12-12-2012 12:00 AM

Roles of PGC-1α/PPARs pathway in regulating insulin sensitivity in mouse skeletal muscle cells under prolonged hypoxia

Yunyan Zhang
The University of Western Ontario

Supervisor
Timothy Regnault
The University of Western Ontario

Graduate Program in Physiology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

ROLES OF PGC-1α/PPARS PATHWAY IN REGULATING INSULIN SENSITIVITY IN MOUSE SKELETAL MUSCLE CELLS UNDER PROLONGED HYPOXIA

(Roles of PGC-1α/PPARs in insulin sensitivity regulation under hypoxia)

(Thesis format: Monograph)

by

Yunyan Zhang

Graduate Program in Physiology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

THE UNIVERSITY OF WESTERN ONTARIO
School of Graduate and Postdoctoral Studies

CERTIFICATE OF EXAMINATION

Supervisor

Dr. Timothy Regnault

Supervisory Committee

Dr. Doug Jones

Dr. Peter Merrifield

Dr. Rommel Tirona

Examiners

Dr. Dean Betts

Dr. Kaiping Yang

Dr. Lynne Postovit

The thesis by

Yunyan Zhang

entitled:

Roles of PGC-1α/PPARs pathway in regulating insulin sensitivity in mouse skeletal muscle cells under prolonged hypoxia

is accepted in partial fulfillment of the
requirements for the degree of
Master of Science

Date

Chair of the Thesis Examination Board

Abstract

Using the C_2C_{12} mouse myoblast cell line, I investigated how prolonged hypoxia affected components of the insulin signalling and FAO/PGC-1α/PPARs pathways, as they might impact insulin sensitivity in skeletal muscle cells.

Hypoxia resulted in lower p-Akt (Thr 308) and higher total cellular GLUT4 protein levels after 7 days of differentiation. This coincided with higher triglyceride content and alterations of the FAO/PGC-1α/PPARs components, both of which could contribute to the changes observed in the components of the insulin signalling pathway. Specifically, cells differentiating in 1% O_2 had lower SIRT1, PPAR- α, FATP4 and MCAD mRNA; accompanied by lower SIRT1, PGC-1α and higher PPAR- γ protein following 7 days of differentiation. Additionally, cells in prolonged hypoxia had significantly higher phosphorylation of PGC-1α, AMPKα and ACC; concurrent with higher PGC-1α acetylation. However, none of these alterations above persisted following an additional 2-day re-oxygenation treatment (recovery).

In conclusion, prolonged hypoxia impairs components of the insulin signalling and FAO/PGC-1α/PPARs pathways, although the degree of this impairment is reduced followed re-oxygenation. An altered FAO/PGC-1α/ PPARs interaction contributes to depress FAO, resulting in increased triglyceride content, which likely impairs insulin signaling, specifically Akt phosphorylation (Thr 308). It is important to note that the alterations of FAO/PGC-1α/ PPARs observed here are similar to those reported in insulin resistant adults. The changes obtained during hypoxia may partly explain the *in utero* factors contributing to decreased insulin sensitivity in intrauterine growth restriction offspring.

Keywords

Intrauterine growth restriction (IUGR), hypoxia, fatty acid β-oxidation (FAO), insulin resistance, insulin signalling pathway, peroxisome proliferator-activated receptor (PPAR), PPAR- γ coactivator 1-α (PGC-1α), silent information regulator T1 (SIRT1)

Acknowledgments

I would like to take this opportunity to extend my deepest appreciation and gratitude to my supervisor, Dr. Timothy Regnault. Your help and guidance throughout this two year has been priceless, and I am very grateful for your unwavering support and positive attitude, and for all the patience your have shown. You provided me with a great deal of encouragement, allowing me to feel confident in my work and abilities. I could not be luckier to have had such an amazing supervisor. I hope that my research was able to set a stable groundwork for your future researches, it is the least I could do for everything that you have done for me. Beyond my research, you made my lab experience a really cheerful and enjoyable one and I have appreciated all of the laughs and jokes (or sarcasm). It is always intimidating for an international student to walk into a new lab, but you made it easy Tim; you have managed to make me laugh every day, I hope you next student would understand your sarcasm as well as I think I have.

Next, I would like to thank the members of my graduate advisory committee members, Dr. Doug Jones, Dr. Peter Merrifield and Dr. Rommel Tirona. Your continuous encouragement, constructive criticism and enthusiastic guidance throughout my project have been invaluable. This research project would not have been possible without your support. There are not enough words to thank all of you!

To the DDT lab members, there are so many individuals I would like to give a big thank you. Jeni, words do not describe how much you've inspired me throughout the past two years. Thank you to be one of my good friends outside the lab and I have enjoyed every minute we have spent together. Andrew, thank you for the guidance that you were always so willing to give me; and your constant care for others, positive outlook on life has influenced me greatly. Jonathan (Teichroeb), I am grateful for every tool and piece of knowledge you gave me. I thank you for your never-ending patience and willingness to answer my questions and help me to solve many problems. Peter, Jonathan (Park), Maya and Jacky, you guys have been there with me from the very beginning. I cannot count the amount of hours we have spent working in the lab together and chatting in the office. I am really appreciated that you guys shared experience with me and always made me laugh. You are part of the best moments of

my master study. Also thanks Lin, Katie and Ousseynou for all their help in my project. I am so happy to have met everyone here and to have made great friends during my last two years; as an international student I never fell lonely in DDT lab.

Last, but not the least, I wish to thank, from the bottom of my heart, my family for being my best people in the world: my husband, my parents and my parents-in-law. All my honors are for my family. I am especially grateful to them who have been continuously supporting me throughout my entire life. Without their optimism, trust, love, motivation, inspiration, sympathy and support, any accomplishment would not have been possible.

I owe my success to everyone who has aided me along these past two years. Thank you all so much.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACC ..... Acetyl-CoA carboxylase

AMPK ..... AMP-activated protein kinase

CD36 ..... Cluster of differentiation 36

CPT1 ..... Carnitine palmitoyltransferase I

DAG ..... diglyceride

FAO ..... Fatty acid β-oxidation

FATP4 ..... Fatty acid transport protein 4

GLUT4 ..... Glucose transporter type 4

HIF1 ..... Hypoxia-inducible factor 1

IR ..... Insulin receptor

IRS ..... Insulin receptor substrate

IUGR ..... Intrauterine growth restriction

JNK ..... C-jun n-terminal kinase

LBW ..... Low birth weight

LOX ..... Lysyl oxidase

MAPK ..... Ras-mitogen-activated protein kinase

MCAD ..... Medium-chain Acyl-CoA dehydrogenase

MRS ..... magnetic resonance spectroscopy

PGC-1α ..... PPAR- γ coactivator-1α

PI3K ..... Phosphatidylinositol 3-kinase

PKB ..... Protein kinase B

PKC ..... Protein kinase C

PPAR- α ..... Peroxisome proliferator-activated receptor- α

PPAR- γ ..... Peroxisome proliferator-activated receptor- γ

PPRE ..... Peroxisome proliferator hormone response elements

PTB ..... Phosphotyrosine binding

RXR ..... Retinoid X receptor

siRNA ..... Small interfering RNAs

SIRT1 ..... Silent information regulator T1

TG ..... Triglyceride

Chapter 1 Introduction

1.1 Metabolic syndrome, insulin resistance and skeletal muscle

The metabolic syndrome (or syndrome X) is a constellation of symptoms/ features, including central obesity, dyslipidemia and hypertension, which predisposes for the development of cardiovascular disease and type II diabetes. The condition of insulin resistance is present before these overt features of the metabolic syndrome are apparent and is believed to be a critical pathophysiological event early in the disease process (Barker 2005). Skeletal muscle is the principal site of glucose uptake under insulin-stimulated conditions, accounting for approximately 75% of glucose disposal after glucose infusion (DeFronzo et al. 1985, Nuutila et al. 1992), and is also the major tissue site affected in insulin resistant individuals (Selak et al. 2003). Insulin resistance is a condition where impaired peripheral tissue response to endogenously secreted insulin occurs, including decreased insulin-mediated glucose uptake (Peppa et al. 2010). Further, in skeletal muscle from animal models of insulin resistance, intracellular lipid metabolites (e.g. triglyceride [TG], long-chain Acyl-CoA and ceramide) are increased, accompanied by a lower fatty acid β-oxidation (FAO) level and insulin signalling defects when compared to healthy adults (Park et al. 2005, Liu et al. 2007, Heydrick et al. 1993). All these studies suggest an association between decreased FAO, defects of insulin signalling cascade and development of insulin resistance in skeletal muscle tissue. The origins of insulin resistance are likely multifactorial, though, emerging evidence suggests the *in utero* environment plays a major role in setting one's susceptibility to developing insulin resistance in postnatal life. This increased susceptibility occurs as a result of reprogramming events that occur in response to *in utero* stresses during pregnant and such activities underlie the concept of the developmental origins of health and disease (Barker 2005).

1.2 Developmental origins of health and disease

The concept of developmental origins of health and disease is the idea that environmental factors (usually *in utero*) acting early in life have crucial effects on the vulnerability to

diseases later in adulthood (Gluckman, Hanson & Mitchell 2010). Many studies have now revealed links between poor early growth *in utero* and susceptibility to adult disease, such as insulin resistance, and later type II diabetes, cardiovascular disease, obesity and cancer (Hales, Barker 1992, Kensara et al. 2005, Osmond et al. 1993). Inadequate maternal and fetal nutrition was commonly associated with poor early human growth, such as low birth weight (LBW) newborns, whose birth weights are less than 2,500g, regardless of their gestational age (Jain, Singhal 2012). Under this substantial developmental challenge, a range of phenotypes that have been called "thrifty" can be induced (Hales, Barker 1992), which refers to a condition where physiological and/or metabolic fetal adaptations occur to enhance the fetus' ability to survive in adverse uterine environments (Fernandez-Twinn, Ozanne 2006). Such adaptations during critical periods may permanently reset and can produce long-term functional and structural changes. This might be because that the fetus predicts the environment into which it is likely to be born in is the same as its poor *in utero* environment, and adapts to gain a competitive advantage when it is born (Gluckman, Hanson 2004). The high incidence of metabolic disease in modern populations has been explained by the selection for "thrifty" metabolism during evolution (Anonymous1989).Various types of insults (such as high altitude pregnancies, placental insufficiency, maternal smoking and malnutrition, placental villous inflammation) have been found to affect birth weight with potential consequence for diseases in later life (Habek et al. 2002, Becroft, Thompson & Mitchell 2005, Ballew, Haas 1986).

Many epidemiological studies have revealed a close relationship between LBW and the subsequent development of insulin resistance leading into type II diabetes in a range of populations worldwide (Hales, Barker 2001). Biopsies from the vastus lateralis muscle of men with LBW showed reduced expression of protein kinase C (PKC) zeta, insulin sensitive glucose transporter type 4 (GLUT4) and other insulin signalling markers (e.g. p85 and p110β), which help to promote insulin resistance (Ozanne et al. 2005). Exploring the key molecular mechanisms underlying early life programming may help to explicate the development of adult diseases and validate potential targets for intervention.

1.3 Intrauterine growth restriction, hypoxia and skeletal muscle

1.3.1 Intrauterine growth restriction

As mentioned above, LBW refers to infants who weigh less than 2, 500g at birth, regardless of gestational age (Jain, Singhal 2012). Intrauterine growth restriction (IUGR) is a term assigned to newborns who have failed to reach their genetically predetermined growth potential *in utero*, which then leads to LBW. The IUGR infants have birth weights and/or lengths below the 10th percentile for their gestational age (Ness, Sibai 2006). The etiology IUGR is multifactorial, including fetal factors (e.g. chromosomal alterations and intrauterine infections), maternal factors (e.g. nutritional disorders, drugs and alcohol abuse) as well as placenta factors (e.g. placental insufficiency).

Placental insufficiency is the most common cause of IUGR (Resnik 2002). Placental insufficiency, which is commonly caused by interference with placenta vascular development, is characterized by reduced nutrition and oxygen supply (Baschat 2004). The resulting fetal undernutrition and hypoxemia are the major stimuli involved in the reduction in fetal growth (Baschat 2004). An early study found that fetal rats exposed to hypoxia showed placental hypertrophy relative to their body weights, while the least hypoxic fetuses showed absolute placental hypertrophy (de Grauw, Myers & Scott 1986). In addition, fetal sheep that were chronic hypoglycemia showed increased protein breakdown, amino acid oxidation and reduced plasma insulin, glucose uptake and fetal growth rate. However, euglycemic correction normalized these parameters within a few days (Limesand, Hay 2003, Limesand et al. 2009). By contrast, in fetal sheep with placental insufficiency (hypoxemic and hypoglycemic), euglycemic recovery failed to restore glucose homeostasis or improve growth rate, but in fact worsened hypoxemia and hypoinsulinemia, resulting in acidosis. Hence, the metabolic alterations related with placenta insufficiency are reliant on placenta oxygen supply and cannot be improved by adding just the nutrition supply (Rozance et al. 2009). All of these studies highlight the fact that hypoxia is a critical regulator of fetal growth, independent of other nutrients, and that it has a primary role in the control of fetal growth (Giussani et al. 2007). Therefore, the focus of this thesis was to investigate the potential impact of hypoxia on skeletal

muscle insulin sensitivity by studying related pathways (e.g. insulin signalling and FAO/PGC-1α/PPARs pathways), aiming to improve our knowledge of *in utero* hypoxia and its possible effects on fetal insulin sensitivity.

1.3.2 Effects of hypoxia on fetal development

Normal maternal arterial PO_2 is 80-100 mmHg (Ang et al. 1969), while the typical fetal umbilical vein PO_2 is approximately 20-30 mmHg in human (Lackman et al. 2001). The fetus requires a moderately low PO_2 for proper development (e.g. vasculogenesis) (Bleiberg, Liron & Feldman 1967, Charnock-Jones, Kaufmann & Mayhew 2004). However, under excessive hypoxia, fetal growth can be reduced and abnormal development is thus incurred.

The fetus is normally able to maintain aerobic metabolism during acute decreases in oxygenation that are common when there is a transient interruption in uterine or umbilical blood flow. Aerobic metabolism will be maintained until the available oxygen in the intervillous space falls to 50% of normal levels. The fetus has several normal compensatory mechanisms for surviving transient hypoxic insults, such as having more hemoglobin per cubic unit of whole blood, redistribution blood flow and increasing cardiac output and heart rate. If these mechanisms are not sufficient to allow the fetus to maintain aerobic metabolism, anaerobic metabolism will ensue. If normal fetal oxygenation does not resume, then asphyxia occurs, where the adaptive mechanisms fail. This failure in auto-regulation will result in brain injury, and all organs can be affected (Blackburn 2007).

A prolonged reduction in uteroplacental perfusion, such as in the condition of placental insufficiency, results in the fetus limiting oxygen-consuming processes, which can result in curtailing oxygen-consuming activities such as protein synthesis, to direct limited resources to more vital functions. Metabolic normality may be maintained by the fetus, which suggests that the fetus is capable of rapid adaptation to limited substrate delivery by decreasing the growth rate. Over time, this adaption results in clinically detectable fetal growth restriction, such as IUGR (Blackburn 2007).

Infants born with IUGR have a reduced umbilical vein PO_2 (as low as 15 mmHg and 20 mmHg in human and sheep, respectively) when compared with normally grown newborns (Baschat 2004, Regnault et al. 2007). During these severe hypoxic conditions, in addition to whole body growth restriction, fetal blood flow is redistributed preferentially to vital organs such as the brain, heart and adrenals to ensure the nutrition and oxygen supply of these organs, while growth of peripheral tissues and organs, such as skeletal muscle is reduced (Sadiq et al. 1999).

1.3.3 Alterations in skeletal muscle mass / function under hypoxia

Various investigations have begun to highlight hypoxia-associated alterations in skeletal muscle. In adults, muscle biopsy samples of climbers who have been exposed to extreme hypoxia (summits higher than 8000 m in the Himalayas), showed a decline in aerobic work capacity, which could be a consequence of muscle mass loss. The degradation of muscle tissue was further characterized by an increase in muscle lipofuscin, which is believed to be the consequence of mitochondrial loss (Howald, Hoppeler 2003).

From a fetal perspective, ultrasound measurements of hypoxia-associated IUGR fetuses showed a reduced muscle mass (Padoan et al. 2004, Larciprete et al. 2005). In fetal sheep with placental insufficiency, skeletal muscle fibers contained fewer myonuclei than those from control fetuses, resulting in 33% less DNA, 40% less RNA, and 76% less protein per fiber (Greenwood et al. 2000, Greenwood et al. 1999), which indicates reduced muscle growth. This is because after myogenesis, muscle growth continues via fiber hypertrophy and requires myoblast incorporation to increase genomic DNA content; myonuclei incorporation precedes protein accumulation, and the size of a muscle fiber is dependent on DNA content (Yates et al. 2012). Furthermore, prolonged hypoxia in culture decreased factors important for muscle FAO, such as peroxisome proliferator-activated receptor- γ coactivator-1α (PGC-1α) (Regnault et al. 2010b). Additionally, the insulin signalling was impaired in animal models of IUGR (Jackson et al. 1993, Maier et al. 1992). Combined, these findings indicate that hypoxia and associated IUGR likely have profound effects on skeletal muscle mass, cell mitochondrial abundance and fatty acid oxidation, and insulin signalling.

1.4 Regulations of insulin sensitivity in skeletal muscle

Insulin sensitivity in skeletal muscle refers to the ability of endogenous and exogenous insulin to lower glucose in extracellular fluid by stimulating the skeletal muscle glucose uptake. It is regulated by a series of enzymes in the insulin signalling pathway (Figure 1.1), through which insulin conducts its signalling. Another important aspect of insulin sensitivity is FAO. Previous researchers have shown that decreased FAO is associated with reduced insulin sensitivity in heart and skeletal muscle (Zhang et al. 2010). The overall FAO capacity is regulated by interactions of FAO/PGC-1α/PPARs (Figure 1.2). Thus, the FAO/PGC-1α/PPARs interactions are also associated with insulin sensitivity, through impacting FAO capacity.

1.4.1 Insulin signalling pathway

Insulin signalling is mediated by a complex, highly integrated network that controls several processes (Taniguchi, Emanuelli & Kahn 2006). Stimulated by insulin, the insulin receptor (IR) phosphorylates insulin receptor substrate (IRS) proteins, which are associated with the activation of 2 signalling pathways: the phosphatidylinositol 3-kinase (PI3K)–Akt/protein kinase B (PKB) pathway and the ras-mitogen-activated protein kinase (MAPK) pathway. The PI3K-Akt pathway bears the responsibility of most insulin metabolic functions, while the MAPK pathway controls cell growth and differentiation (Avruch 1998).

Insulin action is initiated through the binding to and activation of its cell surface receptor: IR, which is composed of 2 α subunits and 2 β subunits (Czech 1985). When insulin binds to the extracellular α subunits, a signal is transmitted across plasma membrane to activate the intracellular tyrosine kinase domain of the β subunit. The receptor then undergoes a series of intra-molecular transphosphorylation reactions in which a β subunit phosphorylates its adjacent partner on specific tyrosine residues (Nystrom, Quon 1999). Certain phosphorylated tyrosine residues on the activated IR protein can be recognized by IRS. Alterations in IR expression, binding, phosphorylation state, and/or kinase activity could account for many insulin resistance phenotypes (Krook, O'Rahilly 1996).

IRS proteins are important intracellular substrates of IR. Activated IR recruits IRS, which binds to the phosphorylated tyrosine residues on the receptor via a phosphotyrosine binding (PTB) domain of IRS (Holgado-Madruga et al. 1996). When IRS is bound to IR, the kinase activity of IR can catalyze phosphorylation of tyrosine residues on IRS. IRS is activated by tyrosine phosphorylation, and is negatively regulated by serine phosphorylation (e.g. phosphorylation at Ser 1101) (Hirosumi et al. 2002). There are 2 predominant IRSs (IRS1 and IRS2) involved in metabolic regulation in skeletal muscle. These IRSs share a highly similar sequence, but appear to have different signalling functions. In L6 myotubes, where small interfering RNAs (siRNAs) were used to decrease either IRS1 or IRS2 expression, IRS1 was found to be more responsible for glucose uptake, whereas IRS2 was more closely associated with MAPK regulation (Huang et al. 2005). Tyrosine phosphorylation of the IRS after insulin stimulation leads to an interaction with and subsequent activation of several downstream substrates along the insulin signalling pathway, ultimately resulting in the activation of Akt (Walker et al. 1998).

Akt is a serine/threonine kinase, also known as protein kinase B (PKB), which is activated by its phosphorylation at Ser 473 and Thr 308(Alessi et al. 1996). There are 3 isoforms of Akt (Akt1, Akt2, and Akt3) expressed in skeletal muscle. Either Akt1 or Akt2 knockout mice demonstrated that the different isoforms have specific roles (Cho et al. 2001b, Cho et al. 2001a). Knockout of Akt2 in mice impaired the ability of insulin to lower blood glucose, because of defects in the action of insulin on liver and skeletal muscle. Thus Akt2 is essential for the maintenance of normal glucose homeostasis (Cho et al. 2001a). In contrast, Akt1 is required for normal growth, but is not essential for glucose homeostasis maintenance in mice (Cho et al. 2001b). Studies have shown that overexpression of constitutively active mutants of Akt in rat adipose cells or 3T3-L1 adipocytes led to increased recruitment of GLUT4 to the cell surface (Cong et al. 1997, Kohn et al. 1996). Furthermore, overexpression of a kinase-deficient inhibitory mutant of Akt inhibited insulin-stimulated translocation of GLUT4 in adipose cells (Cong et al. 1997). GLUT4 is one of several isoforms in a family of facilitative glucose transporter proteins, and available evidence supports the idea that it is the magnitude of GLUT4 translocation which determines the capacity of a tissue to enhance glucose uptake

(Holloszy, Hansen 1996). Thus, these data suggest that Akt has a role in promoting GLUT4 translocation and glucose uptake in adipose tissue. Similar effects of Akt on GLUT4 translocation and glucose uptake were also found in skeletal muscle (Ueki et al. 1998).

As mentioned above, GLUT4, a member of a family of facilitative glucose transporter proteins, is the dominant isoform in skeletal muscle (Birnbaum 1989). In the basal state, GLUT4 slowly recycles between the plasma membrane and vesicular compartments within the cell, where most of the GLUT4 resides (Satoh et al. 1993). Upon insulin stimulation, GLUT4 containing vesicles translocate to fuse with the cell surface membrane, in order to facilitate the transportation of glucose into cells (Holman, Sandoval 2001).Insulin resistance in Type II diabetes is not generally linked to a reduced skeletal muscle GLUT4 protein level (Zierath, Krook & Wallberg-Henriksson 2000). In contrast, GLUT4 protein fails to translocate to the sarcolemma in skeletal muscle under insulin stimulation from subjects with Type II diabetes (Zierath et al. 1996, Ryder et al. 2000). These reports further support the idea that it is the magnitude of GLUT4 protein translocation that determines the capacity of glucose uptake in skeletal muscle. Except insulin, GLUT4 is also translocated to the surface membrane in response to other stimuli, including hypoxia (Holloszy, Narahara 1965, Azevedo et al. 1995, Cartee et al. 1991).

Cartee found hypoxia increased the amount of GLUT4 transporters in the plasma membrane fraction but had little effect on the GLUT4 content of the intracellular fraction in rat skeletal muscle cells (Cartee et al. 1991). Hypoxia also affects other upstream markers of the insulin signalling pathway. Regazzetti and colleagues found in both human and murine adipocytes, that hypoxia (1% O_2) inhibited insulin signalling as revealed by a decrease in the phosphorylation of IR. Furthermore, in murine 3T3-L1 adipocytes, this inhibition of IR phosphorylation is followed by a decrease in the phosphorylation state of protein kinase B/Akt in response to insulin (Regazzetti et al. 2009). Moreover, hypoxia-associated IUGR animal models also have defects in their insulin signalling pathway (Muhlhausler et al. 2009, Camm et al. 2011). A fetal lamb study showed the insulin signalling molecule PKC and GLUT4 protein in the quadriceps muscle of the IUGR fetal lambs were lower than that of the control normal lambs

(Muhlhausler et al. 2009). Also in rats, insulin signalling via Akt is reduced in liver of offspring from dams exposed to IUGR associated hypoxic or malnourished environments during pregnancy (Camm et al. 2011). All these observations suggest that the insulin signalling pathway plays a major role in maintaining normal insulin sensitivity, and hypoxia or hypoxia-related IUGR results in defects in this pathway, which may influence insulin sensitivity promoting insulin resistance.

1.4.2 FAO/PGC-1α/PPARs pathway in regulating FAO

FAO is a crucial component of determining overall insulin sensitivity. FAO capacity is regulated by a number of regulators in skeletal muscle, such as the components of FAO/ PGC-1α/PPARs pathway, including the peroxisome proliferator-activated receptors (PPARs), and PPAR- γ coactivator-1α (PGC-1α), silent information regulator T1 (SIRT1), the AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC). These regulators are directly or indirectly involved in regulating FAO related gene expressions such as carnitine palmitoyltransferase I (CPT1), cluster of differentiation 36 (CD36), fatty acid transport protein 4 (FATP4) and medium-chain Acyl-CoA dehydrogenase (MCAD).

PGC-1α lies at the heart of this regulatory pathway. It was discovered in 1998 as a cold-inducible co-activator of PPAR- γ that promoted adaptive thermogenesis (Puigserver et al. 1998). Since then, it has become clear that PGC-1α can bind to and coactivate most nuclear receptors, as well as many other transcription factors (Lin, Handschin & Spiegelman 2005). As a transcriptional coactivator, PGC-1α functions through direct physical interaction with transcription factors to regulate gene transcription. PGC-1α mRNA and protein levels are directly correlated with muscle FAO capacity (Gerhart-Hines et al. 2007), muscle fiber switching (Lin et al. 2002) and insulin sensitivity (Pagel-Langenickel et al. 2008). In human studies, reduced PGC-1α mRNA in adult skeletal muscle has been linked to the development of insulin resistance and Type II diabetes (Mensink et al. 2007, Patti et al. 2003). Regardless of PGC-1α expression level, post translational modification is also important to its activation. Deacetylation of PGC-1 α by SIRT1 increases the activity of PGC-1α (Sugden, Caton & Holness 2010a).

Silent information regulator T1 (SIRT1) is one of the mammalian homologues of the Sir2 protein in yeast (Rodgers et al. 2005). It is a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase. SIRT1 requires NAD^+ as a cofactor to deacetylate a number of target substrates, including PGC-1α, thus leading to activation of these target substrates. A decrease in the NAD^+/NADH ratio inhibits SIRT1 activity, while an increase promotes its activity. SIRT1 regulates PGC-1α activity through NAD^+-dependent deacetylation of lysine residues (de Lange et al. 2007). Deacetylated PGC-1α is able to combine with a heterodimer, which consists of PPARs and retinoid X receptor (RXR). This protein complex (PGC-1α-PPAR-RXR) regulates genes encoding proteins involved in fatty acid uptake and oxidation (Sugden, Caton & Holness 2010b).

Peroxisome proliferator-activated receptors (PPARs) are transcription factors, which belong to the super-family of nuclear receptors. They act through binding to specific DNA sequences: peroxisome proliferator hormone response elements (PPREs), in the form of heterodimers with the RXR, and together with other cofactors, such as PGC-1α, to regulate genes expression (Kliewer et al. 2001). However, phosphorylation of PGC-1α by Akt2 at Ser 570 can decrease FAO through impairing the recruitment of this protein complex (PGC-1α-PPAR-RXR) to PPREs in the promoter region of PPARs target genes (Li et al. 2007). RXR is another nuclear receptor that functions as PPARs obligatory partner (Schaiff et al. 2005). There are three isoforms of PPAR: α, β and γ.

PPAR- α is highly expressed in skeletal muscle (Su et al. 1998). It regulates genes expression associated with fatty acid transport and oxidation through binding to PPREs (Kliewer et al. 2001). Examples of these target genes include CD36, which is involved in fatty acid uptake (Bonen et al. 2009); CPT1, which is involved in the mitochondrial transfer and oxidation of long-chain fatty acids (Cunningham et al. 2007) and the MCAD, which is an Acyl-CoA dehydrogenase within the FAO cycle with specificity for medium-chain fatty acids (Purushotham et al. 2009). Altered expressions of these genes are directly correlated with altered FAO and insulin resistance (Hulver, Dohm 2004). PPAR-γ, another nuclear receptor in PPAR family, together with RXR, enhance fatty acid uptake and accumulation *in vitro* by promoting genes expression known to be associated with fatty acid uptake and accumulation, such as FATP4 and CD36 (Schaiff et al. 2007).

PPAR- γ is regulated by SIRT1, as a previous study showed that SIRT1 negatively regulated PPAR- γ by interacting with the transcriptional corepressor, NCoR, which then inhibited fatty acid accumulation (Picard et al. 2004).

The acute energy sensor of the cell AMPK also plays an additional regulatory role in FAO. AMPK regulates FAO through its regulation of ACC activity and further downstream control of CPT1.When the energy status of a cell is low (e.g. fasting and acute hypoxia), with a rise in AMP/ATP ratio, the AMPK can be activated by phosphorylation, and consequently phosphorylates its downstream substrate ACC at a number of serine residues, such as Ser 79. ACC, when active, is an enzyme which regulates fatty acid metabolism through its catalyzation of malonyl-CoA. Malonyl-CoA is a building block for new fatty acids and can inhibit the transport of long-chain fatty acids across the mitochondrial membrane by depressing CPT1. These events ultimately inhibit FAO in the mitochondria. Phosphorylation of ACC by activated AMPK (p-AMPK) inactivates this enzyme and prevents the production of malonyl-CoA. This, in turn, releases the inhibitory effect exerted by malonyl-CoA on CPT1and promotes the transport of long-chain fatty acids into the mitochondria for β-oxidation (Kahn et al. 2005, Ruderman et al. 1999).

Besides, AMPK affects GLUT4 protein as well, in an insulin- independent manner (Fryer et al. 2002). Previous studies have shown that chronic chemical activation of AMPK increases total cellular GLUT4 protein level in rat muscle (Holmes, Kurth-Kraczek & Winder 1999), which suggests the possibility of targeting the AMPK as a potential treatment of insulin resistance.

All the above facts highlight that PGC-1α/PPARs pathway plays a major role on controlling FAO genes and through this control regulates overall FAO capacity. A further regulatory input is through AMPK/ACC and together, the AMPK and FAO/PGC-1α/PPARs pathway control the overall FAO capacity and thereby impact insulin sensitivity. Hypoxia, one of the essential components of placental insufficiency, may interfere with these interactions. One of the major basis of this interference is that hypoxia alters the cellular redox balance by reducing NAD^+ concentration, which results

in increased NADH concentration (Wu et al. 2006) . This change could result in decreased SIRT1activity, as SIRT1 requires NAD^+ as a cofactor to deacetylate target substrates. Therefore, PGC-1α activity may be influenced by hypoxia through SIRT1. Besides, a previous study has reported that hypoxia stimulated the increased expression of cardiac membrane fatty acid transporters (e. g. CD36), contributing to lipid accumulation (Chabowski et al. 2006a, Chabowski et al. 2006b). Additionally, the stress of hypoxia likely induces an acute energy deficient status and an alteration in the AMP/ATP ratio thereby promoting AMPK activity. All of these indicate the possibility that hypoxia might impede FAO/PGC-1α/PPARs pathway in skeletal muscle, which ultimately setting the stage for impaired insulin signalling and development of insulin resistance.

1.4.3 Links between lipid metabolism and insulin resistance

A growing body of scientific evidence indicates a strong correlation between lipid metabolism and insulin resistance, and ultimately type II diabetes (Kelley, Goodpaster & Storlien 2002a). Data reported by Pan and colleagues obtained from lipid extractions of human biopsy samples of vastus lateralis showed that TG level was significantly increased in obesity and was directly associated with the severity of insulin resistance (Pan et al. 1995). In another study using light microscopy, Goodpaster observed that the volume of lipid droplets occupied in myocytes was 1.5% in lean volunteers, while this proportion rose to 3-4% in obesity, and slightly higher in type II diabetes (Goodpaster et al. 2000). All of these studies emphasize the importance of lipid accumulation in the development of insulin resistance and its related type II diabetes. But what is the underlying mechanism by which lipids contribute to insulin resistance? Studies have found that excessive intracellular fatty acids, which are the lipid building blocks, have deleterious effects on insulin action (Yu et al. 2002b).

Fatty acids are crucial energy source besides glucose in skeletal muscle. Once entering the myocyte, fatty acids are directed towards either the synthesis of lipid metabolites or mitochondrial β-oxidation. Increases in fatty acids uptake and/or decreases in mitochondrial fatty acids β-oxidation leading to excessive fatty acids in cells, results in accumulation of lipid intermediates. These lipid intermediates, including long-chain

Acyl-CoA, diglyceride (DAG), ceramide and/or TG, can activate a number of different serine kinases, such as PKC and c- jun n-terminal kinase (JNK) (Yu et al. 2002b). These serine kinases phosphorylate serine residues of IRS-1, and decrease phosphorylation of tyrosine residues and activation of IRS-1. Inhibition of IRS-1consequently decreases activation of downstream signalling markers (e.g. PI3 kinase and Akt) along the insulin pathway (Schenk, Saberi & Olefsky 2008), leading to reduce GLUT4 translocation and glucose uptake into cells (Dresner et al. 1999) (Figure 1.3).

1.5 Thesis objectives

Hypoxia is an important component of placental insufficiency, which is the most common cause of IUGR. The IUGR condition has been highlighted as predisposing offspring to metabolic associated diseases in their later life. Reduced insulin sensitivity, is believed to be a critical pathophysiological event early in metabolic associated disease development. Insulin sensitivity is regulated by the insulin signalling pathway and its interaction with FAO: the later is ultimately regulated by FAO/PGC-1α/PPARs interactions. Given that hypoxia may adversely modulate components of these pathways, it is possible that hypoxia as an insult during *in utero* development may impair insulin sensitivity by altering or modulating components of the insulin signalling pathway and or FAO/PGC1-α/PPARs *in utero*. It is further feasible to speculate that these alterations or modulations may persist after birth, making these offspring more likely to suffer from chronic metabolic associated diseases. Therefore, I postulated that prolonged hypoxia, during muscle cell differentiation, induces impairment of the FAO/PGC-1α/PPARs in conjunction with aspects of insulin signalling. Further, these changes will remain following a recovery period in normal oxygen.

Prediction

Mouse skeletal muscle cells that differentiate under prolonged hypoxia will have an increased intramyocyte TG level in conjunction with altered and modified aspects and components of insulin signalling and FAO/PGC-1α/PPARs pathways. I predict that these alterations and modifications will persist following a recovery treatment under normoxia.

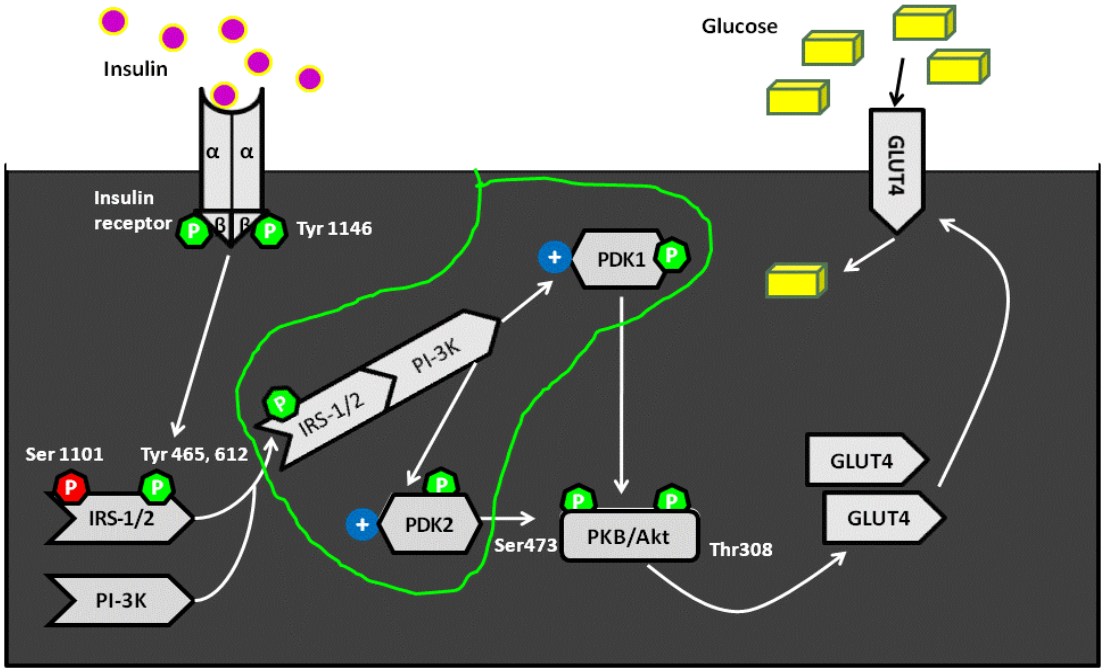

Figure 1.1 Summary of the insulin signalling pathway. Once insulin binds to insulin receptor (IR), IR will auto-phosphorylate a number of tyrosine residues. Certain tyrosine residues are recognized by insulin receptor substrate (IRS), which is recruited to the receptor. IR then activates IRS by phosphorylating IRS molecules at numerous tyrosine residues. Tyrosine phosphorylation of the IRS protein leads to an interaction with and subsequent activation of several downstream substrates along the insulin signalling pathway, ultimately resulting in the activation of Akt. The activated Akt then stimulates glucose uptake by inducing glucose transporter type 4 (GLUT4) translocation from intracellular storage to plasma membrane. Figure was adapted from: www. environmentalhealthnews.org /newscience/2007/2007-0405insulinsignaling.html.

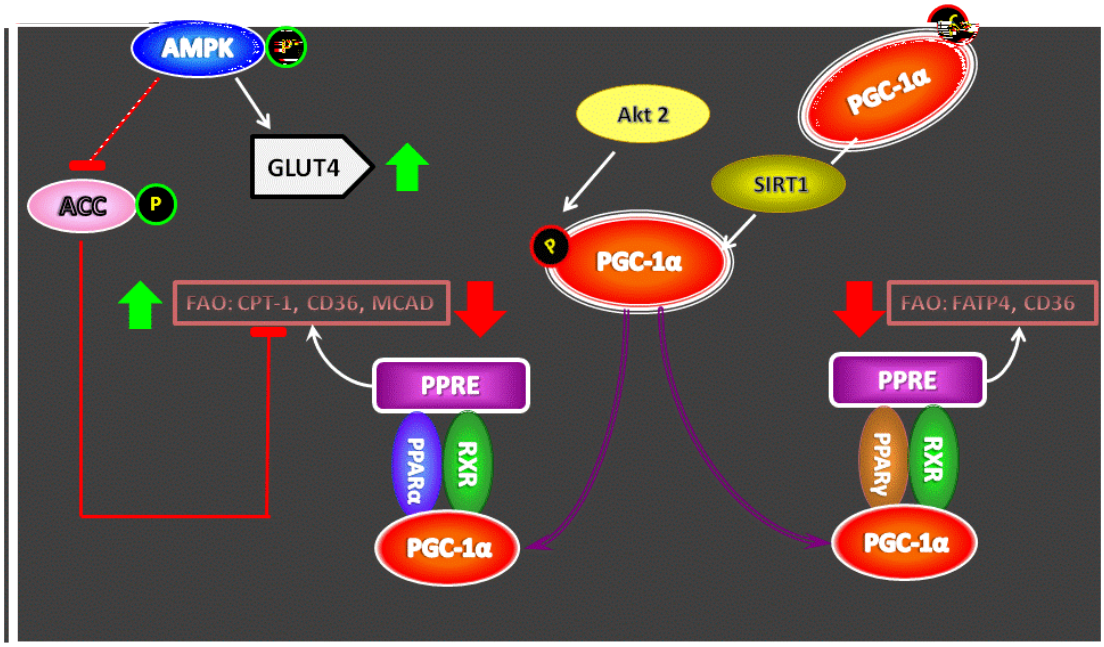

Figure 1.2 Illustration of the FAO/PGC-1α/PPARs interactions. The PPAR- γ coactivator-1α (PGC-1α) lies at the heart of the FAO/PGC-1α/PPARs interactions. It is deacetylated by Silent information regulator T1 (SIRT1), which is a cellular deacetylase. This deacetylated PGC-1α then goes to combine with PPAR-RXR heterodimer. Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors which function as transcription factors. Retinoid X receptor (RXR) is another nuclear receptor which works as PPARs obligated partner. When PGC-1α combines with PPAR-RXR, this protein complex then goes to connect with specific DNA sequence: peroxisome proliferator hormone response elements (PPRE) to regulate gene transcriptions which are involved in fatty acid β-oxidation (FAO), uptake and accumulation. These genes include carnitine palmitoyltransferase I (CPT1), cluster of differentiation 36 (CD36), medium-chain Acyl-CoA dehydrogenase (MCAD) and fatty acid transport protein 4 (FATP4). However, when PGC-1α is phosphorylated by Akt2, the recruitment of the protein complex (PGC-1α-PPAR-RXR) to specific DNA sequence of promoter region will be impaired, which leads to decreased FAO rate. One the other hand, FAO is also regulated by AMP-activated protein kinase (AMPK)/ acetyl-CoA carboxylase (ACC) through CPT1. When ACC is phosphorylated and inhibited by AMPK during fasting or acute hypoxia, the inhibition of ACC to CPT1 can be removed, which consequently increases FAO rate. In addition, the activated AMPK up-regulates total GLUT4 protein content.

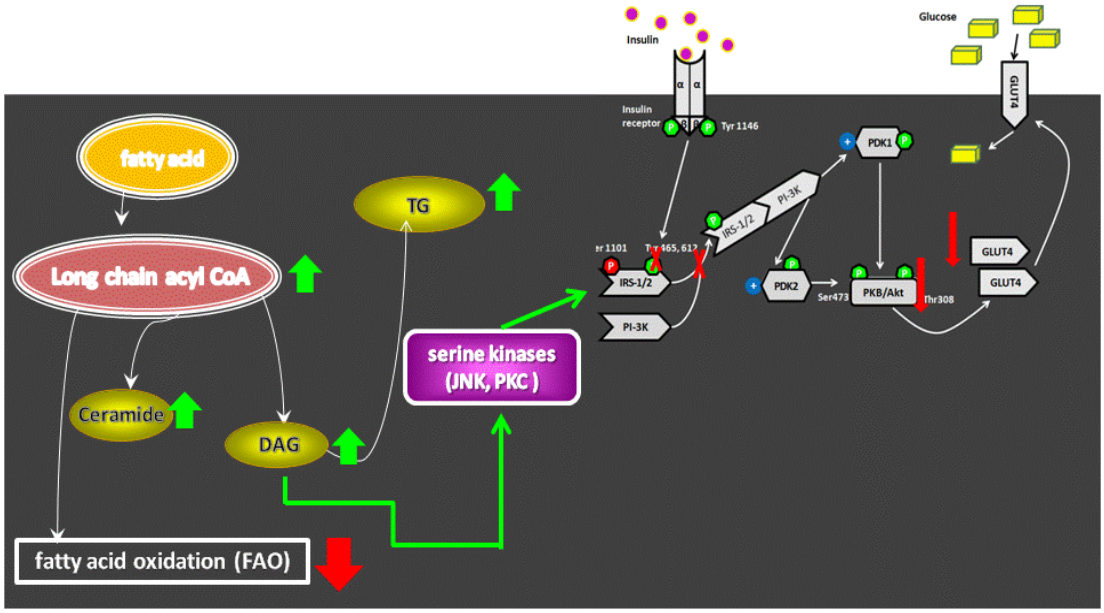

Figure 1.3 Classic model of lipid-induced insulin resistance. Decreased FAO rate will result in accumulation of lipid intermediates including triglyceride (TG), diglyceride (DAG), long-chain Acyl-CoA and ceramide. These intermediates activate serine kinases, which phosphorylate serine residues of IRS. This will decrease tyrosine phosphorylation and activation of IRS, leading to decreased activation of downstream molecules along the insulin signalling pathway (e.g. Akt).

Figure was adapted from:www. environmentalhealthnews .org/newscience/2007/2007-0405insulinsignaling.html and Zhang et al. 2010.

Chapter 2 Materials and Methods

2.1 Experiment design

Animal models using tools such as maternal uterine artery ligation, which mimic placental insufficiency, can display the compounding effects of both hypoxemia and undernutrition. However, it is also important to investigate the separate effect of each regulator, by assessing it directly without any alterations to other regulators. To accomplish this purpose, an *in vitro* system using a mouse muscle cell line C_2C_{12} (ATCC catalog number CRL-1772) was employed in my project. A C_2C_{12} myoblast cell line, which is derived from mice, closely parallels skeletal muscle differentiation from myoblasts to myotubes, and has been widely used in studies of hypoxia, insulin sensitivity, as well as the FAO/PGC-1α/PPARs pathway (Sun et al. 2007, Dressel et al. 2003, Yun, Lin & Giaccia 2005, Arthur, Giles & Wakeford 2000).

C_2C_{12} myoblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin until ~90% confluency. Then cells were induced to form myotubes by then supplementing media with 2% adult horse serum (AHS) instead of FBS, and were incubated in 21%, 5% or 1% O_2, with 5% CO_2 at 37°C. The 5% and 1% O_2 treatments were selected as these values are representative of fetal oxygenation in the normal situation (5% ~ 38 Torr) and in the hypoxic fetal growth-restricted situation (1% ~ 8 Torr) from previous data (Regnault et al. 2007) and as recently described by others for *in utero* culture (Casanello et al. 2009). Hypoxia was attained by placing appropriate cultures in an anaerobic incubator (Modular Incubator Chamber (MIC-10), Billups-Rothberg, Del Mar, CA) flushed and filled with a predetermined oxygen mixture, either 1% or 5% O_2 (with 5% CO_2, balance N_2). After 7 days of differentiation, a subset of cells in each oxygen regime was placed in 21% oxygen as a recovery period for additional 2 days. C_2C_{12} cells were collected, as outlined in Figure 2.1, at various time points through the experiment: Day 0 (cells grown at 21% O_2 before growth medium was replaced with differentiation medium), Day 7 (cells after seven days of differentiation under 1 of 3 O_2 treatments) and

recovery (cells after Day 7 and the 2-day recovery period at 21% oxygen). Collected cells had TG, mRNA, and protein analyses.

2.2 RNA isolation and real-time PCR procedures

2.2.1 RNA isolation

Adherent C_2C_{12} cells were collected in Trizol reagent (Invitrogen, catalog number 15596-026) following 2 PBS washes and stored at -80°C before RNA isolation. Total RNA was then isolated from C_2C_{12} cells using Trizol following the manufacturer's instructions. Briefly, cell samples were thawed, and incubated in Trizol at room temperature for 5 minutes. Two hundred microliters of chloroform was added to the 1 ml of Trizol used initially. Samples were shaken vigorously by hand for 15 seconds and left at room temperature for 3 minutes, and then centrifuged for 15 minutes at 12,000 × g at 4°C. The mixture separated into a lower red phenol-chloroform phase, an inter-phase, and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase. The upper aqueous phase was ~50 % of the total volume. The top chloroform phase was removed and placed in a fresh tube and 500 µl of isopropyl alcohol were added to the top phase for every ml of Trizol used initially. This mixture was incubated at room temperature for 10 minutes prior to centrifugation at 12,000 × g for 15 minutes at 4°C. The chloroform and isopropyl alcohol supernatant was removed, leaving only the RNA pellet. The pellet was washed twice by adding 1 ml of 75% ethanol per 1 ml of Trizol used in the initial cell samples. Samples were vortexed briefly, and then were centrifuged at 7,500 × g for 5 minutes at 4°C. The ethanol was removed with a pipette. These pellets were air dried and dissolved in diethylpyrocarbonate treated water for 5 minutes at room temperature and stored in -80°C before cDNA production.

The quantity of RNA yield was determined by a NanoDrop 2000 spectrophotometer (Thermo-Scientific) and quality was measured using the A260/A280 ratio (≥1.8). To further ensure RNA quality, samples were separated on a 1.2% agarose gel containing ethidium bromide. Samples were screened for degradation by the visualization of the 28S:18S bands; only samples without degradation were used for further analysis.

2.2.2 Reverse transcription and real-time PCR

Two micrograms total RNA was used for reverse transcription. Briefly, samples were treated with deoxyribonuclease (Invitrogen), and then a recombinant ribonuclease inhibitor (Invitrogen) was added to each sample and incubated at 37°C for 30 minutes. Next, samples were incubated with murine leukemia virus reverse transcriptase (Invitrogen) at 37°C for 2 hours to generate cDNA with the use of random primers. cDNA was diluted 1/10 before real-time PCR.

Real-time PCR was performed using Fast EvaGreen Supermix (Invitrogen) on the CFX 384 real-time PCR detection system (Bio-Rad). A total volume of 8 µl in each well contained 3 µl of diluted cDNA, 0.08 µl primer mix, 4 µl SYBR Green and the remaining volume was filled with autoclaved water. Primers sets were designed using the NCBI Primer-BLAST tool based on published *Mus musculus* sequences. Specific primer sets for mouse SIRT1, PGC-1α, PPAR- α, PPAR- γ, FATP4, MCAD, CD36 and ribosomal protein L7 (RL7) are listed in Table 1. All results were standardized by a reporter gene RL7and then expressed relative to the undifferentiated cells (D0) using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001).

2.3 Western blotting analysis

2.3.1 Total protein extraction

Adherent C_2C_{12} cells were washed twice with ice cold PBS in the dish. Ice cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, pH 7.4) supplemented with protease and phosphatase inhibitor cocktail (Thermo Scientific catalog number 78446) was then utilized to extract total proteins from cells. Cells were mixed by triturating, and then were incubated on ice for 10 minutes. Extractions were centrifuged at 4°C at 16,000 g for 10 minutes and supernatants were collected and stored in -80°C for further analyses.

2.3.2 Protein quantification and immunoblotting

Protein quantity was measured using a BCA Protein Assay Reagent Kit (Pierce), and determined using a Sepectramax spectrophotometer (Molecular Devices). Eighteen

micrograms of total protein was mixed with sample reducing agent (10×) and LDS sample buffer (4×), heated for 10 minutes at 90-100°C and loaded onto a 4-12% SDS-PAGE gradient gel. Gel electrophoresis was then undertaken at 175 V for 45-60 minutes in MES buffer until proteins were separated. After the proteins were sufficiently separated, they were transferred from SDS-PAGE gradient gels onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Buckinghamshire, UK) for 2 hours at 100 V. Amino black staining was performed to show efficient transfer and equal loading of protein (as shown in Figure 2.2). Following several TBST washes, membranes were blocked in 5% milk or 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBST) (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.6) for 1 hour at room temperature. And then membranes were incubated with primary antibodies of 1:1000 dilutions overnight at 4°C. Primary antibodies for western blotting are listed in Table 2. Following the overnight primary incubation, membranes were then washed with TBST several times before incubation with a horseradish peroxidase conjugated donkey anti-rabbit secondary antibody (711-0350152, 1:10,000; Jackson Immunoresearch laboratories, Inc. West Grove, PA) at room temperature for 1 hour. Following secondary antibody incubation, membranes were washed several times prior to detection by SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific, part number 34087). VersaDoc Imaging System (BioRad) and Image Lab Software (BioRad) were employed to visualized and quantified protein bands, respectively.

2.4 Immunoprecipitation

Adherent C_2C_{12} cells were washed twice with PBS in the dish. Ice-cold modified RIPA buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol, pH 7.4) supplemented with protease and phosphatase inhibitor cocktail (Thermo cat# 78446) was added to cells. Cells were scraped and transferred into eppendorf tubes. Cell suspensions were sonicated at 30% output amplitude for 4 bursts (burst/second). Protein quantity was measured with a BCA Protein Assay Reagent Kit (Pierce) as described above. Cell lysate was diluted to 1µg/µl. Two hundred microliters of diluted cell lysate was used in each assay. Cell lysate was precleared with 20 µl protein A/G Plus-Agarose Immunoprecipitation Reagent (agrose beads) (Santa Cruz, sc-2003, 0.5 ml agarose in 2.0

ml PBS buffer with 0.02% azide) by rotating for 1 hour at 4°C. Then samples were centrifuged at 1000 g for 2 minutes at 4°C, the supernatants were then collected. One half microgram of PGC-1α antibody (Santa Cruz, sc-13067, Rabbit) was then added to each precleared sample prior to rotation overnight at 4°C. Following the overnight rotation, another 20 µl of protein A/G Plus-Agarose Immunoprecipitation Reagent (agrose beads) were added to samples, and incubated for 1 hour at 4°C. After centrifuging samples at 1,000 g for 2 minutes at 4°C, the agarose beads were collected. Then a series of washes were performed with modified RIPA buffer, except the last wash using TBS (1×) buffer. Samples were centrifuged at 1,000 g for 2 minutes at 4°C after each wash, and beads were collected. To each sample, 40-50 µl of LDS (2×)-10% mercaptoethanol was added before heating at 90-95°C for 5 minutes. Equal fractions of the supernatant were used in western blotting as described above for PGC-1α (calbiochem cat# 516557, Rabbit) and acetylated-lysine (cell signaling, cat# 9441, Rabbit) antibodies.

2.5 Triglyceride assay

To determine intracellular TG level, a TG assay was performed using an adipogenesis detection kit (Abcam, catalog number ab102513). Briefly, after washing adherent C_2C_{12} cells twice with PBS, they were collected and then transferred into eppendorf tubes. Cell suspensions were centrifuged at 1,000 g for 10 minutes at 4°C and the supernatant was removed. One hundred microliters of lipid extraction solution was added to each sample, before sonicating samples twice at 30% output amplitude for 4 bursts (burst/second). Then samples were heated at 90-100°C for 30 minutes, followed by vortexing for 1 minute prior to centrifugation briefly to remove insoluble material. Fifty microliters of the lipid extracts were transferred to a 96-well plate. A standard curve was prepared according to the manufacturer's instructions. Forty microliters of TG standard (1 mM) was diluted into 160 µl assay buffer to generate 0.2 mM standard. 0, 10, 20, 30, 40, 50 µl of the 0.2 mM TG standard was added into a series of wells, volume was adjusted to 50 µl/well with assay buffer to generate 0, 2, 4, 6, 8, 10 nmol/well of TG standard. Two microliters of lipase was added to each well with sample and standard. The plate was mixed well and incubated at room temperature for 10 minutes. Fifty microliters of Reaction Mix (adipogenesis assay buffer 46 µl, probe 2 µl and enzyme mix 2 µl) was

added to each well containing the TG standard or sample. The plate was mixed well and incubated at 37°C for 30 minutes, protected from light. The OD at $_{570nm}$ was measured for colorimetric assay in a microplate reader. Protein concentration of the lipid extract was tested and used as an internal control to normalize the lipid concentration in the samples. Total cellular TG levels were expressed in mol/g.

2.6 Statistical analysis

All data was presented as mean ± SEM, and was analyzed with a Student's two-tailed unpaired t-test or a one-way ANOVA, followed by a Tukey's post-test (Graphpad Prism 5, GraphPad Software, Inc.). Significance was set at a P-value of 0.05 or less. Any sets of data that failed the Shapiro-Wilk normality test or equal variance test (Bartlett's test) were transformed using the log10 and re-tested for normality and equal variance.

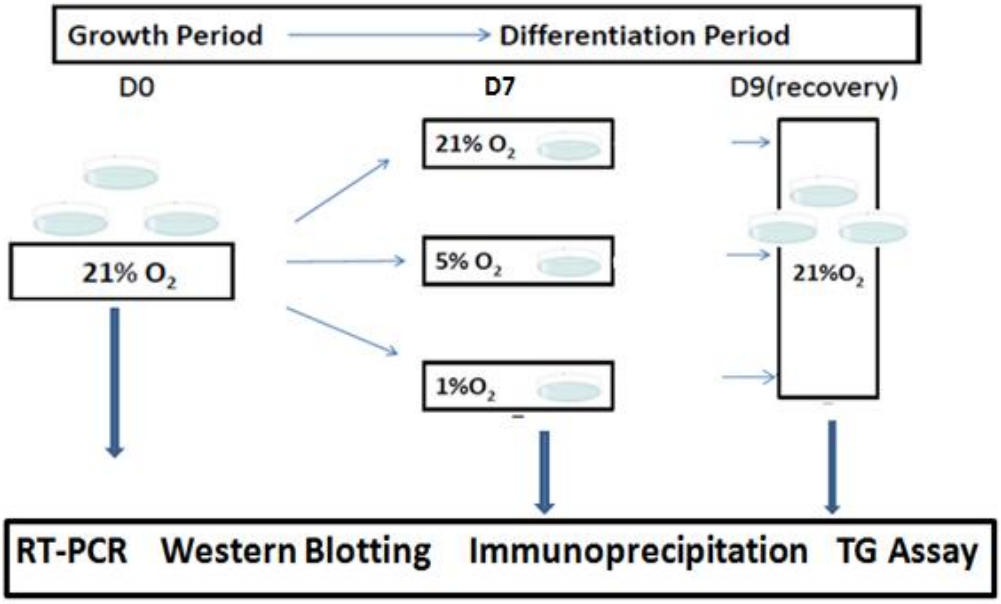

Figure 2.1 Schematic of cell culture procedures and sampling. C_2C_{12} mouse skeletal muscle myoblasts were induced to differentiation under 1%, 5% and 21% O_2 for seven days (D7), after cells reached approximately ~90% confluency in growth medium (D0). After 7 days differentiation, a subset of cells from each O_2 tension was placed in a recovery condition of 21% O_2 for additional 2 days (D9).

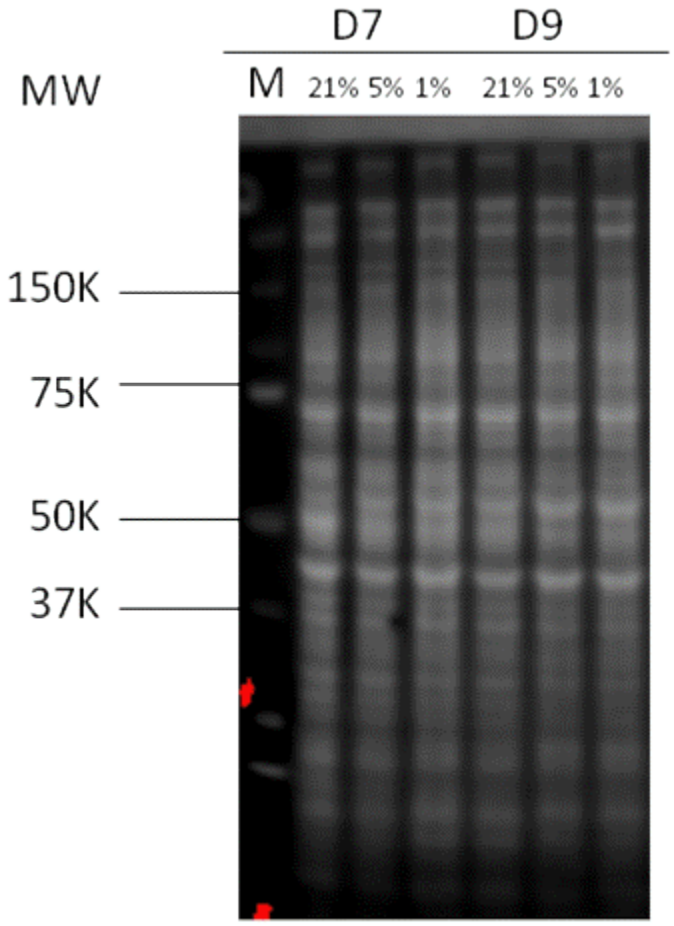

Figure 2.2 Representative amino black stained blot. A representative amino black stained blot was selected to show efficient transfer and equal loading of protein. MW, molecular weight; M, marker; D7, day 7; D9, day 9; 21%, 5% and 1% indicate oxygen tensions.

Table 2.1 Primer sequences for real-time PCR

Primers	Accession No.	Strand	Sequence (5'→3')
SIRT1	NM 019812.2	Forward Reverse	5'-ATATTCCACGGTGCTGAGGT 5'-TCCAAATCCAGATCCTCCAG
PGC-1α	NM 008904.2	Forward Reverse	5'-AACGATGACCCTCCTCACAC 5'-GGGTCATTTGGTGACTCTGG
PPAR- α	NM 011144.6	Forward Reverse	5'-AACCGGAACAAATGCCAGTA 5'-CCGAATCTTTCAGGTCGTGT
PPAR- γ	NM 001127330.1	Forward Reverse	5'-CCAACTTCGGAATCAGCTCT 5'-CAACCATTGGGTCAGCTCTT
FATP4	NM 011989.4	Forward Reverse	5'- CAGCAACTGTGACCTGGAGA 5'- CCTTCCGCAACTCTGTCTTC
MCAD	NM 007382.4	Forward Reverse	5'-ACACAACACTCGAAAGCGGC 5'-CCTCTCTGGCAAACTTGCGG
CD36	NM 001159555.1	Forward Reverse	5'-ATTGGTGCAGTCCTGGCTGT 5'-TCTTTGCCACGTCATCTGGGT
RL7	NM 011291.5	Forward Reverse	5'-GGAGCTCATCTATGAGAAGGC 5'- AAGACGAAGGAGCTGCAGAAC

Table 2.2 Primary antibodies utilized in western blotting

Antibody	Company	Catalogue #	Host
SIRT1	Santa Cruz	sc-15404	Rabbit
PPAR- γ	Santa Cruz	sc 7196	Rabbit
PPAR- α	Abcam	ab24509	Rabbit
GLUT4	Abcam	ab65976	Rabbit
PGC-1α	Calbiochem	516557	Rabbit
p-PGC-1α (S571)	R &D system	AF 6650	Rabbit
AMPK- α	Cell signaling	2603	Rabbit
p-AMPK- α (Thr 172)	Cell signaling	2535	Rabbit
ACC	Cell signaling	3676	Rabbit
p-ACC (ser 79)	Cell signaling	3661	Rabbit
IR- β	Cell signaling	3025	Rabbit
p-IR (Tyr 1146)	Cell signaling	3021	Rabbit
IRS1	Cell signaling	2382	Rabbit
p-IRS1 (Ser 1101)	Cell signaling	2385	Rabbit
Akt (pan)	Cell signaling	4691	Rabbit
p-Akt (Thr 308)	Cell signaling	2965	Rabbit
Akt2	Cell signaling	3063	Rabbit

Chapter 3 Results

3.1 Low oxygen tension partially affected the protein contents of insulin signalling pathway markers

In order to determine if chronic hypoxia impairs the insulin signalling pathway, western blotting was utilized. No significant alterations were identified in the protein content for IR, IRS, p-IR (Tyr 1146) or p-IRS (Ser 1101) among groups (21%, 5% and 1% O_2) (Figure 3.1 and 3.2). However, the basal (without insulin stimulation) levels of p-Akt (Thr 308) and the subsequent p-Akt (Thr 308) to Akt (pan) protein ratio were significantly lower in the 1% O_2 treatment relative to those treated with 21% O_2 at day 7 ($p<0.05$) (Figure 3.3).

3.2 Impact of chronic hypoxia on total GLUT4 protein content

Given that chronic activation of AMPK increased total GLUT4 protein content in rat epitrochlearis and gastrocnemius muscle (Holmes, Kurth-Kraczek & Winder 1999), we determined whether chronic hypoxia, and the subsequent activation of AMPK, increased total cellular GLUT4 protein content in skeletal muscle cells using western blotting. Significantly higher total GLUT4 protein was detected in 1% ($p<0.001$) and 5% ($p<0.001$) O_2 treatments compared to the 21% O_2 treatment; also the protein level in 1% was significantly higher than that in the 5% O_2 treatment ($p<0.001$; Figure 3.4 A) at day 7.

3.3 Cells treated with low oxygen tension displayed higher triglyceride level

In order to determine the effects of prolonged hypoxia on lipid metabolism, the cellular TG level of C_2C_{12} myotubes was determined using an adipogenesis detection kit. The TG level of the 1% O_2 treated cells was significantly higher when compared to that of the 21% O_2 treated cells ($p<0.05$), and was higher than that of the 5% O_2 treated cells by 83% ($p= 0.063$) (Figure 3.5).

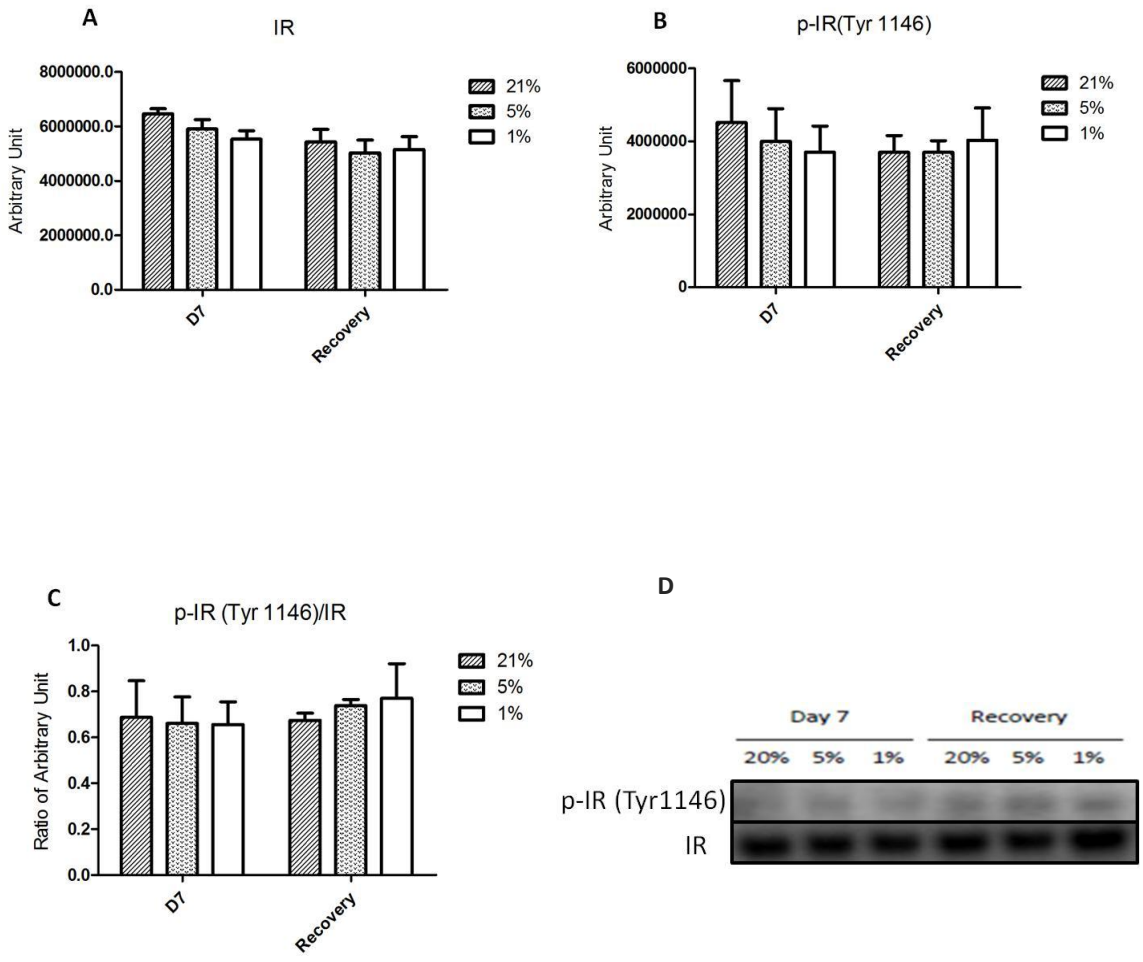

Figure 3.1 IR was not affected in hypoxic treated muscle cells. C_2C_{12} cells were collected and total protein extractions were used in western blotting with antibodies specific to IR and p-IR (Tyr 1146). Densitometry was performed and represented in (A) and (B); ratio of p-IR to IR was calculated and represented in (C). Representative western blots are shown in (D). A one-way ANOVA test was used. All data was presented as mean ± SEM (* $p<0.05$, n=4/experimental group).

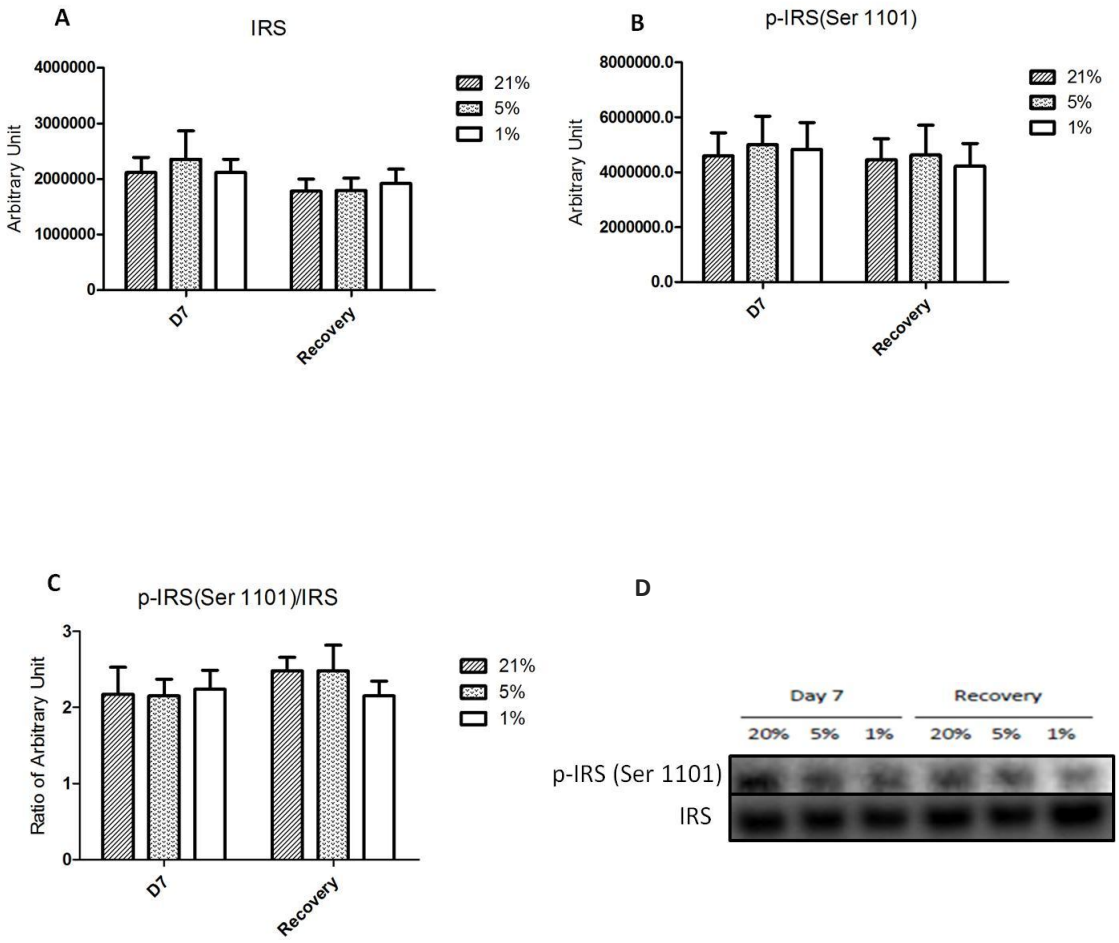

Figure 3.2 IRS was not affected in hypoxic treated muscle cells. C_2C_{12} cells were collected and total protein extractions were used in western blotting with antibodies specific to IRS and p-IRS (Ser 1101). Densitometry was performed and represented in (A) and (B); ratio of p-IRS to IRS was calculated and represented in (C). Representative western blots are shown in (D). A one-way ANOVA test was used. All data was presented as mean ± SEM (* $p<0.05$, n=4/experimental group).

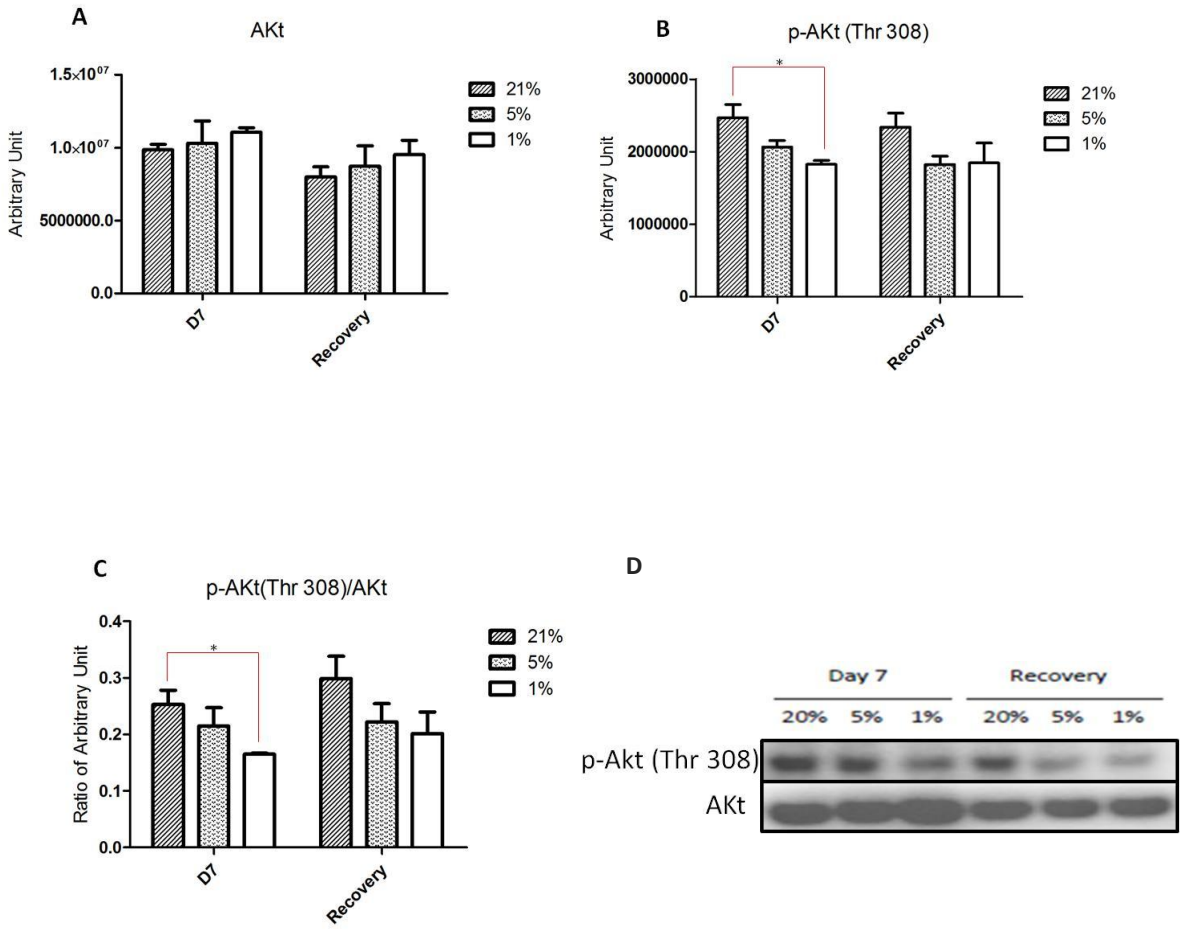

Figure 3.3 p-Akt protein decreased with hypoxic treatment. C_2C_{12} cells were collected and total protein extractions were used in western blotting with antibodies specific to Akt (pan) and p-Akt (Thr 308). Densitometry was performed and represented in (A) and (B); ratio of p-Akt to Akt was calculated and represented in (C). Representative western blots are shown in (D). A one-way ANOVA test, with a Tukey's post-test and a student's two-tailed unpaired t-test were used. All data was presented as mean ± SEM (* $p<0.05$, n=4/experimental group).

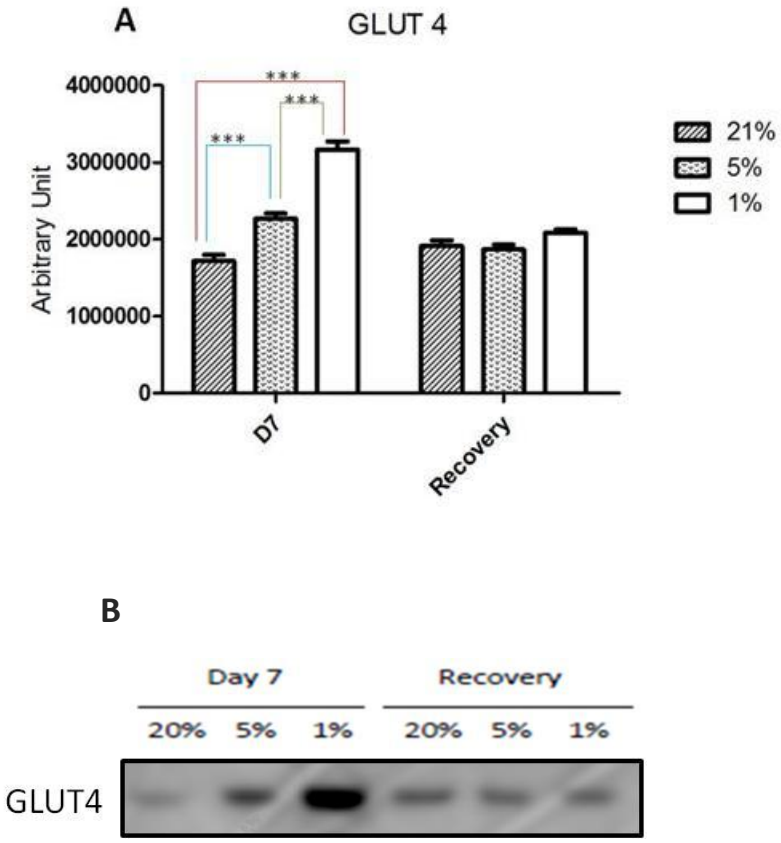

Figure 3.4 GLUT4 protein was elevated in hypoxia. C_2C_{12} cells were collected and total protein extractions were used in western blotting probing with antibody specific to GLUT4. Densitometry was performed and represented in (A). A representative western blot is shown in (B). A one-way ANOVA with a Tukey's post-test was utilized. All data was presented as mean ± SEM (*** $p<0.001$; n=7/experimental group).

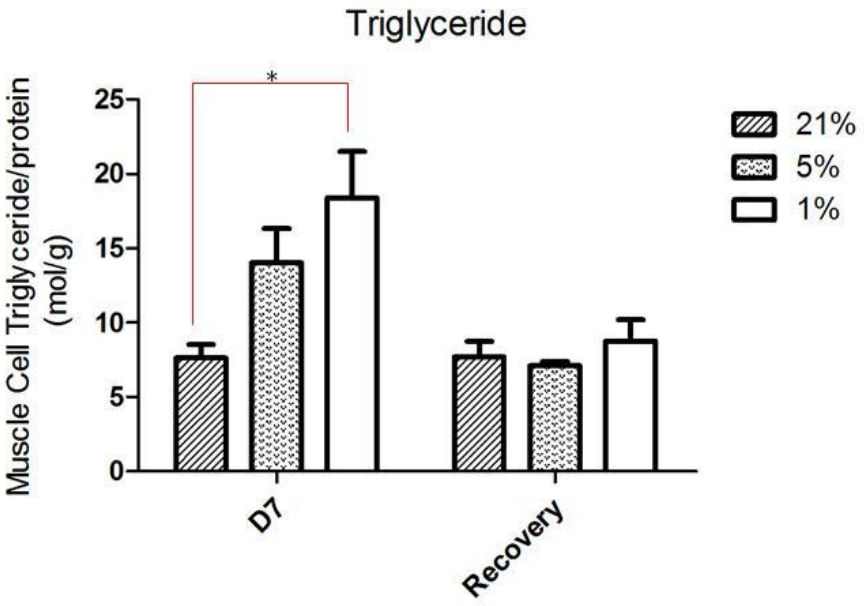

Figure 3.5 Cellular TG content increased in hypoxia. C_2C_{12} cells were collected and total cell TG was extracted and measured. Protein concentration was measured as an internal control. A one-way ANOVA with a Tukey's post-test was utilized. All data was presented as mean ± SEM (* $p<0.05$; n=4~5/experimental group).

3.4 SIRT1 mRNA and protein levels are lower in prolonged hypoxia

SIRT1 is a crucial cellular deacetylase. It activates PGC-1α by deacetylation and allows the formation of the protein complex of PGC-1α-PPAR-RXR. This complex then binds to specific DNA sequences to regulate gene expressions involved in FAO, which ultimately impact upon insulin sensitivity. SIRT1 mRNA was determined using real-time PCR. There were significantly lower SIRT1 mRNA in both 1% and 5% O_2 treatments ($P<0.05$) relative to the 21% O_2 treatment at day 7 (Figure 3.6 A).

Examination of SIRT1 protein by western blotting showed that differentiating C_2C_{12} cells with the 1% and 5% O_2 treatments had protein levels that were 23% ($p<0.01$) and 8% ($p>0.05$) lower, respectively than that with the 21% O_2 treatment at day 7 (Figure 3.6 B).

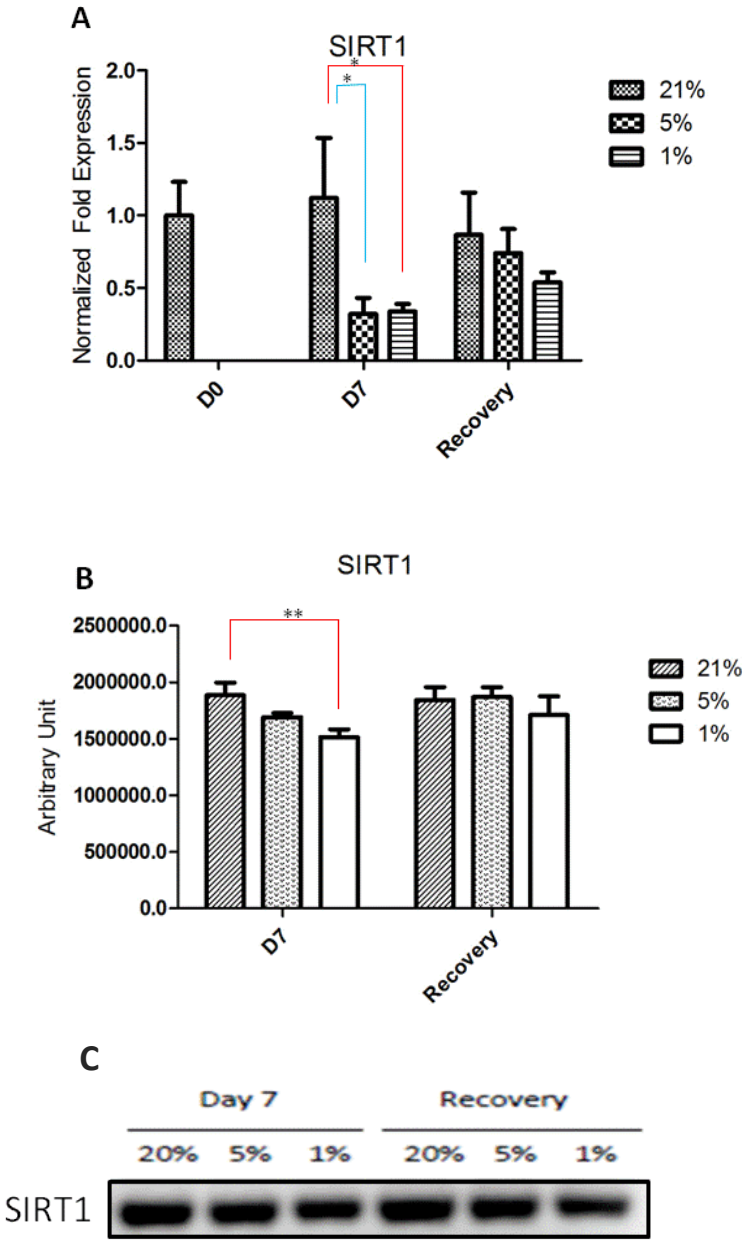

Figure 3.6 SIRT1 mRNA and protein levels decreased in hypoxia. C_2C_{12} cells were collected; SIRT1mRNA (A) and protein (B) were analyzed by real-time PCR and western blotting, respectively. SIRT1 mRNA was standardized to a house keeping gene (RL7), and then expressed relative to the undifferentiated cells (Day 0). A representative western blot of SIRT1 is shown (C). A one-way ANOVA with a Tukey's post-test was employed. All data was presented as mean ± SEM (* $p<0.05$, ** $p<0.01$; n=4~7/experimental group).

3.5 Reduced oxygen tension did not affect PGC-1α mRNA but did affect protein content and post translational modification

3.5.1 Effects of chronic hypoxia on PGC-1α mRNA and protein levels

Real-time PCR and western blotting were utilized in order to examine whether chronic hypoxia alters PGC-1α mRNA and protein levels. C_2C_{12} cells differentiating under hypoxia (1% O_2) had significantly lower PGC-1α protein level (by 35%) when compared to the 21% O_2 treatment at day 7 ($p<0.05$; Figure 3.7 B). However, there was no significant change observed among groups for PGC-1α mRNA levels at day 7 (Figure 3.7 A).

3.5.2 Effect of chronic hypoxia on PGC-1α acetylation status

Since SIRT1 protein level was found to be lower in 1% oxygen at day 7 (Figure 3.1 A), and given the fact that SIRT1 deacetylates PGC-1α, it was reasonable to postulate that the ratio of acetylated PGC-1α to total PGC-1α might be higher in the 1% group at day 7. To examine this hypothesis, immunoprecipitation and western blotting were performed. There was no significant difference between any of the 3 groups (21%, 5% and 1% O_2) when comparing the total PGC-1α precipitates protein level at day 7 (Figure3.8 A). However, under 1% O_2 treatment for 7 days, acetylated PGC-1α protein level was significantly higher ($p<0.05$; Figure 3.8 B); subsequently, the ratio of acetylated PGC-1α to total PGC-1α was higher ($p<0.05$; Figure 3.8 C).

3.5.3 Effect of chronic hypoxia on PGC-1α phosphorylation status

PGC-1α activity is not only regulated through its acetylated status, but also determined by phosphorylation. Phosphorylation of PGC-1α at Ser 570 prevents the recruitment of PGC-1α to the cognate promoters, and thus impairs its ability to activate genes involved in FAO. In order to determine whether PGC-1α phosphorylation status may be altered under low oxygen tension, western blotting was employed with primary antibodies: PGC-1α and p-PGC-1α (Ser 571). As described in 3.5.1, cells that were differentiated under 1% O_2 had a significantly lower PGC-1α protein level when compared to the 21% O_2

treatment at day 7 ($p<0.05$; Figure 3.7 B or Figure 3.9 A). Lower total PGC-1α protein level was accompanied by higher p-PGC-1α (Ser 571) protein content relative to those under 5% ($p<0.05$; Figure 3.9 B) and 21% O_2 ($p<0.01$; Figure 3.9 B) after 7 days of differentiation. Furthermore, when expressed as a ratio (p-PGC-1α to total PGC-1α) the differences are more significant (1% vs. 21%, $p<0.001$and 1% vs. 5%, $p<0.01$; Figure 3.9 C).

3.5.4 Effect of chronic hypoxia on Akt2 protein content

Protein Akt2 is reported to be involved in the phosphorylation of PGC-1α at Ser 570 with insulin stimulation. Given we observed a significantly higher p-PGC-1α (Ser 571) protein content under chronic hypoxia (1% O_2) in present study, we postulated that the level of Akt2 protein would be higher under chronic hypoxia. However, examination of Akt2 protein revealed no alterations among the 3 treatments (21%, 5% and 1% O_2) at day 7 (Figure 3.10).

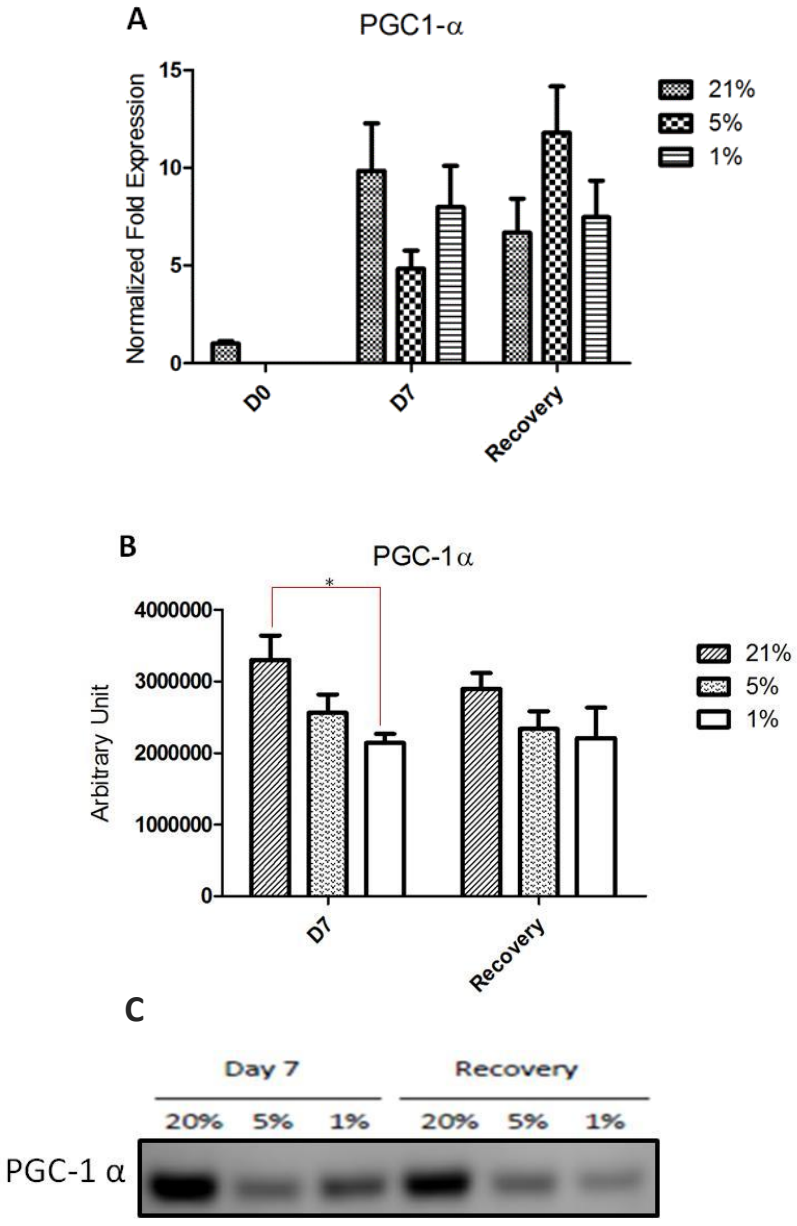

Figure 3.7 Reduced oxygen tension did not affect PGC-1α mRNA, but did affect protein content. C_2C_{12} cells were collected; PGC-1α mRNA (A) and protein (B) were analyzed by real-time PCR and western blotting, respectively. PGC-1α mRNA was standardized to a house keeping gene (RL7), and then expressed relative to the undifferentiated cells (Day 0). A representative western blot of PGC-1α is shown (C). A one-way ANOVA with a Tukey's post-test was employed. All data was presented as mean ± SEM (* $p<0.05$; n=4~6/experimental group).

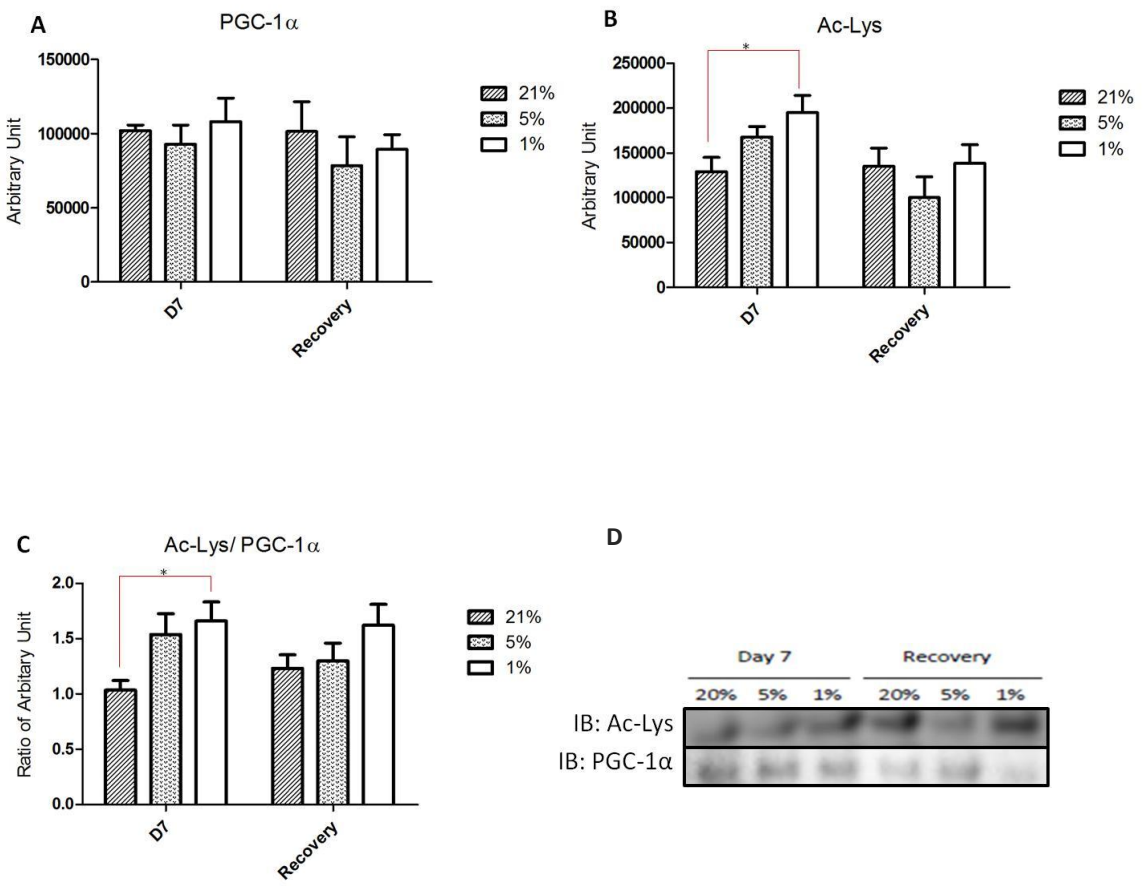

Figure 3.8 Reduced oxygen tension increased acetylated PGC-1α protein. C_2C_{12} cells were collected and immunoprecipitated by antibody specific to PGC-1α. PGC-1α (A) and acetylated PGC-1α (B) in immunoprecipitates were detected using western blotting, with antibodies specific to PGC-1α, and acetylated-lysine. Ratio of Ac-PGC-1α to PGC-1α was calculated and represented in (C). Representative western blots are shown in (D). A one-way ANOVA with a Tukey's post-test and a student's two-tailed unpaired t-test were employed. All data was presented as mean ± SEM (* $p<0.05$; n=5/experimental group).

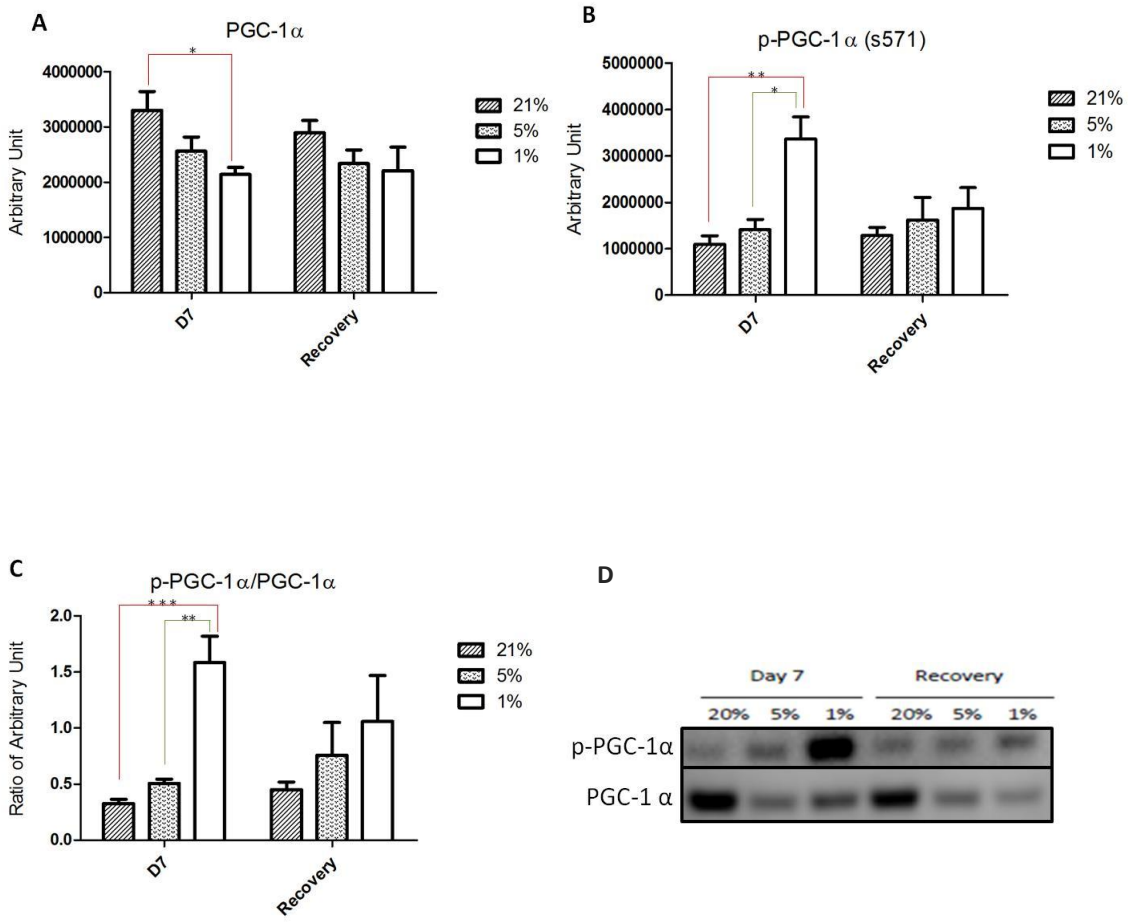

Figure 3.9 Reduced oxygen tension increased p-PGC-1α protein. C_2C_{12} cells were collected and total protein extractions were used in western blotting with antibodies specific to PGC-1α and p-PGC-1α (Ser 571). Densitometry was performed and represented in (A) and (B); ratio of p-PGC-1α to PGC-1α was calculated and represented in (C). Representative western blots are shown in (D). A one-way ANOVA with a Tukey's post-test was utilized. All data was presented as mean ± SEM (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; n=4/experimental group).

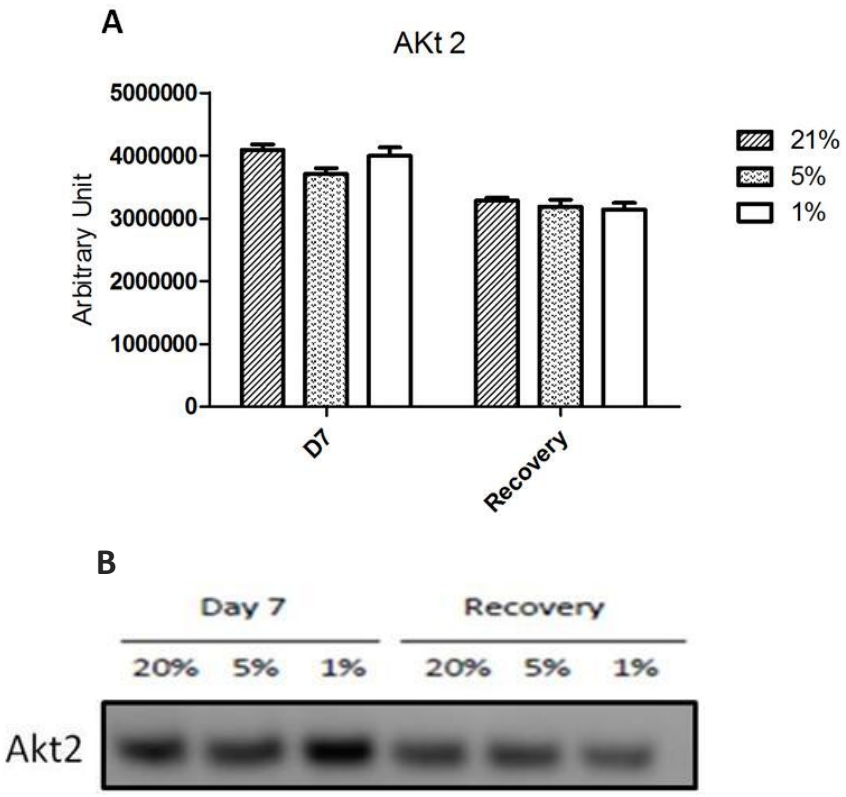

Figure 3.10 Reduced oxygen tension had no effect on Akt2 protein content. C_2C_{12} cells were collected and total protein extractions were used in western blotting probing with antibodies specific to Akt2. Densitometry was performed and represented in (A). A representative western blot is shown in (B). A one-way ANOVA test was utilized. There were no significant alterations among groups at both day 7 and recovery. All data was presented as mean ± SEM, n=7/experimental group.

3.6 Chronic hypoxia altered PPAR- α mRNA and PPAR- γ protein content

PPAR- α and- γ have been identified as important nuclear receptors that regulate FAO related gene expressions. Examination of PPAR- α and PPAR- γ mRNA expression revealed that PPAR- α mRNA level was significantly lower in both 5% ($p<0.001$) and 1% ($p<0.001$) O_2 relative to the 21% O_2 treatment at day 7 (Figure 3.11 A), while no significant alteration was found among the 3 groups for PPAR- γ mRNA levels (Figure 3.12 A). Interestingly, western blotting analysis revealed no significant changes among the 3 oxygen treatments for PPAR- α protein (Figure 3.11 B). However, in contrast, under 1% O_2, PPAR- γ protein was significantly increased ($p<0.05$) compared to the 21% and 5% O_2 treatments at day 7 (Figure 3.12 B).

3.7 FAO genes were altered under chronic hypoxia

FATP4, MCAD and CD36 are all FAO related genes. FATP4 and CD36 are important membrane transporters, which were found to increase FAO rate (Nickerson et al. 2009) and are all PPARs targets (Purushotham et al. 2009). MCAD is an enzyme that functions to catalyze the initial step in each FAO cycle, and was reported to be associated with FAO rate (Purushotham et al. 2009). To determine if reduced oxygen tension impacts its expression, real-time PCR was performed to determine the mRNA level of each gene. FATP4 showed significant lower mRNA levels after exposure to both 5% ($p<0.01$) and 1% O_2 ($p<0.05$) for 7 days than those under 21% O_2 (Figure 3.13 A). MCAD mRNA level was also reduced in hypoxia group (1% O_2) ($p<0.05$) compared to normoxia group (21% O_2) at day 7 (Figure 3.13 B). CD36 mRNA level exhibited a decreasing trend at day 7 from 21% to 1% O_2 treatments, with no significant difference ($p>0.05$) (Figure 3.13 C).

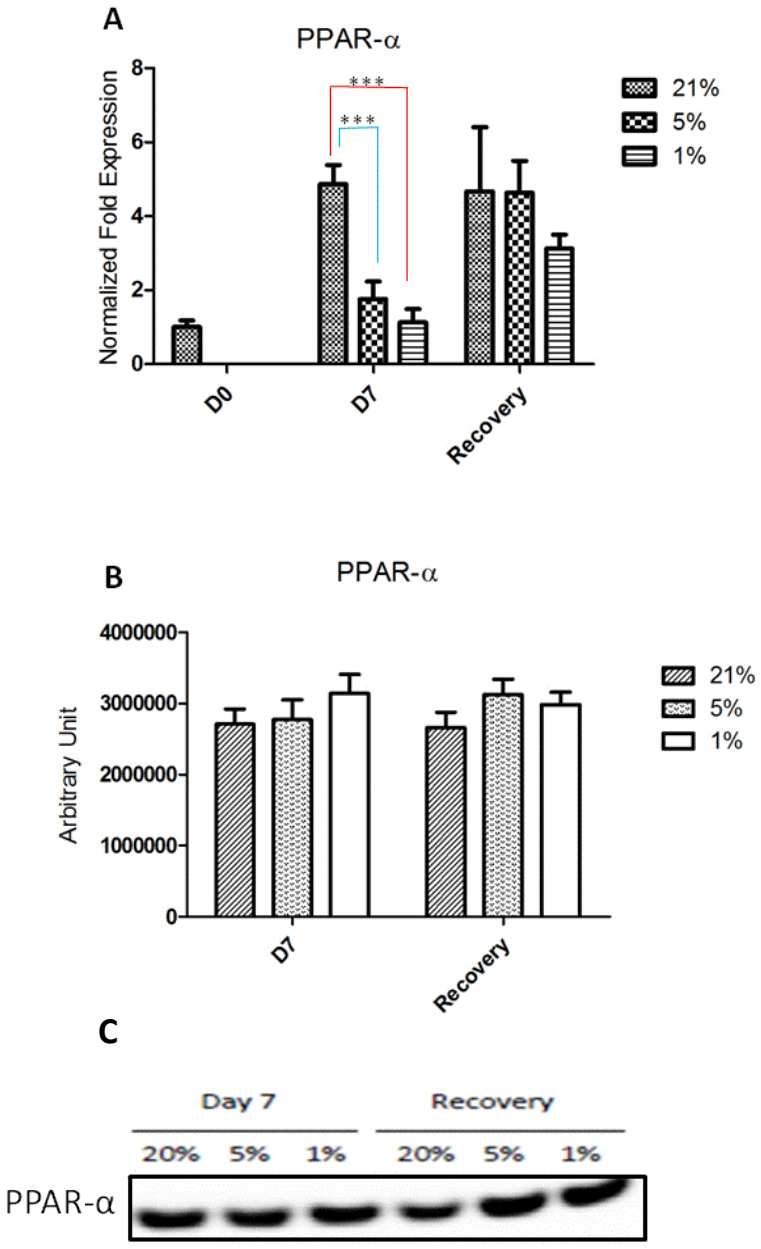

Figure 3.11 PPAR- α mRNA level decreased in hypoxia. C_2C_{12} cells were collected; PPAR- α mRNA (A) and protein (B) were analyzed by real-time PCR and western blotting, respectively. PPAR- α mRNA was standardized to a house keeping gene (RL7), and then expressed relative to the undifferentiated cells (Day 0). A representative western blot of PPAR- α is shown (C). A one-way ANOVA with a Tukey's post-test was employed. All data was presented as mean ± SEM (* $p<0.05$, ** $p<0.01$, ***$p<0.001$; n=5~6/experimental group).

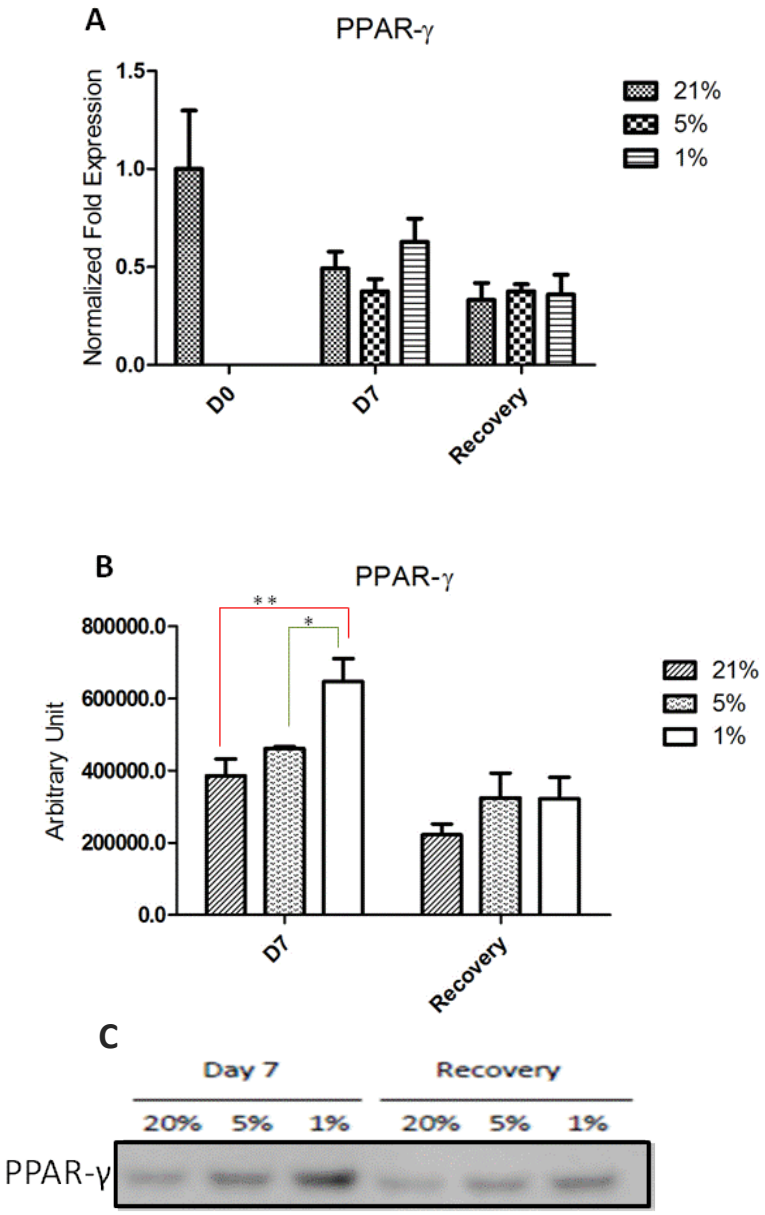

Figure 3.12 PPAR- γ protein content increased in hypoxia. C_2C_{12} cells were collected; PPAR- γ mRNA (A) and protein (B) were analyzed by real-time PCR and western blotting, respectively. PPAR- γ mRNA was standardized to a house keeping gene (RL7), and then expressed relative to the undifferentiated cells (Day 0). A representative western blot of PPAR- γ is shown (C). A one-way ANOVA with a Tukey's post-test was employed. All data was presented as mean ± SEM (* $p<0.05$, ** $p<0.01$; n=5~6/experimental group)

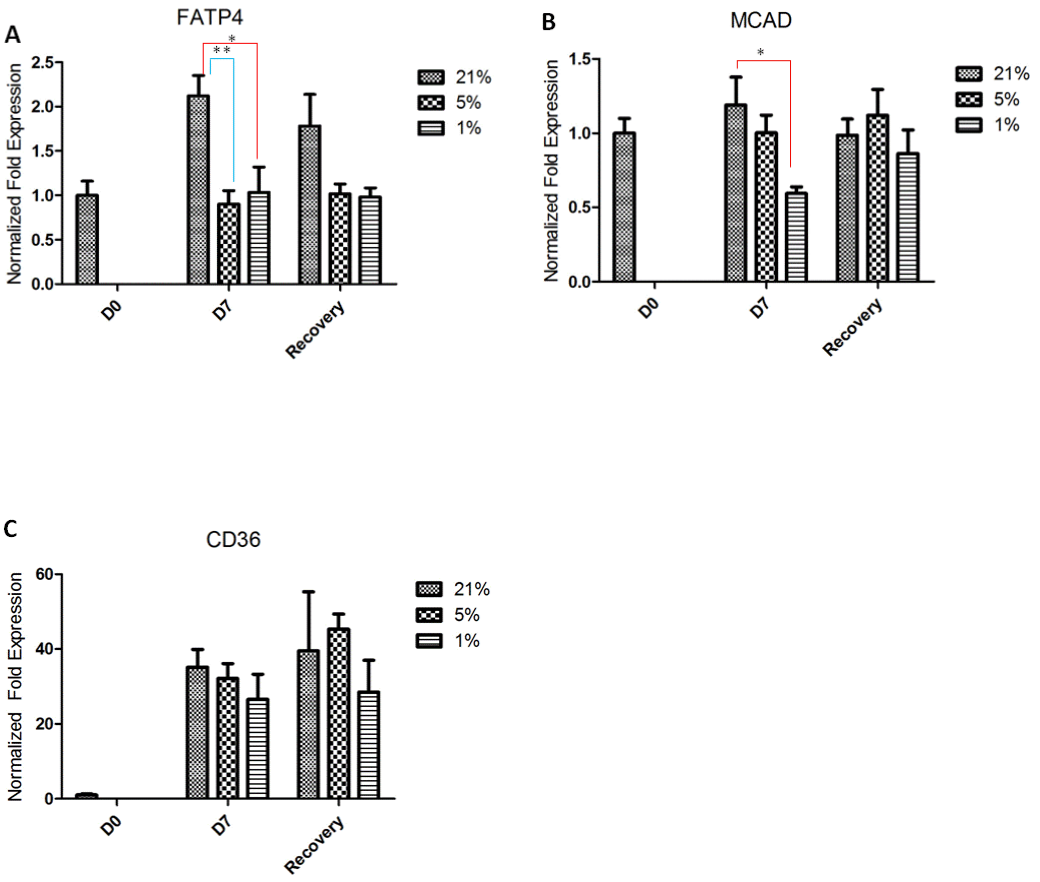

Figure 3.13 FAO genes were suppressed in chronic hypoxia. C_2C_{12} cells were collected; mRNA levels of FATP4 (A), MCAD (B) and CD36 (C) were analyzed by real-time PCR. All mRNAs were standardized to a house keeping gene (RL7), and then expressed relative to the undifferentiated cells (Day 0). A one-way ANOVA with a Tukey's post-test and a student's two-tailed unpaired t-test were utilized. All data was presented as mean ± SEM (* $p<0.05$, ** $p<0.01$; n=6/experimental group).

3.8 The protein ratios /contents of p-AMPK- α to AMPK- α, p-ACC to ACC in chronic hypoxia

3.8.1 Impact of chronic hypoxia on p-AMPK- α and AMPK- α protein content

Acute hypoxia can activate AMPK- α by phosphorylation at a key residue, Thr 172, which is essential to its activity (Park et al. 2002). Whether chronic hypoxia has the same effect on AMPK- α as acute hypoxia is unknown. Investigating the effect of chronic hypoxia, western blotting found no significant difference in AMPK- α protein among the 21%, 5% and 1% O_2 treatments (Figure 3.14 A). However, the p-AMPK- α protein was significantly increased with hypoxia (1% O_2) ($p<0.05$) compared to the 21% O_2 treatment at day 7 (Figure 3.14 B), and the p-AMPK- α/AMPK- α was also higher ($p<0.05$; Figure 3.14 C).

3.8.2 Impact of chronic hypoxia on p-ACC and ACC protein content

ACC is a downstream substrate of AMPK- α and when AMPK- α is activated, ACC phosphorylation occurs, inhibiting ACC activity, which increases FAO. Based on our previous result that protein ratio of p-AMPK- α to AMPK- α was significantly higher with hypoxia, it was reasonable to postulate that p-ACC to ACC would be higher with hypoxia. Surprisingly, ACC protein level was significantly lower with the 1% relative to the 21% O_2 treatments at day 7 (Figure 3.15 A), and the same pattern was also seen for p-ACC (Ser 79) (Figure 3.15 B), although these differences failed to reach statistical significance. However, a significantly higher level was found for the protein ratio of p-ACC (Ser 79) to ACC in hypoxia (1% O_2) at day 7 ($p<0.01$; Figure 3.15 C).

However, all the alterations in mRNA, protein and TG content described above failed to maintain following an additional 2-day re-oxygenation (recovery).

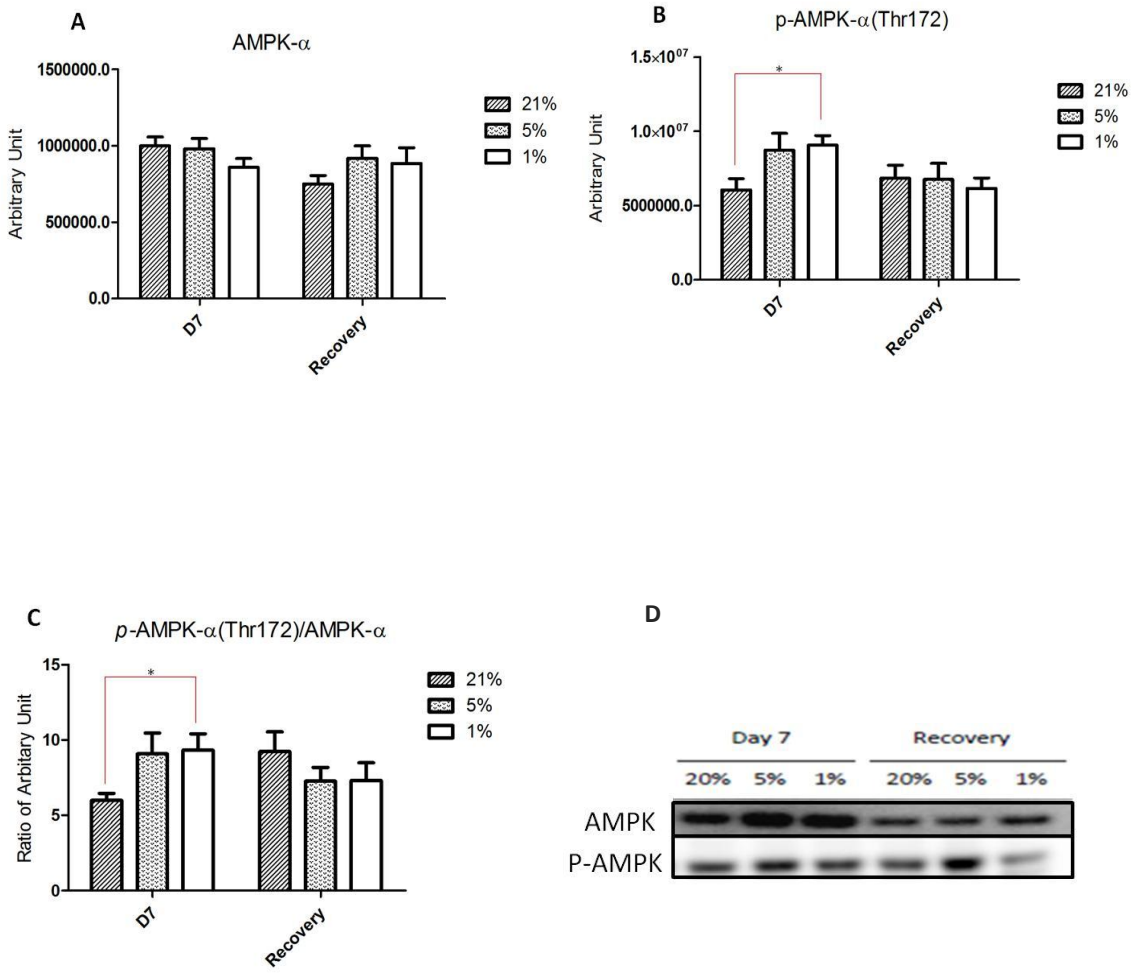

Figure 3.14 p-AMPK- α protein increased in hypoxic treated cells. C_2C_{12} cells were collected and total protein extractions were used in western blotting with antibodies specific to AMPK- α and p-AMPK- α (Thr 172). Densitometry was performed and represented in (A) and (B); ratio of p-AMPK- α to AMPK- α was calculated and represented in (C). Representative western blots are shown in (D). A student two-tailed unpaired t-test was utilized. All data was presented as mean ± SEM (* $p<0.05$, n=5/experimental group)

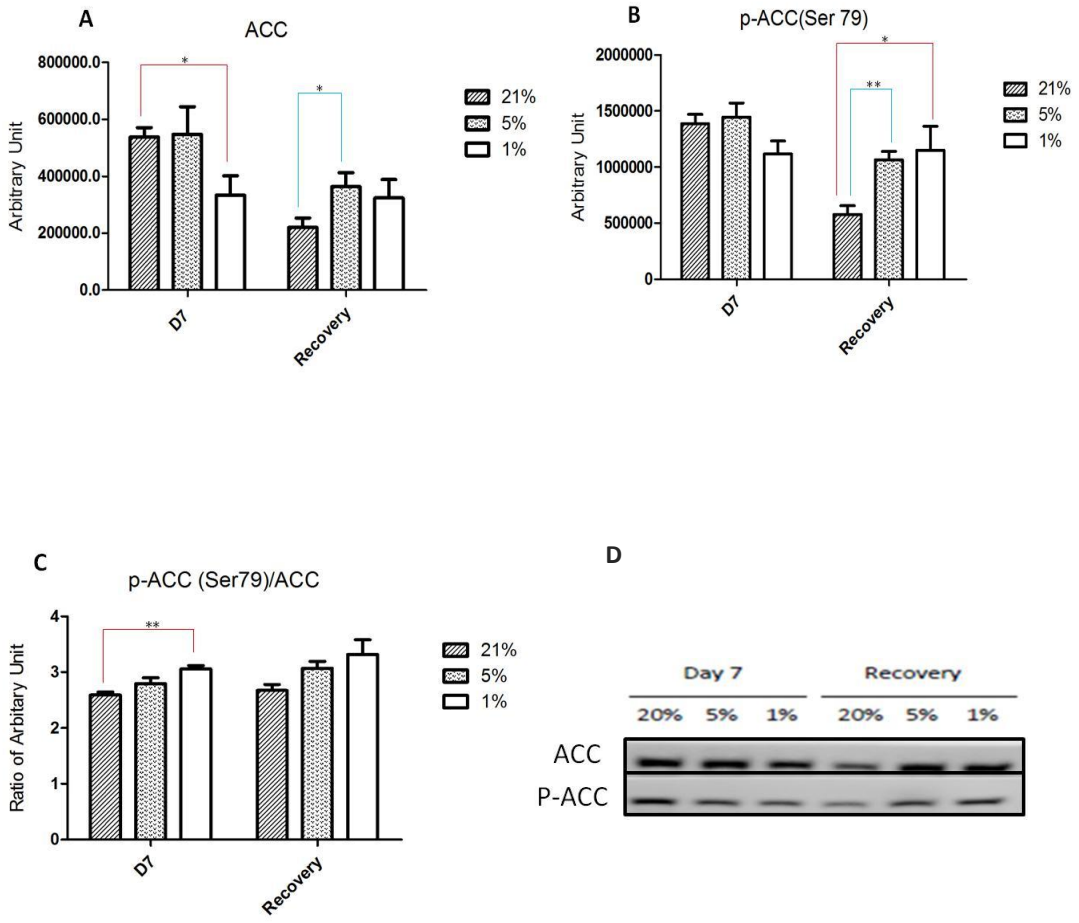

Figure 3.15 Protein ratio of p-ACC to ACC increased in hypoxic treated cells. C_2C_{12} cells were collected and total protein extractions were used in western blotting with antibodies specific to ACC and p-ACC (Ser 79). Densitometry was performed and represented in (A) and (B); ratio of p-ACC to ACC was calculated and represented in (C). Representative western blots are shown in (D). A one-way ANOVA with a Tukey's post-test and a student two-tailed unpaired t-test were utilized. All data was presented as mean ± SEM (* $p<0.05$, n=5/experimental group).

Chapter 4 Discussion

Hypoxia resulted in lower p-Akt (Thr 308) and higher total cellular GLUT4 protein levels after 7 days of differentiation. This coincided with higher TG content and alterations of the FAO/PGC-1α/PPARs components, both of which could contribute to the changes observed in the components of the insulin signalling pathway. Specifically, cells differentiating in 1% O_2 had lower SIRT1, PPAR- α, FATP4 and MCAD mRNA; accompanied by lower SIRT1, PGC-1α and higher PPAR- γ protein following 7 days of differentiation. Additionally, cells in prolonged hypoxia had significantly higher phosphorylation of PGC-1α, AMPK- α, ACC; concurrent with higher PGC-1α acetylation (Figure 4.1). However, none of these alterations above persisted following an additional 2-day re-oxygenation treatment (recovery).

4.1 Roles of prolonged hypoxia on insulin signalling pathway and intracellular TG accumulation in skeletal muscle

IRS and Akt are essential signalling molecules along the insulin signalling pathway, their activation leads to insulin-stimulated glucose uptake. Their activation is stimulated or inhibited by a series of phosphorylation steps. In the present study, a significantly lower in Akt phosphorylation at Thr 308 was found after 7 days of differentiation under hypoxic condition (1% O_2) when compared to control (21% O_2) (Figure 3.3). This was accompanied by a higher in intracellular TG content in hypoxia (Figure 3.5). Previous data that was obtained using magnetic resonance spectroscopy (MRS) showed TG was increased in obesity and was correlated with the severity of insulin resistance (Szczepaniak et al. 1999). Several mechanisms have been proposed to explain the correlation between increasing muscle TG and insulin resistance. A classical one is based on the concept that elevated TG derivatives or metabolites impair the insulin signalling pathway. The enhanced TG derivatives or metabolites (e.g. long-chain Acyl-CoA, DAG, and ceramide) activate a number of serine/threonine kinases, such as PKC. These serine/threonine kinases in turn phosphorylate serine residues of IRS-1, specifically Ser 1101, which inhibits IRS-1 (Bandyopadhyay et al. 2006, Jean-Baptiste et al. 2005,

Sathyanarayana et al. 2002, Yu et al. 2002a), leading to impaired downstream signalling, such as Akt, as observed in our hypoxic treatment (Figure 3.3). Of note, it is TG derivatives or metabolites that activate members of the serine/threonine kinases leading to insulin resistance (Schmitz-Peiffer, Craig & Biden 1999, Itani et al. 2002), thus TG itself does not directly cause insulin resistance. However, TG content is a strong predictor of muscle insulin resistance (Krssak et al. 1999), as studies in sedentary obese and type II diabetics found that higher TG content probably reflects increases in other lipid intermediates, which are factors contributing to insulin resistance (Kelley, Goodpaster & Storlien 2002b, Cooney et al. 2002). Therefore, increased TG content in our hypoxic muscle cells did not directly alter Akt phosphorylation, but may reflect potential increasing levels of its derivatives or metabolites, which could exert direct effects on the upstream (e.g. IRS) and subsequent downstream (e.g. Akt) markers of the insulin signalling pathway. Interestingly, while Akt phosphorylation was lower after 7 days of hypoxic treatment, its upstream marker IRS phosphorylation was not affected (Figure 3.2). Therefore, the depressed Akt phosphorylation (Thr 308) observed here may not be induced by IRS, instead could be directly induced by ceramide, as mentioned in a previous study that ceramide resulted in decrease Akt phosphorylation at Thr 308 in a human glioblastoma cell line (Zinda, Vlahos & Lai 2001).

4.2 GLUT4 expression was up-regulated in chronic hypoxic muscle cells

GLUT4 is final readout of insulin signalling and the dominate glucose transporter in skeletal muscle, which can translocate from intracellular storage to the plasma membrane to uptake glucose into cells (Lizcano, Alessi 2002). The importance of GLUT4 in maintaining insulin sensitivity has been studied extensively in recent years. Muscle specific deletion of GLUT4 results in insulin resistance and glucose intolerance in mice (Zisman et al. 2000). Interestingly, my data have shown that total cellular GLUT4 protein content was significantly higher in both the 1% and 5% O_2 treatments (Figure 3.4), even in the presence of an impaired insulin pathway (reduced p-Akt to Akt ratio) (Figure 3.3). Similar to our data, Viscarra also found that total GLUT4 protein in adipose tissue was up-regulated despite of decreased insulin signalling during prolonged fasting in northern

elephant seal pups (Viscarra et al. 2011). Their data, in conjunction with ours, indicate that the up-regulated GLUT4 in skeletal muscle/adipose tissue during prolonged hypoxia/fasting is insulin signalling pathway independent, and an alternative pathway may be involved. Studies from Holmes and colleagues have demonstrated that chronic activation of AMPK increased total GLUT4 protein content in rat epitrochlearis and gastrocnemius muscle (Holmes, Kurth-Kraczek & Winder 1999). In our study, activated AMPK was significantly higher with prolonged hypoxia, which suggests the possibility that AMPK-dependent but insulin-independent mechanism(s) may determine the up-regulation effect of prolonged hypoxia on total GLUT4 protein content in skeletal muscle cells.

Does this up-regulated total GLUT4 content indicate increases in GLUT4 translocation at basal state (without insulin stimulation) and/or under insulin-stimulated condition? In fact, patients with type II diabetes do not have a deficiency in total GLUT4 in muscle (Garvey et al. 1992), but insulin-induced translocation of GLUT4 to the cell surface is defective (Garvey et al. 1998). The possible mechanisms could be defects in the function of GLUT4, which could include impaired translocation to the plasma membrane, impaired fusion with the plasma membrane, and/or reduced intrinsic activity (Kahn et al. 1991). These findings suggest the possibility that GLUT4 translocation, either in the basal state or under insulin-stimulated condition could still be unaltered or reduced, although the total GLUT4 content was higher in cells treated with diminished oxygen, since GLUT4 defects could occur.

As a result, how prolonged hypoxia would affect GLUT4 translocation with or without insulin stimulation in our hypoxic mouse skeletal muscle cells are still unknown, further experiments (e.g. plasma membrane GLUT4 protein content measurement) are needed to answer these questions.

4.3 Roles of prolonged hypoxia on altering PGC-1α/PPARs interactions

As mentioned in section 1.1, TG was significantly higher in hypoxic muscle cells (Figure 3.5). But what are the factors contributing to this excessive accumulation? To answer this

question, a definition of TG turnover needs to be introduced first. TG turnover is a composite measure of the dynamic balance between lipolysis and lipogenesis; both are influenced by mitochondrial FAO and plasma free fatty acid availability (Moro, Bajpeyi & Smith 2008). In other words, when lipogenesis exceeds lipolysis, in which TG turnover is low, excessive TG accumulation occurs. Mitochondrial FAO is a critical step in lipolysis. Thus, a potentially reduced FAO in our cell culture system may be responsible for the excessive TG accumulation. Then a question will be raised: what mechanism is involved in hypoxia mediated FAO impairment?

The PGC-1α/PPARs pathway is crucial in regulating FAO in skeletal muscle (Sugden, Caton & Holness 2010a, Purushotham et al. 2009). It is most likely that the damaging effect of hypoxia on FAO capacity is through this pathway. In my experiments, we tried to determine whether prolonged hypoxia would alter and/or modulate components of PGC-1α/PPARs pathway in skeletal muscle cells. PGC-1α, which lies in the central part of PGC-1α/PPARs pathway, was explored at both mRNA and protein levels to determine if it plays a role in the potentially decreased FAO rate. PGC-1α protein level was lower, but its mRNA level was not changed with 1% O_2 treatment when compared with 21% O_2 control at day 7 (Figure 3.7). Two possible mechanisms could explain these unparallel changes between mRNA and protein. First, PGC-1α mRNA stability may be improved by hypoxia. Second, hypoxia may induce degradation of PGC-1α protein, without affecting its protein synthesis (translation from mRNA). A previous study showed that phosphorylation of PGC-1α at Ser 570 by Akt2 under insulin stimulation led to a more unstable protein in the primary hepatocytes of mice (Li et al. 2007). Based on this result, it is possible that hypoxia induces PGC-1α protein degradation by increasing phosphorylation at Ser 570 of PGC-1α. In order to confirm this prediction, phosphorylation of PGC-1α at Ser 571 was examined by western blotting. The results showed that in chronic hypoxia PGC-1α phosphorylation (Ser 571) significantly increased after 7 days differentiation (Figure 3.9). This outcome could partially, if not completely explain the unmatched mRNA and protein content of PGC-1α under prolonged hypoxia. Moreover, phosphorylation of PGC-1α at Ser 570 in mice liver prevents the recruitment of PGC-1α to the cognate promoters of its target genes, such as MCAD which is an Acyl-CoA dehydrogenase in FAO cycle (Li et al. 2007). This

prevention impairs the ability of PGC-1α to promote FAO, by preventing its binding to promoter regions of FAO genes such as MCAD. Similar with their data was my observation that PGC-1α had a higher phosphorylation (Ser 571) in response to chronic hypoxia (1% O_2 for 7 days) (Figure 3.9) in mouse skeletal muscle cells, which may suggest an impaired FAO capacity in those cells.

Interestingly, other studies have reported that phosphorylation of PGC-1α leads to a more stable and active protein, which promotes FAO in muscle (Rodgers et al. 2008). These studies, however, used an autoradiography technique to explore effects of another two kinases: P38MAPK and AMPK on PGC-1α phosphorylation status. They found P38MAPK and AMPK phosphorylated multiple threonine and serine residues of PGC-1α (e.g. Thr 262 and Ser 538), activated PGC-1α and increased FAO (Rodgers et al. 2008). These data, in concert with the present study, indicate that phosphorylation of PGC-1α can either promote or inhibit FAO, depending on the upstream kinases and their corresponding phosphorylation locus. Therefore, phosphorylations of PGC-1α by P38MAPK or AMPK at Thr 262 or Ser 538 might be reduced in hypoxic differentiated cells.

With such a higher level of p-PGC-1α (Ser 571) in the hypoxia treated muscle cells, we explored whether hypoxia influenced its upstream kinase, Akt2, after exposure to hypoxia for 7 days. As a previous study found that it is Akt2 that phosphorylates PGC-1α (Ser 570) in mouse liver (Li et al. 2007), thus, it is reasonable to expect an increased Akt2 protein level in these hypoxic cells. Interestingly, hypoxia had no effect upon Akt2 protein content in mouse muscle cells, as there were no differences among treatments (21%, 5% and 1% O_2) at both day 7 and recovery (Figure 3.10). Therefore, we postulate that if Akt2 is the main driver of PGC-1α phosphorylation at Ser 571/570, then possible post translational modifications of Akt2, to increase its activity may play an essential role in deactivating PGC-1α through Ser 571/570 phosphorylation. Further research is necessary to confirm this postulation.

SIRT1, an upstream component of PGC-1α in the FAO/PGC-1α/PPARs pathway, was also measured in this study to verify if it would be affected by prolonged low oxygen

tension. As SIRT1 is a NAD^+-dependent deacetylase; then a high $NADH/NAD^+$ ratio might be expected to decrease SIRT1 activity. Thus, SIRT1 activity would potentially be decreased in prolonged hypoxia, since hypoxia results in increased $NADH/NAD^+$ ratio (Wu et al. 2006). Our results show that SIRT1 mRNA and protein levels are much lower following 7 days of differentiation under hypoxia (1% O_2) than controls (21% O_2) (Figure 3.6). But how might the decreased SIRT1 mRNA and protein influence FAO capacity in the hypoxic muscle cells? Purushotham and colleagues demonstrated that hepatocyte-specific deletion of SIRT1 decreases FAO in mice. In their experiment, liver-specific SIRT1 knockout mice (LKO) on a C57BL/6 background were generated. Although SIRT1 LKO mice were phenotypically normal, q-PCR revealed that a number of PPAR- α target genes that are involved in hepatic FAO were significantly decreased, including MCAD and CD36. Consistent with a reduction in FAO gene expression, β-oxidation of ^{3}H-palmitate in SIRT1-deficient hepatocytes was significantly lower compared to that in control hepatocytes. According to their results, it is rational to predict that decreased SIRT1 level in mouse hypoxic muscle cells in our experiment may cause decreases in messenger RNAs of FAO genes, which contribute to impaired FAO capacity. This is to some extent further confirmed by subsequent real-time PCR analysis in our experiment, where MCAD mRNA level was significantly lower in the hypoxic muscle cells (Figure 3.13), although, no alterations were found among groups for CD36 mRNA (Figure 3.13).

Decreases in SIRT1 mRNA and protein levels suggest that PGC-1α deacetylation and activation may also be inhibited by hypoxia, as SIRT1 is required to deacetylate and thereby activate PGC-1α. To test this possibility, the acetylation level of PGC-1α in the hypoxia treated muscle cells was analyzed by using immunoprecipitation. PGC-1α acetylation level was higher in hypoxia at day 7, while no significant alterations were detected in total PGC-1α immunoprecipitates from C_2C_{12} muscle cells (Figure 3.8). This confirmed the prediction that PGC-1α deacetylation was reduced after 7 days of low oxygen treatment. Similar with our data, Gerhart and colleagues who treated C_2C_{12} cells with nicotinamide, an SIRT1 inhibitor, found that nicotinamide inhibited PGC-1α deacetylation and was blocked with ectopic expression of SIRT1 (Gerhart-Hines et al. 2007).

Another component regulated by SIRT1 is PPAR- γ. PPAR- γ protein level was much higher in hypoxia (1% O_2) than that in control (21% O_2) (Figure 3.12). A previous study showed that SIRT1 interacted with the transcriptional co-repressor NCoR, negatively regulating PPAR- γ in white fat, which promoted fatty acid mobilization and inhibited fatty acid accumulation (Picard et al. 2004). If such a pathway occurs in chronically hypoxic muscle in conjunction with the down-regulated SIRT1, this interaction could be responsible for removing SIRT1 inhibition on PPAR- γ, which resulted in the observed enhanced PPAR- γ protein. Interestingly, in our study while PPAR- γ protein was higher, its mRNA level was similar among groups. The possible reasons of this mismatch between mRNA (unchanged) and protein levels (higher) could be decreased PPAR- γ protein degradation or/and impaired mRNA stability. As the inhibition of SIRT1to PPAR- γ transcription could be relived indicated by a decrease in SIRT1 protein under hypoxia, then enhanced PPAR- γ mRNA and protein levels would be expected. However, PPAR- γ mRNA was unchanged and its protein was higher, which suggests that hypoxia may impair PPAR- γ mRNA stability, and the higher PPAR- γ protein levels are most likely to be due to lower PPAR- γ degradation rather than higher transcription (mRNA) and translation of PPAR- γ coding gene, since the mRNA levels of PPAR- γ among treatments were not changed.

An additional member of the PPAR family is PPAR- α, which has an anti-diabetic effect. Initial studies conducted by Narravula and colleagues using microarray analysis of intestinal epithelial mRNA revealed that hypoxia down-regulates PPAR- α mRNA and protein in epithelial cells *in vitro* and *in vivo* (Narravula, Colgan 2001). Further studies found that there was a DNA consensus motif for the transcription factor hypoxia-inducible factor 1 (HIF1) on the antisense strand of PPAR- α gene. The electrophoretic mobility shift assay (EMSA) revealed that ambient hypoxia (20 Torr) induced HIF1α binding to the HIF1 consensus domain of PPAR- α, which was associated with the inhibitory effect of the hypoxia on PPAR- α (Narravula, Colgan 2001). Similarly, in my cell culture system, the PPAR- α mRNA level was significantly lower in both 5% and 1% O_2 relative to that in 21% O_2 (Figure 3.7). Furthermore, HIF1 activity was suggested to be higher in C_2C_{12} myotubes under hypoxia, indicated by the increased mRNA level of a HIF1α-induced molecular marker, lysyl oxidase (LOX) (Regnault et al. 2010a).

Therefore, it might be assumed that the lower PPAR- α mRNA level observed in our study is attributable to an increasing HIF1α activity, especially an increasing interaction with its consensus domain on the PPAR- α gene. This result suggests HIF1α induced PPAR- α mRNA inhibition under hypoxia is not just limited to intestinal epithelial cells, but at least may also occur in skeletal muscle cells. Nevertheless, protein levels of PPAR- α were not altered among treatments at day 7 (Figure 3.7). A possible mechanism which may explain this unchanged PPAR- α protein could be that hypoxia inhibited PPAR- α protein degradation; thus, the protein level could be unchanged even with a potential reduced protein synthesis.

Target genes of PPARs related to FAO include FATP4 (PPAR- γ), CD36 (PPAR- α, γ) and MCAD (PPAR- α). In our study, FATP4 and MCAD both were significantly lower at the transcriptional levels in the hypoxia groups (1% O_2) (Figure 3.13). These findings suggest that chronic hypoxia might inhibit the FAO gene (FATP4 and MCAD) transcription, leading to a potentially decreased FAO, through reducing fatty acid uptake and dehydrogenase within the FAO cycle. This is in agreement with previous studies which reported that FATP4 increased FAO in rat skeletal muscle (Nickerson et al. 2009) and MCAD mRNA level was associated with a decreased FAO (Purushotham et al. 2009). However, no significant alterations were found among groups for CD36 mRNA (Figure 3.13).

4.4 Activation of the AMPK signalling system in chronic low oxygen tension

In my experiment, I provide novel evidence that 7 days of exposure to hypoxia (1% O_2) increased AMPK activation and consequent ACC phosphorylation in skeletal muscle cells (Figure 3.14 and 3.15). As previously mentioned, under acute low oxygen conditions, AMPK is activated by phosphorylation. The activated AMPK then phosphorylates its downstream substrate, ACC, to inhibit ACC, so as to reduce the production of malonyl-CoA. This, in turn, releases the inhibitory effect exerted by malonyl-CoA on CPT1, which promotes the transport of long-chain fatty acids into the mitochondria for β-oxidation. In our studies, phosphorylation of AMPK was significantly higher after 7 days of differentiation under hypoxia when compared to controls.

Correspondingly, the ratio of p-ACC to total ACC was much higher following hypoxia. Therefore, if the interactions among p-ACC, malonyl-CoA and CPT1 exist in chronic hypoxia, the decreased protein ratio (p-ACC to ACC) would suggest a reduction in malonyl-CoA production, a consequent abolishment of its inhibition on CPT1, and an ultimate increase in FAO in the hypoxic cells. However, a recent study has found decreased CPT1mRNA levels in C_2C_{12} muscle cells after exposure to hypoxia (1% O_2) for 8 days (Regnault et al. 2010a), indicating a potential impairment of FAO capacity. This result is in conflict with what was predicted from my AMPK/ACC data. In fact, Pimenta and colleagues have also demonstrated that FAO impairment occurs despite the activation of AMPK in skeletal muscle cells which were exposed to palmitate (Pimenta et al. 2008). In their experiment, L6 myotubes were exposed to various palmitate concentrations for 8 hours. They found FAO was significantly reduced to different extents without affecting cell viability; this occurred despite significant increases in AMPK and ACC phosphorylation and in malonyl-CoA decarboxylase activity. Their study provides evidence that activation of the AMPK/ACC system is potentially not sufficient to counteract the suppressive effect of palmitate on FAO. Thus, although we have higher AMPK and ACC phosphorylation levels in cells treated with prolonged hypoxia, it is plausible that a decreased FAO may also occur through hypoxic induced inhibition of the PGC-1α/PPARs system. This situation would be further confounded by the fact that TG level was also higher after exposing C_2C_{12} muscle cells to prolonged hypoxia (Figure 3.5). It has been demonstrated that factors resulting in an increasing TG level include: 1) reduction of FAO capacity; 2) decreased TG breakdown; 3) excessive fatty acid uptake, and 4) increased (re)esterification of intermediate products (Moro, Bajpeyi & Smith 2008). Hence, a reduction in FAO capacity is expected to contribute to the increased TG content, especially when the fatty acid uptake was decreased (indicated by decreased FATP4 mRNA), assuming the other 2 factors are not affected. In summary, C_2C_{12} cells in my study could still display a low FAO after 7 days of hypoxia exposure, even if there was a significant increase in AMPK activation. The higher activation of AMPK observed in hypoxic cells may represent the muscle cells' attempt to increase oxidation, as happens in the acute hypoxic condition with declining ATP levels. However, after exposure to prolonged hypoxia, the downstream substrates of the

AMPK/ACC system appear to lose their capacity to respond to or the PGC-1α/PPARs interactions are more dominant players in regulating FAO chronically, resulting in the failure of rescuing the impaired FAO.

4.5 Speculations

In summary, skeletal muscle differentiation under prolonged hypoxia in cell culture caused impairment of components of the PGC-1α/PPARs and markers of insulin signalling pathways, though the degree of these impairments are reduced after normoxic recovery. Altered PGC-1α/PPARs/FAO gene interactions are likely responsible for depressed FAO, resulting in increased TG content. An increased TG level could reflect increases in other lipid intermediates, such as DAG, ceramide and long-chain Acyl-CoA, which contribute to impaired insulin signalling (lower p-Akt to Akt ratio) and likely impair insulin sensitivity.

Our data suggests that hypoxia, distinguished from other insults, is sufficient to alter or modulate PGC-1α/PPARs/FAO and insulin signalling pathways. This is important given that in IUGR fetus' oxygen is limiting and hence reduced oxygen during IUGR muscle development could pre-set the offspring with a defective FAO/PGC-1α/PPARs system, predisposing the offspring to the development of insulin resistance and associated chronic diseases. It is important to note that the alterations of FAO/PGC-1α/PPARs pathway observed here are similar to those reported in insulin resistant adults (Sun et al. 2007). Therefore, the changes observed during prolonged hypoxia may partly explain the intrauterine factors contributing to decreased insulin sensitivity in IUGR offspring.

Significant hypoxic associated changes in the insulin signalling and FAO pathways are described, however upon re-oxygenation, many of these changes returned to levels associated with normoxia. We speculate that in our *in vitro* system a degree of plasticity exists and that following a 7- day period of hypoxic differentiation, C_2C_{12} cells are able to resume normoxic like metabolism upon re-oxygenation. This may be because the hypoxic treatment in my study was not long enough to make permanent changes in the components of the two pathways.

4.6 Future studies

This study has highlighted prolonged hypoxia during differentiation as a major contributor to alterations and modulations of insulin signalling and FAO/PGC-1α/PPARs pathways in skeletal muscle. More importantly, these changes suggest a potentially decreased FAO and impaired insulin sensitivity may occur in hypoxic muscle cells.

Future studies may employ the palmitate oxidation assay to investigate if FAO rate actually decreases in prolonged hypoxia, and more importantly, the aspect of insulin signalling pathway and glucose uptake will be tested under insulin stimulation to determine insulin sensitivity in my system.

Our study found that total cellular GLUT4 protein was increased, despite the defect in the insulin signalling pathway under the basal state (without insulin stimulation) in hypoxia. Further examination into how prolonged hypoxia influences GLUT4 protein translocation in the basal state and insulin-stimulated condition may help to further determine glucose uptake capacity in skeletal muscle, since it is the magnitude of GLUT4 protein translocation rather than total protein content that determines the capacity for glucose uptake in tissues.

Levels of post translational modification (e.g. phosphorylation) of PPAR- α and - γ will also be investigated, as they may have implications in FAO gene regulation. Studies have found that phosphorylation of PPAR- α at both Ser 12 and 21 decreased the transcriptional activity of PPAR- α, whereas dephosphorylation at both sites significantly increased the activity in human hepatoma HepG2 cells (Tamasi et al. 2008). Moreover, Rangwala revealed genetic prevention of PPAR- γ phosphorylation at Ser 112 preserved insulin sensitivity in the setting of diet-induced obesity (Rangwala et al. 2003); Choi and colleagues demonstrated that phosphorylation of PPAR- γ at Ser 273 led to dysregulation of a large number of genes whose expression is altered in obesity (Choi et al. 2010). Examination of these phosphorylation sites on PPAR- α and - γ may help to further reveal the roles of PPAR- α and - γ on prolonged hypoxia induced impairment of FAO capacity.

The duration of hypoxic treatment for skeletal muscle cells will be prolonged, under a precondition of high cell viability, to explore if modifications and alterations in the insulin signalling and the FAO/PGC-1α/PPARs pathways would persist following a recovery treatment in normal oxygen.

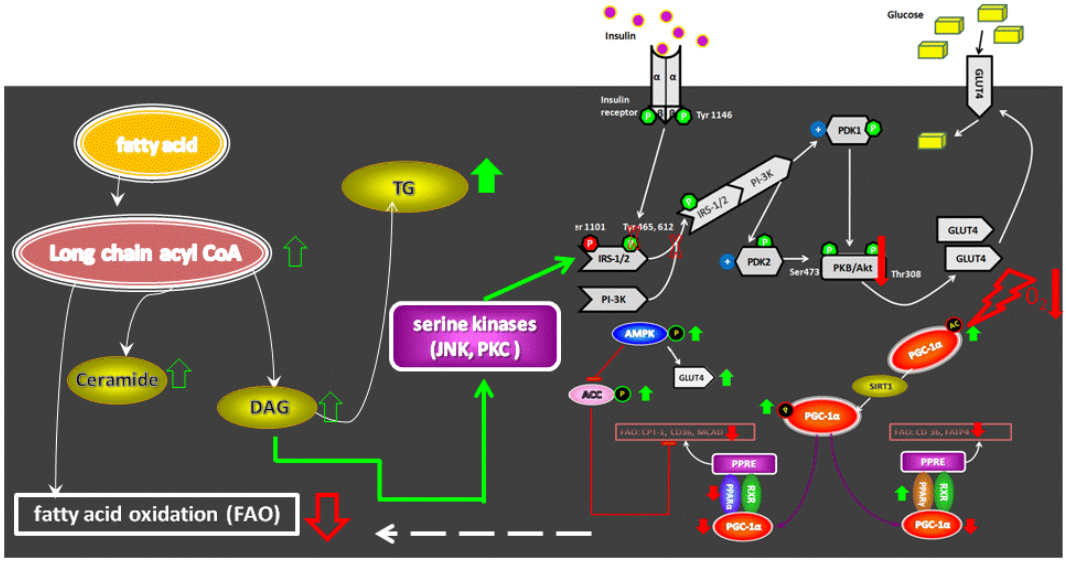

Figure 4.1 Model of prolonged hypoxia induced alterations and modifications in insulin signalling and its interactions with FAO. Hypoxia impaired components of the FAO/PGC-1α/PPARs pathway. This is likely responsible for depressed FAO, which resulted in increase in TG content. An increased TG content could reflect increases in other lipid intermediates, such as DAG, ceramide and long-chain Acyl-CoA, which contribute to impaired makers of insulin signalling pathway(e.g. depressed Akt), and ultimately promoting insulin resistance.

Solid arrow heads indicate actual changes measured in the present experiment; hollow arrow heads indicate potential alterations and further experiments are needed. Figure was adapted from: www.environmentalhealthnews.org /newscience/ 2007/2007-0405 insulinsignaling.html and Zhang et al. 2010.

References

"Thrifty genotype rendered detrimental by progress?", 1989, *Lancet,* vol. 2, no. 8667, pp. 839-840.

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. & Hemmings, B.A. 1996, "Mechanism of activation of protein kinase B by insulin and IGF-1", *The EMBO journal,* vol. 15, no. 23, pp. 6541-6551.

Ang, C.K., Tan, T.H., Walters, W.A. & Wood, C. 1969, "Postural influence on maternal capillary oxygen and carbon dioxide tension", *British medical journal,* vol. 4, no. 5677, pp. 201-203.

Arthur, P.G., Giles, J.J. & Wakeford, C.M. 2000, "Protein synthesis during oxygen conformance and severe hypoxia in the mouse muscle cell line C2C12", *Biochimica et biophysica acta,* vol. 1475, no. 1, pp. 83-89.

Avruch, J. 1998, "Insulin signal transduction through protein kinase cascades", *Molecular and cellular biochemistry,* vol. 182, no. 1-2, pp. 31-48.

Azevedo, J.L.,Jr, Carey, J.O., Pories, W.J., Morris, P.G. & Dohm, G.L. 1995, "Hypoxia stimulates glucose transport in insulin-resistant human skeletal muscle", *Diabetes,* vol. 44, no. 6, pp. 695-698.

Ballew, C. & Haas, J.D. 1986, "Hematologic evidence of fetal hypoxia among newborn infants at high altitude in Bolivia", *American Journal of Obstetrics and Gynecology,* vol. 155, no. 1, pp. 166-169.

Bandyopadhyay, G.K., Yu, J.G., Ofrecio, J. & Olefsky, J.M. 2006, "Increased malonyl-CoA levels in muscle from obese and type 2 diabetic subjects lead to decreased fatty acid oxidation and increased lipogenesis; thiazolidinedione treatment reverses these defects", *Diabetes,* vol. 55, no. 8, pp. 2277-2285.

Barker, D.J. 2005, "The developmental origins of insulin resistance", *Hormone research,* vol. 64 Suppl 3, pp. 2-7.

Baschat, A.A. 2004, "Fetal responses to placental insufficiency: an update", *BJOG : an international journal of obstetrics and gynaecology,* vol. 111, no. 10, pp. 1031-1041.

Becroft, D.M., Thompson, J.M. & Mitchell, E.A. 2005, "Placental villitis of unknown origin: epidemiologic associations", *American Journal of Obstetrics and Gynecology,* vol. 192, no. 1, pp. 264-271.

Birnbaum, M.J. 1989, "Identification of a novel gene encoding an insulin-responsive glucose transporter protein", *Cell,* vol. 57, no. 2, pp. 305-315.

Blackburn, S.T. 2007, *maternal, fetal, Neonatal physiology*, THIRD EDITON edn, ELSEVIER, USA.

Bleiberg, I., Liron, M. & Feldman, M. 1967, "Studies on the regulation of hemopoietic spleen colonies", *Blood,* vol. 29, no. 4, pp. 469-480.

Bonen, A., Holloway, G.P., Tandon, N.N., Han, X.X., McFarlan, J., Glatz, J.F. & Luiken, J.J. 2009, "Cardiac and skeletal muscle fatty acid transport and transporters and triacylglycerol and fatty acid oxidation in lean and Zucker diabetic fatty rats", *American journal of physiology.Regulatory, integrative and comparative physiology,* vol. 297, no. 4, pp. R1202-12.

Camm, E.J., Martin-Gronert, M.S., Wright, N.L., Hansell, J.A., Ozanne, S.E. & Giussani, D.A. 2011, "Prenatal hypoxia independent of undernutrition promotes molecular markers of insulin resistance in adult offspring", *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* vol. 25, no. 1, pp. 420-427.

Cartee, G.D., Douen, A.G., Ramlal, T., Klip, A. & Holloszy, J.O. 1991, "Stimulation of glucose transport in skeletal muscle by hypoxia", *Journal of applied physiology (Bethesda, Md.: 1985),* vol. 70, no. 4, pp. 1593-1600.

Casanello, P., Krause, B., Torres, E., Gallardo, V., Gonzalez, M., Prieto, C., Escudero, C., Farias, M. & Sobrevia, L. 2009, "Reduced l-arginine transport and nitric oxide synthesis in human umbilical vein endothelial cells from intrauterine growth restriction pregnancies is not further altered by hypoxia", *Placenta,* vol. 30, no. 7, pp. 625-633.

Chabowski, A., Gorski, J., Calles-Escandon, J., Tandon, N.N. & Bonen, A. 2006a, "Hypoxia-induced fatty acid transporter translocation increases fatty acid transport and contributes to lipid accumulation in the heart", *FEBS letters,* vol. 580, no. 15, pp. 3617-3623.

Chabowski, A., Momken, I., Coort, S.L., Calles-Escandon, J., Tandon, N.N., Glatz, J.F., Luiken, J.J. & Bonen, A. 2006b, "Prolonged AMPK activation increases the expression of fatty acid transporters in cardiac myocytes and perfused hearts", *Molecular and cellular biochemistry,* vol. 288, no. 1-2, pp. 201-212.

Charnock-Jones, D.S., Kaufmann, P. & Mayhew, T.M. 2004, "Aspects of human fetoplacental vasculogenesis and angiogenesis. I. Molecular regulation", *Placenta,* vol. 25, no. 2-3, pp. 103-113.

Cho, H., Mu, J., Kim, J.K., Thorvaldsen, J.L., Chu, Q., Crenshaw, E.B.,3rd, Kaestner, K.H., Bartolomei, M.S., Shulman, G.I. & Birnbaum, M.J. 2001a, "Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta)", *Science (New York, N.Y.),* vol. 292, no. 5522, pp. 1728-1731.

Cho, H., Thorvaldsen, J.L., Chu, Q., Feng, F. & Birnbaum, M.J. 2001b, "Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice", *The Journal of biological chemistry,* vol. 276, no. 42, pp. 38349-38352.

Choi, J.H., Banks, A.S., Estall, J.L., Kajimura, S., Bostrom, P., Laznik, D., Ruas, J.L., Chalmers, M.J., Kamenecka, T.M., Bluher, M., Griffin, P.R. & Spiegelman, B.M. 2010, "Anti-diabetic drugs inhibit obesity-linked phosphorylation of PPARgamma by Cdk5", *Nature,* vol. 466, no. 7305, pp. 451-456.

Cong, L.N., Chen, H., Li, Y., Zhou, L., McGibbon, M.A., Taylor, S.I. & Quon, M.J. 1997, "Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells", *Molecular endocrinology (Baltimore, Md.),* vol. 11, no. 13, pp. 1881-1890.

Cooney, G.J., Thompson, A.L., Furler, S.M., Ye, J. & Kraegen, E.W. 2002, "Muscle long-chain acyl CoA esters and insulin resistance", *Annals of the New York Academy of Sciences,* vol. 967, pp. 196-207.

Cunningham, J.T., Rodgers, J.T., Arlow, D.H., Vazquez, F., Mootha, V.K. & Puigserver, P. 2007, "mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex", *Nature,* vol. 450, no. 7170, pp. 736-740.

Czech, M.P. 1985, "The nature and regulation of the insulin receptor: structure and function", *Annual Review of Physiology,* vol. 47, pp. 357-381.

de Grauw, T.J., Myers, R.E. & Scott, W.J. 1986, "Fetal growth retardation in rats from different levels of hypoxia", *Biology of the neonate,* vol. 49, no. 2, pp. 85-89.

de Lange, P., Moreno, M., Silvestri, E., Lombardi, A., Goglia, F. & Lanni, A. 2007, "Fuel economy in food-deprived skeletal muscle: signaling pathways and regulatory mechanisms", *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* vol. 21, no. 13, pp. 3431-3441.

DeFronzo, R.A., Gunnarsson, R., Bjorkman, O., Olsson, M. & Wahren, J. 1985, "Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus", *The Journal of clinical investigation,* vol. 76, no. 1, pp. 149-155.

Dresner, A., Laurent, D., Marcucci, M., Griffin, M.E., Dufour, S., Cline, G.W., Slezak, L.A., Andersen, D.K., Hundal, R.S., Rothman, D.L., Petersen, K.F. & Shulman, G.I. 1999, "Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity", *The Journal of clinical investigation,* vol. 103, no. 2, pp. 253-259.

Dressel, U., Allen, T.L., Pippal, J.B., Rohde, P.R., Lau, P. & Muscat, G.E. 2003, "The peroxisome proliferator-activated receptor beta/delta agonist, GW501516, regulates the expression of genes involved in lipid catabolism and energy uncoupling in skeletal muscle cells", *Molecular endocrinology (Baltimore, Md.),* vol. 17, no. 12, pp. 2477-2493.

Fernandez-Twinn, D.S. & Ozanne, S.E. 2006, "Mechanisms by which poor early growth programs type-2 diabetes, obesity and the metabolic syndrome", *Physiology & behavior,* vol. 88, no. 3, pp. 234-243.

Fryer, L.G., Foufelle, F., Barnes, K., Baldwin, S.A., Woods, A. & Carling, D. 2002, "Characterization of the role of the AMP-activated protein kinase in the stimulation of glucose transport in skeletal muscle cells", *The Biochemical journal,* vol. 363, no. Pt 1, pp. 167-174.

Garvey, W.T., Maianu, L., Hancock, J.A., Golichowski, A.M. & Baron, A. 1992, "Gene expression of GLUT4 in skeletal muscle from insulin-resistant patients with obesity, IGT, GDM, and NIDDM", *Diabetes,* vol. 41, no. 4, pp. 465-475.

Garvey, W.T., Maianu, L., Zhu, J.H., Brechtel-Hook, G., Wallace, P. & Baron, A.D. 1998, "Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance", *The Journal of clinical investigation*, vol. 101, no. 11, pp. 2377-2386.

Gerhart-Hines, Z., Rodgers, J.T., Bare, O., Lerin, C., Kim, S.H., Mostoslavsky, R., Alt, F.W., Wu, Z. & Puigserver, P. 2007, "Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha", *The EMBO journal*, vol. 26, no. 7, pp. 1913-1923.

Giussani, D.A., Salinas, C.E., Villena, M. & Blanco, C.E. 2007, "The role of oxygen in prenatal growth: studies in the chick embryo", *The Journal of physiology*, vol. 585, no. Pt 3, pp. 911-917.

Gluckman, P.D. & Hanson, M.A. 2004, "Maternal constraint of fetal growth and its consequences", *Seminars in fetal & neonatal medicine*, vol. 9, no. 5, pp. 419-425.

Gluckman, P.D., Hanson, M.A. & Mitchell, M.D. 2010, "Developmental origins of health and disease: reducing the burden of chronic disease in the next generation", *Genome medicine*, vol. 2, no. 2, pp. 14.

Goodpaster, B.H., Theriault, R., Watkins, S.C. & Kelley, D.E. 2000, "Intramuscular lipid content is increased in obesity and decreased by weight loss", *Metabolism: clinical and experimental*, vol. 49, no. 4, pp. 467-472.

Greenwood, P.L., Hunt, A.S., Hermanson, J.W. & Bell, A.W. 2000, "Effects of birth weight and postnatal nutrition on neonatal sheep: II. Skeletal muscle growth and development", *Journal of animal science*, vol. 78, no. 1, pp. 50-61.

Greenwood, P.L., Slepetis, R.M., Hermanson, J.W. & Bell, A.W. 1999, "Intrauterine growth retardation is associated with reduced cell cycle activity, but not myofibre number, in ovine fetal muscle", *Reproduction, fertility, and development*, vol. 11, no. 4-5, pp. 281-291.

Habek, D., Habek, J.C., Ivanisevic, M. & Djelmis, J. 2002, "Fetal tobacco syndrome and perinatal outcome", *Fetal diagnosis and therapy*, vol. 17, no. 6, pp. 367-371.

Hales, C.N. & Barker, D.J. 2001, "The thrifty phenotype hypothesis", *British medical bulletin*, vol. 60, pp. 5-20.

Hales, C.N. & Barker, D.J. 1992, "Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis", *Diabetologia*, vol. 35, no. 7, pp. 595-601.

Heydrick, S.J., Jullien, D., Gautier, N., Tanti, J.F., Giorgetti, S., Van Obberghen, E. & Le Marchand-Brustel, Y. 1993, "Defect in skeletal muscle phosphatidylinositol-3-kinase in obese insulin-resistant mice", *The Journal of clinical investigation*, vol. 91, no. 4, pp. 1358-1366.

Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C.Z., Uysal, K.T., Maeda, K., Karin, M. & Hotamisligil, G.S. 2002, "A central role for JNK in obesity and insulin resistance", *Nature*, vol. 420, no. 6913, pp. 333-336.

Holgado-Madruga, M., Emlet, D.R., Moscatello, D.K., Godwin, A.K. & Wong, A.J. 1996, "A Grb2-associated docking protein in EGF- and insulin-receptor signalling", *Nature*, vol. 379, no. 6565, pp. 560-564.

Holloszy, J.O. & Hansen, P.A. 1996, "Regulation of glucose transport into skeletal muscle", *Reviews of physiology, biochemistry and pharmacology*, vol. 128, pp. 99-193.

Holloszy, J.O. & Narahara, H.T. 1965, "Studies of tissue permeability. X. Changes in permeability to 3-methylglucose associated with contraction of isolated frog muscle", *The Journal of biological chemistry*, vol. 240, no. 9, pp. 3493-3500.

Holman, G.D. & Sandoval, I.V. 2001, "Moving the insulin-regulated glucose transporter GLUT4 into and out of storage", *Trends in cell biology*, vol. 11, no. 4, pp. 173-179.

Holmes, B.F., Kurth-Kraczek, E.J. & Winder, W.W. 1999, "Chronic activation of 5'-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle", *Journal of applied physiology (Bethesda, Md.: 1985)*, vol. 87, no. 5, pp. 1990-1995.

Howald, H. & Hoppeler, H. 2003, "Performing at extreme altitude: muscle cellular and subcellular adaptations", *European journal of applied physiology*, vol. 90, no. 3-4, pp. 360-364.

Huang, C., Thirone, A.C., Huang, X. & Klip, A. 2005, "Differential contribution of insulin receptor substrates 1 versus 2 to insulin signaling and glucose uptake in l6 myotubes", *The Journal of biological chemistry*, vol. 280, no. 19, pp. 19426-19435.

Hulver, M.W. & Dohm, G.L. 2004, "The molecular mechanism linking muscle fat accumulation to insulin resistance", *The Proceedings of the Nutrition Society*, vol. 63, no. 2, pp. 375-380.

Itani, S.I., Ruderman, N.B., Schmieder, F. & Boden, G. 2002, "Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha", *Diabetes*, vol. 51, no. 7, pp. 2005-2011.

Jackson, B.T., Cohn, H.E., Morrison, S.H., Baker, R.M. & Piasecki, G.J. 1993, "Hypoxia-induced sympathetic inhibition of the fetal plasma insulin response to hyperglycemia", *Diabetes*, vol. 42, no. 11, pp. 1621-1625.

Jain, V. & Singhal, A. 2012, "Catch up growth in low birth weight infants: striking a healthy balance", *Reviews in endocrine & metabolic disorders*, vol. 13, no. 2, pp. 141-147.

Jean-Baptiste, G., Yang, Z., Khoury, C. & Greenwood, M.T. 2005, "Lysophosphatidic acid mediates pleiotropic responses in skeletal muscle cells", *Biochemical and biophysical research communications*, vol. 335, no. 4, pp. 1155-1162.

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 metabolism*, vol. 1, no. 1, pp. 15-25.

Kahn, B.B., Rossetti, L., Lodish, H.F. & Charron, M.J. 1991, "Decreased in vivo glucose uptake but normal expression of GLUT1 and GLUT4 in skeletal muscle of diabetic rats", *The Journal of clinical investigation,* vol. 87, no. 6, pp. 2197-2206.

Kelley, D.E., Goodpaster, B.H. & Storlien, L. 2002a, "Muscle triglyceride and insulin resistance", *Annual Review of Nutrition,* vol. 22, pp. 325-346.

Kelley, D.E., Goodpaster, B.H. & Storlien, L. 2002b, "Muscle triglyceride and insulin resistance", *Annual Review of Nutrition,* vol. 22, pp. 325-346.

Kensara, O.A., Wootton, S.A., Phillips, D.I., Patel, M., Jackson, A.A., Elia, M. & Hertfordshire Study Group 2005, "Fetal programming of body composition: relation between birth weight and body composition measured with dual-energy X-ray absorptiometry and anthropometric methods in older Englishmen", *The American Journal of Clinical Nutrition,* vol. 82, no. 5, pp. 980-987.

Kliewer, S.A., Xu, H.E., Lambert, M.H. & Willson, T.M. 2001, "Peroxisome proliferator-activated receptors: from genes to physiology", *Recent progress in hormone research,* vol. 56, pp. 239-263.

Kohn, A.D., Summers, S.A., Birnbaum, M.J. & Roth, R.A. 1996, "Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation", *The Journal of biological chemistry,* vol. 271, no. 49, pp. 31372-31378.

Krook, A. & O'Rahilly, S. 1996, "Mutant insulin receptors in syndromes of insulin resistance", *Bailliere's Clinical Endocrinology and Metabolism,* vol. 10, no. 1, pp. 97-122.

Krssak, M., Falk Petersen, K., Dresner, A., DiPietro, L., Vogel, S.M., Rothman, D.L., Roden, M. & Shulman, G.I. 1999, "Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study", *Diabetologia,* vol. 42, no. 1, pp. 113-116.

Lackman, F., Capewell, V., Gagnon, R. & Richardson, B. 2001, "Fetal umbilical cord oxygen values and birth to placental weight ratio in relation to size at birth", *American Journal of Obstetrics and Gynecology,* vol. 185, no. 3, pp. 674-682.

Larciprete, G., Valensise, H., Di Pierro, G., Vasapollo, B., Casalino, B., Arduini, D., Jarvis, S. & Cirese, E. 2005, "Intrauterine growth restriction and fetal body composition", *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology,* vol. 26, no. 3, pp. 258-262.

Li, X., Monks, B., Ge, Q. & Birnbaum, M.J. 2007, "Akt/PKB regulates hepatic metabolism by directly inhibiting PGC-1alpha transcription coactivator", *Nature,* vol. 447, no. 7147, pp. 1012-1016.

Limesand, S.W. & Hay, W.W.,Jr 2003, "Adaptation of ovine fetal pancreatic insulin secretion to chronic hypoglycaemia and euglycaemic correction", *The Journal of physiology,* vol. 547, no. Pt 1, pp. 95-105.

Limesand, S.W., Rozance, P.J., Brown, L.D. & Hay, W.W.,Jr 2009, "Effects of chronic hypoglycemia and euglycemic correction on lysine metabolism in fetal sheep", *American journal of physiology.Endocrinology and metabolism,* vol. 296, no. 4, pp. E879-87.

Lin, J., Handschin, C. & Spiegelman, B.M. 2005, "Metabolic control through the PGC-1 family of transcription coactivators", *Cell metabolism,* vol. 1, no. 6, pp. 361-370.

Lin, J., Wu, H., Tarr, P.T., Zhang, C.Y., Wu, Z., Boss, O., Michael, L.F., Puigserver, P., Isotani, E., Olson, E.N., Lowell, B.B., Bassel-Duby, R. & Spiegelman, B.M. 2002, "Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres", *Nature,* vol. 418, no. 6899, pp. 797-801.

Liu, L., Zhang, Y., Chen, N., Shi, X., Tsang, B. & Yu, Y.H. 2007, "Upregulation of myocellular DGAT1 augments triglyceride synthesis in skeletal muscle and protects against fat-induced insulin resistance", *The Journal of clinical investigation,* vol. 117, no. 6, pp. 1679-1689.

Lizcano, J.M. & Alessi, D.R. 2002, "The insulin signalling pathway", *Current biology : CB,* vol. 12, no. 7, pp. R236-8.

Maier, A., McEwan, J.C., Dodds, K.G., Fischman, D.A., Fitzsimons, R.B. & Harris, A.J. 1992, "Myosin heavy chain composition of single fibres and their origins and distribution in developing fascicles of sheep tibialis cranialis muscles", *Journal of muscle research and cell motility,* vol. 13, no. 5, pp. 551-572.

Mensink, M., Hesselink, M.K., Russell, A.P., Schaart, G., Sels, J.P. & Schrauwen, P. 2007, "Improved skeletal muscle oxidative enzyme activity and restoration of PGC-1 alpha and PPAR beta/delta gene expression upon rosiglitazone treatment in obese patients with type 2 diabetes mellitus", *International journal of obesity (2005),* vol. 31, no. 8, pp. 1302-1310.

Moro, C., Bajpeyi, S. & Smith, S.R. 2008, "Determinants of intramyocellular triglyceride turnover: implications for insulin sensitivity", *American journal of physiology.Endocrinology and metabolism,* vol. 294, no. 2, pp. E203-13.

Muhlhausler, B.S., Duffield, J.A., Ozanne, S.E., Pilgrim, C., Turner, N., Morrison, J.L. & McMillen, I.C. 2009, "The transition from fetal growth restriction to accelerated postnatal growth: a potential role for insulin signalling in skeletal muscle", *The Journal of physiology,* vol. 587, no. Pt 17, pp. 4199-4211.

Narravula, S. & Colgan, S.P. 2001, "Hypoxia-inducible factor 1-mediated inhibition of peroxisome proliferator-activated receptor alpha expression during hypoxia", *Journal of immunology (Baltimore, Md.: 1950),* vol. 166, no. 12, pp. 7543-7548.

Ness, R.B. & Sibai, B.M. 2006, "Shared and disparate components of the pathophysiologies of fetal growth restriction and preeclampsia", *American Journal of Obstetrics and Gynecology,* vol. 195, no. 1, pp. 40-49.

Nickerson, J.G., Alkhateeb, H., Benton, C.R., Lally, J., Nickerson, J., Han, X.X., Wilson, M.H., Jain, S.S., Snook, L.A., Glatz, J.F., Chabowski, A., Luiken, J.J. & Bonen, A. 2009, "Greater transport efficiencies of the membrane fatty acid transporters FAT/CD36 and FATP4 compared with FABPpm and FATP1 and differential

effects on fatty acid esterification and oxidation in rat skeletal muscle", *The Journal of biological chemistry,* vol. 284, no. 24, pp. 16522-16530.

Nuutila, P., Koivisto, V.A., Knuuti, J., Ruotsalainen, U., Teras, M., Haaparanta, M., Bergman, J., Solin, O., Voipio-Pulkki, L.M. & Wegelius, U. 1992, "Glucose-free fatty acid cycle operates in human heart and skeletal muscle in vivo", *The Journal of clinical investigation,* vol. 89, no. 6, pp. 1767-1774.

Nystrom, F.H. & Quon, M.J. 1999, "Insulin signalling: metabolic pathways and mechanisms for specificity", *Cellular signalling,* vol. 11, no. 8, pp. 563-574.

Osmond, C., Barker, D.J., Winter, P.D., Fall, C.H. & Simmonds, S.J. 1993, "Early growth and death from cardiovascular disease in women", *BMJ (Clinical research ed.),* vol. 307, no. 6918, pp. 1519-1524.

Ozanne, S.E., Jensen, C.B., Tingey, K.J., Storgaard, H., Madsbad, S. & Vaag, A.A. 2005, "Low birthweight is associated with specific changes in muscle insulin-signalling protein expression", *Diabetologia,* vol. 48, no. 3, pp. 547-552.

Padoan, A., Rigano, S., Ferrazzi, E., Beaty, B.L., Battaglia, F.C. & Galan, H.L. 2004, "Differences in fat and lean mass proportions in normal and growth-restricted fetuses", *American Journal of Obstetrics and Gynecology,* vol. 191, no. 4, pp. 1459-1464.

Pagel-Langenickel, I., Bao, J., Joseph, J.J., Schwartz, D.R., Mantell, B.S., Xu, X., Raghavachari, N. & Sack, M.N. 2008, "PGC-1alpha integrates insulin signaling, mitochondrial regulation, and bioenergetic function in skeletal muscle", *The Journal of biological chemistry,* vol. 283, no. 33, pp. 22464-22472.

Pan, D.A., Lillioja, S., Milner, M.R., Kriketos, A.D., Baur, L.A., Bogardus, C. & Storlien, L.H. 1995, "Skeletal muscle membrane lipid composition is related to adiposity and insulin action", *The Journal of clinical investigation,* vol. 96, no. 6, pp. 2802-2808.

Park, S.H., Gammon, S.R., Knippers, J.D., Paulsen, S.R., Rubink, D.S. & Winder, W.W. 2002, "Phosphorylation-activity relationships of AMPK and acetyl-CoA carboxylase in muscle", *Journal of applied physiology (Bethesda, Md.: 1985),* vol. 92, no. 6, pp. 2475-2482.

Park, S.Y., Kim, H.J., Wang, S., Higashimori, T., Dong, J., Kim, Y.J., Cline, G., Li, H., Prentki, M., Shulman, G.I., Mitchell, G.A. & Kim, J.K. 2005, "Hormone-sensitive lipase knockout mice have increased hepatic insulin sensitivity and are protected from short-term diet-induced insulin resistance in skeletal muscle and heart", *American journal of physiology.Endocrinology and metabolism,* vol. 289, no. 1, pp. E30-9.

Patti, M.E., Butte, A.J., Crunkhorn, S., Cusi, K., Berria, R., Kashyap, S., Miyazaki, Y., Kohane, I., Costello, M., Saccone, R., Landaker, E.J., Goldfine, A.B., Mun, E., DeFronzo, R., Finlayson, J., Kahn, C.R. & Mandarino, L.J. 2003, "Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1", *Proceedings of the National*

Academy of Sciences of the United States of America, vol. 100, no. 14, pp. 8466-8471.

Peppa, M., Koliaki, C., Nikolopoulos, P. & Raptis, S.A. 2010, "Skeletal muscle insulin resistance in endocrine disease", *Journal of biomedicine & biotechnology*, vol. 2010, pp. 527850.

Picard, F., Kurtev, M., Chung, N., Topark-Ngarm, A., Senawong, T., Machado De Oliveira, R., Leid, M., McBurney, M.W. & Guarente, L. 2004, "Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma", *Nature*, vol. 429, no. 6993, pp. 771-776.

Pimenta, A.S., Gaidhu, M.P., Habib, S., So, M., Fediuc, S., Mirpourian, M., Musheev, M., Curi, R. & Ceddia, R.B. 2008, "Prolonged exposure to palmitate impairs fatty acid oxidation despite activation of AMP-activated protein kinase in skeletal muscle cells", *Journal of cellular physiology*, vol. 217, no. 2, pp. 478-485.

Puigserver, P., Wu, Z., Park, C.W., Graves, R., Wright, M. & Spiegelman, B.M. 1998, "A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis", *Cell*, vol. 92, no. 6, pp. 829-839.

Purushotham, A., Schug, T.T., Xu, Q., Surapureddi, S., Guo, X. & Li, X. 2009, "Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation", *Cell metabolism*, vol. 9, no. 4, pp. 327-338.

Rangwala, S.M., Rhoades, B., Shapiro, J.S., Rich, A.S., Kim, J.K., Shulman, G.I., Kaestner, K.H. & Lazar, M.A. 2003, "Genetic modulation of PPARgamma phosphorylation regulates insulin sensitivity", *Developmental cell*, vol. 5, no. 4, pp. 657-663.

Regazzetti, C., Peraldi, P., Gremeaux, T., Najem-Lendom, R., Ben-Sahra, I., Cormont, M., Bost, F., Le Marchand-Brustel, Y., Tanti, J.F. & Giorgetti-Peraldi, S. 2009, "Hypoxia decreases insulin signaling pathways in adipocytes", *Diabetes*, vol. 58, no. 1, pp. 95-103.

Regnault, T.R., de Vrijer, B., Galan, H.L., Wilkening, R.B., Battaglia, F.C. & Meschia, G. 2007, "Development and mechanisms of fetal hypoxia in severe fetal growth restriction", *Placenta*, vol. 28, no. 7, pp. 714-723.

Regnault, T.R., Zhao, L., Chiu, J.S., Gottheil, S.K., Foran, A. & Yee, S.P. 2010a, "Peroxisome Proliferator-Activated Receptor -beta/delta, -gamma Agonists and Resveratrol Modulate Hypoxia Induced Changes in Nuclear Receptor Activators of Muscle Oxidative Metabolism", *PPAR research*, vol. 2010, pp. 129173.

Regnault, T.R., Zhao, L., Chiu, J.S., Gottheil, S.K., Foran, A. & Yee, S.P. 2010b, "Peroxisome Proliferator-Activated Receptor -beta/delta, -gamma Agonists and Resveratrol Modulate Hypoxia Induced Changes in Nuclear Receptor Activators of Muscle Oxidative Metabolism", *PPAR research*, vol. 2010, pp. 129173.

Resnik, R. 2002, "Intrauterine growth restriction", *Obstetrics and gynecology*, vol. 99, no. 3, pp. 490-496.

Rodgers, J.T., Lerin, C., Gerhart-Hines, Z. & Puigserver, P. 2008, "Metabolic adaptations through the PGC-1 alpha and SIRT1 pathways", *FEBS letters,* vol. 582, no. 1, pp. 46-53.

Rodgers, J.T., Lerin, C., Haas, W., Gygi, S.P., Spiegelman, B.M. & Puigserver, P. 2005, "Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1", *Nature,* vol. 434, no. 7029, pp. 113-118.

Rozance, P.J., Limesand, S.W., Barry, J.S., Brown, L.D. & Hay, W.W.,Jr 2009, "Glucose replacement to euglycemia causes hypoxia, acidosis, and decreased insulin secretion in fetal sheep with intrauterine growth restriction", *Pediatric research,* vol. 65, no. 1, pp. 72-78.

Ruderman, N.B., Saha, A.K., Vavvas, D. & Witters, L.A. 1999, "Malonyl-CoA, fuel sensing, and insulin resistance", *The American Journal of Physiology,* vol. 276, no. 1 Pt 1, pp. E1-E18.

Ryder, J.W., Yang, J., Galuska, D., Rincon, J., Bjornholm, M., Krook, A., Lund, S., Pedersen, O., Wallberg-Henriksson, H., Zierath, J.R. & Holman, G.D. 2000, "Use of a novel impermeable biotinylated photolabeling reagent to assess insulin- and hypoxia-stimulated cell surface GLUT4 content in skeletal muscle from type 2 diabetic patients", *Diabetes,* vol. 49, no. 4, pp. 647-654.

Sadiq, H.F., Das, U.G., Tracy, T.F. & Devaskar, S.U. 1999, "Intra-uterine growth restriction differentially regulates perinatal brain and skeletal muscle glucose transporters", *Brain research,* vol. 823, no. 1-2, pp. 96-103.

Sathyanarayana, P., Barthwal, M.K., Kundu, C.N., Lane, M.E., Bergmann, A., Tzivion, G. & Rana, A. 2002, "Activation of the Drosophila MLK by ceramide reveals TNF-alpha and ceramide as agonists of mammalian MLK3", *Molecular cell,* vol. 10, no. 6, pp. 1527-1533.

Satoh, S., Nishimura, H., Clark, A.E., Kozka, I.J., Vannucci, S.J., Simpson, I.A., Quon, M.J., Cushman, S.W. & Holman, G.D. 1993, "Use of bismannose photolabel to elucidate insulin-regulated GLUT4 subcellular trafficking kinetics in rat adipose cells. Evidence that exocytosis is a critical site of hormone action", *The Journal of biological chemistry,* vol. 268, no. 24, pp. 17820-17829.

Schaiff, W.T., Bildirici, I., Cheong, M., Chern, P.L., Nelson, D.M. & Sadovsky, Y. 2005, "Peroxisome proliferator-activated receptor-gamma and retinoid X receptor signaling regulate fatty acid uptake by primary human placental trophoblasts", *The Journal of clinical endocrinology and metabolism,* vol. 90, no. 7, pp. 4267-4275.

Schaiff, W.T., Knapp, F.F.,Jr, Barak, Y., Biron-Shental, T., Nelson, D.M. & Sadovsky, Y. 2007, "Ligand-activated peroxisome proliferator activated receptor gamma alters placental morphology and placental fatty acid uptake in mice", *Endocrinology,* vol. 148, no. 8, pp. 3625-3634.

Schenk, S., Saberi, M. & Olefsky, J.M. 2008, "Insulin sensitivity: modulation by nutrients and inflammation", *The Journal of clinical investigation,* vol. 118, no. 9, pp. 2992-3002.

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", *The Journal of biological chemistry*, vol. 274, no. 34, pp. 24202-24210.

Selak, M.A., Storey, B.T., Peterside, I. & Simmons, R.A. 2003, "Impaired oxidative phosphorylation in skeletal muscle of intrauterine growth-retarded rats", *American journal of physiology.Endocrinology and metabolism*, vol. 285, no. 1, pp. E130-7.

Su, J.L., Simmons, C.J., Wisely, B., Ellis, B. & Winegar, D.A. 1998, "Monitoring of PPAR alpha protein expression in human tissue by the use of PPAR alpha-specific MAbs", *Hybridoma*, vol. 17, no. 1, pp. 47-53.

Sugden, M.C., Caton, P.W. & Holness, M.J. 2010a, "PPAR control: it's SIRTainly as easy as PGC", *The Journal of endocrinology*, vol. 204, no. 2, pp. 93-104.

Sugden, M.C., Caton, P.W. & Holness, M.J. 2010b, "PPAR control: it's SIRTainly as easy as PGC", *The Journal of endocrinology*, vol. 204, no. 2, pp. 93-104.

Sun, C., Zhang, F., Ge, X., Yan, T., Chen, X., Shi, X. & Zhai, Q. 2007, "SIRT1 improves insulin sensitivity under insulin-resistant conditions by repressing PTP1B", *Cell metabolism*, vol. 6, no. 4, pp. 307-319.

Szczepaniak, L.S., Babcock, E.E., Schick, F., Dobbins, R.L., Garg, A., Burns, D.K., McGarry, J.D. & Stein, D.T. 1999, "Measurement of intracellular triglyceride stores by H spectroscopy: validation in vivo", *The American Journal of Physiology*, vol. 276, no. 5 Pt 1, pp. E977-89.

Tamasi, V., Miller, K.K., Ripp, S.L., Vila, E., Geoghagen, T.E. & Prough, R.A. 2008, "Modulation of receptor phosphorylation contributes to activation of peroxisome proliferator activated receptor alpha by dehydroepiandrosterone and other peroxisome proliferators", *Molecular pharmacology*, vol. 73, no. 3, pp. 968-976.

Taniguchi, C.M., Emanuelli, B. & Kahn, C.R. 2006, "Critical nodes in signalling pathways: insights into insulin action", *Nature reviews.Molecular cell biology*, vol. 7, no. 2, pp. 85-96.

Ueki, K., Yamamoto-Honda, R., Kaburagi, Y., Yamauchi, T., Tobe, K., Burgering, B.M., Coffer, P.J., Komuro, I., Akanuma, Y., Yazaki, Y. & Kadowaki, T. 1998, "Potential role of protein kinase B in insulin-induced glucose transport, glycogen synthesis, and protein synthesis", *The Journal of biological chemistry*, vol. 273, no. 9, pp. 5315-5322.

Viscarra, J.A., Vazquez-Medina, J.P., Crocker, D.E. & Ortiz, R.M. 2011, "Glut4 is upregulated despite decreased insulin signaling during prolonged fasting in northern elephant seal pups", *American journal of physiology.Regulatory, integrative and comparative physiology*, vol. 300, no. 1, pp. R150-4.

Walker, K.S., Deak, M., Paterson, A., Hudson, K., Cohen, P. & Alessi, D.R. 1998, "Activation of protein kinase B beta and gamma isoforms by insulin in vivo and by 3-phosphoinositide-dependent protein kinase-1 in vitro: comparison with

protein kinase B alpha", *The Biochemical journal,* vol. 331 (Pt 1), no. Pt 1, pp. 299-308.

Wu, X., Chang, M.S., Mitsialis, S.A. & Kourembanas, S. 2006, "Hypoxia regulates bone morphogenetic protein signaling through C-terminal-binding protein 1", *Circulation research,* vol. 99, no. 3, pp. 240-247.

Yates, D.T., Macko, A.R., Nearing, M., Chen, X., Rhoads, R.P. & Limesand, S.W. 2012, "Developmental programming in response to intrauterine growth restriction impairs myoblast function and skeletal muscle metabolism", *Journal of pregnancy,* vol. 2012, pp. 631038.

Yu, C., Chen, Y., Cline, G.W., Zhang, D., Zong, H., Wang, Y., Bergeron, R., Kim, J.K., Cushman, S.W., Cooney, G.J., Atcheson, B., White, M.F., Kraegen, E.W. & Shulman, G.I. 2002a, "Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle", *The Journal of biological chemistry,* vol. 277, no. 52, pp. 50230-50236.

Yu, C., Chen, Y., Cline, G.W., Zhang, D., Zong, H., Wang, Y., Bergeron, R., Kim, J.K., Cushman, S.W., Cooney, G.J., Atcheson, B., White, M.F., Kraegen, E.W. & Shulman, G.I. 2002b, "Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle", *The Journal of biological chemistry,* vol. 277, no. 52, pp. 50230-50236.

Yun, Z., Lin, Q. & Giaccia, A.J. 2005, "Adaptive myogenesis under hypoxia", *Molecular and cellular biology,* vol. 25, no. 8, pp. 3040-3055.

Zhang, L., Keung, W., Samokhvalov, V., Wang, W. & Lopaschuk, G.D. 2010, "Role of fatty acid uptake and fatty acid beta-oxidation in mediating insulin resistance in heart and skeletal muscle", *Biochimica et biophysica acta,* vol. 1801, no. 1, pp. 1-22.

Zierath, J.R., He, L., Guma, A., Odegoard Wahlstrom, E., Klip, A. & Wallberg-Henriksson, H. 1996, "Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM", *Diabetologia,* vol. 39, no. 10, pp. 1180-1189.

Zierath, J.R., Krook, A. & Wallberg-Henriksson, H. 2000, "Insulin action and insulin resistance in human skeletal muscle", *Diabetologia,* vol. 43, no. 7, pp. 821-835.

Zinda, M.J., Vlahos, C.J. & Lai, M.T. 2001, "Ceramide induces the dephosphorylation and inhibition of constitutively activated Akt in PTEN negative U87mg cells", *Biochemical and biophysical research communications,* vol. 280, no. 4, pp. 1107-1115.

Zisman, A., Peroni, O.D., Abel, E.D., Michael, M.D., Mauvais-Jarvis, F., Lowell, B.B., Wojtaszewski, J.F., Hirshman, M.F., Virkamaki, A., Goodyear, L.J., Kahn, C.R. & Kahn, B.B. 2000, "Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance", *Nature medicine,* vol. 6, no. 8, pp. 924-928.

Curriculum Vitae

MAGGIE (YUNYAN) ZHANG

EDUCATION

Current- 01/2011	Western University, London, Ontario, Canada M.Sc. with focus in physiology (Completion date: December 2012)
07/2009-09/2007	Sichuan University, China Master of Medical Science (Major: Gynecology and Obstetrics)
07/2006-09/2001	Sichuan University, China Bachelor of Medicine (Major: Basic Medicine)

TRAINING EXPERIENCE

02/2009- 3/2009	Internship in the Department of Reproduction and Endocrine, 2nd University Hospital, Sichuan University
09/2008- 2/2008	Internship in the Department of Obstetrics, 2nd University Hospital, Sichuan University
04/2007-09/2007	Internship in the Department of Gynecology, 2nd University Hospital, Sichuan University

SCHOLARSHIPS

12/2012-01/2011	Western Graduate Research Scholarship, Western University, London, ON
08/2012-09/2011	Obstetrics & Gynaecology Graduate Scholarship, Western University, London, ON
08/2012-09/2011	Outstanding Graduate Student Fellowship, Sichuan University, China

PUBLICATIONS

Y.Y. Zhang, A.Zheng, Y.X Chu. Influences of human β-defensin1 on the replication and expression of HPV18 in Hela cell. Journal of Sichuan University (Medical Sciences Edition), 2009, 40 (2), 232-235.

Y.Y. Zhang, X.R. Shi. The advances in the studying of transforming growth factor β. Sichuan Journal of Anatomy, 2006, 3 (14), 30-32.

Y.Q. Bi, ***Y.Y. Zhang***, J.N. Zhao, et.al. A controlled porosity osmotic pump system with biphasic release of theophylline: influence of weight gain on its in vivo pharmacokinetics. Chemical & Pharmaceutical Bulletin, 2008, 56(6), 792-795.

CONFERENCE PRESENTATIONS

Poster Presentation of current research

- Lawson Research Day, London, ON (local)
- Perinatal Investigator Meeting, Kingston, ON (national)
- Perinatal Investigator Meeting, Toronto, ON (national)
- Southern Ontario Reproductive Biology, Guelph, ON (national)
- Paul Harding Research Awards Day, London, ON (local)
- Physiology and Pharmacology Department Research Day, London, ON (local)