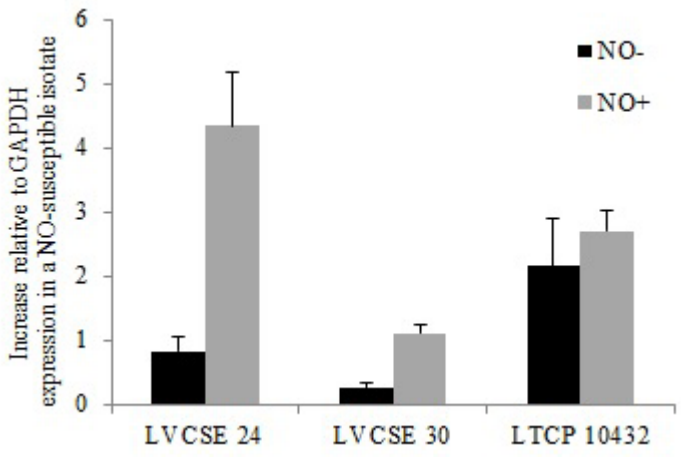

Figure 1. Relative *GAPDH* gene expression in *Leishmania amazonensis* and *L. chagasi* promastigotes. *GAPDH* expression in LTCP 10432 (*L. amazonensis*; NO-resistant) was normalized to LTCP 9667 (NO-susceptible) *GAPDH* expression levels. *GAPDH* expression of LVCSE 24 and LVCSE 30 (*L. chagasi*; NO-resistant) was normalized to LVCSE 19 (*L. chagasi*; NO-susceptible). Parasites were incubated for 2 h in the presence (NO+) or absence (NO-) of 16 mM $NaNO_2$ before RNA extraction. Glucose 6-phosphate dehydrogenase (*G6PDH*) was used as a control for constitutive gene expression in *L. amazonensis* assays and α-tubulin was used in *L. chagasi* assays. The results are reported as means ± SD of three independent experiments.

The relative increase in *GAPDH* expression between treatments (NO- *vs* NO+) was about 5-fold in canine *L. chagasi* isolates, while in human *L. amazonensis,* this relative increase was 1.25-fold (Table 2).

Table 2. Relative increase in *GAPDH* expression in *Leishmania amazonensis* and *Leishmania chagasi* exposed to the NO donor $NaNO_2$.

Isolate	Relative *GAPDH* expression*		Relative increase (NO+/NO-)
	NO+	NO-	
LVCSE 24	4.34 ± 0.85	0.83 ± 0.21	5.23
LVCSE 30	1.11 ± 0.13	0.26 ± 0.08	4.27
LTCP 10432	2.70 ± 0.31	2.16 ± 0.74	1.25

*The relative expression of *GAPDH* was determined as described in the Figure 1 caption. NO+, NO- = exposed, non-exposed to $NaNO_2$ before evaluation, respectively.

GAPDH activity is increased in *L. amazonensis* NO-resistant isolates

GAPDH activity was assayed only in the *L. amazonensis* isolates, exposed or unexposed to the NO donor ($NaNO_2$) before protein extraction. The results obtained showed that GAPDH activity was higher in NO-resistant isolates regardless of NO exposure (Table 3). Interestingly, after exposure to the NO donor, the enzyme activity detected in the resistant isolate was 45% higher compared to the activity in this isolate in the absence of NO. On the other hand, there was no difference in activity measured in the sensitive isolate whether or not it had been exposed to NO.

Table 3. GAPDH activity variation in NO-resistant and NO-sensitive *Leishmania amazonensis* isolates.

	Isolate	Specific activity*	
		NO+	NO-
Resistant	LTCP 10432	0.084 ± 0.003	0.058 ± 0.002
Susceptible	LTCP 9667	0.038 ± 0.010	0.048 ± 0.004

*Specific activity expressed in $\mu mol \cdot min^{-1} \cdot mg$ total $protein^{-1}$. The results are reported as means ± SD of three independent experiments. NO+, NO- = exposed, non-exposed to $NaNO_2$ before evaluation, respectively.

Total protein nitration profile is unchanged in the NO-resistant isolates

A comparative profile of total protein nitration in sensitive and resistant isolates of *L. amazonensis* and *L. chagasi* is presented in Table 4. For both human and canine *Leishmania* isolates the protein nitration levels did not differ, regardless of resistance or exposure to NO.

Table 4. Total protein nitration in NO-resistant and NO-sensitive *Leishmania amazonensis* isolates.

	Isolate	Integrated density value*	
		NO+	NO-
Resistant	LVCSE 30	1.10 ± 0.40	1.00 ± 0.10
	LVCSE 24	0.90 ± 0.30	0.90 ± 0.30
	LTCP 10432	1.17 ± 0.46	0.89 ± 0.30
Susceptible	LVCSE 19	1.20 ± 0.20	1.30 ± 0.30
	LTCP 9667	1.27 ± 0.51	1.10 ± 0.42

*The results are shown as means ± SD of three experiments. NO+, NO- = exposed, non-exposed to $NaNO_2$ before evaluation, respectively.

DISCUSSION

GAPDH (EC 1.2.1.12) is a classical glycolytic enzyme that catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate in the presence of NAD^+ and inorganic phosphate. In addition to this well-known role, GAPDH is known to play a role in diseases such as diabetes, age-related neurodegenerative disorders, and malaria, as well as many cellular processes, including cellular responses to oxidative stress and apoptosis (Sirover, 2011).

The biochemical mechanism of NO action appears to be related to the inhibition of metabolic pathways that are critical for energy production (James, 1995). Indeed, cis-conitase and GAPDH, key enzymes of the Krebs cycle and the glycolytic pathway, have had their ac-

tivities inhibited in *Leishmania* exposed to different NO donors (Lemesre et al., 1997; Mauel and Ransijn, 1997). Interestingly, an increase in the activity of GAPDH and cis-aconitase was demonstrated in *L. infantum* promastigotes showing resistance to NO (Holzmuller et al., 2006). The NO-resistance of these *L. infantum* promastigotes was induced *in vitro* by increasing NO donor pressure in a stepwise fashion. Natural resistance to NO was also demonstrated to occur in *L. amazonensis* (Giudice et al., 2007) and *L. chagasi* (Santos et al., 2012). Here, we demonstrate a slight increase in the GAPDH activity in such an *L. amazonensis* isolate naturally resistant to NO, which became sharper when the parasites were exposed to $NaNO_2$ (16 mM) for two hours. We therefore suggest that an increase in GAPDH activity appears to be related to both *in vitro*-induced and natural resistance in *Leishmania*.

The highest GAPDH activity observed in the *L. amazonensis* NO-resistant isolate might be explained by our observation that the level of *GAPDH* transcripts in this resistant isolate was more than 2-fold higher than that observed in the susceptible isolate, regardless of NO exposure. Considering that an increase in *GAPDH* transcripts was also detected in the *L. chagasi* NO-resistant isolate, we can assume that the increased expression of *GAPDH* is inherent to *Leishmania* NO-resistant isolates. However, in *L. chagasi*, the increase in *GAPDH* transcript levels seemed to be induced by NO; in both resistant isolates evaluated, the relative expression was considerably raised (about 5-fold) after $NaNO_2$ exposure (Table 2). Although the enhancement in GAPDH activity has already been related to NO resistance induced *in vitro*, this is the first report relating both GAPDH activity and expression to natural resistance to NO in *Leishmania*.

The nitration of protein tyrosine residues is one of several possible chemical modifications that can occur when a cell or organism undergoes oxidative stress. Some studies have demonstrated that GAPDH is a target for tyrosine nitration (Palamalai and Miyagi, 2010; Bailey et al., 2011; Guingab-Cagmat et al., 2011). Therefore, any mechanism that reduces the susceptibility of the organism to nitrative stress might contribute to the development of NO resistance. However, this process appears to not be relevant for the NO-resistance observed in *L. amazonensis* and *L. chagasi* clinical isolates, as no difference in the levels of total protein nitration between resistant and sensitive isolates was found. This outcome reinforces the hypothesis that an increase in the activity of an enzyme important in energy metabolism could be one of the key mechanisms for parasite survival. Therefore, our findings allow us to conclude that molecular and biochemical changes in GAPDH should account, at least in part, for the NO resistance observed in *L. amazonensis* and *L. chagasi*.

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