Nitric oxide-releasing indomethacin enhances susceptibility to *Trypanosoma cruzi* infection acting in the cell invasion and oxidative stress associated with anemia

Vera Lucia Hideko Tatakihara a, Aparecida Donizette Malvezi a, Carolina Panis b, Rubens Cecchini c, Nagela Ghabdan Zanluqui a, Lucy Megumi Yamauchi d, Maria Isabel Lovo Martins a, Rosiane Valeriano da Silva a, Sueli Fumie Yamada-Ogatta d, Luiz Vicente Rizzo e,f, Marli Cardoso Martins-Pinge g, Phileno Pinge-Filho a,⇑

aLaboratório de Imunopatologia Experimental, Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, 86051-970 Londrina, Paraná, Brazil
bCentro de Ciências Sociais Aplicadas, Universidade Estadual do Oeste do Paraná – UNIOESTE, Francisco Beltrão 85.605-010, Paraná, Brazil
cLaboratório de Fisiopatologia e Radicais Livres, Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, 86051-970 Londrina, Paraná, Brazil
dLaboratório de Biologia Molecular de Microorganismos, Departamento de Microbiologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, Paraná 86051-970, Brazil
eHospital Israelita Albert Einstein, Av. Albert Einstein 627-701, 2-SS Bloco A, 05651-901 São Paulo, Brazil
fInstituto de Investigação em Imunologia, Instituto Nacional de Ciência e Tecnologia (INCT), Brazil
gLaboratório de Fisiologia e Fisiopatologia Cardiovascular, Departamento de Ciências Fisiológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, Paraná 86051-970, Brazil

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**Abstract**

*Trypanosoma cruzi* is the causative agent of Chagas disease. Approximately 8 million people are thought to be affected with this disease worldwide. *T. cruzi* infection causes an intense inflammatory response, which is critical for the control of parasite proliferation and disease development. Nitric oxide-donating nonsteroidal anti-inflammatory drugs (NO-NSAIDs) are an emergent class of pharmaceutical derivatives with promising utility as chemopreventive agents. In this study, we investigated the effect of NO-indomethacin on parasite burden, cell invasion, and oxidative stress in erythrocytes during the acute phase of infection. NO-indomethacin was dissolved in dimethyl formamide followed by i.p. administration of 50 ppm into mice 30 min after infection with 5 × 10^3 blood trypomastigote forms (Y strain). The drug was administered every day until the animals died. Control animals received 100 μL of drug vehicle via the same route. Within the NO-indomethacin-treatment group, parasitemia and mortality (100%) were higher and oxidative stress in erythrocytes, anemia, and entry of parasites into macrophages were significantly greater than that seen in controls. Increase in the entry and survival of intracellular *T. cruzi* was associated with inhibition of nitric oxide production by macrophages treated with NO-indomethacin (2.5 μM). The results of this study provide strong evidence that NO-NSAIDs potently inhibit nitric oxide production, suggesting that NO-NSAID-based therapies against infections would be difficult to design and would require caution.

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1. Introduction

Chagas disease is caused by the protozoan *Trypanosoma* (*Schizotrypanum*) *cruzi* and affects approximately eight million individuals in Latin America, of whom 30–40% either have or will develop cardiomyopathy, digestive megasymphdromes, or both [1]. More recently, a major concern has been the emergence of Chagas disease in non-endemic areas such as North America and Europe due to the immigration of infected individuals [2]. This disease is characterized by two clinical phases: a short, acute phase defined by patent parasitemia and a long, progressive, chronic phase [1]. In the early stages of *T. cruzi* infection, nitric oxide (NO) and arachidonic acid metabolites could be involved in host resistance,
but their accumulation subsequently causes tissue damage [3]. Cyclooxygenase (COX)-1 and COX-2 are both targets of nonselective nonsteroidal anti-inflammatory drugs (NSAIDs). The relevance of these enzymes, and the bioactive lipids that they produce, are not well understood in the context of parasitic disease, although the role of eicosanoids in the pathogenesis of Chagas disease is becoming more defined [4].

A series of studies have addressed the oxidative status and antioxidant defense capabilities during the course of infection and progression of Chagas disease in human patients and experimental models [5–8]. Recently, Paiva and collaborators [9] demonstrated that oxidative stress enhances T. cruzi infection by a mechanism that may involve facilitating parasite access to iron. In studies involving experimental models of Chagas disease, increased levels of NO, TNF-α, and prostaglandins were shown to be associated with erythrocyte oxidative stress [6,10]. However, our understanding of the role of NO and COX pathways on oxidative stress in erythrocytes during the acute phase of T. cruzi infection remains incomplete.

Nitric oxide (NO)-releasing NSAIDs (NO-NSAIDs) are a newly developed group of NSAIDs, consisting of a traditional NSAID to which a group that donates NO has been covalently attached via spacers [11]. NO-indomethacin is a hybrid molecule of indomethacin (inhibitor of COX-1) and a nitric oxide (NO) donor. Therefore, this drug design combines the anti-inflammatory drug (NSAID) with the effect of NO.

In this study, we aimed to investigate the impact of NO-indomethacin on parasite burden and oxidative stress in erythrocytes during the acute phase of T. cruzi infection.

2. Material and methods

2.1. Animals, parasites, and experimental infection

Female and male C57BL/6 mice, 6–8 weeks old, were purchased from the Multidisciplinary Center for Biological Research (CEMIB/UNICAMP), University of Campinas (Campinas, Brazil), and maintained under standard conditions in the animal house of the Department of Pathological Sciences, Center for Biological Sciences, State University of Londrina. A commercial rodent diet (Nuvilab-CR1, Quintia-Nuvital, Colombo, Paraná, Brazil) and sterilized water were available ad libitum.

Groups of four and five mice were infected with blood-derived T. cruzi trypomastigotes (Y strain, 5,000 parasites/animal, intraperitoneally). Animals were sacrificed during acute infection [12 or 35 days post-infection (dpi)]. For in vitro experiments, we used trypomastigotes grown and purified from a fibroblast cell line (LLC-MK2).

2.2. NO-NSAID

NO-indomethacin (NCX 2121; N-acetyl-D-cysteine-1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid, 4-(nitrooxy) butyl ester). NO-indomethacin is a hybrid molecule of indomethacin and a nitric oxide (NO) donor.

2.3. Treatment of mice with NO-indomethacin

Mice were treated daily with a dose of 100 µL of 50 ppm NO-indomethacin/mouse intraperitoneally for 12 days. Mice were distributed in four groups: non-infected/non-treated (NI–NT), infected/non-treated (I–NT), non-infected/treated (NI–T) and infected/treated (I–T). The dose chosen for these experiments was based on previously published studies demonstrating its efficacy [12] and was administered 30 min after T. cruzi infection. Control mice received phosphate-buffered saline (PBS; 10 mM sodium phosphate, 0.9% NaCl, pH 7.4) alone.

2.4. Parasitemia and survival rates

Blood parasitemia was assessed under standardized conditions by direct microscopic observation at 400× magnification of 50 fields using 5 µL of heparinized tail vein blood on alternate days from the third day after T. cruzi infection. Data are expressed as the number of parasites/mL [13]. Survival rates of control and experimental groups of mice were evaluated daily until 35 dpi; clinical signs (body weight, hackle hair, diarrhea, lethargy, and paralysis) were recorded. Mice that showed clinical signs of severe illness prior to the end of the study were immediately euthanized by cervical dislocation, in accordance with the Committee on the Ethics of Animal Experiments at Londrina State University (CEEA-UEL).

2.5. Hematological analysis

Whole blood was collected under general anesthesia by intracardiac puncture with ethylenediaminetetraacetic acid (EDTA) needles and syringes. The animals were sacrificed by cervical dislocation and hearts were removed. Leukocytes, platelets, and reticulocytes were counted by standard methods [14]. In each case, the plasma was separated and stored at −20 °C until used. Hematocrits were obtained by microcentrifugation of capillary tubes filled with blood. The total number of nucleated cells collected was determined using a manual hemocytometer.

2.6. Measurements for oxidative stress

2.6.1. Determination of oxygen uptake and induction time in erythrocytes

Blood samples from controls and experimental groups of mice (day 12 post-infection) were used for erythrocyte oxidative stress determinations. After removal of plasma and white cells from whole blood, the remaining erythrocytes were washed three times with PBS and then resuspended in the same buffer (1:99, v/v). Oxygen uptake induced by 2 mM t-butyl hydroperoxide (t-BHT) and induction time (Tind) were measured with a Clark-type oxygen electrode at 37 °C [6,14]. The Tind is directly related to the intracellular protective antioxidant capacity, while oxygen uptake is an
indirect measure of the susceptibility of erythrocyte membranes to lipid peroxidation elicited by t-BHT [6,10].

2.6.2. Tert-butyl hydroperoxide-induced chemiluminescence

Mouse erythrocytes from different experimental groups were pelleted by centrifugation (800×g, 10 min) at 25 °C and then washed three times with PBS. A 1% erythrocyte suspension was prepared with PBS at the moment of use. The chemiluminescence (CL) reaction was initiated by the addition of 20 μL t-BHT at a final concentration of 0.6 mM in 1 mL [15,16]. CL was measured in a Luminometer TD20/20 (Turner Biosystems, Sunnyvale CA, USA), and the results are expressed as relative light units (RLU). The integrated area under the curve was used as the relative amount of lipid hydroperoxide pre-existing in erythrocyte membranes.

2.7. Cardiac parasitism

On day 12 of infection, NO-indomethacin-treated and PBS-treated control mice were sacrificed. The heart was removed, fixed in 10% buffered formalin, and then sectioned. Sections were paraffin embedded, stained with hematoxylin/eosin (H&E), and then analyzed by light microscopy. The number of parasite nests was counted in 50 microscope fields (400× magnification) per tissue section. Three sections were evaluated and the data are expressed as the mean of the three sections.

2.8. Macrophage culture

Mice were inoculated intraperitoneally with 2 mL of 5% thioglycollate and, 4 days later, the elicited cells from the peritoneal exudates were harvested in cold PBS. Mouse peritoneum was washed with 5 mL ice-cold, serum-free RPMI. Peritoneal cells from 3 to 6 mice were pooled and left to adhere in complete medium (RPMI, 2 mM glutamine, 1 mM sodium pyruvate, 40 μg/mL gentamicin, and 10 mM HEPES) for 24 h in 24-well plates at 2 × 10^5 cells/well. Each suspension of pooled peritoneal cells was plated in triplicate wells. Then, non-adherent cells were washed away and adherent cells received complete medium. The macrophages were plated onto 13 mm round glass coverslips and washed in warm PBS before the interaction assays. In addition, macrophages were plated onto 96-well dishes at 2 × 10^5/well.

2.9. Treatment of macrophages with NO-indomethacin and macrophage invasion assay

Before the experiments, previously washed peritoneal macrophages were incubated for 30 min at 37 °C in a 5% CO₂ atmosphere in the presence of NO-indomethacin (2.5 μM) to test its effect on internalization of the parasite into the host cell. After incubation, the medium containing NO-indomethacin was removed, and macrophages were allowed to interact with trypomastigotes added at a ratio of 5 parasites per cell. The interaction was allowed to proceed for 2 h at 37 °C in a 5% CO₂ atmosphere. The cells were then washed three times, fixed with Bouin’s fixative, stained with Giemsa (Merck, Darmstadt, Germany) stain, and observed with a light microscope at 1000× magnification. As a positive control for inhibiting T. cruzi infection, some macrophages were incubated with 20 μM indomethacin.

The internalization index was calculated by multiplying the percentage of infected cells by the mean number of parasites per infected cell [17,18]. All internalization indices were normalized to that of untreated macrophages. Experiments were performed in triplicate, and six independent experiments were completed. All experiments included untreated, infected peritoneal macrophages as controls. The quantification was carried out using light microscopy in which 500 cells were randomly counted. The viability of the cells obtained from the cultures before and after incubation experiments was determined using an MTT (Sigma–Aldrich, St. Louis, MO, USA) assay, which indicates the mitochondrial activity of living cells. The culture medium was aspirated, and MTT (0.5 mg/mL) was added to the cells prior to incubation at 37 °C for 4 h. H₂O₂ (1 mM) was used as negative control. The supernatant was aspirated, dimethyl sulfoxide (Sigma–Aldrich) was added to the wells to dissolve insoluble formazan crystals, and the plates were read using a Bio-Rad multiplate reader (Hercules, CA, USA), at a test wavelength of 570 nm and a reference wavelength of 630 nm.

2.10. NO quantification

Nitric oxide (NO) concentration in plasma obtained from control and infected mice (day 12) was estimated by measuring nitrite as described by Navarro-Gonzalez et al. with some modifications [19]. Aliquots (60 μL) of plasma were deproteinized by adding 50 μL of 75 mM ZnSO₄ solution (Merck), followed by vortexing and centrifuging at 10,000 rpm for 2 min at 25 °C. Following this, 70 μL of a 55 mM NaOH solution (Merck) was added to each supernatant. Samples were vortexed and centrifuged at 10,000 rpm for 5 min at 25 °C. The final supernatants were recovered and diluted in glycine buffer solution (45 g/L pH 9.7, Merck, Germany) at a buffer to supernatant ratio of 5:1.

Cadmium granules (Fluka, Poland) stored in 100 mM H₂SO₄ solution (Merck, Germany) were rinsed 3 times in distilled sterile water and added to a 5 mM CuSO₄ solution in glycine–NaOH buffer (15 g/L pH 9.7, Merck, Germany) for 5 min and the copper-coated cadmium granules were used within 10 min. Cadmium treatment is used to convert all nitrate into nitrite in biological samples, thus providing a more accurate estimation of total NO in the original samples. Activated granules (600–1000 mg, approximately 1–2 granules) were added to glycine-buffered, diluted supernatants and gently stirred for 10 min. Aliquots of 200 μL were transferred into microfuge tubes for subsequent nitrite determination. To each tube, 200 μL of Griess reagent was added (Reagent I: 50 mg of NaNaphthylene diamine in 250 mL of distilled water; reagent II: 5 g of sulfanilic acid in 500 mL of 3 M HCl, Sigma–Aldrich). After an incubation of 10 min at room temperature, tubes were centrifuged at 10,000 rpm for 2 min at 25 °C. Finally, 100 μL from each tube was added to triplicate wells in a 96-well microplate.

To determine sample nitrite concentration, a calibration curve was prepared by diluting NaNO₂ (Merck) in distilled sterile water to create concentrations ranging from 125 to 0 μM. Griess reagent (100 μL) was added in triplicate to the wells of the first three columns of each microtiter plate. The absorbance was read at 550 nm using a LabSystems Multiskan EX microplate reader (Thermo Scientific, Waltham, MA, USA), and the final results are expressed in μM nitrite.

Production of NO by macrophages was determined by measuring the level of accumulated nitrite, a metabolite of NO in the culture supernatant using Griess reagent. After 48 h of treatment with NO-indomethacin, the culture supernatants were collected and mixed with an equal volume of Griess reagent in 96-well culture plates and incubated at room temperature for 10 min. The absorbance was measured at 550 nm and nitrite concentrations were calculated by reference to a standard curve generated by known concentrations of sodium nitrite.

2.11. Statistical analysis

Statistical analysis was conducted using one-way ANOVA with the Bonferroni’s multiple comparison test and the Mann–Whitney Test. Values are presented as the mean ± standard error of mean (SEM). The results were considered significant when P < 0.05. Sta-
3. Results

3.1. Effect of NO-indomethacin on parasitemia and survival of T. cruzi-infected mice

The parasitemia curves of T. cruzi-infected mice that had either been treated with NO-indomethacin (the I–T group) or had received no treatment (the I–NT group) are shown in Fig. 2A. Six days following inoculation with T. cruzi, parasitemia was similar between the two groups. Parasitic load in the blood between 7 and 11 dpi was significantly higher in mice from the I–T group than in mice from the I–NT group (Fig. 2A, P < 0.05). There were large and statistically significant differences (P < 0.01) in survival between the two groups (Fig. 2B). It was observed that 100% of the animals in the I–NT group survived, while all mice in the I–T group died on day 27 (Fig. 2B). Parasitic load in the blood between 24 and 27 dpi was significantly higher in mice from the I–T group than in mice from the I–NT group (Fig. 2A, P < 0.05). Cardiac parasitemia in mice from the I–NT group was not significantly different from that in mice from the I–T group (Fig. 2C, P > 0.05).

3.2. Effect of treatment with NO-indomethacin on anemia, thrombocytopenia, and leukopenia during acute infection with T. cruzi

We conducted blood cell counts and measured several hematological values in uninfected and T. cruzi-infected mice at 12 dpi. Based on significant decreases in hematocrit (Fig. 3A) and hemoglobin (Fig. 3B), we concluded that all mice suffered from anemia. The severity of anemia was greater in mice from the I–T group than in mice from the I–NT group (Fig. 3A and B, P < 0.05). Treatment with NO-indomethacin did not affect the extent of leukopenia and thrombocytopenia that developed 12 dpi (Fig. 3C and D). Surprisingly, the number of reticulocytes in mice from the I–T group was substantially higher than that in mice from the I–NT group (Fig. 3E, P < 0.05).

3.3. NO-indomethacin increases oxidative stress

Oxygen uptake by erythrocytes is directly associated with the susceptibility of the erythrocyte membrane to lipid peroxidation elicited by t-BHP and is proportional to previous oxidative stress experienced by erythrocytes in vivo. The oxygen uptake by erythrocytes from infected mice was associated with a significant increase in oxygen uptake at 12 dpi (Fig. 4A). NO-indomethacin treatment enhanced oxygen uptake in the I–T group (Fig. 4A, P < 0.05).

\[ T_{\text{ind}} \] is directly related to the intracellular protective antioxidant capacity of erythrocytes. T. cruzi infection (day 12) resulted in a significant reduction in \( T_{\text{ind}} \) (Fig. 4B, P < 0.05). Treatment with NO-indomethacin did not alter the \( T_{\text{ind}} \) (P > 0.05, Fig. 4B).

An increase in CL is related to previous in vivo oxidative stress, leading to antioxidant consumption and formation of lipoperoxides, with consequent photon emission [15,20]. The profiles of infected mice (I–NT group) displayed significantly higher levels of lipoperoxidation than the profiles of controls did (NI–NT group, Fig. 4C, P < 0.05). Evaluation of the I–T group revealed a significantly higher initial rate of lipoperoxidation than that shown by the controls (I–NT group), as shown in the ascending part of the curve (Fig. 4C, P < 0.05).

3.4. Inhibitory effect of NO-indomethacin on nitrite levels

We investigated if the production of NO in the plasma and by macrophages was influenced by treatment of mice with NO-indomethacin. As shown in Fig. 5A, the level of nitrite in the plasma on day 12 of infection increased in comparison with that of uninfected mice. NO-indomethacin treatment reduced nitrite production.
levels by 33% (Fig. 5A, \( P < 0.01 \)). Consistent with this result, the nitrite level from infected macrophages was elevated (Fig. 5B, \( P < 0.05 \)) and NO-indomethacin treatment decreased nitrite level by 32% in comparison with macrophages infected and not treated (15.76 μM vs 10.62 μM, \( P < 0.05 \)). Taken together, the data in Fig. 5 indicate that NO-indomethacin inhibits NO production, an important cytotoxic effector for \( T. cruzi \).

3.5. NO-indomethacin increases \( T. cruzi \) entry into macrophages

Macrophages were treated with NO-indomethacin (2.5 μM) for 1 h, and after treatment the medium containing the drug was removed before the macrophage invasion assay in order to ensure that the treatment affected only the host cell and not the parasites. After 2 h of incubation with parasites, which provides sufficient time for them to be internalized by macrophages, the free parasites were removed. In some cases, the medium with NO-indomethacin was added every 24 h until the end of the \( T. cruzi \) infection period (6 days, Fig. 6C). Fig. 6A shows that the treatment markedly increased the internalization of trypomastigotes by macrophages at the concentrations tested (\( P < 0.0001 \)). As a positive control, treatment with indomethacin (20 μM) inhibited the internalization of trypomastigotes by macrophages. The drug cytotoxicity in macrophages was evaluated by MTT assay (Fig. 6B). NO-indomethacin did not induce cell death, as the concentration of drug used in all experiments reported were too low to cause cytotoxicity [12].

3.6. NO-indomethacin increases trypomastigote release into culture supernatants from \( T. cruzi \)-infected macrophages

Four days post infection, macrophages began releasing trypomastigotes into the supernatant (Fig. 6C). The release of trypomastigotes into culture supernatants from \( T. cruzi \)-infected macrophages was increased by NO-indomethacin at concentration used.

4. Discussion

NO-NSAIDs are promising chemopreventive agents; unlike conventional NSAIDs, they appear to be free of appreciable adverse effects, while they retain the beneficial activities of their parent compounds [12]. However, reports on the effects of NO-NSAIDs on parasitic diseases have been somewhat limited. The results of this study provide clear evidence that in the early stages of \( T. cruzi \) infection, NO-indomethacin treatment increased parasitemia and reduced survival rate of infected C57BL/6 mice. In fact, pharmacological antagonists of COX-1 (aspirin) and COX-2 (celecoxib) have been found to increase mortality and parasitemia (parasite load in peripheral blood and cardiac tissue) regardless of which mouse or \( T. cruzi \) strains were used [10], [21–24]. Moreover, evidence suggests that administration of NSAIDs may enhance mortality in patients with Chagas disease [21]. Conversely, others have found that inhibition of prostaglandin release reduces parasitemia and extends survival of mice infected with \( T. cruzi \) [25–28]. This observation was often associated with a decrease in the levels of circulating inflammatory cytokines such as TNF-α, IFN-γ, and IL-10 [27]. More recently, treatment with acetylsalicylic acid during chronic infection was found to be beneficial with no increase in mortality and substantial improvement in cardiac function [24]. Our results suggest that treatment with NO-indomethacin tended to increase the number of amastigote nests in cardiac tissue, although this increase was not significantly different from the number in the control group (I–NT).

Acute infection by \( T. cruzi \) has been associated with significant alterations in hematological values in the blood and bone marrow.
NO-indomethacin treatment did affect the extent of anemia but did not alter the level of leukopenia and thrombocytopenia that developed at 12 dpi. 

T. cruzi infection also is associated with a time-dependent increase in blood reticulocytes and a decrease in bone marrow erythroblasts (two markers of anemia). The changes in reticulocytes were greater in the I–T group versus the I–NT group.

Our results confirm that infection with T. cruzi is associated with significantly increased chemiluminescence levels in erythrocytes from C57BL/6 mice [10]. This peroxidative injury was confirmed by the increased oxygen uptake and decreased induction time in T. cruzi-infected mice. Interestingly, conventional NSAIDs (aspirin and indomethacin) did not affect the extent of anemia in C57BL/6 mice infected by T. cruzi (acute phase of infection), but reduced the levels of chemiluminescence and oxygen uptake and increased the induction time [10]. These results indicate that arachidonic acid metabolism via the cyclooxygenase pathway plays a significant role in oxidative damage in erythrocytes of C57BL/6 mice infected by T. cruzi. In contrast, NO-indomethacin caused an increase in oxidative stress. These data suggest that the release of NO by NO-indomethacin promotes oxidative stress in the erythrocyte membrane. In fact, in the complete absence of NO, membrane injury was greatly reduced in erythrocytes isolated from C57BL/6 iNOS−/− mice 14 days after T. cruzi infection [6].

Fig. 4. Erythrocyte oxidative stress on day 12 after T. cruzi infection. (A) Oxygen uptake and (B) induction time, induced by t-butyl hydroperoxide. (C) Time course curve of t-butyl hydroperoxide-initiated chemiluminescence in erythrocytes from C57BL/6 mice. Groups of mice (n = 5/group) were infected with 5 × 10⁷ T. cruzi and treated or not with NO-indomethacin (50 ppm/mouse). Uninfected mice and untreated T. cruzi-infected mice were used as controls. Values represent the mean ± SEM and are representative of two independent experiments. ∗ ∗ P < 0.01, significantly different from values in the infected/non-treated group. ** P < 0.05, significantly different from values in controls (uninfected/non-treated group or uninfected/treated group).

Fig. 5. Effect of NO-indomethacin on nitric oxide (NO) production. Groups of mice (n = 5/group) were infected with 5 × 10⁷ T. cruzi and treated or not with NO-indomethacin (50 ppm/mouse). (A) NO was estimated by measuring nitrite levels in plasma employing the cadmium–copper system followed by the Griess reaction. (B) Production of NO by macrophages was determined by measuring the level of accumulated nitrite, a metabolite of NO in the culture supernatant using Griess reagent. Values are the mean ± SEM and are representative of two independent experiments. ∗ P < 0.05, significantly different when compared with uninfected/non-treated group. ** P < 0.05, significantly different when compared with controls (uninfected/non-treated group or uninfected/treated group). Tc = Trypanosoma cruzi; Infected represents T. cruzi-infected mice.
NO-indomethacin had no apparent adverse effects on the mice during their 12 days of administration at doses used in the efficacy study [12] and did not induce cell death as evaluated by MTT assay. However, its precise biotransformation remains unknown. There are no data available on the metabolism of NO-indomethacin; thus, it is difficult to speculate on how NO-indomethacin affects cyclooxygenases and nitric oxide isoenzymes in our model system. Nevertheless, COX-2 activity was significantly reduced by NO-aspirin oxygenases and nitric oxide isoenzymes in our model system. Nevertheless, COX-2 activity was significantly reduced by NO-aspirin.

To establish the role of NO-indomethacin in the process of parasite invasion, we have studied its effects on the intracellular parasitic load in T. cruzi-infected murine peritoneal macrophages, and our results show that NO-indomethacin (2.5 μM) increased T. cruzi entry into macrophages. As expected, 20 μM indomethacin reduced the parasitic load by 33% [18,31]. In addition, NO-indomethacin increased trypanostigote release into culture supernatants from T. cruzi-infected macrophages. The survival of T. cruzi can be correlated with the reduction in NO production by macrophages treated with NO-indomethacin. This reduction was also observed in plasma of infected mice treated with NO-indomethacin (12 dpi). These observations clearly illustrate that the anti-inflammatory effect of NO-indomethacin (COX inhibitor) combined with the effect of NO (NO donor) alters the immunological patterns of mice and macrophages through a reduction in NO to promote anti-T. cruzi – activity.

Although we do not know precisely how infection causes oxidative stress in erythrocytes, we hypothesize that phagocyte-derived NO and O2 are central to this process. O2 can react with NO to produce ONOO− or by the Fenton reaction to produce OH, both of which could lead to oxidative stress in erythrocytes [6].

In conclusion, the present results indicate that in experimental Chagas disease, oxidative stress occurs in erythrocytes, but the precise nature of this oxidative stress may depend on the balance of NO produced by the host and that released by NO-indomethacin. Our data confirm the importance of NO as part of the host’s defense against T. cruzi infection. However, the impact of a hybrid molecule such as NO-indomethacin on blood cells and macrophages appears to be complex, and NO-NSAIDs-based therapies against infections are therefore difficult to design and demand caution.

Ethical standards

All animal procedures were performed in accordance with the guidelines of the Brazilian Code for the Use of Laboratory Animals. The protocols were approved by the Research and Ethics Committee of the State University of Londrina (process 13269/2013-01) and carried out in accordance with its guidelines. All efforts were made to minimize suffering during surgical procedures.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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