THE EFFECTS OF CALORIC RESTRCTION AND EXERCISE TRAINING ON SKELETAL MUSCLE FUNCTIONS IN OBESE-INSULIN RESISTANT RATS

SINTIP PATTANAKUHAR

DOCTOR OF PHILOSOPHY

IN CLINICAL MEDICAL SCIENCES

GRADUATE SCHOOL

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A THESIS SUBMITTED TO CHIANG MAI UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN CLINICAL MEDICAL SCIENCES

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To

All endeavors existed and existing in this world

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บทคัดย่อ

กล้ามเนื้อโครงร่างเป็นอวัยวะที่มีความสำคัญในภาวะอ้วนที่มีการดื้ออินซูลิน เนื่องจากกล้ามเนื้อโครงร่างควบคุมระดับน้ำตาลในเลือดหลังอาหารถึงร้อยละ 80 ของเนื้อเยื่อทั้งหมดของร่างกาย นอกจากนี้ผู้ที่มีภาวะอ้วน ยังพบความสามารถในหดตัวของกล้ามเนื้อลดลง กล้ามเนื้อโครงร่างประกอบด้วยเส้นใยกล้ามเนื้อหลายเส้นรวมกัน โดยเส้นใยกล้ามเนื้อถูกแบ่งเป็นสองจำพวกใหญ่ ๆ คือ เส้นใยกล้ามเนื้อโครงร่างชนิดที่ใช้พลังงานโดยใช้ออกซิเจนเป็นหลัก และเส้นใยกล้ามเนื้อโครงร่างที่ใช้พลังงานโดยใช้ไกลโคเจนเป็นหลัก โดยพบว่า ภาวะอ้วนสัมพันธ์แบบผกผันกับสัดส่วนของเส้นใยกล้ามเนื้อโครงร่างที่ใช้พลังงานโดยใช้ออกซิเจนลดลง ร่วมกับการทำงานของไมโตคอนเดรียของกล้ามเนื้อลดลง ซึ่งอาจทำให้เกิดการหดตัวของกล้ามเนื้อผิดปกติ กล่าวคือ กล้ามเนื้อจะทนทานต่อการล้าน้อยลง อย่างไรก็ตาม ผลของภาวะอ้วนที่มีการดื้ออินซูลินต่อการหดตัวของกล้ามเนื้อและสัดส่วนของเส้นใยกล้ามเนื้อโครงร่างที่ใช้พลังงานโดยใช้ออกซิเจน รวมทั้งการทำงานของไมโตคอนเดรียในกล้ามเนื้อโครงร่าง โดยเฉพาะด้านจลนภาพของไมโตคอนเดรียยังไม่ชัดเจน จากการทบทวนวรรณกรรม พบว่า ภาวะอ้วนที่มีการดื้ออินซูลินสามารถกระตุ้นให้เกิดภาวะดื้ออินซูลินในระดับเซลล์กล้ามเนื้อ และยังพบอีกว่า ภาวะดื้ออินซูลินในระดับเซลล์กล้ามเนื้อสัมพันธ์กับการทำงานของไมโตคอนเดรียที่ลดลง การศึกษานี้จึงมีสมมติฐานว่า ภาวะอ้วนที่มีการดื้ออินซูลินกระตุ้นให้กล้ามเนื้อทนทานต่อการล้าลดลง ผ่านทางการลดลงของสัดส่วนของเส้นใยกล้ามเนื้อชนิดชนิดที่ใช้พลังงานโดย

ใช้ออกซิเจน และการทำงานที่ผิดปกติของไมโตคอนเดรีย นอกจากนี้ การควบคุมแคลอรีร่วมกับออกกำลังกายเป็นการรักษาหลักของภาวะอ้วน อย่างไรก็ตาม ผลของการรักษานี้ต่อความทนทานต่อการล้าของกล้ามเนื้อ สัดส่วนของเส้นใยกล้ามเนื้อชนิดที่ใช้พลังงานโดยใช้ออกซิเจน และการทำงานของไมโตคอนเดรียในกล้ามเนื้อโครงร่างยังไม่ชัดเจนเช่นเดียวกัน การศึกษานี้มีสมมติฐานที่สองว่า การควบคุมแคลอรีร่วมกับออกกำลังกายสามารถทำให้การทนทานต่อการล้าของกล้ามเนื้อดีขึ้น ผ่านการเพิ่มของสัดส่วนของเส้นใยกล้ามเนื้อชนิดชนิดที่ใช้พลังงานโดยใช้ออกซิเจน และการทำงานของไมโตคอนเดรียของกล้ามเนื้อลายในหนูที่อ้วนที่มีการดื้ออินซูลินดีขึ้นกว่าการควบคุมแคลอรีหรือการออกกำลังกายเพียงอย่างเดียว

การศึกษานี้ศึกษาหนูวิสตา 30 ตัว เป็นเวลา 27 สัปดาห์ โดยหนู 6 ตัวที่อยู่ในกลุ่มควบคุมได้รับอาหารปกติ ขณะที่หนู 24 ตัวได้รับอาหารไขมันสูง เมื่อถึงสัปดาห์ที่ 20 แบ่งหนูกลุ่มไขมันสูงเป็น 4 กลุ่ม ได้แก่ กลุ่มที่ไม่ควบคุมแคลอรีและไม่ออกกำลังกาย กลุ่มที่ไม่ควบคุมแคลอรีแต่ออกกำลังกายแบบเสริมสร้างความทนทาน 5 ครั้งต่อสัปดาห์ กลุ่มที่ควบคุมแคลอรีต่อวันจนเหลือร้อยละ 60 แต่ไม่ออกกำลังกาย และกลุ่มที่ควบคุมแคลอรีร่วมกับออกกำลังกาย โดยการรักษาทุกกลุ่มจะทำเป็นเวลา 7 สัปดาห์ เมื่อครบกำหนดเวลาแล้ว หนูทุกตัวจะถูกนำไปศึกษาการหดตัวของกล้ามเนื้อแกสตรอกนีเมียสและเมื่อเสียชีวิตแล้ว กล้ามเนื้อวาสตัส แลทเทอรัลลิสจะถูกเก็บเพื่อศึกษาสัดส่วนของเส้นใยกล้ามเนื้อ การดื้ออินซูลิน การเสื่อมสลายของเซลล์ และการทำงานของไมโตคอนเดรียของกล้ามเนื้อ ผลการศึกษาพบว่า หนูที่ได้รับอาหารไขมันสูงอ้วนขึ้นและมีภาวะดื้ออินซูลิน รวมถึงมีการทำงานของกล้ามเนื้อโครงร่างผิดปกติ กล่าวคือ มีความทนทานต่อการล้าลดลง มีสัดส่วนของกล้ามเนื้อชนิดชนิดที่ใช้พลังงานโดยใช้ออกซิเจนลดลง มีการดื้ออินซูลินในระดับกล้ามเนื้อมากขึ้น รวมทั้งการเสื่อมสลายของเซลล์มากขึ้น และมีการทำงานของไมโตคอนเดรียลดลง ซึ่งสังเกตได้จาก มีการสร้างอนุมูลอิสระมากขึ้น ศักย์ไฟฟ้าของเยื่อบุลดลง และมีการบวมของไมโตคอนเดรียมากขึ้น รวมทั้งจลนภาพของไมโตคอนเดรียลดลง อนึ่ง พบความสัมพันธ์แบบแปรผันตรงระดับสูงระหว่างสัดส่วนของใยกล้ามเนื้อชนิดที่ใช้พลังงานโดยใช้ออกซิเจนและความเข้มข้นของโปรตีนพีพาร์เดลต้าในกล้ามเนื้อ เนื่องจากโปรตีนพีพาร์เดลต้าเป็นโปรตีนหลักที่ควบคุมการสร้างใยกล้ามเนื้อชนิดที่ใช้พลังงานโดยใช้ออกซิเจน การควบคุมแคลอรีและการออกกำลังกายเพียงอย่างเดียวสามารถแก้ไขความผิดปกติของกล้ามเนื้อได้บางส่วน กล่าวคือ การควบคุมแคลอรีสามารถลดการดื้ออินซูลินของกล้ามเนื้อ ลดการเสื่อมสลายของเซลล์และเพิ่มสมดุลของจลนภาพของไมโตคอนเดรียได้บางส่วน แต่ไม่สามารถเพิ่มการทนทานต่อการล้าของกล้ามเนื้อ และเพิ่มความเข้มข้นของโปรตีนพีพาร์เดลต้าในกล้ามเนื้อได้ ขณะที่การออกกำลังกายสามารถลดการดื้ออินซูลินของกล้ามเนื้อ ลดการเสื่อมสลายของเซลล์ เพิ่ม

สมดุลของจลนภาพของไมโตคอนเดรีย เพิ่มความเข้มข้นของโปรตีนพีพาร์เดลต้าและเพิ่มความทนทานต่อการล้าได้บางส่วน อย่างไรก็ตาม มีเพียงการควบคุมแคลอรีร่วมกับออกกำลังกายเท่านั้นที่สามารถแก้ไขความผิดปกติของการทำงานของกล้ามเนื้อได้ทั้งหมด โดยสังเกตได้จาก มีความทนทานต่อการล้ามากขึ้น มีความเข้มข้นของโปรตีนพีพาร์เดลต้าในกล้ามเนื้อเพิ่มขึ้น มีการดื้ออินซูลินของกล้ามเนื้อลดลง การเสื่อมสลายของเซลล์ลดลง และมีการทำงานของไมโตคอนเดรียเพิ่มขึ้น โดยสังเกตได้จาก มีการสร้างอนุมูลอิสระลดลง ศักย์ไฟฟ้าของเยื่อบุเพิ่มขึ้น และมีการบวมลดลง รวมทั้งจลนภาพของไมโตคอนเดรียเพิ่มขึ้น ผลการศึกษานี้แสดงให้เห็นว่าการควบคุมแคลอรีร่วมกับออกกำลังกายมีผลดีต่อการทำงานกล้ามเนื้อโครงร่างของหนูอ้วนที่มีภาวะดื้ออินซูลิน ทั้งด้านการหดตัวของกล้ามเนื้อ ด้านการเปลี่ยนแปลงของเส้นใยกล้ามเนื้อ และด้านการทำงานของไมโตคอนเดรียมากกว่าการควบคุมแคลอรี่หรือการออกกำลังกายเพียงอย่างเดียว ดังนั้น จึงควรแนะนำผู้ป่วยโรคอ้วนที่มีภาวะดื้ออินซูลินลดน้ำหนักโดยการควบคุมแคลอรีร่วมกับการออกกำลังกายมากกว่าควบคุมแคลอรีหรือออกกำลังกายเพียงอย่างเดียว เพื่อให้เกิดผลดีต่อกล้ามเนื้อโครงร่างให้มากที่สุดต่อไป

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Degree	Doctor of Philosophy (Clinical Medical Sciences)	
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ABSTRACT

Skeletal muscle is an important organ determining obese-insulin resistant condition since it is responsible for 80% of postprandial insulin-stimulated uptake of glucose. In addition, people with obesity have a decrease in contractile function of skeletal muscles. Skeletal muscle consists of several muscle fibers, which can be classified into two main types: oxidative type and glycolytic type. Previous studies reported that an obese insulin-resistant condition was negatively correlated with ratio of oxidative-type/glycolytic muscle fibers and their mitochondrial function. These changes in skeletal muscles might be responsible with a decrease in contractile function, as indicated by an increase in muscle fatigability. However, the effects of obese-insulin resistant condition on muscle contractile functions, ratio of oxidative-type/glycolytic-type fibers, mitochondrial functions and mitochondrial dynamics of skeletal muscles have not been clearly elucidated. Therefore, the present study aimed to test the first hypothesis is that obese-insulin resistant condition leads to early fatigability of skeletal muscle by decreasing the percentage of oxidative-type muscle fibers and mitochondrial function of skeletal muscles.

In addition, caloric restriction, exercise training program and the combined program are proposed to be a mainstream treatment of obesity. However, the effects of those therapies on muscle contractile functions, ratio of oxidative-type/glycolytic-type

fibers, mitochondrial functions and mitochondrial dynamics of skeletal muscles in obese-insulin resistant condition is still underexplored. This research question led to the second aim of the present study. The second aim of the present study aimed to test hypothesis that both exercise training and caloric restriction program in obese-insulin resistant condition attenuate early fatigability of skeletal muscle by muscle contractile functions, ratio of oxidative-type/glycolytic-type fibers, mitochondrial functions and mitochondrial dynamics of skeletal muscles and the combined program has the better efficacy to improve all of those parameters than monotherapy.

To test both hypotheses, 30 Wistar rats were used in this study. For aim 1, rats were fed by normal diet (n=8) and high-fat diet (n=12) for 27 weeks. At the 27th weeks, all rats were applied to in situ muscle contraction studies at gastrocnemius muscle. After euthanatized, the vastus lateralis muscle tissue was collected to determine the percentage of oxidative-type muscle fibers, insulin resistance, apoptosis as well as mitochondrial functions of the muscle. We found that HFD-fed rats without therapy developed obese-insulin resistance and impaired function of skeletal muscles, as indicated by increased fatigability, decreased percentage of oxidative-type muscle fibers, increased skeletal muscle insulin resistance, increased apoptosis and impaired mitochondrial functions, as described by increased mitochondrial ROS production, induced mitochondrial depolarization and increased mitochondrial swelling, as well as more imbalance mitochondrial dynamics. Notice that we found a strong positive correlation between the percentage of oxidative-type muscle fibers and the PPAR delta protein expression of the muscle since PPAR delta is the main control of oxidative-type muscle fiber myogenesis. For aim 2-4, rats were fed by normal diet (n=6) and high-fat diet (n=24) for 20 weeks. At the 21st weeks, the normal diet fed rats continued their diet until the 27th weeks, whereas the high-fat diet fed rats were divided into four groups. The sedentary group continued consuming the high-fat diet and sedentary living until the 27th week. The second group, namely caloric restriction group, received 60% caloric restriction program for six weeks. The third group, namely exercise group, receive regular moderate intensity exercise training for six weeks. The last group, namely combined therapy group, were treated by combined 60% caloric restriction program and

moderate intensity exercise training. At the 27th week, all rats were applied to in situ muscle contraction studies at gastrocnemius muscle. After euthanasia, the vastus lateralis muscle tissue was collected to determine the percentage of oxidative-type muscle fibers, insulin resistance, apoptosis as well as mitochondrial functions of the muscle. The caloric restriction and the exercise training program partially attenuated the impairments of muscle functions. In details, the caloric restriction program could attenuate insulin resistance, decrease apoptosis and balance mitochondrial dynamics, but could not neither reduce fatigability nor increase PPAR delta protein expression. In the other hand, the exercise training program could partially attenuate insulin resistance, decrease apoptosis, balance mitochondrial dynamics, increase PPAR delta protein expression as well as reduce fatigability. However, only the combined exercise training and caloric restriction program could attenuate all aspects of skeletal muscle dysfunctions. These were indicated by reduced muscle fatigability, increased PPAR delta protein expression, decreased insulin resistance, reduced apoptosis and improved mitochondrial functions, as described by decreased mitochondrial ROS production, reduced mitochondrial depolarization and decreased mitochondrial swelling, as well as more balance mitochondrial dynamics. We conclude that a combination between caloric restriction and exercise training program has a beneficial effect on skeletal muscle functions of obese-insulin resistant rats in all aspects, which are contractile function, morphologic function and mitochondrial function. Therefore, physicians should advise patients with obese-insulin resistant condition to apply a combined caloric restriction and exercise training program rather than to use solely caloric restriction or exercise program, to provide the highest beneficial effects for their skeletal muscles.

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LIST of ABBREVIATIONS

Akt	Protein kinase B
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AUC	Area under the curve
Bax	Bcl2 associated X protein
BCA	Bicinchoninic Acid
Bcl2	B-cell lymphoma 2 protein
BMI	Body mass index
BSA	Bovine serum albumin
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CPT1	Carnitine palmitolytransferase-1
CR	Caloric restriction
CVD	Cardiovascular disease
DAG	Diacylglycerol
DCFDA	Dichloro-hydrofluoresceindiacetate
DRP1	Dynamin-1-like protein 1
EDL	Extensor digitorum longus
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
ET	Exercise training
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT	Glucose transporter
HDL	High density lipoprotein
HFD	High fat diet
HFDCb	High-fat diet with combined therapies
HFDCr	High-fat diet with caloric restriction
HFDEx	High-fat diet with exercise

HFDS	High-fat diet with sedentary living
HOMA	Homeostatic model assessment
H_2O_2	Hydrogen peroxide
IHC	Immunohistochemistry
IKK	I kappa B kinase
IR	Insulin receptor
IRS	Insulin receptor substrate
JNK	c-Jun NH2-terminal kinase
LDL	Low density lipoprotein
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MEF	Myocyte-enhancing factor
MEK	Mitogen-activated protein kinase
MetR	Methionine restriction
MFN	Mitofusin
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
MyHC	Myosin heavy chain
ND	Normal diet group;
NEFA	Non-esterified fatty acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institutes of Health
OGTT	Oral glucose tolerance test
pDRP1	Phosphorylated dynamin-1-like protein 1
PGC	Peroxisome proliferator-activated receptor gamma coactivator
pIR	Phosphorylated insulin receptor
PI3K	Phosphatidylinositol-3-kinase
PPAR	Peroxisome proliferator-activated receptor
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
RPM	Repetition power maximum

Ser	Serine
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
SWR	Standard working reagent
TG	Triglyceride
Thr	Threonine
TNF	Tumor necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Tyr	Tyrosine
T2DM	Type 2 diabetes mellitus
VO_2max	Volume of maximum oxygen consumption

LIST OF SYMBOLS

%	Percentage
α	Alpha
β	Beta
µ	Micro
ΔΨ	Mitochondrial membrane potential change
°C	Degree Celsius
g	Gram
kg	Kilogram
mg/g	Milligram per gram
mg/dl	Milligrams per deciliter
min	Minute
mmHg	The millimeter of mercury
mmol/l	Millimoles per liter
mM	Millimolar
ng/ml	Nanograms per milliliter
nM	Nanomolar
pg/ml	Picograms per milliliter
µg/g	Microgram per gram
µm	Micrometer
µM	Micromolar

ข้อความแห่งการริเริ่ม

1. วิทยานิพนธ์นี้แสดงให้เห็นอย่างชัดเจนว่า การบริโภคอาหารไขมันสูงเป็นเวลานานสามารถกระตุ้นให้เกิดภาวะดื้ออินซูลินทั้งในระดับร่างกายและเนื้อเยื่อกล้ามเนื้อโครงร่าง และยังสามารถกระตุ้นให้เกิดการทำงานผิดปกติของกล้ามเนื้อโครงร่าง อันได้แก่ การหดตัวบกพร่อง ลักษณะใยกล้ามเนื้อบกพร่อง และการทำงานของไมโตคอนเดรียบกพร่อง ในกล้ามเนื้อโครงร่างของหนูอ้วนที่มีภาวะดื้ออินซูลิน

2. วิทยานิพนธ์นี้ยังแสดงให้เห็นอย่างชัดเจนว่า การควบคุมแคลอรีสามารถลดภาวะดื้ออินซูลินทั้งในระดับร่างกายและเนื้อเยื่อกล้ามเนื้อโครงร่างได้บางส่วน และยังสามารถเพิ่มการทำงานของไมโตคอนเดรียได้บางส่วน แต่ไม่ช่วยแก้ไขการหดตัวและลักษณะใยกล้ามเนื้อที่บกพร่อง ในกล้ามเนื้อโครงร่างของหนูอ้วนที่มีภาวะดื้ออินซูลิน

3. วิทยานิพนธ์นี้ยังแสดงให้เห็นอย่างชัดเจนว่า การออกกำลังกายสามารถลดภาวะดื้ออินซูลินทั้งในระดับร่างกายและเนื้อเยื่อกล้ามเนื้อโครงร่างได้บางส่วน และยังสามารถแก้ไขการหดตัว ลักษณะใยกล้ามเนื้อ และการทำงานของไมโตคอนเดรียที่บกพร่อง ในกล้ามเนื้อโครงร่างของหนูอ้วนที่มีภาวะดื้ออินซูลินได้บางส่วน

4. วิทยานิพนธ์นี้ยังแสดงให้เห็นอย่างชัดเจนว่า การควบคุมแคลอรีร่วมกับออกกำลังกายสามารถลดภาวะดื้ออินซูลินทั้งในระดับร่างกายและเนื้อเยื่อกล้ามเนื้อโครงร่างได้ทั้งหมด และยังสามารถแก้ไขการทำงานผิดปกติของกล้ามเนื้อโครงร่าง อันได้แก่ การหดตัวบกพร่อง ลักษณะใยกล้ามเนื้อบกพร่อง และการทำงานของไมโตคอนเดรียบกพร่อง ในกล้ามเนื้อโครงร่างของหนูอ้วนที่มีภาวะดื้ออินซูลินได้ทั้งหมด

STATEMENT OF ORIGINALITY

1. This thesis clearly demonstrates that long-term HFD consumption could induce an insulin resistant condition in both peripheral and skeletal muscle tissue. It also induced skeletal muscle dysfunctions, as indicated by impaired contractile functions, morphologic functions and mitochondrial functions of skeletal muscle in obese-insulin resistant rats.

2. This thesis also clearly demonstrates that caloric restriction program could partially improve an insulin resistant condition in both peripheral and skeletal muscle tissue. It also partially attenuate mitochondrial dysfunctions but has no effect on contractile dysfunctions and morphologic dysfunctions of skeletal muscles in obese-insulin resistant rats.

3. This thesis also clearly demonstrates that exercise training program could partially improve an insulin resistant condition in both peripheral and skeletal muscle tissue. It also partially attenuate contractile dysfunctions, morphologic dysfunctions and mitochondrial dysfunctions of skeletal muscles in obese-insulin resistant rats.

4. This thesis also clearly demonstrates that combined caloric restriction and exercise training program could fully restore an insulin resistant condition in both peripheral and skeletal muscle tissue. It also completely restores skeletal muscle functions, as indicated by improved contractile functions, morphologic functions and mitochondrial functions of skeletal muscles in obese-insulin resistant rats.

CHAPTER 1

Introduction

1.1 Historical Background

As the prevalence of overweight and obesity is increasing worldwide [1], the interest in the role of obesity within the skeletal muscles has been growing. Skeletal muscle is the largest metabolic organ in the body [2] and determines 20% of the resting energy expenditure [3]. Moreover, skeletal muscle is responsible for 80% of postprandial insulin-stimulated uptake of glucose [4]. Therefore, roles of skeletal muscle as a cause and an effect of obese-insulin resistant condition are considered. Each skeletal muscle contains of two types of muscle fiber, namely oxidative-type and glycolytic type fiber, but in difference ratio [5]. The oxidative-type fibers play important roles in glucose metabolism as well as fatigue-resistance, leading to prolonged physical activities for individuals. The glycolytic-type fibers play roles in high-power activity and maintaining the mass of skeletal muscles. Both clinical and animal studies demonstrated that skeletal muscle dysfunction in obese-insulin resistant condition remains inconsistent findings. Some studies in obese-insulin resistant models showed skeletal muscle dysfunction, as indicated by decreased percentage of oxidative-type fiber [6-12], decreased mitochondrial function [13-20], decreased insulin signaling [12, 21], and decreased muscle contractile function [22-24], whereas others were reported no changes of oxidative-type fibers [18, 21, 24-27], mitochondrial function [27-29], insulin signaling [12, 21, 30] and muscle contractile function [31, 32] of skeletal muscle. In addition, a study in high-fat diet (HFD) induced obese mice demonstrated an increase in oxidative-type muscle fiber but decreased mitochondrial function of the skeletal muscles [20]. The difference between these findings may be related to the different model of obesity such as genetically obese models or diet-induced models, as well as dietary intervention. Therefore, further studies are required to provide more evidence to strengthen up the skeletal muscle changes in obese-insulin

resistance by using HFD model of obesity. Therefore, the first aim of the present study was aimed to test hypothesis that skeletal muscle dysfunction, as indicated by altered muscle fiber type distribution, mitochondrial dysfunction, increased oxidative stress, impaired insulin signaling, and induced apoptosis in skeletal muscle, occurs in obese-insulin resistant condition.

Caloric restriction and exercise training program are the mainstream interventions of obesity. The beneficial effects of calorie restriction on the improvement of systemic insulin sensitivity and skeletal muscle changes, as indicated by increased muscle function [33], increased insulin signaling [34, 35], as well as decreased apoptosis [36, 37] had been demonstrated. However, evidence from some clinical and animal models of obesity found no effect of calorie restriction on muscle fiber type distribution [18, 38-46] and muscle oxidative capacity [18, 38-48]. The beneficial effects of exercise training on improving systemic insulin sensitivity [12, 47-49], increasing percentage of oxidative-type muscle fiber [47, 48], enhancing oxidative capacity of skeletal muscle [12, 18, 40, 47-49], activating insulin signaling [50, 51] and increasing muscle contractile function [49, 52], but no effect on apoptotic marker in skeletal muscle had been demonstrated in clinical and mice models [53]. However, no significant alterations in muscle fiber type distribution, as well as mitochondrial function of skeletal muscles were reported in some clinical studies [12, 33, 40, 42, 46, 49]. Focusing on the effects of exercise training in obese-insulin resistant models, studies in obese patients found an improved muscle mass as well as mitochondrial function, oxidative capacity, insulin signaling and contractile function of skeletal muscle after diet and exercise training despite of no change of skeletal muscle fiber type distribution [42, 54]. Notice that these inconsistent effects are intensity-specific, dose-dependent, continuation-specific and mode-dependent. It is possible that the effect of combined program in obese-insulin resistant condition is more effective than monotherapy. However, there is still no scientific evidence. Therefore, it is interesting to investigate the effect of combined caloric restriction and exercise on skeletal muscle function in obese-insulin resistant rats. Therefore, the second aim of the present study was aimed to test hypothesis that skeletal muscle dysfunction in obese-insulin resistant condition can be improved by either calories restriction or exercise training, and the combined intervention provides higher efficacy than single intervention.

1.2 Objectives

Aim 1: To investigate the metabolic parameters, skeletal muscle structural and functional changes in obese insulin-resistant rats using HFD-induced obesity.

Hypothesis of aim 1: Skeletal muscle dysfunction, as indicated by altered muscle fiber type distribution, mitochondrial dysfunction, increased oxidative stress, impaired insulin signaling, and induced apoptosis in skeletal muscle, occurs in obese-insulin resistant condition.

Aim 2: To examine the effects of calorie restriction on the metabolic changes, skeletal muscle structure and function in obese insulin-resistance rats.

Hypothesis of aim 2: Skeletal muscle dysfunction in obese-insulin resistant condition can be improved by calories restriction.

Aim 3: To examine the effects of exercise training on the metabolic changes, skeletal muscle structure and function in obese insulin-resistance rats.

Hypothesis of aim 3: Skeletal muscle dysfunction in obese-insulin resistant condition can be improved by exercise training.

Aim 4: To examine the effects of calorie restriction, exercise training and combined calorie restriction and exercise training programs on the metabolic changes, skeletal muscle structure and function in obese insulin-resistance rats.

Hypothesis of aim 4: The combined interventions provides higher efficacy than monotherapy via restoring skeletal muscle function in obese-insulin resistant condition.

1.3 Literature reviews

1.3.1 Effects of high-fat diet on obesity and insulin resistance

Overweight is generally defined as a condition that the body mass index (BMI) is equal to or more than 25 kg/m^2, while obesity is generally defined as a condition that BMI is equal to or more than 30 kg/m^2 [55]. The prevalence and severity of obesity are associated with westernization, particularly high-fat diet consumption [56-58].

Hypertrophic obesity, characterized by expansion of the size of already existing fat cells and excessive weight gain occurring in adulthood, results from increase of high-fat diet consumption [59, 60]. In clinical studies, hypertrophic obesity is positively correlated with many major health issues such as diabetes, hyperlipidemia, hypertension and cardiovascular diseases [61]. Obesity is also positively correlated with an insulin resistant condition, which is proposed to be the major underlying mechanism of those pathological conditions [62]. Evidence from both clinical and animal studies demonstrated that weight gain from excessive caloric consumption significantly induced insulin resistance [62] while weight loss by caloric restriction reduced insulin resistance [63, 64].

Insulin resistance is a pathological condition, in which target cells are resistant to the physiological activities of insulin, especially the effects on glucose and lipid metabolism. In obesity, insulin resistance is characterized by decrease in end-organs response to insulin hormone, resulting in compensatory release of insulin from pancreas to maintain plasma glucose in the near normal levels causing hyperinsulinemia [65, 66]. The combination of insulin resistance and hyperinsulinemia greatly increases risks of abnormal lipid profile as well as hypertension [66]. If the cause of insulin resistance remains, the compensatory hyperinsulinemic process is unable to maintain plasma glucose within the normal level. This is commonly found in an initial, non-insulin dependent phase of type 2 diabetes mellitus (T2DM) [66]. Persistent hyperglycemia leads to micro- and macrovascular complications, e.g. atherosclerotic heart disease or cerebrovascular disease [67]. Finally, failure of the compensatory insulin releases due to dysfunction of overloaded pancreatic beta cell results in decrease of the plasma insulin and severe increase of the plasma glucose [68], which commonly found in the late, insulin-dependent phase of T2DM.

The underlying mechanism of obesity-induced insulin resistance is still elusive, but may associate with lipotoxicity, abnormal adipocyte-derived hormones and adipocyte-related pro-inflammatory cytokines. Excessive plasma non-esterified fatty acid level due to high-fat diet consumption, as well as obesity itself increases accumulation of lipid compounds, particularly triglyceride (TG)-derivatives such as diacylglycerol (DAG) and ceramide, in several non-adipose organs, e.g. heart, liver or

pancreas [69]. Malposition of lipid accumulation can cause cells dysfunction or death via apoptosis and if this process occurs in pancreas, it results in pancreatic β cell dysfunction and eventually, systemic insulin resistance [70]. Abnormal adipose tissue-derived hormones are also proposed to be one of the mechanisms responsible for obesity-related insulin resistance [71]. There is decrease in adiponectin and increase in resistin in obese persons compared with lean persons [71]. Moreover, adipocyte-related pro-inflammatory cytokines, such as tumor necrosis factor alpha, interleukin-6 and interleukin-1 beta, are released from adipose tissue-derived macrophages [72]. It is proposed that macrophage activation is induced by many obesity-related conditions, such as relatively ischemia in rapid-growth adipocytes, endoplasmic reticulum stress response and mitochondrial dysfunction [73]. TG-derivatives, resistin, as well as pro-inflammatory cytokines induce insulin resistance via disturbing phosphorylation process of insulin receptors and their downstream signaling, such as insulin receptor substrate 1 (IRS1) and phosphatidylinositol-3-kinase (PI3K)/Akt [69, 72]. **Figure 1.1** summarizes the possible mechanism of obesity-induced insulin resistance.

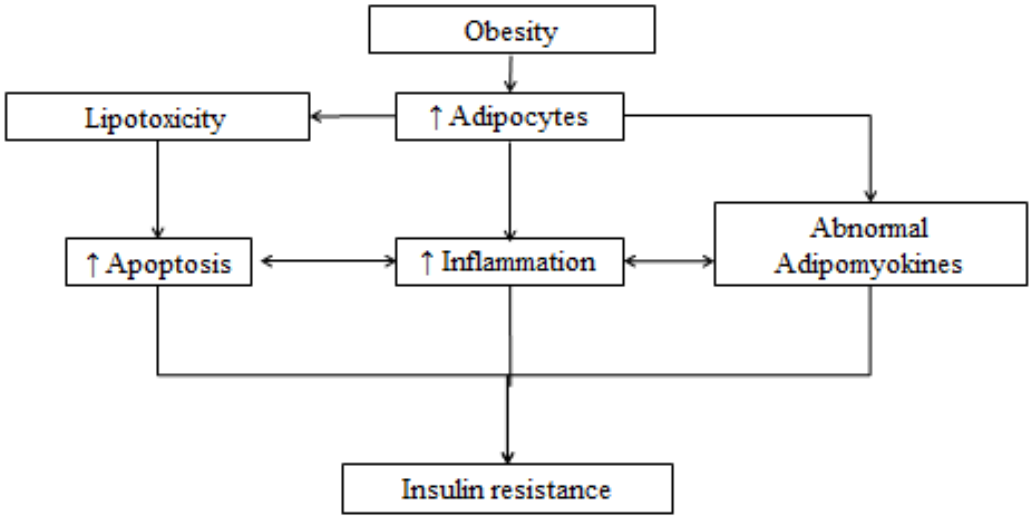

Figure 1.1 Mechanism of obesity-induced insulin resistance. *Obesity could induce insulin resistance via three main mechanisms. Excessive caloric consumption results in obesity, which described by an increase in adipocytes. Excessive adipose tissue increases accumulation of lipid compounds, particularly triglyceride (TG)-derivatives such as diacylglycerol (DAG) and ceramide. Excessive TG and DAG deposition can*

cause cells dysfunction or death via apoptosis and if this process occurs in pancreas, it results in pancreatic β cell dysfunction and eventually, systemic insulin resistance. Moreover, adipocyte-related pro-inflammatory cytokines, such as tumor necrosis factor alpha, interleukin-6 and interleukin-1 beta, are released from adipose tissue-derived macrophages. Abnormal adipose tissue-derived hormones are also proposed to be one of the mechanisms responsible for obesity-related insulin resistance. There is decrease in adiponectin and increase in resistin in obese persons compared with lean persons.

1.3.2 Impaired insulin signaling in skeletal muscle of obese-insulin resistance

Skeletal muscle is a primary site of glucose disposal in the human body and insulin resistance in skeletal muscle can cause insulin resistance of the whole body or systemic insulin resistance [74]. In cellular level, systemic insulin resistance in diet-induced obesity shows disrupted protein expression and activation (mainly in skeletal muscle and liver) of signaling via the IRS-1 and PI3K pathway, resulting in reduction in glucose uptake and utilization [75, 76].

Normal insulin signaling in skeletal muscle is as followed **(Figure 1.2)** Binding of insulin to the receptor induces phosphorylation of its intracellular domains, inducing a recruitment of several IRS, the most important of which are IRS-1 and 2 [77]. These large phosphoproteins serve as a 'platform' that initiates downstream signaling pathways, among which the PI3K/protein kinase B (Akt) pathway and the MEK/ERK (formerly MAP kinase) pathway. The PI3K pathway, which is the key metabolic effector arm of the insulin signaling response, exerts critical metabolic actions in particular through Akt2, one of three isoforms of the serine–threonine kinase Akt that is enriched in insulin-responsive tissues [78]. Evidence demonstrated that the intramuscular lipid intermediates, such as fatty acyl-CoA, ceramides and DAG, inhibit steps in the insulin signaling cascade [79]. Evidence also demonstrates that ceramide activates a protein phosphatase that inhibits phosphorylation of Akt, resulting in decrease of GLUT4 translocation and glycogen synthesis [80, 81].

It was evident that in the 15-week, diet-induced insulin resistant rats, expressions of IRS-1, PI3K and ERK1/2 were significantly increased, while that of insulin receptor beta (IRβ) and Akt were not changed [30]. Moreover, in this study, total signaling

proteins were measured without evaluation of phosphorylated proteins. Interestingly, clinical studies demonstrated the increase in serine-phosphorylated IRS in obese insulin resistance subjects compared with lean subjects [12], and decreased insulin receptor, as well as insulin signaling (Akt and TBC1D4) in muscle from patients with T2DM compared with lean and obese subjects [20]. In conclusion, skeletal muscle insulin signaling in obese-insulin resistant condition is still inconsistent and further study is still needed.

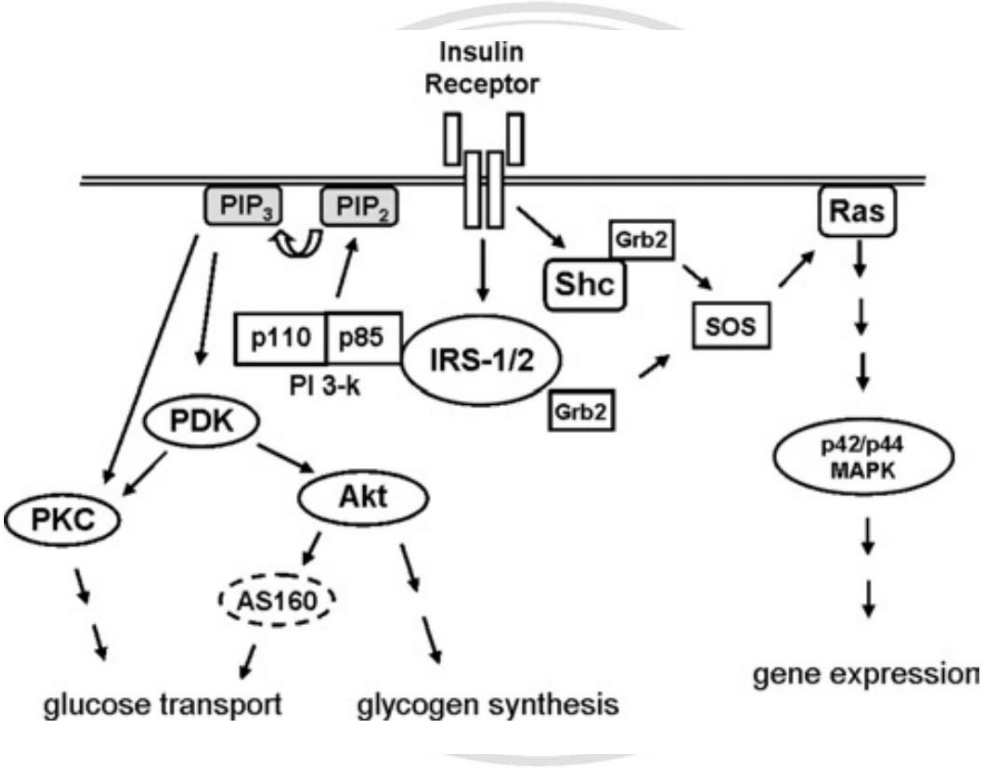

Figure 1.2 Normal insulin pathway in skeletal muscle [82]. *Binding of insulin to the receptor induces phosphorylation of its intracellular domains, inducing a recruitment of several IRS, the most important of which are IRS-1 and 2. IRS systems serve as a 'platform' that initiates downstream signaling pathways, among which the PI3K/protein kinase B (Akt) pathway and the MEK/ERK (formerly MAP kinase) pathway. The PI3K pathway, which is the key metabolic effector arm of the insulin signaling response, exerts critical metabolic actions in particular through three isoforms of the serine–threonine kinase Akt resulting in increased glucose transporatation and glycogen synthesis. After activated by insulin, MEK/ERK (MAPK) induces gene expression resuting in an increase in glycolytic, mitochondrial, as well as oxidative phosphorylation-realted protein expression.*

1.3.3 Skeletal muscle function and obesity

Evidence regarding skeletal muscle contraction parameter in obesity is inconsistent. In obese Zucker rats, peak force of diaphragm was significantly lower than which of the lean controls [83]. In 8-week HFD-induced obese insulin resistant mice, there were trend of decreases in pre- and post-fatigue peak forces of gastrocnemius-plantaris muscle complex [24]. The authors hypothesized that the early adaptations of skeletal muscle to HFD (increased oxidative-type fiber, increased oxidative capacity, and decreased apoptosis) attenuated muscle force loss. They also demonstrated that continued exposure to an HFD would ultimately result in significant decreases in contractile force, consistent with clinical studies [22, 23]. In addition, in mice fed high-fat diet for 5 weeks, muscle contraction study in EDL, a predominate fast-twitch skeletal muscle, demonstrated no change in single twitch and tetanic peak force but significant increase of relaxation time whereas muscle contraction study in soleus, a predominate slow-twitch skeletal muscle, demonstrated significant decrease of single twitch and tetanic peak force and significant decrease of relaxation time [29]. The authors of this study proposed that high-fat diet-induced muscle contractile property change was fiber type specific. In addition, the change in EDL was associated with long chain acycarnitine-induced impairment of calcium homeostasis and the change in soleus was resulted from short chain acycarnitine-induced myofibrillar component change of the muscle from slow- to fast-twitch [29]. In clinical studies, T2DM men had lower grip strength than non-diabetic men, regardless to age, body weight and level of physical activity [22, 23]. However, some studies reported no change of muscle contractile function of lower extremities [31, 32]. However, there has been no study investigating fatigability of tetanic muscle contraction. Fatigability is the most important parameter in clinical setting since it correlates with the duration of activity and determine function, e.g. duration of walking in individuals. In conclusion, evidence regarding skeletal muscle contractile function in obese-insulin resistant condition is inconsistent but having trend to have more dysfunction, but depending on which fiber type is predominate.

1.3.4 Skeletal muscle fiber type change in obese-insulin resistance

Skeletal muscle plays an important role in metabolism, energy expenditure, physical strength, and locomotive activity. Heterogeneity of skeletal muscle fiber types is described by different criteria, for example, using contractile property (slow- and fast-twitch type) or myosin heavy chain (MyHC) structures (type 1, type 2a, type 2x and type 2b) [5]. At present, immunohistochemistry (IHC) of MyHC, using monoclonal antibody specific to each myosin heavy chain, is proposed to be the gold standard method for determining muscle fiber types [5]. A metabolic classification, which categorizes skeletal muscle fibers into 2 types; oxidative-type and glycolytic-type fibers, is frequently used in the studies that focus on their metabolic characters [84]. In human, type 1 skeletal muscle fibers are oxidative-type and type 2 skeletal muscle fiber is glycolytic-type. Each muscle in the human body is composed of both types of fiber, but in different percentages [85].

Skeletal muscle fiber phenotypes are controlled by several mechanisms (comprehensively reviewed in [86] and [84]). Two different mechanisms responsible for obesity-associated muscle fiber type alterations: the metabolic hypothesis and mechanical hypothesis. For the metabolic hypothesis, the alterations in the distribution of skeletal muscle fiber type in obesity results from obesity-induced inflammation and oxidative stress in muscles, leading to changes in the metabolism in muscle fibers. Although skeletal muscle fiber phenotypes are controlled by several mechanisms including p38 mitogen-activated protein kinase (MAPK) calcium/calmodulin-dependent protein kinase IV(CaMKIV), calcineurin and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)- peroxisome proliferator-activated receptor delta (PPAR delta) pathway [87]. Among these, the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1alpha)- peroxisome proliferator-activated receptor delta (PPAR delta) (PGC-1alpha-PPAR delta) pathway is the most described mechanism evident in obesity-related skeletal muscle fiber type redistribution [12, 20, 24, 88-90]. PGC-1alpha is a coactivator interacting with all PPAR isoforms and is an upstream of PPAR-related gene transcription [91], including PPAR delta, which is the most abundant PPAR isoform expressed in the muscle tissue [92]. Studies in mice with genetic modification demonstrated that skeletal muscle PPAR delta and PGC-1alpha

were key regulators of genes involved in mitochondrial fatty acid oxidation, oxidative phosphorylation and oxidative-type skeletal muscle fiber transformation [93, 94]. The main downstream transcription factors of PGC-1alpha that responsible for oxidative - type skeletal muscle fiber transformation is myocyte-enhancing factor 2 (MEF2). Obesity is associated with the decrease of PGC-1alpha-PPAR delta signaling via oxidative stress and inflammatory process resulting from adipogenesis (adipocyte hypertrophy and hyperplasia) [95].

The second proposed mechanism is the mechanical hypothesis. Obesity is associated with increased body weight, resulting in increased skeletal muscle workload, particularly in weight-bearing muscles It has been shown that increased workload enhanced glycolytic-type muscle fiber transformation [96]. In addition, people with obesity show a decrease in physical activity, including walking [97]. Walking ability is associated with an increase in oxidative-type fiber of the muscles, particularly in lower extremities [98]. Those findings suggest that obesity possibly leads to an increase in glycolytic-type fibers from weight gain and a decrease in oxidative-type fiber following decreased ambulation activity. Therefore, this hypothesis seems to suggest that obesity is an indirect cause of the alterations in muscle fiber type. However, a longitudinal clinical study evaluating muscle fiber type together with the monitoring of physical activity in people with obesity is needed to prove this hypothesis.

In obese-insulin resistant models, the change of skeletal muscle fiber type has been studied. Most clinical evidence demonstrated that obesity-related parameters were negatively correlated with the percentage of oxidative-type skeletal muscle fiber and positively correlated with the percentage of glycolytic-type muscle fiber. However, some results remain inconsistent. Studies in mice fed high-fat diet for 4 weeks [24] and 8 weeks [27] showed increase in oxidative-type skeletal muscle fiber transformations, whereas there was no change in skeletal muscle fiber distribution in mice fed by high-fat diet for 12 weeks [99]. On the other hand, the percentage of oxidative-type skeletal muscle fiber was decreased in mice fed by high-fat diet for 52 weeks [100]. The difference in duration of obesity may be responsible for these inconsistent findings. In clinical studies, many cross-sectional comparative studies demonstrated that a decrease in percentages of skeletal muscle type 1 has been shown in overweight (BMI ≥

25)/obese (BMI ≥ 30) subjects, regardless of an insulin-resistant condition, when compared with those of lean subjects [6-12]. However, few studies showed no change in percentage of muscle type 1 in those overweight/obese subjects [18, 21, 26]. Differences in methods for the identification of muscle types (IHC staining or Western blotting) and baseline characteristics of patients in each study may account for these inconsistent findings. The accuracy of IHC and Western blotting depends on which place the muscle tissue is collected and it could change the percentage of fiber type if the sampled place is different, although within the same muscle [5]. **Figure 1.3** summarizes mechanism of obesity-induced skeletal muscle fiber type distribution.

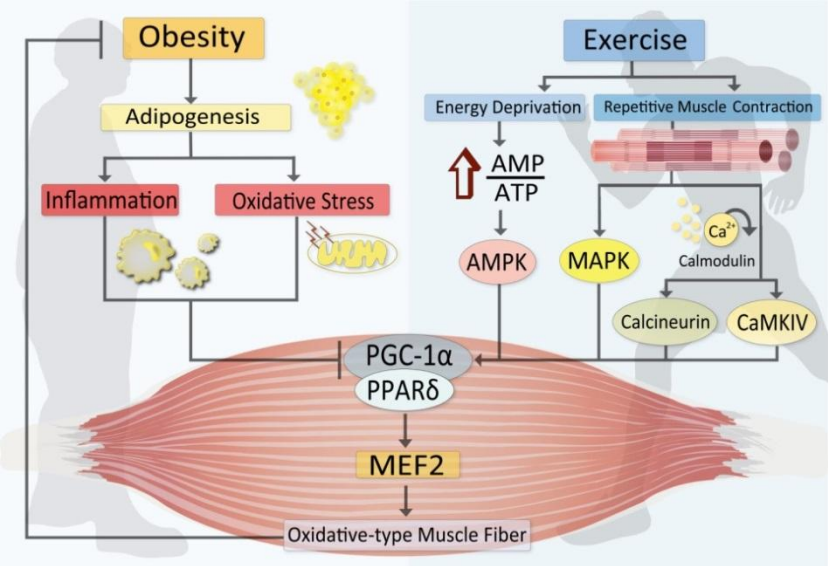

Figure 1.3 Mechanism of obesity-induced skeletal muscle fiber type distribution [87]. *The alterations in the distribution of skeletal muscle fiber type in obesity results from obesity-induced inflammation and oxidative stress in muscles, leading to changes in the metabolism in muscle fibers via the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1alpha)- peroxisome proliferator-activated receptor delta (PPAR delta)*

(PGC-1alpha-PPAR delta) pathway. The main downstream transcription factors of PGC-1alpha that responsible for oxidative -type skeletal muscle fiber transformation is myocyte-enhancing factor 2 (MEF2). Obesity is associated with the decrease of PGC-1alpha-PPAR delta signaling via oxidative stress and inflammatory process resulting from adipogenesis (adipocyte hypertrophy and hyperplasia)

↑ : Increase; AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; AMPK: Adenosine monophosphate-activated protein kinase; MAPK: p38 mitogen-activated protein kinase; Ca2+: Calcium; CaMKIV: Calcium/calmodulin-dependent protein kinase IV; PGC-1 α : Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPARδ: Peroxisome proliferator-activated receptor delta; →: Activation; - - |: Inhibition

In addition, several clinical studies demonstrated the correlation between several obesity-related parameters and the percentage of muscle fiber types. Focusing on type 1 muscle fiber, total body fat was negatively correlated with the percentage of type 1 skeletal muscle fibers [101]. The negative correlation with the percentage of type 1 skeletal muscle fibers was shown, when compared with central body fat [101-103], however, no correlation was found when compared with subcutaneous fat [25, 101]. In addition, a previous study demonstrated that increased adipocytes in central adipose tissues, but not in subcutaneous adipose tissues, led to decreased expression of skeletal muscle contractile proteins, including myosin heavy chain [104]. These studies, along with previous evidence, showed increased infiltration of macrophages, T cells and mast cells in central adipose tissues, but none in subcutaneous adipose tissues in obese condition [105, 106]. Therefore, the inflammatory processes in adipose tissues could be the reason behind the reduction of skeletal muscle fiber type 1 during obesity, since evidence showed that slow-type MyHC was sensitive to inflammation [107]. Some studies demonstrated that body mass index (BMI) was also negatively correlated with the percentage of type 1 muscle fibers [8, 9]. However, this correlation is inconsistent because another study failed to demonstrate this correlation [25]. A possible explanation of these inconsistent findings is that BMI represents both fat and fat-free components of whole body, not just the alterations of adipose tissue [95].

A 19-year follow up longitudinal study found that the percentage of type 1 fiber at the beginning of the study was negatively correlated with weight gain, increased heart rate, hypertension and increased fractional shortening of heart at the end of the study [108]. In addition, several studies demonstrated that aerobic capacity, indicated by volume of maximum oxygen consumption of the body (VO_2max), is the independent protective factor against obesity [109, 110] and cardiovascular diseases [111, 112]. In

addition, two studies demonstrated a positive correlation of the percentage of type 1 fiber and weight loss [113]. It is known that type 1 fibers, the oxidative–type skeletal muscle fibers, are one of the factors determining aerobic capacity [114] and type 1 muscle fibers have greater insulin sensitivity and oxidative capacity, particularly in fatty acid oxidation, than other fiber types [96]. Those findings suggested that the percentage of oxidative-type muscle fiber is proposed to be an important factor protecting against obesity and cardiovascular diseases.

In contrast to alterations in type 1 fibers, several studies demonstrated that total body adiposity [19] and BMI [9] were positively correlated with percentage of type 2x fibers (the glycolytic-type skeletal muscle). Furthermore, two studies reported that plasma non-esterified fatty acid (NEFA) level, representing lipolytic activity was negatively correlated with percentage of type 1 muscle fibers [25, 115]. It is known that the fasting NEFA level is positively correlated with total body adipose tissues [116], while postprandial or post-euglycemic clamp NEFA is negatively correlated with insulin sensitivity [25].

In conclusion, most clinical evidence demonstrated that obesity-related parameters were negatively correlated with the percentage of oxidative-type skeletal muscle fiber and positively correlated with the percentage of glycolytic-type muscle fiber. The association among muscle fiber types and obesity-related parameters could explain that the percentage changes of skeletal muscle fiber types may be the cause and the effect of obesity. However, some evidence did not report any association among muscle fiber types and obesity-related parameters. A possible explanation of this inconsistent finding may be due to a difference in the measurement of obesity-related parameters. Further longitudinal studies, evaluating both anatomical position and quantity of body adiposity by high accuracy and more sensitive multicomponent methods e.g. dual energy X-ray absorptiometry (DEXA) or magnetic resonance imaging (MRI), to confirm this finding in a clinical setting, are required.

1.3.5 Mitochondrial function in skeletal muscle and obese insulin resistance

Evidence demonstrated an increase of H_2O_2 production in the skeletal muscle mitochondria of high-fat-diet induced obese insulin resistant mice [117]. The increase in

H_2O_2 proposed to be the cause of insulin resistance by inducing the stress-sensitive Ser/Thr kinases. c-Jun NH2-terminal kinase 1 (JNK1), which its activation can promote inhibitory serine phosphorylation of the insulin receptor substrate (IRS), eventually decreases response to insulin hormone or insulin resistance [118]. However, the results of skeletal muscle mitochondrial function change in high-fat diet-induced obese-insulin resistance model are inconsistent. Mice fed by high-fat diet for 4 weeks demonstrated no change in mitochondrial swelling [28]. An in vitro study showed decreased mitochondrial membrane potential of skeletal muscle cells with exposure to chronic insulin and saturated fatty acid [119] and an increase in mitochondrial fission with reduction of mitochondrial fusion was detected in mice fed by high-fat diet for 40 weeks [16]. This inconsistency may be resulted from different in obesity models, especially age and duration of high-fat diet feeding, as well as different in measurement tool.

Moreover, mitochondrial dynamics which is a reciprocal change in the morphology between a fission and fusion stage of mitochondria plays an important role in obesity-induced mitochondrial dysfunction. Skeletal muscle mitochondria, regarded as dynamic organelles, undergo a constant structural and morphological cycle involving fusion and fission, which are essential for cell survival as well as cell growth and division during cell differentiation. Mitochondrial fusion can compensate for damaged mitochondria by binding damaged mitochondria to healthy mitochondria, whereas mitochondria fission can maintain mitochondrial function by separating damaged mitochondrial sites from healthy mitochondria. Mitochondrial fusion plays essential role in the regulating the fusion proteins Mitofusins 1 and 2 (MFN1 and MFN2) as well as Optic atrophy 1 (OPA1). MFN1 and MFN2, which are dynamin-related GTPases, are responsible for the fusion of mitochondrial outer membranes while OPA1, also a dynamin-related GTPase, is recruited for the fusion of mitochondrial inner membranes and regulates cristae remodeling. Mitochondrial fission is largely mediated by dynamin-related protein 1 (DRP1), which is mostly localized in the cytoplasm and interacts with several mitochondrial outer membrane receptors such as mitochondrial fission factor (MFF), fission protein 1 (Fis1), and mitochondrial dynamics proteins (Mid49/51) when mitochondria are damaged by loss of membrane potential or oxidative stress. To generate the fission process, Drp1 is recruited from the cytosol to the dysfunctional site

to cleave the damaged mitochondrial site through the receptors Fis1, Mff, and Mid49/51 [210].

The balance between mitochondrial fusion and fission is important for maintaining mitochondrial health in skeletal muscle. However, obesity impairs mitochondrial dynamics and alters the balance between mitochondrial fusion and fission, thereby reducing mitochondrial contents and inducing mitochondrial dysfunction in skeletal muscle [16, 120, 210]. Particularly, a recent study reported that inhibition of MFN2 is related to diminished substrate oxidation, cellular metabolism, and reduction of membrane potential in electron transport chain complexes under obese conditions [211]. In addition, Liu et al. [16] reported that high fat diet consumption for 40 weeks reduced both MFN1 and MFN2 protein levels in skeletal muscle by 20%, whereas protein levels of Fis1 and DRP1 were elevated by 50%. Furthermore, Jheng et al. [120] reported that mitochondrial fusion protein (MFN1, MFN2, and OPA1) levels were unaltered while mitochondrial fission protein (DRP1 and Fis1) levels were significantly increased in genetically induced obese mice (ob/ob) and high fat diet-induced obese mice compared with lean mice, demonstrating the unbalance between fusion and fission in obesity. Evidence demonstrated the role of insulin sensitivity in the homeostasis control in mitochondrial dynamics. Insulin resistance in skeletal muscle could induce an increase in mitochondrial fission, as indicated by increased mitochondrial fission protein DRP1, especially their phosphorylated form Ser616pDRP1 [120] as well as a decrease in mitochondrial fusion, as indicated by increased mitochondrial fusion protein MFN2 [16]. In conclusion, obesity could induce mitochondrial dysfunction, which indicated by increased mitochondrial ROS production and swelling, decreased mitochondrial membrane permeability, as well as imbalance mitochondrial dynamics. **Figure 1.4** summarized the effects of high-fat diet on mitochondrial function of skeletal muscle tissue.

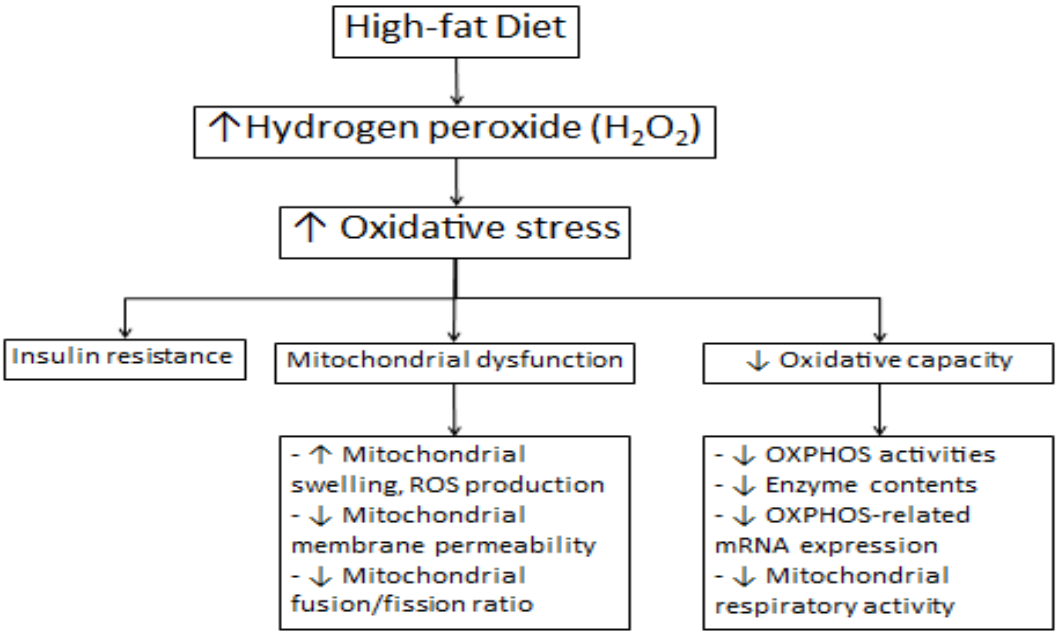

Figure 1.4 Effects of high-fat diet on skeletal muscle mitochondrial function. *High-fat diet consumption induces cellular fatty acid oxidation and oxidative phosphorylation resulting in increased its by product, oxygen radicals. Oxygen radicals react with oxygen, eventually creates reactive oxygen species. Among these, hydrogen peroxide (H_2O_2) is the most important since it is prevalent. Imbalance between reactive oxygen (as well as nitrogen) species and antioxidative system causes oxidative stress to the cell. Oxidative stress induces insulin resistance via creating abnormal phosphorylation of insulin signaling cascade. Simultaneously, oxidative stress induces mitochondrial dysfunction, as indicated by an increase in mitochondrial swelling and ROS production, resulting in a decrease in mitochondrial membrane permeability. Another possible mechanism of mitochondrial function is an imbalance of mitochondrial dynamics, indicated by an increase in mitochondrial fission as well as a decrease in mitochondrial fusion. Eventually oxidative stress also reduces cellular oxidative capacity, as indicated by oxidative phosphorylation (OXPHOS) activity, OXPHOS enzymes protein and mRNA expression, resulting in decreased mitochondrial respiratory capacity. If high-fat diet consumption continues, these detrimental changes would be enhanced and oxidative stress would be increased from abnormal OXPHOS process, causing a vicious cycle.*

1.3.6 Apoptosis of skeletal muscle in obese-insulin resistance

Obesity enhances not only lipid storage in visceral and subcutaneous adipose tissue, but also induces ectopic lipid deposition in non-adipose tissues. This phenomenon is proposed to be a result of increased adipose tissue lipolysis [121], delivery of non-esterified fatty acid (NEFA) and TG to peripheral tissues [122] and an increase in sarcolemmal fatty acid transport [123]. Lipid accumulation in non-adipose cells can cause cell dysfunction or cell death via apoptosis, and these processes have been defined as 'lipotoxic' [70]. DAG and ceramide were proposed to be intermediate signals of lipotoxicity [7]. Although lipotoxic apoptosis is well described for the pancreas, heart and liver [99], it remains poorly described in skeletal muscle [99]. There is in vitro evidence of saturated fatty acids-induced ceramide accumulation causing apoptosis in cultured myotubes [124]. In mice fed high-fat, high sucrose diet for 16 weeks [125], skeletal muscle apoptosis, evident by increase of caspase-3 activity, was demonstrated. However, in ob/ob mice mice fed high-fat diet for 12 weeks, Turpin (2009) demonstrated increase of anti-apoptotic (Bcl-2) signaling [99] with no change of caspase-3, Bax:Bcl2 ratio, TUNEL and decrease of mRNA expression of pro-apoptotic genes except increase of caspase-3 mRNA expression. A study in obese insulin resistant Zucker rats also demonstrated no change of the apoptotic markers in skeletal muscle in soleus and gastrocnemius [53], whereas there were decreased Bcl-2, increased Bax and decreased Bax:Bcl-2 levels in cardiac myocytes. Differences in duration of high-fat diet feeding, as well as the severity of obesity, may account for this inconsistency. The authors proposed that in mild obesity, a molecular adaptation in skeletal muscle which suppressed pro-apoptotic genes, prevented in vivo lipoapoptosis. Interestingly, this adaptive-to-apoptosis in skeletal muscle was compatible with the result of skeletal muscle fiber oxidative capacity. Mice fed high-fat diet for 4 and 8 weeks demonstrated compensatory increase of skeletal muscle oxidative capacity [27, 29] while this compensatory effect was absent in the skeletal muscle of mice fed by high-fat diet for 8 weeks or more, as well as mice or rats with genetic-modified obesity induced increase of skeletal muscle oxidative capacity [24, 27]. In conclusion, apoptosis of skeletal muscle in obese-insulin resistant condition is still inconsistent. Skeletal muscle abnormalities in obese insulin resistant condition are summarized in **Figure 1.5**

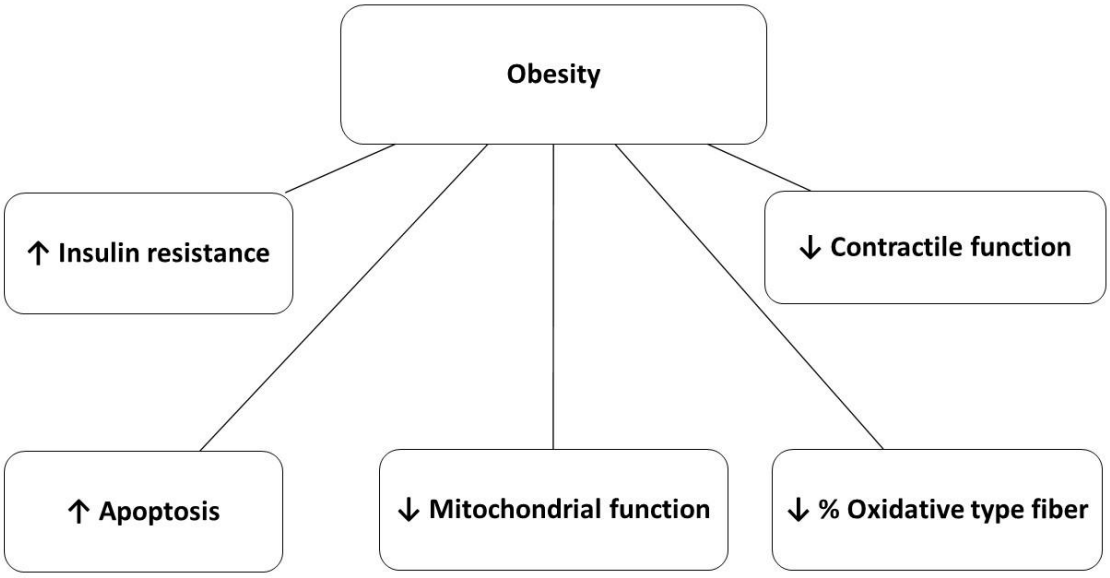

Figure 1.5 Skeletal muscle abnormalities in obese insulin resistant condition. *Obesity induces multiple abnormalities to the skeletal muscle. It increases insulin resistance of the skeletal muscle via pathologic phosphorylation of insulin cascade. Obesity also decreases oxidative-type muscle fiber resulting in early fatigability and contractile dysfunction. Obesity reduces mitochondrial function, as well as mitochondrial dynamics. Eventually, increased apoptosis, as indicated by elevated pro-apoptotic protein Bax and reduced anti-apoptotic protein Bcl-2 is one of the parameters of skeletal muscle dysfunction.*

1.3.7 Effects of caloric restriction program on obese-insulin resistance

Lifestyle modification, such as dietary intervention, is a standard treatment of metabolic syndrome [126]. Evidence demonstrated that food restriction could improve metabolic effects and extended lifespan [127]. Caloric restriction (CR) also decreased body weight, core temperature, heart rate, and motor activity in rats [128]. Moreover, CR reduced cardiovascular (CVD) risk factors [129] and increased maximum and mean life spans, slowing aging [130]. Previous studies in aging obese-insulin resistant rats demonstrated that 55% of CR treatment for 18 months could improve metabolic effects via reducing visceral adipose tissue deposit, resulted in the reduction of hepatic glucose levels [137]. In addition, 85% of CR treatment for 2 weeks in obesity and severe

hypertension rats could reduce body weight, visceral fat, plasma insulin levels, improve lipid profile, as well as up-regulate glucose transporter-2 (GLUT2) in skeletal muscle [138]. Moreover, there were reductions of body weight and visceral fat along with increased ObRb expression in hypothalamus in diet-induced obese rats switch to chow with 60% CR for 30 days, suggesting that CR could improve leptin signaling [139]. In addition, previous studies of caloric and protein restriction or methionine restriction showed decreased ROS production and oxidative damage of mitochondrial rat liver [140, 141]. It might be proposed that CR also has the beneficial effect on the mitochondrial function. However, the effects of CR on mitochondrial dynamics in skeletal muscle of rats in the obese condition have not been investigated.

Several studies found that moderate CR (~60% of daily food intake) improved metabolic parameters including glucose homeostasis, plasma insulin, and insulin sensitivity in both animals [34, 131, 132] and men [133]. In clinical studies, CR intervention has been shown to decrease insulin resistance [39, 42-44]. Increased insulin sensitivity after CR program is correlated with the amount of weight loss which also resulted from the reduction of both fat and fat free mass of the body. More than 10% weight loss can significantly improve insulin sensitivity [39, 42, 46] while 8-10% weight loss can improve insulin sensitivity without reaching significance [43, 44]. CR also decreased plasma TG and LDL [134], increased plasma HDL [135], as well as decreased lipid content in hepatic tissue [136].

1.3.8 Effects of caloric restriction program on skeletal muscle

Caloric restriction increases catabolic metabolism and perhaps results in muscle fiber atrophy in the animal model [142, 143]. De Andrade and colleagues reported that the increased rate of fatty acid oxidation during prolonged caloric restriction occurs via AMPK-mediated signal and its downstream mediators, PGC-1alpha [144]. The expression of PGC-1alpha has been found in oxidative-type muscle fibers, rather than in glycolytic-type muscle fibers, which least found in type 2b/x muscles [144]. According to a lower ability to use fatty acid oxidation of type 2b/x, evidence demonstrates that muscle atrophy predominately presents in type 2b/x muscle fibers [142]. However, this effect depends on amount of protein intake in the program [145-147]. In general, to renew muscle fiber as well as change muscle fiber type, large amount of protein

consumption (at least 1.6 g/kg/day) is needed to produce myosin heavy chain and other contractile proteins [148, 149]. It is also evident that muscle atrophy predominately presents in type 2b/x muscle fibers [142].

Focusing on effect on skeletal muscle fiber type distribution, several studies demonstrated no effect of caloric restriction intervention on the percentage of muscle fiber types, regardless of the intensity and duration of the intervening programs [18, 38-46]. In contrast, Russell and colleagues reported the decreases of not only percentage of type 2b/x muscle fibers but also the oxidative enzymes without any change of type 1 muscle fibers after short-term, severe caloric restriction (400 kcal/day for 2 weeks) [142]. These findings suggest that short-term severe caloric restriction can change the percentages of muscle fiber types and cause harmful effects to muscle, via decreased cross-sectional area as well as the oxidative capacity of the muscle fibers.

Regarding the mitochondrial function and oxidative capacity of the muscle, some clinical studies reported no effect of caloric restriction intervention on the oxidative capacity of the muscle, regardless of the severity and duration of the program [18, 38-46]. However, Russell and colleagues [142] demonstrated decreased oxidative capacity in muscle after 400 kcal/day of a two-week caloric restriction program while Kern and colleagues [41] demonstrated increased oxidative capacity in muscle after 500-800 kcal/day of a three-week caloric restriction program. Therefore, the time-dependent, as well as the dose-dependent effects of caloric restriction program on percentages of muscle fiber type change may account for part of this discrepancy finding. The shorter program resulted in a change of the oxidative capacity in a different direction depending on the severity of caloric restriction whereas metabolic adaptation associated with the longer program resulted in no change in the oxidative capacity.

There was a report of increased IRS-1-associated p110 subunit and Akt-serine phosphorylation in rat skeletal muscle after 60% CR for 8 weeks [34]. Interestingly, study in ovariectomized rats found that CR inhibited the impaired insulin stimulation of IRβ, IRS-1, PI3K, and Akt along with concomitant enhanced p38 MAPK activity in the skeletal muscle [150]. CR also reverse intramyocellular lipid content, which is proposed to be the cause of skeletal muscle lipotoxicity in obese subjects [43, 46] whereas this effect was absent with combination of CR and exercise [42]. Caloric

restriction is also proposed to prevent apoptosis in skeletal muscle in obese [36, 37] and sarcopenic conditions [151].

The effect of CR on skeletal muscle function remains to be investigated. Evidence in aging rats demonstrated no change in peak and tetanic forces [33] after -30%, 8 weeks caloric restriction but another study showed increase in peak and tetanic forces after -40%, 16 weeks caloric restriction [35]. Notice that these effects were present only in skeletal muscles that predominately contain of fast-twitch muscle fibers. In clinical study, evidence demonstrated no change in upper and lower extremity force after 1 year-10% weight loss [152]. Therefore, caloric restriction program may cause either no change or increased muscle contraction force in obese models depending on the severity and the duration of the program. **Figure 1.6** summarized effect of caloric restriction on skeletal muscle in obese insulin resistant condition.

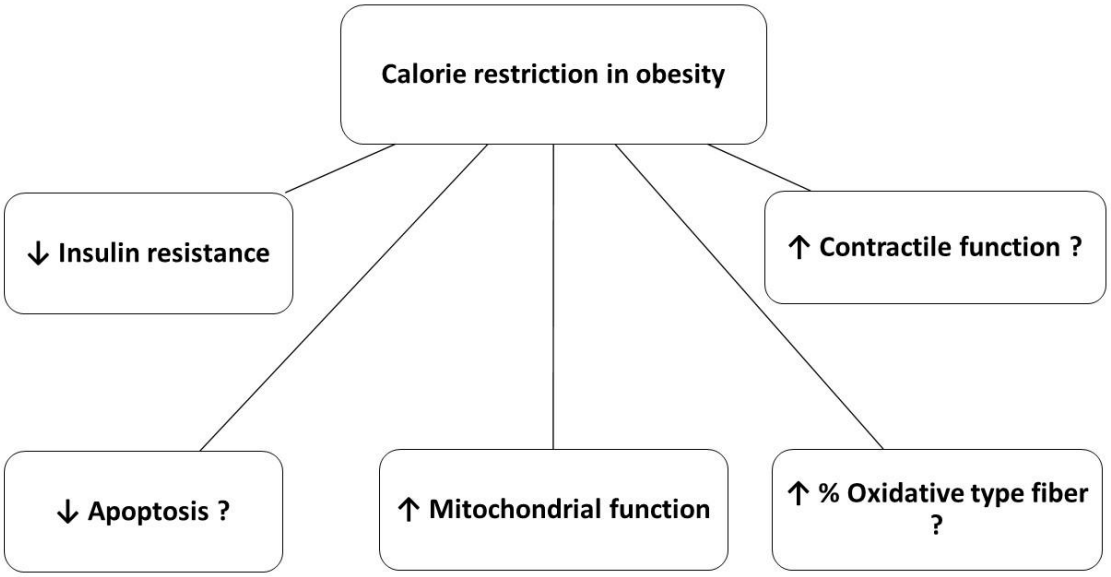

Figure 1.6 Effects of caloric restriction on skeletal muscle in obese-insulin resistant condition. *Calorie restriction (CR) affects skeletal muscle function in obesity in several mechanisms, which are decreased insulin resistance and apoptosis, as well as increased mitochondrial function. However, the effects of CR on skeletal muscle fiber type change as well as the fatigability is still elucidated but it is proposed to be positive.*

1.3.9 Effects of exercise training on obese-insulin resistance

Epidemiological studies have shown that exercise is effective for preventing and improving obesity and T2DM [153]. Several studies also found that there was an increase in insulin sensitivity following exercise training [154-156]. However, insulin resistance commonly returns to near baseline levels after cessation of the exercise. Without weight loss, exercise still influences changes in body composition variables such as fat mass, visceral and subcutaneous adipose tissue, and body fat percentage, which may then translate into improvements in insulin-stimulated glucose disposal [52, 157]. Focusing on mode of exercise (endurance or resistance exercise), one clinical study, all modes of exercise increased insulin sensitivity compared with normal controls but resistance exercise more increased insulin sensitivity than endurance exercise [158]. This finding was proposed to be associated with significant increase of muscle mass resulted from resistance exercise.

In addition, exercise training reduced the white adipose tissue size, resulting in the attenuation of dysregulated expression of adipocyte size-sensitive adipokines, such as leptin and oxidative stress [159]. Exercise training also enhances anti-oxidative system and increased in blood flow [160], which lead to the attenuation of the dysregulated expression of inflammatory adipokines involving TNF-α and MCP-1 [159]. Clinical studies demonstrated that exercise reduced dyslipidemia, intra-abdominal fat, blood platelet adhesiveness and aggregation and inflammation, and improved glucose tolerance [161, 162]. Exercise also increases adiponectin, which correlated with increased insulin sensitivity [163]. The intensity of exercise training appears to be the primary determinant of the degree of metabolic improvement modulating the molecular signaling pathways in a dose-response pattern (at least moderate intensity exercise program), whereas training modality (endurance or resistance) seems to have a secondary role [52, 164].

However, it is still inconsistent whether the benefit of exercise on insulin sensitivity results from weight loss or it is an effect of exercise itself. In clinical studies, the effect of moderate intensity endurance exercise training on insulin sensitivity has been reported [47, 48], and this effect was absent when mild intensity endurance exercise has been combined with resistance exercise training [12, 49]. Also, there was

no additive effect of exercise training on insulin sensitivity when combined with caloric restriction [18, 46]. This finding raised the hypothesis that in obese condition, effects of exercise training and caloric restriction or weight loss on increase of insulin sensitivity are mediated by the similar pathway.

1.3.10 Effects of exercise training on skeletal muscle

Exercise training is commonly included in a weight loss program. There is evidence in both animal and clinical studies that exercise increases PGC-1alpha (144-148) and PPAR delta [165, 166] mRNA expression in affected skeletal muscles. The proposed mechanism was comprehensively reviewed [86]. Briefly, exercise training induces repetitive contractions of the muscles, as well as enhances energy deprivation and increases adenosine monophosphate /adenosine triphosphate ratio (AMP/ATP ratio). Repetitive muscle contractions induce increase of MAPK and Ca-dependent signaling, e.g. calmodulin, calcineurin and CaMK, which are an upstream signaling of PGC-1alpha. Therefore, it is hypothesized that exercise training can induce oxidative-type skeletal muscle fiber transformation.

Effect of exercise training on skeletal muscle fiber type is inconsistent. In rats, endurance exercise by treadmill running for 6 weeks [167] and swimming for 4 weeks [168], as well as resistance training by ladder climbing for 8 weeks [169] caused no change in skeletal muscle fiber type distribution. Two of seven clinical trials demonstrated that exercise training in obesity leads to the alteration in the distribution of muscle fiber type by switching type 2 to type 1 muscle fibers [47, 48] while the other clinical trial failed to demonstrate that effect [12, 40, 42, 46, 49]. These inconsistent findings may result from the heterogeneity of the studies, both in subject characteristics and exercise program. However, another study demonstrated that exercise training in male-type obesity significantly induced a type 2 to 1 fiber switch while this effect was absent in female-type obesity. This finding may be resulted from different body composition change after exercise training, which was more decreased fat mass in male-type than female-type obesity [47].

Furthermore, the studies using resistance [49] or combined of resistance and endurance training [40] demonstrated no effect of exercise training on muscle fiber type

redistribution. As resistance training, which activates a phasic, high-power contraction of the muscle, has been reported to cause hypertrophy and increase percentages of type 2x fiber, and endurance exercise, which activates a tonic, repetitive contraction of the muscles, increases percentages of oxidative-type skeletal muscle fiber [170]. Notice that resistance training program in these studies were moderate intensity, characterized by using 60%–70% of 1-repetition power maximum (RPM) [161, 171]. This intensity mostly causes glycolytic-type skeletal muscle fiber transformation and hypertrophy [172]. Therefore, these resistance exercise-only programs may cause only change in fiber cross-sectional area and the combination of both types of exercise program may cause the increase of both types of muscle fiber, resulted in no change in the percentages of fiber types. These findings were corresponded with the findings in molecular studies, which demonstrated more increase of PGC-1alpha in endurance exercise compared with resistance exercise [173, 174].

Focusing on skeletal muscle oxidative capacity, both clinical [12, 18, 40, 47-49] and animal studies demonstrated that exercise training, regardless of the parameters (type, intensity, duration or frequency), significantly increased the oxidative capacity of the muscle. Several studies also found that increase in skeletal muscle mitochondria biogenesis was observed following exercise training [175, 176]. In addition, studies using genetic modification of mice models demonstrated that muscle fiber phenotype and muscle oxidative capacity were mediated by close but not proximity mechanisms. For example, both muscle fiber phenotype and oxidative capacity were mediated by PGC-1alpha and PPAR delta, while only muscle fiber phenotype is mediated by the adenosine monophosphate-activated protein kinase (AMPK) signaling pathway, which is the main signaling protein responsible for energy control of the body cells [88, 177]. Moreover, the benefits of exercise training to increase the oxidative capacity of muscle were emphasized in the studies of Toledo et al. and Chomentowski et al. demonstrating that the decrease of muscle oxidative capacity and muscle mass with caloric restriction program could be negated in combination with exercise training [18, 46].

Regarding skeletal muscle insulin signaling, exercise has marked acute and chronic effects on insulin action and related inflammatory signaling pathways. Improved insulin action, indicated by increased glucose uptake together with increased

glycogen synthesis, after acute exercise, or electrical stimulation of muscle contraction, was first demonstrated in isolated rat muscles [178, 179]. Although enhanced insulin action after acute exercise resulted from increased membrane translocation of GLUT4 [50, 51], the mechanism that activates GLUT4 expression and translocation is not fully described yet. Furthermore, exercise has the potential to modulate inflammatory processes by decreasing specific inflammatory signaling pathways, for example IKK/NF-κB pathway and inflammasome pathway, which can interfere with signaling pathways of the glucose uptake [164]. In addition, a study in obese insulin resistant Zucker rats demonstrated that after 9 weeks of endurance exercise (treadmill running), there was no change of apoptosis-related markers, which were Bax, Bcl-2 and Bax:Bcl-2 ratio, in skeletal muscle of obese compared with the lean controls [53].

The effect of exercise training on skeletal muscle function is inconsistent. Clinical studies demonstrated increase of not only maximal strength of affected muscle [49, 52], but also aerobic capacity of whole body (indicated by volume of maximum oxygen consumption-VO_2max) [12, 52] after resistance and endurance exercise program, respectively. However, study in rats demonstrated no change in peak single-twitch contraction force after 8 weeks of endurance exercise program [33]. In addition, a previous study reported that ET increased MFN1 protein expression [180], indicating an induction in mitochondrial fusion, as well as decreased DRP1 protein expression, indicating a reduction in mitochondrial fission [181] in the rat model.

Although the effect of mainstream interventions to increase the percentage of oxidative-type skeletal muscle fiber is still inconsistent; an exercise program is proposed to be the only choice of treatment in humans. From reviewed evidence, the preferred exercise program is primarily endurance training, of moderate intensity, at least 150 min/week in volume. Endurance exercise prolongs walking time and distance, increases self-care activity duration, and improves one's quality of life [161, 162]. Although the resistance exercise may negate an effect of exercise to oxidative-type skeletal muscle fiber transformation, it has benefits on increasing muscle mass, enhancing bone mass and reducing the risk of developing musculoskeletal disorders while performing exercise [171]. Since low intensity resistance exercise training can improve fatigability of the skeletal muscle [171], low intensity but high number of

repetitions resistance exercise program, as indicated by resistance weight less than 50% of 1 RPM, 15-25 times/sets, 3-4 sets/day, 2 days/week [171], is hypothetically preferred in people with obesity to prevent benefit on oxidative-type skeletal muscle fiber transformation. However, further investigation to prove this hypothesis is still required. **Figure 1.7** summarized effect of exercise training on skeletal muscle in obese insulin resistant condition.

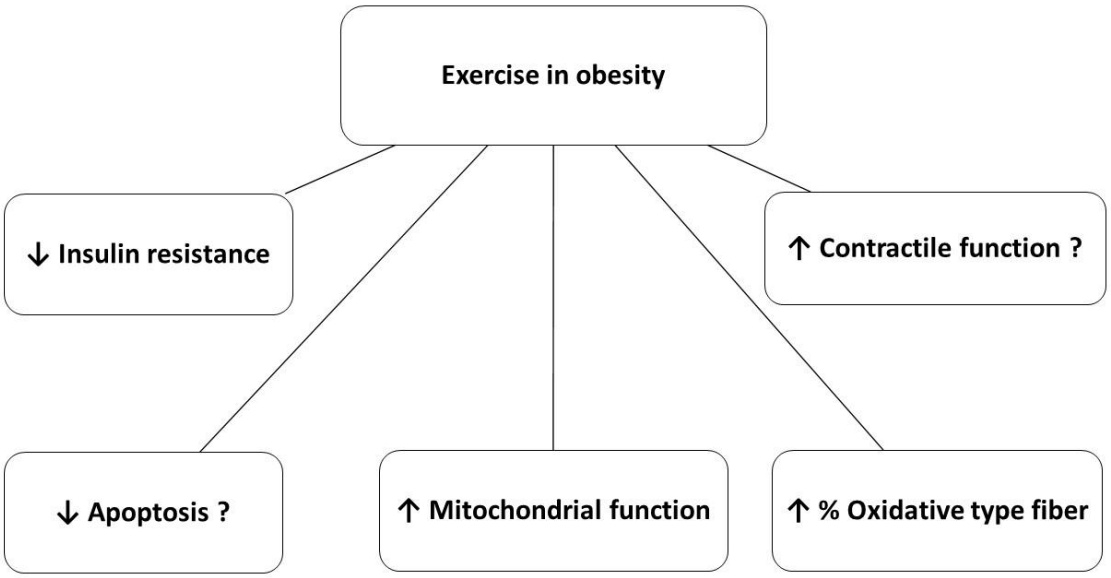

Figure 1.7 Effects of exercise training on skeletal muscle in obese insulin-resistant condition. *Exercise training affects skeletal muscle function in obesity in several mechanisms, which are decreased insulin resistance and improved mitochondrial function, as well as increased oxidative-type muscle fiber. However, the effects of exercise training program on skeletal muscle apoptosis as well as the fatigability is still elucidated but it is proposed to be positive.*

1.3.11 Effects of combined caloric restriction and exercise training program in obese insulin resistance

Both caloric restriction and exercise program provide positive effects to insulin resistant condition [52, 182]. Previous studies demonstrated that combined caloric restriction and exercise training induces comparable [183-187] or more weight loss [188], compared with caloric restriction alone. In addition, clinical studies reported that

caloric restriction in combination with vigorous or moderate exercises can comparably decrease body weight, and improve lipid profiles as well as insulin sensitivity [183], although CR combined with vigorous group had significantly higher $VO2_{max}$ [183, 189] and more decrease of visceral fat [183, 184]. In obese insulin resistant rats, swimming exercise with 50% caloric restriction for 28 days decreased plasma TG, cholesterol, LDL and serum free fatty acid levels along with increased plasma HDL [190].

1.3.12 Effects of combined caloric restriction and exercise training program on skeletal muscle

Aforementioned, caloric restriction program induced negative nitrogen balance [191, 192] and causes muscle wasting. Inadequate protein intake while performing caloric restriction may account for these negative results. Therefore, clinical studies demonstrated that combined caloric restriction and exercise program resulted in no change in skeletal muscle fiber type distribution [18, 46]. However, adding exercise training to caloric restriction program could attenuate decrease of muscle mass compared with caloric restriction alone [46, 54, 187] and aerobic exercise with caloric restriction group demonstrated an increase in mitochondrial density, which supported an improved mitochondrial oxidative capacity, rather than reduction in mitochondrial size observed in the caloric restriction alone group [18]. Combined caloric restriction and exercise program also increased mitochondrial DNA (mtDNA content), corresponding with increased VO_2max [184]. Furthermore, regarding skeletal muscle insulin signaling, clinical study demonstrated that insulin-stimulated glucose disposal increased similarly between caloric restriction alone and combined with exercise, as did phosphorylation of the PI3K/Akt insulin signaling pathway [184]. Focusing on oxidative stress, in obese insulin resistant rats, exercise and combination intervention improved SOD and decreased malondialdehyde (MDA) in soleus muscle while dietary intervention alone had not the effect [193]. In addition, skeletal muscle contraction evaluating by muscle contraction study in rats found that single-twitch contraction force was higher in combined caloric restriction and exercise group than caloric restriction or exercise only groups, as well as obese controlled group [33]. However, the combined effects of an ET with a CR program on mitochondrial dynamics and muscle fatigability in an obese-insulin resistant condition, as well as the comparative effect of an ET, CR and a

combined ET and CR program on functions of skeletal muscles and mitochondria have not yet been investigated. **Figure 1.8** summarized effect of combined caloric restriction and exercise training on skeletal muscle in obese insulin resistant condition.

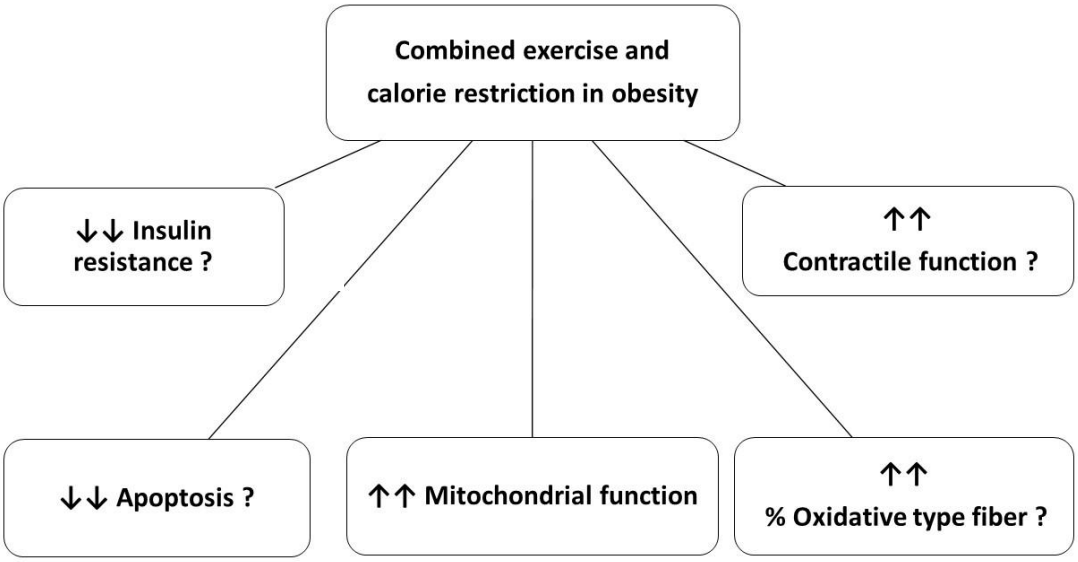

Figure 1.8 Effects of combined caloric restriction and exercise training on skeletal muscle in obese-insulin resistant condition. *Combined caloric restriction and exercise training program affects skeletal muscle function in obesity in several mechanisms, which are decreased insulin resistance and increased mitochondrial function. However, the effects of combined caloric restriction and exercise training on skeletal muscle apoptosis, fiber type change as well as the fatigability is still elucidated but it is proposed to be positive.*

1.4 Theories/principles and rationales

Currently, prevalence of metabolic syndrome, characterized by obesity and insulin resistance, leads to increase an epidemic problem around the world including Thailand. Although several studies demonstrated the beneficial effects of calorie restriction and exercise training on improving systemic insulin sensitivity, skeletal muscle fiber type, skeletal muscle insulin signaling and mitochondrial function, the results of those studies are inconsistent. Moreover, few studies demonstrated effects of these interventions on skeletal muscle apoptosis markers and contractile function. In

addition, the combined effect of calorie restriction and exercise training on skeletal muscle mitochondrial function, skeletal muscle oxidative stress, skeletal muscle apoptosis and skeletal muscle contractile function in obese insulin resistant rats have not been investigated. Therefore, we hypothesize that combined calorie restriction and exercise training increase percentage of oxidative-type fiber of skeletal muscle, improve skeletal muscle mitochondrial function, skeletal muscle oxidative stress, skeletal muscle apoptosis and skeletal muscle contractile function in obese insulin resistant rats.

1.5 Scope of study

As the prevalence of overweight and obesity, particularly in women, has increased up to 40.4% in USA [1], the interest in the effect of obesity on skeletal muscles has been growing. Skeletal muscle is responsible for 80% of postprandial insulin-stimulated uptake of glucose [4]. Therefore, the roles of skeletal muscle in the cause and the effect of the obese-insulin resistant condition need to be considered. In skeletal muscle, the insulin resistant condition can be indicated by a disruption of protein phosphorylation in insulin signaling pathways. This disruption can include decreased tyrosine phosphorylation of insulin receptors (IR) and protein kinase B (Akt), resulting in reduced glucose uptake and utilization [30, 76]. An insulin-resistant condition in skeletal muscles has been associated with mitochondrial dysfunction [18, 48], imbalance of mitochondrial dynamics [16, 120], and increased cell apoptosis [7]. These impairments can lead to abnormal skeletal muscle contractile function, indicated by increased muscle fatigability, the ability to withstand fatigue being one of the most important functions of skeletal muscle [23, 24].

An exercise training program (ET) and caloric restriction (CR) are the conventional interventions for obesity. Previous studies from both animal and clinical studies demonstrated that ET activated insulin signaling [49, 50] and enhanced mitochondrial function of the skeletal muscle [48, 49, 54], leading to an increase in muscle contractile function [49, 52]. In addition, a previous study reported that ET increased MFN1 protein expression [180], indicating an induction in mitochondrial fusion, as well as decreased DRP1 protein expression, indicating a reduction in mitochondrial fission [181] in the rat model. Although ET could ideally attenuate apoptosis in skeletal muscles, a previous study investigating skeletal muscle of rats after

ET failed to demonstrate any significant changes in the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 [53]. Several previous studies showed the beneficial effects of CR in the rat model as regards an increase in insulin sensitivity and function of skeletal muscles [33], increased insulin signaling [34, 35], and also decreased apoptosis [36, 37]. CR has been shown to have no effect on mitochondrial content and mitochondrial oxidative phosphorylation enzymes [42](42). Moreover, the effects of CR on mitochondrial dynamics in skeletal muscle of rats in the obese condition have not been investigated.

Previous clinical study found that the combination of ET with a CR program improved mitochondrial oxidative phosphorylation enzymes, oxidative capacity, insulin signaling and contractile function of skeletal muscle in obese patients [42]. However, the combined effects of an ET with a CR program on mitochondrial dynamics and muscle fatigability in an obese-insulin resistant condition, as well as the comparative effect of an ET, CR and a combined ET and CR program on functions of skeletal muscles and mitochondria have not yet been investigated. Therefore, the hypotheses of the present study were that: 1) in an obese condition skeletal muscles developed insulin resistance, contractile dysfunction, impaired mitochondrial function, mitochondrial dynamics, and biogenesis, as well as there being increased cell apoptosis; and 2) a combination of ET and CR therapy in an obese-insulin resistant condition provides greater efficacy in improving the deleterious effects on skeletal muscles than ET or CR alone.

1.6 Expected benefits

1.6.1 This study will provide novel, basic science knowledge about skeletal muscle dysfunction in obese insulin resistance condition in all modalities, e.g. histology, mitochondrial function, oxidative capacity, apoptosis, insulin signaling and contractile function, as well as effects of caloric restriction, exercise training and combined program in order to treat skeletal muscle dysfunction in obese insulin resistance condition. This knowledge can be applied to manage obese insulin resistant condition in clinical setting.

This study will be published in the international medical journal, which is in the PubMed database, as well as will be presented in the international conference, to transfer these knowledges to the scientific society.

CHAPTER 2

Materials and Methods

2.1 Specific objectives related to the protocol

Aim 1: To investigate the metabolic parameters, skeletal muscle structural and functional changes in obese insulin-resistant rats using HFD-induced obesity.

Hypothesis of aim 1: Skeletal muscle dysfunction, as indicated by altered muscle fiber type distribution, mitochondrial dysfunction, increased oxidative stress, impaired insulin signaling, and induced apoptosis in skeletal muscle, occurs in obese-insulin resistant condition.

Aim 1.1: To investigate the effects of obese insulin resistance on metabolic changes in rats, by measuring plasma insulin level, plasma glucose level, lipid profiles level, Homeostatic model assessment (HOMA) index and oral glucose tolerance test (OGTT)

Aim 1.2: To investigate the effects of obese insulin resistance on skeletal muscle insulin signaling in rats

Aim 1.3: To investigate the effects of obese insulin resistance on skeletal muscle fiber types changes and contractile dysfunction in rats, by measuring the expression of myosin heavy chain isoforms and the contraction parameters of the muscles

Aim 1.4: To investigate the effects of obese insulin resistance on skeletal muscle mitochondrial dysfunction in rats, by measuring skeletal muscle mitochondrial fusion-fission proteins, mitochondrial reactive oxygen species (ROS) production, mitochondrial membrane potential change, mitochondrial swelling and mitochondrial respiratory function

Aim 1.5: To investigate the effects of obese insulin resistance on skeletal muscle apoptosis in rats by measuring the expression of Bax and Bcl2 levels

Rationale: Previous studies regarding changes of skeletal muscle function in obesity are still inconsistent. Both clinical and animal studies demonstrated that skeletal muscle dysfunction , as indicated by decreased percentage of oxidative-type fiber [6-12], decreased mitochondrial function [13-20], decreased insulin signaling [12, 21], and decreased muscle contractile function [22-24] was presented in the obese insulin resistant condition, whereas some studies reported no changes of the oxidative-type fiber [18, 21, 24-27], mitochondrial function [27-29], insulin signaling [12, 21, 30] and muscle contractile function [31, 32] of skeletal muscle of the same pathological condition. The difference between these findings may be related to the different model such as genetically obese mice and rats, as well as HFD feeding mice. Interestingly, a study in HFD-induced obese insulin resistance found an increase in oxidative-type muscle fiber but decreased in mitochondrial function of the skeletal muscles [20]. To strengthen up the effect of obese insulin resistance on the skeletal muscle changes, rats fed HFD for 27 weeks are performed in this study. We hypothesize that high-fat diet induced insulin resistant obese rats have more severe muscle dysfunction compared with non-insulin resistant rats.

Aim 2: To examine the effects of calorie restriction on the metabolic changes, skeletal muscle structure and function in obese insulin-resistance rats.

Hypothesis of aim 2: Skeletal muscle dysfunction in obese-insulin resistant condition can be improved by calories restriction.

Aim 2.1: To examine the effects of caloric restriction on peripheral insulin resistance in obese insulin-resistance rats, by measuring plasma insulin level, plasma glucose level, lipid profiles level, HOMA index and OGTT

Aim 2.2: To examine the effects of caloric restriction on skeletal muscle insulin resistance in obese insulin-resistance rats, by measuring skeletal muscle insulin signaling

Aim 2.3: To examine the effects of caloric restriction on skeletal muscle fiber type changes and contractile dysfunction in obese insulin-resistance rats, by measuring the expression of myosin heavy chain isoforms and the contraction parameters of the muscles

Aim 2.4: To examine the effects of caloric restriction on skeletal muscle mitochondrial dysfunction- in obese insulin-resistance rats, by measuring skeletal muscle mitochondrial fusion-fission proteins, mitochondrial reactive oxygen species (ROS) production, mitochondrial membrane potential change, mitochondrial swelling and mitochondrial respiratory function

Aim 2.5: To examine the effects of caloric restriction on skeletal muscle apoptosis in obese insulin-resistance rats by measuring the expression of Bax and Bcl2 levels

Rationale: Caloric restriction and exercise are mainstream intervention of obesity. Several studies demonstrated the beneficial effects of calorie restriction on improvement of systemic insulin sensitivity [34, 131-133], increase of muscle function [33] and insulin signaling [34, 35], as well as decreased apoptosis [36, 37], but have no effect on muscle fiber type distribution [18, 38-46] and muscle oxidative capacity [18, 38-48]. However, the results of those studies are inconsistent [42, 152] and may be due to different model of obesity. In this study, the effects of obese insulin resistance on skeletal muscle will be investigated by using the model of rat fed HFD for 27 weeks. We hypothesize that caloric restriction attenuates skeletal muscle dysfunction (particularly abnormal insulin signaling), but does not attenuate mitochondrial dysfunction, in obese insulin resistance rats.

Aim 3: To examine the effects of exercise training on the metabolic changes, skeletal muscle structure and function in obese insulin-resistance rats.

Hypothesis of aim 3: Skeletal muscle dysfunction in obese-insulin resistant condition can be improved by exercise training.

Aim 3.1: To examine the effects of exercise training on peripheral insulin resistance in obese insulin-resistance rats, by measuring plasma insulin level, plasma glucose level, lipid profiles level, HOMA index and OGTT

Aim 3.2: To examine the effects of exercise training on skeletal muscle insulin resistance in obese insulin-resistance rats, by measuring skeletal muscle insulin signaling

Aim 3.3: To examine the effects of exercise training on skeletal muscle fiber type changes and contractile dysfunction in obese insulin-resistance rats, by measuring the expression of myosin heavy chain isoforms and the contraction parameters of the muscles

Aim 3.4: To examine the effects of exercise training on skeletal muscle mitochondrial dysfunction- in obese insulin-resistance rats, by measuring skeletal muscle mitochondrial fusion-fission proteins, mitochondrial reactive oxygen species (ROS) production, mitochondrial membrane potential change, mitochondrial swelling and mitochondrial respiratory function

Aim 3.5: To examine the effects of exercise training on skeletal muscle apoptosis in obese insulin-resistance rats by measuring the expression of Bax and Bcl2 levels

Rationale: Several studies demonstrated the beneficial effects of exercise training on improving systemic insulin sensitivity [12, 47-49], increasing percentage of oxidative-type muscle fiber [47, 48], enhancing oxidative capacity of skeletal muscle [12, 18, 40, 47-49], activating insulin signaling [50, 51] and increasing muscle function [49, 52], with no effect on apoptotic marker in skeletal muscle [53]. Notice that these effects are intensity-specific, dose-dependent, continuation-specific and mode-dependent. However, the results of those studies are inconsistent [12, 33, 40, 42, 46, 49]. In this study, the effects of obese insulin resistance on skeletal muscle will be investigated by using the model of rat-fed HFD for 27 weeks. We hypothesize that exercise training attenuates all components of skeletal muscle dysfunction in obese insulin resistant rats.

Aim 4: To examine the effects of calorie restriction, exercise training and combined calorie restriction and exercise training programs on the metabolic changes, skeletal muscle structure and function in obese insulin-resistance rats.

Hypothesis of aim 4: The combined interventions provides higher efficacy than monotherapy via restoring skeletal muscle function in obese-insulin resistant condition.

Aim 4.1: To examine the effects of caloric restriction combined with exercise training on peripheral insulin resistance in obese insulin-resistance rats, by measuring plasma insulin level, plasma glucose level, lipid profiles level, HOMA index and OGTT

Aim 4.2: To examine the effects of caloric restriction combined with exercise training on skeletal muscle insulin resistance in obese insulin-resistance rats, by measuring skeletal muscle insulin signaling

Aim 4.3: To examine the effects of caloric restriction combined with exercise training on skeletal muscle fiber type changes and contractile dysfunction in obese insulin-resistance

rats, by measuring the expression of myosin heavy chain isoforms and the contraction parameters of the muscles

Aim 4.4: To examine the effects of caloric restriction combined with exercise training on skeletal muscle mitochondrial dysfunction- in obese insulin-resistance rats, by measuring skeletal muscle mitochondrial fusion-fission proteins, mitochondrial reactive oxygen species (ROS) production, mitochondrial membrane potential change, mitochondrial swelling and mitochondrial respiratory function

Aim 4.5: To examine the effects of caloric restriction combined with exercise training on skeletal muscle apoptosis in obese insulin-resistance rats by measuring the expression of Bax and Bcl2 levels

Rationale: Effects of combined caloric restriction and exercise program on skeletal muscle in obese insulin resistance are quite consistent. Although skeletal muscle fiber type distribution is not changed [18, 46, 54], muscle mass [46, 54, 187], as well as mitochondrial function and oxidative capacity [18, 46, 54, 184], insulin signaling[184] and contractile function of skeletal muscle are improved [33]. However, these effects on skeletal muscle apoptosis and skeletal muscle contractile function in obese insulin resistant rats have not been investigated yet. We hypothesize that combined caloric restriction and exercise training attenuates skeletal muscle dysfunction and its effect is more than effect of caloric restriction or exercise training alone.

2.2 Study protocol

All experiments were conducted using a protocol approved by the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines.

Aim 1: To investigate the effects of obese insulin resistance on the metabolic changes and skeletal muscle structure and function in rats

Female Wistar rats (n=20, body weight 200-220 g.) were obtained from the National Laboratory Animal Center, Thailand, and were randomly assigned to be fed on either a normal or high-fat diet. The normal-diet (ND) group (n=8) was given standard laboratory chow, which had an energy content of 4.02 kcal/g with 19.77% of the total energy (%E) of the food being from fat (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand) (see Appendix Table A1). The high-fat diet (HFD) group were given a high-fat diet, which had an energy content of 5.35 kcal/g and contained fat mostly from lard (59.28% E) [194] (see Appendix Table A2). Rats in both groups continued consuming their assigned diet for total 27 weeks. Blood samples were collected to determine metabolic parameters at the end of week 26. At the end of week 27, an oral glucose tolerance test (OGTT) was performed on each rat by collecting blood from the tail veins. In the next morning, the animals were deeply anesthetized with xylazine (0.15 ml/kg) and Zoletil (50mg/kg). The gastrocnemius muscles were used to carry out in situ muscle contraction studies for measuring the time to fatigue of tetanic contraction. Then, insulin was injected intramuscularly 10 minutes before the rats were euthanatized and the vastus lateralis muscles were rapidly removed for determining insulin signaling, mitochondrial function, mitochondrial biogenesis, mitochondrial dynamics, oxidative stress and apoptosis, as well as evaluating fiber type component by using immunohistochemistry staining. A summary of the protocol of aim 1 is shown in **Figure 2.1**

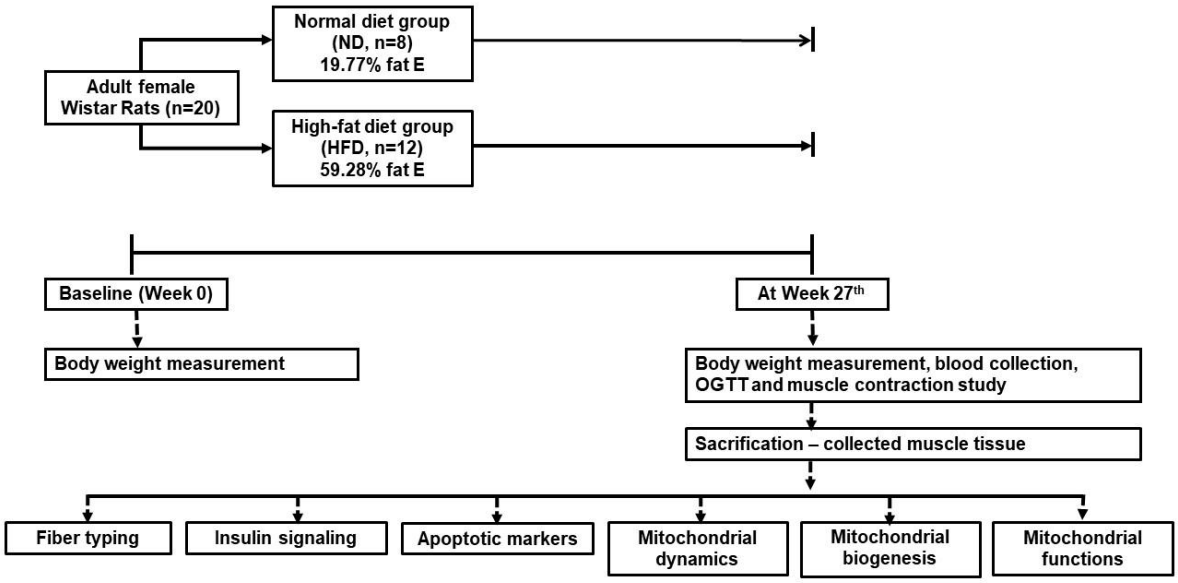

Figure 2.1 Schematic diagram of the experimental design of aim 1

ND, normal diet group; HFD, high-fat diet group; IHC, immunohistochemistry; OGTT, oral glucose tolerance test

Aim 2-4: To investigate the effects of interventions (e.g. exercise training, caloric restriction or combined exercise training and caloric restriction program) on the metabolic changes and skeletal muscle structure and function in obese-insulin resistant rats

Female Wistar rats (n=30, body weight 200-220 g.) were obtained from the National Laboratory Animal Center, Thailand, and were randomly assigned to be fed on either a normal or high-fat diet. The normal-diet (ND) group (n=6) was given standard laboratory chow, which had an energy content of 4.02 kcal/g with 19.77% of the total energy (%E) of the food being from fat (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand) (see Appendix Table A1). The high-fat diet (HFD) group were given a high-fat diet, which had an energy content of 5.35 kcal/g and contained fat mostly from lard (59.28% E) [194] (see Appendix Table A2). Rats in both groups continued consuming their assigned diet for total 27 weeks. At week 21, ND-fed rats continued ingesting ND without any intervention until the 27th week. At week 21, HFD-fed rats were subdivided into four subgroups (n=6 rats per subgroup). Each subgroup

was designated as either: sedentary living (HFDS), exercise training (HFDEx), calorie restriction (HFDCr), or combined exercise and caloric restriction (HFDCb) for 7 weeks. Blood samples were collected to determine metabolic parameters at the end of week 26. At the end of week 27, an oral glucose tolerance test (OGTT) was performed on each rat by collecting blood from the tail veins. In the next morning, the animals were deeply anesthetized with xylazine (0.15 ml/kg) and Zoletil (50mg/kg). The gastrocnemius muscles were used to carry out in situ muscle contraction studies for measuring the time to fatigue of tetanic contraction. Then, insulin was injected intramuscularly 10 minutes before the rats were euthanatized and the vastus lateralis muscles were rapidly removed for determining insulin signaling, mitochondrial function, mitochondrial biogenesis, mitochondrial dynamics, oxidative stress and apoptosis. A summary of the protocol is shown in **Figure 2.2**

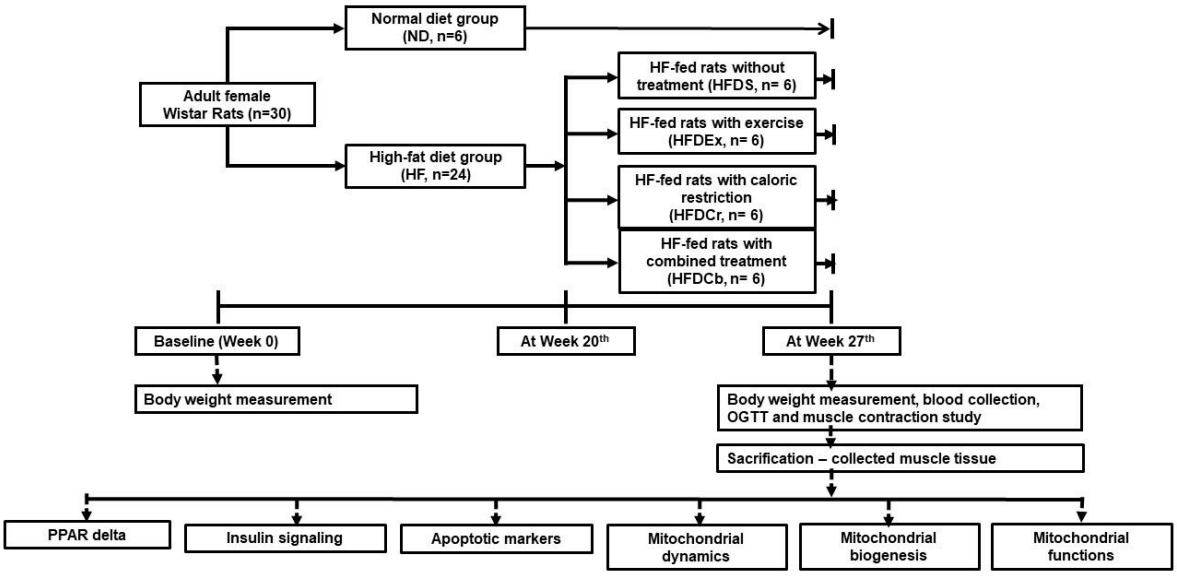

Figure 2.2 Schematic diagram of the experimental design of aim 2-4

ND, normal diet group; HFD, high-fat diet group; IHC, immunohistochemistry; OGTT, oral glucose tolerance test

2.3 Caloric restriction (CR) diet

This CR diet provided 60% energy high fat caloric intake of mean of basal freely available in the form of normal diet chow. By 1 g of high fat diet was 5.35 kcal and normal diet is 4.02 kcal and feeding for 6 weeks [195]. The body weight was monitored every week to prevent excessive body weight loss (not more than 3% per week).

2.4 Exercise training protocol

The exercise training, in terms of endurance training, was performed on a motor-driven rodent treadmill five days/week over a six-week period. The exercise regime was carried out using a motor-driven rodent treadmill (Columbus Instruments Ohio, USA) as previously described [196]. Exercise training was performed five days/week over a 6-week period. The treadmill was equipped with an aversive electrical stimulus (163 V of alternating current and 1.5 mA) in the back region of each lane to force the rats to run. Training sessions were held in the morning. During the running sessions sedentary rats were put in the same room as exercising rats. The intensity of exercise started at 10 min once a day at 22 m/min in the first week to accustom the rats to the equipment. Then, the intensity was increased to 30 min once a day at 25 m/min. This protocol was continued for 6 weeks. The intensity of exercise was 65% VO_2max and the intensity of exercise was classified as moderate intensity as previously described. The exercise program is summarized in **Figure 2.3**

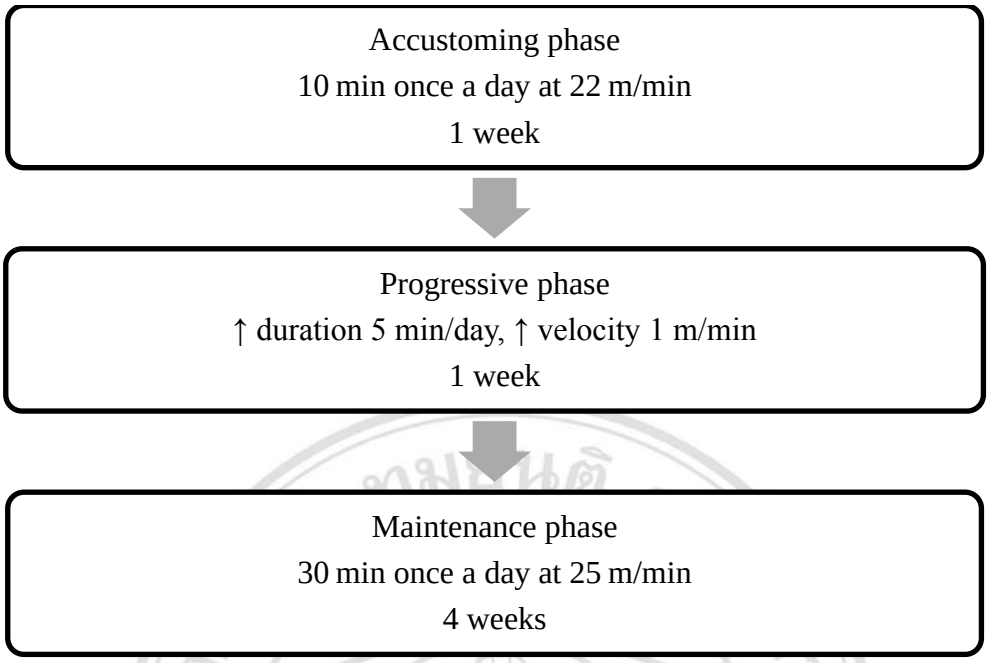

Figure 2.3 Schematic diagram of the exercise protocol.

2.5 Plasma analysis

Plasma glucose, triglyceride, HDL, LDL and cholesterol concentrations were determined using colorimetric assay, a commercially available kit (Biotech, Bangkok, Thailand). Plasma insulin level were measured by Sandwich ELISA (LINCO Research, MO, USA). Plasma estrogen concentration were measured by using Sandwich ELISA (Cayman Chemical Company, MI, USA).

2.6 Determination of insulin resistance (OGTT and HOMA)

Insulin resistance were assessed by oral glucose tolerance test (OGTT) [197, 198] and Homeostasis Model Assessment (HOMA) [199, 200]. OGTT were performed after fasting overnight (12 hrs.). Rats were received a bolus of glucose (2 g/kg BW) via gavage feeding and blood samples were corrected from tail vein at 0, 30, 60, 90 and 120 minutes after glucose administration in NaF microcentrification tube. Then blood samples were centrifuged at 4°C, 6,000 rpm. for 10 minutes. Plasma glucose were estimated by colorimetric assay using a commercially available kit (Biotech, Bangkok, Thailand). HOMA is a mathematical model describing the degree of insulin resistance, calculated from fasting plasma insulin and fasting plasma glucose concentration. A

higher HOMA index indicates a higher degree of insulin resistance. The HOMA index was determined by the following equation:

$$\frac{\text{[Fasting plasma insulin (μU/ml)] x [fasting plasma glucose (mmol/l)]}}{22.5}$$

2.7 Muscle homogenate preparation

Muscles was homogenized in ice-cold lysis buffer: 50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$,1mM $MgCl_2$, 2 mM EDTA, 10 mM NaF, 20 mM sodium pyrophosphate, 20 mMβ-glycerophosphate, 10% glycerol, 1% Triton X-100, 2 mM Na3VO4,10μg/ml aprotinin and leupeptin, and 2 mM PMSF. After 20-min incubation on ice, the homogenates were centrifuged at 13,000 g for 20 min at 4 ^{o}C. Aliquots of supernatant was frozen at−80 ^{o}C, and a portion of these homogenates was used for the determination of total protein (BCA method, SigmaChemical) [150].

2.8 Immunoblotting

Expression of the proteins (antibody), including IR (SC-711, Santa Cruz Biotechnology, Santa Cruz, USA), Tyr1162/1163pIR (SC-25103, Santa Cruz Biotechnology, Santa Cruz, USA), Akt (#9272, Cell Signaling Technology, Danvers, USA), Thr308pAkt (#9271, Cell Signaling Technology, Danvers, USA), Bax (ab 182733, Abcam, Cambridge, UK), Bcl2 (ab 196495, Abcam, Cambridge, UK), PPAR delta (PA5-29678, Thermo Fisher Scientific, Waltham, USA), PGC1alpha (ab 154481, Abcam, Cambridge, UK), CPT1 (SC-393070, Santa Cruz Biotechnology, Santa Cruz, USA), MFN2 (#9482, Cell Signaling Technology, Danvers, USA), DRP1 (#5391, Cell Signaling Technology, Danvers, USA) and Ser616pDRP1 (#3455, Cell Signaling Technology, Danvers, USA), were determined using immunoblotting. The proteins were separated by electrophoresis on 10% polyacrylamide gels (Bio-Rad Laboratories, CA, USA) SDS-Page and transferred into PDVF membranes. Band intensity was quantified by Scion Image program and the results are shown as average signal intensity (arbitrary units) [27, 201]).

2.9 Skeletal muscle mitochondrial isolation

68-150 mg of removed skeletal muscle was kept in 1 ml isolation medium (100 mmol sucrose, 100 mmol KCl, 50 mmol Tris, 1 mmol KH_2PO_4, 0.1 mmol/L EDTA, 0.2% BSA, adjusted pH to 7.4) on ice and cut into small pieces. After that, skeletal muscle tissue was incubated in 1 ml of solution mixed between isolation medium and 0.2 mg of nagrase type XXVII for 2 minutes. The tissue was finely minced and homogenized for 8 minutes in an ice-cooled glass- homogenizer. The homogenate was added 3 ml of isolation medium, and then was centrifuged at 700 ×g for 10 minutes to remove connective tissue. The supernatant was centrifuged at 10,000 ×g for 10 minutes and the pellet was carefully resuspended in 1.3 mL of insolation medium and further centrifuged at 7000 ×g for 3 minutes. All centrifugations were at 4°C. The final mitochondrial pellet was resuspended in 4µL/g of respiratory buffer for measuring mitochondrial RO, membrane potential and mitochondrial swelling. Respiratory buffer contained of 225 mmol/L mannitol, 75 mmol/L sucrose, 90 mmol/L Tris-HCl, 10 mmol/L EDTA, pH 7.4 and brain respiratory buffer contained of mmol KCl, 5 mmol HEPES, 5 mmol $K_2HPO_4*3H_2O$, 2 mmol L-glutamate, 5 mmol Pyruvate sodium salt (183). The volume of the added buffer was 4 uL/mg of skeletal muscle tissue. Concentrations of mitochondrial proteins was determined by the BCA assay [202].

2.10 Protein quantitation by the Bicinchoninic Acid (BCA) assay

Skeletal muscle protein concentration was determined according to the Bicinchoninic Acid (BCA) Assay. Reagent A was composed of sodium bicinchoninate (0.1 g), $Na_2CO_3.H_2O$ (2.0 g), sodium tartrate (dihydrate) 0.16 g, NaOH (0.4 g), and $NaHCO_3$ (0.95 g), made volume up to 100 ml. The pH was adjusted to 11.25 with $NaHCO_3$ or NaOH if necessary. Reagent B was composed of $CuSO_4.5H_2O$ (0.4 g) in 10 ml of water and the standard working reagent (SWR) was a mix of 100 ml of reagent A, with 2 ml of reagent B. The solution was apple green in color and was stable at room temperature for 1 wk. SWR (1 ml) was added to mitochondrial protein (50 µl) and incubate at 60°C for 30 min. The sample was cooled to room temperature and then the absorbance was measured at 562 nm using a spectrophotometer. A calibration curve was constructed using dilutions of a stock 1 mg/ml solution of bovine serum albumin (BSA) [202].

2.11 Reactive oxygen species (ROS) measurement in isolated skeletal muscle mitochondria

ROS in isolated skeletal muscle mitochondria was measured by fluorescent probe and dichloro-hydrofluoresceindiacetate (DCFDA). Protein of skeletal muscle mitochondria (0.4 mg/ml) was incubated with 2 μM DCFDA, 25°C in 20 minutes. ROS was evaluated by fluorescent microplate reader at the wavelength of 485 nm (bandwidth 5 nm) and emission wavelength at 530 nm (bandwidth). The fluorescence was determined using a fluorescent microplate reader (Bio-Tek Instruments, Inc. Winooski, Vermont USA). Increase of fluorescent intensity represented as increased of skeletal muscle ROS [203].

2.12 Mitochondrial membrane potential (ΔΨm) measurement in isolated muscle mitochondria

Mitochondria membrane potential (ΔΨm) change was measured with dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolcarbocyanine iodide (JC-1). Mitochondrial proteins (0.4 mg/ml) was stained with JC-1 (5μM) at 37 °C for 15 minutes. Mitochondrial membrane potential was determined as fluorescence intensity by using a fluorescent microplate reader (Bio-Tek Instruments, Inc. Winooski, Vermont USA. JC-1 monomer (green) fluorescent is excited at wavelength at 485 nm and detects the emission wavelength at 590 nm and JC-1 aggregate form (red) fluorescent is excited wavelength 485 nm and detects the emission wavelength at 530 nm. The change in mitochondrial membrane potential was calculated as the ratio of red to green fluorescence. Mitochondrial depolarization was represented as decrease of red to green ratio [204].

2.13 Isolated skeletal muscle mitochondrial swelling determination

Isolated skeletal mitochondria were measured as its changing in the absorbance of the suspension at 540 nm by using a microplate reader (Bio-Tek Instruments, Inc. Winooski, Vermont USA). Mitochondria (0.4 mg/ml) was incubated in 2 ml respiration buffer Mitochondrial swelling will be represented as decrease of absorbance value [205].

2.14 Skeletal muscle insulin signaling

Rats were fasted 3 h and intraperitoneal injected with human insulin at 1.25 IU/kg body weight. Fifteen minutes later, mice were euthanized, and tissues were collected from bilateral soleus. Total and phosphorylated Akt as well as IR primary antibodies were applied. Peroxidase conjugated immunoglobulin G secondary antibody were used. Tissues were homogenized in cell lysis buffer and a BCA assay was used to determine sample protein concentrations. For western blots, 12 μg of protein was loaded onto a 4 – 20% gradient SDS-PAGE gel for separation. Protein was transferred to nitrocellulose membranes which was then incubated in primary antibody overnight at 4°C (1:1000 for total Akt and phosphorylated Akt). Membranes were washed then incubated in the secondary antibody (1:5000) for 1 hour then washed again. Band intensity was quantified by Scion Image program and the results were shown in average signal intensity (arbitrary units). Data were represented as basal and insulin stimulated phosphorylation as a ratio of phosphorylated Akt to total Akt and phosphorylated insulin receptors to total insulin receptors [206].

2.15 In situ skeletal muscle contraction study

To set up this preparation, the animal was anesthetized, and the left lower extremity of the animal was restrained. Left gastrocnemius muscle was surgically isolated, with the origin intact. Care was taken to maintain the blood and nerve supplies. A long section of the sciatic nerve was cleared of connective tissue and severed proximally. All branches of the distal stump that innervate the medial gastrocnemius muscle was severed. The distal nerve stump was inserted into a cuff lined with stainless steel stimulating wires. The Achilles tendon was attached to the force transducer. The muscle was covered by gauze filled with normal saline to protect myocyte injury and minimizes evaporative heat loss. A heat lamp was placed near the muscle, and the muscle and rat were allowed to warm up to 37°C. While it was warming, maximal voltage and optimal length was determined. Muscle length was adjusted to the resting length, i.e., the length at witch maximal force after single twitch contraction was observed. The muscle length was increased by about 1 mm for another twitch. This was repeated as long as twitch amplitude is increasing. Once twitch amplitude decreased, the length was returned to the one that gave the largest amplitude twitch. Muscles were

allowed to equilibrate for 10 min before starting stimulation protocol. The pulse duration was set at 50 μs. The starting intensity was 0.5 V then the voltage was increased until twitch amplitude did not increase. Maximal voltage was the lowest voltage that activates all motor units. Supramaximal intensity was double of the maximal voltage or 3 V whichever will be higher. Isometric single-twitch contractions were evoked using supramaximal single pulses. Contraction time (time from the baseline to the peak force; ms), relaxation time (time from the peak force to the baseline; ms) and peak force for single-twitch contraction were measured. To minimize the influence of noise, contraction and relaxation times were calculated as the transition time between 10% and 90% of peak force rather than 0% and 100%. Force was normalized to muscle weight in grams [29]. For tetanic contraction protocol, muscle was evoked at 50 Hz until 50% fatigue of the muscle was presented. Peak force and time to fatigue of tetanic contraction was measured. Muscles was allowed to recover for 10 min between different stimulation protocols. At the end of the experiment, tendon-free muscle weight was determined [207]. **Figure 2.4** demonstrates the setting of muscle contraction study used in this research.

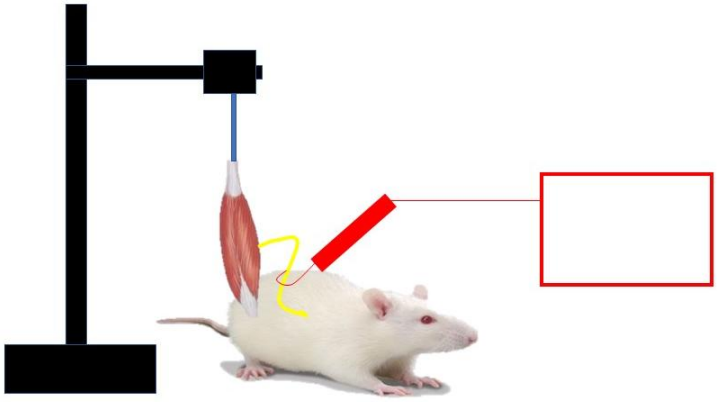

Figure 2.4 The muscle contraction study setting.

2.16 Immunohistochemistry staining

After removed, vastus lateralis muscles will be fixed in 10% neural buffered formalin (NBF) and embedded in paraffin. The tissue will be cut into 10 μm thick with a microtome maintained at -20°C. Immunofluorescence analysis of MHC expression will be performed with primary antibodies against MyHCII. Muscle sections will be blocked in normal goat serum, primary antibody cocktail was added for 2 h. Sections will be then washed and incubated in the appropriate secondary antibody in avidin-biotin peroxidase complex method for 1 h, washed, and cover slipped. Slides will be visualized with a microscope. The Individual images will be taken across the entire cross-section and assembled into a composite panoramic image with Microsoft Image Composite Editor (Microsoft). For fiber type analysis, all fibers within the entire muscle/cross-section will be characterized [208]. Since mammals have only two kinds of skeletal muscle fibers, namely oxidative type and glycolytic type [5], the percentage of oxidative type fibers would be presented as a percentage of non-glycolytic type fibers.

2.17 Statistical analysis

Categorical variables were described using percentages of frequency. Normally-distributed numerical variables were presented using arithmetic mean and standard deviation (SD). Differences of parameters among groups were compared using independent t-test and two-way ANOVA, followed by post-hoc Turkey test. Correlations between parametric continuous parameters were determined by Pearson correlation analysis. Statistical analyses were performed using SPSS version 22 for Windows (SPSS Inc., Chicago, IL, USA). A p-value of less than 0.05 was considered statistically significant.

CHAPTER 3

Results

3.1 Aim 1: To investigate the effects of obese insulin resistance on the metabolic changes and skeletal muscle structure and function in rats

3.1.1 HFD consumption induced obesity, insulin resistance and dyslipidemia

After being fed on HFD for 27 weeks, HFD-fed rats demonstrated metabolic disturbance, as indicated by obesity (increased body weight and visceral fat weight), peripheral insulin resistance, as shown by hyperinsulinemia with euglycemia, increased HOMA index and impaired OGTT, and dyslipidemia (increased plasma cholesterol level, plasma LDL level and decreased plasma HDL level) (**Table 3.1**).

Table 3.1 Effects of high-fat diet on metabolic parameters in rats

Metabolic parameters	**Group**	
	ND	**HFD**
Body weight (g)	281.15 ± 3.50	352.00 ± 4.16*
Visceral fat (g)	9.31 ± 0.69	28.51 ± 1.12*
Plasma glucose (mg/dl)	133.25 ± 3.03	139.66 ± 4.48
Plasma insulin (ng/ml)	1.50 ± 0.17	4.76 ± 0.36*
Plasma glucose AUC from OGTT (AUC)(mg/dl×min×10^4)	1.92 ± 0.07	2.57 ± 0.04*
HOMA index	5 ± 2.4	16 ± 2.1*

Table 3.1 Effects of high-fat diet on metabolic parameters in rats (continued)

Metabolic parameters	Group	
	ND	**HFD**
Plasma total cholesterol (mg/dl)	51.65 ± 6.79	98.55 ± 4.53*
Plasma triglyceride (mg/dl)	61.30 ± 7.39	70.38 ± 5.65
HDL cholesterol (mg/dl)	19.24 ± 1.49	22.64 ± 1.35
LDL cholesterol (mg/dl)	19.29 ± 4.66	57.89 ± 4.66*

*, $p<0.05$ compared with ND; n=6/group;

Abbreviation: AUC, area under the curve; OGTT, oral glucose tolerance test; HOMA, homeostatic model assessment; HDL, high density lipoprotein; LDL, low density lipoprotein; ND, normal diet group; HFD, high-fat diet group

3.1.2 Enhanced early fatigability and decreased PPAR delta protein expression in gastrocnemius muscles occurred in HFD-fed rats

In-situ muscle contraction study of gastrocnemius muscles was used to determine muscle contractile dysfunction. HFD-fed rats in the sedentary living group demonstrated a significant decrease in time-to-fatigue duration, when compared with those of ND-fed rats (**Figure 3.1 A and B**). Muscle fatigability is mainly determined by the predominant type of muscle fiber in the muscle. PPAR delta is part of the PPAR receptor family, which regulates muscle fiber type 1 construction. Corresponding with the results of muscle contraction study, the results of the western blot analysis also showed a significant decrease in PPAR delta protein expression in sedentary living HFD-fed rats, when compared with those of ND-fed rats (**Figure 3.1 C**).

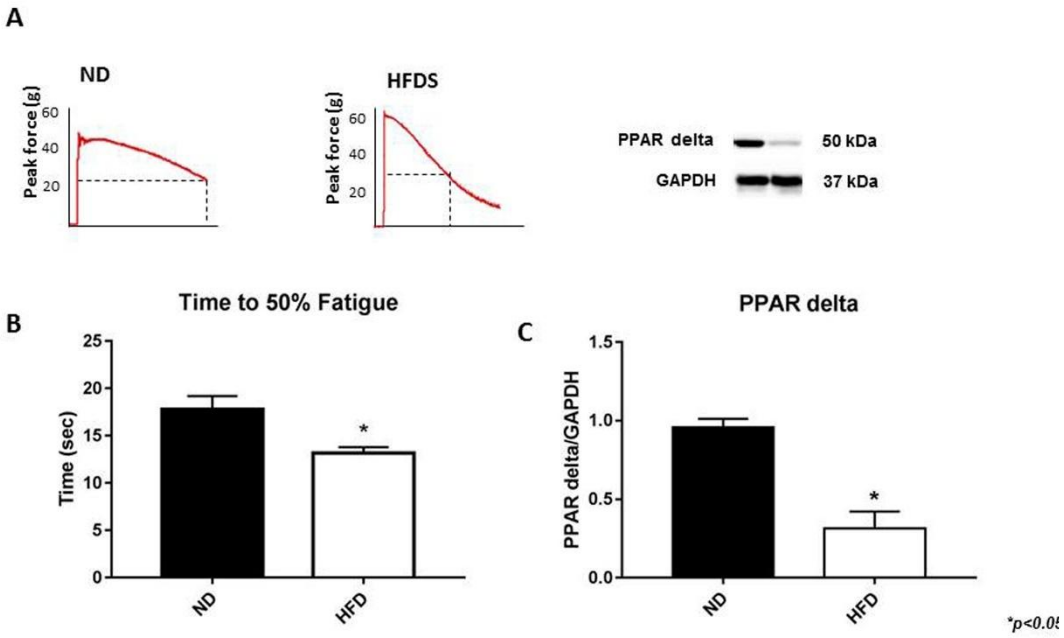

Figure 3.1 The effects of HFD consumption on skeletal muscle fatigability and PPAR delta protein expression. (A): Muscle contraction study tracing in each study group; (B): Comparison of time to fatigue parameter of muscle contraction studies between the study groups; (C): Comparison of PPAR delta protein expression among the study group.

*, $p<0.05$ compared with ND group; n=6/group. ND, normal diet group; HFD, high-fat diet group; PPAR, peroxisome perforator-activated receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

3.1.3 Decreased ratio of oxidative-type skeletal muscle fibers occurred in HFD-fed rats and associated with PPAR delta protein expression.

Immunohistochemistry (IHC) of glycolytic-type muscle fibers was applied to the formalin-fixed muscle tissues from vastus lateralis for determining the ratio between oxidative-type muscle fibers and glycolytic-type muscle fibers in rats from ND and HFS groups. Since mammals have only two kinds of skeletal muscle fibers, namely oxidative type and glycolytic type [5], the percentage of oxidative type fibers would be presented as a percentage of non-glycolytic type fibers.

In ND-fed rats, the percentage of oxidative-type muscle fibers was 27.17±0.83%. HFD-fed rats in the sedentary living group demonstrated a significant reduction in percentage of oxidative-type muscle fibers (20.33±0.76%), when compared with those of ND-fed rats (20.33±0.76% vs 27.17±0.83%, p<0.001; independent t-test). (**Figure 3.2**). Notice that there was a strong positive correlation between the percentage of oxidative-type muscle fibers and PPAR delta protein expression in both ND- and HFD-fed groups (R=0.896, p<0.001; Pearson correlation) (**Figure 3.3**).

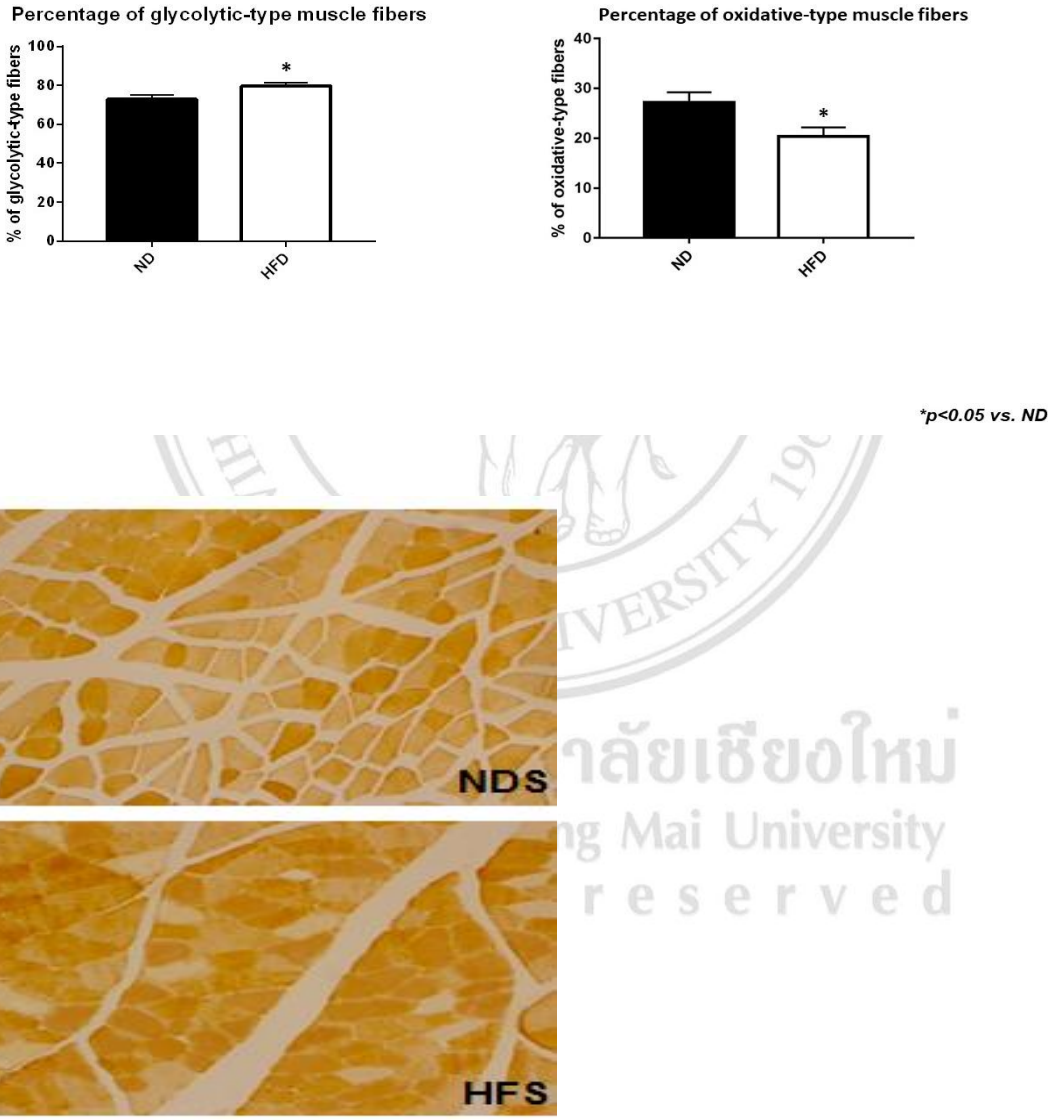

Figure 3.2 The effects of HFD consumption on the percentage of oxidative-type skeletal muscle fibers. *, p<0.05 compared with ND group; n=6/group; ND, normal diet group; HFDS, high-fat diet with sedentary living group

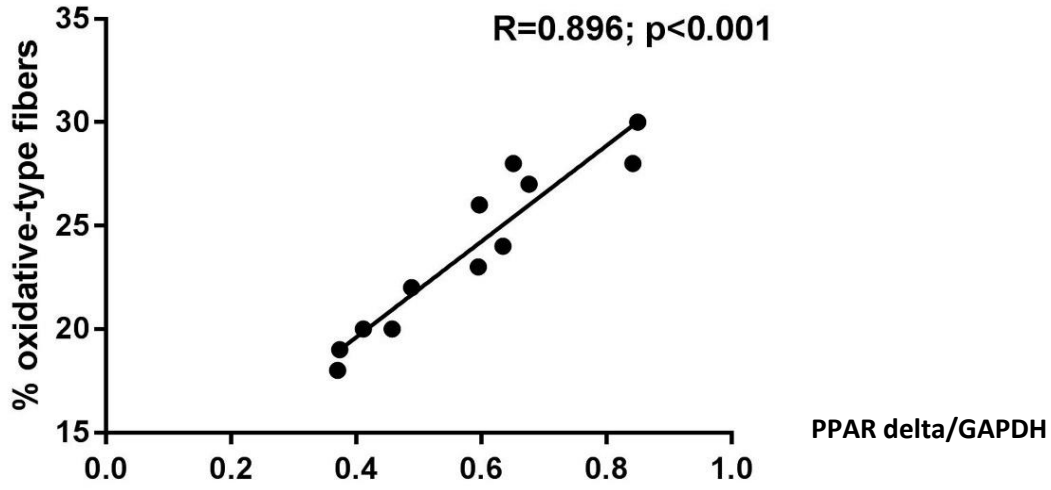

Figure 3.3 The correlation between percentage of oxidative-type muscle fibers and PPAR delta protein expression

Abbreviation: PPAR, peroxisome perforator-activated receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

3.1.4 HFD consumption induced insulin resistance in the skeletal muscle, indicated by decreased Tyr1162/1163pIR/IR ratio and Thr308pAkt/Akt ratio.

We found that the Tyr1162/1163pIR/IR ratio and Thr308pAkt/Akt ratio, which represented the degree of insulin sensitivity, were significantly lower in HFD-fed rats experiencing sedentary living, when compared with those of ND-fed rats (**Figure 3.4 A-B**). These findings indicated that HFD consumption could induce insulin resistance in the skeletal muscle.

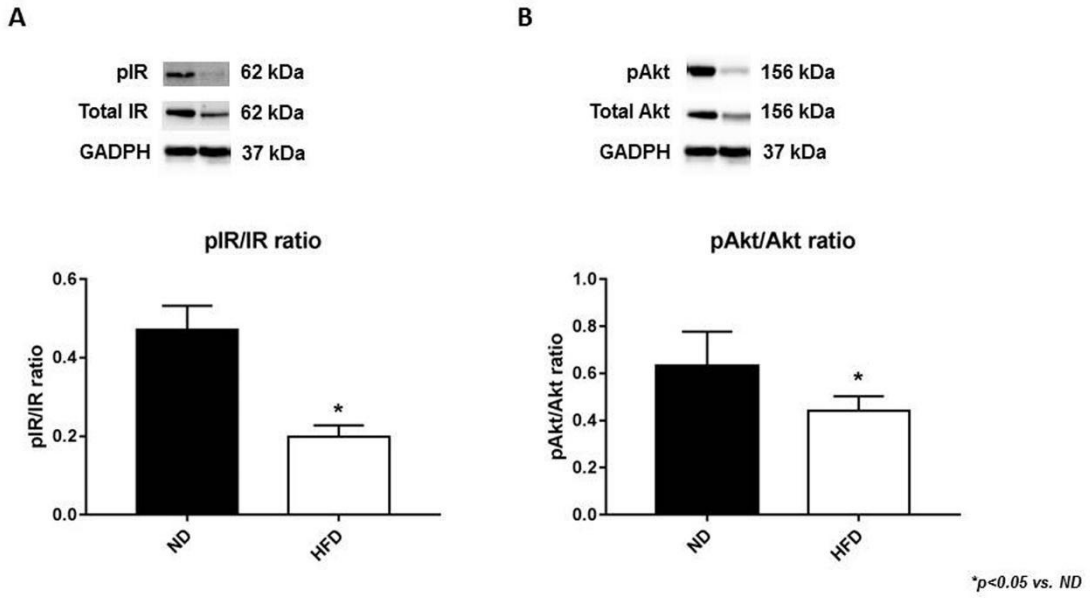

Figure 3.4 The effects of HFD consumption on skeletal insulin signaling. (A): Comparison of Tyr1162/1163pIR/total IR ratio between the study groups; (B): Comparison of Thr308pAkt/total Akt ratio between the study groups.

*, p<0.05 compared with ND group; n=6/group. ND, normal diet group; HFD, high-fat diet group; HFDEx; Tyr1162/1163pIR, tyrosine phosphorylated insulin receptor; IR, insulin receptor; Thr308 pAkt, phosphorylated protein kinase B; Akt, protein kinase B

3.1.5 Impaired skeletal muscle mitochondrial function was found in obese-insulin resistant rats and that dysfunction could be attenuated by combined ET and CR program.

We have found that skeletal muscle was one of the end-organs affected by insulin-resistant condition, and the resulting impact is an impairment of mitochondrial function in the muscle. Mitochondrial ROS production was significantly higher in HFD-fed rats with sedentary living, when compared with those of ND-fed rats (**Figure 3.5A**). This result was compatible with an increase in mitochondrial membrane potential change (**Figure 3.5B**), and mitochondrial swelling (**Figure 3.5C**).

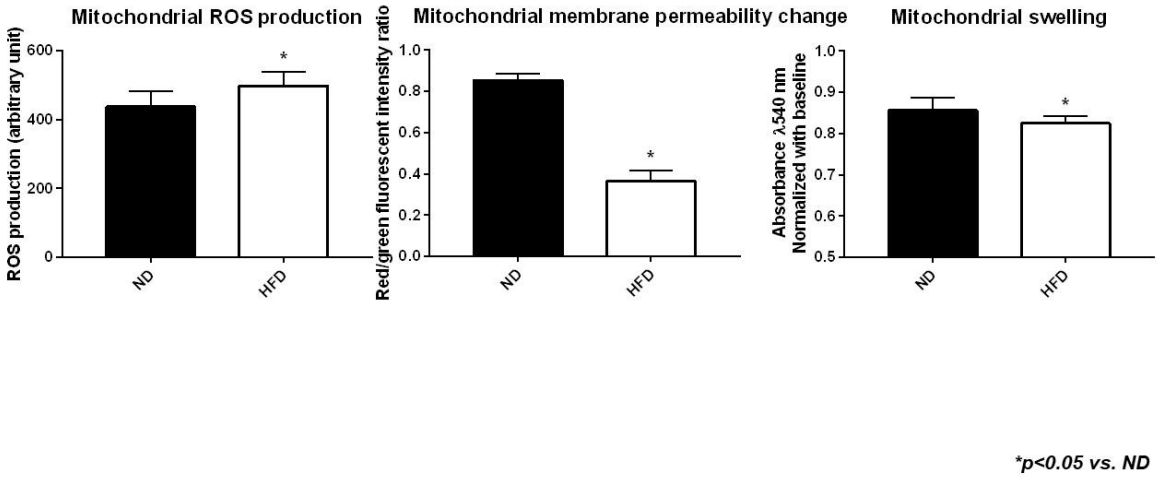

Figure 3.5 The effects of HFD consumption on skeletal muscle mitochondrial function. (A): Comparison of Mitochondrial ROS between the study groups; (B): Comparison of Mitochondrial membrane potential change between the study groups; (C): Comparison of Mitochondrial swelling between the study groups.

*, $p<0.05$ compared with ND group; n=6/group. ND, normal diet group; HFD, high-fat diet group; ROS, reactive oxygen species

3.1.6 An imbalance in mitochondrial dynamics, as shown by an increase in mitochondrial fission and a decrease in mitochondrial fusion were observed in skeletal muscles of obese-insulin resistant rats.

Mitochondrial dynamics involve a reciprocal change in the morphology between a fission and fusion stage of mitochondria. The present study demonstrated an increase in Ser616pDRP1/total DRP1 ratio and a decrease in the MFN2 protein expression of HFD-fed rats experiencing sedentary living, when compared with that of ND-fed rats (**Figure 3.6A-B**). These results indicated that HFD consumption could induce an imbalance of mitochondrial dynamics.

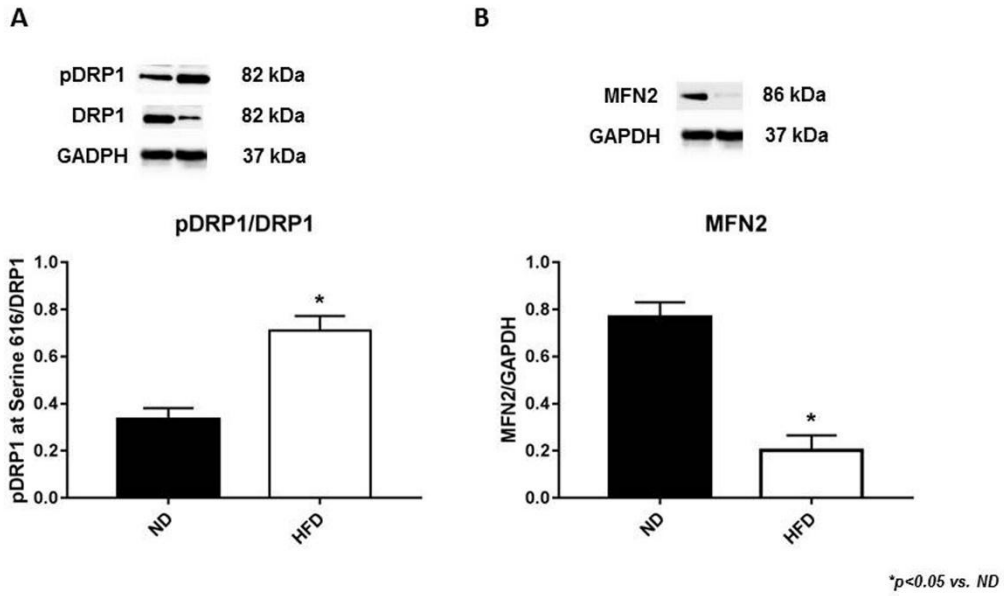

Figure 3.6 The effects of HFD consumption on skeletal muscle mitochondrial dynamics. (A): Comparison of Ser616pDRP1/total DRP1 ratio between the study groups; (B): Comparison of MFN2 protein expression between the study groups.

*, p<0.05 compared with ND group; n=6/group. ND, normal diet group; HFD, high-fat diet group; pDRP1; phosphorylated dynamin-1-like protein 1; DRP1, dynamin-1-like protein 1; MFN2, mitofusin-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

3.1.7 HFD-induced insulin resistance decreased mitochondrial biogenesis and fatty acid oxidation of skeletal muscles.

Compared with ND-fed rats, sedentary living HFD-fed rats had significantly lower PGC-1alpha and CPT1 protein expression, indicating a reduction in fatty acid oxidation metabolism in skeletal muscles (**Figure**. **3.7A and B**). These results indicated that HFD consumption could reduce decreased mitochondrial biogenesis and fatty acid oxidation of skeletal muscles.

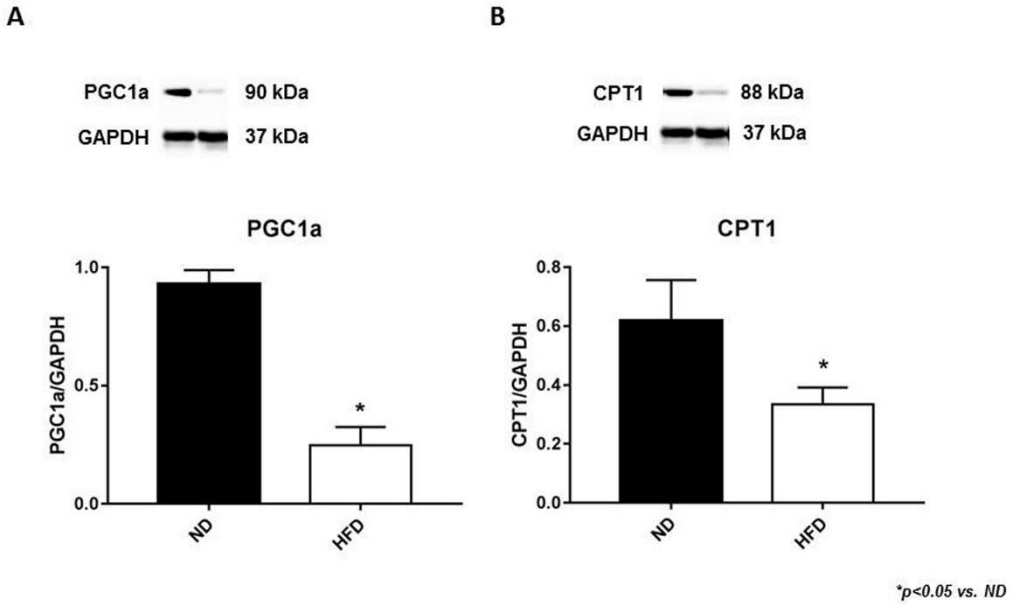

Figure 3.7 The effects of HFD consumption on skeletal muscle mitochondrial biogenesis. (A): Comparison of PGC1alpha protein expression between the study groups; (B): Comparison of CPT1 protein expression between the study groups.

*, p<0.05 compared with ND group; n=6/group. ND, normal diet group; HFD, high-fat diet group; PGC1a, peroxisome perforator-activated receptor coactivator-1 alpha; CPT1, carnitine palmitolytransferase-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

3.1.8 HFD-induced insulin resistance increased skeletal muscle apoptosis, indicated by an increase in pro-apoptotic protein Bax reciprocally with a decrease in anti-apoptotic protein Bcl-2.

Evidence has demonstrated that HFD-induced insulin resistance could enhance apoptosis in skeletal muscle tissue, by increasing pro-apoptotic protein-Bax and decreasing anti-apoptotic protein-Bcl-2 [7]. Compared with ND-fed rats, sedentary living HFD-fed rats had significantly higher Bax protein expression (**Figure 3.8A**), lower Bcl-2 protein expression (**Figure 3.8B**), and a higher Bax/Bcl-2 ratio (**Figure 3.8C**) in skeletal muscles. These results indicated that HFD consumption could induce apoptosis of skeletal muscles.

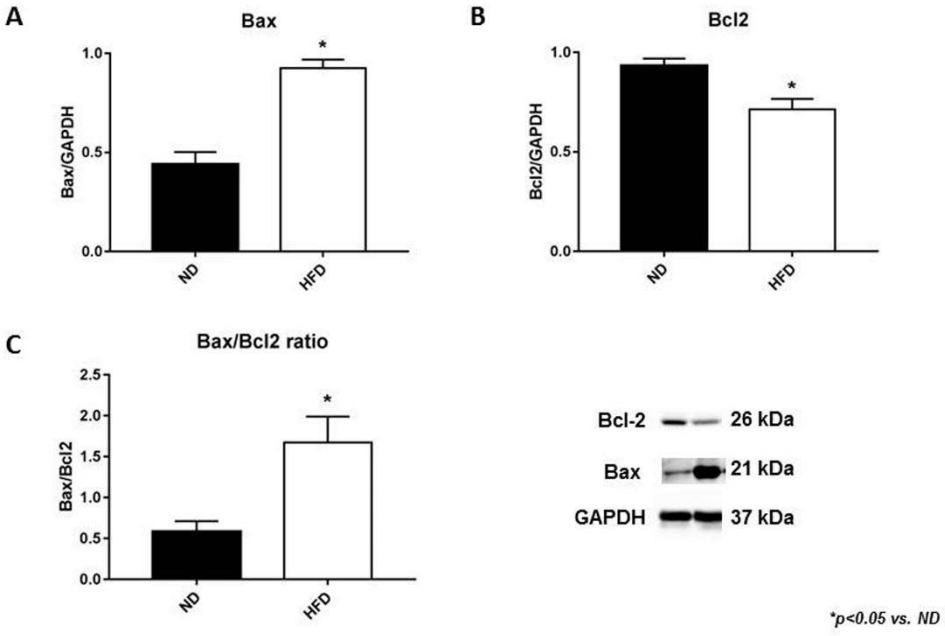

Figure 3.8 The effects of HFD consumption on skeletal muscle apoptosis. (A): Comparison of Bax protein expression between the study groups; (B): Comparison of Bcl2 protein expression between the study groups; (C): Comparison of Bax/Bcl2 ratio between the study groups.

*, p<0.05 compared with ND group; n=6/group. ND, normal diet group; HFD, high-fat diet group; Bax, Bcl2 associated X protein; Bcl2, B-cell lymphoma 2 protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

3.2 Aim 2-4: To investigate the effects of interventions (e.g. exercise training, caloric restriction or combined exercise training and caloric restriction program) on the metabolic changes and skeletal muscle structure and function in obese-insulin resistant rats

3.2.1 ET and CR improved resulting metabolic function, and combined therapies restored the metabolic parameters in HFD consumption induced obese-insulin resistant rats.

After being fed on HFD for 27 weeks, HFD-fed rats demonstrated metabolic disturbance, as indicated by obesity (increased body weight and visceral fat weight), peripheral insulin resistance, as shown by hyperinsulinemia with euglycemia, increased

HOMA index and impaired OGTT, and dyslipidemia. A two-way ANOVA, determining two type of diet (CR, no CR) an physical activity (ET, no ET), revealed a significant effect of ET and CR on body weight ($F [3, 72] = 32.68$, $p<0.001$; $F [1, 72] = 83.65$, $p<0.001$), visceral fat ($F [3, 59] = 35.18$, $p<0.001$; $F [1, 59] = 185.6$, $p <0.001$), area under the curve of OGTT ($F [3, 70] = 3.222$, $p=0.003$; $F [1, 70] = 44.51$, $p<0.001$), plasma insulin ($F [3, 44] = 10.65$, $p<0.001$; $F [1, 44] = 78.35$, $p<0.001$) and HOMA index ($F [2, 44] = 34.65$, $p<0.001$; $F [1, 44] = 72.34$, p , 0.001) (ET; CR, respectively).

The post-hoc analyses revealed that the exercise training and calorie restriction led to equally improved metabolic parameters, as indicated by decreased visceral fat, glucose intolerance from OGTT, plasma insulin, as well as HOMA index, when compared with those of HFD-fed rats experiencing sedentary living (Table 1). A combination of exercise training with calorie restriction showed the greatest benefits in the improvement of metabolic function when compared with either monotherapy (Table 1). Focusing on plasma lipid profiles, a two-way ANOVA, determining two type of diet (CR, no CR) and physical activity (ET, no ET), revealed a significant effect of ET and CR on TC ($F [3, 48] = 7.05$, $p<0.001$; $F [1, 48] = 70.57$, $p<0.001$) and LDL levels ($F [3, 54] = 3.694$, $p=0.017$; $F [1, 54] = 13.06$, $p<0.001$), but not TG ($F [3, 70] = 0.051$, $p=0.985$; $F [1, 70] = 0.340$, $p=0.562$) and HDL level ($F [3, 50] = 0.012$, $p=0.994$; $F [3, 50] = 0.329$, $p=0.804$) (ET; CR, respectively). The post-hoc analyses revealed that HFD-fed rats with all therapies had significantly lower TC and LDL levels while there was no difference in TG and HDL level, when compared with HFD-fed rats experiencing sedentary living (**Table 3.2**).

These findings suggested that: 1) HFD consumption caused an obese-insulin resistant condition; 2) Exercise training and calorie restriction shared similar effects in decreasing metabolic disturbance in obese-insulin resistant rats, and 3) a combination of ET and CR demonstrated the greatest benefits on this improvement.

Table 3.2 Effects of exercise, calorie restriction and combined therapies on metabolic parameters in HFD-fed rats

Metabolic parameters	Groups				
	ND	**HFDS**	**HFSEx**	**HFDCr**	**HFSCb**
Body weight (g)	281.15 ± 3.50	352.00 ± 4.16*	322.86 ± 6.53$^{*,+}$	295.00 ± 8.16$^{*,+}$	269.44 ± 3.27$^{+,\#}$
Visceral fat (g)	9.31 ± 0.69	28.51 ± 1.12*	20.80 ± 1.83$^{*,+}$	15.14 ± 1.06$^{*,+}$	10.14 ± 0.54$^{+,\#}$
Plasma glucose (mg/dl)	133.25 ± 3.03	139.66 ± 4.48	135.73 ± 7.12	125.41 ± 10.97	131.25 ± 7.06
Plasma insulin (ng/ml)	1.50 ± 0.17	4.76 ± 0.36*	3.34 ± 0.20$^{*,+}$	3.01 ± 0.49$^{*,+}$	1.75 ± 0.19$^{+,\#}$
Plasma glucose AUC from OGTT (AUCg)(mg/dl×min×10^4)	1.92 ± 0.07	2.57 ± 0.04*	2.31 ± 0.09$^{*,+}$	2.26 ± 0.09$^{*,+}$	1.97 ± 0.04$^{+,\#}$
HOMA index	5 ± 2.4	16 ± 2.1*	11 ± 1.1$^{*,+}$	9 ± 3.4$^{*,+}$	6 ± 0.8$^{+,\#}$
Plasma total cholesterol (mg/dl)	51.65 ± 6.79	98.55 ± 4.53*	70.33 ± 5.77$^{*,+}$	63.46 ± 2.98$^{*,+}$	56.61 ± 5.70$^{+,\#}$
Plasma triglyceride (mg/dl)	61.30 ± 7.39	70.38 ± 5.65	66.21 ± 7.75	68.37 ± 8.45	63.07 ± 7.47
HDL cholesterol (mg/dl)	19.24 ± 1.49	22.64 ± 1.35	22.24 ± 0.94	22.64 ± 0.82	22.35 ± 0.81

Table 3.2 Effects of exercise, calorie restriction and combined therapies on metabolic parameters in HFD-fed rats (continued)

LDL cholesterol (mg/dl)	19.29 ± 4.66	57.89 ± 4.66*	30.27 ± 6.18*,+	20.26 ± 3.56*,+	28.97 ± 8.89+,#

*, $p<0.05$ compared with ND; +, $p<0.05$ compared with HFDS; #, $p<0.05$ compared with HFDEx and HFDCr groups; n=6/group;

AUC, area under the curve; OGTT, oral glucose tolerance test; HOMA, homeostatic model assessment; HDL, high density lipoprotein; LDL, low density lipoprotein

ND, normal diet group; HFDS, high-fat diet with sedentary living; HFDEx, high-fat diet with exercise; HFDCr, high-fat diet with caloric restriction; HFDCb, high-fat diet with combined therapies

3.2.2 Enhanced early fatigability and decreased PPAR delta protein expression in gastrocnemius muscles of HFD-fed rats were attenuated by ET and combined therapies.

HFD-fed rats in the sedentary living group demonstrated a significant decrease in time-to-fatigue duration, when compared with those of ND-fed rats. A two-way ANOVA, determining two type of diet (CR, no CR) and physical activity (ET, no ET), revealed a significant effect of ET ($F [1, 14] = 44.88$, $p<0.001$), but not CR ($F [1, 14] = 3.878$, $p=0.069$) on time-to-fatigue duration. The post-hoc analyses also revealed that a fatigue-vulnerable property was absent in groups with ET and combined therapies. In addition, HFD-fed rats with combined therapies took a significantly longer time to 50% fatigue than those in the HFD-fed groups with ET (**Figure 3.9A and B**). It is noticeable that the HFD-fed rats with combined therapies were the only group that had no difference in time to 50% fatigue when compared with ND-fed rats. This result suggested that only ET, not CR, attenuated skeletal muscle fatigability and combined therapies prevented fatigability in the obese-insulin resistant condition.

Corresponding with the results of muscle contraction study, the results of the western blot analysis also showed a significant decrease in PPAR delta protein expression in sedentary living HFD-fed rats, when compared with those of ND-fed rats (**Figure 3.9 C**). A two-way ANOVA, determining two type of diet (CR, no CR) and physical activity (ET, no ET), revealed a significant effect of ET ($F [1, 8] = 44.31$, $p=0.010$), but not CR ($F [1, 8] = 1.087$, $p=0.109$) on PPAR delta protein expression. The post-hoc analyses also revealed that a decrease in PPAR delta protein expression was attenuated in HFD-fed rats with ET and with combined therapies. As expected, PPAR delta protein expression of HFD-fed rats with combined therapies was significantly higher than those of HFD-fed rats with ET **(Figure 3.9 C).** It is significant that the combined therapies group was the only group that had no difference in PPAR delta protein expression when compared with those of ND-fed rats.

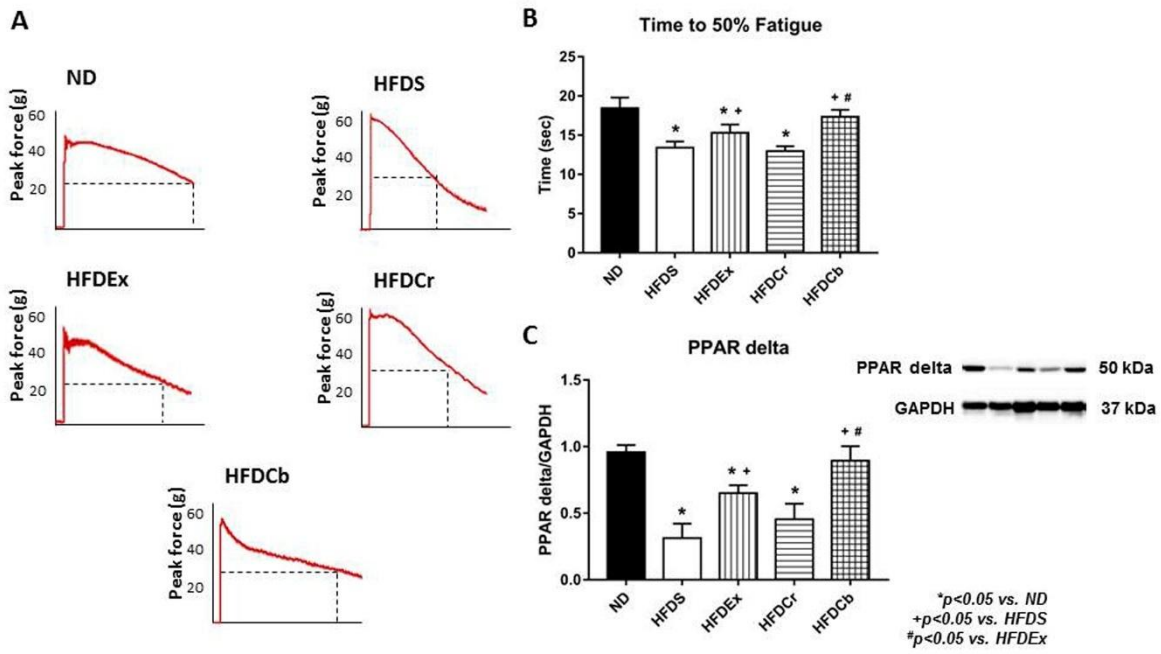

Figure 3.9 The effects of exercise, caloric restriction and a combined program on skeletal muscle fatigability and PPAR delta protein expression in HFD-induced obese-insulin resistant rats. (A): Muscle contraction study tracing in each study group; (B): Comparison of time to fatigue parameter of muscle contraction studies between the study groups; (C): Comparison of PPAR delta protein expression among the study group.

*, $p<0.05$ compared with ND group; †, $p<0.05$ compared with HFDS group; #, $p<0.05$ compared with HFDEx group; n=6/group ND, normal diet group; HFDS, high-fat diet with sedentary living group; HFDEx, high-fat diet with exercise group; HFDCr, high-fat diet with caloric restriction group; HFDCb, high-fat diet with combined therapies group; PPAR, peroxisome perforator-activated receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

3.2.3 Both ET and CR improved insulin resistance and the combined ET and CR program restored insulin sensitivity in skeletal muscles in HFD-induced obese-insulin resistant rats.

It has been proposed that ET and CR increase insulin sensitivity in skeletal muscles. A two-way ANOVA, determining two type of diet (CR, no CR) and physical activity (ET, no ET), revealed a significant effect of ET ($F [1, 8] = 49.33$, $p<0.001$) and

CR (F [1, 8] = 91.76, p<0.001) on Tyr1162/1163pIR/IR ratio. A two-way ANOVA also revealed a significant effect of ET (F [1, 8] = 8.735, p=0.018) and CR (F [1, 8] = 8.444, p=0.020) on Thr308pAkt/Akt ratio. The post-hoc test also revealed that rats with either ET or CR had a significantly higher Tyr1162/1163pIR/IR ratio and Thr308pAkt/Akt ratio, when compared with the sedentary living HFD-fed rat group. The combined therapy HFD-fed rat group had the highest Tyr1162/1163pIR/IR ratio and Thr308pAkt/Akt ratio out of all groups (**Figure 3.10A-B**). However, the combined therapy was the only group that had no difference in the Tyr1162/1163pIR/IR ratio and Thr308pAkt/Akt ratio when compared with those of ND-fed rats. These findings suggest that both ET and CR in the obese-insulin resistant condition improved the insulin sensitivity in the skeletal muscles and the combined ET and CR program in HFD-fed rats restored it.

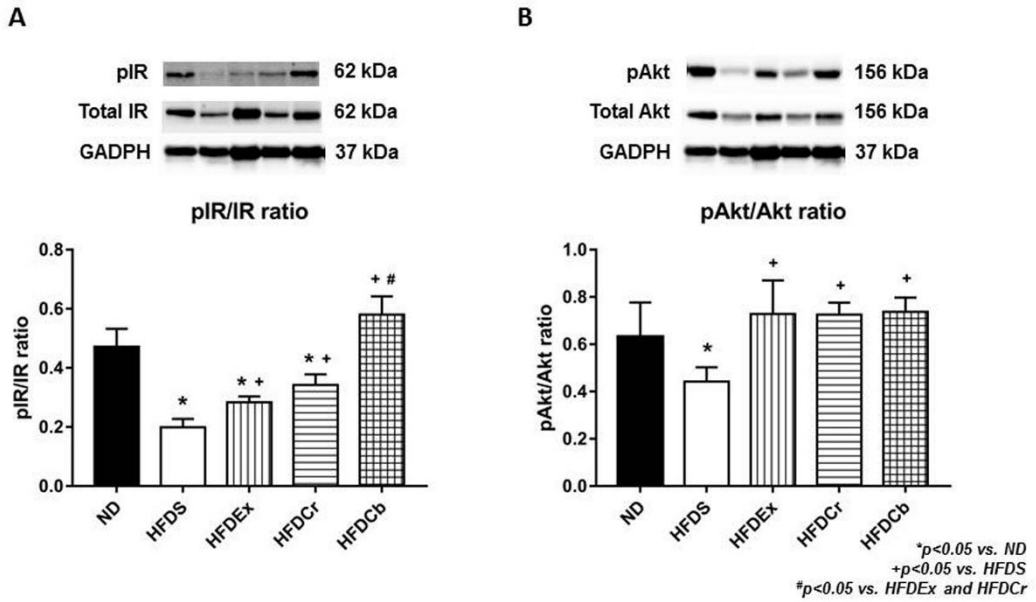

Figure 3.10 The effects of exercise, caloric restriction and combined program on skeletal insulin signaling in HFD-induced obese-insulin resistant rats. (A): Comparison of Tyr1162/1163pIR/total IR ratio among the study groups; (B): Comparison of Thr308pAkt/total Akt ratio among the study groups.

*, p<0.05 compared with ND group; †, p<0.05 compared with HFDS group; #, p<0.05 compared with HFDEx and HFDCr group; n=6/group ND, normal diet group; HFDS,

high-fat diet with sedentary living group; HFDEx, high-fat diet with exercise group; HFDCr, high-fat diet with caloric restriction group; HFDCb, high-fat diet with combined therapies group; Tyr1162/1163pIR, tyrosine phosphorylated insulin receptor; IR, insulin receptor; Thr308 pAkt, phosphorylated protein kinase B; Akt, protein kinase B

3.2.4 Only combined ET and CR program attenuated an impairment of skeletal muscle mitochondrial function in obese-insulin resistant rats.

We have found that there was an impairment of mitochondrial function in the skeletal muscle of obese-insulin resistant rats. A two-way ANOVA, determining two type of diet (CR, no CR) and physical activity (ET, no ET), revealed a significant effect of ET ($F [1, 10] = 7.898$, $p<0.020$) and CR ($F [1, 14] = 14.6$, $p=0.003$) on mitochondrial ROS. It also revealed a significant effect of ET and CR on mitochondrial membrane potential change and mitochondrial swelling ($F [2, 14] = 76.5$, $p<0.001$; $F [1, 14] = 10.24$, $p=0.006$ and $F [1, 9] = 11.16$, $p<0.009$; $F [1, 9] = 10.64$, $p<0.010$ respectively). However, the post-hoc analyses demonstrated that ET or CR program alone did not attenuate any parameters pertinent to mitochondrial dysfunction, but the combined ET and CR program led to restoration of mitochondrial function, represented by a decrease in mitochondrial ROS production, and membrane potential change, as well as mitochondrial swelling (**Figure 3.11 A, B and C**).

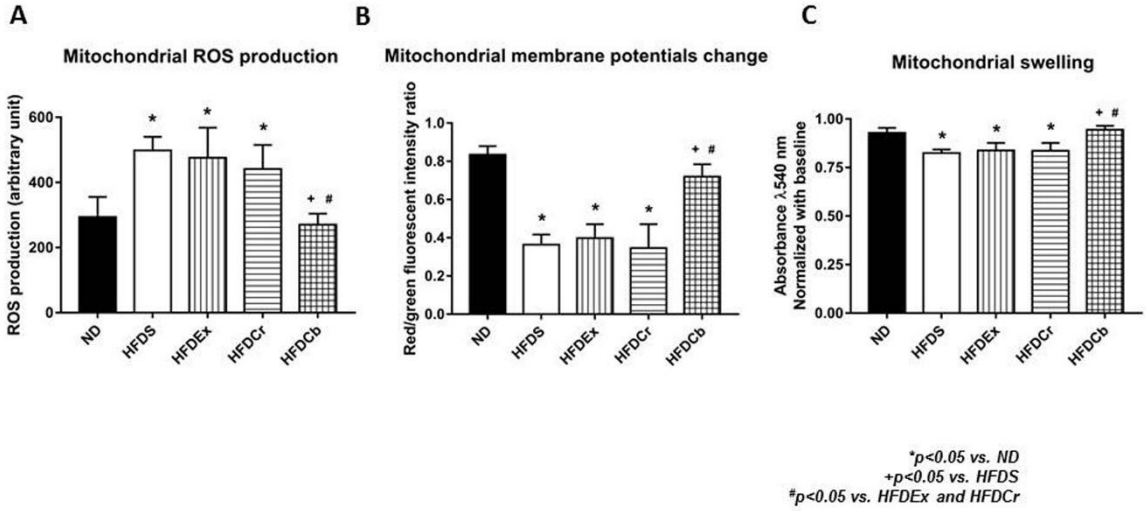

Figure 3.11 The effects of exercise, caloric restriction and combined program on skeletal muscle mitochondrial function in HFD-induced obese-insulin resistant rats. (A): Comparison of Mitochondrial ROS among the study groups; (B): Comparison of Mitochondrial membrane potential change among the study groups; (C): Comparison of Mitochondrial swelling among the study groups.

*, p<0.05 compared with ND group; †, p<0.05 compared with HFDS group; #, p<0.05 compared with HFDEx and HFDCr group; n=6/group ND, normal diet group; HFDS, high-fat diet with sedentary living group; HFDEx, high-fat diet with exercise group; HFDCr, high-fat diet with caloric restriction group; HFDCb, high-fat diet with combined therapies group; ROS, reactive oxygen species

3.2.5 An imbalance in mitochondrial dynamics in skeletal muscle of obese-insulin resistant rats, as shown by an increase in mitochondrial fission and a decrease in mitochondrial fusion were attenuated by either ET or CR, and the dynamics were restored by combined therapies.

Mitochondrial dynamics involve a reciprocal change in the morphology between a fission and fusion stage of mitochondria. The present study demonstrated an imbalance between mitochondrial fission and fusion occurring in HFD-induced obese-insulin resistant rats. A two-way ANOVA, determining two type of diet (CR, no CR)

and physical activity (ET, no ET), revealed a significant effect of ET ($F [1, 8] = 64.73$, $p<0.001$) and CR ($F [1, 8] = 36.1$, $p<0.001$) on Ser616pDRP1/total DRP1 ratio. A two-way ANOVA also revealed a significant effect of ET ($F [1, 8] = 120$, $p<0.001$) and CR ($F [1, 8] = 109.4$, $p<0.001$) on MFN protein expression. The post-hoc analyses demonstrated that all interventions, i.e. ET, CR and combined therapies, led to a decreased Ser616pDRP1/total DRP1 ratio and increased MFN2 protein expression in HFD-fed rats. In addition, the Ser616pDRP1/total DRP1 ratio and MFN2 expression of HFD-fed rats with combined therapies were not significantly different from those of ND-fed rats (**Figure 3.12 A-B**). These results indicated that ET and CR in rats with the obese-insulin resistant condition improved mitochondrial dynamics and combined therapies restored it.

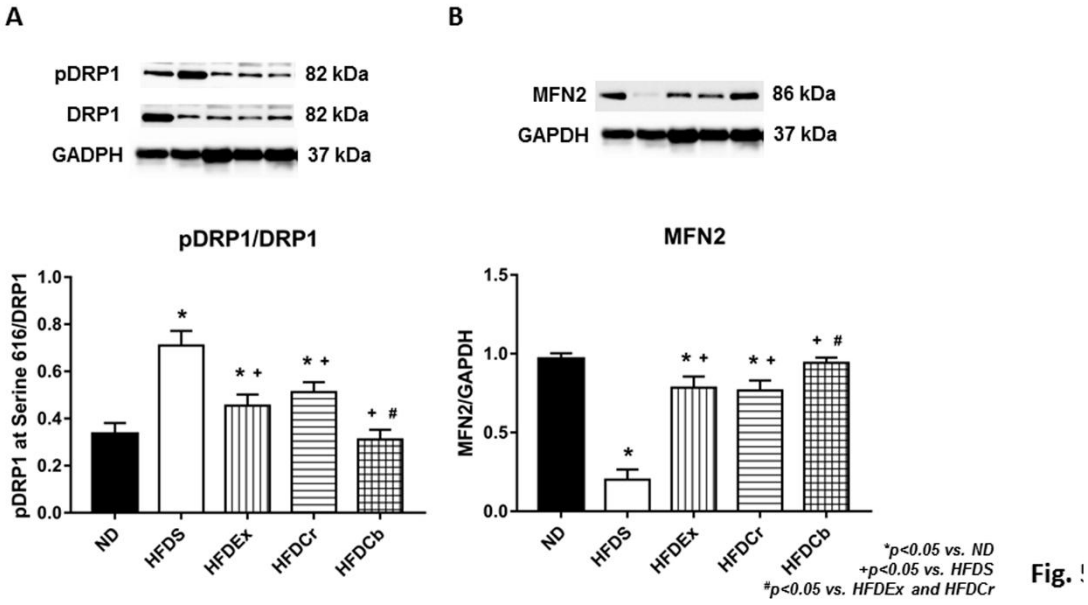

Figure 3.12 The effects of exercise, caloric restriction and combined program on skeletal muscle mitochondrial dynamics in HFD-induced obese-insulin resistant rats. (A): Comparison of Ser616pDRP1/total DRP1 ratio among the study groups; (B): Comparison of MFN2 protein expression between the study groups.

*, $p<0.05$ compared with ND group; †, $p<0.05$ compared with HFDS group; #, $p<0.05$ compared with HFDEx and HFDCr group; n=6/group ND, normal diet group; HFDS, high-fat diet with sedentary living group; HFDEx, high-fat diet with exercise group;

HFDCr, high-fat diet with caloric restriction group; HFDCb, high-fat diet with combined therapies group; pDRP1; phosphorylated dynamin-1-like protein 1; DRP1, dynamin-1-like protein 1; MFN2, mitofusin-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

3.2.6 Decreased mitochondrial biogenesis and fatty acid oxidation in skeletal muscle of HFD-induced obese-insulin resistant rats were attenuated by either ET or CR, and combined therapies restored them.

Compared with ND-fed rats, sedentary living HFD-fed rats had significantly lower PGC-1alpha and CPT1 protein expression, indicating a reduction in fatty acid oxidation metabolism in skeletal muscles. A two-way ANOVA, determining two type of diet (CR, no CR) and physical activity (ET, no ET), revealed a significant effect of ET and CR on PGC-1alpha and CPT1 protein expression ($F [1, 8] = 33.18$, $p<0.001$; $F [1, 8] = 23.8$, $p=0.001$ and $F [1, 8] = 16.89$, $p=0.003$; $F [1, 8] = 14.2$, $p<0.006$ respectively). The post-hoc analyses demonstrated that HFD-fed rats undergoing either ET, CR, or combined therapies had significantly higher PGC1alpha and CPT1 protein expression when compared with sedentary living HFD-fed rats. In addition, the expression of PGC1alpha and CPT1 in the combined therapies group was not significantly different from those of ND-fed rats (**Figure 3.13 A and B**). These results indicated that ET and CR in rats with the obese-insulin resistant condition improved mitochondrial biogenesis as well as the fatty acid oxidation capacity of skeletal muscles, and a combined ET and CR program restored them.

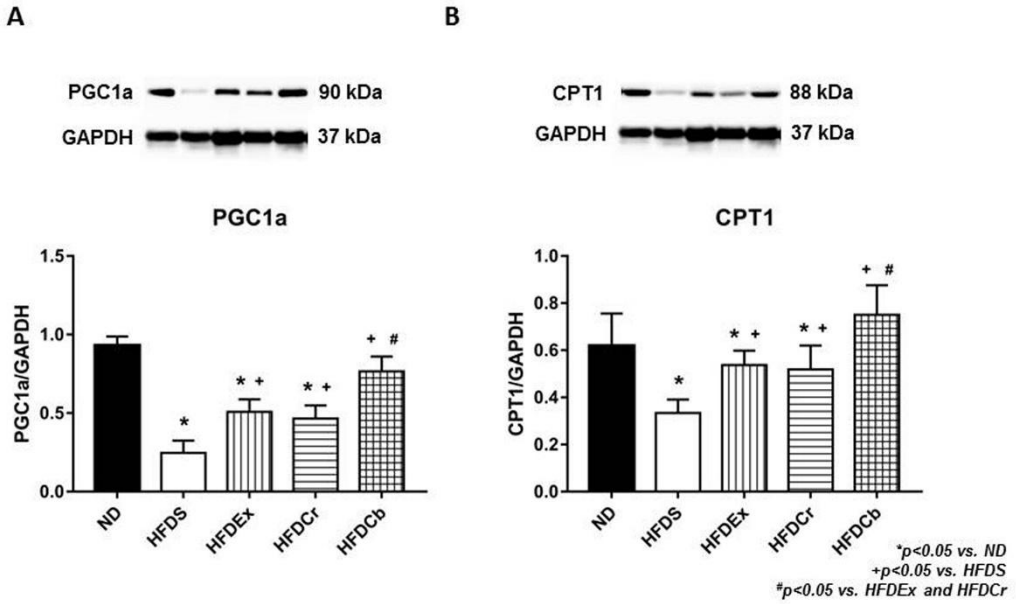

Figure 3.13 The effects of exercise, caloric restriction and combined program on skeletal muscle mitochondrial biogenesis in HFD-induced obese-insulin resistant rats. (A): Comparison of PGC1alpha protein expression among the study groups; (B): Comparison of CPT1 protein expression among the study groups.

*, p<0.05 compared with ND group; †, p<0.05 compared with HFDS group; #, p<0.05 compared with HFDEx and HFDCr group; n=6/group ND, normal diet group; HFDS, high-fat diet with sedentary living group; HFDEx, high-fat diet with exercise group; HFDCr, high-fat diet with caloric restriction group; HFDCb, high-fat diet with combined therapies group; PGC1a, peroxisome perforator-activated receptor coactivator-1 alpha; CPT1, carnitine palmitolytransferase-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

3.2.7 Skeletal muscle apoptosis in HFD-induced obese-insulin resistant rats was attenuated by ET and CR, while combined therapies restored it.

The effects of exercise and caloric restriction were also demonstrated in this study. A two-way ANOVA, determining two type of diet (CR, no CR) and physical activity (ET, no ET), revealed a significant effect of ET and CR on Bax ($F [1, 8] = 36.28$, $p<0.001$; $F [1, 8] = 34.16$, $p=0.001$, respectively) but not Bcl-2 protein expression ($F [1, 8] = 3.308$, $p<0.106$; $F [1, 8] = 3.739$, $p=0.089$, respectively). It also

revealed a significant effect of ET (F [1, 8] = 12.47, p=0.008) and CR (F [1, 8] = 13.65, p=0.006) on Bax/Bcl-2 ratio. The post-hoc analyses demonstrated that HFD-fed rats in the ET or CR groups had significantly lower Bax protein expression, as well as significantly higher Bcl2 protein expression, when compared with those of sedentary living HFD-fed rats. A significant decrease in the Bax/Bcl-2 ratio in combined therapy HFD-fed rats was observed when compared with that of the monotherapy groups and the Bax/Bcl2 ratio of combined therapies group was not different from that of ND-fed rats (**Figure 3.14 A, B and C**). These results indicated that ET and CR attenuated apoptosis in skeletal muscles of HFD-fed rats and combined therapies restored it.

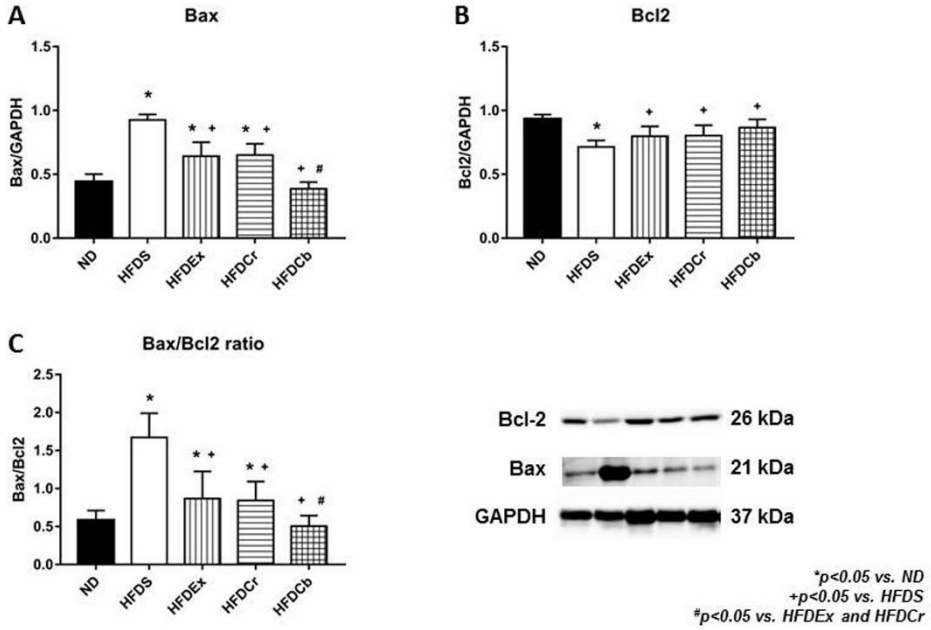

Figure 3.14 The effects of exercise, caloric restriction and combined program on skeletal muscle apoptosis in HFD-induced obese-insulin resistant rats. (A): Comparison of Bax protein expression among the study groups; (B): Comparison of Bcl2 protein expression among the study groups; (C): Comparison of Bax/Bcl2 ratio among the study groups; (D): the representative blots from all groups.

*, p<0.05 compared with ND group; †, p<0.05 compared with HFDS group; #, p<0.05 compared with HFDEx and HFDCr group; n=6/group ND, normal diet group; HFDS, high-fat diet with sedentary living group; HFDEx, high-fat diet with exercise group; HFDCr, high-fat diet with caloric restriction group; HFDCb, high-fat diet with

combined therapies group; Bax, Bcl2 associated X protein; Bcl2, B-cell lymphoma 2 protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

CHAPTER 4

Discussion and Conclusion

4.1 Discussion

The major findings of the present study are as follows: 1) long-term HFD consumption led to obese-insulin resistance and pathological changes in skeletal muscles, including insulin resistance, enhanced early fatigability, increased apoptosis and impaired mitochondrial function, as well as imbalanced mitochondrial dynamics and decreased biogenesis; 2) ET in the induced obese-insulin resistant condition improved metabolic function, insulin signaling, fatigability, apoptosis, mitochondrial biogenesis and mitochondrial dynamics of skeletal muscles; 3) CR in the induced obese-insulin resistant condition also improved metabolic function, insulin signaling, apoptosis and mitochondrial dynamics of skeletal muscles; and 4) combined ET and CR therapies in the induced obese-insulin resistant condition reversed those pathological conditions in skeletal muscles. These major findings are summarized in Table 4.1

Table 4.1 The effect of obesity, exercise training, caloric restriction, and combined program on skeletal muscle function parameters.

Condition	Peripheral insulin resistance	Muscle insulin resistance	Muscle apoptosis	Balance of muscle mitochondrial dynamics	Muscle mitochondrial biogenesis	Muscle fatigue resistance	Muscle PPAR delta (% oxidative type fiber)	Muscle mitochondrial function
Obesity	↑	↑	↑	↓	↓	↓	↓	↓
ET	↓	↓	↓	↑	↑	↑	↑	↔
CR	↓	↓	↓	↑	↑	↔	↔	↔
Combined ET + CR	↓↓	↓↓	↓↓	↑↑	↑↑	↑	↑	↑

PPAR, Peroxisome perforator-activated receptor*;* ET, Exercise training; CR, Caloric restriction

Contractile function is one of the most important functions of skeletal muscle. Although it is dependent on several parameters, the parameter that has the most significant effect on human mobility is fatigability of tetanic contraction [87]. Fatigability is determined by many factors, including the type of muscle fiber. Skeletal muscle fibers can be roughly divided into two types, namely oxidative type and glycolytic type muscle fibers. Oxidative-type fibers have a lower fatigability than glycolytic-type fibers [5]. There is evidence to demonstrate that the fatigability of each skeletal muscle is correlated with the percentage of oxidative-type fiber in the muscle [5]. One of the factors determining the production of oxidative-type fiber is PPAR delta [93, 94]. The percentage of oxidative-type fiber in each muscle has been associated with the amount of PPAR delta protein expression in the muscle [93, 94], which also demonstrated in this study. Therefore, enhanced early fatigability of HFD-fed rats with sedentary living might be as a result of a decrease in the percentage of oxidative-type muscle fiber, which shows a correlation with a decrease in PPAR delta protein expression of the HFD-fed rats undergoing sedentary living, when compared with those of ND-fed rats.

We also demonstrated that HFD-fed rats with sedentary living had impaired mitochondrial function and imbalance of mitochondrial dynamics, as well as increased cell apoptosis, all of which showed a correlation with the degree of insulin-resistant condition. Several previous studies have demonstrated that mitochondrial dysfunction [18, 48], imbalance of mitochondrial dynamics [16, 120], and increased cell apoptosis [7] are associated with an insulin-resistant condition in skeletal muscles.

The ET in this study was the endurance exercise program. In this study, HFD-fed rats with ET had significantly lower peripheral and skeletal muscle insulin resistance, when compared with those that were sedentary living. HFD-fed rats with ET also showed an improvement in metabolic function, insulin sensitivity, muscle fatigability, mitochondrial biogenesis and balance in mitochondrial dynamics, as well as a decreased level in apoptosis of skeletal muscles. These findings might be as a consequence of an activation of adenosine monophosphate kinase (AMPK) during exercise training. AMPK is the major signaling protein responsible for energy control of the body cells [88]. There is evidence to demonstrate that endurance exercise activates

AMPK by causing energy deprivation, and the beneficial effects of exercise are dependent on its activation [86]. AMPK is an upstream signaler of PGC1alpha, which is a coactivator of several proteins for many important metabolisms including CPT1 for fatty acid oxidation [209] and PPAR delta for type 1 skeletal muscle fiber transformation [86].

Another beneficial effect of ET in this study was an improvement in the balance of mitochondrial biogenesis, as indicated by a decrease in mitochondrial fission markers, which occurred simultaneously with an increase in mitochondrial fusion markers. These results were compatible with the result from the previous studies, which also demonstrated a decrease in mitochondrial fission markers [181], as well as an increase in mitochondrial fusion markers [180] after ET. However, the mechanism regarding the effect of exercise on improving the balance of mitochondrial dynamics is still elusive. Although this beneficial effect of exercise training was compatible with the results from the previous studies, this study is the first study that demonstrated the reciprocal change between mitochondrial fission and fusion makers in the same study model.

Apoptosis is programmed cell death, which mostly results from mitochondrial dysfunction. In this study, a decrease in pro-apoptotic proteins and an increase in anti-apoptotic proteins were demonstrated in HFD-fed rats with ET, when compared with those undergoing sedentary living. The mechanism of an exercise-induced reduction of apoptosis might be associated with an improvement in the insulin-resistant condition after ET. This result was different to the results of a previous study [53]. That study in obese-Zucker rats demonstrated that after 9 weeks of endurance exercise (treadmill running), there was no change in apoptosis-related markers, specifically Bax, Bcl2 and the Bax:Bcl2 ratio, in the skeletal muscle of obese rats, when compared with the lean controls [53]. These different findings might be due to the difference in obese modes. Zucker rats developed the insulin- resistant condition from a genetic defect, resulting in prolonged hyperinsulinemia, while the HFD-induced insulin-resistant condition has a shorter period of onset of the hyperinsulinemic condition.

The CR in this study provided 60% of the basal calorie requirement. Earlier evidence has demonstrated that CR could attenuate peripheral insulin resistance and

increase insulin signaling in skeletal muscle tissue [34, 35]. Some of our findings were compatible with the previous studies in that HFD-fed rats with CR demonstrated a significantly lower peripheral and skeletal muscle insulin resistance. HFD-fed rats with CR also exhibited increased skeletal muscle insulin sensitivity, reduced apoptosis and improved mitochondrial dynamics, as well as mitochondrial biogenesis. The mechanism behind these positive results is not clear but might be in association with an improvement in insulin sensitivity. It has been proposed that the underlying mechanism of CR behind improvement in insulin sensitivity is related to the activation of AMPK, which might result from an energy-deprivation stage during caloric restriction [144]. Although we found an increase in CPT1, which is a part of the downstream signaling mechanism of AMPK via PGC-1alpha, we did not find any difference in PPAR delta protein expression or muscle fatigability in HFD-rats with CR, when compared with those undergoing sedentary living. These findings were consistent with the results from a previous study, demonstrating no change in percentage of type 1 muscle fiber of the subjects with caloric restriction, when compared with those without caloric restriction [54]. Since PGC1alpha is induced by several mechanisms, such as an activation of AMPK from energy deprivation as well as an induction of Ca-dependent signaling (CaMKIV, calcineurin) from repetitive muscle contraction) [87]. Distinguished from ET, which induces not only energy deprivation but also repetitive muscle contraction, CR induced only energy deprivation. This might be responsible for the inconsistent findings between the effect of ET and CR on an induction of PPAR delta protein expression and fatigability. We also found that HFD-fed rats with CR had a lower apoptotic process, when compared with those undergoing sedentary living. This result corresponded with the results from a previous study, which demonstrated CR preventing apoptosis in rat skeletal muscle [35]. The possible mechanism of decreased apoptosis of CR might be associated with an improvement in insulin sensitivity [36, 70].

The combined CR and ET therapy in this study included both endurance exercise and 60% caloric restriction program. The study demonstrated that a combination of the therapies resulted in the greatest benefit on almost all of parameters pertaining to the skeletal muscles. The beneficial results on skeletal muscles from the three interventions in obese-insulin resistant condition could be summarized into three main findings. Firstly, the incidence of positive responses to ET and combined

therapies, but not CR, were observed. Secondly, the combined therapies had the synergistic effects from ET and CR. Thirdly, some favorable responses were found only in the combined therapy groups. These different responses from interventions could reflect the underlying mechanisms of each intervention.

The parameters, which were positive responses to ET and the combined therapies, but not CR, were skeletal muscle fatigability and PPAR delta protein expression. The possible mechanisms of these differences could the results from the repetitive muscle contractions in ET and combined therapy groups, but not in CR, led to activation of the Ca-dependent signaling pathway. The next parameters, from which the combined therapies had the synergistic effects from ET and CR, were increased peripheral and skeletal muscle insulin sensitivity, mitochondrial biogenesis, and mitochondrial dynamics, as well as decreased apoptosis. We propose that a combination of ET and CR therapy has a greater beneficial effect than ET or CR alone in term of enhancing the balance of mitochondrial dynamics, and improving mitochondrial biogenesis, as well as attenuating the rate of apoptosis in skeletal muscles. These improvements led to the restoration of peripheral and skeletal muscle insulin sensitivity. The parameters in the third group, specifically the beneficial effects only seen in the combined therapy group, were those relating to mitochondrial function, including attenuation of mitochondrial ROS, mitochondrial membrane potential, and mitochondrial swelling. These exclusive effects might be associated with the synergistic activation of AMPK signaling from both the ET and CR program (86, 123). We proposed that the combined therapies could induce activation of the AMPK pathway at an adequate rate, at a rate at which the ET or CR therapy alone could not adequately maintain. The correlations, as well as the proposed mechanisms of obesity, exercise training, caloric restriction, and combined program affecting skeletal muscle function are summarized in **Figure 4.1**.

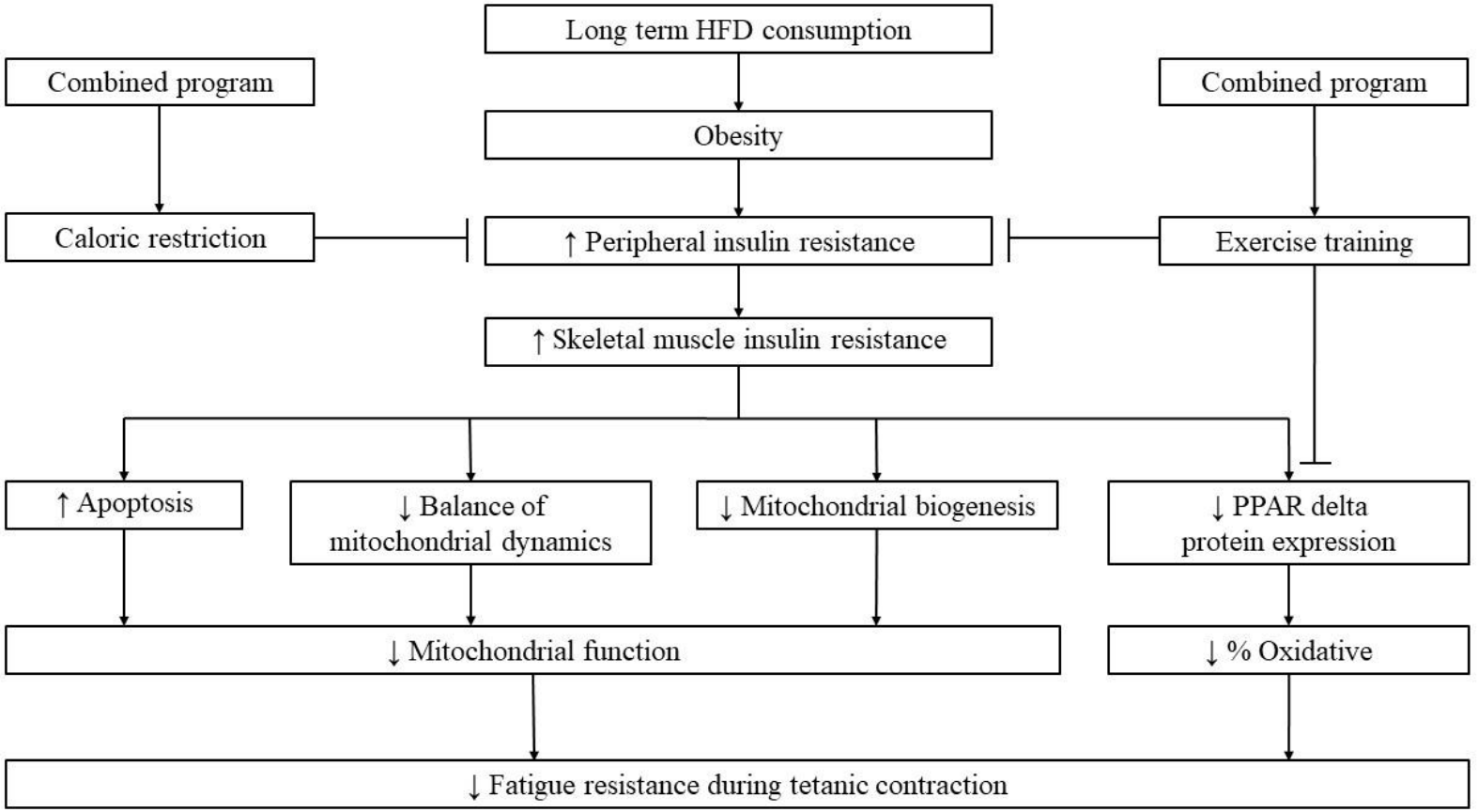

Figure 4.1 The schematic diagram presenting the correlations and the proposed mechanisms of obesity, exercise training, caloric restriction, and combined program affecting skeletal muscle function

Long-term high-fat diet consumption induces obesity. Obesity causes peripheral insulin resistance and eventually enhances insulin resistance in skeletal muscle. Skeletal muscle insulin resistance induces apoptosis, imbalances mitochondrial dynamics as well as decreases mitochondrial biogenesis. These changes eventually result in a reduction in mitochondrial function. In other hand insulin resistance reduces the PPAR delta protein expression of skeletal muscle, resulting in a reduction in the percentage of oxidative-type muscle fiber. Both the reduction of mitochondrial function and the decrease of percentage of oxidative-type muscle fiber eventually result in an early fatigability of the skeletal muscle. Caloric restriction causes an energy deprivation state, resulting in AMPK activation. Increased AMPK signaling improves insulin sensitivity and eventually increases mitochondrial function. However, only caloric restriction could not induce enough PPAR delta expression for improving the percentage of oxidative-type muscle fiber and early fatigability state. Exercise induces both AMPK signaling and PPAR delta expression. Therefore, it could improve both insulin sensitivity and mitochondrial function, as well as increase the percentage of oxidative-type muscle fiber and attenuates the early fatigability state. Combined exercise training and caloric restriction could induce both AMPK signaling and PPAR delta expression in enough levels, resulting the additive effect on improving insulin sensitivity, mitochondrial function, as well as increasing the percentage of oxidative-type muscle fiber and attenuating the early fatigability state to the skeletal muscle.

HFD, High-fat diet; PPAR, Peroxisome perforator-activated receptor; AMPK: adenosine monophosphate-activated protein kinase; →: Activation; - - |: Inhibition

Another point regarding the metabolic parameters is the weight of rats in HFDEx group. Average weight of rats HFDEx group had a trend to lower than rats in HFDCr and HFDCb groups. This might be result from an increase in food intake of rats in HFDEx group, which did not receive the caloric restriction program. Evidence demonstrated that exercise could induce more food intake (212). However, the stress from our negative exercise program (rats would have punishments, which were electrical shock, when they did not perform exercise) might be another cause of elevated food intake since previous evidence demonstrated that the stress-induced behavior of rodents is increased food intake (212). Therefore, applying more in detail on recording the amount of food intake, as well as evaluating the stress-induced behavior such as elevated plus maze test for anxiety behavior, forced swim test for depression, as well as sucrose preference test for anhedonia. Finally, one of the important factors affecting during exercise is autonomic nervous control. Evidence demonstrated that acute exercise induced sympathetic activity (213), whereas chronic exercise elevated parasympathetic activity (214). It was also demonstrated that beta 2 adrenergic receptor type of sympathetic nervous system could induced vasodilation in skeletal muscle (215). Therefore, it could be proposed that the beneficial effects of exercise might be associated with the autonomic nervous control. Further study, evaluating heart rate variability and/or postganglionic muscle sympathetic nerve activity to examine the functions of autonomic nervous system, is needed.

4.2 Conclusion

Long-term consumption of HFD induced peripheral insulin resistance, and decreased cellular insulin signaling, in addition to increasing fatigability, apoptosis and mitochondrial dysfunction in skeletal muscles. ET alone improved cellular insulin signaling, fatigability, apoptosis, mitochondrial biogenesis and mitochondrial dynamics in skeletal muscles. CR alone improved cellular insulin signaling, mitochondrial biogenesis, apoptosis and mitochondrial dynamics in skeletal muscles. A combination of ET and CR therapies improved skeletal muscle fatigability in comparison to ET therapy. In addition, the combined therapies had additive effects on improving insulin signaling, apoptosis, mitochondrial biogenesis and mitochondrial dynamics in skeletal muscles. Furthermore, a combination of ET and CR therapies caused an exclusive effect on mitochondrial function, which ET or CR therapy alone could not instigate. These

results demonstrate the beneficial synergistic effects of the combined ET and CR therapies in an obese-insulin resistant condition on improving the function of skeletal muscle and mitochondria.

4.3 Limitations and suggestions

This study had some limitations. First, the function of glucose uptake in skeletal muscles, for example 2-deoxyglucose uptake study, was not directly determined. Next, although the oral glucose tolerance test, in which is one of the assessments of insulin sensitivity (197), was performed in the present study, the insulin tolerance test should be performed to confirm insulin sensitivity in the future study. Furthermore, there is no positive control group, i.e. normal diet with exercise, normal diet with caloric restriction and normal diet with combined exercise and caloric restriction group. Therefore, it is difficult to identify whether benefits on the metabolic and skeletal muscle are directly from the interventions. In addition, the question whether the beneficial effects on the intervention program is dependent or independent to an effect of weight reduction has not been elucidated. Normalization of the evaluated parameters with the amount of reduced weight should be considered in further study. Finally, since it has been demonstrated that the effects on insulin resistance and skeletal muscle functions between male and female are different (198), therefore we decided to use animals in the only one gender for the present study. Since the period of menopause comes earlier than andropause making female exposed the condition of lack of the sex hormone longer, as well as the prevalence of overweight and obesity, particularly in women, has increased up to 40.4%. Thus, the present study decided to use only female animals. To generalize the results of this study, a further study aims to investigate the effects of exercise training and caloric restriction on skeletal muscle functions in obese-insulin resistant male rats is needed.

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LIST OF PUBLICATIONS

Peer-reviewed Journals

1. **Pattanakuhar S**, Pongchaidecha A, Chattipakorn N, Chattipakorn SC. The Effect of Exercise on Skeletal Muscle Fiber Type Distribution in Obesity: From Cellular Levels to Clinical Application. Obes Res Clin Pract. 2016 Oct 15. (IF=2.09)
2. **Pattanakuhar S**, Phrommintikul A, Tantiworawit A, Konginn S, Srichairattanakool S, Chattipakorn, SC and Chattipakorn N. Increased Sympathovagal Imbalance Evaluated by Heart Rate Variability is Associated with Decreased T2* MRI and Left Ventricular Function in Transfusion Dependent Thalassemia Patients. Biosci Rep 2018 2;38(1). (IF=2.91)
3. Sutham W, Sripetchwandee J, Minta W, Mantor D, **Pattanakuhar S**, Palee S, Pratchayasakul W, Chattipakorn N, Chattipakorn SC. Ovariectomy and obesity have equal impact in causing mitochondrial dysfunction and impaired skeletal muscle contraction in rats. Menopause. 2018 Dec;25(12):1448-1458. (IF=3.361)
4. **Pattanakuhar S**, Sutham W, Sripetchwandee J, Minta W, Mantor D, Palee S, Pratchayasakul W, Chattipakorn N, Chattipakorn SC. Combined exercise and caloric restriction therapies restores contractile and mitochondrial functions in skeletal muscle of obese-insulin resistant rats. Nurtition 2019 Jun 62; 74-84. (IF=3.734)

Peer-reviewed Abstracts

1. **Pattanakuhar S,** Phrommintikul A, Tantiworawit A, Konginn S, Srichairattanakool S, Chattipakorn, SC and Chattipakorn N. Decreased Heart Rate Variability is Associated with Decreased T2* MRI and Left Ventricular Function in Transfusion Dependent Thalassemia Patients. (Poster presentation in the American College of Cardiology Conference

2017 and Peer-reviewed abstract in Journal of the American College of Cardiology. Volume 69, Issue 11 (Supplement). (IF=19.9)

2. **Pattanakuhar S**, Sutham W, Sripetchwandee J, Minta W, Mantor D, Palee S, Pratchayasakul W, Chattipakorn N, Chattipakorn SC. Exercise is better than caloric restriction regarding improving fatigability in muscle of obese rats. (Poster presentation in the 9th Federation of the Asian and Oceanian Physical Society Conference 2019 in Mar. 2019 and Peer-reviewed abstract in The Journal of Physiological Sciences, March 2019, Issue 1 Supplement)

APPENDIX 1

Table A1. Composition of Normal Diet (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand)

Composition	Normal diet (CP 082)		
	g	kcal	%E
Carbohydrate	495.30	1981.20	51.99
Fat	83.70	753.30	19.77
Protein	269.00	1076.00	28.24
Vitamins	65.40	-	-
Fiber	34.30	-	-
Total	947.70	3810.50	100
Kcal/g	4.02 kcal/g		

APPENDIX 2

Table A2. Composition of High-Fat Diet (Pratchayasakul et al 2011) (174)

Composition	High fat diet (modified from Srinivasan et al.)		
	g	kcal	%E
Carbohydrate	190.76	763.04	14.27
Fat	342.24	3080.16	57.60
Protein	353.60	1414.40	26.45
Cholesterol	10	90	1.68
Vitamins	85.19	-	-
DL-Methionine	3	-	-
Fiber	13.21		
Yeast powder	1	-	-
Sodium chloride	1	-	-
Total	1000	5347.60	100
Kcal/g	5.35 kcal/g		

APPENDIX 3

Western Blotting

1. Reagents for western blotting

aCSF

NaCl	125 mM
KCl	2.5 mM
$CaCl_2$	2 mM
$MgCl_2$	1 mM
NaH_2PO_4	1.25 mM
$NaHCO_3$	26 mM
Glucose	25 mM

pH 7.37-7.43, 310-320 mOsm, 34-35oC, bubble with 95%O2/5%CO2

Slicing solution

KCL	2.50 mM
$CaCl_2$	2.00 mM
$MgCl_2$	2.00 mM
KH_2PO_4	1.25 mM
Sucrose	25.20 mM
$NaHCO_3$	26.00 mM
Glucose	10.00 mM

pH 7.37-7.43, 310-320 mOsm, 0-4^{o}C, bubble with 95%O_2/5%CO_2

Slice lysis buffer

EDTA	1 mM
EGTA	1 mM
NP-40	1%
Triton-X	1%
Protease inhibitor	

Non-ionizing lysis buffer

NaCl	100 mM
EDTA	25 mM
Tris	10 mM
Triton X-100	1% v/v
NP-40	1% v/v
Protease inhibitor	

Loading buffer

Tris-HCl, pH6.8	62.5 mM
SDS	2%
Glycerol	20%
Bromophenol blue	0.2%
DTT	100 mM
2-mercaptoethanol	0.2 mM

TBST (10X)

Tris, pH 8	200 mM
NaCl	1.37 M
5% Non-fat milk in TBST	
TBST (TBS+0.5%TWEEN)	

Running buffer

Trizma base, pH 8.3	15.15 g
Glycine	72 g
SDS	5 g
Q to 500 ml with ddH2O	

Transfer buffer

Tris, pH 8.3	25 mM
Glycine	192 mM
Methanol	20%
pH 7.6	

Antibody

Primary antibodies

Anti-GAPDH

Anti-IR (SC-711, Santa Cruz Biotechnology, Santa Cruz, USA)

Anti-Tyr1162/1163pIR (SC-25103, Santa Cruz Biotechnology, Santa Cruz, USA)

Anti-Akt (#9272, Cell Signaling Technology, Danvers, USA)

Anti-Thr308pAkt (#9271, Cell Signaling Technology, Danvers, USA)

Anti-Bax (ab 182733, Abcam, Cambridge, UK)

Anti-Bcl2 (ab 196495, Abcam, Cambridge, UK)

Anti-PPAR delta (PA5-29678, Thermo Fisher Scientific, Waltham, USA),

Anti-PGC1alpha (ab 154481, Abcam, Cambridge, UK)

Anti-CPT1 (SC-393070, Santa Cruz Biotechnology, Santa Cruz, USA)

Anti-MFN2 (#9482, Cell Signaling Technology, Danvers, USA)

Anti-DRP1 (#5391, Cell Signaling Technology, Danvers, USA)

Anti-Ser616pDRP1 (#3455, Cell Signaling Technology, Danvers, USA)

Secondary antibodies

Anti-rabbit, anti-mouse and anti-goat IgG conjugated with horseradish peroxidase

Molecular weight marker

ECL

2. Preparation of gel

Stock A: 1.5M Tris pH 8.8 (200 ml)

Tris base	36.5 g
Deionized water	160 ml
pH 8.8	
Add deionized water to 200 ml	
Filter through membrane	0.2 um
Store at 4^{o}C (light sensitive)	

Stock B: 30% acrylamide/0.8% Bis (25 ml)

Acrylamide	7.3 g
Bisacrylamide	0.2 g
Add deionized water to 25 ml	

Filter through membrane	0.2 um
Store at 4^{o}C (light sensitive)	

Stock C: 0.5M Tris pH 6.8 (200 ml)

Tris base	12.1 g
Deionized water	160 ml
pH 6.8	
Add deionized water to 200 ml	
Filter through membrane	0.2 um
Store at 4^{o}C (light sensitive)	

10% Acrylamide/Bisacrylamide

30% acrylamide/0.8% Bis (Stock B)	3.5 ml
1.5M Tris pH 8.8 (Stock A)	2.5 ml
Deionized water	2.85 ml
10% SDS	100 ul
10% APS*	100 ul
TEMED*	10 ul

4% Stacking gel

Deionized water	3.05 ml
0.5M Tris pH 6.8 (Stock C)	1.25 ml
30% acrylamide/0.8% Bis (Stock B)	650 ul
10% SDS	50 ul
10% APS*	25 ul
TEMED*	5 ul

*should be fresh prepared

3. Gel preparation process

1. Clean glasses for loading gel with 70% Ethanol
2. Load the 10% Acrylamide
3. Fill the space above the gel with propanol, then leave it for 30 minutes

4. Rinsed the gel with distill water and replaced with 4% Stacking gel, leave it for 30 min

4. Immunoblotting process

1. Move gels in to electrophoresis chamber
2. Loading samples
    a. 7 µl for molecular weight marker
    b. 20 µl for protein sample
3. Run gel at 120 Volts, 1.20 hours until the protein arrived the end of the gel
4. Transfer gel to nitrocellulose membrane at 70 Volts, 2 hours
5. Wash the membrane with deionized water for 5 minutes
6. Block membrane with 5%Mlk or 5%BSA in TBST 1 hour on an orbital shaker (room temperature)
7. Add primary antibodies 1:1000 in TBST incubate overnight at 4ºC.
8. Wash the membrane with 10 ml TBST 5 minutes, 5 times
9. Add secondary antibodies 1:4000 in TBST; Anti-rabbit and anti-mouse IgG conjugated with horseradish peroxidase for 1 hour on an orbital shaker
10. Wash the membrane 5 minutes 5 times
11. ECL exposure to film

APPENDIX 4

Reagent for Determination of Muscle Mitochondrial Function

1. Solution for isolated muscle mitochondria proportion

MSE

D-mannitol	225 mM
Sucrose	75 mM
EGTA	1 mM
HEPES	5 mM
BSA	1 mg/ml

pH was adjusted to 7.4 with NaOH (strong base) or HCl (strong acid)

MSE-Nagrase

MSE+Nagrase	0.05%
MSE+Digitonin	0.02%

2. Solution for muscle mitochondrial respiratory buffer

KCl	150 mM
HEPES	5 mM
$K_2HPO_4.3H_2O$	2 mM
$C_5H_8NNaO_4.xH_2O$	5 mM
$CH_3COCOONa$	5 mM

pH was adjusted to 7.2 with NaOH (strong base) or HCl (strong acid)

3. JC-1 dye preparation (310 nM of JC-1)

JC-1	1 mM
DMSO	100%

4. DCFH-DA preparation (2 µM DCF-DA)

DCFH-DA	1 mM
Muscle mitochondrial	100 %
Respiratory buffer	

5. Standard working reagent (SWR) preparation

Reagent A (100 ml)

NAOH	0.40 g
$NaHCO_3$	0.95 g
$Na_2CO_3.H_2O$	2.00 g
Sodium bicinchoninate	0.10 g
Sodium tartrate (dihydrate)	0.16 g
ddH2O	100 ml

Reagent B (10 ml)

$CuSO_4.5H_2O$	0.40 g
ddH_2O	10 ml

SWR

Reagent A	100 ml
Reagent B	2 ml

APPENDIX 5

Immunohistochemistry Staining to Determine Skeletal Muscle Fiber Type

1. Deparaffinization

- Before performing with the staining protocol, the slides must be deparaffinizedand rehydrated.
- Incomplete deparaffinization can result in poor staining of the section.

Materials and reagents

1. Xylene
2. 100% ethanol
3. 95% ethanol

Methods

1. Place the slides in a rack, and perform the following washes:
    1. Xylene: 2 x 3 minutes
    2. Xylene 1:1 with 100% ethanol: 3 minutes
    3. 100% ethanol: 2 x 3 minutes
    4. 95% ethanol: 3 minutes
    5. 70 % ethanol: 3 minutes
    6. 50 % ethanol: 3 minutes
    7. Running cold tap water to rinse
2. Keep the slides in the tap water until ready to perform antigen retrieval.

- After this time, the slides should not be allowed to dry.
- Drying out will result in non-specific antibody binding and high background staining.

2. Antigen retrieval

- Most formalin-fixed tissue needs an antigen retrieval step before immunohistochemical staining can perform. This is due to the formation of methylene bridges during fixation, which cross-link proteins and therefore cover antigenic sites.
- The two methods of antigen retrieval are heat-mediated (also known as heat-induced epitope retrieval, or HIER) and enzymatic. We have used enzymatic method (commercial reagent, Abcam #ab91506).

Enzymatic retrieval, immersion method

Materials and reagents

1. 37^{o}C water bath
2. Slide racks and slide rack containers
3. Enzymatic antigen retrieval solution

Method

1. Be sure to read the manufacturer's instruction and the referred literatures for the enzyme you choose, as some enzymes require specific buffers and cofactors for activity.
2. Set water bath to the optimal temperature for the enzyme you are using. Add water to two containers that can hold slide racks. Place the containers into the water bath to warm. Use an enough volume of water or buffer to cover the slides.
3. Deparaffinize and rehydrate sections as aforementioned. Place slides in a water container to warm. Placing cold slides into the enzyme solution will lower the temperature of the solution, reducing enzyme activity and causing under-retrieval of the antigenic site.
4. Prepare the enzymatic antigen retrieval buffer from the warm water in the other container, and then return the container to the water bath to allow the solution to re-heat. Prepare the enzymatic antigen retrieval solution as quickly as possible to avoid impairing the activity of the enzyme. Allow this solution to return to temperature before introducing the slides.
5. Transfer the warmed slides into the enzyme solution for 10 - 20 minutes. Less than 10 minutes may cause the antigens under retrieved, leading to

weak staining. More than 20 minutes may cause them over retrieved, leading to non-specific background staining and increasing the chances of sections dissociating from the slides or damage to the morphology of the tissue.

6. remove the slides and place them in running tap water for 3 minutes to rinse off the enzyme.
7. Start with immunohistochemical staining protocol.

Note: A control experiment is recommended. Slides of the same tissue section are incubated in the enzyme solution for 10, 15, 20, 25, and 30 minutes before being immunohistochemically stained to evaluate optimum antigen retrieval time for each antibody being used.

3. Immunohistochemical staining

Our protocol is for the laboratory which does not have an automated staining machine or other capillary gap system that allows rapid application and rinsing of reagents (e.g. Shandon Sequenza). Reagents will be applied manually by pipet.

1. General guidelines

All incubations should be performed in a humidified chamber to avoid drying of the tissue. Drying at any stage will lead to non-specific binding and ultimately high background staining. A shallow plastic box with a sealed lid and wet tissue paper in the bottom is an adequate chamber. The slides should be kept off the paper and lay flat so that the reagents don't drain off. A good solution is to cut a plastic serological pipette into lengths to fit your incubation chamber. Glue them in pairs to the bottom of the chamber, with the 2 individual pipette tubes of each pair being placed about 4.0 cm apart. This provides a level and raised surface for the slides to rest on away from the wet tissue paper.

Dilutions of the primary and secondary antibody are listed on the datasheets or are determined by testing. Adjust dilutions appropriately from the results obtained. Adhere strictly to all incubation times in the protocol.

2. Protocol

(Antigen retrieval should be performed before commencing with the following protocols.)

Day 1

1. (If using an HRP conjugate for detection, blocking of endogenous peroxidase can be performed here but we recommend waiting until after the primary antibody incubation.)
2. Wash the slides 2 x 5 minutes in TBS plus 0.025% Triton X-100 with gentle agitation. The use of 0.025% Triton X-100 in the TBS helps to reduce surface tension, allowing reagents to cover the whole tissue section with ease. It will dissolve Fc receptors, therefore reducing non-specific binding. We recommend TBS to give a cleaner background rather than PBS.
3. Block in 10% normal serum with 1% BSA in TBS for 2 hours at room temperature. The primary antibody should be diluted to the manufacturer's instruction or to a previously optimized dilution. Most antibodies will be used in IHC-P at a concentration between 0.5 and 10 µg/ml. Make sure the primary antibody is raised in a species different from the tissue being stained. For example, if you had mouse tissue and your primary antibody was raised in a mouse, an anti-mouse IgG secondary antibody would bind to all the endogenous IgG in the mouse tissue, leading to high background. Use of mouse monoclonals on mouse tissue is discussed in our mouse-on-mouse protocol.
4. Drain slides for a few seconds (do not rinse) and wipe around the sections with tissue paper. Overnight incubation allows antibodies of lower titer or affinity to be used by simply allowing more time for the antibodies to bind. Also, regardless of the antibody's titer or affinity for its target, once the tissue has reached saturation point no more binding can take place. Overnight incubation ensures that this occurs.
5. Apply primary antibody diluted in TBS with 1% BSA.
6. Incubate overnight at 4°C.

Day 2

1. Rinse 2 x 5min TBS 0.025% Triton with gentle agitation.
2. If using an HRP conjugate for detection, incubate the slides in 0.3% $H2O^2$ in TBS for 15 min. H_2O_2 suppresses endogenous peroxidase activity and therefore reduces background staining. Peroxide can be diluted in TBS or water.
3. For enzymatic detection (HRP or AP secondary conjugates): Apply enzyme-conjugated secondary antibody to the slide diluted to the concentration recommended by the manufacturer in TBS with 1% BSA and incubate for 1 hour at room temperature. Apply fluorophore-conjugated secondary antibody to the slide diluted to the concentration recommended by the manufacturer in TBS with 1% BSA and incubate for 1 hour at room temperature. This step should be done in the dark to avoid photobleaching.
4. Rinse 3 x 5min TBS. If visualizing the protein with a chromogen, continue with the following steps.
5. Develop with chromogen for 10 min at room temperature. Develop the colored product of the enzyme with the appropriate chromogen. The choice depends on which enzyme label you are using, the colored end-product you prefer and whether you are using aqueous or organic mounting media.
6. Rinse in running tap water for 5 min.
7. Counterstain (if required). Don't forget that DAB is a suspected carcinogen. Wear the appropriate protection.
8. Dehydrate, clear and mount.

Notes

- The secondary antibody may cross react with endogenous immunoglobulins in the tissue. This is prevented by pre-treating the tissue with normal serum from the species in which the secondary was raised. The use of normal serum before the application of the primary also eliminates Fc receptor binding of both the primary and secondary antibody. BSA is included to reduce non-specific binding caused by hydrophobic interactions.

3. Signal amplification

- To achieve a stronger signal, Avidin-biotin complex (ABC) was applied. This technique developed by Su-Ming Hsu and colleagues (J Histochem Cytochem. 1981 Apr 29 (4):577-80), utilizes the high affinity of avidin, a protein found in chicken egg white, for biotin, an enzyme co-factor in carboxylation reactions. Avidin has four binding sites for biotin and binding is essentially irreversible.

In brief, the primary antibody is bound to the target protein. A biotinylated secondary antibody is then bound to the primary antibody. In a separate reaction, a complex of avidin and biotinylated enzyme is formed by mixing the two in a ratio that leaves some of the binding sites on avidin unoccupied. This complex is then incubated with the tissue section after the antibody incubations. The unoccupied biotin-binding sites on the complex bind to the biotinylated secondary antibody. The result is more enzyme attached to the target than is possible using an enzyme-conjugated secondary or primary antibody.

The components of the avidin-biotin complex are commercially available in kits that provides the two reagents and instructions for combining them in the optimal ratio.

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Publication

1. **Pattanakuhar S**, Kammuang-lue P, Kovindha A. Coping Strategies of Thais with Chronic Spinal Cord Injury and Related Factors. J Thai Rehabil Med 2014; 24(3): 86-94.
2. **Pattanakuhar S**. Effect of Sedentary Activity Reduction on Cardiovascular Risk factors in Individuals with Spinal Cord Injury. J Thai Rehabil Med 2015; 25(1): 3-5.
3. **Pattanakuhar S**, Pongchaidecha A, Chattipakorn N, Chattipakorn SC. The Effect of Exercise on Skeletal Muscle Fiber Type Distribution in Obesity: From Cellular Levels to Clinical Application. Obes Res Clin Pract. 2016 Oct 15. pii: S1871-403X(16)30112-0. (IF=2.09)
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7. Sutham W, Sripetchwandee J, Minta W, Mantor D, **Pattanakuhar S**, Palee S, Pratchayasakul W, Chattipakorn N, Chattipakorn SC. Ovariectomy and obesity have equal impact in causing mitochondrial dysfunction and impaired skeletal muscle contraction in rats. Menopause. 2018 Dec;25(12):1448-1458. (IF=3.361)
8. **Pattanakuhar S**, Kammuang-Lue P, Kovindha A, Komaratat N, Mahachai R, Chotiyarnwong C. Is admission to an SCI specialized rehabilitation facility associated with better functional outcomes? Analysis of data from the Thai Spinal Cord Injury Registry. Spinal Cord 2019 (in press). (IF=1.96)
9. **Pattanakuhar S**, Sutham W, Sripetchwandee J, Minta W, Mantor D, Palee S, Pratchayasakul W, Chattipakorn N, Chattipakorn SC. Combined exercise and caloric restriction therapies restores contractile and mitochondrial functions in skeletal muscle of obese-insulin resistant rats. Nurtition 2019 Jun 62; 74-84. (IF=3.734)

Presentations at International Meetings

1. Poster presentation in the American College of Cardiology Conference 2017 (Washington D.C., USA) in Mar. 2017
2. Oral and poster presentation in Asian Spinal Cord Injury Network (ASCoN) conference 2017 (Chiang Mai, Thailand) in Dec. 2017
3. Oral and poster presentation in International Spinal Cord Society (ISCoS) conference 2018 (Sydney, Australia) in Sep. 2018
4. Oral presentation in Asian Spinal Cord Injury Network (ASCoN) conference 2018 (Yangon, Myanmar) in Nov. 2018
5. Poster presentation in the 9th Federation of the Asian and Oceanian Physical Society Conference 2019 (Kobe, Japan) in Mar. 2019

Peer-reviewed Abstracts

1. **Pattanakuhar S**, Phrommintikul A, Tantiworawit A, Konginn S, Srichairattanakool S, Chattipakorn, SC and Chattipakorn N. Decreased Heart Rate Variability is Associated with Decreased T2* MRI and Left Ventricular Function in Transfusion Dependent Thalassemia Patients. (Poster presentation in the American College of Cardiology Conference 2017 and Peer-reviewed abstract in Journal of the American College of Cardiology. Volume 69, Issue 11 (Supplement). (IF=19.9)
2. **Pattanakuhar S**. The Effect of N-Acetyl Cysteine (NAC) for Improving Neurological Outcomes in Animal Models of Spinal Cord Injury: A Systematic Review (Proceeding in Asian Spinal Cord Injury Network (ASCoN) conference 2017 in Dec. 2017)
3. **Pattanakuhar S**. Laser Therapy for Treating Pressure Ulcer in Spinal Cord Injured Patients: Is It Time to Reconsider? (Proceeding in Asian Spinal Cord Injury Network (ASCoN) conference 2017 in Dec. 2017)
4. **Pattanakuhar S**. Is the Walking Index for Spinal Cord Injury II (WISCI II) Proper for All SCI Persons Walking with Spasticity?: a case report. (Proceeding in Asian Spinal Cord Injury Network (ASCoN) conference 2017 in Dec. 2017)
5. **Pattanakuhar S**. What is Your Choice Between Health or Success?: A Case Series of Thai Spinal Cord Injury Athletes in the 9th ASEAN Paragames. (Proceeding in Asian Spinal Cord Injury Network (ASCoN) conference 2017 in Dec. 2017)
6. **Pattanakuhar S**, Komaratat N, Mahachai R, Chotiyarnwong C, Kammuang-lue P, Kovindha A. Prediction of Functional Improvement after Spinal Cord Injury Rehabilitation in Thailand: Results from Thai Spinal Cord Injury Registry. (Proceeding in International Spinal Cord Society (ISCoS) conference 2018 in Sep. 2018)
7. **Pattanakuhar S**, Komaratat N, Mahachai R, Chotiyarnwong C, Kammuang-lue P, Kovindha A. Comparison of Rehabilitation Outcomes between Traumatic and

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8. Suttinoon L, **Pattanakuhar S**, Tongprasert S, Wongprakaran T. Validity and Reliability of the International Spinal Cord Injury Quality of Life Basic Data Set in Thai Version. (Proceeding in International Spinal Cord Society (ISCoS) conference 2018 in Sep. 2018)

9. **Pattanakuar S**, Kovindha A, Banthadtham H. Early onset of heterotropic ossification might be associated with fracture debridement and external fixation: a case report and literature review. (Proceeding in Asian Spinal Cord Injury Network (ASCoN) conference 2018 in Nov. 2018)

10. **Pattanakuhar S**, Sutham W, Sripetchwandee J, Minta W, Mantor D, Palee S, Pratchayasakul W, Chattipakorn N, Chattipakorn SC. Exercise is better than caloric restriction regarding improving fatigability in muscle of obese rats. (Poster presentation in the 9th Federation of the Asian and Oceanian Physical Society Conference 2019 in Mar. 2019 and Peer-reviewed abstract in The Journal of Physiological Sciences, March 2019, Issue 1 Supplement)

National Grant

1. Thailand Research Fund (TRF) Grant for New Scholar (MRG) Year 2019. The Associations between Gut Microbiotas and Metabolic Parameters in Disabled Patients with Spinal Cord Injury. (Mentor: Prof. Dr. Siriporn Chattipakorn)