Supplemental Materials

STZ-induced type 1-like diabetic rats

Diabetes was induced in fasting rats by intravenously injecting STZ at 65 mg/kg (Sigma-Aldrich Inc., USA)as described previously(1). The animals were considered diabetic if they had a plasma glucose concentration over 350 mg/dL. Diabetic rats received either DAPA (30 mg/kg/day, orally) or insulin (100 mg/kg/day, ip). In both control and STZ rats, body weight, food and water intake were recorded per week. At the end of study, pressure volume loop analysis was performed and rats were thereafter sacrificed. Fresh heart tissues were rapidly removed and stored for histology analysis.

Echocardiography

Standard echocardiography was performed (Vivid E9; GE Vingmed Ultrasound AS, Horten, Norway) with a 3.5-MHz multiphase-array probe in accordance with the recommendations of the American Society of Echocardiography (2). LV ejection fraction (LVEF) was measured using the biplane Simpson's method. In addition, LV diastolic function-associated parameters including trans-mitral and tricuspid early filling velocity (E) to atrial velocity (A) ratio were measured. Tissue Doppler imaging values of left ventricle function parameters, such as early (e′) and late (a′) annular diastolic velocities, were obtained from the apical four-chamber view with sample volume placed at the anterior and lateral sections of the mitral annulus.

Conventional echocardiography in Rats

Rats were first anesthetized with 3% isoflurane mixed with oxygen, and were then placed in a left lateral decubitus position on the pre-warmed rodent platform. Throughout the procedure, heart rate was maintained above 200 beats/min and imaging was performed with ECG gating (3). All cardiac images were recorded at a frame rate of 300–350/s. A single imager procured all the images using the GE Vivid S6 Dimension echocardiography platform with a 10 MHz linear array transducer (GE-Vingmed Ultrasound AS, Horten, Norway). The echo readouts included long- and short-axis views which allowed assessment of both structural and functional parameters including interventricular septum thickness in diastole (IVSd), left ventricular internal diameter in diastole (LVIDd), ejection fraction (EF), and fractional shortening (FS).

STE analysis for deformation in patients and rats

In patients, standard apical 4-, 2-, and 3-chamber views were recorded in digital

loops for the longitudinal strain analysis of the LV. The images were acquired with frame rates of 70–90 frames/s and stored for three cycles. The images were analyzed off-line using EchoPAC computer software (GE-Vingmed Ultrasound AS, Horten, Norway). As described previously, we measured the LV peak systolic GLS in the apical views (4). In rats, with frame rates around 150 frames/s the endocardial border of LV at the parasternal long axis view was tracked and followed by defining the width of myocardium using EchoPAC software (4). Echocardiography including STE was performed at baseline and weekly during the duration of study.

Cell viability

Cell viability was determined by using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-dimethyl-2H-tetrazolium bromide (MTT) assay kit (Sigma-Aldrich Co., St Louis, MO, USA) according to manufacturer's instructions. In brief, H9C2 were cultured in 96-well plates at a density of 2×10^3 per well in 100 μl of complete medium. Subsequently, 50 μl of the MTT solution was added to each well, and then the cells were incubated for an additional 4 hr at 37°C. After removal of the MTT medium, 100 μl of dimethyl sulfoxide. (DMSO; Sigma-Aldrich Co., St Louis, MO, USA) was added to each dish to dissolve the precipitate for 10 min. The absorbance was measured at 450 nm with a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

ROS assay

Intracellular levels of ROS were detected using the fluorescent probe 2',7'-dichlorofluorescindiacetate (H_2DCFDA, Thermo Fisher Scientific, Massachusetts, USA). After indicated treatments, H9C2 cardiomyocytes were incubated with H2DCF-DA at a final concentration of 10 μM for 30 min at 37°C in the dark and the cells were then washed 3 times with Phosphate Buffered Saline (PBS; Sigma-Aldrich Co., St Louis, MO, USA). The levels of fluorescence product was assessed using a fluorescent microplate reader, the excitation wave length was 488 nm (Olympus BX51, Olympus, Optical Co. Ltd, Tokyo, Japan). The mean fluorescence intensity (MFI) from 5 random fields was measured using ImageJ 1.47i software, and the MFI was used as an index for the amount of ROS.

Tunel assay

The terminal deoxynucleotidyl transferase -mediated dUTP nick end labeling (TUNEL) assay was performed using an in situ cell death detection kit (Calbiochem, Merk Biosciences, Germany) according to the manufacturer's protocol. The H9C2 and heart sections were fixed with 4% paraformaldehyde with 0.1% Triton X-100 in

0.1% sodium citrate for 5 min on ice before TUNEL staining. The H9C2 and heart sections digested with fresh diluted proteinase K (1:200) at room temperature for 10 min. Then, TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP was added to each section and allowed to settle at room temperature for 30 min in a dark. The sections were rinsed three times in PBS for 5 min each. The nuclei were stained with DAPI while cardiomyocytes were identified with positive F-actin staining. The sections were examined under a fluorescence microscope (Olympus IX71, Olympus Optical Co. Ltd, Tokyo, Japan) to determine the percentage of apoptotic cells. The number of positive cells and the total number of cells were counted for three fields at 200× magnification. The results are presented as the ratio of positive cells to total cells.

Cell apoptosis detected by flow cytometry

Apoptosis of H9C2 cardiomyocytes was measured using the Annexin V / propidium iodide (PI) double staining method. After treatment, cells were harvested and washed twice with ice-cold PBS. Cell were re-suspended in binding buffer and then incubated with Annexin V and PI working solution for 15 min in the dark at room temperature. Cellular fluorescence was measured by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Western blotting

H9C2 cardiomyocytes were harvested and lysed with ice-cold RIPA buffer (MERCK Millipore, Massachusetts, USA). The total protein concentrations were determined by BCA Protein Assay kit (Thermo Fisher Scientific, Massachusetts, USA). Equal amounts of protein were fractionated by 8-15% SDS polyacrylamide gels. The separated proteins were transferred onto polyvinylidene fluoride (PVDF) microporous membranes (MERCK Millipore, Massachusetts, USA) and blocked in 5% milk. After blocking, membranes were incubated with primary antibodies anti-Bax (1:1000, Cell Signaling, Massachusetts, USA), anti-cleaved caspase 3 (1:1000, Cell Signaling, Massachusetts, USA), anti-eIF2α (1:1,000, Cell Signaling, Massachusetts, USA), anti-ATF4 (1:1000, Cell Signaling, Massachusetts, USA), anti-CHOP (1:500, Cell Signaling, Massachusetts, USA), anti-GRP78 (1:1000, Cell Signaling, Massachusetts, USA), PKCβ II (1:1000, cat no.#25453, anti-rabbit mAb, Cell Signaling, Massachusetts, USA) and PKC beta II (1:1000, cat no.ab67314, anti-rabbit mAb, Abcam, Cambridge, UK) or anti-*β* actin (1:5000, Sigma-Aldrich Co., St Louis, MO, USA) overnight at 4°C. The membranes were then washed and incubated with horseradish peroxidase-conjugated anti-rabbit/mouse IgG (1:5,000, MERCK Millipore, Massachusetts, USA and Sigma-Aldrich Co., St Louis, MO, USA) for 1 hr

at room temperature. Bound antibodies were visualized with the ECL-Western blotting system (AVEGENE CHEMX 400). The protein signals were quantified using a laser densitometer, and the strength of each protein signal was normalized to the corresponding β-actin signal.

Pressure volume loop relationship analysis

Under anesthesia and intubation, invasive hemodynamic assessments were done in rats using a Millar pressure catheter (SPR-838; Millar Instruments, Houston, TX, USA) extended through the right carotid artery (5). After stabilization of heart rate, the hemodynamic parameters of the LV were recorded using a PowerLab converter (Millar Instruments). LV systolic function was evaluated along with following parameters- end-systolic volume (Ves), end-systolic pressure (Pes), maximal velocity of pressure rise (+dP/dt) and fall (-dP/dt), and arterial elastance (Ea), LV diastolic function was evaluated using the end-diastolic volume (Ved), end-diastolic pressure (Ped), time constant of isovolumic pressure decay (tau). To determine conductance, the left jugular vein was cannulated with hypertonic saline (10%) infusion. Also, to obtain the parameters of end-systolic pressure-volume relationship (ESPVR) and end-diastolic pressure-volume relationship (EDPVR), we suppressed the preload using the temporal occlusion of the inferior vena cava using 3-0 surgical silk.

Histology and measurement of fibrosis

At the end of study, rats were sacrificed and fresh heart tissues were rapidly removed. The weight of heart tissue was measured. The lung wet-to-dry (W/D) weight ratio was used as an index of lung water accumulation. The lung tissue was weighed after harvested as wet weight, then dried in an oven at 60°C overnight and re-weighed as dry weight. The W/D weight ratio was calculated by dividing the wet by the dry weight. For histopathological examination, the heart tissue was fixed in 4 % paraformaldehyde and embedded in paraffin. After serial sectioning of heart, 5μm sections were stained with hematoxylin-eosin (HE) and Masson's trichrome stain. The rest of heart tissue was quickly frozen in liquid nitrogen and then stored at -80for further biochemical assays.

Cell cultures

H9C2 rat cardiac myoblast cell lines (H9C2), obtained from the American Tissue Culture Collection (ATCC CRL1446, Manassas, VA, USA were maintained in DMEM medium (DMEM; GIBCO, Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum (FBS, GE, Laboratories Inc, USA), 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mM nonessential amino acids (Invitrogen, Thermo

Fisher Scientific, Massachusetts, USA) and incubated at 37°C in a 5% CO_2. The H9C2 were randomly divided into four groups for treatment as follows (1) control group; (2) high glucose group (HG), in which cells were cultured in high glucose at 30 mM in DMEM for 24 hr; (3) HG+ DAPA treatment group: post 24 hours cultured in high glucose, cells were treated with DAPA at 20 μM for one hour before further studies. Cell associated experiments were addressed in Supplemental materials in details.

Statistical analysis

Differences among the control and diabetic groups before and after DAPA treatment were compared using Student's *t* tests for normally distributed continuous variables, nonparametric tests for non-normally distributed continuous variables, and χ^2 tests for categorical variables. Group differences were analyzed using analysis of variance. A p value < 0.05 was considered significant. All analyses were performed using SPSS version 18 for Windows (SPSS Inc., Chicago, IL, USA).

References

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