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

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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.

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 megasyndromes, 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,

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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, Quimtia-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-1*H*-indole-3-acetic acid, 4-(nitrooxy) butyl ester) was purchased from Cayman Chemical (Ann Arbor, MI, USA) (Fig. 1). NO-indomethacin is sparingly soluble in aqueous buffers. For maximum solubility in aqueous buffers, NO-indomethacin was first dissolved in dimethyl formamide (DMF), purged with nitrogen and then diluted with PBS (solubility of approximately 0.5 mg/mL in a 1:1 solution of DMF-PBS, pH 7.2) using this method. The following NO-indomethacin was diluted only in PBS to obtain the desired concentrations.

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

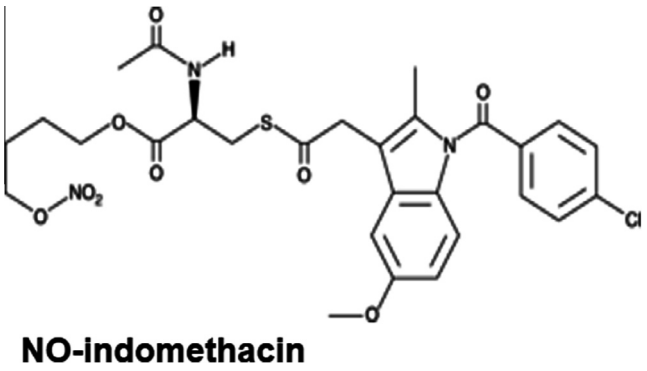

Fig. 1. NO-indomethacin (*N*-acetyl-D-cysteine-1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetic acid, 4-(nitrooxy) butyl esteris). NO-indomethacin is a hybrid molecule of indomethacin and a nitric oxide (·NO) donor.

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 (T^{ind}) were measured with a Clark-type oxygen electrode at 37 °C [6,14]. The T^{ind} is directly related to the intracellular protective antioxidant capacity, while oxygen uptake is an