ENOGENOUS THROMBOSPONDIN-1 PROTECTS THE PRESSURE-OVERLOADED MYOCARDIUM BY MODULATING FIBROBLAST PHENOTYPE AND MATRIX METABOLISM

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ONLINE SUPPLEMENT:
SUPPLEMENTAL METHODS:

1. Animal protocols.

Animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Baylor College of Medicine and Albert Einstein College of Medicine Institutional Review Boards. Male and female, 6-12 month-old WT and TSP-1 null C57/BL6 mice from our colony were genotypes using established protocols. Animals were anesthetized with inhaled isoflurane. Aortic banding was achieved by creating a constriction between the right innominate and left carotid arteries as previously described. The degree of pressure overload was assessed by measuring right-to-left carotid artery flow velocity ratio after constricting the transverse aorta. Only mice with a flow ratio from 5:1 to 10:1 were used for analysis. At the end of the experiment, the heart was excised, fixed in zinc-formalin, and embedded in paraffin for histological studies, or frozen for RNA/protein isolation. Animals used for histology underwent 3, 7, and 28 days of banding (n=8/group). Additional groups of mice were used for RNA and protein extraction after 3 (n=7) or 7 days (n=7) of banding, and for isolation and flow cytometric analysis of cardiac fibroblasts (WT, n=10; TSP-1 /-, n=6). As a control, a "sham" operation without aortic constriction was performed on age-matched mice (histology n=6, RNA n=6, protein n=6).

2. Echocardiographic analysis

Short axis M-mode echocardiography was performed prior to instrumentation and before the end of each experiment (3, 7 or 28 days of TAC) using a Sequoia C256 system (Acuson, Mountain View, CA). The following parameters were measured as indicators of function and remodeling: left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), fractional shortening (FS=[LVEDD-LVESD]x100/LVEDD), interventricular septal (IVS) thickness, posterior wall (PW) thickness and left ventricular mass (LV mass).

3. Immunohistochemistry and quantitative histology.

Sections were cut at 5 μm and stained immunohistochemically as previously described. The collagen network was labeled using picrosirius red staining. Myofibroblasts were identified using staining with an antibody to α-smooth muscle actin (α-SMA) (Sigma, St. Louis, MO) as spindle-shaped cells located outside the vascular media. Macrophages were labeled using a rat anti-mouse Mac-2 antibody (Cedarlane, Burlington NC). Endothelial cells were identified using staining with a rat anti-mouse CD31 antibody (Pharmingen, San Diego CA). Staining was performed with a peroxidase-based technique using the Vectastain ELITE kit (Vector, Burlingame CA) and developed with diaminobenzidine and nickel. The Mouse to Mouse (MOM) kit was used for α-SMA staining. For CD31 staining the Tyramide Signal Amplification (TSA) kit (Perkin Elmer, Boston MA) was used on sections pre-treated with trypsin. Quantitative assessment of myofibroblast and macrophage density was performed by counting the number of cells/myocardial area using Image Pro software. Microvascular density was assessed by counting the number of vascular profiles in the subendocardial and subepicardial area. Eight fields from three different stained sections were used for analysis.

4. Assessment of apoptosis using TUNEL staining and WGA lectin fluorescence.
Identification of apoptotic cardiomyocytes and interstitial cells in pressure-overloaded hearts was performed using fluorescent In situ Cell Death Detection Kit (Roche) and WGA staining of cell membranes. Briefly, paraffin sections were incubated at 55°C for 4h and allowed to cool down for 2h. Subsequently, slides were deparaffinized, rehydrated in graded alcohols and subjected to antigen retrieval with 20 μg/ml Proteinase K (Promega) in modified TE buffer (50mM Tris Base, 1mM EDTA, 0.5% Triton X-100, pH 8.0) for 30 min at 37°C. Slides were washed several times in PBS and stained with 10 μg/ml biotynylated WGA lectin (Vector Labs) in PBS with Ca²⁺ and Mg²⁺ for 1h at RT. After several washes in PBS slides were stained with streptavidin-Texas Red X complex (Invitrogen). Nuclei were stained with DAPI (Invitrogen). Finally, slides were incubated with TUNEL reaction mixture for 1h at 37°C. The densities (cells/mm²) of TUNEL+ cardiomyocytes and TUNEL+ noncardiomyocytes were quantitatively assessed in entire paraffin cross-section of myocardium and averaged using 3 nonadjacent sections from base, mid-myocardium and apex of each heart.

5. Collagen crosslinking assay.
To assess crosslinking of collagen in pressure overloaded hearts we adopted a method described by Mukherjee et al.⁵ In this method, PBS and sodium dodecyl sulfate (SDS) are used to remove the bulk of noncollagen proteins and freshly deposited collagen, leaving insoluble residue of crosslinked collagen. Incubation with cyanogen bromide (CNBr) allows determining the degree of collagen cross-linking based on solubility to CNBr. Briefly, hearts were lyophilized and dry weights were recorded. Subsequently hearts were homogenized with PBS and homogenate was centrifuged at 4000 g for 10 min. Supernatant was retained and remaining pellet was rehomogenized with 10% SDS and incubated at 4°C for 2h. Homogenate was again centrifuged 4,000g for 10 minutes and supernatant was retained. The remaining residue was further extracted three times with PBS to remove the excess SDS. The residue then was rehomogenized in acetone and centrifuged at 4,000g for 10 minutes; the supernatant was discarded. This step was repeated, and the pellet was dried under vacuum. The acetone-dried powder was homogenized with solution of 20 mg/ml cyanogen bromide (Sigma) in 70% vol/vol formic acid. The homogenate was transferred to 5 ml glass ampoules, purged with nitrogen gas and sealed using propane torch. The reaction was allowed to proceed for 18 hours at 25°C. At the completion of the reaction, the digest was centrifuged at 5,000g for 20 minutes and supernatant was retained. At this point all collected supernatants and CNBr insoluble pellet were dried in vacuum centrifuge. Pellets were rehomogenized with 6N HCl, transferred 5 ml glass ampoules, sealed under vacuum and allowed to hydrolyze at 110°C for 16h. Subsequently, samples were evaporated in vacuum centrifuge and were oxidized using 1.27% chloramine T (Sigma, St. Louis, MO), 10% n-propanol, 0.2 M sodium citrate, and 0.5 M sodium acetate, 0.7 M sodium hydroxide at pH 6.5. After 20 min of incubation at room temperature, Erlich's solution (1 M p-dimethylaminobenzaldehyde [Sigma] in 70% n-propanol, 20% perchloric acid) was added and a 15 min incubation at 65°C performed. Absorbance was measured at 550 nm and the amount of hydroxyproline was determined against a standard curve. Total and insoluble collagen was expressed as µg of collagen/mg of dry tissue. The index of collagen crosslinking was defined as ratio of insoluble collagen to total collagen.

6. Ribonuclease protection assay (RPA)

mRNA expression levels of Tumor Necrosis Factor (TNF)-a, Interleukin (IL)-1b and IL-6 was determined using an RPA (RiboQuant; Pharmingen) as previously described⁶.
7. Quantitative PCR

Isolated total RNA from mouse hearts was reverse transcribed to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad) following the manufacturer’s guidelines. Quantitative PCR was performed using the SYBR green (Bio-Rad) method on the iQ™5 Real-Time PCR Detection System (Bio-Rad). Primers were synthesized at the Baylor College of Medicine Child Health Research Center core facility. The following sets of primers were used in the study: TGF-β1 (forward), GACGAGCTGGTTGAGAGAAG, (reverse) CGCAGTGCCAAGAAGTCC; MCP-1 (forward) TGAAGTTGACCCGTAAATCTGAAG, (reverse) AAGGACATCACAGTCCCGAGTC; TSP-1 (forward) AAGACATTTTCAGGAACAAAAGG, (reverse) TGTAGTTGCTGGATGATGC.

8. Protein extraction and western blotting

Protein was isolated from whole hearts. Western blotting was performed as previously described 7 using the following antibodies: rabbit anti-mouse p-Smad2(ser465/467) (Cell Signaling), rabbit monoclonal anti-TGF-β antibody (Cell Signaling), goat anti-MMP-2, rat anti-MMP-3, goat anti-MMP-9 and rat anti-TIMP-1 (all from R&D).

9. Zymography

MMP activity in the pressure overloaded myocardium was examined by gelatin zymography as previously described 4. Myocardial samples were homogenized in 300 µl of an ice-cold extraction buffer containing cacodylic acid (10 mmol/L), NaCl (150 mmol/L), ZnCl₂ (20 mmol/L), NaN₃ (1.5 mmol/L) and 0.01% Triton X-100 (pH 5.0). Subsequently, the homogenate was centrifuged (4°C, 10 min, 10.000g), the supernatant decanted and saved on ice. The protein concentration in tissue extracts was measured using BCA Protein Assay Kit (Thermo Scientific). The myocardial extracts at final protein concentration of 20 µg were mixed in a ratio 1:2 with Zymogram Sample Buffer (Bio Rad) and loaded onto 10% polyacrylamide electrophoretic precast gels (Bio Rad) containing 1 mg/ml of gelatin under non-reducing conditions. The gels were run at 50V/gel through stacking phase and 100v/gel for the separating phase, maintaining a running buffer temperature of 4°C. Subsequently, the gels were renaturated in 2.5% Triton X-100 for 30 min, rinsed in water and incubated for 48h in Zymogram Development Buffer (Bio Rad) at 37°C. After incubation the gels were stained with Coomassie brilliant blue R-250 (Bio Rad) and subsequently destained until clear bands appeared against blue background. Digital images were scanned and the optical density of the bands was measured using ImageJ software.

10. Isolation of cardiac myofibroblasts

Cardiac fibroblasts were isolated from WT and TSP-1 null hearts after 7 days of TAC as previously described 8, fixed for 10 min in 2% paraformaldehyde (Sigma), permeabilized using 0.1% Triton-X (Sigma) and stained using dual immunofluorescence. Slides were stained with an anti-α-SMA antibody (Sigma), followed by incubation with a biotinylated secondary and amplification with Streptavidin Alexa Fluor 488 (Invitrogen). After brief treatment with avidin/biotin blocking kit (Vector) staining with a rabbit anti-type I collagen antibody was performed (Rockland Inc), followed by incubation with a biotinylated secondary antibody and amplification with Streptavidin Alexa Fluor 594 (Invitrogen).
Flow cytometry on cells harvesting of cells from the pressure-overloaded myocardium.

Single cell suspensions were prepared from WT (n=10) and KO (n=6) hearts after 7 days of TAC and underwent flow cytometric analysis as previously described. Flow cytometry was performed using the FITC-conjugated anti-α-SMA (Sigma, Clone 1A4) and purified rabbit anti-collagen I (Rockland Inc.) antibodies. Cell permeable DRAQ5 dye (Alexis Biochemicals) was used to define the gate for nucleated cells. Data analysis was performed using FlowJo (Tree Star, Inc).

Statistical analysis was performed using ANOVA followed by t-test corrected for multiple comparisons (Student-Newman-Keuls). Paired t-test was used to compare echocardiographic endpoints before instrumentation and after TAC. Data were expressed as mean±SEM. Statistical significance was set at 0.05.

REFERENCES