The International Journal of Biochemistry & Cell Biology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

Inhibition of endogenous thioredoxin-1 in the heart of transgenic mice does not confer cardioprotection in ischemic postconditioning

Virginia Perez (Doctoral Fellow of the National Council of Scientific and Technological Research (CONICET))[a,b,1], Verónica D′ Annunzio (Members of the National Council of Scientific and Technological Research (CONICET))[a,b,1], Tamara Mazo (Doctoral Fellow of the National Council of Scientific and Technological Research (CONICET))[a,b], Timoteo Marchini (Postdoctoral Fellow of the National Council of Scientific and Technological Research (CONICET))[c], Lourdes Caceres[c], Pablo Evelson (Members of the National Council of Scientific and Technological Research (CONICET))[c], Ricardo J. Gelpi (Members of the National Council of Scientific and Technological Research (CONICET))[a,b,*]

[a] *Institute of Cardiovascular Physiopathology, Department of Pathology, Faculty of Medicine, University of Buenos Aires, Argentina*
[b] *Institute of Biochemistry and Molecular Medicine (IBIMOL UBA-CONICET), Faculty of Medicine, Argentina*
[c] *Institute of Biochemistry and Molecular Medicine (IBIMOL UBA-CONICET), School of Pharmacy and Biochemistry; University of Buenos Aires, Buenos Aires, Argentina*

ARTICLE INFO

Article history:
Received 13 July 2016
Received in revised form
21 September 2016
Accepted 24 September 2016
Available online xxx

Keywords:
Ischemic postconditioning
Myocardial infarction
Oxidative stress
Thioredoxin-1
Dominant negative of Trx1

ABSTRACT

Thioredoxin-1 maintains the cellular redox status and decreases the infarct size in ischemia/reperfusion injury. However, whether the increase of thioredoxin-1 expression or its lack of activity modifies the protection conferred by ischemic postconditioning has not been yet elucidated. The aim was to evaluate if the thioredoxin-1 overexpression enhances the posctconditioning protective effect, and whether the lack of the activity abolishes the reduction of the infarct size. Wild type mice hearts, transgenic mice hearts overexpressing thioredoxin-1, and a dominant negative mutant (C32S/C35S) of thioredoxin-1 were used. The hearts were subjected to 30 min of ischemia and 120 min of reperfusion (Langendorff) (I/R group) or to postconditioning protocol (PostC group). The infarct size in the Wt-PostC group decreased in comparison to the Wt-I/R group (54.6 ± 2.4 vs. $39.2 \pm 2.1\%$, $p < 0.05$), but this protection was abolished in DN-Trx1-PostC group ($49.7 \pm 1.1\%$). The ischemia/reperfusion and postconditioning in mice overexpressing thioredoxin-1 reduced infarct size at the same magnitude (35.9 ± 2.1 and $38.4 \pm 1.3\%$, $p < 0.05$ vs. Wt-I/R). In Wt-PostC, Trx1-I/R and Trx1- PostC, Akt and GSK3β phosphorylation increased compared to Wt-I/R, without changes in DN-Trx1 groups. In conclusion, given that the cardioprotection conferred by thioredoxin-1 overexpression and postconditioning, is accomplished through the activation of the Akt/GSK3β survival pathway, no synergic effect was evidenced. Thioredoxin-1 plays a key role in the postconditioning, given that when this protein is inactive the cardioprotective mechanism was abolished. Thus, diverse comorbidities or situations modifying the thioredoxin activity, could explain the absence of this strong mechanism of protection in different clinical situations.

Abbreviations: PostC, ischemic postconditioning; ROS, reactive oxygen species; RNS, reactive nitrogen species; I/R, ischemia/reperfusion; Trx1, thioredoxin-1; GSH/GSSG, reduced/oxidized glutathione; DN-Trx1, dominant negative mutant of Trx1; h-Trx1, human Trx1; LVDP, left ventricular developed pressure; $+dP/dt_{max}$, maximal rate of rise of left ventricular pressure; LVEDP, left ventricular end diastolic pressure; CPP, coronary perfusion pressure; LV, Left ventricle; Wt, Wild type; TTC, 2, 3, 5-triphenyltetrazolium chloride; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene fluoride; SEM, standard error of the mean; Nx, normoxic; PC, ischemic preconditioning.

* Corresponding to: Institute of Cardiovascular Physiopathology, Faculty of Medicine, and University of Buenos Aires, JE Uriburu 950–2nd floor, Buenos Aires (1114), Argentina.
E-mail address: rgelpi@fmed.uba.ar (R.J. Gelpi).
[1] Both authors contributed equally to this manuscript.

http://dx.doi.org/10.1016/j.biocel.2016.09.017
1357-2725/

1. Introduction

Ischemic PostC is a potent cardioprotective mechanism that reduces the infarct size (Zhao et al., 2003; Buchholz et al., 2014a; Donato et al., 2010). In the pioneer study of PostC, Zhao et al. (2003) demonstrated that this novel mechanism in open-chest dogs, exerted its protective effect by reducing the endothelial activation and the neutrophil adherence to the vessels, and as a result of these actions, redox-sensible mechanisms were attenuated. Several authors also described that PostC reduced oxidative stress in *in vitro* models (Heusch, 2015a; Kin et al., 2004; Liu et al., 2007; Cai et al., 2011), independent from inflammation and vascular component. Given that it is well known that during reperfusion a burst of ROS and RNS occur, an important target to avoid the deleterious effects is the reduction of oxidative stress (Zhao et al., 2003; Schwartz and Lagranha, 2006; Iliodromitis et al., 2006). Therefore, one of the mechanisms widely attributed to the cardioprotection conferred by PostC against I/R injury, is the ROS and RNS reduction. Also, PostC involves signal transduction pathways that are triggered by several autacoids such as adenosine, acetylcholine, opioids, and also adrenergic receptors that activate protective signaling pathways including RISK signaling, mainly PI3K-Akt and GSK3β (Tsang et al., 2004; Hausenloy et al., 2005; Buchholz et al., 2014a,b). However, it is unclear whether the activation of these intracellular signaling pathways is linked to the changes evidenced in oxidative stress.

Trx1 (12KDa) is an endogenous system that regulates redox homeostasis and has cardioprotective effects against I/R injury (Tsang et al., 2004; Hausenloy et al., 2005; Buchholz et al., 2014b; Aota et al., 1996; Zitta et al., 2015; Tao et al., 2004). In this sense, we previously showed that Trx1 reduced infarct size in young mice, and this cardioprotection included an increase of Akt and GSK3β phosphorylation (D′ Annunzio et al., 2016). Furthermore, we recently demonstrated that PostC decreases the infarct size in young mice due to a preservation of Trx1 levels after I/R. This preservation of Trx1 levels, as expected, showed an improvement in the tissue redox status, evidenced through the GSH/GSSG ratio in young animals (Perez et al., 2016a). This cardioprotection was accompanied by Akt activation and the phosphorylation and inhibition of GSK3β, both proteins related to cell survival pathways, but we were unable to show direct evidence that Trx1 is involved in the PostC cardioprotection mechanism. However, whether the increase of Trx1 expression and/or its lack of activity could modify the PostC cardioprotective effects, has not been described. Thus, the first objective of our study was to evaluate if the Trx1 overexpression enhances the PostC protective effect, and whether the lack of activity of this antioxidant abolishes the reduction of the infarct size. A second objective was to study if PostC modifies the expression and/or activity of Trx1 and the possible changes occurring in the GSH/GSSG ratio. Finally, a third objective was to study if Akt and GSK3β, pro-survival proteins, are involved in the protection conferred by PostC and its relation with Trx1.

2. Material and methods

2.1. Animal care

All procedures performed in the studies involving animals were in accordance with the ethical standards of the Animal Care and Research Committee of the University of Buenos Aires (CICUAL UBA # 0037016/2013). FVB mice were housed in ventilated cages with a 12 h light/dark cycle and controlled temperature (20–22 °C), and fed with normal chow and water ad libitum.

2.2. Transgenic mice

We have used the transgenic mice from the same colonies of Prof. Junichi Sadoshima, who generously donated these mice to us. Two transgenic mice models were used: 1) transgenic mice with cardiac-specific overexpression of Trx1 generated on an FVB background using the α-myosin heavy chain promoter to achieve cardiac-specific expression (Ago and Sadoshima, 2006; Yamamoto et al., 2003), and 2) DN-Trx1 was generated by mutation of 32Cys and 35Cys of hTrx1 to Ser using QuikChange (Stratagene, La Jolla, California, USA). This redox inactive mutant of Trx1 works as a dominant negative for endogenous Trx1 in mice hearts (Ago and Sadoshima, 2006).Wt mice were also used as control group.

2.3. Isolated mice hearts

Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (150 mg/kg body weight) and sodium heparin (500 UI/kg body weight). The hearts were excised and the aorta was immediately cannulated with a 21 gauge cannula. Afterwards, hearts were perfused according to the Langendorff technique with Krebs solution [118.5 mMNaCl, 4.7 mMKCl, 1.2 mM $KH2PO_4$, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 24.8 mM $NaHCO_3$, 10 mM glucose (pH 7.4)] equilibrated with 95% O_2 and 5% CO_2 at 37 °C, as we previously described (Perez et al., 2016a). The coronary flow was constant during all the experimental procedure and was around 4.2 ± 0.3 mL/min. LVDP and LV + dP/dt_{max} were used as contractile state indexes. LVEDP, a myocardial stiffness index in the isovolumic heart and CPP were also measured. We performed the following experimental groups: 1) I/R group: after stabilization, 30 min of global no-flow ischemia and 120 min of reperfusion were performed; 2) PostC group: I/R group was repeated, but at the onset of reperfusion we performed 6 cycles of 10 s of reperfusion followed by 10 s of ischemia (2 min total intervention). Also we performed a normoxic group that was only used as a control group for the western blot and glutathione determinations. Hearts were perfused during 45 min in normoxic conditions, and the LV samples were taken for their further processing. All the experimental groups were performed in Wt, Trx1 and DN-Trx1 mice.

2.4. Infarct size measurement

The assessment of the infarct size was performed using TTC. After 120 min of reperfusion, the hearts were frozen and cut into 1 mm transverse slices from apex to base. Sections were incubated for 20 min in 1% TTC (pH 7.4, 37 °C) and then immersed in 10% formalin. With this technique, viable sections were stained red, while the non-stained sections corresponded to the infarct area. Sections were traced to acetate sheets and planimetered (Image Pro Plus, version 4.5). Infarct size was expressed as a percentage of the left ventricular area.

2.5. Samples preparation

Tissue samples (0.2 g of wet weight) were homogenized with a glass-Teflon homogenizer in a medium consisting of 120 mM KCl, 30 mM phosphate buffer (pH 7.4) (1:5) at 0–4 °C. The suspension was centrifuged at 600*g* for 10 min at 4 °C to remove nuclei and cell debris. The pellet was discarded and the supernatant was used as "homogenate" (Evelson et al., 2001).

2.6. Reduced (GSH) and oxidized (GSSG) glutathione levels

Heart samples were homogenized with a glass-Teflon homogenizer in a solution containing 1 M HClO4–2 mM EDTA (1:1), and

Please cite this article in press as: Perez, V., et al., Inhibition of endogenous thioredoxin-1 in the heart of transgenic mice does not confer cardioprotection in ischemic postconditioning. Int J Biochem Cell Biol (2016), http://dx.doi.org/10.1016/j.biocel.2016.09.017

centrifuged at 16,000g for 20 min at 4 °C. Supernatants were filtered through 0.22 μm cellulose acetate membranes (Corning Inc., NY, US), and frozen at −80 °C until use. HPLC analysis was performed in a Perkin Elmer LC 250 liquid chromatography (Perkin Elmer, Waltham, MA, US), equipped with a Perkin Elmer LC ISS 200 advanced sample processor, and a Coulochem II (ESA, Bedford, MA, US) electrochemical detector. A Supelcosil LC-18 (250 × 4.6 mm ID, 5 μm particle size) column protected by a Supelguard (20 × 4.6 mm ID) precolumn (Supelco, Bellfonte, PA, US) was used for sample separation. GSH and GSSG were eluted at a flow rate of 1.2 mL/min with 20 mM sodium phosphate (pH 2.7), and electrochemically detected at an applied oxidation potential of +0.800 V. Results were expressed as μM (Rodriguez-Ariza et al., 1994).

2.7. Western blot

We performed additional experiments to obtain heart samples (n = 5 in each group). Heart tissue was homogenized in ice for approximately 2 min with extraction buffer (pH 7.4), composed of: Tris 1.2 mM, NaCl 0.36 mM, SDS 0.1%, Triton 1%, DTT 0.2 mM, protease and phosphatase inhibitors cocktail (Thermo Scientific) at a rate of 500 μL buffer every 150 mg of tissue using a PRO 200 Scientific INC homogenizer. Subsequently, homogenates were centrifuged at 12,608g during 20 min at 4 °C. The supernatant protein concentration was quantified with the Bradford method.

After protein quantification with the Bradford method, 50 μg of each sample were separated by 16% Tricine-SDS-PAGE gels (for Trx1 expression), and by 12% Glycine-SDS-PAGE gels (for pAkt and pGSK3β) and after that transferred to a PVDF membrane (Thermo Scientific) that was later blocked with 5% BSA for 2 h at room temperature. Subsequently, the membrane was incubated with anti-Trx1 (1:1000) (Cell Signaling), anti-phospho-Akt for Serine^{473} residue (1:1000) (Cell Signaling) and anti-phospho-GSK3β for Serine^{9} residue (1:1000) (Cell Signalling) overnight at 4 °C with agitation. It was later incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP, 1:15,000) (Millipore) for an hour at room temperature. The membrane was developed with photographic plates (Kodak) and Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific). Trx1 pAkt^{Ser473} and pGSK3β^{Ser9} expression were quantified by densitometry with Image Gauge 4.0 software (Fujifilm) compared to the charge control values, anti-GAPDH (1:1000, Cell Signaling), measured in the same membranes, were used as loading control.

2.8. Statistical analysis

Data are expressed as mean ± SEM. Inter-group comparisons were performed using analysis of variance and then the Bonferroni test for multiple comparisons. $p < 0.05$ was considered statistically significant.

3. Results

Table 1 shows the systolic function behavior at baseline and during different reperfusion times. In all groups, LVDP (mmHg) and LV +dP/dt$_{max}$ (mmHg/s) were significantly lower compared to pre-ischemic values, but showed no significant differences among groups throughout the procedure. LVEDP (mmHg) and CPP (mmHg) increased at 30 min compared to pre-ischemic values without differences among the groups, although LVEDP (myocardial stiffness) were higher in both DN-Trx1 groups compared with Wt and Trx1 groups.

Fig. 1 shows the behavior of the infarct size in all experimental groups. In Wt animals, as expected, PostC reduced the infarct size (I/R: 54.6 ± 2.4 vs. PostC: 39.2 ± 2.1%, $p < 0.05$). In Trx1 mice, I/R mimics the protective effects of PostC, but in the group Trx1

Table 1
Left ventricular systolic and diastolic function and coronary perfusion pressure.

	Groups	Baseline	30 min Rep
LVDP (mmHg)	Wt-I/R	90.5 ± 4.3	21.4 ± 4.1*
	Wt-PostC	92.9 ± 6.2	16.1 ± 3.4*
	Trx1-I/R	93.3 ± 4.8	20.6 ± 6.2*
	Trx1-PostC	93.5 ± 5.5	12.1 ± 1.1*
	DN-Trx1-I/R	92.0 ± 4.0	19.1 ± 2.2*
	DN-Trx1-PostC	92.2 ± 5.8	15.4 ± 5.0*
LVEDP (mmHg)	Wt-I/R	7.6 ± 0.9	18.5 ± 4.6*
	Wt-PostC	8.1 ± 1.0	21.4 ± 5.5*
	Trx1-I/R	7.3 ± 0.9	18.8 ± 4.1*
	Trx1-PostC	8.1 ± 0.8	18.7 ± 3.7*
	DN-Trx1-I/R	7.1 ± 2.4	38.5 ± 5.4*#
	DN-Trx1-PostC	8.0 ± 1.6	32.4 ± 4.6*#
+dP/dt$_{max}$ (mmHg/s)	Wt-I/R	3847 ± 416	836 ± 126*
	Wt-PostC	4112 ± 469	928 ± 139*
	Trx1-I/R	3982 ± 395	917 ± 250*
	Trx1-PostC	4024 ± 492	870 ± 201*
	DN-Trx1-I/R	3854 ± 365	698 ± 119*
	DN-Trx1-PostC	4106 ± 431	779 ± 103*
CPP (mmHg)	Wt-I/R	76.0 ± 2.4	91.6 ± 9.3*
	Wt-PostC	74.8 ± 2.5	108.3 ± 8.0*
	Trx1-I/R	73.4 ± 2.7	98.0 ± 1.7*
	Trx1-PostC	74.3 ± 3.0	102.8 ± 8.2*
	DN-Trx1-I/R	76.7 ± 4.0	96.7 ± 7.9*
	DN-Trx1-PostC	75.7 ± 3.4	95.8 ± 6.6*

LVDP: Left ventricular developed pressure; LVEDP: Left ventricular end diastolic pressure; +dP/dt$_{max}$: Left ventricular maximal rate of pressure increase; CPP: coronary perfusion pressure. Rep: Reperfusion. *: $p < 0.05$ vs. respective baseline value. #: $p < 0.05$ vs. Wt I/R and PostC, respectively. Wt: wild type; Trx1: transgenic mice thioredoxin-1 overexpression; I/R: Ischemia/Reperfusion; PostC: ischemic postconditioning.

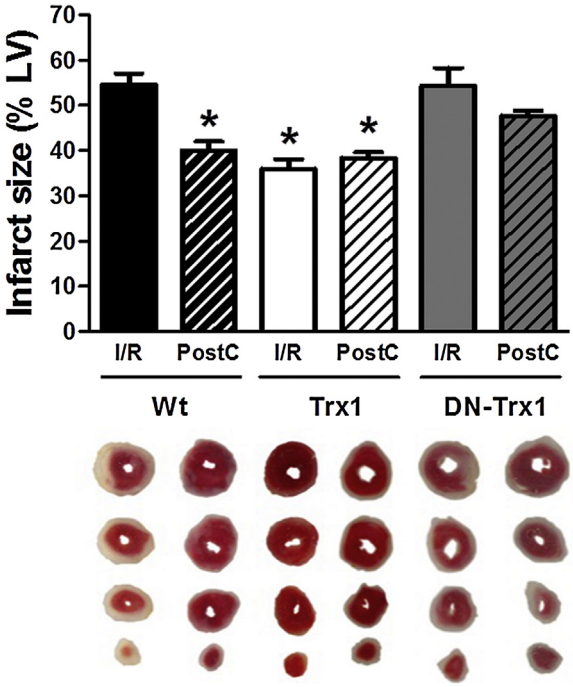

Fig. 1. Panel A shows infarct size expressed as a percentage of the total left ventricular area. Infarct size decreased significantly in Wt-PostC, Trx1-I/R and Trx1-PostC groups compared with Wt-I/R but this cardioprotective effect was abolished in the DN-Trx1-PostC group. There were no differences between Trx1-I/R and Trx1-PostC groups. Panel B shows representative slices of the different experimental groups. *$p < 0.05$ vs. I/R. Wt: wild type; Trx1: thioredoxin-1; DN-Trx1: dominant negative for Trx1; I/R: ischemia/reperfusion; PostC: ischemic postconditioning. n = 7 per group.

Please cite this article in press as: Perez, V., et al., Inhibition of endogenous thioredoxin-1 in the heart of transgenic mice does not confer cardioprotection in ischemic postconditioning. Int J Biochem Cell Biol (2016), http://dx.doi.org/10.1016/j.biocel.2016.09.017

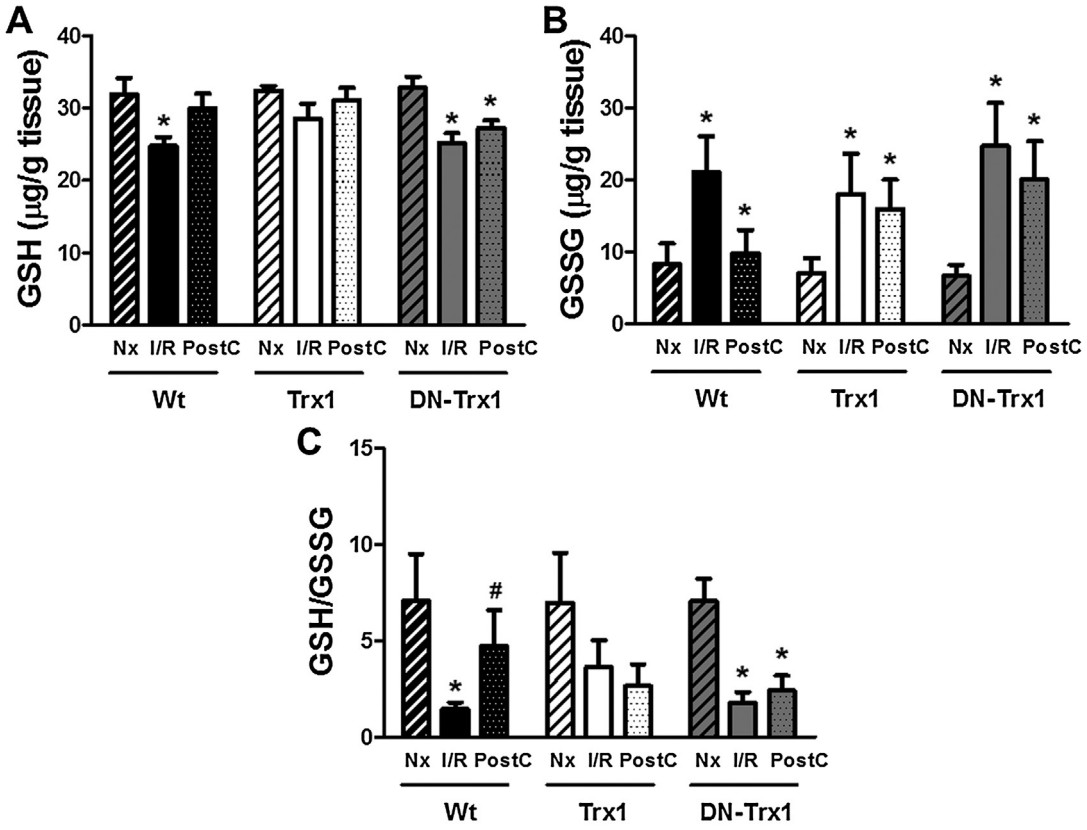

Fig. 2. Oxidative stress. GSH (Panel A), GSSG (Panel B) and GSH/GSSG ratio (panelC). Oxidative stress was significantly increased in Wt-I/R, DN-Trx1-I/R and DN-Trx1-PostC groups, the levels of GSH were decreased and levels of GSSG were increased compared to Nx hearts. However these imbalances in redox homeostasis return to pre-ischemic values after the postconditioning protocol in Wt-PostC groups but not in the DN-Trx1-PostC group. Trx1-I/R and PostC groups did not show differences in oxidative stress. *$p < 0.05$ vs. Nx; #$p < 0.05$ vs. I/R. Wt: wild type; Trx1: thioredoxin-1; DN-Trx1: dominant negative for Trx1; Nx: normoxic; I/R: ischemia/reperfusion; PostC: ischemic postconditioning. n = 5 per group.

with PostC there was not additive cardioprotection (I/R: 35.9 ± 2.1 vs. PostC: 38.4 ± 1.3%, $p < 0.05$). However, in DN-Trx1 the cardioprotective effect of PostC on infarct size reduction was abolished (I/R: 56.3 ± 3.9 vs. PostC: 49.7 ± 1.1%). Panel B shows representative slices of the different experimental groups.

Fig. 2 shows the behavior of oxidative stress in the different mice protocols. We measured the oxidized (GSH) and reduced (GSSG) glutathione concentration (μg/g of tissue) and the ratio between them. In Panel A it can be observed that the GSH concentration significantly reduces after I/R in Wt and DN-Trx1 animals (Wt-Nx: 31.97 ± 2.23 vs. Wt-I/R: 24.76 ± 1.22 and DN-Trx1-Nx: 32.79 ± 1.58 vs. DN-Trx1-I/R: 25.16 ± 1.37, $p < 0.05$) reached to its pre-ischemic values in the PostC only in Wt animals (Wt-PostC: 29.97 ± 2.01 vs. I/R, $p < 0.05$) and in DN-Trx1-PostC it did not modify GSH compared with Nx (27.23 ± 1.09 vs. Nx, $p < 0.05$). In Trx1 animals the GSH concentration did not change between different protocols (Nx: 32.39 ± 0.70, I/R: 28.47 ± 2.17 and PostC: 31.04 ± 1.78). In Panel B it can be observed that the GSSG concentration significantly increases after I/R in Wt, Trx1 and DN-Trx1 animals (Wt-Nx: 8.31 ± 2.86 vs. Wt-I/R: 21.11 ± 4.98; Trx1-Nx: 7.06 ± 2.09 vs. Trx1-I/R: 18.03 ± 5.61 and DN-Trx1-Nx: 6.64 ± 1.60 vs. DN-Trx1-I/R: 24.69 ± 6.00, $p < 0.05$) reaching to its pre-ischemic values in the PostC only in Wt animals (Wt-PostC: 9.82 ± 3.28 vs. I/R, $p < 0.05$), this values did not change when PostC was performed in Trx1 and DN-Trx1 animals (Trx1-PI: 15.90 ± 4.15 and DN-Trx1-PostC: 23.04 ± 5.51 vs. Nx, $p < 0.05$). Finally, in Panel C, the GSH/GSSG ratio can be observed, a reliable indicator of redox status. After I/R, the GSH/GSSG ratio decreases in Wt and DN-Trx1 animals (Wt-Nx: 7.07 ± 2.44 vs. Wt-I/R: 1.47 ± 0.34 and DN-Trx1-Nx: 7.07 ± 1.15 vs. DN-Trx1-I/R: 1.78 ± 0.57, $p < 0.05$) returning to its pre-ischemic values in the PostC only in Wt animals (Wt-PostC: 4.74 ± 1.85 vs. I/R, $p < 0.05$ and DN-Trx1-PostC: 2.43 ± 0.77 vs. Nx, $p < 0.05$). In Trx1 animals the ratio did not change between different protocols (Nx: 6.98 ± 2.58, I/R: 3.64 ± 1.40 and PostC: 2.65 ± 1.13).

Fig. 3 shows, in panel A, that Trx1 expression decreases in the Wt-I/R group (Wt-Nx: 1.00 ± 0.05 vs. Wt-I/R: 0.32 ± 0.09 AUDO, $p < 0.05$), but PostC avoids Trx1 degradation after the I/R protocol (Wt-PostC: 0.65 ± 0.08 AUDO vs. I/R, $p < 0.05$). In both, Trx1 and DN-Trx1 groups, Trx1 expression did not change among Nx, I/R and PostC, but Trx1 levels were 2.5 folds higher in Trx1 and DN-Trx1 compared to Wt Trx1 levels (Trx1-Nx: 2.52 ± 0.14; Trx1-I/R: 2.72 ± 0.07; Trx1-PostC: 2.86 ± 0.11 AUDO and DN-Trx1-Nx: 2.55 ± 0.13; DN-Trx1-I/R: 2.68 ± 0.13; DN-Trx1-PostC: 2.51 ± 0.15 AUDO). In panel B Trx1 activity throughout the time measured in baseline conditions can be observed; at 120 s Trx1 activity was increased in Trx1 animals (Trx1: −0.15 ± 0.02 vs. Wt: −0.07 ± 0.02 nm, $p < 0.05$) and was decreased in DN-Trx1 animals (DN-Trx1: −0.01 ± 0.02 nm vs. Wt, $p < 0.05$) compared with Wt mice.

Fig. 4 shows the expression of p-Akt^{Ser473} (Panel A) and p-GSK3β^{Ser9} (Panel B) normalized by GAPDH. An increased in Akt phosphorylation can be observed in the Wt-PostC group (1.49 ± 0.04 vs. Nx: 1.00 ± 0.04 and I/R: 1.19 ± 0.03 AUDO, $p < 0.05$), and in both protocols of Trx1 mice (I/R: 1.67 ± 0.10 and PostC: 1.49 ± 0.11 vs. Nx: 1.05 ± 0.05 AU and Wt-I/R AUDO, $p < 0.05$). However, we observed a slightly increased in DN-Trx1-I/R mice compared with Nx mice (I/R: 1.29 ± 0.07 vs. Nx: 1.01 ± 0.06 AUDO, $p < 0.05$), but PostC did not improve this augmentation

Please cite this article in press as: Perez, V., et al., Inhibition of endogenous thioredoxin-1 in the heart of transgenic mice does not confer cardioprotection in ischemic postconditioning. Int J Biochem Cell Biol (2016), http://dx.doi.org/10.1016/j.biocel.2016.09.017

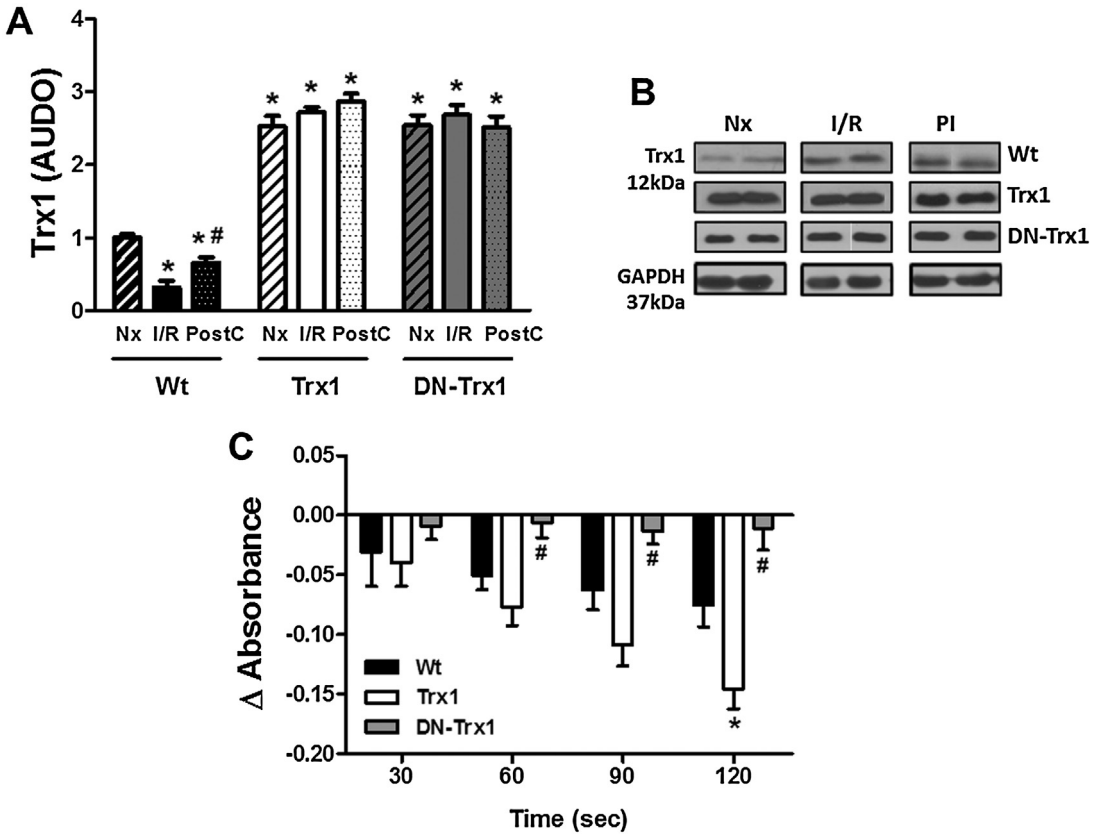

Fig. 3. Trx1 expression (Panel A) and Trx1 activity (Panel C). In Wt animals Trx1 levels were decreased in the I/R group and PostC preserves the protein content. There were no significant changes in Trx1 expression in different protocols of Trx1 and DN-Trx1 groups. Panel B shows representative blots. *p < 0.05 vs. Nx; #p < 0.05 vs. I/R. n = 5 per group. Trx1 activity increased with time in the Trx1 group and was significantly higher than the Wt group at 120 s. Trx1 activity was significantly low in the DN-Trx1 group. *p < 0.05 vs. Wt; #p < 0.05 vs. Trx1. n = 4 per group. Wt: wild type; Trx1: thioredoxin-1; DN-Trx1: dominant negative for Trx1; Nx: normoxic; I/R: ischemia/reperfusion; PostC: ischemic postconditioning. AUDO: Arbitrary units of density optic. n = 6 per group.

(1.33 ± 0.05 AUDO). A similar behavior was observed in relation to GSK3β, but the phosphorylation in Wt-PostC group was higher (PostC: 3.40 ± 0.29 vs. Nx: 1.04 ± 0.02 and I/R: 1.39 ± 0.19 AUDO, p < 0.05) compared with the same intervention in the Trx1 mice (I/R: 1.86 ± 0.14 and PostC: 2.06 ± 0.20 vs. Nx: 1.05 ± 0.04 AUDO, p < 0.05). However, in DN-Trx1-PostC the GSK3β phosphorylation was lower compared to Wt-PostC and Trx1-PostC (DN-Trx1: 1.59 ± 0.38 AUDO vs. Wt and Trx1, p < 0.05). Although we observed a slight increase in DN-Trx1-I/R mice compared with Nx mice (I/R: 1.32 ± 0.28 vs. Nx: 0.93 ± 0.05 AUDO, p < 0.05).

4. Discussion

In the present study we confirmed that Trx1 plays an important role in the PostC protection mechanism. We had indirectly demonstrated previously a possible relation between PostC and Trx1, since PostC avoided the antioxidant's degradation in Wt mice (Perez et al., 2016a). The fact that the reduction of the infarct size that occurs in Wt PostC was abolished in the DN-Trx1 group overexpressing inactive Trx1 strengthens the role of Trx1 in the PostC mechanism. Furthermore Trx1 overexpression decreased the infarct size, as we had previously demonstrated (D' Annunzio et al., 2016), however a PostC protocol did not enhance the cardioprotective effect in these mice. Both PostC and Trx1 overexpression exerted their protective effect through the activation of the Akt/GSK3β cell survival pathway. As we mentioned, these findings were corroborated by the fact that in DN-Trx1 mice, where an overexpression of Trx1 mutated in its active site exists, PostC did not produce a reduction in the infarct size, nor an activation of the Akt/GSK3β cell survival pathway.

The cardioprotective effects of PostC have been demonstrated by several authors, including our previous papers (Zhao et al., 2003; Buchholz et al., 2014a, 2014b; Donato et al., 2010; Tsang et al., 2004; Hausenloy et al., 2005; Perez et al., 2016a). We had previously shown that Trx1 could participate in the PostC mechanism, given that this cardioprotective intervention avoided the Trx1 degradation in Wt mice (Perez et al., 2016a). In the present manuscript, we showed that a direct involvement of Trx1 in PostC could be due to the fact that the PostC cardioprotection was abolished in DN-Trx1 mice, where the activity of Trx1 is almost null.

In mice overexpressing Trx1, PostC did not enhance the protein's protective effect. The lack of synergism when two different cardioprotective interventions were compared had already been demonstrated by other authors (Halkos et al., 2004; Chen et al., 2016). In this sense, Yang et al. (2004) could demonstrate synergism between different cardioprotection mechanisms (PC and PostC) only when they used 45 min of ischemia, where the infarct size was larger, around 65% and therefore it was possible to enhance both protective effects. Furthermore, it was also demonstrated that ischemic remote PC and remote PostC increased the phosphorylation of Akt/GSK3β, with no significant differences between the two procedures in cardioprotection, suggesting that the protective effects of both mechanisms are associated with activation of the RISK pathway. It has been demonstrated that high Trx1 levels inhibit PTEN, and that the PTEN inhibition allows the phosphorylation and activation of the PI3K/Akt complex, triggering a cell survival mechanism. Also, we and other authors evidenced that the activation of cellular membrane receptors coupled with the G protein (Perez et al., 2016a; Buchholz et al., 2014b; Ferdinandy

Please cite this article in press as: Perez, V., et al., Inhibition of endogenous thioredoxin-1 in the heart of transgenic mice does not confer cardioprotection in ischemic postconditioning. Int J Biochem Cell Biol (2016), http://dx.doi.org/10.1016/j.biocel.2016.09.017

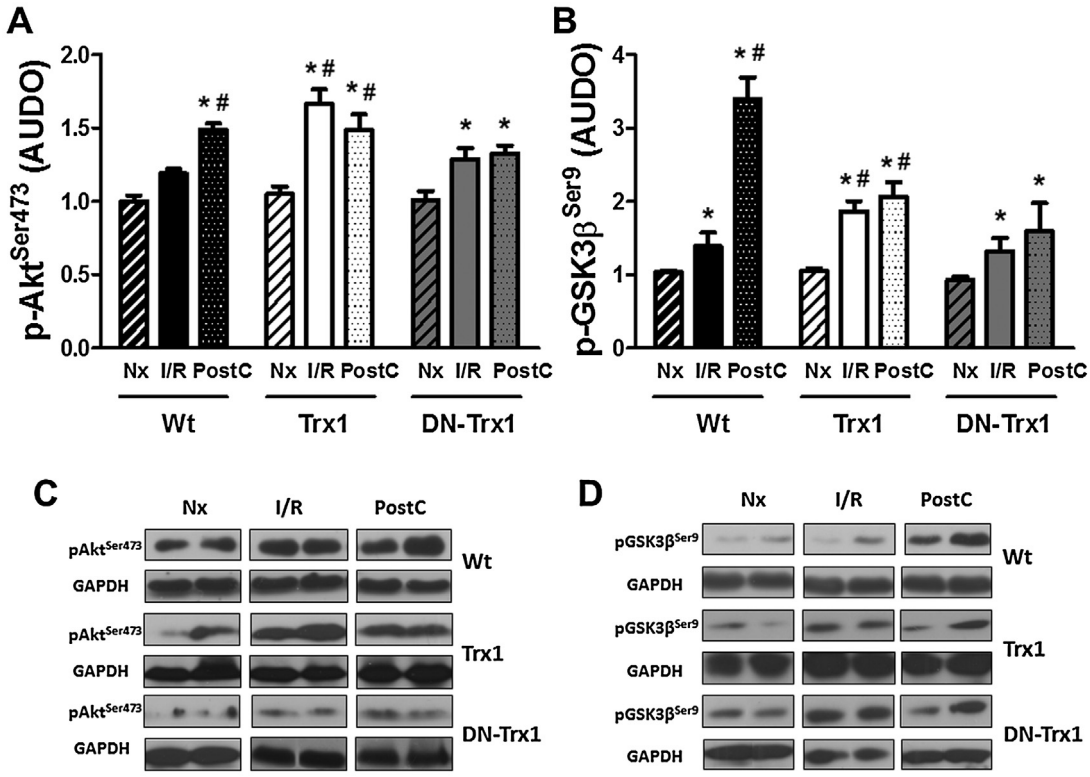

Fig. 4. Akt phosphorylation (Ser^{473}) protein expression (Panel A) and GSK3β phosphorylation (Ser^{9}) protein expression (Panel B). Wt-PostC, Trx1-IR and Trx1-PostC groups show a significantly enhanced Akt and GSK3β phosphorylation compared with Wt-I/R. There was a slight increase in Akt and GSK3β phosphorylation in Wt-I/R and DN-I/R protocols compared with Nx hearts. However in Trx1 and DN-Trx1 groups there were no significant differences between I/R and PostC protocols. Panel C and D show representative blots. GAPDH was used as control loading. *$p < 0.05$ vs. Nx; #$p < 0.05$ vs. Wt-I/R. Wt: wild type; Trx1: thioredoxin-1; DN-Trx1: dominant negative for Trx1; Nx: normoxic; I/R: ischemia/reperfusion; PostC: ischemic postconditioning. AUDO: Arbitrary units' density optic. n = 5 per group.

et al., 2014; Heusch, 2015b), through the performance of a PostC protocol, activate the Akt/GSK3β complex. However, other authors demonstrated that the activation of another intracellular signaling pathway was involved in the PostC protection mechanism. In this sense, Inserte et al. (2013) showed that PostC activates the cGMP/PKG pathway via a mechanism independent of the PI3K/Akt cascade and dependent on the reduction of O_2^- production at the onset of reperfusion. This results in an attenuation of the BH_4 oxidation and a decrease of reduced NOS uncoupling.

The lack of synergism between both cardioprotection mechanisms could be due that in both mechanisms, the Trx1 overexpression and the PostC, exerted their protective effect activating the same pro-survival pathway, in this case Akt/GSK3β. Furthermore, our results reflected that the Akt/GSK3β pathway is not the only intracellular pathway involved in PostC cardioprotection in the Trx1 mice, since we observed a higher phosphorylation in Wt-PostC than in Trx1-PostC. Although, Akt and GSK3β phosphorylation increased in PostC in all mice (Wt, Trx1 and DN-Trx1) compared to their respective Nx values. Only in Wt and Trx1 groups, did Akt and GSK3β phosphorylation increase compared to the Wt-I/R group, consistent with infarct size reduction.

As we have previously demonstrated, the reduction of the infarct size in PostC Wt mice was in concordance with Trx1 levels preservation and with an improvement in the GSH/GSSG ratio, a reliable indicator of redox status, in comparison to the Wt-I/R group (Perez et al., 2016a). However, we did not detect changes in Trx1 levels in the Trx1 and DN-Trx1 mice, compared to their respective baseline values. In a similar manner, we have previously evidenced that Trx1 levels in Trx1 mice were maintained despite having suffered an episode of I/R (D′ Annunzio et al., 2016), and in DN-Trx1 mice, given that the protein is inactive, is irrelevant that Trx1 levels have been preserved after I/R episode. Regarding Wt mice, other authors have evidenced that after an I/R episode, during reperfusion, the RNS concentration increases (Buchholz et al., 2014a; Donato et al., 2010; Schwartz and Lagranha, 2016), and consequently produces cellular damage by lipid peroxidation and DNA fragmentation in the heart. In addition, RNS could decrease endogenous antioxidants (Qin et al., 2011; Dhalla et al., 2000) including Trx1 (Zhang et al., 2007). All these deleterious damages were related with I/R injury could be avoided in the Wt-PostC group.

A better redox status supposedly occurring in Trx1 transgenic mice could increase cardiac performance. Due to this reason, we performed new experiments that would allow us to know the redox status in our model. Taking into consideration that the mitochondrial redox potential is given by the GSH/GSSG ratio, the increase in the cytosolic Trx1 expression in transgenic mice could contribute in reducing the thiol pool reserve. In Wt mice, a decrease of the GSH/GSSG ratio was observed in the I/R group, recovering significantly after the PostC protocol. These results behave similarly to the Trx1 levels, since they decreased during reperfusion after ischemia, and the PostC decreased the protein degradation. In the Trx1 mice, I/R injury did not produce changes in the GSH/GSSG ratio or in the Trx1 protein levels. These findings could explain, at least partially, that the decrease in the infarct size observed in I/R group of the Trx1 was of the same magnitude of those observed in the PostC. Finally, the DN-Trx1 mice have a decreased Trx1 activity at baseline and as consequence an unfavorable redox status. Therefore, when the protective intervention was performed, it was not capable of reducing the infarct size, although the Trx1 levels were preserved after I/R. However, the GSH/GSSG ratio was decreased in the I/R group and it maintained low levels after the PostC protocol. Also, it is clear that the cardioprotective interventions promote a favorable redox sta-

Please cite this article in press as: Perez, V., et al., Inhibition of endogenous thioredoxin-1 in the heart of transgenic mice does not confer cardioprotection in ischemic postconditioning. Int J Biochem Cell Biol (2016), http://dx.doi.org/10.1016/j.biocel.2016.09.017

tus, represented by a decrease of the GSH/GSSG ratio in the groups overexpressing active Trx1 and in the PostC Wt group.

Our data evidences dissociation between the infarct size and the behavior of ventricular function in those groups where a reduction of the infarct size was evidenced (PostC Wt, I/R-Trx1 and PostC-Trx1). However, these findings are not totally surprising since Cohen et al. (2000) demonstrated dissociation between the infarct size reduction and the recovery of ventricular function, conferred by PostC. In Cohen's et al. (2000) study, they evidenced that during the first 48 h of reperfusion there was no improvement in the recovery of ventricular function. The beneficial effect on the ventricular function was achieved at 72 h after the reperfusion of the ischemic myocardium. Probably this is due to the persistence of stunning areas adjacent to the area of necrosis in the first moments of reperfusion. It is probable that this occurs in our experimental model since, due to a limitation of the experimental model, we could not perform the follow up of the ventricular function for several days, as it occurs in Cohen's study. Furthermore, in previous works of our laboratory (D′ Annunzio et al., 2016; Perez et al., 2016a) and of other authors (Cohen et al., 2000; Ashrafian et al., 2012), this dissociation between the behavior of ventricular function and the infarct size in different protection mechanisms has also been evidenced. We only detected and increase in myocardial stiffness in DN-Trx1 mice at 30 min of reperfusion compared with Wt mice. This data are according with our previous study that showed an exacerbation of myocardial stiffness during reperfusion, after a short period of ischemia (myocardial stunning) in DN-Trx1 mice (Perez et al., 2016b). Due to the aforementioned, this dissociation evidenced between the infarct and the ventricular function is possibly associated to the presence of myocardial stunning.

Summarizing, our data suggests that Trx1 plays a key role in the PostC protection mechanism, since the beneficial effect was abolished in DN-Trx1 transgenic mice, in which the activity of endogenous Trx1 is reduced. We were unable to detect synergic effect between both cardioprotection mechanisms, this could be due to the fact that the cardioprotection conferred by Trx1 overexpression and by PostC involve the activation of the Akt/GSK3β cell survival pathway. Our novel finding strongly suggests that Trx1 is an important component in the complex mechanisms of PostC. The description of these new regulatory mechanisms in PostC opens the possibility to new therapeutic strategies in I/R injury.

Funding sources

This study was supported by the University of Buenos Aires (UBACYT# 20020110100159); and the National Agency for Technological and Scientific Promotion (PICT 0258 and PICT 0373).

References

Ago, T., Sadoshima, J., 2006. Thioredoxin and ventricular remodeling. J. Mol. Cell. Cardiol. 41, 762–773, http://dx.doi.org/10.1016/j.yjmcc.2006.08.006.

D′ Annunzio, V., Perez, V., Mazo, T., Muñoz, M.C., Dominici, F.P., Carreras, M.C., Poderoso, J.J., Sadoshima, J., Gelpi, R.J., 2016. Loss of myocardial protection against myocardial infarction in middle-aged transgenic mice overexpressing cardiac thioredoxin-1. Oncotarget 7, 11889–11898, http://dx.doi.org/10.18632/oncotarget.7726.

Aota, M., Matsuda, K., Isowa, N., Wada, H., Yodoi, J., Ban, T., 1996. Protection against reperfusion-induced arrhythmias by human thioredoxin. J. Cardiovasc. Pharmacol. 27, 727–732, http://dx.doi.org/10.1097/00005344-199605000-00016.

Ashrafian, H., Czibik, G., Bellahcene, M., Aksentijević, D., Smith, A.C., Mitchell, S.J., Dodd, M.S., Kirwan, J., Byrne, J.J., Ludwig, C., Isackson, H., Yavari, A., Støttrup, N.B., et al., 2012. Fumarate is cardioprotective via activation of the Nrf2 antioxidant pathway. Cell Metab. 15, 361–367, http://dx.doi.org/10.1016/j.cmet.2012.01.017.

Buchholz, B., D′ Annunzio, V., Giani, J.F., Siachoque, N., Dominici, F.P., Turyn, D., Perez, V., Donato, M., Gelpi, R.J., 2014a. Ischemic postconditioning reduces infarct size through the α1-adrenergic receptor pathway. J. Cardiovasc. Pharmacol. 63, 504–511, http://dx.doi.org/10.1097/FJC.0000000000000074.

Buchholz, B., Donato, M., D'Annunzio, V., Gelpi, R.J., 2014b. Ischemic postconditioning: mechanisms, comorbidities, and clinical application. Mol. Cell. Biochem. 392, 1–12, http://dx.doi.org/10.1007/s11010-014-2014-6.

Cai, M., Li, Y., Xu, Y., Swartz, H.M., Chen, C.L., Chen, Y.R., He, G., 2011. Endothelial NOS activity and myocardial oxygen metabolism define the salvageable ischemic time window for ischemic postconditioning. Am. J. Physiol. Heart Circ. Physiol. 300, H1069–1077, http://dx.doi.org/10.1152/ajpheart.00694.2010.

Chen, K., Yan, M., Wu, P., Qing, Y., Li, S., Li, Y., Dong, Z., Xia, H., Huang, D., Xin, P., Li, J., Wei, M., 2016. Combination of remote ischemic preconditioning and remote ischemic postconditioning fails to increase protection against myocardial ischemia/reperfusion injury, compared with either alone. Mol. Med. Rep. 13, 197–205, http://dx.doi.org/10.3892/mmr.2015.4533.

Cohen, M.V., Yang, X.M., Neumann, T., Heusch, G., Downey, J.M., 2000. Favorable remodeling enhances recovery of regional myocardial function in the weeks after infarction in ischemically preconditioned hearts. Circulation 102, 579–583, http://dx.doi.org/10.1161/01.CIR.102.5.579.

Dhalla, N.S., Elmoselhi, A.B., Hata, T., Makino, N., 2000. Status of myocardial antioxidants in ischemia-reperfusion injury. Cardiovasc. Res. 47, 446–456, http://dx.doi.org/10.1016/S0008-6363(00)00078-X.

Donato, M., D'Annunzio, V., Buchholz, B., Mikysztowicz, V., Carrión, C.L., Valdez, L.B., Zaobornyj, T., Schreier, L., Wikinski, R., Boveris, A., Berg, G., Gelpi, R.J., 2010. Role of matrix metalloproteinase-2 in the cardioprotective effect of ischemic postconditioning. Exp. Physiol. 95, 274–281, http://dx.doi.org/10.1113/expphysiol.2009.049874.

Evelson, P., Travacio, M., Repetto, M., Escobar, J., Llesuy, S., Lissi, E., 2001. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Arch. Biochem. Biophys. 388, 261–266, http://dx.doi.org/10.1006/abbi.2001.2292.

Ferdinandy, P., Hausenloy, D.J., Heusch, G., Baxter, G.F., Schulz, R., 2014. Interaction of risk factors, comorbidities, and comedications with ischemia/reperfusion injury and cardioprotection by preconditioning, postconditioning, and remote conditioning. Pharmacol. Rev. 66, 1142–1174, http://dx.doi.org/10.1124/pr.113.008300.

Halkos, M.E., Kerendi, F., Corvera, J.S., Wang, N.P., Kin, H., Payne, C.S., Sun, H.Y., Guyton, R.A., Vinten-Johansen, J., Zhao, Z.Q., 2004. Myocardial protection with postconditioning is not enhanced by ischemic preconditioning. Ann. Thorac. Surg. 78, 961–969, http://dx.doi.org/10.1016/j.athoracsur.2004.03.033.

Hausenloy, D.J., Tsang, A., Yellon, D.M., 2005. The reperfusion injury salvage kinase pathway: a common target forboth ischemic preconditioning and postconditioning. Trends Cardiovasc. Med. 15, 69–75, http://dx.doi.org/10.1016/j.tcm.2005.03.001.

Heusch, G., 2015a. Treatment of myocardial Ischemia/Reperfusion injury by ischemic and pharmacological postconditioning. Compr. Physiol. 5, 1123–1145, http://dx.doi.org/10.1002/cphy.c140075.

Heusch, G., 2015b. Molecular basis of cardioprotection: signal transduction in ischemic pre-, post-, and remote conditioning. Circ. Res. 116, 674–699, http://dx.doi.org/10.1161/CIRCRESAHA.116.305348.

Iliodromitis, E.K., Georgiadis, M., Cohen, M.V., Downey, J.M., Bofilis, E., Kremastinos, D.T., 2006. Protection from post-conditioning depends on the number of short ischemic insults in anesthetized pigs. Basic Res. Cardiol. 101, 502–507, http://dx.doi.org/10.1007/s00395-006-0606-3.

Inserte, J., Hernando, V., Vilardosa, Ú., Abad, E., Poncelas-Nozal, M., Garcia-Dorado, D., 2013. Activation of cGMP/protein kinase G pathway in postconditioned myocardium depends on reduced oxidative stress and preserved endothelial nitric oxide synthase coupling. J. Am. Heart Assoc. 2 (1), e005975, http://dx.doi.org/10.1161/jaha.112.005975 (2).

Kin, H., Zhao, Z.Q., Sun, H.Y., Wang, N.P., Corvera, J.S., Halkos, M.E., Kerendi, F., Guyton, R.A., Vinten-Johansen, J., 2004. Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. Cardiovasc. Res. 62, 74–85, http://dx.doi.org/10.1016/j.cardiores.2004.01.006.

Liu, X., Chen, H., Zhan, B., Xing, B., Zhou, J., Zhu, H., Chen, Z., 2007. Attenuation of reperfusion injury by renal ischemic postconditioning: the role of NO. Biochem. Biophys. Res. Commun. 359, 628–634, http://dx.doi.org/10.1016/j.bbrc.2007.05.129.

Perez, V., D′ Annunzio, V., Mazo, T., Marchini, T., Caceres, L., Evelson, P., Gelpi, R.J., 2016a. Ischemic postconditioning confers cardioprotection and prevents reduction of Trx1 in young mice, but not in middle-aged and old mice. Mol. Cell. Biochem. 415, 67–76, http://dx.doi.org/10.1007/s11010-016-2677-2.

Perez, V., D'Annunzio, V., Valdez, L.B., Zaobornyj, T., Bombicino, S., Mazo, T., Longo Carbajosa, N., Gironacci, M.M., Boveris, A., Sadoshima, J., Gelpi, R.J., 2016b. Thioredoxin-1 attenuates ventricular and mitochondrial postischemic dysfunction in the stunned myocardium of transgenic mice. Antioxid Redox Signal. 10 (24), 78–88, http://dx.doi.org/10.1089/ars.2015.6459.

Qin, C.X., Williams, S.J., Woodman, O.L., 2011. Antioxidant activity contributes to flavonol cardioprotection during reperfusion of rat hearts. Free Radic. Biol. Med. 51, 1437–1444, http://dx.doi.org/10.1016/j.freeradbiomed.2011.07.003.

Rodriguez-Ariza, A., Toribio, F., López-Barea, J., 1994. Rapid determination of glutathione status in fish liver using high-performance liquid chromatography and electrochemical detection. J. Chromatogr. B Biomed. Appl. 656, 311–318, http://dx.doi.org/10.1016/0378-4347(94)00111-1.

Schwartz, L.M., Lagranha, C.J., 2006. Ischemic postconditioning during reperfusion activates Akt and ERK without protecting against lethal myocardial ischemia-reperfusion injury in pigs. Am. J. Physiol. Heart Circ. Physiol. 290, H1011–1008, http://dx.doi.org/10.1152/ajpheart.00864.200.

Please cite this article in press as: Perez, V., et al., Inhibition of endogenous thioredoxin-1 in the heart of transgenic mice does not confer cardioprotection in ischemic postconditioning. Int J Biochem Cell Biol (2016), http://dx.doi.org/10.1016/j.biocel.2016.09.017

Tao, L., Gao, E., Bryan, N.S., Qu, Y., Liu, H.R., Hu, A., Christopher, T.A., Lopez, B.L., Yodoi, J., Koch, W.J., Feelisch, M., Ma, X.L., 2004. Cardioprotective effects of thioredoxin in myocardial ischemia and reperfusion: role of S-nitrosation. Proc. Natl. Acad. Sci. U. S. A. 101, 11471–11476, http://dx.doi.org/10.1073/pnas.0402941101.

Tsang, A., Hausenloy, D.J., Mocanu, M.M., Yellon, D.M., 2004. Postconditioning: a form of modified reperfusion protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ. Res. 95, 230–232, http://dx.doi.org/10.1161/01.RES.0000138303.76488.fe.

Yamamoto, M., Yang, G., Hong, C., Liu, J., Holle, E., Yu, X., Wagner, T., Vatner, S.F., Sadoshima, J., 2003. Inhibition of endogenous thioredoxin in the heart increases oxidative stress and cardiac hypertrophy. J. Clin. Invest. 112, 1395–1406, http://dx.doi.org/10.1172/JCI200317700.

Yang, X.M., Proctor, J.B., Cui, L., Krieg, T., Downey, J.M., Cohen, M.V., 2004. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. J. Am. Coll. Cardiol. 44, 1103–1110, http://dx.doi.org/10.1016/j.jacc.2004.05.060.

Zhang, H., Tao, L., Jiao, X., Gao, E., Lopez, B.L., Christopher, T.A., Koch, W., Ma, X.L., 2007. Nitrative thioredoxin inactivation as a cause of enhanced myocardial ischemia/reperfusion injury in the aging heart. Free Radic. Biol. Med. 43, 39–47, http://dx.doi.org/10.1016/j.freeradbiomed.2007.03.016.

Zhao, Z.Q., Corvera, J.S., Halkos, M.E., Kerendi, F., Wang, N.P., Guyton, R.A., Vinten-Johansen, J., 2003. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am. J. Physiol. Heart Circ. Physiol. 285, H579–588, http://dx.doi.org/10.1152/ajpheart.01064.2002.

Zitta, K., Meybohm, P., Gruenewald, M., Cremer, J., Zacharowski, K.D., Scholz, J., Steinfath, M., Albrecht, M., 2015. Profiling of cell stress protein expression in cardiac tissue of cardiosurgical patients undergoing remote ischemic preconditioning: implications for thioredoxin in cardioprotection. J. Transl. Med. 27, 13–34, http://dx.doi.org/10.1186/s12967-015-0403-6.

Please cite this article in press as: Perez, V., et al., Inhibition of endogenous thioredoxin-1 in the heart of transgenic mice does not confer cardioprotection in ischemic postconditioning. Int J Biochem Cell Biol (2016), http://dx.doi.org/10.1016/j.biocel.2016.09.017