CHAPTER 2.

MATERIALS AND METHODS

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2.1 INTRODUCTION

In my thesis, I have addressed the novel mechanisms of regulation of cardiac energy metabolism during maturation of a newborn heart and in disease states such as obesity, diabetes, and HF. To address this relationship, I have utilized a number of approaches varying from whole animal studies to evaluate whole body glucose tolerance, to organ level cardiac function *in vivo* using echocardiography and *ex vivo* to measure flux through metabolic pathways, to tissue level using molecular approaches such as western blot to understand how changes in protein expression from various signaling pathways may involve in the development of cardiac diseases. In addition, immunoprecipitation was used to determine levels of protein acetylation in health and disease states. Overall, my studies have employed a number of up to date technologies at the organ, and cellular levels to understand how cardiac energy metabolism is regulated by lysine acetylation during maturation of a newborn heart and in disease states.

2.2 MATERIALS

Low fat diet was obtained from LabDiet® (St. Louis, Montana), while high fat diet was purchased from Research Diets Inc. (New Brunswick, New Jersey). Radiolabeled glucose [U-^{14}C] and D-[5-^{3}H] were obtained from Amersham Canada Ltd. (Oakville, Ontario). Isotopes [9,10-^{3}H] palmitate and [1-^{14}C] palmitate were obtained from NEN research products (Boston, Massachusetts). L-[^{14}C (U)] lactate was obtained from Perkin Elmer (Waltham, Massachusetts). Free fatty acids were prebound to bovine serum albumin (BSA) obtained from Equitech-Bio Inc. (Kerrville, Texas). Human insulin (Novolin ™ ge Toronto) was provided by University of Alberta hospital stores. Scintillation counting fluid was obtained from MP Biomedicals (Solon, Ohio). Hyamine hydroxide was obtained from NEN Research Products (Boston, Massachusetts). For Western Blotting, Enhance Chemiluminescence (ECL) Supelcosil™ Western blotting detection reagents were purchased from GE Healthcare (Piscataway, New Jersey) and Western Lightning® Chemiluminescence Reagents Plus were obtained from Perkin Elmer Life and Analytical Sciences (Woodbridge, Ontario). Nitrocellulose Trans-Blot® Transfer Medium was obtained from BioRad (Richmond, California). Medical X-ray FUJI films were obtained from Mandel Scientific (Guelph, Ontario). Monoclonal and polyclonal primary antibodies for FoxO1 (9454), GSK3β (9315), p-GSK3β (9331), Akt

(9272), P-Akt (9271), PGC1α (4259), AMPK (5831), P-AMPK (2531) and SIRT1 (2028) were obtained from Cell Signaling Technology (Danvers, Massachusetts). Antibodies for β-Actin (sc-47778), VDAC (sc-32063), PKCα (sc-208), and Atrogin1 (sc-33782) were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Antibodies for acetyl-lysine (05-515) were obtained from Millipore (Billerica, Massachusetts). Antibodies for PPARα (ab8934), SIRT3 (ab86671), SIRT6 (ab13697), LCAD (ab128566), and β-HAD (ab93172) were purchased from Abcam (Cambridge, Massachusetts). GCN5L1 was generously provided by Dr. M.N. Sack (NIH, Bethesda, MD). Secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California). All other chemicals used for buffers preparations were obtained from Sigma-Aldrich (St. Louis, Missouri).

2.3 METHODS

2.3.1 Animals

2.3.1.1 Ethics Approval

The use of animals for research in our study protocol was approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. The care of mice conformed to the guidelines of the Canadian Council on Animal Care.

2.3.1.2 Rabbits

Rabbits were housed in groups of 1-2 per cage at the Health Sciences Lab Animal Services (HSLAS) facility, University of Alberta. The room was maintained at ambient temperature (18 – 23 ºC) and humidity (40-70%) and a 12h light/dark cycle was maintained. They were provided food and water ad libitum. They were fed the Laboratory Rabbit Diet (Canadian Lab Diets Inc., Leduc, AB, Canada). One-day (70 g), 7-day (140 g), and 21-day old (400 g) New Zealand White rabbits were used in this study.

2.3.1.3 Mice

Mice were housed in groups of 4-5 per cage at the Health Sciences Lab Animal Services (HSLAS) facility, University of Alberta. The room was maintained at ambient temperature (18 – 23 ºC) and humidity (40-70%) and a 12h light/dark cycle was maintained. They were provided food and water ad libitum. Male C57Bl/6J mice (4-wk of age) were randomly assigned to be fed either standard chow (low fat diet, LFD, 12% Kcal from fat) or high fat diet (HFD, 60% Kcal from fat Cat # D12492; Research Diets Inc., New Brunswick, NJ, USA). Mice in either group underwent a sham or abdominal aortic constriction (AAC) procedure to induce heart failure. Hearts were excised following anesthesia with sodium pentobarbital for isolated working heart perfusion studies.

2.3.1.4 Feeding Protocol

Male C57Bl/6J mice (4-wk of age) were randomly assigned to be fed either standard chow (low fat diet, LFD, 12% Kcal from fat) or high fat diet (HFD, 60% Kcal from fat). The mice were fed their respective diets for 18 weeks before they were sacrificed to obtain hearts for perfusion.

2.3.2 Abdominal Aortic Constriction

Mice were anesthetized with 0.75% isoflurane. A 2 cm medio-lateral incision extending from the level of the 13th rib was made on the ventral side of the left abdominal wall 1.5 cm lateral to the spine. The abdominal aorta was located at the level of the adrenal gland. A titanium vascular clip was applied to constrict the aorta. It was set for a 0.11 mm closure. A silk suture was placed around to constrict the aorta and the titanium clip was removed. Sham operated animals were subjected to an identical surgical procedure except that a clip was not applied to the aorta. The surgical incision was then closed and the animals were allowed to recover under supervision.

2.3.3 Glucose Tolerance Test

In preparation for an oral glucose tolerance test (OGTT) food was removed and mice were introduced into clean cages. They had ad libitum access to water. After an overnight fast for 16h, mice were subjected to OGTT (229). After obtaining a fasting blood glucose sample, mice were

challenged with glucose (2g/Kg body weight) administered by oral gavage. Blood glucose was measured at 15, 30, 60, 90, and 120 min after glucose administration using Accu Check Aviva (Roche Diagnostics). Food was re-introduced at the end of this process.

2.3.4 Magnetic Resonance Imaging

The body composition of fat and lean mass in mice was analyzed after 18 weeks of the feeding protocol using EchoMRI QMNR 4-in-1 Whole Body Composition Analyzer, Echo Medical Systems Inc., Houston, TX, USA.

2.3.5 Echocardiography

Ultrasound echocardiography to assess in vivo cardiac function of the mouse heart was performed using a Visualsonic Vevo 770 high-resolution echocardiography imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, Canada).

Mice were anesthetized with 0.75% isoflurane for the duration of the procedure. M-mode images were obtained for measurements of left ventricular (LV) wall thickness, LV end-diastolic diameter, and LV end-systolic diameter. LV ejection fraction (%EF) and fractional shortening (%FS) were also calculated. Tissue Doppler imaging (TDI) was used to assess diastolic function, where a reduction in E′/A′ and an elevation in E/E′ were considered markers of elevated LV filling pressure and diastolic

dysfunction. TDI was utilized to characterize the inferolateral region in the radial short axis at the base of the LV with the assessment of early (E′) and late diastolic (A′) myocardial velocities.

Diastolic function is determined by flow across the mitral valve (E/A) and tissue doppler (E'/A') as described above. E indicates early diastolic velocity; A, late diastolic mitral flow due to atrial contraction; E′, mitral annulus early diastolic velocity; A′, late diastolic mitral annulus velocity.

2.3.6 Isolated Cardiac Perfusion

2.3.6.1 Rabbit Langendorff Heart Perfusion

One-, 7-, and 21-day old rabbits were anesthetized by intraperitoneal injection with 60mg/kg pentobarbital sodium. After a surgical plane anesthesia achieved, signed by loss of sensation, rabbit's thoracic cavity was opened and the heart was excised and immersed immediately in ice-cold Krebs-Henseleit solution. Hearts were retrogradely perfused with Krebs-Henseleit solution (2.5 mM Ca^{2+}, 5 mM glucose, 0.4 mM palmitate prebound to 3% albumin, 1 mM lactate, and 100 μU/mL insulin) to measure glycolysis, and the oxidation of glucose, lactate, and fatty acids. At the end of the perfusion, hearts were immediately frozen in liquid N_2 and processed for acetylation status. ATP production rates from glycolysis, and the oxidation of glucose, lactate, and palmitate were calculated using the values of 2, 15, 31, and 105 mol ATP/mol of glucose

degraded in glycolysis, and from the oxidation of lactate, glucose, and palmitate, respectively (7-9,21,25,230).

2.3.6.2 Isolated Working Mouse Heart

Mouse hearts were perfused in the working perfused mode as previously described (4,18,231,232). The strain of mice used and treatments with specific diet or any genetic modification are described in the individual chapters. Mice were anesthetized with sodium pentobarbital (60 mg/kg IP), and the hearts were subsequently excised and immersed in ice-cold Krebs-Henseleit bicarbonate solution containing 118 mM NaCl, 25 mM $NaHCO_3$, 5.9 mM KCl, 5 mM EDTA pH = 7.4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 2.5 mM $CaCl_2 \cdot 2H_2O$, 5 mM glucose and gassed with 95% O_2 and 5% CO_2. The aorta was cannulated with 18-G plastic cannula fixed to the perfusion apparatus and the heart was perfused with Krebs-Henseleit solution (37 ºC) at an initial hydrostatic pressure of 60 mmHg. Heart was trimmed off the excess tissue and the opening to the left atrium was cannulated. After equilibration in the Langendorff mode, hearts were switched to the working mode by clamping off the aortic inflow line from the Langendorff reservoir and opening the left atrial inflow line. Oxygenated Krebs-Henseleit solution consisting of 0.4 mM (Normal Fat) or 0.8 mM (High Fat) palmitate bound to 3% fatty acid free BSA, palmitate was pre-bound to the albumin as described previously (233) , 5 mM glucose, 2.5 mM Ca^{2+} in the presence or absence of 100 µU/mL insulin

was delivered to the left atrium at a preload pressure of 11.5 mmHg. The perfusate was ejected from spontaneously beating hearts into a compliance chamber and into the aortic outflow line against a hydrostatic afterload pressure of 50 mmHg. The perfusate was recirculated, and pH was maintained at 7.4 by gassing the perfusate in a glass oxygenator with a gas mixture containing 95% O_2 and 5% CO_2. At the end of perfusion, hearts were immediately frozen in liquid N_2 with Wollenberger tongs, and stored at -80 ºC until further experiments were performed.

2.3.7 Mechanical Function Measurements in Isolated Working Mouse Hearts

Heart rate and aortic pressure (mmHg) were measured using a Gould P21 pressure transducer (Harvard Apparatus) connected to the aortic outflow line. Cardiac output and aortic flow (mL/min) were measured with Transonic T206 (Transonic Systems Inc., New York) ultrasonic flow probes in the preload and afterload lines, respectively. Coronary flow (mL/min) was calculated as the difference between cardiac output and aortic flow.

Coronary flow (mL/min) = Cardiac output (CO, mL/min) – Aortic flow (ml/min)

Cardiac work was calculated as the product of cardiac output times peak systolic pressure (PSP) (aortic systolic pressure minus preload pressure) per unit weight of heart tissue

Cardiac work (joules / min / g) = (PSP – 11.5) X 133.322 X CO X 0.000001 X 60 / 60

Data were collected utilizing an MP100 system from AcqKnowledge (BIOPAC Systems Inc., California).

2.3.8 Measurement of Glycolysis, Glucose, Lactate, and Palmitate Oxidation

Glycolysis, glucose, lactate, and palmitate oxidation were measured by perfusing hearts with [5-^{3}H/U-^{14}C] glucose, [U-^{14}C] lactate, and [9,10-^{3}H] palmitate, respectively. The total myocardial $^{3}H_2O$ production and $^{14}CO_2$ production were determined at 10 min intervals during the 60 min aerobic perfusion period. To measure the rates of glycolysis or palmitate from ^{3}H substrate, $^{3}H_2O$ in perfusate samples was separated from [^{3}H] glucose and [^{3}H] palmitate utilizing a vapor transfer method as previously described (233). This method involves the addition of 500 µL of water into a 5 mL scintillation vial, and then placing a capless 1.5mL microcentrifuge tube inside the scintillation vial. A 200 µL perfusate sample was then added to the microcentrifuge tube, and the scintillation vial was capped. Scintillation vials were then stored initially at 50 ºC for 24 hr to allow vaporization and then cooled at 4ºC for 24 hr. Following storage, the microcentrifuge tube was removed, scintillation fluid (Ecolite, ICN) was added, and the vials were counted for radioactivity in a liquid scintillation counter.

Glucose or lactate oxidation rates were determined by quantitative measurement of $^{14}CO_2$ production including $^{14}CO_2$ released as a gas in the oxygenation chamber and $^{14}CO_2$ dissolved as $H^{14}CO_3^-$ in perfusate. The gaseous $^{14}CO_2$, which exits the perfusion system via an exhaust line, was trapped in hyamine hydroxide solution. The dissolved $^{14}CO_2$ as $H^{14}CO_3^-$ was released and trapped on filter paper saturated with hyamine hydroxide in the central well of 25 mL stoppered flasks after perfusate samples were acidified by the addition of 1 mL of 9*N* H_2SO_4 (233).

2.3.9 Heart Tissue Preparation

Immediately following perfusion, the hearts were flash frozen in liquid nitrogen. Frozen ventricular tissue was homogenized for 30s with a Polytron homogenizer in a homogenization buffer containing 0.05M Tris-HCL, 10% glycerol, 1mM EDTA, 0.02% Brij-35, and 1mM dithiothreitol in the presence of protease and phosphatase inhibitors (Sigma). Homogenized tissues were then centrifuged at 800 g for 10 min to obtain a supernatant lysate. Protein assay was performed using the Bradford method. Lysates from frozen heart tissue were used to assess level of protein expression and acetylation. Samples were boiled for 5 min in a sample preparation buffer containing (0.062M Tris-HCL, 10% glycerol, 0.003% bromphenol blue, 5% 2-β-mercapto-ethanol, 2% SDS, and 6M Urea), and loaded onto 10% SDS-PAGE gels.

2.3.10 Mitochondrial Isolation

Mice were euthanized with sodium pentobarbital. After opening the thoracic cavity, the heart was carefully removed and place on a petridish containing chilled heart homogenization medium (HHM), containing 0.2 M mannitol, 50 mM sucrose, 10 mM KCl, 1 mM EDTA, and 10 mM HEPES (pH 7.4). After briefly rinsing, the heart was minced on ice and homogenized in HHM containing protease inhibitor cocktail, 500 nM trichostatin A (TSA), and 10 mM nicotinamide using a chilled glass dounce. TSA and nicotinamide are class I and class III histone deacetylase inhibitors respectively that prevent the deacetylation of proteins *in vitro*. The heart tissue homogenate was then decanted and stored on ice. All homogenates are first centrifuged for 10 min at 1000g, 4ºC. The supernatant is decanted and then centrifuged for 10 min at 3000g, 4ºC. The resulting supernatant is then aspirated and discarded, leaving a brown mitochondrial pellet. The mitochondrial pellet is then re-suspended in HHM and recentrifuged for 10 min at 3000g, 4ºC. This procedure is repeated for a total of three washes. After the final wash, the mitochondria are re-suspended in the HHM buffer and used for further experiments.

2.3.11 Immunoblot Analysis

Proteins (20 µg/lane) were separated on a 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with

5% skim milk for 1 hour and probed with specific primary antibodies overnight. Membranes were incubated with the appropriate secondary antibodies for 1 hour. Enhanced chemiluminescence (BioRad Inc, Hercules, USA) system was used for band detection. The intensity of band signals was analyzed by Quantity One software (4.4.0).

2.3.12 Immunoprecipitation

One hundred µg of total heart lysate was pre-cleared with 50 µl of protein A-agarose beads and used for immunoprecipitation. Lysates were rotated with acetyl-K antibodies (3µg/100µg lysate) overnight at 4ºC. 50 µl of protein A-agarose beads was added to each sample and incubated on a rotator for 4 hr in a cold room. After 4 hr, samples were washed with a wash buffer containing (0.1% Triton X-100, 50mM Tris.HCL, 300 mM NaCl, and 5 mM EDTA, at pH 7.4) and centrifuged at 16,000 x g for 5 min. This wash was repeated 3 times. After the third wash, proteins were boiled in a sample preparation buffer for 5 min (59).

2.3.13 Assessment of β-hydroxylacyl CoA dehydrogenase (β-HAD) Activity

β-HAD activity was assayed on total heart lysates prepared from frozen heart tissues. Heart lysates were pipetted into a 96 well plate. Each well is brought to a final volume of 190 µL with 160 µL of 50 mM imidazole (pH 7.4) and 20 µL of 1.5 mM NADH. The reaction was initiated by the

addition of 10 μL of 2 mM acetoacetyl CoA and the absorbance at a 340 nM wavelength was followed for 5 min using a spectrophotometer kinetic plate reader, as described previously (17,55).

2.3.14 Assessment of long chain acyl CoA dehydrogenase (LCAD) Activity

LCAD activity was assayed based on the method described by Lehman et al (234). In brief, 20 μg of total heart lysate was added to potassium phosphate buffer containing 200 μM ferriceneum hexafluorophosphate ($Fc^{+}PF6^{-}$), N-ethylmaleimide (500 μM), and EDTA (0.1 μM) at pH 7.2. A base line rate is observed for 3-4 minutes before starting the reaction. The reaction was initiated by the addition of palmitoyl CoA (50 μM) and the absorbance at 300 nM wavelength was followed for 2 min using a spectrophotometer kinetic plate reader.

2.3.15 Statistical Analysis

Data are represented as means ± SE. Comparisons between two groups were performed using the Students t-test. Comparisons between three or more groups were performed using one and two-way ANOVA followed by Bonferroni's multiple-comparison test whenever differences were detected. $P<0.05$ was deemed significant.