Deletion of thioredoxin-interacting protein improves cardiac inotropic reserve in the streptozotocin-induced diabetic heart

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Myers RB, Fomovsky GM, Lee S, Tan M, Wang BF, Patwari P, Yoshioka J. Deletion of thioredoxin-interacting protein improves cardiac inotropic reserve in the streptozotocin-induced diabetic heart. Am J Physiol Heart Circ Physiol 310: H1748–H1759, 2016. First published April 1, 2016; doi:10.1152/ajpheart.00051.2016.—Although the precise pathogenesis of diabetic cardiac damage remains unclear, potential mechanisms include increased oxidative stress, autonomic nervous dysfunction, and altered cardiac metabolism. Thioredoxin-interacting protein (Txnip) was initially identified as an inhibitor of the antioxidant thioredoxin but is now recognized as a member of the arrestin superfamily of adaptor proteins that classically regulate G protein-coupled receptor signaling. Here we show that Txnip plays a key role in diabetic cardiomyopathy. High glucose levels induced Txnip expression in rat cardiomyocytes in vitro and in the myocardium of streptozotocin-induced diabetic mice in vivo. While hyperglycemia did not induce cardiac dysfunction at baseline, β-adrenergic challenge revealed a blunted myocardial inotropic response in diabetic animals (24-wk-old male and female C57BL/6; 129Sv mice). Interestingly, diabetic mice with cardiomyocyte-specific deletion of Txnip retained a greater cardiac response to β-adrenergic stimulation than wild-type mice. This benefit in Txnip-knockout hearts was not related to the level of thioredoxin activity or oxidative stress. Unlike the β-arrestins, Txnip did not interact with β-adrenergic receptors to desensitize downstream signaling. However, our proteomic and functional analyses demonstrated that Txnip inhibits glucose transport through direct binding to glucose transporter 1 (GLUT1). An ex vivo analysis of perfused hearts further demonstrated that the enhanced functional reserve afforded by deletion of Txnip was associated with myocardial glucose utilization during β-adrenergic stimulation. These data provide novel evidence that hyperglycemia-induced Txnip is responsible for impaired cardiac inotropic reserve by direct regulation of insulin-independent glucose uptake through GLUT1 and plays a role in the development of diabetic cardiomyopathy.

NAME: isoproterenol; α-arrestins; GLUT1

NEW & NOTEWORTHY

The pathogenesis of diabetic cardiomyopathy is unclear, but an important clue is that it is strongly linked to hyperglycemia. Here, we provide novel evidence that thioredoxin-interacting protein is responsible for impaired cardiac inotropic reserve by hyperglycemia through regulation of glucose transport in the development of diabetic cardiomyopathy.

Diabetic cardiomyopathy has been defined as “diabetes-associated ventricular dysfunction that occurs independently of coronary artery disease and hypertension” and is supported by extensive clinical data (6). The pathogenesis of diabetic cardiomyopathy is unclear, but an important clue is that it is strongly linked to hyperglycemia (51). Although a specific pathway connecting hyperglycemia to downstream pathophysiological events has remained elusive, potential mechanisms include increased oxidative stress (3, 19), autonomic nervous dysfunction (5), and altered substrate metabolism (3). A predominant change in diabetes is a suppression of cardiac glucose utilization (3), which is tightly regulated by a protein family of glucose transporters (GLUTs). The well-established GLUT isoforms in the heart are GLUT1 and GLUT4, which are known to have distinct regulatory properties. GLUT1 is an insulin-independent glucose transporter responsible for the basal glucose uptake required to sustain energy production, whereas GLUT4 is the rate-limiting transporter regulated by insulin (1). Understanding how cardiomyocytes adapt to changes of extracellular and intracellular glucose concentrations is of critical importance.

Thioredoxin-interacting protein (Txnip) is recognized for important roles in metabolism and redox regulation (39), but its in vivo mechanisms remain unknown. Txnip was initially identified as a protein that stably binds to thioredoxin (42). Thioredoxin is an oxidoreductase that acts as an antioxidant by facilitating the reduction of other proteins via cysteine thiol-disulfide exchange. Txnip binds to the reduced form of thioredoxin, thus suggesting originally that it could inhibit thioredoxin’s antioxidant function to modulate redox status and reactive oxygen species (ROS)-mediated signaling (39).

However, Txnip is now known to be part of the arrestin superfamily of proteins (2). β-Arrestins are well known as important regulators of receptor signaling, including β-adrenergic signaling. In mammals, a related class of proteins called α-arrestins includes Txnip and five other α-arrestin proteins (2), which share the arrestin fold and have predicted structural similarities with β-arrestins. Ancestral α-arrestins can coordinate receptor endocytosis, ubiquitination, and downstream signaling, similar to the functions of β-arrestins (28). Nevertheless, how the arrestin fold contributes to the functions of Txnip and the other mammalian α-arrestins remains unknown.

Importantly, Txnip is a highly glucose-responsive gene via a carbohydrate response element in the promoter of Txnip (38) and one of the most dramatically upregulated genes in response to glucose (57). Hyperglycemia-induced Txnip plays a critical role in diabetes etiology (44, 60) and its complications such as nephropathy (56), retinopathy (50, 59), neuropathy (52), and vasculopathy (17, 55). Glucose-induced Txnip contributes to glucotoxicity, as observed in pancreatic β-cells (12), endothelial cells (17, 49), and cardiomyocytes (22, 61). These data

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suggest that Txnip may serve as a key mechanism in regulating diverse cellular signaling events in diabetic organs. Despite these strong links between Txnip and glucose metabolism, the precise role of myocardial Txnip in the functional adaptation to diabetic stress remains unknown. Therefore, we investigated whether Txnip contributes to the development of hyperglycemia-induced cardiac disorders, using mice with cardiac-selective deletion of Txnip, and discovered a mechanistic link between myocardial glucose metabolism and functional reserve in diabetic cardiomyopathy.

**MATERIALS AND METHODS**

**Diabetic mouse model.** To generate an insulin-deficient diabetic mouse model, 8-wk-old animals were treated with streptozotocin (STZ) according to the low-dose STZ induction protocol (50 mg/kg ip for 5 days) of the Animal Models of Diabetic Complications Consortium. Animals with intraperitoneal injection of 0.1 M Na-citrate (vehicle lacking STZ) served as controls. In each mouse, whole blood was obtained from the tail vein to confirm the increased levels of blood glucose with a glucometer (Bayer). Four weeks after STZ injections, temporally inducible cardiomyocyte-specific Txnip deletion was achieved in αMHC-MerCreMer/Txnip flox/fox (+) mice (C57BL/6J;129Sv strain) injected with 0.5 mg of 4-hydroxytamoxifen per day for 2 wk as described previously (68). Age- and sex-matched littermates of αMHC-MerCreMer/Txnip flox/fox mice treated with vehicle lacking 4-hydroxytamoxifen served as controls; we verified that there was no change in Txnip expression in these mice compared with wild-type (WT) mice (68). Mice were maintained in accordance with the Institutional Animal Care and Use Committees of Harvard Medical School. The protocol was approved by the Harvard Medical Area Standing Committee on Animals.

**Cell culture.** The embryonic rat cardiac H9c2 (CRL-14464), mouse fibroblast L929 (CCL-1), and human embryonic kidney HEK-293 (CRL-1573) and HEK-293T (CRL-3216) cell lines were obtained from American Type Culture Collection (Manassas, VA). Mouse embryonic fibroblasts (MEFs) were prepared from a systemic Txnip-null mouse and its littermate control WT mouse (14). For glucose uptake and expression analyses, cells were treated with the indicated concentrations of d-glucose and/or d-mannitol (Sigma-Aldrich, St. Louis, MO) in Dulbecco’s modified Eagle’s medium (DMEM).

**Gene and protein expression analyses.** Gene expression of Txnip was analyzed by quantitative real-time polymerase chain reaction (PCR) with specific oligonucleotides for the mouse txnip gene (69). Protein expression of Txnip was analyzed by Western blot analysis using the anti-Txnip antibody JY2 (available from MBL International, Woburn, MA) (69). For signaling assays, Western blot analyses were performed with the indicated vector with a hemagglutinin (HA) tag and cotransfected with Txnip or empty control vector into HEK-293T cells with PureFection transfection reagent (System Biosciences, Mountain View, CA) and used in following experiments. GLUT1 protein expression was analyzed by confocal microscopy (Olympus Fluoview 1500). After fixation with 4% paraformaldehyde, cells or heart tissues were stained with GLUT1 (Santa Cruz Biotechnology) antibodies. Signals were quantified by densitometry of scanned autoradiographs by Scion Image 4.02 (Scion, Frederick, MD).

**In vivo assessments of left ventricular performance.** Left ventricular function was followed by serial echocardiographic measurements and by invasive hemodynamic assessment via catheterization at the time of death (30). Echocardiographic acquisition was performed without anesthesia with a Sonos 4500 (Philips) and a 15-MHz transducer. Hemodynamic parameters were acquired under inhalational anesthesia with isoflurane with a Millar pressure catheter (Millar Instruments, Houston, TX), which was advanced retrograde through the aortic valve into the left ventricle (LV) and positioned to obtain LV pressure. Isoproterenol (1.6 mg/g BW) was given to a subset of animals to generate β-adrenergic stimulation by intravenous bolus injection via the right jugular vein cannulated with PE-10 tubing.

**Histopathological examination.** The LVs were paraffin embedded, sectioned at 10 μm, and stained with PicroSirius Red to evaluate collagen deposition and myocyte cross-sectional area as described previously (30). To analyze capillary density in the myocardium, endothelial cells were detected by staining sections with biotinylated GSL-I (100 μg/ml; Vector Laboratories, Burlingame, CA) (29). GSL-I staining was quantified manually by counting the number of vessels per field. Three to five random fields were scanned and quantified for each section.

**GLUT1 protein expression was analyzed by confocal microscopy (Olympus Fluoview 1500). After fixation with 4% paraformaldehyde, cells or heart tissues were stained with GLUT1 (Santa Cruz Biotechnology) antibodies. Signals were quantified by densitometry of scanned autoradiographs by Scion Image 4.02 (Scion, Frederick, MD).**

**Thioredoxin activity and ROS.** Thioredoxin reducing activity was measured using an insulin disulfide reduction assay in whole heart homogenates (47). To evaluate oxidative damages by ROS, tissue levels of lipid peroxide (malondialdehyde) were estimated in whole heart homogenates (68).

**Txnip pull-down assay.** The coding sequences of human Txnip and other arrestins were subcloned into pCDH-CMV-MCS-EF1-GFP-T2A-Puro (System Biosciences, Mountain View, CA) with a dual Strep/FLAG (SF) tag (18). Human GLUT1 (SLC2A1) and β1-adrenergic (ADRB1) receptors were subcloned from commercially available cDNA plasmids (Open Biosystems, Huntsville, AL) into the above mentioned vector with a hemagglutinin (HA) tag and cotransfected with Txnip or empty control vector into HEK-293T cells with PureFection transfection reagent (System Biosciences). Cells were lysed in 0.5% Triton X-100, 500 mM NaCl, 50 mM Tris, 1 mM PMSF, and protease inhibitors (Sigma-Aldrich, pH 7.8). Txnip pull-down was performed with magnetic Strep-Tactin beads (IBA, Göttingen, Germany) according to the manufacturer’s instructions. Western blot analyses of pulled down proteins were performed with anti-FLAG (Sigma-Aldrich) and anti-HA.16 (16B12, Covance, Princeton, NJ) antibodies.

**β-Adrenergic receptor density and internalization assays.** β-Adrenergic receptor density was measured by the methods of Maisel et al. (35) with modifications. Sarcenomural membrane and light vesicle fractions were prepared from the LV. The total β-adrenergic receptor density was determined as the amount of bound radioligand [3H]CGP 12177 (PerkinElmer, Waltham, MA). Filtered radioactivity was counted in a liquid scintillation counter (Beckman), and data were analyzed with a GraphPad Radioactivity Calculator (La Jolla, CA).

β-Adrenergic receptor internalization was evaluated as described previously (34) with modifications. Briefly, HEK-293 cells were plated in complete DMEM and transiently transfected with β1-adrenergic receptor and β-arrestin 1 (as a positive control), Txnip, or empty vector (as a negative control). Cells were then incubated in 125I-cyanopindolol (PerkinElmer) at 37°C for 5 min. Incubations were stopped by placing the cells on ice and rapidly washing twice with ice-cold PBS. The cells were kept on ice for 10 min in acid wash solution (150 mM NaCl and 50 mM acetic acid) to remove the

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surface-bound radioligand. The supernatant containing the acid-released radioactivity was collected, and the cells were treated with 0.5 M NaOH and 0.05% SDS to solubilize the acid-resistant (internalized) radioactivity. Radioactivity was measured, and the percent internalization at each point was calculated from the ratio of the acid-resistant binding to the total binding.

**Glucose uptake measurements.** Arrestin-mCherry fusion constructs were overexpressed in HEK-293 cells with a lentiviral expression system (System Biosciences) as described previously (47). After 2–3 days, expression was verified by red epifluorescence. Four hours prior to labeling, medium was changed to 5.5 mM glucose to reduce endogenous Txnip expression. Cells were then incubated with 100 μM 2-deoxyglucose and 125 μM 2-[14C]deoxyglucose (1 μCi/ml, PerkinElmer Life Sciences) for 30 min. Cells were lysed in 0.2 N NaOH and then neutralized with 6 N HCl.

Zero-trans sugar uptake was measured in MEFs and L929 cells as described previously (15). Briefly, cells were placed in serum-free DMEM for 2 h at 37°C and then placed on ice in glucose-free medium to deplete intracellular sugar levels. Cells were then treated with increasing concentrations of 3-O-[14C]methylglucose (PerkinElmer). Uptake proceeded for 180 s before cells were lysed. Samples were counted by liquid scintillation spectrometry.

**Isolated, perfused heart experiments.** Ex vivo cardiac function was assessed in isolated mouse hearts perfused in the Langendorff mode. The heart was excised and perfused with a constant pressure of 80 mmHg by real-time PCR. Hyperglycemia increased mRNA and/or protein expressions of Txnip in diabetic WT mice after STZ treatments (blood glucose level 392 ± 65 mg/dl vs. 124 ± 18 mg/dl in control; n = 4, P < 0.01) myocardial gene and protein expressions of Txnip increased in vivo (Fig. 1, B–D). These results led us to hypothesize that hyperglycemia-induced Txnip contributes to the development of diabetic cardiomyopathy.

The conditional and inducible approach is applied to test the effects of myocardial Txnip in the diabetic mouse model. To determine whether Txnip contributes to diabetic damages in the heart, cardiomyocyte-specific Txnip deletion was induced by 4-hydroxytamoxifen treatments in STZ-induced diabetic mice. In control animals treated with vehicle only, there were no significant differences in blood glucose levels between Txnip-KO mice (105 ± 5 mg/dl) and their littermate control mice (81 ± 6 mg/dl) at 8 wk after the injections. STZ injections increased blood glucose levels to the same degree (P < 0.01 vs. vehicle) in both Txnip-KO (393 ± 14 mg/dl) and WT (430 ± 12 mg/dl) mice [P = not significant (N.S.) between the genotypes]. Thus the cardiomyocyte-specific Txnip-KO model was able to separate systemic effects of Txnip on blood glucose levels (14).

Of 14 Txnip-KO and 14 WT control mice that were treated with STZ, 4 Txnip-KO (at 8 wk) and 5 WT (between 8 and 16

**RESULTS**

Myocardial Txnip expression is upregulated by glucose in vitro and with diabetes in vivo. We and others have previously found that Txnip’s expression levels are governed by extracellular glucose levels in an insulin-independent fashion (44, 55). Thus we first checked that cardiomyocytes incubated with increasing concentrations of glucose show a concentration-dependent upregulation of Txnip as reported in other cell types (44, 55). In the absence of glucose, H9c2 cardiomyocytes expressed very little endogenous Txnip protein (Fig. 1A). However, incubation with glucose markedly induced expression of Txnip in cardiomyocytes upon stimulation with both 5 mM glucose and 25 mM glucose for 24 h. Identical concentrations of mannitol in cultures did not induce expression of Txnip in a similar fashion, excluding a possible hyperosmolar effect of glucose on this induction. We next showed that in diabetic WT mice after STZ treatments (blood glucose level 392 ± 65 mg/dl vs. 124 ± 18 mg/dl in control; n = 4, P < 0.01) myocardial gene and protein expressions of Txnip increased in vivo (Fig. 1, B–D). These results led us to hypothesize that hyperglycemia-induced Txnip contributes to the development of diabetic cardiomyopathy.

Fig. 1. Txnip expression is upregulated by glucose in cardiomyocytes. A: rat cardiac H9c2 cells were treated with the indicated concentrations of glucose and/or mannitol in DMEM after a starvation period. Western blot analysis was performed with the anti-Txnip antibody JY2. Actin and Coomassie blue staining serve as loading controls in the gel. Higher extracellular glucose levels significantly increase intracellular protein expression of Txnip. B–D: gene (B) and protein (C and D) expressions of Txnip were analyzed in cardiac tissues harvested from streptozotocin (STZ)-induced diabetic or nondiabetic wild-type (Control) mice. Gene expression was analyzed by real-time PCR. Hyperglycemia increased mRNA and protein expressions of Txnip in whole heart homogenates. Values are means ± SE. *P < 0.05, **P < 0.01 vs. control. n = 3 or 4 each.
Insulin-deficient diabetes does not induce cardiac hypertrophy, fibrosis, and basal dysfunction throughout 16 wk after STZ injections in mice. To assess cardiac dysfunction in these diabetic animals, following multiple previous reports (10, 63), echocardiographic parameters were measured at baseline and 8, 12, and 16 wk after STZ injections. There were no differences between the genotypes in left ventricular dimensions or wall thickness (Table 1). Left ventricular mass and fractional shortening were not significantly changed after STZ injections in both genotypes (Fig. 2, A, B, and D). All groups treated with either vehicle or STZ in Txnip-KO and wild-type mice had similar trends in left ventricular wall thickness, dimensions, and fractional shortening throughout the protocol. Invasive hemodynamic analysis at the time of death also showed no significant changes in left ventricular developed pressure (dP/dt) between diabetic and nondiabetic mice at 16 wk after STZ injections in both Txnip-KO and WT mice (Table 1).

Previous studies have suggested that pathological changes of diabetic cardiomyopathy include compensatory cardiomyocyte hypertrophy and interstitial fibrosis (7). In this study, however, prolonged hyperglycemia did not induce cardiac hypertrophy, as measured by myocyte cross-sectional area (Fig. 2F) or heart weight normalized by tibial length in Txnip-KO (KO/STZ 517 ± 36 g/cm vs. KO 596 ± 24 g/cm, $P = N.S.$) and WT (STZ 498 ± 38 g/cm vs. control 495 ± 39 g/cm, $P = N.S.$) mice. The activation of ERK1/2, one of the signal transduction pathways associated with cardiac hypertrophy, was also comparable between WT and Txnip-KO hearts under diabetic conditions (Fig. 2E). Diabetic stress also did not result in interstitial fibrosis in the myocardium from both Txnip-KO and wild-type mice (Fig. 2F).

β-Adrenergic challenge reveals that Txnip-KO hearts manifest greater functional response to β-adrenergic stimulation under diabetic conditions. Resting echocardiographic and hemodynamic analyses showed no significant differences in basal LV functional parameters between diabetic and nondiabetic conditions even in WT mice. Since these techniques may not be sensitive enough to reveal early changes caused by diabetic stress in the myocardium, we next performed an in vivo stress test to evaluate contractile provocation and inotropic reserve. β-Adrenergic challenge by isoproterenol increased heart rate at 3 min after bolus intravenous injection through the jugular vein in all groups: WT nondiabetic control ($119 ± 9\%$, $n = 4$), WT STZ-induced diabetic ($111 ± 5\%$, $n = 9$), Txnip-KO nondiabetic ($115 ± 8\%$, $n = 5$), and Txnip-KO STZ-induced diabetic ($112 ± 6\%$, $n = 4$) mice (Fig. 3A). Cardiac inotropic response, as measured by dP/dt max, was also increased by β-adrenergic stimulation in all groups (Fig. 3, B and C). However, in diabetic mice, the inotropic response was significantly lower than that of control WT mice during β-adrenergic stimulation. Interestingly, in diabetic Txnip-KO hearts, the functional response to β-adrenergic stimulation was preserved compared with diabetic WT hearts (Fig. 3, B and D). Thus we identified that, in contrast to baseline conditions, stress induced by β-adrenergic challenge uncovered masked mechanical dysfunction in the STZ-induced diabetic mouse model. These results demonstrated that Txnip promotes hyperglycemia-induced impairment of myocardial functional reserve in vivo.

The diabetic heart is sometimes characterized by a severe decrease in capillary density. Since capillary density can influence cardiac functional reserve, we evaluated vessel density in the myocardium by lectin staining. In our model, hyperglycemia did not change the number of vessels per field of the myocardium. We found no statistical difference in vessel density between WT ($7.3 ± 0.2 \times 10^3$ vessels/mm$^2$) and Txnip-KO ($7.0 ± 0.2 \times 10^3$ vessels/mm$^2$) hearts under STZ-induced diabetic conditions ($P = N.S.$).

A better functional reserve in Txnip-KO hearts is not associated with level of thioredoxin activity or oxidative stress. Thioredoxin, a binding partner of Txnip, is a potent antioxidant in cardiomyocytes (67). To determine whether alterations of myocardial ROS levels are associated with better cardiac

### Table 1. Echocardiographic and hemodynamic analyses of cardiac function at baseline and at 16 wk after STZ treatment

<table>
<thead>
<tr>
<th>Echocardiography, n</th>
<th>Control</th>
<th>STZ</th>
<th>KO</th>
<th>KO/STZ</th>
<th>Control</th>
<th>STZ</th>
<th>KO</th>
<th>KO/STZ</th>
</tr>
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<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>4</td>
<td>14</td>
<td>5</td>
<td>14</td>
<td>3</td>
<td>9</td>
<td>4</td>
<td>10</td>
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<tr>
<td>Anterior wall thickness, mm</td>
<td>653 ± 31</td>
<td>639 ± 9†</td>
<td>708 ± 12*</td>
<td>662 ± 12</td>
<td>660 ± 1</td>
<td>613 ± 28</td>
<td>690 ± 17*</td>
<td>588 ± 23</td>
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<tr>
<td>Posterior wall thickness, mm</td>
<td>1.0 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>1.2 ± 0.1</td>
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<tr>
<td>End-diastolic dimensions, mm</td>
<td>2.4 ± 0.2</td>
<td>2.5 ± 0.2</td>
<td>2.6 ± 0.3</td>
<td>2.6 ± 0.3</td>
<td>2.8 ± 0.4</td>
<td>3.0 ± 0.4</td>
<td>2.9 ± 0.4</td>
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<tr>
<td>End-systolic dimensions, mm</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.3 ± 0.3</td>
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<tr>
<td>Fractional shortening, %</td>
<td>69 ± 4</td>
<td>66 ± 2</td>
<td>74 ± 1*</td>
<td>60 ± 4</td>
<td>62 ± 7</td>
<td>55 ± 5</td>
<td>64 ± 4</td>
<td>58 ± 3</td>
</tr>
<tr>
<td>Left ventricular mass, g</td>
<td>0.07 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.12 ± 0.01</td>
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### Hemodynamics

| Heart rate, beats/min | N/A | N/A | N/A | N/A | 515 ± 18 | 510 ± 19 | 525 ± 19 | 480 ± 37 |
| End-systolic pressure, mmHg | N/A | N/A | N/A | N/A | 84 ± 8 | 81 ± 5 | 80 ± 10 | 74 ± 5 |
| End-diastolic pressure, mmHg | N/A | N/A | N/A | N/A | 3.5 ± 0.9 | 2.3 ± 0.3 | 3.6 ± 0.8 | 2.7 ± 0.6 |
| dP/dt maximum, mmHg/s | N/A | N/A | N/A | N/A | 6,625 ± 975 | 6,605 ± 933 | 4,890 ± 730 | 5,349 ± 561 |
| dP/dt minimum, mmHg/s | N/A | N/A | N/A | N/A | 6,098 ± 732 | 5,766 ± 683 | 5,322 ± 758 | 5,484 ± 885 |

Values are means ± SE. STZ, streptozotocin; KO, Txnip-knockout mice; N/A, not applicable. *$P < 0.05$ vs. KO/STZ; †$P < 0.01$ vs. KO.
functional reserve in Txnip-KO hearts, we measured cellular thioredoxin activities and ROS levels in whole heart homogenates from Txnip-KO and WT mice. No significant differences in myocardial activities of thioredoxin were seen at baseline (Txnip-KO, 116 ± 22% of WT) or after STZ injections between Txnip-KO (116 ± 22% of WT baseline) and WT (111 ± 22% of WT baseline) mice (Fig. 3E). Levels of cellular lipid peroxide, an indicator of oxidative stress estimated as malondialdehyde, were also comparable between the myocardium from Txnip-KO mice and WT mice at baseline (WT 1.9 ± 0.5 nmol/mg, KO 1.2 ± 0.2 nmol/mg; P = N.S.) or after STZ treatments (WT 1.4 ± 0.2 nmol/mg, KO 1.1 ± 0.1 nmol/mg; P = N.S.) (Fig. 3F). Thus the functional benefits of Txnip-KO hearts under hyperglycemia were not attributed to decreased levels of ROS in the myocardium.

Enhanced sensitivity of Txnip-deletion myocardium to β-adrenergic stimulation is not associated with sarcollemmal expression of β-adrenergic receptors. Members of the arrestin superfamily participate in agonist-mediated desensitization of G protein-coupled receptors. β-Arrestins bind to and desensitize β-adrenergic receptors through clathrin-mediated endocytosis (45, 48). Because Txnip is a member of the arrestin superfamily (48), we sought to determine whether Txnip, like β-arrestin 1, plays a role in desensitization of β1-adrenergic receptor and causes specific dampening of cellular responses to isoproterenol. Txnip and β-arrestin 1 were overexpressed in HEK-293T cells, and the interaction was assayed by coimmunoprecipitation (Fig. 4A). Unlike β-arrestin 1, no interaction between β1-adrenergic receptor and Txnip was detected. While overexpression of β-arrestin 1 resulted in a 57 ± 4% increase in β1-adrenergic receptor internalization compared with empty vector control, overexpression of Txnip showed no significant effects on internalization or endocytosis of the β1-adrenergic receptor (Fig. 4B).

STZ-induced diabetes has been shown to downregulate the expression level of β1-adrenergic receptors in the heart (37). Thus densities of β-adrenergic receptors in sarcolemmal and light vesicle fractions from cardiac tissues were measured based on [3H]CGP-12177 binding studies (Fig. 4C). The calculated β-adrenergic receptor density was 91 ± 18 fmol/mg protein in the sarcolemmal fraction of WT hearts. We observed no statistical difference in β-adrenergic receptor density be-

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**Fig. 2.** Streptozotocin (STZ)-induced diabetes does not induce cardiac hypertrophy, fibrosis, and dysfunction at 16 wk after STZ injections in cardiomyocyte-specific Txnip-knockout (KO) and wild-type (Control) mice. A, B, and D: echocardiographic analysis was performed without anesthesia at baseline and 8, 12, and 16 wk after STZ injections. Representative M-mode echocardiograms at 16 wk after STZ injections are shown. Left ventricular mass and fractional shortening were comparable among 4 groups of wild-type mice without (Control) or with (STZ) diabetes and Txnip-KO mice without (KO) or with (KO/STZ) diabetes. C: cardiac hypertrophy was assessed by histological myocyte cross-sectional area. E: activation of ERK1/2, one of the signal transduction pathways associated with cardiac hypertrophy, was assessed by Western blot analysis. There was no difference in the ERK1/2 phosphorylation state between wild-type and Txnip-KO hearts after STZ injections. F: diabetes did not induce interstitial collagen depositions in the myocardium from both control and Txnip-KO mice as assessed by PicroSirius Red staining. Scale bars, 50 μm. Values are means ± SE. P = N.S. among 4 groups.
tween WT and Txnip-KO samples within nondiabetic and STZ-induced diabetic conditions.

One of the most critical mediators of the cardiac β-adrenergic pathway is phospholamban, a key protein that regulates sarcoplasmic reticular Ca\(^{2+}\) uptake in cardiomyocytes. Recent reports indicate that depressed cardiac performance in diabetes is associated with reduced sarcoplasmic reticular Ca\(^{2+}\) uptake by an impaired regulation of phospholamban (64). We found no difference in the expression level and the phosphorylation state of phospholamban between Txnip-KO and WT hearts (Fig. 4D).

Taken together, these results indicate that the preserved inotropic reserve in Txnip-KO hearts was due to neither higher levels of β1-adrenergic receptor density nor altered regulation of phospholamban under diabetic conditions.

Txnip binds to GLUT1 and inhibits GLUT1-mediated cellular glucose uptake. The rate of glucose transport in cardiomyocytes is GLUT1 dependent under insulin-deficient conditions.
We conducted an unbiased proteomic-based interaction screen to explore signaling downstream of Txnip (26) and identified GLUT1 as a Txnip-interacting protein. We confirmed this interaction with standard coimmunoprecipitation experiments (Fig. 5A). Since a single cysteine-to-serine mutation (C247S) abolishes the ability of Txnip to bind to thioredoxin (47), we used the Txnip C247S mutant and found that thioredoxin binding was not required for Txnip to interact with GLUT1 (Fig. 5B). Overexpression of Txnip decreased glucose uptake to 48 ± 2% of control levels (P < 0.01 vs. mCherry only) in the absence of insulin stimulation (Fig. 5C). Overexpression of Txnip C247S mutant also decreased glucose uptake to 56 ± 1% of control levels (P < 0.001 vs. mCherry only), while overexpression of other related arrestins, β-arrestin 1 (Arrb1) and arrestin domain containing 1 (Arrdc1), had no effects on interaction with GLUT1 as well as on cellular glucose uptake. These data confirm that Txnip’s binding to GLUT1, unlike Arrb1 and Arrdc1, regulates glucose uptake.

We next sought to identify the mechanism of Txnip function on glucose uptake via GLUT1 using Txnip-KO and WT MEFs. Absence of Txnip protein expression was confirmed in Txnip-KO MEFs compared with WT MEFs (Fig. 6A). It has been suggested by in vitro studies that Txnip regulates GLUT1 through endocytosis (66). In our knockout models, however, the GLUT1 levels on the cell surface were comparable between Txnip-KO and WT MEFs (Fig. 6, B and C). Both our in vivo data and those of others also indicate that modulation of glucose uptake by Txnip does not involve increased expression of GLUT1 and GLUT4 in the isolated plasma membrane fraction from mouse hearts in the fed state (Fig. 7, A–C) (4, 68). In addition, confocal microscopic analyses revealed that GLUT1 levels on the cell surface were comparable in vivo tissues between Txnip-KO and WT myocardium within nondiabetic and STZ-induced diabetic conditions (Fig. 7, D and E).

Nevertheless, a sugar uptake assay using 5.5 mM 3-O-methylglucose (3-OMG), a nonmetabolizable transport substrate, showed a 3.0-fold increase in uptake velocity in Txnip-KO MEFs (Fig. 6F) compared with wild-type MEFs (Fig. 6E). Vmax and Km values for 3-OMG transporter were obtained by nonlinear regression analysis of the concentration dependence of sugar uptake assuming that uptake is described by the Michaelis-Menten equation (Fig. 6D). While Txnip deletion increased Vmax for exchange 3-OMG uptake by 3.5-fold, it had no significant effect on Km for exchange 3-OMG uptake (13 ± 3.6 mM in WT vs. 14 ± 2.3 mM in Txnip-KO; P = N.S.). The reciprocal effect on 3-OMG transporter was confirmed by overexpression of Txnip in the L929 cell line, which expresses GLUT1 as the exclusive glucose transporter (31). Txnip overexpression decreased Vmax for exchange 3-OMG uptake by 38 ± 7% (n = 3, P < 0.05), while it had no significant effect on Km for exchange 3-OMG uptake in L929 cells. These results suggest that deletion of Txnip enhances glucose uptake by increasing GLUT1 catalytic turnover more than changing transporter expression or redistribution from a microsomal storage pool to the cell surface (recruitment). This is supported by in vivo studies from another group, who reported that increased glucose utilization in muscle tissues from Txnip-KO mice is not associated with the abundance of glucose transporters on the plasma membrane (4).

Thus these pieces of evidence identify a new mechanism by which Txnip-KO cells have higher capacity of glucose utilization through increased GLUT1 catalytic efficiency.
Enriched glucose utilization by deletion of Txnip may contribute to a greater functional reserve in diabetic cardiomyopathy. The myocardium is generally dependent on glucose metabolism during conditions that stress intracellular calcium homeostasis including β-adrenergic stimulation (40). To determine whether functional reserve afforded by deletion of Txnip was related to increased glucose utilization during isoproterenol exposure, we examined cardiac phenotype by an ex vivo approach with isolated, perfused mouse hearts. In this system, mechanical function was primarily supported by glucose uptake and glycolysis, since the perfusion buffer contained glucose as the sole oxidative energy substrate. Before administration of isoproterenol, there were no significant differences in rate-pressure product (RPP), developed pressure, end-diastolic pressure, or heart rate between WT and Txnip-KO hearts under both nondiabetic and diabetic conditions. Fig. 7, G and H show the changes in mechanical function produced by continuous infusion of isoproterenol. During administration of isoproterenol to WT nondiabetic hearts, RPP rose up to a 3.1 ± 0.5-fold change from baseline within the first 15 min and then mildly decreased to a 2.5 ± 0.6-fold change for the next 15 min (Fig. 7G). Addition of IAA (100 μM), an inhibitor of glycolysis, produced a further decrease in RPP to 1.5 ± 0.3-fold change from baseline. The increase in RPP within the first 15 min was significantly less in STZ-induced diabetic WT hearts compared with nondiabetic WT hearts (Fig. 7, F and G), suggesting an impairment of myocardial glucose utilization in generating left ventricular mechanical force during β-adrenergic stimulation in the diabetic heart. It should be noted that IAA failed to completely block the mechanical function of nondiabetic hearts to the level of diabetic hearts, which may indicate that other mechanisms besides glycolysis are also involved.

Interestingly, RPP in diabetic Txnip-KO hearts remained at the same level of inotropic response to isoproterenol compared with nondiabetic hearts (Fig. 7, F and H). These results demonstrate that glucose utilization improved by deletion of Txnip, at least in part, contributes to preserved myocardial functional reserve during β-adrenergic stimulation under diabetic conditions.

DISCUSSION

The underlying basis of diabetic cardiomyopathy remains elusive, since it is a complex pathological manifestation involving abnormalities in multiple cellular and extracellular compartments, including the coronary microvasculature, extracellular space, autonomic nervous system, and cardiomyocytes (9). These changes are not well understood at the molecular level, nor is it clear how they cooperate to produce cardiac dysfunction.

In this study, we showed that hyperglycemia increases Txnip levels in cardiomyocytes. To determine a role of hyperglycemia-induced Txnip in diabetic cardiomyopathy, we characterized mice with cardiac-selective deletion of Txnip with type 1 diabetes. In animal models of type 1 diabetes, either STZ-induced or genetic rodents, systolic and diastolic cardiac dysfunction have been documented (23, 43, 62). Thus we expected that prolonged hyperglycemia would induce cardiac dysfunction; however, there were no differences in basal cardiac function between baseline and 8–16 wk after STZ injections in both WT and Txnip-deletion mice. This discrepancy between our and previous reports might be due to the age and strain of animals, the timing of onset of functional decline, and/or the severity of hyperglycemia (62). Accordingly, we performed stress tests and found that WT diabetic animals displayed a loss of inotropic reserve during β-adrenergic challenge, uncovering the evidence of early subtle mechanical dysfunction induced by contractile provocation under diabetic conditions. Inotropic reserve is a reflection of the contractile response to the stimulation of the myocardial adrenergic signaling pathways, which provides a measure of the integrity of the myocardial contractile apparatus. Higher inotropic reserve is a marker of the overall health of the LV and is an independent predictor of
shown in diabetic animal models (41, 54). Therefore, we tested

type (E) fraction dependence of zero-trans 3-OMG uptake was

Fig. 6. Deletion of Txnip enhances glucose uptake. A: absence of Txnip protein expression was confirmed in systemic Txnip-KO mouse embryonic fibroblasts (MEFs) compared with wild-type MEFs. B: GLUT1 protein expression in MEFs from wild-type mice (WT-MEF) and Txnip-null mice (KO-MEF). After fixation, cells were stained with GLUT1 with secondary antibody conjugated to Alexa Fluor 594 and were costained with DAPI. Images were acquired by a confocal microscope. Scale bars, 40 μm. Arrows indicate positive staining of GLUT1 in MEF. C: positive staining of GLUT1 on the cell surface was quantified by ImageJ. D: these MEFs were treated with 3-O-[1H]methylglucose (3-OMG), and concentration dependence of zero-trans 3-OMG uptake was analyzed in WT-MEFs and in KO-MEFs. Vmax and Km values for 3-OMG transporter were obtained by nonlinear regression. Txnip-KO MEFs had increased Vmax for exchange 3-OMG uptake without significant changes of Km compared with wild-type cells. E and F: time course of 5.5 mM 3-OMG uptake in wild-type (E) and Txnip-KO MEFs (F) is shown. V indicates the velocity of the reaction. Values are means ± SE. n = 3. *P < 0.05, **P < 0.01 vs. wild-type MEFs.

survival (16). Interestingly, Txnip-KO hearts showed a higher inotropic reserve than WT hearts.

Since Txnip has pleiotropic cellular functions (36), we specifically tested three hypotheses for how Txnip controls cardiac inotropic reserve in the diabetic heart. First, Txnip binds to and inhibits thioredoxin, the key component of a major redox system that detoxifies ROS in the cell. Hyperglycemia causes disorders of the oxidative-antioxidative balance in the cell, leading to increased free radical formation (19). Disturbed redox-sensitive signaling contributes to the development of diabetic cardiomyopathy (3). Hence, the increased Txnip expression resulting from high glucose is expected to reduce thioredoxin activity and increase concentrations of ROS, and thereby Txnip may contribute to oxidative stress. However, in our mouse model hyperglycemia did not lead to significant changes in the levels of thioredoxin activity and ROS in the myocardium. Knockout of Txnip did not alter thioredoxin function and levels of oxidative stress, which is consistent with our earlier findings in vivo and those of others (58, 68).

Second, autonomic neuropathy, which may underlie abnormalities of cardiac performance, is recognized as a complication of chronic diabetes mellitus. An isoproterenol infusion study has shown decreased β-adrenoceptor responsiveness in type 1 insulin-dependent diabetic patients (5). Likewise, a reduction in myocardial β-adrenergic receptor density has been shown in diabetic animal models (41, 54). Therefore, we tested whether interacting with β-adrenergic receptors, a defining characteristic of the β-arrrestins, is a property of Txnip. The data indicate that Txnip interacts with neither β1- nor β2-adrenergic receptors (21, 46) to cause desensitization of the receptor signaling. Nevertheless, we found better contractile reserve by inotropic stimulation in Txnip-KO hearts.

A third critical cellular function of Txnip is serving as a “glucostat” (38, 44). The hallmark of diabetic insult is a glucose transport system that has been either fully or partially compromised. In both type 1 and type 2 diabetes, glucose uptake and glycolysis are impaired, causing the heart to adapt by using fatty acid for ATP generation (3). Chronically, this maladaptation is believed to lead to the development of diabetic cardiomyopathy (3). In type 1 diabetic animals, because of the dramatic decrease in the rate of glucose utilization and glycolysis, the primary metabolic process that provides ATP as an energy source is fatty acid oxidation (11, 65). Compared with glucose, oxidation of fatty acid consumes more oxygen, which increases oxygen demand, making the heart vulnerable during workload such as β-adrenergic challenge. In this way, the cardiac adaptation in Txnip KO hearts appears to be driven by enhanced glucose metabolism by the interaction between Txnip and GLUT1. Increased glucose utilization can confer cardioprotection, as overexpression of GLUT1 increases myocardial glucose utilization and attenuates cardiac dysfunction after aortic constriction in mice (27, 33). Although we found no changes in the expression level of GLUT1
on the cell surface, recent evidence suggests that GLUT1 can be quickly activated without an increase in either GLUT1 expression or total GLUT1 membrane concentration (20). This regulation is sometimes described as “unmasking” of GLUT1 already present in the membrane for activation (20). Because some thiol-reactive compounds maximally activate GLUT1 in L929 fibroblasts (32, 53), Txnip may activate GLUT1 with a thiol-mediated reaction. GLUT1 expression on cell surface was quantified with ImageJ. P = N.S. among groups. F–H: functional reserve afforded by Txnip-KO hearts with diabetes is related to glucose utilization during β-adrenergic stress. Cardiac phenotype was analyzed by isolated, perfused mouse hearts with the perfusion buffer containing glucose as the sole oxidative energy substrate. Thus mechanical function was primarily supported by glucose. Changes in rate-pressure product (RPP) in response to isoproterenol (0.05 μM) were recorded in wild-type (G) or Txnip-KO (H) mice. Glycolytic inhibition with iodoacetate (IAA; 100 μM) caused a decline in mechanical function in both genotypes. The acute increase in RPP at 5 min (F) was significantly less in STZ-induced diabetic wild-type hearts (STZ) than in nondiabetic wild-type hearts (Control) or in Txnip-KO hearts without (KO) and with (KO/STZ) diabetes. Values are means ± SE, n = 3–9. *P < 0.05 vs. control (by 2-way ANOVA and unpaired t-test); †P < 0.05 vs. KO/STZ (by unpaired t-test).

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