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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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. — 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/PKB 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) isofrom 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 transmural filling wave (E-wave) followed by the late filling wave due to atrial contraction (A-wave). E-wave and A-wave were measured and analyzed as a right atrioventricular pressure gradient. Tissue Doppler imaging (TDI) represents a novel and validated technique to assess systolic and diastolic function, with a reduction in E’ and an elevation in E/E’ being considered valid markers of elevated LV filling pressure and diastolic dysfunction (18, 33). TDI was made at the inferolateral region in the radial short axis at the base of the LV and the assessment of peak anular systolic (S’), early diastolic (E’), and late diastolic (A’) myocardial velocities (18, 33). The maximal anteroposterior left atrial diameter was measured by M-mode in the parasternal long-axis view and used as left atrial size.

**Hemodynamic measurements.** In vivo hemodynamic measurements were performed invasively in 10- to 12- wk-old mice of either genotype under 1% isoflurane anesthesia. The right common carotid was exposed and cannulated using a 1.4 French Millar catheter (Millar, Houston, TX), which was then advanced into the proximal aorta and LV for ventricular pressure measurements, as previously described (32). Hemodynamic data were acquired using PowerLab data acquisition system and analyzed.

**Isolated working heart model.** Hearts were perfused using an isolated working heart preparation, as described previously (22). Briefly, hearts were aerobically perfused in an arterograde fashion using an 18-gauge needle at a preload of 7 mmHg and an aortic afterload of 50 mmHg for 30 min with Krebs-Henseleit solution containing 1.2 mM palmitate, 3% BSA, 11 mM glucose, and 2.5 mM Ca²⁺. The perfusate was oxygenated with 95% O₂/5% CO₂, and coronary flows were measured using a transonic flow probe system. Heart function was measured using an inline pressure transducer, and data collection was performed using a BIOPAC data-acquisition system. Specifically, cardiac function was monitored in isolated working hearts using a pressure transducer in the aortic outflow line, as well as flow probes in the left atrial inflow lines and aortic outflow line.

**Insulin treatment in Ins2 WT/C96Y mice.** Insulin pellets (Linshin Canada) were implanted dorsally and subcutaneously in 8-wk-old Ins2 WT/C96Y mice. The pellets release 0.2 U insulin/day for 4 wk. Blood glucose was monitored 2 wk before and every 3 days, starting 1 day after the implant for 4 wk.

**Immunohistological analyses.** For heart morphometry, hearts were arrested in diastole with 1 M KCl, fixed with 10% buffered formalin, and embedded in paraffin. Five-micrometer sections were stained with Masson trichrome and visualized using light microscopy, as previously described (28, 29). Myocyte cross-sectional areas were measured by tracing the cross section of the myocytes in trichrome-stained sections using Image Pro-plus software. The reported average values are from 200 myocytes/five hearts/group. Oil-O red staining was performed using 0.5% oil-O red, as described (26).

**Tagman real-time PCR and Western blot analysis.** mRNA expression levels of procollagen-α type I, procollagen-α type III, and fibronectin were measured using TaqMan real-time PCR, as previously described, using 18S RNA as the internal control (18, 28, 29). The primers and probes for PKD2 and PKD4, mitochondrial medium-chain acyl-CoA dehydrogenase (MCAD), LCAD, acyl-CoA synthase (ACS), CD36, and fatty acid transporter protein (FATP), were purchased from Applied Biosystems Canada. Western blotting was performed to detect phosphorylated and total levels of ERK-1/2, p38, JNK, and Akt/PKB using specific antibodies (Cell Signaling), and PKD4 (Abgent Canada) as before (28, 29). Western blot analyses for SERCA2a, phosphoserine 16)-phospholamban and total phospholamban were carried out using commercial antibodies from Santa Cruz, as previously described (13, 14).

**Blood glucose, plasma insulin, and myocardial triacylglycerol, long-chain fatty acids, ceramide, and diacylglycerol measurements.** Random blood glucose was measured using Ascensia Contour glucometer (Bayer, Canada), and random plasma insulin concentration was measured was measured by ELISA (Alpco Diagnostics, Salem, NH), as previously described (49). Intramyocardial lipids were extracted from 5 mg of flash-frozen freshly excised heart tissue (not perfused), as described previously (45). The dried lipids were redissolved in 50 μl of 3:2 tert-butyl alcohol-Triton X-100-methyl alcohol (1:1 vol/vol) mixture, and cardiac triacylglycerol content was measured with the Wako L-Type TG-H kit (Wako Diagnostics, Osaka, Japan). Identification and quantification of the major long-chain acyl CoA molecular species were performed by high-performance liquid chromatography, as our laboratory has described previously (45). Myocardial ceramide levels (C18) were determined by high-performance liquid chromatography (7, 45). To measure myocardial diacylglycerol (DAG), DAG was converted to [32P]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 Ins2 WT/C96Y and Ins2 WT/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 Ins2 WT/C96Y mice.** The Ins2 WT/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 Ins2 WT/C96Y mice by measuring random plasma insulin (83.8 ± 5.2 pM in Ins2 WT/C96Y vs. 36.2 ± 4.7 pM in Ins2 WT/C96Y, n = 8/group, P < 0.01) and random blood glucose (7.6 ± 1.5 mM in Ins2 WT/C96Y vs. 31.7 ± 4.1 mM in Ins2 WT/C96Y, n = 8/group, P < 0.01) at 3 mo of age. Next, we assessed the cardiac function in Ins2 WT/C96Y and littermate Ins2 WT/WT mice noninvasively using a high-resolution imaging transthoracic echocardiography system equipped with a 30-MHz transducer, invasively by hemodynamic measurements, and ex vivo using an isolated working preparation. M-mode images from Ins2 WT/WT and Ins2 WT/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 Ins2 WT/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 Ins2 WT/C96Y and
Diastolic diameter; systolic diameter; 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.

Fig. 1. Assessment of cardiac systolic function in Ins2WT/WT compared with littermate Ins2WT/WT mice. A: representative M-mode images from Ins2WT/WT and Ins2WT/C96Y hearts at 3 mo of age. B: representative long-axis view of the hearts from Ins2WT/WT and Ins2WT/C96Y mice. C–F: averaged parameters for in vivo hemodynamics (C) and ex vivo working heart preparation (D–F), showing comparable systolic function between Ins2WT/WT and littermate Ins2WT/WT mice. Averaged values are means ± SE (n = 12/group). WT, wild type; LVESD, left ventricular (LV) end-systolic diameter; LVEDD, LV end-diastolic diameter; +dP/dtmax, positive maximum change in pressure over time.

Table 1. Echocardiographic assessment of systolic function in 3- and 6-mo-old mice

<table>
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<tr>
<th></th>
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<th>Ins2WT/C96Y</th>
<th>Ins2WT/WT</th>
<th>Ins2WT/C96Y</th>
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<td>6</td>
<td>6</td>
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<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>5</td>
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<tr>
<td>HR, beats/min</td>
<td>448±13</td>
<td>438±12</td>
<td>459±13</td>
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<td>LVEF, %</td>
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<td>59.8±1.8</td>
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<td>LVFS, %</td>
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<td>29.7±1.33</td>
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<td>SV, μl</td>
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<td>CO, ml/min</td>
<td>19.5±1.26</td>
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<td>LVEDD, mm</td>
<td>3.81±0.07</td>
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<td>LVESD, mm</td>
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<td>LVPWT, mm</td>
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<td>ET, ms</td>
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<td>Vcfc, circ/s</td>
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<td>Vcfc, cm/s</td>
<td>2.39±0.08</td>
<td>2.58±0.09</td>
<td>2.42±0.09</td>
<td>2.61±0.11</td>
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Values are means ± SE; n, no. 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.

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 traditional Doppler technique coupled with TDI. Transmural filling pattern showed reduced E-wave velocity with prolongation of DT, leading to a significant reduction in EWDVs 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, suggesting impaired relaxation (Fig. 2, A and B, Fig. 3B, Table 2). Peak early tissue relaxation (E') was reduced in 3-mo-old Ins2WT/C96Y mice (Fig. 2, E and F, Fig. 3C). To assess for pulmonary edema, we measured wet and dry lung weights in Ins2WT/C96Y and Ins2WT/WT mice, which showed no difference in the lung water content (WT: 0.111 ± 0.002 mg, Akita: 0.097 ± 0.070 mg) or dry-to-wet weight ratio (Ins2WT/WT: 0.36 ± 0.02, Ins2WT/C96Y: 0.33 ± 0.03) between the genotypes. These data collectively demonstrate that Ins2WT/C96Y mice exhibit early diastolic dysfunction at 3 mo of age compared with their littermate Ins2WT/WT mice, but have not reached clinically overt diastolic heart failure.

Impairment of diastolic function in the Ins2WT/C96Y mice was persistent at 6 mo of age. Left atrial size has been shown to positively correlate with the degree and duration of LV dysfunction in the Ins2WT/C96Y mice. Additionally, the left atrial size in the Ins2WT/C96Y mice was significantly larger than that in the Ins2WT/WT mice at 12 mo of age (Fig. 3D).
diastolic dysfunction (36, 42), and we found that the left atrial size in \(\text{Ins2}^{\text{WT/C96Y}}\) mice progressively increased at 3 and 6 mo of age compared with \(\text{Ins2}^{\text{WT/WT}}\) littermates (Table 2). However, the reduction in \(E'/H9270\) and the resulting rise in \(E\)-to-\(E'/H9270\) ratio do not persist in \(\text{Ins2}^{\text{WT/C96Y}}\) 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 \(\text{Ins2}^{\text{WT/C96Y}}\) mice (Fig. 3, D and E), while negative minimum change in pressure over time was reduced in \(\text{Ins2}^{\text{WT/C96Y}}\) mice (Fig. 3F) without alterations in baseline heart rate (524 ± 19 beats/min in \(\text{Ins2}^{\text{WT/WT}}\) vs. 539 ± 23 beats/min in \(\text{Ins2}^{\text{WT/C96Y}}\) mice; \(P = 0.412\)). Overall, our data indicate that \(\text{Ins2}^{\text{WT/C96Y}}\) mice demonstrate early and persistent diastolic dysfunction with preservation of systolic function.

Fig. 2. Echocardiographic assessment of diastolic function in \(\text{Ins2}^{\text{WT/C96Y}}\) compared with \(\text{Ins2}^{\text{WT/WT}}\) mice. A and B: representative transmural Doppler flow profile showing reduced peak E-wave velocity with increased deceleration time and prolongation of isovolumetric relaxation interval in an \(\text{Ins2}^{\text{WT/C96Y}}\) (B) compared with an \(\text{Ins2}^{\text{WT/WT}}\) mouse (A). C and D: representative tissue Doppler images of the basal inferolateral LV wall showing reduced early diastolic tissue velocity (\(E'/H11032\)) in an \(\text{Ins2}^{\text{WT/C96Y}}\) (D) compared with an \(\text{Ins2}^{\text{WT/WT}}\) mouse (C). IVRT, isovolumetric relaxation time.

Fig. 3. Averaged parameters of diastolic dysfunction in \(\text{Ins2}^{\text{WT/C96Y}}\) compared with \(\text{Ins2}^{\text{WT/WT}}\) mice. A–C: echocardiographic assessment showing reduced E-wave deceleration rate (EWDR), prolongation of the IVRT, and reduced early tissue Doppler velocity (\(E'/H11032\)), consistent with diastolic dysfunction (\(n = 12\)/group), respectively. D–F: hemodynamic assessment showing elevated LV end-diastolic pressure (LVEDP), prolonged LV relaxation (\(\tau\)), and reduced negative minimum change in pressure over time (\(-dP/dt_{\text{min}}\)), respectively. *\(P < 0.05\) compared with \(\text{Ins2}^{\text{WT/WT}}\) mice.
Western blot analysis showed a bimodal response of the PKB pathways, which are known to be activated by insulin (6). We evaluated the phosphorylation status of the ERK-1/2 and Akt/survivin signaling pathways in the heart (6, 14, 37, 47). In determining the molecular cause of diastolic dysfunction in Ins2WT/C96Y mice, we examined the mechanisms that have been linked to diastolic cardiomyopathy.

Reduced SERCA2a, metabolic perturbation, and myocardial lipotoxicity as potential mechanisms for diastolic dysfunction in Ins2WT/C96Y mice. In determining the molecular cause of diastolic dysfunction in Ins2WT/C96Y mice, we examined the mechanisms that have been linked to diastolic cardiomyopathy. Downregulation of SERCA2a, the major myocardial sarcoplasmic reticulum Ca2+ pump, is one of the molecular alterations that has been linked to diastolic dysfunction in diabetic cardiomyopathy (8, 14, 37, 47). Phospholamban is a negative regulator of SERCA2a, whose inhibitory function is blocked upon phosphorylation (24). To determine whether our system is altered in Ins2WT/C96Y hearts, we assayed for the phosphorylated and total phospholamban and SERCA2a protein levels. Our results demonstrate that, in Ins2WT/C96Y 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 Ins2WT/C96Y 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 Ins2WT/C96Y 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 Ins2WT/C96Y 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 Ins2WT/C96Y compared with Ins2WT/WT myocardium. These results show that Ins2WT/C96Y 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 Ins2WT/C96Y hearts.

To confirm if fatty acid metabolism was altered in Ins2WT/C96Y 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 Ins2WT/C96Y compared with Ins2WT/WT hearts (Fig. 7B). We also found that expression of fatty acid transporters, FATP and CD36, was significantly elevated in Ins2WT/C96Y hearts (Fig. 7, C and D). Elevated ceramide and DAG levels are markers of myocardial lipotoxicity in Ins2WT/C96Y 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 fibronecin were unaltered in Ins2WT/C96Y compared with Ins2WT/WT mice 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.

Ins2WT/C96Y 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 Ins2WT/C96Y hearts appear smaller compared with Ins2WT/WT hearts (Fig. 4A, Table 1). LV weight-to-tibial length ratio was significantly lower in Ins2WT/C96Y hearts (Fig. 4B), and myocyte cross-sectional areas were significantly smaller in Ins2WT/C96Y hearts compared with Ins2WT/WT hearts (Fig. 4C). These data suggest that type 1 diabetes can limit myocardial growth. The cardiomyopathy in Ins2WT/C96Y mice was further confirmed by elevated expression levels of the disease markers, B-type natriuretic peptide (BNP) (Fig. 4D) and β-miosin heavy chain isoform (B-MHC) (Fig. 4E), further confirming the pathological nature of this phenotype.

Table 2. Echocardiographic assessment of diastolic function in 3- and 6-mo-old mice

<table>
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<tr>
<th>Ins2WT/WT</th>
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<td>n</td>
<td>12</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>E-wave, cm/s</td>
<td>77.5±2.9</td>
<td>66.7±2.3</td>
<td>68.5±2.5</td>
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<tr>
<td>A-wave, cm/s</td>
<td>48.6±2.8</td>
<td>43.9±2.6</td>
<td>44.5±4.7</td>
</tr>
<tr>
<td>E/A ratio</td>
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<td>1.52±0.06</td>
<td>1.6±0.1</td>
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<tr>
<td>IVRT, ms</td>
<td>14.7±0.72</td>
<td>18.87±0.94</td>
<td>15.03±0.66</td>
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<td>DT, ms</td>
<td>22.6±1.45</td>
<td>29.2±1.16*</td>
<td>25.1±2.71</td>
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<tr>
<td>EWDR, cm²</td>
<td>3.67±0.4</td>
<td>2.32±0.124</td>
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<tr>
<td>E', cm/s</td>
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<td>2.48±0.13*</td>
<td>2.63±0.17†</td>
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<tr>
<td>E/E'</td>
<td>24.6±1.72</td>
<td>26.9±0.86*</td>
<td>27.9±1.8</td>
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LA size, mm 1.64±0.08 1.77±0.06* 1.85±0.07 2.17±0.05†

Values are means ± SE; n, no. of mice. E-wave, peak early transmural inflow mitral E velocity; A-wave, mitral Doppler A velocity; IVRT, isovolumetric relaxation time; DT, deceleration time; EWDR, E-wave deceleration rate (E-wave/DT); E', early diastolic tissue Doppler velocity; LA, left atrial. *P < 0.05 compared with age-matched Ins2WT/WT group. †P < 0.05 compared with 3-mo-old group of the corresponding genotype.
Fig. 4. Ins2<sup>WT/C96Y</sup> hearts exhibit limited growth and no hypertrophy. A: trichrome-stained four-chamber views of Ins2<sup>WT/WT</sup> and Ins2<sup>WT/C96Y</sup> 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<sup>WT/C96Y</sup> hearts are smaller than their littermate Ins2<sup>WT/WT</sup> controls. D and E: disease markers for cardiomyopathy, B-type natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC) are elevated in Ins2<sup>WT/C96Y</sup> 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 are shown on the right. Phosphorylation of Akt at serine-473 (S473) and threonine-308 (T308) residues were unchanged at 3 mo, but significantly decreased at 6 mo of age in Ins2<sup>WT/C96Y</sup> mice. n = 6/group. *P < 0.05 compared with Ins2<sup>WT/WT</sup> 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).

Fig. 5. Absence of interstitial fibrosis with downregulation of sarco(endo)plasmic reticulum Ca2+-ATPase 2a (SERCA2a) protein expression in 3- and 6-mo-old Ins2
WT/C96Y mice. A: trichrome staining shows normal myocardial architecture with no accumulation of fibrotic tissue. Scale bar = 100 μm (inset = 50 μm).

B–D: mRNA expression levels of procollagen-α1 type I, procollagen-α1 type III, and fibronectin, respectively, were comparable between Ins2
WT/WT and Ins2 WT/C96Y hearts (n = 6/group). E and F: representative Western blots are shown on the left, and the corresponding quantifications are shown on the right. Phospho (serine 16)-phospholamban (p-PLN)-to-total phospholamban (total-PLN) ratio was comparable between the groups (E), whereas SERCA2a protein levels decreased significantly in Ins2 WT/C96Y hearts (F). α-Tubulin and Coomassie blue staining were used as loading controls (n = 6/group). *P < 0.05 compared with Ins2 WT/WT mice.

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 \( \text{Ins}^2_{\text{WT/WT}} \) myocardium (Fig. 8H), suggesting abolition of lipotoxicity.

**DISCUSSION**

The \( \text{Ins}^2_{\text{WT/C96Y}} \) (Akita) mice harbor a mutation in the insulin 2 gene (\( \text{Ins}^2; \text{Cys96Tyr} \)) 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 \( \text{Ins}^2_{\text{WT/C96Y}} \) 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, \( \text{Ins}^2_{\text{WT/C96Y}} \) 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 \( \text{Ins}^2_{\text{WT/C96Y}} \) mice is an early diastolic dysfunction in the absence of a systolic dysfunction. We evaluated the systolic function in \( \text{Ins}^2_{\text{WT/C96Y}} \) 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 \( \text{Ins}^2_{\text{WT/WT}} \) 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 \( \text{Ins}^2_{\text{WT/C96Y}} \) mice. Using TDI, the early diastolic myocardial velocity (\( \text{E}^\prime \)), which is a sensitive and early marker of diastolic dysfunction (36, 41, 55), was reduced in the \( \text{Ins}^2_{\text{WT/C96Y}} \) model. However, E-to-A ratio was not decreased.
in the \textit{Ins2}^{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}^{WT/C96Y} mice. At 6 mo of age, prolongation of IVRT and LA size enlargement persisted in \textit{Ins2}^{WT/C96Y} compared with \textit{Ins2}^{WT/WT} mice. However, \( E \) was not lowered at this time point, which indicates the complex nature of diabetic cardiomyopathy and requires further investigation.

In addition, \textit{Ins2}^{WT/C96Y} hearts showed no hypertrophy, but with elevated BNP and \( \beta \)-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}^{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 \( \beta \)-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}^{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}^{WT/C96Y} mice results in an early and sustained loss of the insulin-producing \( \beta \)-cells and low plasma insulin levels (17, 54). We found that phosphorylation of ERK and Akt was suppressed in \textit{Ins2}^{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 SERCA2α 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 Ins2 WT/C96Y hearts (9). As such, the loss of myocardial ERK-1/2 and Akt phosphorylation in Ins2 WT/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 Ins2 WT/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 Ins2 WT/C96Y hearts exhibited no myocardial fibrosis, consistent with a lack of oxidative stress in these hearts (9). SERCA2 levels were significantly reduced in Ins2 WT/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 Ins2 WT/C96Y mice at 3 and 6 mo of age and may also contribute to the diastolic dysfunction in the Ins2 WT/C96Y hearts. 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 Ins2 WT/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 Ins2 WT/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 PKD4 levels in Ins2 WT/C96Y hearts is consistent with insulin acting as a negative regulator of PKD4 (25, 50) and correlates with increased myocardial fatty acid oxidation in the Ins2 WT/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, 35, 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 Ins2 WT/C96Y compared with Ins2 WT/WT hearts. In addition, we found lipid deposits in the Ins2 WT/C96Y myocardium, as also reported by others using electron microscopy (9), consistent with lipotoxic cardiomyopathy in Ins2 WT/C96Y hearts. The plasma triglycerol and free fatty acids in Ins2 WT/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 Ins2 WT/C96Y diabetic model.

In this study, we show that, in a mouse model of nonobese type I 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 Ins2 WT/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 Ins2 WT/C96Y hearts strongly suggest myocardial lipotoxicity as the dominant mechanism of the diastolic dysfunction in Ins2 WT/C96Y mice. Ins2 WT/C96Y mice develop secondary peripheral and hepatic insulin resistance (16). In response to insulin treatment, we showed that, in Ins2 WT/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 Ins2 WT/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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