Type 1 diabetic cardiomyopathy in the Akita (Ins2WT/C96Y) mouse model is characterized by lipotoxicity and diastolic dysfunction with preserved systolic function

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Basu R, Oudit GY, Wang X, Zhang L, Ussher JR, Lopaschuk GD, Kassiri Z. Type 1 diabetic cardiomyopathy in the Akita (Ins2WT/C96Y) mouse model is characterized by lipotoxicity and diastolic dysfunction with preserved systolic function. Am J Physiol Heart Circ Physiol 297: H2096–H2108, 2009. First published October 2, 2009; doi:10.1152/ajpheart.00452.2009.—Diabetic cardiomyopathy is an important contributor to diastolic and systolic heart failure. We examined the nature and mechanism of the cardiomyopathy in Akita (Ins2WT/C96Y) mice, a model of genetic nonobese type 1 diabetes that recapitulates human type 1 diabetes. Cardiac function was evaluated in male Ins2WT/C96Y and their littermate control (Ins2WT/WT) mice using echocardiography and tissue Doppler imaging, in vivo hemodynamic measurements, as well as ex vivo working heart preparation. At 3 and 6 mo of age, Ins2WT/C96Y mice exhibited preserved cardiac systolic function compared with Ins2WT/WT mice, as evaluated by ejection fraction, fractional shortening, left ventricular (LV) end-systolic pressure and maximum rate of increase in LV pressure in vivo, cardiac work, cardiac power, and rate-pressure product ex vivo. Despite the unaltered systolic function, Ins2WT/C96Y mice exhibited significant and progressive diastolic dysfunction at 3 and 6 mo of age compared with Ins2WT/WT mice as assessed by tissue and pulse Doppler imaging (E-wave velocity, isovolumetric relaxation time) and by in vivo hemodynamic measurements (LV end-diastolic pressure, time constant of LV relaxation, and maximum rate of decrease in LV pressure). We found no evidence of myocardial hypertrophy or fibrosis in the Ins2WT/C96Y myocardium. Consistent with the lack of fibrosis, expression of procollagen-α type I, procollagen-α type III, and fibronectin were not increased in these hearts. Ins2WT/C96Y hearts showed significantly reduced sarcoplasmic reticulum Ca2+ -ATPase 2a (cardiac sarcoplasmic reticulum Ca2+ pump) levels, elevated β-myosin heavy chain isoform, increased long-chain fatty acids, and triacylglycerol with evidence of lipotoxicity, as indicated by a significant rise in ceramide, diacylglycerol, and lipid deposits in the myocardium. Consistent with metabolic perturbation, and a switch to fatty acid oxidation from glucose oxidation in Ins2WT/C96Y hearts, expression of mitochondrial long-chain acyl-CoA dehydrogenase and pyruvate dehydrogenase kinase isoform 4 were increased. Insulin treatment reversed the diastolic dysfunction, the elevated B-type natriuretic peptide and β-myosin heavy chain, and the reduced sarcoplasmic reticulum Ca2+ -ATPase 2a levels with abolition of cardiac lipotoxicity. We conclude that early type 1 diabetic cardiomyopathy is characterized by diastolic dysfunction associated with lipotoxic cardiac cardiomyopathy with preserved systolic function in the absence of interstitial fibrosis and hypertrophy. Insulin; fibrosis; hypertrophy; sarco(endo)plasmic reticulum calcium-ATPase 2a

Diabetes mellitus has become a major health concern worldwide and is predicted to be the fifth most common cause of deaths globally (36, 39). Diabetes itself has been established to be a strong risk factor for heart failure, independent of age, hypertension, dyslipidemia, and coronary artery disease (8, 37, 40). Although type 2 diabetes is a more common form of diabetes, with an early onset of hyperinsulinemia and a late onset of hyperglycemia, type 1 diabetes affects ~5–10% of the diabetic population globally with early onset of hyperglycemia, and both types can result in severe cardiovascular complications (58).

Diastolic heart failure is now a well-recognized clinical entity often associated with diabetes and hypertension (21, 34, 36, 46). Several factors may contribute to the development and progression of cardiac dysfunction in diabetes mellitus, including increased interstitial fibrosis, suppressed intracellular Ca2+ handling, altered contractile filament properties, and/or lipotoxicity affecting both passive and active relaxation properties of the ventricle (3, 8, 14, 37, 46). The Ins2WT/C96Y (Akita) mouse is a well-validated, nonobese model of human type 1 diabetes, while being free of potential confounding effects of streptozotocin (STZ) administration (17, 48). In this study, we characterized the cardiomyopathy in Ins2WT/C96Y mice and demonstrate that these mice exhibit early and persistent diastolic dysfunction in a setting of preserved systolic function compared with their littermate control, Ins2WT/WT mice. Lack of insulin in Ins2WT/C96Y mice suppressed insulin-dependent signaling pathways, such as phosphorylation of ERK-1/2 and Akt/PI3K in the heart. We found evidence of lipotoxicity in Ins2WT/C96Y hearts coupled with increased expression of long-chain acyl-CoA dehydrogenase (LCAD) and pyruvate dehydrogenase kinase (PDK) isoform 4 (PDK4). We conclude that type 1 diabetic cardiomyopathy is characterized by diastolic dysfunction and preserved systolic function, with evidence of myocardial lipotoxicity and downregulation of the major Ca2+-regulatory protein, sarco(endo)plasmic reticulum Ca2+-ATPase 2a (SERCA2a), in the absence of hypertrophy or interstitial fibrosis.

Materials and Methods

Experimental animals. C57BL/6J wild-type (WT) and diabetic heterozygous Akita (Ins2WT/C96Y) mice were purchased from The Jackson Laboratories. We bred male Ins2WT/C96Y mice with female WT mice at the University of Alberta animal facility. Only male Ins2WT/C96Y and their littermate WT (Ins2WT/WT) mice were used in all experiments. Throughout the period of study, animals were provided with free access to water and standard rodent chow (Harlan Teklad, Madison, WI). All experiments were conducted in accordance with the guidelines of the University of Alberta Animal Care Committee and the Canadian Council of Animal Care. Animal protocols have been reviewed and approved by the Animal Care and Use Committee at the University of Alberta.
Echocardiography and tissue Doppler imaging. Transthoracic echocardiography was performed noninvasively as described previously using a Vevo 770 high-resolution imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, Canada) (56, 57). The temporal resolution for M-mode imaging in this system is a pulse repetition frequency of 8 kHz, with an axial resolution of 55 μm, lateral resolution of 115 μm, focal length of 12.7 mm, and a depth of field of 2.2 mm. Mice were anesthetized with 0.75% isoflurane for the duration of the recordings. M-mode images were obtained for measurements of left ventricular (LV) wall thickness, LV end-diastolic diameter (LVEDD), and LV end-systolic diameter (LVESD) (measures of LV dilation). LV fractional shortening (FS) and LV ejection fraction (EF) were calculated using the following equations: FS (%) = (LVEDD − LVESD/LVEDD) × 100 and EF (%) = LVEDV − LVEDV/LVEDV × 100 as measures of systolic function.

Diastolic function was assessed using pulsed-wave Doppler imaging of the transmitral filling pattern with the early transmitral filling wave (E-wave) followed by the late filling wave due to atrial contraction (A-wave). Isovolumetric relaxation time (IVRT) was calculated as the time from closure of the aortic valve to initiation of the E-wave. The deceleration time of the E-wave (DT) was determined by measuring the time needed for the down-slope of the peak of the E-wave to reach the baseline, while the rate of E-wave deceleration (A-wave). Isovolumetric relaxation time (IVRT) was calculated to [18F]phosphatidic acid by DAG kinase and quantified as before (38). Statistical analysis. Two-way ANOVA followed by multiple-comparison test were performed to compare between the Ins2WT/C96Y and Ins2WT/WT groups at 3 and 6 mo of age. Averaged values are presented as means ± SE. Statistical significance is recognized at P < 0.05.

RESULTS

Diastolic dysfunction in the presence of normal systolic function in Ins2WT/C96Y mice. The Ins2WT/C96Y mouse is a well-established model of nonobese type 1 diabetes and provides a unique opportunity to understand diabetic cardiomyopathy (17). We first established the hypoinsulinemia condition and the diabetic phenotype in Ins2WT/C96Y mice by measuring random plasma insulin (83.8 ± 5.2 pM in Ins2WT/WT vs. 36.2 ± 4.7 pM in Ins2WT/C96Y, n = 8/group, P < 0.01) and random blood glucose (7.6 ± 1.5 mM in Ins2WT/WT vs. 31.7 ± 4.1 mM in Ins2WT/C96Y, n = 8/group, P < 0.01) at 3 mo of age. Next, we assessed the cardiac function in Ins2WT/C96Y and littermate Ins2WT/WT mice noninvasively using a high-resolution imaging transthoracic echocardiography system equipped with a 30-MHz transducer, in vivo, by hemodynamic measurements, and ex vivo using an isolated working preparation. M-mode images from Ins2WT/WT and Ins2WT/C96Y mice at 3 mo of age showed comparable LV contractility (Fig. 1A), while long-axis images from these mice show lack of LV dilation and, in fact, a slight reduction in the LV chamber size in the Ins2WT/C96Y mice (Fig. 1B). Consistently, parameters of cardiac systolic function, including EF, FS, stroke volume (Table 1), as well as maximum change in pressure over time are comparable between Ins2WT/WT and Ins2WT/C96Y.
their littermate Ins2WT/WT mice at 3 and 6 mo of age (Fig. 1C).
Additional systolic function parameters, including the systolic
annular velocity (S') by TDI, are also reported in Table 1. We
further used an ex vivo isolated working heart preparation to
compare the basal systolic function between the genotypes under
controlled conditions. Consistent with echocardiography and
hemodynamic data, Ins2WT/C96Y mice showed unaltered systolic
performance, as determined by cardiac work, cardiac power, and
rate pressure product, compared with Ins2WT/WT mice (Fig. 1D).

We evaluated diastolic function in these mice using tradi-
tional Doppler technique coupled with TDI. Transmitral filling
pattern showed reduced E-wave velocity with prolongation of
DT, leading to a significant reduction in EWDRs in 3-mo-old
Ins2WT/C96Y compared with littermate Ins2WT/WT mice (Fig. 2,
A and B, Fig. 3A, Table 2, Supplemental Fig. 1). (The online
version of this article contains supplemental data.) The
IVRT was also increased markedly in Ins2WT/C96Y mice, sug-
Analyzed and tabulated data from Table 1 is as follows:

<table>
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<th>Parameter</th>
<th>Ins2WT/WT</th>
<th>Ins2WT/C96Y</th>
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<td>6</td>
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<tr>
<td>n</td>
<td>12</td>
<td>12</td>
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<td>HR, beats/min</td>
<td>448±11</td>
<td>438±12</td>
<td>459±16</td>
<td>451±13</td>
</tr>
<tr>
<td>LVEF, %</td>
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<td>57.4±2</td>
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<td>SV, μl</td>
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<td>45±1.11</td>
<td>43.2±3.2</td>
<td>49.2±1.2</td>
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<td>CO, ml/min</td>
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<td>LVEDD, mm</td>
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<td>3.68±0.07</td>
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<td>3.71±0.04</td>
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<td>HR, heart rate</td>
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<td>2.61±0.11</td>
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</tbody>
</table>

Values are means ± SE; n, n, number of mice. HR, heart rate; LVEF, left
ventricular (LV) ejection fraction; LVFS, LV fractional shortening; SV, stroke
volume; CO, cardiac output; LVEDD, LV end-diastolic diameter; LVESD, LV
end-systolic diameter; ET, ejection time; Vcfc, velocity of circumferential
shortening; S’, systolic annular velocity by tissue Doppler imaging.

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diastolic dysfunction (36, 42), and we found that the left atrial size in Ins2\(^{WT/WT}\) mice progressively increased at 3 and 6 mo of age compared with Ins2\(^{WT/WT}\) littermates (Table 2). However, the reduction in E’ and the resulting rise in E-to-E’ ratio do not persist in Ins2\(^{WT/C96Y}\) mice at 6 mo of age. We also used invasive hemodynamic measurements to provide more definitive assessment of the diastolic dysfunction. The key hemodynamic correlates of diastolic dysfunction, LV end-diastolic pressure, and the time constant of LV relaxation were increased in the Ins2\(^{WT/C96Y}\) mice (Fig. 3, D and E), while negative minimum change in pressure over time was reduced in Ins2\(^{WT/C96Y}\) mice (Fig. 3F) without alterations in baseline heart rate (524 ± 19 beats/min in Ins2\(^{WT/WT}\) vs. 539 ± 23 beats/min in Ins2\(^{WT/C96Y}\) mice; P = 0.412). Overall, our data indicate that Ins2\(^{WT/C96Y}\) mice demonstrate early and persistent diastolic dysfunction with preservation of systolic function.

**Fig. 2.** Echocardiographic assessment of diastolic function in Ins2\(^{WT/C96Y}\) compared with Ins2\(^{WT/WT}\) mice. A and B: representative transmitral Doppler flow profile showing reduced peak E-wave velocity with increased deceleration time and prolongation of isovolumetric relaxation interval in an Ins2\(^{WT/C96Y}\) (B) compared with an Ins2\(^{WT/WT}\) mouse (A). C and D: representative tissue Doppler images of the basal inferolateral LV wall showing reduced early diastolic tissue velocity (E’) in an Ins2\(^{WT/C96Y}\) (D) compared with an Ins2\(^{WT/WT}\) mouse (C). IVRT, isovolumetric relaxation time.

**Fig. 3.** Averaged parameters of diastolic dysfunction in Ins2\(^{WT/C96Y}\) compared with Ins2\(^{WT/WT}\) mice. A–C: echocardiographic assessment showing reduced E-wave deceleration rate (EWDR), prolongation of the IVRT, and reduced early tissue Doppler velocity (E’), consistent with diastolic dysfunction (n = 12/group), respectively. D–F: hemodynamic assessment showing elevated LV end-diastolic pressure (LVEDP), prolonged LV relaxation (τ), and reduced negative minimum change in pressure over time (−dP/dτ\(_{\text{min}}\)), respectively. *P < 0.05 compared with Ins2\(^{WT/WT}\) mice.
**Table 2. Echocardiographic assessment of diastolic function in 3- and 6-mo-old mice**

<table>
<thead>
<tr>
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<th>Ins2&lt;sup&gt;WT/C96Y&lt;/sup&gt;</th>
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<th>Ins2&lt;sup&gt;WT/C96Y&lt;/sup&gt;</th>
<th>Ins2&lt;sup&gt;WT/C96Y&lt;/sup&gt;</th>
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<td><strong>n</strong></td>
<td>12</td>
<td>12</td>
<td>9</td>
<td>9</td>
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<tr>
<td><strong>E-wave, cm/s</strong></td>
<td>77.6±2.9</td>
<td>66.7±2.2</td>
<td>68.5±2.5</td>
<td>72.6±3.2</td>
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<tr>
<td><strong>A-wave, cm/s</strong></td>
<td>48.6±2.8</td>
<td>43.9±2.6</td>
<td>44.5±4.7</td>
<td>44.1±4.0</td>
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<tr>
<td><strong>E/A ratio</strong></td>
<td>1.5±0.05</td>
<td>1.52±0.06</td>
<td>1.60±1.01</td>
<td>1.7±0.1</td>
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<td><strong>IVRT, ms</strong></td>
<td>14.7±0.72</td>
<td>18.87±0.94*</td>
<td>15.03±0.66</td>
<td>18.53±0.94*</td>
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<tr>
<td><strong>DT, ms</strong></td>
<td>22.6±1.45</td>
<td>29.2±1.16*</td>
<td>25.1±2.71</td>
<td>23.6±1.59†</td>
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<tr>
<td><strong>E/WDR, cm/s²</strong></td>
<td>3.67±0.4</td>
<td>2.32±0.124*</td>
<td>2.9±0.4†</td>
<td>3.2±0.3†</td>
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<td><strong>E’, cm/s</strong></td>
<td>3.15±0.15</td>
<td>2.48±0.13†</td>
<td>2.63±0.17†</td>
<td>2.82±0.18</td>
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<tr>
<td><strong>E/E’</strong></td>
<td>24.6±1.72</td>
<td>26.9±0.86</td>
<td>27.9±1.8</td>
<td>26.2±1.4</td>
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<tr>
<td><strong>LA size, mm</strong></td>
<td>1.64±0.08</td>
<td>1.77±0.06*</td>
<td>1.85±0.07</td>
<td>2.17±0.05†</td>
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</table>

Values are means ± SE; n, no. of mice. E-wave, peak early transmitral inflow mitral E velocity; A-wave, mitral Doppler A velocity; IVRT, isovolumetric relaxation time; DT, deceleration time; E/WDR, E-wave deceleration rate (E-wave/DT); E’, early diastolic tissue Doppler velocity; LA, left atrial.

*P < 0.05 compared with age-matched Ins2<sup>WT/C96Y</sup> group; †P < 0.05 compared with 3-mo-old group of the corresponding genotype.

**Ins2<sup>WT/C96Y</sup> mice exhibit no myocardial hypertrophy or fibrosis.** To further characterize the cardiomyopathy in this model of type 1 diabetes, we evaluated the cardiac morphometry and expression levels of disease markers. We found that Ins2<sup>WT/C96Y</sup> hearts appear smaller compared with Ins2<sup>WT/WT</sup> hearts (Fig. 4A, Table 1). LV weight-to-tibial length ratio was significantly lower in Ins2<sup>WT/C96Y</sup> hearts (Fig. 4B), and myocyte cross-sectional areas were significantly smaller in Ins2<sup>WT/C96Y</sup> hearts compared with Ins2<sup>WT/WT</sup> hearts (Fig. 4C). These data suggest that type 1 diabetes can limit myocardial growth. The cardiomyopathy in Ins2<sup>WT/C96Y</sup> mice was further confirmed by elevated expression levels of the disease markers, B-type natriuretic peptide (BNP) (Fig. 4D) and β-myosin heavy chain isoform (β-MHC) (Fig. 4E), further confirming the pathological nature of this phenotype.

Insulin is a tyrosine receptor kinase agonist that can activate the ERK-1/2 and Akt/PKB signaling pathways in the heart (6, 30, 31). The genetic defect in Ins2<sup>WT/C96Y</sup> mice results in an early and sustained loss of the insulin-producing β-cells, resulting in very low plasma insulin levels (17, 54). As such, we evaluated the phosphorylation status of the ERK-1/2 and Akt/PKB pathways, which are known to be activated by insulin (6). Western blot analysis showed a bimodal response of the ERK-1/2 pathway in Ins2<sup>WT/C96Y</sup> mice, characterized by an inhibition of ERK-1/2 phosphorylation at 3 mo followed by increased phosphorylation at 6 mo (Fig. 4F). In contrast, the phosphorylation of Akt/PKB at serine-473 (Fig. 4G) and threonine-308 (Fig. 4H) residues remained unaffected at 3 mo of age, but was significantly reduced at 6 mo of age in the Ins2<sup>WT/C96Y</sup> mice, suggesting a time-dependent loss of ERK-1/2 and Akt/PKB signaling with insulin deficiency.

Increased interstitial fibrosis is a characteristic phenotype in diabetic cardiomyopathy, particularly type 2 diabetic cardiomyopathy, and has been proposed to be responsible for the diastolic dysfunction observed in this condition (8, 37). We asked if the diastolic dysfunction in Ins2<sup>WT/C96Y</sup> mice was associated with myocardial fibrosis. We assessed myocardial fibrosis using trichrome staining and light microscopy and found no evidence of increased interstitial or perivascular fibrotic tissue in Ins2<sup>WT/C96Y</sup> hearts (Fig. 5A). We further confirmed the lack of fibrosis by demonstrating that mRNA expression of the extracellular matrix proteins, procollagen-α type I, procollagen-α type III, and fibronectin were unaltered in Ins2<sup>WT/C96Y</sup> compared with Ins2<sup>WT/WT</sup> hearts at 3 or 6 mo of age (Fig. 5, B–D). These results collectively indicate that insulin deficiency, characteristic of type 1 diabetes, limits myocardial growth due to attenuated insulin-dependent signaling without increased interstitial fibrosis.

**Reduced SERCA2a, metabolic perturbation, and myocardial lipotoxicity as potential mechanisms for diastolic dysfunction in Ins2<sup>WT/C96Y</sup> mice.** In determining the molecular cause of diastolic dysfunction in Ins2<sup>WT/C96Y</sup> mice, we examined the mechanisms that have been linked to diabetic cardiomyopathy. Downregulation of SERCA2a, the major myocardial sarcoplasmic reticulum Ca<sub>2+</sub> pump, is one of the molecular alterations that has been linked to diastolic dysfunction in diabetic cardiomyopathy (8, 14, 37). Phospholamban is a negative regulator of SERCA2a, whose inhibitory function is blocked upon phosphorylation (24). To determine whether this system is altered in Ins2<sup>WT/C96Y</sup> hearts, we assayed for the phosphorylated and total phospholamban and SERCA2a protein levels. Our results demonstrate that, in Ins2<sup>WT/C96Y</sup> mice, protein levels of phospholamban and its phosphorylated form did not change (Fig. 5E), while SERCA2a showed an early and marked downregulation (Fig. 5F).

Altered fatty acid metabolism and lipotoxicity have emerged as a unique and important mechanism by which enhanced fatty acid metabolism can generate toxic effects in the heart and lead to diastolic dysfunction (8, 10, 14, 43). We hypothesized that myocardial fatty acid and triacylglycerol levels would be elevated in the insulin-deficient Ins2<sup>WT/C96Y</sup> hearts. Consistent with our hypothesis, myocardial levels of the major long-chain fatty acids, palmitoyl CoA, oleoyl CoA, and steryl CoA (Fig. 6A), were at least doubled in Ins2<sup>WT/C96Y</sup> mice at 3 mo of age, in association with increased myocardial triacylglycerol levels (Fig. 6B). Altered cardiac fatty acid metabolism in diabetic states often correlates with changes in the expression of various key metabolic genes involved in the control of fatty acid metabolism, including PDK and the acyl-CoA dehydrogenase systems (12, 20, 25, 43). While the expression of the MCAD was unchanged (Fig. 6C), LCAD was significantly elevated in 6-mo-old Ins2<sup>WT/C96Y</sup> hearts (Fig. 6D). In addition, expression analysis showed no alteration in levels of PDK2 (Fig. 6E), but a marked early and persistent increase in mRNA and protein levels of PDK4 (Fig. 6, F and G) in Ins2<sup>WT/C96Y</sup> compared with Ins2<sup>WT/WT</sup> myocardium. These results show that Ins2<sup>WT/C96Y</sup> hearts have increased levels of long-chain fatty acids and triacylglycerol, in association with increased expression of PDK4 and LCAD, providing evidence for altered fatty acid metabolism in Ins2<sup>WT/C96Y</sup> hearts.

To confirm if fatty acid metabolism was altered in Ins2<sup>WT/C96Y</sup> mice, we measured palmitate and glucose oxidation in the isolated working heart preparation. While glucose oxidation showed a small decrease, but was not significantly different between the two genotypes (Fig. 7A), palmitate oxidation was significantly increased in Ins2<sup>WT/C96Y</sup> compared with Ins2<sup>WT/WT</sup> hearts (Fig. 7B). We also found that expression of fatty acid transporters, FATP and CD36, was significantly elevated in Ins2<sup>WT/C96Y</sup> hearts (Fig. 7, C and D). Elevated ceramide and DAG levels are markers of myocardial lipotoxic-
Fig. 4. Ins2\textsuperscript{WT/C96Y} hearts exhibit limited growth and no hypertrophy. A: trichrome-stained four-chamber views of Ins2\textsuperscript{WT/WT} and Ins2\textsuperscript{WT/C96Y} hearts at 3 and at 6 mo of age. B and C: LV weight-to-tibial ratio (LVW/TL) and myocyte cross-sectional area (CSA), respectively, show that Ins2\textsuperscript{WT/C96Y} hearts are smaller than their littermate Ins2\textsuperscript{WT/WT} controls. D and E: disease markers for cardiomyopathy, B-type natriuretic peptide (BNP), and \(\beta\)-myosin heavy chain (\(\beta\)-MHC) are elevated in Ins2\textsuperscript{WT/C96Y} hearts. F: representative Western blots for phospho- and total ERK are shown on the left, and the corresponding quantifications are shown on the right. Phosphorylation of ERK-1/2 was reduced at 3 mo, but rebounded at 6 mo of age. G and H: representative Western blots for phospho- and total-Akt are shown on the left, and the corresponding quantifications on the right. Phosphorylation of Akt at serine-473 (G) and threonine-308 (H) residues were unchanged at 3 mo, but significantly decreased at 6 mo of age in Ins2\textsuperscript{WT/C96Y} mice. \(n = 6\) group. *\(P < 0.05\) compared with Ins2\textsuperscript{WT/WT} mice. RE, relative expression; AU, arbitrary units.
We found that myocardial ceramide and DAG levels are elevated in the myocardial tissue of Ins2 WT/C96Y compared with Ins2 WT/WT mice at 3 mo of age (Fig. 7, E and F). In addition, Oil-O red staining of the hearts showed the presence of lipid droplets in the myocardium of Ins2 WT/C96Y mice at 3 mo of age, which became stronger and more prevalent by 6 mo of age compared with the control Ins2 WT/WT hearts (Fig. 7E).

Diastolic cardiomyopathy in Ins2 WT/C96Y mice reversed by insulin replacement therapy. Insulin treatment in 8-wk-old Ins2 WT/C96Y mice lead to a prompt and sustained normalization of the marked hyperglycemia over the ensuing 4-wk period of implantation (Fig. 8A). At 3 mo of age, we found a significant reduction in disease markers, BNP and β-MHC, in insulin-treated Ins2 WT/C96Y hearts to levels comparable to Ins2 WT/WT hearts (Fig. 8B). Importantly, insulin replacement in Ins2 WT/C96Y mice completely abolished the diastolic dysfunction in these mice, as evident by the restoration of IVRT and DT, increased early TDI diastolic myocardial velocity (E’), while improving the deceleration rate (EWDR) (Fig. 8, C–E). Consistent with a lack of diastolic dysfunction in insulin-treated Ins2 WT/C96Y mice, SERCA2a protein levels were restored (Fig. 8G), while ceramide and
DAG levels were reduced to levels seen in \textit{Ins2}^{WT/WT} myocardium (Fig. 8H), suggesting abolition of lipotoxicity.

**DISCUSSION**

The \textit{Ins2}^{WT/C96Y} (Akita) mice harbor a mutation in the insulin 2 gene (Ins2; Cys96Tyr) that results in a disruption of an intramolecular disulfide bond (48). This affects folding of proinsulin in the endoplasmic reticulum, leading to endoplasmic reticulum stress, proteotoxicity in pancreatic β-cells, and cell loss (17, 27, 53). The \textit{Ins2}^{WT/C96Y} mouse provides an ideal nonobese model of type 1 diabetes that is based on a mutation described in human diabetes, while being free of potential confounding effects of STZ-induced type 1 diabetes (17, 48). Moreover, \textit{Ins2}^{WT/C96Y} mice have several advantages over inbred mouse strains that require STZ treatment, including a better defined etiology, along with a more pronounced and durable hyperglycemia (8, 49).

Our study is the first to show that the predominant cardiac phenotypic abnormality in \textit{Ins2}^{WT/C96Y} mice is an early diastolic dysfunction in the absence of a systolic dysfunction. We evaluated the systolic function in \textit{Ins2}^{WT/C96Y} mice by echocardiography, including TDI, in vivo hemodynamic measurements, and ex vivo working heart preparation, and, consistent with previous studies (9), we found it to be comparable to control \textit{Ins2}^{WT/WT} mice at 3 and 6 mo of age. We captured and characterized the diastolic dysfunction using a state-of-the-art echocardiographic technique, including TDI, in combination with invasive hemodynamic assessments. Our data illustrate the typical pattern of elevated LV filling pressures and/or impaired relaxation. IVRT and EWDR were prolonged in the diabetic \textit{Ins2}^{WT/C96Y} mice. Using TDI, the early diastolic myocardial velocity (E'), which is a sensitive and early marker of diastolic dysfunction (36, 41, 55), was reduced in the \textit{Ins2}^{WT/C96Y} model. However, E-to-A ratio was not decreased.
in the \textit{Ins2\textsuperscript{WT/C96Y}} mice, which may reflect altered loading conditions due to the hyperglycemia and accompanying osmotic diuresis (49), leading to subtle changes in preload interacting with the effects of isoflurane on the cardiovascular system. Based on our hemodynamic measurements, negative minimum change in pressure over time, relaxation time constant of LV pressure, and LVEDP were depressed, prolonged, and increased, respectively, which are all consistent with diastolic dysfunction in the \textit{Ins2\textsuperscript{WT/C96Y}} mice. At 6 mo of age, prolongation of IVRT and LA size enlargement persisted in \textit{Ins2\textsuperscript{WT/C96Y}} compared with \textit{Ins2\textsuperscript{WT/WT}} mice. However, E\textsubscript{*/H11032} was not lowered at this time point, which indicates the complex nature of diabetic cardiomyopathy and requires further investigation.

In addition, \textit{Ins2\textsuperscript{WT/C96Y}} hearts showed no hypertrophy, but with elevated BNP and \textit{\beta-MHC} levels. BNP is a disease marker for cardiomyopathy, which has been reported to be elevated in patients with diastolic heart failure (19). The smaller heart size in \textit{Ins2\textsuperscript{WT/C96Y}} mice is consistent with a previous report in mice lacking cardiac-specific insulin receptor, which showed a similar decrease in heart size with persistent expression of \textit{\beta-MHC} (5), further supporting the role of insulin in physiological cardiac growth. Diastolic heart failure is now a well-recognized clinical entity, often associated with hypertension and diabetes, and can lead to marked morbidity and mortality (21, 34, 36, 46). As such, the \textit{Ins2\textsuperscript{WT/C96Y}} diabetic murine model represents a clinically relevant non-obese model of diastolic dysfunction without the confounding effects of systolic dysfunction.

Insulin is a tyrosine receptor kinase agonist, triggering activation of ERK-1/2 and Akt/PKB signaling pathways in the heart (6, 30). The genetic defect in \textit{Ins2\textsuperscript{WT/C96Y}} mice results in an early and sustained loss of the insulin-producing \textit{\beta}-cells and low plasma insulin levels (17, 54). We found that phosphorylation of ERK and Akt was suppressed in \textit{Ins2\textsuperscript{WT/C96Y}} hearts. We found that the changes in phosphorylation of ERK showed
Fig. 8. Diastolic dysfunction and lipotoxicity in Ins2<sup>WT/C96Y</sup> mice is reversed following insulin treatment. A and B: random blood glucose (A) and expression of disease markers, BNP and β-MHC (B), in Ins2<sup>WT/WT</sup>, Ins2<sup>WT/C96Y</sup>, and insulin-treated Ins2<sup>WT/C96Y</sup> (+Ins) mice. C–F: echocardiographic imaging showing representative transmitral Doppler flow profile (C) and tissue Doppler images (D), as well as averaged IVRT, deceleration time (DT), EWDR, and early tissue Doppler velocity (E<sub>D</sub>′) in Ins2<sup>WT/WT</sup>, Ins2<sup>WT/C96Y</sup>, and insulin-treated Ins2<sup>WT/C96Y</sup> (+Ins) mice. F–H: representative Western blot and quantification of SERCA2a protein levels (F), myocardial DAG (G), and ceramide levels (H) in Ins2<sup>WT/WT</sup>, Ins2<sup>WT/C96Y</sup>, and insulin-treated Ins2<sup>WT/C96Y</sup> (+Ins) mice. n = 5/group, *P < 0.05 compared with all other groups.
a bimodal pattern, with a rise at 6 mo. This bimodal change in ERK-1/2 phosphorylation could be due to a number of factors. The initial decrease in ERK-1/2 phosphorylation could be due to insulin deficiency and/or hyperglycemia, while its subsequent rise could be due to progression of disease in the Akita mice, with activation of neurohumoral systems, leading to increased stimulation of G protein-coupled receptors and/or changes in biomechanical stress, such as an increase in blood pressure. Insulin-stimulated phosphorylation of serine-473-Akt is intact in ex vivo Ins2WT/C96Y hearts (9). As such, the loss of myocardial ERK-1/2 and Akt phosphorylation in Ins2WT/C96Y mice is likely primarily driven by reduced activation of insulin receptors secondary to insulin deficiency. The lack of a reduction in serine-473 and threonine-308 phosphorylation of Akt/PKB in Ins2WT/C96Y hearts at 3 mo of age may reflect compensatory changes by other agonists, such as insulin-like growth factor-I and/or adiponectin, which are known to activate the Akt/PKB pathway (23, 30).

Diastolic dysfunction has been linked to increased interstitial fibrosis, SERCA2 downregulation, and/or lipotoxicity, affecting stiffness as well as active relaxation of the ventricle (3, 46). Myocardial fibrosis in diabetic hearts has been shown to be triggered by oxidative stress (2), and we found that Ins2WT/C96Y hearts exhibited no myocardial fibrosis, consistent with a lack of oxidative stress in these hearts (9). SERCA2 levels were significantly reduced in Ins2WT/C96Y hearts, which could result in prolonged Ca2+ transients, leading to delayed relaxation and subsequently diastolic dysfunction. We also found that expression of β-MHC was increased in the LV of Ins2WT/C96Y mice at 3 and 6 mo of age and may also contribute to the diastolic dysfunction in the Ins2WT/C96Y mice. Recently, Flagg et al. (14) showed elegantly that diastolic dysfunction in a mouse model of lipotoxic diabetic cardiomyopathy with cardiac specific overexpression of FATP is due to suppressed myofilament function rather than altered Ca2+ cycling. Interestingly, these authors also observed elevated β-MHC levels and decreased SERCA2 protein levels with diastolic dysfunction and preserved systolic function. In our model, other proteins involved in Ca2+ handling, such as Na+/Ca2+ exchanger, may also be altered, as previously reported in diabetic cardiomyopathy (8). However, the lack of systolic dysfunction, despite reduced SERCA2a and elevated β-MHC, could be due to alterations in the properties of the Ca2+/myofilament interaction, leading to diastolic dysfunction (1, 14).

Under physiological conditions, the heart derives energy from glucose, fatty acids, and/or lactate, depending on substrate availability, circulating hormone levels, and nutritional status. We found that, in Ins2WT/C96Y hearts, there is increased fatty acid utilization, consistent with previous reports (9). Myocardial mRNA expression levels of MCAD was unchanged, while the expression of LCAD was significantly increased, which is consistent with findings in the type 1 nonobese diabetic mouse model (20). Increased myocardial expression of long-chain ACS is sufficient to predispose the heart to lipotoxic cardiomyopathy (11). Impaired pyruvate oxidation is a hallmark of the metabolic defect found in the diabetic heart, including the Ins2WT/C96Y hearts (9, 43). Pyruvate decarboxylation is a key irreversible step in carbohydrate oxidation mediated by pyruvate dehydrogenase, which is negatively regulated by PDK-induced phosphorylation (25, 43, 50). The increase in PDK4 levels in Ins2WT/C96Y hearts is consistent with insulin acting as a negative regulator of PDK4 (25, 50) and correlates with increased myocardial fatty acid oxidation in the Ins2WT/C96Y mice. Lipotoxicity may arise from myocardial triacylglycerol accumulation, increased use of long-chain fatty acids, and increased production of ceramide and DAG, important markers of lipotoxicity in the heart (8, 11, 15, 34, 44, 51). Indeed, we have shown that palmitate oxidation, as well as myocardial levels of fatty acids, triacylglycerol, ceramide, and DAG were all significantly increased in Ins2WT/C96Y compared with Ins2WT/WT hearts. In addition, we found lipid deposits in the Ins2WT/C96Y myocardium, as also reported by others using electron microscopy (9), consistent with lipotoxic cardiomyopathy in Ins2WT/C96Y hearts. The plasma triglycerol and free fatty acids in Ins2WT/C96Y mice have been shown to be lower and similar, respectively, to that in WT mice (16). We observed increased expression of two key molecules, namely CD36 and FATP, involved in fatty acid uptake and cardiac lipotoxicity (14, 52), suggesting that increased uptake of fatty acids, rather than increased delivery, may have also contributed to the cardiac lipotoxicity observed in the Ins2WT/C96Y diabetic model.

In this study, we show that, in a mouse model of nonobese type 1 diabetes, cardiomyopathy is characterized by early diastolic dysfunction in the absence of systolic dysfunction. This cardiomyopathy is associated with elevated levels of disease markers, but lacks myocardial hypertrophy or fibrosis. We propose that the diastolic dysfunction in Ins2WT/C96Y mice could be brought about by a number of factors, including elevated levels of β-MHC isoform and reduced SERCA2a levels, which may have contributed to the impaired relaxation of the LV. The elevated levels of fatty acids, triglycerol, ceramides, DAG, as well as lipid deposits in the Ins2WT/C96Y hearts strongly suggest myocardial lipotoxicity as the dominant mechanism of the diastolic dysfunction in Ins2WT/C96Y mice. Ins2WT/C96Y mice develop secondary peripheral and hepatic insulin resistance (16). In response to insulin treatment, we showed that, in Ins2WT/C96Y mice, hyperglycemia and lipotoxicity were normalized in association with reversal of the diastolic dysfunction and restoration of SERCA2a, BNP, and β-MHC levels, similar to that in Ins2WT/WT mice. Hence, the diastolic dysfunction seen in this type 1 diabetic mouse model is plastic and reversible. The use of insulin therapy and improved glycemic control is of critical importance in minimizing diabetes-induced cardiomyopathy.

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DISCLOSURES

The authors declare that they do not have any conflict of interest.

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concurrent hormonal and metabolic status in type I diabetes mellitus. 


