TRB3 Gene Silencing Alleviates Diabetic Cardiomyopathy in a Type 2 Diabetic Rat Model

Yun Ti, Guo-Lu Xie, Zhi-hao Wang, Xiao-lei Bi, Wen-yuan Ding, Jia Wang, Gui-hua Jiang, Pei-li Bu, Yun Zhang, Ming Zhong, and Wei Zhang

OBJECTIVE—Tribbles 3 (TRB3) is associated with insulin resistance, an important trigger in the development of diabetic cardiomyopathy (DCM). We sought to determine whether TRB3 plays a major role in modulating DCM and the mechanisms involved.

RESEARCH DESIGN AND METHODS—The type 2 diabetic rat model was induced by high-fat diet and low-dose streptozotocin. We evaluated the characteristics of type 2 DCM by serial echocardiography and metabolite tests. Western blot analysis for TRB3 expression, and histopathologic analyses of cardiomyocyte density, lipids accumulation, cardiac inflammation, and fibrosis area. We then used gene silencing to investigate the role of TRB3 in the pathophysiologic features of DCM.

RESULTS—Rats with DCM showed severe insulin resistance, left ventricular dysfunction, aberrant lipids deposition, cardiac inflammation, fibrosis, and TRB3 overexpression. We found that the silencing of TRB3 ameliorated metabolic disturbance and insulin resistance; myocardial hypertrophy, lipids accumulation, inflammation, fibrosis, and elevated collagen I-to-III content ratio in DCM rats were significantly decreased. These anatomic findings were accompanied by significant improvements in cardiac function. Furthermore, with TRB3 gene silencing, the inhibited phosphorylation of Akt was restored and the increased phosphorylation of extracellular signal–related kinase 1/2 and Jun NH2-terminal kinase in DCM was significantly decreased.

CONCLUSIONS—TRB3 gene silencing may exert a protective effect on DCM by improving selective insulin resistance, implicating its potential role for treatment of human DCM.

From the 1Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Chinese Ministry of Health, Qilu Hospital of Shandong University, Jinan, Shandong Province, China; and the 2Department of Cardiology, Qilu Hospital of Shandong University, Jinan, Shandong Province, China.

Corresponding author: Wei Zhang, zhangweisdu@gmail.com, or Ming Zhong, zhongmingzm@gmail.com.

Received 25 April 2011 and accepted 18 August 2011.

DOI: 10.2337/db11-0549

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db11-0549/-/DC1.

© 2011 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See http://creativecommons.org/licenses/by-nc-nd/3.0/ for details.

Diabetic cardiomyopathy (DCM), which occurs in patients with diabetes, carries a substantial risk concerning the subsequent development of heart failure and increased mortality (1). Different pathophysiological stimuli are involved in its development and mediate tissue injury leading to left ventricle (LV) systolic and diastolic dysfunction. Insulin resistance is considered to play a causal role in the pathogenesis and development of DCM (2–4). Insulin resistance is associated with increased LV mass (5) and deterioration of LV diastolic function (6). However, the underlying relevance of insulin resistance leading to altered myocardial structure remains incompletely understood.

Tribbles 3 (TRB3) is a pseudokinase with increased activity in response to fasting that binds to and inhibits the activation of the serine-threonine kinase Akt in the liver (7,8). Indeed, humans with a gain-of-function mutation in TRB3 show increased insulin resistance and diabetes-associated complications (9,10). These observations have led to the suggestion that TRB3 elevation contributes to insulin resistance. TRB3 also serves as a molecular switch and regulates the activation of the three classes of mitogen-activated protein kinases (MAPKs) (11). TRB3 binds to and regulates MAPK kinase, thus controlling the activation of MAPK by incoming signals (11). However, the TRB3/MAPK signaling pathway has not been investigated in vivo on cardiac tissues directly.

Akt and MAPK are the most important pathways involved in selective insulin resistance (12), and activated MAPK contributes to the development of cardiac fibrosis (13–15). Thus, we hypothesized that upregulated TRB3 induced by insulin resistance might participate in the pathophysiological process of DCM. First, we established the type 2 DCM model and determined the relationships among TRB3 expression, cardiac remodeling, and cardiac function in the model. To further elucidate the role of TRB3 in DCM, we used TRB3 gene silencing in vivo to explore the mechanisms of TRB3 in DCM as a potential target for treatment.

RESEARCH DESIGN AND METHODS

Induction of diabetes. Sixty male Sprague–Dawley rats (120–140 g) were purchased from the experimental animal center of Shandong University of Traditional Chinese Medicine (Jinan, China). The animals were housed at 22°C with 12-h light-dark cycles. After 1 week of acclimatization, intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IPITT) were performed. The rats were then randomized into four groups: control, chow + streptozotocin (STZ), high-fat (HF) diet, and diabetes . HF and diabetic groups were fed an HF diet (34.5% fat, 17.5% protein, 48% carbohydrate; Beijing HFK Bio-Technology, China), and the other two groups received normal chow. Four weeks later, IPGTT and IPITT were performed again, and blood was sampled through the jugular vein. Fasting blood glucose (FBG) and fasting insulin were measured, and the insulin sensitivity index [SI = ln(FBG × fasting insulin)1/2] was calculated. Diabetes was induced by a single intraperitoneal injection of STZ (Sigma, St. Louis, MO; 27.5 mg/kg i.p. in 0.1 mol/L citrate buffer, pH 4.5) to rats with insulin resistance. Rats in the chow + STZ group received the same dose of STZ. The control and HF groups received citrate buffer (intraperitoneally) alone. One week after STZ administration, rats with FBG >11.1 mmol/L in two consecutive analyses were considered the diabetic rat model. After 16 weeks of diabetes, rats were killed. All experimental procedures were performed in accordance with animal protocols approved by the Shandong University Animal Care Committee.

IPGTT and IPITT. Glucose tolerance was assessed by IPGTT after rats fasted for 12 h. A bolus of glucose (1 g/kg i.p.) was injected, and blood samples were collected sequentially from the tail vein at 0, 15, 30, 60, and 120 min and tested for glucose. Plasma glucose was measured with a One-Touch Glucometer (LifeScan, Milpitas, CA). The mean area under the receiver operating characteristic curve (AUC) was calculated for glucose.
To evaluate insulin tolerance, IPTTT was performed after rats fasted for 4 h. A bolus of insulin (1 unit/kg i.p.) was administered, and blood samples were taken for glucose measurement as described above.

**Blood analyses.** After rats fasted overnight, we collected jugular blood. Total cholesterol, triglyceride levels, and FBG were analyzed with use of the Bayer 1650 blood chemistry analyzer (Bayer, Tarrytown, NY). Free fatty acid (FFA) concentrations were measured using an enzymatic test kit (CSB-B05770r; HuaMei BIO-TECH, Wuhan, China). Fasting insulin level was measured by enzyme-linked immunosorbent assay. ISI was calculated.

**Echocardiography and measurement of blood pressure.** Echocardiography involved use of the Vevo770 imaging system (VisualSonics, Toronto, Canada). Images were obtained from two-dimensional, M-mode, pulsed-wave Doppler and tissue Doppler imaging. All measurements were performed by the same observer and were the average of six consecutive cardiac cycles. Wall thickness and LV dimensions were obtained from a long-axis view at the level of chordae tendineae. Diameters of left ventricular end diastole, left ventricular end-diastolic posterior wall, and septum thickness, as well as left ventricular ejection fraction (LVEF) and fractional shortening (FS), were measured according to the American Society of Echocardiography guidelines (16). The mitral-valve pulsed Doppler recordings were obtained from the apical four-chamber view. After pulsed Doppler, we evaluated transmural flow velocity variables, including peak E, peak A, and the E-to-A ratio. Isovolumetric contraction time, isovolumetric relaxation time, and ejection time were measured and were used to calculate the Tei index (Tei index = Isovolumetric contraction time + isovolumetric relaxation time/ejection time). The tissue Doppler imaging of the mitral annulus was obtained from the apical four-chamber view. We analyzed early (E') and late (A') diastolic velocity and calculated E'/A' and E/A.

For quantitative analysis of integrated backscatter (IBS) of the LV, we used a commercially available software package (acoustic densitometry; Phillips Medical Systems, Netherlands) (17). Two-dimensional echocardiographic images, including LV long-axis and apical four-chamber views, were obtained. The following variables were measured: time-averaged IBS, cyclic variation of integrated backscatter (CVIB), and standardized BIs (IBS%).

Heart rate, systolic blood pressure, diastolic blood pressure, and mean arterial pressure were measured with a noninvasive tail-cuff system (Softron, Tokyo, Japan). Data were expressed as micrograms collagen per milligram dry weight, with the assumption that collagen contains an average of 13.5% hydroxyproline (21).

**Western blot analysis.** Western blot analysis was described previously (22). We used antibodies against TRB3 (Calbiochem, La Jolla, CA), collagen I, collagen III, TNF-α, and IL-6 antibodies (Abcam, Cambridge, MA) overnight, and then with a matching biotinylated secondary antibody for 30 min at 37°C. Negative controls were omission of the primary antibody. The stained sections were developed with horseradish peroxidase-labeled antibody (Santa Cruz Biotechnology, Dallas, TX). Densitometric analysis was performed using the Quantity One software (version 1.4.2, Bio-Rad, Hercules, CA). The area of the bands was normalized against the loading control (actin). The relationship between the area of the bands of target protein and the loading control was used to determine the amount of protein. The mean protein level was normalized to the control.

**Immunohistochemical staining.** Paraffin sections underwent immunohistochemical analysis, with a microwave-based antigen retrieval method. The sections were incubated with primary rabbit polyclonal antibodies (1:1000). The stained sections were developed with diaminobenzidine (Prolab Diagnostics, Kitchener, ON) as the chromogen. Sections were counterstained with hematoxylin. The results were viewed under a conventional light microscope.
kinase (ERK)/ERK, p-p38/p38 MAPK, p-Jun NH2-terminal kinase (JNK)/JNK, and p-Akt/Akt (Cell Signaling Technology, Beverly, MA), followed by anti-IgG horseradish peroxidase–conjugated secondary antibody. TRB3, collagen I, collagen III, TNF-α, and IL-6 protein levels were normalized to that of β-actin as an internal control and phosphospecific proteins to that of total protein.

Gene silencing of TRB3. Sixty rats were randomized to receive TRB3 small interfering RNA (siRNA) or vehicle treatment. Gene silencing occurred immediately after the appearance of LV diastolic dysfunction. After 12 weeks of diabetes, both E/A and E9/A9 were increased as assessed by echocardiography in diabetic rats. Animals were then injected via the jugular vein with 2.5 × 10^10 plaque-forming units of an adenovirus harboring TRB3 gene (TRB3-siRNA) or a control empty virus (vehicle). Adenovirus transfer was repeated in 2 weeks. According to our present and previous studies (23), TRB3 level was increased in HF diet–fed and high fructose–fed rats. Previous studies have shown that insulin resistance was a hallmark of obesity (24) and metabolic syndrome (25). In light of the interaction between TRB3 and insulin resistance, we also investigated the effect of TRB3-siRNA on HF diet–induced cardiac injury. Four weeks after the first adenovirus injection, rats were killed. The heart was excised and weighed.

Statistical analysis. Values are presented as mean ± SEM. SPSS 16.0 (SPSS, Chicago, IL) was used for statistical analysis. Results were compared by one-way ANOVA, followed by Tukey–Kramer post hoc test and independent samples t test. A P < 0.05 was considered statistically significant.

RESULTS

Generation of type 2 DCM model

General characteristics of diabetic rats. As expected, the rats in the diabetic group had the highest values of water intake, food intake, and urine volume compared with the other three groups (P < 0.01; P < 0.001). Heart weight was significantly higher in the diabetic group than in the control and chow + STZ groups (P < 0.01) (Supplementary Table 1).

Glucose and insulin tolerance. After a 4-week HF diet, insulin resistance was confirmed by IPGTT and IPTT. By IPGTT, the levels of blood glucose in the diabetic group were significantly higher at week 4 than at baseline at all of the time points tested except at 15 min (Supplementary Fig. 1A1). The AUC across the time for glucose level was higher at week 4 than at baseline (25.3 ± 1.27 vs. 21.09 ± 0.73, respectively; P < 0.05) (Supplementary Fig. 1A2). Similarly, IPTT revealed impaired insulin sensitivity

FIG. 2. Deterioration of cardiac function in diabetic (DM) rats. A1: Representative two-dimensional echocardiograms. A2: Representative M-mode echocardiograms. A3: Representative pulsed-wave Doppler echocardiograms of mitral inflow. A4: Representative tissue Doppler echocardiograms. B–F: Sequential evaluations of left ventricular end diastolic diameter (LVEDd) (B), LVEF (C), FS (D), E/A (E), and E9/A9 (F). G: Electrocardiogram of control and DM groups at the end of the experiment. H: Pressure curves of cardiac catheterization. I: Analysis of LVEDP. Data are mean ± SEM; n = 7–10 per group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control; †P < 0.05 vs. HF. W, weeks. (A high-quality digital representation of this figure is available in the online issue.)
Thus, the diabetic group showed insulin resistance after a 4-week HF diet. At the end of the experiment, the diabetic group showed impaired glucose tolerance on IPGTT compared with the other three groups; blood glucose levels especially were significantly elevated at all time points compared with the control ($P < 0.05$) (Supplementary Fig. 1B1). The diabetic group showed the highest mean AUC on both IPGTT and IPITT (Supplementary Fig. 1B2 and B4).

**Total cholesterol, triglyceride, FFA, and FBG concentrations.** The biochemical values are given in Fig. 1. After 4 weeks of a HF diet, serum triglyceride and FFA levels were significantly higher in the HF and diabetic groups than in the control and chow + STZ groups ($P < 0.05$). The ISI was markedly decreased in the HF and diabetic groups ($P < 0.05$). Insulin resistance appeared at week 4 in rats fed a HF diet. One week after STZ injection, FBG was remarkably elevated in the diabetic group and remained elevated until the end of the experiment. ISI consistently decreased in the diabetic group after the onset of diabetes. Simultaneously, in the diabetic group, serum total cholesterol, total triglyceride, and FFA levels were maintained at higher levels than the control ($P < 0.05$) during diabetes. Thus, the diabetic model induced by a HF diet and low-dose STZ was characterized by insulin resistance, moderate hyperglycemia, and hyperlipidemia, resembling the state of chemical diabetes in humans.

**LV dysfunction assessed by echocardiography and catheterization.** We evaluated ejection fraction, FS, E/A, and E'9/A' to investigate changes in systolic and diastolic
function. Similar to the pattern in humans, in rats, diastolic dysfunction precedes systolic dysfunction, beginning from 2 to 3 months after the induction of diabetes (26). In our study, at 6 weeks after the onset of diabetes (at week 11), the diabetic rats showed a moderate decrease in E/A and E/’A/’ compared with the control ($P < 0.05$) (Fig. 2E and F); LVEF and FS were impaired from week 17 (Fig. 2C and D). At the end of the experiment, LVEF, FS, E/A, and E/’A/’ were further decreased in the diabetic group, with the reduction in E/A and E/’A/’ more pronounced. LVEF, FS, E/A, and E/’A/’ were also reduced in the chow + STZ group compared with the control ($P < 0.05$) (Fig. 2C–F) at the end of the experiment. Left ventricular end-diastolic diameter was the highest in the diabetic group (Fig. 2B).

To further confirm the LV diastolic dysfunction, LVEDP was measured by cardiac catheterization. Diabetic rats had the highest LV pressure (Fig. 2H and I). The chow, STZ, and HF groups showed higher LV pressure compared with the control ($P < 0.01$ to $P < 0.001$) (Fig. 2H and I). In summary, both systolic and diastolic dysfunction developed and progressed during DCM, with predominant deterioration of diastolic function.

**Pathology characteristics of diabetic rats.** The heart weight to body weight ratio was 25% higher in the diabetic group than the control group ($P < 0.01$) (Fig. 3B), and LV myocyte size was 40 and 31% higher, respectively, than the control and HF groups ($P < 0.001$) (Fig. 3C). Furthermore, the relative mRNA expression of brain natriuretic protein, a marker of LV hypertrophy (27), was higher in diabetic than control and HF rats ($P < 0.05$) (Fig. 3D).

The diabetic group showed cardiac fibrosis, with a diffuse, small, patchy, and nonuniform pattern, as well as destroyed and disorganized collagen network structure in the interstitial (Fig. 3E1–E3) and perivascular (Fig. 3E4–E6) areas. CVF and collagen content were higher in the diabetic group than the other groups ($P < 0.05$ to $P < 0.001$) (Fig. 3F and G), as was PVCA/LA ($P < 0.001$) (Fig. 3H). These histological changes were confirmed by echocardiographic

**FIG. 4.** Diabetic (DM) rats showed excessive myocardial lipids deposition and inflammation. A: Representative Oil Red O–stained myocardial sections (scale bar: 20 μm). B: Semi-quantification of Oil Red O staining. Data are mean ± SEM of six independent observations in each group. C and D: Relative mRNA expression of myocardial TNF-α and IL-6. E: Immunohistochemical staining for myocardial TNF-α and IL-6 (brown staining considered positive staining; scale bar: 50 μm). F: Representative Western blot of myocardial TNF-α and IL-6. G and H: Western blot analyses of TNF-α (G) and IL-6 (H). Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control; †$P < 0.01$, ‡$P < 0.001$ vs. HF; #P < 0.05, ##P < 0.01 vs. chow + STZ. (A high-quality digital representation of this figure is available in the online issue.)
results. The diabetic group showed increased IB% and decreased CVIB in the interventricular septum and LV posterior and lateral walls compared with the control and HF groups (\(P < 0.05\)) (Supplementary Table 2). Similarly, CVF, collagen content, and PVCA/LA were significantly increased in the chow + STZ and HF groups compared with the control (\(P < 0.01\); \(P < 0.001\)) (Fig. 3F–H).

Coincident with cardiac dysfunction and hypertrophy, myocardial lipid analysis revealed striking myocardial accumulation of triglycerides in diabetic rats (Fig. 4A and B). Diabetic rats had higher Oil Red O-staining areas than other groups (Fig. 4B). The mRNA expression levels of TNF-\(\alpha\) and IL-6 were significantly higher in the diabetic group compared with the control (\(P < 0.05\); \(P < 0.01\)) (Fig. 4C and D). The protein expression levels of TNF-\(\alpha\) and IL-6 were significantly increased in the diabetic group compared with the other three groups (Fig. 4E–H). Likewise, the protein expression of TNF-\(\alpha\) and IL-6 content was increased in the chow + STZ and HF groups compared with the control (\(P < 0.01\) to \(P < 0.001\)) (Fig. 4F–H).

Immunohistochemistry and Western blot analysis revealed the protein expression of collagen I and III content increased in the diabetic group (Fig. 5A and B), and the collagen I-to-III ratio significantly elevated compared with the control group (216 ± 16% vs. 100 ± 6%, respectively; \(P < 0.001\)) (Fig. 5C). Likewise, the protein expression of collagen I and III content was increased in the chow + STZ and HF groups compared with the control (\(P < 0.01\) to \(P < 0.001\)) (Fig. 5A–C).

These results show the established type 2 DCM model, with insulin resistance, severe LV dysfunction, and myocardial remodeling.
**Activated TRB3/MAPK signaling pathway in DCM.** Cardiac TRB3 mRNA and protein expression was significantly increased in diabetic rats (Fig. 5D). We detected Akt expression in the myocardium. The phosphorylation of Akt was significantly lower in the diabetic group than in the control and HF groups (Fig. 5E).

Accompanied by TRB3 overexpression, the phosphorylation of ERK1/2 and JNK was markedly increased, whereas that of p38 MAPK was decreased (Fig. 5E), which suggested that the TRB3/MAPK signaling pathway participates in the pathogenesis of DCM.

**TRB3 gene silencing reverses DCM**

**Detection of cardiac TRB3 expression by Western blot analysis after gene silencing.** When compared with vehicle treatment, TRB3-siRNA treatment conferred downregulated mRNA and protein expression of cardiac TRB3 (Fig. 6A). The mRNA and protein expression of hepatic TRB3 was also downregulated compared with the vehicles (Supplementary Fig. 4).

**TRB3 gene silencing ameliorates metabolism.** Water intake and urine volume were decreased in the diabetic group with TRB3-siRNA treatment (Supplementary Table 3). The elevated serum levels of total cholesterol, triglyceride, FFA, and FBG were greatly reduced after 4-week transfection (Supplementary Fig. 2A–D).

**TRB3 gene silencing improved glucose tolerance and insulin sensitivity.** With TRB3 gene silencing, ISI was higher in the diabetic TRB3-siRNA group than vehicle (25.17 ± 0.14 vs. 25.67 ± 0.14, respectively; P < 0.05) (Supplementary Fig. 2F). With TRB3-siRNA treatment, IPGTT and IPITT results showed a blood glucose level significantly lower for TRB3-siRNA–treated rats than the vehicle group (P < 0.05) (Supplementary Fig. 3A and B). The AUC for glucose and insulin was lower for the diabetic than the vehicle group (P < 0.05) (Supplementary Fig. 3C and D).
Recovery of cardiac function in DCM after TRB3 silencing. During the 4-week follow-up after TRB3-siRNA treatment, FS and E’/A’ were improved in the diabetic group compared with the vehicle group (FS, 37.66 ± 1.09 vs. 34.83 ± 0.57, P < 0.05; E’/A’, 1.08 ± 0.11 vs. 0.67 ± 0.03, P < 0.01) (Fig. 6D and G). This suggests that TRB3-siRNA treatment prevented LV dysfunction. The decreased LVEDP in the TRB3-siRNA diabetic group compared with the vehicle group (12.93 ± 1.77 vs. 17.77 ± 1.29 mmHg, respectively; P < 0.01) (Fig. 6I) reconfirmed the beneficial effect of TRB3-siRNA on improving LV diastolic dysfunction. Likewise, the LVEDP was reduced in the TRB3-siRNA HF group compared with the vehicle group (P < 0.05) (Fig. 6I).

TRB3 gene silencing reverses myocardial remodeling, lipids accumulation, and cardiac inflammation in DCM. With TRB3-siRNA treatment, heart weight-to-body weight ratio and myocyte size were significantly decreased in the diabetic versus the vehicle group (heart weight-to-body weight ratio: 2.67 ± 0.13 vs. 3.49 ± 0.36 mg/g, P < 0.05; myocyte size: 384.32 ± 8.69 vs. 440.14 ± 4.47 μm², P < 0.01) (Fig. 7B and C). The mRNA expression of brain natriuretic protein was lower in both the HF and diabetic groups than in the vehicle control (P < 0.001) (Fig. 7D). CVF and PVCA/LA were reduced (P < 0.001, P < 0.01) (Fig. 7F and H). In addition, collagen content was lower in the diabetic group than the vehicle group (10.17 ± 0.41 vs. 13.84 ± 0.48 µg/mg LV weight, respectively; P < 0.001) (Fig. 7G). Likewise, CVF and PVCA/LA were decreased in the TRB3-siRNA HF group (P < 0.05, P < 0.01) (Fig. 7F and H). In keeping with these observations, the decreased IB% and increased CVIB acquired from the IBS of the LV reconfirmed the improved pathological manifestations (P < 0.05 – P < 0.001) (Supplementary Table 4).

The aberrant lipids accumulation within cardiomyocytes in diabetic rats was markedly reduced after TRB3 silencing (P < 0.001) (Fig. 8A and B). Immunohistochemical staining and Western blot revealed a significant decrease of TNF-α and IL-6 content in the TRB3-siRNA diabetic group compared with those of the vehicle (P < 0.001) (Fig. 8E–H). Similarly, lipids accumulation and cardiac inflammation were reduced in the TRB3-siRNA HF group (P < 0.01, P < 0.001) (Fig. 8B and E–H).
were lower in the diabetic than vehicle group \((P < 0.001)\) (Fig. 9B and C). The decrease in collagen III content was not as great as that of collagen I \((P < 0.01)\) (Fig. 9B and C).

The inhibited phosphorylation of p-Akt in the diabetic group was restored after TRB3 silencing (Fig. 9D).

With TRB3 silencing, the phosphorylation of ERK1/2 and JNK was abolished by 53 and 46%, respectively, in the diabetic group (Fig. 9D); however, the phosphorylation of p38 MAPK was enhanced compared with the vehicle group (Fig. 9D). Thus, TRB3 gene silencing appeared to have differential effects on ERK1/2, JNK, and p38 MAPK: inhibiting ERK1/2 and JNK activation (with a more pronounced effect on ERK1/2) but activating p38 MAPK.

**DISCUSSION**

TRB3 is associated with insulin resistance, an important trigger in the development of DCM. We found TRB3 was involved in the pathogenesis of DCM. TRB3 gene silencing alleviated DCM mainly by modulating MAPK and Akt pathways in type 2 diabetes. Thus, TRB3 has a pivotal role in DCM and could be an attractive drug target for treating type 2 diabetes.

**TRB3, a critical mediator for insulin resistance, is implicated in DCM.** The insulin-Akt signaling pathway regulates mitogenic effects in virtually all metabolic tissues, and proteins that inhibit Akt activity are considered potential inducers of insulin resistance (28). An example of such a protein is the catalytically inactive pseudokinase TRB3 (7). TRB3 is overexpressed in murine models of insulin resistance (7,8). In the current study, diabetic rats showed severe insulin resistance and increased TRB3 level. Akt activation was inhibited. These findings are in accordance with previous studies (7,8,28), which indicates insulin resistance exists in our type 2 diabetic rat model at the molecular level.
In an insulin-resistant state, the phosphatidylinositol 3-kinase/Akt pathway appears to be selectively blunted, compared with the fully activated MAPK pathway (29). This situation is known as pathway-selective insulin resistance (12). Kiss-Toth et al. (11) reported that TRB3 might be an important modulating protein of MAPK. We found TRB3 markedly upregulated in rats with diabetes, which was paralleled by increased ERK1/2 and JNK expression. Diabetic rats showed prominent collagen accumulation in interstitial and perivascular areas on Masson trichrome and Picrosirius red staining. Increased cardiac fibrosis, which can increase LV stiffness, is a common hallmark of DCM (30). Therefore, our diabetic rats showed severe DCM, which was further confirmed by elevated LVEDP. Previous studies have shown that activated MAPK plays an essential role in collagen synthesis and cardiac fibrosis (15,31). Therefore, activation of TRB3 may contribute to the development and progression of DCM, possibly mediated by the MAPK pathway.

Together, these data suggest a pivotal role for TRB3 in selective insulin resistance by modulating Akt and MAPK pathways and implicated in the development of DCM. 

**Effect of TRB3 silencing on DCM mediated by MAPK and Akt pathways.** In light of the pivotal role of TRB3 in the development of DCM, we wondered whether downregulation of TRB3 could reverse the progression of DCM. According to our previous study (14), we used TRB3-siRNA in vivo in rats. TRB3-siRNA treatment produced no notable adverse effects and no deaths in rats. TRB3 mRNA levels were significantly decreased, and the protein expression was reduced by ~60% with TRB3-siRNA treatment in diabetic rats. Thus, global silencing of TRB3 was feasible and effective.

With TRB3 silencing, the impaired glucose tolerance and decreased insulin sensitivity were improved, and the inhibited activation of Akt was restored. So TRB3 silencing could improve insulin resistance and lower blood glucose tolerance.
glucose, which was attributed to increased phosphorylation of Akt (32).

In addition to improving metabolic disturbance, TRB3 silencing reduced the aberrant interstitial and perivascular collagen accumulation, as well as total collagen content. Echocardiography and catheterization results revealed that the LV diastolic dysfunction was improved, which was attributed to decreased cardiac fibrosis and reduced collagen I-to-collagen III ratio (33). Thus, TRB3 silencing attenuated DCM.

As discussed above, the MAPK pathway likely mediated TRB3-related cardiac fibrosis. Intriguingly, we found a generalized decrease in phosphorylation of JNK and especially ERK1/2 in with TRB3 silencing. Combined with previous studies (12,14,34), the improvements in cardiac fibrosis were primarily attributed to the decreased activation of ERK1/2. So TRB3 is an effective target to ameliorate DCM mainly through the ERK/MAPK pathway.

With silencing of TRB3, enhanced phosphorylation of ERK/MAPK was suppressed, whereas the impaired Akt activity was upregulated. These alterations resulted in overall improvement of selective insulin resistance. In accordance with these changes at the molecular level, TRB3 silencing attenuated the cardiac fibrosis and metabolic disturbance in type 2 diabetic rats.

Furthermore, lipids accumulation (35) and inflammation (36) in the cardiac tissue play a pivotal role in the pathophysiology of DCM. In the current study, we also referred to these aspects. TRB3 silencing reduced the aberrant lipids deposition and cardiac inflammation. However, the underlying mechanisms remain unclear. Further studies have to reveal the impact of TRB3 silencing on these effects.

Conclusions. TRB3, as a critical regulator for selective insulin resistance, was implicated in DCM. The cardioprotective effects with TRB3 silencing suggest a potential role for TRB3 inhibitors in treating DCM in type 2 diabetes.

ACKNOWLEDGMENTS

This work was supported by the research grants from Key Technologies R & D Program of Shandong Province (No. 2006GG2202020 and 2010GG0020262), the Natural Science Foundation of Shandong Province (ZR2006CM022, ZR2009CM025, and BS2009YY026), the National Natural Science Foundation of China (30670874, 30871038, 30971215, 81070192, and 81070141), and the National Basic Research Program of China (973 Program, grant 2009CB521904).

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

Y.T. and G.-L.X. researched data and wrote the manuscript. Z.-H.W. researched data and contributed to discussion. X.-J.B., W.-Y.D., J.W., and G.-H.J. researched data. P.-L.B., M.Z. and W.Z. wrote and reviewed and edited the manuscript. Y.Z. reviewed and edited the manuscript.

REFERENCES

34. Olson ER, Shamhart PE, Naugle JE, Meszaros JG. Angiotensin II-induced extracellular signal-regulated kinase 1/2 activation is mediated by protein kinase Cdelta and intracellular calcium in adult rat cardiac fibroblasts. Hypertension 2008;51:704–711