Targeted Deletion of Thioredoxin-Interacting Protein Regulates Cardiac Dysfunction in Response to Pressure Overload


Abstract—Biomechanical overload induces cardiac hypertrophy and heart failure, and reactive oxygen species (ROS) play a role in both processes. Thioredoxin-Interacting Protein (Txnip) is encoded by a mechanically-regulated gene that controls cell growth and apoptosis in part through interaction with the endogenous dithiol antioxidant thioredoxin. Here we show that Txnip is a critical regulator of the cardiac response to pressure overload. We generated inducible cardiomyocyte-specific and systemic Txnip-null mice (Txnip-KO) using Flp/frt and Cre/loxP technologies. Compared with littermate controls, Txnip-KO hearts had attenuated cardiac hypertrophy and preserved left ventricular (LV) contractile reserve through 4 weeks of pressure overload; however, the beneficial effects were not sustained and Txnip deletion ultimately led to maladaptive LV remodeling at 8 weeks of pressure overload. Interestingly, these effects of Txnip deletion on cardiac performance were not accompanied by global changes in thioredoxin activity or ROS; instead, Txnip-KO hearts had a robust increase in myocardial glucose uptake. Thus, deletion of Txnip plays an unanticipated role in myocardial energy homeostasis rather than redox regulation. These results support the emerging concept that the function of Txnip is not as a simple thioredoxin inhibitor but as a metabolic control protein. (Circ Res. 2007;101:1328-1338.)

Key Words: cardiac hypertrophy  ■  reactive oxygen species  ■  glucose

Heart failure is among the most prevalent diseases worldwide and frequently results from sustained biomechanical overload. After a prolonged period of compensatory adaptation of cardiac hypertrophy, myocardium undergoes functional and histological deterioration. A large body of literature suggests that mechanical left ventricular (LV) wall stress induces cardiac hypertrophy and failure in part through induction of reactive oxygen species (ROS).\(^1\) In addition to structural ventricular remodeling, myocardium changes utilization of metabolic fuels from fatty acid to glucose, a process called “metabolic remodeling”.

Multiple antioxidant systems—including the two major thiol reductase systems, glutathione and thioredoxin—can protect cells by scavenging ROS. The thioredoxin system is a thiol-reducing mechanism expressed in almost all living cells that functions through the reversible oxidation of vicinal cysteines of thioredoxin and through reduction by thioredoxin reductase. Indeed, cytosolic thioredoxin1 can protect the heart against oxidative stress and inhibit cardiac hypertrophy via its antioxidant activity.\(^2\) In addition to its antioxidant properties, thiol-disulfide exchange reactions serve as control mechanisms for signal transduction. For example, thioredoxin modulates transcription factor activation, stimulates growth, and inhibits apoptosis through interaction with several binding partners.\(^3\)

Thioredoxin-interacting protein (Txnip), also known as thioredoxin binding protein 2 or vitamin D\(_3\) upregulated protein 1, probably interacts with thioredoxin via a disulfide bond, reducing thioredoxin activity.\(^4\) Mechanical or oxidative stress suppresses Txnip expression without affecting thioredoxin activity.\(^5\) Mechanical or oxidative stress suppresses Txnip expression without affecting thioredoxin activity.\(^3\) Forced overexpression of Txnip decreases thioredoxin activity, increases oxidative stress, and inhibits cell growth.\(^5,6\) In addition, glucose strongly induces Txnip in multiple cell types, suggesting possible physiological roles of Txnip in glucose metabolism.\(^7\) Thus, Txnip acts as a redox-sensitive signaling protein that participates in a variety of biological functions, although it is unclear if these roles of Txnip are mediated solely by inhibition of thioredoxin function.
Because Txnip can inhibit thioredoxin function, we hypothesized that deletion of Txnip would protect the myocardium by allowing greater thioredoxin antioxidant activity. To test this hypothesis, we generated mice with targeted deletion of Txnip using Flp/frt and Cre/loxP technologies.

Materials and Methods

An expanded Materials and Methods section can be found in the online data supplement at http://circres.ahajournals.org.

Generation of Conditional Txnip Deletion Mice

A genomic BAC containing the Txnip locus, RPCI-22 clone 496G18, from a mouse 129S6/SvEvTac genomic library was obtained. We used a 6.5-kb plasmid vector containing the Txnip gene exon1 and its flanking genomic sequences including the Txnip promoter region and exons 2 to 6 (Figure 1A). The construct was electroporated into J1 ES cells, and chimeric mice were generated by injection of clones into C57BL/6 blastocysts. The successful homologous recombination was confirmed in genomic DNA from Txnip(FrtneoFrtloxFrt) mice (Figure 1B). We used the Flp/frt system to eliminate the neomycin gene by crossing Txnip(FrtneoFrtloxFrt) mice to mice expressing FLPe recombinase from the Rosa26 locus.8

Thioredoxin Activity and ROS

The tissue levels of thioredoxin activity, \( \text{O}_2^- \) production (ferri-cytochrome c reduction), lipid peroxides (malondialdehyde), and the ratio of reduced (GSH) to oxidized glutathione (GSSG) were measured in whole heart homogenates. Cryosections were stained with 5-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCFDA).

Transverse Aortic Constriction

Transverse aortic constriction (TAC) surgery, echocardiography, and hemodynamic acquisition were performed in in vivo studies using blinded protocols with respect to genotype.

Nuclear Magnetic Resonance Spectroscopy

Levels of phosphorylated metabolites were monitored based on the intensities of the \(^1\text{H}-\text{nuclear magnetic resonance (NMR)}\) responses in a perfused Langendorff heart model system. LV mechanical function was monitored during the NMR spectroscopy.

Statistical Analysis

All data are presented as mean±SEM. Statistical analysis was performed with the paired \( t \) test, unpaired Student \( t \) test or Mann-Whitney test between groups.

Results

Generation of Txnip-Null Mice

We first generated mice with systemic deletion of Txnip. Males were generated with both the Protamine-Cre transgene and the Txnipbox allele; the endogenous mouse protamine genes are expressed during the haploid stages of spermatogenesis.9 These males were then mated to wild-type females. Protamine-Cre mediated recombination of the Txnipbox allele resulted in a heterozygous Txnip-null and subsequent intercrossing led to homozygous Txnip-null mice. After confirmation of the recombined gene in the male germ line by Protamine-Cre, mice without the Cre transgene were used for further breeding to avoid the potential effect of ectopic Cre activity on phenotypes. For each of the crosses, progeny were healthy and viable at birth and genotypes were present at the normal Mendelian frequencies. Txnipboxnull mice did not display any evident gross phenotypes up to 36 weeks old compared with wild-type mice. Southern analysis and PCR of genomic DNA confirmed the targeted gene deletion in Txnip-null mice (Figure 1B and 1C). Although an ATG methionine accompanied with a Kozak sequence was predicted at exon 6 in the shifted-frame (2 base pairs), Northern analysis on RNA extracts from hearts and skeletal muscle with a specific probe encoding Txnip exon7 and exon8 showed that the gene targeting strategy abolished expression of the 3’ exons (Figure 1D). Because the Cre-loxP strategy deleted only exon1 of the Txnip gene and because the putative thioredoxin-binding site of Txnip is 3’ to exon1, we confirmed that no aberrant protein was synthesized from the targeted allele. Txnip protein expression was confirmed by Western analysis using monoclonal mouse anti-human Txnip antibodies generated in our laboratory. Epitope-mapping using various deletion constructs demonstrated that the antibodies (clone JY1 and JY2) bound to regions in the C terminus of the Txnip protein after amino acid 302 (Figure 2A). Western analyses using these antibodies indicated that Txnip protein was robustly expressed in the heart, lung, spleen, and skeletal muscle of wild-type mice, but no significant expression was observed in these tissues from Txnip-null mice, confirming deletion of Txnip protein in mice (Figure 2B and 2C).

Txnip-Null Mice Exhibit No Changes in Thioredoxin Activity

We then examined whether systemic deletion of Txnip led to compensatory changes in other components of the thioredoxin system in mice. Interestingly, immunoblot analysis and insulin disulfide reduction assay revealed that neither expression levels (Figure 2D) nor activities (Figure 2E) of thioredoxin were significantly different between Txnip-null mice and their wild-type littermates. Because the thioredoxin system can regulate glutathione reductase,10 the ratio of GSH to GSSG was measured in tissues. The GSH/GSSG ratio was not different between Txnip-null and wild-type mice in whole blood (Figure 2G) or in the heart (Figure 2H). Western analysis of ventricular protein from Txnip-null mice showed no changes in the protein levels of thioredoxin1 reductase, catalase, or superoxide dismutase, indicating no compensatory up- or downregulation of other antioxidants in the heart (Figure 2F).

Txnip-Null Mice Have Normal Lipid Profiles and Hypoglycemia

Previously, a nonsense mutation in the mouse Txnip gene (Hyplip1) was identified as a possible cause of hyperlipidemia, implicating the roles of Txnip in lipid metabolism.11 In contrast with previous reports of hyperlipidemia,11,12 serum levels of total cholesterol and triglycerides were within the normal range in 12-week-old Txnip-null mice, and not significantly different from wild-type controls in the 18-hour fasted state (total cholesterol 112±8 mg/dL, n=11 in null mice versus 114±9 mg/dL, n=7 in wild-type, triglycerides 48±7 mg/dL, n=11 in null mice versus 36±7 mg/dL, n=5 in wild-type). Blood glucose levels were lower in Txnip-null mice than in littermate controls (fasting blood glucose 59±5
Figure 1. Dual-recombinase strategy for conditional inactivation of Txnip in mice. A, Targeting vector pPGKneoF2L2DTA contains two loxP and two frt sites. B, The successful homologous recombination events created the additional SpeI site. SpeI and NcoI-digested DNA was hybridized with the 5' probe (8-kb and 11-kb bands represent the deletion and wild-type allele, respectively), and the 3' probe (2.3-kb and 11-kb bands represent the deletion and wild-type allele, respectively). C, Homozygous and heterozygous (het) Txnip-null mice were characterized by PCR with primers F1, F2, and R, allowing the amplification of the loxP-containing deletion region; a 220-bp fragment for the Txnip-null and 644- and 128-bp fragments for the wild-type allele. D, No significant Txnip mRNA was expressed in Txnip-null mice on Northern analysis probed by Txnip exon7 and exon8.
mg/dL, n=11 in null mice versus 109±6 mg/dL, n=7 in wild-type; P<0.01). These results show that Txnip-null mice have lower blood glucose levels than wild-type mice but no clear changes in thioredoxin activity.

**Txnip Deletion and the Response to Pressure Overload**

Because thioredoxin was initially identified as a growth factor, we examined whether deletion of Txnip, a potential inhibitor of thioredoxin, promotes myocardial hypertrophy in mice. Systemic blood pressure and cardiac parameters were measured noninvasively and followed up to the age of 25 weeks in non-TAC mice (Table). There were no differences between Txnip-null and wild-type mice in systolic blood pressure, LV wall thickness, and LV mass to tibial length ratio (LVM/TL). However, echocardiography revealed that LV dimensions were slightly larger and % fractional shortening (%FS) was lower in Txnip-null mice at 8 to 15 weeks of age compared with wild-type littermates. Thus, Txnip-deficient hearts showed mild cardiac dysfunction at baseline.

To determine whether compensatory changes in the thioredoxin system contribute to these baseline cardiac phenotypes, we examined redox state in these mice. Intracellular ferricytochrome c reduction (O$_2^*$ production; 17±7 nmol/mg,
n=10 in null mice versus 16±4 nmol/mg, n=9 in wild-type; P=NS), and myocardial lipid peroxides (malondialdehyde; 0.85±0.09 nmol/mg, n=11 in null mice versus 0.96±0.13 nmol/mg, n=9 in wild-type; P=NS) were not altered in Txnip-null hearts compared with wild-type hearts.

Next, to evaluate a potential role for Txnip as a regulator of the response to cardiac pressure overload, 8- to 10-week-old Txnip-null mice were subjected to TAC. Echocardiographic LVM/TL increased in wild-type mice after TAC, but progressive hypertrophy was reduced in Txnip-null mice (ΔLVM/TL from baseline to 4 weeks after TAC: +56±9%, n=27 for wild-type versus +37±9%, n=22 for null mice, P<0.05; Figure 3A). Postmortem examination independently confirmed that the increase in heart weight/tibial length (HW/TL) caused by TAC was reduced in Txnip-null mice at 4 weeks (32±11% from sham, n=10 for wild-type versus 10±6% from sham, n=6 for null mice, P<0.05). Histological examination revealed that TAC caused

Table. Blood Pressure and Cardiac Parameters in Non-TAC Mice

<table>
<thead>
<tr>
<th>Age</th>
<th>Wild-Type</th>
<th>Txnip-Null</th>
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<tbody>
<tr>
<td>n</td>
<td>12–59</td>
<td>6–8</td>
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<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>113±2</td>
<td>117±2</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>574±11</td>
<td>582±11</td>
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<td>Anterior wall thickness, mm</td>
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<td>0.90±0.01</td>
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<tr>
<td>Posterior wall thickness, mm</td>
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<td>0.87±0.01</td>
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<tr>
<td>End-diastolic dimensions, mm</td>
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<td>2.87±0.07</td>
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<tr>
<td>End-systolic dimensions, mm</td>
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<td>1.27±0.05**</td>
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<tr>
<td>Fractional shortening, %</td>
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<td>56±1**</td>
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<td>LV mass/tibial length, g/m</td>
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<td>3.7±0.1</td>
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<tr>
<td>Heart weight/tibial length, g/m</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>23±1</td>
<td>25±0.4*</td>
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</tbody>
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*P<0.05, **P<0.01 vs wt.

Figure 3. Echocardiographic (A) and histological assessments revealed wild-type (wt) mice had progressive increases in left ventricular (LV) mass over tibial length, myocyte cross-sectional area with Periodic acid Schiff (PAS) staining (B and C), and collagen content with Picro-sirius red staining (D and E) after transverse aortic constriction (TAC) comparing with the sham group. Txnip-null mice had reduced hypertrophy at 4 weeks (+32±11% from sham, n=10 for wild-type versus +10±6% from sham, n=6 for null mice, P<0.05). Histological examination revealed that TAC caused

Scale bars represent 20 μm.
a greater increase in myocyte cross-sectional area in wild-type mice compared with Txnip-null mice at 4 weeks (Figure 3B and 3C). However, at 8 weeks after TAC, heart weight and myocyte cross-sectional areas of Txnip-null hearts increased to the same level of wild-type hearts (HW/TL 82±8 g/m, n=11 for wild-type, 90±12 g/m, n=10 for null mice, P=NS). Txnip-null mice had consistently less interstitial fibrosis as assessed by collagen content at 4 and 8 weeks after TAC (Figure 3D and 3E). Close-chest invasive hemodynamic assessments revealed no difference in peripheral vascular resistance between wild-type mice (9.6±2.4×10³ dynes·s·cm⁻¹, n=8) and Txnip-null mice (8.9±1.6×10³ dynes·s·cm⁻¹, n=6, P=NS) at baseline. Four weeks after TAC, no significant differences in peak LV pressure (126±9 mm Hg, n=24 for wild-type versus 133±6 mm Hg, n=31 for null mice) and peak pressure gradient (43±5 mm Hg, n=8 for wild-type versus 43±9 mm Hg, n=8 for null mice) were found. Thus, reduction in hypertrophy in Txnip-null mice during an early phase of pressure overload was not attributable to lower systemic blood pressure or a difference in the transverse aortic pressure gradient.

One week after TAC, %FS was significantly depressed in wild-type mice, but Txnip-null mice subjected to TAC exhibited no significant depression in %FS from baseline to 1 week (Figure 4A). At echocardiography, there was only a trend toward an increase in %FS at 4 weeks after TAC in Txnip-null mice compared with wild-type mice, but the trend toward an increase in %FS at 4 weeks after TAC in both wild-type and Txnip-null hearts, but there was no significant difference between genotypes (Figure 5C and 5D). Neither ferricytochrome c reduction nor malondialdehyde level was altered in Txnip-null hearts at 4 weeks after TAC. These survival rates were not different by Fisher’s exact test. Thus, Txnip deletion led to less cardiac hypertrophy and prevented cardiac dysfunction during an early phase of pressure overload, but these benefits were not sustained and Txnip deletion led to worse cardiac function at the later phase of pressure overload.

We hypothesized that the regulatory effect of Txnip deletion on cardiac performance could be attributable to alterations of myocardial ROS levels after TAC. However, we found no significant difference in thioredoxin activity between Txnip-null and wild-type hearts both at 4 and 8 weeks after TAC (Figure 5A and 5B). Myocardial oxidative stress, assessed by DCFDA, was increased significantly at 4 and 8 weeks after TAC in both wild-type and Txnip-null hearts, but there was no significant difference between genotypes (Figure 5C and 5D). Neither ferricytochrome c reduction nor malondialdehyde level was altered in Txnip-null hearts at 4 weeks after TAC (Figure 5E and 5F). These results suggest that deletion of Txnip regulates the acute development of cardiac hypertrophy and the biphasic functional response by the mechanism independent of global levels of thioredoxin activity or ROS in the myocardium.

To examine whether the changes in LV function after TAC were related to cardiomyocyte apoptosis, triple staining with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL), anti-α-sarcomeric actin anti-
body, and DAPI was performed. TAC increased the number of TUNEL-positive cardiomyocytes in both wild-type and Txnip-null mice, but there was no significant difference between genotypes at 4 weeks after TAC (Figure 5G). At 8 weeks after TAC, Txnip-deficient mice had an increased number of TUNEL-positive cardiomyocytes compared with wild-type mice. These data suggest that Txnip deletion promotes cardiomyocyte apoptosis after prolonged mechanical stress by a mechanism independent of thioredoxin activity.

Deletion of Txnip Increases Myocardial Glucose Utilization

Txnip-null mice exhibited lower blood glucose levels without apparent hyperlipidemia. Therefore, we hypothesized that increasing myocardial glucose utilization could be an alternative mechanism by which Txnip deletion modulates cardiac function. In isolated perfused hearts from 17- to 20-week-old mice, we found that deletion of Txnip dramatically increased the uptake of 2-deoxyglucose (2-DG), a glucose analogue, during insulin-free perfusion (Figure 6A and 6B). The accumulation ratio of sugar phosphate (d[SP]/dt) was significantly higher in Txnip deletion mice than in wild-type mice in both fed (0.49±0.07 area/g/min, n=6 for Txnip-null versus 0.15±0.03 area/g/min, n=5 for wild-type, P<0.01) and fasted states (0.40±0.09 area/g/min, n=4 for Txnip-null versus 0.15±0.03 area/g/min, n=4 for wild-type, P<0.05).

At baseline, cardiac phosphocreatine (PCr)/ATP ratio, a marker of cardiac bioenergetic status, was comparable between Txnip-null and wild-type hearts (1.8±0.1, n=6 in null mice versus 2.1±1.8, n=5 in wild-type; P=NS). When all glucose in the perfusate was switched to 2-DG, myocardial PCr, ATP, and mechanical function declined with the accumulation of 2-DG-6-phosphate in both genotypes (Figure 6A). At baseline, LV developed pressure in Txnip-null mice was slightly lower (96±7 mm Hg, n=6) but not significantly different from that (116±9 mm Hg, n=5) in wild-type. However, Txnip-null hearts maintained LV developed pressure during metabolic inhibition by 2-DG perfusion (Figure 6C). Despite the higher LV developed pressure in Txnip-null hearts, there was a significant decrease in PCr and ATP levels in Txnip-null hearts (Figure 6A, and online supplemental results and discussion).

There were no significant changes in the total protein expression levels of glucose transporter 1 (GLUT1) and GLUT4, but myocardial glycogen storage was significantly greater in Txnip-null hearts than in wild-type hearts (Figure 6D). The tissue level of triglycerides was not different between Txnip-null and wild-type hearts (Figure 6E).
β-adrenoreceptor stimulation induces glucose transport through cAMP-dependent PKA. To examine whether abnormal cardiac responses to β-adrenergic stimulation might account for progression of cardiac dysfunction and augmented myocardial glucose uptake by deletion of Txnip, we assessed the acute effect of a β-adrenoreceptor agonist in isolated perfused hearts. Isoproterenol (5 nmol/L) increased the rate-pressure product in Txnip-null hearts similar to wild-type hearts (Figure 6F). Txnip-null hearts exhibited greater glucose uptake than wild-type under β-adrenergic stimulation (Δ[dSP]/Δt 0.51±0.08 area/g/min, n=4 for null mice versus 0.18±0.04 area/g/min, n=3 for wild-type;...
Mer/Txnip flox/flox mice were injected with 0.5 mg of 4-hydroxytamoxifen (4-OHT; n=7), and wt mice treated with vehicle (n=9). D. Myocardial glucose uptake assessed by 2-deoxyglucose (2-DG) 6-phosphate accumulation using 31P-NMR spectroscopy in cardiac-KO (n=4) and control mice (n=4). Values represent mean±SEM. **P<0.01 vs pre, †P<0.05 vs control.

4-OHT. At 2 weeks after TAC, pressure overload markedly increased LV mass in control mice, wild-type mice with 4-OHT, and wild-type mice with vehicle (Figure 7C). However, the cardiac-KO mice exhibited a smaller increase in LV mass compared with controls (−22±7% over control TAC; n=7, P<0.05). Postmortem examination independently confirmed that the heart weight/body weight was smaller in cardiac-KO mice (5.5±0.4 g/m, n=7) than in control (6.3±0.3 g/m, n=7), wild-type mice with vehicle (6.3±0.1 g/m, n=5), and wild-type mice with 4-OHT (6.6±0.6 g/m, n=5) after TAC (P<0.05). Thus, cardiac-specific deletion of Txnip reduced cardiac hypertrophy in the early response to pressure overload.

Similar to the findings in Txnip-null mice, cardiac-KO mice exhibited a robust increase in myocardial glucose uptake (d[SP]/dt 1.22±0.19 area/g/min, n=4, for cardiac-KO versus 0.48±0.17 area/g/min, n=4, for controls; P<0.05), indicating that changes in glucose metabolism in the heart were not secondary to systemically abnormal glucose/insulin metabolism (Figure 7D). Invasive hemodynamic assessments showed that cardiac output (9.1±1.9 mL/min for cardiac-KO versus 6.4±0.9 mL/min for control) and preload-recruitable stroke work (110±13 mm Hg, n=5 for cardiac-KO versus 87±7 mm Hg, n=10 for control) tended to be higher in cardiac-specific KO hearts at 2 weeks after TAC, but these differences did not reach statistical significance. There was no significant difference in interstitial fibrosis between cardiac-specific KO and control mice at 2 weeks after TAC (% sirius red positive area 11±5%, n=7 for cardiac-KO versus 13±4%, n=12 for control). These results support the concept that myocardial Txnip plays a critical role in regulation of cardiac metabolism.
Discussion
Although Txnip participates in a variety of biological functions, the physiological roles of Txnip in the myocardium have not been defined. In this study, we explored the in vivo role of Txnip in the heart by using Txnip deletion in mice. Our results indicate that deletion of Txnip induces mild cardiac dysfunction in the basal state with a biphasic functional response to pressure overload. In the early phase of hemodynamic stress, deletion of Txnip provides cardioprotection with a reduced propensity to cardiac hypertrophy. At the later transition to cardiac failure, deletion of Txnip leads to worse cardiac function. Because a direct linkage between ROS generation and cardiac contractile defect has been proposed,15 we anticipated that these cardiac phenotypes in Txnip deletion mice were mediated by increased thioredoxin activity to antagonize oxidative stress. However, we did not detect changes in overall redox state in Txnip-null mice; instead, our data support a role of Txnip in cardiac metabolism.

Under normal conditions, the heart generates energy primarily by oxidizing fatty acids with smaller contributions from glycolysis and oxidation of pyruvate. In contrast, under conditions of energy supply-demand imbalance such as pressure overload, glucose oxidation in mitochondria contributes significantly to the energy synthesis in hypertrophied hearts. Enhancing glucose utilization, therefore, may promote a favorable mode of energy supply that enables Txnip-null hearts to maintain higher contractile performance in the acute pressure overload. However, it remains unclear whether the increase in glucose utilization in hypertrophied hearts represents a beneficial adaptation or is ultimately maladaptive.16 Indeed, alteration of glucose metabolism by deletion of Txnip was not coupled to LV contractile reserve after prolonged pressure overload. One possibility is that the augmentation of glucose uptake in Txnip-null mice may be an intrinsic compensatory mechanism of impaired energy metabolism. Our preliminary studies suggest that mitochondrial ATP synthetic machinery, assessed by respiratory control ratio, is reduced in Txnip-null mice at baseline and after TAC. This could possibly explain worse contractility in the sham and late TAC mice by deletion of Txnip, but this alone could not explain the improved function early after TAC. Further investigations are necessary to clarify the non-redox mechanisms to regulate cardiac metabolism and biphasic functional response to pressure overload by deletion of Txnip in the myocardium.

We previously reported that adenoviral overexpression of Txnip blunted protein synthesis in response to hypertrophic stimuli in rat neonatal cardiomyocytes.6 In the present study, targeted deletion of Txnip in the heart attenuated the early hypertrophic response to pressure overload, creating an apparent paradox. To address this discrepancy, we measured protein synthesis in neonatal cardiomyocytes isolated from Txnip-null mice and wild-type mice as previously described.6 Stimulation with angiotensin II (0.1 μmol/L, 24 hours) increased protein synthesis in cells from both genotypes, but the response was not significantly different between Txnip-null mice (+26±8%, n=13) and wild-type mice (+21±4%, n=17, P=NS). The apparent paradox of blunted hypertrophic response in both the knockout and overexpression models may be attributable to the nonphysiological protein levels of Txnip by adenoviral vector (14±2-fold expression of Txnip protein relative to the level of endogenous protein). Although overexpression of Txnip robustly enhances cellular levels of ROS through the inhibition of thioredoxin activity after hypertrophic stimuli, deletion of Txnip does not appear to change thioredoxin activity or redox state dramatically. These data suggest that whereas forced overexpression of Txnip can inhibit thioredoxin activation and downstream signaling events that depend on its activation, physiological levels of Txnip may not regulate thioredoxin activity and thus inhibit hypertrophy through a different mechanism.

Other groups have generated systemic Txnip-mutant mice with no reports of increases in thioredoxin activity in tissues.11,12,17 Because endogenous Txnip primarily exists in nuclei and mitochondria in mammalian cells,18 the Txnip-thioredoxin interactions may be localized within the cell in the nucleus or the mitochondria. Thus, deletion of Txnip in mice might regulate the inhibitory effects of thioredoxin in a nuclear interaction with transcriptional activators or mitochondrial oxidative phosphorylation, rather than scavenging global levels of ROS in the cytoplasm. Although our experiments cannot define the precise molecular mechanisms by which Txnip regulates cardiac metabolism or contractile function, our data strongly suggest that control of redox state is not the dominant mechanism, despite the ability of Txnip to bind to and inhibit thioredoxin. Instead, Txnip may function in a manner similar to the related family of proteins, the arrestins.3 The arrestins have multiple signaling mechanisms, including control of G protein signaling and binding of MAP kinases.19 Although we speculate that Txnip may also have multiple roles, further studies in proteomics, for example, will be necessary to define these roles.

In conclusion, conditional deletion of Txnip regulates cardiac dysfunction in response to pressure overload. Txnip deletion may render hypertrophied hearts more tolerant to acute hemodynamic stress through adaptive metabolic changes rather than regulation of thioredoxin activity. However, these beneficial adaptations are not sustained, and Txnip deletion can ultimately lead to maladaptive LV remodeling under the prolonged hemodynamic stress. The present study provides a basis for further investigations of the effects of Txnip on cardiac metabolism and as a potential link between thioredoxin and myocardial energetics in cardiac hypertrophy and heart failure.

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Disclosures
None.

References


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EXPANDED MATERIALS AND METHODS

Generation of Conditional Txnip Deletion Mice

A genomic BAC containing the Txnip locus, RPCI-22 clone 496G18, from a mouse 129S6/SvEvTac genomic library was obtained (Invitrogen, Huntsville, AL). To generate a Txnip targeted allele suitable for tissue-specific deletion, we used a 6.5kb plasmid vector pPGKneoF2L2DTA (kind gift of Philippe Soriano) containing the Txnip gene exon1 and its flanking genomic sequences including the Txnip promoter region and exons2-6 (Fig.1A). The targeting vector contained a neo-cassette driven by the phosphoglycerate kinase (PGK) promoter inserted in intron1. The PGK-neo was flanked by two frt sites for Flp-mediated excision. Two loxP sites were also introduced in the targeting construct for Cre-mediated conditional deletion of Txnip gene exon1. The first loxP site was located after the Txnip promoter region, 168 bp upstream of the first ATG codon. The other loxP site was located in the 3’ side of the PGK-neo cassette, immediately following the 3’ frt site. Thus, neither the loxP sites nor the frt-flanked PGK-neo cassette interrupted the Txnip coding sequence. The construct was electroporated into J1 ES cells, and homologous recombination of the targeting vector was confirmed by Southern blots. Chimeric mice were generated by injection of clones into C57BL/6 blastocysts. Germine transmission of the Txnip^{(Frt-neo-Frt)lox+/-} allele was obtained. Txnip^{(Frt-neo-Frt)lox+/-} mice were intercrossed to generate mice homozygous on a mixed 129/SV and C57BL/6 background. The successful homologous recombination was confirmed in genomic DNA from tails of Txnip^{(Frt-neo-Frt)lox+/-} mice by Southern analysis (Fig.1B). Since the neomycin phosphotransferase (neo) can frequently interfere with the transcription and splicing of the target and neighboring genes, resulting in an ambiguous
genotype-phenotype relationship, we employed the Flp/frt system to eliminate the neomycin gene flanked by Flp recognition target (frt) sites. The PGK-neo cassette was excised at frt sites by crossing Txnip^{(Frt-neo-Frt)lox+/Frt-neo-Frt)lox+} mice with mice expressing FLPe recombinase from the Rosa26 locus\(^1\) to generate Txnip^{lox/+} mice. These mice were intercrossed to generate homozygous Txnip^{lox/lox} mice. Thus, we successfully generated a selection marker-free Txnip mutant allele suitable for efficient conditional gene deletion studies. Protocols for generation and initial breeding of mice were approved by the MIT Committee on Animal Care.

Then, ablation of exon1 was achieved by crossing Txnip^{lox/lox} mice with Protamine-Cre transgenic mice to create systemic Txnip null mice\(^2\). To generate temporally regulated cardiac-specific Txnip deletion mice, a transgenic mouse overexpressing MerCreMer in cardiomyocytes (kindly provided by Jeffrey Molkentin) was mated with Txnip^{lox/lox} mice. 4-Hydroxytamoxifen (Sigma) was dissolved in 2% ethanol with peanut oil at a concentration of 5mg/mL and injected intraperitoneally to induce Cre recombination in adult MerCreMer transgene mice. Primers used for PCR genotyping were F1 sense 5’-TTTCGTTTGGGGTTTTCAAGC-3’; F2 sense, 5’-CTTCACCCCCCTAGAGTGAT-3’; and R antisense, 5’-CCCAGAGCACTTTCTTGAGC-3’.

**Southern and Northern Analysis**

For Southern analysis, genomic DNA was digested with NcoI and SpeI, and hybridized with the radiolabeled 5’ probe and 3’ probe (external probes). The following oligonucleotide primers were used: 5’ probe sense 5’-TCTCTTCAGCTCCTCCCTGGAC-3’
and antisense 5’-TCCGAGAAAGTGGTCAGGTC-3’; 3’ probe sense 5’-ACTGGTGCTTTGTGGTGAC-3’ and antisense 5’-CTGCTGCCCAACTGTGAGTA-3’. For Northern blots, total RNA was isolated by TRIzol reagent (Life Technologies), and hybridized with a cDNA fragment corresponding to Txnip exon7 and exon8 labeled with [γ^{32}P]dCTP.

**Immunoblot Analysis**

Protein samples were subjected to immunoblotting analysis using antibodies against thioredoxin1 (Cell Signaling, Danvers, MA), thioredoxin1 reductase (TxnRD1; Abcam, Cambridge, MA), catalase (Abcam), superoxide dismutase (BD Biosciences), V5 epitope (Invitrogen), β-tubulin (Thermo Fisher Scientific, Waltham, MA), and Actin (A5060, Sigma). Immunodetection of Txnip was performed with custom-made mouse monoclonal antibodies raised against full-length human Txnip (clone JY1 and JY2, available from MBL Intl., Woburn, MA)

**Epitope Mapping of the Txnip Monoclonal Antibody**

HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium with low glucose, no sodium pyruvate, 10% fetal bovine serum, and 100 U/mL penicillin and streptomycin. Full-length human Txnip cDNAs with or without V5 tag, and truncated Txnip cDNAs (Fig.2A) were subcloned into a mammalian expression vector (pcDNA3.1, Invitrogen). Cells were transfected using FUGENE transfection reagent (Roche Applied Biosystems) and harvested in lysis buffer.
**Thioredoxin Reducing Activity, ROS, Biochemical Assay, and Blood Sampling**

Hearts were homogenized with a buffer containing 20 mM HEPES (pH 7.9), 300 mM NaCl, 100 mM KCl, 10 mM EDTA, 0.1% Nonidet P-40, and protease inhibitors. Thioredoxin reducing activity was measured using the insulin disulfide reduction assay in tissue homogenates as described. Briefly, 50 μg of protein homogenates were incubated at 37 °C for 15 min. After addition of reaction buffer, the reaction was started with 2 μl thioredoxin reductase (American Diagnostica Inc., Greenwich, CT) and incubated at 37 °C for 20 min. The reaction was terminated by adding 250 μl of stopping buffer followed by absorption measurement at 412 nm. To evaluate tissue levels of ROS, the production of O$_2^•$ in the left ventricle was measured by superoxide dismutase-inhibitable reduction of ferricytochrome c. Rates of O$_2^•$ production were calculated as described. Tissue levels of lipid peroxides malondialdehyde were estimated by measuring the chromophore formed by the reaction of N-methyl-2-phenylindole and methanesulfonic acid as described. To prevent sample oxidation during the assay, 5mM of butylated hydroxytoluene was added to homogenates prior to the homogenization. The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) was measured in whole blood samples or in whole heart homogenates using a Bioxytech GSH/GSSG-412 kit (Oxis International Inc., Foster City, CA).

Myocardial glycogen content was determined by measuring the amount of glucose released from glycogen with a glucose assay kit (Sigma). An alkaline extraction procedure was used to separate glycogen and exogenous glucose in the tissue. Myocardial triglycerides content was measured with an enzymatic quantitative analysis as previously described.
Blood samples were obtained by an apical puncture of mouse hearts. The samples were centrifuged for 5 min at 1500 g to separate the serum and analyzed for metabolic profile and biochemistry (IDEXX Laboratories Inc., North Grafton, MA).

**Transverse Aortic Constriction (TAC), Echocardiography and Hemodynamic Assessment**

TAC surgery and echocardiographic acquisition were performed as described previously. The left ventricular (LV) mass by echocardiography was calculated as described. Systolic blood pressure and heart rate were measured using a noninvasive computerized tail-cuff system (BP-2000, Visitech Systems) as described. Hemodynamic parameters were acquired with the close-chest invasive method using a Millar Pressure Volume Catheter (Millar Instruments) through the right carotid artery and analyzed by PowerLab Chart5 (ADInstruments, Inc., Colorado Springs, CO). All surgeries and subsequent analyses were performed as a blinded study with regard to Txnip genotype. Mice were anesthetized with pentobarbital (30-70 mg/kg, i.p.). The Harvard Medical School Standing Committee on Animal Research approved the study protocols.

**Histological Analysis**

Paraffin-mounted histologic sections were stained with Picro-sirius red or Periodic acid Schiff (PAS). Picro-sirius red-stained sections were quantified for percent area stained positive for collagen. Staining, scanning, and quantitation were all performed in a blinded manner using a minimum of 5 randomly-selected sections from the heart, and
the amount of fibrosis was expressed as an average for each animal. Myocyte cross-sectional area was determined with Periodic acid Schiff (PAS) staining and analyzed by ImagePro (Media Cybernetics, Inc., Silver Spring, MD). Triple staining with TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling), anti-α-sarcomeric actin antibody (Sigma), and DAPI was performed with ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA). The number of TUNEL-positive myocytes was analyzed by Image J (NIH). To evaluate tissue levels of ROS, heart tissue was embedded in Tissu-Tek OCT compound (Electron Microscopy Science, Hatfield, PA) and snap-frozen in acetone chilled in dry ice. Cryosections of left ventricle were incubated for 1 h at 37 °C with 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCFDA, 4 µM; Molecular Probes) as described 5. The positive DCFDA staining in the myocardium was quantified in Matlab (The Mathworks, Inc., Natick, MA). Data from each heart was normalized with background fluorescence intensity to calculate the DCFDA intensity.

Nuclear Magnetic Resonance (NMR) Spectroscopy

Levels of phosphorylated metabolites were monitored based on the intensities of the 31P-NMR resonances in a perfused Langendorff murine heart model system. The rate of glucose transport was assessed based on the uptake of 2-deoxy-D-glucose (2-DG) as determined from the intensity of the 2-deoxyglucose-6-phosphate resonance, as described elsewhere12. Briefly, isolated hearts were first perfused with Krebs-Henseleit buffer containing 5mM glucose and 5mM pyruvate. Left ventricular developed pressure, ±dP/dt, and heart rate were continuously monitored via a water-filled latex balloon inserted into
the left ventricle. After stabilization, the perfusate was switched to a solution containing 5mM 2-DG and 5mM pyruvate with 1.2mM KH₂PO₄. Pyruvate and KH₂PO₄ were supplied to replenish the inorganic phosphate pool and maintain ATP synthesis. To avoid the effects of substrate selectivity due to different affinities for hexokinase between glucose and 2-DG, all glucose in the perfusate was switched to 2-DG. The accumulation of sugar phosphate (SP) was assessed in hearts isolated from Txnip null mice and their wild type littermates.

**Statistical Analysis**

All data are presented as mean±SEM. Statistical analysis was performed with the paired t test to the same group comparison, and unpaired Student’s t test or Mann-Whitney test between groups. Statistical significance was achieved when P<0.05.

**SUPPLEMENTAL RESULTS AND DISCUSSION**

Supplemental Figure 1 shows quantitative assessment of myocardial PCr, ATP levels and LV developed pressure (LVDP) from fed (Supplemental Fig.1A, 1C, and 1E) and fasted animals (Supplemental Fig.1B, 1D, and 1F) during metabolic inhibition by 2-DG infusion. At baseline, LV developed pressure (mmHg) in Txnip null mice was slightly lower but not significantly different at fed (96±7, n=6) and fasted states (92±7, n=4) from that in wild type hearts from fed (116±9, n=5) and fasted (103±12, n=4) mice. However, Txnip KO hearts maintained LVDP during 2-DG perfusion with the accumulation of 2-DG-6-phosphate. Interestingly, despite the higher LVDP in Txnip KO hearts, there was a dramatic decrease in PCr and ATP levels in Txnip KO hearts at both
fed and fasted states. We speculate that the increase in contractility led to decreased ATP levels in Txnip KO hearts during metabolic inhibition. Because there was greater 2-DG uptake, there should be more phosphate trapped in the 2-DG-6-phosphate in Txnip null hearts. This could result in a lower inorganic phosphate (Pi) in Txnip null hearts with higher 2-DG uptake. It has been shown that there is a strong dependence of relative isometric force with Pi concentration in skinned cardiac trabeculae\textsuperscript{13}. Since lower Pi concentrations lead to higher cardiac mechanical force\textsuperscript{13}, a lower Pi in Txnip null hearts might result in increased cardiac contractility. The increased work in Txnip KO hearts, therefore, could result in the decrease ATP levels through reducing Pi levels.

The finding showing differences in the development of interstitial fibrosis was observed in systemic Txnip null mice at 4 and 8 weeks after TAC, but not in cardiomyocyte-specific Txnip null mice at 2 weeks after TAC. These results could be attributed to several possibilities. First, the Txnip gene is deleted in fibroblasts from systemic Txnip KO mice but not in fibroblasts from cardiomyocyte-specific KO mice. Second, we assessed collagen content in systemic Txnip KO mice at 4 weeks and 8 weeks after TAC, but at the earlier phase only (2 weeks after TAC) for cardiac-specific Txnip KO mice. Third, whereas deletion of Txnip in cardiomyocytes should be achieved completely in systemic KO mice, deletion by tamoxifen-inducible αMHC-MerCreMer mice was up to 85±5% of cardiomyocytes with no recombination detectable in 15% of cardiomyocytes. Fourth, systemic metabolic alterations might affect the development of fibrosis in the heart during pressure overload. Thus, while both systemic and cardiomyocyte-specific Txnip deletion changed myocardial glucose uptake, the
development of interstitial fibrosis during pressure overload may also be related to mechanisms other than the adaptive metabolic regulation by Txnip in cardiomyocytes.

REFERENCES


