Na\(^{+}/\text{Ca}^{2+}\) exchanger-1 protects against systolic failure in the Akita\(^{ins2}\) model of diabetic cardiomyopathy via a CXCR4/NF-κB pathway

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LaRocca TJ, Fabris F, Chen J, Benhayon D, Zhang S, McCollum L, Schecter AD, Cheung JY, Sobie EA, Hajjar RJ, Lebeche D. Na\(^{+}/\text{Ca}^{2+}\) exchanger-1 protects against systolic failure in the Akita\(^{ins2}\) model of diabetic cardiomyopathy via a CXCR4/NF-κB pathway. Am J Physiol Heart Circ Physiol 303: H353–H367, 2012. First published May 18, 2012; doi:10.1152/ajpheart.01198.2011.—Diabetic cardiomyopathy is characterized, in part, by calcium handling imbalances associated with ventricular dysfunction. The cardiac Na\(^{+}/\text{Ca}^{2+}\) exchanger 1 (NCX1) has been implicated as a compensatory mechanism in response to reduced contractility in the heart; however, its role in diabetic cardiomyopathy remains unknown. We aimed to fully characterize the Akita\(^{ins2}\) murine model of type 1 diabetes through assessing cardiac function and NCX1 regulation. The CXCL12/CXCR4 chemokine axis is well described in its cardioprotective effects via progenitor cell recruitment postacute myocardial infarction; however, it also functions in regulating calcium dependent processes in the cardiac myocyte. We therefore investigated the potential impact of CXCR4 in diabetic cardiomyopathy. Cardiac performance in the Akita\(^{ins2}\) mouse was monitored using echocardiography and in vivo pressure-volume analysis. The Akita\(^{ins2}\) mouse is protected against ventricular systolic failure evident at both 5 and 12 mo of age. However, the preserved contractility was associated with a decreased sarco(end)plasmic reticulum Ca\(^{2+}\)/ATPase (SERCA2a)/phospholamban ratio and increased NCX1 content. Direct myocardial injection of adenovirus encoding anti-sense NCX1 significantly decreased NCX1 expression and induced systolic failure in the Akita\(^{ins2}\) mouse. CXCL12 and CXCR4 were both upregulated in the Akita\(^{ins2}\) heart, along with an increase in I\(\text{CaL}\)-o and NF-κB p65 phosphorylation. We demonstrated that CXCR4 activation upregulates NCX1 expression through a NF-κB-dependent signaling pathway in the cardiac myocyte. In conclusion, the Akita\(^{ins2}\) type 1 diabetic model is protected against systolic failure due to increased NCX1 expression. In addition, our studies reveal a novel role of CXCR4 in the diabetic heart by regulating NCX1 expression via a NF-κB-dependent mechanism.

systolic function; calcium cycling; action potential

DIABETES MELLITUS is a leading risk factor in causing premature illness and death worldwide (10). Importantly, diabetes mellitus is a known independent risk factor for cardiovascular disease and heart failure and more than half of diabetic inviduals will succumb to a cardiovascular event (11, 42). Diabetic cardiomyopathy is defined in part by diastolic and systolic impairment, myocardial remodeling, and inflammation absent from other independent risk factors such as hypertension and coronary artery disease (17, 35). Myocardium exposed to hyperglycemia not only alters the energetic efficiency of the cardiac myocyte, which relies heavily on fatty acid oxidation in the diabetic state, but also leads to significant alterations in activity and expression of Ca\(^{2+}\) transporters including cardiac sarco(endo)plasmic reticulum Ca\(^{2+}\)/ATPase (SERCA2a), L-type Ca\(^{2+}\) channel (LTCC), and cardiac Na\(^{+}/\text{Ca}^{2+}\)-exchanger-1 (NCX1) (21, 31, 48).

The molecular mechanisms of diabetic cardiomyopathy are not well understood; however, impairment of Ca\(^{2+}\) homeostasis is a significant feature of type 1 and type 2 diabetic cardiomyopathies (7, 49). In normal excitation-contraction (EC) coupling, Ca\(^{2+}\) entry via LTCC triggers the release of approximately two-thirds of Ca\(^{2+}\) stored in sarcoplasmic reticulum (SR), thereby acutely raising cytosolic Ca\(^{2+}\), which activates the contractile apparatus (1, 3). During diastole, ~95% of cytosolic Ca\(^{2+}\) in murine myocytes is resequenced into the SR by SERCA2a [under the control of phospholamban (PLB)], thereby lowering intracellular Ci\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and allowing myocyte relaxation (3). To maintain beat-to-beat Ca\(^{2+}\) balance, Ca\(^{2+}\) that has entered via LTCC is extruded by NCX1, with minor contributions from the plasmalemmal Ca\(^{2+}\)/ATPase (3, 4). The exact role of NCX1 in disease and whether it participates as a compensatory or maladaptive mechanism remain controversial. In the initial stages of clinical and experimental heart failure, SERCA2a Ca\(^{2+}\) reuptake activity and expression are decreased leading to reduced SR Ca\(^{2+}\) load and increased diastolic [Ca\(^{2+}\)]\(_i\) (13, 15, 25, 38). Increased NCX1 expression and forward NCX current (\(I_{\text{ncx}}\)) is thought to contribute up to 50% of cytoplasmic calcium efflux to maintain diastolic [Ca\(^{2+}\)]\(_i\) levels as a compensatory mechanism to decreased SERCA2a activity (34, 43, 46). In addition, reverse \(I_{\text{ncx}}\) may contribute to Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) by directly contributing to local Ca\(^{2+}\) for CICR and through refilling SR Ca\(^{2+}\) content (26). However, this may come at the cost of increased arrhythmogenesis at the cellular level (41, 45).

We utilized the Akita\(^{ins2}\) mouse, a genetic model of type 1 diabetes, in which diabetes is evident by 4 wk of age with...
blood glucose levels consistently >600 mg/dl in males (47). Several groups (2, 6, 24, 28) have reported strikingly different results on the systolic and diastolic capacity of the Akitains2 hearts ranging from overt heart failure to minimal cardiac dysfunction. The conflicting data led us to initiate an in-depth cardiac physiological analysis of the Akitains2 mouse to accurately determine the exact nature of type 1 diabetic cardiac dysfunction and to identify potential mechanisms involved.

The CXCL12/CXCR4 chemokine pair has been identified as being cardioprotective in acute myocardial infarction. Augmenting the CXCL12/CXCR4 axis promotes endothelial progenitor cell recruitment, angiogenesis, and possibly cardiogenesis maintaining and preserving ventricular function postmyocardial infarction (5, 14). However, the CXCL12/CXCR4 chemokine axis may participate in additional mechanisms promoting cardiac myocyte survival and function. CXCR4 has direct signaling consequences in the cardiomyocyte (32) and may provide beneficial regulation of cardiac homeostatic mechanisms. Due to the presence of Ca2+ handling imbalances, altered Ca2+ cycling proteins expression, and activity in diabetic cardiomyopathies, we sought to determine the role of the CXCL12/CXCR4 axis in this process.

MATERIALS AND METHODS

Animals, In Vivo Studies, and Virus Injection

Animals were handled as approved by the Mount Sinai Institutional Animal Care and Use Committees in accordance with the Principles of Laboratory Animal Care by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86–23, revised 1996).

Heterozygous male Akitains2 and wild-type controls were acquired from Jackson laboratories at 6–8 wk of age. Twenty- and fifty-two-week-old mice were anesthetized with intraperitoneal ketamine (100 μg/g) for echocardiographic analysis. Two-dimensional images and M-mode tracings were recorded on the short-axis at the level of the papillary muscle to determine percent fractional shortening and ventricular dimensions (GE Vivid 7 Vision). One day after echocardiography, in vivo hemodynamics were performed using a 1.2-Fr (2-electrode) pressure-volume (PV) conductance catheter Advantage System (Scisense, Canada). Mice were anesthetized with an intraperitoneal injection of urethane (1 mg/g), etomidate (10 μg/g), and morphine (1 μg/g) combination and intubated via a tracheotomy and mechanically ventilated at 7 μl/g tidal volume and 125 respirations/min. A central jugular venous cannula was placed for vascular access, and a thoracotomy was performed to expose the heart. The PV catheter was placed in the left ventricle via an apical stab approach as previously described (27). Hemodynamics data were obtained at baseline and after isoproterenol administration (20 μg·kg−1·min−1 for 5 min; Sigma-Aldrich, St. Louis, MO). PV data were analyzed using IOX2 software (EMKA Technologies, Falls Church, VA).

For viral injection, wild-type and Akitains2 mice (20 wk old; n = 7) were anesthetized with 50 μl of ketamine/xylazine (50/5 μg/g) mix intraperitoneal injection, intubated, and ventilated. The chest was opened, and 30 μl of 1.5 × 10⁸ particles/μl of either Ad.GFP or Ad.asNCX1 were directly injected into left ventricular free wall at five to six injection sites with 30-g needle-1-cc syringe. After 7 days postinjection (a window which constitutes the optimal time for Adeno gene expression), wall motion and wall thickening, percent fractional shortening, and ventricular dimensions were evaluated by echocardiography. One day after echocardiography in vivo hemodynamics were performed using a 1.2-Fr PV conductance catheter (Scisense) as described above.

Culture of Adult Rat Ventricular Myocytes

Adult rat ventricular myocytes (ARVM) were isolated from Sprague-Dawley rats. Hearts were excised and quickly cannulated in the ascending aorta. The hearts were initially perfused for 5 min with a low calcium Tyrode’s buffer (containing in mM: 120.0 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, and 25.0 HEPES, pH 7.40 at 37°C) and followed by an enzyme solution containing collagenase (0.7 mg/ml; Worthington Type II, 258 μg/mg), and the hearts were perfused for another 10–15 min. The hearts were minced, filtered, and underwent a step-wise calcium challenge reaching a final calcium concentration of 1.2 mM. ARVMs were plated at a density of 2 × 10⁴ cells/ml onto laminin-precovers on coverslips (1 μg/cm², Invitrogen) and cultured for 24 h in MEM (Sigma) containing 50 IU/ml penicillin/streptomycin, 0.1% BSA, 10 mM 2,3-butanediol monoxime, and 1× insulin/transferrin/selenium.

Isolated Myocyte Calcium Transient Studies

Adult mouse cardiomyocytes from the Akitains2 mouse and wild-type controls were isolated as previously described (23). Freshly isolated mouse cardiomyocytes plated onto laminin-coated coverslips were loaded with fura 2-AM (Invitrogen) for 5 min at 27°C and subsequently washed. Cells included in the study were rod shaped with a clear striation pattern and were quiescent in the absence of electrical stimulation. Cells were placed in a chamber and paced at 1 Hz with a 4-ms duration. Sarcomeric shortening and whole cell calcium transients were determined on individual cardiomyocytes using a dual excitation spectrophotometer and video-edge detection system (Ionoptix), as described previously (22). Three animals per group were used measuring ≥15 cardiomyocytes per animal to generate average sarcomeric shortening and whole cell calcium transients.

Immunoblotting

Isolated cardiomyocytes, cultured for 24 h, and whole left ventricular tissues, harvested from the Akitains2 and wild-type littermate controls, were lysed with RIPA buffer plus protease inhibitors (Roche) and protein concentration was determined using a standard Bradford assay (Bio-Rad). Cytoplasmic and nuclear fractions of adult cardiomyocytes were prepared using the NE-PER nuclear and cytoplasmic extraction reagents kit (Thermo Scientific) according to the manufacturer’s instructions. Western blots were performed using antibodies against SERCA2a (1:5,000; custom-made in our laboratory); CXCR4 (1:200; Abcam); CXCL12 (1:500, Cell Signaling); phospholamban (1:5,000; Barilla); Cav1.2 (1:500; Antibodies, Inc.); NCX1 (1:1,000; Invitrogen); β1-adrenergic receptor (β1-AR), β2-AR, and Gs, (1:1,000; Santa Cruz Biotechnology); and IκBα and NF-κB (Cell Signaling Technology). Signal intensities were visualized by Chemiluminescence (Pierce). Films from at least three independent experiments were scanned, and densities of the immunoreactive bands were evaluated using NIH ImageJ software. Protein loadings were verified against GAPDH densities.

Quantitative Real-Time PCR

RNA was extracted from isolated ARVMs using TRIzol reagent (Invitrogen). Total RNA (250 ng) was used for cDNA synthesis using SuperScript reverse transcriptase (BioRad) following manufacturer’s protocol. CXCL12 and CXCR4 expression levels were determined using SYBR Green PCR Master Mix (Applied Biosystems). One microliter of cDNA synthesis was used with a final primer concentration of 0.4 μM in each 25-μl PCR reaction. Real-time PCR conditions included a 95°C for a 10-min denaturation, followed by 40 amplification cycles: 15 s at 95°C (denaturation) and 60 s at 59°C (annealing and extension). Postamplification dissociation curves were performed to verify the presence of a single amplification product in the absence of genomic DNA.
contamination or contribution from primer dimers. Fold changes in gene expression were determined using the ΔΔCt method with normalization to endogenous controls. Primers sequences include the following: CXCL12 forward: 5’-CTCTTATCCCCATCTCTCTCA-3’; CXCL12 reverse: 5’-GACTCTGCTCTGGTGGAAGG-3’ (NM_021704); CXCR4 forward: 5’-CTCGTGCTGCA-CAGTGGAATCT-3’; CXCR4 reverse: 5’-GGTACGGCAGG-GACAGGAGG-3’ (NM_022205); 28S rRNA forward: 5’-CTCG CTGGCCCTGAAATCC-3’; and 28S rRNA reverse: 5’-CCCAGCCCTTAGACCACTTCTA-3’ (NR_046246).

**Histology and Fluorescent Immunoassaying**

Heart tissue. Whole ventricular tissue was frozen in optimum cutting temperature using liquid nitrogen (TissueTek). Frozen sections (10 μm) were mounted and subsequently stained with hematoxylin-eosin using routine procedures. For CXCL12 immunostaining, sections were incubated at 37°C for 30 min.Slides were then fixed in 100% cold acetone for 10 min at −20°C; nonspecific binding sites were blocked using 10% goat serum (DAKO) for 30 min at 37°C in a humid chamber. Ventricular sections were coincubated with anti-CXCL12 (1:100; Santa Cruz Biotechnology) and anti-α sarcomeric actin (1:100; Abcam) in blocking buffer overnight at 4°C in a humid chamber. CXCL12 and sarcomeric actin were visualized with goat anti-rabbit-FITC and goat anti-mouse-Cy3, respectively. Sections were counterstained with DAPI to visualize cell nuclei and mounted with a coverslip (Vector Labs) for confocal microscopy.

Isolated myocytes. Adult rat cardiomyocytes, isolated as described above, were fixed with 4% formaldehyde and stained with an anti-NF-κB p65 monoclonal antibody (Cell Signaling; 1:400). Primary antibodies were detected with an anti-mouse Alexa Fluor 555 IgG secondary antibodies (Invitrogen). Images were collected using a Olympus IX71 fluorescence microscope. Cells were counterstained with DAPI to visualize cell nuclei and mounted with a coverslip (Vector Labs). Nonspecific staining for NF-κB was assessed by omission of the primary antibody and examination of the cells in the presence of the secondary antibody alone.

**Assessment of [Ca2+] in SR and Na+-Ca2+ Exchange Activity**

All chemicals were purchased from the Sigma-Aldrich unless otherwise indicated. Normal Tyrode (NT) solution was composed of the following (in mM): 140 NaCl, 1.2 CaCl2, 1 MgCl2, 10 glucose, and 5 HEPES, pH 7.4 with NaOH. The Na+ free NT had the same concentrations of the aforementioned chemicals, with the exception of no added CaCl2, the addition of 10 EGTA, and 140 LiCl substituted for NaCl; the solution was brought up to a pH of 7.4 with LiOH.

Isolated mouse ventricular myocytes were loaded with 10 μM fluo-3 AM (Biotium, Hayward, CA), a single-wavelength cytosolic calcium dye, for 30 min at room temperature (20–22°C) and allowed to deesterify for at least 30 min. Cells were then placed in a laminin-coated experimental chamber and left to settle for 5 min so that the cells could adhere.

Changes in [Ca2+] were recorded using a laser-scanning confocal microscope (Zeiss 5 Exciter) operating in line-scan mode, with excitation at 488 nm and emission >505 nm. Cells were perfused with NT and electrically stimulated at 0.5 Hz for 2 min to establish a steady-state SR Ca2+ load. Stimulation was halted, a line-scan recording was initiated, and NT containing 20 mM caffeine was rapidly perfused onto the cells after ~10 s. After again being perfused with NT and electrically stimulated for ≥2 min to reload the SR, a second application of 20 mM caffeine was performed. In this case, after stimulation was ceased, cells were perfused with 0 Na+ 0 Ca2+ solutions for 30 s, and then 0 Na+ 0 Ca2+ solution containing 20 mM caffeine was applied.

These measurements served multiple purposes. The peak increase in fluorescence upon application of 20 mM caffeine in 0 Na+ 0 Ca2+ solutions was used to estimate the total Ca2+ content of the SR [see below for a more detailed explanation of the calculation of [Ca2+] in SR ([Ca2+]SR)]. The difference in the rates of Ca2+ decay in the presence vs. the absence of extracellular Na+ was used as an index of NCX activity in each cell. Analysis of these data was performed using routines written in the MATLAB programming environment that performed the following steps: 1) average the line-scan image over the cell area; 2) convert from fluorescence to intracellular [Ca2+]; 3) determine the maximum intracellular [Ca2+] and convert to the equivalent [Ca2+]SR; and 4) fit the decaying phase of the Ca2+ signal to a decaying exponential curve.

**Analysis and Calculation of [Ca2+]SR**

The data analysis performed using MATLAB incorporates fluorescence data taken from images recorded via confocal microscopy with well-established mathematical equations to calculate [Ca2+]SR accurately. Initially, ΔF/F0 is determined by subtracting baseline fluorescence from the peak of the caffeine-induced calcium transient. Total cellular Ca2+ bound to fluo-3 AM ([Ca2+]i) can be determined from fluorescence and established buffering properties using the following formula (in nM):

$$[\text{Ca}^{2+}] = \frac{\Delta F}{F_0} \cdot \frac{K_{d_{\text{fluor}}} \cdot [\text{Ca}^{2+}]_{SR}}{K_{d_{\text{fluor}}} \cdot [\text{Ca}^{2+}]_{SR} - \Delta F / F_0 + 1}$$

Total cellular Ca2+ calculation incorporates [Ca2+]i, with additional properties of binding and dissociation of other buffering properties in the cell (in μM):

$$[\text{Ca}^{2+}]_{\text{tot}} = [\text{Ca}^{2+}] + [\text{Ca}^{2+}]_{\text{SR}} + [\text{Ca}^{2+}]_{\text{SR}} = [\text{Ca}^{2+}]_{\text{SR}} + [\text{Ca}^{2+}]_{\text{SR}} = [\text{Ca}^{2+}]_{\text{SR}}$$

Total [Ca2+]SR can be calculated by dividing the total cellular calcium by the percentage of cellular volume occupied by the SR (in μM):

$$[\text{Ca}^{2+}]_{\text{SR}} = \frac{[\text{Ca}^{2+}]_{\text{tot}}}{\text{SR ratio}}$$

Finally, with the use of known buffering properties of [Ca2+]SR, [Ca2+]SR-free can be determined (in μM):

$$[\text{Ca}^{2+}]_{\text{SR-free}} = -\frac{K_{d_{\text{SR}}} + [\text{Ca}^{2+}]_{\text{SR}} - B_{\text{max}_{\text{SR}}} \cdot [\text{Ca}^{2+}]_{\text{SR}}}{2K_{d_{\text{SR}}} + 2 + 2 \cdot K_{d_{\text{SR}}} \cdot [\text{Ca}^{2+}]_{\text{SR}} \cdot [\text{Ca}^{2+}]_{\text{SR}} + 2 \cdot K_{d_{\text{SR}}} \cdot B_{\text{max}_{\text{SR}}} \cdot [\text{Ca}^{2+}]_{\text{SR}} \cdot [\text{Ca}^{2+}]_{\text{SR}} - 2 \cdot [\text{Ca}^{2+}]_{\text{SR}} \cdot B_{\text{max}_{\text{SR}}} + B_{\text{max}_{\text{SR}}}^2}{2}$$

where [Ca2+]SR is total cellular Ca2+ bound to fluo-3 AM (nM); [Ca2+]SR is SR Ca2+ concentration (μM); [Ca2+]SR-free is unbound [Ca2+]SR (μM); [Ca2+]tot is total cellular [Ca2+] (μM); Bmax_{fluor} is binding capacity of fluo-3 AM to Ca2+ (150 μM/I cytosol); Bmax_{SR} is binding capacity for Ca2+ binding within the cell (150 μM/I) cytosol; Bmax_{SR} is binding capacity for Ca2+ binding within the SR (3.25 mM/I SR); K_d_{fluor} is cytosolic [Ca2+] at rest (100 nM); F/F0 is relative fluorescence (arbitrary units); K_d_{fluor} is dissociation constant between fluo-3 AM and Ca2+ (1.1 μM); K_d_{SR} is dissociation constant of Ca2+ binding within cell (1.1 μM); K_d_{SR} is dissociation constant of Ca2+ binding...
within the SR (0.63 mM/l); and SR\textsubscript{ratio} is %cellular volume attributed to SR (0.05).

**Action Potential Recording and Analysis**

An additional group of myocytes were loaded with 5 µM Di-8-ANEPPS (Biotium, Hayward, Ca), a membrane-bound voltage indicator dye, for 20 min at 20–22°C. While membrane potential voltage cannot be quantified, the changes in fluorescence observed relative to resting fluorescence directly correlate to changes in membrane voltage relative to resting membrane potential, thus enabling an accurate measurement of action potential duration (APD). Cells were placed in a laminin-coated chamber and left to settle and adhere for 5 min before initiating gravity-driven perfusion with NT. This solution additionally contained 20 µM blebbistatin, an inhibitor of myosin’s ATPase activity, to prevent motion artifacts. Fluorescence was excited with an He-Ne laser at 543 nm, and fluorescence was recorded. Line scan images reflecting changes in transmembrane potential were obtained during steady-state pacing at 1 Hz. Data were analyzed using a MATLAB routine that averages the change in fluorescence of the series of APs recorded in a single cell, normalizes all cells to a uniform amplitude, and calculates the time it takes for a cell to repolarize to a predetermined percentage of the amplitude.

**Statistics**

Numeric data are presented as means ± SD. One-way ANOVA and Student’s t-test were utilized with P values <0.05 considered statistically significant.

**RESULTS**

**Echocardiography Indicates Normal Systolic Function in the Akitains² Mouse**

We first utilized noninvasive transthoracic echocardiography in the Akitains² and age-matched wild-type controls at 20 weeks of age. Echocardiographic analysis and gross examination of the Akitains² heart at 20 weeks of age compared with wild-type (WT) controls. A: representative M-mode images of WT and Akitains² mice. B: gross examination of the hearts of WT and Akitains² shows decreased cardiac size as well as significantly decreased heart weight/body weight ratio. C: ventricular indexes: left ventricular inner diameter diastole and systole (LVIDd/s) are smaller in the Akitains² heart. D: cardiac function was not significantly different between the Akitains² and WT controls as determined by percent fractional shortening (%FS). E: heart rates were not significantly different (n = 5 WT; n = 7 Akita). F: pressure-volume analysis of Akitains² mice and WT controls. Representative baseline pressure-volume loops of the Akitains² mouse (blue) compared with WT (black) at 5 and 12 mo of age (n = 4–5 Wild-type; n = 4–7 Akita) are shown. G: percent survival rates of Akitains² mice; the Akitains² cumulative survival drastically decreases at 52 wk of age. *P < 0.05; **P < 0.01; ns, nonspecific.
1. When we administered the nonspecific proterenol wild-type mice responded immediately with in-weight ratio in the Akitains2 mouse (Fig. 1) modest cardiac atrophy with a diminished heart weight:body weight ratio in the Akitains2 mouse (Fig. 1B).

In Vivo Hemodynamics Reveals Diastolic Dysfunction with Enhanced Inotropy in the Akitains2 Mouse

To further examine Akitains2 cardiac function, we conducted left ventricular PV analysis. PV analysis of the Akitains2 at 5 mo of age (12-mo) revealed abnormalities in diastolic measurements including increased relaxation time and tau (Table 1). Upon inferior vena cava occlusion to determine load-independent indexes of contraction and relaxation, an increased end-diastolic PV relationship was observed (Table 1). Lusitropic defects are classical indicators of cardiac dysfunction accompanying type 1 diabetes. In addition to the diastolic abnormalities observed in the Akitains2, the systolic function was enhanced with increased dP/dmax and ESPVR (Table 1). These results are in concordance with diastolic dysfunction observed in the Akitains2 in vivo. Importantly, we observed no alterations in peak Ca2+ or peak sarcomeric shortening. Similar peak [Ca2+] transient amplitudes but decreased tau of [Ca2+] transient decline suggest reduced SR Ca2+ uptake activity in Akitains2 myocytes.

Isolated Cardiomyocytes from the Akitains2 Have Diastolic Dysfunction with Preserved Contractility

We isolated cardiomyocytes from 20-wk-old Akitains2 mice and their wild-type controls. Upon field stimulation, Ca2+ transient analysis indicated increased Ca2+ reuptake time and prolonged myocyte relaxation (Fig. 3A). There was a significant increase in tau and time to 10, 50, and 90% relaxation (Table 2). These results are in concordance with diastolic dysfunction observed in the Akitains2 in vivo. Importantly, we observed no alterations in peak Ca2+ or peak sarcomeric shortening. Similar peak [Ca2+] transient amplitudes but decreased tau of [Ca2+] transient decline suggest reduced SR Ca2+ uptake activity in Akitains2 myocytes.

Analysis of Ca2+ Handling Proteins Expression Profile in the Akitains2 Heart

Alterations in key Ca2+ transporters influence ventricular function in diabetes. We examined how LTCC, NCX1, SERCA2a, and PLB expressions were affected in the Akitains2 heart. LTCC (Ca1.2) expression was unaffected between the

<table>
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<th>Hemodynamics</th>
<th>WT (n = 5)</th>
<th>Akitains (n = 7)</th>
<th>WT (n = 4)</th>
<th>Akitains (n = 4)</th>
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<tbody>
<tr>
<td>Pes, mmHg</td>
<td>87.1 ± 5.9</td>
<td>94.2 ± 6.8</td>
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<td>Ped, mmHg</td>
<td>5.1 ± 1.1</td>
<td>4.8 ± 1.3</td>
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<td>dPmax, mmHg/s</td>
<td>6,091 ± 617</td>
<td>9,111 ± 978‡</td>
<td>4,474 ± 152</td>
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<td>dPmin, mmHg/s</td>
<td>−4,777 ± 998</td>
<td>−5,008 ± 1,001</td>
<td>−3,560 ± 185</td>
<td>−4,238 ± 401</td>
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<td>RT, ms</td>
<td>16.0 ± 1.2</td>
<td>22.3 ± 2.1†</td>
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<td>Tau, ms</td>
<td>4.21 ± 0.42</td>
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<td>EDV, μl</td>
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<td>6.7 ± 5.1</td>
<td>34.0 ± 9.1</td>
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<td>SV, μl</td>
<td>29.9 ± 3.8</td>
<td>32.2 ± 4.1</td>
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<td>CO, μl/min</td>
<td>15,299 ± 1,840</td>
<td>14,445 ± 2,001</td>
<td>14,825 ± 2,277</td>
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<td>EF, %</td>
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<td>2133 ± 122</td>
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<td>HR, beats/min</td>
<td>576 ± 31</td>
<td>554 ± 42</td>
<td>467 ± 37</td>
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</table>

Values are means ± SD. WT, wild type; Pes and Ped, end-systolic and -diastolic pressure; dPmax and dPmin, maximum and minimum changes in pressure; RT, relaxation time; EDV and ESV, end-diastolic and end-systolic volume; SV, stroke volume; CO, cardiac output; EF, ejection fraction; SW, stroke work; HR, heart rate; ESPVR and EDPVR, end-systolic and -diastolic pressure-volume relationship; PRSW, preload recruitable stroke work. *P < 0.05; †P < 0.01; ‡P < 0.001.
two groups; however, SERCA2a expression levels were significantly decreased in the Akita<sup>ins2</sup> myocardium (Fig. 3B). PLB expression was unchanged but serine-16 phosphorylation level of PLB (Fig. 3B) was significantly decreased, leading to a decreased SERCA2a:PLB ratio and further inhibition of SERCA2a, consistent with diastolic dysfunction of the Akita<sup>ins2</sup> cardiomyocytes seen in vitro and in vivo. Notably, NCX1 expression was significantly increased in the Akita<sup>ins2</sup> mouse (Fig. 3B). This led us to hypothesize that increased expression of NCX1 was a potential mechanism to compensate for the decreased activity of SERCA2a in the Akita<sup>ins2</sup> heart and maintain ventricular contractility.

**Akita<sup>ins2</sup> Cardiomyocytes Have Reduced SR Ca<sup>2+</sup> Content and Prolonged Action Potential Duration**

To measure NCX1 activity in cells and determine the effects of decreased SERCA2a expression on SR Ca<sup>2+</sup> content, we rapidly applied 20 mM caffeine to quiescent ventricular myocytes in the absence or presence of extracellular Na<sup>+</sup>/H<sup>+</sup> (Fig. 3C). From these measurements we determined that Akita<sup>ins2</sup> myocytes have a 27% reduction in SR Ca<sup>2+</sup> load compared with controls (655 ± 74 vs. 899 ± 77 μM; P < 0.05; Fig. 3C), likely resulting from decreased SERCA2a activity. Surprisingly, however, we observed no change in NCX1 activity, as assessed by the decay rate of caffeine-induced Ca<sup>2+</sup> transients in the presence of extracellular Na<sup>+</sup> (Fig. 3C), suggesting increased NCX1 expression in Akita<sup>ins2</sup> hearts does not result in increased NCX1 function in myocytes.

Therefore, we investigated how the Akita<sup>ins2</sup> has preserved systolic function in the presence of depressed SERCA2a function and decreased SR Ca<sup>2+</sup> load. One potential mechanism is that during each cardiac cycle NCX1 spends more time operating in reverse (Ca<sup>2+</sup> entry) mode, as previously observed in failing human myocytes (9). To evaluate this possibility, we optically measured action potentials in isolated myocytes. APD at 80% was significantly prolonged 51% (115 ± 16 vs. 174 ± 19 ms; P < 0.05) in the Akita<sup>ins2</sup> cardiomyocyte compared with wild type (Fig. 3D). Importantly, the early part of the APD at 10%, when NCX1 operates in reverse mode, was prolonged in the Akita<sup>ins2</sup> cardiomyocyte by 66% (6.2 ± 1.1 vs. 10.3 ± 1.6 ms; P < 0.05). Increased Ca<sup>2+</sup> entry via NCX1 during the early phase of the AP may support calcium homeostasis and contractility in Akita<sup>ins2</sup> cardiomyocytes.

**NCX1 Knockdown Using Adenovirus Encoding Antisense-NCX1 Induced Systolic Failure in the Akita<sup>ins2</sup> Mouse**

We tested the functional significance of NCX1 in the Akita<sup>ins2</sup> heart through direct myocardial injection of adenoviral vectors engineered to encode antisense-NCX1 (Ad.asNCX1). In wild-type and Akita<sup>ins2</sup> mice, Ad.asNCX1 achieved ~50% reduction in NCX1 content compared with
Ad.GFP controls 7 days postinfection (Fig. 4A). In wild-type mice treated with Ad.asNCX1, there were mild elevations in contractile parameters indicated by echocardiography and PV analyses; however, peak calcium or sarcomeric shortening were unchanged in isolated wild-type GFP/H11001 cardiomyocytes treated with Ad.asNCX1. Strikingly, in Akita ins2 mice receiving gene transfer of Ad.asNCX1, echocardiography revealed a significant decrease in ventricular function with decreased percent fractional shortening compared with Akita + Ad.GFP controls (33.8 ± 4.9 vs. 58.2 ± 4.4%, n = 7; P < 0.001; Fig. 4B; Table 3). In addition, PV analysis indicated depressed myocardial contractility with reductions in ESPVR (3.07 ± 1.82 vs. 8.53 ± 0.05).
CXCL12/CXCR4 Axis Is Upregulated in the Akitains2 Mouse

Several studies (30) have demonstrated a role for CXCL12 in type 1 diabetes. CXCR4/CXCL12 signaling pathway has been shown to protect NOD mice from autoimmune diabetes via inhibition of T-cell-mediated inflammation and pancreatic islet cell death. How CXCL12/CXCR4 signaling network functions in other organ systems in the diabetic state is unknown. CXCL12 is a critical paracrine signal during acute cardiac ischemic events for circulating progenitor cells to initiate the myocardial repair process. However, CXCL12 may also act in an autocrine fashion on the cardiomyocyte regulatingCa2+ homeostasis and promoting cell survival. In the setting of the diabetic heart with calcium perturbations, the CXCL12/CXCR4 axis could represent a novel protective mechanism in maintaining ventricular function and calcium homeostasis. In the myocardium of the Akitains2 mouse, we observed a significant increase in CXCL12 (Fig. 5, A and B) and CXCR4 expression (Fig. 5B). Importantly, the Akitains2 heart has been reported to have low levels of myocardial inflammation and we also show no significant inflammatory infiltration (Fig. 5C). This suggests that the upregulated CXCL12/CXCR4 expression is not involved in chemotactic, inflammatory mechanisms but potentially is enhanced due to hyperglycemia and calcium-handling disturbances in the cardiomyocyte. We tested if hyperglycemia in vitro induces CXCL12/CXCR4 expression in cardiomyocytes. Isolated ARVMs were cultured in a high glucose (25 mM) media for 24 h, and CXCL12 and CXCR4 expression was determined by quantitative PCR. We demonstrate both CXCL12 and CXCR4 expression was upregulated seven and ninefold, respectively (Fig. 5D). The upregulation of CXCL12/CXCR4 in the Akitains2 myocardium in the absence of acute inflammation details an additional, potentially cardioprotective, mechanism of the CXCL12/CXCR4 axis within diabetic cardiomyopathy.

CXCR4 Regulates the Expression of NCX1 via NF-κB

Our data reveal the increased NCX1 expression in Akitains2 cardiomyocytes may potentially be regulated by a CXCR4-dependent mechanism. To test this hypothesis, we treated isolated ARVMs with CXCL12 (SDF-1; 100 ng/ml) for 24 h. CXCL12 exposure generated a significant increase in NCX1 expression in the ARVM (Fig. 6A). Additionally, adenoviral overexpression of CXCR4 (Ad.CXCR4) in ARVM resulted in a significant increase in NCX1 expression compared with control adenovirus encoding β-gal (Ad.β-gal; Fig. 6A). These data demonstrate CXCR4 as a novel regulator of NCX1 expression.

NF-κB is a transcriptional mediator involved in the regulation of stress-induced genes related to cardiomyocyte hypertrophy and cell survival (12). Importantly, Sirabela et al. (40) reported NF-κB is involved in transcriptional regulation of NCX1 in cortical neurons. We sought to determine if CXCR4 could activate NF-κB in cardiomyocytes and if this pathway was involved in regulating NCX1 expression. Indeed, we show CXCR4 activation by CXCL12/SDF-1 induces phosphorylation of IkB-α (an endogenous inhibitor of NF-κB) in cardiomyocytes (Fig. 6B). Phosphorylation and subsequent ubiquitination and degradation of IkB-α are essential for NF-κB activation and nuclear translocation. Furthermore, cardiac myocytes treated with CXCL12 and separated into cytoplasmic and nuclear fractions indicated significant NF-κB p65 nuclear translocation (Fig. 6B). Additionally, immunostaining revealed CXCL12 enhanced NF-κB p65 localization within the cardiomyocyte nuclei (Fig. 6B). Cardiomyocytes were treated with the IkB-α inhibitor Bay11–7082, which selectively inhibits NF-κB activation through inhibition of IkB-α phosphorylation. When IkB-α phosphorylation is inhibited, CXCL12 failed to increase NCX1 expression (Fig. 6C). Likewise, treatment with the CXCR4 antagonist AMD3100 prevented CXCL12-induced NCX1 upregulation. These results suggest NCX1 expression is regulated by CXCR4 activation of NF-κB. Finally, in the Akitains2 myocardium there is significantly increased IkB-α and NF-κB p65 total and phosphorylation levels (Fig. 7). Therefore, elevated CXCL12/CXCR4 and the increased NF-κB may be a potential mechanism for the increased NCX1 expression and systolic preservation in the Akitains2 heart.

DISCUSSION

Diabetic cardiomyopathy is an important clinical entity predisposing individuals to ventricular dysfunction and ar-

Table 2. Calcium transients and contractility parameters of cardiomyocytes isolated from control or Akita mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Akitains2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calcium Transient</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.74 ± 0.05</td>
<td>1.73 ± 0.02</td>
</tr>
<tr>
<td>Peak height</td>
<td>0.11 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Time to peak, ms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td>36.0 ± 1.6</td>
<td>41.5 ± 3.0*</td>
</tr>
<tr>
<td>50%</td>
<td>63.9 ± 1.3</td>
<td>73.7 ± 4.1*</td>
</tr>
<tr>
<td>90%</td>
<td>96.1 ± 5.0</td>
<td>110.7 ± 6.4*</td>
</tr>
<tr>
<td>Time to baseline, ms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td>145.2 ± 5.1</td>
<td>178.8 ± 9.5*</td>
</tr>
<tr>
<td>50%</td>
<td>178.9 ± 3.8</td>
<td>225.7 ± 6.0*</td>
</tr>
<tr>
<td>90%</td>
<td>244.1 ± 20.4</td>
<td>357.8 ± 29.4*</td>
</tr>
<tr>
<td><strong>Sarcomeric Shortening</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.54 ± 0.06</td>
<td>1.57 ± 0.05</td>
</tr>
<tr>
<td>Peak height</td>
<td>0.15 ± 0.02</td>
<td>0.13 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.05.

2.44, n = 7; P < 0.001) with Akita + Ad.asNCX1 (Fig. 4C, Table 4). Additionally, isolated GFP+ cardiomyocytes from the Akitains2 mouse treated with Ad.asNCX1 displayed a significant reduction in peak calcium and myocyte shortening compared with Ad.GFP controls (Fig. 4D). These in vivo results, coupled with the biochemical and electrophysiological data, determine NCX1 is functioning as a compensatory mechanism to maintain ventricular function in the Akitains2 mouse.

(See Table 2.)
Fig. 4. Adenovirus knockdown of NCX1 leads to systolic failure in the Akita<sup>min2</sup>. Direct myocardial injection of adenovirus encoding anti-sense NCX1 (Ad.asNCX1) knocked down NCX1 protein expression ~50% compared with Ad.GFP controls (A). Seven days postinjection echocardiography was performed on WT and Akita<sup>min2</sup>. Representative M-mode images and fractional shortening (%FS; B) and in vivo hemodynamic occlusion studies and ESPVR analysis (C) of WT and Akita<sup>min2</sup> treated with vehicle only, Ad.GFP, or Ad.asNCX1. *P < 0.05. D: calcium transients and sarcomeric shortening parameters of GFP<sup>+</sup> ventricular myocytes from WT and Akita<sup>min2</sup> mice infected with Ad.GFP or Ad.asNCX1 for 7 days. Histograms comparing means (±SD) peak Ca<sup>2+</sup> and peak shortening determined in GFP<sup>+</sup> myocytes are shown. *P < 0.05; **P < 0.01.
However, in the 5-mo Akitains2 mice, there is only modest presenting with consistent blood glucose levels.

Ped, mmHg 3.6

Vt, mm 1.96 ± 0.40

LVIDs, mm 1.41 ± 0.03

LVPWs, mm 2.00 ± 0.28

%FS 54.3 ± 2.4

HR, beats/min 553 ± 25

LVPWs, mm 2.00

LVIDs, mm 1.16 ± 0.07

LVPWs, mm 1.75 ± 0.03

%FS 58.2 ± 4.4

HR, beats/min 528 ± 30

Values are means ± SD. Seven days postinjection echocardiography was performed. WT or Akita mice received gene transfer of Ad.GFP or Ad.asNCX1. IVSd, intraventricular septum in diastole; LVIDd and LVIDs, left ventricular internal diameter at diastole and systole; LVPWd and LVPWs, left ventricular posterior wall diastole and systole. *P < 0.05; †P < 0.01; ‡P < 0.001.

Table 3. Echocardiographic analyses

<table>
<thead>
<tr>
<th></th>
<th>WT + Ad.GFP</th>
<th>WT + Ad.asNCX1</th>
<th>Akita + Ad.GFP</th>
<th>Akita + Ad.asNCX1</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSd, mm</td>
<td>1.31 ± 0.19</td>
<td>1.13 ± 0.12</td>
<td>1.29 ± 0.14</td>
<td>1.13 ± 0.12</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>3.09 ± 0.53</td>
<td>2.96 ± 0.31</td>
<td>3.13 ± 0.19</td>
<td>3.09 ± 0.53</td>
</tr>
<tr>
<td>LVPWd, mm</td>
<td>1.29 ± 0.19</td>
<td>1.33 ± 0.19</td>
<td>1.33 ± 0.19</td>
<td>1.29 ± 0.19</td>
</tr>
<tr>
<td>IVSs, mm</td>
<td>1.96 ± 0.40</td>
<td>1.94 ± 0.15</td>
<td>1.94 ± 0.15</td>
<td>1.96 ± 0.40</td>
</tr>
<tr>
<td>LVIDs, mm</td>
<td>1.41 ± 0.03</td>
<td>0.99 ± 0.30</td>
<td>0.99 ± 0.30</td>
<td>1.41 ± 0.03</td>
</tr>
<tr>
<td>LVPWs, mm</td>
<td>2.00 ± 0.28</td>
<td>2.19 ± 0.06*</td>
<td>2.19 ± 0.06*</td>
<td>2.00 ± 0.28</td>
</tr>
<tr>
<td>%FS</td>
<td>54.3 ± 2.4</td>
<td>67 ± 7.4‡</td>
<td>67 ± 7.4‡</td>
<td>54.3 ± 2.4</td>
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<tr>
<td>HR, beats/min</td>
<td>553 ± 25</td>
<td>559 ± 19</td>
<td>559 ± 19</td>
<td>553 ± 25</td>
</tr>
<tr>
<td>LVIDs, mm</td>
<td>1.16 ± 0.07</td>
<td>2.31 ± 0.32‡</td>
<td>2.31 ± 0.32‡</td>
<td>1.16 ± 0.07</td>
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<tr>
<td>LVPWs, mm</td>
<td>1.75 ± 0.03</td>
<td>1.35 ± 0.12‡</td>
<td>1.35 ± 0.12‡</td>
<td>1.75 ± 0.03</td>
</tr>
<tr>
<td>%FS</td>
<td>58.2 ± 4.4</td>
<td>33.8 ± 4.9‡</td>
<td>33.8 ± 4.9‡</td>
<td>58.2 ± 4.4</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>528 ± 30</td>
<td>541 ± 32</td>
<td>541 ± 32</td>
<td>528 ± 30</td>
</tr>
</tbody>
</table>

Table 4. Left ventricular invasive hemodynamics 7 days post-Ad.GFP or Ad.asNCX1 gene transfer in 20 wk WT or Akita mice

<table>
<thead>
<tr>
<th></th>
<th>WT + Ad.GFP</th>
<th>WT + Ad.asNCX1</th>
<th>Akita + Ad.GFP</th>
<th>Akita + Ad.asNCX1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pes, mmHg</td>
<td>88.6 ± 3.4</td>
<td>99.9 ± 5.1*</td>
<td>102.0 ± 8.4</td>
<td>101.5 ± 9.7</td>
</tr>
<tr>
<td>Ped, mmHg</td>
<td>3.6 ± 0.5</td>
<td>5.2 ± 1.6</td>
<td>6.0 ± 3.1</td>
<td>4.8 ± 2.6</td>
</tr>
<tr>
<td>dPmax, mmHg/s</td>
<td>5,870 ± 400</td>
<td>6,864 ± 378</td>
<td>7,133 ± 188</td>
<td>7,331 ± 954</td>
</tr>
<tr>
<td>dPmax, mmHg/s</td>
<td>−3,664 ± 181</td>
<td>−4,932 ± 624</td>
<td>−4,864 ± 391</td>
<td>−5,605 ± 757</td>
</tr>
<tr>
<td>Tau, ms</td>
<td>4.96 ± 0.2</td>
<td>4.38 ± 0.50*</td>
<td>6.00 ± 0.2</td>
<td>5.25 ± 0.75</td>
</tr>
<tr>
<td>EDV, ul</td>
<td>41.6 ± 8.6</td>
<td>42.8 ± 8.2</td>
<td>44.7 ± 8.6</td>
<td>54.8 ± 9.79</td>
</tr>
<tr>
<td>ESV, ul</td>
<td>10.0 ± 2.4</td>
<td>9.6 ± 4.0</td>
<td>10.9 ± 2.4</td>
<td>22.1 ± 6.0†</td>
</tr>
<tr>
<td>SV, ul</td>
<td>23.0 ± 4.0</td>
<td>32.6 ± 5.2</td>
<td>38.8 ± 5.2</td>
<td>32.6 ± 8.5</td>
</tr>
<tr>
<td>CO, ul/min</td>
<td>11.851 ± 2.357</td>
<td>15.784 ± 1613</td>
<td>17.688 ± 2.800</td>
<td>16.317 ± 1.613</td>
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<tr>
<td>EF, %</td>
<td>72.6 ± 8.2</td>
<td>79.8 ± 9.7</td>
<td>79.6 ± 3.2</td>
<td>59.8 ± 2.1‡</td>
</tr>
<tr>
<td>SW, mmHg/µl</td>
<td>1,533 ± 347</td>
<td>2,614 ± 480</td>
<td>3,313 ± 470</td>
<td>2,722 ± 256*</td>
</tr>
<tr>
<td>ESPVR</td>
<td>4.78 ± 0.54</td>
<td>9.03 ± 2.47*</td>
<td>8.53 ± 2.44</td>
<td>3.07 ± 1.82‡</td>
</tr>
<tr>
<td>EDPVR</td>
<td>0.10 ± 0.01</td>
<td>0.13 ± 0.07</td>
<td>0.23 ± 0.03</td>
<td>0.30 ± 0.08‡</td>
</tr>
<tr>
<td>PRSW</td>
<td>80.1 ± 9.53</td>
<td>121 ± 27.22*</td>
<td>116.3 ± 11.1</td>
<td>93 ± 17.45</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>508 ± 26</td>
<td>538 ± 35</td>
<td>519 ± 26</td>
<td>489 ± 35</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.05; †P < 0.01; ‡P < 0.001.

rhythmogenesis. Calcium cycling is disturbed in the diabetic heart and understanding the involvement of calcium cycling in the cardiomyocyte will give insight into the pathogenesis of diabetic cardiomyopathy. This study identifies an important compensatory function of NCX1 in the diabetic heart mediated by a CXCR4-NF-kB pathway.

The Akita min2 mouse is a genetic model of type 1 diabetes presenting with consistent blood glucose levels >600 mg/dl (47). However, in the 5-mo Akita min2 mice, there is only modest evidence of myocardial dysfunction. Echocardiography revealed no difference in fractional shortening between Akita min2 and wild-type littermate controls. However, hemodynamic measurements using a PV conductance catheter indicated elevated diastolic parameters, including relaxation time tau, and end-diastolic pressure volume relationship, correlating with diastolic dysfunction. Interestingly, we did observe enhanced systolic function in the Akita min2 mouse with increased dP/dt max, and ESPVR; however, there was no correlative increase in peak calcium transient or sarcomeric shortening of isolated Akita min2 myocytes. Of note, the Akita min2 survival significantly diminishes at 12 mo, ~50%. Echocardiography and PV analysis at 12 mo indicated no systolic impairment, thus the decrease in the 12-mo survival is not due to a worsening cardiac systolic function or failure. Grossly, there was mild cardiac atrophy; however, evidence of fibrosis or inflammation that could also lead to decreased survival at 12 mo was not detected. In agreement with our findings, Basu et al. (2) have also reported that Akita min2 mouse exhibits diastolic dysfunction with preserved systolic function at 3 and 6 mo of age. Maintained ventricular function with such prolonged exposure to severe diabetes is surprising as other models of type 1 and type 2 diabetes generally present with significant systolic deterioration (33).

Maintenance of cardiac systolic function during diabetes includes not only adaptive alterations in metabolic glucose and free-fatty acid metabolism but also initiates adaptive mechanisms involving calcium cycling proteins (8, 48). Type 1 and type 2 diabetes both decrease myocardial SERCA2a content and activity leading to diastolic and systolic dysfunction shown in animal models and in human hearts (7, 19, 30). In the streptozotocin (STZ)-induced type 1 diabetes model, mice progress into systolic failure within weeks to months postdiabetes onset (7, 49), although other studies showed preserved function (8). In both the STZ and the Akita min2 models, there are reductions in SERCA2a expression; however, a key difference between the STZ model and the Akita min2 is the expression of NCX1, which is decreased in the former (7) but significantly elevated in the latter. In Akita min2 myocytes, reduced SR Ca2+ content was primarily a result of decreased SR Ca2+ uptake due to decreased SERCA2 expression and/or increased SERCA inhibition by unphosphorylated PLB. Despite decreased SR Ca2+ content, [Ca2+]i transient amplitudes were unchanged. Three possibilities to account for this result include 1) increased Ca2+ entry via reverse-mode NCX, 2) an increase in the gain of CICR, and 3) an increase in intracellular [Na+], which would alter the electrochemical potentials that dictate the direction of NCX1 activity. We did not address the second or third possibilities, but the prolonged action potentials observed in the Akita min2 myocytes support increased reverse-mode NCX1 ac-
Slower phase 1 repolarization in the Akita<sup>ins2</sup> myocytes allows more time for Ca<sup>2+</sup> entry via NCX, and this Ca<sup>2+</sup> influx may both contribute to CICR and help maintain SR load, as seen in developing mouse myocytes (19). The prolonged action potential likely results from decreased I<sub>Ko</sub> potassium channel currents (3) and can be interpreted as a potential adaptive mechanism regulating NCX1 activity and preventing contractile deterioration. It is also interesting to note that in the absence of both NCX1 and SERCA2a function the time constant of Ca<sup>2+</sup> decay was faster in Akita<sup>ins2</sup> myocytes. This observation suggests that the activities of mitochondrial Ca<sup>2+</sup> uniporter and sarcolemmal Ca<sup>2+</sup>-ATPase are also increased, perhaps as an additional compensatory response to decreased SERCA2a function in Akita<sup>ins2</sup> myocytes. However, the elevated NCX1 activity and expression providing systolic support to the Akita<sup>ins2</sup>, along with the prolonged action potential duration, may be at the cost of generating an arrhythmogenic substrate in the cardiac myocyte (41). The possibility of sudden cardiac death as a contributing factor to the decreased 12-mo survival in the Akita<sup>ins2</sup> mouse is currently being investigated.

The observation that NCX1 expression was increased in Akita<sup>ins2</sup> hearts without a detectable change in NCX1 function suggests increased NCX1 expression might represent an important compensatory mechanism, required simply to maintain normal NCX1 function. To evaluate this possibility, we utilized a direct myocardial injection of anti-sense NCX1. NCX1 reduction in the Akita<sup>ins2</sup> mouse heart exhibited a marked impairment in systolic function, in stark contrast to wild-type hearts.
control, with significantly depressed percent fractional shortening, ejection fraction, and ESPVR. Importantly, cardiac-specific NCX1 knockout in otherwise healthy, nondiabetic mice display normal Ca\textsuperscript{2+} dynamics and have little to no contractile depression (18). This indicates that in the Akitains\textsuperscript{2} mouse elevated NCX1 is compensating and stabilizing cardiac contractility in the setting of reduced SR Ca\textsuperscript{2+} and SERCA2a expression. This observation is relevant since Hasenfuss et al. (16) have also shown the compensatory mechanism of increased NCX1 in the setting of reduced SERCA2a expression. However, the increased NCX1 expression in the Akitains\textsuperscript{2} mouse did not correlate with increased NCX1 forward mode function as there was no alteration in caffeine-induced Ca\textsuperscript{2+} decay rates, but the prolonged APD suggests increased NCX1 reverse mode activity. Compared with failing human myocytes, NCX1 operating in the reverse mode contributes to maintenance of systolic function (9). This indicates the Akitains\textsuperscript{2} mouse is upregulating NCX1 transcription and translation to maintain basal NCX1 activity and myocyte contractility. Thereby, reducing NCX1 expression in the setting of reduced SERCA2a is deleterious, as is observed in the STZ model (49), and NCX1 upregulation indicates a compensatory mechanism of the cardiomyocyte. Importantly, even though manipulating the expression of NCX1 in vivo by direct myocardial gene transfer of antisense NCX1 has significant impact on cardiac performance and isolated cardiac myocyte contractility, this does not address the question of which cell types are affected by the transgene. It is well known that local injection of adenoviral transgenes would certainly transduce myocytes, fibroblasts, smooth muscle cells, as well as endothelial cells. As such, the impact of antisense NCX1 on cardiac function in vivo is more likely a global effect.
NF-κB nuclear translocation. We determined in cardiomyocyte NCX1 is also regulated by NF-κB through a CXCR4-dependent mechanism. The Akita<sup>ins2</sup> heart has significantly elevated IkB-α and NF-κB p65 total and phosphorylation levels. Therefore, the increased CXCL12/CXCR4 expression in vivo is potentially a source for IkB-α phosphorylation and p65 nuclear translocation resulting in the upregulation of NCX1 expression in the Akita<sup>ins2</sup> heart, promoting cardiac systolic function. The functional role of NF-κB within the cardiac myocyte has remained difficult to define as it participates in hypertrophy, myocyte survival, apoptosis, and myocardial inflammatory responses. The adaptive and maladaptive implications of NF-κB signaling have been well documented, and NF-κB downstream targets are considered to be dependent on the contextual myocardial microenvironment. Several studies have shown activation of NF-κB postmyocardial infarction is a cardioprotective mechanism leading to enhanced myocyte survival and a reduction in infarct size. Specifically, NF-κB p50 null mice have been shown to have worsening cardiac function postmyocardial infarction with increased apoptosis (44). Also, p65 has been described as antiapoptotic through repression of Bnip3 (39). Additionally, patients diagnosed with NYHA III/VI heart failure, having the p50 ATTG1/1 polymorphism with diminished myocardial p50 expression, have worse cardiac performance and decreased survival (36). Here we show the prolonged activation of NF-κB irrespective of acute ischemia in the Akita myocardium is not cardiotoxic and does not lead to increased apoptosis, inflammation, or ventricular dysfunction. This study shows long-term NF-κB activation in diabetic cardiomyopathy is cardioprotective and may be cardioprotective through its influence on NCX1 and promotion of calcium homeostasis.

In summary, our results define an adaptive role for NCX1 in promoting contractile function in diabetic cardiomyopathy. They also reveal a novel mechanism for CXCR4 in the Akita<sup>ins2</sup> diabetic heart by regulating NCX1 expression via NF-κB. The identification of CXCR4 involvement in NCX1 regulation further establishes CXCR4 as a central mediator of calcium homeostasis in the cardiac myocyte. The CXCR4/NF-κB/NCX1 module represents a potential therapeutic target in diabetic cardiomyopathy.
ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS


REFERENCES


