

Thrombospondin-1 Induction in the Diabetic Myocardium Stabilizes the Cardiac Matrix in Addition to Promoting Vascular Rarefaction Through Angiopoietin-2 Upregulation

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Rationale: Diabetes mellitus is associated with cardiac fibrosis. Matricellular proteins are induced in fibrotic conditions and modulate fibrogenic and angiogenic responses by regulating growth factor signaling.

Objective: Our aim was to test the hypothesis that the prototypical matricellular protein thrombospondin (TSP)-1, a potent angiostatic molecule and crucial activator of transforming growth factor- β , may play a key role in remodeling of the diabetic heart.

Methods and Results: Obese diabetic db/db mice exhibited marked myocardial TSP-1 upregulation in the interstitial and perivascular space. To study the role of TSP-1 in remodeling of the diabetic heart, we generated and characterized db/db TSP-1^{-/-} (dbTSP) mice. TSP-1 disruption did not significantly affect weight gain and metabolic function in db/db animals. When compared with db/db animals, dbTSP mice had increased left ventricular dilation associated with mild nonprogressive systolic dysfunction. Chamber dilation in dbTSP mice was associated with decreased myocardial collagen content and accentuated matrix metalloproteinase-2 and -9 activity. TSP-1 disruption did not affect inflammatory gene expression and activation of transforming growth factor- β /small mothers against decapentaplegic signaling in the db/db myocardium. In cardiac fibroblasts populating collagen pads, TSP-1 incorporation into the matrix did not activate transforming growth factor- β responses, but inhibited leptin-induced matrix metalloproteinase-2 activation. TSP-1 disruption abrogated age-associated capillary rarefaction in db/db mice, attenuating myocardial upregulation of angiopoietin-2, a mediator that induces vascular regression. *In vitro*, TSP-1 stimulation increased macrophage, but not endothelial cell, angiopoietin-2 synthesis.

Conclusions: TSP-1 upregulation in the diabetic heart prevents chamber dilation by exerting matrix-preserving actions on cardiac fibroblasts and mediates capillary rarefaction through effects that may involve angiopoietin-2 upregulation. (*Circ Res*. 2013;113:1331-1344.)

Key Words: diabetic cardiomyopathies ■ fibrosis ■ matrix metalloproteinases ■ thrombospondins
■ ventricular remodeling

Diabetes mellitus and obesity are associated with increased susceptibility to cardiovascular disease.^{1,2} Data derived from the Framingham study suggest that men with diabetes mellitus have a 2.4-fold higher incidence of heart failure; the risk of heart failure is even higher (5.1-fold) in diabetic women.³ The increased prevalence of heart failure in diabetes mellitus is only in part because of the increased risk of atherosclerotic coronary disease and its complications. Diabetics also develop a unique cardiomyopathic condition, termed diabetic cardiomyopathy,⁴⁻⁶ that is independent of coronary artery disease. Diabetic cardiomyopathy is characterized by extensive fibrotic

changes, expansion of the cardiac interstitium,⁷ and profound alterations in the cardiac interstitial matrix⁸ leading to increased myocardial stiffness and development of diastolic dysfunction.⁹ Despite its significance, the pathophysiologic basis of cardiac fibrosis in diabetes mellitus remains poorly understood.

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Tissue fibrosis requires the dynamic participation of the extracellular matrix and is regulated by a family of structurally unrelated macromolecules called matricellular proteins.¹⁰ Matricellular proteins are generally not expressed in the

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Nonstandard Abbreviations and Acronyms

MMP	matrix metalloproteinase
Smad	small mothers against decapentaplegic
TGF-β	transforming growth factor- β
TSP-1	thrombospondin-1
VEGF	vascular endothelial growth factor

normal heart, but are markedly upregulated in the remodeling myocardium, and, through binding to structural matrix proteins, serve as molecular bridges between the matrix and the cells, transducing or modulating growth factor signals.^{11–14} Thrombospondin (TSP)-1 is a prototypical matricellular protein that is not part of the normal cardiac matrix network, but is upregulated in cardiac remodeling because of myocardial infarction¹⁵ or pressure overload.¹⁶ In the pressure-overloaded heart, TSP-1 modulates fibroblast phenotype by activating transforming growth factor (TGF)- β and preserves the matrix by inhibiting matrix metalloproteinase (MMP) activity.¹⁶ In addition to its profibrotic and matrix-preserving actions, TSP-1 is also a potent angiostatic mediator,^{17,18} which promotes endothelial cell apoptosis through activation of a CD36/p59fyn/p38 mitogen-activated protein kinase pathway.¹⁹

Experimental and clinical studies have demonstrated that obesity and diabetes mellitus are associated with marked upregulation of TSP-1 in the adipose tissue and in the cardiovascular system.^{20,21} In adipose tissue harvested from obese patients, TSP-1 expression was markedly increased and was strongly associated with insulin resistance and inflammatory activity.²¹ Moreover, in obese diabetic Zucker rats, TSP-1 protein expression was markedly upregulated in the vascular adventitia and in the cardiac interstitium²⁰; TSP-1 induction in diabetic vessels was associated with reduced density of vasa vasorum. In vitro, hyperglycemia potently upregulated TSP-1 synthesis; high glucose levels induced a 30-fold increase in TSP-1 expression by isolated endothelial cells, smooth muscle cells, and fibroblasts.²⁰

We hypothesized that, through its profibrotic and angiostatic properties, TSP-1 may play an important role in remodeling of the diabetic heart. Incorporation of TSP-1 in the interstitial matrix of the diabetic myocardium may locally activate TGF- β signaling, inducing fibroblast activation and promoting fibrotic remodeling. Moreover, through its potent angiostatic properties, TSP-1 may be responsible for the impaired angiogenic responses in the diabetic heart. To test this hypothesis, we used the db/db mouse, a model of genetic obesity and diabetes mellitus due to central leptin resistance that is associated with progressive cardiac fibrosis.²² A marked TSP-1 upregulation in db/db hearts predominantly localized in perivascular and interstitial areas was demonstrated. Through the generation and systematic study of db/db TSP-1^{-/-} (dbTSP) mice, we found that TSP-1 loss attenuates diabetes mellitus-associated cardiac fibrosis and enhances myocardial protease activity, without significantly improving diastolic function. In contrast, loss of the matrix-preserving actions of TSP-1 resulted in mild left ventricular dilation and modest nonprogressive systolic dysfunction. In vitro, TSP-1 incorporation in collagen gels did not activate latent TGF- β , but exerted matrix-preserving actions

by inhibiting leptin-induced MMP activation. Assessment of the cardiac vasculature demonstrated that TSP-1 loss attenuated diabetes mellitus-associated vascular rarefaction, documenting that TSP-1 upregulation is an important angiostatic mediator in the diabetic myocardium. Our *in vivo* and *in vitro* studies demonstrated that TSP-1 induction in the diabetic heart upregulates angiopoietin-2 expression, suggesting a novel TSP-1-mediated angiostatic pathway.

Methods

Detailed methodology is provided in the Online Data Supplement.

Generation of db/db TSP-1^{-/-} Mice

Animal studies were approved by the Institutional Animal Care and Use Committees at Albert Einstein College of Medicine and at Baylor College of Medicine. Lep^{r^dm}/+ on a C57BL6J background (db/+) were purchased from Jackson Laboratories, Bar Harbor, ME. TSP-1^{-/-} mice (on a C57BL6J background) were obtained from our own colony.^{15,16,23} TSP-1^{-/-} db/db mice were generated by crossing db/+ mice and TSP-1^{-/-} animals. Genotyping was performed through established PCR protocols.

Assessment of Adiposity

Lean and fat tissue mass was measured in db/db (male, n=9; female, n=7) and dbTSP mice (male, n=11; female, n=5) at 2 and 4 months of age by dual-energy x-ray absorptiometry.

Assessment of Metabolic Profile

Animals used for assessment of metabolic parameters in the serum (n=5 per group) were euthanized at 2 months of age and blood was collected by aortic puncture. Plasma glucose, insulin, cholesterol, leptin, adiponectin, triglyceride, free fatty acid, and glycerol levels were assessed at the Baylor College of Medicine Diabetes and Endocrinology Research Center Core.

Echocardiography

For echocardiographic analysis, db/db (n=14) and dbTSP (n=19) mice were imaged at 4, 6, 8, and 12 months of age using a Vevo770 system (Visualsonics, Toronto, Canada).

Assessment of Contractile Function and Reserve in Isolated Perfused Mouse Hearts

Wild-type (WT; n=7), db/db (n=10), and dbTSP (n=14) mice were used for functional analysis in an isolated perfused heart system. Hearts were perfused in the Langendorff mode and subjected to dobutamine infusion to assess inotropic and lusitropic responses.

Immunohistochemistry and Histology

For histopathologic analysis, zinc/formalin-fixed, paraffin-embedded sections were obtained from WT, db/db, and dbTSP hearts at 6 and 12 months of age. Collagen fibers were identified by Picosirius red staining. For visualization of myocardial capillary network, CD31 immunohistochemistry was performed using the rat antimouse CD31 antibody (BD Pharmingen) as previously described.²⁴ To identify cardiac arterioles, immunohistochemistry with an anti- α -SMA antibody (Sigma) was performed as previously described.²⁴ TSP-1 immunostaining was performed using a mouse monoclonal antibody to TSP-1 (NeoMarkers). Assessment of capillary and arteriolar density was performed. Capillary and arteriolar densities were expressed as vessels/mm².

RNA Extraction and Quantitative Real-Time PCR

Gene expression in hearts and cultured fibroblasts was assessed using quantitative PCR. The sequences of primers used in the study are listed in Online Table I.

Protein Extraction and Western Blotting

Protein was isolated from whole WT, TSP-1^{-/-}, db, and dbTSP hearts (n=8 per group) and Western blotting was performed as previously described.

Hydroxyproline Assay

To assess collagen deposition in WT, db/db, and dbTSP hearts (n=8 per group), we used a previously described method²⁵ that uses hydroxyproline content as surrogate measure of collagen.

Zymography

MMP activity in murine WT, db, and dbTSP hearts (n=8 per group) was assessed by gelatin zymography as previously described.²⁶

Isolation and Stimulation of Cardiac Fibroblasts in Collagen Pads

WT mouse cardiac fibroblasts were isolated by enzymatic digestion as previously described.²⁵ To study the effects of matrix-bound TSP-1 on cardiac fibroblasts, an *in vitro* assay of cardiac fibroblasts populating collagen pads in the presence or absence of TSP-1, leptin, and active or latent TGF- β was used as previously described.^{25,27} At the end of the experiment, gel area was assessed using Image Laboratory 3.0 software to measure the contractile activity of fibroblasts. Subsequently, each collagen pad was divided in half and used for analysis of MMP activity by gelatin zymography and for gene expression studies by quantitative PCR.

Isolation and Stimulation of Mouse Splenic Macrophages

Splenic macrophages were isolated from WT mice using immunomagnetic bead sorting for CD11b as previously described.²⁸

Stimulation of Mouse Cardiac Microvascular Endothelial Cells

Mouse cardiac microvascular endothelial cells (MCMECs) were purchased from Cell Biologics and were stimulated with TSP-1 to assess expression of angiogenesis-related genes.

Statistical Analysis

Data are expressed as means \pm SEM. Statistical analysis was performed using unpaired, 2-tailed Student *t* test or nonparametric *t* test for non-Gaussian distributions (Mann–Whitney) and 1-way ANOVA with Tukey multiple comparison test or nonparametric ANOVA (Kruskal–Wallis) for non-Gaussian distributions. Statistical analyses were performed using Prism 6.0 software. *P*<0.05 was considered to be significant. Mortality was compared using the log-rank test.

Results

Cardiac Fibrosis in db/db Mice Is Associated With TSP-1 Upregulation

Obese diabetic db/db mice exhibited cardiac fibrosis. At 2 months of age, db/db animals had higher myocardial collagen I mRNA expression compared with lean WT animals (Figure 1A). At 6 months of age, myocardial collagen content, measured through a hydroxyproline biochemical assay, was significantly higher in db/db animals (Figure 1B). Sirius red staining identified collagen fibers in the myocardium showing expansion of the cardiac interstitium and extensive deposition of collagen in db/db mouse hearts (Figure 1C and 1D). Cardiac fibrosis in db/db animals was associated with significant TSP-1 mRNA upregulation (Figure 1E). Immunohistochemical staining demonstrated low levels of TSP-1 immunoreactivity in WT mice (Figure 1F); in contrast, db/db animals had intense TSP-1 staining in perivascular and interstitial areas (Figure 1G). TSP-1^{-/-} mice were used as negative controls and had no myocardial staining for TSP-1 (Figure 1H).

Weight Gain and Adiposity in db/db TSP-1^{-/-} Mice

To study the role of TSP-1 in fibrotic remodeling of the db/db heart, we generated dbTSP mice. During a 12-month follow-up, mortality was comparable between db/db and dbTSP

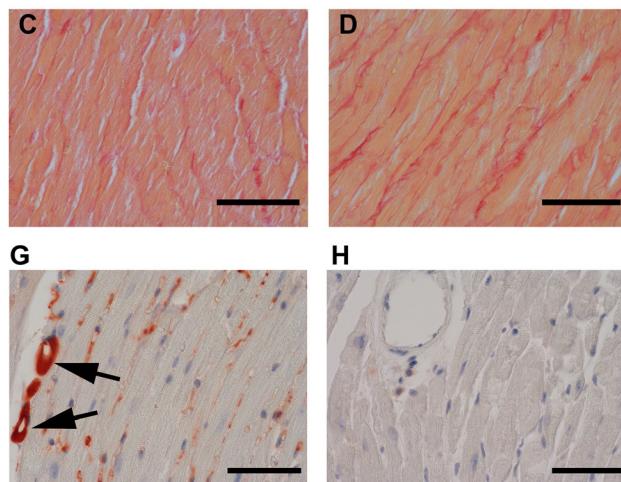
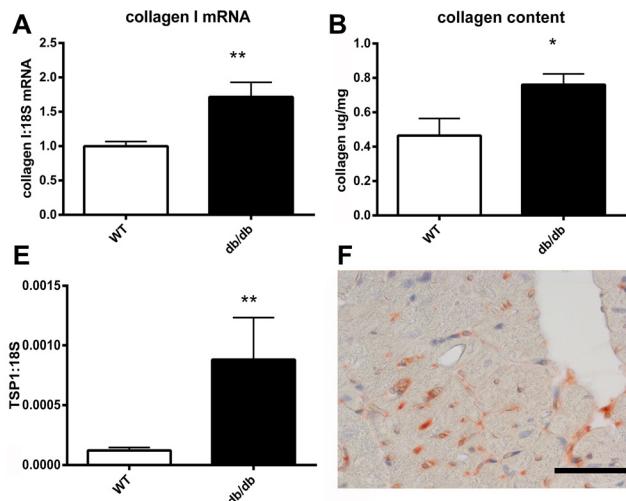


Figure 1. Cardiac fibrosis in the db/db myocardium is associated with thrombospondin (TSP)-1 upregulation. A to D, db/db mice had significant fibrotic remodeling of the myocardium. Quantitative PCR analysis showed marked upregulation of collagen I mRNA in db/db hearts at 2 months of age (A). A hydroxyproline biochemical assay showed that 6-month-old db/db mice had significantly higher myocardial collagen content when compared with age-matched wild-type (WT) animals (B). Sirius red staining shows increased deposition of collagen in the cardiac interstitium of db/db animals (D), when compared with lean WT mice (C). E, Quantitative PCR shows that TSP-1 mRNA expression was markedly increased in db/db hearts. F and G, Immunohistochemical staining of 6-month-old mouse hearts showed weak immunoreactivity in lean WT mice (F). In contrast, in the db/db myocardium, intense TSP-1 staining was noted (G) and was predominantly localized in perivascular and interstitial areas (arrows). TSP-1^{-/-} mice showed negligible staining for TSP-1 (H); **P*<0.05 vs WT; ***P*<0.01 vs WT. Scale bar, 50 μ m.

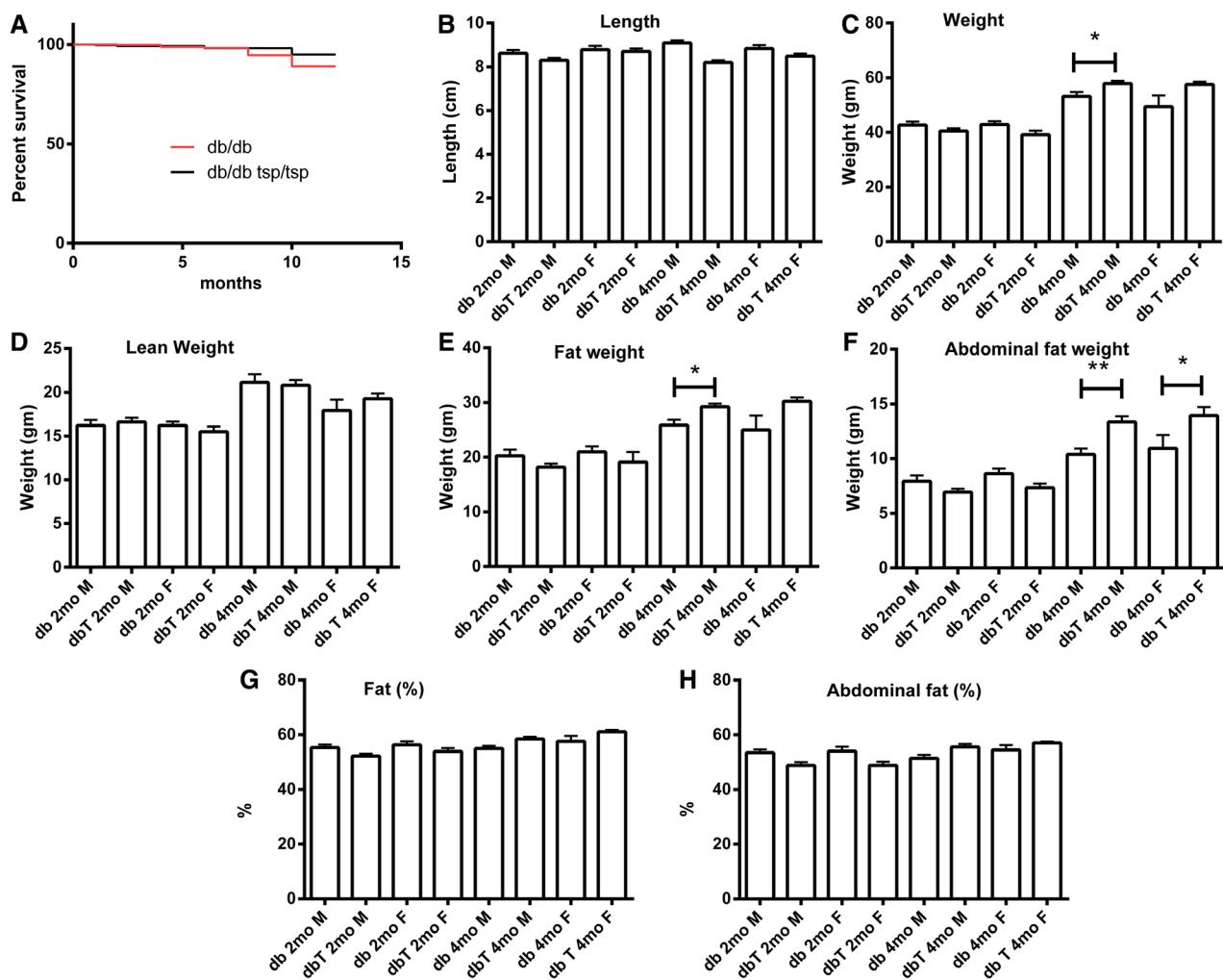


Figure 2. Effects of thrombospondin (TSP)-1 loss on survival, weight gain, and adiposity of db/db mice. **A**, db/db and db/db TSP-1^{-/-} mice had comparable survival curves after 12 months of follow-up. **B**, TSP-1 loss did not affect mouse size (length). **C**, TSP-1 loss had subtle sex-specific effects on weight gain in db/db mice. At 4 months of age, male db/db TSP-1^{-/-} animals (dbT) had significantly increased body weight; however, in female mice no statistically significant difference was noted between db/db and db/db TSP-1^{-/-} animals. **D**, Lean weight was comparable between groups. **E** and **F**, At 4 months of age, TSP-1 loss was associated with increased fat weight in male db/db mice and with higher abdominal fat weight in both male and female animals. **G** and **H**, However, fat percent content was comparable between db/db and db/db TSP-1^{-/-} mice. *P<0.05 and **P<0.01 vs corresponding db/db mice.

mice (Figure 2A). In db/db mice, TSP-1 loss was associated with an increase in body weight at 4 months of age that was statistically significant only for male mice; in contrast, mouse length was comparable between groups (Figure 2B and 2C). Dual-energy x-ray absorptiometry analysis showed that in db/db mice TSP-1 disruption did not affect lean weight (Figure 2D), but resulted in a modest increase in total fat weight (Figure 2E) and abdominal fat weight (Figure 2F) in both male and female animals. However, no sex-specific differences in weight and fat gain were noted between 2 and 4 months of age (Online Table II), and the percentage of total and abdominal fat content in db/db animals was not affected by TSP-1 loss (Figure 2G and 2H).

Effects of TSP-1 Loss on the Metabolic Profile in db/db Mice

Next, we examined whether TSP-1 loss had an effect on the development of metabolic dysfunction in db/db mice. At 2 months of age, db/db and dbTSP mice had comparable

plasma glucose and insulin levels; HOMA IR was also not significantly different between groups (Figure 3A–3C). TSP-1 disruption in db/db animals was associated with a significant increase in plasma cholesterol levels (Figure 3D). However, plasma glycerol, free fatty acids, and triglycerides were not significantly affected by TSP-1 loss (Figure 3E–3G). Plasma levels of the adipokines leptin and adiponectin were also comparable between db/db and dbTSP mice (Figure 3H and 3I).

dbTSP Mice Exhibit Nonprogressive Ventricular Dilation Accompanied by Modest Systolic Dysfunction

Because cardiac TSP-1 upregulation may play a role in remodeling of the diabetic heart, we examined the effects of TSP-1 loss on function and geometry of db/db heart. As they age, db/db mice exhibit marked cardiac hypertrophy in the absence of significant systolic dysfunction. At 4 to 12 months of age, dbTSP mice had increased left ventricular dimensions and volumes when compared with db/db animals (Figure 4A and

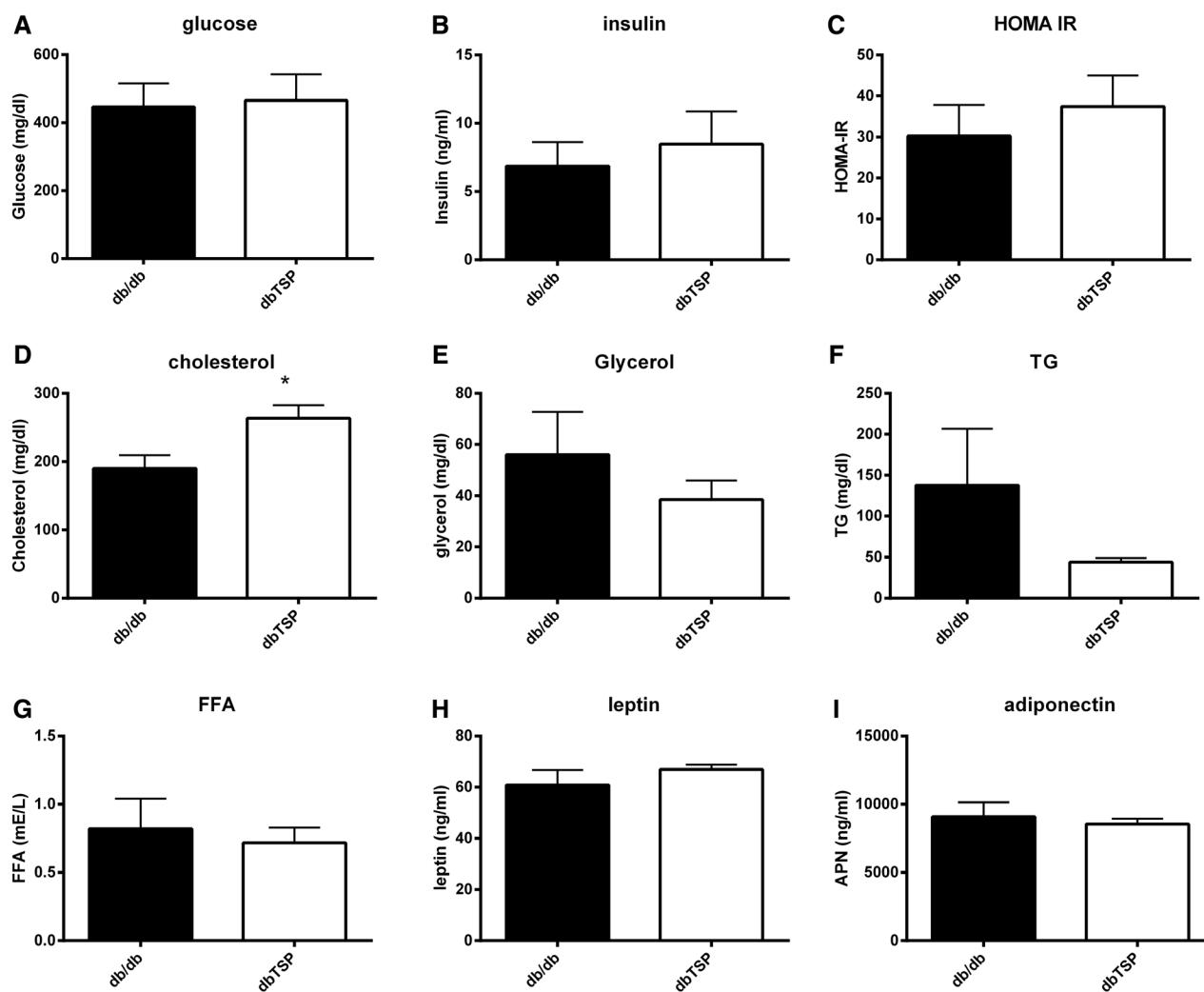


Figure 3. Effects of thrombospondin (TSP)-1 loss on the metabolic profile in db/db mice. Serum glucose (A), insulin (B), HOMA IR (C), glycerol (E), triglyceride (F), free fatty acids (FFA) (G), leptin (H), and adiponectin (I) levels were comparable between db/db and dbTSP animals at 2 months of age. However, TSP-1 loss was associated with an increase in serum cholesterol levels (D). *P<0.05 vs db/db mice.

4B; Online Table II), suggesting that TSP-1 absence induces chamber dilation. At 4 months of age, dbTSP mice had mildly depressed systolic function, evidenced by a reduction in ejection fraction (Figure 4C; Online Table II). However, systolic dysfunction in dbTSP mice was not progressive; at later time points, ejection fraction was not affected by TSP-1 loss. dbTSP animals also had lower LV mass compared with db/db mice and reduced anterior and posterior wall thickness, suggesting attenuated cardiac hypertrophy (Figure 4D and 4F).

When Compared With db/db Hearts, dbTSP Hearts Exhibit Delayed Responses to Dobutamine

We studied the effects of TSP-1 loss on contractile reserve in dobutamine-stimulated isolated working hearts. Lean WT and TSP-1^{-/-} mouse hearts had comparable systolic and diastolic function at baseline and on stimulation with dobutamine (Online Table III). At 6 months of age, db/db mice and dbTSP animals had no evidence of systolic dysfunction; baseline dp/dt_{max} and left ventricular developed pressure were comparable between WT, db/db, and dbTSP hearts (Figure 4G and 4H). During dobutamine infusion, db/db mice and WT

animals had comparable increases in systolic contractile function. On infusion of low-dose dobutamine (7.5 ng/minute), dbTSP hearts exhibited a trend toward a blunted contractile response, evidenced by lower dp/dt_{max} and left ventricular developed pressure (Figure 4I and 4J). Moreover, with high doses of dobutamine infusion (37.5–75 ng/minute), dbTSP hearts had a marked temporal delay in mounting a peak contractile response (Figure 4K).

Effects of TSP-1 Loss on the Development of Diastolic Dysfunction in the db/db Heart

Diastolic function was compared between WT, db/db, and dbTSP mice by assessing left atrial size, by performing mitral inflow Doppler echocardiography, and through hemodynamic assessment of the isolated perfused heart. At 6 months of age, db/db mice had increased left atrial size compared with WT animals. TSP-1 loss in db/db animals was associated with a reduction in left atrial dimensions (Figure 4G). Mitral inflow Doppler echocardiography showed that, compared with WT mice, db/db animals had a reduced E/A ratio; however, TSP-1 loss did not significantly affect the E/A ratio in db/db mice

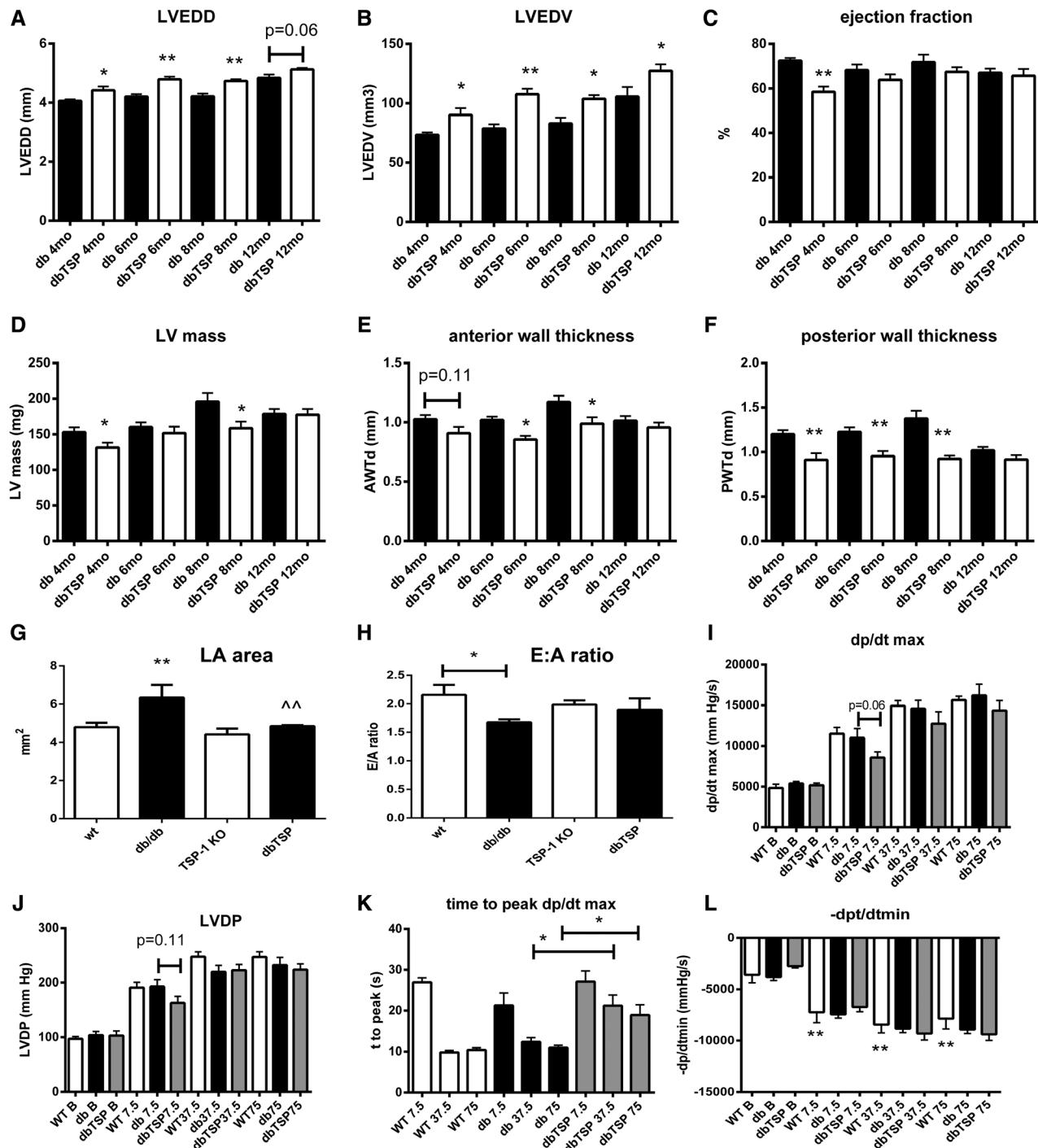


Figure 4. Thrombospondin (TSP)-1 loss in db/db mice results in dilative cardiac remodeling and decreased left ventricular wall thickness. Echocardiographic imaging showed that db/db TSP-1^{-/-} mice (dbTSP) had increased left ventricular end-diastolic diameter (LVEDD; **A**) and left ventricular end-diastolic volume (LVEDV; **B**) when compared with db/db animals (db), suggesting increased dilative remodeling; * $P<0.05$, ** $P<0.01$ vs age-matched db/db mice; db/db, n=14; dbTSP, n=19. **C**, At 4 months of age, systolic function was mildly depressed in dbTSP mice; however, loss of TSP-1 did not cause prolonged or progressive systolic ventricular dysfunction. **D** to **F**, TSP-1 absence in db/db animals attenuated cardiac hypertrophy leading to reductions in left ventricular mass (**D**), anterior wall end-diastolic thickness (AWT; **E**), and posterior wall end-diastolic thickness (PWT; **F**). **G**, At 6 months of age, db/db mice had significantly increased left atrial area when compared with wild-type (WT) animals; ** $P<0.01$. TSP-1 loss significantly abrogated the increase in LA size observed in diabetic animals. **H**, Mitral inflow Doppler echocardiography showed that db/db animal had a significant reduction in the E/A ratio, suggesting impaired relaxation; * $P<0.05$ vs WT. TSP-1 loss did not affect the E/A ratio in lean or obese mice. **I** to **L**, Assessment of left ventricular contractile reserve in a Langendorff model demonstrated that at 6 months of age obese diabetic db/db mice and WT lean animals had no difference in systolic function, assessed through measurements of dp/dt_{max} (**I**) and left ventricular developed pressure (LVDP; **J**), both at baseline and after infusion of dobutamine. TSP-1 loss did not significantly affect systolic function in db/db mice; only trends toward reduced LVDP and dp/dt_{max} were observed with low-dose dobutamine infusion. However, TSP-1 loss in db/db mice significantly delayed the peak systolic response after dobutamine infusion (**K**). **L**, Diastolic function, measured through assessment of -dp/dt_{min} was not significantly affected by TSP-1 loss; ** $P<0.01$ vs baseline -dp/dt_{min}.

(Figure 4H). Assessment of diastolic function in isolated perfused hearts showed that $-dp/dt_{max}$ was comparable between db/db and dbTSP animals (Figure 4L). Thus, TSP-1 absence was not associated with consistent evidence of altered diastolic function in diabetic animals.

dbTSP Mice Exhibit Reduced Myocardial Collagen Content and Increased MMP Activation

Next, we explored the basis for dilative remodeling in dbTSP hearts. Because the extracellular matrix plays a key role in determining cardiac geometry, we studied the effects of TSP-1 loss on cardiac matrix metabolism. Using a hydroxyproline assay, we demonstrated that db/db hearts have increased myocardial collagen deposition; TSP-1 loss significantly reduced collagen content in db/db animals (Figure 5A). Reduced collagen content in dbTSP hearts was not because of lower collagen mRNA synthesis (Figure 5B) but was associated with marked increases in MMP activity. dbTSP mice had a 2-fold increase in MMP-2

activity and a 5-fold increase in MMP-9 activity when compared with db/db animals (Figure 5C–G). Increased protease activity in the absence of TSP-1 was not associated with increased MMP mRNA or protein levels. Quantitative PCR showed that myocardial MMP-2 and MMP-9 mRNA levels were comparable between db/db and dbTSP mice (Figure 5F and 5G). Moreover, Western blotting demonstrated that db/db and dbTSP mice had comparable myocardial MMP-2 and MMP-9 protein levels (Online Figure I).

Dilation and Increased Matrix Remodeling in dbTSP Mice Is Not Because of Impaired TGF- β Activation

TGF- β promotes matrix preservation and critically regulates cardiac remodeling.²⁹ Because TSP-1 is an essential activator of TGF- β , we hypothesized that the effects of TSP-1 disruption in db/db hearts may be because of impaired TGF- β signaling. When compared with WT animals, db/db mice had significantly increased myocardial expression of p-Smad2 and

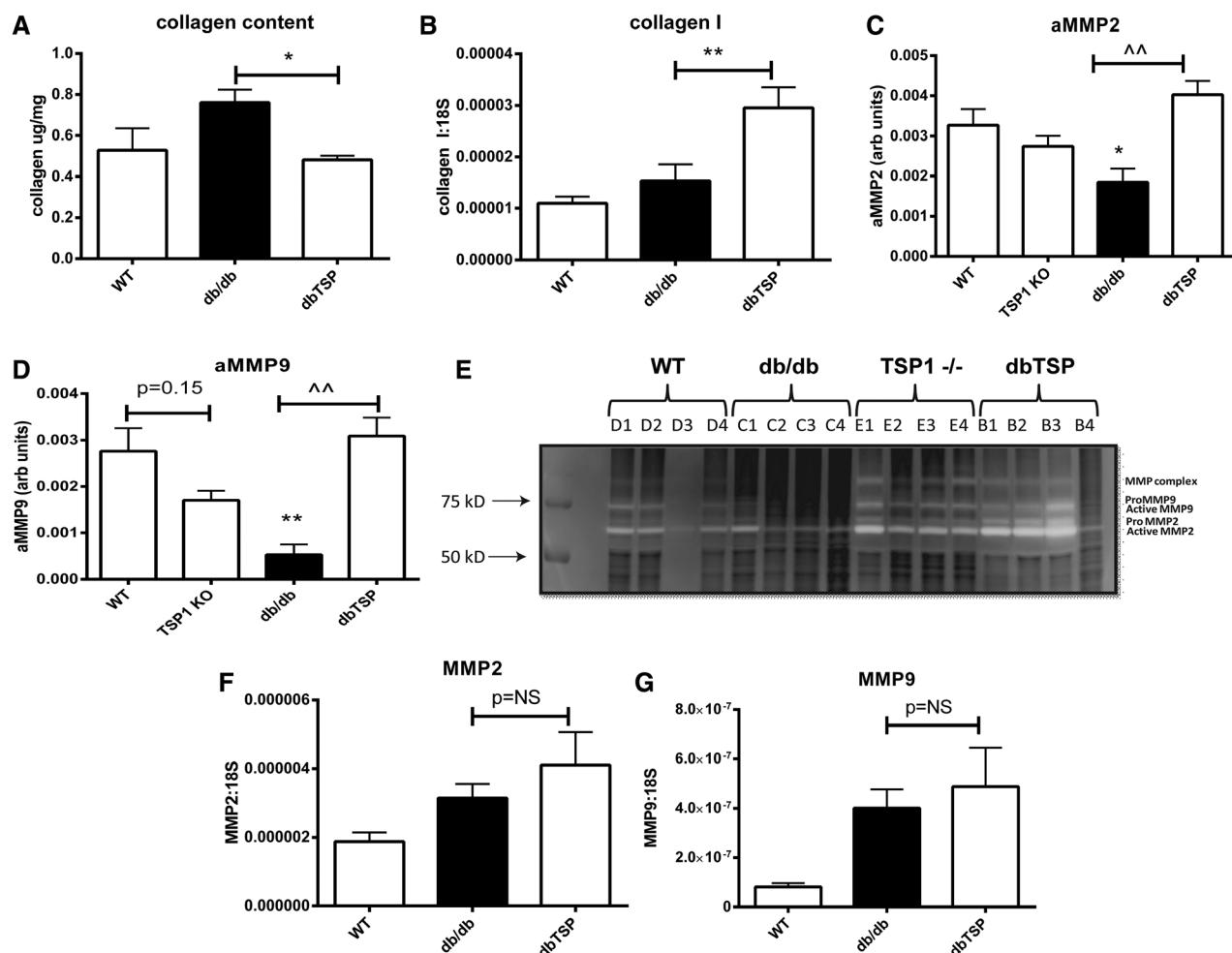


Figure 5. Dilative remodeling of the thrombospondin (TSP)-1-deficient db/db heart is associated with decreased collagen content and enhanced matrix metalloproteinase (MMP) activity. **A**, Assessment of collagen content using a hydroxyproline biochemical assay demonstrated that TSP-1 loss significantly reduced collagen content in 6-month-old db/db animals; * $P<0.05$ vs db/db; db/db, n=8 per group. **B**, Reduced collagen content was not associated with attenuated collagen mRNA levels; myocardial collagen I mRNA levels were higher in db/db TSP-1^{-/-} (dbTSP) hearts. **C** to **E**, Zymography showed that TSP-1 absence resulted in significantly increased MMP-2 and MMP-9 activity in db/db animals; * $P<0.05$ vs wild-type (WT); ** $P<0.01$ vs WT; ^ $P<0.01$ vs db/db. **F** and **G**, Enhanced MMP activity in dbTSP hearts was not associated with increased MMP-2 or MMP-9 mRNA levels.

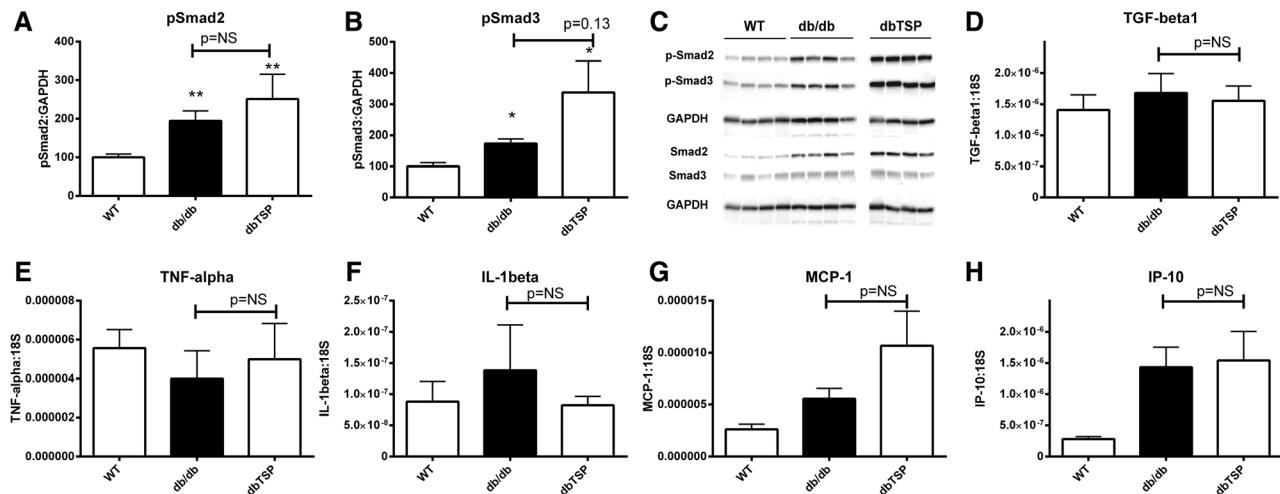


Figure 6. Thrombospondin (TSP)-1 deficiency does not affect myocardial inflammation and activation of TGF- β /Smad signaling in the db/db myocardium. **A** to **C**, Western blotting showed that myocardial expression of phosphorylated Smad2 (**A**) and phosphorylated Smad3 (**B**) was significantly increased in db/db animals when compared with lean wild-type mice. db/db TSP-1^{-/-} (dbTSP) mice had comparable myocardial p-Smad2 levels and showed a trend toward increased pSmad3 expression when compared with db/db mice. Moreover, db/db and dbTSP mice had comparable myocardial mRNA expression of the cytokines transforming growth factor (TGF)- β (**D**), tumor necrosis factor (TNF)- α (**E**), interleukin (IL)-1 β (**F**), and the chemokines monocyte chemoattractant protein (MCP)-1/CCL2 (**G**) and interferon- γ -inducible protein (IP)-10/CXCL10 (**H**; n=8 mice per group).

p-Smad3, suggesting activation of the TGF- β /small mothers against decapentaplegic (Smad) signaling pathway. p-Smad2 and p-Smad3 expression was comparable between db/db and dbTSP mice (Figure 6A–6C), suggesting that TSP-1 loss does not affect activation of canonical TGF- β signaling. Moreover, db/db and dbTSP mice had comparable expression of TGF- β 1 mRNA in the myocardium (Figure 6D).

Dilation of dbTSP Heart Is Not Because of Accentuated Inflammation

Proinflammatory mediators, such as interleukin-1 β and tumor necrosis factor- α , mediate cardiac dilation and dysfunction by inducing and activating MMPs. Because TSP-1 down-modulates proinflammatory responses, we examined whether dilation and accentuated matrix metabolism in dbTSP hearts is because of increased inflammatory activity. Myocardial expression of the proinflammatory cytokines tumor necrosis factor- α and interleukin-1 β (Figure 6E and 6F), and of the chemokines monocyte chemoattractant protein-1 and interferon- γ -inducible protein-10 (Figure 6G and 6H), was comparable between db/db and dbTSP mice, suggesting that TSP-1 loss does not affect inflammatory activity in db/db mice.

Cardiac Fibroblasts Harvested From dbTSP Mice Exhibit Attenuated Matrix Synthetic Capacity

To explore the basis for accentuated matrix-degrading activity in the absence of TSP-1, we isolated cardiac fibroblasts from db/db and dbTSP hearts and compared baseline expression of genes associated with matrix synthesis and metabolism. dbTSP fibroblasts had reduced collagen I and III mRNA expression and showed trends for increased expression of MMP-2 and MMP-3 mRNA (Figure 7A–7D). Tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 mRNA expression was comparable between groups (Figure 7E). Altered expression of genes associated with matrix metabolism in dbTSP

cardiac fibroblasts was not associated with impaired activation of TGF- β /Smad signaling. Western blotting demonstrated that p-Smad2 expression was comparable between fibroblasts isolated from db/db and dbTSP hearts (Figure 7F).

When Bound to the Matrix, TSP-1 Does Not Activate TGF- β Responses But Enhances Matrix Preservation, Inhibiting Leptin-Induced MMP Activation

As a matricellular protein, TSP-1 modulates cellular phenotype when bound to the structural matrix. Accordingly, we tested the hypothesis that incorporation of TSP-1 into the matrix may induce a matrix-preserving phenotype in cardiac fibroblasts using a cell biological assay of fibroblast-populated collagen pads in the presence or absence of TSP-1. Because TSP-1 is considered an essential activator of TGF- β , we examined whether incorporation of TSP-1 into the matrix promotes TGF- β -mediated actions in fibroblasts stimulated with latent TGF- β . Active, but not latent, TGF- β induced gel contraction in fibroblast-populated collagen pads (Figure 7G), reflecting the prominent effects of the growth factor on myofibroblast transdifferentiation. Incorporation of TSP-1 into the matrix had no effect on gel contraction, both in the presence or absence of latent TGF- β (Figure 7G), suggesting that TSP-1 exerts no significant TGF- β -activating effects in this model. Whereas, leptin stimulation induced modest, but significant, gel contraction in the presence or absence of TSP-1 (Figure 7H). Zymography demonstrated that leptin markedly enhanced MMP-2 activity in fibroblast-populated collagen pads (Figure 7I). When incorporated into the matrix, TSP-1 attenuated the matrix-degrading effects of leptin. The effects of leptin and TSP-1 on MMP-2 activity were not because of modulation of MMP-2 mRNA levels (Figure 7J). Because membrane-type MMP-1 and TIMP-2 are implicated in MMP-2 activation, we examined whether they are involved in

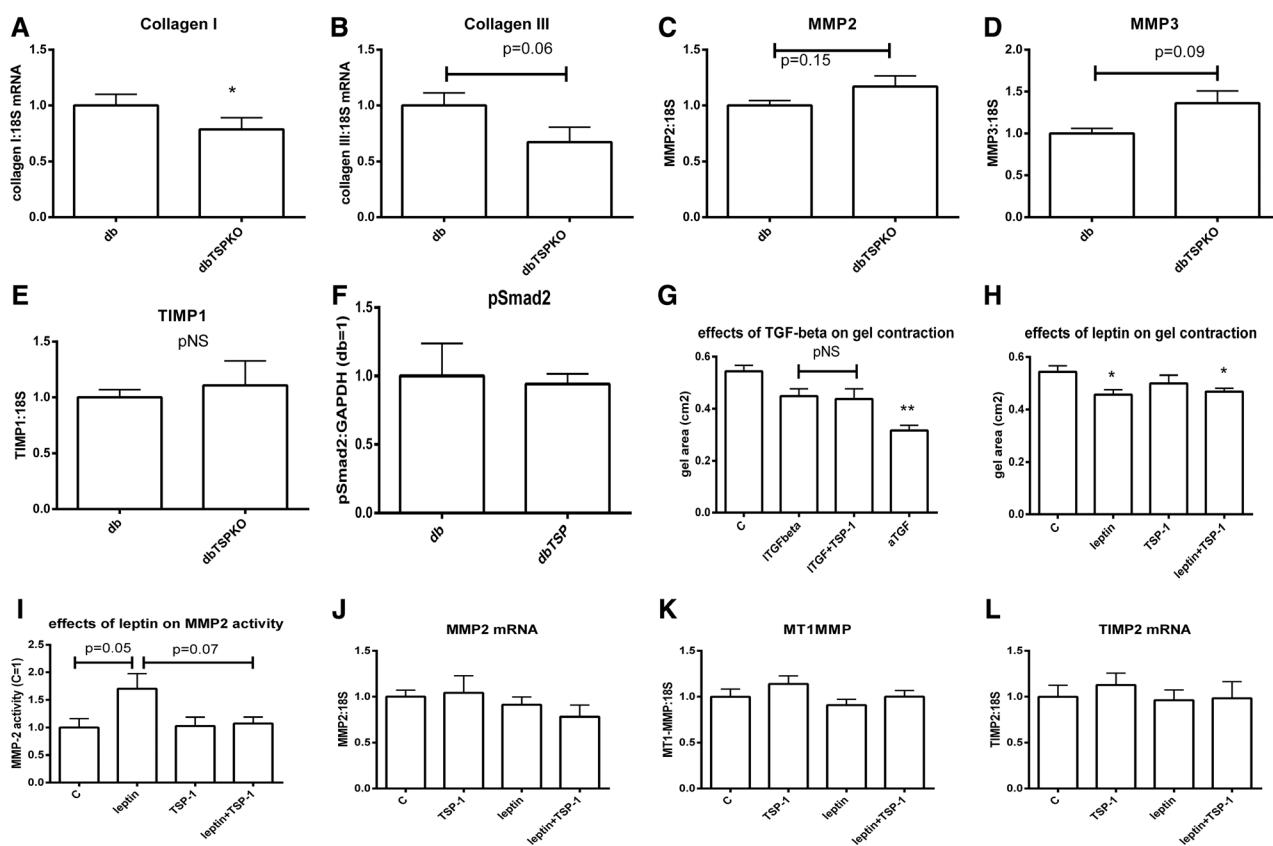


Figure 7. Matrix-preserving effects of thrombospondin (TSP)-1 in isolated cardiac fibroblasts. Cardiac fibroblasts isolated from db/db TSP-1^{-/-} hearts had reduced collagen I (A) and III (B) mRNA expression and showed trends toward increased matrix metalloproteinase (MMP)-2 (C) and MMP-3 (D) synthesis when compared with fibroblasts harvested from db/db hearts. In contrast, tissue inhibitor of metalloproteinase (TIMP)-1 mRNA synthesis (E) and expression of p-Smad2 protein (F) was comparable between db/db and dbTSP fibroblasts. G, In fibroblasts populating collagen pads, incorporation of TSP-1 into the matrix did not activate TGF- β responses. Active TGF- β (aTGF) induced marked gel contraction in fibroblast-populated pads, reflecting myofibroblast transdifferentiation. In contrast, TSP-1 incorporation into the pad failed to increase the effects of latent TGF- β (ITGBeta) on gel contraction, suggesting that in this model TSP-1 cannot activate TGF- β . H, Leptin stimulation modestly, but significantly, increased gel contraction in fibroblast-populated pads. TSP-1 did not affect the effects of leptin on gel contraction. I, In fibroblast-populated collagen pads, TSP-1 incorporation into the matrix abrogated the stimulatory effects of leptin on MMP-2 activity. J to L, Inhibitory actions of TSP-1 on MMP-2 activation were not because of modulation of MMP-2 (J), membrane-type matrix metalloproteinase-1 (MT1-MMP; K), and TIMP-2 (L) synthesis (n=6 per experimental condition).

mediating the inhibitory effects of TSP-1 on MMP-2 activity. We found that leptin and TSP-1 had no effects on fibroblast-derived membrane-type MMP-1 and TIMP-2 mRNA synthesis (Figure 7K and 7L).

TSP-1 Loss Abrogates Capillary, But Not Arteriolar, Rarefaction in db/db Mice

Through its potent angiostatic effects, TSP-1 may be responsible for impaired angiogenesis in the diabetic heart. To study the effects of TSP-1 loss on the microvascular network in diabetic hearts, we compared vascular density between db/db and dbTSP animals. At 6 and 12 months of age, db/db mice exhibited significantly lower capillary and arteriolar density when compared with WT animals (Figure 8A and 8B). TSP-1 disruption attenuated capillary rarefaction in db/db animals but had no significant effect on arteriolar density (Figure 8A–8E).

TSP-1 Mediates Myocardial Angiopoietin-2 Upregulation in db/db Mice

Angiogenesis is regulated through dynamic interactions between vascular endothelial growth factor (VEGF) and

angiopoietins.³⁰ Because diabetes mellitus is associated with overexpression of angiopoietin-2, a mediator that promotes vascular regression,^{31,32} we examined whether attenuation of capillary rarefaction in dbTSP mice is associated with reduced angiopoietin-2 expression. Western blotting showed that 12-month-old db/db mice had significantly higher myocardial angiopoietin-2 expression compared with WT animals (Figure 8G). TSP-1 absence markedly reduced myocardial angiopoietin-2 expression in 6- and 12-month-old animals (Figure 8F and 8G). In contrast, TSP-1 loss did not affect myocardial VEGF protein expression (Online Figure II).

TSP-1 Stimulation Upregulates Angiopoietin-2 Expression in Macrophages

We next examined whether TSP-1 directly induces angiopoietin-2 expression. In isolated mouse macrophages, TSP-1 stimulation increased angiopoietin-2 mRNA synthesis (Figure 8H). Moreover, cardiac fibroblasts harvested from db/db mice showed increased angiopoietin-2 mRNA expression;

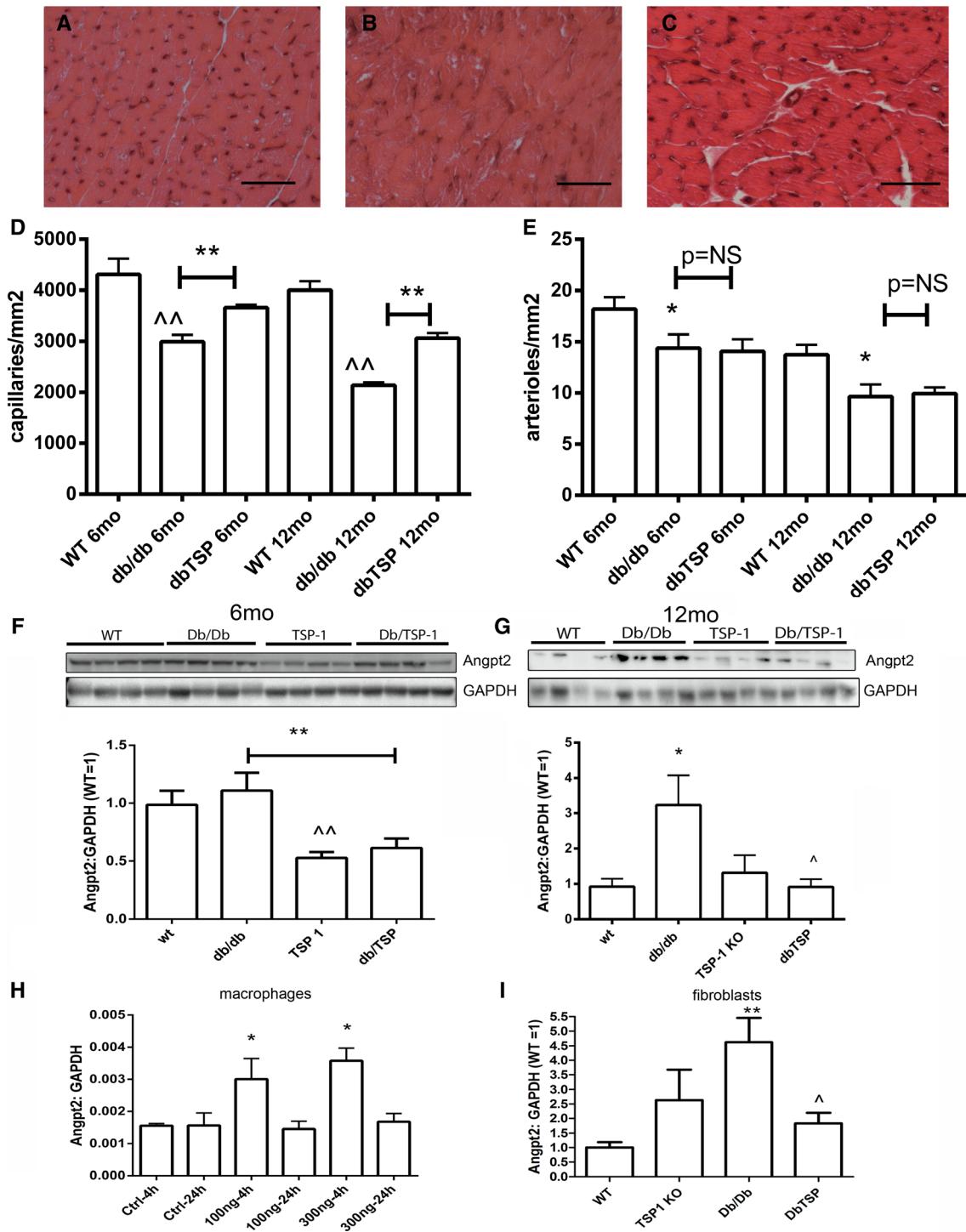


Figure 8. Thrombospondin (TSP)-1 upregulates angiopoietin-2 and mediates capillary rarefaction in the db/db myocardium. **A** to **C**, Myocardial capillaries were identified using staining with the endothelial cell marker CD31. Representative images of stained sections from 6-month-old wild-type (WT; **A**), db/db (**B**), and db/bb TSP-1^{-/-} (dbTSP; **C**) hearts are shown. **D**, Quantitative analysis demonstrated that db/db hearts exhibited significant reduction in vascular density at 6 and 12 months of age; $^{\wedge\wedge}P<0.01$ vs corresponding WT mice. dbTSP mice were partially protected from microvascular loss; $^{**}P<0.01$ vs db/db. **E**, Myocardial arterioles were identified using α -SMA staining. Quantitative analysis showed that db/db mice had reduced myocardial arteriolar density when compared with WT animals; $^{*}P<0.05$ vs WT. However, TSP-1 absence did not affect the progression of arteriolar loss in db/db mice (6-month group: WT, n=7; db/db, n=12; dbTSP, n=5; 12-month group: WT, n=10; db/db, n=12; dbTSP, n=5). **F** and **G**, TSP-1 mediates angiopoietin-2 upregulation in the db/db heart. Western blotting was used to measure myocardial angiopoietin-2 expression levels in 6-month-old (**F**) and 12-month-old (**G**) animals. At 12 months of age, db/db mice exhibited marked myocardial angiopoietin-2 upregulation; $^{*}P<0.05$ vs WT. dbTSP-1 mice had significantly reduced myocardial angiopoietin-2 expression when compared with age-matched db/db animals; $^{*}P<0.05$, $^{\wedge\wedge}P<0.01$ vs db/db. **H**, In splenic macrophages, TSP-1 stimulation for 4 hours significantly upregulated angiopoietin-2 mRNA synthesis; $^{*}P<0.05$ vs control; n=6. **I**, Cardiac fibroblasts harvested from db/db mice had significantly increased angiopoietin-2 mRNA expression levels; TSP-1 loss abrogated the increase in angiopoietin-2 synthesis; $^{*}P<0.01$ vs WT; $^{\wedge}P<0.05$ vs db/db; n=6; scalebar, 40 μ m.

the increase in angiopoietin-2 levels was abrogated in cells isolated from dbTSP hearts (Figure 8I). In contrast, TSP-1 had no effect on macrophage-derived VEGF-A mRNA synthesis, and TSP-1 loss did not affect VEGF-A mRNA expression by db/db cardiac fibroblasts. Mouse cardiac microvascular endothelial cells constitutively expressed VEGF-A mRNA but did not express angiopoietin-2 in the presence or absence of TSP-1 (Online Figure III).

Discussion

Our study provides the first direct evidence supporting the role of the prototypical matricellular protein TSP-1 in matrix remodeling and regulation of angiogenesis in the diabetic heart. We report several novel observations: (1) Diabetic cardiac fibrosis is associated with marked TSP-1 upregulation and deposition in the cardiac interstitium. (2) In the diabetic heart, TSP-1 exerts matrix-preserving actions preventing dilative remodeling. (3) The matrix-preserving effects of TSP-1 seem to be due to direct actions on MMP activation and not to activation of TGF- β signaling. (4) TSP-1 induction in the diabetic heart upregulates angiopoietin-2 expression, exerting potent angiostatic actions and mediating rarefaction of the capillary network.

The development of interstitial and perivascular fibrosis is a consistent characteristic of diabetic cardiomyopathy.⁶ In nonhypertensive patients with familial diabetes mellitus, increased collagen deposition was noted in the cardiac perivascular and interstitial space.³³ Fibrotic cardiac remodeling in subjects with diabetes mellitus has important functional implications and may be involved in the pathogenesis of diastolic dysfunction. In patients with early type 2 diabetes, collagen synthesis, assessed through measurement of serum levels of the carboxy-terminal propeptide of procollagen type I, correlated with the severity of diastolic impairment.³⁴ Animal models of type 2 diabetes also exhibit significant cardiac fibrosis. Using quantitative PCR, histochemical staining, and a hydroxyproline biochemical assay, we found that obese diabetic db/db mice exhibit a marked increase in myocardial collagen content (Figure 1). Collagen deposition in the db/db heart is associated with marked TSP-1 upregulation. We localized TSP-1 immunoreactivity in vascular cells and in perivascular areas, suggesting that endothelial cells and pericytes may be important sources of TSP-1 in the diabetic myocardium (Figure 1). Hyperglycemia is a potent inducer of TSP-1 in both endothelial and vascular smooth muscle cells³⁵ and may mediate TSP-1 upregulation in the db/db heart. Glucose-mediated activation of the hexosamine pathway induces TSP-1 upregulation in vascular smooth muscle cells.³⁶ A similar mechanism for glucose-induced TSP-1 upregulation is operative in endothelial cells³⁷; however, the transcription factors activated by high glucose seem to be cell type-specific.³⁵

What is the function of TSP-1 in the diabetic myocardium? Systematic assessment of cardiac dimensions and function in dbTSP mice demonstrated that TSP-1 plays an important role in preservation of the geometry of the diabetic heart. As they age, db/db mice develop cardiac hypertrophy with preserved left ventricular chamber dimensions. TSP-1 loss in db/db mice was associated with

a 20% increase in left ventricular end-diastolic volume, reflecting significant dilative remodeling of the ventricle. Dilatation of the left ventricle in dbTSP mice was associated with modest, nonprogressive systolic dysfunction and impairment of contractile reserve (Figure 4). Dilatation and dysfunction of the dbTSP heart was associated with significant loss of myocardial collagen and increased MMP activity (Figure 5). Taken together, these findings suggest that loss of TSP-1 deprives the db/db myocardium from an important matrix-preserving signal leading to geometric remodeling of the ventricle.

TSP-1 may exert matrix-preserving actions through several distinct pathways. First, as a potent activator of TGF- β ,³⁸ TSP-1 may enhance growth factor activity in the cardiac interstitium, promoting matrix deposition and increasing expression of protease inhibitors such as TIMP-1. To test this hypothesis, we measured activation of the profibrotic, canonical TGF- β /Smad pathway by assessing Smad2/3 phosphorylation in the myocardium. db/db mice had markedly increased myocardial p-Smad2 and p-Smad3 expression; however, TSP-1 disruption did not affect TGF- β /Smad activation. Moreover, in an *in vitro* model using collagen pads populated with cardiac fibroblasts, incorporation of TSP-1 in the collagenous matrix did not stimulate gel contraction and did not upregulate TGF- β -inducible genes in cells stimulated with latent TGF- β . These observations suggest that the matrix-preserving actions of TSP-1 in the remodeling diabetic heart are not because of TGF- β activating effects. Second, TSP-1 may preserve the matrix by attenuating the inflammatory response, thus preventing cytokine-induced protease synthesis and activation. However, TSP-1 absence in db/db mice did not affect myocardial cytokine and chemokine synthesis, suggesting that overactive inflammation is not responsible for the increased chamber dilation in db/db TSP-1^{-/-} hearts. Third, TSP-1 may directly inhibit MMP activity.³⁹ TSP-1 loss in db/db mice was associated with a 2-fold increase in myocardial MMP-2 activity and a 5-fold increase in MMP-9 activity (Figure 5). In vitro, TSP-1 incorporation into the matrix inhibited leptin-induced MMP-2 activation; the inhibitory effects of TSP-1 on MMP activity were not because of transcriptional regulation of MMPs (Figure 6). Thus, our *in vivo* and *in vitro* observations suggest that the matrix-preserving actions of TSP-1 in the diabetic heart are likely because of direct inhibition of protease activity and may not involve activation of growth factors that stimulate matrix synthesis, such as TGF- β .

In addition to its effects in matrix metabolism, TSP-1 is also involved in regulation of myocardial angiogenesis in diabetic mice. In both human patients and in experimental animal models, diabetes mellitus is associated with impaired angiogenesis. Patients with diabetes mellitus exhibit attenuated angiogenic responses to myocardial ischemia⁴⁰ and have poor development of collateral coronary vessels.⁴¹ Moreover, microvascular rarefaction is a characteristic of the diabetic heart and may be responsible for development of contractile dysfunction and for increased susceptibility to myocardial ischemia.^{42,43} The molecular signals responsible for impaired angiogenesis in subjects with diabetes mellitus remain poorly understood; animal model studies and investigations in human

patients have produced somewhat conflicting results. In diabetic animals, expression of angiogenic growth factors (such as hypoxia-inducible factor-1 α and VEGF) was reduced, suggesting that diabetes mellitus-associated impairment in the angiogenic response may be because of attenuation of growth factor synthesis.⁴⁰ In contrast, in human patients with coronary disease and type 2 diabetes, myocardial VEGF levels were increased; however, VEGF receptor phosphorylation was reduced and downstream myocardial VEGF signaling was impaired, contributing to reduced angiogenesis.⁴⁴ A potential mechanism of impaired angiogenesis in diabetes mellitus may involve activation of angiostatic pathways. Our findings suggest that upregulation of the angiostatic matricellular protein TSP-1 is in part responsible for the age-related rarefaction of the cardiac microvascular network in db/db hearts (Figure 8). Published evidence suggests that the antiangiogenic effects of TSP-1 may involve several distinct biological processes.⁴⁵ First, TSP-1 may antagonize VEGF signaling through direct binding and inhibition of its release from the matrix and via inhibition of VEGF receptor phosphorylation.⁴⁶ Second, TSP-1 may induce endothelial cell apoptosis through a CD36/fyn-mediated pathway.¹⁹ Third, TSP-1 may directly suppress endothelial cell cycle progression in a CD36-independent manner or inhibit endothelial cell migration through interactions involving β 1 integrins.⁴⁷ Fourth, TSP-1-induced suppression of NO signaling may also be involved in activation of angiostatic signaling⁴⁸ in endothelial cells. Our experiments identified angiopoietin-2 induction as a new mechanism that may be implicated in the angiostatic actions of TSP-1 in the diabetic myocardium. Angiopoietin-2 potently regulates angiogenesis in a context-dependent manner. Angiopoietin-2 promotes endothelial cell death if endogenous VEGF activity is inhibited⁴⁹ and promotes vascular regression by inhibiting pericyte recruitment.⁵⁰ We show that angiopoietin-2 protein is upregulated in db/db hearts; loss of TSP-1 is associated with marked reduction of angiopoietin-2 levels (Figure 8). Thus, the angiostatic effects of TSP-1 in the diabetic myocardium may be in part because of upregulation of angiopoietin-2 expression, in an environment where VEGF signaling is also attenuated (Online Figure II). Our in vitro experiments demonstrated cell-specific stimulatory effects of TSP-1 on angiopoietin-2 expression. TSP-1 induced angiopoietin-2 synthesis in macrophages, but not in cardiac microvascular endothelial cells (Figure 8).

In addition to its role in tissue remodeling, angiogenesis, and fibrosis, TSP-1 has been recently identified as an adipokine that is highly expressed in fat tissue harvested from obese, insulin-resistant subjects and is associated with adipose inflammation.²¹ Our recent work demonstrated that TSP-1 $^{-/-}$ mice have attenuated fat accumulation and reduced weight gain when fed a high-fat diet or a high-carbohydrate low-fat diet⁵¹ and suggested effects of TSP-1 in promoting adipose tissue inflammation and adipocyte proliferation. Li et al⁵² also showed that TSP-1 $^{-/-}$ mice fed a high-fat diet exhibited reduced adipose inflammation and improved insulin sensitivity. In contrast, Olerud et al⁵³ found that TSP-1 $^{-/-}$ mice were glucose-intolerant because of islet dysfunction; reconstitution of the TGF- β -activating effects of TSP-1 prevented the development of insulin resistance in TSP-1 $^{-/-}$

mice. In our current study, db/db mice that lacked TSP-1 had only subtle alterations in adipose tissue gain and in metabolic function when compared with db/db animals. The percentage of total and abdominal fat, glucose, insulin levels, and HOMA IR were not significantly affected by TSP-1 loss; however, db/db TSP-1 $^{-/-}$ mice had higher serum cholesterol levels compared with db/db animals. The differences in the findings between investigations may reflect the distinct mechanisms of obesity in various experimental models. db/db mice develop morbid obesity and severe metabolic dysfunction because of central nervous system leptin resistance that results in hyperphagia and rapid weight gain; the very high caloric intake in these animals may induce metabolic dysfunction regardless of the presence or absence of TSP-1. An alternative possibility is that any effects of TSP-1 on metabolic function may require intact leptin signaling.

Conclusions

TSP-1 does not play a significant role in weight gain and metabolic dysfunction in db/db mice, but is highly induced in the db/db myocardium where it exerts matrix-preserving and angiostatic actions. TSP-1-mediated matrix preservation protects the diabetic heart from dilation by locally inhibiting MMP activity; however, dilative remodeling in the absence of TSP-1 is not progressive and is associated with mild systolic dysfunction. In addition to its effects in maintaining chamber geometry, TSP-1 exerts potent angiostatic actions mediating vascular rarefaction in the aging diabetic heart. Because the antiangiogenic effects of TSP-1 are mediated through binding of specific angiostatic domains with CD36 or CD47,^{45,54} selective inhibition of the angiostatic effects of TSP-1 may be a promising approach to correct impaired angiogenic responses in the diabetic myocardium.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Patients with diabetes mellitus develop diastolic heart failure associated with interstitial fibrosis and exhibit impaired myocardial angiogenesis.
- Matricellular proteins are critically involved in fibrotic and angiogenic responses by modulating growth factor and cytokine signaling.
- Hyperglycemia upregulates matricellular protein thrombospondin (TSP)-1, which might play an important role in remodeling of the diabetic heart by modulating growth factor signaling, inhibiting protease activation, and exerting angiostatic actions.

What New Information Does This Article Contribute?

- Obese diabetic db/db mice exhibit marked upregulation of myocardial TSP-1, which is localized in interstitial and perivascular areas.
- Upregulation of TSP-1 in the diabetic myocardium increases myocardial collagen content, but preserves chamber geometry and systolic function by inhibiting matrix metalloproteinase activation.
- TSP-1 induction in the diabetic heart mediates age-related capillary rarefaction by upregulating expression of angiopoietin-2.

Matricellular protein TSP-1, which is markedly induced in the heart in diabetes mellitus, is implicated in fibrotic cardiac

remodeling in diabetes mellitus. Moreover, through its potent angiostatic actions, TSP-1 may be responsible for impaired angiogenesis in the diabetic myocardium. Obese diabetic db/db mice exhibit marked upregulation of TSP-1 in the myocardium. To investigate the role of TSP-1 in diabetic cardiomyopathy, we generated db/db TSP-1^{-/-} mice. Our findings show, for the first time, that: (1) loss of TSP-1 in db/db mice does not significantly affect weight gain and metabolic function; (2) TSP-1 disruption in db/db animals results in cardiac chamber enlargement and modest systolic dysfunction, associated with reduced collagen content and enhanced matrix metalloproteinase activity in the myocardium; (3) effects of TSP-1 on the matrix are not mediated through activation of transforming growth factor- β responses, but may reflect inhibition of matrix metalloproteinase activity; (4) TSP-1 mediates age-associated capillary rarefaction in the diabetic heart by upregulating expression of angiopoietin-2, a mediator that induces vascular regression. Our findings identify TSP-1-mediated angiopoietin-2 induction as a crucial mechanism responsible for impaired preservation of the cardiac microvascular network in diabetes mellitus. Inhibition of the angiostatic actions of TSP-1 may have therapeutic potential in diabetic heart disease.