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Mechanisms of vascular dysfunction in acute phase of *Trypanosoma cruzi* infection in mice

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ABSTRACT

Vascular disorders have a direct link to mortality in the acute phase of *Trypanosoma cruzi* infection. However, the underlying mechanisms of vascular dysfunction in this phase are largely unknown. We hypothesize that *T. cruzi* invades endothelial cells causing dysfunction in contractility and relaxation of the mouse aorta. Immunodetection of *T. cruzi* antigen TcRBP28 was observed in endothelial cells. There was a decreased endothelial nitric oxide synthase (eNOS)-derived NO-dependent vascular relaxation, and increased vascular contractility accompanied by augmented superoxide anions production. Endothelial removal, inhibition of cyclooxygenase 2 (COX-2), blockade of thromboxane A_2 (TXA_2) TP receptors, and scavenger of superoxide normalized the contractile response. COX-2, thromboxane synthase, inducible nitric oxide synthase (iNOS), p65 NFκB subunit and $p22^{phox}$ of NAD(P)H oxidase (NOX) subunit expressions were increased in vessels of chagasic animals. Serum TNF-α was augmented. Basal NO production, and nitrotyrosine residue expression were increased. It is concluded that *T. cruzi* invades mice aorta endothelial cells and increases TXA_2/TP receptor/NOX-derived superoxide formation. Alongside, *T. cruzi* promotes systemic TNF-α increase, which stimulates iNOS expression in vessels and nitrosative stress. In light of the heart failure that develops in the chronic phase of the disease, to understand the mechanism involved in the increased contractility of the aorta is crucial.

1. Introduction

The agent of Chagas' disease *Trypanosoma cruzi* affects millions of people in rural areas of Central and South America, with great economic burden [1]. Infecting parasites or trypomastigotes, by entering the bloodstream, infect a wide range of susceptible host cells such as macrophages [2,3], cardiomyocytes [4], skeletal [5] and smooth muscle cells [6,7], human endothelial cell lines such as HUVEC and EAhy926 [8–10].

The acute phase of Chagas' disease is characterized by high levels of parasitemia, fever, lymphadenopathy and hepatosplenomegaly [10]. Despite the absence of cardiac remodeling and heart failure in this phase, some studies showed the existence of myocarditis, increased platelet aggregation, fibrin microthrombi formation, spasm and vasculitis of coronary microcirculation [4,10,11].

In the chronic phase, there is activation of the acquired immunity leading to a gradual reduction in parasitemia [10,12,13]. In some patients the low-grade of infection masks the symptoms for many years (silent infection). Differently from the asymptomatic patients, about 30% of symptomatic patients show a progressive active chagasic cardiomyopathy associated with cardiomegaly and disturbance in heart rhythm [12,14], increasing morbidity and mortality.

While the cardiac manifestations of Chagas' disease are well known, the involvement of the vasculature in its pathogenesis is commonly neglected. Some studies suggested that inflammation of cardiac microvessels related to *T. cruzi* infection induces the synthesis of several pro-inflammatory cytokines, vascular adhesion molecules and some vasoactive molecules such as endothelin (ET-1) and thromboxane A_2 (TXA_2) [15–17]. Moreover, vasculitis [18] and structural changes of the aorta endothelial layer were reported in the acute phase of Chagas' disease [19]. The vascular function of vessels removed from animals infected with *T. cruzi* is so far unknown.

Aortic pulse-wave velocity has been shown to be associated with cardiovascular risk [20]. Moreover, aortic stiffness has been shown as an independent predictor of all-cause and cardiovascular mortality [21]. Therefore, the study of aorta function of animals infected with

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T. cruzi is crucial. Thus, the aim of the present study was to investigate the reactivity of the aorta during the acute phase of Chagas' disease and the underlying mechanism.

2. Methods

2.1. Animals

All animal procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health (United States), as well as, the guidelines for the humane use of laboratory animals at our Institute. These studies were approved by the ethics committee of the Federal University of Minas Gerais (UFMG; protocol # 72/2010). Male C57bl/6 mice were used. All animals were obtained from Rene Rachou Research Center (CPqRR-Fiocruz; Belo Horizonte, MG, Brazil) and maintained in the animal facilities of the Cellular and Molecular Parasitology Laboratory. Free access was allowed to standard diet and filtered water was supplied ad libitum. All mice were maintained at five per cage and in a constant temperature (24 ± 2 °C), with a 12-h dark/light cycle.

2.2. Protocol of infection

Animals (eight-week-old) were infected intraperitoneally with 50 bloodstream trypomastigote forms of Colombian *T. cruzi* strain [22], which was maintained by serial passages. Animals were sacrificed 30 days post-infection, and the aorta and serum were collected.

2.3. Vascular function studies

Experiments to assay vascular function were performed in an organ bath system, as previously described [23]. Briefly, thoracic aortic rings (3–4 mm length) from infected and control mice were obtained, mounted in an organ bath system containing Krebs–Henseleit solution (in $mmol \cdot L^{-1}$: 110.8 NaCl, 5.9 KCl, 25.0 $NaHCO_3$, 1.07 $MgSO_4$, 2.49 $CaCl_2$, 2.33 NaH_2PO_4 and 11.51 glucose, pH 7.4), for 60 min. Concentration–response curves to acetylcholine (ACh) were constructed in vessels pre-contracted to the same tension level (approximately 2.5 $mN \cdot mm^{-1}$) with submaximal concentrations of phenylephrine (0.03–0.1 $\mu mol \cdot L^{-1}$). Vascular contractions were evaluated by concentration–response curves to phenylephrine. When necessary, some vessels were incubated for 20 min with the indicated drugs before the construction of curves. Mechanical activity was recorded isometrically by a force transducer (World Precision Instruments, Inc.) connected to an amplifier-recorder (Model TBM-4; World Precision Instruments, Inc.) and to a personal computer equipped with an analog-to-digital converter board (DI-720; Dataq Instruments, Inc.), using Windaq data acquisition/recording software (Dataq Instruments, Inc.).

2.4. Superoxide and nitric oxide detection

Superoxide anions and nitric oxide (NO) production were measured by fluorescence microscopy, in aortic rings [24]. For superoxide measurements, the rings were incubated for 20 min at 37 °C in Krebs–Henseleit solution containing or not 10 $\mu mol \cdot L^{-1}$ tiron, a superoxide scavenger (Merck-Millipore, USA). Then, the superoxide sensitive dye dihydroethidium (DHE; 10 $\mu mol \cdot L^{-1}$; Invitrogen, USA), was added for 30 min, protected from light. After that, the excess of dye was washed out, the rings were embedded in Tissue-Tek® O.C.T.™ freezing medium (Sakura®, USA) and quickly frozen in liquid nitrogen. For NO measurements, aortic rings were incubated for 20 min at 37 °C in Krebs–Henseleit solution containing or not 10 $\mu mol \cdot L^{-1}$ of the iNOS inhibitor N(6)-(1-iminoethyl)-l-lysine dihydrochloride (L-NIL; Merck-Millipore, USA). The intracellular NO sensitive dye 4-amino-5-methylamino-2′,7′-difluorescein diacetate (DAF-FM; 10 $\mu mol \cdot L^{-1}$; Invitrogen, USA) was added for 30 min, protected from light. After washing, rings were frozen, as described above. Frozen rings were sliced in a cryostat (10 μm; Leica 1850, Leica, USA) and mounted in slides with DAPI/antifade-containing medium (Santa Cruz Biotechnology, USA). Slides were imaged with an inverted fluorescence microscope (Eclipse Ti, Nikon, USA). Fluorescence intensity (Ex/Em: 518/605 and 495/515, to DHE and DAF-FM, respectively) was calculated from at least 8 fields from 4 different experiments using ImageJ software (NIH, USA).

2.5. Determination of tumoral necrosis factor alpha (TNF-α) in serum by ELISA

Blood was collected during the sacrifice and the serum was separated by centrifugation (1500 × *g* for 10 min). Quantification of TNF-α level was performed by the use of colorimetric kits (R&D Systems). The concentration of TNF-α in each sample was determined by a standard curve with known concentrations of TNF-α.

2.6. Western-blot analysis

Western blot was performed as previously described [25] with some modifications. Briefly, the frozen aorta segments (~50 mg) were homogenized in lysis buffer (in $mmol \cdot L^{-1}$): 150 NaCl, 50 Tris–HCl, 5 EDTA.2Na, and 1 $MgCl_2$ containing 1% Triton X-100 and 0.5% SDS plus protease inhibitors (SigmaFAST; Sigma-Aldrich, MO, USA). Equal amounts of proteins (30 μg) were denatured and separated in denaturing SDS/7.5% polyacrylamide gel. Proteins were transferred onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore, MA). Blots were blocked at room temperature with 5% BSA in TBS enriched with 0.1% Tween 20 before incubation with rabbit polyclonal anti-eNOS (1:1000; Sigma-Aldrich), goat polyclonal anti-phospho-eNOS^{Ser1177} (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat polyclonal anti-phospho-eNOS^{Thr495} (1:1000; Santa Cruz Biotechnology), mouse monoclonal anti-iNOS (1:2000; Santa Cruz Biotechnology), mouse monoclonal anti-nitrotyrosine (1:2000; Santa Cruz Biotechnology), mouse polyclonal anti-COX-2 (1:1000; Cayman Chemical), mouse monoclonal anti-NADPH p-22^{phox} (1:1000; Santa Cruz Biotechnology), rabbit polyclonal anti-thromboxane synthase (1:500; Abcam, Cambridge, UK), rabbit polyclonal anti-NF-κB p65 (1:1000; Santa Cruz Biotechnology), mouse monoclonal anti-β-actin (1:8000; Santa Cruz Biotechnology) or mouse monoclonal anti-GAPDH (1:8000; Santa Cruz Biotechnology), at room temperature. The immunocomplexes were detected by chemiluminescent reaction (ECL Plus kit; Amersham, Les Ulis, France) followed by densitometric analyzes with software ImageJ.

2.7. T. cruzi immunostaining

T. cruzi localization was performed in aortic rings (3–4 mm length) by immunofluorescence. Aortic rings were collected as described above, washed in Krebs–Henseleit solution, imbibed in Tissue-Tek® O.C.T.™ freezing medium (Sakura®, USA) and quickly frozen in liquid nitrogen. Frozen rings were sliced in a cryostat (10 μm; Leica 1850, Leica, USA), fixed and permeabilized with cold acetone and 0.5% Triton X-100 (in PBS, pH 7.4). After block procedure (5% bovine serum albumin in PBS), slices were incubated with mouse polyclonal antibody against *T. cruzi* antigen TcRBP28 [26], followed by Alexa Fluor 647 donkey anti-mouse (Invitrogen, USA, OR). Slides were mounted with DAPI/antifade-containing medium (Santa Cruz Biotechnology) and imaged in an inverted fluorescence microscope (Eclipse Ti, Nikon, USA) at Ex/Em: 495/515 and 647/665, to elastin and TcRBP28, respectively.

2.8. Data analysis

Results are expressed as mean ± SEM. Two-way ANOVA was used to compare concentration–response curves. Student's *t*-test was used in the other experiments. All statistical analyzes were considered significant when $p < 0.05$.