CHAPTER 2

Materials and Methods

2.1 Animal preparation

All experiments were approved by the Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. Thirty domestic pigs (30-35 kg) were anesthetized by an intramuscular injection of a combination of 4.4 mg/kg zoletil® (Vibbac Laboratories, Carros, France) and 2.2 mg/kg xylazine (Laboratorios Calier, S.A., Barcelona, Spain). After endotracheal intubation, anesthesia were be maintained by 1.5–3.0% isoflurane (Abbott Laboratories Ltd., Queenborough, UK) delivered in 100% oxygen. Electrocardiogram (ECG: lead II), femoral arterial blood pressure (BP), heart rate (HR), and rectal temperature were continuously monitored, and all data were recorded for subsequent analysis. Arterial blood gases and electrolytes were also monitored every 30 minutes and maintained within acceptable physiological condition [90]. Furthermore, under fluoroscopic guidance, platinum coated titanium coil electrodes (34- and 68-mm) were advanced into and positioned at the right ventricular apex and the junction between the right atrium and superior vena cava, respectively, to deliver electrical shocks when malignant ventricular arrhythmias spontaneously occurred during ischemia/reperfusion (I/R) [90].

2.2 Ischemia/reperfusion (I/R) protocol

The chest was opened through a left thoracotomy. The left anterior descending artery (LAD) were isolated and occluded by ligature (3-0 silk) three centimeters from the left main coronary artery. Ischemia was confirmed by an ST elevation on the ECG and the change in color of myocardial tissues on the ischemic area. I/R were performed by 60 minutes of a complete LAD occlusion followed by 120 minutes of reperfusion.

2.3 Vagus nerve stimulation (VNS) protocol

The left vagus nerve was surgically isolated (~ 3 cm length, at C5-6 level) from the carotid sheath. A VNS lead (Model 304, Cyberonics, Houston, TX, USA) with bipolar electrodes (platinum-iridium, 4 mm^2 surface area, 6-mm interelectrode spacing) were attached to the vagus nerve using helical fixation elements to assure electrode stability. The cathodic electrode was oriented closest to the heart. The proximal terminal pin of VNS lead was attached to a pulse generator (Demipulse, Model 103, Cyberonics) for delivery of VNS. Prior to onset of ischemia, the mean PR interval were determined from an average of ten consecutive sinus beats. We verified that VNS were engaging the autonomic nervous system by briefly stimulating the vagus nerve and observing a significant increase in the PR intervals. The VNS parameters (3.5 mA, 500 μs pulse width and 20 Hz) are based on FDA-approved VNS parameters and our previous study [91].

2.4 Experimental protocols

Pigs were randomly divided into five groups (n = 6/group) as shown in Figure 2-1 All pigs in each group were undergone a 60 minutes of ischemia follow by 120 minutes of reperfusion.

1. Group 1: the control group without VNS (I/R).
2. Group 2: pigs were received intermittent VNS (continuous recurring cycles of 21-sec ON and 30-seconds OFF) at the onset of LAD occlusion and continue until the end of reperfusion (LC-VNS).
3. Group 3: pigs were subjected to left vagus nerve transection at middle cervical level and received intermittent VNS 2 cm under the point of cut (LtVNX).
4. Group 4: pigs were subjected to right vagus nerve transection at middle cervical level and received intermittent VNS at left side (RtVNX).
5. Group 5: pigs were subjected to a similar protocol as Group 2 except that Atropine (1 mg/kg) was administered by intravenous 15 minutes prior to initiation of VNS to inhibit parasympathetic actions on the heart (Atropine).

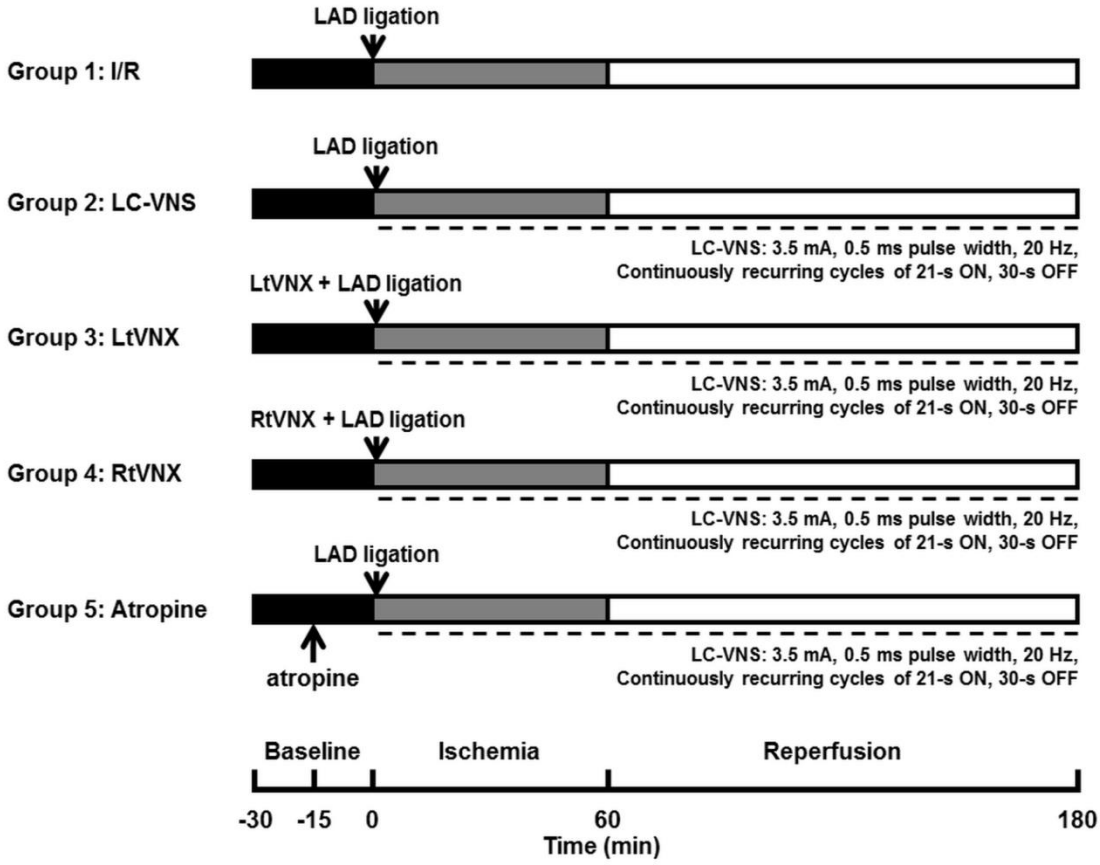

Figure 2-1 Schematic design of experimental protocols

2.5 Evaluation of cardiac functions

Heart rate (HR), PR interval, QRS complex duration (an indicator of ventricular activation time), QT interval (an indicator of ventricular repolarization time), time from T-wave peak to end (Tpe; an indicator of transmural dispersion of repolarization), and T-wave peak to end per QT interval ratio (Tpe/QT ratio; an indicator of dispersion of repolarization relative to the total duration of repolarization) were measured. ECG traces were analyzed with Chart 6 (AD Instruments). The mean baseline all of parameters were determined from an average of twenty sinus beats just prior to LAD occlusion. The mean parameters during the ischemia and reperfusion periods were analyzed from an average of twenty consecutive beats before the end of occlusion and the end of reperfusion, respectively.

2.6 Evaluation of rhythm disturbances

Premature ventricular contractions (PVC), Ventricular tachycardia (VT), and Ventricular fibrillation (VF) were defined according to the Lambeth Convention criteria [92] with more rigorous modifications for the entire 180 minutes I/R period. Specifically, PVC was defined as ventricular contractions without atrial depolarization. VT was defined as more than six consecutive PVC. VF was characterized by a loss of synchronicity of electrocardiogram plus decreased amplitude and a precipitous fall in blood pressure (BP) for more than one second. ECG traces were analyzed with Chart 6 (AD Instruments). Furthermore, the arrhythmia scores were determined. The arrhythmia scores all correlated with the incidences of PVC, VT, and VF.

Table 2-1: Criteria of arrhythmia score [93]

0	< 50 ventricular premature beat
1	50-499 ventricular premature beats
2	> 500 ventricular premature beats and/or 1 episode of spontaneously reverting ventricular tachycardia or ventricular fibrillation
3	> 1 episode of spontaneously reverting ventricular tachycardia or fibrillation (< 1 min total combined duration)
4	1-2 min of total combined ventricular tachycardia or fibrillation
5	> 2 min of ventricular tachycardia or fibrillation

2.7 Evaluation of left ventricular (LV) functions parameters

During the I/R study in each pig, the left ventricular function including stroke volume (SV), ejection fraction (EF), end-systolic pressure (ESP), end-diastolic pressure (EDP), and stroke work (SW) were continuously monitored and recorded using the pressure-volume (P-V) loop recording system (Model ADV500/ADVantage System, Scisense Inc., London, Canada) as described previously [94].

2.8 Infarct size determination

After 120 minutes of reperfusion, the LAD was re-occluded by the LAD ligation, and the heart was removed and injected normal saline to wash out blood from chambers and vessels. The infarct size was assessed with 0.5% Evans Blue and 1.0% Triphenyltetrazolium Chloride (TTC) staining as previously described [13]. The area at risk (AAR) were defined as the area not stained by the Evan blue dye, and the infarct area were defined as the area not stained by TTC. An area measurement was performed using the Image Tool software version 3.0. The area of infarct size was normalized to the AAR, and calculated as % infarct size/AAR as described previously [13].

2.9 Isolated cardiac mitochondria

Cardiac mitochondria were isolated from the ischemic and non-ischemic regions, using the technique previously described [95], and the protein concentrations were determined according to the bicinchoninic acid assay. Cardiac mitochondrial function were be determined by measuring the cardiac mitochondrial reactive oxygen species (ROS) production, cardiac mitochondrial membrane potential change ($\Delta\Psi m$) and cardiac mitochondrial swelling.

1. Cardiac mitochondrial ROS production was determined using a fluorescent microplate reader in all groups. The dye dichlorohydro-fluorescein diacetate (DCFDA) were used to determine the level of ROS production in cardiac mitochondria. The DCFDA can pass through the mitochondrial membrane, and were oxidized by ROS in the mitochondria into the fluorescent form of DCF. Thus, increased fluorescent intensity indicates increased ROS production in the mitochondria.
2. Cardiac mitochondrial membrane potential change was determined using a fluorescent microplate reader in all groups. The dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanine iodide (JC-1) were used to determine the changes in the mitochondrial membrane potential. JC-1 is characterized as a cation and remains in the mitochondrial matrix as a monomer (green fluorescence) form. However, it can interact with anions in the mitochondrial matrix to form an aggregate (red fluorescence) form.

Cardiac mitochondrial depolarization was indicated by a decrease in the red/green fluorescence intensity ratio.

3. Cardiac mitochondrial swelling was be assessed by measuring changes in the absorbance of the suspension wavelength at 540 nm using a microplate reader. Cardiac mitochondria (0.4 mg/ml) were be incubated in 2 ml of respiration buffer: KCl 150 mM, HEPES 5 mM, $K_2HPO_4.3H_2O$ 5 mM, L-glutamate 2 mM and pyruvate sodium salt 5 mM. Mitochondrial swelling were be indicated by a decrease in the absorbance of the mitochondrial suspension. Isolated cardiac mitochondrial morphology were be confirmed by using a transmission electron microscope.

2.10 Transmission electron microscopy for cardiac mitochondrial morphology

Cardiac mitochondrial morphology was determined by transmission electron microscopy. Isolated cardiac mitochondria from both ischemic and remote areas were fixed overnight by mixing 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 4 ºC. Then, the pellets were postfixed in 1% cacodylate-buffered osmium tetroxide for two hours at room temperature. The pellets were dehydrated in a graded series of ethanol and embedded in Epon-Araldite and cut by a diamond knife into ultra-thin sections (60-80 nm thick), placed on copper grids and stained with the combination of uranyl acetate and lead citrate. Finally, the mitochondrial morphology was observed with a transmission electron microscope [96].

2.11 Western blot analysis

At the end of each experiment, the hearts were rapidly excised, and then the remote and ischemic areas of ventricular tissues were collected, quickly frozen in liquid nitrogen, and stored at -80 ºC until analysis. Heart proteins were lysed with extraction buffer (Tris 20 mmol/L, Na_3VO_4 1 mmol/L, NaF 5 mmol/L) and separated by electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then were transferred onto nitrocellulose membranes. After immunoblots were blocked for one hour with 5% nonfat dry milk in Tris-buffer saline (pH 7.4) containing 0.1% Tween 20, they were probed overnight at 4ºC with the primary antibodies that recognize phospho-connexin43 (P-Cx43)(Ser368) (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA); a marker of intercellular electrical communication, Bax, Bcl-2,

Cleaved caspase-3 and Pro caspase-3 (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA); a marker of apoptosis, Mitofusin-2 (MFN2), optic atrophy protein 1 (OPA1), dynamin related protein 1 (DRP1), phospho-dynamin related protein 1 at serine 616 (P-DRP1 Ser 616) and serine 637 (P-DRP1 Ser 637) (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA); a marker of mitochondrial fission and fusion, Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1α), Carnitine palmitoyltransferase 1 (CPT1) (1:200 dilution, Santa Cruz biotechnology, TX, USA); a marker of mitochondrial biogenesis and fatty acid oxidation and actin (1:4000 dilution, Santa Cruz biotechnology, TX, USA); a loading control, followed by one hour of incubation at room temperature with the horseradish peroxidase–conjugated secondary antibody (1:2000 dilution, Cell Signaling Technology, Danvers, MA, USA). The blots were visualized by ECL reagent (Bio-Rad Laboratories, CA, USA). The western blot pictures were carried out using the ChemiDoc Touching system (Bio-Rad Laboratories, CA, USA). The densitometric analysis was performed using NIH Image J analysis software. For quantitation of the proteins of interest, the ratio of ischemic (I) area per remote (R) area was determined, and normalized with actin.

2.12 HPLC-based assay of malondialdehyde (MDA) concentration

Malondialdehyde (MDA) concentration in cardiac tissue was measured by HPLC system [97]. A 0.5 ml aliquot of samples were mixed with 1.1 ml of 10% trichloroacetic acid (TCA) containing BHT (50 ppm), heated at 90 °C for 30 minutes and cooled down to room temperature. The mixture was centrifuged at 6,000 rpm, 10 minutes. The supernatant (0.5 ml) were be mixed with 0.44 M H_3PO_4 (1.5 ml) and 0.6% thiobabituric acid (TBA) solution (1.0 ml) and then incubated at 90°C for 30 minutes to generate a pink-colored products called thiobarbituric acid reactive substances (TBARS). The solution was filtered through a syringe filter (polysulfone type membrane, pore size 0.45 µm, Whatman International, Maidstone, United Kingdom) and analyzed with HPLC system. The TBARS was fractionated on the adsorption column (Water Spherosorb ODS2 type, 250×4.3 mm, 5 µm), eluted with mobile-phase solvent of 50 mM KH_2PO_4: methanol at flow rate 1.0 ml/min and online detected at 532 nm. Data was recorded and analyzed with BDS software (BarSpec Ltd., Rehovot, Israel). A standard curve was constructed from the peak from height of standard 1,1,3,3-tetramethoxypropane (standard reagent for malondialdehyde) at

different concentrations (0-10 µM). TBARS concentration was determined directly from standard curve and reported as MDA equivalent concentration. MDA concentration was expressed in µM [97].

2.13 Cardiac inflammatory and anti-inflammatory cytokine assay

Myocardial protein was extracted by the homogenization of myocardial tissues in a homogenization buffer (PBS containing 0.5% Triton X-100 and a protease inhibitor cocktail, pH 7.2 at 4ºC), and subsequently be centrifuged at 14,359 g for ten minutes. Then, the supernatant and plasma were collected to measure the level of tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) by using an enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, Inc., Camarillo, CA, USA).

2.14 TUNEL assay

To determine cardiomyocyte apoptosis quantitatively, pigs were studied in an additional experiment using TUNEL staining (terminal Deoxynucleotidyl transferase-mediated dUTP nick end labeling). TUNEL staining of cardiomyocyte was performed with a TdT-Blue Label apoptosis detection kit. The enzyme terminal deoxynucleotidyl transferase was used to incorporate biotinylated-conjugated dUTP to the ends of DNA fragments. At the end of the experiment, the hearts were perfused first with 0.9% NaCl for 5 minutes and then with 4% paraformaldehyde in PBS (pH 7.4) for 20 minutes. The ventricles were removed and further fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 hours at room temperature. The ventricles were cut into 10µm sections for the TUNEL assay in a cryostat. Immunohistochemical procedures for detecting apoptotic cardiomyocytes were performed using an In Situ Apoptosis Detection Kit (Trevigen, Maryland, USA) according to the manufacturer's instructions. For the negative control, TdT was omitted from the reaction mixture. After washing, the label incorporated at the damaged sites of the DNA was visualized by fluorescence microscopy. Five images per heart (3-5 hearts per genotype group) were acquired and positive cells were counted individually. Results were expressed as the percentage of apoptotic cells among the total cell population [98].

2.15 Statistical Analysis

Data were expressed as mean ± standard error. The normality and equality of variance was tested using the Shapiro-Wilk test and Levene's test, respectively. The mean values between the two groups were compared using the paired student's t-test. One-way ANOVA with Dunnett's multiple-comparison or LSD tests using the statistical program SPSS22 (SPSS, Inc., Chicago, IL, USA) were used for multiple sets of data. A value of $P < 0.05$ was considered statistically significant.