TRB3 Function in Cardiac Endoplasmic Reticulum Stress

Joan Avery, Sharon Etzion, Brian J. DeBosch, Xiaohua Jin, Traian S. Lupu, Bassel Beitinjaneh, Jacob Grand, Attila Kovacs, Nandakumar Sambandam, Anthony J. Muslin

Rationale: Tribbles (TRB)3 is an intracellular pseudokinase that modulates the activity of several signal transduction cascades. TRB3 has been reported to inhibit the activity of Akt protein kinases. TRB3 gene expression is highly regulated in many cell types, and amino acid starvation, hypoxia, or endoplasmic reticulum (ER) stress promotes TRB3 expression in noncardiac cells. 

Objective: The objective of this work was to examine TRB3 expression and function in cultured cardiac myocytes and in mouse heart.

Methods and Results: Agents that induced ER stress increased TRB3 expression in cultured cardiac myocytes while blocking insulin-stimulated Akt activation in these cells. Knockdown of TRB3 in cultured cardiac myocytes reversed the effects of ER stress on insulin signaling. Experimental myocardial infarction led to increased TRB3 expression in murine heart tissue in the infarct border zone suggesting that ER stress may play a role in pathological cardiac remodeling. Transgenic mice with cardiac-specific overexpression of TRB3 were generated and they exhibited normal contractile function but altered cardiac signal transduction and metabolism with reduced cardiac glucose oxidation rates. Transgenic TRB3 mice were also sensitized to infarct expansion and cardiac myocyte apoptosis in the infarct border zone after myocardial infarction.

Conclusions: These results demonstrate that TRB3 induction is a significant aspect of the ER stress response in cardiac myocytes and that TRB3 antagonizes cardiac glucose metabolism and cardiac myocyte survival. (Circ Res. 2010;106:1516-1523.)

Key Words: ER stress ■ Akt ■ TRB3 ■ myocardial infarction ■ signal transduction

Many cellular proteins are produced by ribosomes that coat the endoplasmic reticulum (ER), and stress of the rough ER occurs when proteins within its lumen are misfolded.1,2 This can occur as a result of reduced calcium levels, insufficient concentrations of molecular chaperones, altered protein glycosylation machinery, altered redox status, and other factors within the ER lumen. ER stress leads to the initiation of the unfolded protein response (UPR), an adaptive mechanism that initially promotes organelle recovery.1,2 However, if ER stress is prolonged or if the UPR is unsuccessful in promoting recovery, apoptotic cell death can ensue.2 The UPR promotes global translational blockade, but also leads to the selective transcription and translation of several genes.1,2

In response to protein misfolding, the glucose regulated protein 78 (GRP78, also called BiP) chaperone is released by the ER transmembrane proteins PERK (a protein kinase), IRE-1 (an endoribonuclease), and activating transcription factor (ATF)6, so that it can directly bind to the misfolded proteins within the ER lumen.1,2 Release of GRP78 leads to altered activity of its transmembrane binding partners, and this triggers various aspects of the UPR. Release of GRP78 allows PERK to become activated through dimerization, and active PERK phosphorylates the ribosomal elongation factor eIF-2α, resulting in the inhibition of most protein translation, with the exception of certain proteins such as GRP78 and ATF4.1,2 Release of GRP78 allows IRE-1 to splice an mRNA encoding XBP1 (X-box-binding protein 1), creating a new open reading frame that encodes an active transcription factor. Finally, release of GRP78 allows ATF6 to migrate to the Golgi where S1P and S2P cleave the protein releasing the N-terminal cytosolic portion of the protein, called N-ATF6 which has a transcriptional activation domain, DNA-binding domain, and nuclear localization sequence. XBP1active and N-ATF6 bind to ER stress response elements in various ER stress response genes, including GRP78, CHOP (CHOP10, GADD153, DDIT3, C/EBP), and XBP1.1,2

ER stress occurs in cardiac myocytes and cardiac tissue in response to various stressors, including ischemia, inflammation and exposure to alcohol.3-5 Indeed, experimental autoimmune cardiomyopathy induced by injection of a peptide corresponding to a portion of the β1 adrenergic receptor was associated with cardiac ER stress as measured by induction of GRP78 and CHOP gene expression.4 Furthermore, autoimmune cardiomyopathy was associated with inhibition of the
phosphatidylinositol 3-kinase/Akt protein kinase signaling cascade. Similarly, treatment of animals with alcohol resulted in the development of a cardiomyopathy associated with ER stress and inhibition of Akt activity. In earlier work with other cell types, the relationship between ER stress and Akt activation appears to be biphasic: acute Akt activation occurs during the initial phase of the UPR to promote cell survival, and this is followed by delayed Akt inhibition that contributes to apoptosis. Indeed, Akt and TOR (target of rapamycin) activation was transiently observed 2 and 4 hours after treatment of cultured MCF-7 breast cancer and H1299 lung cancer cells with the glycosylation inhibitor tunicamycin or the sarcoplasmic/endoplasmic reticulum calcium-ATPase (SERCA) inhibitor thapsigargin, and acute Akt activation was essential to promote cell survival in the acute phase. Similar transient Akt activation followed by Akt inhibition was observed in cultured primary glial cells treated tunicamycin or thapsigargin.

Tribbles (TRB3) is a pseudokinase that modulates several signaling pathways, including the phosphatidylinositol 3-kinase/Akt cascade, the nuclear factor κ-light-chain-enhancer of activated B cells cascade, and the ATF4/CHOP pathway. TRB3 binds to and inhibits the kinase activity of Akt and TOR (target of rapamycin) activation was transiently observed 2 and 4 hours after treatment of cultured MCF-7 breast cancer and H1299 lung cancer cells with the glycosylation inhibitor tunicamycin or the sarcoplasmic/endoplasmic reticulum calcium-ATPase (SERCA) inhibitor thapsigargin, and acute Akt activation was essential to promote cell survival in the acute phase. Similar transient Akt activation followed by Akt inhibition was observed in cultured primary glial cells treated tunicamycin or thapsigargin.

In previous work, alcohol exposure and autoimmune myocarditis were found to be associated with cardiac ER stress and inhibition of Akt signaling. We initially wished to confirm whether agents that induce ER stress in cardiac myocytes would inhibit Akt signaling. Cultured HL-1 murine atrial cardiac myocytes were treated with thapsigargin and then stimulated with insulin. HL-1 cells were treated with thapsigargin (2 μmol/L), and 24 hours later were treated with insulin (10 nmol/L) or control buffer and Akt activation was examined by immunoblotting with an anti–phospho-Akt antibody (Figure 1A and 1B). Thapsigargin-treated HL-1 cells were resistant to insulin-stimulated Akt activation. Furthermore, thapsigargin-treated HL-1 cells exhibited increased protein levels for GRP78 and CHOP, 2 markers of ER stress (Figure 1A).

To determine whether the impaired insulin-stimulated Akt activation in thapsigargin-treated HL-1 cells was correlated with TRB3 induction, TRB3 protein levels were determined by immunoblotting (Figure 1C). HL-1 cells treated with thapsigargin (2 μmol/L) for 24 hours exhibited a nearly 2-fold increase in TRB3 protein levels. Furthermore, HL-1 cells treated with thapsigargin (2 μmol/L) or the glycosylation inhibitor tunicamycin (2 μg/mL) for 24 hours exhibited significantly increased TRB3 mRNA levels when determined by quantitative real-time PCR (Figure 1D).

To determine whether TRB3 induction in response to agents that cause ER stress was responsible for defective insulin signaling, we performed knock down experiments. HL-1 cells were transfected with a TRB3 siRNA that is effective at reducing TRB3 levels in murine cultured cardiac myocytes. Insulin-stimulated phosphorylation of Akt1/2 was attenuated by pretreatment with thapsigargin, but the inhibitory effect of thapsigargin was reversed by knockdown of TRB3 by use of siRNA at a dosage of 50 or 100 nmol/L (Figure 1E). Treatment of HL-1 cells with 100 nmol/L scrambled siRNA did not reverse the effect of thapsigargin on insulin signaling (Figure 1E).

To further evaluate whether TRB3 induction in response to agents that cause ER stress specifically affected Akt signaling, we performed rescue experiments with adenoviral-mediated Akt2 overexpression in HL-1 cells. Cultured HL-1 cells were infected with recombinant adenoviruses encoding β-galactosidase (ad-βGal, control) or wild-type Akt2 (ad-Akt2) and 48 hours later were treated with thapsigargin for 24 hours. Insulin-stimulated phosphorylation of glycogen synthase kinase 3β, an indicator of Akt activation, was increased in thapsigargin-treated HL-1 cells infected with ad-Akt2 compared to those infected with ad-GFP (Online Figure I).

**Results**

**ER Stress Inhibits Akt Activation and Promotes TRB3 Expression in Cultured Cardiac Myocytes**

In previous work, alcohol exposure and autoimmune myocarditis were found to be associated with cardiac ER stress and inhibition of Akt signaling. We initially wished to confirm whether agents that induce ER stress in cardiac myocytes would inhibit Akt signaling. Cultured HL-1 murine atrial cardiac myocytes were treated with thapsigargin and then stimulated with insulin. HL-1 cells were treated with thapsigargin (2 μmol/L), and 24 hours later were treated with insulin (10 nmol/L) or control buffer and Akt activation was examined by immunoblotting with an anti–phospho-Akt antibody (Figure 1A and 1B). Thapsigargin-treated HL-1 cells were resistant to
models of cardiomyopathy were found to be associated with cardiac ER stress. For example, transgenic mice with cardiac-specific overexpression of monocyte chemoattractant protein 1 developed a form of ischemic cardiomyopathy that was associated with myocardial ER stress and TRB3 expression. In another mouse model system, experimental MI resulted in ER stress in the infarct border zone. Increased levels of GRP78 protein were detected in the infarct border zone of mice subjected to experimental MI 4 days earlier. Finally, pressure overload by transverse aortic constriction in mice

Figure 1. Cardiomyocyte ER stress inhibits Akt activation caused by TRB3 induction. A, Treatment of HL-1 atrial myocytes with thapsigargin results in ER stress and reduced insulin-stimulated Akt activation. HL-1 cells were pretreated with thapsigargin (THAPS) (2 μmol/L) or DMSO for 24 hours. HL-1 cells were treated with insulin (10 nmol/L) or control buffer for 10 minutes. Protein lysates were generated from HL-1 cells and proteins were separated by SDS-PAGE followed by immunoblotting with primary antibodies directed against phospho-Akt1/2 (p-Akt), total-Akt1/2 (t-Akt), GRP78, and CHOP. B, Densitometric analysis of Akt activation in HL-1 cells incubated with thapsigargin or DMSO and then stimulated with insulin (Ins) or buffer as depicted in A. Phospho-Akt levels were normalized by total Akt levels for each sample. *P < 0.001 vs HL-1 cells that were not treated with thapsigargin or insulin by Student's t test; ^P < 0.004 vs HL-1 cells treated with DMSO and insulin by Student’s t test. C, TRB3 protein levels increase in HL-1 in response to ER stress. HL-1 cells were treated with DMSO or thapsigargin (THAPS) (2 μmol/L) for 24 hours, and protein lysates were obtained. Protein lysates were separated by SDS-PAGE followed by immunoblotting with an anti-TRB3 primary antibody. The anti-TRB3 antibody specifically recognizes a 42 kilodalton band or a doublet (depending on the resolution of the gel). Blots were reprobed with an anti–β-actin antibody to control for protein loading. The TRB3 protein levels were measured by computerized densitometry and were normalized by β-actin protein levels. The TRB3/actin levels are indicated below each lane in arbitrary units. The mean TRB3/actin level was 1.0 ± 0.19 for control DMSO-treated cells and was 1.88 ± 0.14 for thapsigargin-treated cells (P = 0.021 by Student’s t test). D, Agents that promote ER stress cause induction of TRB3 mRNA in HL-1 cardiac myocytes. HL-1 cells were treated with tunicamycin (2 μg/mL), thapsigargin (2 μmol/L), or DMSO (control) for 24 hours. RNA was purified from HL-1 cells and TRB3 and GAPDH mRNA levels were analyzed by quantitative real-time PCR. *P < 0.05 by Kruskal-Wallis 1-way ANOVA on ranks; ^P < 0.05 by Kruskal–Wallis 1-way ANOVA on ranks. E, ER stress–mediated blockade of Akt activation is dependent on TRB3. HL-1 cells analyzed in this figure were treated with thapsigargin (THAPS) (2 μmol/L) or DMSO for 24 hours. Some cells were also pretreated with TRB3 siRNA at the indicated doses (20, 50, or 100 nmol/L) or with scrambled siRNA (100 nmol/L) for 24 hours before the addition of thapsigargin. Top, Cells were serum-starved for 6 hours and then insulin (10 nmol/L) was added to all HL-1 cells for 10 minutes and protein lysates were obtained for SDS-PAGE followed by immunoblotting. Immunoblots depict the levels of activated Akt (p-Akt), total Akt (t-Akt). Under the blots, a computerized densitometry analysis of phospho-Akt levels normalized by total Akt protein levels for each lane is provided in arbitrary units. Bottom, HL-1 cells were cultured in parallel with those used for the immunoblot experiment, and were treated with TRB3 siRNA (20, 50, or 100 nmol/L) or with scrambled siRNA (100 nmol/L) for 24 hours. RNA was purified from HL-1 cells and TRB3 and GAPDH mRNA levels were analyzed by quantitative real-time PCR. *P < 0.05 by 1-way ANOVA (Holm–Sidak method) vs scrambled siRNA.
resulted in cardiac ER stress, as measured by increased levels of GRP78 and CHOP.20

To evaluate whether provocative stimuli that promote cardiac ER stress in vivo also induce TRB3 expression, we performed experimental MI surgery in mice.21–23 Anesthetized and ventilated wild-type C57BL/6J mice were subjected to ligation of the left anterior descending coronary artery or to a sham operation, and then left ventricular tissue was obtained 4 and 24 hours after surgery. GRP78 and CHOP mRNA levels did not change 4 hours after MI, but exhibited a trend toward increased levels after 24 hours (Figure 2A and 2B). Analysis of TRB3 mRNA levels from cardiac samples similarly revealed that TRB3 mRNA did not increase significantly 4 hours after MI, but increased by 2.5-fold 24 hours after MI when compared to sham tissue (*P<0.05 by 1-way ANOVA (Holm–Sidak method) vs sham operation). D, Infarct border zone (defined as one-quarter circumference on either side of the infarct edge) and remote left ventricular tissue was isolated 24 hours after MI surgery for RNA isolation. In addition, the entire left ventricle was isolated 24 hours after a sham operation for RNA isolation. Quantitative real-time PCR analysis of TRB3 was performed. *P<0.05 by 1-way ANOVA (Holm–Sidak method) vs remote left ventricle.

Figure 2. ER stress markers and TRB3 expression is induced in cardiac tissue after MI. Wild-type C57BL/6J mice were subjected to experimental MI by ligation of the left anterior descending coronary artery. A, Total left ventricle was isolated 4 and 24 hours after MI or sham operation for RNA isolation. Quantitative real-time PCR analysis of GRP78 was performed. B, Total left ventricle was isolated 4 and 24 hours after MI or sham operation for RNA isolation. Quantitative real-time PCR analysis of CHOP was performed. C, Total left ventricle was isolated 4 and 24 hours after MI or sham operation for RNA isolation. Quantitative real-time PCR analysis of TRB3 was performed. *P<0.05 by 1-way ANOVA (Holm–Sidak method) vs sham operation. D, Infarct border zone (defined as one-quarter circumference on either side of the infarct edge) and remote left ventricular tissue was isolated 24 hours after MI surgery for RNA isolation. In addition, the entire left ventricle was isolated 24 hours after a sham operation for RNA isolation. Quantitative real-time PCR analysis of TRB3 was performed. *P<0.05 by 1-way ANOVA (Holm–Sidak method) vs remote left ventricle.

Generation of Transgenic Mice With Cardiac-Specific Overexpression of TRB3

In vitro experiments showed that ER stress induced TRB3 expression in cultured cardiac myocytes, and that TRB3 blocked cardiac myocyte insulin signaling in vitro. To evaluate the ability of TRB3 to modulate cardiac metabolism in vivo, transgenic mice with cardiac-specific overexpression of TRB3 were generated. A DNA construct was made that included the α-myosin heavy chain promoter linked to a myc-1 epitope-tagged cDNA encoding murine TRB3 corresponding to GenBank_NM_175093. This DNA construct was injected into the male pronucleus of one-cell murine embryos to generate transgenic mice. Multiple lines were obtained and lines designated 1×-TRB3 (low expression), 2×-TRB3 (medium expression), and 4×-TRB3 (high expression) based on differential genomic DNA expression by PCR were selected for initial evaluation. Mice from all lines appeared grossly normal at birth and were fertile. Overexpression of TRB3 in transgenic mice was confirmed by immunoblotting of cardiac protein lysates (Figure 3A).

To evaluate cardiac signal transduction in transgenic mice, anesthetized 2×-TRB3 mice were administered 0.1 U/kg body weight insulin by jugular vein injection. After 5 minutes, hearts were excised and left ventricular lysates obtained.
Analysis of Akt1/2 and GSK-3β phosphorylation status by immunoblotting revealed that insulin-stimulated signaling pathway activation was reduced in 2×-TRB3 hearts (Figure 3B).

Morphometric analysis of 4- and 12-month-old 2×-TRB3 hearts demonstrated that these mice do not spontaneously develop cardiac hypertrophy (Online Table I). The biventricular weight-to-body weight ratio was nearly identical in 12-month-old 2×-TRB3 mice (4.02±0.11 mg/g) when compared to wild-type littermates (4.00±0.47 mg/g, P=0.871). Similarly, the biventricular weight-to-body weight ratio was similar in 12-month-old 2×-TRB3 mice (3.78±0.17 mg/g) when compared to wild-type littermates (4.00±0.25 mg/g, P=0.477). Similar relationships were observed for the left ventricular-to-body weight ratios (Online Table I).

Echocardiographic evaluation of awake 12-week-old 2×-TRB3 and 4×-TRB3 mice demonstrated that they had normal left ventricular contractile function when compared to wild-type mice as measured by fractional shortening (Online Table II). Furthermore, the left ventricular (LV) mass index, which normalizes calculated LV mass by body weight, did not show a significant difference between 12-week-old 2×-TRB3, 4×-TRB3 and wild-type mice (Online Table II).

Although 2×-TRB3 transgenic mice do not spontaneously develop cardiac hypertrophy and have normal LV contractile function, we evaluated genetic markers of cardiac stress in these animals. Cardiac ventricular tissue was obtained from 12-month-old 2×-TRB3 mice and was isolated from 12-week-old nontransgenic C57BL/6J littermates. Ventricular protein lysates were analyzed by immunoblotting for TRB3 protein content. Blots were reprobed with anti-14-3-3β primary antibody to control for protein loading. Reduced insulin-stimulated intracellular signal transduction in 2×-TRB3 cardiac tissue, RNA was purified from ventricular tissue isolated from 12- to 16-week-old 2×-TRB3 mice and their nontransgenic wild-type littermates (WT). ANF, β-MHC, SERCA2, GRP78, and CHOP gene expression was analyzed by quantitative real-time PCR. *P=0.006 by Student’s t test vs WT heart.

Analysis of Akt1/2 and GSK-3β phosphorylation status by immunoblotting revealed that insulin-stimulated signaling pathway activation was reduced in 2×-TRB3 hearts (Figure 3B).

Morphometric analysis of 4- and 12-month-old 2×-TRB3 hearts demonstrated that these mice do not spontaneously develop cardiac hypertrophy (Online Table I). The biventricular weight-to-body weight ratio was nearly identical in 4-month-old 2×-TRB3 mice (4.02±0.14 mg/g) when compared to wild-type littermates (4.01±0.14 mg/g, P=0.917). Similarly, the biventricular weight-to-body weight ratio was similar in 12-month-old 2×-TRB3 mice (3.78±0.17 mg/g) when compared to wild-type littermates (4.00±0.25 mg/g, P=0.477). Similar relationships were observed for the left ventricular-to-body weight ratios (Online Table I).

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Although 2×-TRB3 transgenic mice do not spontaneously develop cardiac hypertrophy and have normal LV contractile function, we evaluated genetic markers of cardiac stress in these animals. Cardiac ventricular tissue was obtained from wild-type and 2×-TRB3 12- to 16-week-old mice and was examined by quantitative real-time PCR for the expression of atrial natriuretic factor (ANF), β-myosin heavy chain (β-MHC), and SERCA2 (Figure 3C). The expression of ANF was increased 5-fold (P=0.11), the expression of β-MHC was increased by 4.7-fold (P=0.006), and the expression of SERCA2 was unchanged in 2×-TRB3 cardiac tissue (Figure 3C). The expression of the ER stress marker genes GRP78 and CHOP were unchanged in 2×-TRB3 cardiac tissue (Figure 3C).

Given the ability of TRB3 overexpression to antagonize insulin-stimulated cardiac Akt signaling, we evaluated cardiac metabolism in transgenic mice. An ex vivo working heart analysis was performed on 2×-TRB3 and 4×-TRB3 12-week-old mice and their nontransgenic littermates. Peak systolic pressure, cardiac output and cardiac work levels were similar between wild-type and transgenic genotypes of mice, confirming the echocardiographic finding that transgenic mice have normal cardiac systolic function at baseline (Online Tables III and IV). Analysis of cardiac metabolism revealed that both 2×-TRB3 and 4×-TRB3 mice had significantly reduced glucose oxidation rates, but normal palmitate oxidation rates when compared to nontransgenic littermates (Figure 4A and 4B). These results demonstrate that overexpression of TRB3 in heart inhibits glucose metabolism.

**Evaluation of Remodeling After Experimental Myocardial Infarction in 2×-TRB3 Mice**

The cardiac ER stress response, including TRB3 gene induction, is activated in response to myocardial ischemia. TRB3 is an Akt inhibitor and we previously demonstrated that akt2-/- mice exhibit increased pathological cardiac remodeling after experimental MI. To determine whether cardiac-specific overexpression of TRB3 would sensitise myocardium to pathological cardiac remodeling, we performed experimental...
MI surgery with 2×-TRB3 mice and their wild-type littermates at 12 weeks of age. One day after MI surgery, mice were evaluated by transthoracic echocardiography and this confirmed that the initial infarct sizes were similar for the 2 genotypes (Figure 5A). Seven days after MI surgery, the hearts were isolated for histological examination of scar area and cardiac myocyte apoptosis.

Histological examination of LV sections obtained 7 days after MI surgery showed that the infarct scar area was significantly greater in 2×-TRB3 mice when compared to wild-type littermates (Figure 5B and 5C). Indeed, the scar area as a percentage of total LV area was 25.0±1.8% in wild-type mice, but was increased to 35.9±3.7% in 2×-TRB3 mice (P=0.009). Furthermore, cardiac myocyte apoptosis in the infarct border zone was evaluated by TUNEL, and this demonstrated that the number of apoptotic cardiac myocytes was significantly greater in 2×-TRB3 mice (Figure 5D). Therefore, 2×-TRB3 mice are sensitized to the development of pathological cardiac remodeling after MI.

**Discussion**

ER stress occurs in response to nascent protein misfolding in the ER lumen and a complex variety of cellular responses to this stress initially act to promote ER recovery.1,2 If the initial mechanisms that are designed to promote ER functional recovery are inadequate, however, prolonged activation of stress responses lead to apoptosis of the cell. TRB3 is a pseudokinase that inhibits the activity of several signal transduction cascades, including the Akt pathway and the ATF4/CHOP pathway.8–10 Inhibition of all Akt signaling impacts the cellular response to insulin and other growth factors, in part, by reducing glucose uptake and metabolism, inhibiting protein synthesis, and promoting apoptosis.

In this work, we investigated the role of TRB3 in the UPR of cardiac myocytes. We found that various chemical inducers of ER stress, including tunicamycin and thapsigargin, strongly upregulated TRB3 expression in cultured cardiac myocytes. Furthermore, nutrient deprivation and hypoxia also induced the expression of TRB3 in cardiac myocytes. Inducers of ER stress blocked Akt activation in cultured cardiac myocytes. Experimental MI, a physiological stimulus of ER stress, led to increased TRB3 expression in the infarct border zone of the left ventricle after 24 hours. In previous work, another group performed a comprehensive analysis of gene expression at several time points after MI in mice.24 Inspection of the microarray data from that study shows that TRB3 and CHOP mRNA levels rose at 12 and 24 hours after MI in the ischemic/infarcted area. This rise in TRB3 was exactly paralleled by increased CHOP mRNA levels. To determine the physiological consequences of increased TRB3 gene expression in vivo, we generated transgenic mice with cardiac-specific overexpression of TRB3 and showed that these mice had reduced cardiac insulin-stimulated signal transduction and reduced cardiac glucose oxidation rates. Furthermore, we demonstrated that these transgenic mice were sensitized to pathological cardiac remodeling after MI.

Cardiac insulin signaling results in Akt2 activation that is known to promote GLUT4 transporter translocation to the plasma membrane resulting in increased glucose uptake by cardiac myocytes.22 Insulin signaling also leads to Akt2-mediated inactivation of the FoxO1 transcription factor that regulates cardiac metabolism.23 The UPR-mediated induction of TRB3 is predicted to block insulin-mediated glucose uptake and oxidation. It is not entirely clear why this might be beneficial to promote ER recovery, although it is possible that reduced glucose oxidation might generate less reactive oxygen species or other toxins. Alternatively, TRB3 induction may be a purely deleterious aspect of the UPR after ER recovery is deemed to be impossible. TRB3 induction as a component of the UPR is predicted to lower the apoptotic threshold of cardiac myocytes by blocking Akt activity.

Despite the fact that the acute UPR is designed to reduce protein synthesis, and that TRB3 is induced as a component of the UPR, we observed that 2×-TRB3 and 4×-TRB3 transgenic hearts showed significantly greater in 2×-TRB3 mice when compared to wild-type littermates (Figure 5B and 5C). Indeed, the scar area as a percentage of total LV area was 25.0±1.8% in wild-type mice, but was increased to 35.9±3.7% in 2×-TRB3 mice (P=0.009). Furthermore, cardiac myocyte apoptosis in the infarct border zone was evaluated by TUNEL, and this demonstrated that the number of apoptotic cardiac myocytes was significantly greater in 2×-TRB3 mice (Figure 5D). Therefore, 2×-TRB3 mice are sensitized to the development of pathological cardiac remodeling after MI.

Cardiac insulin signaling results in Akt2 activation that is known to promote GLUT4 transporter translocation to the plasma membrane resulting in increased glucose uptake by cardiac myocytes.22 Insulin signaling also leads to Akt2-mediated inactivation of the FoxO1 transcription factor that regulates cardiac metabolism.23 The UPR-mediated induction of TRB3 is predicted to block insulin-mediated glucose uptake and oxidation. It is not entirely clear why this might be beneficial to promote ER recovery, although it is possible that reduced glucose oxidation might generate less reactive oxygen species or other toxins. Alternatively, TRB3 induction may be a purely deleterious aspect of the UPR after ER recovery is deemed to be impossible. TRB3 induction as a component of the UPR is predicted to lower the apoptotic threshold of cardiac myocytes by blocking Akt activity.

Despite the fact that the acute UPR is designed to reduce protein synthesis, and that TRB3 is induced as a component of the UPR, we observed that 2×-TRB3 and 4×-TRB3 transgenic hearts.
mice did not exhibit cardiac atrophy at any time point. Therefore, it appears that TRB3 induction is not a significant mechanism to reduce protein synthesis in the myocardium during the UPR.

Because the UPR promotes cellular survival through reparative mechanisms in its early phase, and promotes cell death through apoptosis in its later phases, it is difficult to predict the overall consequences of antagonizing the overall ER stress pathway in cardiac tissue. Previous work suggests that several factors induced in response to ER stress are cardioprotective.25,26 For example, ATF6 and RCAN1 (regulator of calcineurin 1) are cardioprotective factors that are induced in response to cardiac ER stress.25,26 The TRB3 knockout mouse has no obvious phenotype at baseline, but this is not entirely surprising given that TRB3 is largely an inducible factor in the ER stress pathway.27 Further work is required to delineate the physiological effects of various aspects of the UPR in the heart.

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**Disclosures**

None.

**References**

What Is Known?

- The intracellular Akt protein kinases regulate cardiac myocyte growth, survival, and glucose metabolism.
- Cardiac myocyte endoplasmic reticulum (ER) stress occurs in response to several provocative stimuli that result in protein misfolding within the ER.
- ER stress in cardiac myocytes and other cell types leads to activation of a complicated response mechanism that initially promotes ER recovery but, if unsuccessful, promotes cell death.

What New Information Does This Article Contribute?

- Activation of the ER stress response in cultured cardiac myocytes and murine myocardium results in increased expression of the Tribbles (TRB3) pseudokinase.
- Activation of the ER stress response in cultured cardiac myocytes inhibits Akt protein kinase activity in a TRB3-dependent fashion.
- Cardiac-specific overexpression of TRB3 in transgenic mice results in abnormal cardiac glucose metabolism and insulin signaling and also results in increased sensitivity to ER stress pathway–mediated cardiac myocyte death.

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Novelty and Significance

Akt protein kinases play a critical role in cardiac myocyte growth, survival, and metabolism. Many proteins are produced by ribosomes that coat the ER, and ER stress occurs when luminal proteins are misfolded. Studies of ER stress in noncardiac cells have shown that ER stress results in the induction of TRB3, a pseudokinase that inhibits Akt activity. This study demonstrated that cardiac ER stress leads to TRB3 induction, Akt inhibition, and cardiac myocyte death. In response to various stimuli that are known to promote ER stress, cultured cardiac myocytes exhibited reduced Akt activity dependent on increased expression of TRB3. Experimental myocardial infarction in mice resulted in the induction of TRB3 and other ER stress markers in the infarct border zone. Cardiac-specific overexpression of TRB3 in mice was associated with reduced glucose metabolism and insulin signaling. Furthermore, TRB3 overexpression sensitized animals to pathological cardiac remodeling after myocardial infarction, with increased apoptosis in the border zone. We conclude that myocardial infarction results in cardiac ER stress with induction of TRB3 and that TRB3 antagonizes Akt activity and promotes cell death. Agents that inhibit TRB3 expression or activity may lead to reduced pathological cardiac remodeling in patients.
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SUPPLEMENTAL MATERIAL

TRB3 Function in Cardiac Endoplasmic Reticulum Stress

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Detailed Methods

All research involving the use of mice was performed in strict accordance with protocols approved by the Animal Studies Committee of Washington University School of Medicine.

Cell Culture and Chemicals

HL-1 murine cardiomyocytes, derived from murine AT-1 atrial cardiomyocyte primary culture, were generously provided by Dr. William Claycomb (Louisiana State University Health Sciences Center, New Orleans, LA). Cells were essentially cultured in the manner described by Claycomb et al. In brief, all culture dishes and flasks were pre-coated with gelatin and fibronectin (Sigma, St. Louis, MO). Cells were maintained in medium designated as HL-1 complete medium: Claycomb medium (Sigma, St. Louis, MO) supplemented with 10% FBS (SAFC BioSciences, Lenexa, KS), 2mM (1%) L-glutamine (Invitrogen, Carlsbad, CA), 100U/ml penicillin: 100ug/ml streptomycin (1% pen/strep) (Invitrogen, Carlsbad, CA), and 0.1 mM norepinephrine (Sigma, St. Louis, MO) in 5% CO₂ at 37°C. The medium was changed approximately every 24-48 hours and cells were passaged every 3 days.

Cultured HL-1 cells were treated with thapsigargin (2uM), tunicamycin (2ug/ml), DMSO (vehicle control), and insulin (10nM), all purchased from Sigma-Aldrich (St. Louis, MO). For experiments involving insulin stimulation, cells were washed 3 times in αMEM (Mediatech, Inc., Manassas, VA) only, before incubating the cells in 5% CO₂ at 37°C for 6 hours in αMEM supplemented 1% L-glutamine, 1% pen/strep with no serum. Cells were then stimulated for 10 minutes 37°C in this same medium supplemented with 10nM insulin.

siRNA treatment of HL-1 cells

HL-1 TRB3 siRNA experiments were performed using murine TRB3 ON-TARGETplus siRNA (Catalog #L-040943 for Genbank NM_175093), ON-TARGETplus non-targeting siRNA #2 (both from Dharmacon, Lafayette, CO), and Lipofectamine 2000 (Invitrogen, Carlsbad, CA). HL-1 cells were plated in 12-well tissue culture dishes at a density of 250,000 cells/well and incubated overnight in complete HL-1 medium (Claycomb medium, 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 0.1 mM norepinephrine) in 5% CO₂ at 37°C. Medium was removed, cells were washed 3 times with αMEM (Mediatech, Inc., Manassas, VA) only, replacement medium containing αMEM, 10% FBS, 1% L-glutamine without antibiotics was added, and cells were again incubated for 4 hours before being treated with TRB3 siRNA. The Lipofectamine 2000 protocol was followed according to manufacturer’s instructions using 20 nM, 50 nM, and 100 nM TRB3 siRNA or 100 nM non-targeting siRNA. After 4 hours, an equal volume of HL-1 complete medium was added and cells were incubated for 24 hours. Cells were then treated with HL-1 complete medium with either 2 μM thapsigargin or vehicle (DMSO) for an additional 24 hours. Cells were washed 3 times in αMEM only before incubation in 5% CO₂ at 37°C for 6 hours in αMEM supplemented with 1% L-glutamine, 1% pen/strep with no serum. Cells were then stimulated for 10 minutes 37°C in this same medium supplemented with 10 nM insulin. Protein lysates were isolated for further western blotting analysis.

Adenoviral Infection of HL-1 cells

HL-1 cells were plated in 12-well tissue culture dishes at a density of 200,000 cells/well and incubated overnight in complete HL-1 medium (Claycomb medium, 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 0.1 mM norepinephrine) in 5% CO₂ at 37°C. Cells were infected at a multiplicity of infection (MOI) of 20 with CMV-driven wild type HA-tagged Akt2 or control β-GAL adenovirus (Vector Biolabs, Philadelphia, PA). After 48 hours, cells were treated with 2 μM thapsigargin in complete HL-1 medium supplemented with 10 nM insulin for
24 hours. Cells were then washed 3 times in αMEM only before incubation in 5% CO₂ at 37°C for 6 hours in αMEM supplemented with 1% L-glutamine, 1% pen/strep with no serum, after which cells were stimulated for 10 minutes at 37°C in this same medium supplemented with 12.5 nM insulin or buffer. Protein lysates were isolated for further western blotting analysis.

**Simulated ischemia/reperfusion injury of HL-1 cells**

HL-1 cells were plated in 12-well tissue culture dishes at a density of 500,000 cells/well and incubated overnight in complete HL-1 medium (Claycomb medium, 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 0.1 mM norepinephrine) in 5% CO₂ at 37°C. For simulation of ischemia, the cell medium was replaced with Hepes Tyrode’s buffer without glucose (126 mM NaCl, 5.4 mM KCl, 10 mM Heps, 1 mM MgCl₂, 2 mM CaCl₂, 0.39 mM NaH₂PO₄, pH 7.4) and the plates were placed in hypoxic pouches (BBL GasPak EZ Pouch System, BD Biosciences, San Jose, CA) for 2 hours at 37°C. For control plates, the medium was replaced with fresh HL-1 medium and incubated in 5% CO₂ at 37°C for 2 hours. At the end of the ischemic period, the medium from both the ischemic and control plates were replaced with fresh HL-1 medium and were further incubated for an additional 2 hours in 5% CO₂ at 37°C. After reperfusion, the cells were harvested using TRIZOL (Invitrogen, Carlsbad, CA) for RNA/cDNA analysis by real-time PCR.

**Isolation of RNA and quantitative real-time PCR**

Total RNA was isolated from cultured cardiomyocytes or from snap-frozen cardiac tissue using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions, followed by cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using real-time detection technology and analyzed on an ABI Prism 7500 Sequence Detector (Applied Biosystems) and relative quantification of gene expression was performed using the comparative threshold (C_T) method as described by the manufacturer. Power SYBER Green PCR Master Mix (Applied Biosystems, Foster City, CA) and gene-specific primers (TRB3 forward, 5’cagtagagagatgagcctgagc3’; TRB3 reverse, 5’ccccatgggtcttcgtgaaa3’) were used to analyze TRB3 expression. TaqMan Fast Universal PCR Master Mix (2x) and gene-specific primers/fluorescent probe sets were purchased from Applied Biosystems (Foster City, CA) to analyze the following genes: β-myosin heavy chain [(β-MHC), Mm00600555_m1], sarcoplasmic–endoplasmic reticulum calcium ATPase [(SERCA2), Mm00437634_m1], atrial natriuretic factor (ANF), Mm00492097_m1], and 78 kDa glucose-regulated protein [(GRP78, aka Hspa5 or BiP), Mm00517691_m1]. Glyceraldehyde-3-phosphate dehydrogenase [(GAPDH), Applied Biosystems 4308313] was used as an internal control to normalize the mRNA levels for each gene.

**Western blotting**

SDS-PAGE and Western blotting was performed according to standard procedures as previously described.²³ PVDF Membranes were blocked with 5% non-fat dried milk in TBST (10 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20). Total Akt1/2, phospho-specific Akt1/2 (Ser473), phospho-specific GSK3β (Ser9), and BiP (GRP78) antibodies were obtained from Cell Signaling Technologies (Beverly, MA). Primary antibodies for GADD 153 (CHOP10) and 14-3-3β (Santa Cruz Biotechnologies, Santa Cruz, CA), β-actin (Sigma, St. Louis, Missouri), and TRB3 (Calbiochem/EMD Chemicals, Inc., Gibbstown, NJ) were also used. ECL anti-rabbit IgG, horseradish peroxidase (HRP)-linked whole antibody (from donkey) (GE Healthcare, Piscataway, NJ) and anti-mouse IgG HRP-linked antibodies were used as secondary antibodies. Either the Millipore (Billerica, MA) Immobilon Western Chemiluminescent HRP Substrate or GE Healthcare/Amersham (Piscataway, NJ) ECL Blotting Detection Reagents were
used to visualize protein bands. Densitometry was performed using the ImageJ software developed by Wayne Rasband (National Institutes of Health, Bethesda, MD).

**Generation of TRB3 transgenic mice**

A cDNA encoding myc epitope-tagged murine TRB3 (Genbank NM_175093) was inserted into a plasmid vector containing the murine α-myosin heavy chain promoter (the generous gift of Dr. Jeffrey Robbins, University of Cincinnati) as previously described. Linearized plasmid DNA was provided to the Mouse Genetics Core of Washington University in St. Louis and was injected into the male pro-nucleus of one-cell C57BL/6J x CBA hybrid embryos and then the embryos were inserted into pseudo-pregnant female mice. Progeny were screened for genomic insertion of the transgenic DNA by PCR of tail-prep DNA. Three F0 mice were obtained with insertion of the transgene and they were used to propagate three independent lines of transgenic mice, designated 1x-TRB3, 2x-TRB3, and 4x-TRB3 to reflect the differential genomic DNA expression of the TRB3 transgene determined by PCR. All lines of mice were viable, fertile, and appeared grossly normal throughout life. Transgenic mice were bred with C57BL/6J purchased from The Jackson Laboratories for 3-4 generations. Transgenic mouse lines 2x-TRB3 and 4x-TRB3, with intermediate and higher expression of TRB3 mRNA in cardiac tissue, respectively, were analyzed in this work.

**Acute insulin administration in mice**

2x-TRB3 mice and their WT littermates were fasted overnight (12 hours) in a clean cage on hardwood bedding with access to water. Mice were anesthetized with a mixture of ketamine (80mg/kg) and xylazine (10mg/kg) administered i.p. The jugular vein was exposed and 0.1units/kg insulin or saline (control) in a volume of 50 µl was injected intravenously. After 5 minutes, the heart was harvested and the left ventricle isolated and snap-frozen in liquid nitrogen for later protein isolation and analysis.

**Experimental murine myocardial infarction**

Murine myocardial infarction surgery was performed as previously described. Briefly, adult C57BL/6J anesthetized and ventilated mice were subjected to thoracotomy, and a single 8-0 Prolene suture was tied around the proximal left coronary artery, ~1 mm distal to the atrioventricular junction. Successful occlusion was confirmed by the appearance of pallor of the anterior left ventricular wall. The incision was closed, and the animal was allowed to recover on a heating pad. The surgeon was blinded to the genetic status of the mice.

To evaluate ER stress markers and the upregulation of TRB3 in WT mice after MI, C57BL/6J mice underwent myocardial infarction and were sacrificed by CO2 asphyxiation 4 and 24 hours after surgery. The left ventricle was isolated and snap-frozen in liquid nitrogen for RNA analysis.

Myocardial infarction was performed on 2x-TRB3 mice and their WT littermates. Transthoracic echocardiography was performed one day after myocardial infarction to verify that the area of infarct size was the same between animals. Follow-up echocardiography was performed one week after MI to analyze cardiac remodeling and cardiac function. The initial infarct size, indicated by the segmental wall motion score index (SWMSI), was determined as previously described. Mice were sacrificed by CO2 asphyxiation and the hearts were dissected out, weighed, and prepared for histologic examination.

**Murine echocardiography**

Mice were imaged in the left lateral decubitus position on a Sequoia cardiac echocardiography machine (Acuson Co.) equipped with a 15-MHz linear transducer.
parasternal long- and short-axis views were recorded, as was 2-dimensional targeted M-mode tracings throughout the anterior and posterior LV walls. Echocardiographic procedures were performed by the Mouse Cardiovascular Phenotyping Core at Washington University in St. Louis.

**Isolated working heart analysis of cardiac metabolism**

Isolated mouse working heart perfusions were performed as previously described. Adult wild type, 2x-TRB3, and 4x-TRB3 male mice (8 weeks old) were heparinized (100 units i.p) 10 min prior to anesthesia. Animals were then deeply anesthetized with 5-10 mg of Na\(^+\)-pentobarbital (i.p.). Hearts were excised and placed in an ice-cold Krebs-Henseleit bicarbonate (KHB) solution (118mM NaCl, 25 mM NaHCO3, 4.7 mM KCl, 1.2 mM K2HPO4, 2.5 mM CaCl2, 5.0 mM glucose, and 10 μU/ml insulin; pH 7.4). Hearts were cannulated first via the aorta and perfused retrogradely by the Langendorff method. Following left atrial cannulation, perfusion was switched to working heart perfusion with KHB solution containing 1.2 mM palmitate bound to 3% fatty acid free BSA with a preload pressure of 11.5 mmHg and an afterload pressure of 50 mmHg for 60 minute with oxygenated buffer solution. To determine palmitate and glucose oxidation rates, trace amounts of \(^3\)H-palmitate (0.1 μCi/ml) and [U-\(^14\)C]-glucose (0.1 μCi/ml) were used, respectively. Samples were collected every 10 minutes for \(^{14}\)CO\(_2\) trapped in 1M hyamine hydroxide solution for glucose oxidation and \(^3\)H\(_2\)O released into the buffer due to palmitate oxidation, and the radioactivity was counted.

Functional measurements including cardiac output, aortic flows, peak systolic pressure and heart rate were acquired every ten minutes for 10 seconds with inline flow probes (Transonic Systems, Inc) and MP100 system from AcqKnowledge (BIOPAC Systems, Inc) and a pressure transducer (TSD 104A, BIOPAC System, Inc) respectively. Cardiac hydraulic work (expressed in J·s\(^{-1}\)·g wet wt\(^{-1}\)) was calculated as the product of peak systolic pressure and cardiac output, normalized to heart wet weight. At the end of each perfusion, hearts were frozen immediately in liquid nitrogen. A small piece of heart tissue was used to determine the dry-to-wet weight ratio.

**Histological Analysis**

Adult hearts obtained 7 days after myocardial infarction were fixed in formalin, embedded in paraffin, and sectioned with a microtome. Tissue sections were stained with Masson’s trichrome to evaluate fibrosis (infarct, scar). To quantitate the size of the infarct, the area of blue staining (scar) was normalized by the entire ventricular area (red and blue) for each of approximately 6 left ventricular sections per animal. Apoptosis was detected by in situ by terminal deoxynucleotidyl transferase assay (TUNEL). TUNEL in the infarct border zones was defined as one-quarter circumference and either side of the scar edge.

**Statistics**

Normality and equal variance assumptions were assessed by Levene’s test using the SigmaStat Statistical Analysis Package (version 3.1). Rank sum tests with were used in comparisons for which normality or equal variance assumptions were invalid. Where indicated, data sets were compared by two-sample, two-tailed homoscedastic T-tests with Bonferroni post-hoc correction. In other instances, 1-way ANOVA was calculated by SigmaStat. Data is expressed as mean ± SEM (unless indicated otherwise).
**Online Figures**

**Online Figure I.** Akt2 overexpression promotes insulin-stimulated GSK-3β phosphorylation in thapsigargin-treated HL-1 cardiac myocytes. HL-1 cells were infected with ad-β-Gal (control) or ad-Akt2. 48 hours later, cells were treated with thapsigargin for 24 hours. HL-1 cells were then treated with 12.5 nM insulin (+ins) or buffer (−) for 10 minutes. Protein lysates were separated by SDS-PAGE and immunoblotting was performed to evaluate the phosphorylation status of GSK-3β, an Akt substrate. Blots were re-probed with anti-14-3-3β primary antibody to control for protein loading. Depicted in the figure are the computerized densitometry results that indicate the relative phospho-GSK3β/14-3-3β protein levels for each sample.
Online Figure II. TRB3 expression is induced in response to low glucose. H9C2 cardiac myocytes were cultured in DMEM in the presence of 0.5 mM, 5.5 mM or 25 mM glucose for 24 hours. RNA was purified from cardiomyocytes and TRB3 and GAPDH mRNA levels were determined by quantitative real-time RT-PCR. *, P<0.05 versus cells cultured in 5.5 mM or 25 mM glucose; ^, P<0.05 versus cells cultured in 25 mM glucose by one-way ANOVA.
Online Figure III. TRB3 expression is induced in response to an in vitro model of ischemia/reperfusion injury. HL1 cardiac myocytes were cultured under hypoxic and hypoglycemic conditions for 2 hours, and then were cultured for an additional 2 hours under normoxic and normoglycemic conditions (I/R). Control HL1 cells (C) were cultured solely under normoxic and normoglycemic conditions (4 hours). RNA was purified from cardiomyocytes and TRB3, GRP78, CHOP and GAPDH mRNA levels were determined by quantitative real-time RT-PCR. *, P<0.05 compared to control HL1 cells in each group by one-way ANOVA.
Online Tables

Online Table I. Cardiac Chamber Weight of 2x-TRB3 Mice.

<table>
<thead>
<tr>
<th></th>
<th>WT 4 mo. (n=6)</th>
<th>2x-TRB3 4 mo. (n=10)</th>
<th>P value 4 mo.</th>
<th>WT 12 mo. (n=7)</th>
<th>2x-TRB3 12 mo. (n=8)</th>
<th>P value 12 mo.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>25.0 ± 0.71</td>
<td>27.5 ± 0.68</td>
<td>0.033</td>
<td>34.9 ± 2.0</td>
<td>39.1 ± 1.8</td>
<td>0.144</td>
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<td>BVW (g)</td>
<td>100.2 ± 2.6</td>
<td>110.1 ± 3.2</td>
<td>0.050</td>
<td>138.2 ± 8.3</td>
<td>146.4 ± 4.8</td>
<td>0.396</td>
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<tr>
<td>LV (mg)</td>
<td>79.7 ± 1.2</td>
<td>89.3 ± 2.7</td>
<td>0.116</td>
<td>110.0 ± 7.1</td>
<td>117.1 ± 3.8</td>
<td>0.374</td>
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<tr>
<td>RV (mg)</td>
<td>20.5 ± 1.5</td>
<td>20.8 ± 1.0</td>
<td>0.877</td>
<td>28.3 ± 1.3</td>
<td>29.3 ± 1.4</td>
<td>0.596</td>
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<tr>
<td>BV/BW (mg/g)</td>
<td>4.01 ± 0.07</td>
<td>4.02 ± 0.14</td>
<td>0.871</td>
<td>4.00 ± 0.25</td>
<td>3.78 ± 0.17</td>
<td>0.477</td>
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<tr>
<td>LV/BW (mg/g)</td>
<td>3.19 ± 0.05</td>
<td>3.26 ± 0.11</td>
<td>0.648</td>
<td>3.18 ± 0.21</td>
<td>3.02 ± 0.13</td>
<td>0.523</td>
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<tr>
<td>RV/BW (mg/g)</td>
<td>0.82 ± 0.049</td>
<td>0.76 ± 0.047</td>
<td>0.459</td>
<td>0.82 ± 0.049</td>
<td>0.76 ± 0.046</td>
<td>0.371</td>
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</tbody>
</table>

Cardiac chamber weight analysis of 4 month-old and 12 month-old wild type and 2x-TRB3 transgenic male mice. BW, body weight; BV, biventricular weight; LV, left ventricular weight; RV, right ventricular weight; BVW/BW, biventricular weight-to-body weight ratio; LV/BW, left ventricular weight-to-body weight ratio; RV/BW, right ventricular weight-to-body weight ratio. Data is expressed as mean ± SEM. The P value was determined by Student’s t-test or by Rank Sum test (if the data failed a normality or an equal variance test).
Online Table II. Echocardiographic Analysis of 2x-TRB3 and 4x-TRB3 Mice.

<table>
<thead>
<tr>
<th></th>
<th>Wild Type (n=5)</th>
<th>2x-TRB3 (n=3)</th>
<th>4x-TRB3 (n=4)</th>
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</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>698 ± 9.7</td>
<td>648 ± 16</td>
<td>663 ± 14</td>
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<td>LVIDd (mm)</td>
<td>3.31 ± 0.18</td>
<td>2.83 ± 0.11</td>
<td>3.30 ± 0.08</td>
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<td>LVIDs (mm)</td>
<td>1.67 ± 0.23</td>
<td>1.25 ± 0.05</td>
<td>1.51 ± 0.10</td>
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<td>LVM (mg)</td>
<td>82.2 ± 6.0</td>
<td>84.7 ± 4.8</td>
<td>96.9 ± 3.0</td>
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<tr>
<td>LVMI (mg/g)</td>
<td>2.96 ± 0.14</td>
<td>3.37 ± 0.42</td>
<td>3.08 ± 0.06</td>
</tr>
<tr>
<td>FS (%)</td>
<td>50.5 ± 3.6</td>
<td>55.9 ± 0.2</td>
<td>54.4 ± 1.5</td>
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</table>

Transthoracic echocardiographic analysis of awake 12-week-old wild type, 2x-TRB3 and 4x-TRB3 transgenic male mice. HR, heart rate (bpm); LVIDd, left ventricular (LV) internal dimension at end-diastole (mm); LVIDs, LV internal dimension at end systole; LVM, calculated LV mass (mg); LVMI, calculated LV mass (mg) divided by body weight (g); FS, fractional shortening of the LV in systole. Data is expressed as mean ± SEM. None of the comparisons reached statistical significance.
**Online Table III. Ex Vivo Analysis of 2x-TRB3 Cardiac Function in the Working Mode.**

<table>
<thead>
<tr>
<th></th>
<th>Wild Type (n=7)</th>
<th>2x-TRB3 (n=5)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>268 ± 22.2</td>
<td>244 ± 9.8</td>
<td>0.053</td>
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<tr>
<td>PSP (mmHg)</td>
<td>98.4 ± 4.1</td>
<td>97.7 ± 8.8</td>
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<tr>
<td>CO (ml/min)</td>
<td>11.1 ± 1.9</td>
<td>10.4 ± 0.18</td>
<td>0.546</td>
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<tr>
<td>CW (ml*mmHg/min)</td>
<td>10.9 ± 2.0</td>
<td>10.3 ± 2.5</td>
<td>0.640</td>
</tr>
</tbody>
</table>

Evaluation of cardiac function during ex vivo working heart experiments in 12-week-old wild type and 2x-TRB3 transgenic male mice. HR, heart rate; PSP, peak systolic pressure; CO, cardiac output; CW, cardiac work. Data is expressed as mean ± SD.
Online Table IV. Ex Vivo Analysis of 4x-TRB3 Cardiac Function in the Working Mode.

<table>
<thead>
<tr>
<th></th>
<th>Wild Type (n=6)</th>
<th>2x-TRB3 (n=5)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>259 ± 14.0</td>
<td>257 ± 18.0</td>
<td>0.934</td>
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<tr>
<td>PSP (mmHg)</td>
<td>118 ± 7.0</td>
<td>127 ± 8.2</td>
<td>0.439</td>
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<tr>
<td>CO (ml/min)</td>
<td>13.0 ± 1.1</td>
<td>12.7 ± 0.8</td>
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<tr>
<td>CW (ml*mmHg/min)</td>
<td>15.4 ± 1.5</td>
<td>14.7 ± 1.5</td>
<td>0.761</td>
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</table>

Evaluation of cardiac function during ex vivo working heart experiments in 12-week-old wild type and 4x-TRB3 transgenic male mice. HR, heart rate; PSP, peak systolic pressure; CO, cardiac output; CW, cardiac work. Data is expressed as mean ± SD.
Supplemental References


