Acta Tropica 174 (2017) 153–157

Contents lists available at ScienceDirect

Acta Tropica

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

Trypanosoma cruzi modulates gene expression of plasma membrane repair-related proteins

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ARTICLE INFO

Article history:
Received 21 March 2016
Received in revised form 11 May 2016
Accepted 7 June 2016
Available online 8 June 2016

Keywords:
Trypanosoma cruzi
Membrane repair
Dysferlin
Acid sphingomyelinase
Transcriptional factor EB
Gene expression
Host interaction

ABSTRACT

Plasma membrane injury and repair is particularly prevalent in muscle cells. Here, we aimed to verify *dysferlin*, *acid sphingomyelinase* and *transcriptional factor EB* gene expression during *Trypanosoma cruzi* infection *in vitro* and *in vivo*. Our results showed that the parasite modulates gene expression of these proteins in a way dependent on the number of plasma membrane interacting parasites and in a rapamycin-sensitive manner.

1. Introduction

Trypanosoma cruzi invasion of non-muscular cell lines mimics a process of plasma membrane injury and repair that involves Ca^{2+}-dependent exocytosis of lysosomes, delivery of acid sphingomyelinase (ASM) to the outer leaflet of the plasma membrane, and a rapid form of endocytosis that internalizes membrane lesions (Fernandes et al., 2011). The transcription factor EB (TFEB) regulates lysosomal biogenesis, induces plasma membrane lysosome anchorage and fusion. Moreover, it promotes autophagosomes formation in a variety of cell types (Sardiello et al., 2009; Settembre and Ballabio, 2011; Settembre et al., 2011). The mechanisms by which TFEB regulates these biological processes depends on its interaction with mTOR, a lysosomal kinase (Zoncu et al., 2011; Cortez et al., 2016).

Dysferlin is protein specifically expressed in muscular cells which regulates lysosome and ASM exocytosis during plasma membrane repair (Defour et al., 2014). The role of dysferlin in membrane repair of muscle cells upon *T. cruzi* infection is completely unknown. In addition, the impact of *T. cruzi* infection over gene expression of these membrane repair related protein has been neglected. Thus, we aimed to verify *dysferlin*, *acid sphingomyelinase* and *transcriptional factor EB* gene expression during *T. cruzi* infection *in vitro* and *in vivo*.

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http://dx.doi.org/10.1016/j.actatropica.2016.06.008
0001-706X/

2. Material and methods

2.1. Cell lines and parasites

C2C12 (murine myoblast) and Vero (monkey fibroblast) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2,5% fetal bovine serum (FBS), 10 mg/mL streptomycin (Sigma), 100 U/mL penicillin (Sigma), and 40 mg/mL gentamycin (Sigma) at 37 °C and 5% CO_2. Tissue culture-derived trypomastigotes (TCT) from *T. cruzi* Y strain were obtained in the supernatant of previously infected VERO cells.

2.2. Animals and ethics

C57BL/6 mice, six to eight weeks old were maintained at the Centro de Bioterismo de Experimentação Animal (CEBEA) at the Universidade Federal de Uberlândia (UFU, Uberlândia, Brazil) under standard conditions on a 12-h light-dark cycle in a temperature-controlled room (25 ± 2 °C) with water and food *ad libitum.* All experimental protocols were approved by the Ethics Committee from Universidade Federal de Uberlândia. Maintenance and care of animals complied with the guidelines of the Laboratory Animal Ethics Committee from the Universidade Federal de Uberlândia. Animal euthanasia was performed based on international welfare grounds according to the American Veterinary Medical Association Guidelines on Euthanasia.

2.3. Animal infection and immunosuppression

C57BL/6 mice were inoculated with 2×10^5 of TCT from Y strain into the peritoneum cavity. Non-infected animals were used as a control. After 1 month and 3 months, the animals were euthanized and their heart collected and processed for qPCR. Chronically infected mice were immunosuppressed with dexamethasone phosphate administered *ad libitum* in drinking water at a dosage level of 12 μg/mL during 10 days (Kim and Healey, 2001). Parasitaemia was followed from the 1th day up to 10th day post-infection (acute phase) and post-immunosuppression (acute relapse). Bloodstream forms of *T. cruzi* were assessed under standardized conditions by direct microscopic observation.

2.4. Invasion assay

C2C12 cell line was seeded in 6-well plates (1×10^6 cells per well) and 24-well plates (1×10^5 cells per well) contained a 13 mm round coverslips. After 18 h of incubation, TCT from Y strain were added to the cell culture in a ratio of 50 parasites per cell. After different time points, cells were gently washed with PBS fixed with Bouin solution, and stained with Giemsa. The percentage of cells containing parasites was determined by counting the number of internalized parasites in 100 cells per coverslip. The experiment was performed in triplicate and three times. Experiments performed for qPCR were performed as described. After PBS washing, cells were removed by adding Ribozol (AMRESCO), and kept under −80 °C until use. To assess the activity of mTOR during the invasion, cells were treated with 10 nM Rapamycin (Cell Signaling Technology®) by two hours, before parasite cell invasion.

2.5. DNA extraction and quantitative PCR

To extract DNA of myoblast and heart tissues, we used PureLink® Genomic DNA Kit (Invitrogen, Carlsbad, CA, USA), according to the recommendations of manufacturer. The concentration and quality of DNA was determined at 260/280 nm and were amplified using the primer pair P21fw (5′- AACGCCACCATCAATCTTTTG −3′) and P21rv (5′-CGTCGCATTCCTCATTTCTTC-3′), and probe P21p (5′-ACGCCATCGTCATGTGCGCAG-3′), which result in the amplification of a 65 bp fragment of *T. cruzi* genomic DNA (XM_812182.1). qPCR reactions were performed with a final volume of 12.5 μL containing 2 μL (~50 ng) of the DNA extracted from C2C12 cells and heart samples. The reactions were processed in ABI7300 equipment (Applied Biosystems) under the following conditions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 45 s and 72 °C for 30 s. After the final elongation of qPCR, samples were submitted to variation of temperature from 50 to 95 °C, with a gradual increase of 0.5 °C/s to obtain the melting temperature (Tm) and non-specific products.

2.6. RNA extraction, cDNA synthesis, and RT-qPCR

RiboZol™ Plus RNA Purification Kit (Amresco) was used to RNA extraction according to the recommendations of manufacturer. The concentration and quality of RNA was determined at 260/280 nm. The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was applied for the reverse transcription reaction according to the manufacturer's recommendations. Only samples with an OD260/280 ratio from 2.0 +/− 0.1 were analyzed. The RT-PCR was performed with 2 μg RNA in a final volume of 20 μL and under the following conditions: temperature of 25 °C, kept for 10 min, followed by 37 °C for 120 min. Afterwards the samples were heated up to 85 °C for 5 min and finally cooled to 4 °C. cDNA was stored at −20 °C. The gene quantification was determined in ABI 7300 equipment (Applied Biosystems). Standard cycling conditions were used as recommended by the manufacturer: 95 °C for 10 min, (95 °C for 15 s, 60 °C for 1 min) × 40 cycles, and the melt curve analysis at 95 °C for 15 s then 60 °C for 1 min. Each PCR reaction was conducted in triplicate. In addition, melting curve analysis was performed in each assay in order to detect non-specific amplification. The relative levels of gene expression was analyzed with $2^{-\Delta\Delta Ct}$ method, where ΔΔCt = ΔCt infected group (Ct gene target − Ct gene endogenous) − ΔCt not infected group (Ct gene target − Ct gene endogenous). The GAPDH was used as reference gene (endogenous). The extreme values were not included in statistical analysis. Table 1 indicates the pair of primers used.

2.7. Statistical analysis

The relative gene expressions were compared using Kruskal Wallis test, followed by Dunnett's multiple comparisons test, and was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA. The level of significance was set at $P < 0.05$.

3. Results and discussion

Firstly, we aimed to address the parasite load in *in vitro* infected myoblasts at different time points. Our results showed higher number of intracellular parasites at later time points of the kinetic (Fig. 1A–J). These observations correlated to higher gene expression of ASM, TFEB and dysferlin at the same time points (Fig. 1 K–M). Thus, we suggest that *T. cruzi*-host cell interaction leads to a cascade of events that culminate in up-regulation of ASM, TFEB and dysferlin gene expression *in vitro*. The correlation between the number of plasma membrane interacting parasites and up-regulation of ASM, TFEB and dysferlin gene expression is in accordance to the paradigm that postulated that TCT utilized the plasma membrane repair machinery for cell invasion (Tardieux et al., 1992).

In order to gain insights into gene expression levels of these proteins *in vivo*, we infected mice, determined heart parasitism at 30 (acute infection), 90 days (chronic infection) and 10 days after immunosuppression of chronically infected animals. The gene expression of ASM, TFEB and dysferlin was not altered during acute