Microbial Pathogenesis 73 (2014) 47–52

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Cardiopathogenic mediators generated by GATA4 signaling upon co-activation with endothelin-1 and *Trypanosoma cruzi* infection

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ARTICLE INFO

Article history:
Received 7 March 2014
Received in revised form
26 May 2014
Accepted 3 June 2014
Available online 11 June 2014

Keywords:
Chagas cardiomyopathy
Trypanosoma cruzi
Endothelin-1
GATA4

ABSTRACT

Trypanosoma cruzi (Tc), the etiological agent of Chagas disease, triggers multiple responses in the myocardium, a central organ of infection and pathology in the host. Parasite-driven induction of diverse regulators of cardiovascular function, including the vasoconstrictor endothelin-1 (ET-1), the inducible form of nitric oxide synthase (iNOS) and the B-type natriuretic peptide (BNP), has been linked to the development of severe chagasic cardiomyopathy. Our current goal was to analyze the participation of the zinc finger transcription factor GATA4, critically implicated in pathological cardiac hypertrophic response, in the generation of key mediators involved in the pathogenesis of Tc-elicited heart dysfunction. In this study, we found that the combined effects of Tc and ET-1 on atrial myocytes promoted the protein expression, phosphorylation and DNA-binding activity of GATA4, leading to augmented protein levels of iNOS and increased nitric oxide release. Moreover, Tc- and ET-1-co-activation of cardiomyocytes resulted in enhanced GATA4-dependent secretion of BNP. Accordingly, mice with chronic chagasic cardiomyopathy showed increased expression of GATA4, iNOS and BNP at inflammatory lesions in cardiac muscle. Our findings support a role for the GATA4 signaling pathway in the myocardial production of pathogenic mediators associated with Chagas heart disease, and may help define novel therapeutic targets.

1. Introduction

Chagas disease, caused by the hemoflagellate protozoan *Trypanosoma cruzi*, is one of the most serious public health and socio-economic problems in Latin America. The overall prevalence of chronic infection is about 8–10 million cases, with 50,000 people estimated to die from the disease each year [1]. In recent decades, this parasitosis has spread around the globe through human migration to non-endemic areas [2]. Upon *T. cruzi* infection, individuals undergo an acute phase which displays frequently as a non-apparent form with a few or no symptoms. Thereafter, the majority of chagasic subjects enter into an asymptomatic, indeterminate stage, which lasts throughout life. The remaining 20–30% of chronically infected patients develop clinical complications, typically years or decades after infection. Chronic cardiomyopathy is the most common and severe manifestation of Chagas disease, causing congestive heart failure, arrhythmias and conduction abnormalities, which often result in stroke and sudden death. This type of dilated cardiomyopathy is associated with chronic inflammation and fibrosis, cardiac hypertrophy and thrombo-embolic events [3].

The pathogenesis of *T. cruzi*-driven cardiomyopathy is still matter of intense debate. However, three main pathogenic mechanisms have been identified: cardiac dysautonomy, inflammatory/immunological tissue damage and disorders of the microvascular circulation [4]. Among other factors, elevated levels of the vasoactive peptide endothelin-1 (ET-1) play a pivotal role in the development of Chagas heart disease contributing to vascular injury, cardiac remodeling and enhanced liberation of inflammatory agents [5]. Recently, we found that the combined effect of *T. cruzi* infection and ET-1 activates the Ca^{2+}/calcineurin (Cn)/nuclear factor of activated T cells-c4 (NFATc4) signaling pathway in atrial myocytes, leading to cyclooxygenase-2 (Cox-2) overexpression and increased eicosanoid release [6].

The discovery of cooperation between the NFATc4 protein and the zinc finger transcription factor GATA binding protein 4 (GATA4), critically involved in pathological cardiac hypertrophic response, has revealed the importance of Ca^{2+} signaling in the activation of this GATA family member [7]. We therefore hypothesized that, upon ET-1 treatment and parasite infection, stimulation of the intracellular Ca^{2+}-dependent cascade may trigger GATA4

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http://dx.doi.org/10.1016/j.micpath.2014.06.001
0882-4010/

Glossary

Tc	Trypanosoma cruzi
ET-1	endothelin-1
Cn	calcineurin
NFAT	nuclear factor of activated T cells
Cox-2	cyclooxygenase-2
GATA4	GATA binding protein 4
phospho-GATA4	phosphorylated form of GATA4
Ser-105	amino acid serine at position 105 in the GATA4 protein
BNP	B-type natriuretic peptide
iNOS	inducible form of nitric oxide synthase
PMA	phorbol 12-myristate 13-acetate
siRNA	small interfering RNA
Wt	wild-type
Mut	mutant

phosphorylation in cardiac muscle cells. GATA4 downstream target genes include B-type natriuretic peptide (BNP) and inducible nitric oxide synthase (iNOS), both implicated in Chagas pathogenesis [8,9]. Nevertheless, contribution of GATA4 activity to *T. cruzi*-elicited myocardial dysfunction remains unexplored so far. Our current goal was to examine whether the cooperative action of the pathogen and ET-1 on atrial myocytes leads to GATA4-regulated induction of critical mediators of cardiovascular pathology in Chagas disease.

2. Materials and methods

2.1. Ethics statement

The protocol of this study was reviewed and approved by the Research & Teaching Committee from Hospital de Niños "Dr. Ricardo Gutiérrez" (Buenos Aires City Government, Argentina).

2.2. Trypanosoma cruzi infection of cardiac myocytes

Mouse HL-1 cardiomyocytes were cultured as described previously [10]. Cells (5×10^5) were infected with *T. cruzi* trypomastigotes (cell:parasite ratio 1:5), Tulahuén strain, routinely propagated in Vero cells. In some experiments, cell cultures were starved for 18 h and then treated with endotoxin-free 1 nM ET-1 (Sigma–Aldrich) for 2 h before infection. Cultures were rinsed to remove free parasites and further incubated at 37 °C under 5% CO_2 for the indicated times.

2.3. Immunoblot analysis

Immunoblotting was carried out as described elsewhere [11]. HL-1 cells were stimulated for 2 h with ET-1 and/or infected with *T. cruzi* for 15 min (GATA4 analysis) or 3 h (iNOS expression). Phorbol 12-myristate 13-acetate (PMA, Sigma–Aldrich, 0.5 nM) was used as standard stimulus for GATA4 induction. Untreated samples were included as controls. Myocytes were disrupted and solubilized whole cell extracts (50 μg) were electrophoretically separated in 10% sodium dodecyl sulfate-polyacrylamide gels, and transferred to nitrocellulose filters. The membranes were probed 2 h at 37 °C with rabbit polyclonal antibodies (1 μg/ml) against dephosphorylated murine GATA4 (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated (Ser-105)-GATA4 (phospho-GATA4, Thermo Scientific Pierce, Waltham, MA), or iNOS (Santa Cruz Biotechnology), and mouse monoclonal antibody against α-tubulin (0.5 μg/ml, Sigma–Aldrich). The filters were washed and incubated with the corresponding secondary antibody linked to horseradish peroxidase (Thermo Scientific Pierce) and the stained bands were visualized by a chemiluminescent peroxide substrate (GE Healthcare, Pittsburgh, PA). Band intensity was analyzed using NIH Image J software [12].

2.4. Preparation of nuclear extracts

Nuclear extracts from stimulated (2 h)/infected (3 h) HL-1 cardiomyocytes were prepared as described previously [13]. Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA).

2.5. GATA4 DNA-binding activity

Nuclear extracts (15 μg) were applied to a GATA4 transcription factor assay kit (TransAM, Active Motif, Carlsbad, CA) according to the manufacturer's instructions [14]. Activated GATA4 present in nuclear extract binds specifically to an oligonucleotide containing the consensus binding site 5′-AGATAA-3′ immobilized on the microplate. In some experiments, a competitor oligonucleotide (wild-type or mutant) was added to confirm specific reactivity. The GATA4 antibody recognizes an epitope on the protein that is accessible upon DNA binding. Addition of a secondary peroxidase-conjugated antibody and the corresponding substrate provided a sensitive colorimetric readout quantified by spectrophotometry.

2.6. Small interfering RNA (siRNA)-mediated knockdown

HL-1 cardiomyocytes were transfected with 40 pmol of GATA4-specific Stealth siRNA (Invitrogen™, Life Technologies, Carlsbad, CA) or scramble siRNA (control) for 48 h using Lipofectamine 2000 (Invitrogen™) following the instructions of the supplier. This protocol has been proved to efficiently interfere with GATA4 gene expression [15].

2.7. Quantification of cardiomyocyte-derived soluble mediators

For BNP measurements, 24-h supernatants from ET-1-stimulated and/or *T. cruzi*-infected HL-1 cells were analyzed by ELISA (Kamiya Biomedical, Seattle, WA) according to the manufacturer's instructions. The assay sensitivity was 5.3 pg/ml. Further, nitrite accumulation in the culture supernatants was used as an indicator of NO production and was determined by the Griess reaction with sodium nitrite as a standard, as reported previously [16]. Supernatants (50 μl) were incubated, in the dark and at room temperature, with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4). The absorbance at 540 nm was read 10 min later.

2.8. Chronic Trypanosoma cruzi infection of mice

Eight-week-old female BALB/c mice were infected intraperitoneally with 25 Tulahuén strain trypomastigotes. At 100 days post-infection, hearts were removed and sectioned. Immunohistochemical analysis of formalin-fixed, paraffin-embedded cardiac muscle specimens from infected and uninfected mice was performed as described previously [17]. BNP immunostaining was accomplished using a rabbit polyclonal antibody specific to the mouse peptide (Bioss, Woburn, MA).

2.9. Statistical analysis

Statistical analysis was performed by using GraphPad Prism (San Diego, CA) 5.0 software. Arithmetic means and standard error of the means (s.e.m.) were calculated. Significant differences among groups were made by using the one-way analysis of variance test followed by Tukey's test. A difference between groups of $P < 0.05$ was considered significant.

3. Results

3.1. Endothelin-1 and Trypanosoma cruzi cooperatively activate GATA4 in cardiomyocytes

As shown in Fig. 1A, the combined effect of parasite infection and ET-1 treatment (Tc + ET-1) on atrial HL-1 myocytes induced early (15 min) GATA4 phosphorylation (Ser-105). In contrast, *T. cruzi*-infected cardiomyocytes as well as ET-1-stimulated uninfected cells displayed low protein expression of phospho-GATA4. Activation of the transcription factor in cardiac muscle cells was further confirmed after transfection of GATA4 siRNA. Maximum level of GATA4 interference was obtained in 40 mM siRNA-transfected myocytes, as determined by RT-PCR analysis (data not shown). Fig. 1B shows that GATA4 silencing resulted in a great decrease in (Tc + ET-1)-elicited phospho-GATA4 levels, thus providing strong evidence of myocardial GATA4 induction in response to stimulation with the vasoactive peptide and *T. cruzi* invasion. Augmented GATA4 protein expression promoted by (Tc + ET-1) was associated with enhanced DNA-binding activity. We found that (Tc + ET-1) induced three-fold and eight-fold increases in GATA4 DNA-binding activity compared to those generated independently by ET-1 and *T. cruzi* infection, respectively. Specificity of the assay was confirmed by competitive binding studies, in which oligonucleotides containing a wild-type (Wt competitor), but not a mutant (Mut competitor), GATA-binding site attenuated binding to the immobilized probe (Fig. 1C).

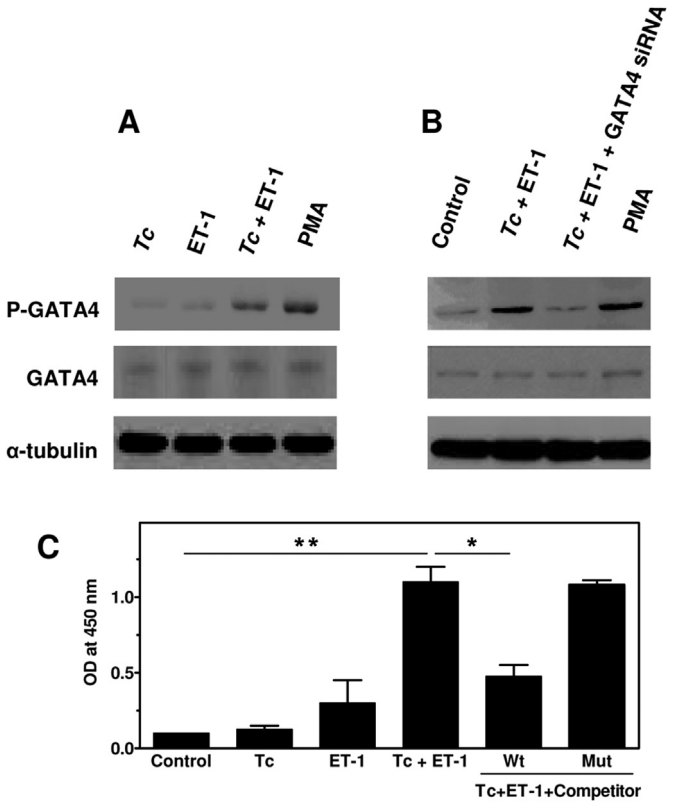

Fig. 1. *Trypanosoma cruzi* infection of endothelin-1-pre-treated HL-1 cardiomyocytes induces GATA4 activation. A–B. Effects of endothelin-1 (ET-1) pre-treatment and *T. cruzi* (Tc) infection of HL-1 cardiomyocytes on GATA4 protein expression and phosphorylation (Ser-105). The cells were stimulated with 1 nM ET-1 for 2 h, and/or infected with Tc trypomastigotes for 15 min, and the levels of GATA4 [dephosphorylated (GATA4) and phosphorylated (P-GATA4) forms] and α-tubulin (loading marker) proteins were analyzed by immunoblotting. Phorbol 12-myristate 13-acetate (PMA, 0.5 nM) was used as standard stimulus for GATA4 induction. Untreated/uninfected samples (Control) were also assayed. Transfection of small interfering RNA was used for GATA4 knockdown (GATA4 siRNA). Results show a representative experiment of three performed. C. ELISA for GATA4 DNA-binding activity. Activated GATA4 present in nuclear extracts (15 μg) from ET-1-stimulated and/or Tc-infected atrial HL-1 myocytes recognizes an oligonucleotide containing the consensus binding motif immobilized on the microplate. Specificity of the assay was confirmed by competition of GATA4 binding by addition of unbound wild-type (Wt) *versus* mutant (Mut) oligonucleotide. OD, optical density. Data are the means ± s.e.m. of three independent experiments, each performed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$.

3.2. Trypanosoma cruzi infection of endothelin-1-stimulated cardiomyocytes triggers GATA4-mediated overexpression of inducible nitric oxide synthase and B-type natriuretic peptide

Our findings showing (Tc + ET-1)-dependent GATA4 activation in HL-1 cardiac cells prompted us to examine the expression of some GATA4 downstream targets, such as iNOS and BNP, both implicated in the development of Chagas heart disease. Interestingly, stimulation with *T. cruzi* plus ET-1 induced a significant ($P < 0.01$) increase in iNOS protein expression detected by immunoblotting, even more pronounced than that achieved by the independent effect of peptide and infection (Fig. 2A). Further, Fig. 2B depicts myocardial iNOS expression in response to (Tc + ET-1) and the decrease upon GATA4 siRNA transfection suggesting, at least in part, GATA4-dependent control of the enzyme levels. Supporting these observations, we found that (Tc + ET-1) boosted NO production in cardiomyocytes, reaching nitrite concentrations significantly ($P < 0.01$) higher than those raised by *T. cruzi* and ET-1 separately. Also, NO release was reduced ($P < 0.05$) in GATA4 siRNA-transfected cells (Fig. 2C). In addition, parasite infection promoted a three-fold increased BNP production compared to that observed in uninfected controls. More important, BNP levels in the cell supernatants were significantly ($P < 0.05$) augmented by the cooperative action of (Tc + ET-1). GATA4 siRNA transfection reduced natriuretic peptide secretion from cardiomyocytes pointing out that GATA4 is likely to be involved in the regulation of (Tc + ET-1)-triggered BNP synthesis (Fig. 2D). Altogether, these results suggest that phospho-GATA4 is a cis-acting element of myocardial iNOS and BNP genes under (Tc + ET-1)-stimulation.

3.3. Mice with chronic chagasic cardiomyopathy show increased expression of myocardial GATA4, inducible nitric oxide synthase and B-type natriuretic peptide

To confirm the in vitro observations in an in vivo model, we next examined the hearts of BALB/c mice with long-term *T. cruzi* infection. At 100 days post-infection, histopathological studies revealed intense mononuclear cell inflammation in the cardiac tissue of chagasic mice (data not shown). Concomitantly, we verified strong immunostaining for GATA4, iNOS and BNP in the lesions of the myocardium from chronically infected animals. On the other hand, heart sections from uninfected mice displayed no enhanced expression of either molecule (Fig. 3).

4. Discussion

Advances in knowledge of *T. cruzi*-cardiomyocyte interactions have contributed to a better understanding of the biological events

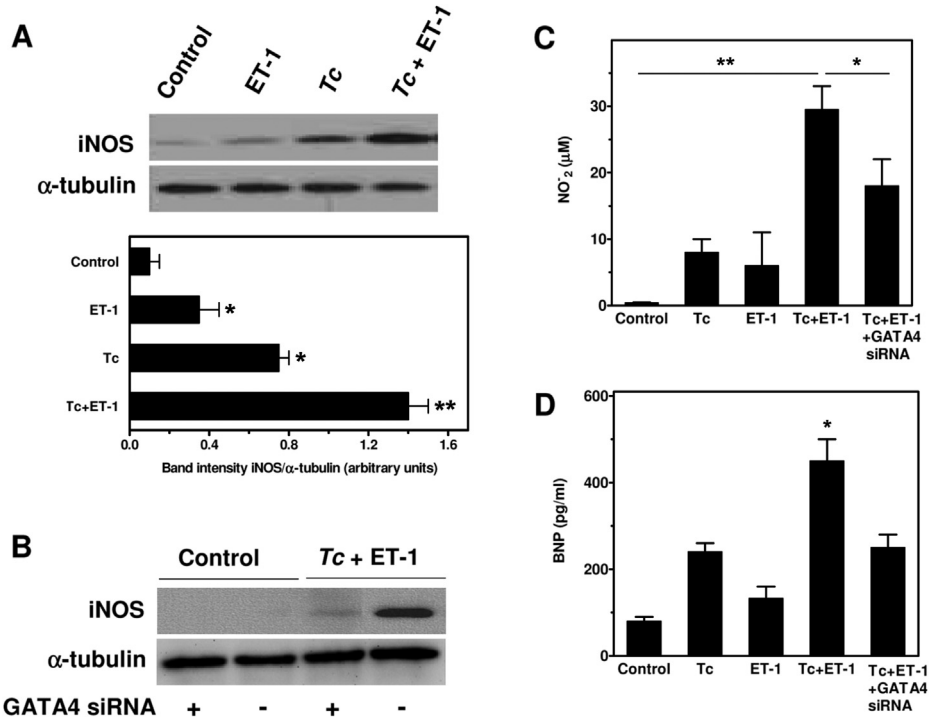

Fig. 2. *Trypanosoma cruzi* infection of endothelin-1-stimulated HL-1 cardiomyocytes promotes GATA4-mediated expression of inducible nitric oxide synthase (iNOS), nitric oxide (NO) production and secretion of B-type natriuretic peptide (BNP). A–B. Effects of ET-1 pre-treatment and Tc infection of atrial HL-1 myocytes on GATA4-dependent induction of iNOS. The cells were stimulated with 1 nM ET-1 for 2 h, and/or infected with Tc trypomastigotes for 3 h, and the levels of iNOS and α-tubulin proteins were analyzed by immunoblotting. Band intensity was analyzed using NIH Image J software. Untreated/uninfected samples (Control) were also assayed. Transfection of small interfering RNA was used for GATA4 silencing (GATA4 siRNA). Results show a representative experiment of three performed. $^{*}P < 0.05$ *versus* Tc + ET-1; $^{**}P < 0.01$ *versus* Control. C. Nitrite accumulation was measured in the culture medium by the Griess reaction. NO levels are expressed as mean ± s.e.m. of three independent experiments, each performed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$. D. BNP measurements. BNP concentration (mean ± s.e.m.) in 24-h culture supernatants was quantified by ELISA The results are representative of three independent experiments performed in quadruplicate. $^{*}P < 0.05$ *versus* all other groups.

involved in the pathophysiology of Chagas disease. So far, the pathogenesis of *T. cruzi*-driven myocarditis represents interplay of many parasite and host factors. A growing body of evidence suggests that the combined effects of infection, immune mediators and regulators of cardiovascular function on heart muscle cells influence the progressive evolution of chagasic cardiomyopathy [18–21]. In a previous study, we demonstrated that HL-1 atrial myocytes respond to ET-1 stimulus and *T. cruzi* infection by induction of Cox-2 expression via the Ca^{2+}/Cn/NFATc4 signaling pathway leading to enhanced release of prostaglandins E_2 and $F_{2\alpha}$, and thromboxane A_2 [6]. GATA4 is also a key marker and a downstream effector of Ca^{2+}/Cn cascade, and co-regulation of myocardial

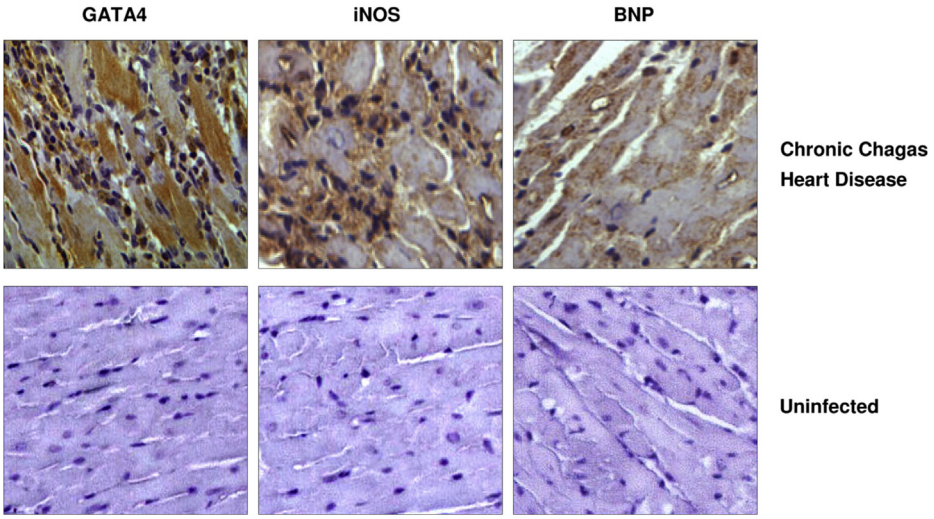

Fig. 3. Myocardial expression of GATA4, inducible nitric oxide synthase (iNOS) and B-type natriuretic peptide (BNP) in mice with chronic *Trypanosoma cruzi*-elicited cardiomyopathy. Representative results of immunohistochemical analysis of GATA4, iNOS and BNP expression in cardiac tissue specimens from BALB/c mice with chronic Chagas heart disease (100 days post-infection) and uninfected controls (top and bottom panels, respectively) are shown. Original magnification for all microphotographs, ×400.

gene expression by NFATc4 and GATA4 has been well documented [22–24]. Interestingly, our current findings suggest that the cooperation between the pathogen and ET-1 is also capable of triggering GATA4 activation that contributes to up-regulate iNOS and BNP protein levels in this target cell type.

GATA4 is abundantly expressed in cardiomyocytes from early embryonic stages to adulthood where it modulates cardiac-specific gene expression. This transcription factor has important roles in the processes of cardiac development and remodeling [25]. Besides these physiological functions, GATA4 activity is drastically altered by a panel of pathological stressors including pressure overload, hypoxia, sympathetic nerve discharge, angiotensin II, isoproterenol, ET-1 and PMA [26]. Our results indicate that *T. cruzi* and ET-1 cooperatively activate GATA4 in cardiomyocytes. We observed early phosphorylation of Ser-105 of GATA4 and enhanced DNA-binding activity induced by (Tc + ET-1). Activation of this transcription factor is known to be regulated by protein phosphorylation at Ser-105, mainly via extracellular signal-regulated and p38 mitogen-activated protein kinases [27]. Importantly, increased GATA4 expression and DNA-binding activity in myocardium have been associated with the progression to an impaired cardiac function [28].

A number of cardiac structural genes induced during hypertrophic response possess functional GATA-binding motifs in their promoter region, most notably α-myosin heavy chain, atrial natriuretic factor and BNP [8]. Recently, iNOS was uncovered as another GATA4 target gene in heart tissue [9]. We found that *T. cruzi* infection plus ET-1-stimulation boosted the GATA4-dependent expression of iNOS and the production of NO in HL-1 atrial cells. The parasite is capable of triggering NO production in cardiomyocytes via up-regulation of iNOS, further enhanced by co-activation with interleukin-1β or tumor necrosis factor-α [29,30]. Apart from its trypanocidal activity, the iNOS/NO pathway may participate in *T. cruzi*-induced myocardial cell lesion and heart injury in chronically infected hosts [31–33]. Our findings suggest that myocardial iNOS induction generated by the cooperative actions of the vasoactive agent and the infection is mediated, at least in part, by activated GATA4. Nevertheless, protein expression of this pro-inflammatory enzyme and nitrite levels were not totally abolished under GATA4 silencing, which may be indicative of other signaling pathway involvement, probably nuclear factor kappa B [34].

Several studies show that BNP is elevated in the blood and heart tissues of patients with chagasic cardiomyopathy and ventricular disorders [35,36]. Our results suggest that GATA4 activation by (Tc + ET-1) contributes to trigger the production of this neurohormone in cardiomyocytes. As for iNOS, BNP levels in HL-1 cell supernatants were not reduced to zero upon GATA4 si RNA-mediated knockdown, reflecting potential induction of peptide secretion by alternative underlying mechanisms such as p38 kinase [37]. We also found augmented GATA4, iNOS and BNP protein levels at the inflammatory sites in the heart from mice with chronic *T. cruzi*-elicited cardiomyopathy. However, the cardiopathogenic consequences of myocardial overexpression of these mediators in patients with Chagas disease have not been fully determined yet.

5. Conclusions

In summary, this study shows for the first time that the cooperation between ET-1-stimulation and *T. cruzi* infection of cardiac myocytes leads to the exacerbated production of pathogenic mediators associated with chagasic cardiomyopathy, such as iNOS/NO and BNP, through the activation of the GATA4 signaling pathway. These findings open new perspectives for understanding the complex host-parasite interplay and reveal potential novel targets for modulating the progression of Chagas heart disease.

Acknowledgments

RSC is a Member of Research Career from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina). This study was supported by a grant from CONICET (112-201201-00518CQ, RSC). The funding source had no role in study design, data collection and analysis, preparation of the manuscript or decision to publish. The authors would like to thank Dr. Manuel Fresno (CBMSO, Madrid, Spain) for valuable suggestions and discussions, and Ruth Zimmerman for helpful technical assistance with RNA silencing.

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