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Diastolic dysfunction in prediabetic male rats: Role of mitochondrial oxidative stress

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Diabetic and prediabetic animals have increased free radical production (1), which is thought to contribute to the pathophysiological changes characteristic of insulin resistance and type 2 diabetes mellitus (2). However, the impact of prediabetes on cardiac function and to investigate the effect of prediabetes on cardiac function and to characterize parameters and pathways associated with deteriorated cardiac performance. Long-Evans rats were fed with either control or high-fat chow for 21 wk and treated with a single low dose (20 mg/kg) of streptozotocin at week 4. High-fat and streptozotocin treatment induced prediabetes as characterized by slightly elevated fasting blood glucose, impaired glucose and insulin tolerance, increased visceral adipose tissue and plasma leptin levels, as well as sensory neuropathy. In prediabetic animals, a mild diastolic dysfunction was observed, the number of myocardial lipid droplets increased, and left ventricular mass and wall thickness were increased; however, no molecular sign of fibrosis or cardiac hypertrophy was shown. In prediabetes, production of reactive oxygen species was elevated in subsarcomemmal mitochondria. Expression of mitofusin-2 was increased, while the phosphorylation of phospholamban and expression of Bcl-2/adenoavirus E1B 19-kDa protein-interacting protein 3 (BNIP3, a marker of mitophagy) decreased. However, expression of other markers of cardiac auto- and mitophagy, mitochondrial dynamics, inflammation, heat shock proteins, Ca2+/calmodulin-dependent protein kinase II, mammalian target of rapamycin, or apoptotic pathways were unchanged in prediabetes. This is the first comprehensive analysis of cardiac effects of prediabetes indicating that mild diastolic dysfunction and cardiac hypertrophy are multifactorial phenomena that are associated with early changes in mitochondria, cardiac lipid accumulation, and elevated oxidative stress and that prediabetes-induced oxidative stress originates from the subsarcomemmal mitochondria.

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TYPE 2 DIABETES MELLITUS is a common civilization disease with a growing prevalence worldwide (2, 68, 81). It is well established that type 2 diabetes mellitus is a risk factor of cardiovascular diseases, such as heart failure and myocardial infarc-
tion, contributing to their increased morbidity and mortality (6, 68). However, before the development of overt diabetes, a period of prediabetic state (i.e., impaired glucose and insulin tolerance, insulin and leptin resistance, oscillations of normo- and hyperglycemic states, mild to moderate obesity) occurs (54), which may also promote cardiovascular complications (21, 30, 45). Although cardiac pathophysiological alterations are relatively well characterized in fully developed diabetes (i.e., diabetic cardiomyopathy), information about prediabetes is quite limited. It has been reported that prediabetes induces mild diastolic dysfunction in OLETF rats, which is a genetic model for spontaneous long-term hyperglycemia (51); however, cardiac consequences of prediabetes and their molecular mechanism are unknown in non-genetic prediabetic settings.

Contractile dysfunction in diabetic cardiomyopathy has been attributed to numerous factors and pathways (i.e., increased oxidative stress or activated apoptosis (13, 78), which could be connected to an impaired mitochondrial function (24), autophagy (41, 78), or to an imbalance in the calcium homeostasis (60)). Although these pathways are well studied in diabetes, their role in prediabetes has not been uncovered. Furthermore, because mitochondrial function is heavily influenced by mitochondrial dynamics including mitochondrial biogenesis, fusion, fission, and autophagy-mitophagy and because these processes have been linked to the development of diabetic cardiomyopathy (13, 32, 41, 78), we hypothesized that altered mitochondrial dynamics might be involved in the mechanism of deteriorated cardiac functions in prediabetes. Moreover, development of diabetes leads to systemic sensory neuropathy that has been shown to result in diastolic dysfunction in the rat heart (7, 86).

Therefore, here we aimed to systematically characterize the cardiac effect of prediabetes on functional, morphological, and molecular levels in a nongenetic rodent model.

**MATERIALS AND METHODS**

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and was approved by the animal ethics committee of the Semmelweis University, Budapest, Hungary (registration numbers: XIV-1-001/450-6/2012). Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

**Animal model and experimental design.** Male Long-Evans rats of 5–7 wk of age were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed in a room maintained in a 12-h:12-h light/dark cycle and constant temperature of 21°C. Animals were allowed access to food and water ad libitum. After 1 wk of acclimatization, rats were divided into two groups: control (CON; n = 20) and prediabetic group (PRED; n = 20) (Fig. 1). The control group was fed control chow, whereas the prediabetic group was fed a chow supplemented with 40% lard as a high-fat diet. Body weights were measured weekly. Blood was taken, and fasting blood glucose levels were measured from the saphenous vein every second week with a glucose monitoring system (Accu-Check; Roche, Basel, Switzerland). To facilitate the development of prediabetes and to avoid hypoinsulinemia, animals on high-fat diet received 20 mg/kg streptozotocin (STZ) at week 4 (PRED) to induce diabetes.

Body weights were measured weekly, and blood samples were taken from the saphenous vein every second week. Sensory neuropathy was measured at week 15. Oral glucose tolerance test (OGTT), insulin tolerance test (ITT), and computer tomography (CT) were performed at week 20.

Echocardiography, hemodynamic analysis, and parameters of mitochondrial function were measured at week 21 of diet. Tissue sampling was performed after terminal procedures.

Cardiac function by echocardiography. Before euthanasia, to measure cardiac function, echocardiography was performed as previously described (42, 64). Briefly, anesthetized animals were placed on a controlled heating pad, and the core temperature, measured via rectal probe, was maintained at 37°C. Transthoracic echocardiography was performed with animals in the supine position by one investigator.
blinded to the experimental groups. Two-dimensional and M-mode echocardiographic images of long and short (midpapillary muscle level) axis were recorded, using a 13-MHz linear transducer (GE 12L-RS; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), connected to an echocardiographic imaging unit (Vivid i, GE Healthcare). The digital images were analyzed by a blinded investigator using an image analysis software (EchoPac, GE Health-care). On two-dimensional recordings of the short axis at the midpapil- lary level, left ventricular (LV) anterior (LVAWT) and posterior wall thickness (LVPWT) in diastole (index: d) and systole (index: s) as well as LV end-diastolic (LVEDD) and end-systolic diameter (LVESD) were measured. In addition, end-diastolic and end-systolic LV areas were planimetrized from two-dimensional recordings of short and long axis. End-systole was defined as the time point of minimal LV dimensions and end-diastole as the time point of maximal dimensions. All values were averaged over three consecutive cycles. The following parameters were derived from these measurements (65). Fractional shortening (FS) was calculated as 
\[ \text{FS} = \frac{\text{LVEDD} - \text{LVESD}}{\text{LVEDD}} \times 100. \]
LV mass was calculated according to the following formula: \[ \text{LV mass} = \text{LVESD} + \text{AWTd} + \text{PWTd} \times 3 - \text{LVEDD} \times 3.04 \times 0.8 + 0.14. \]

**Hemodynamic measurements, LV pressure-volume analysis.** After echocardiographic measurements, hemodynamic measurement was performed as previously described (62, 63). Briefly, rats were tracheotomized, intubated, and ventilated, while core temperature was maintained at 37°C. A median laparotomy was performed. A poly- ethylene catheter was inserted into the left external jugular vein. A 2-Fr microtip pressure-conductance catheter (SPR-838; Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the ascending aorta. After stabilization for 5 min, mean arterial blood pressure (MAP) was recorded. After that, the catheter was advanced into the LV under pressure control. After stabilization for 5 min, signals were continuously recorded at a sampling rate of 1,000/s using a pressure-volume (P-V) conductance system (MPVSS-Ultra, Millar Instruments) connected to the PowerLab 16/30 data acquisition system (AD Instruments, Colorado Springs, CO), stored, and displayed on a personal computer by the LabChart5 Software System (AD Instruments). After positioning the catheter, we registered baseline P-V loops. With the use of a special P-V analysis program (PVAN, Millar Instruments), LV end-systolic pressure and end-diastolic pressure, the maximal slope of LV systolic pressure increment (dP/dmax) and diastolic pressure decrement, time constant of LV pressure decay (τ, according to the Glantz method), ejection fraction (EF), stroke work (SW), and LV maximal power were computed and calculated. Stroke volume and cardiac output (CO) were calculated and corrected according to in vitro and in vivo volume calibrations using the PVAN software. Total peripheral resistance (TPR) was calculated by the following equation: \[ \text{TPR} = \frac{\text{MAP}}{\text{CO}}. \]
In addition to the above parameters, P-V loops recorded at different preloads can be used to derive other useful systolic function indexes that are less influenced by loading conditions and cardiac mass (37, 58). Therefore, LV P-V relations were measured by transiently compressing the inferior vena cava (reducing preload) under the diafragm with a cotton-tipped applicator. The slope of the LV end-systolic P-V relationship (according to the parabolic curvilinear model), preload recruitable stroke work, and the slope of the dP/dmax-end-diastolic volume (EDV) relationship (dP/dmax-EDV) were calculated as load-independent indexes of LV contractility. The slope of the LV end-diastolic P-V relationship (EDPVR) was calculated as a reliable index of LV stiffness (37). At the end of each experiment, 100 μl of hypertonic saline was injected intravenously, and, from the shift of P-V relations, parallel conductance volume was calculated by the software and used for the correction of the cardiac mass volume. The volume calibration of the conductance system was performed as previously described (37).

**Adipokine array from rat plasma.** Adipokine array was performed from 1 ml rat plasma according to manufacturer’s instructions (Pro- teome Profiler Rat Adipokine Array Kit; R&D Systems, Abingdon, United Kingdom).

**Biochemical measurements.** Serum cholesterol, high-density lipoprotein (HDL), and triglyceride levels were measured by colorimetric assays (Diagnosticum, Budapest, Hungary) as previously described (19). Plasma leptin (Invitrogen, Camarillo, CA), tissue inhibitor of matrix metalloprotease (TIMP) metalloproteinase inhibitor 1 (TIMP-1; R&D System, Minneapolis, MN), and angiotensin-II (Phoenix Pharmaceuti- cals, Karlsruhe, Germany) were measured by ELISA according to manufacturer’s instructions. Urea, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, low-density lipoprotein, C-reactive protein (CRP), cholesterol, uric acid, and creatinine were measured by automated clinical laboratory assays (Diagnostics).

**Histology.** Heart, liver, and pancreas samples were fixed in 4% neutral-buffered formalin. After 24 h, samples were washed with PBS and stored in 70% ethanol in PBS until embedded in paraffin. Samples were stained with hematoxylin-eosin (HE) and Masson’s trichrome (MA) staining. LV samples were analyzed to examine histopathological differences and evaluate cardiomyocyte hypertrophy and fibrosis. The level of fibrosis was measured on MA-stained LV sections, and transverse transmural width (cardiomyocyte diameter) was assessed on longitudinally oriented cardiomyocytes on HE-stained LV sections by a Zeiss microscope (Carl Zeiss, Jena, Germany). Digital images were acquired using an imaging software (QCapture Pro 6.0; Qimaging, Surrey, British Columbia, Canada) at ×200 magnification. Quantification of cardiomyocyte diameter and fibrosis was performed with ImageJ Software (v1.48; NIH, Bethesda, MD). Liver samples were evaluated for hepatic steatosis/fibrosis and scored as previously de- scribed (40).

**Nitrotyrosine immunostaining of LV samples.** After embedding and cutting 5-μm-thick sections, heat-induced antigen epitope retrieval was performed (95°C, 10 min, in citrate buffer with a pH of 6.0). Sections were stained with rabbit polyclonal anti-nitrotyrosine antibody (5 μg/ml; Cayman Chemical, Ann Arbor, MI) by using the ABC kit of Vector Laboratories (Burlingame, CA) according to the manu- facturer’s protocol. Nitrotyrosine-stained sections were counterstained with hematoxylin. Specific staining was visualized, and images were acquired using a BX-41 microscope (Olympus, Tokyo, Japan).

**QuantitativeRT-PCR.** Total RNA was isolated from LV tissue with Reliaprep RNA Tissue Miniprep kit (Promega, Madison, WI) according to the manufacturer’s instructions. cDNA was synthesized using Tetro CDNA Synthesis Kit (Bioline, London, UK) according to the manufacturer’s protocol. PCR reaction was performed with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) or TaqMan Universal PCR MasterMix (Thermo Fisher Scientific, Waltham, MA) and 3 μl forward and reverse primers for collagen type 1 and 3 (COL1 and COL3), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) (Integrated DNA Technologies, Leuven, Belgium), assay mixes for β-actin (assay ID: Rn00665696_m1), GAPDH (assay ID: Rn01267877_m1), β2-Microglobulin or GAPDH (assay ID: Rn17755363_g1) were used as reference genes. Quantitative real-time PCR was performed with the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). Expression levels were calculated using the CT comparative method (2⁻ⁿCt).

**Measurement of pancreatic insulin.** Freeze-clamped and pulverized pancreas samples were used to determine pancreatic insulin content. Analysis was performed with insulin (I-125) IRMA Kit (Izotop Kitz, Budapest, Hungary) according to the manufacturer’s instructions.

**Electron microscopy.** LV tissue samples (1 × 1 mm) were placed in modified Karnovsky fixative (2% paraformaldehyde, 2.5% glutar- aldehyde, 0.1 M Na-cacodylate buffer, pH 7.4, and 3 mM CaCl₂). After being washed in cacodylate buffer, samples were incubated in 1% osmium tetroxide in 0.1 M PBS for 35 min. Samples were then washed in buffer several times for 10 min and dehydrated in an

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ascending ethanol series, including a step of uranyl acetate (1%) solution in 70% ethanol to increase contrast. Dehydrated blocks were transferred to propylene oxide before being placed into Durcupan resin. Blocks were placed in a thermostat for 48 h at 56°C. From the embedded blocks, 1-µm-thick semithin and serial ultrathin sections (70 nm) were cut with a Leica ultramicrotome and mounted either on mesh or on Collodion-coated (Parlodion; Electron Microscopy Sciences, Fort Washington, PA) single-slot copper grids. Additional contrast was provided to these sections with uranyl acetate and lead citrate solutions, and they were examined with a JEOL1200EX-II electron microscope. Areas of subsarcolemmal (SSM), interfibrillar (IFM), and the resulting mitochondrial pellet was finally resuspended in buffer A and used for mitochondrial respiration, membrane potential, H2O2 production, and Ca2+ uptake measurements. For Western blots, the resulting SSM and IFM pellets were finally resuspended in a 200 µl volume of Buffer B, which were layered on 30% Percoll solution and ultracentrifuged (Rotor type: Beckman Type 70.1 Ti) for 30 min at 18,700 g at 4°C. After ultracentrifugation, lower rings were collected (100 µl/tube) and filled with 1 ml Buffer B and centrifuged for 10 min at 12,200 g, 4°C. After being washed, pellets were stored at −80°C.

Measurement of mitochondrial respiration. Protein concentration of SSM and IFM samples was determined by the Biuret method (11). Mitochondrial oxygen consumption was measured by high-resolution respirometry with Oxygraph-2K (Orboros Instruments, Innsbruck, Austria), a Clark-type O2 electrode, for 40 min. The mitochondrial protein content was 0.1 mg/ml in the measurements. Measuring mitochondrial respiration followed the substrate-uncoupler-inhibitor titration protocol. Mitochondria were energized with 5 mM glutamate and 5 mM malate. Mitochondrial respiration was initiated with 2 mM adenosine diphosphate. Cytochrome c (4 µM), succinate (5 mM), rotenone (1 µM), and carboxyatractyloside (2 µM) were used as indicated. Measurements were performed in an assay medium containing 125 mM KCl, 20 mM HEPES, 100 µM EGTA, 2 mM K2HPO4, 1 mM MgCl2, and 0.025% BSA. Data were digitally recorded using DatLab4 software.

Measurement of mitochondrial membrane potential. To detect mitochondrial membrane potential, we used the fluorescent, cationic dye, safranine O (2 µM), which can bind to the protein possessing negative charge in the inner mitochondrial membrane depending on the mitochondrial membrane potential. The excitation/emission wavelengths were 495/585 nm, respectively. Fluorescence was recorded at 37°C by Hitachi F-4500 spectrofluorometer (Hitachi High Technologies, Maidenhead, UK). The reaction medium was the following: 125 mM KCl, 20 mM HEPES, 100 µM EGTA, 2 mM K2HPO4, 1 mM MgCl2, and 0.025% BSA.

Detection of H2O2 formation in mitochondria. H2O2 production from SSM and IFM was assessed by Amplex UltraRed fluorescent dye method (52). Horseradish peroxidase (2.5 U/ml) and Amplex UltraRed reagent (1 mM) and then 0.05 mg/ml mitochondria were added to the incubation medium. H2O2 formation was initiated by the addition of 2.6-dichlorophenolindophenol and phenazine methosulfate to a final concentration of 34,900 µM and 1.625 mM, respectively. The activity of cytochrome c-oxidase was measured in 550 nm in the presence of 0.08% reduced cytochrome c. The activity of succinate-dehydrogenase was measured at 600 nm in the presence of 0.1 mM EDTA, 2.5 mM KCN, 0.1% bovine serum albumin, and 4 mM succinate. The reaction mix was incubated at 30°C for 10 min (except for cytochrome c oxidase). Enzyme activities were expressed relative to citrate synthase activity or total protein levels (measured with bicinchoninic acid assay).

Preparation of isolated mitochondria. SSM and IFM fractions were isolated according to a protocol described previously (74) with the use of homogenization buffer (buffer A) containing the following (in mM): 100 KCl, 50 MOPS, 5 MgSO4, 1 EGTA, and pH 7.4 Tris.HCl. Isolation buffer (buffer B) contained the following (in mM): 250 sucrose, 10 HEPES, 1 EGTA, and pH 7.4 Tris.HCl. Before the isolation, 1 mM ATP was added freshly to the homogenization buffer. All steps were carried out on ice. After Langendorff perfusion of the heart, LV samples were cut to small species with scissors and washed in buffer A, then homogenized with five strokes of Teflon pistils in a glass potter. The homogenate was centrifuged for 10 min at 800 g, 4°C. For isolation of SSM, the supernatant was centrifuged for 10 min at 8,000 g. This pellet was suspended in buffer A and centrifuged for 10 min at 8,000 g, and the resulting sediment was resuspended in a small volume of buffer A. The pellet of the first centrifugation was used for isolation of IFM fraction and resuspended in buffer B (10 ml/g tissue) and after addition of 8 U/g of bacterial protease incubated for 1 min on ice and then homogenized with five strokes of Teflon pistil in a glass potter and centrifuged for 10 min at 800 g. The supernatant was centrifuged for 10 min at 8,000 g, and the resulting mitochondria.
For tropomyosin oxidation analysis, tissue samples were homogenized in ice-cold PBS, pH 7.2, containing an antiprotease mixture (Complete, Roche) and 5 mM EDTA. Just before use, the protein samples were stirred under vacuum and bubbled with argon to maximally reduce the oxygen tension. The protein suspension was centrifuged at 12,000 g for 10 min at 4°C. The resulting pellet was resuspended in sample buffer (2% SDS, 5% glycerol, 1% β-mercaptoethanol, 125 mM Tris-HCl, pH 6.8) and denatured by 10 min of boiling. This procedure referred to as reducing condition was compared with the nonreducing condition obtained without the addition of β-mercaptoethanol. To avoid artifacts attributable to the oxidation of thiol groups in vitro, nonreducing conditions were performed in the presence of 1 mM N-ethylmaleimide.

Protein samples were resolved on precast 4–20% Criterion TGX gels (Bio-Rad) or Bis-Tris gels depending on the protein of interest and transferred to nitrocellulose or Immun-Blot PVDF membranes (Bio-Rad). Quality of transfer was verified with Ponceau staining. Membranes were blocked with 5% nonfat milk (Bio-Rad) or 2–5% BSA (Santa Cruz Biotechnology) in Tris-buffered saline with 0.05% Tween 20 (TBS-T) for 0.5–2.0 h. Membranes were incubated with primary antibodies in 1–5% nonfat milk or BSA in TBS-T: anti-tropomyosin (Tm; 1:250), anti-phospho-phospholamban (PLB-Ser16; 1:500), p-PLB (Thr17; 1:500), anti-sarco/endoplasmic reticulum Ca2+-ATPase II (SERCA2A; 1:5,000; Badrilla, Leeds, United Kingdom), anti-heat shock protein-60 (HSP-60; 1:500), anti-HSP-70 (1:500), anti-HSP-90 (1:500), anti-B-cell lymphoma 2 (Bcl-2; 1:500), anti-caspase-3 (1:500), anti-Ca2+/calmodulin-dependent protein kinase II (CaMKII; 1:2,000), anti-Parkin (1:5,000, Santa Cruz Bio-technology), anti-β2 (1:1,000), anti-dynamin-related/lk protein 1 (DRP1/DLP1; 1:500), anti-optic atrophy 1 protein (OPA1; 1:2,500, BD Biosciences, Franklin Lakes, NJ), anti-mitofusin-2 (MFN2; 1:2,500, Abcam, Cambridge, UK), anti-phospho-CaMKII (Thr286; 1:2,000), anti-phospho-HSP-27 (Ser22; 1:1,000), anti-HSP-27 (1:1,000), anti-Bax (1:1,000), anti-sequestosome 1 (SQSTM1/p62; 1:1,000), anti-microtubule-associated protein 1 light chain 3 A/B (LC3 A/B; 1:5,000), anti-Beclin-1 (1:1,000), anti-Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP3; 1:5,000), anti-phospho-Akt (Ser473; 1:1,000), anti-Akt (1:1,000), anti-phospho-eukaryotic translation initiation factor 2 alpha (eIF2α) (S51; 1:1,000), anti-phospho-AMP-activated protein kinase α (AMPKα1-Thr172; 1:1,000), anti-AMPKα2 (1:1,000), anti-phospho-ribosomal S6 (Ser235/236; 1:1,000), anti-ribosomal S6 (1:1,000), anti-phospho-glycogen synthase kinase-3β (GSK3β; 1:1,000), anti-GSK3β (1:1,000), and anti-GAPDH (1:5,000) as loading control (Cell Signaling Technology).

For isolated mitochondria, the following primary antibodies were used in 5% nonfat milk in TBS-T: anti-OPA1 (1:2,500) from BD Biosciences, anti-ribosomal S6 (1:1,000), anti-ribosomal S6 kinase (1:1,000), anti-AMPKα1-Thr172; 1:1,000), anti-phospho-eukaryotic translation initiation factor 2 alpha (eIF2α) (S51; 1:1,000), anti-phospho-AMP-activated protein kinase α (AMPKα1-Thr172; 1:1,000), anti-AMPKα2 (1:1,000), anti-phospho-ribosomal S6 (Ser235/236; 1:1,000), anti-ribosomal S6 (1:1,000), anti-phospho-glycogen synthase kinase-3β (GSK3β; 1:1,000), anti-GSK3β (1:1,000), and anti-GAPDH (1:5,000) as loading control (Cell Signaling Technology). After three washes with TBS-T, membranes were incubated with primary antibodies in 1–5% nonfat milk or BSA in TBS-T: anti-OPA1 (1:2,500) from BD Biosciences, anti-ribosomal S6 (1:1,000), anti-ribosomal S6 kinase (1:1,000), anti-phospho-eukaryotic translation initiation factor 2 alpha (eIF2α) (S51; 1:1,000), anti-phospho-AMP-activated protein kinase α (AMPKα1-Thr172; 1:1,000), anti-AMPKα2 (1:1,000), anti-phospho-ribosomal S6 (Ser235/236; 1:1,000), anti-ribosomal S6 (1:1,000), anti-phospho-glycogen synthase kinase-3β (GSK3β; 1:1,000), anti-GSK3β (1:1,000), and anti-GAPDH (1:5,000) as loading control (Cell Signaling Technology). After three washes with TBS-T, horseradish peroxidase-conjugated secondary antibody was added for 2 h at room temperature (1:5,000 in 5% nonfat milk in TBS-T). Signals were detected with an enhanced chemiluminescence kit (Bio-Rad) by Chemidoc XRS+ (Bio-Rad). For the analysis of tropomyosin oxidation, the density of the additional band with higher molecular weight reflecting the formation of disulfide cross bridges was normalized to densitometric values of the respective tropomyosin monomer. Antibodies against phosphorylated epitopes were removed with Pierce Stripping Buffer (Thermo Fisher Scientific) before incubation with antibodies detecting the total protein.

Statistical analysis. Values are expressed as means ± SE. Statistical analysis was performed between groups by unpaired two-tailed t-test or by Mann-Whitney U-test by using GraphPad Prism 6 software. A P < 0.05 value was considered significant.

RESULTS

Moderately increased adiposity in prediabetic animals. To determine the effect of high-fat diet and the single, low-dose STZ injection, we measured body weight, fat tissue volumes, and plasma lipid parameters. We found that body weights of the prediabetic animals were moderately but statistically significantly elevated from week 9 compared with the control group and that this difference reached 18% at the end of the diet period (Fig. 2, A and B). At week 20, plasma leptin level was significantly increased in prediabetes; however, CRP level was decreased, and plasma cholesterol, HDL cholesterol, triglyceride levels, and parameters of liver and kidney function were unchanged (Table 1). To characterize prediabetes-induced changes in further obesity-related molecules, we performed an adipokine array measurement, which revealed that the circulating level of TIMP-1 might be influenced by prediabetes; however, we could not confirm these results by ELISA (data not shown). CT scan showed that body fat volume of prediabetic rats was substantially increased at the end of the diet (Fig. 2, C and D). Epididymal fat tissue weight, which is an indicator of total body adiposity, was increased in the prediabetic group; however, the weight of interscapular brown adipose tissue was not changed (Fig. 2, G–I). Histological score analysis of HE- and MA-stained liver samples showed the development of hepatic steatosis in the prediabetic group (CON: 0.5 ± 0.3 vs. PRED: 2.25 ± 0.5; P < 0.05); however, no signs of hepatic fibrosis were detected (Fig. 2H). Furthermore, electron microscopy showed an increased number of lipid droplets in the myocardium of prediabetic animals compared with controls (Fig. 2, E and F). These results demonstrated a moderately increased adiposity and hepatic and cardiac fat deposits without signs of hyperlipidemia in the prediabetic group.

Impaired glucose tolerance, insulin resistance, and sensory neuropathy evidenced disturbed carbohydrate metabolism in prediabetes. We aimed to characterize the glucose homeostasis in our rat model of prediabetes. At week 20 of the diet, fasting blood glucose levels were slightly elevated in prediabetes from week 10; however, they remained in the normoglycemic range (Fig. 3, A and B). OGTT and ITT demonstrated impaired glucose tolerance and insulin resistance in the prediabetic group (Fig. 3, C–F); however, there was no difference in pancreatic insulin content (Fig. 3G) or in pancreatic islet morphology (data not shown) between groups. These results demonstrate prediabetic conditions in the present model and reveal that type 1 diabetes did not develop due to the STZ treatment. Sensory neuropathy is a well-accepted accompanying symptom of diabetes (80). Accordingly, here we have found a decrease in the mechanical hind limb withdrawal threshold at week 15 (CON: 48 ± 1 g vs. PRED: 42 ± 2 g; P < 0.05) of diet in the prediabetic group, which indicates a moderate sensory neuropathy in this model of prediabetes.

Diastolic dysfunction and hypertrophy in prediabetes with no sign of fibrosis. To determine the cardiac effect of prediabetes, we measured morphological and functional parameters of the hearts. Heart weights were significantly increased (Fig. 4A); however, heart weight/body weight ratio was decreased in prediabetes (CON: 0.27 ± 0.01% vs. PRED: 0.24 ± 0.01%; P < 0.05), plausibly attributable to obesity. LV mass, LVAWTs, LVPWtS, and LVPWtD were increased in the prediabetic group as assessed with echocardiography; however, other cardiac dimensional parameters were unchanged (Table 2). The slope of EDPRV, which is a very early and sensitive marker of diastolic dysfunction, was significantly
Fig. 2. High-fat feeding with a single low-dose STZ treatment increased adiposity. Changes in body weight during the experiment (A) and body weight data after 21 wk (B) are shown. Axial representative CT slice shows the middle of the 4th lumbar spine. C: red color indicates the segmented volumes of interest (VOIs) showing the volume of fat in the control (CON) and prediabetic (PRED) rats. D: whole body fat volume:body weight ratio at week 20. Representative transmission electron micrographs of myocardial lipid droplets (E; white arrows) and the number of lipid droplets in CON and PRED cardiomyocytes (F) are shown; magnification ×7,500; scale bar = 1 μm. Epididymal fat tissue (G) and interscapular brown adipose tissue (BAT) (I) weight:body weight ratios are shown. Hematoxylin-Eosin (HE) and Masson’s trichrome (MA) staining of liver sections (H) are shown; magnification ×200; scale bar = 100 μm. Data are means ± SE, n = 3–19 per group (*P < 0.05).
Data are means ± SE for 12 rat per group (*P < 0.05). HDL, high-density lipoprotein; LDL, low-density lipoprotein; GPT, glutamate pyruvate transaminase; GOT, glutamate pyruvate transaminase; CRP, C-reactive protein; CON, control; PRED, prediabetes group.

elevated in prediabetes although other hemodynamic parameters, including blood pressure, were unchanged, demonstrating the lack of systolic dysfunction or hypertension (Table 3, Fig. 4B). To uncover the molecular background of the observed mild diastolic dysfunction, we performed measurements on the common mechanistic contributors of heart failure (38). On HE-stained LV sections, increased cardiomyocyte diameter was detected in prediabetes (Fig. 4, C and D). To characterize components affecting diastolic function, we analyzed MHC expression. Interestingly, the gene expression of β-MHC was decreased, and α-MHC also showed a tendency to decrease (P = 0.17), the ratio of which resulted in a strong tendency to decrease in prediabetes. No increase in ANP or BNP gene expressions (Fig. 4, G and H) or in angiotensin-II level (data not shown) was detected in prediabetes. To evaluate the extent of fibrosis, MA-stained LV sections were analyzed, which revealed no difference between groups (Fig. 4E). Similarly, we found that gene expression of type I (COL1) and III (COL3) collagen isoforms were unchanged in the LV (Fig. 4F). These results indicate that mild diastolic dysfunction developed in prediabetic animals, which was associated with a mild hypertrophy (increased LV mass, LVAWT, LVPWT, and cardiomyocyte diameter) without signs of fibrosis.

Elevated reactive oxygen species formation in cardiac subsarcolemmal mitochondria in prediabetic rats. To investigate whether cardiac mitochondrial disturbances contribute to the observed diastolic dysfunction, mitochondrial morphology and enzyme activity were analyzed from LVs of prediabetic rats. Our electron microscopy results showed that there is no major difference in the number of IFM between the groups (Fig. 5, A and B). However, area (CON: 0.43 ± 0.01 vs. PRED: 0.39 ± 0.01 μm²; P < 0.05), perimeter (CON: 2.69 ± 0.02 vs. PRED: 2.63 ± 0.03 μm; P < 0.05), and sphericity (CON: 0.35 ± 0.01 vs. PRED: 0.31 ± 0.01; P < 0.05) of IFM are decreased in the prediabetes group. Previous studies indicated that IFM and SSM are affected by diabetes differentially (35, 82). Therefore, we analyzed our electron microscopy imagery containing SSM and found no difference in SSM size, perimeter, or sphericity (data not shown) although the statistical power of these analyses was not high enough (n = 2 for CON and n = 4 for PRED). Furthermore, we have not seen any major difference in mitochondrial oxygen consumption, enzyme activities (Tables 4 and 5), Ca uptake, or membrane potential (data not shown).

However, we have found that hydrogen peroxide production was increased in the cardiac SSM fraction with glutamate-malate as a substrate (Fig. 5C) although there was no difference when succinate was used as substrate. Interestingly, there was no increase in reactive oxygen species (ROS) production of the IFM isolated from LV supported either with glutamate-malate or with succinate (Fig. 5, D–F). As leukocytes are one of the main sources of ROS, inflammatory mediators were measured. We could find no significant difference in TNF-α (CON: 1 ± 0.27 vs. PRED: 0.59 ± 0.07; ratio normalized to

Table 1. Plasma parameters at week 20

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CON</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma leptin, ng/ml</td>
<td>2.51 ± 0.33</td>
<td>5.91 ± 0.60*</td>
</tr>
<tr>
<td>Plasma cholesterol, mmol/l</td>
<td>1.88 ± 0.06</td>
<td>1.72 ± 0.08</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>2.75 ± 0.14</td>
<td>2.75 ± 0.10</td>
</tr>
<tr>
<td>Plasma triglyceride, mmol/l</td>
<td>1.20 ± 0.10</td>
<td>1.31 ± 0.09</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>0.44 ± 0.02</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>1.61 ± 0.05</td>
<td>1.51 ± 0.08</td>
</tr>
<tr>
<td>GOT, U/l</td>
<td>82.00 ± 15.00</td>
<td>58.00 ± 4.00</td>
</tr>
<tr>
<td>GPT, U/l</td>
<td>50.00 ± 14.00</td>
<td>49.00 ± 5.00</td>
</tr>
<tr>
<td>Uric acid, mmol/l</td>
<td>24.00 ± 16.00</td>
<td>16.00 ± 1.00</td>
</tr>
<tr>
<td>Creatinine, μmol/l</td>
<td>46.00 ± 3.00</td>
<td>40.00 ± 5.00</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>109.56 ± 1.24</td>
<td>94.96 ± 3.87*</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. CON.
Fig. 4. Characterization of cardiac function, myocardial morphology, and fibrosis in prediabetic rats. A: quantification of heart weights after 21 wk. B: representative pressure-volume loops and slope of end diastolic pressure-volume relationship (EDPVR) in CON and PRED group. HE and MA staining of myocardial sections (C), quantification of cardiomyocyte diameter (D), and level of fibrosis (E) in control (CON) and prediabetic (PRED) rats are shown; magnification \times 200; scale bar = 100 \mu m. Quantifications of collagen type I (COL1), COL3 (F), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) (G), \( \alpha \)-myosin heavy chain (\( \alpha \)-MHC), \( \beta \)-MHC, and \( \alpha \)-\( \beta \)-MHC ratio (H) in CON and PRED group are shown. Data are means \pm SE, \( n = 5 \)–19 per group (*\( P < 0.05 \)).
with control animals (Fig. 6) expression of SERCA2A in our model of prediabetes compared to wild-type animals. However, the Bcl-2/Bax ratio was unchanged (Fig. 7A). We also characterized the effect of prediabetes on apoptosis in the heart. Prediabetes did not affect the expression of proapoptotic caspase-3 and Bax in LVs. On the other hand, the antiapoptotic Bcl-2 was downregulated in prediabetic animals. However, the Bcl-2/Bax ratio was unchanged (Fig. 7F; Table 6).

No changes in cardiac HSPs in prediabetes. We also characterized the effect of prediabetes on the expression and/or phosphorylation of HSPs in the LV. Our results showed no differences in the expression of HSP-60, HSP-70, and HSP-90 or in either phosphorylation or expression of HSP-27 (Fig. 7E; Table 6).

**DISCUSSION**

This is the first comprehensive analysis of the cardiac effects of prediabetes in a nongenic rodent model in which we assessed cardiac functions, parameters of hypertrophy, fibrosis, oxidative and nitrative stress, inflammation, mitochondrial dynamics, autophagy, mitophagy, markers of myocardial calcium handling, apoptosis, and expression of HSPs. In this model of prediabetes, we demonstrated an impaired glucose and insulin tolerance, increased adiposity and myocardial lipid accumulation, and altered cardiac morphology and function.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV mass, g</td>
<td>1.01 ± 0.04</td>
<td>1.22 ± 0.07*</td>
</tr>
<tr>
<td>LVAWTd, mm</td>
<td>1.89 ± 0.12</td>
<td>2.07 ± 0.12</td>
</tr>
<tr>
<td>LVAWTs, mm</td>
<td>2.86 ± 0.17</td>
<td>3.42 ± 0.11*</td>
</tr>
<tr>
<td>LVWPTd, mm</td>
<td>1.86 ± 0.07</td>
<td>2.05 ± 0.04*</td>
</tr>
<tr>
<td>LVWPTs, mm</td>
<td>2.72 ± 0.12</td>
<td>3.25 ± 0.15*</td>
</tr>
<tr>
<td>LVDD, mm</td>
<td>7.71 ± 0.22</td>
<td>7.75 ± 0.17</td>
</tr>
<tr>
<td>LVDS, mm</td>
<td>4.93 ± 0.31</td>
<td>4.96 ± 0.31</td>
</tr>
<tr>
<td>FS, %</td>
<td>36.00 ± 2.20</td>
<td>37.40 ± 3.80</td>
</tr>
<tr>
<td>HR, 1/min</td>
<td>335.00 ± 13.00</td>
<td>348.00 ± 10.00</td>
</tr>
</tbody>
</table>

Data are means ± SE for 10 rats per group (*P < 0.05). LV, left ventricle; LVAWTd, LV anterior wall thickness, diastolic; LVAWTs, LV anterior wall thickness, systolic; LVWPTs, LV posterior wall thickness, systolic; LVWPTd, LV posterior wall thickness, diastolic; FS, fractional shortening; HR, heart rate.

Table 2. Characterization of cardiac morphology and function in prediabetes by means of echocardiography

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mmHg</td>
<td>110.100 ± 7.300</td>
<td>113.600 ± 6.100</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>116.600 ± 5.600</td>
<td>120.000 ± 6.800</td>
</tr>
<tr>
<td>LVESV, μl</td>
<td>292.800 ± 14.500</td>
<td>280.200 ± 9.600</td>
</tr>
<tr>
<td>SV, μl</td>
<td>130.700 ± 6.900</td>
<td>127.500 ± 4.500</td>
</tr>
<tr>
<td>CO, μl/min</td>
<td>162.100 ± 9.100</td>
<td>152.800 ± 7.700</td>
</tr>
<tr>
<td>EF, %</td>
<td>59.500 ± 3.200</td>
<td>56.600 ± 2.300</td>
</tr>
<tr>
<td>SW, mmHg/ml</td>
<td>55.300 ± 1.300</td>
<td>54.400 ± 1.300</td>
</tr>
<tr>
<td>Dp/dmax, mmHg/s</td>
<td>14.500 ± 0.500</td>
<td>13.600 ± 0.600</td>
</tr>
<tr>
<td>Dp/dmin, mmHg/s</td>
<td>7226.000 ± 487.000</td>
<td>7387.000 ± 401.000</td>
</tr>
<tr>
<td>τ (Glanz), ms</td>
<td>12.600 ± 0.300</td>
<td>12.100 ± 0.400</td>
</tr>
<tr>
<td>TPR, (mmHg/min)/ml</td>
<td>1.900 ± 0.190</td>
<td>2.000 ± 0.120</td>
</tr>
<tr>
<td>Slope of ESPVR, mmHg/μl</td>
<td>2.680 ± 0.120</td>
<td>2.710 ± 0.060</td>
</tr>
<tr>
<td>Slope of EDVPR, mmHg/μl</td>
<td>0.026 ± 0.001</td>
<td>0.037 ± 0.004*</td>
</tr>
<tr>
<td>PRSW, mmHg</td>
<td>100.500 ± 5.200</td>
<td>98.900 ± 4.100</td>
</tr>
<tr>
<td>Slope of dp/dmax-EDV, (mmHg/s)/μl</td>
<td>34.300 ± 2.300</td>
<td>35.200 ± 2.200</td>
</tr>
<tr>
<td>Maximal power, mW</td>
<td>91.800 ± 8.200</td>
<td>98.200 ± 12.500</td>
</tr>
</tbody>
</table>

Data are means ± SE for 10 rats per group (*P < 0.05). MAP, mean arterial pressure; LVEDP, LV end-diastolic pressure; LVEDP, LV end-diastolic pressure; LVESV, LV end-systolic volume; LVEDV, LV end-diastolic volume; SV, stroke volume; CO, cardiac output; EF, ejection fraction; SW, stroke work; Dp/dmax, maximal slope of LV systolic pressure increment; Dp/dmin, maximal slope of LV diastolic pressure decrement; τ, time constant of LV pressure decay; TPR, total peripheral resistance; ESPVR, end-systolic pressure-volume relationship; EDVPR, end-diastolic pressure-volume relationship; PRSW, preload recruitable SW; dp/dmax-EDV, slope of the dp/dmax-end-diastolic volume relationship.
tion, a mild diastolic dysfunction, and sensory neuropathy despite normal fasting plasma glucose and lipid levels. We also observed elevated ROS production in the SSM, nitrative stress, elevated expression of MFN2, decreased expression of \( \beta \)-H9252-MHC, and phosphorylation of PLB. Furthermore, here we found early signs of dysregulated mitophagy and decreased mitochondrial size in prediabetes; however, other major markers of mitochondrial dynamics, autophagy, mitophagy, inflammation, or myocardial expression of apoptotic proteins or HSPs, were not modulated by prediabetes.

In this study, we used high-fat chow-fed Long-Evans rats treated with a single, low-dose STZ. This setting allowed us to investigate cardiac consequences of a moderate metabolic derangement, prediabetes, rather than of a severely disturbed glucose and lipid homeostasis, such as seen in genetically modified models of diabetes, e.g., in \( db/db \) or \( ob/ob \) mice (36, 67). Because it has been reported that LV hypertrophy had a higher prevalence in patients with diabetes and that 40–75% of patients with type 1 or type 2 diabetes mellitus presented with diastolic dysfunction (12, 73), we aimed to investigate whether cardiac function is affected by prediabetes. Previously, it has been shown that diastolic dysfunction was developed in several pathological conditions; however, the underlying mechanisms are still not clearly understood (38). Here, we demonstrated that the deterioration of diastolic function and sensory neuropathy

Table 4. Quantification of cardiac mitochondria enzyme activity in LV

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate-synthase activity, U/mg protein</td>
<td>223.12 ± 9.98</td>
<td>220.44 ± 8.32</td>
</tr>
<tr>
<td>NADH:ubiquinone-oxidoreductase activity, U/mg protein</td>
<td>40.52 ± 2.55</td>
<td>36.48 ± 2.99</td>
</tr>
<tr>
<td>NADH:cytochrome c-oxidoreductase activity, U/mg protein</td>
<td>7.85 ± 1.18</td>
<td>8.47 ± 1.31</td>
</tr>
<tr>
<td>Succinate:cytochrome c-oxidoreductase activity, U/mg protein</td>
<td>21.09 ± 1.49</td>
<td>23.57 ± 1.61</td>
</tr>
<tr>
<td>Succinate-dehydrogenase activity, U/mg protein</td>
<td>84.06 ± 5.83</td>
<td>80.09 ± 3.42</td>
</tr>
<tr>
<td>Cytochrome-c-oxidase activity, U/mg protein</td>
<td>38.74 ± 3.15</td>
<td>40.36 ± 2.33</td>
</tr>
</tbody>
</table>

Data are means ± SE for 5–9 rat per group.
athy occurs well before overt diabetes develops, which is accompanied by early signs of cardiac hypertrophy. These findings are in agreement with previous reports showing that neuropathy might precede the development of full-fledged diabetes (48) and that high-fat diet-induced prediabetes increased heart weights and decreased contractile function, as assessed by a diminished aortic output (23, 29). However, in contrast to our report, plasma triglycerides and insulin levels

<table>
<thead>
<tr>
<th></th>
<th>CON Subsarcolemmal (pmol/ml)</th>
<th>PRED Subsarcolemmal (pmol/ml)</th>
<th>CON Interfibrillar (pmol/ml)</th>
<th>PRED Interfibrillar (pmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate-malate</td>
<td>25.08 ± 3.60</td>
<td>21.06 ± 3.53</td>
<td>58.60 ± 19.31</td>
<td>61.78 ± 21.41</td>
</tr>
<tr>
<td>ADP</td>
<td>203.31 ± 32.57</td>
<td>194.03 ± 42.16</td>
<td>304.23 ± 25.75</td>
<td>287.90 ± 22.35</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>260.80 ± 28.27</td>
<td>287.96 ± 54.91</td>
<td>335.96 ± 25.79</td>
<td>345.41 ± 28.17</td>
</tr>
<tr>
<td>Succinate</td>
<td>306.20 ± 25.19</td>
<td>289.74 ± 23.23</td>
<td>367.76 ± 15.74</td>
<td>393.56 ± 18.82</td>
</tr>
<tr>
<td>Rosentone</td>
<td>143.12 ± 18.19</td>
<td>139.70 ± 23.64</td>
<td>238.40 ± 18.44</td>
<td>220.73 ± 16.19</td>
</tr>
<tr>
<td>CAT</td>
<td>105.95 ± 8.89</td>
<td>106.50 ± 12.22</td>
<td>159.78 ± 6.86</td>
<td>156.60 ± 8.03</td>
</tr>
</tbody>
</table>

Data are means ± SE for 9 rats per group. CAT, carboxyatractyloside.

Fig. 6. Characterization of oxidative and nitrative stress in prediabetes. Representative immunostaining of nitrotyrosine in the left ventricle (A) is shown; magnification ×200; scale bar = 200 μm. Representative Western blots (B) and quantification (C) of tropomyosin oxidation are shown. Representative Western blots (D) and quantification (E) of cardiac p66Shc expression are shown. Representative Western blots (F), quantification of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (G), phospholamban (PLB) phosphorylation on Thr^{17} (H) and Ser^{16} (I), and sarco/endoplasmic reticulum Ca^{2+} ATPase II (SERCA2A) (J) expression are shown. Tm, tropomyosin; Ox. Tm, oxidized tropomyosin. Data are means ± SE, n = 6–8 per group.
were elevated in these studies, highlighting that a substantial difference can be observed between different diet-induced models and stages of prediabetes (29). Furthermore, it has been described that obesity promoted the hypertrophy-inducing effect of diabetes regardless of hypertension (26), which could be attributed to adipokines, such as leptin and resistin (5, 39). Similarly, here we showed that even mild obesity (only 18% increase in body weight was observed in the present study) with an elevated leptin level is sufficient to induce hypertrophy even without impairment of fasting plasma glucose and lipid levels or hypertension, which is in agreement with previous reports (23, 29). However, clinical data seem to contradict these findings because no increase in the prevalence of LV hypertrophy was observed in overweight prediabetic patients with impaired fasting glucose and impaired glucose tolerance (66). Mechanistic studies on how obesity abrogates cardiac function are scarce. Increased myocardial triglyceride content is associated with diastolic dysfunction in ob/ob mice (18), which is well in line with our findings that the number of lipid particles increased in the myocardium in prediabetes. Although microRNA-451 has been demonstrated to promote cardiac hypertrophy and diminished contractile reserves in mice on high-fat diet (44), further studies are warranted to describe the relationship between cardiac dysfunction and the disturbed cardiac lipid metabolism in prediabetes. Interestingly, unlike in genetic models of prediabetes (20), diet-induced prediabetes did not result in an elevation in classical molecular markers of hypertrophy or conventional signs of fibrosis in the heart, as expected in the case of hypertrophy. Moreover, this is the first evidence on decreased β-MHC in prediabetes. Although the vast majority of publications demonstrate an increase in β-MHC in diabetes (4, 83), a small number of studies indicate a downregulation of MHC expression in animals with diverse cardiac or metabolic challenges. For instance, in cardiomyocytes from STZ-treated rats, total MHC expression was significantly decreased (25). These results indicate that, although in most cardiometabolic derangements expression of the slow MHC isoform increases, in certain conditions, such as in prediabetes, a general suppression of MHC expression might be present. The reduction in MHC expression might also contribute to the observed cardiac dysfunction in prediabetes; however, to uncover its significance and mechanism, further experiments are warranted.

Oxidative stress has a major role in the development of diabetic cardiomyopathy (10, 31); however, it has not been well described whether it is responsible for the decreased cardiac function in prediabetes. Here we found elevated hydrogen peroxide production in SSM, increased nitrotyrosine

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Fig. 7. Cardiac expression of mitochondrial dynamics, autophagy/mitophagy, heat shock proteins (HSPs), and apoptosis-related proteins in prediabetes. Representative Western blots of autophagy/mitophagy-related proteins and upstream modulators of autophagy (A) and mitochondrial fission- and fusion-related protein (B) in whole left ventricles are shown. Representative Western blots of mitochondrial dynamics- and mitophagy-related proteins in isolated SSM (C) and IFM (D) are shown. Representative Western blots of HSP-related (E) and apoptosis-related (F) proteins in whole left ventricle are shown. DRP1/DLP1, dynamin-related/protein 1; MFN2, mitofusin-2; OPA1, optic atrophy 1; COX4, cytochrome c oxidase subunit 4, mitochondrial; LC3, 1 microtubule-associated protein 1 light chain 3; SQSTM1/p62, sequestosome 1; BNIP3, Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3; AMPKα, AMP-activated protein kinase α; GSK3β, glycogen synthase kinase-3β.
Beclin1/GAPDH ratio 0.810
Parkin/GAPDH ratio 2.250
MFN2/GAPDH ratio 0.270
prediabetic mitochondria (see Table 5), showing no impair-
production (8, 43). Although here we observed a moderately
amine oxidase also have important roles in mitochondrial ROS
formation of ROS (22), and NADPH oxidase 4 and mono-
that p66Shc translocation to mitochondria can increase the
produce ROS in mitochondria (17). For instance, it is known
ROS; however, several other intracellular components can
form ROS in mitochondria. It is presently unknown
what mechanism leads to the increased ROS production exclu-
sively in SSM in prediabetes. In mice on a high-fat diet, cardiac
mitochondrial ROS production was elevated, and, similar to
our results, mitochondrial oxygen consumption did not change
substantially, whereas a significant amount of cardiac lipid
accumulation was observed (1). However, the source of ROS
has not been identified in this study either. Thus, to reveal the
direct connection between elevated ROS production and mito-
chondrial and cardiac dysfunction, further studies are war-
anted.

Molecular mechanisms that contribute to hypertrophy and
cardiac dysfunction in prediabetes have not been investigated in
detail. In our previous studies on diet-induced hypercholes-
terolemia or metabolic syndrome in ZDF rats, we have shown by
DNA and miRNA microarrays that a multitude of cardiac
cellular processes is modulated by these conditions (69, 79).
Similarly, in this study, we showed changes in several cellular
processes, suggesting that hypertrophy and deteriorated dia-
static function in prediabetes may be consequences of numer-
ous concurrent alterations in the cardiac homeostasis (see Fig. 8).
Characterizing active components of the contractile
apparatus and Ca²⁺ homeostasis, here we observed a tendency for
a decrease in the Ser16 phosphorylation of PLB in predia-
betes. In previous studies, decreased phosphorylation of PLB
on Ser16 was demonstrated to be associated with abnormalities
in contraction and relaxation in the diabetic heart (57, 85). This
notion is further supported by the findings of Abdurrachim et
al. (1), who demonstrated that phosphorylation of PLB was
reduced in the heart of mice with diastolic dysfunction induced
by a high-fat diet. Therefore, decreased phosphorylation of
PLB may also contribute to the development of early diastolic
dysfunction that we uncovered in prediabetes. Increased activity
and expression of CaMKIIα and reduced phosphorylation of
PLB by CaMKIIα in the heart of STZ-treated diabetic rats (71, 76)
although, in these reports, a significant hyperglycemia was
increased in the heart of diabetic rats (71, 76) although, in these reports, a significant hyperglycemia was
present, which was shown to facilitate the activation of
CaMKIIβ (28). Apoptosis is considered to be one of the
hallmarks of diabetic cardiomyopathy, and it is induced by
oxidative stress in diabetes (10, 78). It has been described that
experimental diabetes induces upregulation of proapoptotic
and downregulation of antiapoptotic proteins (3, 84); however,
no data have been available on the cardiac apoptosis in pre-
diabetes. In the present study, we show a modest downregulation
of Bcl-2; however, no change in Bcl-2/Bax ratio and caspase-3
expression was detected in prediabetic animals. Thus our data
suggest an early dysregulation of pro- and antiapoptotic pro-
teins in prediabetes; however, they do not show a gross
induction of apoptosis in prediabetes. Tropomyosin is prone to
loss of function by oxidative modifications that are associated
with the severity of heart failure in humans (14, 15). In this
study, oxidized tropomyosin content of the heart was not
modulated by prediabetes. These data suggest that neither the
CaMKIIβ pathway, apoptosis induction, nor tropomyosin oxi-
formation, and elevated cardiac expression of MFN2. These
findings are in agreement with previous reports in which
elevated oxidative stress, such as seen in our model of pre-
diabetes, leads to an increase in MFN2 in rat vascular smooth
muscle cells (33), and its robust overexpression induced apop-
totic cell death in neonatal rat cardiomyocytes (72). Similarly,
in another study, high-fat diet induced oxidative stress and
MFN2 overexpression in the liver of C57BL/6 mice after 16
wk (27). However, in a previous study on diet-induced pre-
diabetes, no sign of cardiac mitochondrial oxidative stress was
shown in male Wistar rats after 16 wk (29), which may suggest
that mitochondrial oxidative stress might not be present in all
models and stages of prediabetes and that it might not be the
primary driving force of prediabetes-induced cardiac func-
tional alterations.

It is well established that mitochondria, especially the mito-
ochondrial electron transport chain, is one of the main sources of
ROS; however, several other intracellular components can
produce ROS in mitochondria (17). For instance, it is known that
p66Shc translocation to mitochondria can increase the
formation of ROS (22), and NADPH oxidase 4 and mono-
amine oxidase also have important roles in mitochondrial ROS
production (8, 43). Although here we observed a moderately
increased ROS production in SSM, no difference can be seen
in mitochondrial oxygen consumption between normal and
prediabetic mitochondria (see Table 5), showing no impair-

Table 6. Quantification of HSPs, apoptosis, mitochondrial
dynamics-related, and mitophagy-related protein expressions
in isolated mitochondrial fractions and whole LVs

<table>
<thead>
<tr>
<th></th>
<th>Total LV</th>
<th>CON</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNIP3/GAPDH ratio</td>
<td>0.590 ± 0.030</td>
<td>0.440 ± 0.020*</td>
<td></td>
</tr>
<tr>
<td>MFN2/GAPDH ratio</td>
<td>0.270 ± 0.010</td>
<td>0.360 ± 0.020*</td>
<td></td>
</tr>
<tr>
<td>OPAL1/GAPDH ratio</td>
<td>1.470 ± 0.110</td>
<td>1.630 ± 0.100</td>
<td></td>
</tr>
<tr>
<td>DRP1/GAPDH ratio</td>
<td>1.160 ± 0.080</td>
<td>1.340 ± 0.140</td>
<td></td>
</tr>
<tr>
<td>LC3-II/GAPDH ratio</td>
<td>0.560 ± 0.070</td>
<td>0.560 ± 0.050</td>
<td></td>
</tr>
<tr>
<td>p62/GAPDH ratio</td>
<td>3.050 ± 0.180</td>
<td>3.450 ± 0.230</td>
<td></td>
</tr>
<tr>
<td>Parkin/GAPDH ratio</td>
<td>2.250 ± 0.140</td>
<td>2.430 ± 0.170</td>
<td></td>
</tr>
<tr>
<td>Beclin1/GAPDH ratio</td>
<td>0.810 ± 0.070</td>
<td>0.820 ± 0.070</td>
<td></td>
</tr>
<tr>
<td>Phospho AKT(Ser473)/GSK3 ratio</td>
<td>0.320 ± 0.040</td>
<td>0.290 ± 0.020</td>
<td></td>
</tr>
<tr>
<td>Phospho AMPK(Thr172)/AMPK ratio</td>
<td>0.120 ± 0.020</td>
<td>0.210 ± 0.060</td>
<td></td>
</tr>
<tr>
<td>Phospho S6k(Thr235/240)/S6 ratio</td>
<td>2.620 ± 1.080</td>
<td>2.160 ± 0.610</td>
<td></td>
</tr>
<tr>
<td>Phospho GSK3β(Ser9)/GSK3β ratio</td>
<td>0.800 ± 0.090</td>
<td>0.720 ± 0.100</td>
<td></td>
</tr>
<tr>
<td>Bcl-2/GAPDH ratio</td>
<td>0.230 ± 0.010</td>
<td>0.200 ± 0.003*</td>
<td></td>
</tr>
<tr>
<td>Bcl-2/Bax ratio</td>
<td>0.150 ± 0.020</td>
<td>0.150 ± 0.020</td>
<td></td>
</tr>
<tr>
<td>Caspase-3/GAPDH ratio</td>
<td>0.050 ± 0.001</td>
<td>0.040 ± 0.003</td>
<td></td>
</tr>
<tr>
<td>P-HSP-27(Ser56)/T-HSP-27 ratio</td>
<td>0.340 ± 0.050</td>
<td>0.280 ± 0.020</td>
<td></td>
</tr>
<tr>
<td>HSP-60/GAPDH ratio</td>
<td>0.850 ± 0.080</td>
<td>0.840 ± 0.070</td>
<td></td>
</tr>
<tr>
<td>HSP-70/GAPDH ratio</td>
<td>0.530 ± 0.010</td>
<td>0.510 ± 0.010</td>
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</tr>
<tr>
<td>HSP-90/GAPDH ratio</td>
<td>0.460 ± 0.010</td>
<td>0.500 ± 0.020</td>
<td></td>
</tr>
</tbody>
</table>

| Subsarcolemmal mitochondria    |                 |           |           |
| OPA1/COX4 ratio               | 1.340 ± 0.070   | 1.320 ± 0.060 |          |
| LC3-II/COX4 ratio             | 0.220 ± 0.100   | 0.250 ± 0.040 |          |
| p62/COX4 ratio                | 0.130 ± 0.020   | 0.120 ± 0.030 |          |

| Interfibrillar mitochondria    |                 |           |           |
| OPA1/COX4 ratio               | 1.440 ± 0.150   | 1.520 ± 0.140 |          |
| LC3-II/COX4 ratio             | 0.220 ± 0.090   | 0.290 ± 0.070 |          |
| p62/COX4 ratio                | 0.350 ± 0.060   | 0.360 ± 0.040 |          |

Data are means ± SE for 8 rat per group (*P < 0.05). HSP, heat shock protein; BNIP3, Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3; MFN2, mitofusin-2; OPA1, optic atrophy 1; DRP1, dynamin-related protein 1; LC3, microtubule-associated protein 1 light chain 3; AMPK, AMP-activated protein kinase; GSK3β, glycogen synthase kinase-3β; COX4, cytochrome c oxidase subunit 4, mitochondrial.
duction are responsible for the diastolic dysfunction observed in prediabetes.

Here we also demonstrate an early dysregulation of mitochondrial fusion and mitophagy first in the literature, as shown by elevated MFN2 and attenuated BNIP3 expression in prediabetes; however, other canonical markers of autophagy, mitophagy, and apoptosis were unaffected. Similarly, right atrial myocardial samples of type-2 diabetic patients presenting no signs of overt cardiomyopathy, the expression of the majority of mitochondrial dynamics, and autophagy-related proteins were not elevated, except for that of ATG5 and MFN1 (53). Therefore, we can assume that only major disturbances in glucose and lipid homeostasis, such as seen in untreated patients or in genetic models of diabetes, might be a powerful enough signal to extensively modulate cardiac autophagy, mitophagy, or mitochondrial dynamics, which might result in grossly deteriorated cardiac function. Moreover, experimental systemic sensory neuropathy by itself has been previously shown to cause diastolic dysfunction and global gene expression changes in the rat heart (7, 86). Therefore, prediabetes-induced sensory neuropathy observed in the present study might also contribute to the diabetic cardiomyopathy.

Furthermore, this is the first report to show that prediabetes does not modulate cardiac expression of HSP-60, HSP-70, HSP-90, and phosphorylation or expression of HSP-27. In contrast, in STZ-induced diabetes, increased levels of HSP-70 have been detected in the rat heart (7), and increased levels of circulating HSP-60 were found in diabetic patients (70), suggesting that, in advanced stages of diabetes, HSPs might be involved in the development of cardiac dysfunction. However, our data suggest no role of HSPs in prediabetes in the heart.

A limitation of this study is that the prediabetic condition was not analyzed in female rats. Because pathophysiological processes might substantially differ between sexes (see NIH notice NOT-OD-15-102), it cannot be excluded that performing the study with the inclusion of both sexes might allow different conclusions to be drawn. Furthermore, because other studies showed that MHC protein expression is influenced by certain factors such as Foxo1 (61) or miR-27a (55), assessing α- and β-MHC protein levels might have helped to understand the molecular background of the mild diastolic dysfunction and hypertrophy observed in prediabetes better.

Taken together, this study emphasizes that parallel occurrence of several abnormalities of metabolic, oxidative, and contractile functions might trigger cardiac pathological changes characteristic of prediabetes well before hyperglycemia or major metabolic derangements occur and that preventing these abnormalities might be of importance for future therapies of cardiac pathologies observed in early metabolic diseases such as prediabetes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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