

Markers of oxidative stress in adipose tissue during *Trypanosoma cruzi* infection

Jian-Jun Wen · Fnu Nagajyothi · Fabiana S. Machado ·
Louis M. Weiss · Philipp E. Scherer ·
Herbert B. Tanowitz · Nisha Jain Garg

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Abstract The protozoan parasite *Trypanosoma cruzi* causes Chagas disease. Cardiac and adipose tissues are among the early targets of infection and are sites of persistent infection. In the heart and adipose tissue, *T. cruzi* infection results in an upregulation of pro-inflammatory mediators. In the heart, infection is associated with an increase in the markers of oxidative stress. To date, markers of oxidative stress have not been evaluated in adipose tissue in this infection. Brown and white adipose tissues were obtained from CD-1 mice infected with the Brazil strain of *T. cruzi* for 15, 30, and 130 days post infection. Protein carbonylation and lipid peroxidation assays were performed on these samples. There was an upregulation of these markers of oxidative stress at all time-

points in both white and brown adipose tissue. Determinants of anti-oxidative stress were downregulated at similar time-points. This increase in oxidative stress during *T. cruzi* infection most likely has a deleterious effect on host metabolism and on the heart.

Keywords *Trypanosoma cruzi* · Chagas disease · Adipose tissue · Oxidative stress · Protein carbonylation · Lipid peroxidation

Introduction

For over a century research in Chagas disease, caused by *Trypanosoma cruzi*, the heart and the gastrointestinal tract have understandably received the greatest scrutiny. However, *T. cruzi* also has significant effects on other organs such as adipose tissue and the pancreas (Combs et al. 2005; Nagajyothi et al. 2008, 2010, 2012a, b, 2013). Adipose tissue is both an early target as well as a reservoir of latent *T. cruzi* infection (Combs et al. 2005; Nagajyothi et al. 2012a, b). Our laboratory group has demonstrated that *T. cruzi* infection of adipose tissue leads to an intense inflammatory reaction that extends into the chronic phase of infection (Combs et al. 2005). This, in some aspects, is similar to the obese state where adipose tissue displays a chronic inflammatory phenotype (Ferrante 2007) which contributes, in part, to heart disease (Turer et al. 2012) and other host metabolic disorders, such as insulin resistance.

Adipose tissue is the largest endocrine organ in the body accounting for 10 to 50 % of body composition, depending on the host, and contributes to energy homeostasis and fulfills critical roles in host immune responses (Halberg et al. 2008). Among the first experimental observations examining the relationship of infection and adipose tissue was the demonstration that injection of LPS into mice that were rendered

Jian-Jun Wen and Fnu Nagajyothi are the co-first-authors.

J.-J. Wen · H. B. Tanowitz · N. J. Garg (✉)
Department of Microbiology and Immunology,
University of Texas Medical Branch, Galveston, TX, USA
e-mail: nigarg@utmb.edu

F. Nagajyothi · L. M. Weiss · H. B. Tanowitz (✉)
Department of Pathology, Albert Einstein College of Medicine,
1300 Morris Park Avenue, Bronx, NY 10461, USA
e-mail: herbert.tanowitz@einstein.yu.edu

P. E. Scherer
Departments of Internal Medicine and Cell Biology and the
Touchstone Diabetes Center, University of Texas Southwestern,
Dallas, TX, USA

L. M. Weiss · H. B. Tanowitz
Department of Medicine, Albert Einstein College of Medicine,
Bronx, NY, USA

F. S. Machado
Program in Health Sciences: Infectious Diseases and Tropical
Medicine/Interdisciplinary Laboratory of Medical Investigation,
Faculty of Medicine and Department of Biochemistry and
Immunology, Institute of Biological Science, Federal University of
Minas Gerais, Belo Horizonte, MG, Brazil

fatless did not result in immediate death, as is observed in control mice with a normal component of adipose tissue (Pajvani et al. 2005). The major component of adipose tissue is the adipocyte which exerts its influence through the synthesis and release of cytokine-like proteins known as adipokines. It is now appreciated that the intense pro-inflammatory potential of adipose tissue indicates that it plays an important role both in the innate and adaptive immune responses during infection and that its absence reduces inflammatory markers (Kaminski and Randall 2010; Sell et al. 2012; Bondia-Pons et al. 2012).

Adipose tissue obtained from *T. cruzi*-infected mice and *T. cruzi*-infected cultured adipocytes displays an upregulation in traditional markers of inflammation such as cytokines, chemokines, Toll-like receptors, and components of the mitogen-activated protein kinase (MAPK) pathway (Combs et al. 2005; Nagajyothi et al. 2008, 2010, 2012a, b).

In *T. cruzi*-infected adipose tissue samples and cultured adipocytes, there is a reduction in the adipokine, adiponectin, and in peroxisome proliferator-activated receptor (PPAR- γ) (Combs et al. 2005; Nagajyothi et al. 2008). *T. cruzi*-infected mice display a reduction in serum adiponectin levels (Combs et al. 2005). Both adiponectin and PPAR- γ are inversely associated with upregulation of inflammation; a situation reminiscent of the obese state (Ferrante 2007; Shetty et al. 2009; Hotamisligil 2006; Weisberg et al. 2003; Bondia-Pons et al. 2012).

The accumulating data strongly suggests that there are some similarities between adipose tissue in the obese state and in adipose tissue in Chagas disease. For example, in both disease states, there is an influx of macrophages into adipose tissue and an upregulation of pro-inflammatory pathways. In the obese state, markers of oxidative stress are increased (Murdolo et al. 2013; Crujeiras et al. 2013; Furukawa et al. 2004; Bondia-Pons et al. 2012). Oxidative stress is also increased in the myocardium of mice infected with *T. cruzi* (Wen et al. 2008, 2010). To date, however, no study has been performed in adipose tissue obtained from *T. cruzi*-infected mice or patients. Therefore, we examined the level of oxidative stress in white (WAT) and brown (BAT) adipose tissue of mice infected with *T. cruzi*. Markers of oxidative stress were increased during both the acute and chronic stages of infection, and these observations are consistent with and likely involved in the host metabolic changes observed in infected mice and similar changes seen in humans (Bondia-Pons et al. 2012).

Materials and methods

Parasitology In all experiments, 8 to 10-week-old male CD-1 mice (Charles River, Wilmington, MA) were used. They were injected IP with 5×10^4 trypomastigotes of the Brazil strain of *T. cruzi*. After the initial injection, there is a gradual increase in

parasitemia which peaked at 30 to 40 days post infection (dpi). During this time, the mortality rate is approximately 50 to 60 %. The parasitemia then wanes, and by 90 to 100 dpi, there is morphological and functional evidence of cardiomyopathy (Jelicks and Tanowitz 2011). Both WAT and BAT were removed 15, 30, 130, and 160 dpi. Epididymal WAT and interscapular BAT were collected and stored at -80°C , until used. These animal protocols were approved by the Institutional Animal Care and Use Committees of the Albert Einstein College of Medicine and of the University of Texas Medical Branch.

Protein carbonylation Protein carbonyls were measured as described (Levine et al. 2000) and modified by Wen et al. (2006a, 2008). Briefly, 20 μg proteins was denatured and derivatized in 3 % sodium dodecyl sulfate (SDS), 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 10 % trifluoroacetic acid (20- μl volume). After neutralization with 7.5 μl of 2 M Tris, 30 % glycerol, DNP-derivatized protein samples were resolved by 10 % of SDS-PAGE. Gels were transferred to PVDF membranes. Carbonylized proteins were probed with rabbit anti-DNP antibody (1:2,000, Sigma-Aldrich, St Louis, MO), followed by HRP-conjugated goat anti-rabbit IgG (1:5,000, Sigma-Aldrich), and signal was developed by using an enhanced chemiluminescence detection system (GE Healthcare, Pittsburgh, PA). Images were visualized, digitized, and quantified by densitometry using a FluorChem 8800 (Alpha Innotech, San Jose, CA) image analyzing system.

Lipid peroxidation We evaluated the extent of lipid peroxidation products, i.e., malonyldialdehyde (MDA) contents, by a thiobarbituric acid reactive substances (TBARS) assay as described (Ohkawa et al. 1979) and modified by Wen et al. (2008). Tissue lysates (1:10 w/v) were mixed with 0.2 ml of 8.1 % SDS, 1.5 ml of 20 % acetic acid, pH 3.5, and 1.5 ml of 0.8 % thiobarbituric acid (TBA). The mixture was diluted to 4 ml with distilled H_2O and heated at 95°C for 60 min. After cooling on ice, samples were extracted with 5 ml n-butanol:pyridine (15:1, v/v), and the concentration of lipid peroxides was calculated as an MDA equivalent using the extinction coefficient for the MDA-TBA complex of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ at 532 nm.

Quantitative real-time PCR RNA was isolated from adipose tissue using TRIzol (Invitrogen). Total RNA was purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). First-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA in a final volume of 50 μl using SuperScript III transcriptase (Invitrogen). A real-time PCR was performed on an iQ5 thermal cycler (Bio-Rad) with SYBR Green Supermix (Qiagen) using Mouse Oxidative Stress PCR Array (PAM 065Z, SA Biosciences) and cDNA template. The

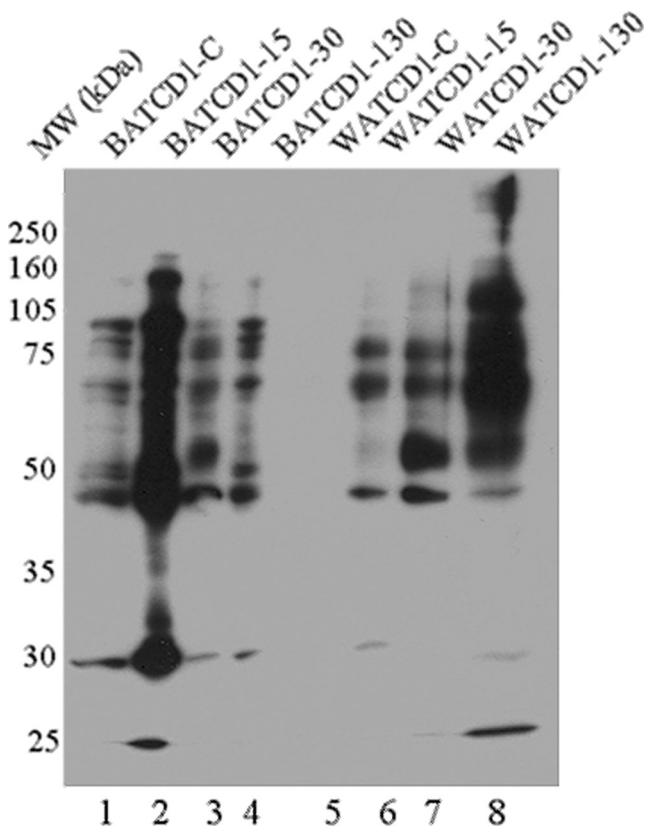


Fig. 1 Protein carbonylation in adipose tissue of chagasic mice. CD1 mice were sacrificed at 15, 30, and 130 dpi corresponding to early, acute, and chronic phases, respectively, of *T. cruzi* infection and disease development. Carbonylated proteins in the adipose tissue homogenates were derivatized with DNP, and the DNP-reactive proteins were identified by Western blot analysis using DNP-specific antibody. A representative Western blot is shown. Lane 1: BAT CD1-control; Lane 2: BAT CD1-D15; Lane 3: BAT CD1-D30; Lane 4: BAT CD1-D130; Lane 5: WAT CD1-control; Lane 6: WAT CD1-D15; Lane 7: WAT CD1-D30; Lane 8: WAT CD1-D130 (BAT brown adipose tissue, WAT white adipose tissue)

threshold cycle (Ct) values for each target messenger RNA (mRNA) were normalized to 18S rRNA mRNA, and the relative expression level of each target gene was calculated

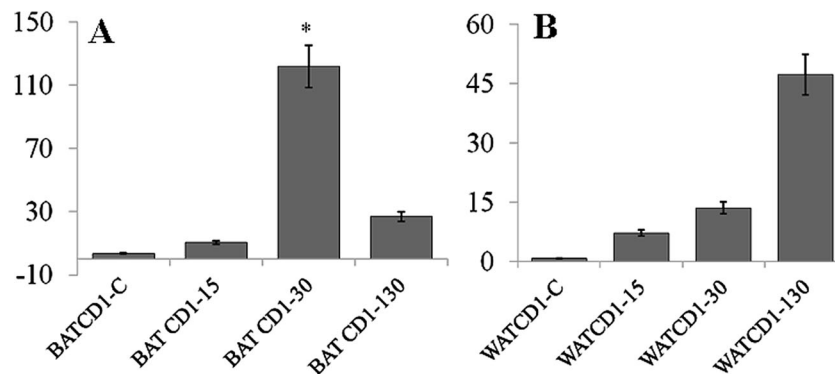


Fig. 2 Bar graphs showing the levels of malonyldialdehydes (MDA) in adipose tissue of chagasic mice. Mice were harvested as in Fig. 1. MDA contents, indicative of lipid peroxidation products in BAT (a) and WAT (b)

with the formula $n\text{-fold change} = 2^{-\Delta\text{Ct}}$, where ΔCt represents $\text{Ct}(\text{infected}) - \text{Ct}(\text{control})$.

Results

The Western blot shown in Fig. 1 demonstrates that the level of protein carbonylation, a biomarker of oxidative stress, was increased in BAT and WAT of mice harvested at the early, 15 days post infection (dpi); acute (30 dpi); and chronic (130 dpi) stages of *T. cruzi* infection and disease development. Similarly, there was an upregulation in lipid peroxidation in both BAT and WAT of chagasic mice at all time-points post infection (Fig. 2). Together, these data suggest that oxidative stress is increased in the BAT and WAT of mice during the course of infection and chronic disease development.

We evaluated the mRNA levels of the genes involved in the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) to determine the source of oxidative stress (Fig. 3). Testing of a ROS-generating enzyme NADPH oxidase (NOX) revealed different expression levels of NOX1 and NOX4 between BAT and WAT during infection. We did not observe significant differences in the expression of NOX1 in BAT during acute and chronic infection (data not shown); however, BAT displayed an increased expression of neutrophil cytosolic factor (a subunit of neutrophil NADPH oxidase) and NOX4 during both acute and chronic infection. In WAT tissue, we observed a higher expression of NOX1 (5-fold) during acute infection which significantly decreased (−2.8-fold) during chronic infection. WAT revealed no significant changes in NOX4 expression. These data suggested that ROS production, most likely due to activation of NADPH oxidase, is enhanced in BAT and WAT of *T. cruzi*-infected mice.

Previously, we demonstrated that WAT undergoes significant lipolysis during acute infection compared to BAT (Nagajyothi et al. 2012a, b). It has been shown that NOS2

tissue of infected mice at 15, 30, and 130 dpi was determined by a TBARS assay as described in “Materials and methods”. Data are presented as n ml/mg protein and represents mean \pm SD (* $p < 0.05$; ** $p < 0.01$)

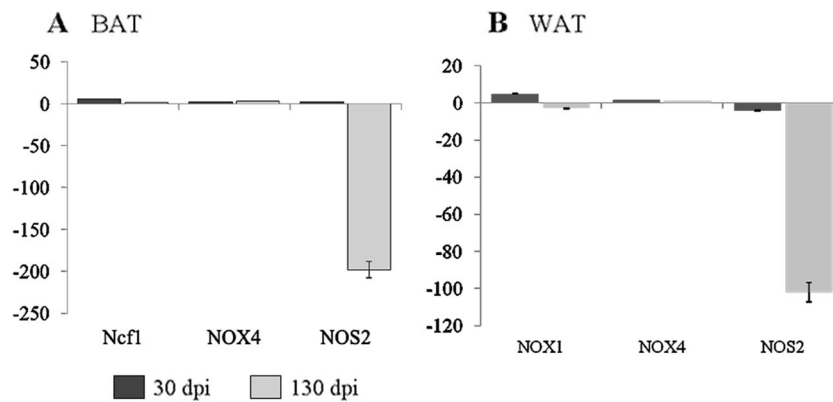


Fig. 3 The mRNA level for subunits of NADPH oxidase complex is enhanced in adipose tissue of mice infected by *T. cruzi*. Adipose tissue (**a** BAT and **b** WAT) samples from normal and infected mice were harvested at acute and chronic stages of infection and disease development, and total RNA isolated as discussed in “Materials and methods”. A

quantitative real-time PCR was performed to evaluate the mRNA levels of selected genes. Transcript levels were normalized against 18S rRNA, and derived from at least four mice per group. Data are presented as fold change ($*p < 0.05$, normal vs. infected; SD < 12 %)

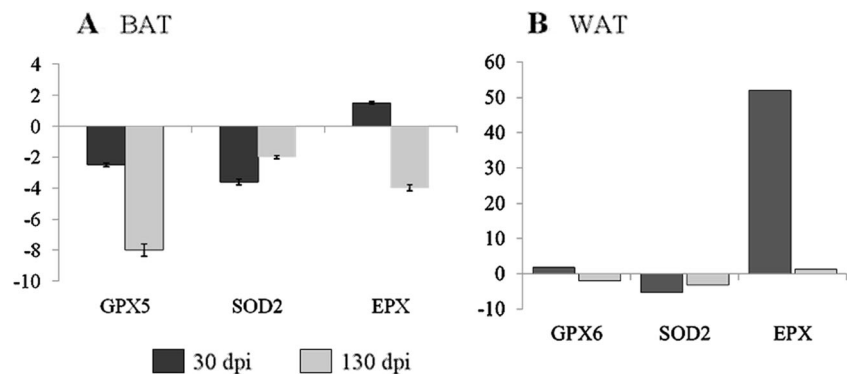
modulates lipolysis in adipocytes (Penforis and Marette 2005). NOS2 mediates the synthesis of nitric oxide, a reactive free radical which acts as a biologic mediator in several processes, including neurotransmission and antimicrobial and anti-tumoral activities. NOS2 expression was downregulated in both BAT and WAT of chronically infected mice which could contribute to increased lipolysis during infection (Fig. 3).

Finally, we examined if the antioxidant capacity of the BAT and WAT in chagasic mice is altered (Fig. 4). The expression levels of glutathione peroxidase (GPX) and superoxide dismutase (SOD) isoforms was significantly reduced in BAT (Fig. 4a) and WAT (Fig. 4b) of acutely and chronically infected mice. The mRNA levels of eosinophil peroxidase (EPX) antioxidant in BAT and WAT was increased by 2-fold and >50-fold, respectively, during the acute phase of infection. With the progression to chronic phase, the expression level of EPX in BAT and WAT was decreased by 4-fold or became non-responsive, respectively. These data suggest that despite an increase in oxidative stress, the antioxidant response is compromised in BAT and WAT of chagasic mice.

Discussion

Many human diseases have been linked to the generation of oxidative stress such as neurodegenerative diseases, obesity, and heart disease (Bondia-Pons et al. 2012; Enns 2003; Luczak and Anderson 2014). In recent years, oxidative stress has been linked to *T. cruzi*-associated heart disease (reviewed in Machado et al. 2012; Nagajyothi et al. 2012a, b). The potential role of adipose tissue in the pathogenesis of Chagas disease including cardiomyopathy has only recently received attention (Nagajyothi et al. 2009). Herein, we demonstrate that both BAT and WAT obtained from *T. cruzi*-infected mice during acute and chronic infection displayed significant up-regulation of markers of oxidative stress including protein carbonylation and lipid peroxidation. The quantitative PCR (qPCR) analysis demonstrated that increased oxidative stress was associated with an increase in the expression of genes that encode components of NADPH oxidase complex, indicating that the production of ROS might be enhanced in BAT and WAT during infection. In addition, some antioxidant markers were reduced in BAT and WAT, consistent with the increase in oxidative stress in acutely and chronically infected mice.

Fig. 4 The expression level of genes encoding antioxidants is compromised in adipose tissue of mice infected by *T. cruzi* (**a** BAT and **b** WAT). A quantitative real-time PCR was performed as in Fig. 3 to evaluate the mRNA levels for antioxidants. The data are presented as fold change in expression ($*p < 0.05$, normal vs. infected; SD < 12 %)



In general, oxidative stress is defined as a steady-state condition where ROS (free radicals) flux is not balanced by antioxidant defense. It is now accepted that inflammation of adipose tissue and the upregulation of oxidative stress play an important role in the pathogenesis of obesity, atherosclerosis, and diabetes and insulin resistance. Adipose tissue is also considered a major source of free radicals and of ROS. This has led to the notion that the increase in oxidative stress may result in the obesity-associated complications. However, it is noteworthy that the mechanistic basis for the linkage of oxidative stress-associated tissue damage to adipose tissue dysfunction is not entirely known.

ROS are critical signaling intermediates linking the innate and adaptive immune systems by triggering the production of pro-inflammatory cytokines (TNF- α , IL-1 β) by macrophages and dendritic cells (DCs) of the innate immune system. Inhibition studies with cultured and primary macrophages revealed that NOX/ROS was a critical regulator of cytokine production in response to *T. cruzi* infection (Dhiman and Garg 2011). In vivo studies using splenocytes of *T. cruzi*-infected mice, with or without in vitro stimulation with parasite antigens, validated the above observations and demonstrated that the inhibition of NOX by apocynin or use of ROS scavengers substantially blocked the activation and proliferation of phagocytes and inflammatory mediators such as IL-1, IL-6, IFN- γ , and TNF- α (Dhiman and Garg 2011). Subsequently, inhibition of NOX/ROS resulted in an increased susceptibility to *T. cruzi*, a finding suggesting that redox status plays an important role in immune activation and control of *T. cruzi* (Garg, unpublished).

A chronic upregulation of pro-oxidants affects the cardiac function in the setting of Chagas disease. Interestingly, studies in experimental models and infected humans demonstrate that an infected host sustains oxidative stress due to *T. cruzi*-elicited splenic NOX/ROS and the enhanced mitochondrial release of ROS in the myocardium (Gupta et al. 2011). Our studies demonstrated that the host responds to acute *T. cruzi* infection by upregulating its glutathione antioxidant defense constituted by GPX, GSR, and GSH. However, in the chronic phase, the pro-oxidant milieu of the heart was evidenced by increased ROS levels, decreased activity of MnSOD, insensitivity of glutathione defense to oxidative stress and increased GSSG, and lipid (MDA) and protein (carbonyl) oxidation products (Wen et al. 2004). A similar pro-oxidant status in seropositive humans has been reported and demonstrated by increased GSSG and MDA contents; decreased MnSOD, GPX activity, and GSH contents (Perez-Fuentes et al. 2003; de Oliveira et al. 2007); and inhibition of CIII activity (Wen et al. 2006b). Importantly, the treatment of *T. cruzi*-infected animals with an antioxidant tipped the balance in favor of preserving mitochondrial and cardiac function. *T. cruzi*-infected mice and rats, treated with an antioxidant, exhibited a significant decline in the myocardial accumulation of

oxidative adducts concurrent with improved mitochondrial function as evidenced by increased ATP synthesis and decreased ROS production (Wen et al. 2006b). Thus, preventing oxidative injuries during chronic infection preserved the cardiac hemodynamic state that otherwise was compromised in chagasic rats Wen et al (2010), and others have demonstrated a decline in oxidative stress in chagasic patients given vitamin A (Macao et al. 2007). Collectively, these observations support the idea that antioxidant depletion and inefficient scavenging of ROS, resulting in sustained oxidative stress, are important in the pathogenesis of Chagas disease and in the progression to chagasic cardiomyopathy.

Oxidative stress in adipose tissue has been examined in the setting of obesity and as noted, in the chagasic heart. It has not been investigated in adipose tissue in *T. cruzi* infection either in animals or humans. Herein, we demonstrate that both in BAT and in WAT obtained from infected mice, markers of oxidative stress were significantly increased as determined by protein carbonylation and lipid peroxidation studies. Previously, we reported that at 15 dpi, when mice are clinically well and there is often no visible parasitemia, pro-inflammatory markers are already elevated and associated with presence of parasites in adipose tissue (Nagajyothi et al. 2012a, b). Similarly, during the chronic stage of the disease in infected CD-1 mice, pro-inflammatory markers as well as markers of oxidative stress are upregulated. Therefore, these findings are associated with evidence of parasite persistence as determined by qPCR (Combs et al. 2005). We believe that the persistence of the parasite in adipose tissue even into the chronic stage is responsible, in part, for the persistence of inflammation and oxidative stress, similar to what has been reported in the obese state.

The relationship between adipose tissue and host metabolism and heart disease has been well-described (Turer et al. 2012). Well-functioning adipose tissue is important to the host. For example, adiponectin, synthesized by healthy adipose tissue, has anti-inflammatory properties. Low levels of adiponectin in adipose tissue and in the blood is associated with an increased pro-inflammatory phenotype as observed in the obese state and in the serum and adipose tissue of *T. cruzi*-infected mice. Low levels of adiponectin are associated with heart disease and insulin resistance (Turer and Scherer 2010). It is noteworthy that impairment of insulin release has been reported in individuals with chronic Chagas disease (dos Santos et al. 1999; Oliveira et al. 1993; Guariento et al. 1993; Long et al. 1980). Although inflammation and oxidative stress are observed in chagasic heart disease, it is likely that the increased levels of inflammation and oxidative stress in adipose tissue contribute to the cardiac dysfunction observed in Chagas disease.

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