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Role of NO Synthase in the Development of *Trypanosoma cruzi*-Induced Cardiomyopathy in Mice

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Abstract

Trypanosoma cruzi infection results in an increase in myocardial NO and intense inflammation. NO modulates the *T. cruzi*-induced myocardial inflammatory reaction. NO synthase (NOS)1-, NOS2-, and NOS3-null mice were infected with *T. cruzi* (Brazil strain). Infected NOS1-null mice had increased parasitemia, mortality, and left ventricular inner diameter (LVID). Chronically infected NOS1- and NOS2-null and wild-type mice (WT) exhibited increased right ventricular internal diameter (RVID), although the fold increase in the NOS2-null mice was smaller. Infected NOS3-null mice exhibited a significant reduction both in LVID and RVID. Reverse transcriptase-polymerase chain reaction showed expression of NOS2 and NOS3 in hearts of infected NOS1-null and WT mice, whereas infected NOS2-null hearts showed little change in expression of other NOS isoforms. Infected NOS3-null hearts showed an increase only in NOS1 expression. These results may indicate different roles for NOS isoforms in *T. cruzi*-induced cardiomyopathy.

INTRODUCTION

Infection with the parasite *Trypanosoma cruzi* causes Chagas disease. The important manifestations of clinical Chagas disease are acute myocarditis and chronic dilated cardiomyopathy. Chagas disease is endemic in many areas of Latin America, affecting ~20 million infected people. Chagasic heart disease, which accounts for thousands of deaths annually, is manifested by congestive heart failure, conduction abnormalities, and thrombo-embolic events.^{1,2} We and others have shown that mice infected with *T. cruzi* develop many of the structural and functional alterations observed in the human disease.^{1,3–7} Our laboratory

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has pioneered the application of cardiac magnetic resonance imaging (MRI) in the evaluation of the heart in mice infected with *T. cruzi*.

Infection with *T. cruzi* results in an intense inflammatory response in many organs including the heart, associated with an increase in the expression of inflammatory mediators that facilitates parasite killing in part through enhanced release of NO.^{5,8} However, *T. cruzi*-induced increase in myocardial inducible NO synthase (NOS2) is often associated with cardiac dysfunction. This has also been observed in cardiomyopathies of other etiologies.^{4,9–11}

NO contributes to myocardial function in several ways including exerting a negative inotropic effect and influencing the regulation of blood pressure, coronary tone, myocardial contractility, and platelet aggregation.^{12–19} The major source of endogenous NO in the normal heart is endothelial NOS (NOS3), which is present in endothelial cells, cardiac myocytes, and platelets. Mice deficient in NOS3 have low body weight, hypertension, heart rate variability, and age-dependent left ventricular hypertrophy. In ischemia-reperfusion studies NOS3-null mice have significantly larger infarcts than wild-type (WT) mice, suggesting a protective role for NOS3 in reperfusion injury.²⁰ Neuronal NOS (NOS1) is expressed in the sarcoplasmic reticulum and in cardiac mitochondria.^{21,22} NOS1-null mice display left ventricular hypertrophy and enlargement of the stomach²³ and do not display any difference in infarct size compared with WT mice, although left ventricular remodeling after myocardial infarction was observed.²⁴

The increase in the production of NO in response to *T. cruzi* infection may contribute to the myocardial dysfunction observed in chagasic patients and animal models of Chagas disease. Differential expression of NOS isoforms has been reported in a rat model of acute chagasic cardiomyopathy and that data suggested a role for NOS2 and NOS3 early in infection.²⁵ However, the rat and mouse models of infection are not comparable.

Mice deficient in NOS1, NOS2, or NOS3 are novel models for studying their roles in the development of chronic chagasic heart disease. Here we evaluate the roles of the different NOS isoforms in *T. cruzi*-induced cardiomyopathy in NOS-null mice, using non-invasive cardiac MRI. Although previous studies have shown a role for NOS2 in murine chagasic heart disease,³ this is the first extensive report studying the roles of NOS1 and NOS3 in the development of *T. cruzi*-induced cardiomyopathy in a murine model using MRI.

MATERIALS AND METHODS

Parasitology and pathology

The Brazil strain of *T. cruzi* was maintained in C3H mice (Jackson Laboratories, Bar Harbor, ME). NOS-null mice (NOS1-, NOS2-, and NOS3-null mice) and wild-type C57BL/6J (WT) mice were obtained from Jackson Laboratories. Mice were infected at 6–8 weeks of age with 1×10^4 trypomastigotes of the Brazil strain. Parasitemia was determined beginning on day 20 after infection. All mice were housed in our Animal Institute Facility and handled with protocols approved by our Animal Care and Use Committee. Hearts were fixed in 10% buffered formalin, and paraffin-embedded sections were stained with hematoxylin–eosin (H&E).

Semi-quantitative RT-PCR for NOS isoform expression

Total RNA was extracted from infected and uninfected mouse hearts on day 30 after infection by Trizol reagent (Invitrogen, Carlsbad, CA) as recommended by the manufacturer and quantitated by a Nanodrop ND1000 spectrophotometer (Wilmington, DE) before subjecting to reverse transcriptase-polymerase chain reaction (RT-PCR). One microgram of the purified RNA was used to amplify NOS mRNA isoforms using GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA).²⁶ We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping control gene and amplified it using the following primer pair:

forward, 5-TGAAGGTCGGTGTGAACGGAT-3, and reverse, 5-CA TGTAGGCCATGAGGTCCAC-3. PCR products were vacuum-dried and electrophoresed in a 0.9% agarose gel containing ethidium bromide. The gel image was captured in FluorChem (Alpha Innotech, San Leandro, CA), and the bands were quantitated in Image J software.

Magnetic resonance imaging

Imaging was performed on mice anesthetized through intraperitoneal injection with ketamine (150 mg/kg)–xylazine (10 mg/kg) solution. Once anesthetized, a set of standard electrocardiographic (ECG) leads was attached to the mouse limbs. The mouse was wrapped in a small insulated Teflon tube and positioned head up in the MRI coil (35-mm inner diameter). MRI was accomplished using a 9.4-T 400-MHz vertical wide bore GE Omega spectrometer (Fremont, CA). The S50 shielded micro-imaging gradient coils were maintained at 30°C with a Neslab water cooling/heating unit to maintain body temperature during the imaging experiment. A 5-V R-wave trigger was used to gate the MRI acquisition as previously described.²⁷ A routine cardiac gated multi-slice (90° sinc pulse) spin echo sequence was used. An echo time (TE) of 18 ms and repetition time (TR) of ~100–200 ms (depending on heart rate) was used for short axis images of the heart, with a 40-mm field of view (FOV) and a 128 × 256 matrix size (interpolated to 256 × 256 pixels). As previously described, the heart was imaged in a series of transverse (short axis) image sets from the base to the apex of the heart. MRI data were analyzed with MRI analysis software running in MATLAB. Heart measurements included the left ventricular inner chamber dimension (LVID), left ventricle wall thickness (LV wall), and right ventricular inner chamber dimension (RVID).^{27,28}

Statistical analysis

Statistical significance ($P < 0.05$) was evaluated using the t test parametric test. All data are presented as mean ± SEM.

RESULTS

Parasitology and pathology

The mean parasitemia observed in infected WT and NOS2- and NOS3-null mice ranged between 1×10^5 and 4×10^5 trypomastigotes/mL 20 days after infection. The mean parasitemia was higher (2×10^6) in infected NOS1-null mice, and these mice had the highest initial mortality, which resulted in the smaller number of mice available in this group for chronic studies. Infected NOS3-null mice also exhibited high mortality even though parasitemia was not significantly different from levels in infected WT mice. Parasitemia waned by day 60 in all mice that survived the acute stage. Uninfected WT (data not shown) and NOS2- and NOS3-null mice had normal myocardial morphology (Figure 1). Hearts obtained from uninfected NOS1-null mice showed focal interstitial and replacement fibrosis. Infected NOS1-null mouse hearts exhibited inflammation, necrosis, and numerous pseudocysts. There were fewer parasite pseudocysts and a reduction in inflammation and necrosis in hearts obtained from infected NOS2-null mice compared with NOS1-null mice. Similarly, infected NOS3-null hearts showed less inflammation and no pseudocysts (Figure 1).

NOS isoforms by RT-PCR

Expression of NOS isoforms in the hearts of WT and NOS1-, NOS2-, and NOS3-null mice was evaluated by RT-PCR (Figure 2 and Figure 3). Infected WT mice exhibited increased levels of expression of both NOS2 and NOS3 but no change in NOS1 compared with uninfected controls. Infected NOS1-null mice also exhibited increased expression of both NOS2 and NOS3. There was no significant change in expression of NOS1 in the hearts of infected NOS2- and NOS3-null mice compared with respective uninfected controls (Figure 2 and Figure 3).

MRI studies of the heart

Previously, we observed that WT mice surviving the acute phase of infection gradually develop chronic cardiomyopathy. Those studies were performed at ~100 days after infection.^{3–5,29} In this report, we evaluated infected and uninfected WT (C57BL/6J) together with NOS1-, NOS2-, and NOS3-null mice, using cardiac MRI 6 months after infection. Infected WT mice exhibited significant dilation of the right ventricle (RV) (Figure 4 and Figure 5). Dilation of the RV was also observed in the infected NOS2-null mice, but the fold increase was smaller than that observed in infected WT mice, similar to our previous results acquired at 100 days after infection.⁴ Dilation of the RV was not observed in the chronically infected NOS3-null mice; however, we observed a significant reduction in the left ventricle (LV) chamber dimension in NOS3-null mice compared with uninfected NOS3-null mice and WT mice (Figure 5). Infected and uninfected NOS3-null mice had the thickest LV walls (Table 1) consistent with the hypertrophy and hypertension generally observed in NOS3-null mice.²¹ Chronically infected NOS1-null mice exhibited significant dilation of the RV (Figure 4 and Figure 5), suggesting that the absence of NOS1 does not ameliorate right ventricular dilation in chronic *T. cruzi* infection. These mice also exhibited LV chamber dilation, which was not observed in the other infected mice.

DISCUSSION

Although our previous studies have shown that infection with *T. cruzi* results in an intense inflammatory reaction in the myocardium accompanied by increased expression of NOS2, this is the first report studying the roles of NOS1 and NOS3 in the development of chronic chagasic cardiomyopathy in mice. Upregulation of cytokines and chemokines in infected cultured cardiac myocytes and in the whole heart obtained from infected mice has been reported.^{4,30–32} Infected inbred WT mice (C57BL/6J) develop acute myocarditis and a transient parasitemia but do not die during the acute phase.⁴ During the chronic phase (100 days after infection), we previously reported marked right ventricular dilation, cardiac myocyte hypertrophy, chronic inflammation, and fibrosis in WT mice.^{3–5} Infected NOS2-null mice show attenuation of *T. cruzi*-induced right ventricular dilation and mortality.^{3,4}

In this study, we examined the role of each NOS isoform in a murine model of *T. cruzi*-induced cardiomyopathy. There was a transient low-level parasitemia in infected WT and NOS2- and NOS3-null mice. The parasitemia was higher in infected NOS1-null mice and the acute disease mortality was the highest in infected NOS1- and NOS3-null mice. At 30 days after infection, the most significant pathologic features were found in the hearts of infected NOS1-null mice. Infected NOS3-null mice also had significant pathology, whereas infected NOS2-null and WT mice showed less pathology (data not shown). These findings remained stable over 6 months after infection.

Similar to infected WT, we observed a large increase in the expression of both NOS2 and NOS3 in the myocardium of infected NOS1-null mice. RT-PCR results also showed an increased expression of NOS3 in NOS2-null mice, suggesting compensatory mechanisms in the absence of NOS2. However, there is no increased expression of NOS1 in these mice, which contrasts with what we recently observed in the colon of infected NOS2-null mice.²⁶

These MRI studies were conducted 6 months after infection. We observed left ventricular hypertrophy in both chronically infected and uninfected NOS3-null mice as evidenced by the increased LV wall thickness compared with WT mice. These findings are consistent with compensatory LV wall hypertrophy associated with the hypertension observed in this phenotype,^{21,33,34} which seems to be exacerbated by *T. cruzi* infection. The LV chamber size of the NOS3-null mice was decreased during chronic infection, whereas infection had no effect on LV chamber size in the WT and NOS2-null mice. Infected NOS3-null mice also lost

significant body weight during the acute stage and remained significantly smaller than uninfected NOS3-null mice throughout the course of this study (data not shown). Interestingly, infected NOS3-null mice did not exhibit right ventricular dilation, suggesting that NOS3 contributes to the right ventricular remodeling observed in *T. cruzi*-infected WT mice, a mechanism not previously reported.

Because NOS1-null mice exhibited a high mortality during acute infection, we were only able to image the few mice that survived to the chronic stage. These infected NOS1-null mice exhibited dilation in both LV and RV, but there were no significant differences in thickness of the LV wall between the infected and uninfected groups. Additionally, chronically infected NOS2-null mice exhibited no significant change in LV wall thickness or chamber dimension but developed mild dilation of the RV 6 months after infection. Similar to our previous findings in these mice at 100 days after infection, the degree of dilation was less than that observed in WT mice. Although the LV chamber was enlarged in infected NOS1-null mice, it was reduced in infected NOS3-null mice. There were no significant changes in LV chamber size in the infected WT or NOS2-null mice.

Several studies suggest that NOS3-derived NO limits the development and progression of LV remodeling after myocardial infarction, whereas other data suggest that NOS1-derived NO may have important functions in the pathophysiology of cardiac remodeling.^{35,36} Mice deficient in both NOS1 and NOS3 (double KO) exhibit hypertension (as observed in NOS3-null mice) but develop a unique age-related phenotype characterized by concentric remodeling, with a marked increase in wall thickness.³⁷ Thus, it seems that the disruption of both NOS1 and NOS3 leads to a phenotype that is additive from that observed in either single NOS knockout. These observations support independent roles for each NOS isoform in maintaining normal cardiac structure. The results of these studies with infected NOS1- and NOS3-null mice suggest potentially opposing roles for NO derived from either isoform in maintenance of normal LV structure. When both isoforms are present, LV chamber size is within normal values; however, when NOS1 is absent, there is left ventricular enlargement, and when NOS3 is absent, there is reduction of the LV chamber.

This is the first study evaluating NOS1 and NOS3 in the pathogenesis of murine chagasic heart disease, and the data suggest different roles for the three NOS isoforms. This study also highlights the non-invasive use of cardiac MRI in the evaluation of myocardial structure in chronic cardiomyopathies in mice such as that induced by *T. cruzi* infection. Our results suggest that NOS2 and NOS3 have the most active roles in the right ventricular cardiac syndrome of this disease, whereas NOS1 deficiency seems to affect remodeling of the left ventricle.

The role of NO in the pathogenesis in human Chagas disease remains unclear. As noted, NO in the heart is derived from the three NOS isoforms.³⁸ Although NOS1 and NOS3 are constitutively expressed low output enzymes, there is increased expression of NOS2 in disease states such as infection and congestive heart failure.^{22,23} The three isoforms exist in different compartments in the heart. In the mouse model of *T. cruzi* infection, NO can be regarded as a “doubleedged” sword. Although the large amounts of NO generated from the infection-associated increase in NO from NOS2 is important in the killing of the parasite, it may cause myocardial depression. Interestingly, infected NOS2-null mice do not die, and there is an amelioration of the infection-associated increase in the RVID. There is, however, increased pathology and mortality despite the lack of a significant increase in the LV and RV in infected NOS3-null mice. *T. cruzi* infection is associated with increased expression of endothelin-1³⁹ and thromboxane A₂⁴⁰ both of which cause vasospasm. Thus, the two vasoactive agents can exert their effect in the absence of the counteracting vasodilator effect of NOS3-derived NO. This may result in increased ischemia, inflammation, and necrosis of the myocardium. The use

of selective inhibitors of NOS isoforms may prove to be beneficial as adjunctive therapy in the management of chagasic heart disease.

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FIGURE 1.

Representative myocardial sections of uninfected and *T. cruzi*-infected NOS-null (KO) mice 21 days post-infection stained with H&E (original magnification, $\times 20$). Uninfected NOS1-null mice showed fibrosis, whereas infected NOS1-null mice showed marked inflammation and numerous pseudocysts (arrow). Infected NOS2-null mice had minimal inflammation, whereas in infected NOS3-null mice, there was inflammation and scattered areas of myonecrosis in the myocardium. Uninfected NOS2- and NOS3-null mice had no significant pathology. This figure appears in color at www.ajtmh.org.

FIGURE 2.

Semi-quantitative RT-PCR for NOS isoforms in murine heart of control and *T. cruzi*-infected mice from wild-type and various NOS-null (KO) backgrounds at day 30 after infection.

Representative gels showing NOS isoform expression in wild-type control (Con) and infected (Inf) mice (**A**). Representative RT-PCR gels showing NOS2 and NOS3 in NOS1-null mice (**B**), NOS1 and NOS3 in NOS2-null mice (**C**), and NOS1 and NOS2 in NOS3-null (**D**) mice. Data from two independent control (Con1 and Con2) and infected (Inf1 and Inf2) mice from wild-type and each null background are shown. In **B–D**, the respective NOS-null isoform was also amplified as a negative control. **E**, GAPDH RT-PCR as an internal control ($N = 4$ for each group).

FIGURE 3.

Densitometry of the semi-quantitative RT-PCR data for NOS isoforms in *T. cruzi*-infected (Inf) murine hearts. In infected WT mice, there was an increase in the expression of all NOS isoforms compared with control (Con). In infected NOS1-null (KO) mice, expression of both NOS2 and NOS3 was significantly increased. In infected NOS2 KO mice, there was no significant increase in NOS1 or NOS3 isoforms, whereas infected NOS3 KO mice showed increased expression of NOS1 isoform. $N = 3$ for each group and column values represents the average, error bars, and the maximum and minimum values.

FIGURE 4.

MRI measurements of end-diastolic right ventricle inner diameter (RVID) of chronically (6 months) infected and uninfected WT (C57BL/6J), NOS1-null (KO), NOS2-null and NOS3-null mice. *N* values are stated in Table 1.

FIGURE 5.

Transverse cardiac gated end-diastolic MR images of hearts of infected and uninfected WT and NOS1- and NOS3-null (KO) mice. Images of the NOS2-null mice are not shown, because they were similar to WT mice, although with a less robust increase in RVID. Left (LV) and right ventricle (RV) are indicated by arrows. Images were acquired with a spin-echo sequence, FOV of 40 mm, and with TE = 18 ms, TR = 100–200 ms, number of transients = 4, and 128 × 256 matrix size interpolated to 256 × 256.

TABLE 1

MRI measurements at end-diastole of the hearts of chronically (6 months) infected (I) and uninfected (U) NOS1-, NOS2-, and NOS3-null (KO) and C57BL/6J mice

Mouse	N	Body mass (g)	LVID (mm)	LVwall (mm)
U NOS1 KO	4	26.5 ± 2.1	3.1 ± 0.1	1.2 ± 0.1
I NOS1 KO	4	29.8 ± 2.0	3.5 ± 0.2 ^{*†}	1.1 ± 0.1
U NOS2 KO	8	29.2 ± 1.5	3.1 ± 0.08	1.1 ± 0.05
I NOS2 KO	4	28.9 ± 0.9	3.1 ± 0.08	1.2 ± 0.1
U NOS3 KO	11	28.3 ± 1.1	3.2 ± 0.2	1.3 ± 0.08
I NOS3 KO	13	24.1 ± 1.2 [*]	2.5 ± 0.1 [*]	1.4 ± 0.1
U C57BL/6J	5	25.9 ± 0.5	3.1 ± 0.1	1.1 ± 0.1
I C57BL/6J	6	24.9 ± 0.9	3.0 ± 0.1	1.2 ± 0.1

Left ventricle inner diameter (LVID) and left ventricle wall thickness (LVwall) are included.

^{*} Significantly different ($P < 0.05$) compared with uninfected mice of same genotype.

[†] Significantly different ($P < 0.05$) compared with I C57BL/6J value.