Role of Thioredoxin-1 on Myocardial Stunning in Transgenic Mice

Rol de la tiorredoxina-1 en el atontamiento cardíaco en ratones transgénicos

TAMARA MAZO1, VIRGINIA PÉREZ1,2, ANABELLA GÓMEZ1, CLARA LLAMOSAS1, LAURA VALDEZ2, TAMARA ZAobORnyJ2, LILIANA NICoLOs2MTAc, 3, MARÍA C. RUBIo2MTAc, 3, VERÓNICA D’ANNUNZIoMTAc, 1,2, RICARDO J. GELPI1MTAc, 1,2

ABSTRACT

Background: Postischemic ventricular dysfunction (myocardial stunning) involves increased oxidative stress. In this sense, the cell has defense mechanisms, as thioredoxin-1, an antioxidant that protects the myocardium from ischemia/reperfusion injury, reducing infarct size.

Objective: The aim of this study was to evaluate systolic and diastolic ventricular function, specifically analyzing myocardial stiffness and isovolumic relaxation, during myocardial stunning in different transgenic mice.

Methods: Hearts from mice overexpressing thioredoxin-1 and transgenic mice overexpressing thioredoxin-1 with gene mutation in its active site (dominant negative) were compared with hearts from non-transgenic mice after 15-minute global ischemia and 30-minute reperfusion using the Langendorff technique. Systolic and diastolic ventricular function was evaluated and t63 was calculated as ventricular relaxation index.

Results: At 30-minute reperfusion, thioredoxin-1 mice showed a significantly improved contractile state (57.4±4.9 mmHg; p ≤ 0.05 vs. non-transgenic mice) and stiffness (11.8±2.9 mmHg; p ≤ 0.05 vs. non-transgenic mice). Conversely, at the same reperfusion time, dominant negative mice exhibited increased stiffness (37.7±5.5 mmHg; p ≤ 0.05 vs. non-transgenic mice) and slower relaxation (78.2±9.8 ms; p ≤ 0.05 vs. non-transgenic mice).

Conclusion: This study reveals the protective role of thioredoxin-1 on myocardial stunning and its pathophysiological importance in mice overexpressing this antioxidant.

Key words: Myocardial Stunning - Oxidative Stress - Thioredoxins - Ventricular Function

RESUMEN

Introducción: La disfunción ventricular posisquémica (miocardio atontado) involucra un aumento del estrés oxidativo. En este sentido, la célula cuenta con mecanismos de defensa, como la tiorredoxina-1, un antioxidante que protege al miocardio de la lesión por isquemia/reperfusión, reduciendo el tamaño del infarto.

Objetivo: Evaluar el comportamiento de la función ventricular sistólica y diastólica, particularmente estudiando la rigidez miocárdica y la relajación isovolumécnica en el miocardio atontado en diferentes ratones transgénicos.

Material y métodos: Se utilizaron corazones de ratones que sobreexpresan tiorredoxina-1 y de ratones transgénicos que sobreexpresan tiorredoxina-1 mutada en su sitio activo (dominante negativo), comparados con los de ratones no transgénicos, los cuales fueron sometidos a 15 minutos de isquemia global y 30 minutos de reperfusión utilizando la técnica de Langendorff. Se evaluó la función ventricular sistólica y diastólica y se calculó el t63 como índice de relajación isovolumécnica.

Resultados: Las mediciones a los 30 minutos de reperfusión mostraron una mejoría significativa del estado contráctil en los ratones tiorredoxina-1 (57,4 ± 4,9 mm Hg; p ≤ 0.05 vs. no transgénicos) y también en la rigidez (11,8 ± 2,9 mm Hg; p ≤ 0.05 vs. no transgénicos). Por otra parte, en los ratones dominantes negativos se observó un aumento de la rigidez (37,7 ± 5,5 mm Hg; p ≤ 0.05 vs. no transgénicos) y un enlentecimiento de la relajación a los 30 minutos de la reperfusión (78,2 ± 9,8 mseg; p ≤ 0.05 vs. no transgénicos).

Conclusion: Este trabajo evidencia el rol protector de la tiorredoxina-1 en el miocardio atontado y su importancia fisiopatológica en ratones que sobreexpresan este antioxidante.

Palabras clave: Aturdimiento miocárdico - Estrés oxidativo - Tiorredoxinas - Función ventricular
INTRODUCTION
Myocardial stunning is a reversible post-ischemic ventricular dysfunction occurring after a short ischemic period followed by reperfusion, characterized by decreased contractile state together with abnormal diastolic function. (1) It is important to emphasize that this pathophysiological entity is present in several clinical and surgical situations, as patients submitted to reperfusion therapies with thrombolytic agents, angioplasty and coronary artery bypass graft surgery. (2) Therefore, due to the clinical relevance of myocardial stunning, it is important to study the involved pathophysiological mechanisms.

After a period of ischemia-reperfusion (I/R), post-ischemic ventricular dysfunction, known as myocardial stunning, involves abnormal calcium ($Ca^{2+}$) homeostasis together with increased stress and oxidative injury. (3, 4) One of the particularly damaged organelles is the mitochondrion, which in turn produces more oxidative stress. Numerous authors have pointed out that during reperfusion there is a marked increase in the mitochondrial production of superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$), leading to oxidative stress and damage and cellular injury. (5) Boveris et al. established that the production of $O_2^-$ and $H_2O_2$ occurs in physiological conditions, (6, 7) and later it was acknowledged that they are increased in I/R. (8, 9) In this sense, previous experiments performed in our laboratory in an isolated rabbit heart model of myocardial stunning showed that ventricular dysfunction is associated to mitochondrial dysfunction known as “complex I syndrome”, which includes decreased tissue and mitochondrial oxygen consumption, and a significant increase in the production of $H_2O_2$ and peroxynitrites (ONOO-). (10) Consequently, it is clear that during I/R injury there is increased stress and oxidative injury, and that it is of interest to examine the role of antioxidant systems in this pathophysiological entity.

The cell has several antioxidant protective mechanisms, among which the thioredoxin (Trx) system is one of the most important antioxidant systems known so far. (11) Specifically, Trx1 (12 kDa) is an important component of cell defense against myocardial injury having antiapoptotic, (12) anti-inflammatory (13) and protective effects against I/R injury. (14, 15) However, there is no sufficient experimental evidence demonstrating the effects of the Trx system on ventricular function, especially on the contractile state and diastolic function in a protocol of myocardial stunning. Regarding Trx and myocardial stunning, Yoshioka et al. reported that deficiency in thioredoxin-interacting protein (TXNIP), a protein that binds and inhibits cytosolic Trx1 and mitochondrial Trx2, improves mitochondrial and ventricular function recovery in the stunned myocardium. (16) They found a clear improvement in ventricular function contractility, but did not study diastolic function in detail or the relationship between mechanical dysfunction and Trx1.

Therefore, the purpose of this work was to assess the behavior of systolic and diastolic ventricular function, particularly analyzing myocardial stiffness and isovolumic relaxation during myocardial stunning in transgenic mice overexpressing Trx1 and in transgenic mice overexpressing Trx1 with mutation in its active site (DN-Trx1), compared with non-transgenic mice (NTG).

METHODS
Male, three-month old non-transgenic (NTG) FVB mice and transgenic mice on FVB background, weighing 24.2±1.5 g, were used. Transgenic mice had cardiac-specific Trx1 overexpression through the α-myosin heavy chain promoter, and Trx1 with mutation in the active site (dominant negative, DN-Trx1), respectively (Stratagene, La Jolla, California, USA). DN-Trx1 mice had virtually no Trx1 enzymatic activity.

Experimental model
Mice were anesthetized with an intravenous injection of pentobarbital (150 mg/kg) and sodium heparin (500 IU/kg). Then, following thorax opening, the aorta was isolated and was immediately cannulated with a 21G catheter. Then the hearts were mounted in a Langendorff system and perfused with Krebs-Henseleit solution, composed of (in mM): NaCl 118.5, KCl 4.7, NaHCO$_3$ 24.8, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, CaCl$_2$ 1.5, and glucose 10. The solution was continuously bubbled with carbogen (95% $O_2$, 5% $CO_2$ at pH 7.4 and 37º C) to maintain oxygenation and physiological pH. A latex balloon filled with an aqueous solution was connected through a thin plastic catheter (P50) to a pressure transducer (Deltram II, Utah Medical System); the balloon was then inserted into the left atrium into the left ventricle and filled to attain an end-diastolic pressure of 10 mmHg. Two electrodes were placed at the base of the atra and connected to a pacemaker to obtain continuous heart rate throughout the experiment. Coronary perfusion pressure (CPP) was also recorded using a pressure transducer connected to the perfusion catheter. All hearts were perfused at constant flow. Real time left ventricular pressure and CPP were digitally recorded using a computer with data acquisition hardware. Left ventricular developed pressure (LVDP) was calculated as the difference between left ventricular maximum systolic pressure and end-diastolic pressure (LVEDP).
left ventricular pressure rise, or time derivative of pressure (+dP/dt$_{max}$) were measured. After 20 minutes (min) of stabilization, all hearts were submitted to 15 min global ischemia followed by 30 min reperfusion. Left ventricular developed pressure and +dP/dt$_{max}$ were used as indicators of contractile state, and LVEDP as indicator of myocardial stiffness. The t63 index and the t93 index, defined as the time required for 63% and 93% drop in left ventricular pressure, respectively, were calculated as isovolumic relaxation indexes.

Statistical analysis
The Kolmogorov-Smirnov test was used to test normal distribution of data. Results were expressed as mean±standard error of the mean. Comparison among groups was performed using one way analysis of variance (ANOVA) followed by the Bonferroni test for multiple comparisons. A p value ≤0.05 was considered statistically significant.

Ethical considerations
The study was performed according to the American Psychological Society “Guide for the Care and Use of Laboratory Animals” (NIH Publication 85-23, 1996). The experimental protocol was approved by the Committee for the Care and Use of Laboratory Animals of the University of Buenos Aires, according to Argentine regulations (ANMAT) for the use of experimental animals (Resolution N° 1-47-1113-800-13 July 9, 2013).

RESULTS
Table 1 shows mean baseline hemodynamic variables. Table 2 shows that there were no significant differences in baseline mean systolic (LVDP and +dP/dt$_{max}$) and diastolic (LVEDP, -dP/dt$_{min}$, t63 and t93) ventricular function among NTG, Trx1 and DN-Trx1 groups.

Figure 1 illustrates the contractile behaviour represented by left ventricular developed pressure (LVDP, panel A) and time derivative of pressure (+dP/dt$_{max}$, panel B). No significant differences among groups were found in baseline LVDP; however, at 30 min of reperfusion there was a clear improvement in the contractile state of the Trx1 group compared with NTG and DN-Trx1 groups (Trx1: 57.4±4.9 mmHg, p≤0.05 NTG: 27.1±6.3 y DN-Trx1: 29.2±7.1 mmHg). The contractile behavior of dP/dt$_{max}$ was similar to that of LVDP.

Figure 2 illustrates left ventricular end-diastolic pressure as index of myocardial stiffness (LVEDP, panel A). At 30 min of reperfusion, a marked increase in LVEDP was observed in the NTG group (24.5±4.8 mmHg), whereas Trx1 mice evidenced a significant improvement of myocardial stiffness (11.8±2.9 mmHg, p≤0.05 vs. NTG and DN-Trx1). In turn, the DN-Trx1 group showed exacerbated LVEDP at 30 min of reperfusion (37.7±5.5 mmHg, p≤0.05 vs. NTG and Trx1). Regarding t63 (panel B), slower relaxation could be seen at the onset of reperfusion (1.5 s) in NTG and DN-Trx1 groups (NTG: 63.3±3.2 and DN-Trx1: 65.4±5.2 ms). However, Trx1 mice improved ventricular relaxation compared with NTG and DN-Trx1 groups (Trx1: 51.4±1.9 ms, p<0.05 vs. NTG and DN-Trx1). At the end of reperfusion (30 min) both NTG and Trx1 groups returned to similar preischemic t63 values (NTG: 57.1±2.1 and Trx1: 47.5±2.5 ms). Yet, an exacerbated slowing of relaxation was seen in the DN-Trx1 group compared with NTG and Trx1 mice (78.2±9.8 ms, p<0.05 vs. NTG and Trx1).

DISCUSSION
Results showed improved contractile function in the group of mice overexpressing Trx1, compared with NTG and DN-Trx1 groups. Trx1 mice also evidenced lower diastolic stiffness than NTG and DN-Trx1 groups; in turn, DN-Trx1 mice displayed increased stiffness compared with the NTG group. Finally, myocardial relaxation was slower in the DN-Trx1 group compared with NTG and Trx1 groups.

It is known that stunned myocardium presents diastolic dysfunction, with different behavior for each of the two subphases, i.e. relaxation and myocardial stiffness. This dissociation occurs because in early reperfusion there is abnormal relaxation, while stiffness remains within normal ranges. Conversely, during late reperfusion, lusotropic abnormalities return to normal, while myocardial stiffness increases. (16,

### Table 1. Mean baseline hemodynamic variables

<table>
<thead>
<tr>
<th></th>
<th>Coronary flow</th>
<th>Heart rate</th>
<th>Coronary perfusion pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>4.0±0.2 ml/min</td>
<td>472.1±30.2 lat/min</td>
<td>73.1±3.1 mmHg</td>
</tr>
</tbody>
</table>

### Table 2. Mean baseline hemodynamic variables in each mice group

<table>
<thead>
<tr>
<th>Strains</th>
<th>LVDP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>+dP/dt$_{max}$ (mmHg/s)</th>
<th>-dP/dt$_{min}$ (mmHg/s)</th>
<th>t63 (ms)</th>
<th>t93 (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTG</td>
<td>92.4±6.7</td>
<td>9.1±0.8</td>
<td>3,259±344</td>
<td>2,786±282</td>
<td>44.5±2.1</td>
<td>62.9±2.8</td>
</tr>
<tr>
<td>Trx1</td>
<td>91.9±4.2</td>
<td>8.3±0.7</td>
<td>3,234±251</td>
<td>2,698±196</td>
<td>44.3±2.0</td>
<td>65.1±2.8</td>
</tr>
<tr>
<td>DN-Trx1</td>
<td>95.8±4.2</td>
<td>8.2±0.7</td>
<td>3,490±400</td>
<td>2,950±370</td>
<td>42.3±2.1</td>
<td>60.8±3.2</td>
</tr>
</tbody>
</table>

LVDP: Left ventricular developed pressure. LVEDP: Left ventricular end-diastolic pressure. +dP/dt$_{max}$: Maximum time derivative of pressure. -dP/dt$_{min}$: Minimum time derivative of pressure. t63: Relaxation index, time for 63% ventricular relaxation. t93: Relaxation index, time for 93% ventricular relaxation. NTG: Non-transgenic group. Trx1: Thioredoxin-1 group. DN-Trx1: Dominant negative group, with Trx1 mutation in the active site.
17) This diastolic behavior was confirmed in our model in the NTG group with abnormal relaxation during early reperfusion and increased stiffness during late reperfusion. It is interesting to observe that in the DN-Trx1 group, abnormal relaxation and increased stiffness occurred during the whole reperfusion period, without respecting the afore-mentioned periods. Conversely, abnormal relaxation and increased stiffness were lower in the Trx1 group than in NTG mice, with a tendency to return to preischemic values towards end-reperfusion.

In addition, the present data show a direct relationship between Trx1 overexpression and systolic ventricular function, since the Trx1 group showed improved contractile recovery of the stunned myocardium compared with NTG and DN-Trx1 groups. The DN-Trx1 group of mice, where overexpressed Trx1 has a mutation of its active state, and therefore, practically null activity, did not evidence either improved or impaired contractile state with respect to the NTG group, different from diastolic function parameters. This dissociation between contractile state and diastolic function is perhaps due to different mechanisms involved in Trx1 cardioprotection against I/R injury.

Regarding ventricular function, other authors have demonstrated improved recovery in myocardial stunning protocols with exogenous antioxidant administration. (18, 19) However, there are no studies evaluating ventricular function without antioxidant activity, as in the DN-Trx1 group. Asimakis et al., using two models of transgenic mice, one with cytosolic SOD deficiency (SOD1) and the other with mitochondrial SOD deficiency (SOD2), found that only partial SOD2 deficiency negatively affected ventricular function recovery compared with NTG control mice. (20) They attributed this difference to the possible greater mitochondrial contribution to oxidative stress generation, and hence, only observed changes in the model with mitochondrial SOD deficit. A difference between this work and the present
study is that they used 30 min of ischemia, where the presence of necrotic tissue masks correct ventricular function assessment. In our model, although the ischemic time was lower (15 min), cytosolic Trx1 deficiency appeared to have a deleterious effect on diastolic ventricular function recovery, without changes in contractile state recovery. This is a novel finding, as it postulates dissociation between systolic and diastolic mechanisms involving Trx1. Also, Yoshioka et al. (21) studied the effects of the protein system regulating the activity of Trx-1 and Trx-2 on ventricular function in a model of myocardial stunning. Different from our study, these authors did not study diastolic ventricular function in detail; moreover, they used a transgenic model with deficiency in thioredoxin interacting protein (TXNIP), inhibiting both Trx1 as Trx2, so that it was not possible to identify whether the beneficial effects on ventricular function were due to cytosolic or mitochondrial Trx. Consequently, our work extends this knowledge, by studying in detail the effects of cytosolic Trx1 on diastolic ventricular function, and proposes the analysis of protein signaling involved in relaxation and its association with mitochondrial function.

As already mentioned, abnormal Ca2+ homeostasis as well as generation of oxidative stress are involved in myocardial stunning ventricular dysfunction. In abnormal Ca2+ homeostasis, the pathophysiological mechanisms of stunning are associated with intracellular Ca2+ overload and altered response of contractile proteins to Ca2+. In turn, the latter could be due to covalent modifications produced by reactive oxygen species (ROS) that damage contractile proteins, modifying their Ca2+ sensitivity. (22) Regarding generation of oxidative stress, Bolli et al. (23, 24) were the first to demonstrate ROS participation in the pathophysiological mechanism of myocardial stunning, strongly suggesting that increased ROS elicits lipid peroxidation and consequently favors protein denaturation, with the ensuing alteration of membrane permeability and enzyme and organelle functioning. (25-27)

CONCLUSION
This study shows the protective role of Trx1 in stunned myocardium in mice overexpressing this antioxidant. Moreover, it shows the importance of this protein, as on the one hand, Trx1 overexpression improves both systolic and diastolic ventricular function, but on the other, deficiency in its performance alters diastolic function recovery compared with NTG control mice. Further studies are necessary to elucidate the intracellular mechanisms involved in the protection conferred by Trx1, in order to postulate possible new treatments for the stunned myocardium of patients submitted to reperfusion therapies, thus avoiding risks and complications.

Conflicts of interest
None declared

REFERENCES
27. Wills ED. Effects of lipid peroxidation on membrane-bound enzymes of the endoplasmic reticulum. Biochem J 1971;123:983-91