Thioredoxin-2 protects mice against experimental myocardial infarction

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Supplemental methods

Cloning of human Trx-2

Full-length human Trx-2 cDNA was amplified by PCR from human peripheral blood mononuclear cells using the forward primer: 5'-CGCTCCGTGGGACCT (15 bp extension homologous to vector end) *AAGCTT* (*HindIII*) TAGGCTGTGCATCCCTCCGC-3' and the reverse primer 3'-TATCTTATCATGTCT (15 bp extension homologous to vector end) *GGATCC* (*BamHI*) CCCACGCGGGCAAGGGAACC-5'. The PCR product was purified and directionally cloned between *HindIII* and *BamHI* sites in the vector pTR-ETG using In-Fusion® HD Cloning Kit (Clontech) as recommended by the supplier. The construct was used after confirmation of its sequence (Eurofins Genomics). Plasmid DNA was prepared using the EndoFree® Plasmid Maxi Kit (Qiagen).

Adeno-associated viruses (AAV) production

The pTR-ETG plasmid for AAV-Trx-2 and AAV-Luciferase was a generous gift of Pr. Roger Hajjar (Phospholamban Foundation, Amsterdam, Netherlands). This plasmid contained the *Luciferase* gene under the transcriptional control of the chicken cardiac Troponin T promoter which is specific to CM (Supplemental Fig. I). The viral preparation of AAV2/9 serotype was manufactured by the Centre de Production de Vecteurs (Nantes, France). Briefly, vectors were produced in HEK293 cells by standard helper free transfection method. Recombinant viral particles were purified by CsCl ultracentrifugation and phosphate-buffered saline (PBS) dialysis. Particles were quantified by real-time PCR and vector titers were expressed as viral genomes per milliliter (vg/mL). The AAV infection efficiency was verified at the mRNA and protein levels in neonatal mice CMs.

Experimental animals

Care of the animals and surgical procedures were performed according to the Directive 2010/63/EU of the European Parliament. The project was submitted to the French Ethic Committee CEEA (*Comité d'Ethique en Expérimentation Animale*) and obtained the authorization APAFIS#24071-2020020611035354 v4. Animals were housed in an environmentally-controlled facility for the duration of the experiment. All animals had access ad libitum to food and water. *In vitro* experiments were conducted with C57BL/6JRj gestating female mice (Janvier Labs, Le Genest St Isle, France) in order to use the neonatal mice. *In vivo* experiments were conducted on C57BL/6JRj adult (8 week old) male mice. Mice were randomly divided into two groups: the first one with an experimental myocardial infarction (MI) and the second one without it (SHAM). Each group was next divided into two subgroups: AAV-Luciferase (control) and AAV-Trx-2. AAV (1×10^{11} vg/100 µL) were intravenously injected one month before experimental myocardial infarction. Untreated control mice received saline solution.

Coronary artery ligation

Myocardial infarction (MI) was performed in mice by left anterior descending coronary artery permanent ligation. Mice were anesthetized with 2% isoflurane in O_2 and maintained at 37°C. After intubation, mice were connected to Minivent Mouse ventilator (type 845, Harvard Apparatus, respiratory frequency 170 strokes/min and respiratory volume 200 µL). A left thoracotomy was performed in the fourth intercostal space to induce left ventricular infarction (MI) by ligation of the left anterior descending coronary or for sham operation (SHAM) with an 8-0 Prolene. Myocardial ischemia was confirmed by the occurrence of regional blanching. An injection of Metacam was administrated to avoid pain before surgery. The chest was

closed in layers and mice were hydrated with saline solution. Mice were controlled daily for three weeks.

Echocardiography

Echocardiography was performed on lightly anesthetized animals under isoflurane (0.5 %, 2L/min oxygen flow rate), placed on a heating pad (37°C), with a probe emitting ultrasounds from 9- to 14-MHz frequency (Vivid7 PRO apparatus; GE Medical System Co). The two-dimensionally guided Time Motion mode recording (parasternal long-axis view) of the left ventricle (LV) provided the following measurements: end-diastolic and end-systolic interventricular septum (IVSd and IVSs), posterior wall thicknesses (PWd and PWs), internal diameter (LVEDD and LVESD) and heart rate (HR). Each set of measurements was obtained from the same cardiac cycle. At least three sets of measurements were registered from three different cardiac cycles. Fractional shortening (FS): ([LVEDD − LVESD]/LVEDD) × 100, ejection fraction (EF): (stroke volume/end-diastolic volume) x 100, and h/r: (left ventricle diastolic wall thickness/radius) were calculated. The first echocardiography (T_0) was performed one month after AAV injections and one day before the experimental MI. The second one (T_1) was performed 3 weeks post-infarction to compare the morphometric and hemodynamic parameters between T_0 and T_1.

Histochemistry

Hearts were harvested and embedded into OCT embedding matrix (Optimal Cutting Temperature Medium) in cold isopentane (between -80°C and -100°C). 5-µm-thick sections were stained with Trichrome (Abcam, Trichrome Stain) or Picro-Sirius Red (Abcam, Picro-Sirius Red Stain kit), according to the manufacturer's protocol.

TUNEL Assay

Sections were fixed in 4% PFA at room temperature for 10 min and washed with PBS. Saturation of non-specific binding sites was assessed with 10% of donkey serum, 30% glycine and PBS/0.2% Triton X100 for 1 hour. After three washes with PBS, sections were incubated overnight at 4°C with anti-cardiac Troponin T (BD Pharmingen, 564766; 1:200) diluted in PBS/2% BSA. DNA fragmentation, such as in apoptosis, was then detected with the TUNEL Assay Kit - BrdU-Red (Abcam), as recommended by the supplier. Heart sections were washed and probed with the Alexa Fluor™ 488 goat anti-mouse IgG (Invitrogen, A-11001; 1:1000) in a light-protected chamber for 1h at room temperature. A mounting with Fluoroshield™ coupled with DAPI (Sigma-Aldrich) was performed. Heart tissue sections were observed under a fluorescence microscope (Leica DMi8). All imaging was performed with 10x objective and appropriate dichroic filters. The whole section was recorded per sample in a non-overlapping manner and without binning.

Measurement of DHE (dihydroethidium) reactive ROS

Heart sections (5 µm) were fixed in 4% PFA at room temperature for 10 min and washed with PBS. To detect superoxide, sections were incubated with the fluorescent dye dihydroethidium (DHE, 10µM) (Invitrogen, D11347) in a light-protected chamber for 20 min at room temperature. After three washes with PBS, heart tissue sections were mounted with Fluoroshield™ coupled with DAPI and observed under a fluorescence microscope (Leica DMi8). All imaging was performed with 10x objective and appropriate dichroic filters. The whole section was recorded per sample in a non-overlapping manner and without binning.

Reactive oxygen species assessment by HPLC

Cells were incubated with 100µM DHE at 37°C protected from light for 30 min. They were washed and harvested with cold acetonitrile into tubes. Cells were then sonicated and centrifuged at 12000 rpm at 4°C for 5 min. Supernatant was collected and lyophilized at 40°C for 1 hour in a speed vacuum. Pellet was maintained at -80°C until analysis. Lyophilized cells were resuspended in 120µl PBS/DTPA 100µM and injected (100µl) into a HPLC system. Detection of DHE and derived oxidation products (EOH and Ethidium) was realized using respectively ultraviolet and fluorescence as described in (Laurindo et al., 2008). DHE-derived products were expressed as ratios generated of EOH and Ethidium per DHE consumed (initial DHE concentration minus remaining DHE): EOH/DHE and Ethidium/DHE). Data were normalized with protein level or cell number.

Proliferation Assay

Cultured CMs were treated with 10 µM 5-ethynyl-2′-deoxyuridine (EdU) (Invitrogen) for 24h. Cells were fixed in 4% PFA at room temperature for 10 min, followed by permeabilization in PBS/3% Triton X100 for 1 hour. The blocking was assessed with 3% BSA in PBS for 1 hour at room temperature. Cells were then incubated overnight at 4°C with a primary antibody against cardiac Troponin T (BD Pharmingen, 564766; 1:500). Cells were washed with PBS and probed with the Alexa Fluor™ 555 goat anti-mouse IgG1 (Invitrogen, A21127; 1:1000), in the presence of Alexa Fluor™ 488 Phalloidin (Invitrogen, A12379; 1:500) and Hoescht 33342 dye (Invitrogen, C10337 G; 1:20000). EdU was detected by using the Click-iT™ EdU Alexa Fluor™ 488 Imaging Kit (Invitrogen), according to the manufacturer's instructions.

For mononucleation assessments, frozen hearts were dissected into 1 mm wide cubes and fixed in 3.7% formaldehyde solution at room temperature for 100 min. Subsequently, tissue

blocks were washed with PBS for 30 min and then digested in the presence of collagenase B (Roche, 11088815001) (3.6 mg/mL) and collagenase D (Roche, 11088858001) (4.8 mg/mL) in PBS for 24h at 37°C with gentle rotation. Isolated CMs were gently centrifuged at 1 x g and the supernatant was removed. Isolated CMs were allowed to settle for 15 min and were resuspended in 500 µL of blocking buffer with primary antibodies against mouse-α-actinin (Sigma-Aldrich, A7811; 1:500) and rabbit-connexin-43 (Sigma-Aldrich, C6219; 1:1000) for 30 min. CMs were washed once with PBS for 15 min and incubated with secondary antibodies Alexa Fluor™ 555 anti-mouse (Jackson Immuno Research, 711-546-152; 1:1000) and Alexa Fluor™ 488 anti-rabbit (Abcam, ab150110; 1:1000) and Hoechst 33342 dye (ThermoFisher, 62249; 1:1000) for 30 min. Cells were washed with PBS, resuspended in a small volume of PBS and pipetted onto a glass slide. CMs on the glass slide were allowed to settle and to adhere before mounting with ProLong™ Gold Antifade Reagent (ThermoFisher, P36930). All imaging was performed on a Keyence BZ-X800E with 20x objectives and appropriate dichroic filters. At least 12 fields of view were recorded per sample in a non-overlapping manner.

Isolation and treatment of neonatal mice cardiomyocytes

Hearts from 1-day old mice pups were harvested and digested mechanically and enzymatically with specific enzymes using Neonatal Heart Dissociation Kit (Miltenyi Biotec) as recommended by the supplier. Erythrocytes were lysed with Red Blood Cell Lysis Solution (Miltenyi Biotec). CMs were then isolated and purified through specific columns (MS Columns, Miltenyi Biotec). Purified cells were cultured in DMEM containing glucose 4.5g/L (Gibco) and supplemented with 10% horse serum, 20% fetal bovine serum and 100U/ml penicillin/100 µg/ml streptomycin in a humidified 5% CO_2 incubator at 37°C. At day 1,

AAV-Luciferase or AAV-Trx-2 (1 x 10^5 vg) was added in the culture medium for 24 h. Cells were harvested and RNA or protein extractions were performed.

Western blot analysis

Proteins were extracted from cultured CMs or heart tissue with CelLytic™ M (Sigma-Aldrich) or CelLytic™ MT (Sigma-Aldrich) respectively, containing Protease and Phosphatase Inhibitor Cocktails (Thermo Scientific). Heart tissue was milled with Ultraturrax T25 (Janke & Kunkel). Protein content was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) as recommended by the supplier. Proteins were separated on Any kD™ Criterion™ TGX™ Precast Gels (Bio-Rad) and electro-transferred onto Nitrocellulose Membranes 0.45µm (Bio-Rad). The blocking was assessed with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1h at room temperature. The blots were then incubated overnight at 4°C with a primary antibody against either Trx-2 (Invitrogen, PA5-81102; 1:500), Bax (Proteintech, 60267-1-Ig; 1:5000) or Bcl-2 (Proteintech, 26593-1-AP; 1:2000). GAPDH (mouse, Invitrogen, MA5-15738; 1:5000 or rabbit, Abcam, ab181602; 1:50000) was used for normalization. The membranes were probed with a horseradish peroxidase-conjugated secondary antibody for 1h at room temperature. Membranes were revealed with the Clarity™ Western ECL Substrate (Bio-Rad) and the ChemiDoc™ MP Imaging System (Bio-Rad). Levels of protein expression were calculated as the relative band intensity with the ImageJ software.

Quantitative reverse Transcription-PCR analysis

Total RNA was extracted from CMs using TRI Reagent® (Sigma-Aldrich) and 1-Bromo-3-chloropropane (Sigma-Aldrich). Total RNA was extracted from heart tissue only with TRI Reagent® using an Ultraturrax T25. Reverse transcription was realized using the RevertAid H

Minus First Strand cDNA Synthesis Kit (Thermo Scientific) with random hexamers. Real time qPCR analysis was performed on a LightCycler 480 with the Go Taq qPCR Master Mix (Promega) using a SYBR Green I dye for product detection. Primer sequences used in PCR reactions are listed in Table 1. Relative quantification of target RNA using *36B4* as reference was realized with the $\Delta\Delta C_T$ method.