Metallothionein Preserves Akt2 Activity and Cardiac Function via Inhibiting TRB3 in Diabetic Hearts

Junlian Gu,1,2 Xiaoqing Yan,1,2 Xiaozhen Dai,1,2,3 Yuehui Wang,4 Qian Lin,2,5 Jian Xiao,1 Shanshan Zhou,2,4 Jian Zhang,2,4 Kai Wang,1,2 Jun Zeng,1,2 Ying Xin,2,6 Michelle T. Barati,7 Chi Zhang,1 Yang Bai,2,4 Yan Li,8 Paul N. Epstein,2,5,9 Kupper A. Wintergerst,2,9,10 Xiaokun Li,1 Yi Tan,1,2,5,9 and Lu Cai1,2,5,9

Diabetes 2018;67:507–517 | https://doi.org/10.2337/db17-0219

Cardiac insulin resistance is a key pathogenic factor for diabetic cardiomyopathy (DCM), but the mechanism remains largely unclear. We found that diabetic hearts exhibited decreased phosphorylation of total Akt and isoform Akt2 but not Akt1 in wild-type (WT) male FVB mice, which was accompanied by attenuation of Akt downstream glucose metabolic signal. All of these signal changes were not observed in metallothionein cardiac-specific transgenic (MT-TG) hearts. Furthermore, insulin-induced glucose metabolic signals were attenuated only in WT diabetic hearts. In addition, diabetic hearts exhibited increased Akt-negative regulator tribbles pseudokinase 3 (TRB3) expression only in WT mice, suggesting that MT may preserve Akt2 function via inhibiting TRB3. Moreover, MT prevented tert-butyl hydroperoxide (tBHP)–reduced insulin-stimulated Akt2 phosphorylation in MT-TG cardiomyocytes, which was abolished by specific silencing of Akt2. Specific silencing of TRB3 blocked tBHP inhibition of insulin-stimulated Akt2 phosphorylation in WT cardiomyocytes, whereas overexpression of TRB3 in MT-TG cardiomyocytes and hearts abolished MT preservation of insulin-stimulated Akt2 signals and MT prevention of DCM. Most importantly, supplementation of Zn to induce MT preserved cardiac Akt2 signals and prevented DCM. These results suggest that diabetes-inhibited cardiac Akt2 function via TRB3 upregulation leads to aberrant cardiac glucose metabolism. MT preservation of cardiac Akt2 function by inhibition of TRB3 prevents DCM.

Almost all insulin-induced effects on glucose and fatty acid metabolism (1) require activation of phosphatidylinositol 3-kinase (PI3K) and its downstream substrate, protein kinase B (Akt). Insulin-induced activation of both PI3K and Akt is impaired in diabetes, which is detrimental to cardiomyocytes: diabetes-induced damage, such as superoxide generation, cardiomyocyte death, and cardiomyopathy, is reduced by PI3K overexpression (2). Akt isoforms 1 and 2 are abundant in myocardium. Akt1 regulates cardiomyocyte growth in response to exercise, whereas Akt2 plays a major role in the metabolic response to insulin (3,4). Negative regulators of Akt promote cardiac damage by reducing Akt-mediated cell survival and metabolic effects (4–6).

Tribbles pseudokinase 3 (TRB3) is a newly discovered Akt-negative regulator that directly binds to and inhibits Akt phosphorylation (5–9). TRB3 has been linked to diabetic insulin resistance and is overexpressed in the skeletal muscle of obese patients and patients with type 2 diabetes, db/db diabetic mice, streptozotocin (STZ)–diabetic, and Zucker fatty rats (5,6,10,11). The upregulation of TRB3 in
Diabetic hearts are damaged because small interfering RNA (siRNA) silencing of TRB3 alleviates metabolic disturbance, insulin resistance, and cardiomyopathy in diabetic rats (12). These findings suggest that the Akt-negative regulator TRB3 may be an important target under diabetic and insulin-resistant conditions.

Glycogen synthase kinase-3β (GSK-3β) is a downstream target of Akt that plays a critical role in insulin regulation of glycogen and fatty acid synthesis (13). Recently, we demonstrated that diabetic hearts exhibit elevated GSK-3β activity and inactivation with a specific inhibitor of GSK-3β or overexpression of the potent antioxidant metallothionein (MT) completely prevents diabetes-induced derangement of glucose/lipid metabolism and associated pathological changes (14). Therefore, induction of MT may be a therapeutic approach to preventing diabetic cardiomyopathy (DCM). However, the mechanism by which MT inactivates GSK-3β and protects the diabetic heart remains elusive.

Given the critical role of Akt in regulating GSK-3β-modulated energy metabolism, we hypothesized that MT prevents diabetes-induced insulin resistance and cardiomyopathy by promoting Akt inactivation of GSK-3β. The current study, therefore, investigated diabetic downregulation of cardiomyocytes, which, as an isoform of Akt, is critical to reduced inactivation of GSK-3β in diabetes and whether preservation of Akt activity is the mechanism for MT protection from diabetes-induced impairment of cardiac insulin signaling.

**RESEARCH DESIGN AND METHODS**

**Diabetic Models**
Male MT cardiac-specific transgenic (MT-TG) mice on the FVB background (8–10 weeks) and age-matched wild-type (WT) controls were induced diabetic by five consecutive STZ (Sigma-Aldrich, St. Louis, MO) injections (40 mg/kg) as described previously (14–16). Five days after the last injection of STZ, mice with blood glucose levels >250 mg/dL were considered diabetic. Mice receiving the same volume of sodium citrate buffer were used as controls. Three studies were performed in WT and MT-TG diabetic mice and age-matched control mice. The first study investigated the effects of diabetes on cardiac Akt and TRB3 signaling changes 3 months after diabetes onset. The second study investigated the direct effects of diabetes on cardiac insulin signaling. Three months after diabetes onset, mice received an insulin injection (1 unit/kg, i.p.; Humulin R; Eli Lilly and Company). Fifteen minutes later, blood glucose was measured, and mice were sacrificed for collection of heart tissue to assay insulin signaling. The third study investigated the effects of TRB3 overexpression on MT protection from diabetes impairment of cardiac Akt signaling and cardiac damage. TRB3 was overexpressed by intramyocardial gene delivery to MT-TG mice 2 months after diabetes onset. Mice were sacrificed 1 month after gene delivery to collect heart tissue after assaying cardiac function by echocardiography (echo) using a Vevo 770 high-resolution imaging system (Visual Sonics, Toronto, Ontario, Canada) as previously described (17).

Male db/db and littermate WT control mice (FVB background) at the age of 10–12 weeks were fed a high-zinc (HZ) diet (rodent diet with 10% kcal% fat plus zinc, 90 mg/4,057 kcal; Research Diets, New Brunswick, NJ) or normal-zinc diet (NZ; rodent diet with 10% kcal% fat plus zinc, 30 mg/4,057 kcal; Research Diets). After 3 months of diet treatment, mice were sacrificed to collect heart tissue after cardiac function assay, as described above.

All animal procedures were approved by the Animal Policy and Welfare Committee of Wenzhou Medical University and the Institutional Animal Care and Use Committee of the University of Louisville.

**Western Blot Analysis**
Western blot analysis using specific antibodies against Akt, Akt1, Akt2, GSK-3β, and GS, phosphorylated (p)Akt (Ser473), -Akt1 (Ser473), -Akt2 (Ser474), -GSK-3β, and -GS, hexokinase II (HK II; Cell Signaling Technology, Danvers, MA), TRB3 (EMD Millipore, Billerica, MA), connective tissue growth factor (CTGF), transforming growth factor-β1 (TGF-β1), plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor-α (TNF-α), peroxisome proliferator–activated receptor γ coactivator-1α (PGC-1α), GAPDH, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), and MT (DakoCytomation, Santa Clara, CA) was performed as described previously (14–16,18,19).

**Adenoviral Vectors**
TRB3 or Akt1 high-expressing adenovirus was constructed using an Ad-EASY adenoviral vector system following published guidelines (20). Adenovirus expressing only GFP (Ad-GFP) was used as control. Efficacy of adenovirus overexpression of TRB3 (Ad-TRB3) or Akt1 (Ad-Akt1) was determined in adult cardiomyocytes obtained from MT-TG mice in pilot studies (Supplementary Fig. 1).

To knockdown TRB3, Akt1, or Akt2 expression in adult cardiomyocytes, cardiomyocytes were infected with the adenoviruses containing short hairpin RNA (shRNA) against TRB3, Akt1, Akt2, or nonsense shRNA constructed by Shanghai Sunbio Medical Biotechnology (Shanghai, China). We selected three target sequences for each gene (provided in Supplementary Table 1). After infection for 48 h, expression of each gene was determined by Western blot. Adenoviruses containing shRNA against each gene were screened in a pilot study (Supplementary Fig. 2) for knockdown efficiency in adult cardiomyocytes obtained from WT or MT-TG mice.

**Intramyocardial Gene Delivery**
Intramyocardial gene delivery was performed as previously reported (21–23). Briefly, 2 months after diabetes onset, diabetic and age-matched control mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and intubated. After the chest was opened through a midline sternotomy, mice received an intramyocardial injection in the anterior left ventricular wall with Ad-GFP or Ad-TRB3 (5 × 10⁸
plaque-forming units). Each mouse received four-point injection in a total volume of 100 μL. One month after intra-myocardial injection, mice were sacrificed for collecting heart tissues following cardiac function assay. We chose a 1-month time point because adenovirus-mediated cardiac gene expression peaks at ~1 week and declines gradually within ~2 weeks after infection (21). Efficacy of the intra-myocardial gene delivery was validated in a pilot study in MT-TG mice (Supplementary Fig. 3).

**Cardiac Oxidative Stress and Fibrosis Assay**

Thiobarbituric acid–reactive substances (TBARS) assay was used to measure relative malondialdehyde production as an index of lipid peroxidation as described previously (24). Cardiac fibrosis was examined by Sirius Red staining of collagen accumulation (14,15,19).

**Primary Adult Cardiomyocyte Isolation, Cell Culture, and Tert-butyl Hydroperoxide or Lipopolysaccharide Treatment**

Adult cardiomyocytes from 2-month-old WT and MT-TG mice were isolated as we previously described (25). The effects of tert-butyl hydroperoxide (tBHP; 100 μmol/L) exposure for 2 h or lipopolysaccharide (LPS; 10 μg/mL) for 4 h on insulin-stimulated Akt, Akt1, Akt2, GS-3β, and GS phosphorylation and TRB3, HK II, and PGC-1α expression were measured by Western blot. The effects of 2-h exposure to tBHP (100 μmol/L) on insulin-stimulated glucose uptake and ATP production were examined using a Glucose Uptake Assay Kit and an ATP Assay Kit (Abcam, Cambridge, MA) following the manufacturer’s protocols.

**Statistical Analysis**

Data are presented as mean ± SD. Significant differences were assessed by one-way or two-way ANOVA followed by the Bonferroni test. A P value <0.05 was considered statistically significant.

**RESULTS**

**MT Preserves Akt2 Function Along With Inhibition of Akt-Negative Regulator TRB3**

Consistent with our previous study (14), WT and MT-TG diabetic mice showed similar elevation in blood glucose, and there were no significant differences in body weight between WT and MT-TG mice in either diabetic or nondiabetic mice during the 3-month period after diabetes onset (Supplementary Fig. 4). Because Akt is the immediate upstream mediator of GS-3β phosphorylation, we examined expression and phosphorylation of total Akt in WT and MT-TG hearts 3 months after diabetes onset. The results showed that Akt phosphorylation was decreased in WT hearts but preserved in MT-TG hearts (Fig. 1A and B). It is especially noteworthy that the phosphorylation responses of Akt to diabetes and MT paralleled that of diabetic GS-3β activation as previously reported (14).

Because both Akt1 and Akt2 are abundant in myocardium, we next examined Akt1 and Akt2 expression and phosphorylation to determine which Akt isoformal changes in parallel with GS-3β activation under diabetic and MT overexpression conditions. The results showed that Akt1 phosphorylation in hearts of WT or MT-TG mice was not significantly changed by diabetes (Fig. 1A and C). In contrast, diabetes significantly decreased Akt2 phosphorylation in WT, but not in MT-TG hearts (Fig. 1A and D). Most importantly, preservation of Akt2 activation in MT-TG diabetic hearts was accompanied by inhibition of downstream target GS-3β (Fig. 1A and E) and preservation of GS activation (Fig. 1A and F), along with normalization of cardiac HK II (Fig. 1A and G) and PGC-1α (Fig. 1A and H) expression.

To investigate how MT preserves Akt2 function in the diabetic heart, we determined if diabetes upregulates the Akt-negative regulator TRB3 and assessed whether MT overexpression prevented diabetes upregulation of TRB3. Diabetes was found to significantly upregulate TRB3 expression in WT but not MT-TG hearts (Fig. 1A and I). This suggests that diabetic downregulation of Akt2 activity may be because of upregulation of Akt-negative regulator TRB3 and that blocking induction of MT TRB3 may be the basis for preservation of Akt2 activity in diabetic MT-TG hearts.

**MT Prevents Diabetic Attenuation of Akt2-Mediated Cardiac Insulin Signaling**

To directly assess the interaction of diabetes and MT on insulin-induced Akt2 signaling we investigated cardiac insulin signaling after systemic insulin injection to WT
and MT-TG mice, with and without diabetes. Insulin-induced blood glucose clearance was impaired to the same extent in WT and MT-TG mice (Fig. 2A), but this was not true for cardiac insulin signaling. In WT hearts, but not in MT-TG hearts, diabetes significantly attenuated insulin-stimulated phosphorylation of Akt2 (Fig. 2B and C) and GSK-3β (Fig. 2B and D) and dephosphorylation of GS (Fig. 2B and E). These results confirm diabetes-induced insulin resistance on cardiac Akt2 function and that cardiac MT overexpression prevented this insulin resistance.

**Akt2 Plays a Critical Role in MT Preserving Cardiac Insulin-Stimulated Metabolic Signaling**

Considering that diabetic insulin resistance is predominantly mediated by diabetic oxidative stress (26,27), adult WT and MT-TG cardiomyocytes were exposed to tBHP, an organic oxidant, to mimic diabetic oxidative stress in cultured cells. Paralleling the effects of diabetes in vivo on diabetic hearts (Figs. 1 and 2), tBHP exposure of WT cardiomyocytes significantly inhibited insulin-stimulated total Akt (Fig. 3A and B), Akt2 (Fig. 3A and C), and GSK-3β phosphorylation (Fig. 3A and D) and GS dephosphorylation (Fig. 3A and E), as well as inhibiting HK II (Fig. 3A and F) and PGC-1α (Fig. 3A and G) expression. Inhibition of insulin-stimulated Akt and Akt2 phosphorylation by tBHP was accompanied by increased expression of Akt-negative regulator TRB3 in WT cardiomyocytes. Importantly, as seen for diabetic hearts, all of these effects of tBHP on WT cardiomyocytes were prevented by elevated MT in MT-TG cardiomyocytes (Fig. 3A and H).

Chronic inflammation-induced oxidative stress in diabetes is considered a major cause of insulin resistance (28). This study directly tested if inflammatory stress contributes to impaired insulin signaling in cardiomyocytes. Adult cardiomyocytes from WT and MT-TG hearts were treated with LPS to mimic diabetes-induced cardiac inflammatory stress. Similar to what was observed with exposure to tBHP treatment, LPS treatment also inhibited insulin-stimulated Akt2 (Fig. 3I and J), GSK-3β phosphorylation (Fig. 3I and K), and GS dephosphorylation (Fig. 3I and L), downregulated HK II (Fig. 3I and M) and PGC-1α (Fig. 3I and N) expression, and upregulated TRB3 expression (Fig. 3I and O) in WT cardiomyocytes, but not in MT-TG cardiomyocytes (Fig. 3I–O).

Although these studies confirmed our previous finding (14) of a close association between activation of Akt2 by elevated MT and prevention of diabetic GSK-3β activation, we did not demonstrate causality. Therefore, we tested whether knockdown of Akt1 or Akt2 genes could eliminate MT preservation of cardiomyocyte insulin signaling. Transfection of MT-TG adult cardiomyocytes with specific Akt1-shRNA or Akt2-shRNA dramatically reduced Akt1 (Fig. 4A and B) or Akt2 (Fig. 4A and C) expression and phosphorylation. But only Akt2 knockdown completely blocked MT’s ability to protect from tBHP inhibition of insulin signaling, including insulin-stimulated GSK-3β (Fig. 4A and D) phosphorylation, GS (Fig. 4A and E) dephosphorylation, reduced HK II (Fig. 4A and F) and PGC-1α (Fig. 4A and G) expression, and reduced glucose uptake (Fig. 4H) and ATP production (Fig. 4I). These findings demonstrate that Akt2, but not Akt1, is essential for MT preservation of cardiac insulin signaling.

**MT Effects on TRB3 Expression Are Critical to MT Preservation of Akt2 Function in Cardiomyocytes**

Results presented in Figs. 1 and 3 showed an association between reduced Akt2 activation and upregulation of Akt-negative regulator TRB3 in diabetic myocardium and cardiomyocytes under oxidative or inflammatory stress. Furthermore, they showed that MT preservation of Akt2 function was accompanied by inhibition of TRB3 expression. However, they did not directly test if TRB3 upregulation inhibits Akt2 activity under these conditions nor did they test if MT effects on TRB3 are critical to MT preservation of Akt2 function. Therefore, we directly manipulated TRB3 expression in adult cardiomyocytes from WT and MT-TG hearts.

---

**Figure 2**—MT prevents diabetes-induced cardiac insulin signal impairment in STZ-induced type 1 diabetes. Three months after diabetes onset, mice were fasted for 4 h, weighed, and then injected with insulin (Humulin R; Eli Lilly and Company, i.p.) at a dose of 1 unit/kg body weight. Blood glucose at 15 min after insulin injection was measured by using a FreeStyle complete blood glucose monitor (A), then mice were sacrificed, and cardiac tissue was collected immediately for detecting the phosphorylation of Akt2 (B and C), GSK-3β (B and D), and GS (B and E). Results were normalized to WT control without insulin treatment. n ≥ 5. Data shown in graphs represent the mean ± SD. *P < 0.05. C, vehicle control; Ctrl, control; DM, diabetes mellitus; I, insulin; ns, not significant.
First, we determined if inhibition of TRB3 expression by specific shRNA can upregulate Akt2 phosphorylation in WT cardiomyocytes exposed to tBHP, either with or without insulin stimulation. As shown in Fig. 5, shRNA downregulation of TRB3 expression in WT cardiomyocytes (Fig. 5A and B) significantly upregulated phosphorylation of Akt2 (Fig. 5A and C) and GSK-3β (Fig. 5A and D), dephosphorylation of GS (Fig. 5A and E), and decreased expression of HK II (Fig. 5A and F) and PGC-1α (Fig. 5A and G) under basal conditions. Downregulation of TRB3 also abolished the ability of tBHP to inhibit insulin-stimulated upregulation of Akt2 and GSK-3β phosphorylation, GS dephosphorylation, and HK II and PGC-1α expression (Fig. 5A–G). This finding demonstrates that TRB3 is critical to the ability of tBHP to inhibit insulin activation of Akt2.

Secondly, we upregulated TRB3 expression with Ad-TRB3 in MT-TG cardiomyocytes to determine if forced overexpression of TRB3 blocked MT preservation of Akt2 function and further test if MT protection was rescued by elevated Akt1. These experiments were performed with or without tBHP exposure and with or without insulin stimulation. Compared with MT-TG cardiomyocytes infected with Ad-GFP, transfection with Ad-TRB3 produced about a twofold increase in TRB3 protein expression (Fig. 5H and I). Forced overexpression of TRB3 completely blocked the ability of MT to protect insulin-stimulated Akt2 (Fig. 5H and J) and GSK-3β (Fig. 5H and K) phosphorylation, GS (Fig. 5H and L) dephosphorylation, and HK II (Fig. 5H and M) and PGC-1α (Fig. 5H and N) upregulation. MT protection could not be rescued by forced overexpression of Akt1.
Akt downstream signals in MT-TG cardiomyocytes. The efficacy of shRNA interference of Akt1 and Akt2 expression cannot rescue MT protection or compensate for lost inhibition of TRB3 expression. Furthermore, Akt1 overexpression in vivo that MT prevents most diabetic damage to the heart, other than oxidative stress, by preventing upregulation of TRB3 and that MT probably blocks TRB3 induction in vivo that MT prevents most diabetic damage to the heart, other than oxidative stress, by preventing upregulation of TRB3 and that MT probably blocks TRB3 induction.

Figure 4—Effects of Akt isoform knockdown on MT preservation of Akt downstream signals in MT-TG cardiomyocytes. The efficacy of shRNA interference of Akt1 and Akt2 expression in MT-TG adult cardiomyocytes was validated in a pilot study (Supplementary Fig. 2B and C). Then the effects Akt1 and Akt2 knockdown on the phosphorylation of total Akt1 (A and B), Akt2 (A and C), GSK-3β (A and D), and GS (A and E) and expression of HK II (A and F) and PGC-1α (A and G) were detected by Western blot in adult cardiomyocytes from MT-TG mice. GAPDH was used for loading control. Results were normalized to control treatment. The effects of Akt1 and Akt2 knockdown on glucose uptake (H) and ATP production (I) were detected by a Glucose Uptake Assay Kit and an ATP Assay Kit (Abcam), respectively. Three independent experiments were performed. Data shown in graphs represent the means ± SD. *P < 0.05 vs. control; $P < 0.05 vs. tBHP treatment for each subgroup; #P < 0.05 vs. Ctrl-shRNA+INS treatment; $P < 0.05 vs. Ctrl-shRNA+tBHP+INS treatment. Ctrl, control; 2-DG6P, 2-deoxy-D-glucose-6-phosphate; INS, insulin.

Supplementation With Zn Preserves Akt2 Function and Prevents DCM in db/db Type 2 Diabetic Mice
To demonstrate that our findings on STZ diabetic mice apply to other diabetic models, we also studied type 2 diabetic db/db mice. Zn treatment was used to elevate cardiac MT in db/db mice, because we previously demonstrated that Zn is an effective inducer of cardiac MT in STZ type 1 diabetes, and Zn prevented DCM (19). Littermate WT and diabetic db/db mice received an NZ or HZ diet for 3 months. Zn treatment did not affect body weight or blood glucose in db/db diabetic or age-matched WT mice (Table 1). Consistent with our previous study (19), Zn treatment for 3 months significantly induced cardiac MT expression, which was more obvious in db/db mice because diabetic...
**DISCUSSION**

We previously proposed that MT prevents DCM by blocking activation of GSK-3β (14). However, the mechanism underlying MT inhibition of GSK-3β remained uncertain. In this study, we decisively connect beneficial effects of MT on DCM and GSK-3β activation to MT’s ability to inhibit diabetic induction of the Akt-negative regulator TRB3, thereby preserving Akt2 activity to block activation of GSK-3β. This connection was suggested by diabetic induction of TRB3 in STZ and db/db diabetes coincident with inhibition of insulin signaling, including reduced Akt2 phosphorylation. In vitro and in vivo studies to directly manipulate cardiomyocyte and cardiac TRB3 levels confirmed that inhibition of TRB3 induction was essential to MT cardiac protection and that TRB3 induction was sufficient to induce insulin resistance. Either transgenic overexpression of MT in STZ diabetes or Zn induction of MT in db/db diabetes blocks TRB3 induction and protects from almost all measures of diabetic damage to the heart. These results present a new mechanism for MT protection of the heart and emphasize the key role of TRB3 induction and Akt2 inhibition in DCM.

The first innovative findings are that the Akt isoform Akt2 is critical to MT prevention from insulin resistance and protection of cardiomyocytes. The importance of Akt2 to cardiomyocyte function has been shown in earlier studies.
Table 1—Heart function assay 3 months after diabetes onset in STZ-induced type 1 diabetes or 3 months after zinc treatment in db/db type 2 diabetes

| Heart function assay | WT/Ctrl (n = 16) | WT/DM (n = 12) | MT-TG/Ctrl + Ad-GFP (n = 10) | MT-TG/DM + Ad-GFP (n = 11) | MT-TG/DM + Ad-TRB3 (n = 7) |
|----------------------|------------------|----------------|-----------------------------|-----------------------------|----------------------------|
| 3 months after diabetes onset in STZ-induced type 1 diabetes | Body weight (g) | 33.2 ± 3.0 | 33.7 ± 2.9 | 34.0 ± 2.8 | 34.2 ± 3.6 | 35.8 ± 3.7 |
| Blood glucose (mg/dL) | 108 ± 21 | 453 ± 38 | 129 ± 13 | 416 ± 65 | 385 ± 46 |
| IVS.d (mm) | 0.63 ± 0.01 | 0.62 ± 0.02 | 0.63 ± 0.01 | 0.64 ± 0.01 | 0.60 ± 0.01 |
| LVID.d (mm) | 3.80 ± 0.07 | 4.10 ± 0.19* | 3.87 ± 0.15* | 3.90 ± 0.14 | 3.86 ± 0.12 |
| LV PW.d (mm) | 0.82 ± 0.01 | 0.97 ± 0.04* | 0.822 ± 0.01 | 0.81 ± 0.02 | 0.74 ± 0.03†† |
| IVS.s (mm) | 1.09 ± 0.03 | 1.08 ± 0.01 | 1.10 ± 0.02 | 1.08 ± 0.01 | 1.08 ± 0.01 |
| LVID.s (mm) | 1.81 ± 0.06 | 2.38 ± 0.11* | 1.84 ± 0.06 | 1.98 ± 0.13 | 2.14 ± 0.10†† |
| LV PW.s (mm) | 1.45 ± 0.05 | 1.28 ± 0.12* | 1.44 ± 0.05 | 1.41 ± 0.02 | 1.31 ± 0.03†† |
| LV Vol.d (µL) | 62.20 ± 2.77 | 74.82 ± 7.92* | 64.63 ± 5.92 | 65.71 ± 5.42 | 64.35 ± 4.56 |
| LV Vol.s (µL) | 9.96 ± 0.85 | 19.92 ± 2.17* | 10.28 ± 0.90 | 12.32 ± 1.98 | 15.22 ± 1.74†† |
| EF,% (%) | 84.01 ± 1.08 | 73.37 ± 1.16* | 84.06 ± 1.02 | 82.05 ± 1.61 | 75.83 ± 1.15†† |
| FS,% (%) | 52.32 ± 1.25 | 41.60 ± 1.02* | 52.43 ± 1.23 | 50.20 ± 1.72 | 43.95 ± 1.16†† |
| LV mass (mg) | 94.81 ± 3.66 | 102.81 ± 11.04 | 97.82 ± 6.73 | 97.79 ± 6.53 | 87.86 ± 5.83 |
| LV mass corrected (mg) | 75.84 ± 2.93 | 82.25 ± 8.83 | 78.26 ± 5.38 | 78.23 ± 5.22 | 70.29 ± 4.66 |

| Heart function assay | WT-NZ (n = 7) | WT-HZ (n = 8) | db/db-NZ (n = 7) | db/db-HZ (n = 8) |
|----------------------|--------------|--------------|----------------|----------------|
| 3 months after zinc treatment in db/db type 2 diabetes | Body weight (g) | 34.9 ± 2.8 | 34.3 ± 2.7 | 68.3 ± 7.3** | 72.3 ± 8.0** |
| Blood glucose (mg/dL) | 123 ± 23 | 104 ± 28 | 462 ± 48 | 469 ± 50 |
| IVS.d (mm) | 0.60 ± 0.02 | 0.62 ± 0.02 | 0.63 ± 0.02 | 0.62 ± 0.02 |
| LVID.d (mm) | 3.61 ± 0.16 | 3.56 ± 0.16 | 4.01 ± 0.24** | 3.73 ± 0.07†† |
| LV PW.d (mm) | 0.65 ± 0.03 | 0.70 ± 0.06 | 0.72 ± 0.05 | 0.69 ± 0.06 |
| IVS.s (mm) | 0.83 ± 0.04 | 0.85 ± 0.06 | 0.87 ± 0.04 | 0.86 ± 0.04 |
| LVID.s (mm) | 2.07 ± 0.08 | 2 ± 0.14 | 2.56 ± 0.18*** | 2.24 ± 0.08†† |
| LV PW.s (mm) | 0.9 ± 0.07 | 0.9 ± 0.07 | 0.96 ± 0.05 | 0.92 ± 0.07 |
| LV Vol.d (µL) | 54.91 ± 5.73 | 53.31 ± 5.55 | 70.76 ± 9.11** | 59.12 ± 2.61†† |
| LV Vol.s (µL) | 13.93 ± 1.29 | 12.87 ± 2.22 | 23.88 ± 4.08** | 17.02 ± 1.36†† |
| EF,% (%) | 74.22 ± 2.03 | 75.94 ± 2.62 | 66.35 ± 2.01** | 71.29 ± 2.11†† |
| FS,% (%) | 42.33 ± 1.80 | 43.85 ± 2.34 | 36.16 ± 1.48** | 39.91 ± 1.02†† |
| LV mass (mg) | 71.54 ± 7.91 | 75.04 ± 6.02 | 93.96 ± 10.16** | 79.46 ± 5.28†† |
| LV mass corrected (mg) | 57.23 ± 6.32 | 60.04 ± 4.82 | 75.17 ± 8.13** | 63.94 ± 4.06†† |

Data are mean ± SD. At 3 months after diabetes onset in STZ-induced type 1 diabetes or at 3 months after zinc treatment in db/db type 2 diabetes, transthoracic echo was performed to detect heart function. Ctrl, control; DM, diabetes mellitus; IVS.d, end-diastolic interventricular septum; IVS.s, end-systolic interventricular septum; LVID.d, LV end-diastolic diameter; LVID.s, LV end-systolic diameter; LV PW.d, LV end-diastolic posterior wall; LV PW.s, LV end-systolic posterior wall; LV Vol.d, end-diastolic LV volume; LV Vol.s, end-systolic LV volume.

*P < 0.05 vs. WT/Ctrl; †P < 0.05 vs. MT-TG/Ctrl+Ad-GFP; ‡P < 0.05 vs. MT-TG/DM+Ad-GFP; **P < 0.05 vs. WT-NZ; ††P < 0.05 vs. db/db-NZ.

with Akt2-knockout mice, which have reduced cardiac glucose oxidation, increased fatty acid oxidation, and increased cardiac damage in response to acute myocardial infarction (3). Li et al. (29) reported that cardiomyocytes from Akt2-knockout mice display glucose intolerance and compromised contractile function. In this study, we provide direct evidence (Fig. 4) that downregulation of cardiomyocyte Akt2 but not Akt1 function resulted in insulin signaling impairment on GSK-3β and GS phosphorylation and metabolism, as well as loss of MT protection of insulin signaling. We also demonstrated that chronic diabetes attenuated insulin-stimulated cardiac Akt2 activation (Fig. 2) and Akt2 downstream targets including GSK-3β phosphorylation and GS dephosphorylation. MT overexpression prevented these deficits. These results confirm that Akt2 is a direct target for MT preservation of cardiac insulin signaling.

Our second innovative finding is that TRB3 is a direct target for MT prevention of DCM. TRB3 is an Akt-negative regulator that directly binds to and blocks Akt phosphorylation (5,7,8). Substantial evidence implicates elevated TRB3 in cardiac pathology; TRB3 was shown to upregulate in response to oxidative stress (30). TRB3-knockout mice are protected from high-fat diet–induced insulin resistance (5). Transgenic mice with cardiac-specific overexpression of TRB3 exhibited abnormal cardiac signal transduction and metabolism (31). TRB3 expression is increased in the heart of type 2 diabetic rats (12) and patients with type 2 diabetes.
Figure 6—Adenovirus-mediated TRB3 overexpression abolishes MT’s preservation of cardiac Akt signaling and prevention of cardiac damage in STZ-induced type 1 diabetes. The efficacy of the intramyocardial gene delivery was validated in 2-month-old MT-TG mice in a pilot study (Supplementary Fig. 3). Two months after diabetes onset, Ad-TRB3 or Ad-GFP control was delivered by myocardial injection. One month after intramyocardial injection, these mice were sacrificed, and cardiac tissues were collected following cardiac function examination by echo (Table 1). Cardiac oxidative stress was detected by a TBARS assay (A), and the phosphorylation of total Akt (B and C), Akt2 (B and D), and GSK-3β (B and E) and the expression of TRB3 (B and F), CTGF (B and G), TGF-β1 (B and H), PAI-1 (B and I), and TNF-α (B and J) were detected by Western blot. GAPDH was used for loading control. Results were normalized to the WT-Control. The cardiac collagen accumulation was detected by Sirius Red staining (K). n = 7. Scale bars: 50 μM. Data shown in graphs represent the means ± SD. *P < 0.05 vs. WT-Control; #P < 0.05 vs. WT-DM; $P < 0.05 vs. MT-TG-DM-Ad-GFP. Ctrl, control; DM, diabetes mellitus.

Figure 7—Supplementation with Zn preserves cardiac Akt signaling and prevents DCM in db/db type 2 diabetic mice. db/db and littermate WT control mice at age of 10–12 weeks were fed an NZ or NZ diet for 3 months. Then, these mice were sacrificed for collecting heart tissues following cardiac function assay by echo (Table 1). Cardiac MT expression (A and B), the phosphorylation of total Akt (A and C), Akt2 (A and D), GSK-3β (A and E), and GS (A and F), and the expression of HK II (A and G), PGC-1α (A and H), and TRB3 (A and I) were detected by Western blot. GAPDH was used for loading control. Results were normalized to the WT control (WT-NZ). n ≥ 7. Data shown in graphs represent the means ± SD. *P < 0.05 vs. WT-NZ; #P < 0.05 vs. db/db-NZ. J: A mechanistic illustration of diabetes-induced impairment of and MT preservation of cardiac Akt2-mediated glucose metabolic signaling. Diabetic oxidative stress upregulation of TRB3 inhibits insulin-induced phosphorylation of Akt2 and GSK-3β and dephosphorylation of GS and eventually inhibits cardiac glucose metabolism and induces oxidative stress and inflammation, resulting in cardiac damage (cardiomyopathy). MT-TG or Zn induction of MT can prevent these pathological and functional changes. The blue font and → or ↓ indicate the findings in our previous reports (5,14,35); the black font and ← or ↑ indicate the findings in the current study; and ← or ↑ indicates stimulation, ↓ indicates inhibition, IR, insulin receptor.
resistance, and cardiomyopathy in diabetic rats (12). These results and our own show that TRB3 Ad-siRNA is a possible approach to prevent DCM. However, these methods are problematic for clinical use. Alternatively, prevention of TRB3 induction by upregulation of endogenous MT expression may be a more practical approach to prevent DCM. We previously demonstrated that upregulation of cardiac MT by Zn supplementation provided significant protection from cardiomyopathy in STZ-induced type 1 diabetic mice (19). In this study, we further validated that approach by Zn induction of endogenous MT expression in db/db type 2 diabetic mice, which preserved Akt2 metabolic signaling and prevented DCM (Fig. 7A–I and Table 1).

The mechanism by which Zn preserves cardiac glucose metabolic signals remains largely unknown. Our recent study showed that Zn supplementation partially rescued Akt2 deficiency— but not MT deficiency-induced cardiac metabolic signal impairment (32). Our previous findings and the present results indicate that, in addition to improving the antioxidant potency via enhancing MT expression to inhibit Akt-negative regulator such as TRB3, Zn supplementation improvement of cardiac metabolic signals may also be mediated by Zn induction of MT to store and release Zn to directly activate cardiac glucose metabolic signals in an Akt2-dependent or -independent manner (32–34), which has to be further explored in future studies.

In summary, the current study provided several findings to support a novel scheme of DCM, MT protection, and possible therapeutic use of Zn supplementation (Fig. 7J) (35): 1) diabetic-induced oxidative stress upregulates TRB3 expression, leading to downregulation of Akt2 function and GSK-3β activation. This leads to aberrant cardiac glucose metabolism, which contributes to the development of DCM. 2) MT prevention of DCM is mediated predominantly by inhibiting TRB3 induction, thus preserving Akt2 function. 3) Zn induction of cardiac endogenous antioxidant MT blocks TRB3 induction, preserving Akt2 signaling and preventing DCM. It is important to develop pharmacological inducers of cardiovascular MT for prevention of cardiomyopathy in patients with diabetes.

Acknowledgments. The authors thank Dr. Robyn Cunard, Research and Medicine Services, Division of Nephrology, Veterans Affairs San Diego Healthcare System, La Jolla, San Diego, CA, for generating the pcDNA3-TRB3 expression plasmid.

Funding. This study was supported in part by the National Key R&D Program of China (2017YFA0506000), a Starling-Up Fund from Wenzhou Medical University (QTJ 13007), National Natural Science Foundation of China projects (81273509, 81070189, 81200239, and 81370917), Basic Research Awards (1-11-BS-017 and 1-15-BS-018) and Junior Faculty Award (1-13-JF-53) from the American Diabetes Association, a Foundation for the National Institutes of Health grant (1R01-HL-125877-01), and a Clinical and Translational Science Award from the National Institutes of Health (UL1-TR-000117).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. J.G. and X.Y. researched data, wrote, reviewed, and edited the manuscript. X.D., Y.W., Q.L., J.X., S.Z., J.Zh., K.W., J.Ze., Y.X., C.Z., Y.B., and Y.L. researched data. M.T.B. provided research material and techniques. P.N.E., K.A.W., and X.L. contributed to data interpretation, discussion, and/or edited the manuscript. Y.T. and L.C. directed the project, contributed to discussion, and wrote, reviewed, and edited the manuscript. Y.T. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Palomer X, Salvadó L, Barroso E, Vázquez-Carrera M. An overview of the crosstalk between inflammatory processes and metabolic dysregulation during diabetic cardiomyopathy. J Am Coll Cardiol 2013;62:1360–3172
2. Ritchie RH, Love JE, Huyynh K, et al. Enhanced phosphoinositide 3-kinase/110α activity prevents diabetes-induced cardiomyopathy and superoxide generation in a mouse model of diabetes. Diabetologia 2012;55:3369–3381
3. DelBosch B, Sambandam N, Weinheimer C, Courtois M, Muslin AJ. Akt2 regulates cardiac metabolism and cardiomyocyte survival. J Biol Chem 2006;281:32841–32851
4. Muslin AJ, Akt2: a critical regulator of cardiomyocyte survival and metabolism. Pediatr Cardiol 2011;32:317–322
5. Koh HJ, Toyoda T, Didesch MM, et al. Tribbles 3 mediates endoplasmic reticulum stress-induced insulin resistance in skeletal muscle. Nat Commun 2013;4:1871
6. Yu J, Xiao F, Guo Y, et al. Hepatic phosphoserine aminotransferase 1 regulates insulin sensitivity in mice via tribbles homolog 3. Diabetes 2015;64:1591–1602
7. Du K, Herzog S, Kulkarni RN, Montminy M. TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. Science 2003;300:1574–1577
8. Salazar M, Lorente M, García-Taboada E, et al. Loss of Tribbles pseudokinase 3 promotes Akt-driven tumorigenesis via FOXO inactivation. Cell Death Differ 2015;22:131–144
9. Jeong HW, Choi RH, McClellan JL, et al. Tribbles 3 inhibits brown adipocyte differentiation and function by suppressing insulin signaling. Biochem Biophys Res Commun 2016;470:783–791
10. Prudente S, Hribal ML, Flex E, et al. The functional Q84R polymorphism of mammalian Tribbles homolog TRB3 is associated with insulin resistance and related cardiovascular risk in Caucasians from Italy. Diabetes 2005;54:2807–2811
11. Liu J, Wu X, Franklin JL, et al. Mammalian Tribbles homolog 3 impairs insulin action in skeletal muscle: role in glucose-induced insulin resistance. Am J Physiol Endocrinol Metab 2010;298:E565–E576
12. Ti Y, Xie GL, Wang ZH, et al. TRB3 gene silencing alleviates diabetic cardiomyopathy in a type 2 diabetic rat model. Diabetes 2011;60:2963–2974
13. Biensø RS, Ringholm S, Kiilerich K, et al. GLUT4 and glycogen synthase are key players in bed rest-induced insulin resistance. Diabetes 2012;61:1090–1099
14. Wang Y, Feng W, Xue W, et al. Inactivation of GSK-3βeta by metformin prevents diabetes-related changes in cardiac energy metabolism, inflammation, nitrosative damage, and remodeling. Diabetes 2009;58:1391–1402
15. Cai L, Wang Y, Zhou G, et al. Attenuation by metformin of early cardiac cell death via suppression of mitochondrial oxidative stress results in a prevention of diabetic cardiomyopathy. J Am Coll Cardiol 2006;48:1688–1697
16. Cai L, Wang J, Li Y, et al. Inhibition of superoxide generation and associated nitrosative damage is involved in metformin prevention of diabetic cardiomyopathy. Diabetes 2005;54:1829–1837
17. Zhou G, Li X, Hein DW, et al. Metallothionein suppresses angiotensin II-induced nicotinamide adenine dinucleotide phosphate oxidase activation, nitrosative stress, apoptosis, and pathological remodeling in the diabetic heart. J Am Coll Cardiol 2008;52:655–666
18. Liu Q, Wang G, Zhou G, et al. Angiotensin II-induced p53-dependent cardiac apoptotic cell death: its prevention by metallothionein. Toxicol Lett 2009;191:314–320
19. Wang J, Song Y, Elsherif L, et al. Cardiac metallothionein induction plays the major role in the prevention of diabetic cardiomyopathy by zinc supplementation. Circulation 2006;113:544–554
20. He TC, Zhou S, da Costa LT, Yu J, Kindzierski KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A 1998;95:2509–2514
21. Guzman RJ, Lemarchand P, Crystal RG, Epstein SE, Finkel T. Efficient gene transfer into myocardium by direct injection of adenovirus vectors. Circ Res 1993;73:1202–1207
22. Li Q, Guo Y, Xuan YT, et al. Gene therapy with inducible nitric oxide synthase protects against myocardial infarction via a cyclooxygenase-2-dependent mechanism. Circ Res 2003;92:741–748
23. Li Q, Guo Y, Tan W, et al. Cardioprotection afforded by inducible nitric oxide synthase gene therapy is mediated by cyclooxygenase-2 via a nuclear factor-kappaB dependent pathway. Circulation 2007;116:1577–1584
24. Sun W, Zhang Z, Chen Q, et al. Magnolia extract (b153) protection of heart from lipid accumulation caused cardiac oxidative damage, inflammation, and cell death in high-fat diet fed mice. Oxid Med Cell Longev 2014;2014:205849
25. Zhang C, Huang Z, Gu J, et al. Fibroblast growth factor 21 protects the heart from apoptosis in a diabetic mouse model via extracellular signal-regulated kinase 1/2-dependent signalling pathway. Diabetologia 2015;58:1937–1948
26. Song P, Wu Y, Xu J, et al. Reactive nitrogen species induced by hyperglycemia suppresses Akt signaling and triggers apoptosis by upregulating phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) in an LKB1-dependent manner. Circulation 2007;116:1585–1595
27. Tan Y, Ichikawa T, Li J, et al. Diabetic downregulation of Nrf2 activity via ERK contributes to oxidative stress-induced insulin resistance in cardiac cells in vitro and in vivo. Diabetes 2011;60:625–633
28. Stienstra R, Tack CJ, Kanneganti TD, Joosten LA, Netea MG. The inflammasome puts obesity in the danger zone. Cell Metab 2012;15:10–18
29. Li L, Hua Y, Ren J. Short-chain fatty acid propionate alleviates akt2 knockout-induced myocardial contractile dysfunction. Exp Diabetes Res 2012;2012:851717
30. Kanwar YS. TRB3: an oxidant stress-induced pseudokinase with a potential to negatively modulate MCP-1 cytokine in diabetic nephropathy. Am J Physiol Renal Physiol 2010;299:F963–F964
31. Avery J, Etzion S, DeBosch BJ, et al. TRB3 function in cardiac endoplasmic reticulum stress. Circ Res 2010;106:1516–1523
32. Sun W, Miao X, Zhou S, et al. Zinc rescue of Akt2 gene deletion-linked murine cardiac dysfunction and pathological changes is metallothionein-dependent. J Mol Cell Cardiol 2014;74:88–97
33. Ilouz R, Kaidanovich O, Gurwitz D, Eldar-Finkelman H. Inhibition of glycogen synthase kinase-3beta by bivalent zinc ions: insight into the insulin-mimetic action of zinc. Biochem Biophys Res Commun 2002;295:102–106
34. Tang X, Shay NF. Zinc has an insulin-like effect on glucose transport mediated by phosphoinositol-3-kinase and Akt in 3T3-L1 fibroblasts and adipocytes. J Nutr 2001;131:1414–1420
35. Xu J, Wang G, Wang Y, et al. Diabetes- and angiotensin II-induced cardiac endoplasmic reticulum stress and cell death: metallothionein protection. J Cell Mol Med 2009;13(8A):1499–1512