Supplementary materials

Animals, diet and drug preparation

Sixty adult male Wistar rats weighing 180–200 g were obtained from the National Animal Center, Salaya Campus, Mahidol University, Bangkok, Thailand. All rats were housed in an environment with controlled temperature, humidity and a light–dark (12:12 h) cycle. Rats were fed either a ND or HFD. The ND is a standard laboratory pelleted diet (Mouse Feed Food No.082, C.P. Company, Bangkok, Thailand) containing 19.77% energy from fat and gives a total energy of 4.02 kcal/g food, whereas the HFD contains 59.28% energy from fat and gives a total energy of 5.35 kcal/g food (Pratchayasakul et al 2011; Tanajak et al 2017).

SGLT2-i dapagliflozin (FORXIGA, AstraZeneca Pharmaceuticals LP, Bangkok, Thailand) and DDP4-i vildagliptin (Galvus, Novartis, Bangkok, Thailand) were used in this study. Dapagliflozin (10 mg) and Vildagliptin (50 mg) were ground and dissolved in 0.9% normal saline solution (0.9% NSS). Rats were treated with either vildagliptin at a dosage of 3 mg/kg/day (Pongkan et al 2016; Tanajak et al 2017), dapagliflozin at a dosage of 1 mg/kg/day (Han et al 2008; Shin et al 2016) or combined drugs (vildagliptin 3 mg/kg/day in combined with dapagliflozin 1 mg/kg/day) via oral gavage feeding once a day for 28 days (Lee et al 2017; Tanajak et al 2017).

Blood chemistry, metabolic parameters and urinary parameters assessments

Plasma glucose levels, total cholesterol (TC) levels, triglyceride (TG) levels and urine glucose levels were determined using a colorimetric assay kit (ERBA Mannheim, Mannheim, Germany) (Apaijai et al 2014; Pratchayasakul et al 2011). Plasma HDL-c level was determined using a commercial colorimetric assay kit (Biovision, California, USA) (Apaijai et al 2013). Plasma LDL-c level was calculated from the Friedewald equation (Apaijai et al
Plasma insulin levels were determined using a commercial sandwich ELISA kit (LINCO research, Missouri, USA) (Apaijai et al. 2013). Homeostasis Model Assessment (HOMA) index was calculated using the following equation: [Fasting plasma insulin (µU/ml)] x [fasting plasma glucose (mmol/l)/22.5] (Pipatpiboon et al. 2012). An increase in the HOMA index represents an increasing degree of insulin resistance (Pipatpiboon et al. 2012). Plasma glucagon and plasma FGF21 levels were determined using the quantitative sandwich enzyme immunoassay technique by ELISA kit for mouse/rat glucagon and mouse/rat FGF21 (R&D systems Inc, Minneapolis, MN, USA) respectively.

Blood pressure measurement

Rats were placed into the restraining chamber which allows the tail of the animal to extend out of the chamber. The restrainer was placed on a warming platform at a controlled temperature between 32-35°C (Tanajak et al. 2017). The occlusion cuff was placed near the base of the rat’s tail, whereas volume-pressure recording (VPR) tail-cuff were placed at 1 inch distal to the occlusion cuff. Both occlusion cuff and VPR cuff were connected to the CODA 2 system for measuring and recording blood pressure (Kent Scientific Corporation, Torrington, CT, USA) (Feng et al. 2008; Tanajak et al. 2017). An average value of systolic and diastolic blood pressures was taken from 20 consecutive measured cycles, after 5 consecutive warming cycles (Tanajak et al. 2017; Tanajak et al. 2016).

Heart rate variability (HRV) assessment

Rats were anesthetized by 3% isoflurane inhalation and O₂ flow rates of 4 liters per minute, and then needle electrodes were subcutaneously inserted in the right arm, trunk and left leg of the animal. Rats were allowed to gain full consciousness prior to ECG recording.
Lead II ECG was recorded continuously for 15 minutes using PowerLab 4/25T (AD Instruments, Colorado Springs, CO, USA) equipped with the Chart 5.0 program. Time-domain and frequency-domain analyses were performed. The relationship between the RR interval and the beat numbers (Tachogram) from a stable ECG was determined using the MATLAB program (Tanajak et al 2016). Time-domain HRV parameters including the standard deviation (SD) of all normal to normal (NN) intervals, (SDNN), and square root of the mean squared differences of successive NN-intervals (rMSSD) were analyzed. The Fast Fourier transform (FFT) algorithm was used to determine RR interval variability (power spectra) (Camm AJ 1996; Chattipakorn et al 2007). Total power (TP; 0.0-3.0 Hz) was identified from spectral bands and TP was presented in absolute units (ms²). Frequency domain parameters under each peak of TP were calculated. The spectral bands in each peaks, including very low frequency (VLF; 0-0.2 Hz), low frequency (LF; 0.2-0.6 Hz) and high frequency (HF; 0.6-3.0 Hz) were analyzed (Camm AJ 1996; Tanajak et al 2016). LF and HF were presented as normalized units by TP to minimize the effect of changes in TP (Camm AJ 1996; Moheimani et al 2017). The HF component is an indicator of vagal activity, whereas LF component is a mixture of both parasympathetic and sympathetic activities (Camm AJ 1996; Moheimani et al 2017). The LF/HF ratio was also determined, and used as an index of cardiac sympathovagal balance (Camm AJ 1996; Moheimani et al 2017).

**Echocardiography for LV function assessment**

A probe (12S) was used in the echocardiography procedure. LV morphometric and LV systolic function were performed using M-mode echocardiography (Vivid-i, GE Medical Systems, USA). Parasternal short axis at the papillary muscle level was identified and used as the positional landmark (Apaijai et al 2014; Pongkan et al 2016). Ejection fraction (%EF)
was derived from the examination, and an increase in %EF was considered to be an indicator of increased LV function.

Non-invasive assessments of LV diastolic function and filling pressures were performed using M-mode echocardiography. After an apical 4-chamber view was obtained, the Pulsed Wave Doppler (PWD) cursor was placed between the tips of the open mitral leaflets and PWD was used to study the flow in this area. LV diastolic functions were determined by using the mitral valve study mode to calculate the E wave, A wave, E/A ratio, LV deceleration time (DT) and LV isovolumetric relaxation time (IVRT). An increase in E/A ratio and a decrease in DT and IVRT are indicators of increasing LV diastolic function (Das et al 2015; Nagueh et al 2016).

**Western blot analysis**

LV tissues and cardiac mitochondrial fractions were obtained from fresh tissues in both the remote and ischemic areas of myocardium. For the preparation of cardiac mitochondrial fraction, cardiac mitochondria were isolated using a differential centrifugation technique which is the protocol used in a previous cardiac mitochondrial study (Thummasorn et al 2011). Cardiac tissue proteins and cardiac mitochondrial fractions were used to determine FGF21 receptor complex, apoptotic pathway, gap junction protein and myocardial metabolic pathway. The general procedures for protein preparation and western blot analysis have been described in previous studies (Tanajak et al 2017). The samples were loaded into 10% SDS-polyacrylamide gels and the proteins were transferred to nitrocellulose membranes in glycine/methanol transfer buffer in a Wet/Tank blotting system (Bio-Rad Laboratories, CA, USA). Then, 5% skim milk or 5% bovine serum albumin in Tris-Buffered Saline and Tween (TBST) buffer was used for membrane blocking for 1 hour before adding primary antibody (Pongkan et al 2016; Tanajak et al 2017).
The expression levels of fibroblast growth factor 21 (FGF21) receptors complex, FGF receptors 1 (FGFR1; 1:200 dilution) expression, FGFR1 at Tyrosine154 (p-FGFR1 Tyr154; 1:200 dilution) phosphorylation and β-Klotho (1:200 dilution, Sigma-Aldrich, Singapore) were evaluated using tissue homogenization from both remote- and ischemic myocardium (Tanajak et al 2017). Moreover, proteins regulated by cardiac metabolism proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α; 1:200 dilution, Santa Cruz Biotechnology, TX, USA) and carnitine palmitoyltransferase 1 levels (CPT1; 1:200 dilutions, Santa Cruz Biotechnology, TX, USA) were determined using myocardial tissue homogenization and mitochondrial fraction proteins, respectively.

For the apoptotic signaling pathway, we determined the expression of B-cell lymphoma 2 (Bcl-2; 1:1,000 dilutions, Cell signaling technologies, MA, USA), Bcl-2 Associated X (Bax; 1:200 dilutions, Santa Cruz Biotechnology, TX, USA) and Cleaved caspase 3 (1:1,000 dilutions, Cell signaling technologies, MA, USA) using homogenized myocardial tissue from both remote and ischemic areas.

For the study of cardiac mitochondrial dynamics protein expression and phosphorylation, we determined cardiac mitochondrial fission proteins such as dynamin related protein 1 (DRP1), DRP1 phosphorylation at Serine616 (p-DRP1 Ser616) and DRP1 phosphorylation at Serine637 (p-DRP1 Ser637) (1:1,000 dilutions, Cell signaling technologies, MA, USA). We also determined mitofusin 2 (MFN2 1:500 dilutions, Cell signaling technologies, MA, USA) and optic atrophy type 1 (OPA1; 1:1,000 dilutions, Cell signaling technologies, MA, USA) expression for the evaluation of cardiac mitochondrial fusion. The levels of p-DRP1 Ser616 and p-DRP1 Ser637 were also determined. However, DRP1, MFN2 and OPA1 protein expressions were determined using mitochondrial fraction proteins and tissue homogenation from both remote- and ischemic myocardial tissues.
Oxidative phosphorylation (OXPHOS) protein expression (1:1,000 dilutions, Abcam, TX, USA) was determined using remote and ischemic mitochondrial fraction proteins. The gap junction protein expression was examined by detecting the expressions of connexin 43 (Cx43) and phosphorylation Cx43 at Serine368 (p-Cx43 Ser\textsubscript{368}; 1:1,000 dilutions, Cell Signaling Technology, MA, USA) in remote- and ischemic tissue homogenation. GAPDH (1:10,000 dilutions, Abcam, TX, USA) was used as a loading control for protein expression studies in tissue homogenization from remote- and ischemic myocardium, whereas VDAC (1:1,000 dilutions, Cell Signaling Technology, MA, USA) was used as a loading control in remote- and ischemic mitochondrial fraction protein studies. The secondary antibody for β-Klotho was detected using a rabbit anti-goat IgG conjugate HRP-linked antibody (1:2,000 dilution, Santa Cruz Biotechnology, TX, USA). The secondary antibodies for FGFR1, p-FGFR1 Tyr\textsubscript{154}, Bax, Bcl-2, Cleaved caspase-3, PGC-1\textalpha, CPT-1, VDAC, CX43, p-CX43 Ser\textsubscript{368}, DRP1, p-DRP1 Ser\textsubscript{616}, p-DRP1 Ser\textsubscript{637}, MFN2, OPA1 and GAPDH were detected using a goat anti-rabbit IgG conjugate HRP-linked antibody (1:1,000 dilution, Cell Signaling Technology, MA, USA). The secondary antibody for OXPHOS was detected using a horse anti-mouse IgG conjugate HRP-linked antibody (1:1,000 dilution, Cell Signaling Technology, MA, USA). Enhanced chemiluminescence (ECL) detection reagents were used to visualize peroxidase reaction products (Clarity ECL Western blotting substrate, Bio-Rad, CA, USA) (Pongkan et al 2016; Tanajak et al 2017). The membrane chemiluminescence was developed in the ChemiDoc touch imaging system (Bio-Rad Laboratories, CA, USA) (Tanajak et al 2017). Densitometric analysis was done using the image J program (Chinda et al 2014; Tanajak et al 2017). Each protein expression that used the myocardial tissue homogenation as a sample, was normalized with GAPDH expression, whereas each protein expression that used the mitochondrial fraction proteins as a samples, was normalized with VDAC expression. The results from protein expression studies were
shown using normalization of ischemic area by remote area.
References


