THE ROLE OF BIOACTIVE LIPIDS AND LIPID BINDING PROTEINS IN SKELETAL MUSCLE

Mohd Fadly Mohd Noor, MBBS, MMDSc

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

December 2018

Abstract

Obesity and diabetes mellitus have been identified as major worldwide health problems. There is a growing body of evidence which suggests lipid signalling molecules including fatty acids, endocannabinoids and fatty acid amides may play a role in the development of obesity and insulin resistance. Much of the current research has focused upon the synthesis and release of such molecules by adipose tissue and their effects upon the endocrine system. Interestingly, cannabinoid (CB) receptors and other endocannabinoid components are also found to be expressed in human and animal skeletal muscle cells. However, there is minimal information regarding the role of these fatty acid derivatives in skeletal muscle and the lack of these data represents a significant gap in our knowledge. The aims of this thesis were to investigate the effects of cannabinoid receptor CB1 modulation upon insulin-stimulated glucose uptake and its effects upon regulation of selected genes expression in primary skeletal muscle myotubes. In addition, we would like to investigate the synthesis and secretion of Lipocalin 2 (LCN2), a type of lipid binding protein as a novel myokine.

Our previous laboratory data demonstrated that the level of GLUT4 was expressed at approximately 3000-fold lower in non-contracting primary skeletal muscle cell cultures as compared to skeletal muscle tissue. Since the mechanism of glucose uptake in contracting skeletal muscle is determined by the translocation of GLUT4 to the surface membrane, we have developed a contractile model of skeletal muscle myotubes by electrical pulse stimulation (EPS) which is sensitive to insulin-stimulated glucose uptake for further studies on CB1 receptor characteristics. CB1

activation demonstrated a significant reduction in insulin-stimulated glucose uptake in contractile primary skeletal muscle myotubes by inhibition of Akt phosphorylation and alteration in p85/p110 ratio in insulin signalling cascade. Apart from that, CB1 modulation in primary skeletal muscle showed no effects on regulations of selected genes that known to be related to obesity and insulin resistance namely LCN2, Neuropeptide Y, NR4A1, NR4A2 and NR4A3. On the other hand, the upregulation of these genes were influenced by low free fatty acid levels and muscle contractions. In addition, this study also demonstrated that LCN2, a lipid binding protein was found to be expressed in skeletal muscle. LCN2 protein were synthesized and secreted by rat myotubes post-EPS and human skeletal muscle cells following acute eccentric exercise *in vivo*.

In conclusion, the development of this contractile myotubes model provides an important tool not only for the study on CB1 receptor characteristics as in this thesis, but also for other aspect in future such as oxidative phosphorylation and exercise. This thesis provides a new understanding of the CB1 characteristic in skeletal muscle, in which it inhibit insulin-stimulated glucose uptake by interrupting the important steps in insulin signalling cascade. Even though the current study has demonstrated that skeletal muscle is able to synthesize and secrete LCN2, further future works need to be done to investigate its role in mediating other metabolic changes or exerting specific endocrine effects to other organs such as the liver and the adipose tissue, in order to classify LCN2 as a novel myokine.

Presentations

Mohd Fadly MN, Kostas Tsintzas and Andrew J. Bennett. Effects of Cannabinoid CB1 Receptor Activation upon Exercise-induced Insulin Stimulated Glucose Uptake in Human Primary Skeletal Muscle Myotubes. Life Sciences Postgraduate Symposium 2016, School of Life Sciences, University of Nottingham UK. **Oral Presentation**.

Mohd Fadly MN, Kostas Tsintzas and Andrew J. Bennett. Molecular Pharmacology of the Cannabinoid Receptor Type-1 in Primary Skeletal Muscle. Life Sciences Postgraduate Symposium 2015, School of Life Sciences, University of Nottingham UK. **Poster Presentation**.

Acknowledgements

Praise be to Allah

First and foremost, I would like to express my gratitude to my supervisor, Associate Professor Dr. Andrew Bennett for his expert guidance, dedication, support and encouragements throughout my PhD studies. My sincere appreciation and innumerable thank you also goes to my co-supervisor, Associate Professor Dr. Kostas Tsintzas for the additional supervisory support, guidance of the laboratory works, constructive comments and encouragements.

My countless thank also goes to all FRAME Alternatives laboratory members especially Monika, Nikki, Elke, Rachael, Suvik, Louis, Richard, Fateha, Fatema, Amer, Razan and Hazulin for their technical assistant and social support.

Special thanks to the School of Life Sciences, University of Nottingham UK and the FRAME Alternatives laboratory for giving me the opportunity to further my study and to perform my research works. I am grateful to the Ministry of Higher Education Malaysia and my employer International Islamic University Malaysia for providing me the scholarship.

My deepest gratitude to my beloved parents for all the prayers, love, encouragements and supports throughout the years of my study. To my dear wife Noraihan, thank you for all the time that we've spent together. We've faced challenges that we never dreamed of. Together, we've made life-changing decisions. Every step of the way, you've been there to support me and our family. To our wonderful children, Ain, Adam and Alya, thank you for being our inspiration; our source of joys and smiles.

Declaration

I hereby declare that the work presented in this thesis, has not been submitted for any degree or diploma, at this, or any other university, and that all of the experiments, unless otherwise stated, were performed by me.

Dr. Mohd Fadly Mohd Noor

Table of Contents

List of Figures

List of Tables

Abbreviations

2-AG: 2-arachidonoyl glycerol

2DOG: 2-Deoxy-D-glucose

ACC: acetyl-coA carboxylase

ACEA: Arachidonyl-2'-chloroethylamide

ADP: Adenosine diphosphate

AEA: N-arachidonoylethanolamine (anandamide)

AKT: Serine threonine kinase/ protein kinase B

AMP: Adenosine monophosphate

AMPK: AMP-activated protein kinase

APS: Ammonium persulfate

ATP: Adenosine triphosphate

BCA: Bicinchoninic acid

BCP: 1-Bromo-3-chloro-propane

BDNF: brain-derived neurotrophic factor

BSA: Bovine serum albumin

cAMP: Cyclic adenosine monophosphate

CB1: Cannabinoid receptor type-1

CB2: Cannabinoid receptor type-2

cDNA: Complementary deoxyribonucleic acid

CNS: Central nervous system

CPT-1: Carnitine palmitoyl transferase

CXCR1: CXC chemokine receptor 1

CXCR2: CXC chemokine receptor 2

DAPI: 4',6-diamidino-2-phenylindole

DEPC: Diethylpyrocarbonate

DIO: Diet-induced obesity

DTT: Dithiothreitol

ECS: Endocannabinoid system

EDTA: Ethylene diaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

ERK: Extracellular signal–regulated kinase

EPS: Electrical pulse stimulation

FAAH: Fatty acid amide hydrolase

FABP: Fatty acid binding protein

FAK: Focal adhesion kinase

FAT: Fatty acid translocase

FFA: Free fatty acids

G6P: Glucose-6-phosphate

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GEF2: GLUT4 enhancer factor

GLUT4: Glucose transporter type-4

GOD: Glucose oxidase

GPCR: G-protein coupled receptors

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HK: Hexokinase

HK2: Hexokinase-2

IEG: Immediate early genes

IL: Interleukin

IR: Insulin receptor

IRS: Insulin receptor complex

JNK: c-Jun N-terminal kinases

LCN2: Lipocalin 2

LDH: Lactate dehydrogenase

MAGL: Monoacylglycerol lipase

MAPK: Mitogen activated protein kinase

MEF2: Myocyte enhancer factor 2

MEK: MAP kinase kinase

mRNA: Messenger ribonucleic acid

MyHC: Myosin heavy chain

NAD: Nicotinamide adenine dinucleotide

NADH: Nicotinamide adenine dinucleotide hydride

NEFA: Non-esterified fatty acids

NPY: Neuropeptide Y

NR4A: Nuclear receptor 4A

pAKT: Phospho-AKT

PBS: Phosphate buffered saline

PDC: Pyruvate dehydrogenase complex

PDK-1: Phosphoinositide-dependent kinase-1

PFK: Phosphofructokinase

PI3K: Phosphatidylinositol 3’ kinase

PIP2: PI 4,5-biphosphate,

PIP3: PI 3,4,5-triphosphate

PGC1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PKA: Protein kinase A

PKC: Protein kinase C

PLO: Palmitic acid, linoleic acid and oleic acid

qRTPCR: Quantitative real-time polymerase chain reaction

RBP: Retinol binding proteins

RIM: Rimonabant

SDS: Sodium dodecyl sulfate

SEM: Standard error of mean

SERCA: Sarcoplasmic reticulum Ca2+-ATPase

SNARE: Soluble N-ethylmaleimide-sensitive factor-attachment protein receptors

T2DM: Type-2 diabetes mellitus

TBS: Tris-buffered saline

TBST: Tris-buffered saline with Tween 20

TCA: Trichloroacetic acid

TEMED: Tetramethylethylenediamine

THC: Tetrahydrocannabinol

TNFα: Tumour necrosis factor alpha

CHAPTER ONE

GENERAL INTRODUCTION

Chapter 1: General Introduction

1.1 Skeletal Muscle

Muscle tissues are generally divided into three types namely cardiac muscle, smooth muscle and skeletal muscle. The integrated function of cardiac, skeletal, and vascular smooth muscles is essential for oxygen delivery and utilization, especially during exercise. Cardiac muscle, which is striated and involuntarily control type of tissue, produces the driving force to transport blood-borne oxygen to the periphery. Smooth muscle, a non-striated and involuntarily controlled type of tissue, is the major component of the arterial system that is involved in the distribution of blood flow and oxygen transport to the whole body. Whereas skeletal muscle, a striated and voluntary type of tissue, utilize the oxygen for the movements and metabolic requirements (Park et al., 2014).

Skeletal muscle is largely composed of heterogeneous populations of muscle fibres. On the basis of metabolic and contractile properties and myosin heavy chain (MyHC) isoforms, muscle fibres can be broadly classified as type I, oxidative, slow-twitch fibres and type II, fast-twitch fibres. Type II muscle fibres can be further classified into type IIa fibres, which possess a more oxidative type of energy metabolism, or type IIx and IIb fibres, which are more glycolytic in nature (Shi et al., 2007).

1.1.1 Skeletal muscle metabolism

Skeletal muscle is one of the most metabolically active tissues in the body. It is considered the largest tissue in the body which constitutes ~40% of the human body mass (Delbono et al., 2007) and represent 35-40 % of

the total body mass in the rat (James, Jenkins, & Kraegen, 1985). Skeletal muscle is a contractile tissue that important in energy homeostasis due to the large amount of energy required in performing its activities (Abdul-Ghani & DeFronzo, 2010). Due to this fact, skeletal muscle was found to be the primary site of glucose and fatty acid oxidation accounting for up to 30% of basal energy requirements (Zurlo, Larson, Bogardus, & Ravussin, 1990). Skeletal muscle is also a major site of action for insulin involving the regulation of glucose entrance into the cells. Subsequent impairment of glucose uptake and transport, glucose oxidation or glucose synthesis in muscles might be the major determinant of the severity of type-2 diabetes mellitus (T2DM) (Abdul-Ghani & DeFronzo, 2010).

Apart from that, recent data showed that several humoral factors or cytokines have been synthesized and secreted by contracting muscle cells which were found to play important roles in metabolism. Due to this fact, skeletal muscle can be considered as an endocrine organ that is able to secrete hormone-like factors or myokines such as interleukin (IL)-6, IL-8 and IL-15 that may influence metabolism in other tissues or organs (Pedersen, 2011b).

Skeletal muscle rapidly adapts to changing energy needs such as following exercise or reduced energy intake by increasing fatty acids oxidation (Henriksson, 1995). Increased fatty acid oxidation delays the consumption of glycogen stores within skeletal muscle and conserves circulating plasma glucose (Henriksson, 1995). The capacity for skeletal muscle to adapt appropriately depends upon insulin sensitivity, leanness and aerobic fitness (Ukropcova et al., 2005).

In order to perform its activity, skeletal muscle utilizes adenosine triphosphate (ATP) as the energy source. The skeletal muscle fuel metabolism requires continuous source of ATP in order to match the ATP supply and demand. Under normal physiological condition, skeletal muscle depends on both glucose and lipids (fatty acids and ketone bodies) as the substrate for oxidative metabolism. Around 55 years ago, Randle and his colleagues hypothesised the interaction and competition between glucose and fatty acids for oxidation, known as the glucose-fatty acid cycle or Randle cycle. The study revealed that, in starvation or diabetic state, the availability of free fatty acids (FFA) will be the main source for oxidation. The increase in FFA oxidation will raise the production of acetyl-coA, increase the mitochondrial ratio of acetyl-CoA/CoA, resulting in the inhibition of pyruvate dehydrogenase complex (PDC) activity and elevation of citrate levels in tricarboxylic acid cycle. Elevated citrate levels together with an increase in ATP/ADP ratio can inhibit the activity of phosphofructokinase (PFK) and the flux of glucose through the glycolytic pathway resulting in glucose-6-phosphate accumulation, hexokinase inhibition and consequently reduced glucose uptake (Randle, 1998; Randle, Garland, Hales, & Newsholme, 1963).

Interestingly, a study by Sidossis and his colleague suggested that the intracellular availability of glucose could reverse the glucose-fatty acid cycle in human skeletal muscle. Their study demonstrated that high concentrations of glucose lead to increased glycolysis, formation of pyruvate (as the end product of glycolysis), stimulation of PDC and formation of acetyl-coA. This in turn would result in increased formation of malonyl-CoA, a potent inhibitor of carnitine palmitoyl transferase 1 (CPT-

I). Thus accelerated glucose metabolism may inhibit fatty acid oxidation by limiting long-chain fatty acids uptake by the mitochondria, thus reducing the β-oxidation rates (Sidossis & Wolfe, 1996). The ability of skeletal muscle to switch from fat oxidation during the fasting state to glucose oxidation during the postprandial state has been referred to as metabolic flexibility (Kiens, 2006). Both mechanisms are described in Figure 1-1.

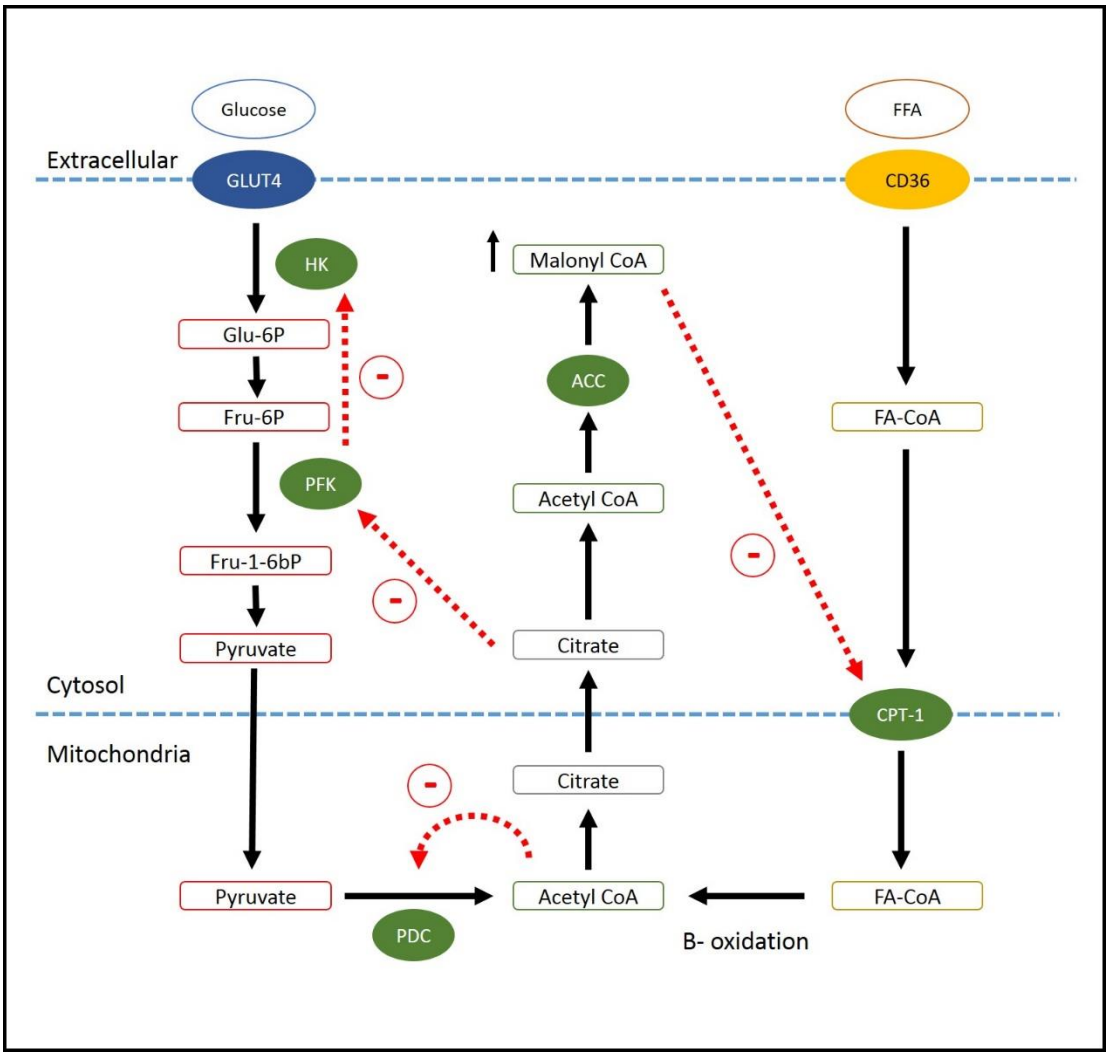


Figure 1- 1: Summary of 'glucose-fatty acid cycle' and reversal of glucose-fatty acid cycle.

Glucose metabolism inhibition by fatty acids or 'glucose-fatty acid cycle' as proposed by Randle et al 1963, and reversal of glucose-fatty acid cycle as proposed by Sidossis & Wolfe 1996. Glucose transporter type-4 (GLUT4), hexokinase (HK), phosphofructokinase (PFK), pyruvate dehydrogenase complex (PDC), acetyl-coA carboxylase (ACC), fatty acid translocase (FAT/CD36) and carnitine palmitoyl transferase (CPT-1).

1.1.2 Skeletal muscle glucose uptake and insulin signalling

Skeletal muscle is a contractile tissue that is important in energy homeostasis due to the large amount of energy required in performing its activities (Abdul-Ghani & DeFronzo, 2010). Glucose is an important source of energy for skeletal muscle and its uptake into the cells is mainly stimulated by contraction and insulin.

Glucose transport is mediated by carriers known as glucose transporters (GLUT) family which facilitate glucose movement into the cells. There are 13 members of GLUT family but the best-characterized are the class 1 GLUT (GLUT 1-4). The GLUT1 transporters are commonly responsible for basal glucose uptake. The GLUT2 is mainly expressed in beta cells and liver, and has relatively low affinity for glucose. GLUT3 has the highest affinity and only expressed during fetal development and in adult neurons. Finally GLUT4 is predominantly responsible for insulin-stimulated glucose uptake in fat and muscle (Khan & Pessin, 2002), and it acts as a major facilitative glucose transporter in skeletal muscle (Zisman et al., 2000)

As mentioned above, both GLUT1 and GLUT4 are expressed in skeletal muscle, however there is a significant difference in the level of each transporter. Even though GLUT4 was identified as the major facilitative glucose transporter, several studies have shown that the level of GLUT1 is higher in skeletal muscle. Sarabia et al. demonstrated that skeletal muscle myotubes expressed both the GLUT1 and GLUT4 glucose transporter protein isoforms at an average molar ratio of 7:1 (Sarabia, Lam, Burdett, Leiter, & Klip, 1992). In another study by Al-Khalili et al., the protein and mRNA expressions of GLUT1 were found to be significantly reduced in myotubes

as compared to myoblasts. In contrast, protein and mRNA expression of GLUT4 increased with differentiation. Despite the differentiation-induced increase, GLUT4 expression remained lower than GLUT1 in which in myoblasts, GLUT1:GLUT4 expression was 37:1 and in myotubes, 12:1. Furthermore, the level of GLUT4 in differentiated cultured myotubes was significantly reduced as compared to mature skeletal muscle (53 fmol/mg protein in cultured myotubes vs 1128 fmol/mg protein in rat skeletal muscle homogenates, and 4130 fmol/mg protein in human skeletal muscle) (Al-Khalili et al., 2003).

The insulin stimulation of glucose uptake in muscle tissue occurs via a signalling pathway through the insulin receptor tyrosine kinase. The main effect is to promote the movement of GLUT4 from the intracellular space to the plasma surface membrane. Therefore, the rate limiting step in insulin-stimulated glucose uptake in muscles is the translocation of GLUT transporters to the cell surface membrane (Khan & Pessin, 2002).

The insulin receptor (IR) is a heterotetrameric membrane protein which composed of two identical α and β subunits. Insulin binds to the α subunit of IR and subsequently activates intrinsic kinase activity in β subunit. This leads to intramolecular transautophosphorylation reaction in which one β subunit tyrosine phosphorylates the other β subunit. Then the insulin receptor substrate (IRS) protein family interacts with the phosphorylated IR through a phosphotyrosine binding module (Saltiel & Kahn, 2001). This IRS protein becomes a docking site for proteins with Src Homology 2 (SH2) domains in which p85 becomes the most important regulatory subunit of the type 1A phosphatidylinositol 3’ kinase (PI3K). PI3K

exists as a dimer consisting of a regulatory p85 subunit and a catalytic p110 subunit (Fruman, Meyers, & Cantley, 1998).

The catalytic p110α subunit is brought to the cell membrane and catalyzes the phosphorylation of 3’ position in the inositol ring of phosphoinositide (PI) lipids. Specifically the type 1A PI3K catalyzes the transformation of PI(4,5)-biphosphate to PI(3,4,5)-triphosphate and PI(4)-phosphate to PI(3,4)-biphosphate (Fruman et al., 1998). The phosphorylation of the 3’ further activates 3’phosphoinositide-dependent kinase-1 (PDK-1) and Akt (protein kinase B) (Rameh & Cantley, 1999). Subsequently, PDK-1 phosphorylates and activates Akt and protein kinase C (PKC) ζ/λ. Akt and PKC ζ/λ promote the GLUT4 translocation from the intracellular into the cell membrane (Khan & Pessin, 2002) (Figure 1-2).

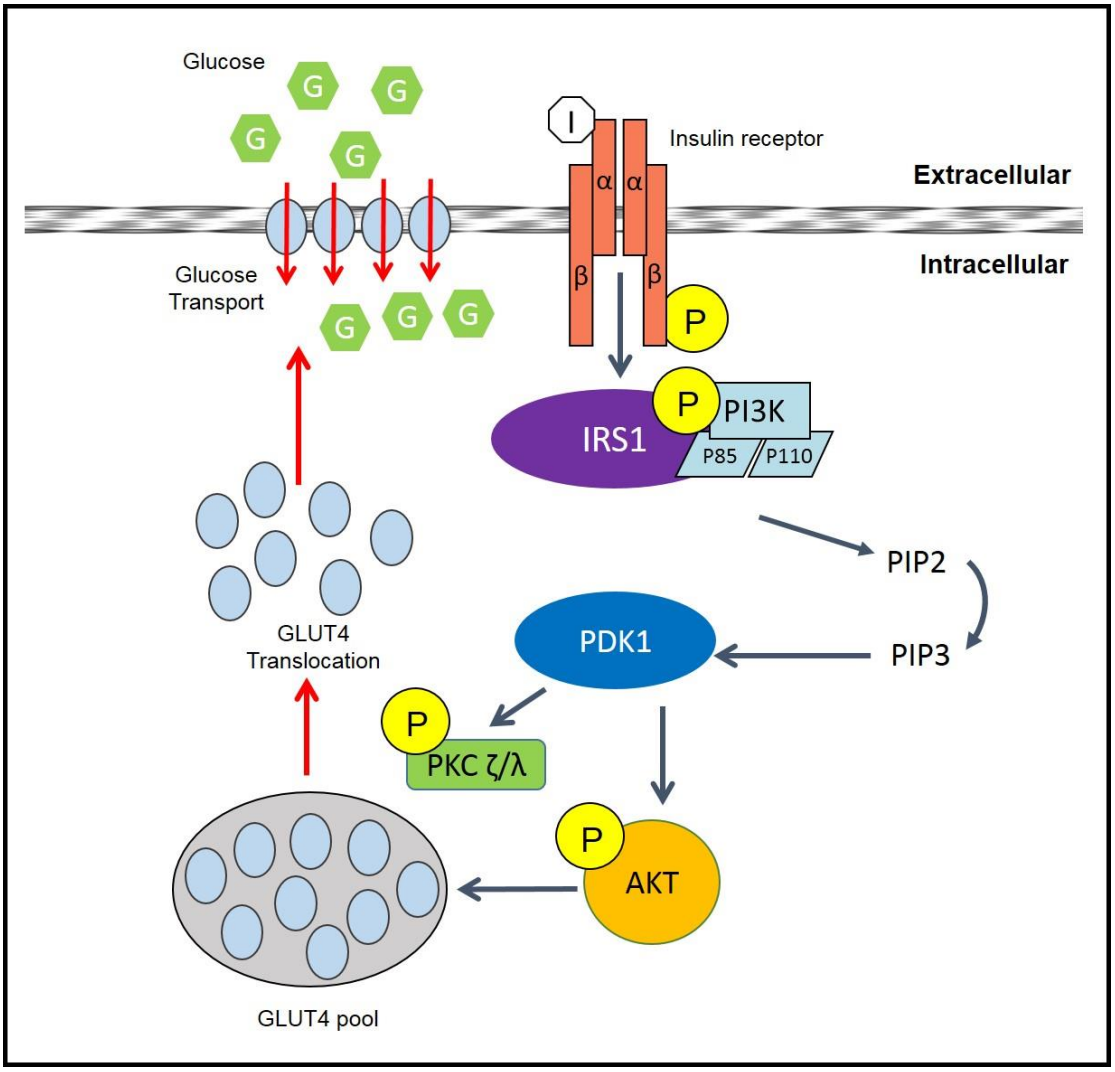


Figure 1- 2: Insulin signalling and glucose uptake in skeletal muscle.

Insulin (I), glucose (G), insulin receptor (IR), insulin receptor substrate (IRS), phosphatidylinositol 3' kinase (PI3K), p85 regulatory subunit (p85), p110 catalytic subunit (p110), phosphoinositide (PI), PI (4,5)-biphosphate (PIP2), PI(3,4,5)-triphosphate (PIP3) phosphoinositide-dependent kinase-1 (PDK-1) and Akt (protein kinase B), protein kinase C (PKC) and glucose transporter type-4 (GLUT4).

1.1.3 Skeletal muscle glucose uptake during exercise

In order to maintain its function, contracting muscle tissues require FFA and glucose as their major source of energy. During low intensity exercise (25% of maximal oxygen uptake [VO_2 max]), an intensity comparable to walking, most of the energy requirements can be met from plasma fatty acid oxidation, with a small contribution from the oxidation of plasma glucose. With an increase in exercise intensity from 25% to 65% of VO_2 max (the pace that could be sustained by a trained person for up to eight hours), total fat oxidation reaches its peak, despite a slight decline in the rate of appearance of plasma fatty acid. During high intensity exercise at 85% of VO_2 max (race pace for endurance events lasting approximately one hour) there is a decline in total fatty acid oxidation compared to moderate intensity exercise. This continuous high intensity exercise is associated with high rates of glycogenolysis and the concomitant production of lactic acid which accumulates in muscle and blood. The increased glycolytic flux also acts to inhibit fatty acid oxidation by skeletal muscle. Thus, this progressive decline in plasma FFA turnover with increasing exercise intensity appears to be offset by progressively increasing blood glucose turnover; therefore the contribution of plasma substrates to caloric expenditure remains remarkably constant over this wide range of exercise intensities (Romijn et al., 1993).

During prolonged, strenuous exercise, muscle glucose uptake is mainly determined by exercise intensity, duration and also glucose supply (Richter & Hargreaves, 2013).

There are three main sites of glucose uptake that can be regulated; the glucose delivery to the skeletal muscle cell, glucose transport through the surface membrane and glucose intracellular metabolism (Rose & Richter, 2005) as shown in Figure 1-3.

There are several differences in glucose uptake between resting and exercise. Since GLUT4 is kept within intracellular storage sites and GLUT1 expression is relatively low, glucose transport is believed to be the rate limiting step for glucose uptake at rest. On the other hand, the limiting step of glucose uptake during exercise (in particular intense exercise) is mainly glucose phosphorylation since limitations in glucose delivery and transport can be overcame by skeletal muscle hyperaemia, capillary recruitment and GLUT4 translocation (Wasserman, 2009).

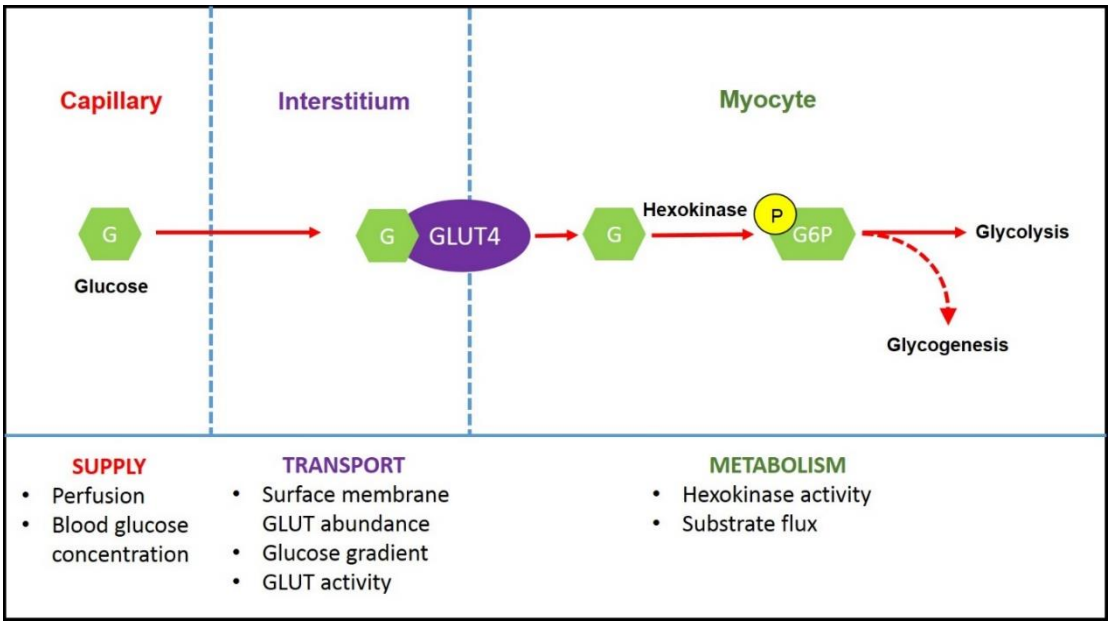


Figure 1- 3: Potential sites of regulation of muscle glucose uptake during exercise.

Glucose transporter type-4 (GLUT4) and glucose-6-phosphate (G6P). Adapted with modification from (Rose & Richter, 2005).

1.1.3.1 Glucose delivery

Glucose delivery or supply to a tissue vascular bed is expressed as the product of blood flow and arterial blood glucose concentration. Since muscle blood flow will increase up to 20-fold during intense, dynamic exercise (Andersen & Saltin, 1985), the increase in muscle blood flow is important for the increase in glucose supply (Rose & Richter, 2005). Apart from an increase in blood flow to the contracting muscles during exercise, there is also capillary recruitment that increases the surface area for the glucose delivery and exchange (Inyard, Clerk, Vincent, & Barrett, 2007).

In vitro studies of glucose uptake during electrical stimulation of perfused rat hindlimb muscle have demonstrated the importance of an increase in perfusion for the rate of glucose uptake during contractions (Hespel, Vergauwen, Vandenberghe, & Richter, 1995). Other than that, the other important factor for muscle glucose uptake during exercise is the arterial glucose level. This is because, during exhaustive prolonged exercise, there is a gradual depletion of glycogen in the liver and gluconeogenesis is unable to compensate. Thus, there is a reduction in the liver glucose output that may lead to hypoglycaemia which may limit the glucose uptake (Ahlborg, Felig, Hagenfeldt, Hendler, & Wahren, 1974).

1.1.3.2 Glucose transport

The augmentation in translocation of GLUT4 from the intracellular sites to the sarcolemma and T-tubules increases the muscle glucose transport during exercise (Richter & Hargreaves, 2013). A study conducted by Ryder and his colleagues demonstrated that, contraction-induced glucose uptake was fourfold lower in the skeletal muscle of GLUT4 knockout

mice when compared to the wild-type group. This result suggests the important role of GLUT4 as the primary mediator of exercise-induced glucose uptake in skeletal muscle (Ryder et al., 1999). In addition, mice with muscle-specific GLUT4 deletion demonstrated a significant reduction in muscle glucose uptake and exercise tolerance during exercise (Fueger et al., 2007).

1.1.3.3 Glucose metabolism

Glucose phosphorylation to glucose-6-phosphate (G6P) occurs after the glucose has been transported into the cell across the sarcolemma. This reaction is catalysed by hexokinase-2 (HK2). G6P is the first substrate in glucose metabolism in which it is catalysed for energy production during exercise via glycolysis or stored as glycogen during recovery from exercise. In maximal, dynamic exercise, HK2 activity is inhibited by elevated levels of intramuscular G6P as the result of an increase in muscle glycogenolytic rate. At the start of submaximal exercise, the limitation of glucose uptake and utilization is the inhibition of HK2 by G6P. As exercise continues, the HK2 inhibition is resolved by the lowering of G6P concentration that leads to reduction in intramuscular glucose concentration and increase in glucose uptake (Hargreaves, Meredith, & Jennings, 1992).

1.1.4 Exercise-induced GLUT4 translocation

GLUT4 is mainly retained in intracellular vesicle structures in resting muscle (Foley, Boguslavsky, & Klip, 2011) and several studies showed that both insulin as well as muscle contractions can translocate GLUT4 to the sarcolemma and T-tubular system (Derave, Hansen, Lund, Kristiansen, &

Richter, 2000; Derave et al., 1999; Douen et al., 1990). It is believed that there are two different intracellular pools of GLUT4 which are recruited by insulin and muscle contraction respectively (Coderre, Kandror, Vallega, & Pilch, 1995).

During insulin-stimulated GLUT4 translocation to the surface membrane, the event is controlled by proteins known as SNARE proteins (soluble N-ethylmaleimide-sensitive factor-attachment protein receptors) and proteins that regulate SNAREs. (v-) SNARES and (t-) SNARES are the proteins that are located in the GLUT4 vesicles and at the cell membrane respectively. Once interaction between v-SNARES and t-SNARES occurs, it leads to the formation of SNAREpin complex which consists of four SNARE motifs assembled into a twisted parallel four-helical bundle (Hong, 1998). This helical structure catalyzes the fusion of vesicles with the target membrane. The specific SNARE proteins that are known to be involved in this process include VAMP2, syntaxin 4 and SNAP23. These proteins are regulated by other proteins namely munc18C, synip and synaptotagmin (Foley et al., 2011).

On the other hand, it is generally believed that the contraction-stimulated GLUT4 translocation is different form insulin-stimulated mechanism (A. D. Lee, Hansen, & Holloszy, 1995). Calcium (Ca^{2+}) was found to activate glucose transport by indirect effect. By inducing muscle contraction and activation of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump (Norris, Bombardier, Smith, Vigna, & Tupling, 2010), it results in metabolic stress to the muscle cell via activation of AMP-activated protein kinase (AMPK), which increases muscle glucose uptake (Jensen, Rose, Hellsten, Wojtaszewski, & Richter, 2007).

Other than that, mitogen activated protein kinase (MAPK) are also involved in glucose uptake in skeletal muscle during exercise. The MAPK extracellular signal–regulated kinase (ERK)1, ERK2, p38 and c-Jun N-terminal kinases (JNK) are activated during muscle contraction (Aronson et al., 1997; Richter & Hargreaves, 2013; Ryder et al., 2000). However, JNK knockout mice were found to have no effect on glucose uptake (Witczak et al., 2006). Furthermore, blockade of ERK by inhibiting MAP kinase kinase (MEK) had no effect on contraction-induced glucose uptake in rat muscle (Hayashi, Hirshman, Dufresne, & Goodyear, 1999). The role of p38 MAPK in regulating contraction-induced glucose uptake in skeletal muscle is not yet well established (Richter & Hargreaves, 2013).

The actin cytoskeleton also found to be associated with glucose uptake in contracting muscle. Inhibition of Rac1 in contracting mouse muscle was found to impair the glucose uptake process (Sylow et al., 2013). Myo1c an actin based motor protein is also involved in glucose uptake and Myo1c mutated mice showed reduction in contraction-stimulated glucose uptake (Toyoda et al., 2011).

In addition, glucose uptake in skeletal muscle during exercise may also involve nitric oxide (in fast-twitch muscle) (Ross, Wadley, Clark, Rattigan, & McConell, 2007) and reactive oxygen species (in very intense electrical stimulation in vitro) (Richter & Hargreaves, 2013).

1.2 Endocannabinoids

1.2.1 Cannabis and cannabinoids

Cannabis is known to be among the first plants that have been used for a broad range of medical and non-medical applications (Pertwee, 2006). *Cannabis sativa* is one of the oldest plants cultivated by man and it is a member of the Cannabaceae family (Russo, 2007). Besides being applied as a therapeutic compounds, *Cannabis sativa* is widely consumed as a recreational drug through its various products such as marijuana, hashish and hash oil (Elsohly & Slade, 2005).

It is believed that cannabis has been used for more than 4000 years (Pagotto, Marsicano, Cota, Lutz, & Pasquali, 2006) and is the third most commonly used drug after tobacco and alcohol (D. Baker, Pryce, Giovannoni, & Thompson, 2003). Several reviews have been written on the use of cannabis in therapeutic drugs such as anticonvulsive, analgesics, antianxiety and antiemetic agents (D. Baker et al., 2003; Kumar, Chambers, & Pertwee, 2001). Apart from that, the acute effects of cannabis were clearly recognised such as euphoria, psychoactive, relaxation and intoxication and these mild altering effects may cause misuse or abuse as an illicit drug especially among youngsters (Hall & Degenhardt, 2009; Kumar et al., 2001).

In some cases, acute unwanted effects of cannabis may arise including impairment in short term memory, alteration in orientation to time and space, anxiety, and it may lead to acute psychosis involving hallucinations (Hall & Degenhardt, 2009; Kumar et al., 2001). Apart from that, there are numerous chronic effects of cannabis that have been identified which include respiratory symptoms such as bronchitis and

malignancies (if consumed by smoking), reproductive effects such as impairment in sperm production and disturbance of ovulatory cycle, psychotic symptoms including delusions, hallucinations and amnesia; dependence and withdrawal symptoms (Hall & Degenhardt, 2009; Kumar et al., 2001). Despite of all these unpleasant and adverse side effects, cannabis does not seem to be significantly associated with an increase in mortality (Degenhardt & Hall, 2012).

Cannabis is a complex plant containing more than 400 compounds (Ashton, 2001) and among those more than 60 compounds have been identified and collectively referred to as cannabinoids (Ashton, 2001; Hall & Solowij, 1998).

In the early 1960's, the structure and ingredients of cannabis were discovered and among those, the main psychoactive compound delta-9-tetrahydrocannabinol (Δ^9-THC) was identified and isolated (D. Baker et al., 2003; Mechoulam, Devane, Breuer, & Zahalka, 1991; Mechoulam, Fride, & Di Marzo, 1998). Other important natural cannabinoids include delta-8-tetrahydrocannabinol (Δ^8-THC), cannabidiol and cannabinol (Pertwee, 2006; Thakur, Duclos, & Makriyannis, 2005). The structures of these compounds are shown in Figure 1-4.

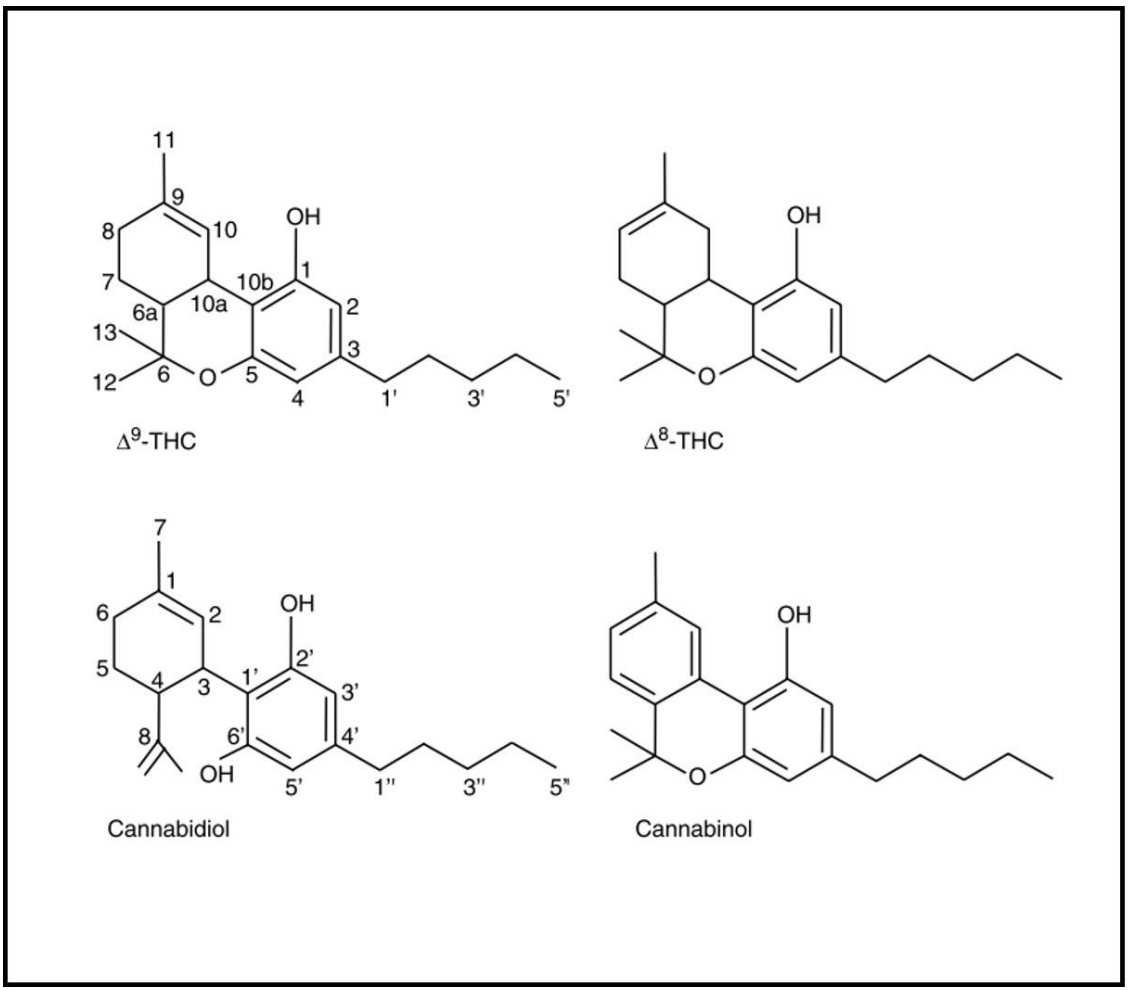


Figure 1- 4: The structures of Delta-9-tetrahydrocannabinol (Δ^9-THC), delta-8-tetrahydrocannabinol (Δ^8-THC), cannabidiol and cannabinol.

Adapted with minor modification from (Pertwee, 2006).

1.2.2 Endocannabinoid system

The endocannabinoid system (ECS) has been identified to play an important role in human regulation of energy balance and metabolism (Butler & Korbonits, 2009; Nogueiras et al., 2009; Silvestri, Ligresti, & Di Marzo, 2011).

This system consists of G-protein coupled receptors (GPCR), their lipid signalling ligands and enzymes involved in ligand generation and metabolism (Silvestri et al., 2011). It is known to act both at central and

peripheral sites; it modulates calorie intake centrally and affects the nutrients transport, cellular metabolism and energy storage peripherally (Silvestri et al., 2011).

Upon stimulation, the ECS may increase food intake causing weight gain, promoting lipogenesis and impairing glucose tolerance. There is increasing evidence in humans that the ECS is overactive in obesity, thus targeting and supressing this system may help in treating obesity, T2DM and metabolic syndrome (Scheen & Paquot, 2009).

Two types of cannabinoid G-protein couple receptors have been discovered, namely cannabinoid receptor type-1 (CB1) in year 1990 and cannabinoid receptor type-2 (CB2) in year 1993 (Howlett et al., 2002). These G-protein-coupled receptors cause the inhibition of the enzymatic activity of adenylate cyclase responsible for the production of cyclic adenosine monophosphate (cAMP) in the cell. In addition, the CB1 receptor is reported to have an effect on calcium and potassium channels (Howlett, 2002; Howlett et al., 2004).

The discovery of CB receptors led to the identification of their ligands, endogenous lipid mediators named endocannabinoids that bind to CB receptors. Anandamide (the ethanolamide of arachidonic acid, AEA) was the first endocannabinoid identified in year 1992 and the second endocannabinoid was isolated in 1995 named 2-arachidonoyl glycerol (2-AG) (Begg et al., 2005). Three other endocannabinoids, albeit not well-characterised, have also been identified, namely nolandin ether, virhodamine and N-arachidonoyldopamine (Cota, 2007).

CB1 was initially described as “brain type” receptor because it was first thought to be expressed predominantly in the central nervous system.

CB1 was identified in the brain cortex, hippocampus, amygdala, pituitary and hypothalamus. Subsequently, the CB1 receptor was found in other peripheral organs and tissues namely gastrointestinal tract, adipose tissue, thyroid, adrenals, skeletal muscle, hepatocytes, kidneys, reproductive organs and endocrine cells of the pancreas (O'Keefe, Simcocks, Hryciw, Mathai, & McAinch, 2013; Pagotto et al., 2006). Conversely, CB2 receptors are present mainly in immune and blood cells, where they may participate in immune responses (Howlett, 2002; Pagotto et al., 2006).

In the brain, endocannabinoids work at the synaptic level as neuromodulators. They are synthesized from cell membrane phospholipids and are immediately released, usually from the post-synaptic side and act in a retrograde manner by binding to presynaptic CB1 receptors (Freund, Katona, & Piomelli, 2003; Piomelli, 2003).

1.2.3 CB1 receptor

CB1 is located in the cell membrane in which it is coupled to the intracellular effector system via G-proteins. As mentioned earlier, CB1 is a typical GPCR. Thus it contains seven transmembrane domains connected by three extracellular and three intracellular loops with an extracellular N-terminal tail, and an intracellular C-terminal tail (Turu & Hunyady, 2010).

At cellular level, CB1 has been found to modulate several functions such as inhibiting voltage-gated Ca^{2+} channels, activating K^{+} currents, phosphorylating and activating MAPK such as p42/p44 MAPK, p38 MAPK and JNK, inhibiting adenynyl cyclase in most tissues and cells (Turu & Hunyady, 2010) and increasing NO signalling (O'Keefe et al., 2013).

CB1 is an inhibitory type of GPCR. Activation of CB1 results in the stimulation of $G_{i/o}$ protein that inhibits the edenynyl cyclase-mediated conversion of ATP to cAMP. In the normal state, cAMP molecules can bind to the regulatory subunits of protein kinase A (PKA) and cause the release of the catalytic subunits. Furthermore, activated PKA phosphorylates the A-type potassium (K^+_A) channels, which in turn, cause a reduction in the current. Thus the negative effects of CB1 on adenynyl cyclase results in the activation of K^+ channels. $G_{i/o}$ activated by CB1 can also directly inhibit N- or P/Q-type Ca^{2+} channels and activate inwardly rectifying potassium (K_{ir}) channels. These last two effects are controlled by protein kinase C (PKC), which upon activation can phosphorylate CB1 in the third cytoplasmic loop and uncouple the receptor from the ion channels. Activation of CB1 can also stimulate several intracellular kinases, such as focal adhesion kinase (FAK), phosphatidyl inositol 3-kinase (PI3-K) and its downstream effector protein kinase B (PKB)/AKT, ERKs, JNK and p38 MAPK. Stimulation of cytoplasmic kinases could also mediate the CB1-induced expression of the immediate early genes (IEG), such as the transcription factors c-fos, c-jun, and zif268, and the brain-derived neurotrophic factor (BDNF) (Pagotto et al., 2006; Svizenska, Dubovy, & Sulcova, 2008) as shown in Figure 1-5.

CB1 has gained more attention than CB2 in studies investigating aspects of energy balance regulation mainly due to the discovery of the specific CB1 receptor antagonist Rimonabant and the development of CB1 knockout (CB1 (-/-)) mice models (Nogueiras et al., 2009).

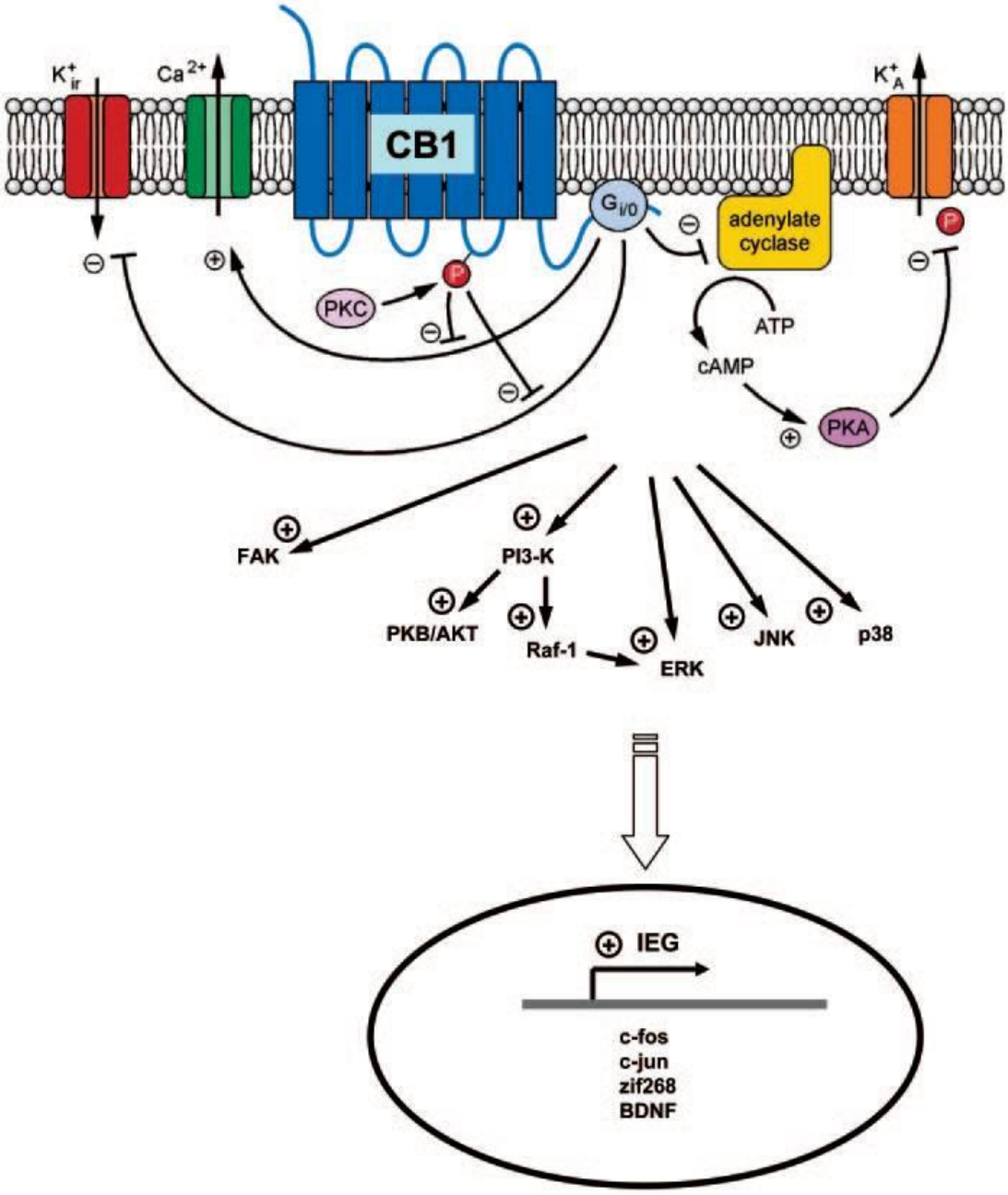


Figure 1- 5: Schematic representation of the main effects of CB1 on intracellular signalling cascades.

Adapted from (Pagotto et al., 2006).

1.2.4 CB2 receptor

In contrast to CB1 receptors, CB2 receptors are present mainly in immune and blood cells, where they may participate in immune responses (Howlett, 2002; Pagotto et al., 2006). CB2 are also expressed in immune tissues including spleen, tonsils, monocytes, B and T cells (Galiegue et al., 1995; Hohmann & Suplita, 2006). Besides that, CB2 has been implicated recently in the regulation of liver fibrosis and atherosclerosis (Ofek et al., 2006). On the other hand, CB2 receptors are also found in central nervous system (CNS) but only in low levels (Munro, Thomas, & Abu-Shaar, 1993). In addition, CB2 messenger RNA (mRNA) has been detected in the lumbar dorsal horn together with appearance of activated microglia in pathological states (J. Zhang et al., 2003).

There is also evidence that CB2 receptors play an essential role in the maintenance of bone mass with these receptors expressed in both cells of osteoblasts and osteoclasts lineages. Exposures of those cells to CB2-specific agonists suggested CB2 signalling contributes to the bone-mass maintenance by at least two mechanisms (i) direct stimulation of stromal cells/osteoblasts and (ii) inhibition of monocytes/osteoclasts (Ofek et al., 2006).

1.2.5 Endocannabinoid ligands

The discovery of CB receptors led to the identification of their ligands, endogenous lipid mediators named endocannabinoids that bind to CB receptors.

As mentioned earlier, anandamide (ethanolamide of arachidonic acid/AEA) was the first endocannabinoid identified (Devane et al., 1992)

and the name adapted from the Sanskrit word ‘ananda’ means bliss. Subsequently, the second endocannabinoid 2-arachidonoyl glycerol (2-AG) was discovered (Sugiura et al., 1995).

Both of these compounds are the derivatives of arachidonic acid and have the ability to bind to CB1 and CB2 receptors, however with different affinity and activation efficacies (Howlett, 2002). Unlike other neurotransmitters, endocannabinoids are very lipophilic in nature. Due to this property, they are rapidly produced and degraded, thus they are not stored in vesicles (Freund et al., 2003). The regulation of endocannabinoid signalling is controlled by their synthesis, release, uptake and degradation (Piomelli, 2003).

In the brain, these endocannabinoids work at the synaptic level as neuromodulators. They are systhesized from phospholipids in the cell membranes and immediately released from the post-synaptic side in retrograde manner by binding to pre-synaptic CB1 receptors (Piomelli, 2003).

Neurons produce both anandamide and 2-AG following depolarization of the membrane and an increase in intracellular calcium. Anandamide is formed by hydrolysis of N-arachidonoyl phosphatidyl ethanolamine by phospholipase D, whereas 2-AG is formed following hydrolysis of phosphatidylinositol by phospholipase C and diacylglycerol lipase. After synthesis, anandamide and 2-AG are released into the extracellular space and bind to CB1 receptors. Activation of pre-synaptic CB1 receptors leads to modulation of neurotransmitter release thus affecting synaptic activity (Freund et al., 2003; Piomelli, 2003).

Endocannabinoids are rapidly metabolized. Inactivation of anandamide occurs by cellular re-uptake and subsequent hydrolysis by a fatty acid amide hydrolase (FAAH). Meanwhile, 2-AG degradation in neurons is primarily due to hydrolysis by monoacylglycerol lipase (MAGL). Both FAAH and MAGL are abundantly expressed in the brain and in peripheral organs (Dinh et al., 2002; Thomas, Cravatt, Danielson, Gilula, & Sutcliffe, 1997).

1.2.6 Role of ECS in central regulation of energy balance

There is growing evidence that both CB1 receptor agonists and antagonists have an effect on food intake. Treatment with oral Δ^9-THC can cause hyperphagia and increased food intake in rats (Brown, Kassouny, & Cross, 1977; Williams & Kirkham, 2002). Furthermore, animals given anandamide via intraperitoneal (IP) and subcutaneous routes also showed an increase in food intake (Hao, Avraham, Mechoulam, & Berry, 2000; Williams & Kirkham, 1999). It is believed that these effects are mediated by activation of the CB1 receptor since pre-treatment with the CB1 receptor antagonist SR 141716 (rimonabant) was found to inhibit hyperphagia (Williams & Kirkham, 1999, 2002). In addition, CB1 receptor blockage was found to have a significant reduction in the consumption of both bland and palatable foods in different animal models (Colombo et al., 1998; Verty, McGregor, & Mallet, 2004; Werner & Koch, 2003; Williams & Kirkham, 1999).

Furthermore, CB1 receptor knockout mice (CB1 (-/-)) were found to be slightly hypophagic and have a lean phenotype associated with reduced body weight and fat mass percentage (CB1–/– fat mass, 9.7% ± 0.55% vs.

CB1+/+ 12.3% ± 0.51%, P<0.005); and increased in lean mass percentage (CB1–/– lean mass, 73.1% ± 0.43% vs. CB1+/+ 71.6% ± 0.44%, P<0.05) (Cota et al., 2003). This supports the involvement of CB1 receptors in feeding behaviour and transient anorexia. Moreover, the CB1 receptor antagonist rimonabant has no effect on the eating behaviour of CB1 (-/-) mice (Di Marzo et al., 2001). In addition, CB2 receptor antagonism does not affect eating behaviour in mice (Wiley et al., 2005).

Intrahypothalamic administration of CB1 receptor agonists and antagonists provides direct evidence for the involvement of ECS in the hypothalamus in regulating food intake. Administration of anandamide through the ventromedial nucleus of the hypothalamus showed stimulation of eating in pre-satiated rats and pre-treatment with CB1 receptor antagonist rimonabant successfully blocked anandamide-induced overeating (Jamshidi & Taylor, 2001). Furthermore, treatment with rimonabant (20mg/day) in human subjects reported reduction in appetite and their desire for sweets and high-fat food consumption (Scheen et al., 2006).

1.2.7 Role of ECS in peripheral metabolism

Initially, it was thought that the ECS acted solely centrally through its effects on appetite and food intake. However, CB1 receptors have been identified in a number of peripheral organs and tissues involved in the regulation of metabolism including adipose tissue, muscle, pancreas, liver and gut. CB1 receptors innervating the gastrointestinal tract affect gastric emptying, gut motility and peristalsis thus play a role in satiety signalling (Massa, Storr, & Lutz, 2005).

A human study showed an increase in calorie intake and weight gain in subjects who smoked cannabis when compared to control. They observed that the weight of the participants continued to increase throughout the 3-week study, despite of cessation of increase in food intake after just a few days (Greenberg, Kuehnle, Mendelson, & Bernstein, 1976). In another study, chronic oral administration (5 weeks) of the CB1 antagonist SR 141716/rimonabant in mice with diet-induced obesity (DIO) caused a transient reduction of food intake but weight reduction persisted even after food intake returned to the level of normal untreated group (Ravinet Trillou et al., 2003). Both of the above studies suggest that endocannabinoids promote weight gain by other peripheral effects such as direct adipogenesis and inhibiting adiponectin (Matias et al., 2006), rather than only by stimulating appetite.

In addition, neurotoxin capsaicin-induced destruction of sensory afferent fibres of the gut showed absence of hyperphagia and hypophagia after peripheral administration of CB1 receptor agonist and antagonist respectively, suggesting that these agents modulate food intake by acting on CB1 receptors located on capsaicin-sensitive sensory terminals (Gomez et al., 2002).

The levels of endocannabinoids in adipose tissue were found to be similar to those in the brain and experimental evidence has shown that besides the existence of CB1 receptors, adipose tissue also contains enzymes regulating the synthesis and degradation of the endocannabinoids (Pagano et al., 2007). Moreover, there was a 3-fold higher CB1 receptor mRNA expression in the adipose tissue of obese rats when compared to lean rats (Bensaid et al., 2003).

The CB1 receptors are also expressed in the liver (Osei-Hyiaman et al., 2005; Pagotto et al., 2006), pancreas (Juan-Pico et al., 2006) and skeletal muscle (Crespillo et al., 2011; Eckardt et al., 2009; Pagotto et al., 2006). Diet-induced obesity is associated with increased hepatic CB1-mediated fatty acid synthesis and increased levels of CB1 receptor mRNA and anandamide were reported in the liver of wild-type mice on high fat diet (Osei-Hyiaman et al., 2005). CB1 and CB2 receptors are also expressed in the islet of Langerhans in rat and mouse pancreas, and CB2 receptors were identified in both alpha and beta cells of the islet (Bermudez-Silva et al., 2007; Juan-Pico et al., 2006).

1.2.8 ECS in adipose tissues

The adipose tissue is a central metabolic organ in the regulation of whole-body energy homeostasis. It is originally considered as a passive reservoir for energy storage (Frayn, Karpe, Fielding, Macdonald, & Coppack, 2003) and plays a crucial role in obesity and its co-morbidities (Fain, Madan, Hiler, Cheema, & Bahouth, 2004). Lately, it was reported that adipose tissues also act as an endocrine organ in which it secretes various hormones and adipokines that control systemic energy balance by regulating appetitive signals from the central nerve system as well as metabolic activity in peripheral tissues (Choe, Huh, Hwang, Kim, & Kim, 2016; Silvestri et al., 2011).

Cultured primary adipose cells from mice treated with a CB1 agonist showed increased levels of lipoprotein lipase activity in dose dependent manner (Cota et al., 2003). Lipoprotein lipase is an essential enzyme in

adipose tissue that serves the function of fatty acid uptake and storage (Gonzales & Orlando, 2007).

On the other hand, the upregulation of lipoprotein lipase in adipose tissue was blocked following the treatment with the CB1 receptor antagonist rimonabant (Jbilo et al., 2005).

In a study by Bluher et al., adipose tissue samples from both lean and obese individuals following open abdominal surgery were investigated for the presence of endocannabinoids. The findings from that study showed that the mRNA expression of FAAH was higher in adipose tissue of lean subjects and exhibited a negative correlation with visceral obesity. Furthermore, there was higher CB1 expression in visceral compared to subcutaneous tissue in both groups (Bluher et al., 2006).

1.2.9 ECS hyperactivity in obesity

Experimental data demonstrated that ECS is upregulated in obesity in both humans and animals (Di Marzo & Matias, 2005). The amount of 2-AG was found to be significantly higher in the visceral fat of obese and overweight subjects when compared to normal-weight control subjects (Matias et al., 2006). Furthermore increased levels of anandamide and 2-AG, together with a reduced expression of FAAH, were observed in obese, post-menopausal women compared to control subjects, which suggest impairment of endocannabinoid degradation may play an important role in the upregulation of ECS in obesity (Engeli et al., 2005).

In addition, specific gene polymorphisms affecting the regulation of the ECS activity may also contribute to the incident of obesity. Indeed, a missense polymorphism in FAAH, which occurs at higher frequency of obese

individuals, was found to correlate with body mass index (BMI). Furthermore, FAHH activity was reduced in individuals with this type of polymorphism when compared with control subjects, thus suggesting that upregulation of ECS due to a defect in their degradation might contribute to the development of obesity (Sipe, Waalen, Gerber, & Beutler, 2005).

1.2.10 ECS in muscles

CB receptors and other ECS components are expressed in human and animal muscle cells (Crespillo et al., 2011; Eckardt et al., 2009). Furthermore, in a study by Eckardt et al. using primary skeletal muscle cells, anandamide was able to induce ERK 1/2, p38 and insulin receptor substrate 1 (IRS1(Ser307)) phosphorylation, which inhibits insulin-dependent PKB/Akt phosphorylation and glucose uptake (Eckardt et al., 2009).

ECS was also seen to alter muscle glucose transport; isolated soleus muscle from ob/ob Leptin mutant mice treated with rimonabant demonstrated increased skeletal muscle glucose uptake (Y. L. Liu, Connoley, Wilson, & Stock, 2005). Furthermore, by using rat myotubes *in vitro*, Esposito et al. found that direct CB1 inhibition with rimonabant at a low concentration increases glucose uptake without affecting the expression levels of the glucose transporters GLUT1 and GLUT4. However, CB2 blockage had no effect on the alteration in glucose uptake (Esposito et al., 2008).

1.3 Skeletal muscle myokines

1.3.1 Overview of the myokines

Skeletal muscle is the largest organ in human body and recent data showed that several humoral factors or cytokines have been synthesized and secreted by contracting muscle cells which were found to play some roles in metabolism. Due to this fact, skeletal muscle can be considered as an endocrine organ which is able to secrete hormone-like factors that may influence metabolism in other tissues or organs (Pedersen, 2011b). In accordance with this, it was suggested that any cytokines and other peptides that are synthesized, expressed and secreted by muscle fibres and exert either paracrine or endocrine effects are classified as 'myokines' (Pedersen, Akerstrom, Nielsen, & Fischer, 2007).

For decades, researchers have tried to reveal a relationship between muscle contraction and changes in other organs in the form of myokine release which can mediate several exercise-induced metabolic changes in other organs especially liver and adipose tissue (Pedersen, 2011b).

Recent research revealed that skeletal muscle has the capacity to express several myokines including interleukin (IL)-6, IL-8 and IL-15 (Pedersen et al., 2007). Specifically, research has shown that IL-6 (Febbraio & Pedersen, 2002, 2005; Pedersen, Steensberg, Fischer, et al., 2003; Pedersen, Steensberg, Keller, et al., 2003) and IL-8 (Chan, Carey, Watt, & Febbraio, 2004; Nieman et al., 2003) mRNA and protein expression was regulated by concentric muscle contractions, whereas IL-15 mRNA was upregulated in human skeletal muscle following strength training (Nielsen et al., 2007).

1.3.2 Interleukin 6 (IL-6)

IL-6 belongs to a family of cytokines that also includes leukemia inhibitory factor, interleukin-11, ciliary neurotrophic factor, cardiotrophin-1, and oncostatin M. In addition to structural similarities, these cytokines share the gp130 receptor subunit (Kamimura, Ishihara, & Hirano, 2003; Kishimoto, Akira, Narazaki, & Taga, 1995).

Skeletal muscle contractions are the main source of IL-6 in the systemic circulation in response to exercise. In the resting state, IL-6 mRNA content in skeletal muscle is very low, whereas small amounts of IL-6 protein may be detected using sensitive immunohistochemical methods in type I fibres (Plomgaard, Penkowa, & Pedersen, 2005). The level of circulating IL-6 rises in an exponential manner (up to 100-fold) in response to exercise; and the peak of IL-6 is reached at the end or shortly after exercise followed by a rapid decline to the level seen before exercise (Pedersen & Fischer, 2007).

IL-6 may be produced by both immune cells, specifically macrophages, and skeletal muscle. However, the IL-6 synthesized by macrophages leads to an inflammatory response, whereas IL-6 synthesized and produced by muscle cells do not activate classical pro-inflammatory pathways (Pedersen & Febbraio, 2008).

In terms of systemic effects, a number of studies demonstrated that IL-6 is able to induce insulin resistance *in vitro* (Lagathu et al., 2003; Rotter, Nagaev, & Smith, 2003; Senn, Klover, Nowak, & Mooney, 2002; Senn et al., 2003) and in rodents *in vivo* (Kim et al., 2004; Klover, Clementi, & Mooney, 2005; Klover, Zimmers, Koniaris, & Mooney, 2003). In addition, a study in both primary mouse hepatocytes and the human hepatocarcinoma

cell line, HepG2 suggested that insulin resistance is induced by IL-6 through its adverse effects on the liver in which it inhibits both insulin receptor (IR) signal transduction and Akt phosphorylation (Senn et al., 2002). Furthermore, the IL-6 induced insulin resistance seems to be associated with an increase in the expression of suppressor of cytokine signalling 3 (SOCS-3) (Senn et al., 2003), which may directly inhibit insulin receptor activity (Ueki, Kondo, & Kahn, 2004).

However, there are also several studies that suggested positive effects of IL-6 on insulin stimulated glucose uptake. Acute treatment of muscle cells *in vivo* with IL-6 may increase basal and insulin-stimulated glucose uptake through the increase in GLUT4 translocation from intracellular compartments to the plasma membrane (Carey et al., 2006). In addition, IL-6 was also suggested to have a link with AMP-activated protein kinase (AMPK). AMPK activation leads to the induction of fatty acid oxidation and enhancement in glucose uptake (Kahn, Alquier, Carling, & Hardie, 2005), and IL-6 was found to increase AMPK activity in skeletal muscle and adipose tissue (Kelly et al., 2004). IL-6 was also identified as a lipolytic factor on the basis of evidence showing that infusion of recombinant human IL-6 into healthy humans (to achieve physiological concentrations) stimulates lipolysis without alterations in cathecolamines, insulin, glucagon or any other adverse effects in healthy individuals (Lyngso, Simonsen, & Bulow, 2002; van Hall et al., 2003).

1.3.3 Interleukin 8 (IL-8)

IL-8 is a known myokine that attracts primarily neutrophils and acts as an angiogenic factor (Pedersen, 2011b; Pedersen et al., 2007). There is

an increase in plasma concentration of IL-8 following exhaustive eccentric exercise such as marathon running in trained individuals (Nieman et al., 2003; Nieman et al., 2002; Nieman et al., 2001; Ostrowski, Rohde, Asp, Schjerling, & Pedersen, 2001). In contrast, there was no increment of plasma IL-8 in response to concentric exercise such as rowing (Henson et al., 2000) or bicycle ergometry (Chan et al., 2004). However, another study reported a local low amount of IL-8 secretion by contracting limb muscle following concentric exercise, detected by arterio-venous concentration differences, without a measurable effect on systemic plasma IL-8 levels (Pedersen, 2011b).

The primary role of muscle-derived IL-8 appears to be stimulation of angiogenesis. IL-8 associates with CXC chemokine receptor 1 (CXCR1) to induce chemotactic effects and CXC chemokine receptor 2 (CXCR2), which is present in human microvascular endothelial and mediates the induction of angiogenesis (Bek, McMillen, Scott, Angus, & Shaftan, 2002; Brat, Bellail, & Van Meir, 2005; Koch et al., 1992).

1.3.4 Interleukin 15 (IL-15)

IL-15 is a four α-helix cytokine which has structural similarities to IL-2 (Bamford et al., 1994; Grabstein et al., 1994). IL-15 mRNA was shown to be upregulated following a single bout of strength training in human skeletal muscle (Nielsen et al., 2007) and was found to exert anabolic effects on skeletal muscle both *in vitro* and *in vivo* (Pedersen et al., 2007). A study by Furmanczyk demonstrated that IL-15 stimulates the accumulation of protein myosin heavy chain in differentiated muscle cells in skeletal myogenic cultures. Since the accretion of this major myofibrillar

protein, myosin heavy chain, was used as a measure of muscle anabolism, the result supports the role of IL-15 as an anabolic factor in muscle growth (Furmanczyk & Quinn, 2003).

On the other hand, IL-15 was shown to play a role in reducing adipose tissue mass (Pedersen et al., 2007). Indeed, a 33% reduction in white adipose tissue mass was observed following 7 days of IL-15 administration in adult rats (Carbo et al., 2001). In addition, another study reported a negative association between IL-15 concentration and trunk fat mass. This was demonstrated by a reduction in truncal fat mass upon overexpression of IL-15 in mice, however there was no alteration in subcutaneous fat mass (Nielsen et al., 2008).

1.4 Lipocalin 2

1.4.1 Overview of Lipocalin 2

Lipocalin 2 (LCN2), also known as neutrophil gelatinase-associated lipocalin, was originally identified as a 25-kDa protein secreted from human neutrophils (Kjeldsen, Bainton, Sengelov, & Borregaard, 1994; Kjeldsen, Johnsen, Sengelov, & Borregaard, 1993). It was recently identified as an adipokine that belongs to the superfamily of lipocalins that appear to affect glucose metabolism and insulin sensitivity (Kjeldsen et al., 1994).

Lipocalins are small proteins with a hydrophobic ligands binding pocket (Flower, 1996). As a member of lipocalin family, LCN2 possesses common crystal structures of an eight-stranded antiparallel β-barrel and participate in various biological processes. This structure indicates that lipocalins are able to bind and transport a wide variety of small hydrophobic

molecules such as retinol, fatty acids, steroids and thyroid hormone (Flower, 1996; LaLonde, Bernlohr, & Banaszak, 1994)

The structure of LCN2 is similar to fatty acid binding proteins (FABPs) and retinol binding proteins (RBPs), whereas the cavity in LCN2 is unusually large with more polar and positively charged amino acids compared with other proteins in the lipocalin family (Kjeldsen, Cowland, & Borregaard, 2000), suggesting that LCN2 may have unique functions in lipid binding and transport (Kjeldsen et al., 1993).

LCN2 has been implicated in diverse actions, such as apoptosis and innate immunity and is expressed in several tissues including neutrophils, liver, kidney, adipocytes and macrophages (Y. Wang et al., 2007). LCN2 is also highly expressed in uterus, bone marrow, and granulocytes immune cells (Aigner et al., 2007; H. L. Huang, Chu, & Chen, 1999). Mice at 3 weeks of age also express LCN2 in liver, spleen, testis and lungs; however, the expression declines gradually with age, particularly in the liver, kidney and spleen with complete disappearance by the age of 11 weeks in adult mice (Garay-Rojas, Harper, Hraba-Renevey, & Kress, 1996). In most of the early studies, LCN2 expression was examined in non-adipose tissues. In 2005, LCN2 was first reported to be secreted from adipocytes, and since then the role of LCN2 as a new adipokine in metabolism has been widely examined (X. Chen, Cushman, Pannell, & Hess, 2005; Yan et al., 2007).

1.4.2 Regulation of Lipocalin 2

LCN2 expression is regulated by environmental stress such as hypoxia and infection, as well as metabolic conditions like hyperlipidaemia, obesity and insulin resistance (Li & Chan, 2011).

The promoter region of LCN2 contains the binding sites of the transcription factors nuclear factor-κB (NFκB), CCAAT/enhancer-binding protein (C/EBP) (Shen, Hu, Goswami, & Gaffen, 2006) and glucocorticoid response element (Garay-Rojas et al., 1996), indicating the expression of LCN2 may be controlled by inflammatory and metabolic conditions.

Expression of LCN2 is markedly induced by a variety of pro-inflammatory stimuli, including lipopolysaccharide, interleukin-1β, interleukin-17, TNFα and hyperglycaemia (Bu et al., 2006; Pawluczyk, Furness, & Harris, 2003; Yan et al., 2007). Dexamethasone and retinoic acid were also reported to increase LCN2 expression in mouse L cells (sarcoma cell line isolated from mouse fibroblasts) through an autocrine mechanism (Garay-Rojas et al., 1996).

1.4.3 Lipocalin 2 in obesity and insulin resistance

A study in humans by Wang et al demonstrated that serum concentrations of LCN2 were significantly higher in obese persons when compared to lean individuals in both adult males and females. Furthermore, LCN2 concentrations in the serum were found to have a positive association with waist circumference, waist-to-hip ratio, body fat percentage, systolic blood pressure, fasting triglycerides, fasting glucose and insulin concentrations, and also markers for chronic inflammation specifically C-reactive protein (Y. Wang et al., 2007). Other studies demonstrated upregulation of LCN2 gene expression in adipose tissue in genetic and dietary-induced obese rodents (Y. Wang et al., 2007; J. Zhang et al., 2008), as well as in obese humans (Y. H. Lee et al., 2010; Panidis et al., 2010; Y. Wang et al., 2007).

A cross-sectional study of 2519 Chinese individuals revealed serum LCN2 to be significantly higher in impaired glucose metabolism and newly diagnosed T2DM patients compared to normoglycaemic individuals. In addition, LCN2 was also positively associated with the insulin resistance index of homeostasis model assessment (HOMA-IR). The possible mechanism of this insulin resistance that has been proposed is by the influence of LCN2 on β cell function and insulin sensitivity partially through mediation of iron (Y. Huang et al., 2012). Since LCN2 has been suggested to be an iron delivery protein (Yang et al., 2002), it will delivers iron to the cytoplasm. These iron-loaded LCN2 increases intracellular iron level and the iron excess might result in β cell oxidative stress and impairment insulin secretary capacity (Cooksey et al., 2004).

In an animal study, circulating LCN2 levels were found to be increased in obese animals, and LCN2 mRNA was increased in the liver of ob/ob mice (Yan et al., 2007). On the other hand, LCN2-deficient mice showed a significant decrease in fasting glucose and insulin levels, and improved insulin sensitivity in ageing, dietary-induced obesity or genetic-induced obesity compared with their wild-type littermates (Law et al., 2010). Moreover, thiazolidinedione administration reduces LCN2 gene expression in adipose tissue of obese animals (Yan et al., 2007; J. Zhang et al., 2008). Based on one of the study, it has been proposed that LCN2 acts as an adipocyte-derived mediator of insulin resistance. Thus, the administration of insulin-sensitizing compounds- thiazolidinedione will reduce the expression of LCN2 in adipocytes (Yan et al., 2007).

1.5 Aim of thesis

Obesity and T2DM have been identified as major worldwide health problems (Reilly et al., 2003; Scarborough et al., 2011; Y. C. Wang, McPherson, Marsh, Gortmaker, & Brown, 2011). There is a growing body of evidence which suggests lipid signalling molecules including fatty acids, endocannabinoids and fatty acid amides may play a role in the development of obesity and insulin resistance (Adams et al., 2004; Bermudez-Siva et al., 2006; Chavez & Summers, 2003; Di Marzo, 2008; Kahn & Flier, 2000; Storz, Doppler, Wernig, Pfizenmaier, & Muller, 1999). Much of the current research has focused upon the synthesis and release of such molecules by adipose tissue and their effects upon the endocrine system. Interestingly, in addition to adipose tissue, CB receptors and other ECS components are also expressed in human and animal skeletal muscle cells. However, there is minimal information regarding the role of these fatty acid derivatives in skeletal muscle – which is primarily responsible for determining the metabolic rate of mammals. Given the growing obesity epidemic and the potential for receptors such as G protein couple receptors (GPCRs) and nuclear receptors to act as therapeutic targets, this lack of data represents a significant gap in our knowledge.

The main mechanism of glucose uptake in contracting skeletal muscle is by facilitated diffusion and this process is controlled by the translocation of GLUT4 glucose transporter to the surface membrane. Our laboratory has previously demonstrated that the level of GLUT4 is extremely low in non-contracting primary skeletal muscle cell cultures, expressed at approximately 3000-fold lower as compared to skeletal muscle tissue. Due

to the fact that the GLUT4 translocates from intracellular storage depots to the plasma membrane upon muscle contraction, it is essential to develop a contractile model of myotubes that may resemble the properties of skeletal muscle during exercise *in vivo* in order to study the effects of ECS and lipocalin 2 on insulin stimulated glucose uptake in skeletal muscle.

Therefore, the aims of this thesis are:

1. To develop a contractile model of skeletal muscle myotubes which is sensitive to insulin-stimulated glucose uptake for further studies on CB1 receptor characteristics.
2. To investigate the effects of cannabinoid CB1 modulation upon contraction-induced improvement in insulin-stimulated glucose uptake in primary skeletal muscle myotubes.
3. To examine the effects of cannabinoid CB1 modulation in skeletal muscle upon molecular adaptations related to the development of obesity and insulin resistance.
4. To investigate the potential role of LCN2 as a novel myokine both *in vitro* and *in vivo*.

CHAPTER TWO

MATERIALS AND METHODS

Chapter 2: Materials and Methods

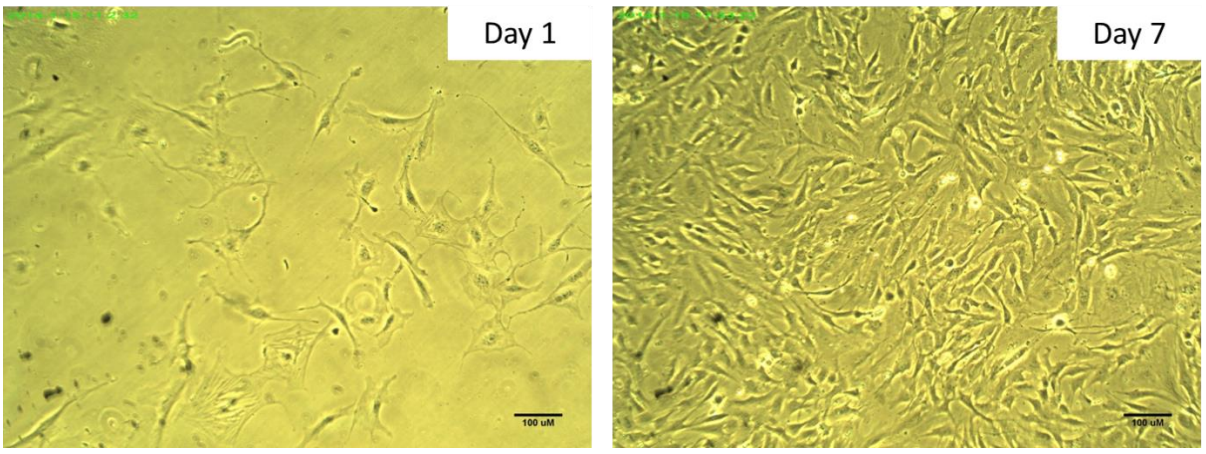
2.1 Tissue Collection

Animal muscle samples were obtained from Wistar rats, weighted 200g-250g. The muscle samples were taken from soleus muscles from the hindlimb. The rats were killed by cervical dislocation without anaesthesia, a method approved by the University of Nottingham and the Animal Scientific Procedures Act (ASPA) and the muscles were carefully excised within 10 minutes of death. Human muscle samples were obtained from the vastus lateralis muscle. The samples were taken from healthy male volunteers with the age between 18-35 years old using the needle biopsy technique (Bergstrom needle) under local anaesthesia with full ethical approval- Ref No: A10112015 SoLS, approved on 11/12/2015 by University of Nottingham Faculty of Medicine & Health Sciences Research Ethics Committee. The skeletal muscle cell samples from rats and human were then subjected for primary skeletal muscle cell culture that will be explained in the next section.

2.2 Skeletal Muscle Cell Culture

The fresh skeletal muscle samples were immersed in phosphate buffered saline (PBS) (Oxoid, Fischer Scientific, USA) to wash out the residual blood. The muscles were placed on a petri dish and homogenized using scalpel blade. The tissues were transferred into a universal tube and 5ml of trypsin/EDTA (0.5 g/l porcine trypsin and 0.2 g/l EDTA) was added, an autoclaved stirrer magnet was placed and the sample was gently mixed for 15 minutes at 37^{o}C. Then the supernatant was transferred to a fresh

falcon tube, an equal volume of Ham F-10 medium (Sigma-Aldrich, USA) was added and centrifuged for 5 minutes at 1700 rpm. The supernatant layer was removed and the cell pellet (satellite cells) was resuspended in HAM's F10 (Sigma-Aldrich, USA) growth medium, pre-plated on an uncoated flask for 10 minutes at 37°C to purify the satellite cells from existing fibroblasts in the extract, and then non-adherent cells were transferred to 6-well plates coated with 0.2% (W/V) gelatine. The satellite cells were then grown to confluence as myoblasts and differentiated into myotubes in growth medium. 20% (V/V) fetal bovine serum (FBS) (Thermo Scientific, USA) and 5ml of antibiotics (10,000 units penicillin and 10mg streptomycin/ml in 0.9% NaCl) (Sigma-Aldrich, USA) were added to the Ham's F10 medium. After 48 hours, the cells were fed with fresh medium and the medium changed every subsequent 48 hours. The cells were fed with 20% (V/V) FBS fresh medium for 1 week, then reduced to 10% (V/V) FBS in fresh medium for the next 1 week and then changed to 6% horse serum (Gibco, Life Technologies, USA) for the subsequent week until the cells fused to form myotubes. Horse serum contains lower level of growth factors and it promoted the process of cell differentiation. The progress of the cell growth is shown in Figure 2-1:



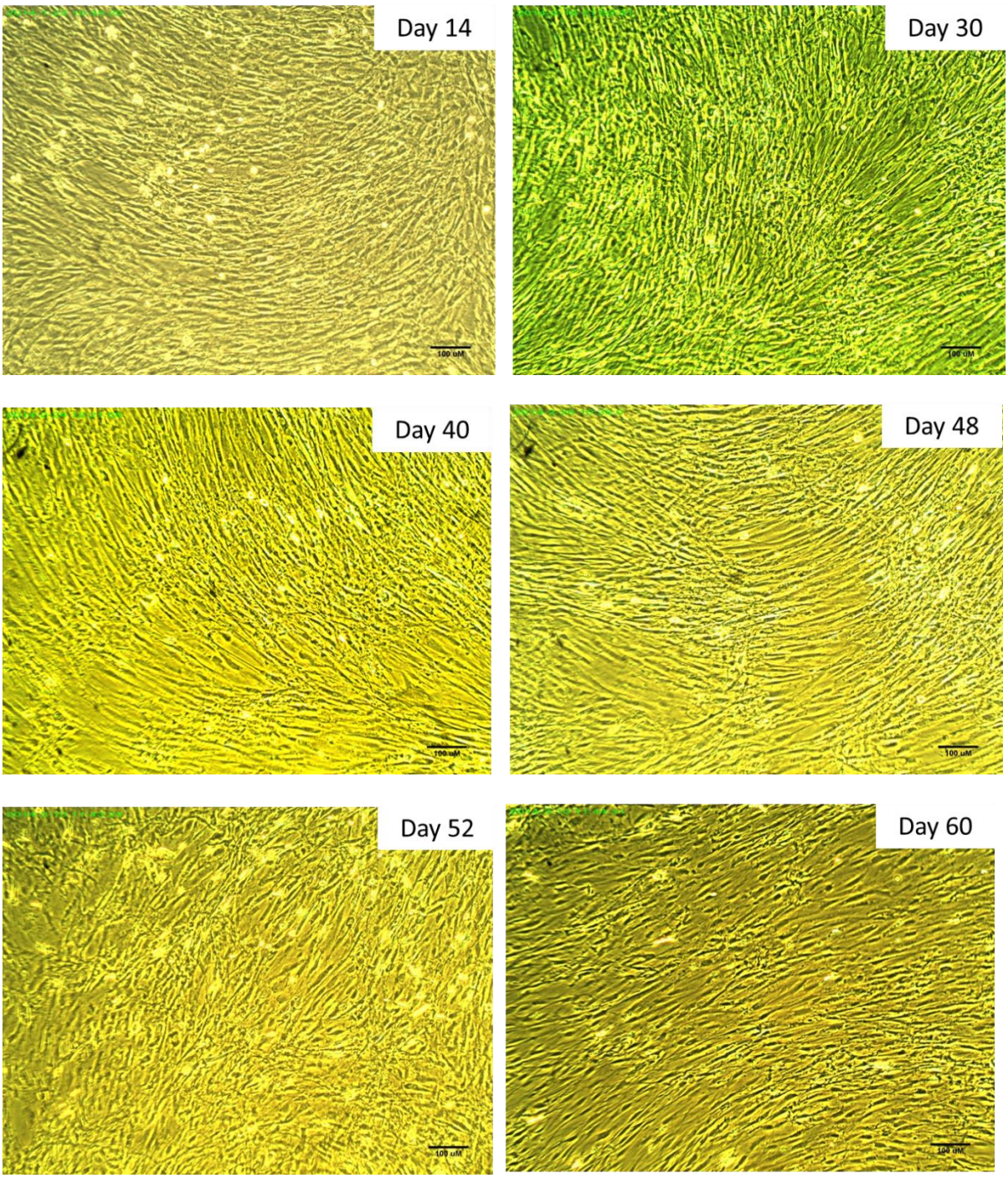


Figure 2- 1: Representative images for rat satellites cells (day 1), myoblasts (day 7) and myotubes (day 30 onwards).

2.3 Electrical Pulse Stimulation

Electrical pulse stimulation (EPS) was applied to fully differentiated myotubes in six-well dishes using a C-Dish Culture Dish Electrode System combined with a C-Pace EP pulse generator (IonOptix, Milton, MA, USA)

(Figure 2-2). The instrument emits bipolar stimuli to the carbon electrodes of the C-dish, which are placed in the cell culture medium. The myotubes were stimulated at 1Hz, 2ms and 11.5V for 24 hours and 48 hours. The medium was changed directly before stimulation. After completed 24 hours and 48 hours, the media were stored and the cells were collected in TRI Reagent (Sigma-Aldrich, USA) for further analysis.

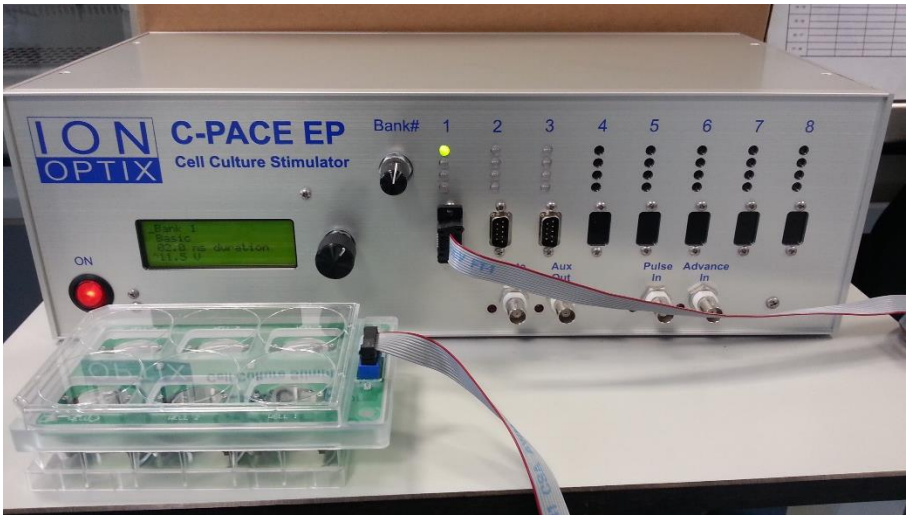


Figure 2- 2: Electrical pulse stimulator and six-well culture dish electrode system used in the study.

2.4 Human Subject Exercise Protocol

Human volunteers were subjected to right lower limb isotonic eccentric exercise with the total of 75 contractions which is divided to 3 sets of 25 reps. The contralateral limb acted as control. The muscle samples from the vastus lateralis muscles of both limbs were taken pre- and post-exercise as described in table 2-1.

Table 2- 1: Timeframe of skeletal muscle sample collection after exercise

Samples	Baseline (Pre-exercise)	Post- exercise Immediate	Post-exercise 3 hours	Post-exercise 48 hours
Exercise leg	✓	✓	✓	✓
Control leg	✓	–	–	✓

Blood samples from arterial and venous circulations were also taken at pre- and post-exercise as described in table 2-2.

Table 2- 2: Timeframe of serum collection after exercise

		Post-exercise			
Samples	Pre-exercise	Immediate	120 min	180 min	48 Hours
Arterial	✓	✓	✓	✓	✓
Venous	✓	✓	✓	✓	✓

Eccentric exercise was chosen as the exercise protocol in this study since other several established myokines such as IL-6 was found to be increased in the serum (Bruunsgaard et al., 1997); IL-8 mRNA (Nieman et al., 2003) together with IL-15 mRNA and protein (Nielsen et al., 2007) were found to be upregulated in skeletal muscles following eccentric exercise.

2.5 Delipidation of FBS/ Horse Serum

125ml FBS/horse serum was mixed with 100ml N-butanol and 150ml di-isopropyl ether (40:60 volume ratio) in a 500ml Nalgene Translucent Polypropylene Copolymer (PPCO) centrifuge bottle (Thermo Scientific, USA) and was mixed thoroughly by end to end rotation on a daisy-wheel for 40 minutes. Then the aqueous and organic phases were separated by centrifugation at 2500rpm for 5 minutes on Avanti-J series (Beckman Coulter, USA) centrifuge by using JLA-10.500 rotor. The aqueous phase (bottom layer, straw coloured) was carefully extracted by using a glass pipette tip and transferred to a clean 500ml Nalgene PPCO centrifuge bottle. The solvent extraction was repeated by adding 50ml di-isopropyl ether and mix thoroughly on the daisy wheel for another 40 minutes. The aqueous phase was again extracted by using glass pipette tip and was transferred to a clean 250ml conical flask. The flask was then put on the perforator in order to remove any trace of solvent for 5-6 hours. Next, the mixture was transferred to a round bottomed flask and froze in a thin layer using liquid nitrogen before put in a freeze dryer for overnight.

On the next morning, 50ml of HPLC water was added into the flask to dissolve the freeze dried serum. After that, the dialysis bag was prepared by soaking it in distilled water for a minimum of 1 hour at 4^{o}C to allow it to soften. After tying a knot at the end of the dialysis bag, the dissolved FBS was transferred into the dialysis bag using a glass funnel. Then another knot was tied at the top of the bag and a dialysis clip was added across of the bag in order to avoid any leakage. The dialysis bag then placed in a 2 litre conical flask which filled with PBS. A magnetic stirrer was put at the bottom to stir and it was left at 4^{o}C for a minimum of 12 hours during which

the PBS was replaced 2-3 times in between. Finally the delipidated serum was sterilised by passing through a 0.8µm acrodisc syringe filter.

2.6 Preparation of Fatty Acids in Complex with BSA

24% (w/v) bovine serum albumin (BSA) was prepared by gradually adding 6g of fatty-acid-free BSA (cat. No. A6003, Sigma-Aldrich, USA) into 17.5ml 150mM NaCl in a beaker, over time at room temperature with stirring. The pH was adjusted to 7.4 with 5M NaOH, and the volume made up to 25ml with 150mM NaCl. This solution was filtered through a 0.45µm filter and frozen in aliquots at –20°C. The fatty acid/BSA solution was then prepared by a modification of the method of (Van Harken, Dixon, & Heimberg, 1969). Briefly, 39.6 mg sodium oleate (Sigma-Aldrich, USA) or 36mg palmitic acid (Sigma-Aldrich, USA) was transferred to a glass beaker containing 0.5ml ethanol, and the ethanol was evaporated under a stream of nitrogen. Then 7ml 150mM NaCl and 48µl of 5M NaOH were added. The solution heated gently with stirring on a hot plate for 5 minutes. The beaker then was removed from the heat and 7 ml 24% (w/v) ice-cold BSA was added and stirred for 10 minutes. The emulsion was formed then the fatty acids were stored at –20°C until use. The 10mM PLO stock was prepared by combining each fatty acid at a ratio of 4:3:3 (palmitic, linoleic and oleic acid respectively).

2.7 Molecular biology

2.7.1 RNA isolation from skeletal muscle myotubes

The RNA from the myotubes was isolated using TRI Reagent (Sigma-Aldrich, USA) according to the manufacturer's instruction. RNA

concentration and quality were measured using an LVis Plate and read using a FLUOstar Omega spectrometer-based microplate reader (BMG Labtech, Germany) by determining the ratio of absorbance at 260nm/280nm and 260nm/230nm respectively. Ratios of (1.8 - 2.0) for purity of RNA were considered acceptable.

RNA extraction for some samples were performed using Direct-zol RNA Kits (Zymo Research, USA) according to the manufacturer's instruction.

2.7.2 Complementary DNA (cDNA) synthesis

Materials for cDNA synthesis

Affinity Script Reverse Transcriptase (200U/µl) (Agilent Technologies, CA, USA) 10X first strand buffer (Invitrogen, UK)

25 mM deoxynucleotide triphosphates (dNTPs) mix (Promega, UK)

100ng/ul Random hexamers (Promega, UK)

RNAse out (40U/µl) (Invitrogen, UK)

0.1M dithiothreitol (DTT) (Invitrogen, UK)

Diethylpyrocarbonate (DEPC) treated water

2.7.2.1 Methods for cDNA synthesis

cDNA was synthesized from 500ng of total RNA per sample using AffinityScript Multiple Temperature Reverse Transcriptase (Agilent Technologies, USA). 500ng RNA was first incubated with 50ng random primer and 1µl of 25mM dNTP mix at 65^{o}C for 5 minutes to denature RNA secondary structure and then immediately chilled on ice for at least 1 minute to let the primer anneal to the RNA. 2µl of 10x AffinityScript RT

Buffer, 2µl of 100mM DTT, 1µl RNase out and 1µl AffinityScript Multiple Temperature Reverse Transcriptase were then added. The reaction mixture was mixed gently and pre-incubated at 25°C for 10 minutes and further incubated at 55°C for 60 minutes. Inactivation of the enzyme was achieved by further heating the mixture to 70°C for 15 minutes and finally cooling to 4°C to stop the reaction. The cDNA was then used as a template for amplification in RT-PCR.

2.7.3 Taqman Real-time quantitative PCR

2.7.3.1 Sample preparation

The cDNA was then used as a template and amplified by using the Taqman Universal PCR fast Master Mix Kit (Applied Biosystem, USA). For the standard curve, an equal aliquot of undiluted cDNA from each sample was pooled together. This cDNA pool (super neat) was serially diluted four-fold with DEPC treated water (0.1% diethylpyrocarbonate in water) to produce a five-point standard curve (neat, 1:4, 1:16, 1:64, 1:256). DEPC treated water was used as no-template control. The samples for quantification were diluted in range of 1:8 to 1:16. Each PCR reaction was performed in a total volume of 13µl per-reaction containing 6.5µl of Taqman Universal PCR Master Mix, 0.4µl of 400pM forward and reverse primers, 0.25µl of 250pM of TaqMan probe, 2.45 µl of DEPC treated water and 3µl of cDNA sample (Table 2-3).

Table 2- 3: Sample preparation for Taqman QRT-PCR reaction

Reagent	Volume
Taqman Universal Fast PCR Master Mix	6.5 µl
Forward primer (400 pM)	0.4 µl
Reverse primer (400 pM)	0.4 µl
Probe (250 pM)	0.25 µl
DEPC treated H_2O	2.45 µl
Sample cDNA	3 µl
Total Reaction Volume	13 µl

2.7.3.2 Primer and Probe Design

The primer and probe sets for Taqman qRT-PCR were designed using Primer Express 3 software (Applied Biosystems) and synthesized commercially (Eurofins MWG Operon, Germany). The probe was labelled with a reporter fluorescent dye (FAM) at the 5'-end and a fluorescent dye quencher (TAMRA) at the 3'-end. The sequences of the primers and probes are detailed in Table 2.4:

Table 2- 4: Primer and probe sequences for Taqman qRTPCR

No	Oligo Name	Sequence (5'-3')		Tm (°C)	GC-content
1	Rat Beta-actin	Forward	AGCCATGTACGTAGCCATCCA	59.8	52.4%
		Reverse	TCTCCGGAGTCCATCACAATG	59.8	52.4%
		Probe	TGTCCCTGTATGCCTCTGGTCGTACCAC	69.5	57.1%
2	Rat GAPDH	Forward	TCTGCTCCTCCCTGTTCTAGAGA	62.4	52.2%
		Reverse	CGACCTTCACCATCTTGTCTATGA	61.0	45.8%
		Probe	ATCTTCTTGTGCAGTGCCAGCCTCGT	66.4	53.8%
3	Rat	Forward	CCAGAAGACCTCTGAAAACAAACA	59.3	41.7%

	Lipocalin 2	Reverse	CGAATCGCTCCTTCAGTTCAT	57.9	47.6%
		Probe	CTTCAAAGTCACCCTGTACGGAAGAACCAA	66.8	46.7%
4	Rat Neuro-peptide Y	Forward	GCAGAGGACATGGCCAGATAC	61.8	57.1%
		Reverse	TGGATCTCTTGCCATATCTCTGTCT	61.3	44.0%
		Probe	ACTCCGCTCTGCGACACTACATCAATCTC	68.1	51.7%
5	Rat TRIB 3	Forward	CAAGCACGGGCTCATCTTG	58.8	57.9%
		Reverse	CACCAACTTCGTCCTCTCACAGT	62.4	52.2%
		Probe	CCTCAAGTTGCGTCGATTTGTCTTCAGC	66.6	50.0%
6	Rat NR4A1	Forward	TGTTGCTAGAGTCCGCCTTTC	59.8	52.4%
		Reverse	CAGGCCTGAGCAGAAGATGAG	61.8	57.1%
		Probe	TTATCCTCCGCCTGGCCTACCGATCTAA	68	53.6%
7	Rat NR4A2	Forward	TTGCAGAATATGAACATCGACATTT	56.4	32.0%
		Reverse	CCCGTGTCTCTCTGTGACCAT	61.8	57.1%
		Probe	TCTCCTGCATTGCTGCCCTGGC	65.8	63.6%
8	Rat NR4A3	Forward	CGCCCTTGTCCGAGCTTTA	58.8	57.9%
		Reverse	CGGTGGGACAGTATCTGGAGTAA	62.4	52.2%
		Probe	CAGACGCAACGCCCAGAGACCTTG	67.8	62.5%
9	Rat GLUT4	Forward	ATACCTCTACATCATCCGGAACCT	61.0	45.8%
		Reverse	GCCAGTGCATCAGACACATCA	59.8	52.4%
		Probe	CTGCCCGAAAGAGTCTAAAGCGCCTG	68.0	57.7%
10	Rat PGC1α	Forward	AGACAAATGTGCTTCCAAAAAGAA	55.9	33.3%
		Reverse	GTTGTTGGTTTGGCTTGAGCAT	58.4	45.5%
		Probe	TCCCATACACAACCGCAGTCGCA	64.2	56.5%
11	Human Beta-Actin	Forward	CCTGGCACCCAGCACAAT	58.2	61.1%
		Reverse	GCCGATCCACACGGAGTACT	61.4	60.0%
		Probe	ATCAAGATCATTGCTCCTCCTGAGCGC	66.5	51.9%
12	Human Lipocalin 2	Forward	CCTCCGTCCTGTTTAGGAAAAA	58.4	45.5%
		Reverse	CTCGTTAATCCAGGGTAACTCTTAATG	61.9	40.7%
		Probe	TTGCCAGCCCGGCGAGTTCA	63.5	65%

2.7.3.3 Taqman Rt-QPCR Protocol

All samples were run in TaqMan RNase P 96-well plates (Applied Biosystem, USA) and qRTPCR was carried out using an Applied Biosystem Step OnePlus™ Thermocycler for 40 cycles. This was performed in two consecutive stages as follows (Figure 2-3):

Stage 1: *95°C (enzyme activation) for 20 seconds*

Stage 2: *95°C (denaturation) for 1 second and 60°C (annealing/ elongation) for 20 sec (repeated for 40 cycles)*

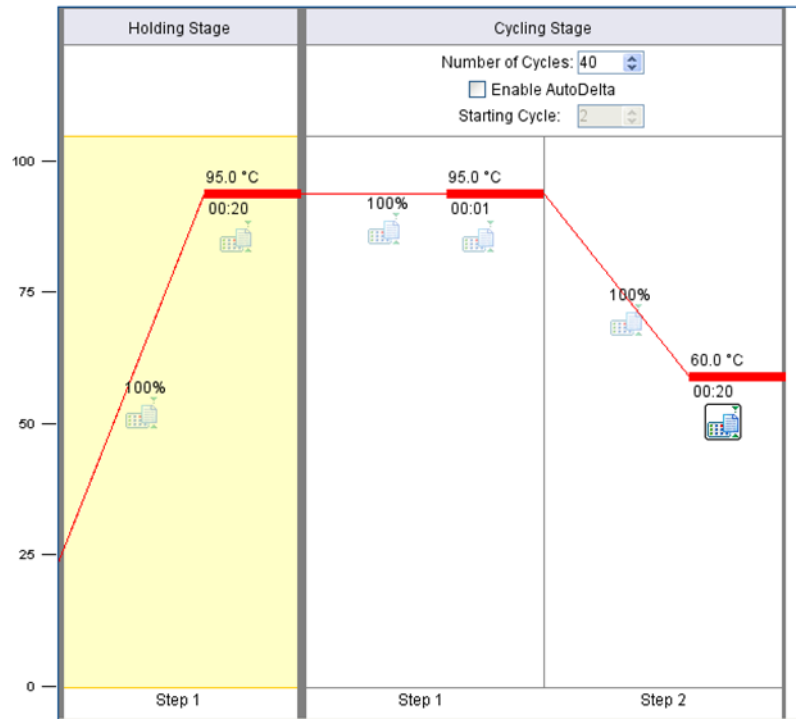


Figure 2- 3: Taqman qRTPCR protocol

Enzyme activation at 95°C, denaturation at 95°C and annealing/elongation at 60°C.

All standards, samples and negative controls were assayed in triplicate to ensure accurate results. In this study, relative expression levels were determined using the relative standard curve method (Bustin, 2000). A standard curve was then constructed by plotting Ct values versus the

logarithm of the serial cDNA dilutions. The slope of this standard curve is an indication of the efficiency of the RT-PCR reaction. For these experiments, assays were deemed acceptable if there was no more than 0.5 cycle threshold (Ct) difference between the values of a triplicate and the standard curve slope within the range (-3.2 to -3.6) were considered acceptable (Figure 2-4). The mRNA expression levels of specific genes were normalized to the mRNA expression level of reference genes, ß-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

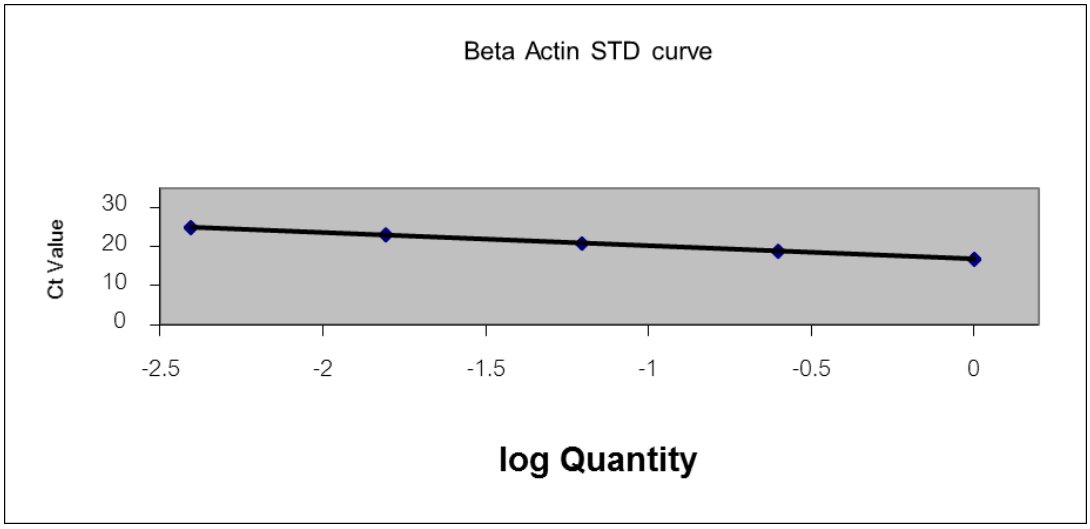


Figure 2- 4: Serial standard curve with slope of -3.37 and r^2 of -0.99

2.8 Free Fatty Acid Assay

Free fatty acid assay of horse serum and lipid-free horse serum was performed using NEFA-HR Assay kit (Wako Diagnostic, USA) according to manufacturer's instructions.

2.9 Triglyceride assay

A stock of 500µg/ml of Nile red was prepared in acetone and subsequently the working solution of 5.5µg/ml of Nile red was prepared in PBS. Standards were prepared for 8 points of 1:2 serial dilutions using olive oil (diluted in isopropanol) starting from 0.4% concentration (4µg/ml). Assay performed by loading 10µl of standards, blanks and samples. Then 90µl of working solution was added to each well. The plate was covered from light and shook for 5 minutes before read Ex: 485 and Em: 590 using FLUOstar Omega spectrometer-based microplate reader (BMG Labtech, Germany).

2.10 Protein Assay

Protein assays were performed using Pierce BCA Protein Assay Kit (Thermo Scientific, USA). The samples were prepared as 1:50 dilution. 20µl of standards and samples together with 200µl of working reagent were used in the assay. The absorbance was read at 560nm using FLUOstar Omega spectrometer-based microplate reader (BMG Labtech, Germany).

2.11 Lactate Assay

The lactate determination method was based on LDH- mediated conversion of lactate to pyruvate which in the process converts NAD^+ to NADH. The enzyme rate was quantified by following the increase in NADH at 340nm.

Reaction mix was prepared by adding 20u/ml L-Lactate Dehydrogenase from rabbit muscle (Sigma Aldrich, USA) and 3mM β-Nicotinamide adenine dinucleotide sodium salt (Sigma Aldrich, USA) in 20ml

aqueous glycine-EDTA-hydrazine buffer pH 9.0 (200mM glycine, 2mM EDTA, 250mM hydrazine hydrate). Standards were prepared in 1:2 serial dilution of 5mM lactic acid (Fisher Scientific, USA) in HAM's F10 culture media.

Assays were performed by loading 20µl standards, samples and blanks respectively in triplicates into a 96-well plate. Then 100µl reaction mix was added to each well and the absorbance was measured at 340nm at once (t0) and after 30 minutes (t30) using FLUOstar Omega spectrometer-based microplate reader (BMG Labtech, Germany). The optical density values of t30-t0 were taken to determine the lactic acid concentration in the samples by reference to the standard curve.

2.12 Glucose Assay

The principle of this glucose determination assay is based on the oxidation of glucose by glucose oxidase (GOD) to glucoronic acid and hydrogen peroxide. The hydrogen peroxide is then quantified stoichiometrically by horse radish peroxide-linked oxidation of and acceptor to a chromogen (tetra-amino benzidine to a blue-yellow product measured at 450nm or dianisidine to a pink product measured at 540nm). The reagent mix was prepared by adding 0.4mg glucose oxidase (Sigma-Aldrich, USA) and 1mg horseradish peroxidase (Sigma-Aldrich, USA) into 10ml TrisHCl 1M, pH7.4, then 0.1 tetramethylbenzidine (Sigma-Aldrich, USA) was added to the mix. The standards were prepared in 1:2 serial dilution of HAM's F10 (Sigma-Aldrich, USA) culture media (6.1 mM glucose concentration). Assay was performed by loading 10µl standards and samples into 96-well plate and 100µl of reaction mix was added to each well. After 10 minutes, 0.2M

sulphuric acid was added to each well to stop the reaction and the absorbance was read at 450nm using FLUOstar Omega spectrometer-based microplate reader (BMG Labtech, Germany). The standard curve was prepared by curve fit 'single site binding' using PRISM 5 and the samples concentration were derived from the standard curve.

2.13 Enzyme-linked Immunosorbent Assay (ELISA)

Lipocalin 2 protein levels in human serum were quantified using a Quantikine ELISA kit (cat. no. DLCN20, R&D Systems, USA) and assayed according to manufacturer's instructions. The ELISA plate was read using a FLUOstar Omega multi-mode microplate reader (BMG LABTECH, Germany) set to 450 nm and using wavelength correction set at 570 nm. Quantification and analysis of Lipocalin 2 in the samples was carried out using MARS Data Analysis Software (BMG LABTECH, Germany).

2.14 Protein expression analysis by Western Blotting

Western blotting was used as a qualitative and semi-quantitative approach for visualizing and measuring the expression pattern of total intracellular proteins in primary skeletal muscle myotubes and extracellular secreted proteins released by the myotubes into the media.

Reagents and solutions

8M SDS-Urea lysis buffer

8M urea

5% SDS (w/v)

1M DTT

1.5M tris-HCL pH6.8

100% Trichloroacetic Acid (TCA)

10g of trichloroacetic acid (TCA) in a final volume of 10 ml H2O

10% Ammonium Persulfate (APS)

0.1g of Ammonium persulfate was dissolved in 1ml of distilled water immediately prior to use.

4X Separation gel buffer (pH 8.8)

1.5M Tris Base; 0.4% (w/v) SDS, pH 8.8

4X Stacking gel buffer (pH 6.8)

0.5M Tris Base; 0.4% (w/v) SDS, pH 6.8

10% Separation gel

3.35ml 30% (w/v) Acrylamide; 3.25ml distilled water; 2.5ml 4X separation buffer pH 8.8, 100µl 10% (w/v) Ammonium persulfate (APS); 10µl TEMED.

4X Stacking gel (for 4 mini gels)

0.5ml 30% (w/v) Acrylamide; 3.25ml distilled water; 1.25ml 4X Stacking buffer pH6.8; 50µl 10% (w/v) Ammonium persulfate (APS); 5µl TEMED.

10X Running Buffer (500ml)

72g Glycine; 15g Tris Base; 50ml 10% (w/v) SDS, made up to 500ml with distilled water

10X Transfer Buffer (500ml)

72g Glycine; 15g Tris Base, made up to 500ml with distilled water

Transfer Buffer working solution (1X)

80ml 10X Transfer Buffer; 120ml Methanol, made up to 800ml with distilled water

10X Tris Buffered Saline (TBS) (1000ml)

24.23g Tris Base; 87.66g Sodium Chloride (NaCl) pH 7.4 with concentrated HCl

1X Tris Buffered Saline-Tween 20 (TBS-T)

100ml 10X TBS with 1ml of Tween 20, made up to 1litre with distilled water

Blocking buffer

5% (w/v) non-fat dry milk dissolved in 1X TBST

2.14.1 Protein extraction from primary skeletal muscle myotubes for Western blotting analysis

The culture media was aspirated and the cells washed with ice-cold PBS. 1ml of ice-cold monophasic lysis reagent -TriReagent (Sigma-Aldrich, USA) was added per well and the homogenates were incubated for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. Then, the cells were scraped using the cell scraper and each sample was divided and transferred into two separate

1.5ml tubes. Next, 0.2ml of 1-Bromo-3-chloro-propane (BCP) was added to the sample and vigorously mixed. The samples were then separated into two phases by centrifugation at 10,000 x g for 15 minutes at 4°C and the upper aqueous phase was transferred into a fresh tube (for RNA extraction). The lower aqueous phase was then used for protein extraction.

Next, 0.3ml 100% ethanol was added into the lower aqueous phase, mixed by inversion and allowed to stand at room temperature for 2-3 minutes followed by centrifugation at 2000 x g for 5 minutes at 4°C. Then, the supernatant was transferred into 5ml Falcon tubes, 1.5ml of isopropanol was added and the samples mixed and allowed to stand at room temperature for 10 minutes before centrifugation at 12000 x g for 10 minutes at 4°C. The supernatants were discarded and the pellets were washed 3 times in 0.3M guanidine hydrochloride/95% ethanol solution. During each wash, the samples were stored in wash solution for 20 minutes at room temperature before being centrifuged at 7,500 × g for 5 minutes at 4°C. After 3 washes, 2 ml of 100% ethanol was added and the samples vortexed. The sample was again allowed to stand for 20 minutes at room temperature before being centrifuged at 7,500 x g for 5 minutes at 4°C. The supernatant was than discarded and the pellets were allowed to dry for 5-10 minutes before being dissolved in 200µl 8M SDS-urea lysis buffer and 5µl of 1% (w/v) bromophenol blue was added. The samples were then stored at -20°C until later use.

2.14.2 Protein extraction from primary myotube conditioned media using Trichloroacetic acid (TCA)

Secreted proteins and factors in primary skeletal myotubes media were precipitated using a Trichloroacetic acid (TCA) precipitation method. Briefly, 250µL of 100% TCA was added to 1ml culture media, vortexed and incubated for 30 minutes on ice. Samples were centrifuged at 13000 rpm for 30 minutes at 4^{o}C, and the supernatant discarded. The remaining protein pellets were washed two times with ice cold acetone and left to dry for 5 minutes. Protein pellets were the dissolved in 300µl 8M SDS-urea lysis buffer and 5µl of 1% (w/v) bromophenol blue was added. Samples were incubated at 95^{o}C for 5 minutes and then stored at -20^{o}C until later use.

2.14.3 Western blotting gel electrophoresis

Protein samples from skeletal muscle myotubes or media were incubated for 5 minutes at 95°C before being loaded onto 10% SDS-PAGE gels. 5µL of protein molecular weight marker (Precision Plus Protein TM Standards, Kaleidoscope, Bio-Rad) was also loaded into each gel for target protein identification. The gel electrophoresis for protein samples was run at 80V for 15 minutes to allow gentle migration of protein from the stacking gel, and then switched into a constant voltage of 150V for about 1 hour.

For protein transfer, nitrocellulose membranes (GE Healthcare Life Sciences) were pre-soaked in 1X transfer buffer, and protein transferred to the nitrocellulose membrane using a Mini Trans-Blot Cell Tank (BioRad, USA) filled with 1X transfer buffer according the manufacturer's guidelines (Bio-Rad, USA), at 100v for 1 hour. Protein bands were visualized by staining the membrane with Ponceau Red (diluted 1:10) for 5 minutes; this was washed off with TBS-T before further steps. The nitrocellulose

membrane was incubated in a blocking solution of 5% skimmed milk (w/v) in TBS-T for 1 hour at room temperature under gentle shaking.

2.14.4 Immunodetection following western blotting and densitometry analysis

Blots were removed from the blocking solution and incubated in the presence of a specific primary antibody in 5% milk (w/v) in TBS-T, overnight at 4°C with gentle shaking. Subsequently, the membrane was washed three times with TBS-T, and incubated with the appropriate IR-Dye®-conjugated secondary antibody (LI-COR® Biosciences, 1:3000 dilution in 5% (w/v) skimmed milk). Details of primary antibodies are described in Table 2.5. The blot was washed five times with TBS-T the target protein was visualised using the Odyssey® Infrared Image system (LICOR Biosystems, USA) at 700nm or 800nm. Densitometry was performed using Odyssey® Imaging System Software (LICOR Biosystems, USA). Densitometric analysis was performed by measuring the sum of the individual pixel intensity values (Total) for a shape (protein band) minus the product of the average intensity values of the pixels in the background (BKgnd), and total number of pixels enclosed by the shape (Area).

Densitometry value (Signal) = Total-(Background x Area)

Table 2- 5: List of primary and secondary antibodies used in Western Blot experiments

Antibody (Catalog number)	Company	Species raised in	Concentration	Condition	Secondary	Approx. weight (kDa)
Monoclonal-Akt (pan)-(#2920)	Cell Signalling	Mouse	1:2000	Overnight incubation	1:3000	60
Polyclonal-Phospho-Akt (Ser473)-(#9271)	Cell Signalling	Rabbit	1:1000	Overnight incubation	1:3000	60

Polyclonal-RPLPO-(ab154970)	Abcam	Rabbit	1:1000	Overnight incubation	1:3000	34
Polyclonal-PI3K p85-(#4292)	Cell Signalling	Rabbit	1:1000	Overnight incubation	1:3000	85
Polyclonal-PI3K p110α-(#4255)	Cell Signalling	Rabbit	1:1000	Overnight incubation	1:3000	110
Polyclonal-Rat Lipocalin 2/NGAL-(AF3508)	R&D Systems	Goat	1:200	Overnight incubation	1:3000	25
Polyclonal-Human Lipocalin 2/NGAL (AF1757)	R&D Systems	Goat	1:1000	Overnight incubation	1:3000	25

2.15 Immunocytochemistry

Rat myotubes were grown on a coated cover slip (0.2% (w/V) gelatine) and fixed with 3-4% paraformaldehyde in PBS, pH 7.4 for 15 min at room temperature. The cells were then washed twice with ice cold PBS. Cells were permeabilized by incubation of the samples in PBS containing 0.25% Triton X-100 for 10 min and cells were then washed in PBS three times for 5 min. To block unspecific binding of the antibodies cells were incubated with 1% (w/v) BSA in PBST for 30 min. Overnight incubation with primary antibodies was carried out at 4°C. The primary antibody solution was then decanted and the cells washed three times in PBS (5min each) before incubation with the secondary antibody in 1% (w/v) BSA, PBS for 1 hr at room temperature in the dark. The cells were then washed three times with PBS for 5 min each in the dark. Before mounting the cover slip on a slide the nuclei were stained with DAPI (1μg/mL) for 1 min. Images were obtained using a Leica DMRB fluorescent microscope with an oil or glycerol immersion lens at 40x and 64x magnification, respectively. Images were captured using Openlab software (Improvision-Perkin Elmer, UK).

Table 2- 6: Primary and secondary antibodies used in immunofluorescence experiment

Primary antibody (source)	Dilution/ conditions	Secondary antibody/ wavelenght	Dilution/ conditions
Rabbit polyclonal anti-GLUT4 (Abcam ab33780)	1:100 4°C overnight	Goat anti-rabbit Cross-adsorbed Dylight 488 (Thermo Sc. #35553)	1:750 RT 1hr

2.16 Glucose Uptake Assay

Reagents and solutions

[^{3}H] 2-Deoxy-DGlucose (2-DOG) specific activity 20-50 Ci (740 - 1850 GBq)/mmol) was purchased from PerkinElmer. 2-Doexy-D-Glucose and D-Glucose were purchased from Sigma-Aldrich, USA.

Reaction buffer:

-138 mM NaCl

- 1.85 mM $CaCl_2$

-1.3 mM MgSO4

-4.8 mM KCl

-0.2% (w/v) BSA

-50 mM HEPES pH 7.4

2.16.1 Glucose uptake in skeletal muscle myotubes

Myotubes in 6 well plates with 6% horse serum media (Ham-F10) were given electrical pulse stimulation (2ms, 1 Hz, 11.5V) for 48 hours. They were then washed twice in PBS and incubated in serum free medium (Hams-F10 only) for 2 hours. The myotubes were then washed twice in PBS and incubated in 2ml reaction buffer for 45 minutes at 37°C. Myotubes were

then treated with 2 µl 100 nM insulin for 15 min in reaction buffer at 37°C. Following the incubation with insulin, 250 µl of (27.8 kBq [3H] 2-DOG and 10 µM of 2-DOG) was added in each well and incubated for another 15 minutes. Glucose uptake was terminated by washing the cells with ice cold PBS containing 10 mM glucose three times and then cells were solubilised in 500 µL 0.5 M NaOH and 0.1% SDS. Cells were then scraped and the lysates transferred to scintillation vials and 5ml liquid scintillant (Perkin-Elmer, USA) was added. Radioactivity was measured using a liquid scintillation analyser Tri-Carb 2100TR (Packard Instrument Company, USA).

2.17 Presentation of data and statistical analysis

Data are presented as mean ±SEM. Unpaired two-tailed Student's t test, one-way ANOVA or two-way ANOVA (Bonferroni's post-hoc test) were used to determine statistical significance. All statistical analyses were performed using Prism5 (Graph-Pad, La Jolla, CA, USA), with a value of $p<0.05$ considered to be statistically significant. Corresponding significance levels are as indicated.

CHAPTER THREE

DEVELOPMENT OF CONTRACTILE MYOTUBES MODEL

Chapter 3: Development of Contractile Myotubes Model

3.1 Introduction

Exercise has been shown to have a positive impact on a number of diseases in humans including obesity and T2DM. It is well established that physical inactivity causes accumulation of visceral fat and that the health consequences of both are related to systemic low-grade inflammation (Handschin & Spiegelman, 2008; Pedersen, 2009; Yudkin, 2007). Study shown that an appropriate amount of physical exercise exerts a wide variety of biological effects, such as an enhancement of glucose disposal, changes in muscle fibre types, induction of angiogenesis, and regulation of the immune system (Jessen & Goodyear, 2005).

For over 300 years, scientists have understood that stimulation, in the form of an electrical impulse, is required for normal muscle function (Donnelly et al., 2010). Apart from that, during development of skeletal muscle cell myotubes in vitro, the transition from primary myotubes to secondary myotubes is dependent on electrical activity. In the absence of electrical activity, secondary myotubes and adult muscle fibers fail to form (Wilson & Harris, 1993). Therefore, if the electrical stimulation pattern can be reproduced *in vitro*, it may promote the transition toward adult myofibers within engineered muscles (Donnelly et al., 2010).

Even though the use of electrical stimulation of muscle tissue has a long history but the control of important stimulation parameters such as pulse widths, frequency, and voltage amplitude has been poorly understood (Dennis, Smith, Philp, Donnelly, & Baar, 2009). It is only within the last few decades that the effect of different stimulation patterns on muscle function

has been fully appreciated (Dow et al., 2004). It is well known that physiological electrical impulses can be modelled in vitro by tuning EPS parameters such as voltage amplitude, pulse width, and frequency. However, methodology for controlling these stimulation parameters to develop in vitro functional skeletal muscle tissues remains to be established. Inappropriate EPS parameters can result in electrochemical damage to the tissues. Too high voltages typically cause electroporation (W. Chen, 2005), which plays a greater role in electrochemical damage than pulse width.

A muscle twitch is usually divided into three phases: 1) the latent period; 2) the contraction period; 3) the relaxation period. The latent period is the time from when the stimulus is delivered to the first indications of contraction in the muscle. The contraction period, or contraction time, is the time it takes the muscle to reach its peak contraction after the latent period. The relaxation period is the time the muscle takes to return to resting tension after reaching its peak contraction (Sandow, 1952).

An interesting study by Ito et al. demonstrated the effects of chronic continuous EPS upon skeletal muscle culture physiological parameters such as peak twitch force (Pt) and excitability. They investigated continuous EPS parameter ranges of 0.1-0.5 V/mm pulse amplitudes, 2-10ms pulse widths and 0.5-50Hz frequencies that were given to mouse C2C12 cell cultures for up to 14 days. Results revealed the skeletal muscle tissue culture began to generate contractile force in response to EPS after 4 days of cell differentiation. On the day-4 of EPS, low frequency electrical stimulation at 0.5, 1, and 2 Hz induced individual twitch contractions, while the additional activation of contractile elements, called tetanus, was observed after repeated stimulation with high-frequency electrical pulses at 50 Hz. The

twitch force at various voltages and pulse widths was measured and results showed %Pt increased proportionately within the range of electric pulses tested (voltage: 0.1–0.5 V/mm; width: 2–10 ms). On the day-4 to day-7 of EPS, the tissue culture generated the highest peak twitch force which was about 24.5%Pt by electrical pulse of 1Hz, 0.3V/mm, and 4ms. On day-10 onwards, highest peak twitch force was 54.1% by electrical pulse of 1Hz, 0.3V/mm, and 4ms. The changes in force production were dependent on %Pt, regardless of electrical signal parameters such as pulse voltage and width (Ito et al., 2014).

Apart from that, several studies have shown that EPS on skeletal muscle elicits GLUT4 translocation (Derave et al., 1999; Nedachi, Fujita, & Kanzaki, 2008) and promotes the increase in glucose uptake (Aas, Torbla, Andersen, Jensen, & Rustan, 2002; Derave et al., 1999; Hespel et al., 1995; Lambernd et al., 2012; Manabe et al., 2012; Nedachi et al., 2008; Nikolic et al., 2012; Ploug, Galbo, & Richter, 1984).

However, previous data from our laboratory showed that the level of GLUT4 was extremely low, approximately 3000-fold lower levels in non-contracting primary skeletal muscle cell cultures compared to muscle tissue and this may results in ineffective glucose uptake. Due to the fact that glucose enters muscle cells via facilitated diffusion through GLUT4 which translocates from intracellular storage depots to the plasma membrane and T-tubules upon muscle contraction (Richter & Hargreaves, 2013), we began to develop a contractile model of myotubes that may resemble the properties of muscle cells during exercise. The contractile myotubes model should demonstrate several properties such as high in GLUT4, sensitive to insulin and able to perform insulin-stimulated glucose uptake effectively in

order to be used for further experiments. For this purpose, we have decided to apply EPS on the myotubes.

The frequency of 1Hz has been selected to be applied to our myotubes since it was shown to be the optimum frequency level for the skeletal muscle culture to produce the maximum peak twitch force (Ito et al., 2014). In relation to this, our EPS was performed according to protocol by Lambernd et al. (Lambernd et al., 2012) 1Hz, 2ms and 11.5V since this protocol has shown to be able to elicit insulin-stimulated glucose uptake in human primary skeletal muscle myotubes.

3.2 Objectives

3.2.1 General objective

To develop a contractile model of skeletal muscle myotubes which is sensitive to insulin stimulated glucose uptake.

3.2.2 Specific objectives

- To compare biochemical changes after contractile stimulation namely lactic acid production and glucose consumption in culture media in the stimulated and non-stimulated myotubes.
- To compare GLUT4 expression in the stimulated and non-stimulated myotubes.
- To compare mitochondrial biogenesis markers, specifically PGC1α in the stimulated and non-stimulated myotubes.
- To compare the insulin stimulated glucose uptake between the stimulated and non-stimulated myotubes.

3.3 Experiment designs and methods

3.3.1 Lactic acid concentration in culture media of rat myotubes post-EPS

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 4 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. The myotubes from 2 of the plates were then given EPS for 24 hours and 48 hours respectively as described in Chapter 2 section 2.3, and the other 2 plates acted as control. Lactic acid levels in the culture media were determined by lactate assay method as described in Chapter 2 section 2.11.

3.3.2 Glucose concentration in culture media of rat myotubes post-EPS

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 4 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. The myotubes from 2 of the plates were then given EPS for 24 hours and 48 hours respectively as described in Chapter 2 section 2.3, and the other 2 plates acted as control. Glucose levels in the culture media were determined by glucose assay method as described in Chapter 2 section 2.12.

3.3.3 GLUT-4 mRNA gene expression in rat myotubes post-EPS

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 4 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. The myotubes from 2 of the plates were then given

EPS for 24 hours and 48 hours respectively as described in Chapter 2 section 2.3, and the other 2 plates acted as control. The RNA was extracted and the GLUT4 mRNA was measured by Taqman qRTPCR as described in Chapter 2 section 2.7.

3.3.4 PGC1α mRNA gene expression in rat myotubes post-EPS

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 2 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. The myotubes from one of the plates were then given EPS for 48 hours as described in Chapter 2 section 2.3, and the other plate acted as control. The RNA was extracted and the PGC1α mRNA was measured by Taqman qRTPCR as described in Chapter 2 section 2.7.

3.3.5 Insulin stimulated glucose uptake post-EPS in rat myotubes

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 4 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. The myotubes from 2 of the plates were then given EPS for 48 hours as described in Chapter 2 section 2.3, and the other 2 plates acted as control. Glucose uptake was determined by the glucose uptake assay as described in Chapter 2 section 2.16 with and without insulin treatment.

3.3.6 Insulin stimulated glucose uptake post-EPS in human myotubes

$1x10^5$ of satellite cells/well (from a single individual) were seeded into 4 units of 6-well plates and grown to form matured myotubes as

described in Chapter 2 section 2.2. The myotubes from 2 of the plates were then given EPS for 48 hours as described in Chapter 2 section 2.3, and the other 2 plates acted as control. Glucose uptake was determined by the glucose uptake assay as described in Chapter 2 section 2.16 with and without insulin treatment.

3.3.7 Optimum time for insulin stimulated glucose uptake post-EPS in rat myotubes

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 4 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. The myotubes from 3 of the plates were then given EPS for 48 hours as described in Chapter 2 section 2.3, and the other 1 plate acted as control. Glucose uptake was determined by the glucose uptake assay as described in Chapter 2 section 2.16 at 3 different timeframe: immediately post-EPS, 24 hours post-EPS, 48 hours post-EPS and 48 hours control respectively. The glucose uptake assay was performed with the treatment of insulin (n=3) and vehicle (n=3) from the same plate for each set of the experiment.

3.3.8 GLUT-4 translocation following EPS in rat myotubes

For the immunocytochemistry study, $1x10^5$ of satellite cells (from a single rat) were seeded on cover slip pre-coated with 0.2% (w/v) gelatine which were placed in 2 units of 6-well plates. The myotubes from 1 of the plates were then given EPS for 48 hours as described in Chapter 2 section 2.3, and the other plate acted as control (non-stimulated). Then the myotubes were treated either with 100nM insulin or vehicle.

- Non-stimulated myotubes with vehicle: n=3
- Non-stimulated myotubes with 100nM insulin: n=3
- EPS myotubes with vehicle: n=3
- EPS myotubes with 100nM insulin: n=3

The immunofluorescence staining was performed as described in Chapter 2 section 2.15. The primary and secondary antibodies used together with their dilutions and conditions were as described in Table 2-6.

3.4 Results

3.4.1 Lactic acid concentration in culture media of rat myotubes post-EPS

Lactic acid concentrations in the culture media increased from 24 hours to 48 hours in both control and electrical pulse stimulation groups. There was significant increase in lactic acid concentration in the culture media of the electrical pulse stimulated myotubes at 24-hours ($p<0.05$, $n=6$) and 48-hours ($p<0.001$, $n=6$) (Figure 3-1).

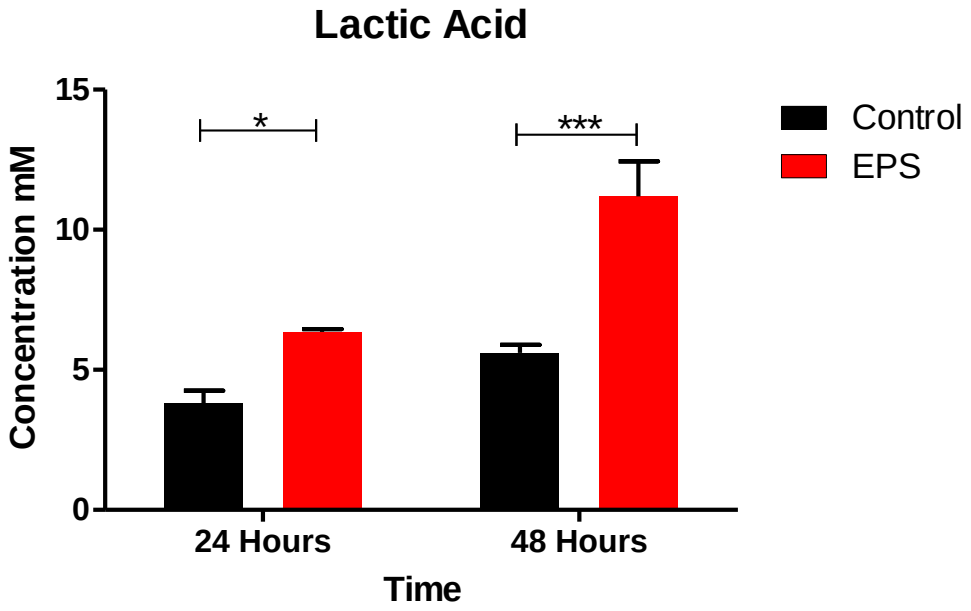


Figure 3- 1: Effect of EPS on lactic acid accumulation in the culture media of rat myotubes.

Cultured myotubes were electrically stimulated (1Hz, 2ms pulses, 11.5V) for 24h and 48h continuously. Data presented as mean ± SEM (*$p<0.05$, ***$p<0.001$, Two-way ANOVA with Bonferronis's post-hoc, $n=6$).

3.4.2 Glucose concentration in culture media of rat myotubes post-EPS

Glucose concentrations in the culture media decreased over 24 to 48 hours in both control and electrical stimulation groups. There was significantly increased reduction of glucose in the culture media of 48-hour electrical pulse stimulated myotubes as compared to 48-hour control ($p<0.01$, $n=6$) (Figure 3-2)

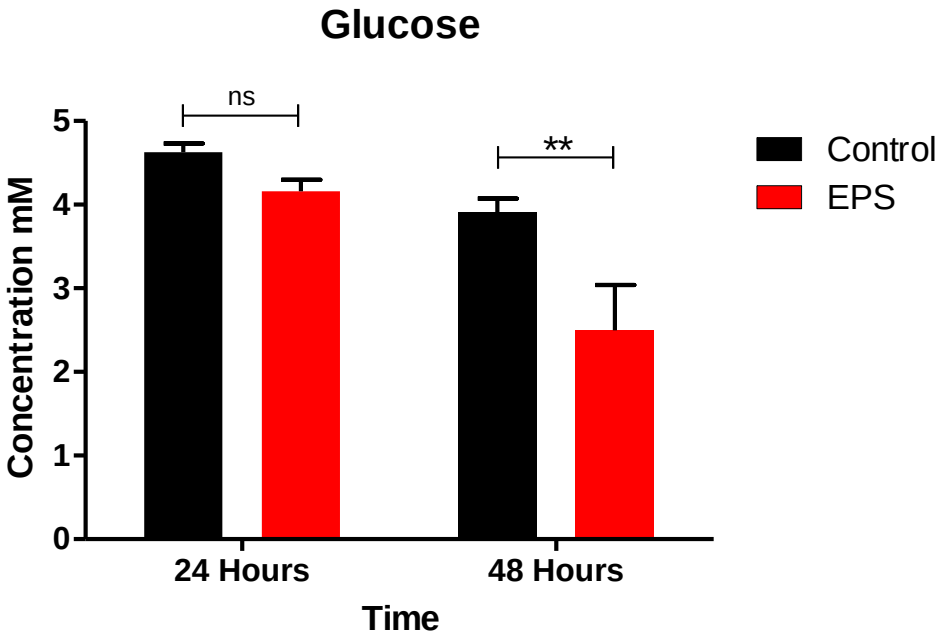


Figure 3- 2: Effects of EPS on glucose concentration in the culture media of rat myotubes.

Cultured myotubes were electrically stimulated (1Hz, 2ms pulses, 11.5V) for 24h and 48h continuously. Data presented as mean ± SEM (**p<0.01, Two-way ANOVA with Bonferronis's post-hoc, n=6).

3.4.3 GLUT-4 mRNA gene expression in rat myotubes post-EPS

GLUT4 mRNA expression in the electrical pulse stimulated groups increased from 24-hour to 48-hour. There was a significant increase of GLUT4 mRNA expression in 48-hour electrical pulse stimulation group as compared to 48-hour control ($p<0.05$, $n=6$) (Figure 3-3).

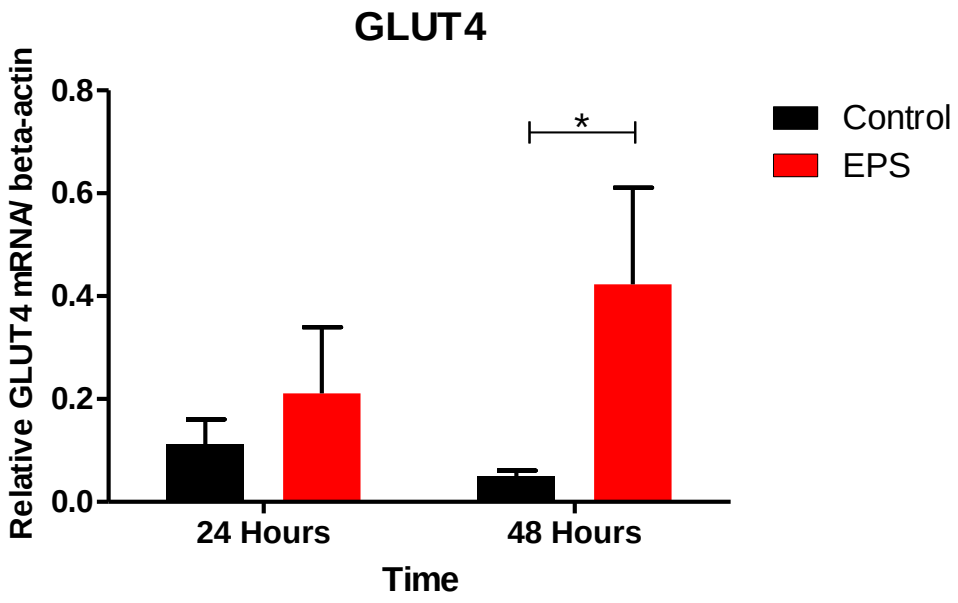


Figure 3- 3: Effects of EPS on GLUT4 mRNA expression in rat myotubes.

Cultured myotubes were electrically stimulated (1Hz, 2ms pulses, 11.5V) for 24h and 48h continuously. Data presented as mean ± SEM (*$p<0.05$, Two-way ANOVA with Bonferronis's post-hoc, $n=6$).

3.4.4 PGC1α mRNA gene expression in rat myotubes post-EPS

PGC1α mRNA expression was significantly elevated in the electrical pulse stimulated groups as compared to control after 48-hours ($p<0.01$, $n=9$) (Figure 3-4).

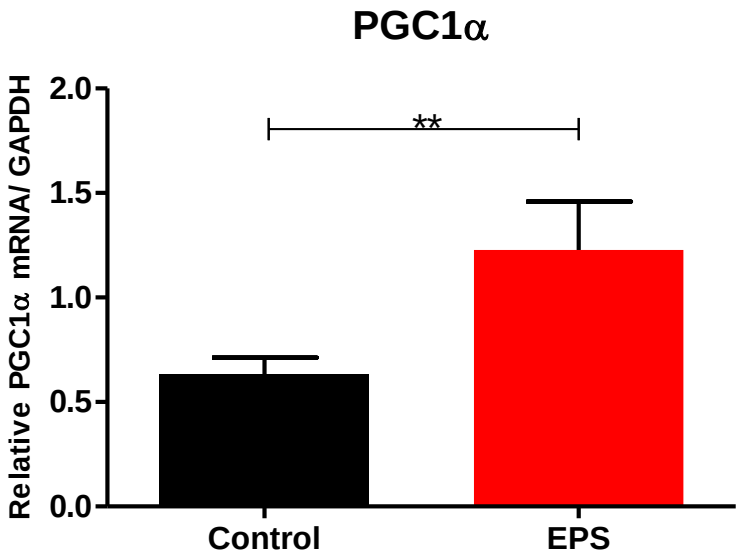


Figure 3- 4: PGC1α gene expression in rat primary skeletal muscle myotubes in control and post-electrical pulse stimulation.

Data expressed as mean ± SEM (**p<0.01, t-test, EPS: electrical pulse stimulation, n=9).

3.4.5 Insulin stimulated glucose uptake post-EPS in rat myotubes

From the results before, EPS for 48 hours is sufficient to produce a significant increase in GLUT-4 mRNA expression in myotubes and to increase lactate accumulation in the medium. Furthermore, the glucose level in the medium was shown to be declined following 48 hours of EPS, which was indicative of glucose uptake by the cells. These data suggest that post-EPS, myotubes may show increased sensitivity to insulin in terms of glucose uptake.

Result below showed that EPS alone is capable of increasing glucose uptake by the cells in the absence of insulin. Furthermore, the combination of EPS with 100nM insulin for 15 minutes further increased glucose uptake. On the other hand, without EPS, the cells did not show any changes in glucose uptake in the presence of insulin (Figure 3-5)

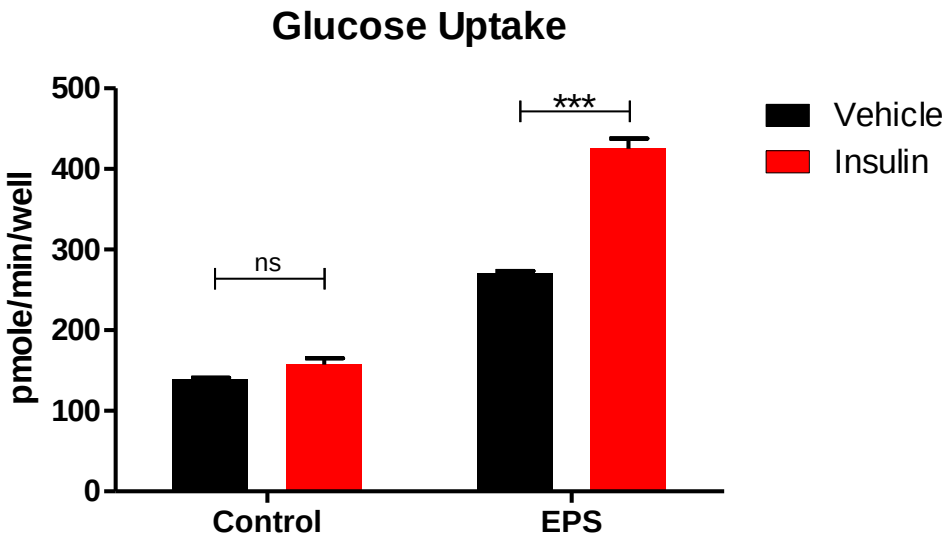


Figure 3- 5: Glucose uptake rates in rat primary skeletal muscle myotubes in control and post-electrical pulse stimulation in response to insulin.

Data expressed as mean ± SEM (***p<0.001, Two-way ANOVA with Bonferronis's post-hoc, EPS- electrical pulse stimulation, n=3).

3.4.6 Insulin stimulated glucose uptake post-EPS in human myotubes

After observing the increase of insulin stimulated glucose uptake in rat myotubes, we repeated the same experiment in human myotubes. Result showed a similar pattern in which EPS alone increases glucose uptake by the cells in the absence of insulin. In addition, combination of EPS with 100nM insulin resulted in a further increase in glucose uptake. On the other hand, without EPS, the cells did not show any changes of glucose uptake despite in the presence of insulin (Figure 3-6).

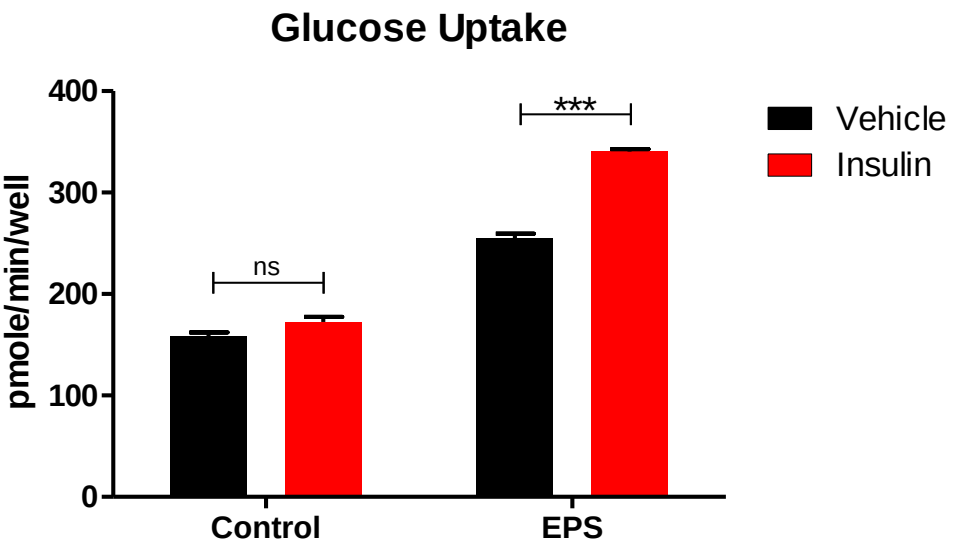


Figure 3- 6: Glucose uptake assay in human primary skeletal muscle myotubes in control and post-electrical pulse stimulation in response to insulin.

Data expressed as mean ± SEM (***p<0.001, Two-way ANOVA with Bonferronis's post-hoc, EPS- electrical pulse stimulation, n=3).

3.4.7 Optimum time for insulin stimulated glucose uptake post-EPS in rat myotubes

Apart from studying the effects of EPS upon insulin stimulated glucose uptake in the myotubes, we were also interested in observing the rate of glucose uptake in different time-frame post-EPS. Thus we designed 3 different time frames namely immediate, 24 hours and 48 hours post-EPS, with or without insulin. This experiment is to determine the optimum time which may give the greatest difference between insulin and non-insulin stimulated glucose uptake so that it can be used as a guide for later experiments. Results showed that insulin stimulated glucose uptake is at the optimum rate only immediately after EPS. 24 and 48 hours post-EPS showed no significant difference between insulin and non-insulin treated groups (Figure 3-7).

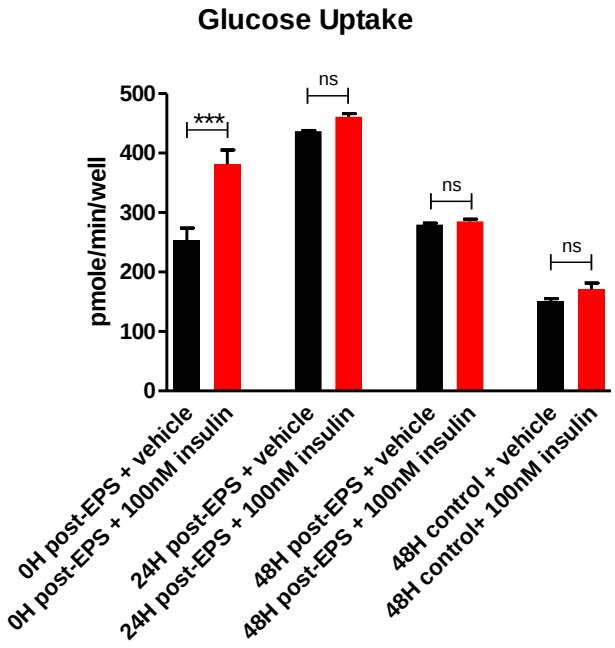


Figure 3- 7: Glucose uptake assay in rat myotubes at 3 different time points post-electrical pulse stimulation.

Data expressed as mean ± SEM (***p<0.001, One way ANOVA with Bonferronis's post-hoc, EPS- electrical pulse stimulation, n=3).

3.4.8 GLUT-4 translocation following EPS in rat myotubes

Immunofluorescence analysis of GLUT4 in non-EPS stimulated rat primary skeletal muscle myotubes showed that treatment with 100nM insulin for 15 minutes did not induce significant changes in GLUT4 immunoreactivity when compared to vehicle treated group. On the other hand, EPS for 48 hours increased the overall levels of GLUT4protein expression. Interestingly, the EPS with 100nM insulin treated group showed further increase in GLUT4 expression. However, the recruitment of GLUT4 to the cell membrane was not clearly seen (3-8).

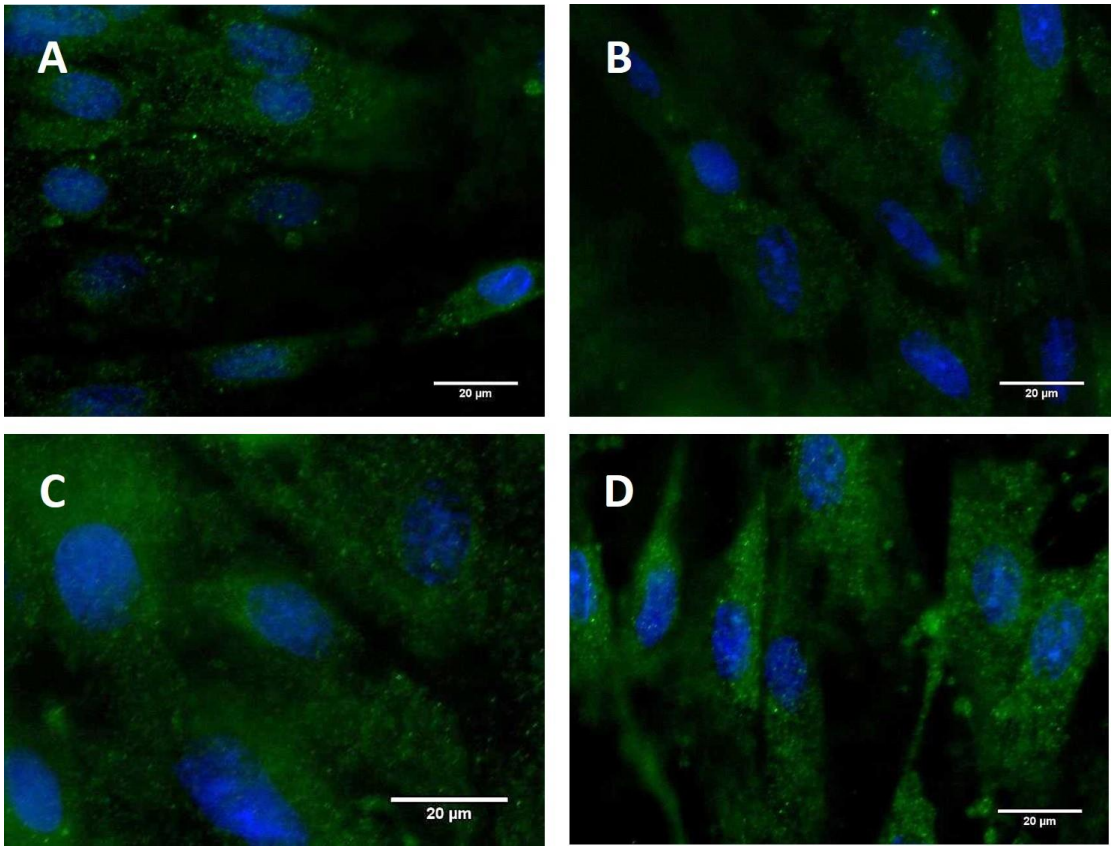


Figure 3- 8: GLUT4 immunofluorescence in rat primary skeletal muscle myotubes.

Images were taken at 40x magnification. Scale bar 20 µm. GLUT4 green fluorescence and DAPI blue fluorescence. A) Non-stimulated myotubes. B) Non-stimulated myotubes treated with 100nM insulin. C) Myotubes treated with EPS without insulin. D) Myotubes treated with EPS and 100nM insulin.

3.5 Discussion

We observed a significant increase in lactic acid and reduction in glucose concentration in the culture media following EPS that suggests the occurrence contractile activities. During intense exercise, glucose is catabolised and pyruvate is substantially generated. When the mitochondrial activity is exceeded, pyruvate is reduced to lactate, resulting in oxidation of $NADH/H^+$ to NAD^+ (J. S. Baker, McCormick, & Robergs, 2010).

In this study, analysis of the culture media showed that the level of lactic acid was elevated significantly as early as 24 hours after EPS and the level was increased to almost 2-fold after 48-hours of EPS when compared to non-stimulated group. On the other hand, the glucose level in the culture media was found to be significantly reduced just after 48-hours of EPS as compared to non-stimulated group. This might suggest that in the early phase of EPS, the cells might consume the internal source specifically glycogen as the source of energy through glycogenolysis. A study showed that, during a maximal dynamic exercise, there will be an increase rate in muscle glycogenolysis that may lead to escalation in intramuscular glucose concentration. This condition will results in hexokinase inhibition due to elevated glucose-6-phosphate, which will lead to reduction in glucose uptake and utilization. As exercise continues, hexokinase inhibition by glucose-6-phosphate will be relieved as the result from reduction in intramuscular glucose concentration. Thus, it will lead to increase in glucose uptake from the external source (Katz, Broberg, Sahlin, & Wahren, 1986).

Additionally, the EPS-stimulated myotubes exhibited an increase in GLUT4 mRNA expression from 24-hour to 48-hours with a significant

increase of GLUT4 mRNA expression in 48-hour stimulated myotubes as compared to 48-hour non-stimulated cells. This upregulation of GLUT4 mRNA is important since the main mechanism of glucose uptake in contracting skeletal muscle is by facilitated diffusion and this process determined by the translocation of GLUT4 to the surface membrane (Richter & Hargreaves, 2013). In GLUT4 knockout mice, muscle contraction has been shown to have a negligible effect on glucose uptake (Ryder et al., 1999). In addition, mice with muscle-specific GLUT4 deletion demonstrated a significant reduction in muscle glucose uptake and exercise tolerance during exercise (Fueger et al., 2007).

Another positive parameter of contractile activities in the myotubes is the increase in PGC1α mRNA expression. PGC1α is a transcriptional coactivator that interacts with a variety of transcription factors that are involved in biological responses including mitochondrial biogenesis (Liang & Ward, 2006). Thus, it can be considered as a marker for mitochondrial biogenesis. In this study, there was a significant increase in PGC1α mRNA expression in the myotubes after 48-hours EPS as compared to non-stimulated group. This indicates the contractile muscles generate more mitochondria production in order to cope with the increase in the exercise workload. This result is consistent with a study done by Baar et al that showed significant increase in PGC1 mRNA expression and protein in rats muscle after exercise (Baar et al., 2002).

Furthermore, PGC-1α has also has been shown to induce the expression of GLUT4. In a study by Michael et al, they demonstrated that PGC-1 was able to restore GLUT4 mRNA levels in cultured muscle cells up to the level observed *in vivo*, in which PGC-1 mediates the increase in GLUT4

expression by binding to and coactivating the muscle-selective transcription factor: myocyte enhancer factor 2 (MEF2) (Michael et al., 2001). Indeed, MEF2 binding activity is mandatory for regulation of the GLUT4 gene promoter in muscle and adipose tissue (Thai, Guruswamy, Cao, Pessin, & Olson, 1998).

The GLUT4 promoter is regulated through the cooperative function of two different regulatory elements, domain 1 which binds a transcription factor: GLUT4 enhancer factor (GEF) (Oshel, Knight, Cao, Thai, & Olson, 2000) , and MEF2 domain that binds isoforms of the MEF2 family of transcription factors (Thai et al., 1998) .

Study by Knight and his colleagues has showed a restricted pattern of GEF expression which overlaps with MEF2A only in tissues expressing high levels of GLUT4, suggesting the hypothesis that GEF and MEF2A function together to activate GLUT4 transcription. In addition, neither GEF nor MEF2A alone significantly activates GLUT4 promoter activity, but when they expressed together, the promoter activity was increased up to 4- to 5-folds. In addition, deletion of the GEF-binding domain (domain I) and the MEF2-binding domain prevents the GLUT4 activation, which suggests that the promoter regulation occurs through these elements and strongly supports the mechanism of GLUT4 transcription activation that depends on this protein–protein interaction (Knight, Eyster, Griesel, & Olson, 2003).

Interestingly, both rat and human myotubes exhibit the similar pattern of glucose uptake post-EPS, with and without pre-treatment with insulin. As showed in the results, EPS alone was able to induce the increase in glucose uptake, without the presence of insulin. Furthermore, pre-

treatment with insulin has the synergistic effect in glucose uptake when combined with EPS. This suggest that EPS is not only induce the expression of GLUT4 mRNA and protein as suggested by other study (Kuo, Hunt, Ding, & Ivy, 1999), but it also increase the sensitivity of the myotubes to insulin by increasing the GLUT4 levels.

In addition, the results also indicate that muscle contractions and insulin activate muscle glucose transport by two different mechanisms. This is in line with several studies that suggest muscle contraction and insulin activate muscle glucose transport by different molecular mechanism (A. D. Lee et al., 1995; Lund, Holman, Schmitz, & Pedersen, 1995; Wallberg-Henriksson & Holloszy, 1984). It is generally believed that in skeletal muscle, glucose transport can be activated by two distinct pathways, first is by insulin stimulation and second by muscle contraction/exercise. Phosphatidylinositol 3-kinase (PI3K) is involved in insulin-stimulated but not contraction-stimulated glucose transport and GLUT4 translocation. Whereas, 5-AMP-activated kinase, as well as increases in cytoplasmic calcium concentrations could be involved in the contraction response because they both lead to insulin-independent increases in glucose transport (Richter & Hargreaves, 2013; Zierath, Krook, & Wallberg-Henriksson, 2000).

As reported by Lee et al., wortmannin (PI3K inhibitor) completely blocked insulin-stimulated glucose transport in muscle. In contrast, wortmannin had no effect on the stimulation of glucose transport by contractions, providing evidence that PI3K activity is not involved in the activation of glucose transport by these stimuli. Furthermore, this study

supports the evidence that two distinct pathways exist for the activation of skeletal muscle glucose transport (A. D. Lee et al., 1995).

In vivo studies showed that there will be an increase in GLUT4 mRNA expression (Kraniou, Cameron-Smith, & Hargreaves, 2006; McGee & Hargreaves, 2004) and GLUT4 protein (Kraniou et al., 2006) in human skeletal muscle immediately after exercise. The elevation in GLUT4 mRNA and protein remains for several hours post-exercise (Kraniou et al., 2006). Due to fact that the level of GLUT4 protein may fluctuates by time, it is essential to investigate when is the best time after EPS that will elicit the optimum insulin-stimulated glucose uptake in vitro, specifically in our contractile myotubes model. We have showed that the maximum different in rate of insulin stimulated glucose uptake was achieved immediately after EPS, which increased about 50% when compared to vehicle treated group. Interestingly after 48-hours of EPS, the glucose uptake rate declined dramatically for both insulin and non-insulin treated groups. Thus, we have decided to perform our future experiments on this contractile model of myotubes immediately after EPS.

Finally, the immunofluorescence study revealed the increase in GLUT4 protein expression in EPS and EPS with insulin treatment compared to non-stimulated myotubes. This indicates EPS protocol used in this study is able to stimulate the increase in GLUT4 protein expression even though the recruitment of GLUT4 to the cell membrane was not clearly seen in this experiment.

The application of EPS on the myotubes have been described in several studies specifically in murine C2C12 cells (Burch et al., 2010; Fujita, Nedachi, & Kanzaki, 2007; Nedachi et al., 2008), in L6 cells (Yano et al.,

2011), in primary rat skeletal muscle cells (Silveira, Pilegaard, Kusuhara, Curi, & Hellsten, 2006) and in human myotubes (Aas et al., 2002; Lambernd et al., 2012; Nikolic et al., 2012).

In an acute muscle contraction model by Nedachi et al., they introduced a contraction system in which C2C12 myotubes were cultured in culture dishes and electrically stimulated with commercially available carbon electrodes and stimulator (Nedachi et al., 2008). This system is practical, easy to manipulate and is suitable for analyzing the biochemical and molecular biological changes induced by muscle contraction. However in some of the experiments, the protocol requires 24-hour pre-stimulation with low-voltage electrical pulses for myotube contraction, before subjected to a second EPS challenge. To avoid diminishing contractile ability after complete discontinuation of the pre-stimulation, application of a very low frequency of EPS (0.1 Hz) was given even during the resting period to obtain strong contractile activity in response to the subsequent second EPS session. Since control myotubes were also required to be placed under pre-stimulation conditions before the main contraction session, any changes that override pre-stimulation in the main contraction session must be observed and recorded as a contraction-induced effect. Therefore, primary contraction without pre-stimulation would be more suitable for detecting contraction-induced effects in skeletal muscle myotubes.

Study by Nikolic et al. reported that human primary skeletal muscle myotubes were electrically stimulated using two protocols namely acute high-frequency EPS (pulse train of bipolar pulses of 100Hz for 200ms given every 5 second, 30V for 5-60 minutes) and chronic low-frequency EPS (single bipolar pulses of 2ms, 30V and 1Hz continuously for 24 or 48 hours).

They demonstrated that, acute high-frequency EPS increased both lactate production and insulin-stimulated glucose uptake by the myotubes; and the amount of glucose uptake was positively correlated with the duration of the stimulation. Furthermore, in chronic low-frequency EPS group, insulin-stimulated glucose uptake was significantly increased as early as 24 hours and 48 hours by 96% and 145% respectively but no additional effect of insulin and EPS on glucose uptake was observed. Surprisingly, the GLUT4 mRNA did not appear to be affected by chronic low-frequency EPS. However GLUT4 mRNA expression was not measured in acute high-frequency EPS (Nikolic et al., 2012). Even though the chronic low frequency EPS protocol of this study is almost similar to our stimulation protocol, except with the lower voltage used (11.5V), the increased in insulin-stimulated glucose uptake in our model only can be seen after 48 hours of EPS. However, we were able to demonstrate a significant increase in GLUT4 mRNA expression after 48 hours and it correlates with the increase in glucose uptake at the same time point.

Lambernd et al. also reported the similar EPS protocol with our study (single bipolar pulses of 2ms, 11.5V and 1Hz) but with the shorter duration (2 to 24 hours) on human skeletal muscle myotubes. They reported a significant increase in lactate production in the media from 8 to 24 hours following EPS and a significant 2.4 fold increase in insulin-stimulated glucose uptake just after 7.5 hours of EPS. However the level of GLUT4 protein was not affected by EPS. They hypothesized that, this finding might be due to the contractile activity affects the GLUT4 trafficking machinery, mediating a more efficient mobilisation of the transporter by insulin despite a constantly low level of GLUT4 (Lambernd et al., 2012).

In conclusion, by applying a chronic continuous low-frequency EPS to myotubes, several important aspects of in vivo effects of exercise were observed. We were able to demonstrate an elevation in lactic acid and reduction of glucose in the culture media; significant rise in GLUT4 and PGC1α mRNA expression; increment in glucose uptake and increase in protein expression by immunofluorescence. Thus we can conclude that this model of EPS in myotubes represents a physiologically relevant in vitro model of insulin stimulated glucose uptake, which can be used for future study that relates to insulin signalling in exercise.

CHAPTER FOUR

EFFECTS OF CB1 MODULATION UPON INSULIN STIMULATED GLUCOSE UPTAKE IN PRIMARY SKELETAL MUSCLE

Chapter 4: Effects of CB1 Modulation upon Insulin Stimulated Glucose Uptake in Primary Skeletal Muscle

4.1 Introduction

ECS has been identified to play an important role in human regulation of energy balance and metabolism (Butler & Korbonits, 2009). It is a lipid signalling system which includes the cannabinoid receptors, their lipid signalling ligands, and the enzymes that involve in the ligands metabolism (Matias & Di Marzo, 2007).

There is a growing body of evidence which suggests these lipids signalling molecules including fatty acids, endocannabinoids and fatty acid amides may play a role in the regulation of intermediary metabolism. In particular it has been demonstrated that the circulating levels of some of these fatty acid derivatives are elevated in the obese state and are reduced by weight loss (Engeli et al., 2005; Matias et al., 2006). Cannabinoid receptor type-1 (CB1) has been identified as a target for obesity therapy by a number of major pharmaceutical companies by using its antagonists to reduce food intake (Di Marzo & Matias, 2005; Pacher, Batkai, & Kunos, 2006).

Endocannabinoid system consists of G-protein coupled receptors (GPCR), their lipid signalling ligands and enzymes that involved in ligand generation and metabolism (Silvestri et al., 2011). It is known to act both at central and peripheral in which it modulates calorie intake centrally and affects the nutrients transport, cellular metabolism and energy storage peripherally (Silvestri et al., 2011).

CB1, which is a typical GPCR, is located in the cell membrane in which it coupled to intracellular effector system via G-proteins. It contains seven transmembrane domains connected by three extracellular and three intracellular loops with an extracellular N-terminal tail, and an intracellular C-terminal tail (Turu & Hunyady, 2010). CB1 has been found to modulate several functions at cellular level such as inhibiting voltage-gated Ca^{2+} channels, activating K^{+} currents, phosphorylation and activation of mitogen-activated protein kinases (MAPK) such as p42/p44 MAPK, p38 MAPK and c-Jun N-terminal kinase, inhibiting adenylyl cyclase in most tissues and cells (Turu & Hunyady, 2010) and increasing NO signalling (O'Keefe et al., 2013).

There two main ligands of endocannabinoid system, Anandamide (the ethanolamide of arachidonic acid- AEA) was the first endocannabinoid identified in 1992 and the second endocannabinoid was isolated in 1995 named 2-arachidonoyl glycerol (2-AG) (Begg et al., 2005).

Much of the current research has focused upon the synthesis and release of such compounds by adipose tissue and their effects upon the endocrine system (Gonthier et al., 2007; Kyrou, Valsamakis, & Tsigos, 2006; Pagotto et al., 2006). Apart from adipose tissues, CB receptors and other ECS components were also found to be expressed in human and animal muscle cells (Crespillo et al., 2011; Eckardt et al., 2009) However, there is minimal information regarding the role of these fatty acid derivatives in skeletal muscle – which is primarily responsible for determining the metabolic rate of mammals. Given the growing obesity epidemic and the potential for receptors such as G protein couple receptors

(GPCRs) and nuclear receptors as therapeutic targets, this lack of data represents a significant gap in our knowledge.

In this chapter, our aim is to observe the effects of CB1 activation and inhibition on exercise-induced insulin stimulated glucose uptake in primary skeletal muscle myotubes by using ACEA, a synthetic CB1 receptor agonist and an antagonist, Rimonabant. In addition, we investigated the underlying mechanism of inhibition by these endocannabinoids on the insulin signalling cascade.

4.2 Objectives

4.2.1 General Objective

- To investigate the effects of cannabinoid CB1 activation and inhibition upon contraction-induced insulin stimulated glucose uptake in primary skeletal muscle myotubes.

4.2.2 Specific Objectives

- To investigate the effects of ACEA and Rimonabant on contraction-induced insulin stimulated glucose uptake in primary skeletal muscle myotubes.
- To investigate the mechanism of inhibition by ACEA on contraction-induced insulin-stimulated glucose uptake in primary skeletal muscle myotubes.
- To investigate the effects of palmitate in relation to ACEA and Rimonabant on contraction-induced insulin stimulated glucose uptake in primary skeletal muscle myotubes.

4.3 Experiment design and methods

4.3.1 Effects of CB-1 activation and inhibition on insulin stimulated glucose uptake in rat myotubes

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 3 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. The myotubes were then given EPS for 48 hours as described in Chapter 2 section 2.3. Treatment with 10nM ACEA with or without 30 minutes pre-treatment of 100nM rimonabant were given to the myotubes after 24 hours of commencement of EPS.

In this study, the dosages of 10nM ACEA and 100nM Rimonabant we selected based on their effect upon glucose uptake, as shown in several studies. Lipina et al. demonstrated that 10nM ACEA was able to cause inhibition in insulin-stimulated ERK1/2 activity in L6 myotubes. On the other hand, 100nM rimonabant prevented the inhibitory effect of 10nM ACEA upon ERK1/2 and enhanced both insulin-stimulated Akt and ERK activities (Lipina et al., 2010). Esposito et al. and Lindborg et al. also showed the concentration of 100nM rimonabant led to increase in glucose uptake by L6 cells and rat skeletal muscle in vitro respectively (Esposito et al., 2008; Lindborg, Teachey, Jacob, & Henriksen, 2010).

Glucose uptake was determined by the glucose uptake assay as described in Chapter 2 section 2.16 with and without insulin treatment.

4.3.2 Effects of CB-1 activation and inhibition on insulin stimulated glucose uptake in human myotubes

$1x10^5$ of satellite cells/well (from a single individual) were seeded into 3 units of 6-well plates and grown to form matured myotubes as

described in Chapter 2 section 2.2. The myotubes were then given EPS for 48 hours as described in Chapter 2 section 2.3. Treatment with 10nM ACEA with or without 30 minutes pre-treatment of 100nM rimonabant were given to the myotubes after 24 hours of commencement of EPS.

Glucose uptake was determined by the glucose uptake assay as described in Chapter 2 section 2.16 with and without insulin treatment.

4.3.3 Mechanism of endocannabinoid-mediated inhibition of glucose uptake in human myotubes

$1x10^5$ of satellite cells/well (from a single individual) were seeded into 6 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. Then the plates were divided into several conditions and treatments:

- Plate 1: Non-EPS + vehicle
- Plate 2: Non-EPS + 100nM insulin
- Plate 3: EPS + vehicle
- Plate 4: EPS + 100nM insulin
- Plate 5: EPS + 10nM ACEA +/- 100nM insulin
- Plate 6: EPS + 100nM Rimonabant + 10nM ACEA +/- 100nM insulin

EPS was given for 48 hours as described in Chapter 2 section 2.3. Insulin was given for 15 minutes prior to cells collection. For Plate 6, pre-treatment with Rimonabant was given 30 minutes before the treatment of ACEA. The proteins were extracted and analysed by western blotting as described in Chapter 2 section 2.14. The primary and secondary antibodies used together with their dilutions and conditions were as described in Table 2-5.

4.3.4 Inhibition of glucose uptake by endocannabinoids and palmitate in human myotubes

$1x10^5$ of satellite cells/well (from a single individual) were seeded into 3 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. The myotubes were then given EPS for 48 hours as described in Chapter 2 section 2.3. Treatment with 10nM ACEA, 100nM Rimonabant or 200μM Palmitate were given to the myotubes after 24 hours of commencement of EPS. Pre-treatment with rimonabant was given 30 minutes before the treatment of ACEA

Glucose uptake was determined by the glucose uptake assay as described in Chapter 2 section 2.16 with and without insulin treatments.

4.4 Results

4.4.1 Effects of CB-1 activation and inhibition on insulin stimulated glucose uptake in rat myotubes

Insulin caused a significant increase in glucose uptake after EPS in differentiated rat myotubes. ACEA inhibited glucose uptake in insulin treated group and the effect was blocked by pre-treatment with Rimonabant. However, ACEA and Rimonabant had no effect upon glucose uptake in non-insulin treated cells (Figure 4-1).

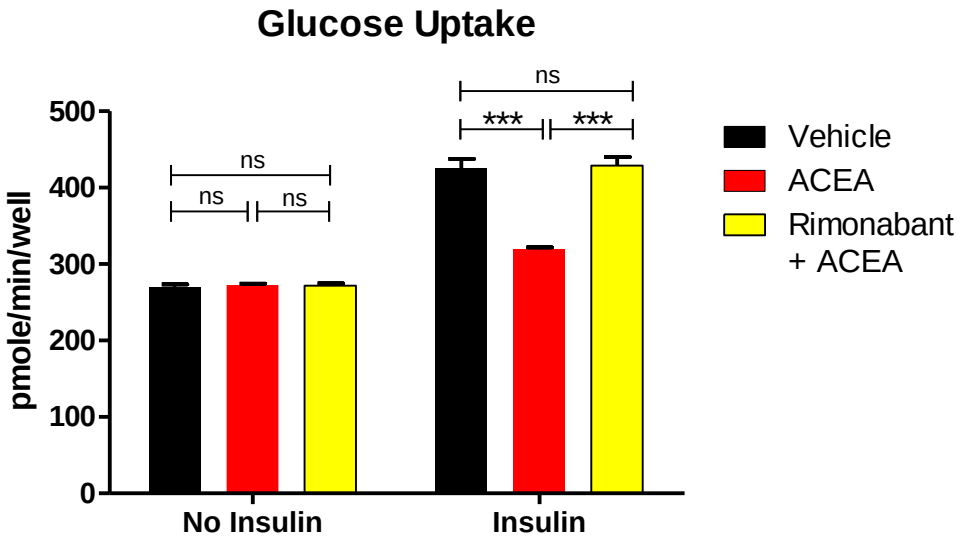


Figure 4- 1: Glucose uptake assay in rat primary skeletal muscle myotubes after ACEA and Rimonabant treatment post-electrical pulse for 48 hours.

All groups were subjected to EPS for 48 hours. Data expressed as mean ± SE (**p<0.01, ***p<0.001, Two-way ANOVA with Bonferronis's post-hoc, n=3).

4.4.2 Effects of CB-1 activation and inhibition on insulin stimulated glucose uptake in human myotubes

Similar effects of ACEA and Rimonabant upon insulin stimulated glucose uptake were seen in EPS human myotubes. Treatment with insulin caused a significant increase in glucose uptake after EPS when compared to non-insulin treated group. Moreover, treatment with ACEA inhibited glucose uptake in the insulin treated group. Pre-treatment with Rimonabant blunted the effects of ACEA. ACEA and Rimonabant had no effect upon glucose uptake in the non-insulin treated group (Figure 4-2).

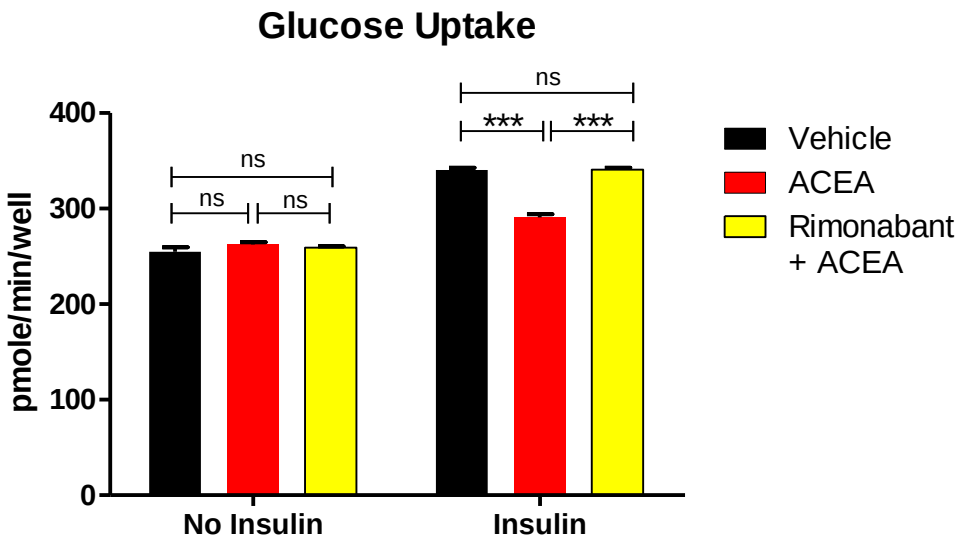


Figure 4- 2: Glucose uptake assay in human primary skeletal muscle myotubes in after ACEA and Rimonabant treatment post-electrical pulse stimulation for 48 hours.

All groups were subjected to EPS for 48 hours. Data expressed as mean ± SE (***p<0.001, Two-way ANOVA with Bonferronis's post-hoc, n=3).

4.4.3 Mechanism of endocannabinoid-mediated inhibition of glucose uptake in human myotubes

i) Effects of ACEA and RIM on insulin stimulated AKT Phosphorylation

In order to investigate the underlying mechanism of the inhibition of insulin-stimulated glucose uptake by CB1 activation, we first observed the effects of EPS on AKT phosphorylation in human myotubes. Results showed that treatment with insulin increased the phosphorylation of AKT regardless of the contraction status. (Figure 4.3).

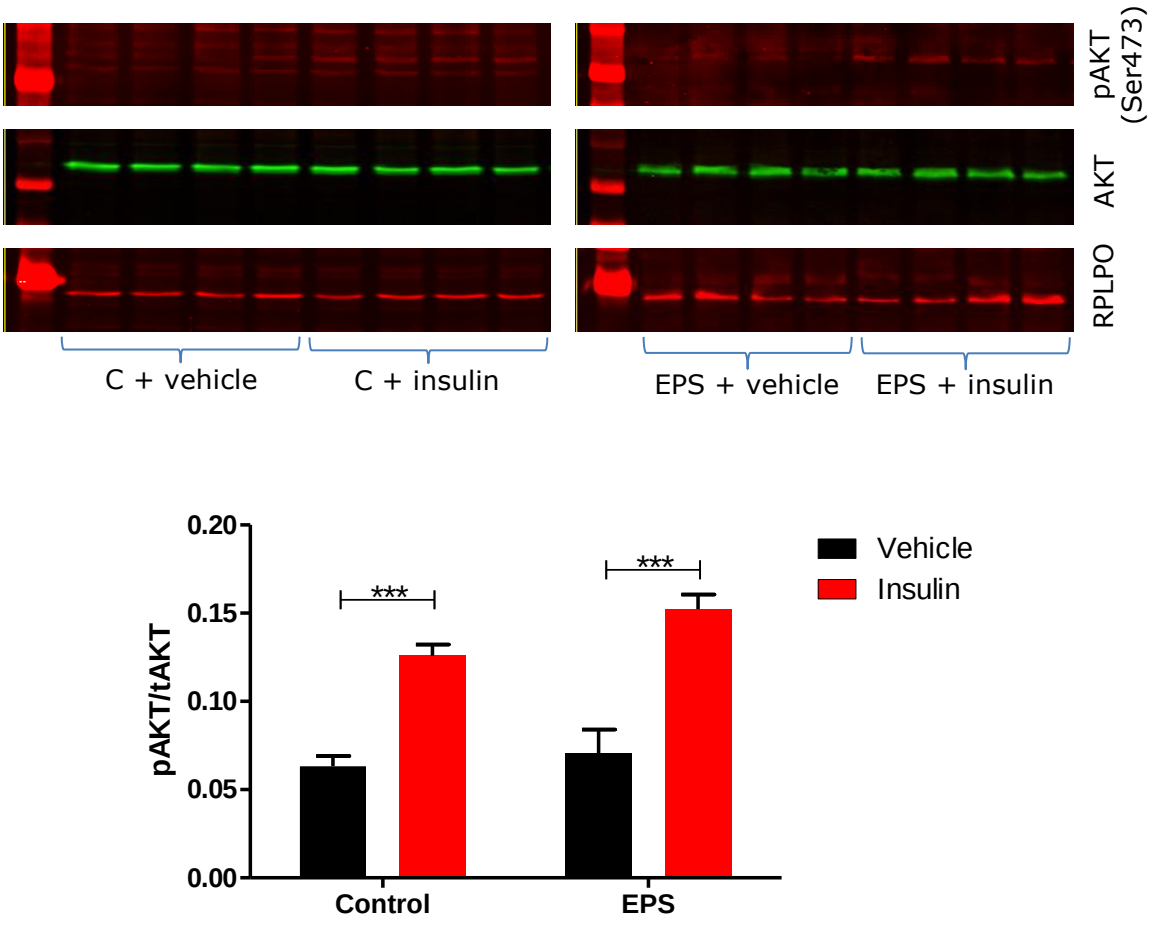


Figure 4- 3: Representative western blot showing pAKT/AKT ratio in unstimulated and stimulated human skeletal muscle myotubes

Quantified values of pAKT/AKT ratio are shown in lower panels. Data expressed as mean ± SEM (**p<0.01, ***p<0.001, Two-way ANOVA with Bonferronis's post-hoc, n=4).

The effects of CB1 activation and antagonism by 10nM ACEA and 100nM Rimonabant respectively upon AKT phosphorylation in human myotubes was then investigated. Results indicated that ACEA inhibited insulin stimulated AKT phosphorylation and the action of ACEA was blunted by pre-treatment with Rimonabant (Figure 4.4).

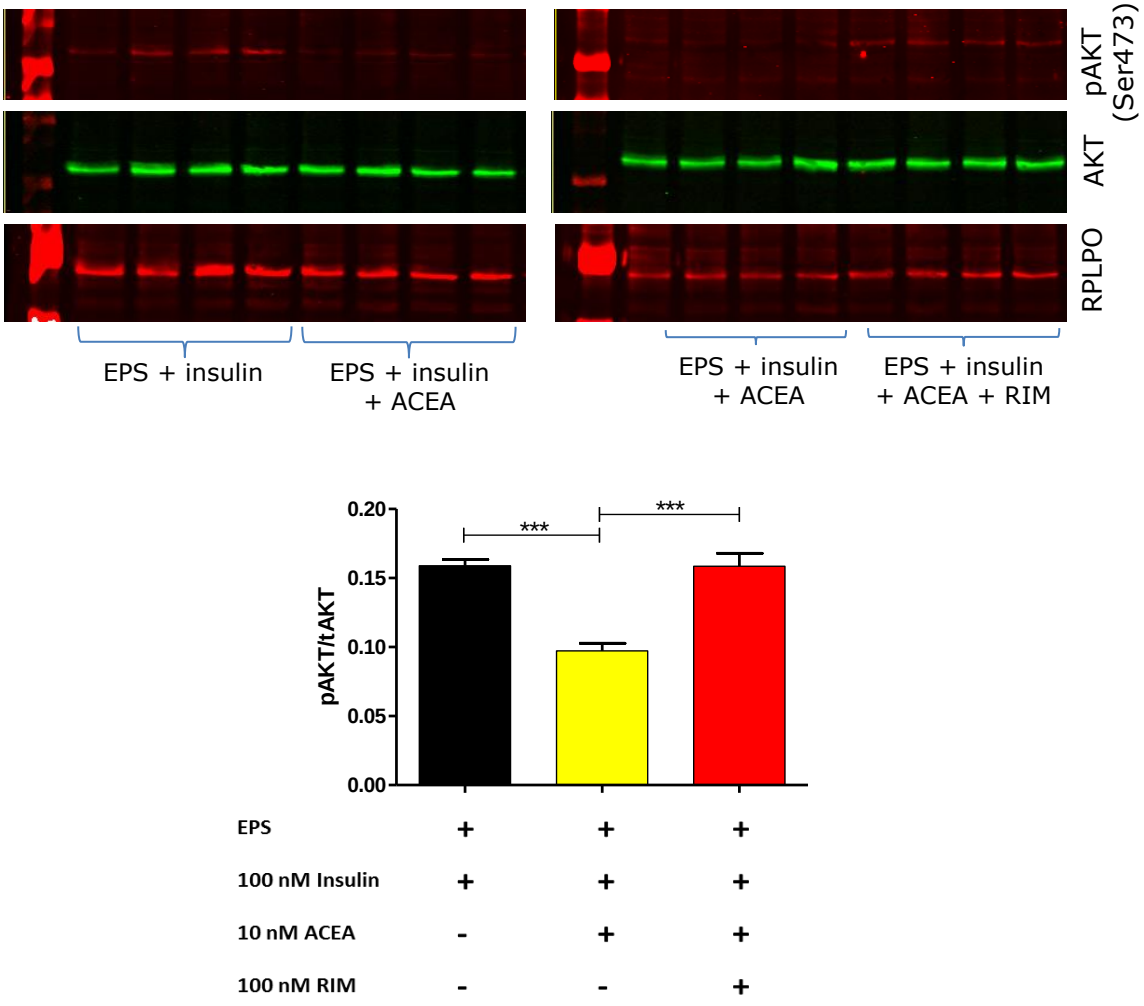


Figure 4- 4: Representative blot showing effects of ACEA and RIM upon pAKT/AKT ratio in stimulated human skeletal muscle myotubes

Quantified values of pAKT/AKT ratio are shown in lower panels. Data expressed as mean ± SEM (***p<0.001, One-way ANOVA with Bonferronis's post-hoc, n=4).

ii) Effects of ACEA and RIM on phosphorylation status of PI3K subunits: p85 and p110

Another important protein in insulin signalling cascade is phosphoinositide 3-kinase (PI3K), a heterodimer composed of a p85 regulatory and a p110 catalytic subunit. Next we would like to investigate the effects of EPS and insulin on p85/p110 ratio before we observe the effects of endocannabinoids on this protein. Results showed that EPS and insulin has no significant effects on p85/p100 ratio of PI3K subunits (Figure 4-5). The molecular weight of the bands of p85, p110 and RPLPO were detected at 85 kDa, 110 kDa and at 34 kDa respectively.

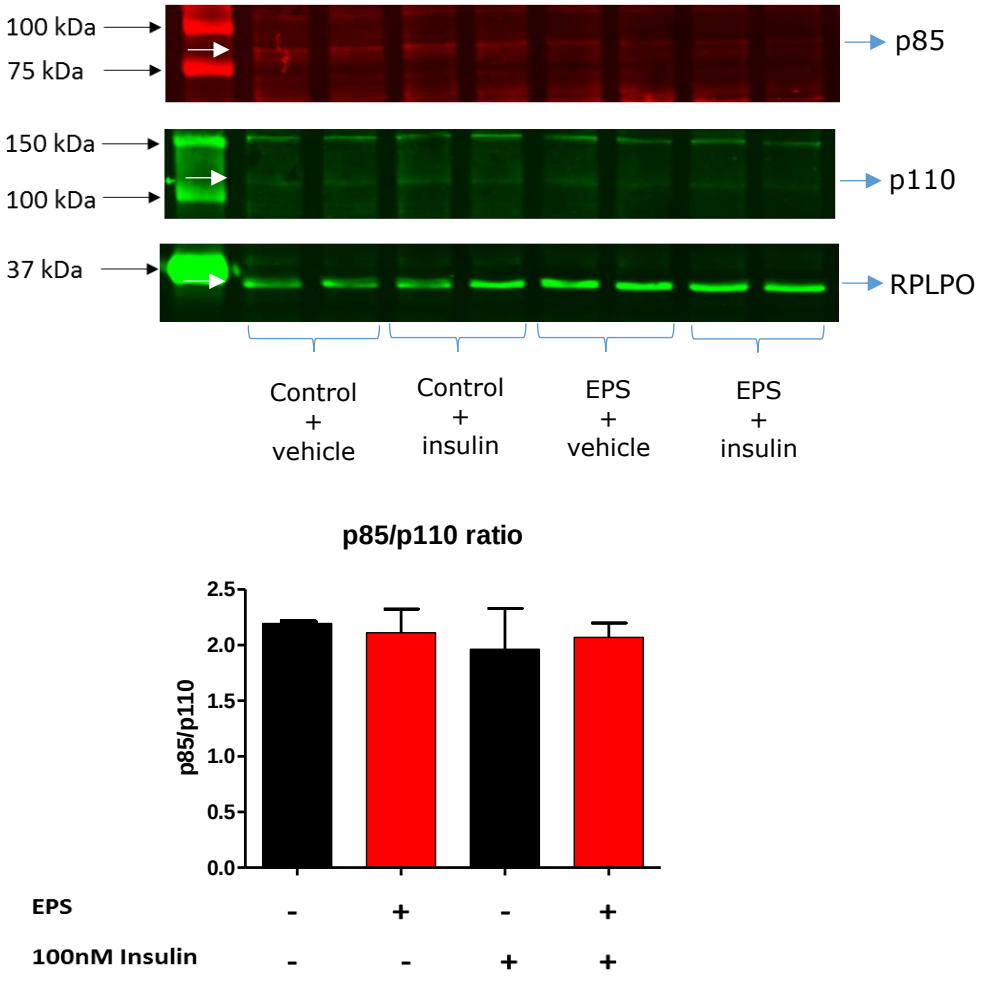


Figure 4- 5: Representative blot showing p85/p110 ratio in unstimulated and stimulated human skeletal muscle

Quantified values of p85/p110 ratio are shown in lower panels by direct comparison.

Furthermore, treatment with 10nM ACEA and insulin increases p85/p110 ratio and pre-treatment with 100nM Rimonabant reverted the p85/p100 ratio to the level seen in non-endocannabinoid treatment group (Figure 4-6).

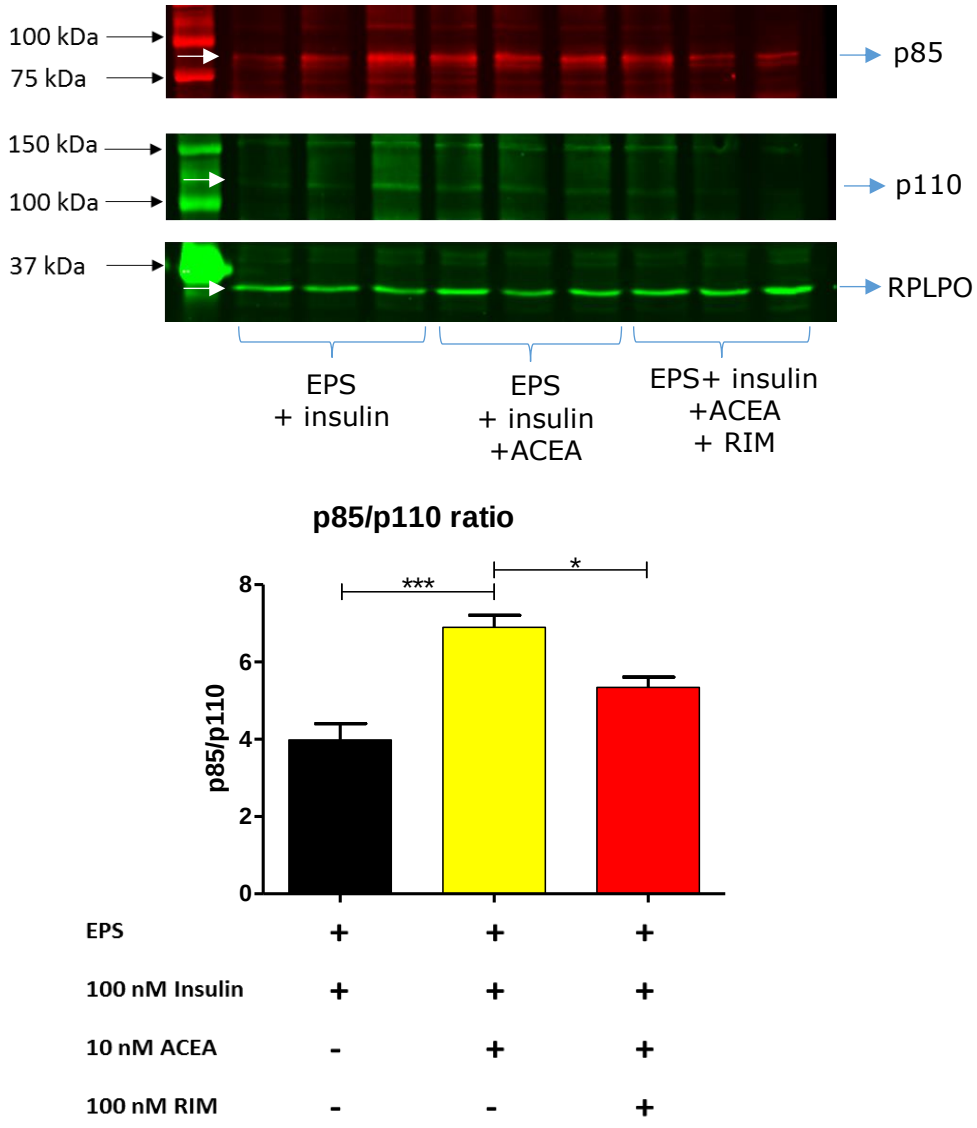


Figure 4- 6: Representative blot showing effects of ACEA with/without pre-treatment with RIM upon p85/p110 ratio in stimulated human skeletal muscle myotubes.

Quantified values of p85/p110 ratio are shown in lower panels. * denotes P<0.05, *** denotes P<0.001. Data were analysed by One-way ANOVA with Bonferronis's post-hoc (n=3).

4.4.4 Inhibition of glucose uptake by endocannabinoids and palmitate in human myotubes

Palmitate is a non-esterified fatty acids that known to have an inhibitory effect on insulin stimulated glucose uptake in skeletal muscle. Palmitate was found to promote accumulation of ceramide in skeletal muscle; and synthesis of this ceramide is crucially dependent on the activity of serine palmitoyl transferase (SPT). This ceramide in turn was found to play a role in insulin resistance in skeletal muscle by inhibit the phosphorylation of of Akt. Study showed that CB1 antagonism may inhibit de novo synthesis of ceramide by inhibition of the activity of SPT. After we have observed the effects endocannabinoids, next we would like to investigate the effects of palmitate with comparison to ACEA and combination with Rimonabant upon exercise-induced insulin stimulated glucose uptake in human myotubes.

Result showed the treatment with Palmitate inhibited insulin-stimulated glucose uptake. Furthermore, treatment with ACEA reduced insulin-stimulated glucose uptake at a similar level. Interestingly, treatment with a combination of Palmitate and ACEA caused an additive reduction of insulin-stimulated glucose uptake in the myotubes. On the other hand, pre-treatment with Rimonabant blunted the inhibitory effect of combination of Palmitate and ACEA to a level; observed with palmitate alone. (Figure 4-7). This suggest the inhibitory effect of Rimonabant only acts on ACEA but not on palmitate (as seen in previous experiment: Figure 4-2)

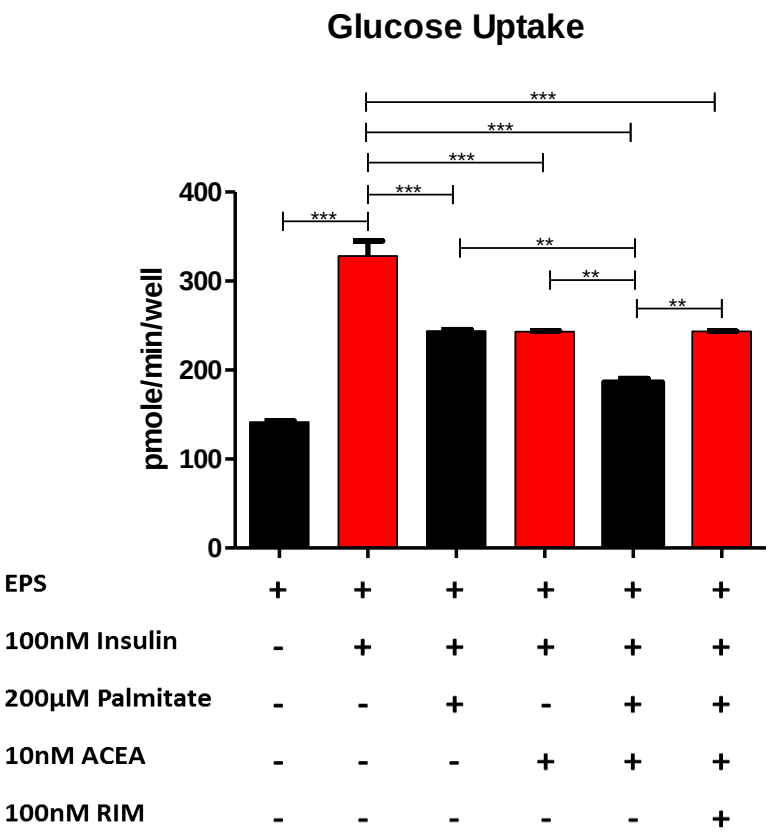


Figure 4- 7: Glucose uptake assay in human primary skeletal muscle myotubes after Palmitate, ACEA and Rimonabant treatment post-electrical pulse stimulation.

Data expressed as mean ± SEM (**p<0.01, ***p<0.001, One-way ANOVA with Bonferronis's post-hoc, n=3).

4.5 Discussion

The endocannabinoid system (ECS) is one of the important systems in our body and it has been identified to play an essential role in the regulation of energy balance and metabolism It is known to act both at central and peripheral sites and modulates calorie intake centrally whilst affecting the nutrient transport, cellular metabolism and energy storage peripherally (Silvestri et al., 2011).

In the first part of the current study, treatment with ACEA inhibit glucose uptake in insulin treated group, meanwhile pre-treatment with Rimonabant blunted the inhibitory effect of ACEA. These results are in line with several studies that suggest the similar effects of CB1 activation and blockade upon insulin stimulated glucose uptake. Adipocyte-conditioned medium from overweight and obese human subjects that contained naturally occurring levels of AEA was found to induce insulin resistance in skeletal muscle cell cultures and the effect was inhibited by Rimonabant (Eckardt et al., 2009). In the other study, activation of CB1 receptors with anandamide and ACEA induced glucose intolerance in rats as revealed by the elevation in blood glucose level. On the other hand, pre-treatment with CB1 antagonist AM251 inhibit the glucose intolerance that induced by anandamide (Bermudez-Siva et al., 2006). Furthermore, peripheral CB1 blockade by Rimonabant in diet-induced obese rats not only caused reduction in food intake and body weight but, in addition enhanced insulin sensitivity in skeletal muscle (Nogueiras et al., 2008).

The insulin receptor (IR) is a heterotetrameric membrane protein which composed of two identical α and β subunits. Insulin will bind to the α subunit of IR and subsequently activates intrinsic kinase activity in β

subunit. This will lead to intramolecular transautophosphorylation reaction in which one β subunit tyrosine phosphorylates the other β subunit. Then the insulin receptor substrate (IRS) protein family will interact with the phosphorylated IR through a phosphotyrosine binding module (Saltiel & Kahn, 2001). This IRS protein will become a docking site for proteins with Src Homology 2 (SH2) domains in which p85 become the most important regulatory subunit of the type 1A phosphatidylinositol 3' kinase (PI3K). PI3K exist as a dimer of a regulatory p85 subunit and a catalytic p110 subunit (Fruman et al., 1998).

Then the catalytic p110α subunit will be brought to the cell membrane and catalyzes the phosphorylation of 3' position in the inositol ring of phosphoinositide (PI) lipids. Specifically the type 1A PI3K catalyzes the transformation of PI(4,5)-biphosphate to PI(3,4,5)-triphosphate and PI(4)-phosphate to PI(3,4)-biphosphate (Fruman et al., 1998). The phosphorylation of the 3' will further activates 3'phosphoinositide-dependent kinase-1 (PDK-1) and Akt (protein kinase B) (Rameh & Cantley, 1999). Subsequently, PDK-1 phosphorylates and activates Akt and protein kinase C (PKC) ζ/λ. Akt and PKC ζ/λ will then promote the GLUT4 translocation from the intracellular into the cell membrane and increased glucose transport (Bae, Cho, Mu, & Birnbaum, 2003; Khan & Pessin, 2002; Kotani et al., 1998).

From the above description, it is clear that Akt and PI3K are among the important proteins in insulin signalling cascade. In current study, we observed the treatment with insulin increases the phosphorylation of AKT regardless of the contraction status. There was a significant reduction in AKT phosphorylation in non-insulin treated groups for both EPS and non-

EPS when compared to insulin treated groups. This shows that the insulin signalling pathway has been activated in the presence of insulin. Furthermore, ACEA inhibit AKT phosphorylation and the action of ACEA was blunted by pre-treatment with Rimonabant.

Similar results was demonstrated by Eckardt et al., in which the treatment with adipocyte-conditioned medium from overweight and obese human subjects that contained naturally occurring levels of anandamide, was found to impair insulin-stimulated AKT (Ser473) phosphorylation in human skeletal muscle cell cultures. On the other hand, pre-incubation with Rimonabant or AM251 significantly reduced the inhibiting effect of the conditioned media upon Akt phosphorylation. Apart from that, incubation of the human skeletal muscle cell culture with the endogenous cannabinoid, anandamide significantly reduced the insulin-stimulated Akt (Ser473) phosphorylation and the reduction was prevented with pre-incubation with AM251 (Eckardt et al., 2009).

As mentioned above, PI3K is another key protein in insulin signalling pathway. PI3K is a heterodimer consist of a p110 catalytic subunit and a p85 regulatory subunit. In the present of insulin, it will cause tyrosine phosphorylation of insulin receptor substrate (IRS) proteins, which in turn associate with PI3K through the p85 regulatory domain, leading to elevation in PI3K activity (Saltiel & Kahn, 2001; Shepherd, Withers, & Siddle, 1998)

According to several studies, transcription of the p85 gene leads to the expression of its splice variants p85α, p55α and p50α; all these three can bind to p110 and to phosphotyrosine domain (Antonetti, Algenstaedt, & Kahn, 1996; Fruman, Cantley, & Carpenter, 1996; Inukai et al., 1997; Ueki et al., 2002). Under normal state, PI 3-kinase activity associated with

p50α was greater than that associated with p85α or p55α. Increasing the level of p85α or p55α, but not p50α, inhibited both phosphotyrosine-associated and p110-associated PI 3-kinase activities (Ueki, Algenstaedt, Mauvais-Jarvis, & Kahn, 2000). Furthermore, when p85 subunit is more abundant than p110, leading to competition between the p85 monomer and the p85-p110 dimer for binding to phosphorylated IRS proteins and results in ineffective signalling (Ueki et al., 2002). It could be postulated that increased levels of p85 subunits might inhibit PI 3-kinase activity by competing with phosphotyrosine targets. Thus, the excess of these subunits may contribute to insulin resistance.

In current study, we observed the effects of endocannabinoids upon insulin-stimulated expression of PI3K subunits p85 and p110 in term of p85/p110 ratio. The result revealed treatment with 10nM ACEA and insulin increases p85/p110 ratio and pre-treatment with 100nM Rimonabant reverted the p85/p100 ratio to the level seen in non-endocannabinoid treatment group. This suggest that elevation in p85 regulatory subunit may lead to insulin resistance.

This result is consistent with other study in which a p85 knockout mouse exhibit an increased in insulin sensitivity (Mauvais-Jarvis et al., 2002; Terauchi et al., 1999). Furthermore, overexpression of the p85 SH2 domains or deletion mutants of p85 that unable to bind to p110 leads to a decrease in PI3K activity and disruption in insulin signalling (Jhun et al., 1994; Ueki et al., 2000). A model of insulin resistance which induced by chronic exposure to placental growth hormone also showed overexpression of p85 (Barbour et al., 2004).

From the above results, we can suggest that the mechanism of inhibition of CB1 activation upon exercise induced insulin-stimulated glucose uptake is by inhibition of Akt and increase in p85 regulatory subunit of PI3K. However, this inhibition of insulin cascade by endocannabinoids is a complicated process, we believe that there are a lot of other mechanism involving another steps of the pathway such as inhibition of PKC and indirect inhibition by PKA and adenynyl cyclase that need to be explored.

Apart from that, we have also demonstrated the effects of palmitate with comparison to ACEA upon exercise-induced insulin stimulated glucose uptake in human myotubes. Palmitate is a non-esterified fatty acids that found to has an inhibitory effect on insulin stimulated glucose uptake in skeletal muscle (Alkhateeb, Chabowski, Glatz, Luiken, & Bonen, 2007; Storz et al., 1999). In current study, treatment with Palmitate or ACEA significantly inhibit insulin-stimulated glucose uptake at almost similar level. Interestingly, treatment with combination of Palmitate and ACEA caused a synergistic effect in reduction of insulin-stimulated glucose uptake. On the other hand, pre-treatment with Rimonabant blunted half of the inhibitory effect of combination of Palmitate and ACEA. This suggest that Rimonabant only reverted the effect of ACEA, but not Palmitate. Several studies found that Palmitate inhibit insulin-stimulated glucose uptake by inhibiting Akt phosphorylation (Chavez & Summers, 2003; Peng et al., 2011; Storz et al., 1999). Since ACEA also inhibit Akt phosphorylation as observed in current study, it may has the similar site of action with palmitate in inhibiting insulin-stimulated glucose uptake, however the underlying pathway of Akt inhibition might be different.

Previous studies also suggested that saturated fatty acids such as palmitate promote accumulation of ceramide (Chavez & Summers, 2003, 2012; Pickersgill, Litherland, Greenberg, Walker, & Yeaman, 2007; Schmitz-Peiffer, Craig, & Biden, 1999; Watson, Coghlan, & Hundal, 2009) in skeletal muscle; and synthesis of this ceramide is crucially dependent on the activity of serine palmitoyl transferase (SPT) which catalyses the first and rate-limiting step of *de novo* biosynthesis involving the condensation of amino acid L-serine with palmitoyl-CoA to form 3-Ketosphinganine (Levy & Futerman, 2010). This ceramide in turn was found to play a role in insulin resistance in skeletal muscle by inhibiting the phosphorylation of of Akt (Adams et al., 2004; Hajduch et al., 2001; Schmitz-Peiffer et al., 1999; Watson et al., 2009). Interestingly, as demonstrated by Cinar et al, peripheral CB1 blockade by JD5037 appear to reverse the elevation of long-chain ceramide in muscle. Furthermore, CB1 antagonism also inhibit de novo synthesis of ceramide by inhibition of the activity of SPT and ceramide synthase (CerS); and promote the degradation of ceramide in liver microsomes of diet-induced obese mice (Cinar et al., 2014). These findings has open up a new perspectives on the study of these fatty acid derivatives, and further future work need to be done to study the relationship between endocannabinoid and ceramide activities in regulating insulin-stimulated glucose uptake in skeletal muscle.

CHAPTER FIVE

EEFECTS OF CB1 MODULATION UPON SELECTED GENES EXPRESSION

Chapter 5: Effects of CB1 Modulation upon Selected Genes Expression

5.1 Introduction

The main objective of this chapter is to determine the molecular pharmacological properties of the cannabinoid receptor type-1 (CB1) in primary skeletal muscle. In order to study the effects of CB1 agonism and antagonism upon gene expression, qRTPCR was conducted. The myotubes were then treated with the CB1 agonist-ACEA and antagonist-Rimonabant.

Apart from observing the effects of the treatments, we were also would like to determine the influence of the lipid products in the serum media on the CB1 agonism and antagonism. Thus, the experiments were performed under various serum media conditions. In most of the experiments, the myotubes were exposed and compared between normal serum and lipid-free serum- in order to exclude the influence of naturally occurring endocannabinoids, triglycerides, cholesterol and phospholipids in the serum.

The effects of CB1 agonism and antagonism were observed on LCN2, Neuropeptide Y (NPY), TRIB3, NR4A1, NR4A2 and NR4A3 genes expressions. These genes were suggested to be related to obesity and insulin resistance.

Previous studies have shown that inhibition of CB1 leads to the upregulation of these genes. Study by Haddad demonstrated that rimonabant upregulated the mRNA content of LCN2 and NPY, and down-regulated NR4A1 mRNA in rats' myotubes (Haddad, 2014). Furthermore, a

study by Ruby et al. showed that CB1 inhibition by AM251, a rimonabant analog, upregulated LCN2 mRNA expression in rat's liver (Ruby et al., 2011).

In addition, previous data from our laboratory also has shown that the gene expression of LCN2 and NPY were upregulated by Rimonabant. Meanwhile the gene expression of TRIB3, NR4A1, Nr4A2 and NR4A3 were downregulated by the same treatment via microarray. So in this part, we would like to validate the previous data via Taqman real-time PCR.

5.2 Objectives

5.2.1 General Objective

- To observe the effects of cannabinoid CB1 activation and antagonism upon selected gene expression related to obesity and insulin resistance.

5.2.2 Specific Objectives

- To observe the effects of ACEA and Rimonabant upon gene expression in primary skeletal muscle myotubes specifically LCN2, NPY, TRIB3, NR4A1, NR4A2 and NR4A3.
- To observe the effects of exercise contraction on gene expression namely LCN2, NPY, TRIB3, NR4A1, NR4A2 and NR4A3.

5.3 Experiment designs and methods

5.3.1 Preparation of lipid-free serum

The delipidation of the fetal bovine serum and horse serum in this chapter was performed according to the method of Cham and Knowles with a slight modification (Cham & Knowles, 1976) as described in chapter 2 section 2.5.

5.3.2 Effect of CB1 modulation in rat myotubes upon genes expression in horse serum and lipid-free FBS media

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 4 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. The wells of the myotubes were then divided into 6 groups of different media environments and treatments:

- Normal horse serum media + vehicle (0.01 ethanol) (n=4)
- Normal horse serum media + 10nM ACEA (n=4)
- Normal horse serum media + 100nM Rimonabant (n=4)
- Lipid-free FBS media + vehicle (0.01% ethanol) (n=4)
- Lipid-free FBS media + 10nM ACEA (n=4)
- Lipid-free FBS media + 100nM Rimonabant (n=4)

6% of lipid-free horse serum in Ham's F10 media was introduced 48 hours prior to cells collection. Treatment by ACEA or Rimonabant was given 24 hours prior to cells collection. The RNA was extracted and the mRNA expression of 6 different genes namely LCN2, NPY, TRIB3, NR4A1, NR4A2 and NR4A3 were measured by Taqman qRTPCR as described in Chapter 2

section 2.7. The sequences of the primers and probes are detailed in Table 2-4.

5.3.3 Effect of CB1 modulation in rat myotubes upon genes expression in lipid-free horse serum media

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 2 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. 6% of lipid-free horse serum in Ham's F10 media was introduced to all wells 48 hours prior to cells collection. The wells of the myotubes were then divided into 3 groups of different treatments (n=4) namely vehicle (0.01% ethanol), 10nM ACEA or 100nM Rimonabant which was given 24 hours prior to cells collection. The RNA was extracted and the mRNA expression of 6 different genes namely LCN2, NPY, TRIB3, NR4A1, NR4A2 and NR4A3 were measured by Taqman qRTPCR as described in Chapter 2 section 2.7. The sequences of the primers and probes are detailed in Table 2-4.

5.3.4 Genes expression in rat myotubes in normal horse serum and lipid-free horse serum media

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 2 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. Lipid-free horse serum media was introduced 48 hours prior to cells collection. The RNA was extracted and the mRNA expression of 6 different genes namely LCN2, NPY, TRIB3, NR4A1, NR4A2 and NR4A3 were measured by Taqman qRTPCR as described in Chapter 2

section 2.7. The sequences of the primers and probes are detailed in Table 2-4.

5.3.5 Effect of CB1 modulation in rat myotubes upon genes expression in normal horse serum, lipid-free horse serum and charcoal stripped FBS media

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 5 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. 6% of lipid-free horse serum in Ham's F10 media or 6% charcoal stripped FBS (Sigma-aldrich, USA) in Ham's F10 media was introduced 48 hours prior to cells collection. Charcoal stripped FBS is a type of serum that is prepared by filtration through charcoal, which reduce the levels of hormones and steroids such as progesterone, cortisol, corticosterone, testosterone and estradiol without affecting the FFA content. 100nM Rimonabant which was given 24 hours prior to cells collection. The RNA was extracted and the mRNA expression of 6 different genes namely LCN2, NPY, TRIB3, NR4A1, NR4A2 and NR4A3 were measured by Taqman qRTPCR as described in Chapter 2 section 2.7. The sequences of the primers and probes are detailed in Table 2-4.

5.3.5 Effect of fatty acid content in media upon genes expression in rat myotubes

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 4 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. The wells of the myotubes were then divided into 6 groups of different media environments and treatments:

- Normal horse serum media (n=4)
- Lipid-free horse serum media (n=4)
- Lipid-free horse serum media + 0.4% PLO (n=4)
- Lipid-free horse serum media + 0.4% PLO + vehicle (0.01% ethanol) (n=4)
- Lipid-free horse serum media + 0.4% PLO media + 100nM Rim (n=4)
- Lipid-free horse serum media + 0.4% PLO + 200nM Rimonabant (n=4)

PLO (palmitic acid + oleic acid + linoleic acid) in BSA was prepared as described in Chapter 2 section 2.6. Different types of media were introduced 48 hours prior to cells collection. Treatment by Rimonabant was given 24 hours prior to cells collection. The RNA was extracted and the mRNA expression of 6 different genes namely LCN2, NPY, TRIB3, NR4A1, NR4A2 and NR4A3 were measured by Taqman qRTPCR as described in Chapter 2 section 2.7. The sequences of the primers and probes are detailed in Table 2-4.

5.3.6 Genes expression in rat myotubes after EPS in normal horse serum

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 3 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. The myotubes were then given EPS for 48 hours as described in Chapter 2 section 2.3. The RNA was extracted and the mRNA expression of 6 different genes namely LCN2, NPY, TRIB3, NR4A1, NR4A2 and NR4A3 were measured by Taqman qRTPCR as described in Chapter 2

section 2.7. The sequences of the primers and probes are detailed in Table 2-4.

5.3.7 Genes expression in rat myotubes in horse serum, lipid-free horse serum and lipid-free horse serum supplemented with PLO after EPS

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 4 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. The wells of the myotubes were then divided into 6 groups of different media environments and conditions:

- Normal horse serum media (n=4)
- Lipid-free horse serum media (n=4)
- Lipid-free horse serum media + 0.4% PLO (n=4)
- Normal horse serum media + EPS (n=4)
- Lipid-free horse serum media + EPS (n=4)
- Lipid-free horse serum media + 0.4% PLO + EPS (n=4)

PLO (palmitic acid + oleic acid + linoleic acid) in BSA was prepared as described in Chapter 2 section 2.6. Different types of media were introduced 48 hours prior to cells collection. The myotubes were given EPS for 48 hours as described in Chapter 2 section 2.3. The RNA was extracted and the mRNA expression of 6 different genes namely LCN2, NPY, TRIB3, NR4A1, NR4A2 and NR4A3 were measured by Taqman qRTPCR as described in Chapter 2 section 2.7. The sequences of the primers and probes are detailed in Table 2-4.

5.4 Results

5.4.1 Effect of CB1 modulation in rat myotubes upon genes expression in horse serum and lipid-free FBS media

i) Lipocalin 2

There were no significant differences in LCN2 mRNA expression between treatment groups in normal horse serum or lipid-free FBS conditions. On further analysis, LCN2 mRNA expression was significantly higher in lipid-free FBS in all groups when compared to normal horse serum (Figure 5-1).

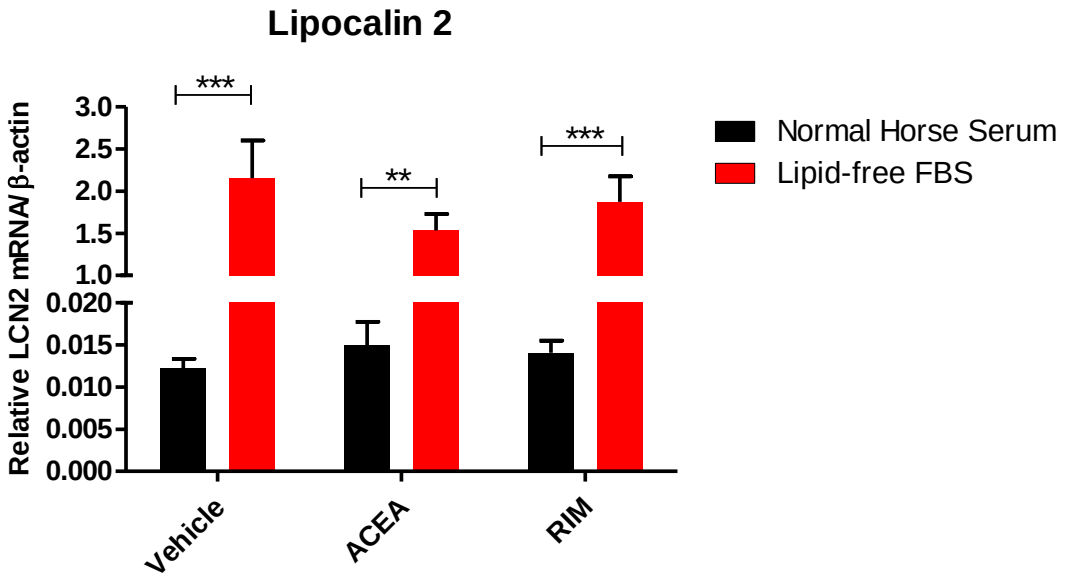


Figure 5- 1: Lipocalin 2 mRNA expression in rat myotubes in horse serum and lipid-free FBS media.

Comparison of LCN2 mRNA expression in vehicle, ACEA and Rimonabant treatment between normal horse serum and lipid-free FBS media. Data expressed as mean ± SEM (**p<0.01, ***p<0.001, Two-way ANOVA with Bonferronni's post-hoc, n=4).

ii) Neuropeptide Y

In a similar pattern to LCN2, NPY expression was not affected by either ACEA or RIM, but expression was significantly increased in the lipid-free FBS culture conditions (Figure 5-2).

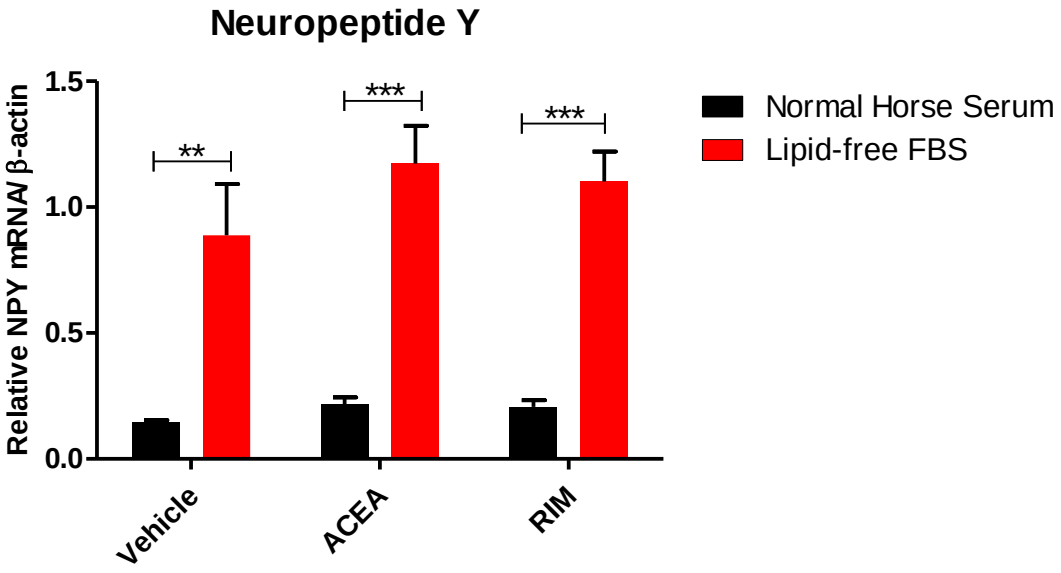


Figure 5- 2: Neuropeptide Y mRNA expression in rat myotubes in horse serum and lipid-free FBS media.

Comparison of NPY mRNA expression in vehicle, ACEA and Rimonabant treatment between normal horse serum and lipid-free FBS media. Data expressed as mean ± SEM (**p<0.01, ***p<0.001, Two-way ANOVA with Bonferronni's post-hoc, n=4).

iii) TRIB-3

In contrast, TRIB3, whilst again being unnafected by ACEA or RIM was expressed at significantly lower levels in lipid-free FBS as compared to horse serum (Figure 5-3).

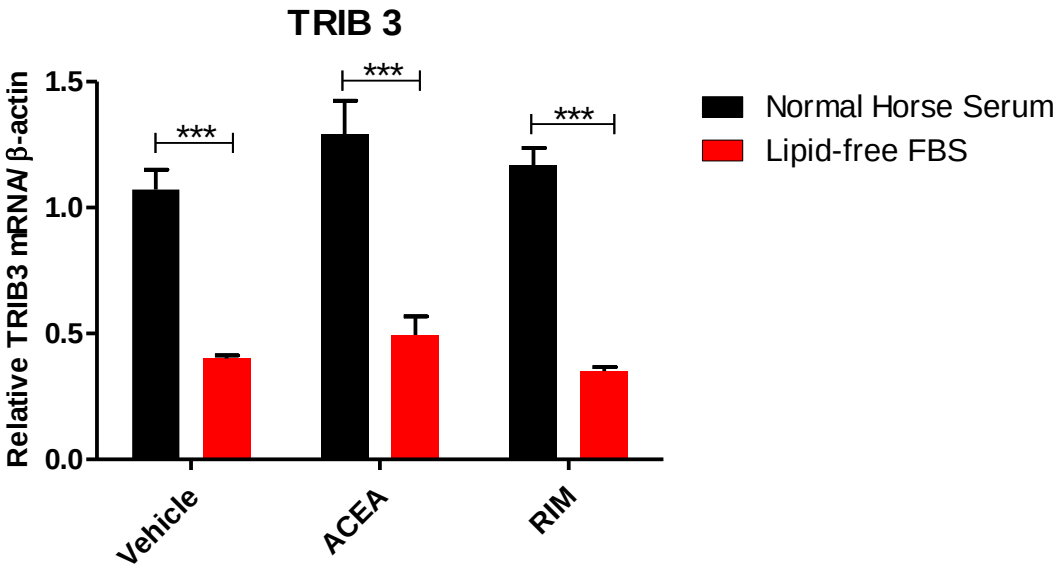


Figure 5- 3: TRIB 3 mRNA gene expression in rat myotubes in horse serum and lipid-free FBS media.

Comparison of TRIB 3 mRNA expression in vehicle, ACEA and Rimonabant treatment between normal horse serum and lipid-free FBS media. Data expressed as mean ± SEM (***p<0.001, Two-way ANOVA with Bonferronni's post-hoc, n=4).

iv) NR4A1

Whereas, NR4A1 expression showed an increasing pattern (but not significantly so) in lipid-free FBS and also unaffected by ACEA or RIM (Figure 5-4).

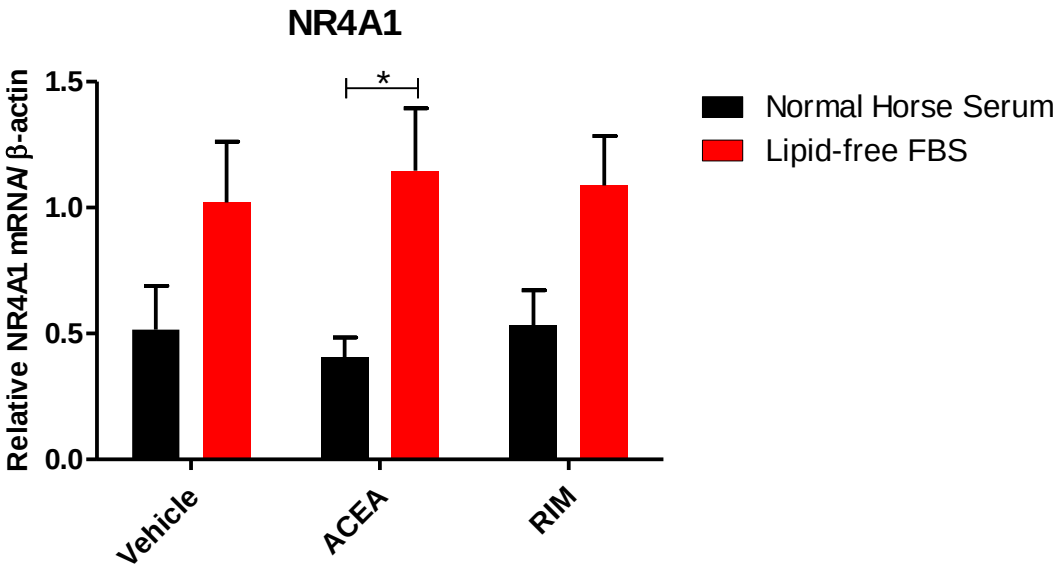


Figure 5- 4: NR4A1 mRNA gene expression in rat myotubes in horse serum and lipid-free FBS media.

Comparison of NR4A1 mRNA expression in vehicle, ACEA and Rimonabant treatment between normal horse serum and lipid-free FBS media. Data expressed as mean ± SEM (Two-way ANOVA with Bonferronni's post-hoc, n=4).

v) NR4A2

As seen with LCN2 and NPY, NR4A2 mRNA gene expression was also not affected by either ACEA or RIM, but expression was significantly increased in the lipid-free FBS culture conditions (Figure 5-5).

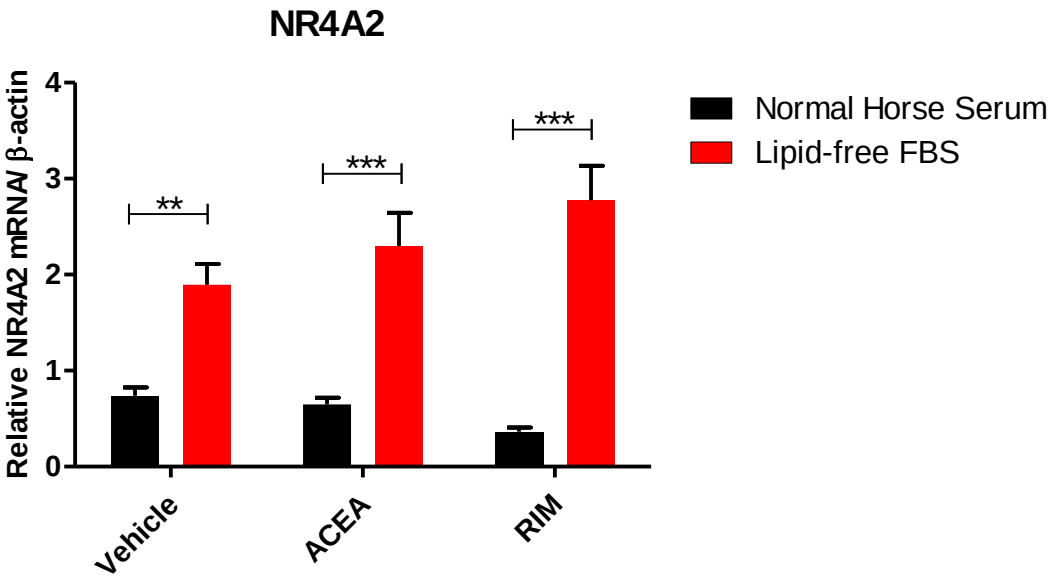


Figure 5- 5: NR4A2 mRNA gene expression in rat myotubes in horse serum and lipid-free FBS media.

Comparison of NR4A2 mRNA expression in vehicle, ACEA and Rimonabant treatment between normal horse serum and lipid-free FBS media. Data expressed as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001, Two-way ANOVA with Bonferronni's post-hoc, n=4).

vi) NR4A3

In addition, NR4A3 expression was again higher in lipid-free FBS and also unnafected by ACEA or RIM (Figure 5-6).

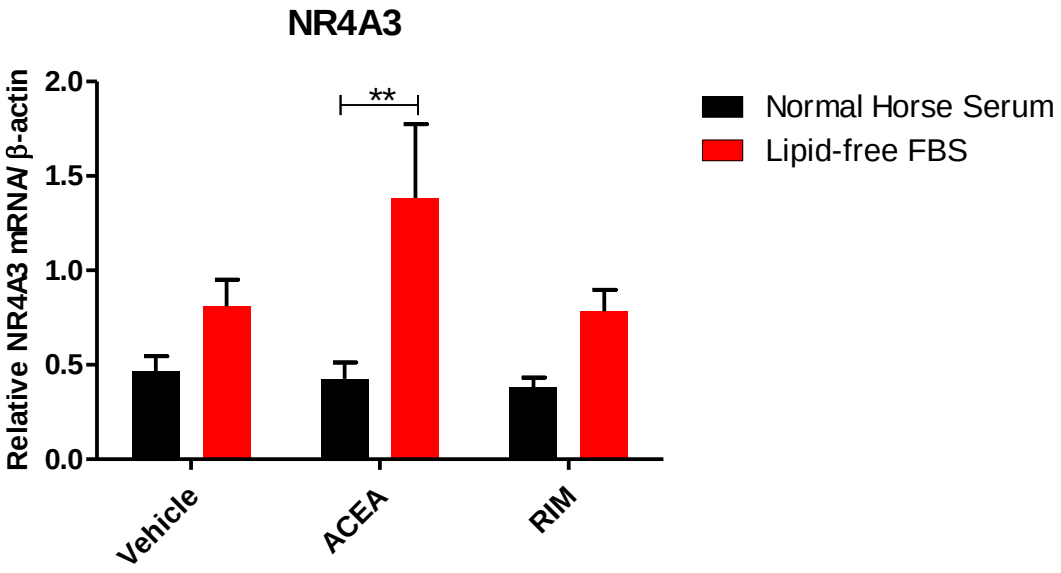


Figure 5- 6: NR4A3 mRNA gene expression in rat myotubes in horse serum and lipid-free FBS media.

Comparison of NR4A3 mRNA expression in vehicle, ACEA and Rimonabant treatment between normal horse serum and lipid-free FBS media. Data expressed as mean ± SEM (*p<0.05, Two-way ANOVA with Bonferronni's post-hoc, n=4).

5.4.2 Effect of CB1 modulation in rat myotubes upon genes expression in lipid-free horse serum media

After observing the previous results in 4.4.5, we thought that the significant different in gene expressions between normal horse serum and lipid-free FBS media conditions might be due to the higher protein content in lipid-free FBS; since FBS known to have more growth factors (Gstraunthaler, 2003) and this property may influence the expressions of those genes. Next, we performed the experiment with in a single lipid-free horse serum condition. There were no significant differences in LCN2, NPY, TRIB3, NR4A1, NR4A2 and NR4A3 mRNA expressions between treatment groups in lipid-free horse serum (Figure 5-7).

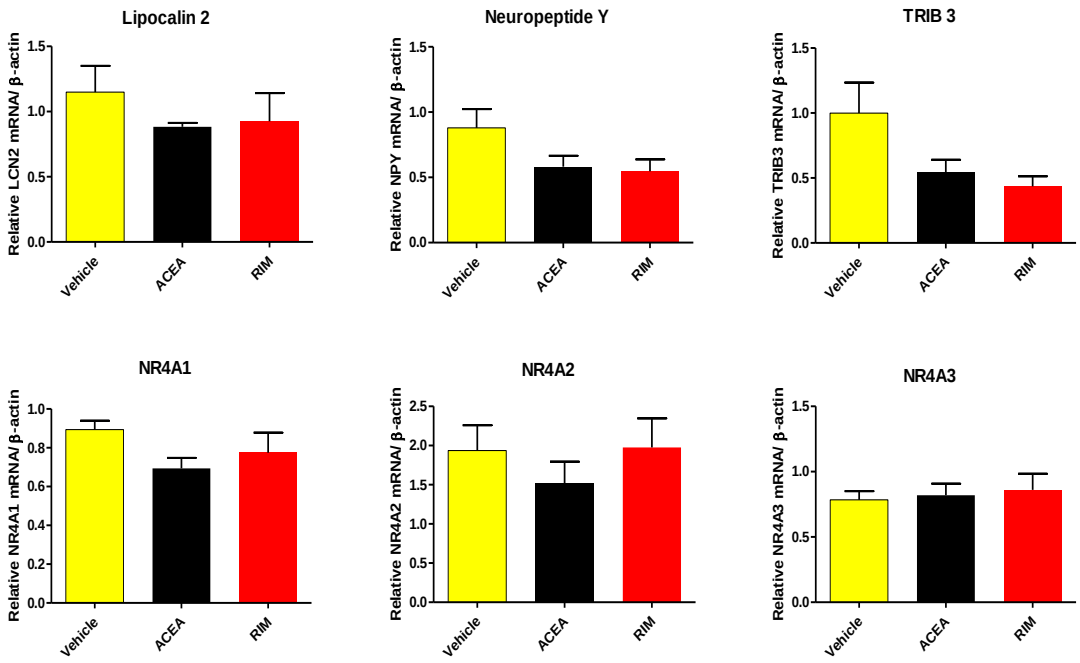


Figure 5- 7: Lipocalin 2, neuropeptide Y, TRIB3, NR4A1, NR4A2 and NR4A3 mRNA gene expression in primary skeletal muscle myotubes.

Data expressed as mean ± SEM (One-way ANOVA with Bonferronis's post-hoc, n=4).

5.4.3 Genes expression in rat myotubes in normal horse serum and lipid-free horse serum media

After we have observe no significant different in ACEA and Rimonabant upon gene expression, next we would like to investigate the gene expression in normal horse serum media as compared to lipid-free horse serum media; in which the lipid components such as naturally occurring endocannabinoids, triglycerides, cholesterol and phospholipids were removed.

There was a significant increase in LCN2, NPY, NR4A1, NR4A2 and NR4A3 mRNA expression in lipid-free horse serum media as compared to normal horse serum (Figure 5-8).

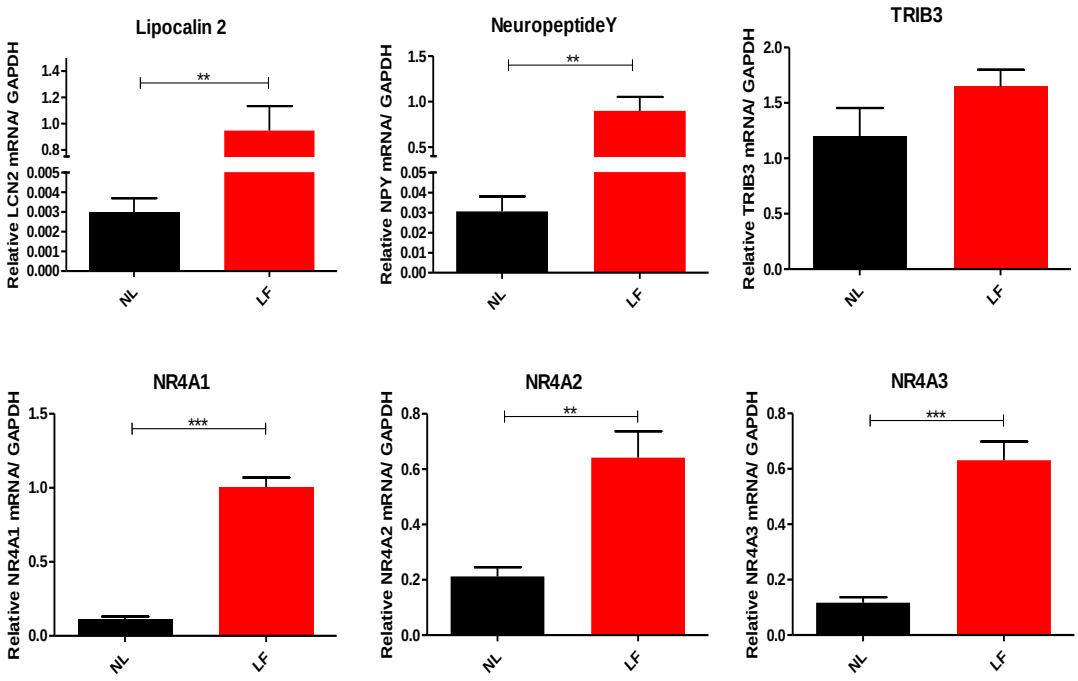


Figure 5- 8: Lipocalin 2, neuropeptide Y, TRIB3, NR4A1, NR4A2 and NR4A3 mRNA gene expression in primary skeletal muscle myotubes in normal horse serum and lipid-free horse serum.

Data expressed as mean ± SEM (**p<0.01, ***p<0.001, t-test, NL- normal horse serum, LF- lipid free horse serum, n=4).

5.4.4 Effect of CB1 modulation in rat myotubes upon genes expression in normal horse serum, lipid-free horse serum and charcoal stripped FBS media

Based on previous data, the effects of Rimonabant were observed in myotubes following incubation in charcoal stripped FBS. In charcoal stripped serum, the concentration of steroid hormones such as estradiol, progesterone, cortisol, testosterone, T3 and T4 is reduced; as compared to delipidisation method, it reduces mainly free fatty acids and triglycerides. In the next experiment, we would like to determine whether gene expression is regulated by endocannabinoids or other lipids products in the media. There were significant increase of LCN2, NPY, NR4A1, NR4A2 and NR4A3 mRNA expressions in lipid-free horse serum condition as compared to normal horse serum and charcoal strip FBS and the expressions were not affected by RIM (Figure 5-9).

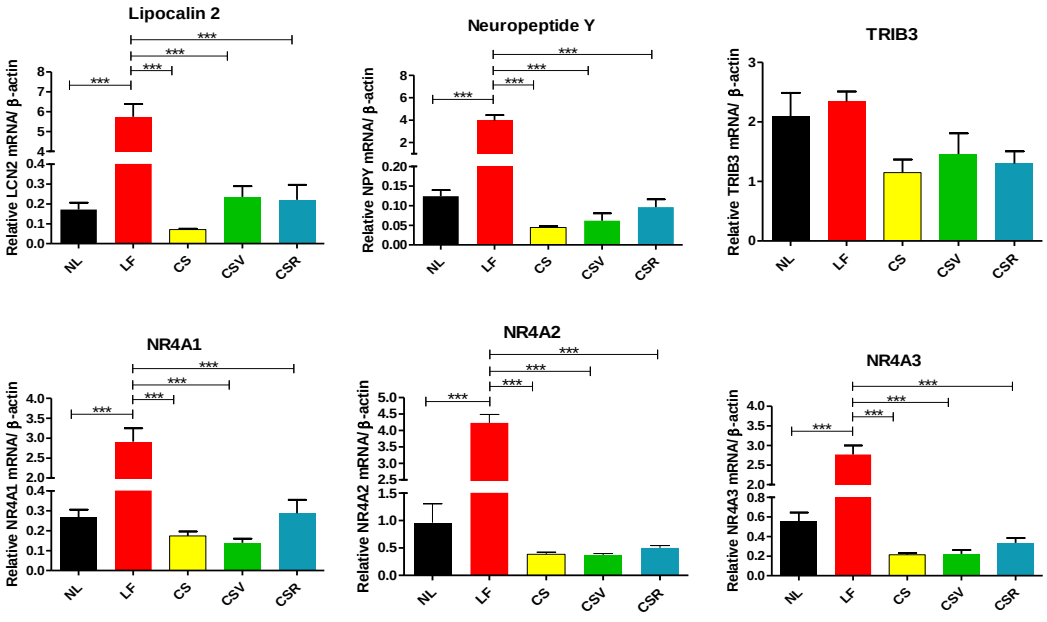


Figure 5- 9: Lipocalin 2, neuropeptide Y, TRIB3, NR4A1, NR4A2 and NR4A3 mRNA gene expression in primary skeletal muscle myotubes.

Data expressed as mean ± SEM (***p<0.001, One-way ANOVA with Bonferronis's post-hoc, NL- normal horse serum, LF- lipid free horse serum, CS- charcoal stripped fetal bovine serum, CSV- CS + vehicle, CSR- CS + 100nM rimonabant, n=6).

5.4.5 Effect of fatty acid content of media upon gene expression in rat myotubes

There was a significant reduction in NPY, TRIB3, NR4A1, NR4A2 and NR4A3 mRNA expressions with reducing pattern of LCN2 after supplementation of lipid free medium with free fatty acids in lipid-free horse serum media as compared to lipid-free horse serum media only condition. These significant reductions showed no significant different as compared to normal horse serum media. All of the genes expressions were not affected by either 100nM or 200nM RIM (Figure 4-17).

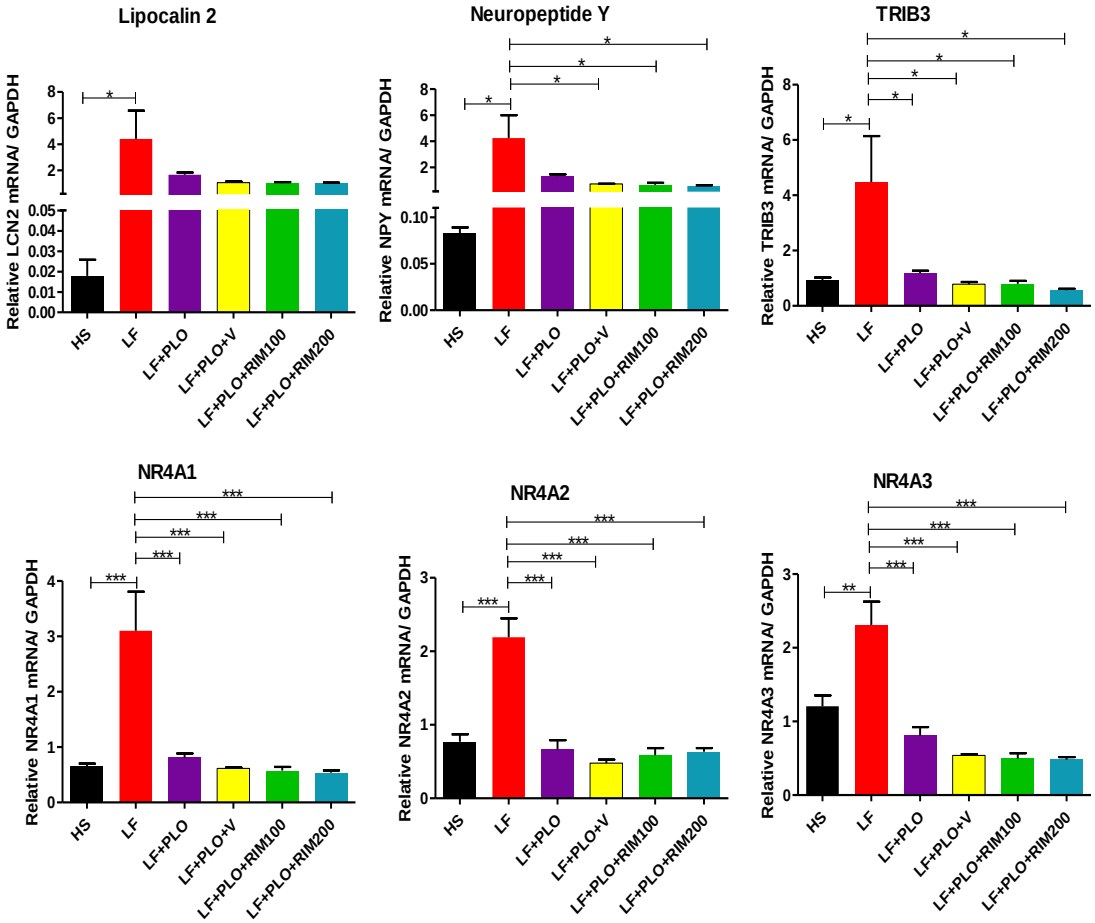


Figure 5- 10: Lipocalin 2, neuropeptide Y, TRIB3, NR4A1, NR4A2 and NR4A3 mRNA gene expression in primary skeletal muscle myotubes: effect of free fatty acids.

Data expressed as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001, One-way ANOVA with Bonferronis's post-hoc, HS- normal horse serum, LF- lipid free horse serum, lipid free horse serum supplemented with 10mM PLO- palmitic, linoleic and oleic acids (ratio of 4:3:3 respectively), V- vehicle, RIM100- rimonabant 100nM, RIM200- rimonabant 200nM, n=4).

5.4.6 Serum fatty acids and triglycerides

For validation of the delipidisation process, the free fatty acid and triglyceride contents were measured in FBS, lipid-free FBS, normal horse serum and lipid-free horse serum. Results showed both components were much lower after delipidisation of both sera (Table 5-1).

Table 5- 1: Free fatty acids and triglycerides levels in sera used in the experiments

FFA (mmol/L)	FBS	Lipid-free FBS	% Reduction
	0.084	0.016	81%
	Horse Serum	Lipid-free Horse Serum	% Reduction
	0.502	0.100	80%

Triglycerides (mmol/L)	FBS	Lipid-free FBS	% Reduction
	0.745	0.045	94%
	Horse Serum	Lipid-free Horse Serum	% Reduction
	0.250	0.065	74%

5.4.7 Serum protein

Protein level in the sera were also measured after delipidisation process in order to exclude any influence of protein content on the mRNA gene expression. Results showed that the protein levels were similar in horse serum, lipid-free horse serum and after reintroduction of PLO in lipid-free horse serum (Table 5-2):

Table 5- 2: Serum protein level in horse serum and lipid-free horse serum

Serum	Protein (µg/ml)
Horse serum	1098.920
Lipid free HS	1370.077

5.4.8 Genes expression in rat myotubes after EPS in normal horse serum

There were significant increase in LCN2, NPY, NR4A1, NR4A2 and NR4A3 mRNA expression in EPS stimulated cells (48-hour stimulation 1Hz, 2ms pulses, 11.5V) as compared to control (Figure 5-11).

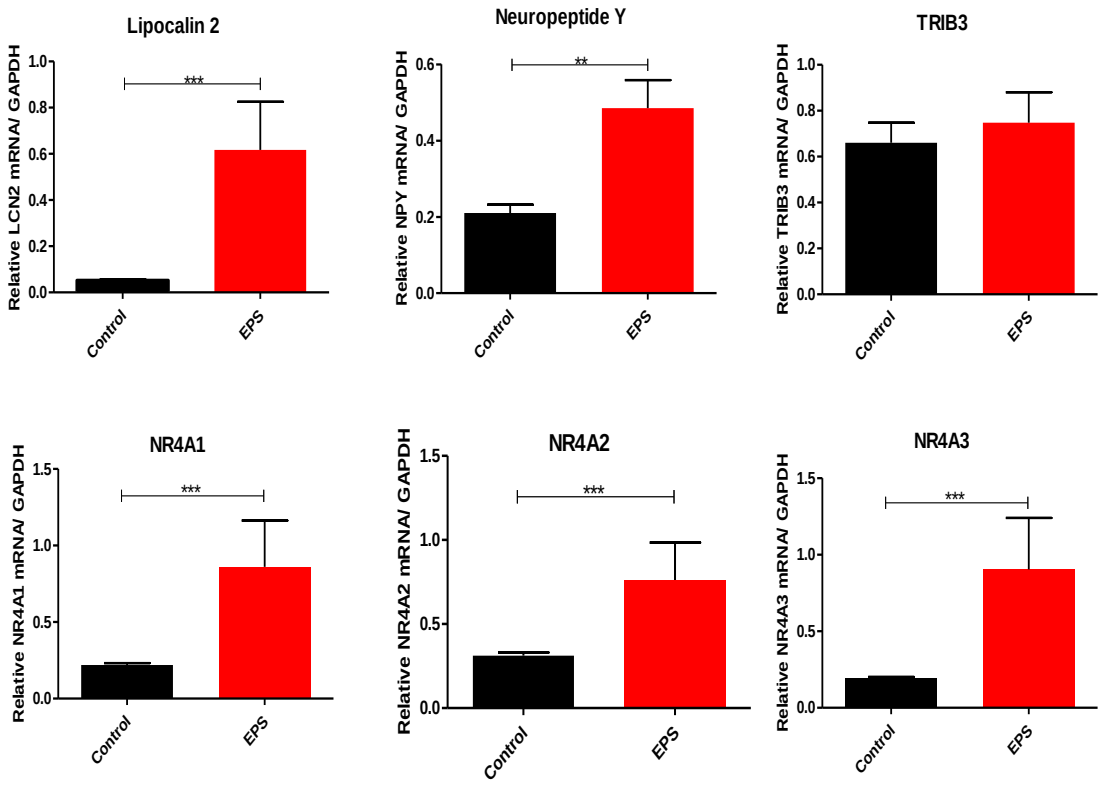


Figure 5- 11: Lipocalin 2, neuropeptide Y, TRIB3, NR4A1, NR4A2 and NR4A3 mRNA gene expression in primary skeletal muscle myotubes after 48-hour electrical pulse stimulation.

Data expressed as mean ± SEM (**p<0.01, ***p<0.001, t-test, n=9).

5.4.9 Genes expression in rat myotubes in horse serum, lipid-free horse serum and lipid-free horse serum supplemented with PLO after EPS

There were significant reductions in LCN2, NPY, NR4A1, NR4A2 and NR4A3 mRNA expression after reintroduction of PLO in lipid-free horse serum media. There was a significant upregulation of LCN2, NPY, NR4A1, NR4A2 and NR4A3 mRNA after electrical pulse stimulation for 48 hours regardless of the contents of the medium (Figure 5-12).

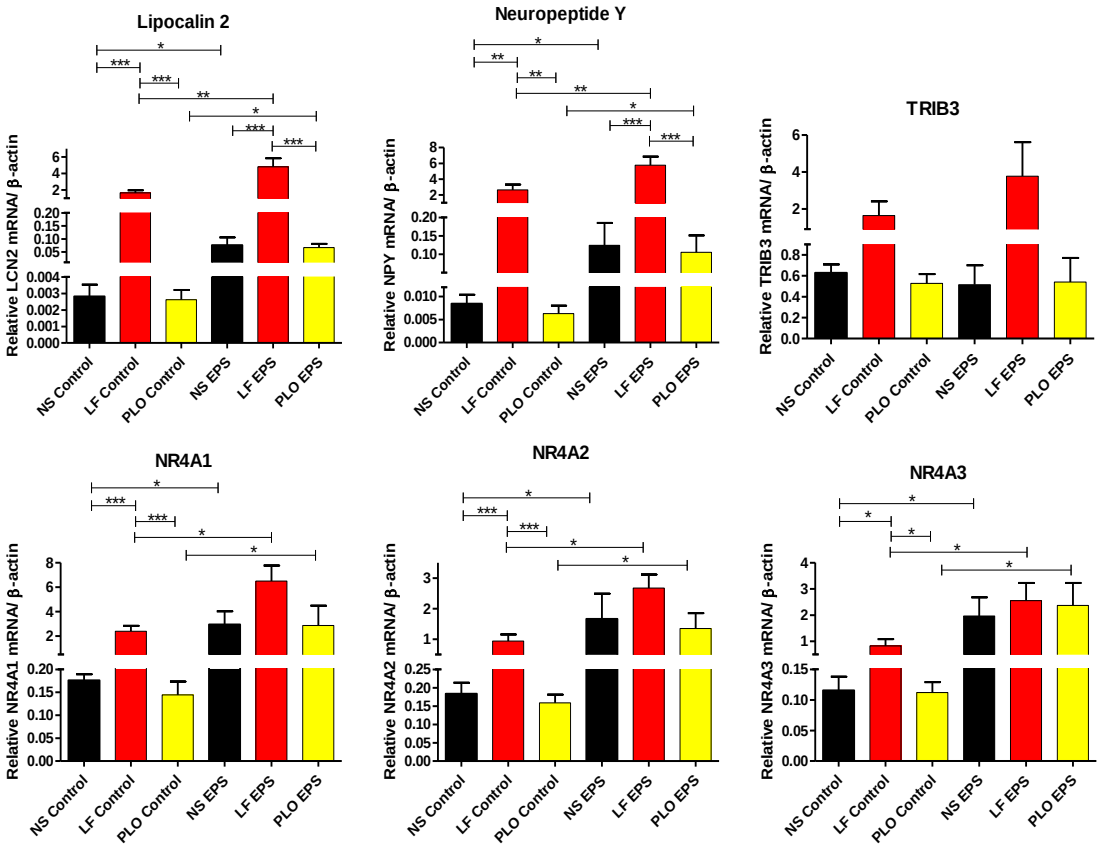


Figure 5- 12: Gene expression in primary skeletal muscle myotubes in various serum conditions in control and post-electrical pulse stimulation.

Data expressed as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001, One-way ANOVA with Bonferronis's post-hoc, HS- normal horse serum, LF- lipid free horse serum, PLO- palmitic, linoleic and oleic acids, EPS- electrical pulse stimulation, n=4).

5.5 Discussion

The main objective of this chapter is to observe the effects of ACEA and Rimonabant upon selected gene expression that relates to obesity and insulin resistance in primary skeletal muscle myotubes, specifically LCN2, NPY, TRIB3, NR4A1, NR4A2 and NR4A3.

LCN2 also known as neutrophil gelatinase-associated lipocalin, a 25-kDa protein secreted from human neutrophils (Kjeldsen et al., 1994; Kjeldsen et al., 1993) which initially identified as an adipokine that belongs to the superfamily of lipocalins that appear to affect glucose metabolism and insulin sensitivity (Kjeldsen et al., 1994). It is a small protein with a hydrophobic ligands binding pocket (Flower, 1996) which is able to bind and transport a wide variety of small hydrophobic molecules such as retinol, fatty acids, steroids and thyroid hormone (Flower, 1996; LaLonde et al., 1994).

Serum concentrations of LCN2 were found to significantly higher in obese persons when compared to lean individuals; and have a positive association with waist circumference, waist-to-hip ratio, body fat percentage, systolic blood pressure, fasting triglycerides, fasting glucose and insulin concentrations, and also markers for chronic inflammation specifically C-reactive protein (Y. Wang et al., 2007). Circulating LCN2 levels were found to be increased in obese animals (Yan et al., 2007) and LCN2-deficient mice showed a significant decrease in fasting glucose and insulin levels, and improved insulin sensitivity in ageing, dietary-induced obesity or genetic-induced obesity compared with their wild-type littermates (Law et al., 2010).

Until now, there is minimal information regarding the synthesis of LCN2 by skeletal muscle and its role in skeletal muscle is not widely understood. In fact, there is a study demonstrated that LCN2 is not expressed in skeletal muscle (Mosialou et al., 2017). However in current study, we have observed the expression of LCN2 mRNA in lipid-free condition and post-chronic exercise in skeletal muscle myotubes. This suggest that skeletal muscle might produce LCN2 in a different condition compared to adipocytes and it may act as a signal of 'energy deprivation'. Further study of LCN2 will be discussed in the next chapter.

NPY is a 36-amino acid peptide that acts as a neurotransmitter in the brain. It is widely distributed within neurons of the central and peripheral nervous system in mammals (Gray & Morley, 1986). As studied by Zarjevski et al, there were significant increase in food intake, increased in body weight, increased in fat storage and increased basal insulinaemia in rats after intracerebroventricular administration of NPY (Zarjevski, Cusin, Vettor, Rohner-Jeanrenaud, & Jeanrenaud, 1994). Stanley and Leibowitz demonstrated that direct injection of NPY into the paraventricular nucleus of the hypothalamus increased food and water intake, together with decrease in grooming during the absent of food (Stanley & Leibowitz, 1984). Apart from that, Levine and Morley found that NPY not only increased food intake during the daytime in satiated animals, but also significantly increased food intake in starved animals and at night when the animals were normally fed (Levine & Morley, 1984). NPY also increased water intake in the absence of food and this effect of NPY is highly selective for carbohydrate-rich foods (Jhanwar-Uniyal, Beck, Jhanwar, Burlet, & Leibowitz, 1993; Stanley, Anderson, Grayson, & Leibowitz, 1989; Stanley,

Daniel, Chin, & Leibowitz, 1985). These results suggest the remarkable potency of NPY as an orexigenic agent. In our study, the upregulation of NPY in both lipid-free condition and post-chronic contractions might be as a compensatory mechanism in respond to 'energy deprivation' state.

TRIB3 is a protein family influences proliferation, motility, metabolism, and oncogenic transformation. In study conducted by Liew et al, TRIB3 was found to be associated with T2DM in human subjects. TRIB3 also was found to be elevated in β-cells in islets of T2DM patient; and in high-fat fed and insulin receptor-deficient mice (Liew et al., 2010). In other study by Liu and his colleagues found that skeletal muscle TRIB3 protein levels are significantly elevated in T2DM human subjects. Furthermore, muscle TRIB3 protein content is inversely correlated with glucose disposal rates and positively correlated with fasting glucose. In animal study, skeletal muscle TRIB3 protein levels were found to be increase in STZ-diabetic rats, db/db mice, and Zucker fatty rats. In L6 muscle cells, TRIB3 hyperexpression inhibits insulin-stimulated glucose transport and glucose transporter 4 (GLUT4) translocation and impairs phosphorylation of Akt, ERK, and insulin receptor substrate-1 in insulin signal transduction. Interestingly, TRIB3 mRNA and protein levels were significantly low in the physiological glucose range (5 mM), whereas TRIB3 expression increased dramatically in high glucose concentrations (10-15 mM), as well as by glucose deprivation (0-2.5 mM) in L6 muscle cells. These data identify TRIB3 induction as a novel molecular mechanism in human insulin resistance and diabetes. TRIB3 acts as a nutrient sensor and could mediate the component of insulin resistance attributable to hyperglycemia (i.e., glucose toxicity) in diabetes (J. Liu et al., 2010). In current work, the

expression of TRIB3 was not consistent throughout the study. It was downregulated in lipid-free FBS, but showed an increasing pattern in lipid-free horse serum condition and post-chronic contraction. This suggest that the expression of TRIB3 might indicate the imbalance of nutrient in skeletal muscle myotubes by its increasing or decreasing trend.

NR4A1, NR4A2 and NR4A3 are protein from the nuclear receptor family which regulate many aspects of development, reproduction, metabolism and homeostasis. NR4A receptors are involved in hepatic glucose metabolism, specifically they are induced in the liver by different physiological stimuli, including glucagon stimulation and fasting (Oita, Mazzatti, Lim, Powell, & Merry, 2009; Pei et al., 2006). Adenovirus-mediated overexpression of NR4A1 in the mouse liver activates multiple genes involved in gluconeogenesis and stimulates hepatic glucose production (Pei et al., 2006).

NR4A receptors also participate in lipid metabolism. NR4A1 promotes lipolysis in muscle (Maxwell et al., 2005), decreases hepatic triglyceride content and modulates plasma lipoprotein profiles by increasing plasma low density lipoprotein cholesterol and by decreasing high density lipoprotein cholesterol (Pols et al., 2008). In addition, NR4A receptors may participate in adipogenic differentiation (Chao, Bensinger, Villanueva, Wroblewski, & Tontonoz, 2008; Fumoto, Yamaguchi, Hirose, & Osumi, 2007) and central regulation of energy homeostasis (Nonogaki et al., 2009). In a study conducted by Veum et al, adipose tissue samples were obtained from extremely obese individuals before and 1 year post-bariatric surgery; and from healthy lean controls that were going for hernia repair and laparotomy. Results demonstrated that NR4A1, NR4A2 and NR4A3 were highly

expressed in the adipose tissue of the extremely obese individuals before going for the bariatric surgery. However after 1 year of fat loss post-surgery, the expression of those genes was normalized back to the level observed in the normal healthy individuals (Veum et al., 2012).

In skeletal muscle, the NR4A receptors are induced by β-adrenergic signaling (Pearen et al., 2008) and endurance exercise (Mahoney, Parise, Melov, Safdar, & Tarnopolsky, 2005). NR4A1 KO mice showed increased susceptibility to diet-induced obesity and insulin resistance in skeletal muscle and liver (Chao et al., 2009).

In our study, all NR4A subfamily members were upregulated following chronic contraction by EPS (increase glucose uptake by the cells). This might be explained by a study which demonstrated that NR4A1 is an important regulator of gene expression linked to glucose metabolism in skeletal muscle. In their in vivo study, denervation of rats' hind limb leads to the reduced expression of NR4A1 that resulted in interruption in glucose and glycogen metabolism. They have showed that reduction in NR4A1 level interfere with glucose uptake, glycolysis, glycerophosphate shuttle and glycogenolysis by downregulating the genes involved such as GLUT4, muscle phosphofructokinase (Pfkm); phosphoglycerate mutase 2 (Pgam2); 2,3-biphosphoglycerate mutase (Bpgm); glycerol-3-phosphate dehydrogenase 1 (Gpd1); phosphorylase kinase γ (Phkg1) and muscle glycogen phosphorylase (Pygm). Furthermore, ectopic expression of NR4A1 in both rat skeletal muscle and C2C12 myotubes induce expressions of most of the above genes. In addition, selective knockdown of NR4A1 in rats skeletal muscle by small hairpin RNA or genetic deletion in mice reduce the expression of the above genes. These results suggest that NR4A1 is

required for physiological expression of glucose metabolic genes and as an important regulator of glucose metabolism in skeletal muscle (Chao et al., 2007). This might be the main underlying mechanism for the increased expression of NR4A1 in our study, as the response to increase in glucose uptake in order to facilitate further glucose metabolism following chronic contractions.

CHAPTER SIX

LIPOCALIN 2 AS A NOVEL MYOKINE

Chapter 6: Lipocalin 2 as a Novel Myokine

6.1 Introduction

LCN2 was originally identified as a 25-kDa protein secreted from human neutrophils (Kjeldsen et al., 1994; Kjeldsen et al., 1993). It is recently identified adipokine that belongs to the superfamily of lipocalins which seems to affect glucose metabolism and insulin sensitivity (Kjeldsen et al., 1994).

Lipocalins are small generally secreted proteins with a hydrophobic ligands binding pocket (Flower, 1996). As a member of lipocalin family, LCN2 possess common crystal structures of an eight-stranded continuously hydrogen-bonded antiparallel β-barrel and participate in various biological processes. This structure indicates that lipocalins is able to bind and transport a wide variety of small hydrophobic molecules such as retinol, fatty acids, steroids and thyroid hormone (Flower, 1996; LaLonde et al., 1994)

LCN2 protein has been implicated in diverse actions, such as apoptosis and innate immunity and is expressed in several tissues including neutrophils, liver, kidney, adipocytes and macrophages (Y. Wang et al., 2007). LCN2 also was found to be highly expressed in uterus, bone marrow, and granulocytes immune cells (Aigner et al., 2007; H. L. Huang et al., 1999). Mice at 3 weeks of age also expressed LCN2 in liver, spleen, testis and lungs; however, the expression declined gradually with age, particularly in the liver, kidney and spleen with complete disappearance by the age of 11 weeks in adult mice (Garay-Rojas et al., 1996). In most of the early studies, LCN2 expression was examined in non-adipose tissues. In 2005, LCN2 was first reported to be secreted from adipocytes, since then the role

of LCN2 as a new adipokine in metabolism has been widely discussed (X. Chen et al., 2005; Yan et al., 2007).

LCN2 expression is regulated by environmental stress such as hypoxia and infection, as well as metabolic conditions like hyperlipidemia, obesity and insulin resistance (Li & Chan, 2011).

The promoter region of LCN2 contains the binding sites of transcription factors nuclear factor-κB (NFκB), CCAAT/enhancer-binding protein (C/EBP) (Shen et al., 2006) and glucocorticoid response element (Garay-Rojas et al., 1996), indicating that the expression of LCN2 may be controlled by inflammation and metabolic conditions.

Expression of LCN2 was markedly induced by a variety of pro-inflammatory stimuli, including lipopolysaccharide, interleukin-1β, interleukin-17, TNFα and hyperglycaemia (Bu et al., 2006; Pawluczyk et al., 2003; Yan et al., 2007). Dexamethasone and retinoic acid were also reported to increase LCN2 expression in mouse L cells (sarcoma cell line isolated from mouse fibroblasts) through an autocrine mechanism (Garay-Rojas et al., 1996).

Recent studies demonstrated that LCN2 gene expression in adipose tissue was upregulated in genetic and dietary-induced obese rodents (Y. Wang et al., 2007; J. Zhang et al., 2008), as well as in obese humans (Y. H. Lee et al., 2010; Panidis et al., 2010; Y. Wang et al., 2007); thiazolidinedione administration reduces LCN2 gene expression in adipose tissue of obese animals (J. Zhang et al., 2008). Furthermore, circulating LCN2 levels increased in obese animals, and LCN2 mRNA was increased in the liver of ob/ob mice (Yan et al., 2007). LCN2-deficient mice showed significant decreased in fasting glucose, decreased insulin levels and

improved insulin sensitivity under condition of aging, dietary-induced obesity or genetic-induced obesity compared with their wild-type littermates (Law et al., 2010).

Much of the current research has focused upon the synthesis and release of LCN2 by adipose tissue and its regulation by inflammation, metabolic stress, obesity and insulin resistance (Yan et al., 2007; J. Zhang et al., 2008; Y. Zhang et al., 2014). However, there is minimal information regarding the role of LCN2 in skeletal muscle, which is one of the important systems that primarily responsible for determining the metabolic rate of mammals.

In previous chapter, we have shown that there is an upregulation of LCN2 mRNA expression in low lipid environment and during chronic low-frequency EPS. Due to the fact that this early data suggest that the regulation of LCN2 in skeletal muscle might differ from adipocytes, the synthesis and release of LCN2 in skeletal muscle is interesting to be explored.

In this chapter, our aim is to investigate the production of LCN2 protein in skeletal muscle following various nutritional conditions and exercise. In addition, we also would like to observe the release of LCN2 as a novel myokine in the culture media and human serum.

6.2 Objectives

6.2.1 General Objective

To investigate the release of LCN2 protein as a novel myokine by skeletal muscle.

6.2.2 Specific Objectives

- To investigate the release of LCN2 in primary skeletal muscle myotubes and culture media following various nutritional conditions.
- To investigate the release of LCN2 following EPS in the primary skeletal muscle myotubes and culture media.
- To investigate the synthesis of LCN2 protein following eccentric exercise in human skeletal muscle and its secretion in the serum.

6.3 Experiment designs and methods

6.3.1 LCN2 mRNA and protein expressions in rat myotubes following various nutritional conditions

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 4 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. For this experiment, several nutritional conditions were created namely postprandial, fasting and diabetic/metabolic syndrome; and the regulation of LCN2 mRNA and protein were observed. Rats myotubes were be divided into 4 groups (n=6):

i) Postprandial

- Increased in glucose, increased in insulin & low free fatty acids (FFA)

Media composition:

- Increased glucose – Ham F10 (already contain 6mM glucose) + 4mM glucose= total 10mM
- Insulin 10nM
- Low-lipid horse serum 6%: FFA= 0.1mmol/L

ii) Fasting

- Low/normal glucose, low insulin & increased FFA

Media composition:

- Low/normal glucose: Ham F10= 6mM
- No insulin given
- Normal horse serum 6%: FFA= 0.5mmol/L

iii) Diabetic/metabolic syndrome

- Increased glucose, increased insulin increased FFA

Media composition:

- Increased glucose: Ham F10 (already contain 6mM glucose) + 4mM glucose= total 10mM
- Insulin 10nM
- Normal horse serum media 6%: FFA= 0.5mmol/l

iv) Lipid-free (control)

- Low/normal glucose, no insulin, low FFA

Media composition:

- Low/normal glucose: Ham F10= 6mM
- No insulin given
- Low-lipid horse serum 6%: FFA= 0.1mmol/L

The media were supplemented 48 hours before cell collection. The cells were collected in TRI Reagent (Sigma-Aldrich, USA) for LCN2 mRNA and protein expression, and the media was collected for Lipocalin 2 protein expression.

The RNA was extracted from the cells and the mRNA expression of LCN2 was measured by Taqman qRTPCR as described in Chapter 2 section 2.7. The sequences of the primers and probes are detailed in Table 2-4.
The proteins from the cells were extracted and analysed by western blotting as described in Chapter 2 section 2.14. The primary and secondary antibodies used together with their dilutions and conditions were as described in Table 2-5.

The proteins from the media were extracted by TCA precipitation methods and analysed by western blotting as described in Chapter 2 section 2.14. The primary and secondary antibodies used together with their dilutions and conditions were as described in Table 2-5.
Summary of the media preparation is described in Table 6-1.

Table 6- 1: Culture media preparation and composition for LCN2 level following various nutritional condition experiment

Status	Condition	Media composition (suggestion)
Postprandial	Increased glucose	Increased glucose: Ham F10 (already contain 6mM glucose) + 4mM glucose= total 10mM
	Increased insulin	Insulin 10nM
	Low FFA	Low-lipid horse serum 6%: FFA= 0.1mmol/L
Fasting	Low/normal glucose	Low/normal glucose: Ham F10= 6mM
	Low insulin	No insulin given
	Increased FFA	Normal horse serum 6%: FFA= 0.5mmol/L
Diabetic/ Metabolic syndrome	Increased glucose	Increased glucose: Ham F10 (already contain 6mM glucose) + 4mM glucose= total 10mM
	Increased insulin	Insulin 10nM
	Increased FFA	Normal horse serum media 6%: FFA= 0.5mmol/l
Lipid-free (control)	Low/normal glucose	Low/normal glucose: Ham F10= 6mM
	No insulin	No insulin given
	Low FFA	Low-lipid horse serum 6%: FFA= 0.1mmol/L

6.3.2 LCN2 protein expression in rat primary skeletal muscle myotubes pre- and post-EPS

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 4 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. The wells of the myotubes were then divided into 6 groups of different media environments and conditions:

- Normal horse serum media (n=4)
- Lipid-free horse serum media (n=4)
- Lipid-free horse serum media + 0.4% PLO (n=4)
- Normal horse serum media + EPS (n=4)
- Lipid-free horse serum media + EPS (n=4)

- Lipid-free horse serum media + 0.4% PLO + EPS (n=4)

PLO (palmitic acid + oleic acid + linoleic acid) in BSA was prepared as described in Chapter 2 section 2.6. Different types of media were introduced 48 hours prior to cells collection. The myotubes were given EPS for 48 hours as described in Chapter 2 section 2.3. The proteins were extracted and analysed by western blotting as described in Chapter 2 section 2.14. The primary and secondary antibodies used together with their dilutions and conditions were as described in Table 2-5.

6.3.3 LCN2 protein secretion in the culture media of rat primary skeletal muscle myotubes pre- and post-EPS

$1x10^5$ of satellite cells/well (from a single rat) were seeded into 4 units of 6-well plates and grown to form matured myotubes as described in Chapter 2 section 2.2. The wells of the myotubes were then divided into 6 groups of different media environments and conditions:

- Normal horse serum media (n=4)
- Lipid-free horse serum media (n=4)
- Lipid-free horse serum media + 0.4% PLO (n=4)
- Normal horse serum media + EPS (n=4)
- Lipid-free horse serum media + EPS (n=4)
- Lipid-free horse serum media + 0.4% PLO + EPS (n=4)

PLO (palmitic acid + oleic acid + linoleic acid) in BSA was prepared as described in Chapter 2 section 2.6. Different types of media were introduced 48 hours prior to cells collection. The myotubes were given EPS for 48 hours

as described in Chapter 2 section 2.3. The proteins from the media were extracted by TCA precipitation methods and analysed by western blotting as described in Chapter 2 section 2.14. The primary and secondary antibodies used together with their dilutions and conditions were as described in Table 2-5.

6.3.4 LCN2 protein expression in human muscle pre- and post-exercise

For this study, 6 human volunteers were recruited. The exercise protocol and tissue collection were performed as described in Chapter 2 section 2.4. The proteins were extracted and analysed by western blotting as described in Chapter 2 section 2.14. The primary and secondary antibodies used together with their dilutions and conditions were as described in Table 2-5.

6.3.5 LCN2 protein expression in human serum pre- and post-exercise

For this study, the same 6 human volunteers as previous experiment were recruited. The exercise protocol and blood samples collection from the local arterial and venous circulations pre- and post-exercise were performed as described in Chapter 2 section 2.4. The LCN2 protein levels in the sera were quantified using ELISA method as described in Chapter 2 section 2.13.

6.4 Results

6.4.1 LCN2 mRNA expression in rat myotubes following various nutritional conditions

There was a significant increase in LCN2 mRNA expression in postprandial condition when compared to fasting and diabetic/metabolic condition. The upregulation of LCN2 mRNA is almost the same level seen in lipid-free condition. Furthermore, there was no significant different in LCN2 mRNA expression when compared between fasting and diabetic/metabolic conditions (Figure 6-1).

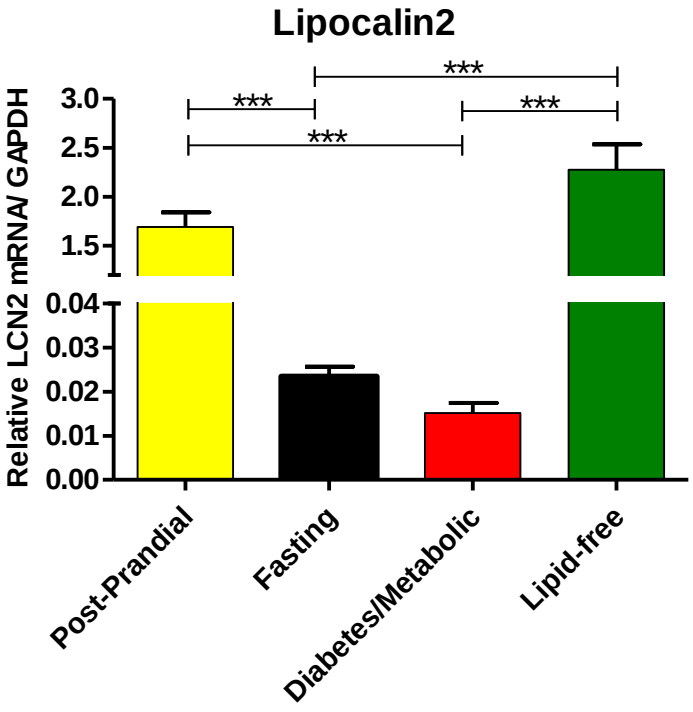
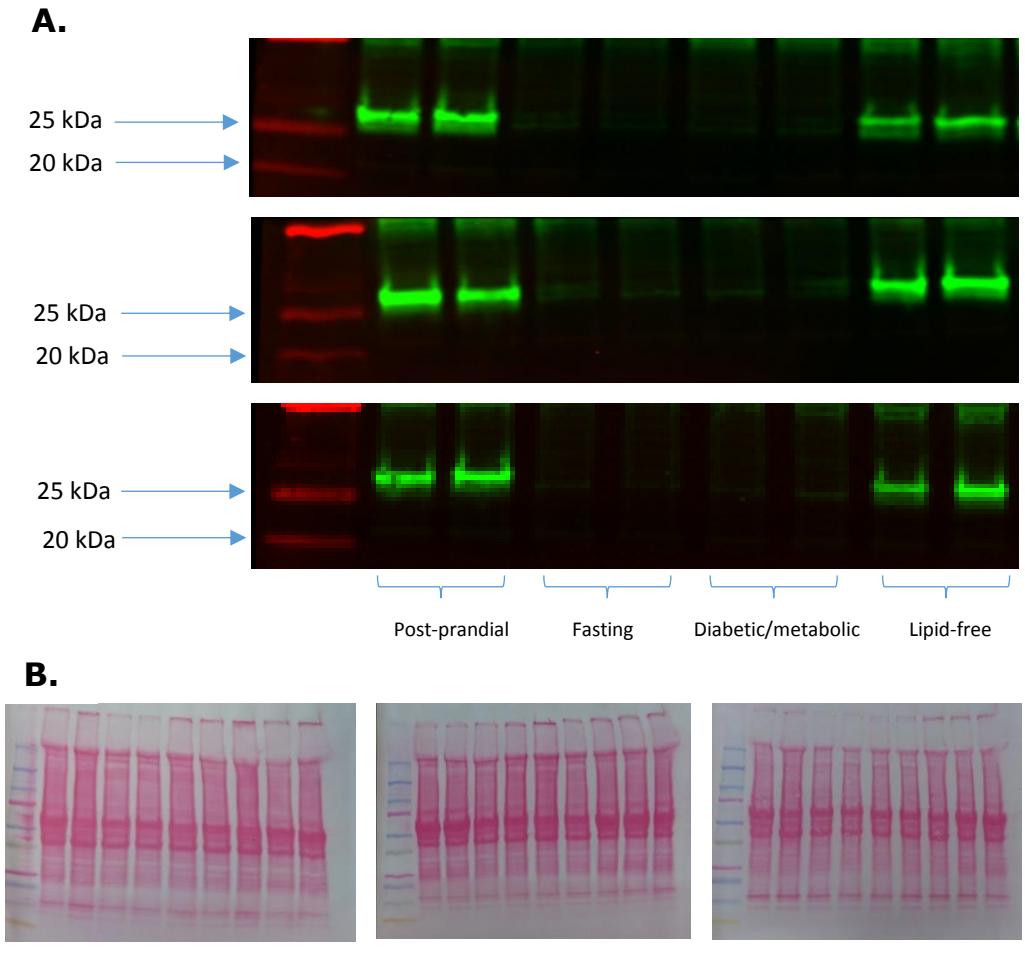


Figure 6- 1: LCN2 mRNA gene expression in rat primary skeletal muscle myotubes in several nutritional conditions after 48 hours.

Data expressed as mean ± SEM (***p<0.001, One way ANOVA with Bonferronis's post-hoc, n=6).

6.4.2 LCN2 protein expression in rat myotubes following various nutritional conditions

After we observed the upregulation of LCN2 mRNA in post-prandial condition, next we would like to investigate the expression of LCN2 protein in rat myotubes following various nutritional conditions. Results showed a significant increase in LCN2 protein expression in postprandial condition when compared to fasting and diabetic/metabolic condition. The upregulation of LCN2 protein in post-prandial was as high as in lipid-free condition with no significant different. Furthermore, there was no significant different in LCN2 protein expression when compared between fasting and diabetic/metabolic conditions (Figure 6-2). The molecular weight of the bands of lipocalin 2 were detected at 25 kDa.



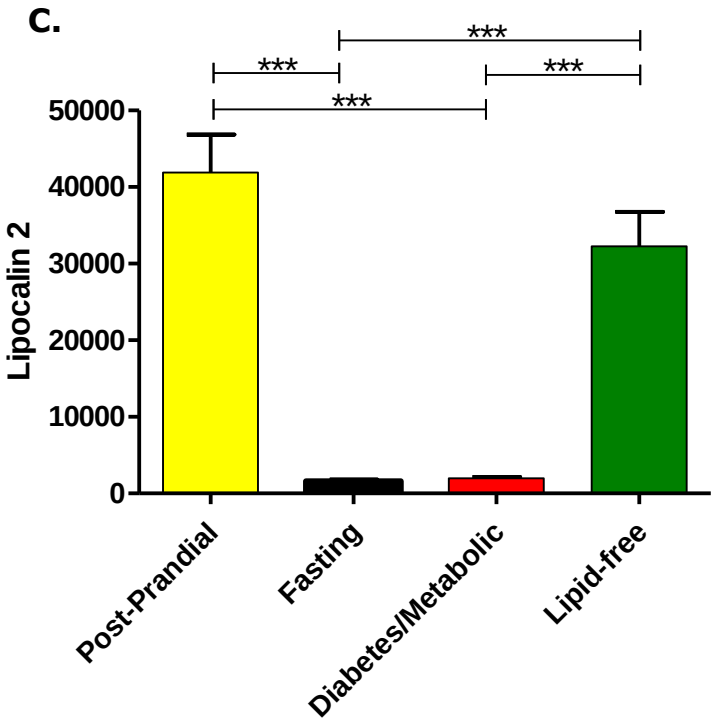
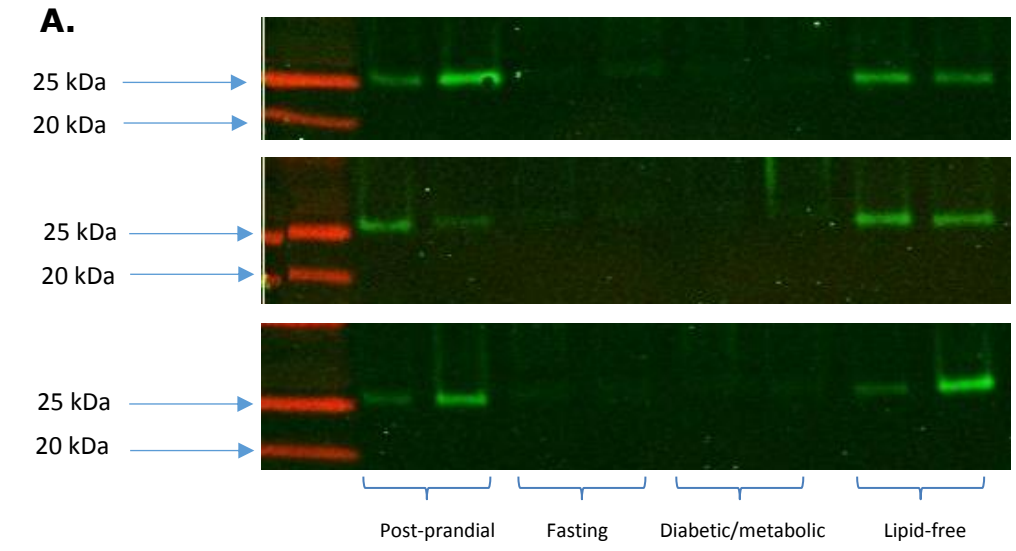


Figure 6- 2: LCN2 protein expression in rat myotubes following several nutritional conditions.

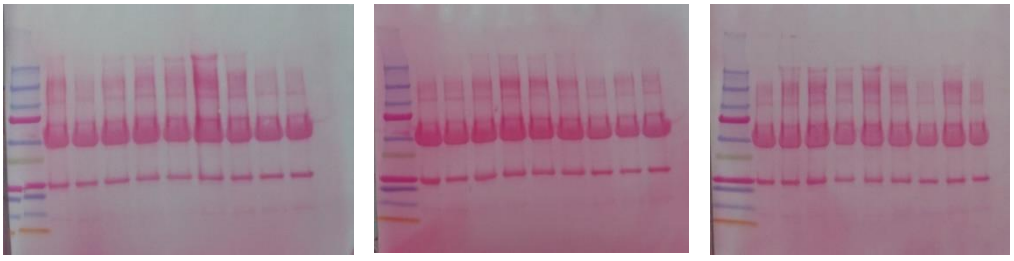
Representative blot showing LCN2 protein expression in rat myotubes following several nutritional conditions namely post-prandial, fasting, diabetic/metabolic and control (low-lipid serum media) after 48 hours (A). The protein blots density is normalised to respective protein band on the membrane after stained by ponceau red (B). Quantified values of lipocalin 2 are shown in lower panels (C). *** denotes P<0.001. Data were analysed by One way ANOVA with Bonferronis's post-hoc (n=6).

6.4.3 LCN2 protein secretion in the media of rat myotubes following various nutritional conditions

Next, we would like to observe LCN2 protein expression in the culture media. This represent the secretion of LCN2 from the rat myotubes into the culture media. Results showed a similar pattern as seen in the myotubes, in which a significant increase in LCN2 protein expression in postprandial condition when compared to fasting and diabetic/metabolic condition. The upregulation of LCN2 protein in post-prandial was as high as in lipid-free condition with no significant different. Furthermore, there was no significant different in LCN2 protein expression when compared between fasting and diabetic/metabolic conditions (Figure 6-3). The molecular weight of the bands of lipocalin 2 were detected at 25 kDa.



B.



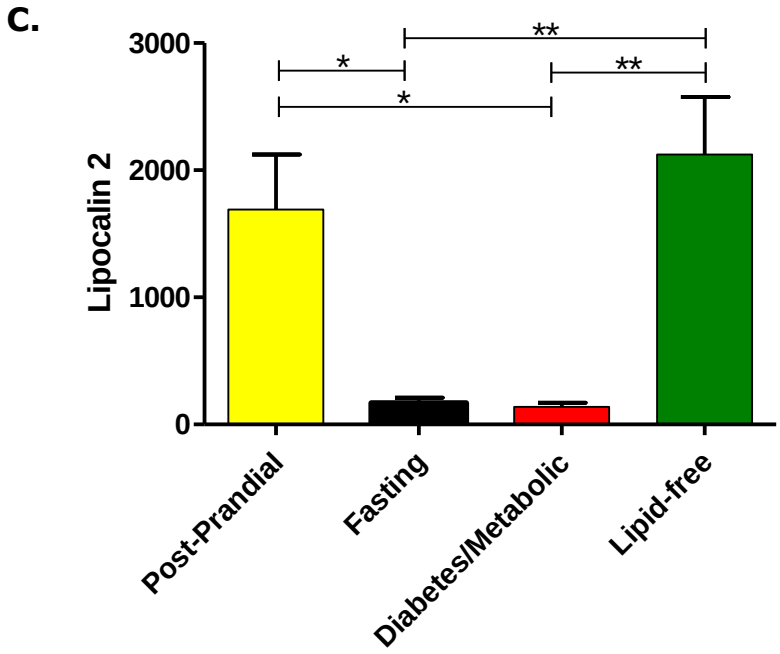
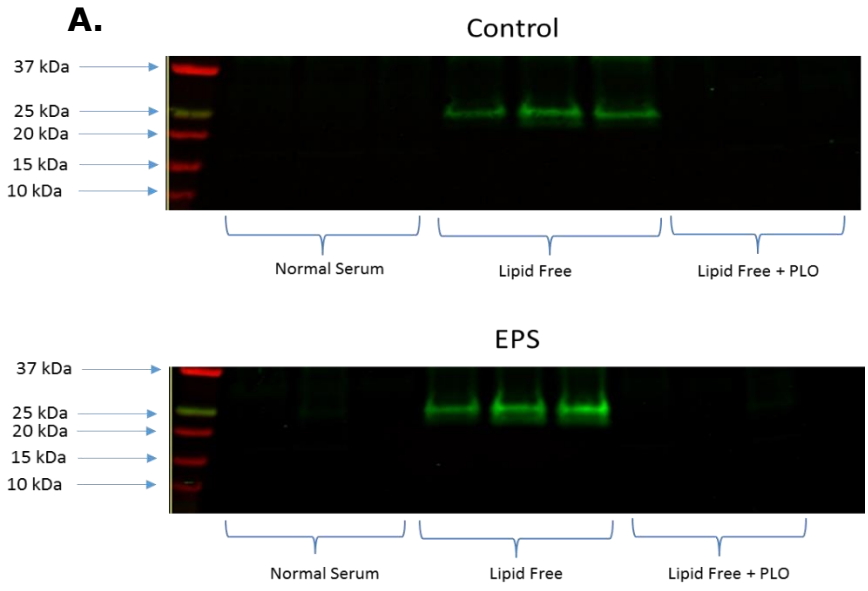


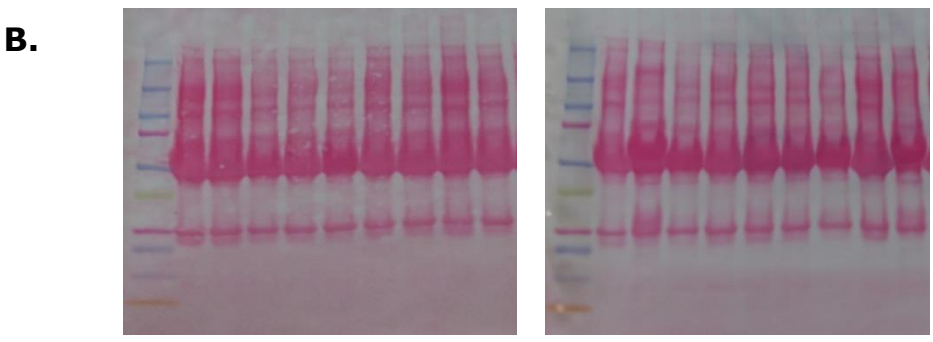
Figure 6- 3: LCN2 protein expression in the culture media following several nutritional conditions.

Representative blot showing LCN2 protein expression in the culture media following several nutritional conditions namely post-prandial, fasting, diabetic/metabolic and control (low-lipid serum media) for 48 hours (A). The protein blots density is normalised to respective protein band on the membrane after stained by ponceau red (B). Quantified values of lipocalin 2 are shown in lower panels (C). * denotes $P<0.05$ and ** denotes $P<0.01$. Data were analysed by One way ANOVA with Bonferronis's post-hoc (n=6).

6.4.4 LCN2 protein expression in rat primary skeletal muscle myotubes pre- and post-EPS

In the previous chapter, we have observed the upregulation of LCN2 mRNA following removal of lipid of the serum in the culture media and after EPS. In this experiment, we would like to observe the LCN2 protein expression in rat myotubes in various media conditions namely normal horse serum, lipid-free and lipid-free + PLO, together with the combination of effects of 48-hour EPS. Results revealed there was a huge increase in lipocalin 2 protein expression in lipid-free media in both unstimulated and EPS groups. In contrast, Lipocalin 2 protein was not expressed both normal horse serum and lipid-free + PLO group in control or even after 48 hours of EPS (Figure 6-4). The molecular weight of the bands of lipocalin 2 were detected at 25 kDa.





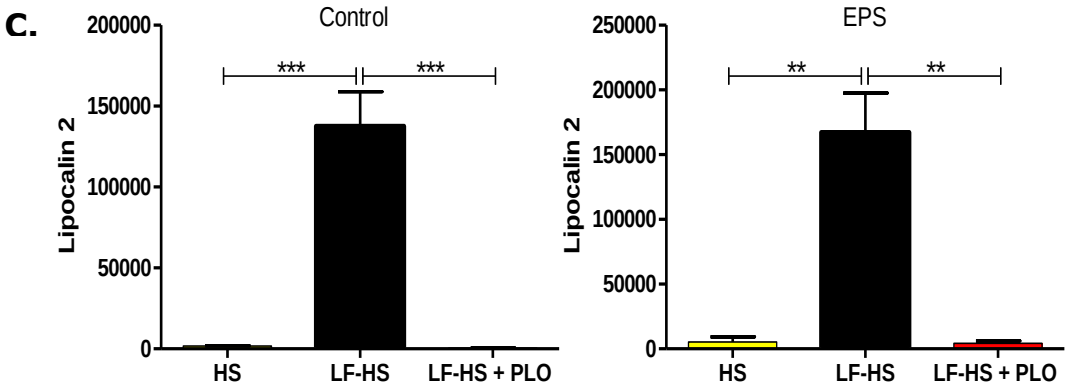
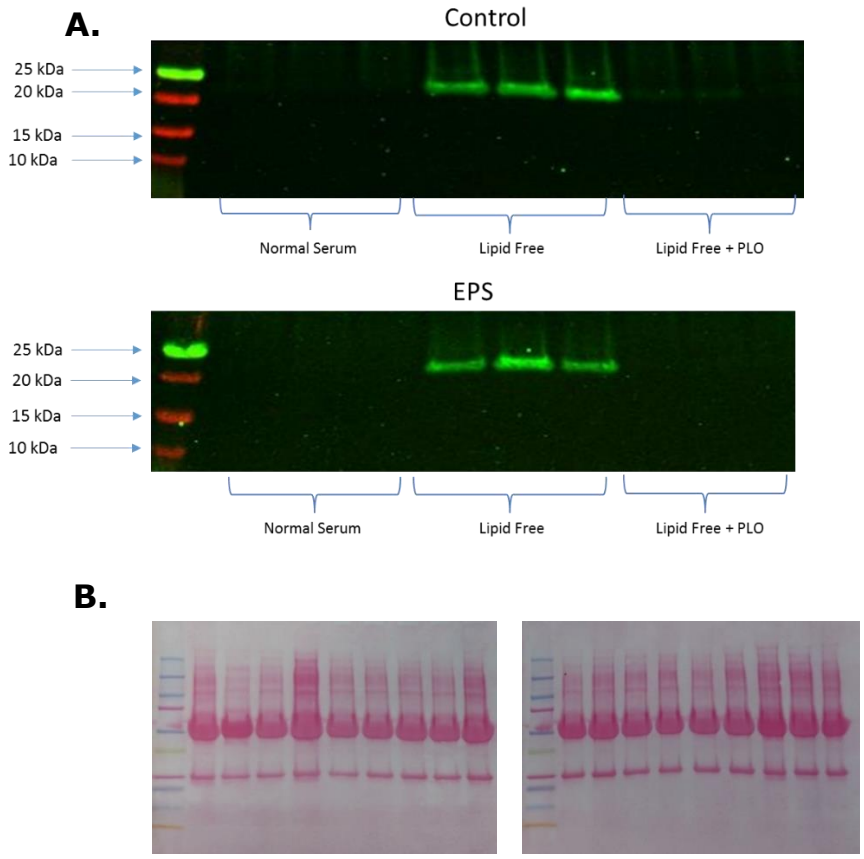


Figure 6- 4: LCN2 protein expression in rat myotubes in various media conditions with the effects of 48-hour EPS.

Representative blot showing LCN2 protein expression in the rat myotubes in various media conditions namely normal horse serum, lipid-free and lipid-free + PLO, together with the effects of 48-hour EPS (A). The protein blots density is normalised to respective protein band on the membrane after stained by ponceau red (B). Quantified values of lipocalin 2 are shown in lower panels (C). ** denotes $P<0.01$ and *** denotes $P<0.001$. Data were analysed by One way ANOVA with Bonferronis's post-hoc (HS- normal horse serum, LF- lipid free horse serum, LF-HS+PLO- lipid free horse serum supplemented with 10mM PLO- palmitic, linoleic and oleic acids (ratio of 4:3:3 respectively), n=3).

6.4.5 LCN2 protein secretion in the culture media of rat primary skeletal muscle myotubes pre- and post-EPS

In order to observe the effects of lipid content in the media; and EPS upon the secretion of LCN2 by the myotubes into the culture media, the proteins were precipitated from the culture media using TCA protein precipitation method and secreted LCN2 was measured by western blotting. Results showed that LCN2 protein was secreted in the culture media in lipid-free condition in both control and after 48-hour EPS. However, LCN2 protein was not secreted in the culture media of both normal horse serum and lipid-free + PLO group in control or even after 48 hours of EPS (Figure 6-5). The molecular weight of the bands of LCN2 were detected at 25 kDa.



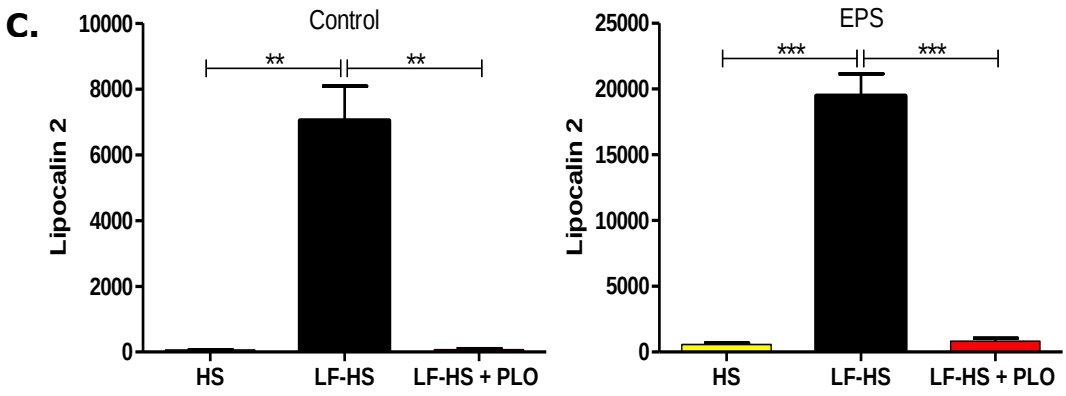
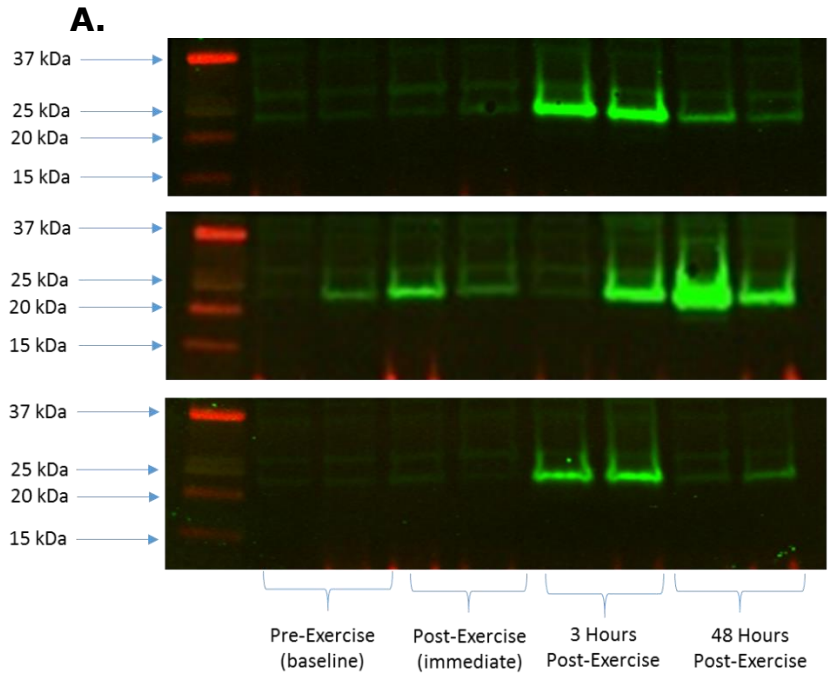


Figure 6- 5: LCN2 protein expression in the culture media of rat myotubes in various media conditions with the effects of 48-hour EPS.

Representative blot showing LCN2 protein expression in the culture media of rat myotubes in various media conditions namely normal horse serum, lipid-free and lipid-free + PLO, together with the effects of 48-hour EPS (A). The protein blots density is normalised to respective protein band on the membrane after stained by ponceau red (B). Quantified values of LCN2 are shown in lower panels (C). ** denotes P<0.01 and *** denotes P<0.001. Data were analysed by One way ANOVA with Bonferronis's post-hoc (HS- normal horse serum, LF- lipid free horse serum, LF-HS+PLO- lipid free horse serum supplemented with 10mM PLO- palmitic, linoleic and oleic acids (ratio of 4:3:3 respectively) n=3).

6.4.6 LCN2 protein expression in human muscle pre- and post-exercise

In the previous chapter, we have observed the increased in LCN2 mRNA expression in rat skeletal muscle myotubes following EPS. In this experiment, we would like to investigate the LCN2 protein expression in human skeletal muscle following exercise. 6 human volunteers were subjected to right lower limb isotonic eccentric exercise with the total of 75 contractions which is divided to 3 sets of 25 repetitions. The muscle samples from the vastus lateralis muscles of both limbs were taken pre- and post-exercise as described in Chapter 2 section 2.4. Result showed that there is a significant increase in LCN2 protein expression 3 hours after exercise in 5 out of 6 samples. Furthermore, the lipocalin 2 protein expression reduced gradually within 48 hours post-exercise (Figure 6-6). The molecular weight of the bands of lipocalin 2 were detected at 25 kDa.



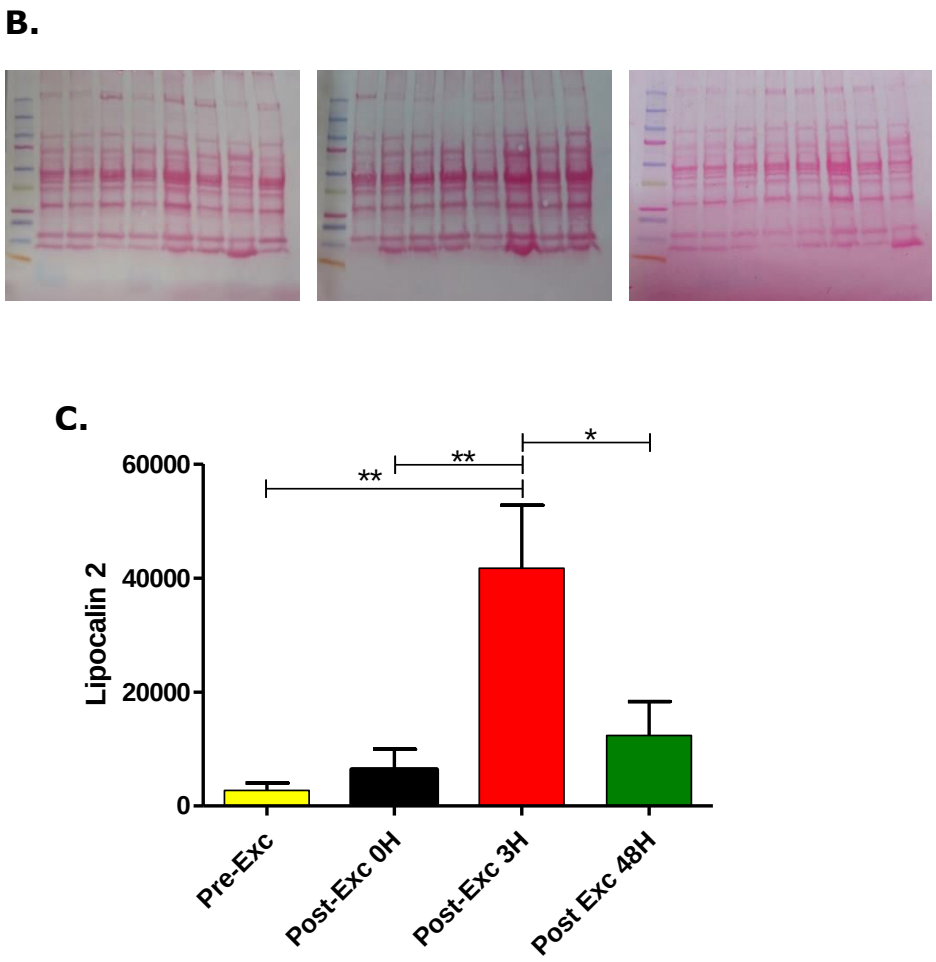


Figure 6- 6: LCN2 protein expression in the human vastus lateralis muscle pre- and post-exercise.

Representative blot showing LCN2 protein expression in the human vastus lateralis muscle pre- and post-exercise (immediate, 3 hours and 48 hours) in 6 different individuals (A). The protein blots density is normalised to respective protein band on the membrane after stained by ponceau red (B). Quantified values of lipocalin 2 are shown in lower panels (C). * denotes P< 0.05, ** denotes P<0.01 and *** denotes P<0.001. Data were analysed by One way ANOVA with Bonferronis's post-hoc (n=6).

6.4.7 LCN2 protein expression in human serum pre- and post-exercise

In previous experiment, we have observed the increased in LCN2 protein in human muscle 3 hour after exercise. In this experiment, we would like to investigate the release of LCN2 protein by the skeletal muscle into the serum following exercise by ELISA. The same 6 human volunteers were subjected to right lower limb isotonic eccentric exercise with the total of 75 contractions which is divided to 3 sets of 25 repetitions. The blood samples from the local arterial and venous circulations were also taken at pre- and post-exercise as described in Chapter 2 section 2.4. Results showed that, there was a gradual increase in pattern of LCN2 protein level in the venous serum following exercise (Figure 6-7A). Furthermore, there was a significant increase in LCN2 protein in the venous serum at 3 hours post-exercise as compared to pre-exercise (Figure 6-7B).

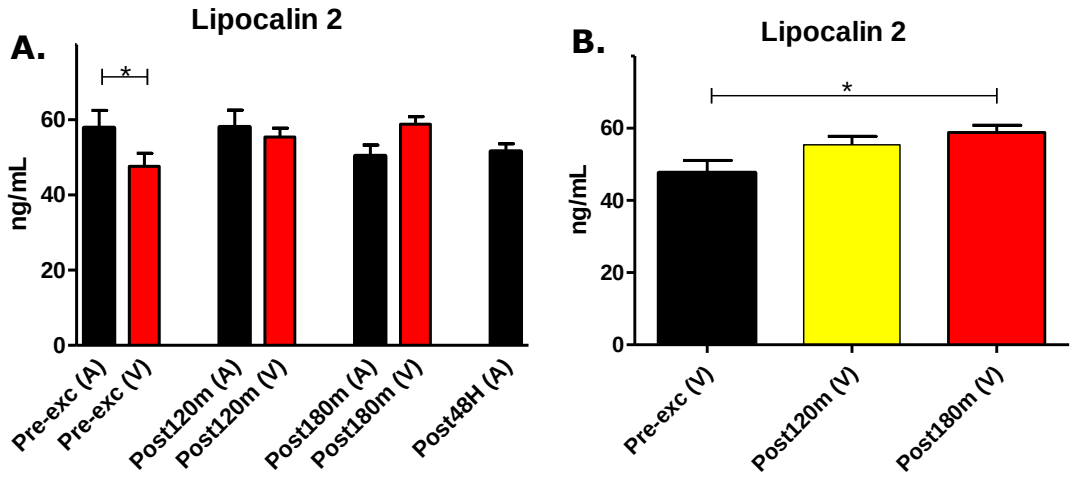


Figure 6- 7: LCN2 protein level in the serum of human vastus lateralis muscle in pre- and post-exercise.

The LCN2 level comparison between arterial and venous serum of vastus lateralis muscle from 6 human volunteers in pre- and post-exercise (A) and the comparison of LCN2 level only in venous serum (B) in pre- and post-exercise. * denotes $P < 0.05$. Data were analysed by One way ANOVA with Bonferronis's post-hoc (n=6).

On further analysis, based on the LCN2 level difference in arterial and venous serum, we analysed the uptake/release of LCN2 level by the skeletal muscle specifically vastus lateralis following exercise. Result showed that, there was an uptake of LCN2 by the muscle in pre-exercise state. At 2 hours post exercise, there was a reduction of LCN2 uptake by the muscle from the circulation and interestingly, the muscle started to secrete LCN2 protein into the venous circulation at 3 hours after exercise (Figure 6-8).

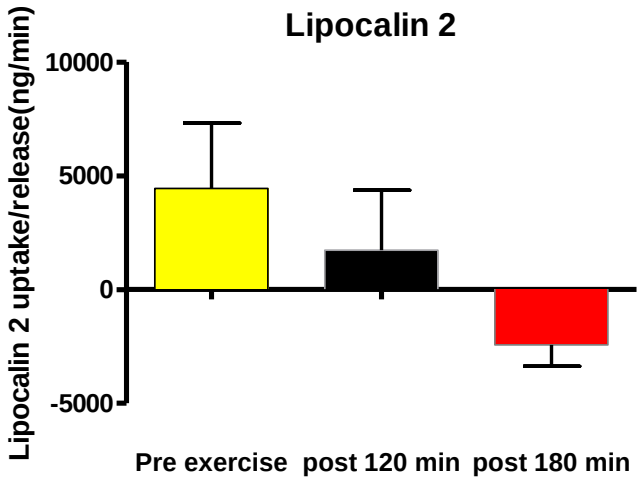


Figure 6- 8: LCN2 protein uptake/release by vastus lateralis muscle in pre- and post-exercise.

LCN2 protein uptake/release by vastus lateralis muscle from 6 human volunteers in pre- and post-exercise (2 hours and 3 hours). Data were analysed by One way ANOVA with Bonferronis's post-hoc (n=6).

6.5 Discussion

Although LCN2 was identified more than 2 decades ago, the regulation and synthesis of this protein by skeletal muscle remain poorly understood. In fact, there is a study demonstrated that lipocalin 2 is not expressed in skeletal muscle (Mosialou et al., 2017). Much of the previous research has focused upon the synthesis and release of LCN2 by adipose tissue and its regulation by inflammation, metabolic stress, obesity and insulin resistance. Skeletal muscle is the largest organ in human body and studies showed that contracting muscle cells are able to synthesize and secrete several humoral factors or cytokines (Pedersen, 2011b). In line with this fact, our current study demonstrated that LCN2 mRNA and protein are expressed and synthesized by skeletal muscle.

In the early part of this chapter, the upregulation of LCN2 mRNA together with LCN2 protein synthesis and secretion by primary skeletal muscle was seen in post-prandial (increased glucose, increased insulin and low lipid) but not in fasting (normal glucose, low insulin and normal lipid) or diabetic/metabolic (high glucose, high insulin and normal lipid) condition. This might be as the response to the low-lipid environment but not influenced by the glucose content.

However in other tissues specifically in adipose tissue and liver, LCN2 mRNA was found to be significantly upregulated in diabetic and obese mice (Y. Wang et al., 2007) . Apart from been highly expressed in the adipocytes of obese mice, LCN2 expression was also been upregulated during adipogenesis of 3T3-L1 cells (Yan et al., 2007). Furthermore, in human subjects, serum LCN2 concentrations were positively correlated with adiposity, waist-to-hip ratio, waist circumference, hypertriglyceridemia,

hyperglycaemia and insulin resistance index (Y. Wang et al., 2007). These above studies demonstrated that LCN2 was highly expressed in adipose tissues and serum of obese and diabetic subjects. This suggests that the regulation of LCN2 in skeletal muscle is quite different compared to adipocytes.

In the next part, we have observed the LCN2 protein expression in primary skeletal muscle myotubes in various media conditions with the effects of 48-hour EPS. In previous chapter, we have showed a significant increase in LCN2 mRNA expression post-EPS in the myotubes in normal horse serum and after reintroduction of PLO in lipid-free horse serum media condition. However in current chapter, the protein expression of LCN2 in the same group of myotubes post-EPS was not detected by western blotting. This is because the rise in the mRNA level post-EPS was not high enough to be translated into protein.

The upregulation of LCN2 mRNA after EPS and expression of LCN2 protein post-exercise support the evidence that contracting skeletal muscle is able to synthesize and secrete LCN2. The expression of LCN2 post-exercise in this study is in line with other established myokines such as IL-6, IL-8 and IL-15 which also have shown to be upregulated after exercise. As mentioned by Pederson and Fischer, the level of circulating IL-6 may rise up to 100-fold in response to exercise; and the peak of IL-6 is reached at the end or shortly after exercise followed by a rapid decline to the level seen before exercise (Pedersen & Fischer, 2007). Another myokine, IL-8 was shown to be increased in plasma concentration following exhaustive eccentric exercise (Nieman et al., 2003; Nieman et al., 2002; Nieman et al., 2001; Ostrowski et al., 2001) and IL-15 mRNA also was shown to be

upregulated following a single bout of strength training in human skeletal muscle (Nielsen et al., 2007).

As compared to our in vivo study, the level of LCN2 protein in skeletal muscle cells was not significantly increased immediately after exercise. However, the LCN2 protein level was tremendously increased after that and reached its peak for about 20-fold at 3 hours post-exercise and significantly decline after 48-hours. Furthermore, we also showed that contracting skeletal muscle cells are able to secrete LCN2 protein to the circulation, evidence by a significant net of release of LCN2 protein in the local venous system at 3 hours post-exercise.

On the other aspect, strenuous exercise also has been documented to cause muscle inflammation. Exercise-induced muscle inflammation may represented by muscle soreness (Clarkson & Hubal, 2002; Proske & Morgan, 2001; Smith, 1991) depends on the intensity of exercise together with inflammatory cell infiltration (Camus et al., 1993; Clarkson, 1997; Clarkson & Hubal, 2002; Shek & Shephard, 1998).

Apart from the local changes observed at the muscle, inflammatory reaction to the muscle injury including systemic response will also taking place. This response which known as the acute-phase reaction, is initiated by substances produced by several cell types, including monocytes or macrophage action at the site of injury (Camus et al., 1993; Shek & Shephard, 1998). These substances are called cytokines, and they play central roles in control of immune response, acute-phase response, inflammatory reactions, and tissue repair process (Camus et al., 1993).

Several inflammatory mediators have been identified to be increased following exercise. Significant rise in serum concentration of IL-6 were

observed 2 hours after eccentric exercise. This elevation of IL-6 was significantly correlated to Creatinine kinase (CK) concentration which indicates association with muscle damage. (Bruunsgaard et al., 1997). Another important mediator namely IL-1β, a protein released from blood monocytes and related cells in response to inflammatory stimuli; is also elevated in the muscle fibres as early as 45 minutes and lasted for 5 days after eccentric exercise detected by specific immunohistochemical tissue staining (Cannon et al., 1989). Furthermore, a significant increase in plasma concentration of tumor necrosis factor (TNF) was also observed 1 h after the end of exercise (Dufaux & Order, 1989).

The above studies demonstrated that inflammation process might taking place in the muscle after exercise. Since the expression of LCN2 was shown to be induced by a variety of pro-inflammatory stimuli, including lipopolysaccharide, interleukin-1β, interleukin-17, TNFα and hyperglycaemia (Bu et al., 2006; Pawluczyk et al., 2003; Yan et al., 2007), the elevation of LCN2 post-exercise in this study might be influenced by these inflammatory mediators. However in this study, the rise in the inflammatory mediators post-exercise were not measured. The level of these mediators should be take into account in future in order to investigate the relationship between the expression of LCN2 and muscle inflammation.

In general, the term "myokines" is defined as cytokines and other peptides that are produced, expressed, and released by muscle fibers and exert either paracrine or endocrine effects to other organs (Pedersen, 2011a, 2011b; Pedersen et al., 2007). Even though the current study has demonstrated that skeletal muscle is able to synthesize and secrete LCN2, further future works need to be done to investigate its role in mediating

other metabolic changes or exerting specific endocrine effects to other organs such as the liver and the adipose tissue, in order to classify LCN2 as a novel myokine.

CHAPTER SEVEN

GENERAL DISCUSSION AND FUTURE ASPECTS

Chapter 7: General Discussion and Future Aspects

Skeletal muscle is a contractile tissue that plays an important role in energy homeostasis due to high amount of energy consumed in performing its activities (Abdul-Ghani & DeFronzo, 2010). It is the primary site of glucose and fatty acid oxidation that accounting up to 30% of basal energy requirements (Zurlo et al., 1990). Therefore, the energy is acquired from the metabolism of carbohydrates and fat, thus it makes muscle as a major site of action for insulin involving the regulation of glucose entrance into the cells. Subsequent impairment of glucose uptake and transport, glucose oxidation or glucose synthesis in muscles might be the major determinant of the severity of T2DM (Abdul-Ghani & DeFronzo, 2010). Skeletal muscle rapidly adapts to changing energy needs such as following exercise or reduced energy intake by increasing fatty acids oxidation (Henriksson, 1995). Increased fatty acid oxidation delays the consumption of glycogen stores within skeletal muscle and conserves circulating plasma glucose (Henriksson, 1995). The capacity for skeletal muscle to adapt appropriately is depends upon insulin sensitivity, leanness and aerobic fitness (Ukropcova et al., 2005).

Glucose is the source of energy for skeletal muscle and its uptake into the cells is mainly stimulated by insulin. In skeletal muscle, glucose transport is mediated by carriers known as glucose transporters (GLUT) family which facilitate glucose movement into the cells. At the moment, there are 13 members of GLUT family but the best-characterized are the class 1 GLUT (GLUT 1-4). The GLUT1 transporters are commonly responsible for basal glucose uptake. The GLUT2 is mainly expressed in beta cells and liver, and has relatively low affinity for glucose. GLUT3 has the

highest affinity and only expressed during foetal development and in adult neurons. Finally GLUT4 is predominantly responsible for insulin-stimulated glucose uptake in fat and muscle.

The insulin stimulation of glucose uptake in muscle tissue occurs via a signalling pathway through the insulin receptor tyrosine kinase. The main effect is to promote the movement of GLUT4 from the intracellular space to the plasma membrane. Therefore, the rate limiting step in insulin-stimulated glucose uptake in muscles is the translocation of GLUT transporters to the cell membrane (Khan & Pessin, 2002).

GLUT4 is mainly retained in intracellular vesicle structures in resting muscle (Foley et al., 2011) and several studies showed that insulin as well as muscle contractions will translocate GLUT4 to the sarcolemma and T-tubular system (Derave et al., 2000; Derave et al., 1999). It is believed that there are two different intracellular pools of GLUT4 which are recruited by insulin and muscle contraction respectively (Coderre et al., 1995).

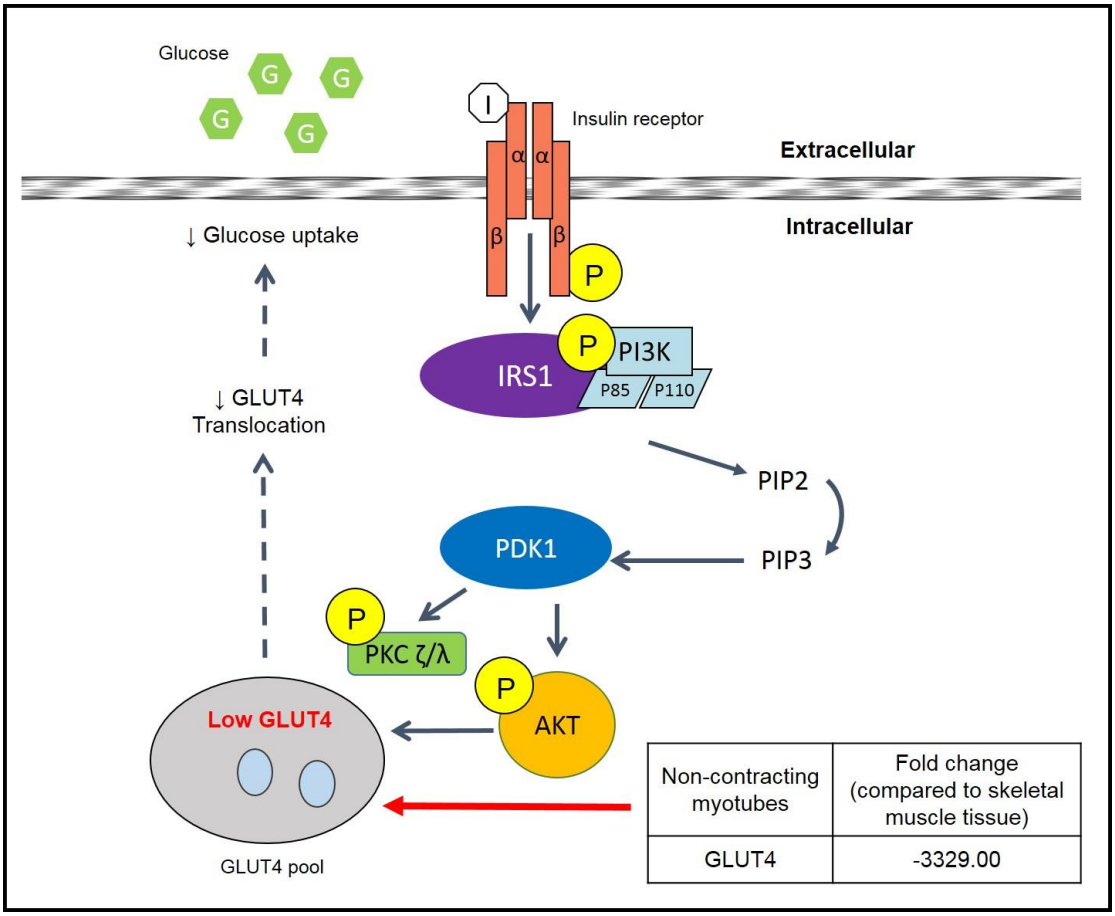
In the aspect of *in vitro* study, isolated skeletal muscle preparations are short lived for experimental manipulation, and they are invasive for human studies. Several muscle cell lines of animal origin are currently available namely rat L6 and mouse C2C12 of skeletal muscle origin and mouse BC3H1 of brain tumour origin. However, there are no muscle cell lines of human origin that have been characterized. The introduction of primary skeletal cell cultures offer an excellent model for performing experiments under standardized conditions since they express metabolic and protein characteristics of tissue origins. Furthermore, it is able to maintain the response to alterations in substrates and hormones that resembles skeletal muscle *in vivo* (Gaster, Rustan, Aas, & Beck-Nielsen,

2004; Steinberg et al., 2006). Thus, cultured myotubes is accepted as a valuable tool for investigation of metabolic processes in skeletal muscle (Nikolic et al., 2012). Therefore, the primary skeletal muscle cell cultures were used in this study.

In current study, primary skeletal muscle satellite cells were collected and cultured from both rat and human. As for the optimization, the experiments were initially done on rat primary skeletal muscle myotubes. Rat hind limb muscle was selected and soleus muscle was preferred in view of the fact that soleus muscle was found to has more satellite cell nuclei (Gibson & Schultz, 1983). These satellite cells are the source of myoblasts for regeneration of mammalian skeletal muscle (Snow, 1977) thus it makes soleus a better choice for cell culture. As for the human study, vastus lateralis muscle was selected since this muscle is characterised by a mixed type composition and distribution of muscle fibres namely slow (type I fibres) and fast (type II fibres); and is considered as the site of choice for biopsies in research work and for clinical diagnosis (Kadi, Charifi, & Henriksson, 2006).

Our laboratory previous data showed that the level of GLUT4 was approximately 3000-fold lower levels in non-contracting primary skeletal muscle cell cultures as compared to skeletal muscle tissue and this leads to minimal glucose uptake by the cells (Figure 7-1). Due to the facts that glucose enters muscle cells via facilitated diffusion through the GLUT4 glucose transporter which translocates from intracellular storage depots to the plasma membrane and T-tubules upon muscle contraction (Richter & Hargreaves, 2013), we began to develop a contractile model of myotubes

that may resemble the properties of muscle cells during exercise by applying electrical pulse stimulation (EPS).



Non-contracting myotubes	Fold change (compared to skeletal muscle tissue)
GLUT4	-3329.00

Figure 7- 1: Insulin signalling and glucose uptake in non-contracting primary skeletal muscle cell culture.

In non-contracting primary skeletal muscle cell culture, the level of GLUT4 is extremely low, approximately 3000-fold lower levels as compared to skeletal muscle tissue (as shown in the table- data not published). Thus, there will be minimal GLUT4 translocated to the membrane and results in low glucose uptake by the cells. IRS- insulin receptor substrate, PI3K- phosphatidylinositol 3’ kinase, P85- regulatory p85 subunit, P110- catalytic p110 subunit, PDK1- 3’phosphoinositide-dependent kinase-1, AKT- protein kinase B, PKC- protein kinase C.

Development of Contractile Myotubes Model

In the present study, we demonstrated how a cell model of skeletal muscle myotubes can be established as an *in vitro* model of exercise, which can be used to study some of the adaptations seen in trained skeletal muscles. We have applied a chronic, low frequency EPS (bipolar pulse train of 2ms, 1Hz at 11.5V) for 48 hours to simulate regular exercise in order to study the effects of CB1 modulation upon insulin stimulated glucose uptake. Even though we are unable to visualize the muscle contraction via light microscope, we successfully observed a significant increase in lactic acid and reduction in glucose concentration in the culture media that suggest the occurrence contractile activities. During intense exercise, glucose is catabolised and pyruvate is substantially generated. When the mitochondrial activity is exceeded, pyruvate is reduced to lactate, resulting in oxidation of $NADH/H^+$ to NAD^+ (J. S. Baker et al., 2010).

Apart from that, the stimulated myotubes exhibited a significant increase of GLUT4 mRNA expression in 48 hours of EPS. This upregulation of GLUT4 mRNA is important since the main mechanism of glucose uptake in contracting skeletal muscle is by facilitated diffusion and this process determined by the translocation of GLUT4 to the surface membrane (Richter & Hargreaves, 2013). As studied in GLUT4 knockout mice, muscle contraction has shown to have a negligible effect on glucose uptake and almost similar to the level seen in basal glucose uptake in unstimulated wild-type mice (Ryder et al., 1999). In addition, mice with muscle-specific GLUT4 deletion demonstrated a significant reduction in muscle glucose uptake and exercise tolerance during exercise (Fueger et al., 2007).

Furthermore, another positive parameter of contractile activities in our contractile myotubes model is the increase in PGC1α mRNA expression. PGC1α is a transcription coactivator that interacts with a variety of transcription factors that are involved in biological responses including mitochondrial biogenesis (Liang & Ward, 2006). Thus, it can be considered as a marker for mitochondrial biogenesis. In this model, there was a significant increase in PGC1α mRNA expression in the myotubes after 48 hours of EPS. This indicates the contractile muscles generate more mitochondria production in order to cope with the increase in the exercise workload. This result is consistent with a study done by Baar et al that showed significant increase in PGC1 mRNA expression and protein in rats muscle after exercise (Baar et al., 2002). Furthermore, PGC-1α also has been shown to induce the expression of GLUT4 which in turn stimulates both basal and insulin-stimulated glucose transport in cultured muscle cells (Michael et al., 2001).

The most important parameter in this model is the ability of the myotubes to resemble skeletal muscle *in vivo* in term of insulin sensitivity and glucose uptake. By applying EPS, both rat and human myotubes exhibit an increase in glucose uptake, with or without the presence of insulin. This suggest that EPS is not only induce the expression of GLUT4 mRNA and protein as suggested by other study (Kuo et al., 1999), but it also increase the sensitivity of the myotubes to insulin. In addition, the results also suggest that muscle contractions and insulin activate muscle glucose transport by two different mechanisms. This is in line with several studies that suggest muscle contraction and insulin activate muscle glucose transport by different molecular mechanism (A. D. Lee et al., 1995; Lund

et al., 1995; Wallberg-Henriksson & Holloszy, 1984). The summary of the results is described in Figure 7-2.

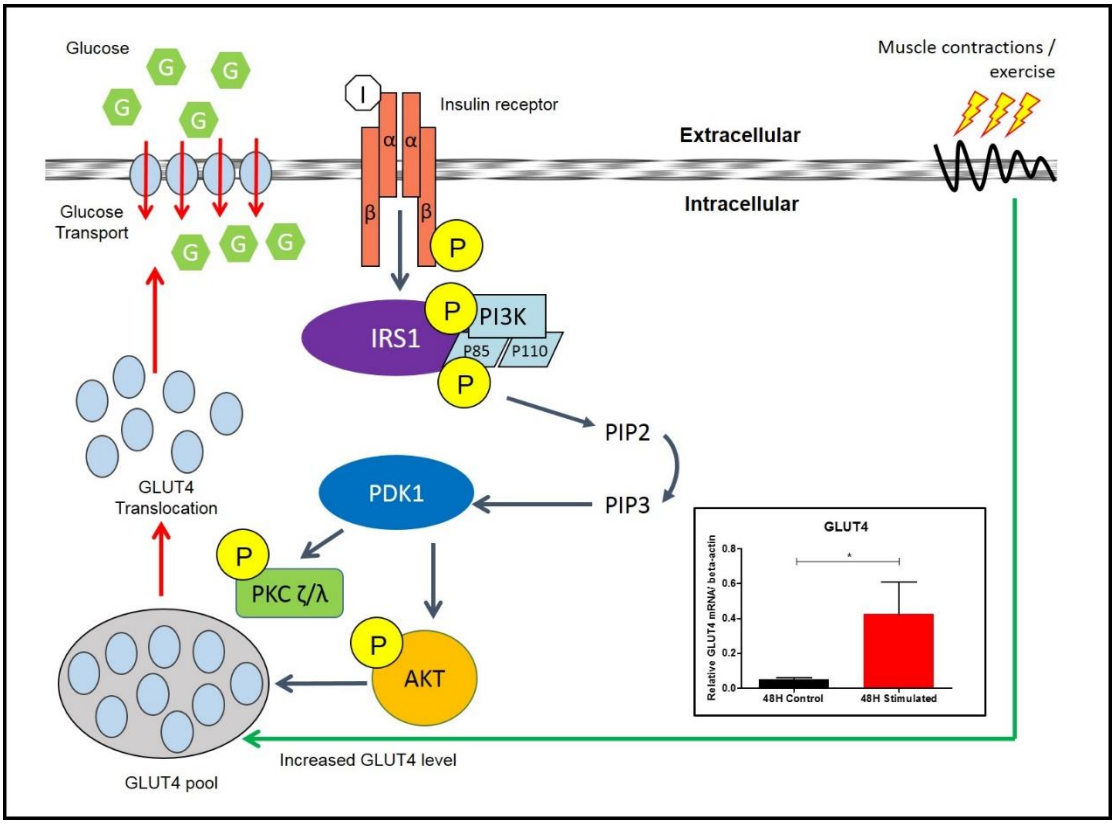


Figure 7- 2: Insulin signalling and glucose uptake in contractile primary skeletal muscle myotube model.

In the presence muscle contractions by electrical pulse stimulation (EPS), it will leads to the rise in the production of GLUT4 in the vesicle pool (as shown in the graph). Akt and PKC ζ/λ will then promote the GLUT4 translocation from the GLUT4 pool to the cell membrane to facilitate glucose uptake. Furthermore, the muscle contraction alone is suffice to stimulate the translocation of GLUT4 to the cell membrane. IRS- insulin receptor substrate, PI3K- phosphatidylinositol 3' kinase, P85- regulatory p85 subunit, P110- catalytic p110 subunit, PDK1- 3'phosphoinositide-dependent kinase-1, AKT- protein kinase B, PKC- protein kinase C.

Effects of CB1 Modulation upon Insulin Stimulated Glucose Uptake in Primary Skeletal Muscle

Recent data from our laboratory has clearly demonstrated that CB1 is expressed in human skeletal muscle and that activation with a specific agonist activates the p42/44 and p38 mitogen-activated protein kinase (MAPK) pathways.

After we successfully develop a contractile model of skeletal muscle myotubes as described in chapter 3, our next aim is to observe the effects of CB1 activation and inhibition on exercise-induced insulin stimulated glucose uptake in the contractile primary skeletal muscle myotubes by using ACEA, a synthetic anandamide analogue and its antagonist, Rimonabant.

As discussed in chapter 4, treatment with insulin caused a significant increase in glucose uptake in exercise model of both rat and human myotubes. Moreover, treatment with ACEA inhibit glucose uptake in insulin treated group. On the other hand, pre-treatment with Rimonabant reverted the inhibitory effect of ACEA. However, ACEA and Rimonabant not given any effect upon glucose uptake in non-insulin treated group. This results clearly revealed that the inhibitory effects of CB1 upon glucose uptake in contractile myotubes is only visible in the present of insulin (as shown in Chapter 4 Figure 4-1 and Figure 4-2). Furthermore, this indicates that CB1 inhibition is specifically through insulin signalling cascade and not through contraction-stimulated glucose uptake; since ACEA did not give any effects in non-insulin EPS group.

In the aspect of underlying mechanism of inhibition upon insulin stimulated glucose uptake, current study revealed activation of CB1 by ACEA caused inhibition of Akt phosphorylation and the action of ACEA was

attenuated by pre-treatment with Rimonabant. Furthermore, ACEA also was found to reduce PI3K activity by increasing its p85/p110 subunit ratio and again, pre-treatment with Rimonabant reverted the effect of ACEA. Therefore, the inhibition of these proteins may lead to inhibition of insulin stimulated glucose uptake, since activation of both Akt and PI3K are among the important steps in insulin signalling cascade (Fruman et al., 1998; Khan & Pessin, 2002; Rameh & Cantley, 1999). This mechanism is described in Figure 7-3.

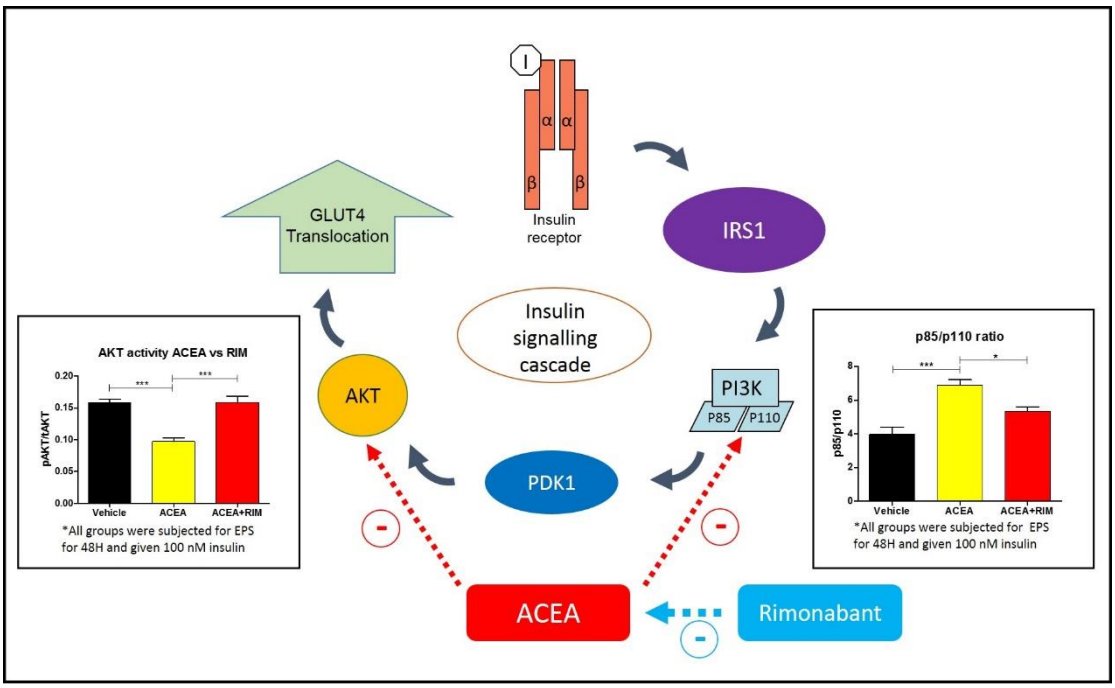


Figure 7- 3: Effects of endocannabinoids upon insulin signalling cascade.

CB1 activation by ACEA treatment inhibit insulin stimulated glucose uptake by inhibiting several steps in insulin signalling cascade. ACEA inhibit PI3K by elevating p85 regulatory subunit and this results in increase p85/p110 ratio (as shown in the graph on the right). Furthermore, ACEA inhibit the activity of Akt by suppressing phosphorylation of Akt (as shown in the graph on the left). These will lead to inhibition of GLUT4 translocation from the vesicles to the cell membrane. Pre-treatment with CB1 antagonist, Rimonabant attenuates the effects of ACEA. IRS- insulin receptor substrate, PI3K- phosphatidylinositol 3' kinase, P85- regulatory p85 subunit, P110- catalytic p110 subunit, PDK1- 3'phosphoinositide-dependent kinase-1, AKT- protein kinase B.

Apart from that, we have also demonstrated the effects of palmitate with comparison to ACEA upon exercise-induced insulin stimulated glucose uptake in human myotubes. Palmitate is a non-esterified fatty acids that found to has an inhibitory effect on insulin stimulated glucose uptake in skeletal muscle (Alkhateeb et al., 2007; Storz et al., 1999). In current study, treatment with Palmitate or ACEA significantly inhibit insulin-stimulated glucose uptake at almost similar level. Interestingly, treatment with combination of Palmitate and ACEA caused a synergistic effect in reduction of insulin-stimulated glucose uptake. On the other hand, pre-treatment with Rimonabant blunted half of the inhibitory effect of combination of Palmitate and ACEA. This suggest that Rimonabant only reverted the effect of ACEA, but not Palmitate.

Several studies found that Palmitate inhibit insulin-stimulated glucose uptake by inhibiting Akt phosphorylation (Chavez & Summers, 2003; Peng et al., 2011; Storz et al., 1999). Since ACEA also inhibit Akt phosphorylation as mention in previous result, it may has the similar site of action with palmitate in inhibiting insulin-stimulated glucose uptake, however the underlying pathway of Akt inhibition might be different. Previous studies also suggested that saturated fatty acids such as palmitate promote accumulation of ceramide (Chavez & Summers, 2003, 2012; Pickersgill et al., 2007; Schmitz-Peiffer et al., 1999; Watson et al., 2009) in skeletal muscle; and synthesis of this ceramide is crucially dependent on the activity of serine palmitoyl transferase (SPT) which catalyses the first and rate-limiting step of *de novo* biosynthesis involving the condensation of amino acid L-serine with palmitoyl-CoA to form 3-Ketosphinganine (Levy & Futerman, 2010). This ceramide in turn was found to play a role in insulin

resistance in skeletal muscle by inhibit the phosphorylation of of Akt (Adams et al., 2004; Hajduch et al., 2001; Schmitz-Peiffer et al., 1999; Watson et al., 2009). Interestingly, as demonstrated by Cinar et al, peripheral CB1 blockade by JD5037 appear to reverse the elevation of long-chain ceramide in muscle. Furthermore, CB1 antagonism also inhibit de novo synthesis of ceramide by inhibition of the activity of SPT and ceramide synthase (CerS); and promote the degradation of ceramide in liver microsomes of diet-induced obese mice (Cinar et al., 2014). These findings has open up a new perspectives on the study of these fatty acid derivatives, and further future work need to be done to study the relationship between endocannabinoid and ceramide activities in regulating insulin-stimulated glucose uptake in skeletal muscle.

Effects of CB1 Modulation upon Selected Genes Expression

In chapter 5, the main objective of the experiment is to determine the molecular pharmacological properties of the cannabinoid receptor CB1 in primary skeletal muscle. In order to study the effects of CB1 agonism and antagonism upon gene expression, qRTPCR was conducted. The experiments were performed on rats' myotubes under various serum media conditions. Most of the experiments were done with comparing normal lipid serum and lipid-free serum media condition. The aim of using lipid-free serum is we would like to remove the lipid components including natural occurring endocannabinoids so that it will not interfere with the endocannbinoids treatment that we give prior to the experiments. Recent research from our laboratory has shown that the gene expression of LCN2 and Neuropeptide Y were upregulated; and TRIB3, NR4A1, NR4A2 and

NR4A3 were downregulated by Rimonabant treatment via microarray. Thus, the current study is done in view of to validate this previous data.

However, current study revealed that expression of the above genes is regulated by the levels of FFA in the serum and not by the endocannabinoids treatments. Furthermore, LCN2, Neuropeptide Y, NR4A1, NR4A2 and NR4A3 mRNA expression were also upregulated after given EPS. This might suggest that the gene expressions of LCN2, Neuropeptide Y, NR4A1, NR4A2 and NR4A3 were consistently upregulated in 'energy deprivation' state, either in lipid-free or in chronic exercise.

LCN2 as a Novel Myokine

LCN2, also known as neutrophil gelatinase-associated lipocalin (NGAL), is recently identified adipokine that belongs to the superfamily of lipocalins which seems to affect glucose metabolism and insulin sensitivity (Kjeldsen et al., 1994). Lipocalins is a lipid-binding protein that is able to bind and transport a wide variety of small hydrophobic molecules such as retinol, fatty acids, steroids and thyroid hormone (Flower, 1996; LaLonde et al., 1994). Until now, there is minimal information regarding the synthesis of LCN2 by skeletal muscle and its role in skeletal muscle is not widely understood. In fact, there is a study demonstrated that LCN2 is not expressed in skeletal muscle (Mosialou et al., 2017). In chapter 5, we have shown that there is an upregulation of LCN2 mRNA expression in low lipid environment and during chronic low-frequency EPS. Due to the fact that this early data suggest that the regulation of LCN2 in skeletal muscle might differ from adipocytes, the synthesis and release of LCN2 in skeletal muscle is interesting to be explored. In chapter 6, our aim is to investigate the

production of LCN2 protein in skeletal muscle following various nutritional conditions and exercise. In addition, we also would like to observe the release of LCN2 as a novel myokine in the culture media (*in vitro*) and serum (*in vivo*).

As discussed in chapter 6, we began the study with the investigation of the synthesis and release of LCN2 by skeletal muscle myotubes in respond to various nutritional status namely post-prandial, fasting, diabetic/metabolic conditions. We incubated the rat myotubes in culture media that has been modified to suit the mentioned condition for 48 hours prior to collection. We assigned 'low-lipid' condition as control in this experiment, in view of LCN2 mRNA was found to be upregulated in low-lipid condition as mentioned in the previous chapter. Results showed upregulation of LCN2 mRNA together with increase in LCN2 protein production and secretion in post-prandial condition. However LCN2 mRNA or protein was not increase in fasting and diabetic/metabolic condition which suggests that the increment in LCN2 protein is not due to glucose content, but most probably due to the respond to the low-lipid environment.

The next experiment also revealed almost similar findings. We have observed the LCN2 protein expression in rat myotubes and secretion (in the culture media) in various media conditions namely normal horse serum, lipid-free and lipid-free + PLO, together with the combination of effects of 48-hour EPS. Results revealed there was a huge significant increase in LCN2 protein expression in cells and LCN2 secretion in culture media; in lipid-free media in both unstimulated and EPS groups. In contrast, LCN2 protein was not expressed or secreted in both normal horse serum and lipid-free + PLO group in non-stimulated or even after 48 hours of EPS. This results also

suggest that the elevation in LCN2 in skeletal muscle is in response to low-lipid environment.

These results however contradict with previous studies which stated that LCN2 was elevated in obese and hyperlipidaemic status. Studies demonstrated that LCN2 gene expression in adipose tissue was upregulated in genetic and dietary-induced obese rodents (Y. Wang et al., 2007; J. Zhang et al., 2008), as well as in obese humans (Y. H. Lee et al., 2010; Panidis et al., 2010; Y. Wang et al., 2007). Furthermore, circulating LCN2 levels increased in obese animals, and LCN2 mRNA was increased in the liver of ob/ob mice (Yan et al., 2007). These results showed that the regulation and secretion of LCN2 is a complicated process; and is suggest that the elevation of LCN2 in obese individuals might due to secretion from other organs such as adipose tissue and liver but not from skeletal muscle.

Human study showed a significant increase in LCN2 protein expression 3 hours after exercise when compared to pre-exercise state in skeletal muscle. Furthermore, the LCN2 protein expression reduced gradually within 48 hours post-exercise. Meanwhile, the analysis of the blood serum revealed a significant increase in LCN2 protein in the venous serum at 3 hours post-exercise as compared to pre-exercise. Further analysis based on arterial and venous serum difference revealed that there is an uptake of LCN2 by the skeletal muscle from the systemic circulation in pre-exercise state. Then, there was a reduction of LCN2 uptake by the muscle from the circulation at 2 hours post-exercise and interestingly, the muscle started to secrete LCN2 protein into the venous circulation after 3 hours of exercise. Current results suggest that LCN2 is also being synthesized and secreted by skeletal muscle in response to exercise, similar

to several other myokines that were found to be influenced and increased by physical activity such as IL-6 (Fischer, 2006; Pedersen & Febbraio, 2008; Pedersen & Fischer, 2007), IL-8 (Pedersen & Febbraio, 2008) and IL-15 (Nielsen et al., 2007).

Apart from that, exercise was documented to cause inflammatory reaction to the muscle. Several inflammatory mediators have been identified to be increased following exercise such as IL-6 (Bruunsgaard et al., 1997), IL-1β (Cannon et al., 1989) and TNF (Dufaux & Order, 1989). Since the expression of LCN2 was shown to be induced by a variety of pro-inflammatory stimuli, including lipopolysaccharide, interleukin-1β, interleukin-17, TNFα and hyperglycaemia (Bu et al., 2006; Pawluczyk et al., 2003; Yan et al., 2007), the elevation of LCN2 post-exercise in this study might be influenced by these inflammatory mediators. However in this study, the rise in the inflammatory mediators post-exercise were not measured. The level of these mediators should be take into account in future in order to investigate the relationship between the expression of LCN2 and muscle inflammation.

The term "myokines" is defined as cytokines and other peptides that are produced, expressed, and released by muscle fibers and exert either paracrine or endocrine effects to other organs (Pedersen, 2011a, 2011b; Pedersen et al., 2007). The current study has demonstrated that the skeletal muscle synthesize and secrete LCN2 in response to low-lipid environment and post-exercise. In order to classify LCN2 as a novel myokine, further future works need to be done to investigate its role in mediating other metabolic changes or exerting specific endocrine effects to other organs such as the liver and the adipose tissue.

Experimental Limitations

In LCN2 experiments, we encounter several problems especially in western blot procedures. There was a huge unspecific band blotted in between 37kDa and 50 kDa. Thus, the blotting of control protein such as RPLPO (34 kDa), GAPDH (36 kDa) and β-actin (42 kDa) is quite difficult to be visualize. Thus, we used the ponceau red-stained membrane as the control band for densitometry quantification in the cells. Furthermore, we also used the ponceau red-stained membrane to quantify loading control for the secretion of LCN2 in the media, since we extracted the protein from 1 ml media and loaded the similar amount into the separating gel. In addition, there was also a huge unspecific band blotted in between 37kDa and 50 kDa for the membrane of the protein from the media and this might be due to a high protein content in the horse serum.

Apart from that, we were unable to successfully yield the LCN2 protein band from human myotubes in western blot, even after applying 2 EPS protocols: acute high frequency (pulse train of bipolar pulses 100Hz for 200ms given every 5^{th} second, 30V for 30 min, 1 hour and 2 hours) and chronic low frequency (single, bipolar pulses of 2ms, with 11.5V and 1Hz continuously for 48 hours). There are several possibilities that may lead to this condition, first the EPS protocol given to the human myotubes is not optimum enough to stimulate the cells to produce LCN2, even though the EPS protocol is suffice in rat myotubes. Second, there might be several steps in the western blot protocol that need to be optimized and modified especially sample preparation. Thus, we collected human fresh muscle samples and blood serum post-exercise to investigate the LCN2 production and secretion in the blood.

References

Aas, V., Torbla, S., Andersen, M. H., Jensen, J., & Rustan, A. C. (2002). Electrical stimulation improves insulin responses in a human skeletal muscle cell model of hyperglycemia. *Ann N Y Acad Sci, 967*, 506-515.

Abdul-Ghani, M. A., & DeFronzo, R. A. (2010). Pathogenesis of insulin resistance in skeletal muscle. *J Biomed Biotechnol, 2010*, 476279. doi: 10.1155/2010/476279

Adams, J. M., 2nd, Pratipanawatr, T., Berria, R., Wang, E., DeFronzo, R. A., Sullards, M. C., & Mandarino, L. J. (2004). Ceramide content is increased in skeletal muscle from obese insulin-resistant humans. *Diabetes, 53*(1), 25-31.

Ahlborg, G., Felig, P., Hagenfeldt, L., Hendler, R., & Wahren, J. (1974). Substrate turnover during prolonged exercise in man. Splanchnic and leg metabolism of glucose, free fatty acids, and amino acids. *J Clin Invest, 53*(4), 1080-1090. doi: 10.1172/JCI107645

Aigner, F., Maier, H. T., Schwelberger, H. G., Wallnofer, E. A., Amberger, A., Obrist, P., . . . Troppmair, J. (2007). Lipocalin-2 regulates the inflammatory response during ischemia and reperfusion of the transplanted heart. *Am J Transplant, 7*(4), 779-788. doi: 10.1111/j.1600-6143.2006.01723.x

Al-Khalili, L., Chibalin, A. V., Kannisto, K., Zhang, B. B., Permert, J., Holman, G. D., . . . Krook, A. (2003). Insulin action in cultured human skeletal muscle cells during differentiation: assessment of cell surface GLUT4 and GLUT1 content. *Cell Mol Life Sci, 60*(5), 991-998. doi: 10.1007/s00018-003-3001-3

Alkhateeb, H., Chabowski, A., Glatz, J. F., Luiken, J. F., & Bonen, A. (2007). Two phases of palmitate-induced insulin resistance in skeletal muscle: impaired GLUT4 translocation is followed by a reduced GLUT4 intrinsic activity. *Am J Physiol Endocrinol Metab, 293*(3), E783-793. doi: 10.1152/ajpendo.00685.2006

Andersen, P., & Saltin, B. (1985). Maximal perfusion of skeletal muscle in man. *J Physiol, 366*, 233-249.

Antonetti, D. A., Algenstaedt, P., & Kahn, C. R. (1996). Insulin receptor substrate 1 binds two novel splice variants of the regulatory subunit of phosphatidylinositol 3-kinase in muscle and brain. *Mol Cell Biol, 16*(5), 2195-2203.

Aronson, D., Violan, M. A., Dufresne, S. D., Zangen, D., Fielding, R. A., & Goodyear, L. J. (1997). Exercise stimulates the mitogen-activated protein kinase pathway in human skeletal muscle. *J Clin Invest, 99*(6), 1251-1257. doi: 10.1172/JCI119282

Ashton, C. H. (2001). Pharmacology and effects of cannabis: a brief review. *Br J Psychiatry, 178*, 101-106.

Baar, K., Wende, A. R., Jones, T. E., Marison, M., Nolte, L. A., Chen, M., . . . Holloszy, J. O. (2002). Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. *FASEB J, 16*(14), 1879-1886. doi: 10.1096/fj.02-0367com

Bae, S. S., Cho, H., Mu, J., & Birnbaum, M. J. (2003). Isoform-specific regulation of insulin-dependent glucose uptake by Akt/protein kinase B. *J Biol Chem, 278*(49), 49530-49536. doi: 10.1074/jbc.M306782200

Baker, D., Pryce, G., Giovannoni, G., & Thompson, A. J. (2003). The therapeutic potential of cannabis. *Lancet Neurol, 2*(5), 291-298.

Baker, J. S., McCormick, M. C., & Robergs, R. A. (2010). Interaction among Skeletal Muscle Metabolic Energy Systems during Intense Exercise. *J Nutr Metab, 2010*, 905612. doi: 10.1155/2010/905612

Bamford, R. N., Grant, A. J., Burton, J. D., Peters, C., Kurys, G., Goldman, C. K., . . . Waldmann, T. A. (1994). The interleukin (IL) 2 receptor beta chain is shared by IL-2 and a cytokine, provisionally designated IL-T, that stimulates T-cell proliferation and the

induction of lymphokine-activated killer cells. *Proc Natl Acad Sci U S A, 91*(11), 4940-4944.

Barbour, L. A., Shao, J., Qiao, L., Leitner, W., Anderson, M., Friedman, J. E., & Draznin, B. (2004). Human placental growth hormone increases expression of the p85 regulatory unit of phosphatidylinositol 3-kinase and triggers severe insulin resistance in skeletal muscle. *Endocrinology, 145*(3), 1144-1150. doi: 10.1210/en.2003-1297

Begg, M., Pacher, P., Batkai, S., Osei-Hyiaman, D., Offertaler, L., Mo, F. M., . . . Kunos, G. (2005). Evidence for novel cannabinoid receptors. *Pharmacol Ther, 106*(2), 133-145. doi: 10.1016/j.pharmthera.2004.11.005

Bek, E. L., McMillen, M. A., Scott, P., Angus, L. D., & Shaftan, G. W. (2002). The effect of diabetes on endothelin, interleukin-8 and vascular endothelial growth factor-mediated angiogenesis in rats. *Clin Sci (Lond), 103 Suppl 48*, 424S-429S. doi: 10.1042/CS103S424S

Bensaid, M., Gary-Bobo, M., Esclangon, A., Maffrand, J. P., Le Fur, G., Oury-Donat, F., & Soubrie, P. (2003). The cannabinoid CB1 receptor antagonist SR141716 increases Acrp30 mRNA expression in adipose tissue of obese fa/fa rats and in cultured adipocyte cells. *Mol Pharmacol, 63*(4), 908-914.

Bermudez-Silva, F. J., Sanchez-Vera, I., Suarez, J., Serrano, A., Fuentes, E., Juan-Pico, P., . . . Rodriguez de Fonseca, F. (2007). Role of cannabinoid CB2 receptors in glucose homeostasis in rats. *Eur J Pharmacol, 565*(1-3), 207-211. doi: 10.1016/j.ejphar.2007.02.066

Bermudez-Siva, F. J., Serrano, A., Diaz-Molina, F. J., Sanchez Vera, I., Juan-Pico, P., Nadal, A., . . . Rodriguez de Fonseca, F. (2006). Activation of cannabinoid CB1 receptors induces glucose intolerance in rats. *Eur J Pharmacol, 531*(1-3), 282-284. doi: 10.1016/j.ejphar.2005.12.016

Bluher, M., Engeli, S., Kloting, N., Berndt, J., Fasshauer, M., Batkai, S., . . . Stumvoll, M. (2006). Dysregulation of the peripheral and adipose tissue endocannabinoid system in human abdominal obesity. *Diabetes, 55*(11), 3053-3060. doi: 10.2337/db06-0812

Brat, D. J., Bellail, A. C., & Van Meir, E. G. (2005). The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. *Neuro Oncol, 7*(2), 122-133. doi: 10.1215/S1152851704001061

Brown, J. E., Kassouny, M., & Cross, J. K. (1977). Kinetic studies of food intake and sucrose solution preference by rats treated with low doses of delta9-tetrahydrocannabinol. *Behav Biol, 20*(1), 104-110.

Bruunsgaard, H., Galbo, H., Halkjaer-Kristensen, J., Johansen, T. L., MacLean, D. A., & Pedersen, B. K. (1997). Exercise-induced increase in serum interleukin-6 in humans is related to muscle damage. *J Physiol, 499 (Pt 3)*, 833-841.

Bu, D. X., Hemdahl, A. L., Gabrielsen, A., Fuxe, J., Zhu, C., Eriksson, P., & Yan, Z. Q. (2006). Induction of neutrophil gelatinase-associated lipocalin in vascular injury via activation of nuclear factor-kappaB. *Am J Pathol, 169*(6), 2245-2253.

Burch, N., Arnold, A. S., Item, F., Summermatter, S., Brochmann Santana Santos, G., Christe, M., . . . Handschin, C. (2010). Electric pulse stimulation of cultured murine muscle cells reproduces gene expression changes of trained mouse muscle. *PLoS One, 5*(6), e10970. doi: 10.1371/journal.pone.0010970

Bustin, S. A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol, 25*(2), 169-193.

Butler, H., & Korbonits, M. (2009). Cannabinoids for clinicians: the rise and fall of the cannabinoid antagonists. *Eur J Endocrinol, 161*(5), 655-662. doi: 10.1530/EJE-09-0511

Camus, G., Deby-Dupont, G., Deby, C., Juchmes-Ferir, A., Pincemail, J., & Lamy, M. (1993). Inflammatory response to strenuous muscular exercise in man. *Mediators Inflamm, 2*(5), 335-342. doi: 10.1155/S0962935193000468

Cannon, J. G., Fielding, R. A., Fiatarone, M. A., Orencole, S. F., Dinarello, C. A., & Evans, W. J. (1989). Increased interleukin 1 beta in human skeletal muscle after exercise. *Am J Physiol, 257*(2 Pt 2), R451-455. doi: 10.1152/ajpregu.1989.257.2.R451

Carbo, N., Lopez-Soriano, J., Costelli, P., Alvarez, B., Busquets, S., Baccino, F. M., . . . Argiles, J. M. (2001). Interleukin-15 mediates reciprocal regulation of adipose and muscle mass: a potential role in body weight control. *Biochim Biophys Acta, 1526*(1), 17-24.

Carey, A. L., Steinberg, G. R., Macaulay, S. L., Thomas, W. G., Holmes, A. G., Ramm, G., . . . Febbraio, M. A. (2006). Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase. *Diabetes, 55*(10), 2688-2697. doi: 10.2337/db05-1404

Cham, B. E., & Knowles, B. R. (1976). A solvent system for delipidation of plasma or serum without protein precipitation. *J Lipid Res, 17*(2), 176-181.

Chan, M. H., Carey, A. L., Watt, M. J., & Febbraio, M. A. (2004). Cytokine gene expression in human skeletal muscle during concentric contraction: evidence that IL-8, like IL-6, is influenced by glycogen availability. *Am J Physiol Regul Integr Comp Physiol, 287*(2), R322-327. doi: 10.1152/ajpregu.00030.2004

Chao, L. C., Bensinger, S. J., Villanueva, C. J., Wroblewski, K., & Tontonoz, P. (2008). Inhibition of adipocyte differentiation by Nur77, Nurr1, and Nor1. *Mol Endocrinol, 22*(12), 2596-2608. doi: 10.1210/me.2008-0161

Chao, L. C., Wroblewski, K., Zhang, Z., Pei, L., Vergnes, L., Ilkayeva, O. R., . . . Tontonoz, P. (2009). Insulin resistance and altered systemic glucose metabolism in mice lacking Nur77. *Diabetes, 58*(12), 2788-2796. doi: 10.2337/db09-0763

Chao, L. C., Zhang, Z., Pei, L., Saito, T., Tontonoz, P., & Pilch, P. F. (2007). Nur77 coordinately regulates expression of genes linked to glucose metabolism in skeletal muscle. *Mol Endocrinol, 21*(9), 2152-2163. doi: 10.1210/me.2007-0169

Chavez, J. A., & Summers, S. A. (2003). Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes. *Arch Biochem Biophys, 419*(2), 101-109.

Chavez, J. A., & Summers, S. A. (2012). A ceramide-centric view of insulin resistance. *Cell Metab, 15*(5), 585-594. doi: 10.1016/j.cmet.2012.04.002

Chen, W. (2005). Electroconformational denaturation of membrane proteins. *Ann N Y Acad Sci, 1066*, 92-105. doi: 10.1196/annals.1363.028

Chen, X., Cushman, S. W., Pannell, L. K., & Hess, S. (2005). Quantitative proteomic analysis of the secretory proteins from rat adipose cells using a 2D liquid chromatography-MS/MS approach. *J Proteome Res, 4*(2), 570-577. doi: 10.1021/pr049772a

Choe, S. S., Huh, J. Y., Hwang, I. J., Kim, J. I., & Kim, J. B. (2016). Adipose Tissue Remodeling: Its Role in Energy Metabolism and Metabolic Disorders. *Front Endocrinol (Lausanne), 7*, 30. doi: 10.3389/fendo.2016.00030

Cinar, R., Godlewski, G., Liu, J., Tam, J., Jourdan, T., Mukhopadhyay, B., . . . Kunos, G. (2014). Hepatic cannabinoid-1 receptors mediate diet-induced insulin resistance by increasing de novo synthesis of long-chain ceramides. *Hepatology, 59*(1), 143-153. doi: 10.1002/hep.26606

Clarkson, P. M. (1997). Eccentric exercise and muscle damage. *Int J Sports Med, 18 Suppl 4*, S314-317. doi: 10.1055/s-2007-972741

Clarkson, P. M., & Hubal, M. J. (2002). Exercise-induced muscle damage in humans. *Am J Phys Med Rehabil, 81*(11 Suppl), S52-69. doi: 10.1097/01.PHM.0000029772.45258.43

Coderre, L., Kandror, K. V., Vallega, G., & Pilch, P. F. (1995). Identification and characterization of an exercise-sensitive pool of glucose transporters in skeletal muscle. *J Biol Chem, 270*(46), 27584-27588.

Colombo, G., Agabio, R., Diaz, G., Lobina, C., Reali, R., & Gessa, G. L. (1998). Appetite suppression and weight loss after the cannabinoid antagonist SR 141716. *Life Sci, 63*(8), PL113-117.

Cooksey, R. C., Jouihan, H. A., Ajioka, R. S., Hazel, M. W., Jones, D. L., Kushner, J. P., & McClain, D. A. (2004). Oxidative stress, beta-cell apoptosis, and decreased insulin secretory capacity in mouse models of hemochromatosis. *Endocrinology, 145*(11), 5305-5312. doi: 10.1210/en.2004-0392

Cota, D. (2007). CB1 receptors: emerging evidence for central and peripheral mechanisms that regulate energy balance, metabolism, and cardiovascular health. *Diabetes Metab Res Rev, 23*(7), 507-517. doi: 10.1002/dmrr.764

Cota, D., Marsicano, G., Tschop, M., Grubler, Y., Flachskamm, C., Schubert, M., . . . Pagotto, U. (2003). The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. *J Clin Invest, 112*(3), 423-431. doi: 10.1172/JCI17725

Crespillo, A., Suarez, J., Bermudez-Silva, F. J., Rivera, P., Vida, M., Alonso, M., . . . Rodriguez de Fonseca, F. (2011). Expression of the cannabinoid system in muscle: effects of a high-fat diet and CB1 receptor blockade. *Biochem J, 433*(1), 175-185. doi: 10.1042/BJ20100751

Degenhardt, L., & Hall, W. (2012). Extent of illicit drug use and dependence, and their contribution to the global burden of disease. *Lancet, 379*(9810), 55-70. doi: 10.1016/S0140-6736(11)61138-0

Delbono, O., Xia, J., Treves, S., Wang, Z. M., Jimenez-Moreno, R., Payne, A. M., . . . Zorzato, F. (2007). Loss of skeletal muscle strength by ablation of the sarcoplasmic reticulum protein JP45. *Proc Natl Acad Sci U S A, 104*(50), 20108-20113. doi: 10.1073/pnas.0707389104

Dennis, R. G., Smith, B., Philp, A., Donnelly, K., & Baar, K. (2009). Bioreactors for guiding muscle tissue growth and development. *Adv Biochem Eng Biotechnol, 112*, 39-79. doi: 10.1007/978-3-540-69357-4_3

Derave, W., Hansen, B. F., Lund, S., Kristiansen, S., & Richter, E. A. (2000). Muscle glycogen content affects insulin-stimulated glucose transport and protein kinase B activity. *Am J Physiol Endocrinol Metab, 279*(5), E947-955.

Derave, W., Lund, S., Holman, G. D., Wojtaszewski, J., Pedersen, O., & Richter, E. A. (1999). Contraction-stimulated muscle glucose transport and GLUT-4 surface content are dependent on glycogen content. *Am J Physiol, 277*(6 Pt 1), E1103-1110.

Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., . . . Mechoulam, R. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science, 258*(5090), 1946-1949.

Di Marzo, V. (2008). The endocannabinoid system in obesity and type 2 diabetes. *Diabetologia, 51*(8), 1356-1367. doi: 10.1007/s00125-008-1048-2

Di Marzo, V., Goparaju, S. K., Wang, L., Liu, J., Batkai, S., Jarai, Z., . . . Kunos, G. (2001). Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature, 410*(6830), 822-825. doi: 10.1038/35071088

Di Marzo, V., & Matias, I. (2005). Endocannabinoid control of food intake and energy balance. *Nat Neurosci, 8*(5), 585-589. doi: 10.1038/nn1457

Dinh, T. P., Carpenter, D., Leslie, F. M., Freund, T. F., Katona, I., Sensi, S. L., . . . Piomelli, D. (2002). Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc Natl Acad Sci U S A, 99*(16), 10819-10824. doi: 10.1073/pnas.152334899

Donnelly, K., Khodabukus, A., Philp, A., Deldicque, L., Dennis, R. G., & Baar, K. (2010). A novel bioreactor for stimulating skeletal muscle in vitro. *Tissue Eng Part C Methods, 16*(4), 711-718. doi: 10.1089/ten.TEC.2009.0125

Douen, A. G., Ramlal, T., Rastogi, S., Bilan, P. J., Cartee, G. D., Vranic, M., . . . Klip, A. (1990). Exercise induces recruitment of the "insulin-responsive glucose transporter". Evidence for distinct intracellular insulin- and exercise-recruitable transporter pools in skeletal muscle. *J Biol Chem, 265*(23), 13427-13430.

Dow, D. E., Cederna, P. S., Hassett, C. A., Kostrominova, T. Y., Faulkner, J. A., & Dennis, R. G. (2004). Number of contractions to maintain mass and force of a denervated rat muscle. *Muscle Nerve, 30*(1), 77-86. doi: 10.1002/mus.20054

Dufaux, B., & Order, U. (1989). Plasma elastase-alpha 1-antitrypsin, neopterin, tumor necrosis factor, and soluble interleukin-2 receptor after prolonged exercise. *Int J Sports Med, 10*(6), 434-438. doi: 10.1055/s-2007-1024939

Eckardt, K., Sell, H., Taube, A., Koenen, M., Platzbecker, B., Cramer, A., . . . Eckel, J. (2009). Cannabinoid type 1 receptors in human skeletal muscle cells participate in the negative crosstalk between fat and muscle. *Diabetologia, 52*(4), 664-674. doi: 10.1007/s00125-008-1240-4

Elsohly, M. A., & Slade, D. (2005). Chemical constituents of marijuana: the complex mixture of natural cannabinoids. *Life Sci, 78*(5), 539-548. doi: 10.1016/j.lfs.2005.09.011

Engeli, S., Bohnke, J., Feldpausch, M., Gorzelniak, K., Janke, J., Batkai, S., . . . Jordan, J. (2005). Activation of the peripheral endocannabinoid system in human obesity. *Diabetes, 54*(10), 2838-2843.

Esposito, I., Proto, M. C., Gazzerro, P., Laezza, C., Miele, C., Alberobello, A. T., . . . Bifulco, M. (2008). The cannabinoid CB1 receptor antagonist rimonabant stimulates 2-deoxyglucose uptake in skeletal muscle cells by regulating the expression of phosphatidylinositol-3-kinase. *Mol Pharmacol, 74*(6), 1678-1686. doi: 10.1124/mol.108.049205

Fain, J. N., Madan, A. K., Hiler, M. L., Cheema, P., & Bahouth, S. W. (2004). Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology, 145*(5), 2273-2282. doi: 10.1210/en.2003-1336

Febbraio, M. A., & Pedersen, B. K. (2002). Muscle-derived interleukin-6: mechanisms for activation and possible biological roles. *FASEB J, 16*(11), 1335-1347. doi: 10.1096/fj.01-0876rev

Febbraio, M. A., & Pedersen, B. K. (2005). Contraction-induced myokine production and release: is skeletal muscle an endocrine organ? *Exerc Sport Sci Rev, 33*(3), 114-119.

Fischer, C. P. (2006). Interleukin-6 in acute exercise and training: what is the biological relevance? *Exerc Immunol Rev, 12*, 6-33.

Flower, D. R. (1996). The lipocalin protein family: structure and function. *Biochem J, 318 (Pt 1)*, 1-14.

Foley, K., Boguslavsky, S., & Klip, A. (2011). Endocytosis, recycling, and regulated exocytosis of glucose transporter 4. *Biochemistry, 50*(15), 3048-3061. doi: 10.1021/bi2000356

Frayn, K. N., Karpe, F., Fielding, B. A., Macdonald, I. A., & Coppack, S. W. (2003). Integrative physiology of human adipose tissue. *Int J Obes Relat Metab Disord, 27*(8), 875-888. doi: 10.1038/sj.ijo.0802326

Freund, T. F., Katona, I., & Piomelli, D. (2003). Role of endogenous cannabinoids in synaptic signaling. *Physiol Rev, 83*(3), 1017-1066. doi: 10.1152/physrev.00004.2003

Fruman, D. A., Cantley, L. C., & Carpenter, C. L. (1996). Structural organization and alternative splicing of the murine phosphoinositide 3-kinase p85 alpha gene. *Genomics, 37*(1), 113-121. doi: 10.1006/geno.1996.0527

Fruman, D. A., Meyers, R. E., & Cantley, L. C. (1998). Phosphoinositide kinases. *Annu Rev Biochem, 67*, 481-507. doi: 10.1146/annurev.biochem.67.1.481

Fueger, P. T., Li, C. Y., Ayala, J. E., Shearer, J., Bracy, D. P., Charron, M. J., . . . Wasserman, D. H. (2007). Glucose kinetics and exercise tolerance in mice lacking the GLUT4 glucose transporter. *J Physiol, 582*(Pt 2), 801-812. doi: 10.1113/jphysiol.2007.132902

Fujita, H., Nedachi, T., & Kanzaki, M. (2007). Accelerated de novo sarcomere assembly by electric pulse stimulation in C2C12 myotubes. *Exp Cell Res, 313*(9), 1853-1865. doi: 10.1016/j.yexcr.2007.03.002

Fumoto, T., Yamaguchi, T., Hirose, F., & Osumi, T. (2007). Orphan nuclear receptor Nur77 accelerates the initial phase of adipocyte differentiation in 3T3-L1 cells by promoting mitotic clonal expansion. *J Biochem, 141*(2), 181-192. doi: 10.1093/jb/mvm018

Furmanczyk, P. S., & Quinn, L. S. (2003). Interleukin-15 increases myosin accretion in human skeletal myogenic cultures. *Cell Biol Int, 27*(10), 845-851.

Galiegue, S., Mary, S., Marchand, J., Dussossoy, D., Carriere, D., Carayon, P., . . . Casellas, P. (1995). Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem, 232*(1), 54-61.

Garay-Rojas, E., Harper, M., Hraba-Renevey, S., & Kress, M. (1996). An apparent autocrine mechanism amplifies the dexamethasone- and retinoic acid-induced expression of mouse lipocalin-encoding gene 24p3. *Gene, 170*(2), 173-180.

Gaster, M., Rustan, A. C., Aas, V., & Beck-Nielsen, H. (2004). Reduced lipid oxidation in skeletal muscle from type 2 diabetic subjects may be of genetic origin: evidence from cultured myotubes. *Diabetes, 53*(3), 542-548.

Gibson, M. C., & Schultz, E. (1983). Age-related differences in absolute numbers of skeletal muscle satellite cells. *Muscle Nerve, 6*(8), 574-580. doi: 10.1002/mus.880060807

Gomez, R., Navarro, M., Ferrer, B., Trigo, J. M., Bilbao, A., Del Arco, I., . . . Rodriguez de Fonseca, F. (2002). A peripheral mechanism for CB1 cannabinoid receptor-dependent modulation of feeding. *J Neurosci, 22*(21), 9612-9617.

Gonthier, M. P., Hoareau, L., Festy, F., Matias, I., Valenti, M., Bes-Houtmann, S., . . . Roche, R. (2007). Identification of endocannabinoids and related compounds in human fat cells. *Obesity (Silver Spring), 15*(4), 837-845. doi: 10.1038/oby.2007.581

Gonzales, A. M., & Orlando, R. A. (2007). Role of adipocyte-derived lipoprotein lipase in adipocyte hypertrophy. *Nutr Metab (Lond), 4*, 22. doi: 10.1186/1743-7075-4-22

Grabstein, K. H., Eisenman, J., Shanebeck, K., Rauch, C., Srinivasan, S., Fung, V., . . . et al. (1994). Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. *Science, 264*(5161), 965-968.

Gray, T. S., & Morley, J. E. (1986). Neuropeptide Y: anatomical distribution and possible function in mammalian nervous system. *Life Sci, 38*(5), 389-401.

Greenberg, I., Kuehnle, J., Mendelson, J. H., & Bernstein, J. G. (1976). Effects of marihuana use on body weight and caloric intake in humans. *Psychopharmacology (Berl), 49*(1), 79-84.

Gstraunthaler, G. (2003). Alternatives to the use of fetal bovine serum: serum-free cell culture. *ALTEX, 20*(4), 275-281.

Haddad, M. (2014). What does Rimonabant do in rat primary skeletal muscle cells. *Biomed Pharmacol J, 7*, 81-92.

Hajduch, E., Balendran, A., Batty, I. H., Litherland, G. J., Blair, A. S., Downes, C. P., & Hundal, H. S. (2001). Ceramide impairs the insulin-dependent membrane recruitment of protein kinase B leading to a loss in downstream signalling in L6 skeletal muscle cells. *Diabetologia, 44*(2), 173-183. doi: 10.1007/s001250051596

Hall, W., & Degenhardt, L. (2009). Adverse health effects of non-medical cannabis use. *Lancet, 374*(9698), 1383-1391. doi: 10.1016/S0140-6736(09)61037-0

Hall, W., & Solowij, N. (1998). Adverse effects of cannabis. *Lancet, 352*(9140), 1611-1616. doi: 10.1016/S0140-6736(98)05021-1

Handschin, C., & Spiegelman, B. M. (2008). The role of exercise and PGC1alpha in inflammation and chronic disease. *Nature, 454*(7203), 463-469. doi: 10.1038/nature07206

Hao, S., Avraham, Y., Mechoulam, R., & Berry, E. M. (2000). Low dose anandamide affects food intake, cognitive function, neurotransmitter and corticosterone levels in diet-restricted mice. *Eur J Pharmacol, 392*(3), 147-156.

Hargreaves, M., Meredith, I., & Jennings, G. L. (1992). Muscle glycogen and glucose uptake during exercise in humans. *Exp Physiol, 77*(4), 641-644.

Hayashi, T., Hirshman, M. F., Dufresne, S. D., & Goodyear, L. J. (1999). Skeletal muscle contractile activity in vitro stimulates mitogen-activated protein kinase signaling. *Am J Physiol, 277*(4 Pt 1), C701-707.

Henriksson, J. (1995). Muscle fuel selection: effect of exercise and training. *Proc Nutr Soc, 54*(1), 125-138.

Henson, D. A., Nieman, D. C., Nehlsen-Cannarella, S. L., Fagoaga, O. R., Shannon, M., Bolton, M. R., . . . Schilling, B. K. (2000). Influence of carbohydrate on cytokine and phagocytic responses to 2 h of rowing. *Med Sci Sports Exerc, 32*(8), 1384-1389.

Hespel, P., Vergauwen, L., Vandenberghe, K., & Richter, E. A. (1995). Important role of insulin and flow in stimulating glucose uptake in contracting skeletal muscle. *Diabetes, 44*(2), 210-215.

Hohmann, A. G., & Suplita, R. L., 2nd. (2006). Endocannabinoid mechanisms of pain modulation. *AAPS J, 8*(4), E693-708. doi: 10.1208/aapsj080479

Hong, W. (1998). Protein transport from the endoplasmic reticulum to the Golgi apparatus. *J Cell Sci, 111 (Pt 19)*, 2831-2839.

Howlett, A. C. (2002). The cannabinoid receptors. *Prostaglandins Other Lipid Mediat, 68-69*, 619-631.

Howlett, A. C., Barth, F., Bonner, T. I., Cabral, G., Casellas, P., Devane, W. A., . . . Pertwee, R. G. (2002). International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev, 54*(2), 161-202.

Howlett, A. C., Breivogel, C. S., Childers, S. R., Deadwyler, S. A., Hampson, R. E., & Porrino, L. J. (2004). Cannabinoid physiology and pharmacology: 30 years of progress. *Neuropharmacology, 47 Suppl 1*, 345-358. doi: 10.1016/j.neuropharm.2004.07.030

Huang, H. L., Chu, S. T., & Chen, Y. H. (1999). Ovarian steroids regulate 24p3 expression in mouse uterus during the natural estrous cycle and the preimplantation period. *J Endocrinol, 162*(1), 11-19.

Huang, Y., Yang, Z., Ye, Z., Li, Q., Wen, J., Tao, X., . . . Hu, R. (2012). Lipocalin-2, glucose metabolism and chronic low-grade systemic inflammation in Chinese people. *Cardiovasc Diabetol, 11*, 11. doi: 10.1186/1475-2840-11-11

Inukai, K., Funaki, M., Ogihara, T., Katagiri, H., Kanda, A., Anai, M., . . . Asano, T. (1997). p85alpha gene generates three isoforms of regulatory subunit for phosphatidylinositol 3-kinase (PI 3-Kinase), p50alpha, p55alpha, and p85alpha, with different PI 3-kinase activity elevating responses to insulin. *J Biol Chem, 272*(12), 7873-7882.

Inyard, A. C., Clerk, L. H., Vincent, M. A., & Barrett, E. J. (2007). Contraction stimulates nitric oxide independent microvascular recruitment and increases muscle insulin uptake. *Diabetes, 56*(9), 2194-2200. doi: 10.2337/db07-0020

Ito, A., Yamamoto, Y., Sato, M., Ikeda, K., Yamamoto, M., Fujita, H., . . . Kamihira, M. (2014). Induction of functional tissue-engineered skeletal muscle constructs by defined electrical stimulation. *Sci Rep, 4*, 4781. doi: 10.1038/srep04781

James, D. E., Jenkins, A. B., & Kraegen, E. W. (1985). Heterogeneity of insulin action in individual muscles in vivo: euglycemic clamp studies in rats. *Am J Physiol, 248*(5 Pt 1), E567-574.

Jamshidi, N., & Taylor, D. A. (2001). Anandamide administration into the ventromedial hypothalamus stimulates appetite in rats. *Br J Pharmacol, 134*(6), 1151-1154. doi: 10.1038/sj.bjp.0704379

Jbilo, O., Ravinet-Trillou, C., Arnone, M., Buisson, I., Bribes, E., Peleraux, A., . . . Casellas, P. (2005). The CB1 receptor antagonist rimonabant reverses the diet-induced obesity phenotype through the regulation of lipolysis and energy balance. *FASEB J, 19*(11), 1567-1569. doi: 10.1096/fj.04-3177fje

Jensen, T. E., Rose, A. J., Hellsten, Y., Wojtaszewski, J. F., & Richter, E. A. (2007). Caffeine-induced Ca(2+) release increases AMPK-dependent glucose uptake in rodent soleus muscle. *Am J Physiol Endocrinol Metab, 293*(1), E286-292. doi: 10.1152/ajpendo.00693.2006

Jessen, N., & Goodyear, L. J. (2005). Contraction signaling to glucose transport in skeletal muscle. *J Appl Physiol (1985), 99*(1), 330-337. doi: 10.1152/japplphysiol.00175.2005

Jhanwar-Uniyal, M., Beck, B., Jhanwar, Y. S., Burlet, C., & Leibowitz, S. F. (1993). Neuropeptide Y projection from arcuate nucleus to parvocellular division of paraventricular nucleus: specific relation to the ingestion of carbohydrate. *Brain Res, 631*(1), 97-106.

Jhun, B. H., Rose, D. W., Seely, B. L., Rameh, L., Cantley, L., Saltiel, A. R., & Olefsky, J. M. (1994). Microinjection of the SH2 domain of the 85-kilodalton subunit of phosphatidylinositol 3-kinase inhibits insulin-induced DNA synthesis and c-fos expression. *Mol Cell Biol, 14*(11), 7466-7475.

Juan-Pico, P., Fuentes, E., Bermudez-Silva, F. J., Javier Diaz-Molina, F., Ripoll, C., Rodriguez de Fonseca, F., & Nadal, A. (2006). Cannabinoid receptors regulate Ca(2+) signals and insulin secretion in pancreatic beta-cell. *Cell Calcium, 39*(2), 155-162. doi: 10.1016/j.ceca.2005.10.005

Kadi, F., Charifi, N., & Henriksson, J. (2006). The number of satellite cells in slow and fast fibres from human vastus lateralis muscle. *Histochem Cell Biol, 126*(1), 83-87. doi: 10.1007/s00418-005-0102-0

Kahn, B. B., Alquier, T., Carling, D., & Hardie, D. G. (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab, 1*(1), 15-25. doi: 10.1016/j.cmet.2004.12.003

Kahn, B. B., & Flier, J. S. (2000). Obesity and insulin resistance. *J Clin Invest, 106*(4), 473-481. doi: 10.1172/JCI10842

Kamimura, D., Ishihara, K., & Hirano, T. (2003). IL-6 signal transduction and its physiological roles: the signal orchestration model. *Rev Physiol Biochem Pharmacol, 149*, 1-38. doi: 10.1007/s10254-003-0012-2

Katz, A., Broberg, S., Sahlin, K., & Wahren, J. (1986). Leg glucose uptake during maximal dynamic exercise in humans. *Am J Physiol, 251*(1 Pt 1), E65-70.

Kelly, M., Keller, C., Avilucea, P. R., Keller, P., Luo, Z., Xiang, X., . . . Ruderman, N. B. (2004). AMPK activity is diminished in tissues of IL-6 knockout mice: the effect of exercise. *Biochem Biophys Res Commun, 320*(2), 449-454. doi: 10.1016/j.bbrc.2004.05.188

Khan, A. H., & Pessin, J. E. (2002). Insulin regulation of glucose uptake: a complex interplay of intracellular signalling pathways. *Diabetologia, 45*(11), 1475-1483. doi: 10.1007/s00125-002-0974-7

Kiens, B. (2006). Skeletal muscle lipid metabolism in exercise and insulin resistance. *Physiol Rev, 86*(1), 205-243. doi: 10.1152/physrev.00023.2004

Kim, H. J., Higashimori, T., Park, S. Y., Choi, H., Dong, J., Kim, Y. J., . . . Kim, J. K. (2004). Differential effects of interleukin-6 and -10 on skeletal muscle and liver insulin action in vivo. *Diabetes, 53*(4), 1060-1067.

Kishimoto, T., Akira, S., Narazaki, M., & Taga, T. (1995). Interleukin-6 family of cytokines and gp130. *Blood, 86*(4), 1243-1254.

Kjeldsen, L., Bainton, D. F., Sengelov, H., & Borregaard, N. (1994). Identification of neutrophil gelatinase-associated lipocalin as a novel matrix protein of specific granules in human neutrophils. *Blood, 83*(3), 799-807.

Kjeldsen, L., Cowland, J. B., & Borregaard, N. (2000). Human neutrophil gelatinase-associated lipocalin and homologous proteins in rat and mouse. *Biochim Biophys Acta, 1482*(1-2), 272-283.

Kjeldsen, L., Johnsen, A. H., Sengelov, H., & Borregaard, N. (1993). Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J Biol Chem, 268*(14), 10425-10432.

Klover, P. J., Clementi, A. H., & Mooney, R. A. (2005). Interleukin-6 depletion selectively improves hepatic insulin action in obesity. *Endocrinology, 146*(8), 3417-3427. doi: 10.1210/en.2004-1468

Klover, P. J., Zimmers, T. A., Koniaris, L. G., & Mooney, R. A. (2003). Chronic exposure to interleukin-6 causes hepatic insulin resistance in mice. *Diabetes, 52*(11), 2784-2789.

Knight, J. B., Eyster, C. A., Griesel, B. A., & Olson, A. L. (2003). Regulation of the human GLUT4 gene promoter: interaction between a transcriptional activator and myocyte enhancer factor 2A. *Proc Natl Acad Sci U S A, 100*(25), 14725-14730. doi: 10.1073/pnas.2432756100

Koch, A. E., Polverini, P. J., Kunkel, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M., . . . Strieter, R. M. (1992). Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science, 258*(5089), 1798-1801.

Kotani, K., Ogawa, W., Matsumoto, M., Kitamura, T., Sakaue, H., Hino, Y., . . . Kasuga, M. (1998). Requirement of atypical protein kinase clambda for insulin stimulation of glucose uptake but not for Akt activation in 3T3-L1 adipocytes. *Mol Cell Biol, 18*(12), 6971-6982.

Kraniou, G. N., Cameron-Smith, D., & Hargreaves, M. (2006). Acute exercise and GLUT4 expression in human skeletal muscle: influence of exercise intensity. *J Appl Physiol (1985), 101*(3), 934-937. doi: 10.1152/japplphysiol.01489.2005

Kumar, R. N., Chambers, W. A., & Pertwee, R. G. (2001). Pharmacological actions and therapeutic uses of cannabis and cannabinoids. *Anaesthesia, 56*(11), 1059-1068.

Kuo, C. H., Hunt, D. G., Ding, Z., & Ivy, J. L. (1999). Effect of carbohydrate supplementation on postexercise GLUT-4 protein expression in skeletal muscle. *J Appl Physiol (1985), 87*(6), 2290-2295.

Kyrou, I., Valsamakis, G., & Tsigos, C. (2006). The endocannabinoid system as a target for the treatment of visceral obesity and metabolic syndrome. *Ann N Y Acad Sci, 1083*, 270-305. doi: 10.1196/annals.1367.024

Lagathu, C., Bastard, J. P., Auclair, M., Maachi, M., Capeau, J., & Caron, M. (2003). Chronic interleukin-6 (IL-6) treatment increased IL-6 secretion and induced insulin resistance in adipocyte: prevention by rosiglitazone. *Biochem Biophys Res Commun, 311*(2), 372-379.

LaLonde, J. M., Bernlohr, D. A., & Banaszak, L. J. (1994). The up-and-down beta-barrel proteins. *FASEB J, 8*(15), 1240-1247.

Lambernd, S., Taube, A., Schober, A., Platzbecker, B., Gorgens, S. W., Schlich, R., . . . Eckel, J. (2012). Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways. *Diabetologia, 55*(4), 1128-1139. doi: 10.1007/s00125-012-2454-z

Law, I. K., Xu, A., Lam, K. S., Berger, T., Mak, T. W., Vanhoutte, P. M., . . . Wang, Y. (2010). Lipocalin-2 deficiency attenuates insulin resistance associated with aging and obesity. *Diabetes, 59*(4), 872-882. doi: 10.2337/db09-1541

Lee, A. D., Hansen, P. A., & Holloszy, J. O. (1995). Wortmannin inhibits insulin-stimulated but not contraction-stimulated glucose transport activity in skeletal muscle. *FEBS Lett, 361*(1), 51-54.

Lee, Y. H., Lee, S. H., Jung, E. S., Kim, J. S., Shim, C. Y., Ko, Y. G., . . . Ha, J. W. (2010). Visceral adiposity and the severity of coronary artery disease in middle-aged subjects with normal waist circumference and its relation with lipocalin-2 and MCP-1. *Atherosclerosis, 213*(2), 592-597. doi: 10.1016/j.atherosclerosis.2010.09.012

Levine, A. S., & Morley, J. E. (1984). Neuropeptide Y: a potent inducer of consummatory behavior in rats. *Peptides, 5*(6), 1025-1029.

Levy, M., & Futerman, A. H. (2010). Mammalian ceramide synthases. *IUBMB Life, 62*(5), 347-356. doi: 10.1002/iub.319

Li, C., & Chan, Y. R. (2011). Lipocalin 2 regulation and its complex role in inflammation and cancer. *Cytokine, 56*(2), 435-441. doi: 10.1016/j.cyto.2011.07.021

Liang, H., & Ward, W. F. (2006). PGC-1alpha: a key regulator of energy metabolism. *Adv Physiol Educ, 30*(4), 145-151. doi: 10.1152/advan.00052.2006

Liew, C. W., Bochenski, J., Kawamori, D., Hu, J., Leech, C. A., Wanic, K., . . . Kulkarni, R. N. (2010). The pseudokinase tribbles homolog 3 interacts with ATF4 to negatively regulate insulin exocytosis in human and mouse beta cells. *J Clin Invest, 120*(8), 2876-2888. doi: 10.1172/JCI36849

Lindborg, K. A., Teachey, M. K., Jacob, S., & Henriksen, E. J. (2010). Effects of in vitro antagonism of endocannabinoid-1 receptors on the glucose transport system in normal and insulin-resistant rat skeletal muscle. *Diabetes Obes Metab, 12*(8), 722-730. doi: 10.1111/j.1463-1326.2010.01227.x

Lipina, C., Stretton, C., Hastings, S., Hundal, J. S., Mackie, K., Irving, A. J., & Hundal, H. S. (2010). Regulation of MAP kinase-directed mitogenic and protein kinase B-mediated signaling by cannabinoid receptor type 1 in skeletal muscle cells. *Diabetes, 59*(2), 375-385. doi: 10.2337/db09-0979

Liu, J., Wu, X., Franklin, J. L., Messina, J. L., Hill, H. S., Moellering, D. R., . . . Garvey, W. T. (2010). Mammalian Tribbles homolog 3 impairs insulin action in skeletal muscle: role in glucose-induced insulin resistance. *Am J Physiol Endocrinol Metab, 298*(3), E565-576. doi: 10.1152/ajpendo.00467.2009

Liu, Y. L., Connoley, I. P., Wilson, C. A., & Stock, M. J. (2005). Effects of the cannabinoid CB1 receptor antagonist SR141716 on oxygen consumption and soleus muscle glucose uptake in Lep(ob)/Lep(ob) mice. *Int J Obes (Lond), 29*(2), 183-187. doi: 10.1038/sj.ijo.0802847

Lund, S., Holman, G. D., Schmitz, O., & Pedersen, O. (1995). Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin. *Proc Natl Acad Sci U S A, 92*(13), 5817-5821.

Lyngso, D., Simonsen, L., & Bulow, J. (2002). Interleukin-6 production in human subcutaneous abdominal adipose tissue: the effect of exercise. *J Physiol, 543*(Pt 1), 373-378.

Mahoney, D. J., Parise, G., Melov, S., Safdar, A., & Tarnopolsky, M. A. (2005). Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise. *FASEB J, 19*(11), 1498-1500. doi: 10.1096/fj.04-3149fje

Manabe, Y., Miyatake, S., Takagi, M., Nakamura, M., Okeda, A., Nakano, T., . . . Fujii, N. L. (2012). Characterization of an acute muscle contraction model using cultured C2C12 myotubes. *PLoS One, 7*(12), e52592. doi: 10.1371/journal.pone.0052592

Massa, F., Storr, M., & Lutz, B. (2005). The endocannabinoid system in the physiology and pathophysiology of the gastrointestinal tract. *J Mol Med (Berl), 83*(12), 944-954. doi: 10.1007/s00109-005-0698-5

Matias, I., & Di Marzo, V. (2007). Endocannabinoids and the control of energy balance. *Trends Endocrinol Metab, 18*(1), 27-37. doi: 10.1016/j.tem.2006.11.006

Matias, I., Gonthier, M. P., Orlando, P., Martiadis, V., De Petrocellis, L., Cervino, C., . . . Di Marzo, V. (2006). Regulation, function, and dysregulation of endocannabinoids in models of adipose and beta-pancreatic cells and in obesity and hyperglycemia. *J Clin Endocrinol Metab, 91*(8), 3171-3180. doi: 10.1210/jc.2005-2679

Mauvais-Jarvis, F., Ueki, K., Fruman, D. A., Hirshman, M. F., Sakamoto, K., Goodyear, L. J., . . . Kahn, C. R. (2002). Reduced expression of the murine p85alpha subunit of phosphoinositide 3-kinase improves insulin signaling and ameliorates diabetes. *J Clin Invest, 109*(1), 141-149. doi: 10.1172/JCI13305

Maxwell, M. A., Cleasby, M. E., Harding, A., Stark, A., Cooney, G. J., & Muscat, G. E. (2005). Nur77 regulates lipolysis in skeletal muscle cells. Evidence for cross-talk between the beta-adrenergic and an orphan nuclear hormone receptor pathway. *J Biol Chem, 280*(13), 12573-12584. doi: 10.1074/jbc.M409580200

McGee, S. L., & Hargreaves, M. (2004). Exercise and myocyte enhancer factor 2 regulation in human skeletal muscle. *Diabetes, 53*(5), 1208-1214.

Mechoulam, R., Devane, W. A., Breuer, A., & Zahalka, J. (1991). A random walk through a cannabis field. *Pharmacol Biochem Behav, 40*(3), 461-464.

Mechoulam, R., Fride, E., & Di Marzo, V. (1998). Endocannabinoids. *Eur J Pharmacol, 359*(1), 1-18.

Michael, L. F., Wu, Z., Cheatham, R. B., Puigserver, P., Adelmant, G., Lehman, J. J., . . . Spiegelman, B. M. (2001). Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. *Proc Natl Acad Sci U S A, 98*(7), 3820-3825. doi: 10.1073/pnas.061035098

Mosialou, I., Shikhel, S., Liu, J. M., Maurizi, A., Luo, N., He, Z., . . . Kousteni, S. (2017). Corrigendum: MC4R-dependent suppression of appetite by bone-derived lipocalin 2. *Nature, 546*(7658), 440. doi: 10.1038/nature22808

Munro, S., Thomas, K. L., & Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature, 365*(6441), 61-65. doi: 10.1038/365061a0

Nedachi, T., Fujita, H., & Kanzaki, M. (2008). Contractile C2C12 myotube model for studying exercise-inducible responses in skeletal muscle. *Am J Physiol Endocrinol Metab, 295*(5), E1191-1204. doi: 10.1152/ajpendo.90280.2008

Nielsen, A. R., Hojman, P., Erikstrup, C., Fischer, C. P., Plomgaard, P., Mounier, R., . . . Pedersen, B. K. (2008). Association between interleukin-15 and obesity: interleukin-15 as a potential regulator of fat mass. *J Clin Endocrinol Metab, 93*(11), 4486-4493. doi: 10.1210/jc.2007-2561

Nielsen, A. R., Mounier, R., Plomgaard, P., Mortensen, O. H., Penkowa, M., Speerschneider, T., . . . Pedersen, B. K. (2007). Expression of interleukin-15 in human skeletal muscle effect of exercise and muscle fibre type composition. *J Physiol, 584*(Pt 1), 305-312. doi: 10.1113/jphysiol.2007.139618

Nieman, D. C., Davis, J. M., Henson, D. A., Walberg-Rankin, J., Shute, M., Dumke, C. L., . . . McAnulty, L. S. (2003). Carbohydrate ingestion influences skeletal muscle cytokine mRNA and plasma cytokine levels after a 3-h run. *J Appl Physiol (1985), 94*(5), 1917-1925. doi: 10.1152/japplphysiol.01130.2002

Nieman, D. C., Henson, D. A., McAnulty, S. R., McAnulty, L., Swick, N. S., Utter, A. C., . . . Morrow, J. D. (2002). Influence of vitamin C supplementation on oxidative and immune changes after an ultramarathon. *J Appl Physiol (1985), 92*(5), 1970-1977. doi: 10.1152/japplphysiol.00961.2001

Nieman, D. C., Henson, D. A., Smith, L. L., Utter, A. C., Vinci, D. M., Davis, J. M., . . . Shute, M. (2001). Cytokine changes after a marathon race. *J Appl Physiol (1985), 91*(1), 109-114.

Nikolic, N., Bakke, S. S., Kase, E. T., Rudberg, I., Flo Halle, I., Rustan, A. C., . . . Aas, V. (2012). Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise. *PLoS One, 7*(3), e33203. doi: 10.1371/journal.pone.0033203

Nogueiras, R., Diaz-Arteaga, A., Lockie, S. H., Velasquez, D. A., Tschop, J., Lopez, M., . . . Tschop, M. H. (2009). The endocannabinoid system: role in glucose and energy metabolism. *Pharmacol Res, 60*(2), 93-98. doi: 10.1016/j.phrs.2009.04.004

Nogueiras, R., Veyrat-Durebex, C., Suchanek, P. M., Klein, M., Tschop, J., Caldwell, C., . . . Tschop, M. H. (2008). Peripheral, but not central, CB1 antagonism provides food intake-independent metabolic benefits in diet-induced obese rats. *Diabetes, 57*(11), 2977-2991. doi: 10.2337/db08-0161

Nonogaki, K., Kaji, T., Ohba, Y., Sumii, M., Wakameda, M., & Tamari, T. (2009). Serotonin 5-HT2C receptor-independent expression of hypothalamic NOR1, a novel modulator of food intake and energy balance, in mice. *Biochem Biophys Res Commun, 386*(2), 311-315. doi: 10.1016/j.bbrc.2009.06.023

Norris, S. M., Bombardier, E., Smith, I. C., Vigna, C., & Tupling, A. R. (2010). ATP consumption by sarcoplasmic reticulum Ca2+ pumps accounts for 50% of resting metabolic rate in mouse fast and slow twitch skeletal muscle. *Am J Physiol Cell Physiol, 298*(3), C521-529. doi: 10.1152/ajpcell.00479.2009

O'Keefe, L., Simcocks, A. C., Hryciw, D. H., Mathai, M. L., & McAinch, A. J. (2013). The cannabinoid receptor 1 and its role in influencing peripheral metabolism. *Diabetes Obes Metab*. doi: 10.1111/dom.12144

Ofek, O., Karsak, M., Leclerc, N., Fogel, M., Frenkel, B., Wright, K., . . . Bab, I. (2006). Peripheral cannabinoid receptor, CB2, regulates bone mass. *Proc Natl Acad Sci U S A, 103*(3), 696-701. doi: 10.1073/pnas.0504187103

Oita, R. C., Mazzatti, D. J., Lim, F. L., Powell, J. R., & Merry, B. J. (2009). Whole-genome microarray analysis identifies up-regulation of Nr4a nuclear receptors in muscle and liver from diet-restricted rats. *Mech Ageing Dev, 130*(4), 240-247. doi: 10.1016/j.mad.2008.12.004

Osei-Hyiaman, D., DePetrillo, M., Pacher, P., Liu, J., Radaeva, S., Batkai, S., . . . Kunos, G. (2005). Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *J Clin Invest, 115*(5), 1298-1305. doi: 10.1172/JCI23057

Oshel, K. M., Knight, J. B., Cao, K. T., Thai, M. V., & Olson, A. L. (2000). Identification of a 30-base pair regulatory element and novel DNA binding protein that regulates the human GLUT4 promoter in transgenic mice. *J Biol Chem, 275*(31), 23666-23673. doi: 10.1074/jbc.M001452200

Ostrowski, K., Rohde, T., Asp, S., Schjerling, P., & Pedersen, B. K. (2001). Chemokines are elevated in plasma after strenuous exercise in humans. *Eur J Appl Physiol, 84*(3), 244-245. doi: 10.1007/s004210170012

Pacher, P., Batkai, S., & Kunos, G. (2006). The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol Rev, 58*(3), 389-462. doi: 10.1124/pr.58.3.2

Pagano, C., Pilon, C., Calcagno, A., Urbanet, R., Rossato, M., Milan, G., . . . Vettor, R. (2007). The endogenous cannabinoid system stimulates glucose uptake in human fat cells via phosphatidylinositol 3-kinase and calcium-dependent mechanisms. *J Clin Endocrinol Metab, 92*(12), 4810-4819. doi: 10.1210/jc.2007-0768

Pagotto, U., Marsicano, G., Cota, D., Lutz, B., & Pasquali, R. (2006). The emerging role of the endocannabinoid system in endocrine regulation and energy balance. *Endocr Rev, 27*(1), 73-100. doi: 10.1210/er.2005-0009

Panidis, D., Tziomalos, K., Koiou, E., Kandaraki, E. A., Tsourdi, E., Delkos, D., . . . Katsikis, I. (2010). The effects of obesity and polycystic ovary syndrome on serum lipocalin-2 levels: a cross-sectional study. *Reprod Biol Endocrinol, 8*, 151. doi: 10.1186/1477-7827-8-151

Park, S. Y., Gifford, J. R., Andtbacka, R. H., Trinity, J. D., Hyngstrom, J. R., Garten, R. S., . . . Richardson, R. S. (2014). Cardiac, skeletal, and smooth muscle mitochondrial respiration: are all mitochondria created equal? *Am J Physiol Heart Circ Physiol, 307*(3), H346-352. doi: 10.1152/ajpheart.00227.2014

Pawluczyk, I. Z., Furness, P. N., & Harris, K. P. (2003). Macrophage-induced rat mesangial cell expression of the 24p3-like protein alpha-2-microglobulin-related protein. *Biochim Biophys Acta, 1645*(2), 218-227.

Pearen, M. A., Myers, S. A., Raichur, S., Ryall, J. G., Lynch, G. S., & Muscat, G. E. (2008). The orphan nuclear receptor, NOR-1, a target of beta-adrenergic signaling, regulates gene expression that controls oxidative metabolism in skeletal muscle. *Endocrinology, 149*(6), 2853-2865. doi: 10.1210/en.2007-1202

Pedersen, B. K. (2009). The diseasome of physical inactivity--and the role of myokines in muscle--fat cross talk. *J Physiol, 587*(Pt 23), 5559-5568. doi: 10.1113/jphysiol.2009.179515

Pedersen, B. K. (2011a). Exercise-induced myokines and their role in chronic diseases. *Brain Behav Immun, 25*(5), 811-816. doi: 10.1016/j.bbi.2011.02.010

Pedersen, B. K. (2011b). Muscles and their myokines. *J Exp Biol, 214*(Pt 2), 337-346. doi: 10.1242/jeb.048074

Pedersen, B. K., Akerstrom, T. C., Nielsen, A. R., & Fischer, C. P. (2007). Role of myokines in exercise and metabolism. *J Appl Physiol (1985), 103*(3), 1093-1098. doi: 10.1152/japplphysiol.00080.2007

Pedersen, B. K., & Febbraio, M. A. (2008). Muscle as an endocrine organ: focus on muscle-derived interleukin-6. *Physiol Rev, 88*(4), 1379-1406. doi: 10.1152/physrev.90100.2007

Pedersen, B. K., & Fischer, C. P. (2007). Beneficial health effects of exercise--the role of IL-6 as a myokine. *Trends Pharmacol Sci, 28*(4), 152-156. doi: 10.1016/j.tips.2007.02.002

Pedersen, B. K., Steensberg, A., Fischer, C., Keller, C., Keller, P., Plomgaard, P., . . . Saltin, B. (2003). Searching for the exercise factor: is IL-6 a candidate? *J Muscle Res Cell Motil, 24*(2-3), 113-119.

Pedersen, B. K., Steensberg, A., Keller, P., Keller, C., Fischer, C., Hiscock, N., . . . Febbraio, M. A. (2003). Muscle-derived interleukin-6: lipolytic, anti-inflammatory and immune regulatory effects. *Pflugers Arch, 446*(1), 9-16. doi: 10.1007/s00424-002-0981-z

Pei, L., Waki, H., Vaitheesvaran, B., Wilpitz, D. C., Kurland, I. J., & Tontonoz, P. (2006). NR4A orphan nuclear receptors are transcriptional regulators of hepatic glucose metabolism. *Nat Med, 12*(9), 1048-1055. doi: 10.1038/nm1471

Peng, G., Li, L., Liu, Y., Pu, J., Zhang, S., Yu, J., . . . Liu, P. (2011). Oleate blocks palmitate-induced abnormal lipid distribution, endoplasmic reticulum expansion and stress, and insulin resistance in skeletal muscle. *Endocrinology, 152*(6), 2206-2218. doi: 10.1210/en.2010-1369

Pertwee, R. G. (2006). Cannabinoid pharmacology: the first 66 years. *Br J Pharmacol, 147 Suppl 1*, S163-171. doi: 10.1038/sj.bjp.0706406

Pickersgill, L., Litherland, G. J., Greenberg, A. S., Walker, M., & Yeaman, S. J. (2007). Key role for ceramides in mediating insulin resistance in human muscle cells. *J Biol Chem, 282*(17), 12583-12589. doi: 10.1074/jbc.M611157200

Piomelli, D. (2003). The molecular logic of endocannabinoid signalling. *Nat Rev Neurosci, 4*(11), 873-884. doi: 10.1038/nrn1247

Plomgaard, P., Penkowa, M., & Pedersen, B. K. (2005). Fiber type specific expression of TNF-alpha, IL-6 and IL-18 in human skeletal muscles. *Exerc Immunol Rev, 11*, 53-63.

Ploug, T., Galbo, H., & Richter, E. A. (1984). Increased muscle glucose uptake during contractions: no need for insulin. *Am J Physiol, 247*(6 Pt 1), E726-731. doi: 10.1152/ajpendo.1984.247.6.E726

Pols, T. W., Ottenhoff, R., Vos, M., Levels, J. H., Quax, P. H., Meijers, J. C., . . . de Vries, C. J. (2008). Nur77 modulates hepatic lipid metabolism through suppression of SREBP1c activity. *Biochem Biophys Res Commun, 366*(4), 910-916. doi: 10.1016/j.bbrc.2007.12.039

Proske, U., & Morgan, D. L. (2001). Muscle damage from eccentric exercise: mechanism, mechanical signs, adaptation and clinical applications. *J Physiol, 537*(Pt 2), 333-345.

Rameh, L. E., & Cantley, L. C. (1999). The role of phosphoinositide 3-kinase lipid products in cell function. *J Biol Chem, 274*(13), 8347-8350.

Randle, P. J. (1998). Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. *Diabetes Metab Rev, 14*(4), 263-283.

Randle, P. J., Garland, P. B., Hales, C. N., & Newsholme, E. A. (1963). The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet, 1*(7285), 785-789.

Ravinet Trillou, C., Arnone, M., Delgorge, C., Gonalons, N., Keane, P., Maffrand, J. P., & Soubrie, P. (2003). Anti-obesity effect of SR141716, a CB1 receptor antagonist, in diet-induced obese mice. *Am J Physiol Regul Integr Comp Physiol, 284*(2), R345-353. doi: 10.1152/ajpregu.00545.2002

Reilly, J. J., Methven, E., McDowell, Z. C., Hacking, B., Alexander, D., Stewart, L., & Kelnar, C. J. (2003). Health consequences of obesity. *Arch Dis Child, 88*(9), 748-752.

Richter, E. A., & Hargreaves, M. (2013). Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiol Rev, 93*(3), 993-1017. doi: 10.1152/physrev.00038.2012

Romijn, J. A., Coyle, E. F., Sidossis, L. S., Gastaldelli, A., Horowitz, J. F., Endert, E., & Wolfe, R. R. (1993). Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *Am J Physiol, 265*(3 Pt 1), E380-391. doi: 10.1152/ajpendo.1993.265.3.E380

Rose, A. J., & Richter, E. A. (2005). Skeletal muscle glucose uptake during exercise: how is it regulated? *Physiology (Bethesda), 20*, 260-270. doi: 10.1152/physiol.00012.2005

Ross, R. M., Wadley, G. D., Clark, M. G., Rattigan, S., & McConell, G. K. (2007). Local nitric oxide synthase inhibition reduces skeletal muscle glucose uptake but not capillary blood flow during in situ muscle contraction in rats. *Diabetes, 56*(12), 2885-2892. doi: 10.2337/db07-0745

Rotter, V., Nagaev, I., & Smith, U. (2003). Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. *J Biol Chem, 278*(46), 45777-45784. doi: 10.1074/jbc.M301977200

Ruby, M. A., Nomura, D. K., Hudak, C. S., Barber, A., Casida, J. E., & Krauss, R. M. (2011). Acute overactive endocannabinoid signaling induces glucose intolerance, hepatic steatosis, and novel cannabinoid receptor 1 responsive genes. *PLoS One, 6*(11), e26415. doi: 10.1371/journal.pone.0026415

Russo, E. B. (2007). History of cannabis and its preparations in saga, science, and sobriquet. *Chem Biodivers, 4*(8), 1614-1648. doi: 10.1002/cbdv.200790144

Ryder, J. W., Fahlman, R., Wallberg-Henriksson, H., Alessi, D. R., Krook, A., & Zierath, J. R. (2000). Effect of contraction on mitogen-activated protein kinase signal transduction in skeletal muscle. Involvement Of the mitogen- and stress-activated protein kinase 1. *J Biol Chem, 275*(2), 1457-1462.

Ryder, J. W., Kawano, Y., Galuska, D., Fahlman, R., Wallberg-Henriksson, H., Charron, M. J., & Zierath, J. R. (1999). Postexercise glucose uptake and glycogen synthesis in skeletal muscle from GLUT4-deficient mice. *FASEB J, 13*(15), 2246-2256.

Saltiel, A. R., & Kahn, C. R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature, 414*(6865), 799-806. doi: 10.1038/414799a

Sandow, A. (1952). Excitation-contraction coupling in muscular response. *Yale J Biol Med, 25*(3), 176-201.

Sarabia, V., Lam, L., Burdett, E., Leiter, L. A., & Klip, A. (1992). Glucose transport in human skeletal muscle cells in culture. Stimulation by insulin and metformin. *J Clin Invest, 90*(4), 1386-1395. doi: 10.1172/JCI116005

Scarborough, P., Bhatnagar, P., Wickramasinghe, K. K., Allender, S., Foster, C., & Rayner, M. (2011). The economic burden of ill health due to diet, physical inactivity, smoking, alcohol and obesity in the UK: an update to 2006-07 NHS costs. *J Public Health (Oxf), 33*(4), 527-535. doi: 10.1093/pubmed/fdr033

Scheen, A. J., Finer, N., Hollander, P., Jensen, M. D., Van Gaal, L. F., & Group, R. I.-D. S. (2006). Efficacy and tolerability of rimonabant in overweight or obese patients with type 2 diabetes: a randomised controlled study. *Lancet, 368*(9548), 1660-1672. doi: 10.1016/S0140-6736(06)69571-8

Scheen, A. J., & Paquot, N. (2009). Use of cannabinoid CB1 receptor antagonists for the treatment of metabolic disorders. *Best Pract Res Clin Endocrinol Metab, 23*(1), 103-116. doi: 10.1016/j.beem.2008.09.001

Schmitz-Peiffer, C., Craig, D. L., & Biden, T. J. (1999). Ceramide generation is sufficient to account for the inhibition of the insulin-stimulated PKB pathway in C2C12 skeletal muscle cells pretreated with palmitate. *J Biol Chem, 274*(34), 24202-24210.

Senn, J. J., Klover, P. J., Nowak, I. A., & Mooney, R. A. (2002). Interleukin-6 induces cellular insulin resistance in hepatocytes. *Diabetes, 51*(12), 3391-3399.

Senn, J. J., Klover, P. J., Nowak, I. A., Zimmers, T. A., Koniaris, L. G., Furlanetto, R. W., & Mooney, R. A. (2003). Suppressor of cytokine signaling-3 (SOCS-3), a potential mediator of interleukin-6-dependent insulin resistance in hepatocytes. *J Biol Chem, 278*(16), 13740-13746. doi: 10.1074/jbc.M210689200

Shek, P. N., & Shephard, R. J. (1998). Physical exercise as a human model of limited inflammatory response. *Can J Physiol Pharmacol, 76*(5), 589-597.

Shen, F., Hu, Z., Goswami, J., & Gaffen, S. L. (2006). Identification of common transcriptional regulatory elements in interleukin-17 target genes. *J Biol Chem, 281*(34), 24138-24148. doi: 10.1074/jbc.M604597200

Shepherd, P. R., Withers, D. J., & Siddle, K. (1998). Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. *Biochem J, 333 (Pt 3)*, 471-490.

Shi, H., Zeng, C., Ricome, A., Hannon, K. M., Grant, A. L., & Gerrard, D. E. (2007). Extracellular signal-regulated kinase pathway is differentially involved in beta-agonist-induced hypertrophy in slow and fast muscles. *Am J Physiol Cell Physiol, 292*(5), C1681-1689. doi: 10.1152/ajpcell.00466.2006

Sidossis, L. S., & Wolfe, R. R. (1996). Glucose and insulin-induced inhibition of fatty acid oxidation: the glucose-fatty acid cycle reversed. *Am J Physiol, 270*(4 Pt 1), E733-738.

Silveira, L. R., Pilegaard, H., Kusuhara, K., Curi, R., & Hellsten, Y. (2006). The contraction induced increase in gene expression of peroxisome proliferator-activated receptor (PPAR)-gamma coactivator 1alpha (PGC-1alpha), mitochondrial uncoupling protein 3 (UCP3) and hexokinase II (HKII) in primary rat skeletal muscle cells is dependent on reactive oxygen species. *Biochim Biophys Acta, 1763*(9), 969-976. doi: 10.1016/j.bbamcr.2006.06.010

Silvestri, C., Ligresti, A., & Di Marzo, V. (2011). Peripheral effects of the endocannabinoid system in energy homeostasis: adipose tissue, liver and skeletal muscle. *Rev Endocr Metab Disord, 12*(3), 153-162. doi: 10.1007/s11154-011-9167-3

Sipe, J. C., Waalen, J., Gerber, A., & Beutler, E. (2005). Overweight and obesity associated with a missense polymorphism in fatty acid amide hydrolase (FAAH). *Int J Obes (Lond), 29*(7), 755-759. doi: 10.1038/sj.ijo.0802954

Smith, L. L. (1991). Acute inflammation: the underlying mechanism in delayed onset muscle soreness? *Med Sci Sports Exerc, 23*(5), 542-551.

Snow, M. H. (1977). Myogenic cell formation in regenerating rat skeletal muscle injured by mincing. II. An autoradiographic study. *Anat Rec, 188*(2), 201-217. doi: 10.1002/ar.1091880206

Stanley, B. G., Anderson, K. C., Grayson, M. H., & Leibowitz, S. F. (1989). Repeated hypothalamic stimulation with neuropeptide Y increases daily carbohydrate and fat intake and body weight gain in female rats. *Physiol Behav, 46*(2), 173-177.

Stanley, B. G., Daniel, D. R., Chin, A. S., & Leibowitz, S. F. (1985). Paraventricular nucleus injections of peptide YY and neuropeptide Y preferentially enhance carbohydrate ingestion. *Peptides, 6*(6), 1205-1211.

Stanley, B. G., & Leibowitz, S. F. (1984). Neuropeptide Y: stimulation of feeding and drinking by injection into the paraventricular nucleus. *Life Sci, 35*(26), 2635-2642.

Steinberg, G. R., McAinch, A. J., Chen, M. B., O'Brien, P. E., Dixon, J. B., Cameron-Smith, D., & Kemp, B. E. (2006). The suppressor of cytokine signaling 3 inhibits leptin activation of AMP-kinase in cultured skeletal muscle of obese humans. *J Clin Endocrinol Metab, 91*(9), 3592-3597. doi: 10.1210/jc.2006-0638

Storz, P., Doppler, H., Wernig, A., Pfizenmaier, K., & Muller, G. (1999). Cross-talk mechanisms in the development of insulin resistance of skeletal muscle cells palmitate rather than tumour necrosis factor inhibits insulin-dependent protein kinase B (PKB)/Akt stimulation and glucose uptake. *Eur J Biochem, 266*(1), 17-25.

Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., . . . Waku, K. (1995). 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun, 215*(1), 89-97.

Svizenska, I., Dubovy, P., & Sulcova, A. (2008). Cannabinoid receptors 1 and 2 (CB1 and CB2), their distribution, ligands and functional involvement in nervous system structures--a short review. *Pharmacol Biochem Behav, 90*(4), 501-511. doi: 10.1016/j.pbb.2008.05.010

Sylow, L., Jensen, T. E., Kleinert, M., Mouatt, J. R., Maarbjerg, S. J., Jeppesen, J., . . . Richter, E. A. (2013). Rac1 is a novel regulator of contraction-stimulated glucose uptake in skeletal muscle. *Diabetes, 62*(4), 1139-1151. doi: 10.2337/db12-0491

Terauchi, Y., Tsuji, Y., Satoh, S., Minoura, H., Murakami, K., Okuno, A., . . . Kadowaki, T. (1999). Increased insulin sensitivity and hypoglycaemia in mice lacking the p85 alpha subunit of phosphoinositide 3-kinase. *Nat Genet, 21*(2), 230-235. doi: 10.1038/6023

Thai, M. V., Guruswamy, S., Cao, K. T., Pessin, J. E., & Olson, A. L. (1998). Myocyte enhancer factor 2 (MEF2)-binding site is required for GLUT4 gene expression in transgenic mice. Regulation of MEF2 DNA binding activity in insulin-deficient diabetes. *J Biol Chem, 273*(23), 14285-14292.

Thakur, G. A., Duclos, R. I., Jr., & Makriyannis, A. (2005). Natural cannabinoids: templates for drug discovery. *Life Sci, 78*(5), 454-466. doi: 10.1016/j.lfs.2005.09.014

Thomas, E. A., Cravatt, B. F., Danielson, P. E., Gilula, N. B., & Sutcliffe, J. G. (1997). Fatty acid amide hydrolase, the degradative enzyme for anandamide and oleamide, has selective distribution in neurons within the rat central nervous system. *J Neurosci Res, 50*(6), 1047-1052.

Toyoda, T., An, D., Witczak, C. A., Koh, H. J., Hirshman, M. F., Fujii, N., & Goodyear, L. J. (2011). Myo1c regulates glucose uptake in mouse skeletal muscle. *J Biol Chem, 286*(6), 4133-4140. doi: 10.1074/jbc.M110.174938

Turu, G., & Hunyady, L. (2010). Signal transduction of the CB1 cannabinoid receptor. *J Mol Endocrinol, 44*(2), 75-85. doi: 10.1677/JME-08-0190

Ueki, K., Algenstaedt, P., Mauvais-Jarvis, F., & Kahn, C. R. (2000). Positive and negative regulation of phosphoinositide 3-kinase-dependent signaling pathways by three different gene products of the p85alpha regulatory subunit. *Mol Cell Biol, 20*(21), 8035-8046.

Ueki, K., Fruman, D. A., Brachmann, S. M., Tseng, Y. H., Cantley, L. C., & Kahn, C. R. (2002). Molecular balance between the regulatory and catalytic subunits of phosphoinositide 3-kinase regulates cell signaling and survival. *Mol Cell Biol, 22*(3), 965-977.

Ueki, K., Kondo, T., & Kahn, C. R. (2004). Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. *Mol Cell Biol, 24*(12), 5434-5446. doi: 10.1128/MCB.24.12.5434-5446.2004

Ukropcova, B., McNeil, M., Sereda, O., de Jonge, L., Xie, H., Bray, G. A., & Smith, S. R. (2005). Dynamic changes in fat oxidation in human primary myocytes mirror metabolic characteristics of the donor. *J Clin Invest, 115*(7), 1934-1941. doi: 10.1172/JCI24332

van Hall, G., Steensberg, A., Sacchetti, M., Fischer, C., Keller, C., Schjerling, P., . . . Pedersen, B. K. (2003). Interleukin-6 stimulates lipolysis and fat oxidation in humans. *J Clin Endocrinol Metab, 88*(7), 3005-3010. doi: 10.1210/jc.2002-021687

Van Harken, D. R., Dixon, C. W., & Heimberg, M. (1969). Hepatic lipid metabolism in experimental diabetes. V. The effect of concentration of oleate on metabolism of triglycerides and on ketogenesis. *J Biol Chem, 244*(9), 2278-2285.

Verty, A. N., McGregor, I. S., & Mallet, P. E. (2004). Consumption of high carbohydrate, high fat, and normal chow is equally suppressed by a cannabinoid receptor antagonist in non-deprived rats. *Neurosci Lett, 354*(3), 217-220.

Veum, V. L., Dankel, S. N., Gjerde, J., Nielsen, H. J., Solsvik, M. H., Haugen, C., . . . Mellgren, G. (2012). The nuclear receptors NUR77, NURR1 and NOR1 in obesity and during fat loss. *Int J Obes (Lond), 36*(9), 1195-1202. doi: 10.1038/ijo.2011.240

Wallberg-Henriksson, H., & Holloszy, J. O. (1984). Contractile activity increases glucose uptake by muscle in severely diabetic rats. *J Appl Physiol Respir Environ Exerc Physiol, 57*(4), 1045-1049.

Wang, Y., Lam, K. S., Kraegen, E. W., Sweeney, G., Zhang, J., Tso, A. W., . . . Xu, A. (2007). Lipocalin-2 is an inflammatory marker closely associated with obesity, insulin resistance, and hyperglycemia in humans. *Clin Chem, 53*(1), 34-41. doi: 10.1373/clinchem.2006.075614

Wang, Y. C., McPherson, K., Marsh, T., Gortmaker, S. L., & Brown, M. (2011). Health and economic burden of the projected obesity trends in the USA and the UK. *Lancet, 378*(9793), 815-825. doi: 10.1016/S0140-6736(11)60814-3

Wasserman, D. H. (2009). Four grams of glucose. *Am J Physiol Endocrinol Metab, 296*(1), E11-21. doi: 10.1152/ajpendo.90563.2008

Watson, M. L., Coghlan, M., & Hundal, H. S. (2009). Modulating serine palmitoyl transferase (SPT) expression and activity unveils a crucial role in lipid-induced insulin resistance in rat skeletal muscle cells. *Biochem J, 417*(3), 791-801. doi: 10.1042/BJ20081149

Werner, N. A., & Koch, J. E. (2003). Effects of the cannabinoid antagonists AM281 and AM630 on deprivation-induced intake in Lewis rats. *Brain Res, 967*(1-2), 290-292.

Wiley, J. L., Burston, J. J., Leggett, D. C., Alekseeva, O. O., Razdan, R. K., Mahadevan, A., & Martin, B. R. (2005). CB1 cannabinoid receptor-mediated modulation of food intake in mice. *Br J Pharmacol, 145*(3), 293-300. doi: 10.1038/sj.bjp.0706157

Williams, C. M., & Kirkham, T. C. (1999). Anandamide induces overeating: mediation by central cannabinoid (CB1) receptors. *Psychopharmacology (Berl), 143*(3), 315-317.

Williams, C. M., & Kirkham, T. C. (2002). Reversal of delta 9-THC hyperphagia by SR141716 and naloxone but not dexfenfluramine. *Pharmacol Biochem Behav, 71*(1-2), 333-340.

Wilson, S. J., & Harris, A. J. (1993). Formation of myotubes in aneural rat muscles. *Dev Biol, 156*(2), 509-518. doi: 10.1006/dbio.1993.1097

Witczak, C. A., Hirshman, M. F., Jessen, N., Fujii, N., Seifert, M. M., Brandauer, J., . . . Goodyear, L. J. (2006). JNK1 deficiency does not enhance muscle glucose metabolism in lean mice. *Biochem Biophys Res Commun, 350*(4), 1063-1068. doi: 10.1016/j.bbrc.2006.09.158

Yan, Q. W., Yang, Q., Mody, N., Graham, T. E., Hsu, C. H., Xu, Z., . . . Rosen, E. D. (2007). The adipokine lipocalin 2 is regulated by obesity and promotes insulin resistance. *Diabetes, 56*(10), 2533-2540. doi: 10.2337/db07-0007

Yang, J., Goetz, D., Li, J. Y., Wang, W., Mori, K., Setlik, D., . . . Barasch, J. (2002). An iron delivery pathway mediated by a lipocalin. *Mol Cell, 10*(5), 1045-1056.

Yano, S., Morino-Koga, S., Kondo, T., Suico, M. A., Koga, T., Shimauchi, Y., . . . Kai, H. (2011). Glucose uptake in rat skeletal muscle L6 cells is increased by low-intensity electrical current through the activation of the phosphatidylinositol-3-OH kinase (PI-3K) / Akt pathway. *J Pharmacol Sci, 115*(1), 94-98.

Yudkin, J. S. (2007). Inflammation, obesity, and the metabolic syndrome. *Horm Metab Res, 39*(10), 707-709. doi: 10.1055/s-2007-985898

Zarjevski, N., Cusin, I., Vettor, R., Rohner-Jeanrenaud, F., & Jeanrenaud, B. (1994). Intracerebroventricular administration of neuropeptide Y to normal rats has divergent effects on glucose utilization by adipose tissue and skeletal muscle. *Diabetes, 43*(6), 764-769.

Zhang, J., Hoffert, C., Vu, H. K., Groblewski, T., Ahmad, S., & O'Donnell, D. (2003). Induction of CB2 receptor expression in the rat spinal cord of neuropathic but not inflammatory chronic pain models. *Eur J Neurosci, 17*(12), 2750-2754.

Zhang, J., Wu, Y., Zhang, Y., Leroith, D., Bernlohr, D. A., & Chen, X. (2008). The role of lipocalin 2 in the regulation of inflammation in adipocytes and macrophages. *Mol Endocrinol, 22*(6), 1416-1426. doi: 10.1210/me.2007-0420

Zhang, Y., Foncea, R., Deis, J. A., Guo, H., Bernlohr, D. A., & Chen, X. (2014). Lipocalin 2 expression and secretion is highly regulated by metabolic stress, cytokines, and nutrients in adipocytes. *PLoS One, 9*(5), e96997. doi: 10.1371/journal.pone.0096997

Zierath, J. R., Krook, A., & Wallberg-Henriksson, H. (2000). Insulin action and insulin resistance in human skeletal muscle. *Diabetologia, 43*(7), 821-835. doi: 10.1007/s001250051457

Zisman, A., Peroni, O. D., Abel, E. D., Michael, M. D., Mauvais-Jarvis, F., Lowell, B. B., . . . Kahn, B. B. (2000). Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. *Nat Med, 6*(8), 924-928. doi: 10.1038/78693

Zurlo, F., Larson, K., Bogardus, C., & Ravussin, E. (1990). Skeletal muscle metabolism is a major determinant of resting energy expenditure. *J Clin Invest, 86*(5), 1423-1427. doi: 10.1172/JCI114857