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Cardiac-specific overexpression of thioredoxin 1 attenuates mitochondrial and myocardial dysfunction in septic mice

Juana P. Sánchez-Villamil[a], Verónica D'Annunzio[b], Paola Finocchietto[a,c], Silvia Holod[d], Inés Rebagliati[a], Hernán Pérez[a], Jorge G. Peralta[c], Ricardo J. Gelpi[b], Juan J. Poderoso[a,c], María C. Carreras[a,d,*]

[a] *INIGEM-CONICET-UBA, Laboratory of Oxygen Metabolism, Buenos Aires, Argentina*
[b] *IBIMOL-CONICET-UBA and Institute of Cardiovascular Physiopathology, Departamento de Patología, Facultad de Medicina, Universidad de Buenos Aires, Argentina*
[c] *Universidad de Buenos Aires, Facultad de Medicina, Departamento de Medicina, Buenos Aires, Argentina*
[d] *Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Departamento de Bioquímica Clínica, INFIBIOC, Buenos Aires, Argentina*

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ABSTRACT

Sepsis-induced myocardial dysfunction is associated with increased oxidative stress and mitochondrial dysfunction. Current evidence suggests a protective role of thioredoxin-1 (Trx1) in the pathogenesis of cardiovascular diseases. However, it is unknown yet a putative role of Trx1 in sepsis-induced myocardial dysfunction, in which oxidative stress is an underlying cause. Transgenic male mice with Trx1 cardiac-specific overexpression (Trx1-Tg) and its wild-type control (wt) were subjected to cecal ligation and puncture or sham surgery. After 6, 18, and 24 h, cardiac contractility, antioxidant enzymes, protein oxidation, and mitochondrial function were evaluated. Trx1 overexpression improved the average life expectancy (Trx1-Tg: 36, wt: 28 h; $p = 0.0204$). Sepsis induced a decrease in left ventricular developed pressure in both groups, while the contractile reserve, estimated as the response to β-adrenergic stimulus, was higher in Trx1-Tg in relation to wt, after 6 h of the procedure. Trx1 overexpression attenuated complex I inhibition, protein carbonylation, and loss of membrane potential, and preserved Mn superoxide dismutase activity at 24 h. Ultrastructural alterations in mitochondrial cristae were accompanied by reduced optic atrophy 1 (OPA1) fusion protein, and activation of dynamin-related protein 1 (Drp1) (fission protein) in wt mice at 24 h, suggesting mitochondrial fusion/fission imbalance. PGC-1α gene expression showed a 2.5-fold increase in Trx1-Tg at 24 h, suggesting mitochondrial biogenesis induction. Autophagy, demonstrated by electron microscopy and increased LC3-II/LC3-I ratio, was observed earlier in Trx1-Tg. In conclusion, Trx1 overexpression extends antioxidant protection, attenuates mitochondrial damage, and activates mitochondrial turnover (mitophagy and biogenesis), preserves contractile reserve and prolongs survival during sepsis.

Abbreviations: ANOVA, analysis of variance; ATP, adenosine triphosphate; CK-MB, creatine kinase MB; Drp1, dynamin-related protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LC3, microtubule associated protein 1A/1 B light chain 3; Mfn1, 2 mitofusin 1 2; MOMP, mitochondrial outer membrane permeability; NADPH, nicotinamide adenine nucleotide phosphate; NF-κB, nuclear factor-κB; Nrf, nuclear respiratory factor; OPA 1, optic atrophy protein 1; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PGC-1, peroxisome proliferator-activated receptor-α-coactivator-1; ROS, reactive oxygen species; SOD, superoxide dismutase; TFAM, mitocondrial transcription factor A; Trx, thioredoxin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; UCP, uncoupling protein; VDAC, voltaje-dependent anion channel.

* Corresponding author at: INIGEM-CONICET-UBA, Laboratory of Oxygen Metabolism, Buenos Aires, Argentina.
E-mail addresses: juanipsan3@gmail.com (J.P. Sánchez-Villamil), vdannunzio@gmail.com (V. D'Annunzio), pfinocchietto@hotmail.com (P. Finocchietto), silviaholod@yahoo.com.ar (S. Holod), inerreba@yahoo.com (I. Rebagliati), hernanperez82@gmail.com (H. Pérez), jorghighpear@yahoo.com.ar (J.G. Peralta), rgelpi@fmed.uba.ar (R.J. Gelpi), jpoderos@fmed.uba.ar (J.J. Poderoso), carreras@ffyb.uba.ar (M.C. Carreras).

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1. Introduction

Mortality related to sepsis mainly results from the dysfunction and failure of vital organs, including the heart. Current evidence indicates that there are many mechanisms involved in myocardial dysfunction, including inflammatory response, alterations in intracellular calcium homeostasis and adrenergic signaling, mitochondrial dysfunction, and apoptosis (Flierl et al., 2008; Flynn et al., 2007; Rudiger and Singer, 1992). Oxidative stress seems to play a major role among the pathophysiological mechanisms of sepsis, and in the development and progression of sepsis-induced organ failure. There is plenty of evidence supporting a positive correlation between oxidative damage and organ injury (Gonzalez et al., 2014; Ritter et al., 2003; Wheeler, 2011). Moreover, many studies demonstrate outcome improvement in sepsis after antioxidant supplementation and reinforcement of endogenous antioxidant defenses (Baumgart et al., 2009; Ceylan-Isik et al., 2010; Lowes and Galley, 2011).

In experimental models of sepsis, the impact of antioxidant therapy has been extensively evaluated, but little specific information on cardiac dysfunction in sepsis is available, or it is inconclusive. The myocardium exhibits two thiol-based major antioxidant defenses to maintain a reduced intracellular redox state, such as thioredoxin (Trx) and glutaredoxin (Grx) systems (Berndt et al., 2007). The Trx system consists of Trx, NADPH, and Trx reductase (TrxR). Trx is a small multifunctional redox-active protein involved in cellular redox homeostasis and cell survival (Holmgren and Lu, 2010). There are three isoforms of Trx depending on their localization, such as Trx1 with its cytosolic and nuclear forms, Trx2 mitochondrial form, and Sp-trx, which is expressed in testes (Miranda-Vizuete et al., 2001). Trx1 is the most intensively studied, because it has many biological functions. It reduces peroxiredoxin or oxidized proteins, interacts with transcription factors (Powis and Montfort, 2001), participates in control of apoptosis through binding to apoptosis signal regulating kinase-1 (ASK-1), and modulates other redox-regulated proteins like caspases through the control of protein S-nytrosilation and denitrosylation (Li et al., 2013).

Several responses allow the mitochondrial network to adapt to stress and loss of membrane potential. These include mitochondrial fission and fusion, mitophagy, and mitochondrial biogenesis (Youle and van der Bliek, 2012). Mitochondrial dynamics is a recent topic in cardiac physiology (Song and Dorn, 2015; Ikeda et al., 2015; Cimolai et al., 2015). The proteins involved in mitochondrial fusion (mitofusins [Mfn 1 and 2] and optic atrophy protein 1 [OPA1]) and fission (dynamin related protein 1 [Drp1]) are highly expressed in cardiomyocytes, and are required for mitochondrial biogenesis and the quality control of the organelles. In particular, mitochondrial fission allows for selective segregation of damaged mitochondria, which are afterwards eliminated by autophagy (or mitophagy).

Some molecular research studies on cardiac-specific overexpression of Trx1 have demonstrated that proteins associated to mitochondrial permeability transition pore (MPTP), and contractile apparatus are selective targets for facilitating muscle contraction (Fu et al., 2009), and Trx1 even upregulates mitochondrial proteins and enhances mitochondrial function, possibly by activating genes related with mitochondrial biogenesis (Ago et al., 2006).

Additional evidence has emphasized the importance of Trx1 in cardiovascular diseases, including heart ischemia-reperfusion injury, contractile dysfunction, atherosclerosis and heart failure (Ahsan et al., 2009; Mahmood et al., 2013; Nicholson et al., 2013).

The aim of this study was to evaluate the protective role of Trx1 in CLP-induced myocardial dysfunction, focusing on cardiac contractility, mitochondrial function, dynamics and biogenesis.

2. Materials and methods

2.1. Cardiac-specific thioredoxin1-overexpressing mice

Transgenic male mice with Trx1 cardiac-specific overexpression (Trx1-Tg) (22–32 g) were used. The mice were donated for research purposes to the Institute of Cardiovascular Physiopathology, Department of Pathology, School of Medicine, Universidad de Buenos Aires, Argentina (courtesy of Sadoshima J., New Jersey Medical School, Rutgers University, Newark, USA). As previously described (Yamamoto et al., 2003), Trx1-Tg mice were generated on an FVB background, using the α-myosin heavy chain promoter to achieve cardiac-specific expression.

2.2. Cecal ligation and puncture (CLP) model

The model of cecal ligation and puncture (CLP) in rodents has been extensively used to research the clinical settings of sepsis and septic shock. This model produces a hyperdynamic, hypermetabolic state that can lead to a hypodynamic, hypometabolic stage, and eventual death (Hubbard et al., 2005). It is based on the disruption of the intestinal barrier by means of surgical procedures. Animal experiments were performed in accordance with the Principles of Laboratory Animal Care. The Institutional Animal Care and Research Committee of the Universidad de Buenos Aires approved all animal procedures. We made every possible effort to minimize animal suffering and to reduce the number of animals used. Animals were given access to food and water *ad libitum*. All the animals were fasted for 16 h before any surgical procedure. The mice were anesthetized with an intraperitoneal ketamine ($100\ mg\ kg^{-1}$) and xylazine ($5\ mg\ kg^{-1}$) mixture. Under aseptic conditions, a 1.5–2.0 cm midline incision was performed to allow exposure of the cecum. The cecum was tightly ligated, and perforated twice with a 21-gauge needle. The cecum was then gently squeezed to extrude a small amount of fecal content from the perforation sites to the abdominal cavity. A sham surgery (laparotomy and cecum exposure) was performed as control. The animals were resuscitated with normal saline (NaCl 0.9%, 1 ml subcutaneous) and pain medication (tramadol 20 μg/g BW) was administered immediately after CLP. The animals were sacrificed 6, 18, or 24 h after CLP or the sham operation and the hearts were isolated for posterior analyses, in order to evaluate the hyperdynamic (6 h) and hypodynamic (18–24 h) stages of sepsis.

2.3. Survival experiments

Survival rate was tested in seven animals per group, which underwent the CLP procedure described above. Mortality was monitored over a period of 72 h.

2.4. Biochemical parameters

Blood samples were taken from mammary arteries without anticoagulants. Serum aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) activity were determined by the rate of decrease in NADH measured at 340 nm. Creatine kinase, CK, and CK-MB were assessed by CK-NAC UV method. Lactate dehydrogenase activity, LDH, was evaluated by direct reduction of NADH. All previous measurements were carried out by bioanalytical standard Wiener lab diagnostic kits. Serum biochemical parameters were used as markers of sepsis-related organ damage and evolution.

2.5. Myocardial contractility

After 6, 18, or 24 h of the surgically induced peritonitis or sham procedures, the mice were anesthetized by an intraperitoneal injec-

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tion of sodium pentobarbital (150 mg/kg body weight) and sodium heparin (500 UI/kg body weight). After ensuring sufficient depth of anesthesia, the hearts were excised and the aorta was immediately cannulated with a 21 gauge cannula. Afterwards, retrograde heart perfusion was performed according to the Langendorff technique with Krebs medium equilibrated with 95% O_2 and 5% CO_2 at 37 °C. A small latex fluid-filled balloon connected by way of a thin plastic catheter (P50) to a Deltram II pressure transducer (Utah Medical System, UT, US) was inserted into the left ventricle via the left atrium. The catheter with the transducer was positioned in such a way that it secured the position of the balloon in the left ventricle. Two electrodes were sutured and connected to a pacemaker in order to induce a constant heart rate of 470 ± 30 beats/min. Coronary perfusion pressure (CPP) was recorded by means of a pressure transducer connected to the perfusion line. The hearts were perfused at constant flow in order to obtain a CPP of 73 ± 3 mm Hg during the initial stabilization period and then they were kept constant throughout the experiment. Left ventricular developed pressure (LVDP) was calculated as the difference between peak systolic pressure and left ventricular end-diastolic pressure. The inotropic contractile reserve was evaluated as the delta LVDP, before and after a β-adrenergic stimulus through the addition of isoproterenol (ISO) (1 μM) to the perfusion line as described by Marchini et al. (2013).

2.6. Tissue cubes preparation

The hearts were excised and placed in ice-cold assay medium (118 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 25 mM $NaHCO_3$). By assay, 50 ± 10 mg of tissue were minced into 1 mm^3 cubes by means of a scalpel.

2.7. Mitochondrial isolation

The hearts were extracted, and immediately placed in MSHE buffer [0.22 M mannitol, 70 mM sucrose, 0.5 mM EGTA, 2 mM KHEPES], and the mitochondria were isolated by gradient centrifugation in a Sorvall centrifuge. Fresh mitochondria were used to determine membrane potential. To determine complex activities, MnSOD activity and Western blotting, the mitochondria were stored at −80 °C, and then subjected to three freeze/thaw cycles followed by a homogenization step by passage through a 29 G hypodermic needle.

2.8. RNA extraction and quantitative real-time-PCR

The total RNA was extracted with TRIzol Reagent (Invitrogen Corp.). Previous DNAse treatment, 2 μg of sample was reverse-transcribed in duplicate using Taq polymerase and mouse gene-specific primer sequences: Trx2, sense 5′-GTGGACTTTCATGCACAGTGG-3′ and antisense 5′-GTTCTTGATGGCTAGCACGG-3′; UCP2, sense 5′-CAGCCTGAGACCTCAAAGCA-3′ and antisense 5′-GACCTTGGCGGTATCCAGAG-3′; UCP3, sense 5′-ATGCCTACAGAACCATCGCC-3′; and antisense 5′-CTTTGRAGAAGGCCGTGGGT-3′. We also used oligonucleotide primers common to mouse, rat and human SOD2 sense 5′-CAGATCATGCAGCTGCACCAC-3′ and antisense 5′-TTCAGTGCAGGCTGAAGAGC-3′; GADPH sense 5′-CTGCACCACCAACTGCTTAG-3′ and antisense 5′-AGGGGCCATCCACAGTCTTC. The cycling conditions were 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s for 40 cycles. The integrity of amplified DNA was confirmed using the determination of the melting temperature. The data were expressed as fold change over wt sham-group samples, and standardized by those of GAPDH.

2.9. Heart oxygen consumption

Tissue cubes were incubated in an all-glass thermostatic chamber with magnetic stirring at 30 °C, in assay medium with 5.5 mM glucose. The oxygen uptake was registered polarographycally with a Clark-type oxygen electrode. The results were expressed in nmolesO_2 min^{-1} mg tissue $^{-1}$. In order to quantify the O_2 uptake by non-mitochondrial sources, 4 mM KCN was added to the assay measurement medium to inhibit mitochondrial cytochrome oxidase (Villani and Attardi, 2007).

2.10. Mitochondrial respiratory chain complexes activity

The enzymatic activity of complexes I, II, III and IV was measured spectrophotometrically (Hitachi F-3010, Tokyo, Japan). The mitochondrial preparation (0.025 mg/ml) was incubated in reaction buffer (potassium phosphate 100 mM, pH 7.2) at 30 °C. Complex I was measured at 340 nm monitoring the oxidation of NADH, inhibited by rotenone, and the results were expressed as nmol NADH oxidized/min. mg mitochondrial protein. For complex II–III, the reaction buffer was supplemented with 10 mM succinate as substrate, and the cytochrome c^{3+} reduction rate inhibited by antimycin was followed at 550 nm. The results were expressed as nmol reduced cytochrome c^{3+}/min. mg protein. For complex IV, the reaction buffer was supplemented with 50 μM cytochrome c^{2+} monitoring oxidation rate at 550 nm, which was calculated from pseudo-first reaction constant (*k*') and expressed as *k*'/mg protein.

2.11. Mitochondrial membrane potential (Δψ)

Mitochondrial membrane potential was estimated by flow cytometry (FACScalibur) using fluorescent dye 3,3′-Dihexyloxacarbocyanine iodide ($DiOC_6$) (200 nM), which selectively accumulates in mitochondria based on the membrane potential. Fresh heart mitochondria (50 μg protein/ml) were suspended in 0.5 ml of respiration buffer [120 mM KCl, 5 mM KH2PO4, 1 mM EGTA, 3 mM HEPES and 1 mg/ml fatty acid-free BSA (pH 7.4)]. This assay was performed in the dark at 37 °C for 15 min. After the incubation period, the content of the cuvette was centrifuged for 5 min at 10,000*g* to pellet mitochondria, suspended in 200 μl, and then, the fluorescence intensity was recorded. In order to quantify changes in membrane potential, the $DiOC_6$ signal was analyzed in rest state respiration in the presence or absence of 6 mM succinate. As a positive control, total depolarization was induced by 2 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP).

2.12. Antioxidant enzymes activities

The activity of Trx in the heart was determined by the insulin reduction assay, according to the method described by Holmgren and Bjornstedt with a slight modification (Berndt et al., 2007). Tissue homogenates containing 25 μg of protein, in DTT activation buffer (100 mM HEPES, 2 mM EDTA, 1 mg/ml BSA, 2 mM DTT), were incubated in 96-well plates at 37 °C for 15 min, to reduce and activate endogenous Trx. The samples were mixed with reaction mixture (100 mM HEPES pH 7.6, 2 mM EDTA, 0.2 mM NADPH, and 140 μM insulin.) The reaction was started by the addition of 0.5 UTrx reductase or an equal volume of water for negative controls, and maintained at 37 °C for 20 min. The reaction was stopped by the addition of 250 μL250 μl of stop solution (0.4 mg/ml DTNB/6 M guanidine hydrochloride in 0.2 M Tris–HCl, pH 8.0). The 96-well plates were measured at 405 nm. Changes in the absorbance in the absence of Trx reductase were subtracted from those in the presence of the reductase. The Trx1 activity was expressed as μmol SH_2 insulin/g tissue.

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Table 1
Biochemical parameters in developing sepsis.

Groups	Wildtype			Tg-Trx1		
	SHAM	CLP 6 h	CLP 24 h	SHAM	CLP 6 h	CLP 24 h
AST	63 ± 7	292 ± 21*	1142 ± 259*	62 ± 10	220 ± 20	633 ± 55*,**
ALT	34 ± 2	122 ± 6*	292 ± 12*	36 ± 3	73 ± 9	370 ± 56*,**
Total CK	480 ± 160	3018 ± 626	4537 ± 1315*	428 ± 96	2003 ± 566	5551 ± 1627*
CK-MB	90 ± 16	540 ± 88	2607 ± 338	155 ± 35	356 ± 125	2451 ± 545
CK-MB/Total	12.5 ± 1.1	27.4 ± 3.4	78.4 ± 8.1*	30 ± 2.4	29.6 ± 6.9	50.2 ± 6.9**
LDH	714 ± 68	1512 ± 210	6671 ± 1194*	673 ± 48	1354 ± 268	4589 ± 467*,**
Urea	71 ± 3	83 ± 5	159 ± 11*	76 ± 4	82 ± 3	152 ± 12*

Enzyme activities are expressed in IU/L and urea in mg/dL. Data are mean ± SEM (n = 6 per group).
* P < 0.05 vs sham of the same phenotype by ANOVA-Dunnett's test.
** P < 0.05 between groups at the same time-period of sepsis by ANOVA-Bonferroni's multiple comparisons test.

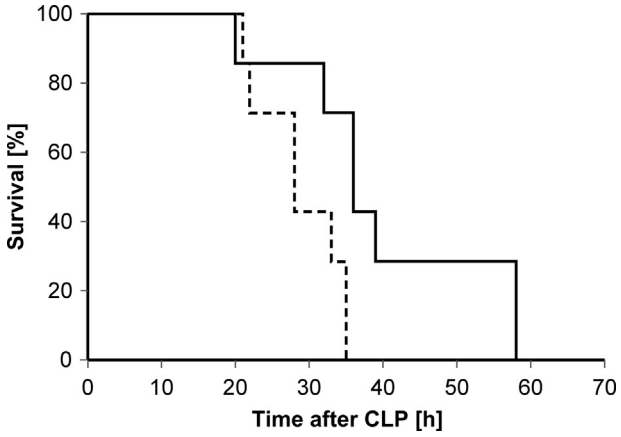

Fig. 1. Prolonged survival time in Trx1-Tg septic mice. No deaths occurred in sham animals that underwent laparotomy. The data are presented as percentage of survival and Kaplan-Meier survival curves were compared using a log-rank test to determine significance. Dashed lines correspond to wt mice and solid lines correspond to Trx1-Tg mice. (n = 7 per group).

Manganese superoxide dismutase (MnSOD) activity was examined by enzymatic determination. One unit of SOD inhibited the reduction of cytochrome c by 50% competing for the superoxide radical. The rate of reduction was followed spectrophotometrically at 550 nm and results were expressed as pmoles SOD per mg of protein.

Catalase (CAT) activity was assayed in cytosolic fraction according to Cohen (Cohen et al., 1970), with some modifications. The consumption rate for H_2O_2 was followed by the decrease in the absorbance at 240 nm and 25 °C of 0.025, 0.05 and 0.1 mg of protein. The pseudo-first reaction constant (*k*') expressed as *k*'/mg protein was calculated by each concentration of protein and then extrapolated to a calibration curve to obtain U CAT/mg protein. One unit of CAT decomposed 1.0 μmole of H_2O_2 per minute.

2.13. Protein carbonyl assay

Protein oxidation was determined by Oxyblot kit (Millipore). Tissue homogenates were obtained and prepared to a final concentration of 3 mg/ml of protein, and immediately frozen until assessment. 15 μg of protein were used for each determination.

2.14. Western blot analysis

Myocardial protein levels were measured in mitochondria and homogenate samples, using SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked with 5% milk in T-TBS and incubated overnight at 4°C with anti-thioredoxin1 (C63C6 rabbit mAb, Cell Signaling Technology, 1:1000), anti-thioredoxin2 ([H-75], Santa Cruz Biotechnology, 1:3000), anti-manganese SOD (Stress Gen,1:3,000), anti-actin ([I-19], Santa Cruz Biotechnology, 1:4000, homogenate loading control) and anti-VDAC1 ([N-18] Santa Cruz Biotechnology, 1:4000, mitochondrial loading control) antibodies.

2.15. Statistical analysis

The statistical analyses were performed using Graph Pad Prism 5.01. The data are presented as means ± SEM and significant differences between groups were assessed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test or Dunnett's test. Kaplan-Meier survival curves were compared using a log-rank test to determine significance.

3. Results

3.1. Effects of Trx1 cardiac overexpression on survival, morphometric, and biochemical parameters

In the survival curve, the average life expectancy in septic mice was 28 h for wt and 36 h for Trx1-Tg (Fig. 1). Differences between curves were statistically significant. No deaths occurred in the sham-operated group, in wt or Trx1-Tg.

Age, body and organ weights were comparable between groups and not affected by CLP (Suppl. Table 1). The mice displayed piloerection, loss of interest in their surroundings, lethargy and ocular drainage after CLP procedures.

Most of the biochemical parameters reflected sepsis-related organ damage in both groups as sepsis progressed (Table 1). In septic wt mice, AST and ALT levels increased significantly as early as 6 h post-CLP; they also remained significantly higher, as well as the lactate dehydrogenase (LDH), after 24 h compared to Trx1-Tg (p = 0.002). At 24 h, the ratio of CK-MB/total CK was 56% higher in wt than in Trx1-Tg mice, revealing more severe heart damage in wt mice. Urea levels were not different between groups during any time of sepsis.

3.2. Trx1 overexpression attenuates the early drop of contractile reserve

Cardiac contractility was determined at baseline and after β-adrenergic stress in shams, and 6 and 24 h after CLP (Fig. 2). The left ventricular developed pressure (LVDP) was reduced after CLP without changes in baseline conditions. As depicted in Fig. 2A, neither basal nor isoproterenol (ISO) differences were observed. On the other hand, as expected for a normal myocardium, LVDP increased significantly by 29.4% in wt and by 32.4% in Trx1-Tg mice, when comparing basal with ISO-induced LVDP. Interestingly, in septic mice 6 h after CLP, Trx1-Tg mice showed a better response to ISO-

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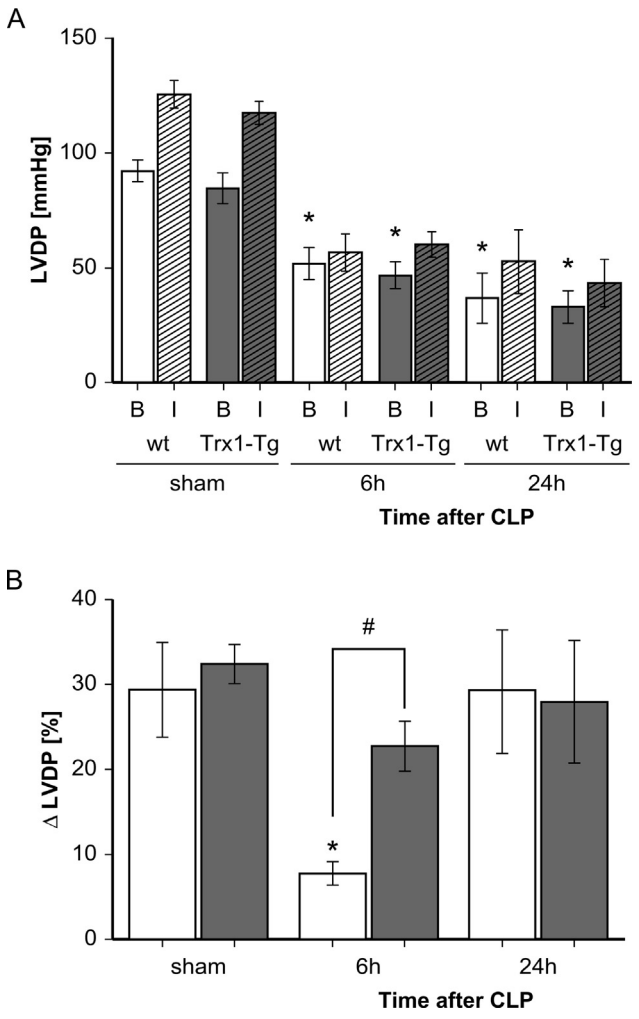

Fig. 2. Effects of Trx1 overexpression on cardiac contractile function in CLP-induced sepsis. Measurements were performed on sham, 6 and 24 h post CLP according to the Langendorff technique at constant flow, in basal conditions (plain bars) and after isoproterenol addition – Iso 1 μM – (weft bars). Values are expressed as mean ± SEM of 5 independent experiments. (A) Left ventricular developed pressure (LVDP) of isolated perfused hearts from wt (white bars) and Trx1-Tg mice (dark bars). (B) Increase in LVDP (Delta LVDP), shown as the relative percentage of difference between basal and Iso-stimulated LVDP. * $P < 0.05$ compared with wt sham group; # $P < 0.05$ comparison between groups at the same time-period of sepsis.

induced LVDP compared with wt. Contractile reserve was improved by 15% between wt and Trx1-Tg mice ($p = 0.0441$) (Fig. 2B).

3.3. *Trx1 overexpression attenuates CLP-induced cardiac oxidative stress*

First, we evaluated total Trx activity in heart homogenates corresponding to cytosolic Trx1 and mitochondrial Trx2 activities (Fig. 3A). We observed a progressive increase in Trx activity along sepsis in both groups, which was significant in wt mice at 24 h, while in Trx1-Tg, it was significantly higher from 6 h after CLP.

Taking into account that Trx1 can regenerate other cellular antioxidant defense systems, and in order to evaluate possible actions of Trx1 on other cytosolic and mitochondrial antioxidant systems, catalase and MnSOD enzymatic activities were measured. We found that Trx1 overexpression did not counteract the decrease in catalase activity in the progression of sepsis, which was significantly reduced in both groups 24 h after CLP. On the contrary, MnSOD activity progressively increased in Trx1-Tg, but decreased at 24 h in wt mice (Table 2).

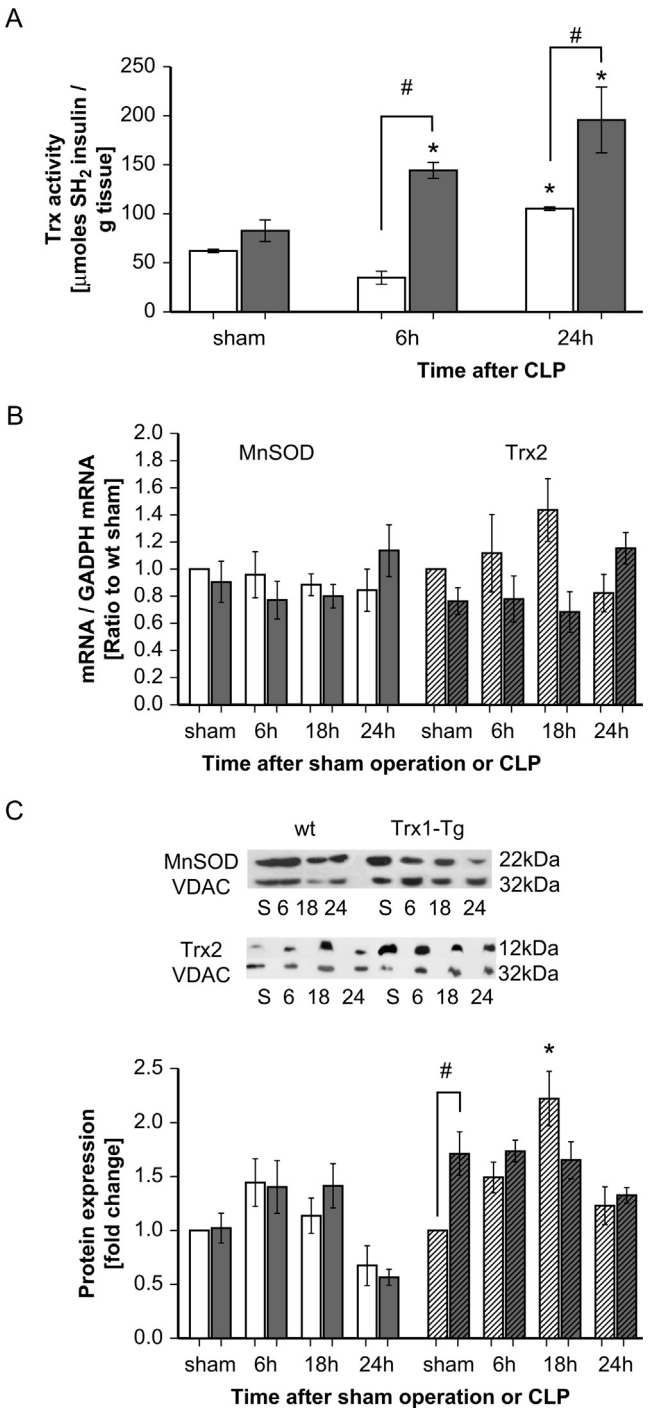

Fig. 3. Effects of Trx 1 overexpression on antioxidant defenses in CLP-induced sepsis. (A) Specific activity of total Trx, determined by insulin reduction assay (n = 3). Tissue homogenates were prepared from total cardiac muscle, extracted from wt (white bars) and Trx1-Tg group (dark bars). (B) mRNA/Real-time PCR analyses of MnSOD and Trx2; (C) Western blot analyses of MnSOD and Trx2 expression. Data represented three independent experiments done in duplicate. *The data represent the mean ± SEM. $P < 0.05$ compared with sham surgery; # $P < 0.05$ comparison between groups at the same time-period of sepsis.

No significant changes were observed in MnSOD and Trx2 at transcriptional level (Fig. 3B), while Trx2 protein expression was differentially modulated in wt mice, showing a significant increase 18 h after CLP (Fig. 3C). Overexpression of Trx1 resulted in higher levels of Trx2 compared to wt only in the sham group.

In order to evaluate protein oxidation along the course of sepsis, protein carbonylation was analyzed (Fig. 4). Oxidation of mitochon-

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Table 2
Antioxidant enzymes activities in developing sepsis.

	Groups	MnSOD pmol SOD/mg protein	Catalase pmol H_2O_2/mg protein
Wildtype	Sham	366 ± 23	0.45 ± 0.04
	6 h	437 ± 64	0.38 ± 0.05
	18 h	504 ± 33*	0.24 ± 0.06
	24 h	270 ± 21*	0.29 ± 0.03**
Tg-trx1	Sham	319 ± 65	0.45 ± 0.02
	6 h	362 ± 53	0.34 ± 0.03
	18 h	473 ± 24	0.33 ± 0.03
	24 h	494 ± 83	0.24 ± 0.02**

Data are mean ± SEM (n = 6 per group).
* P < 0.05 vs sham of the same genotype by ANOVA-Dunnett's test.
** P < 0.05 between groups at the same time-period of sepsis by ANOVA- Bonferroni's multiple comparisons test.

drial and cytosolic proteins was observed in both wt and Trx1-Tg mice, being significantly lower 6 h after CLP in Trx1-Tg as compared to wt mice. Protein carbonylation was higher in cytosolic proteins than in mitochondrial ones in both groups. In wt mice, cytosolic protein oxidation increased significantly during late sepsis.

3.4. Trx1 overexpression attenuates CLP-induced mitochondrial dysfunction

To evaluate mitochondrial function we measured O_2 consumption, membrane potential and mitochondrial complex activities (Fig. 5 and Table 3).

Heart tissue cubes from wt septic mice did not show significant changes in the O_2 consumption rate compared to those that underwent sham surgery. On the other hand, in Trx1-Tg, we found that the total rate of oxygen consumption augmented significantly at 6 h after CLP (50%) (Fig. 5A). Mitochondrial and non-mitochondrial O_2 consumption source rates increased at 6 h, with mitochondrial O_2 consumption being significantly higher (p = 0.0339) in relation to wt (Fig. 5B). At 24 h, O_2 consumption returned to baseline rates.

Afterwards, we measured the respiratory chain complex activities in isolated mitochondria (Table 3). Mitochondria from the hearts of septic wt mice exhibited no changes in the activity of any of the studied complexes in early sepsis, but showed reduction in complex I and IV activity during late sepsis, while in Trx1-Tg, only inhibition of complex IV was observed 24 h after CLP.

When the mitochondrial membrane potential was evaluated, we could evidence significant depolarization at 18 h in wt mice, with 50 and 36% reduction in wt and Trx1-Tg, respectively (Fig. 5C and D).

A lot of evidence suggests that high $\Delta\psi$ seems to favor the production of ROS, particularly at complex III, and that mitochondrial uncoupling lowers ROS production (Korshunov et al., 1997). UCP2 can influence the mitochondrial membrane potential, and thus, alter the production of reactive oxygen species. With the objective of establishing if uncoupling proteins (UCPs) were operating to reduce the formation of ROS in our model, we evaluated mRNA UCP2 and UCP3 expression. We found significantly decreased mRNA levels in UCP2 (Fig. 5E) and UCP3 (Fig. 5F) isoforms, but a sudden rise in UCP2 at 24 h only in Trx1-Tg mice, which was significantly higher compared with wt.

3.5. Trx1 overexpression attenuates mitochondrial ultrastructure alterations, and stimulates mitochondrial biogenesis and autophagy

Alterations in mitochondrial morphology, cristae architecture, and mitochondrial outer membrane damage were observed by electron microscopy, mostly at 24 h post-CLP (Fig. 6D). At 6 h, only

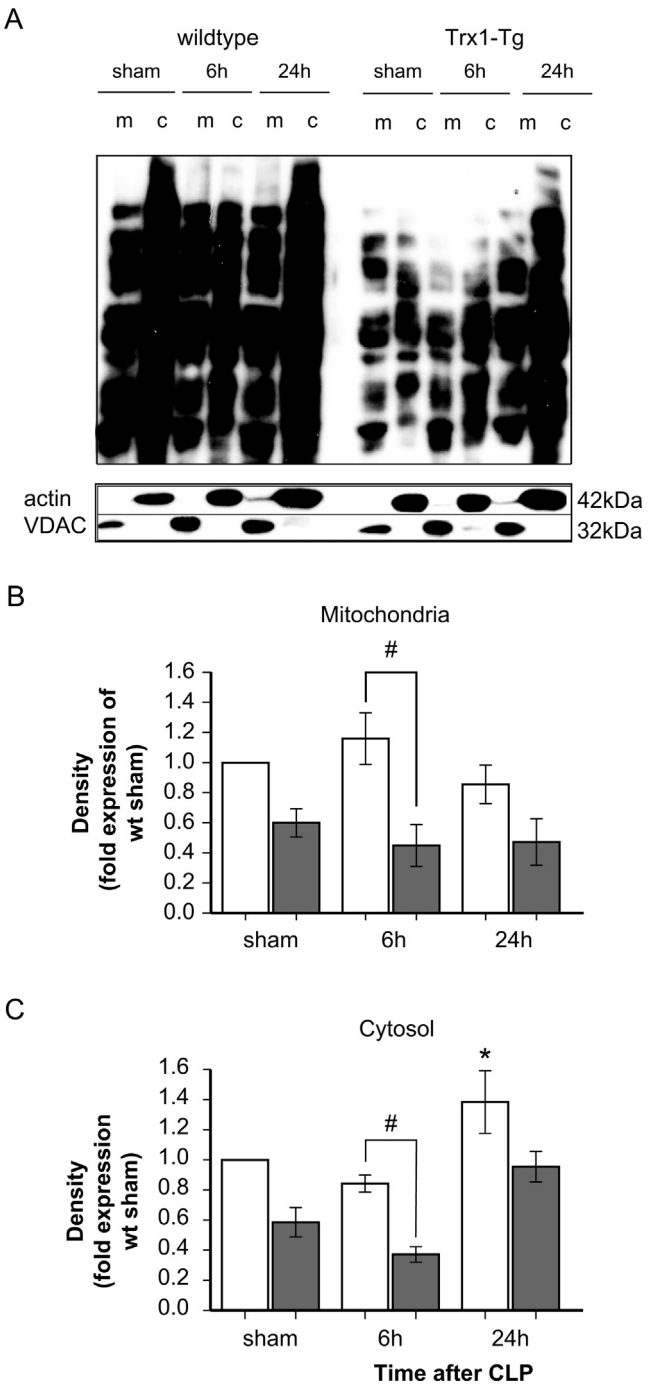

Fig. 4. Trx1 overexpression decreases protein carbonylation in CLP-induced sepsis. (A) Anti-DNP Oxyblot (15 μg protein); representative Western blot of 4–5 independent experiments are shown; not treated proteins with carbonylation inductor did not show bands. (B) Mitochondrial and (**C**) cytosolic protein carbonyls analysis. Values are density mean ± SEM. * P < 0.05 compared with sham surgery; # P < 0.05 comparison between groups at the same time-period of sepsis.

a few mitochondria of wt ventricular tissue showed vacuolization, cristae disorganization and/or mitochondrial outer membrane disruption. In Trx1-Tg, alterations in the mitochondria ultrastructure appeared later, and were significantly lower. Lipid droplets were observed in both groups (Trx1 and wt).

In order to evaluate mitochondrial dynamics, we measured the transcriptional regulation of mitochondrial fusion and fission proteins (Mfn2, OPA 1 and Drp1, and OPA1 and Drp1 protein expression.)

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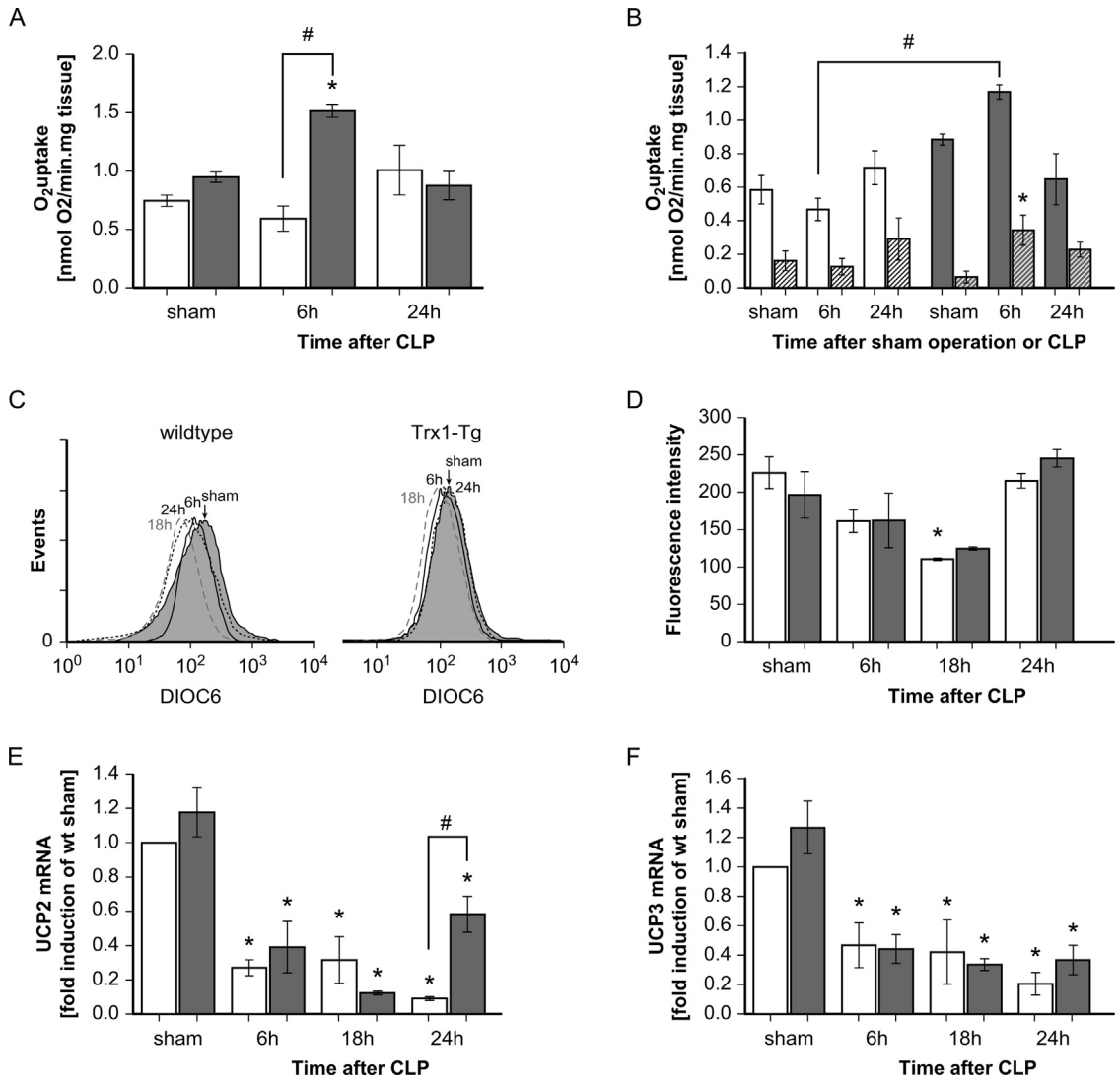

Fig. 5. Trx1 overexpression protects heart mitochondria in CLP-induced sepsis. (A) Rate of oxygen consumption in heart tissue cubes. (B) Rate of mitochondrial (plain bars) and other non-mitochondrial O_2 consumption sources in presence of KCN (weft bars). (C) Evaluation of heart mitochondrial membrane potential by flow cytometry. Overlaid histograms of gated (R1) mitochondrial events versus $DiOC_6$ fluorescence intensity by sepsis time (color lines); left histograms correspond to wt mice, and right histograms to Tg-Trx1 mice. (D) $DiOC_6$ fluorescence histograms quantification in wt (white bars) and Tg-Trx1 mice (dark bars). (E, F) Real time PCR analyses of uncoupling protein 2 (UCP2) and UCP3. Data represented 4–6 independent experiments made by duplicate. Results are mean ± SEM. * $P < 0.05$ compared with sham surgery; # $P < 0.05$ compared between groups at the same time-period of sepsis.

Table 3
Cardiac mitochondrial respiratory chain complexes activities.

	Groups	Complex I (nmol/min mg protein)	Complex II-III (nmol/min mg protein)	Complex IV [k (min^{-1})/mg protein]
Wildtype	Sham	341 ± 32	225 ± 23	39 ± 2
	6 h	344 ± 38	236 ± 32	43 ± 2
	18 h	231 ± 13	194 ± 15	35 ± 4
	24 h	130 ± 22*	146 ± 20	30 ± 1*
Tg-trx1	Sham	346 ± 35	168 ± 33	40 ± 2
	6 h	318 ± 45	219 ± 27	37 ± 1
	18 h	216 ± 28	207 ± 28	38 ± 4
	24 h	198 ± 26	137 ± 23	30 ± 1*

Data are mean ± SEM (n = 6 per group).
* $P < 0.05$ vs sham of each group by ANOVA-Dunnett's test.

Mfn2 was not transcriptionally modulated by either the septic process or Trx1 overexpression. OPA 1 mRNA decreased in both groups after CLP, while protein levels only decreased significantly 6 h after CLP in wt mice hearts (Fig. 6A and B).

As Drp1 is post-transcriptionally modulated, Drp1 activity was quantified by pDrp1 Ser616 (active form) to total Drp1 ratio by Western blot (Fig. 6C). At 24 h post-CLP, an increase in this ratio was observed in wt animals, while it did not change in Trx1-Tg.

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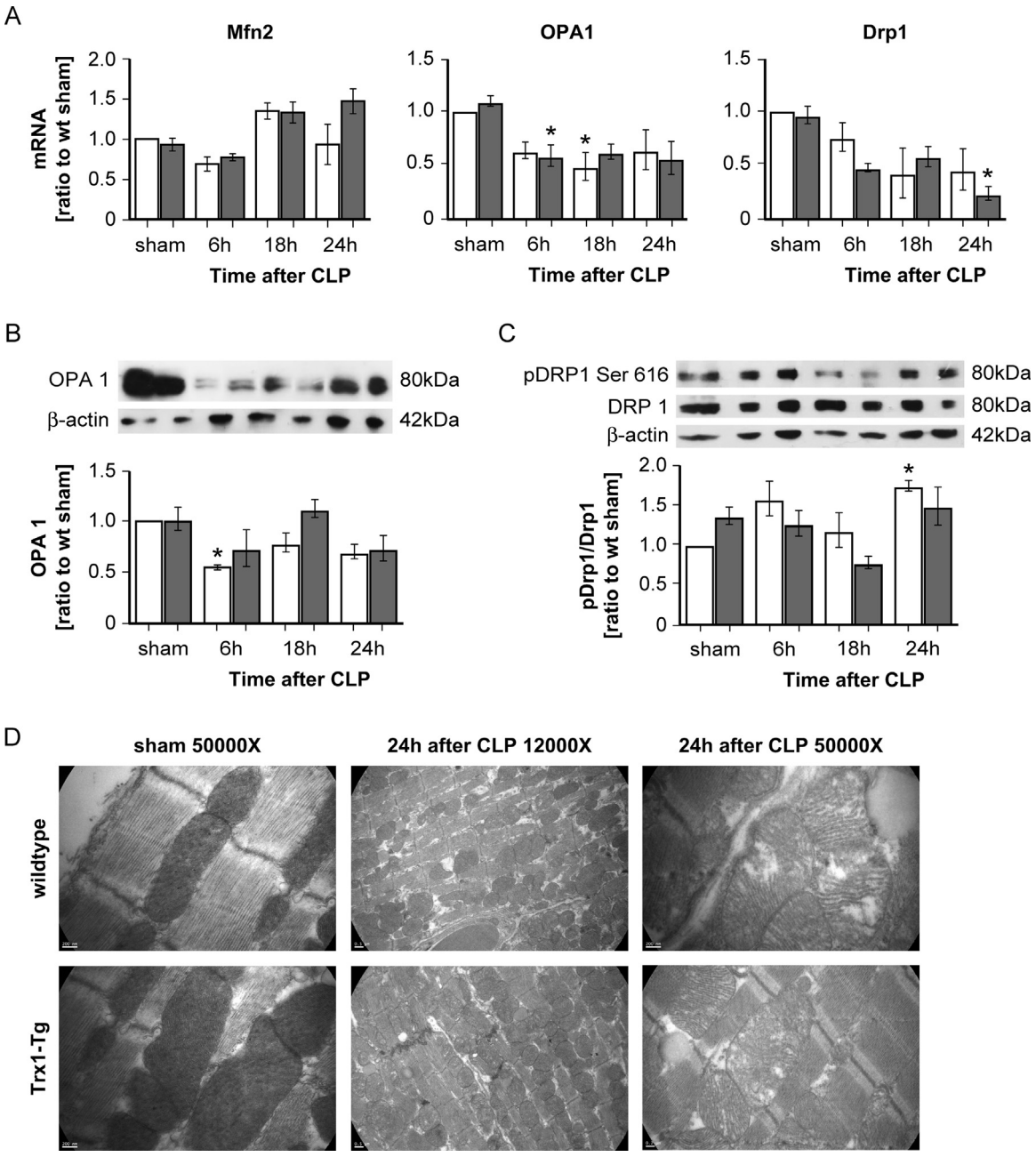

Fig. 6. Effects of Trx1 overexpression on mitochondrial dynamics and morphology in CLP-induced sepsis. (A) Real time PCR analysis of Mfn2, OPA1 and Drp1 expression in cardiac tissue. (B) OPA1 and (C) pDRP1 protein expression with their respective examples of Western blots. β-actin was used as loading control. Quantification analyses of pDrp1/Drp1 ratio was determined to estimate Drp1 activation. (D) Representative transmission electron micrographs of myocardium after CLP. Sham displayed typical organization of mitochondrial internal and outer membranes; 24 h after CLP, tissue disorganization was observed, and mitochondria displayed swelling, disruption of cristae and lipid droplets (see micrographs on the right at 50,000×). Data of Western blots represented four independent experiments made by duplicate. Results are mean ± SEM. * $P < 0.05$ compared with sham surgery. Scale bars: 0.5 μm at 12,000× and 0.2 μm at 50,000×.

In addition, a progressive decrease in Drp1 mRNA was observed in both groups, accompanied by a significant decrease of Drp1 in wt mice at 24 h.

Recovery of organ function in sepsis is dependent on the removal of dysfunctional mitochondria by mitophagy and the generation of new, functionally competent mitochondria by stimulation of mitochondrial biogenesis. We studied transcriptional activation of the main nuclear-encoded activator PGC-1α, and the TFAM and Nrf 1 transcription factors (Fig. 7A and B). PGC-1 α mRNA levels were 50% higher in Trx1-Tg sham mice in relation to wt ones. During the progression of sepsis, there was a decrease in PGC-1α and TFAM mRNA between 6 and 18 h post-CLP, which was significantly reverted at 18 h in Trx1-Tg hearts. No modifications were observed in Nrf 1 mRNA levels in either group.

Autophagosome formation was assessed by evaluating the conversion of the soluble form of microtubule-associated protein light chain 3 (LC3-I) to lipidated and autophagosome-associated form (LC3-II) by Western blot of heart homogenates (Fig. 7C). We observed a significant increase in LC3-II to LC3-I ratio in the Trx1-Tg group 6 h after CLP with a lower increase in wt hearts. These results coincided with a lower number of mitochondria with alterations of the inner and/or outer membranes observed by electron microscopy in the hearts that overexpressed Trx1, suggesting mito-

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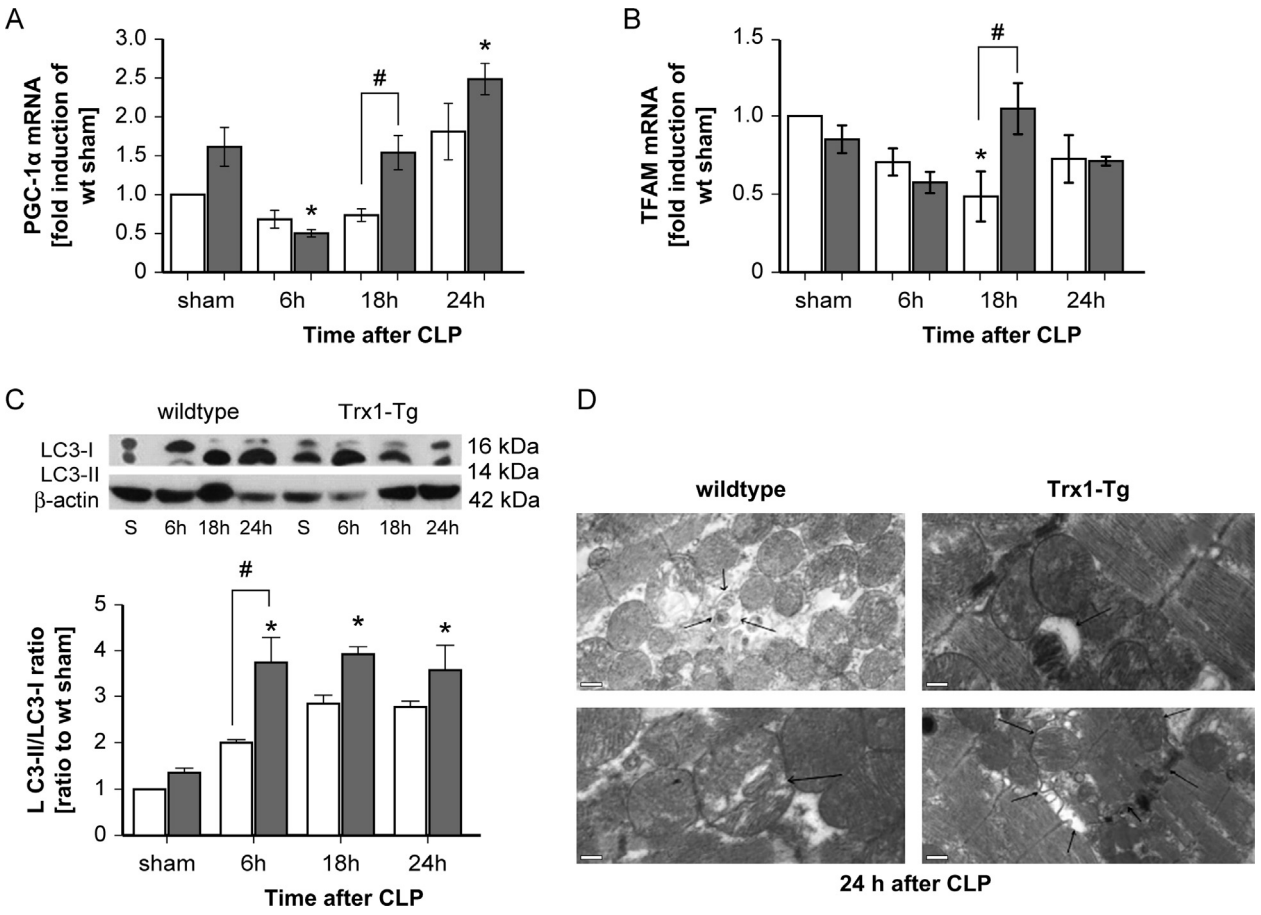

Fig. 7. Effects of Trx 1 overexpression on mitochondrial biogenesis and autophagy in CLP-induced sepsis. (A) Real time PCR analysis of PGC-1α and (B) TFAM expression in cardiac tissue. (C) Western blots of LC3 in cardiac homogenates samples and quantification analyses of LC3-II/LC3-I ratio (n = 3). β-actin was used as loading control. When autophagy occurs, conversion from LC3-I to LC3-II is observed. (D) Representative transmission electron micrographs of left ventricule myocardium at 20,000×, showing ultrastructural findings of autophagy at 24 h of sepsis. Black arrows indicate ultrastructures suggestive of mitophagy and autophagic vacuoles containing damaged mitochondria. Data of mRNA levels analysis represented four independent experiments done in duplicate. Results are mean ± SEM. * $P < 0.05$ compared with sham surgery. Scale bar: 0.5 μm.

chondrial recycling by mitophagy and mitochondrial biogenesis (Figs. 6 D and 7 D).

No evidence of apoptosis was observed in either group assessed by TUNEL or cytochrome *c* release (data not shown).

4. Discussion

Cardiac-specific Trx1 overexpression has been shown to protect against heart disease, although no studies on sepsis-induced myocardial dysfunction have been reported yet. Our results indicated that cardiac-specific overexpression of Trx1: (1) prolongs survival in septic mice; (2) attenuates the early drop in cardiac contractile reserve; (3) delays exhaustion of the mitochondrial antioxidant defense; (4) preserves mitochondrial complex activities and membrane potential; and (5) attenuates myocardial damage characterized by fiber disruption and mitochondrial injury. Our findings highlight that there is no significant increase in oxidative stress in the heart at early sepsis in Trx1-Tg mice, as evidenced by the absence of alterations of mitochondrial complex activities, protein oxidation, or antioxidant systems.

It has been demonstrated that systemic overexpression of Trx1 results in resistance against oxidative stress and a possible extension of life span in healthy animals (Mitsui et al., 2002; Perez et al., 2011; Yamamoto et al., 2003). Accordingly, our findings show that cardiac-specific overexpression of Trx1 increases survival time of septic mice compared to septic wt animals, supporting the protective role of Trx1 in stress-associated pathophysiological conditions like sepsis.

In addition to its local cardiac effects, a systemic action of Trx1 would be possible as supported by the lower and later rise in hepatic marker enzymes (AST and ALT) in Trx1-Tg mice during sepsis. Furthermore, previous works have shown that Trx1 is released to the extracellular medium, and plasma Trx1 increases in response to inflammation, and oxidative stress (Callister et al., 2006). In human sepsis, Hofer et al. found that extracellular levels of Trx1 are raised as a consequence of increased oxidative stress (Hofer et al., 2009); while in experimental models, the intraperitoneal administration of Trx1 promotes animal survival, and its neutralization increases mortality in septic shock.

Myocardial depression is a recognized manifestation of organ dysfunction in sepsis. Alteration in myocardial contractile function has been demonstrated previously (Tao et al., 2004; Vincent et al., 1992), and accordingly, our present data showed reduction in LVDP and loss of myocardial contractile reserve either in Trx1-Tg or wt septic mice. Nevertheless, we observed that the overexpression of Trx1 helped to maintain a better performance in inotropic responsiveness to beta-adrenoceptors in early sepsis.

Current data indicate that there is an altered inotropic response in early stages of sepsis, and that it depends on increased levels of catecholamines (Bernardin et al., 1998). Multiple factors have been pointed out (Romero-Bermejo et al., 2011; Rudiger and Singer, 2007), but the most suggested ones are the modulation

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of signaling of β −adrenergic receptors, and the alteration in the calcium flux in cardiac sarcoplasmic reticulum (Bernstein et al., 2011; de Montmollin et al., 2009). Trx1 was shown to reduce sarcomeric proteins that participate in the myocardial contractile function (Fu et al., 2009); then, it could be feasible that Trx1 in the cardiomyocytes creates a better redox control and function of protein components in calcium flux that preserve a better myocardial contraction (Reyes-Juarez and Zarain-Herzberg, 2006). Another possible action could be that Trx1 attenuates sepsis-induced production of proinflammatory cytokines, similar to other antioxidants previously studied like resveratrol (Hao et al., 2013).

Sepsis is characterized by antioxidant systems depletion, rendering organs vulnerable to oxidative injury (MacDonald et al., 2003). Trx1 is a multi-functional protein that has a redox-active site through which it interacts with other proteins to regenerate proteins damaged by ROS, and regulates several transcriptions factors such as NF-kB. This redox- regulated factor is also able to increase the expression of genes associated with the immune response and antioxidant systems (Hirota et al., 1999). In such a way, we examined whether Trx1 protects or affects other cytosolic and mitochondrial antioxidant enzymes in heart tissue. We found a decreasing trend in catalase activity, analogous to that reported previously by other authors in sepsis (Llesuy et al., 1994; Ritter et al., 2003), which was not protected in transgenic mice. On the contrary, Trx1 overexpression seems to preserve MnSOD activity even at late stages of sepsis. In the same way, our results are comparable to those previously reported in other oxidative stress models by Li et al. (2006).

Regarding Trx2, our data shows that Trx1 overexpression appears to increase Trx2 expression levels at baseline. Preceding evidence has indicated that Trx2 plays a crucial role in scavenging ROS in mitochondria and in the regulation of mitochondrial apoptosis signaling pathways (Tanaka et al., 2002), and correlates with the metabolic activity of the tissue. Our results showed an increasing expression of Trx2 as a response to sepsis only in wt mice, in a period where rising oxidative stress seemed to be controlled in the mitochondria. Then, a drop at 24 h post-CLP was observed, which coincides with a significant reduction in mitochondrial complexes I and IV, increased protein oxidation, and exhaustion of MnSOD activity.

Both thioredoxin isoforms significantly contribute to antioxidant cell defense due not only to their capability to repair catalytic activity of peroxiredoxins and glutathione peroxidases decomposing hydroperoxides and H_2O_2, but also to the ability of Trx1 to directly reduce both H_2O_2 and oxidized glutathione (Kalinina et al., 2008). It has been previously shown that CLP increases mitochondrial lipid peroxidation and increases the oxidized glutathione/glutathione ratio (Escames et al., 2007). Thus, it could be possible that overexpression of Trx1 attenuated the increase of oxidized glutathione levels in our model. More studies should be done to support this hypothesis.

Recently, Benhar reported that thioredoxin plays an active role in attenuating nitric oxide (NO) signaling as well as ameliorating nitrosative stress (Benhar, 2015). At the same time, Trx can also support the activity of NO synthases, thus promoting NO production. We measured NO synthase activity and expression in heart homogenates, but we could not detect any differences between the transgenic and wt tissues at any time of sepsis (data not shown). In our knowledge, there are no reports on the effects of Trx1 overexpression on cardiac NO production.

Mitochondrial dysfunction is a well-known component of the pathogenic mechanisms in sepsis. Membrane integrity damage, increasing oxidative stress, altered antioxidant defenses (Zang et al., 2007), and ultrastructure abnormalities are included (Azevedo, 2010; Singer, 2007, 2013). We demonstrated alterations in the heart mitochondrial function with decreasing complex activities during the progression of sepsis with significant reduction in complex I and IV activity after 24 h. Other studies in cardiac mitochondria following the CLP model have shown similar findings, such as decreasing myocardial cytochrome oxidase activity and oxygen consumption after 24–48 h of severe sepsis (Groening et al., 2011; Verma et al., 2009).

The fall in mitochondrial membrane potential may indicate several potential situations. First, a response mechanism to β-adrenergic stimulation, which slightly dissipates proton motive force in correlation with accelerated electron flux and highest O_2 consumption (Nickel et al., 2013); and second, a mechanism in which ROS formation is reduced possibly by dissipation of $\Delta\psi_m$ mediated by ROS–induced activation of ion channels in the inner mitochondrial membrane (Zorov et al., 2000). Mild uncoupling activity by UCP isoforms UCP2 and UCP3, could also participate in it. Even though it has been accepted that the uncoupling of mitochondrial respiration by mitochondrial UCPs inhibits ROS production in cardiomyocytes (Wheeler, 2011; Korshunov et al., 1997), some authors have not detected UCP2 expression in polymicrobial sepsis (Roshon et al., 2003). However, they have found myocardial UCP2 mRNA increased expression at 12 h compared with control hearts. Our results do not support the uncoupling theory. Reduced transcription of UCP2 gene at early sepsis could be related with the prolonged β-adrenergic stimulation; and then, at very late sepsis stages, a sudden rise in mRNA expression levels at 24 h in Trx1-Tg could be a cardioprotective mechanism (Kukat et al., 2014).

The protection of mitochondrial integrity and quality is the task of cellular programs that monitor and replace dysfunctional mitochondria with new organelles. This process of mitochondrial quality control involves the transcription and replication of mitochondrial DNA, mitochondrial protein synthesis, mitochondrial fusion and fission, mitochondrial autophagy and mitochondrial proliferation, as well as reorganization of the cytoskeleton. Disruption of these mechanisms involving mitochondrial dynamics and mitophagy has recently been linked to various cardiac diseases (Ikeda et al., 2015).

In this study, we observed a decrease in OPA 1 protein expression and an increase in Drp1 activation in wt mice at late sepsis, which were concomitant with alterations in cristae architecture and mitochondrial membrane disruption, vacuolization and cytoskeletal disorganization. Trx1 overexpression delayed and attenuated these structural abnormalities. Reduction of OPA 1 levels in the inner mitochondrial membrane of depolarized mitochondria could contribute to the prevention of the damaged mitochondria to be fused, targeting them for mitophagy. Evidence of mitochondrial autophagy was observed by electron microscopy in the left ventricle in both groups at 24 h post-CLP. These findings coincided with other reports (Hsieh et al., 2011; Takahashi et al., 2013), which showed the formation of autophagosomes also at 24 h in mice hearts. We observed that LC3-I lipidation appeared at early sepsis in transgenic mice and that it was significantly higher than in wt animals in the progression of sepsis. At the same time, a 2.5- fold increase of PGC-1α mRNA in Trx1-Tg 24 h after CLP, suggests the activation of mitochondrial biogenesis only in these mice. Similar results were obtained by administration of a CO-releasing compound (Lancel et al., 2009).

Mitophagy induced by cardiac stress can remove damaged dysfunctional mitochondria, thus preventing oxidative damage, which can otherwise initiate apoptosis and, ultimately, heart failure. In this study, we did not observe signs of apoptosis either by the TUNEL assay, or mitochondrial cytochrome *c* release (data not shown).

In summary, Trx1 cardiac-specific overexpression extended antioxidant protection and preserved myocardial contractile reserve by attenuating mitochondrial damage and by activating mechanisms of mitochondrial turnover (mitophagy and mitochon-

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drial biogenesis), which could be related to increased survival in septic mice.

Conflicts of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2016.08.045.

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