Activation of Foxo1 by Insulin Resistance Promotes Cardiac Dysfunction and β–Myosin Heavy Chain Gene Expression

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Background—Heart failure is a leading cause of morbidity and mortality in the USA and is closely associated with diabetes mellitus. The molecular link between diabetes mellitus and heart failure is incompletely understood. We recently demonstrated that insulin receptor substrates 1, 2 (IRS1, 2) are key components of insulin signaling and loss of IRS1 and IRS2 mediates insulin resistance, resulting in metabolic dysregulation and heart failure, which is associated with downstream Akt inactivation and in turn activation of the forkhead transcription factor Foxo1.

Methods and Results—To determine the role of Foxo1 in control of heart failure in insulin resistance and diabetes mellitus, we generated mice lacking Foxo1 gene specifically in the heart. Mice lacking both IRS1 and IRS2 in adult hearts exhibited severe heart failure and a remarkable increase in the β-isofrom of myosin heavy chain (β-MHC) gene expression, whereas deletion of cardiac Foxo1 gene largely prevented the heart failure and resulted in a decrease in β-MHC expression. The effect of Foxo1 deficiency on rescuing cardiac dysfunction was also observed in db/db mice and high-fat diet mice. Using cultures of primary ventricular cardiomyocytes, we found that Foxo1 interacts with the promoter region of β-MHC and stimulates gene expression, mediating an effect of insulin that suppresses β-MHC expression.

Conclusions—Our study suggests that Foxo1 has important roles in promoting diabetic cardiomyopathy and controls β-MHC expression in the development of cardiac dysfunction. Targeting Foxo1 and its regulation will provide novel strategies in preventing metabolic and myocardial dysfunction and influencing MHC plasticity in diabetes mellitus. (Circ Heart Fail. 2015;8:198-208. DOI: 10.1161/CIRCHEARTFAILURE.114.001457.)

Key Words: diabetic cardiomyopathies • Forkhead transcription factors • insulin receptor substrate-1 • insulin receptor substrate-2 • insulin resistance

Heart failure is a clinical syndrome evidenced by decreased ability of the heart to provide sufficient cardiac output to support the normal functions of peripheral tissues, resulting from structural and functional impairment of ventricular filling or ejection of blood. Given the prevalence of type 2 diabetes mellitus and obesity, insulin resistance is a significant risk factor promoting cardiac dysfunction. Two thirds of patients with type 2 diabetes mellitus have died of heart failure during the past decades. Diabetic cardiomyopathy was initially referred to as hyperglycemia-induced heart failure independent from hypertension and coronary artery diseases. Insulin is a primary and effective therapy to decrease hyperglycemia and reduce the risk of cardiovascular dysfunction in patients with type 1 diabetes mellitus. However, intensive insulin therapy lowers blood glucose levels but increases body weight and cardiovascular risk in patients with type 2 diabetes mellitus, and hyperinsulinemia itself can result in insulin resistance. Thus, insulin resistance is recognized as an important mechanism for cardiac dysfunction. The molecular mechanisms of cardiac dysfunction from insulin resistance and diabetes mellitus include increased oxidative stress, altered substrate metabolism, mitochondrial dysfunction, activation of sympathetic nervous activity and the renin angiotensin system, and impaired calcium homeostasis, but the molecules responsible for insulin action, resistance, and association with cardiac dysfunction are incompletely understood.

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The heart is responsive to insulin that activates the insulin receptor on the cell membrane. The insulin receptor has tyrosine kinase activity and phosphorylates and recruits IRS1 to 4 and other scaffold proteins, including SHC, CBL, APS, SH2B, GAB1, and DOCK1, that trigger downstream signaling cascades, including phosphatidylinositol-3-kinase (PI3K)
and mitogen-activated protein (MAP) kinases. Activation of PI3K generates phosphatidylinositol (3,4,5)-triphosphate, recruiting the 3-phosphoinositide–dependent protein kinase-1 and 2 (PDK1 and PDK2) and Akt to the plasma membrane, where Akt is activated by PDK1-mediated phosphorylation at T308 and PDK2-mediated phosphorylation at S473. Akt phosphorylates downstream targets, including inhibitors of macromolecular synthesis, such as glycogen synthase kinase-3β (glycogen synthesis), tuberous sclerosis protein-2 and p70S6K (protein synthesis), and forkhead transcription factor Foxo1 (gene transcription). Foxo1, a member of the forkhead/winged helix transcription factor family, was first identified as an Akt substrate for phosphorylation. Recently, we have demonstrated that insulin receptor substrate-1, 2 (IRS1, 2) are major mediators of insulin in the activation of PI3K and Akt in mouse liver and heart. Loss of IRS1 and IRS2 in the liver resulted in hyperglycemia and hyperinsulinemia, and loss of IRS1 and IRS2 in the heart prevented endogenous PI3K and Akt activation, promoted Foxo1 activation, and resulted in cardiac failure. Moreover, reduced IRS1 and IRS2 gene expression and functionality and activation of Foxo1 are widely present in the heart of animals with insulin resistance or type 2 diabetes mellitus. Thus, loss of IRS1 and IRS2 and associated Akt inactivation and Foxo1 activation may provide a fundamental mechanism for insulin-resistant cardiomyopathy. In this study, we hypothesize that activation of Foxo1, after inhibition of IRS1 and IRS2, insulin resistance, and type 2 diabetes mellitus, has a central role in promoting cardiac dysfunction and expression of β-MHC gene, a heart failure marker involving cardiac contractile dysfunction.

Methods

Mice

All animal experiments were performed following procedures approved by the Texas A&M Health Science Center Institutional Animal Care and Use Committee. The floxed IRS1 mice (IRS1L/L), IRS2 mice (IRS2L/L), and Foxo1 (Foxo1L/L) were described previously. MHC-merCremer, MHC-Cre, and db/+ mice were purchased from Jackson Laboratory. All mice on a C57BL/6 and 129 Sv mixed background were maintained on regular chow (Prolab Isopro 5P76). The high-fat diet (HFD) mice were fed with chow (Research Diet, D12451) for 6 months, after 12 weeks of age. The HFD consists of 45% calories from fat, 25.6% carbohydrate, and 16.4% protein and the normal diet contains 11.4% fat, 62.8% carbohydrate, and 25.8% protein. For streptozotocin-induced diabetes mellitus, adult male mice were intraperitoneally injected with streptozotocin (50 mg/kg of body weight per day; Zanosar) for 5 consecutive days. Control mice were injected with the same volume of vehicle (0.1 mol/L sodium citrate). Male mice were used at the age of 1 to 8 months in all experiments, as indicated.

Chemicals and Antibodies

Insulin, wortmannin, PD98059, SP600125, and β-MHC antibody (cat#M8421) were purchased from Sigma. Antibodies against Foxo1 (cat#9454), pFoxo1-S256 (cat#9461), Akt (cat#2972), pAkt-S473 (cat#9271), and α-actin (cat#6498) were from Cell Signaling Technology (Danvers, USA). IRS1 (cat#606–248) and IRS2 (cat#MAB51S) were from EMD Millipore. Foxo1 antibody used for chromatin immunoprecipitation was from Santa Cruz Biotech Inc (cat#sc11350, Dallas, USA).

Echocardiography

Echocardiograms were performed on anaesthetized mice using a VisualSonics Vevo 2100 system, equipped with a 40-MHz linear probe. Left ventricular end-diastolic diameter and left ventricular end-systolic diameter were measured from eight 2-dimensional short axis views of M-mode recordings, as we previously described. Fractional shortening (FS) was calculated as (left ventricular end-diastolic diameter–left ventricular end-systolic diameter)/left ventricular end-diastolic diameter and expressed as a percentage.

Electron Microscope

Hearts were fixed in 2.5% formaldehyde/glutaraldehyde with 0.1-mol/L sodium cacodylate buffer pH 7.4 overnight, followed by osmication and uranyl acetate staining, dehydration in alcohols and embedded in Taab 812 Resin (Marivac Ltd, Nova Scotia, Canada). Eighty-nanometer sections were cut with a Leica ultratrac microscope, picked up on 300 mesh formvar/carbon-coated Cu grids, stained with 0.2% lead citrate and viewed and imaged under the Philips Technai BioTwin Spirit Electron Microscope, as we previously described.

Glucose and Insulin Measurements

Blood samples were collected from mice, blood glucose measured with a glucometer (Elite, XL, Byer), and serum analyzed for insulin, using a commercial kit (Crystal Chem, Inc).

Protein Analysis and Western Blot

Tissue or cellular proteins were prepared, resolved by SDS-PAGE, and transferred to nitrocellulose membrane for immunoblotting analysis, using specific antibodies and signal intensity measured and analyzed by NIH Image J software, as previously described.

Isolation and Cell Culture of Primary Cardiomyocyte and Cardiomyoblast

Primary cultures of neonatal rat ventricular cardiomyocytes (NRVMs) were prepared from hearts of 1- to 2-day-old Sprague–Dawley rat pups, as previously described.

Adenovirus Infection of Cardiomyocytes

NRVMs were cultured in DMEM/M199 medium with serum for 48 hours, and then transfected by adding adenovirus to the culture medium with a dose as indicated as multiplicity of infection. Cells were infected with adenovirus expressing green fluorescent protein or green fluorescent protein–fused-Foxo1wild-type, as previously described. After 8 hours of infection, cells were changed to fresh medium with serum for 8 hours and then serum starved for 8 hours, before further treatment or collection of cell lysates.

RNA Isolation and Real-Time Polymerase Chain Reaction Analysis

RNA from hearts or NRVMs was extracted with Trizol reagent (Invitrogen), cDNA synthesis used the SuperScript first-strand synthesis system (Bio-Rad), and quantity of cDNA for each transcript was measured using real-time polymerase chain reaction with the SYBER green supermix (Bio-Rad) and the primer sequences of polymerase chain reaction for α-MHC, β-MHC, Foxo1, and cyclophilin as previously described.
Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assay was performed as described previously.\textsuperscript{23,25} Immunoprecipitated DNA was analyzed by polymerase chain reaction with primers specific to the promoters of the β-MHC gene: 5′-ACCATCTGACATTCTACAGTCT-3′ and 5′-AGAGCTCATCCTTTCTGTCAT-3′.

Statistical Analysis

All results are presented as the mean±SEM and analyzed by ANOVA to determine P values. P<0.05 was considered to be statistically significant, as previously described.\textsuperscript{9,13} Freedom from cardiac death in mice was estimated by the Kaplan–Meier method and compared by the log-rank test.

Results

Generation of Tamoxifen-Inducible Heart-Specific IRS1, IRS2, and Foxo1 Knockout Mice

Deletion of both IRS1 and IRS2 genes in the hearts of double knockout mice (H-DKO mice) resulted in animal sudden death from heart failure, at the ages of 6 to 8 weeks, with cardiac Akt inactivation and Foxo1 activation.\textsuperscript{9} To further assess the role of Foxo1 activation in the adult hearts, we created mouse models of temporally regulated inactivation of IRS1 and IRS2, with or without Foxo1, in adult hearts. We crossed IRS1\textsuperscript{L/L}::IRS2\textsuperscript{L/L} and IRS1\textsuperscript{L/L}::IRS2\textsuperscript{L/L}::Foxo1\textsuperscript{L/L} mice with transgenic mice expressing tamoxifen-inducible Cre-recombinase protein fused to 2 mutant estrogen-receptor ligand-binding domains under control of the α-MHC promoter (α-MHC-merCREmer).\textsuperscript{26} In the resulting IRS1\textsuperscript{L/L}::IRS2\textsuperscript{L/L}::MerCre* (H-DKO-merCREmer) and IRS1\textsuperscript{L/L}::IRS2\textsuperscript{L/L}::Foxo1\textsuperscript{L/L}::MerCre* (H-TKO-merCREmer) mice at the age of 10 weeks (Figure 1A), we administered tamoxifen daily for 5 days. We confirmed by immunoblot analysis that IRS1 and IRS2 protein expression was almost undetectable in the heart of both H-DKO and H-TKO (heart-specific triple IRS1, IRS2, and Foxo1 knockout) mice and that Foxo1 was reduced by 40% in H-TKO mice after the initiation of tamoxifen treatment (Figure 1B). The H-DKO hearts demonstrated diminished Akt-mediated Foxo1 phosphorylation at S\textsuperscript{253}, indicative of Foxo1 activation, and strikingly enhanced the heart failure marker β-MHC gene expression, which is largely attenuated in H-TKO hearts (Figure 1B).

Lethal Heart Failure in H-DKO Mice and Rescue by Foxo1 Deficiency in H-TKO Mice

Without tamoxifen treatment, H-DKO-merCREmer and H-TKO-merCREmer mice survived and were indistinguishable

Figure 1. Cardiac function and myosin heavy chain (MHC) gene expression in hearts of double knockout (H-DKO) mice lacking cardiac insulin receptor substrate (IRS) 1 and IRS2 and heart-specific triple knockout (H-TKO) mice lacking cardiac IRS1, IRS2, and forkhead transcription factor (Foxo1). A, Strategies for generation of H-DKO and H-TKO mice. B, Western blot analysis of protein from hearts. Seven days after mice were treated with tamoxifen, hearts were collected and protein prepared and analyzed using antibodies against IRS1, IRS2, Foxo1, pFoxo1–S\textsuperscript{253}, β-MHC, and α-actinin. Data from 2 different mice per group are shown. C, Kaplan–Meier curves for survival of H-DKO and H-TKO mice (n=16 male mice per group). D, Expression of heart failure marker genes in hearts of mice after 6 months of tamoxifen treatment in control, H-DKO and H-TKO mice. Values represent the mean±SEM (n=6–8; *P<0.05 vs control; #P<0.05 vs H-DKO). E, Ultrastructures of hearts were analyzed by electron microscopy. Heart sections from control, H-DKO, and H-TKO mice after 6 months of tamoxifen treatment were prepared. Representative images of hearts with scale bars for 500 and 100 nm are shown. CNTR indicates control (triple floxed and Cre mice).
Cardiac Inactivation of Foxo1 Rescues Cardiac Dysfunction in db/db and HFD Mice

Foxo1 is persistently activated and mainly located in the nucleus of myocardium in db/db or HFD mice, and cardiac Foxo1 deficiency prevented cardiac dysfunction in HFD mice. To further examine whether cardiac Foxo1 deficiency improves cardiac function in db/db mice, we crossed Foxo1L/L mice with transgenic mice expressing a noninducible Cre-recombinase under control of an α-MHC promoter, as we previously reported, generating heart-specific Foxo1 knockout mice (F1KO) mice. Breeding F1KO mice with db/+ mice resulted in the generation of F1KO in db/db genetic background (Figure 2A). In addition, F1KO and control mice were fed with a HFD for 4 months before cardiac functional analysis.

Cardiac dysfunction in db/db and HFD hearts was clearly evident, when compared with that in control heart, and the dysfunction was significantly improved by Foxo1 deficiency (Figure 2B and 2D): EF and FS were reduced in db/db hearts by 40% and 45%, respectively. However, EF and FS were only reduced by 10% and 8%, respectively, in db/db/F1KO mice when compared with those in control hearts. Similarly, EF and FS were reduced by 32% and 38%, respectively, in HFD hearts when compared with that in control, whereas EF and FS were reduced by 15% and 10%, respectively, in HFD/F1KO mice (Figure 2B and 2D). RNA analysis indicated that β-MHC increased 2.8- and 12.3-fold in the hearts of db/db and HFD mice, respectively, when compared with that in control. However, cardiac Foxo1 deletion in db/db and HFD mice markedly decreased β-MHC expression (Figure 2C and 2E). Together, these results indicate that cardiac Foxo1 activation, after type 2 diabetes mellitus (db/db mice) or insulin resistance (HFD mice), significantly contributes to cardiac dysfunction as well as increases β-MHC gene expression.

in appearance from controls. Death of H-DKO-merCremer mice was observed beginning from 10 weeks after initiation of tamoxifen treatment. Kaplan–Meier analysis showed that the death event was significant higher in H-DKO mice than in H-TKO mice (log-rank test; P<0.05). None of H-TKOMerCremer mice died after 24 weeks of tamoxifen treatment (Figure 1C).

Twenty-four weeks after tamoxifen treatment, cardiac function of surviving H-DKO, H-TKO, and control mice was analyzed by echocardiography. H-DKO mice had a significant reduction in ejection fraction (EF), FS, and left ventricular posterior wall thickness at systole and an increase in left ventricular internal dimension at systole/diastole, exhibiting a dilated cardiomyopathy, compared with controls (Table). However, H-TKO mice demonstrated significant improvement of cardiac function, although cardiac ventricular mass was insignificantly improved, compared with H-DKO mice (Table). In H-DKO hearts, β-MHC, atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP) mRNAs were significantly increased, whereas myocardial Foxo1 deletion in H-TKO hearts significantly reduced β-MHC mRNA and barely affected ANP and BNP mRNA levels (Figure 1D).

We further examined the cardiac ultrastructure of these mice. A well-organized morphology in sarcomeres, mitochondria, and Z-line is exhibited in control hearts (Figure 1E). However, H-DKO hearts exhibited enlarged sarcomeres, a loss of mitochondria, and cristae of inner membranes. Strikingly, T-DKO hearts displayed nearly normal structures of sarcomeres and mitochondria, in which Foxo1 deficiency rescued the H-DKO myocardial morphology (Figure 1E).

### Table. Echocardiographic Parameters of H-DKO, H-TKO, and F1KO Mice vs Control Mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>CNTR (n=11)</th>
<th>H-DKO (n=10)</th>
<th>H-TKO (n=10)</th>
<th>CNTR (n=8)</th>
<th>F1KO (n=8)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>6 Mo After Tamoxifen Injection</td>
<td>4-Mo Old</td>
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<td></td>
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<tr>
<td>BW, g</td>
<td>37.2±4.3</td>
<td>39.4±4.2</td>
<td>37.0±2.1</td>
<td>29.1±1.5</td>
<td>27.3±1.8</td>
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<td>HVW, mg</td>
<td>122.8±4.4</td>
<td>104.9±5.8*</td>
<td>108.7±5.2*</td>
<td>125.2±10.3</td>
<td>128.2±1.1</td>
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<td>HVW/BW, mg/g</td>
<td>3.43±0.35</td>
<td>2.73±0.22*</td>
<td>3.07±0.21*</td>
<td>4.31±0.21</td>
<td>4.75±0.22</td>
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<tr>
<td>HVW/TL, mg/mm</td>
<td>67.3±3.1</td>
<td>57.8±2.3*</td>
<td>60.8±2.1*</td>
<td>67.6±3.7</td>
<td>69.2±1.4</td>
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<td>LVPW-s, mm</td>
<td>0.91±0.07</td>
<td>0.82±0.11*</td>
<td>0.82±0.07*</td>
<td>0.94±0.05</td>
<td>0.96±0.08</td>
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<tr>
<td>EF, %</td>
<td>53.80±3.74</td>
<td>42.58±4.44*</td>
<td>48.44±4.28†</td>
<td>59.45±8.31</td>
<td>56.83±9.97</td>
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<tr>
<td>FS, %</td>
<td>27.56±2.46</td>
<td>20.27±2.56*</td>
<td>23.02±2.51†</td>
<td>31.60±5.48</td>
<td>29.75±6.96</td>
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<td>CO, mL/min</td>
<td>18.75±1.55</td>
<td>12.58±1.67*</td>
<td>16.01±2.6†</td>
<td>17.66±1.00</td>
<td>16.80±4.14</td>
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<td>LVID-d, mm</td>
<td>2.72±0.30</td>
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<td>3.00±0.19</td>
<td>2.77±0.18</td>
<td>2.83±0.21</td>
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<td>LVID-d, mm</td>
<td>3.68±0.38</td>
<td>4.06±0.38*</td>
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<td>MV Decl, ms</td>
<td>19.94±0.92</td>
<td>21.59±3.86*</td>
<td>19.11±3.2†</td>
<td>20.88±1.54</td>
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<td>IVRT, ms</td>
<td>18.30±1.75</td>
<td>21.08±3.00*</td>
<td>19.34±1.26†</td>
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<td>E/A</td>
<td>1.50±0.12</td>
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<td>1.55±0.19†</td>
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<tr>
<td>MV E/E'</td>
<td>45.11±5.31</td>
<td>55.42±4.62*</td>
<td>42.69±6.72†</td>
<td>45.57±8.31</td>
<td>49.84±1.57</td>
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Unless noted otherwise in the text, measurements come from male mice. Values are mean±SEM. BW indicates body weight; CNTR, control; CO, cardiac output; E/A, ratio of early and late ventricular filling velocity; EF, ejection fraction; F1KO, Foxo1 knockout mice; FS, fractional shortening; H-DKO mice, heart-specific double knockout mice; H-TKO mice, heart-specific triple knockout mice; HVW, heart ventricular weight; IVRT, isovolumic relaxation time; LVID-d, left ventricular internal dimension at diastole; LVID-s, left ventricular internal dimension at systole; LVPW-s, left ventricular posterior wall thickness at systole; MV Decl, mitral valve E wave deceleration time; MV E/E', ratio of mitral valve E velocity to E' velocity; and TL, tibial length.

*P<0.05 vs control mouse; and †P<0.05 vs H-DKO mice.
Insulin Deficiency Increases β-MHC Gene Expression

We hypothesize that β-MHC, the expression level of which is inversely proportional to cardiac contractility,22 is a target gene of insulin and Foxo1. We treated control and F1KO mice with streptozotocin, a compound known to destroy pancreatic β-cells and deplete insulin secretion, resulting in myocardial Akt inactivation.27 Without streptozotocin treatment, both control and F1KO mice exhibited normal blood glucose and serum insulin levels, with a blood glucose of 110±10 mg/dL and normal insulin level of 3.01±0.2 ng/dL, in random-fed mice. However, 2 weeks after streptozotocin treatment, hyperglycemia and hypoinsulinemia were observed in both control and F1KO mice, with a blood glucose level of 500±35 mg/dL and insulin level of 0.015±0.005 ng/dL (n=6; *P<0.05 versus non-streptozotocin group).

Streptozotocin treatment increased β-MHC gene expression by 6.8-fold in control hearts compared with the non–streptozotocin-treated group (Figure 3A), an effect which was attenuated in F1KO hearts, demonstrating an increase by 3.6-fold, compared with the non–streptozotocin-treated group (Figure 3A). Similar results were observed at the β-MHC protein level, as determined by Western blot (Figure 3B). The results demonstrate that an increase of β-MHC expression in the hearts of mice with type 1 diabetes mellitus and that Foxo1 deficiency partially normalized β-MHC expression in hearts of type 1 diabetic mice. These results suggest that Foxo1 stimulates β-MHC gene expression at 2 weeks of streptozotocin-treated hearts, before overt cardiac dysfunction that was evident 10 weeks after streptozotocin treatment.12

Figure 2. Cardiac function and myosin heavy chain (MHC) expression in db/db and high-fat diet (HFD) mice lacking cardiac forkhead transcription factor (Foxo1). A, Strategies for generation of db/db mice lacking cardiac Foxo1 gene. B, Echocardiographic analysis of 8-month-old control, db/db, and db/db::F1KO mice. Values represent the mean±SEM (n=8; *P<0.05 vs control; #P<0.05 vs db/db). C, mRNA levels of hearts isolated from 8-month-old random-fed controls, db/db and db/db::F1KO mice. Values represent the mean±SEM (n=8; *P<0.05 vs control; #P<0.05 vs db/db mice). D, Echocardiographic analysis of 8-month-old control, HFD, F1KO, and HFD::F1KO mice. Values represent the mean±SEM (n=6; *P<0.05 vs control; #P<0.05 vs F1KO). E, mRNA levels of hearts isolated from 8-month-old random-fed control, HFD, F1KO, and HFD::F1KO mice. Values represent the mean±SEM (n=6; *P<0.05 vs control; #P<0.05 vs F1KO). CNTR indicates control; EF, ejection fraction; and FS, fractional shortening.

Foxo1 Deficiency Decreases β-MHC Gene Expression in Cardiac Foxo1 Gene Knockout Mice

We further examined whether Foxo1 regulates β-MHC gene expression at a physiological level. Because Foxo1 is phosphorylated at S253 and inactivated by insulin during the random-fed state in mice,13 in which the β-MHC mRNA changes in the hearts of control and F1KO mice were not evident (Figure 3A and 3B). Thus, we measured Foxo1 and β-MHC mRNA and protein levels in hearts of mice after an overnight fast, where Foxo1 is dephosphorylated and activated on a lower amount of insulin in controls (0.38±0.2 ng/dL). In F1KO hearts, Foxo1 mRNA and protein were decreased by 70% and 60%, respectively, when compared with those in control hearts (Figure 4A and 4B). Likewise, β-MHC mRNA and protein levels were also decreased by 40% and 35%, respectively, whereas the α-MHC mRNA level was significantly increased by 31% in F1KO hearts (Figure 4A). The control and F1KO hearts did not exhibit significant differences in EF and FS and other cardiac parameters (Table). However, mRNA expression levels of ANP and BNP were significantly increased in the hearts of F1KO mice without cardiac dysfunction (Figure 4C; Table). The results suggest that Foxo1 is required for β-MHC expression in vivo.

Foxo1 Is Required for Insulin Suppression of β-MHC Expression

We next determined whether myocardial β-MHC expression can be physiologically regulated during fasting and feeding conditions, through Foxo1 or insulin which is a major
suppressor of Foxo1 via Akt activation. We examined the effect of overnight fasting, random-fed, or insulin stimulation on β-MHC expression, in the hearts of control and F1KO mice. In control mice, hearts from overnight fasting animals had an ≈2- or 1.6-fold increase of β-MHC mRNA and protein, respectively, compared with random-fed hearts (Figure 5A and 5C). Hearts with random feeding and insulin stimulation demonstrated markedly increased Akt that inhibited Foxo1 by enhancing phosphorylation of S253, compared with hearts from fasting control mice (Figure 5B). In F1KO mice, the fasting-feeding regulation of β-MHC expression in the hearts was abolished and insulin administration had little effect on suppressing cardiac β-MHC gene expression (Figure 5D). These results suggest that Foxo1 is necessary for feeding or insulin, to suppress cardiac β-MHC gene expression.

Insulin Suppresses and Foxo1 Stimulates β-MHC Gene Expression in Cardiomyocytes

We next examined whether β-MHC is a direct target gene of insulin, via Foxo1 regulation in vitro. Using primary cardiomyocyte NRVMs, we previously showed that insulin inhibited β-MHC mRNA levels.9 We next examined which insulin signaling pathway was involved in suppressing β-MHC expression in cardiomyocytes. Insulin decreased β-MHC mRNA levels by 40% in NRVMs, compared with nontreatment (Figure 6A). Pretreatment with the PI3K inhibitor wortmannin completely blocked the inhibitory effect of insulin on β-MHC gene expression, whereas the extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor (PD98059) and c-Jun N-terminal kinases (JNKs) inhibitor (SP600125) did not block the inhibitory effect of insulin. The stimulatory effect

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**Figure 3.** Myosin heavy chain (MHC) gene expression in mice lacking cardiac Foxo1 gene (Foxo1 knockout mice [F1KO]) with type 1 diabetes mellitus. A and B, Two weeks after streptozotocin (STZ) treatment and non-STZ treatment, mRNA and protein were isolated from hearts of 10-week-old random-fed control and F1KO mice. The mRNA expression of β-MHC and α-MHC was analyzed by real-time polymerase chain reaction (A) and protein expression of β-MHC by Western blot (B). Values represent the means±SEM (n=4; *P<0.05 vs control [CNTR]; #P<0.05 vs CNTR+STZ). Representative results of Western blots from hearts of 2 mice in each group are shown in B.

**Figure 4.** Cardiac β-myosin heavy chain (β-MHC) gene expression in mice lacking cardiac forkhead transcription factor (Foxo1). A, mRNA and protein expression of β-MHC (B) in hearts of 10-week-old F1KO mice, after an 18-hour fast. Values are expressed as the means±SEM (n=6–8; *P<0.05 vs control [CNTR] heart). Representative results of Western blot from hearts of 2 mice are shown in B. C, Expression of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were analyzed by real-time polymerase chain reaction in hearts from A. *P<0.05 vs CNTR hearts.
of insulin on α-MHC expression was blocked completely by the PI3K inhibitor.

To further assess the role of Foxo1 in regulating β-MHC expression in a cell-specific manner, NRVMs and cardiofibroblasts were simultaneously isolated and infected with adenovirus expressing Foxo1 wild-type or control green fluorescent protein and treated with or without insulin for 12 hours. Overexpression of Foxo1-wild-type significantly increased β-MHC mRNA and protein levels by 2.2- and 1.5-fold, respectively, compared with control cardiomyocytes expressing green fluorescent protein (Figure 6B–6D). The stimulatory effect of Foxo1-wild-type on β-MHC, at both mRNA and protein expression levels, was completely suppressed by insulin treatment in cardiomyocytes (Figure 6B–6D). However, the expression level of β-MHC in cardiofibroblasts was <2% to 3% of that in NRVMs and was insensitive to insulin suppression or Foxo1 stimulation (Figure 6B). These data suggest that insulin suppresses β-MHC gene expression via a PI3K and Foxo1-dependent pathway in cardiomyocytes, rather than fibroblasts.

**F**oxo1 **I**nteracts With the **P**romoter **R**egion of the **β**-**MHC** **G**ene

We finally examined whether the promoter region of β-MHC has a consensus Foxo1 binding site. A region of 10 kb of mouse β-MHC promoter was analyzed with the ENSEMBL genome database and 3 consecutive copies of conserved Foxo1-binding sequence or insulin response element, CAAACAAC, were identified, located 9.0 kb upstream of the transcriptional initiation site, with consensus in rodent species (Figure 6E). To determine whether Foxo1 interacts with the promoter region of the β-MHC gene, chromatin immunoprecipitation experiments were performed, the results of which indicated that endogenous Foxo1 interacted with the consensus DNA sequence on the β-MHC promoter region (Figure 6F). Together, our data suggest that Foxo1 is an endogenous transcription factor that interacts with the promoter region of β-MHC for transcriptional activation.

**D**iscussion

In this study, we present 3 important findings: (1) Foxo1 deficiency in the heart prevented the cardiac dysfunction in mouse models with insulin resistance (H-DKO mice) and type 2 diabetes mellitus (db/db mice), reducing the heart failure marker β-MHC gene expression; (2) Foxo1 activation stimulates β-MHC gene expression in cardiomyocytes and interacts with a Foxo1-binding site on the promoter region of β-MHC gene; and (3) β-MHC is a target of insulin signaling that suppresses its expression via PI3K activation, whereas hearts lacking Foxo1 demonstrate decreased β-MHC expression by 50%, and the inhibitory effect of insulin or feeding is prevented.

In this study, we provide a new target gene of Foxo1, β-MHC, in cardiomyocytes, which may potentially contribute to reduction of cardiac contractility (Figure 7). Replacing all α-MHC by β-MHC in mice did not result in sudden death, but increasing cardiac β-MHC expression in vivo is mal-adaptive reducing myocardial contractility. In many forms of cardiac stress resulting from diabetes mellitus, pressure overload, or aging, a transition from α- to β-MHC occurs, and the shift from fast to slow isomyosin was disadvantageous under cardiac stress. Moreover, hypertrophied or aged hearts displayed insulin resistance, by reduced IRS1 tyrosine phosphorylation and associated PI3K activity, which may also activate Foxo1 and subsequently increase β-MHC gene expression. Thus, increasing β-MHC and decreasing α-MHC...
gene expression by IRS suppression and in turn Foxo1 activation, resulting from insulin resistance, may link metabolic and mechanic stresses for cardiac contractile dysfunction.

Foxo1 activation is suppressed by insulin that reduces β-MHC and increases α-MHC gene expression. A transient response of β-MHC gene expression is <2-fold, on fasting and feeding conditions in normal hearts. However, the MHC homeostasis is largely disrupted on Foxo1 activation when insulin deficiency or resistance occurs. Moreover, cardiac β-MHC gene expression is tightly controlled by the developmental and hormonal factors and is species dependent.

During the fetal developmental period of rodent hearts, β-MHC is the principal isoform expressed in the ventricles when insulin is mostly absent; α-MHC, however, is the principal isoform expressed in adult animals when insulin dominates, to control energy homeostasis. Although a rise of circulating thyroid hormone shortly after birth is thought to suppress β-MHC and increase α-MHC gene expression, rodent hearts continue to mature to adulthood where β-MHC is re-expressed, accounting for 10% to 15% of the total MHC

Figure 6. Effects of insulin and forkhead transcription factor (Foxo1) on β-myosin heavy chain (β-MHC) gene expression in cardiomyocytes. A. Neonatal rat ventricular cardiomyocytes (NRVMs) were cultured and pretreated with the kinase inhibitors-wortmannin (100 nmol/L), SP600125 (10 μmol/L), or PD98059(20 μmol/L), for 30 minutes, before 18 hours of 100 nmol/L insulin stimulation. Cellular RNA was prepared for real-time analysis. Graphs indicate quantification of mRNA normalized to cyclophilin, from ≥3 independent experiments. Data are expressed as the mean±SEM (*P<0.05 vs control [CNTR]; #P<0.05 vs insulin group). B–D, NRVMs and cardiofibroblasts (CFs) were infected with 25 multiplicity of infection adenovirus expressing green fluorescent protein (GFP) or GFP-Foxo1-wild-type (wt) for 8 hours, and then serum starved for 8 hours before addition of 100 nmol/L insulin for 18 hours and mRNA (B) or protein in NRVMs (C and D) was analyzed by real-time polymerase chain reaction (PCR) or Western blot, respectively. Data are expressed as the mean±SEM (*P<0.05 vs GFP; #P<0.05 vs Foxo1-wt). Representative results of Western blot (C) are shown, in which endogenous Foxo1 (70 kDa) and overexpressed Foxo1 fused with GFP (109 kDa) are indicated. In D, based on C, pFoxo1 represents phosphorylation of endogenous Foxo1-S253 normalized by total endogenous Foxo1 in GFP and GFP+insulin group or phosphorylation of overexpressed GFP-Foxo1-S253 normalized by overexpressed total GFP-Foxo1 in Foxo1wt and Foxo1wt+insulin group. t-Foxo1 represent the relative total endogenous Foxo1 in GFP and GFP+insulin group or the total overexpressed GFP-Foxo1 normalized by total endogenous Foxo1 in the GFP group. E, Potential Foxo1 binding sites or insulin response elements are located 9.0 kb upstream of the β-MHC gene transcriptional initiation site and a comparison between mouse and rat is shown. F, Binding of endogenous Foxo1 in the hearts of wt mice to the potential Foxo1 binding site was confirmed by chromatin immunoprecipitation assay. Immunoprecipitation of heart chromatin was isolated from fasted control mice with anti-Foxo1 antibody and control IgG. Occupancy of the Foxo1 site in the β-MHC promoter was determined by PCR, in which control immunoprecipitation with nonrelevant IgG demonstrates specificity of the assay. Representative results from 3 independent experiments are shown.
pool, likely because of an increase in mechanical stress and insulin resistance. Moreover, activation of fetal genes such as \(\beta\)-MHC was observed in the failing human hearts, in which overexpression of Foxo1 has been reported. Unlike mice, normal human adult ventricles express \(\alpha\)-MHC on a background of 90% \(\beta\)-MHC, which is consistent with a lower heart rate (70 versus 600 beats per minute in mice). However, the distribution further shifts to 100% \(\beta\)-MHC in the failing heart. Thus, manipulating MHC plasticity to suppress \(\beta\)-MHC is thought to be a strategy in preventing heart failure, and we expect that targeting Foxo1 after insulin resistance or deficiency may provide a novel mechanism suppressing fetal gene expression and improving cardiac dysfunction in failing and aged hearts.

Gene expression of cardiac MHC genes is controlled by a complex regulatory program, although few transcription factors regulating MHC gene expression have been identified. The \(\beta\)-MHC and \(\alpha\)-MHC genes are 93% homologous and products of 2 distinct genes, situated in tandem, in a head-to-tail position, on mouse chromosome 2, providing an antithetical manner for expression of the genes. Myocardial deletion of Foxo1 reduced \(\beta\)-MHC and increased \(\alpha\)-MHC gene expression, suggesting that Foxo1 has an important role in regulating cardiac MHC. Binding of Foxo1 to the promoter region of \(\beta\)-MHC stimulates activity, possibly by recruiting \(p300/CBP\), which contains histone acetylase activity, as observed for insulin-like growth factor binding protein-1 (IGFBP-1). Alternatively, Foxo1 may act as a corepressor for the \(\alpha\)-MHC promoter, by recruiting transcriptional repressors and Foxo1 can recruit histone deacetylase or nuclear suppressor N-CoR to suppress gene expression.

Myocardial insulin resistance in H-DKO and db/db or HFD mice indicates that distinct cardiac remodeling may develop in type 2 diabetes mellitus. H-DKO mice did not develop cardiac hypertrophy as is often seen in type 2 diabetes mellitus but rather displayed a dilated cardiomyopathy, indicating that loss of IRS1 and IRS2, which closely associate with inactivation of PI3K and Akt, promotes cardiac dysfunction and loss of ventricular mass. Other signaling is required for cardiac hypertrophy in type 2 diabetes mellitus and it is likely that activation of sympathetic nerve activity or the renin–angiotensin system may be involved in cardiac hypertrophy, which often coexists with type 2 diabetes mellitus before heart failure and animal death, such as in db/db or HFD mice. Thus, the compensation of cardiac hypertrophic cardiomyopathy was lost in H-DKO hearts. Regardless, that inactivation of Foxo1 significantly improved cardiac function and promoted survival of animals with either dilated cardiomyopathy (H-DKO) or hypertrophic cardiomyopathy (db/db or HFD) underscores the key role of Foxo1 in promoting cardiac dysfunction on insulin resistance (Figure 7). However, patients with heart failure often have suppression of insulin release and action by an increase in catecholamine secretion. Clinically, insulin has been used to treat patients with severe heart failure and catecholamine antagonists reduce morbidity and mortality from heart failure, partially by increasing pancreatic insulin release. Thus, activation of Foxo1 secondary to insulin deficiency, resistance, or other activation mechanisms via catecholamines, may significantly promote its expression and functionality resulting in a remarkable increase in \(\beta\)-MHC gene expression and cardiac contractile dysfunction.

In addition to the regulation of MHC plasticity, inactivation of PI3K and Akt through inhibition of IRS1 and IRS2 and activation of Foxo1 may provide a key mechanism for insulin-resistant cardiomyopathy (Figure 7). First, Foxo1 promotes loss of mitochondrial by activating gene expression of hemeoxygenase-1, an enzyme that catalyzes heme degradation. Heme is an essential component of mitochondrial complex III and IV, as we identified in hepatocytes. The death of H-DKO mice was likely from heart failure secondary to mitochondrial abnormality and H-TKO largely normalized the mitochondrial morphology and function, similar to hepatocytes we observed previously. Second, Foxo1 promotes cell death and inhibits myocardial proliferation. Overexpression of Foxo1 in the heart resulted in premature death in mice. A significant increase in cell apoptosis was found in H-DKO hearts, which was nearly undetectable in H-TKO hearts (data not shown). Third, Foxo1 has other target genes that may promote cardiac dysfunction. For example, we recently identified angiotensinogen as a Foxo1 target gene, which stimulates angiotensin II generation controlling blood pressure and cellular apoptosis. Finally, Foxo1 activation is involved in metabolic remodeling. It has been shown recently that cardiac inactivation of Foxo1 in HFD mice promotes animal survival by protecting cardiac function, in which a major mechanism is that Foxo1 inactivation enhances myocardial glucose utilization via increasing glucokinase gene expression. Of note is that Foxo1 activation can provide benefit under certain conditions, by increasing hepatic glucose production for survival or promoting cardioprotective autophagy when animals experience prolonged fasting. However, the unrestrained autophagy in cardiomyocytes lacking IRS1 and IRS2 can result in myocyte loss, heart failure, and premature death.
In summary, we found that Foxo1 controls cardiac homeostasis and MHC plasticity, which is moderately, dynamically, and metabolically regulated by insulin signaling at a physiological level, a process which is markedly dysregulated on insulin resistance. Thus, we think that targeting Foxo1 and its regulatory system may provide novel strategies for therapeutic intervention in preventing metabolic and myocardial dysfunction and in influencing MHC plasticity in patients with diabetes mellitus and associated heart failure.

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Disclosures
None.

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receptor substrate 1, 2 and associated PI3K/Akt activation, as being involved in myocardial mitochondrial biogenesis, cardiomyopathy, and type 2 diabetes mellitus.

β-cardiac homeostasis, and myosin heavy chain gene expression. This study demonstrates that β-myosin heavy chain gene is a new target gene of Foxo1, providing a novel mechanism of insulin-resistant cardiomyopathy by which Foxo1 may potentially promote cardiac contractile dysfunction via motor and myocardial structural gene expression after insulin resistance and type 2 diabetes mellitus.

**CLINICAL PERSPECTIVE**

The risk of heart failure and cardiac sudden death is significantly increased by insulin resistance and type 2 diabetes mellitus, a major component of the metabolic syndrome; however, the molecular link between insulin resistance and cardiac dysfunction is unclear. Here, we have identified Foxo1, a known insulin-suppressed transcription factor through insulin receptor substrate 1, 2 and associated PI3K/Akt activation, as being involved in myocardial mitochondrial biogenesis, cardiac homeostasis, and myosin heavy chain gene expression. This study demonstrates that β-myosin heavy chain gene is a new target gene of Foxo1, providing a novel mechanism of insulin-resistant cardiomyopathy by which Foxo1 may potentially promote cardiac contractile dysfunction via motor and myocardial structural gene expression after insulin resistance and type 2 diabetes mellitus.