

The University of Nottingham

UNITED KINGDOM · CHINA · MALAYSIA

Investigation of molecular mechanisms regulating palmitate-induced metabolic inflexibility in a cell-based skeletal muscle model: physiological and pharmacological interventions.

Hung-Che Chien

BSc. (2010), MSc. (2014)

This thesis is submitted to the University of Nottingham for the degree of

Doctor of Philosophy

July 2020

"An experiment is a question, which science poses to Nature, and a measurement is the recording of Nature's answer."

.'

MAX PLANCK, 1858-1947

Theoretical Physicist

Abstract

Background

Present evidence indicates that increased systemic circulating fatty acids following chronic high-fat dietary intake leads to skeletal muscle (SKM) metabolic inflexibility, and this is associated with an impairment of the mitochondrial pyruvate dehydrogenase complex (PDC) controlled carbohydrate (CHO) oxidation. However, the molecular mechanisms behind the reduction in the PDC controlled CHO oxidation during chronic high-fat dietary intake are poorly understood. Nevertheless, previously published evidence advocated that transcription factors such as PPARα, PPARδ, and FOXO1 might contribute to the PDK4 mediated PDC inhibition.

This thesis aimed, therefore, (1) to elucidate molecular and metabolic evidence supporting this contention in a metabolic inflexible C2C12 SKM cell model, and (2) to determine whether activators of CHO oxidation, such as exercise (delivered as electrical pulse stimulation - EPS), dichloroacetate (DCA) or R-α-lipoic acid (RALA) could rescue CHO oxidation in the presence of palmitate by altering the same molecular and metabolic events identified at (1).

Results

In chapter 3, we established what concentration of palmitate could be tolerated by our *in vitro* SKM cell model (500 μM) without jeopardising the viability of the muscle cells. It was found in our SKM cell-based model that palmitate supplementation replicated well-defined markers

of *in vivo* impaired cellular CHO oxidation: i.e. decreased glucose uptake, lower acetylcarnitine accumulation, lower pyruvate mediated mitochondrial ATP production rate, increased media lactate concentration and decreased PDC activity. It was also found that palmitate significantly increased the gene and protein expression of PDK4, PPARα, PPARδ, and FOXO1.

In chapter 4, it was found that the palmitate-induced reduction in PDC activity could be reversed in tandem with a reduction in the PDK4 protein expression by siRNA PPARδ and FOXO1 gene silencing. It appeared, however, that although PPARα gene silencing restored the palmitate-induced reduction in PDC activity, this occurred independently from any changes in the PDK4 protein expression. siRNA PPARα, PPARδ, and FOXO1 gene silencing rescued the palmitate-induced reduction in the cellular glucose uptake. In contrast, only PPARδ and FOXO1 gene silencing reversed the palmitate-induced changes in media lactate and acetylcarnitine concentrations. The latter findings, which are in line with the contention these transcription factors are directly targeting PDC, also provide further evidence that the effects of PPARα on PDC activity occur independently of the involvement of PDK4 protein.

Additionally, siRNA silencing of PPARα and FOXO1 restored the palmitate-induced reductions in the mitochondrial maximal ATP production rates (MAPR). In contrast, PPARδ silencing did not, surprisingly, rescue the palmitate-induced decline in MAPR. It was also found that the siRNA PPARδ gene silencing decreased FOXO1 protein expression. In contrast, the siRNA gene silencing of FOXO1 did not change the PPARδ protein expression, which

collectively suggests that FOXO1 may be a PPARδ downstream target, thereby modulating PDK4 protein expression.

In chapter 5, it was shown that administration of RALA or DCA rescued the palmitate-induced inhibition of PDC activity and the increase in media lactate concentration. However, only RALA corrected the deleterious effects of palmitate on cell glucose uptake and MAPR. At the molecular level, DCA, but not RALA, reduced PDK4 protein expression in the presence of palmitate. We also found that DCA and RALA reversed the palmitate-induced upregulation of PPARα and PPARδ and total-FOXO1 protein expression.

In chapter 6, it was demonstrated that the treatment of palmitate-supplemented cells with electrical pulse stimulation (EPS) rescued the palmitate-induced dysregulation of glucose uptake, PDC activity, and its flux, and MAPR. Furthermore, these events were accompanied by a restoration/lowering of the protein expression levels of PPARδ and its downstream target FOXO1, but not those of PPARα and the p-FOXO1/t-FOXO1 ratio normalised to that of nuclear to cytoplasmic ratio. These molecular findings indicate benefits of sustained muscle contraction in maintaining CHO flux in the presence of palmitate.

Conclusions

This thesis provides persuasive evidence that the mechanism by which palmitate downregulates the PDC-mediated reduction in CHO oxidation in muscle occurs according to the following series of events:

Palmitate → PPARδ ⊣ FOXO1 ⊣ PDK4 ⊣ PDC → CHO oxidation.

However, the action of PPARα seems not to involve the upregulation of the PDK4 protein. This thesis also provides evidence that DCA, RALA, and EPS can rescue the palmitate-induced reduction in CHO oxidation. Collectively, these findings are of clinical relevance as they open the path for potential therapy to the lifestyle-induced metabolic inflexibility and insulin resistance.

Oral Presentations

1. **Hung-Che Chien**, Paul L. Greenhaff and Dumitru Constantin-Teodosiu (2019). PPARδ and FOXO1 mediate palmitate-induced inhibition of pyruvate dehydrogenase and mitochondrial fuel selection in C2C12 myotubes, which is reversed by continuous electrical pulse stimulation. SOLS Post-graduate Symposium, University of Nottingham, Nottingham, UK.

Poster Presentations

1. **Hung-Che Chien**, Paul L. Greenhaff, and Dumitru Constantin-Teodosiu (2018). Reversing palmitate-induced inhibition of pyruvate dehydrogenase complex activity and mitochondrial ATP production in C2C12 muscle cells by electric pulse stimulation. ACSM Integrative Physiology of Exercise Conference. San Diego, USA.
2. **Hung-Che Chien**, Paul L. Greenhaff, and Dumitru Constantin-Teodosiu (2019). PPARδ and FOXO1, but not PPARα, are involved in palmitate-induced PDK4 upregulation in C2C12 skeletal muscle cell. Exercise Metabolism, Cell symposium. Sitges, Spain.
3. **Hung-Che Chien,** Despina Constantin, Paul Greenhaff, Dumitru Constantin-Teodosiu (2020). Effect of dichloroacetate and R-alpha-lipoic acid on palmitate-induced inhibition of pyruvate dehydrogenase complex and mitochondrial ATP production in C2C12 myotube. European College of Sport Science (ECSS) Congress, Seville, Spain.

Acknowledgements

The work detailed in this thesis is the culmination of four years' work within the Metabolic and Molecular Physiology Research group based within the School of Life Sciences at the University of Nottingham. The scientific knowledge and enthusiasm for research, which I have been exposed to in Nottingham has inspired me to pursue a career in scientific research. I would like to express my sincere gratitude to the following people:

Dr Tim Constantin-Teodosiu for his constant supervision and training through my PhD life. His knowledge of cellular metabolism and biochemistry is outstanding, and he always could answer my questions in daily life and meetings. Also, he gave instructive advice and useful suggestions for my thesis. I am deeply thankful for his help in the completion of this thesis and my PhD life. **Professor Paul Greenhaff** for his supervision in my PhD life. He gave many instructive suggestions and helped me to design my experiments. I am grateful for his guidance. Also, I would like to express my thanks to my colleagues **Robert Jones, Simon Platt, Pardeep Pabla, and Yangfan Gao,** for their encouragement and help in my daily life.

I would like to thank my **parents** for their support throughout my life and during my PhD training. Also, most importantly, I would like to express my profound thanks to my wife, **Belinda Su**, for her continuous support and encouragement. This thesis is dedicated to her. At

last, I would like to express my thanks to the **government of Taiwan** for providing the scholarship that allowed me to study for the PhD degree at the University of Nottingham.

Declaration

The data presented in this thesis were collected and analysed by me.

I declare that this thesis has been constructed by me and that all the data presented are my work unless otherwise stated. All published literature within this thesis has been appropriately referenced. None of the data presented in this thesis has previously been submitted towards the assessment of a higher degree.

Hung-Che Chien

May 2020

List of Abbreviations

ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CHO	Carbohydrate
CoASH	Coenzyme A
DCA	Dichloroacetate
EPS	Electrical pulse stimulation
FOXO	Forkhead transcription factors
GLUT4	Glucose transporter type 4
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinases
MAPR	Maximal ATP Production Rates
MMP	Mitochondrial membrane potential
mRNA	Messenger ribonucleic acid
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)

NADH	Nicotinamide adenine dinucleotide (reduced form)
PDC	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PI3K	Phosphoinositide 3-kinase
PPARα	Peroxisome proliferator-activated receptor alpha
PPARδ	Peroxisome proliferator-activated receptor delta
PTP	Pyruvate dehydrogenase phosphatase
RALA	R-Alpha lipoic acid
ROS	Reactive oxygen species
siRNA	small interfering Ribonucleic Acid
TBST	Tris-buffered saline and Tween-20

Table of Contents

Abstract 3
Oral Presentations 7
Acknowledgements 8
Declaration 10
List of Abbreviations 11
Table of Contents 13
Chapter 1 20
Introduction 20
1-1. Metabolic flexibility in the development of insulin resistance and type 2 diabetes 21
1-2. Excess free fatty acid and palmitate's role in the metabolic inflexibility 24
1-3. Mitochondria 26
1-3-1. Structure and function 26
1-3-2. Mitochondrial life cycle. 28
1-3-3. Metabolism of mitochondria and their regulatory contribution 29
1-4. The mitochondrial pyruvate dehydrogenase complex (PDC) 30
1-4-1. structure and function. 30
1-4-4. PDC flux and acetyl-CoA, acetylcarnitine and free carnitine, ... 35
1-4-5. The role of pyruvate dehydrogenase kinase 4 (PDK4) in the regulation of PDC and glucose homeostasis in skeletal muscle 37
1-5. Methods to assess mitochondrial function 38
1-5-1. Mitochondrial Metabolic Function Assessed *In Vivo* and *In Vitro* 39
1-5-1.1. *In vivo* non-invasive methods 39
1-5-1.2 *In vitro* invasive methods 39
1-5-2. High-resolution respirometry versus bio-luminometric measurements of the ATP formation rates 41
1-5-3. Mitochondrial electron and proton uncoupling 42
1-6. Mechanism of PDK4 activation by PPARs/FOXO1 44
1-7. Physiological and pharmacological interventions aimed at increasing activity of PDC. 48
1-7-1. Dichloroacetate (DCA) 49
1-7-2. R-α-lipoic acid 49
1-7-3. Exercise 50
1-8. Hypothesis & Aims 51

Chapter 2 53
Materials and general methods 53
2-1. Materials. 54
2-2. Cell culture. 55
2-3. Stock palmitate preparation 56
2-4. MTT assay 57
2-5. Glucose uptake assay 58
2-6. Media lactate level measurement 58
2-7. Pyruvate dehydrogenase complex activity 59
2-8. Muscle cell acetylcarnitine content 60
2-9. Measurement of maximal rates of mitochondrial ATP production (MAPR) in C2C12 myotubes. 61
2-10. Making ATP standard 64
2-11. RNA extraction and Real-time PCR 64
2-12. Small interfering RNA (siRNA) transfection. 65
2-13. Western blotting. 68
2-14. Electrical pulse stimulation. 70
2-15. Bradford protein assay 71
2-16. Statistical analyses 73
Chapter 3 74
Metabolic inflexibility in an *in vitro* palmitate-induced C2C12 skeletal muscle cell model： Molecular responses associated with impairment of mitochondrial CHO oxidation 74
3-1 Introduction 75
3-1-1.Metabolic inflexibility 75
3-1-2. Fatty acid and PPARs 79
3-1-3. PPARs activity and mitochondrial function 80
3-2. Methods 82
3-3. Results 84
3-3-1. Cell viability in C2C12 myotubes treated with increasing levels of palmitate 84
3-3-2. Glucose uptake and lactate concentration in the presence or absence of palmitate in C2C12 myotubes 86
3-3-3. PDC activity, acetyl-CoA, and acetylcarnitine content in C2C12 myotubes in the presence or the absence of palmitate. 87
3-3-4. Pyruvate based maximal generated rates of ATP production in C2C12 myotubes in the presence or absence of palmitate 89
3-3-5. mRNA gene expression in C2C12 myotubes in the presence or the absence of palmitate. 90

3-3-6. Protein expression in C2C12 myotubes in the presence or the absence of palmitate.....91
3-4. Discussion.....93
Chapter 4.....100
The impact of siRNA PPARα, PPARδ and FOXO1 gene silencing on palmitate-induced impairment of mitochondrial CHO oxidation.....100
4-1. Introduction 101
4-1-1. RNA interference 101
4-1-2. *In vitro* use of siRNAs.....101
4-1-3. PPARα.....102
4-1-4. PPARδ.....103
4-1-5. FOXO1 105
4-1-6. Aim of this chapter.....107
4-2. Methods 108
4-3. Results 110
4-3-1. Study 1: The effect of siRNA PPARα gene silencing on the palmitate-induced impairment of CHO oxidation in C2C12 myotubes. 110
4-3-1.1. Validation of siRNA PPARα gene silencing in C2C12 myotubes in the presence or the absence of palmitate. 110
4-3-1.2. The siRNA PPARα gene silencing reversed the palmitate-induced impairment of cellular glucose uptake in C2C12 myotubes. 112
4-3-1.3. The siRNA PPARα gene silencing reversed the palmitate-induced inhibition of PDC activity in C2C12 myotubes.....113
4-3-1.4. The siRNA PPARα gene silencing did not change the acetylcarnitine, and acetyl-CoA content in the palmitate treated C2C12 myotubes.....115
4-3-1.5. The siRNA PPARα gene silencing restored the palmitate-induced reduction in the pyruvate derived maximal mitochondrial ATP production rates in C2C12 myotubes. 116
4-3-1.6. The siRNA PPARα gene silencing did not change the PDK4 protein expression in the presence of palmitate in C2C12 myotubes. 118
4-3-1.7. The siRNA PPARα gene silencing decreased phosphorylated FOXO1 and total FOXO1 protein expressions in the presence of palmitate in C2C12 myotubes. 119
4-3-2 Study 2: the effects of siRNA PPARδ gene silencing on the palmitate-induced impairment of CHO oxidation 122

4-3-2.1. Validation of siRNA PPARδ gene silencing in C2C12 myotubes in the presence or the absence of palmitate. 122

4-3-2.2. The siRNA PPARδ gene silencing reversed the palmitate-induced reduction in the cellular glucose uptake and the increase in media lactate levels in C2C12 myotubes. 124

4-3-2.3. The siRNA PPARδ gene silencing reversed the palmitate-induced reduction in PDC activity in C2C12 myotubes. 125

4-3-2.4. The siRNA PPARδ gene silencing reversed the palmitate-induced reduction in acetylcarnitine content in palmitate treated C2C12 myotubes. 127

4-3-2.5. The siRNA PPARδ gene silencing reversed the palmitate-mediated reduction in pyruvate derived maximal mitochondrial ATP production in C2C12 myotubes 128

4-3-2.6. The siRNA PPARδ gene silencing reversed the palmitate-mediated increase in PDK4 protein expression in C2C12 myotubes 130

4-3-2.7. The siRNA PPARδ gene silencing reversed the palmitate-mediated increase in PPARα, phosphorylated FOXO1 and total-FOXO1 protein expression in C2C12 myotubes 131

4-3-3 Study 3: the effect of siRNA FOXO1 gene silencing on the palmitate-induced impairment of CHO oxidation in C2C12 myotubes in the presence or the absence of palmitate. 134

4-3-3.1. Validation of the siRNA FOXO1 gene silencing in C2C12 myotubes in the presence or the absence of palmitate. 134

4-3-3.2. The siRNA FOXO1 gene silencing reversed the palmitate-induced impairment of the cellular glucose uptake and increase in media lactate level in C2C12 myotubes. 136

4-3-3.3. The siRNA FOXO1 gene silencing reversed the palmitate-induced reduction in PDC activity in C2C12 myotubes 137

4-3-3.4. The siRNA FOXO1 gene silencing reversed the palmitate-induced reduction in the acetylcarnitine content in C2C12 myotubes. 139

4-3-3.5. The siRNA FOXO1 gene silencing reversed the palmitate-induced reduction in the pyruvate based maximal mitochondrial ATP production rates in C2C12 myotubes 140

4-3-3.6. The siRNA FOXO1 gene silencing reversed the palmitate mediated increase in PDK4 mRNA and protein expression in C2C12 myotubes 142

4-3-3.7. The siRNA FOXO1 gene silencing reversed the palmitate-induced decrease in PPARα, but not PPARδ, gene and protein expression. 143

4-4. Discussion....145
Chapter 5....151
The impact of the pharmacological intervention on the palmitate-induced impairment of mitochondrial CHO oxidation....151
5-1. Introduction....152
5-1-1. Dichloroacetate (DCA)....152
5-1-2. R-Alpha Lipoic acid (RALA)....154
5-1-3 Aim of this chapter....156
5-2. Methods....156
5-3. Result....158
5-3-1. Study 4: The effect of dichloroacetate (DCA) treatment on the palmitate-induced impairment of CHO oxidation....158
5-3-1.1. Effect of DCA titration curve on the activity of PDC. DCA treatment rescued the palmitate-induced reduction in PDC activity in C2C12 myotubes....158
5-3-1.2. DCA did not change the cellular glucose uptake with or without palmitate but reversed the palmitate-mediated increase in media lactate concentration in C2C12 myotubes....161
5-3-1.3. DCA reversed the palmitate-induced reduction in the cellular acetylcarnitine and acetyl-CoA content in C2C12 myotubes....162
5-3-1.4. DCA decreased the pyruvate based maximal mitochondrial ATP production rates in the presence of palmitate in C2C12 myotube....164
5-3-1.5. DCA treatment reversed the palmitate-induced increase in PDK4 protein expression in C2C12 myotubes....165
5-3-1.6. DCA treatment decreased the palmitate-induce increase in PPARα and PPARδ protein expression....167
5-3-1.7. DCA decreased the total FOXO1 protein expression in the presence of palmitate....168
5-3-2. Study 5: The effect of R-α-lipoic acid (RALA) treatment on the palmitate-induced impairment of CHO oxidation....171
5-3-2.1. RALA intervention reversed the palmitate-induced reduction in PDC activity in C2C12 myotubes....171
5-3-2.2. RALA intervention reversed the palmitate-induced inhibition of glucose uptake and increase in media lactate concentration....173
5-3-2.3. There was no change in the acetylcarnitine, and acetyl-CoA levels in the palmitate treated C2C12 myotubes with RALA as a co-treatment....174

5-3-2.4. RALA reversed the palmitate-induced reduction in the pyruvate fuelled maximal mitochondrial ATP production rates in C2C12 myotubes....176

5-3-2.5. RALA increased the PDK4 protein expression in the presence of palmitate in C2C12 myotubes....177

....177

5-3-2.6. RALA decreased the PPARα and PPARδ protein expression in C2C12 myotubes in the presence of palmitate....178

5-3-2.7. RALA decreased phosphorylated and total FOXO1 protein expression in C2C12 myotubes in the presence of palmitate. 180

5-4. Discussion....183

Chapter 6....190

The impact of chronic low-frequency electrical pulse stimulation (EPS) on palmitate-induced impairment of mitochondrial CHO oxidation....190

6-1. Introduction 191

6-1-1. Electrical pulse stimulation (EPS)....191

6-1-2. Aim of this chapter....193

6-2. Methods 193

6-3. Results of Study 6: The effect of EPS treatment on the palmitate-induced impairment of CHO oxidation 195

6-3-1. Palmitate reduced the C2C12 myotube glucose uptake, which was restored by the low-frequency electrical pulse stimulation, but with no change in the media lactate concentration....195

6-3-2. Palmitate reduced the C2C12 myotube's PDC activity, which was rescued by EPS 197

6-3-3. EPS reversed the palmitate-induced reduction in the cellular acetylcarnitine content in C2C12 myotubes....198

6-3-4. Palmitate reduced the C2C12 myotube' s maximal mitochondrial ATP production rates from pyruvate, which was restored by electrical pulse stimulation. 199

6-3-5. Palmitate increased the C2C12 myotube PDK4 protein expression, which was reversed by EPS 201

6-3-6. Palmitate increased the PPARδ protein expression in C2C12 myotubes, and this was restored by electrical pulse stimulation 202

6-3-7. EPS decreased the phosphorylated and total FOXO1 protein expression in C2C12 myotubes in the presence of palmitate....204

6-4. Discussion....208

Chapter 7 214
General discussion 214
7-1. Primary findings and discussion 215
7-1-1 Primary findings and discussion in chapter 3 215
7-1-2. Primary findings and discussion in chapter 4 220
7-1-3. Primary findings and discussion in chapter 5 223
7-1-4. Primary findings and discussion in chapter 6 227
7-2. The advantage and limitation of in vitro cell model 231
7-3. Conclusion 233
7-4. Future directions 234
References 238

Chapter 1
Introduction

1-1. Metabolic flexibility in the development of insulin resistance and type 2 diabetes

Metabolic flexibility is the capacity of the organism to adapt fuel oxidation according to fuel availability. Conversely, metabolic inflexibility is defined as the impairment of the capacity to increase fat oxidation upon increased fatty acid availability or to switch from fat to glucose as the primary fuel source after a meal (Corpeleijn, Saris et al. 2009). Fatty acid availability, uptake, and oxidation all play a role in the induction of the metabolic flexibility and impairment of insulin-stimulated glucose uptake, also named insulin resistance. Insulin resistance is a critical component of the metabolic inflexibility that can develop in many tissues and organs. The metabolic inflexibility can also be viewed as an umbrella of several pathological conditions where organs such as muscle, liver, and adipose lose sensitivity to the effects of insulin, which broadly translates to lower whole-body capacity to dispose of glucose following a diet intake (Figure 1-1).

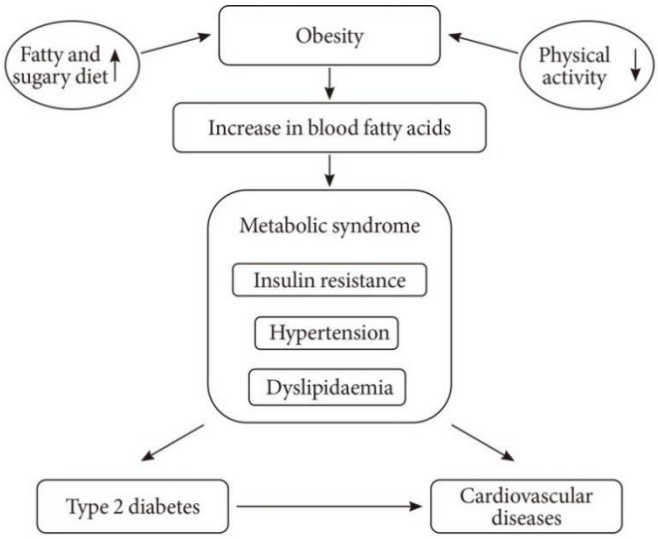


Figure 1-1. Metabolic inflexibility and type 2 diabetes

Insulin resistance (IR), which is associated with obesity and chronic high levels of circulating free fatty acids, can be related to the metabolic inflexibility, in which nutrient overload, especially in the form of fat, results in mitochondrial function disturbances, impaired CHO switching from fat to carbohydrate, and energy dysregulation (Muoio 2014). The pancreas begins to release insulin when glucose is released into the blood from the digestion of carbohydrates. Proteins in food and other hormones produced by the gut in response to food also stimulate insulin release. Insulin, which is produced by beta-cells of the pancreas, helps to (1) transport glucose into tissues, mainly skeletal muscle and adipose tissue, (2) increase the rate of glucose disposal by either (2a) increasing the rate of glucose oxidation following insulin-mediated activation of the mitochondrial pyruvate dehydrogenase complex, which controls the rate-limiting step of glucose oxidation or (2b) by increasing the rate of glucose deposition as glycogen or fat, and (3) inhibit endogenous hepatic glucose production by inhibiting gluconeogenesis. All these processes, collectively, reduce the circulating blood glucose concentrations. However, when our body sustains high-level of circulating free fatty acids, this leads to the impairment of the metabolic pathways controlled by insulin (exemplified above) despite that beta cells would initially respond by releasing more insulin to rebalance the circulation levels of blood glucose levels.

The main clinical feature of type 2 diabetes is a chronic elevation in circulating blood glucose levels. Chronic systemic hyperglycemia is toxic and can lead to cellular dysfunction that may

become irreversible over time due to deterioration of the pericyte cells' ability to provide vascular stability and control to endothelial proliferation. Therefore, it may not be surprising that Type 2 diabetes's complications are mainly macrovascular and microvascular related, i.e., neuropathy, retinopathy, nephropathy, coronary artery, and peripheral vascular diseases.

On a particular note, skeletal muscles are quantitatively the primary site of whole-body oxidative and non-oxidative glucose disposal (mainly in the form of glycogen) (Jue, Rothman *et al.* 1989, Shulman, Rothman *et al.* 1990) (Figure 1-2). Insulin resistance is also associated with reduced glucose transporter translocation in muscle and interruption of the molecular signalling pathway, as well as enzymatic reactions like that controlled by the mitochondrial pyruvate dehydrogenase complex reaction, which collectively results in an inability to keep blood glucose concentrations within normal ranges (4.-5.4 mmol/L).

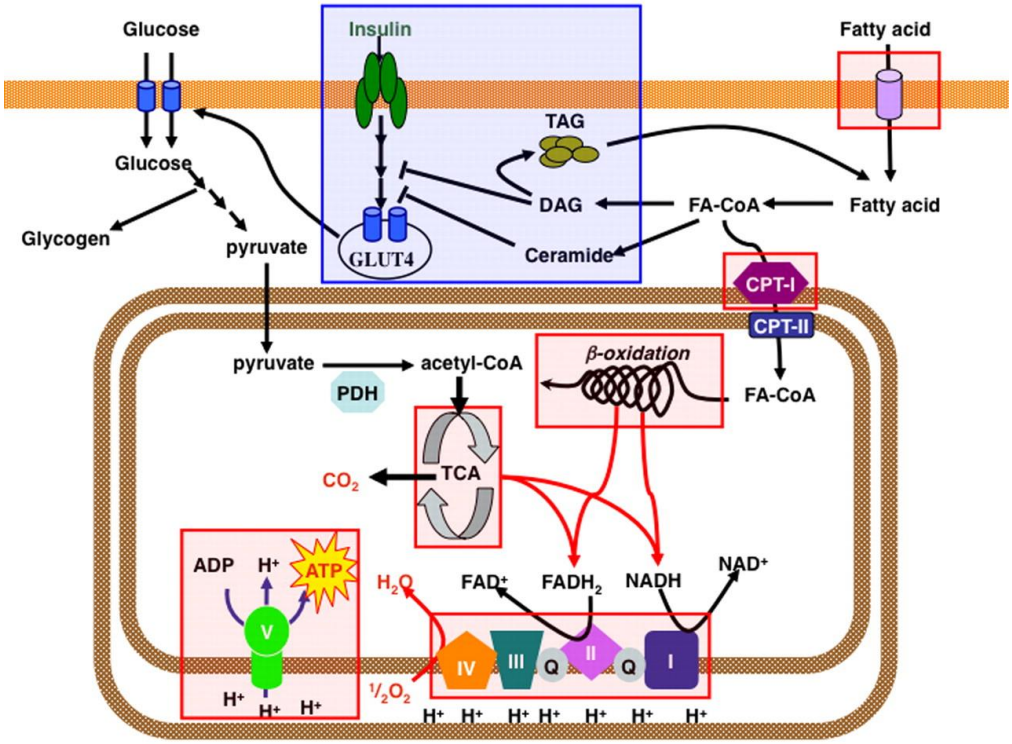


Figure 1-2. Muscle and metabolic inflexibility: A case of fat overconsumption, not mitochondrial dysfunction (Kraegen, Cooney et al. 2008).

1-2. Excess free fatty acid and palmitate's role in the metabolic inflexibility

Evidence suggests that chronic fatty acids excess plays a significant role in the progress of skeletal muscle metabolic inflexibility (Shulman 2000, Boden, Lebed *et al.* 2001). Excess free long-chained fatty acids (FFA) delivery to skeletal muscle encourages ectopic fat accumulation, leading to metabolic inflexibility, defined as the inability to switch from lipid to glucose oxidation during the fasting-to-feeding transition (Capel, Cheraiti *et al.* 2016). Randle was the first scientist who proposed that excess free fatty acid (FFA) decreases glucose disposal in the muscle (Randle, Garland *et al.* 1963). This study described that elevated FFA resulted in an increased rate of fatty acid oxidation relative to carbohydrate oxidation, which led to acetyl-CoA and citrate accumulation and then resulted in decreased glucose utilisation. Excess FFA also impaired insulin sensitivity and signalling. Intramyocellular triacylglycerol (IMTG), diacylglycerol (DAG), and ceramide, which are metabolites of lipids, are involved in the inhibition of insulin sensitivity and insulin signalling, such as IRS-1 and PI3K/AKT, in the skeletal muscle in mice and obese human. Also, elevated FFAs activate inflammatory pathways that generate pro-inflammatory cytokines, such as tumour necrosis factor α (TNFα) and interleukin 6 (IL-6), which further deteriorate insulin signalling and insulin sensitivity (Amati,

Dube *et al.* 2011, Eckardt, Taube *et al.* 2011, Turner, Kowalski *et al.* 2013). Besides, excess FFAs led to mitochondria dysfunction. Thus, Sparks LM *et al.* showed that high-fat diet in human downregulates genes responsible for mitochondria oxidative phosphorylation in skeletal muscle (Sparks, Xie *et al.* 2005). Besides, increased reactive oxygen species (ROS) production decreased the generation of ATP in cultured skeletal muscle cells (Lambertucci, Hirabara *et al.* 2008, Hirabara, Curi *et al.* 2010).

Palmitate is the most common long-chained (C16) saturated fatty acid found in animals, plants, and microorganisms. There is abundant evidence that increased palmitate availability induces skeletal muscle insulin resistance by impairing insulin signalling pathway through the production of ROS, apoptosis, and mitochondrial dysfunction. Also, inflammatory cytokines such as TNF-α, IL-6, inflammatory pathways such as JNK and NF-κB pathways are activated after administration of palmitate (Coll, Palomer *et al.* 2010, Gorgani-Firuzjaee, Ahmadi *et al.* 2014, Khodabandehloo, Gorgani-Firuzjaee *et al.* 2016). Alternatively, increasing the availability of circulation FFAs/palmitate may directly exert an inhibitory effect on the muscle mitochondrial pyruvate dehydrogenase complex activity, which is the rate-limiting step in carbohydrate oxidation (Tsintzas, Chokkalingam *et al.* 2007). Therefore, palmitate is often used to treat cells to induce in *in intro* models of metabolic inflexibility/insulin resistance.

1-3. Mitochondria

1-3-1. Structure and function

Mitochondria are double membrane-bound organelles found in nearly all eukaryotes. These structures are "the powerhouse of the cell" because they generate most of the cell's supply of adenosine triphosphate (ATP), used as a source of cellular chemical energy. Mitochondria are involved in other tasks (signalling, cellular differentiation, and cell death as maintaining control of the cell cycle and cell growth). Mitochondria range from 0.5 to 1.0 μm in diameter. The number and location are according to the cell type (1 in the unicellular organism up to 2,000 in the human liver cell). The cellular occupied mitochondrial volume varies from 2-5% in muscle up to 20% in the liver. In muscle, mitochondria can be found nestled between the myofibrils (IMF) and in the subsarcolemmal (SS) region. Morphologically, mitochondria consist of four compartments: the outer membrane, the intermembrane space, the folded inner membrane, and

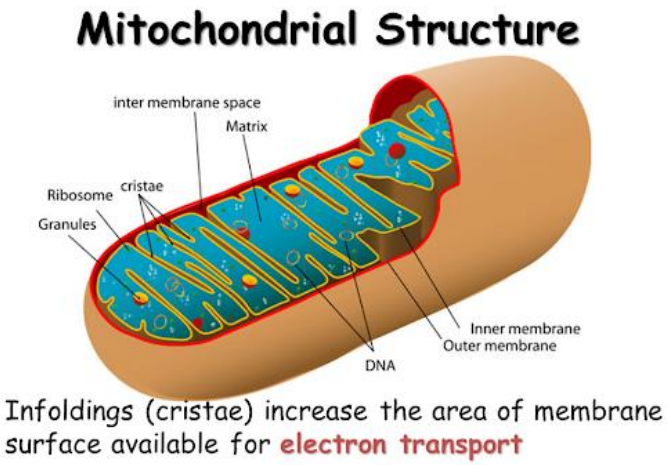


Figure 1-3. Mitochondrial structure

the matrix (Figure 1-3).

The mitochondrion has often been named the powerhouse of the cell, because it is the site where most of the chemical energy of the cell is produced, in the form of adenosine triphosphate (ATP), through oxidative phosphorylation system. The latter multipart is an assembly of several protein complexes that use the energy linked to the flow of electrons under cover of $NADH_2$ to support the extrusion of H^+ ions out of the mitochondrial matrix into the space between the two mitochondrial membranes, thereby creating a proton gradient/membrane potential $\Delta\Psi$. The force of the proton gradient is then used by the ATP synthase complex to generate ATP from ADP and phosphate (Figure 1-4).

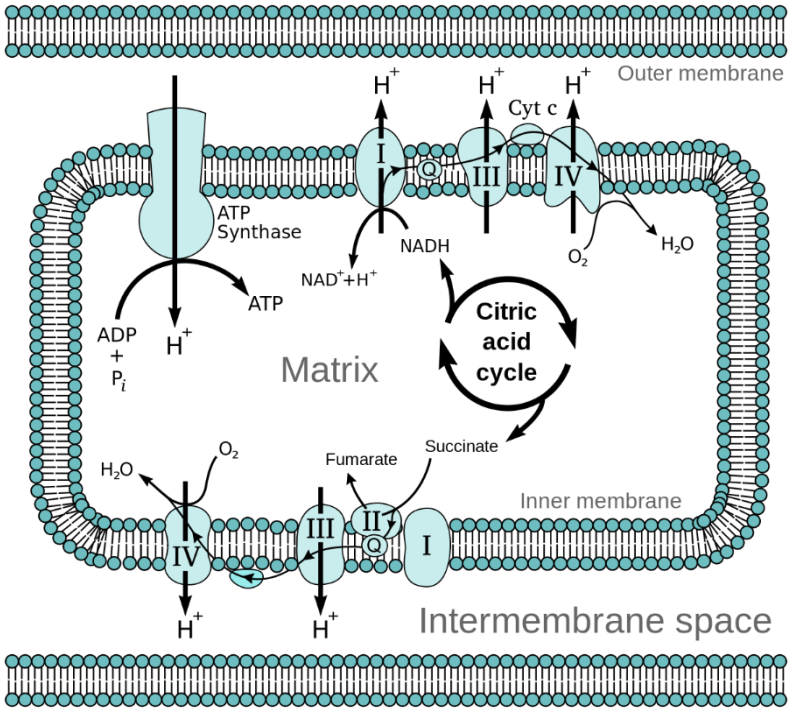


Figure 1-4. Mitochondrial electron transport chain

1-3-2. Mitochondrial life cycle.

Mitochondria are lively organelles that endure fusion and fission events (Figure 1-5). These paired-events are regulated by mitochondria-shaping proteins. Changes in the expression and/or localization of these proteins lead to an active mitochondrial impairment and may encourage apoptosis. Ample experimental and clinical data established that inhibition of either fusion or fission results in worsening of mitochondrial bioenergetics. While fusion may benefit mitochondrial function by allowing the spreading of metabolites, protein, and DNA throughout the network, the functional benefit of fission is not as instinctive. Remarkably, studies that tracked individual mitochondria through fusion and fission found that the two events are paired, and that fusion triggers fission. On average, each mitochondrion would go through ~ 5 fusion: fission cycles every hour. Measurement of $\Delta\psi_m$ during single fusion and fission events demonstrates that fission may yield uneven daughter mitochondria where the depolarised daughter is less likely to become involved in a subsequent fusion and is, therefore, more likely to be targeted by autophagy. Based on these observations, a mechanism by which the integration of mitochondrial fusion, fission, and autophagy form a quality preservation mechanism was proposed. According to this hypothesis, pairs of fusion and fission allow for the reorganization and sequestration of damaged mitochondrial components into daughter mitochondria that are segregated from the viable networking pool and then becoming eliminated by autophagy.

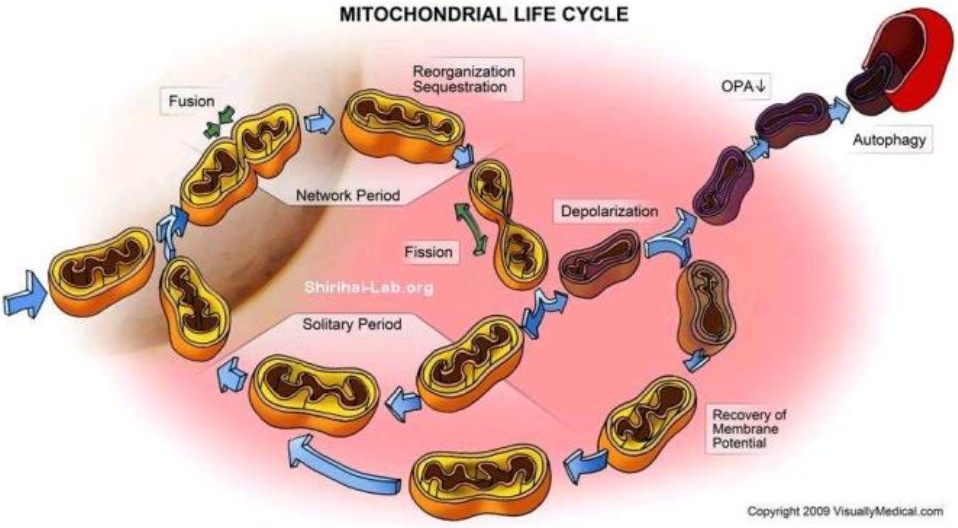


Figure 1-5. Mitochondrial life cycle

1-3-3. Metabolism of mitochondria and their regulatory contribution

In addition to producing ATP, mitochondria are responsible for the synthesis of countless vital metabolites and cofactors such as α-ketoacids, steroids, lipoic acid tetrahydrofolate, acetyl-CoA, iron-sulfur clusters, and ubiquinone. (McBride, Neuspiel et al. 2006). At the cellular regulatory level, mitochondria also play a central role in cellular signalling and differentiation, and apoptosis (Joza, Susin et al. 2001). From a physiological perspective, mitochondria and their metabolism have been implicated in ageing and several non-transmissible human diseases, including inherited mitochondrial disorders, cardiac dysfunction, heart failure, neurodegenerative diseases, metabolic inflexibility and diabetes. In the background of the above, there is, therefore, a great interest in understanding the complicated relationship that exists between the mitochondrial function/metabolism and other cellular processes.

1-4. The mitochondrial pyruvate dehydrogenase complex (PDC)

1-4-1. structure and function.

Pyruvate dehydrogenase complex (PDC) is positioned on the mitochondrial inner membrane and consists of three catalytic components, pyruvate dehydrogenase (E1), dihydrolipoamide transacetylase (E2), and dihydrolipoamide dehydrogenase (E3). Each PDC unit consists of a core of sixty E2 subunits, to which thirty E1 subunits are attached (at the E1-binding domain of E2). Six to twelve E3 subunits are tightly bound to the E2 core through the E3-binding protein (Figure 1-6).

Pyruvate is oxidised by PDC in mitochondria to generate acetyl-CoA according to the reaction: pyruvate + nicotinamide adenine dinucleotide (NAD^+) + coenzyme A (CoASH) → acetyl-CoA + nicotinamide adenine dinucleotide reduced ($NADH_2$) + CO_2. Through this reaction, the activity of PDC in skeletal muscle plays a vital role in the whole-body glucose homeostasis by enhancing glucose oxidation in response to mainly insulin stimulus and exercise, which are the primary physiological activators of PDC.

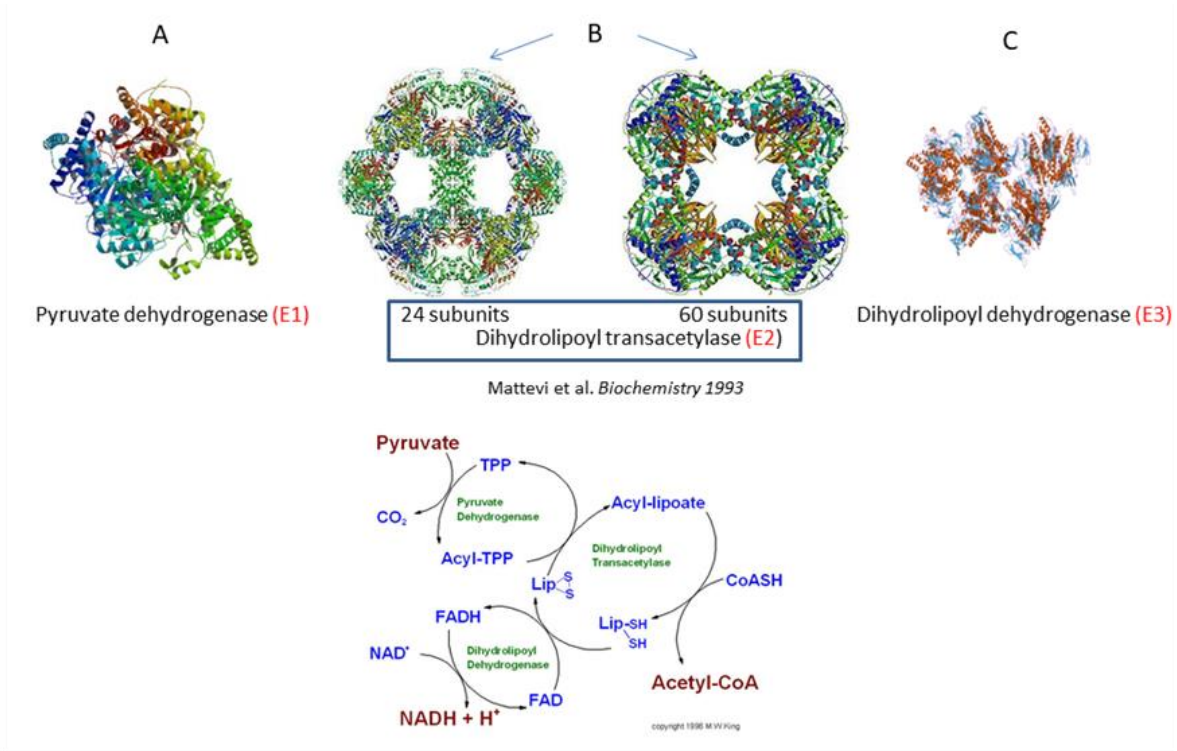


Figure 1-6. Pyruvate dehydrogenase structure

The activity of PDC is controlled by a pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PTP) tandem (Figure 1-7). The PDK family consists of four isoforms (PDK1-4), whereas PDP consists of two isoforms (PTP1-2). PDK2 and PDK4 mRNA are expressed in most tissues, including skeletal muscles, but the specific activity of PDK4 is eightfold greater than that of PDK2. PDK1 and PDK3 are more prevalent in heart, kidney and pancreatic islets. The activation of PDC is controlled by phosphorylation-dephosphorylation of three serine residues (Ser^{232}, Ser^{293} and Ser^{300}) by the PDKs and PTPs, respectively (Yeaman, Hutcheson *et al.* 1978). Also, high ratios of acetyl-CoA/CoA and NADH/NAD^+ have been shown to activate PDKs allosterically and inhibit PDC activity (Harris, Bowker-Kinley

et al. 2002). However, calcium release mediated by muscle contraction can activate PDC, despite the levels of intracellular acetyl-CoA and NADH being increased. This suggests that muscle contraction can override the inhibitory effects that acetyl-CoA and NADH accumulation can have on PDC activity in resting muscle. The PDC activation induced by muscle contraction appears to be attained through contraction mediated accumulation of intra-mitochondrial calcium and pyruvate, which inhibit PDK2 and PDK4 (Constantin-Teodosiu, Peirce *et al.* 2004). Nevertheless, after several days of the high-fat diet, pyruvate and calcium cannot increase muscle PDC activity during exercise to the same extent like that observed after a normal diet, showing that calcium/pyruvate cannot override the effect of excess fatty acid on muscle PDC activation (Putman, Spriet *et al.* 1993).

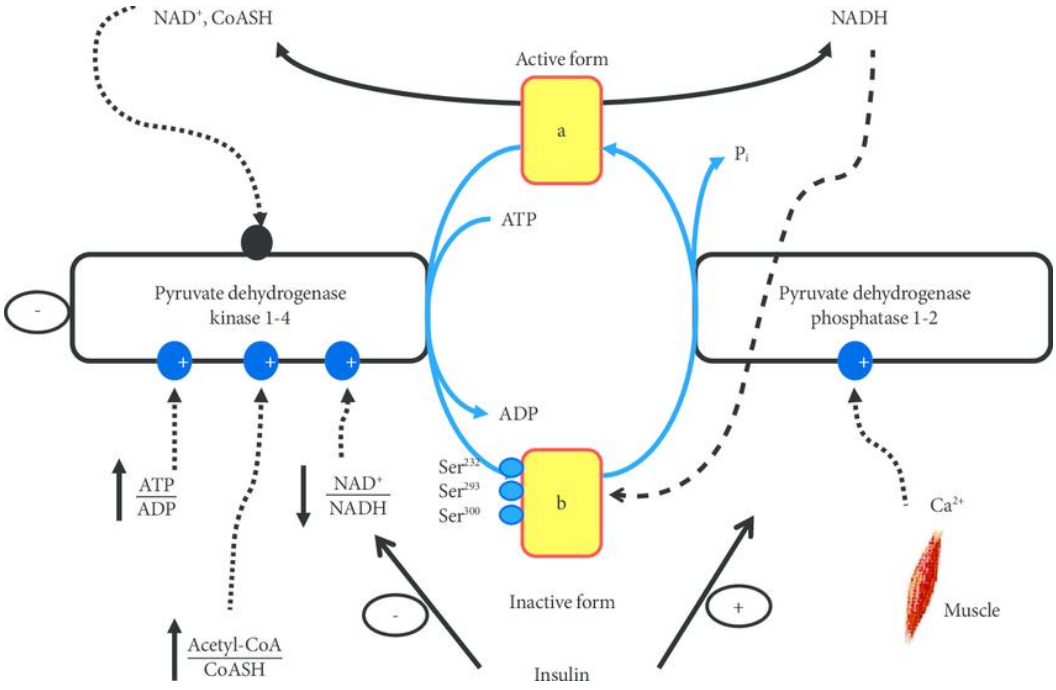


Figure 1-7. A schematic illustration of the mechanisms regulating the pyruvate dehydrogenase complex (PDC) by PDK, PDP, and physiological factors.

1-4-2. PDK4 structure and regulation

PDK4 protein covers 294 amino acids in its sequence. To form the active protein, two of the polypeptide chains come together to form an open conformation (Fig.1-8). Specifically, the two subunits come together to form a nucleotide-binding pocket, which is targeted most often by inhibitors. PDK4 is located in the matrix of the mitochondria and inhibits PDC by phosphorylating one of its subunits (E1 - at three serine residues) (Kukimoto-Niino, Tokmakov et al. 2011). This phosphorylation renders the complex inactivate. Rosiglitazone, which is a thiazolidinedione known to activate the glycerol biogenesis pathway, increases PDK4 gene expression in white adipose tissue, but not in liver or muscle tissue (Cadoudal, Distel et al. 2008). Farnesoid X receptor, or FXR, inhibits glycolysis and increases fatty acid oxidation by increasing PDK4 expression and inactivating the PDC (Savkur, Bramlett et al. 2005). Other modulators, such as insulin, can directly downregulate PDK4 gene expression. This is done through the phosphatidylinositol 3-kinase (PI3K)-dependent pathway. Even when cells are exposed to dexamethasone to increase mRNA expression, insulin blocks this effect (Kwon, Huang et al. 2004). Peroxisome proliferator-activated receptors also regulate PDK4 expression.

Thus, PPARα and δ were found to upregulate PDK4 mRNA, but PPAR gamma activation downregulated expression (Abbot, McCormack et al. 2005).

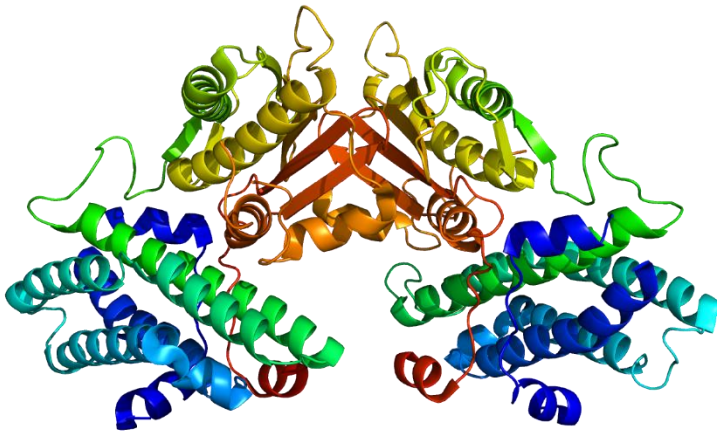


Figure 1-8. Crystallographic structure of PDK4 protein molecule

1-4-3. The heterogeneous nature of the genes coding for the most critical components involved in the regulation of mitochondrial pyruvate dehydrogenase complex

The genetic location of the most important constitutive and regulatory components of human PDC covers many sites of human karyotype as it is exemplified below:

PDHA1 ($E_1\alpha$) gene - pyruvate dehydrogenase E1 alpha 1 subunit is located on chromosome **X**

PDHB ($E_1\beta$) gene - pyruvate dehydrogenase E1 beta subunit is located on chromosome **3**.

DLD (E2) gene - dihydrolipoamide dehydrogenase is located on chromosome **7**.

DLAT (E3) gene - dihydrolipoamide S-acetyltransferase is located on chromosome **11.**

PDHX gene - provides instructions for making a protein called E3 binding protein that is located on chromosome **11**.

PDK4 gene - pyruvate dehydrogenase kinase isoform is located on chromosome **7.**

PDP1 gene - pyruvate dehydrogenase phosphatase 1 is located on chromosome **8**.

PDP2 gene - pyruvate dehydrogenase phosphatase 2 is located on chromosome **16**.

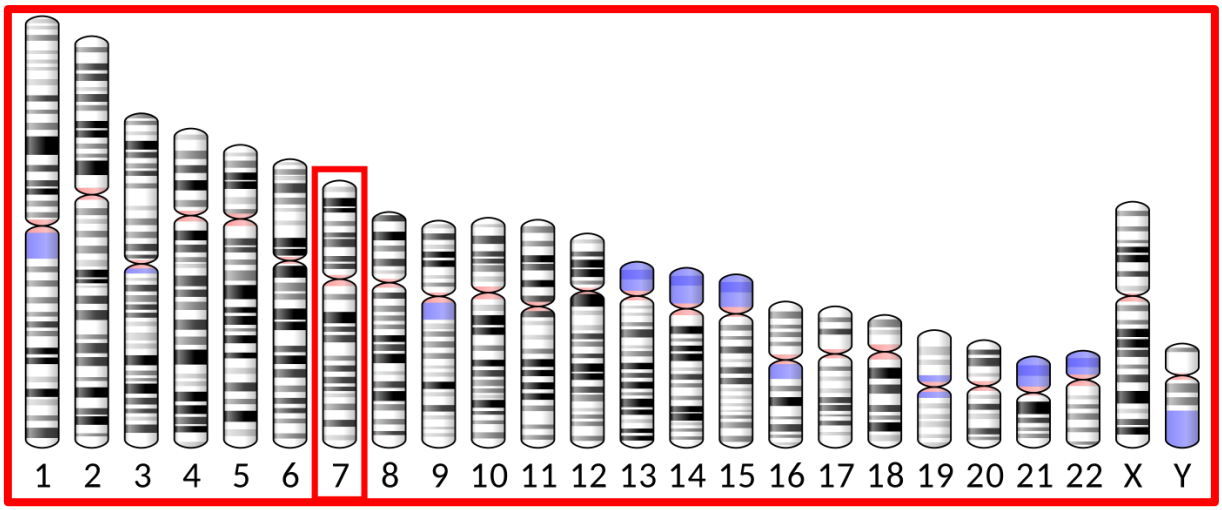


Figure 1-9. Graphical representation of an idealised human karyotype, showing the organization of the genome into chromosomes. Here, PDK4 is located on chromosome 7.

1-4-4. PDC flux and acetyl-CoA, acetylcarnitine and free carnitine,

Previous evidence suggests there is a strong relationship between the PDC flux and the intracellular levels of acetyl-CoA, acetylcarnitine, and free carnitine (Figure 1-10). When the rate of PDC mediated mitochondrial acetyl-CoA formation exceeds the rate of acetyl-CoA entry into the Krebs Cycle, the cellular acetyl-CoA and acetylcarnitine content will increase,

and a stochiometric decline in the cell-free carnitine content will occur. This acetyl group buffering role of carnitine may help to maintain a pool of free CoASH under conditions where the rate of acetyl-CoA condensation with oxaloacetate is less than its rate of formation from PDC mediated pyruvate decarboxylation. In addition to maintaining a viable pool of CoASH, the accumulation of acetylcarnitine itself provides a store of acetyl groups, which is readily available for transacetylation back to acetyl-CoA for utilisation by the Krebs cycle. A previous study showed that within a few minutes of high-intensity exercise, skeletal muscle free carnitine content was reduced to approximately 75% of total muscle carnitine pool (Foster and Harris 1987). Also, an increase in acetylcarnitine formation during high-intensity exercise, which occurs to a greater extent in type I muscle fibres, is directly related to an increase in muscle acetyl-CoA (Constantin-Teodosiu, Carlin *et al.* 1991, Constantin-Teodosiu, Howell *et al.* 1996), showing that the greatest capacity to buffer acetyl group accumulation during exercise lies within slow aerobic muscle types.

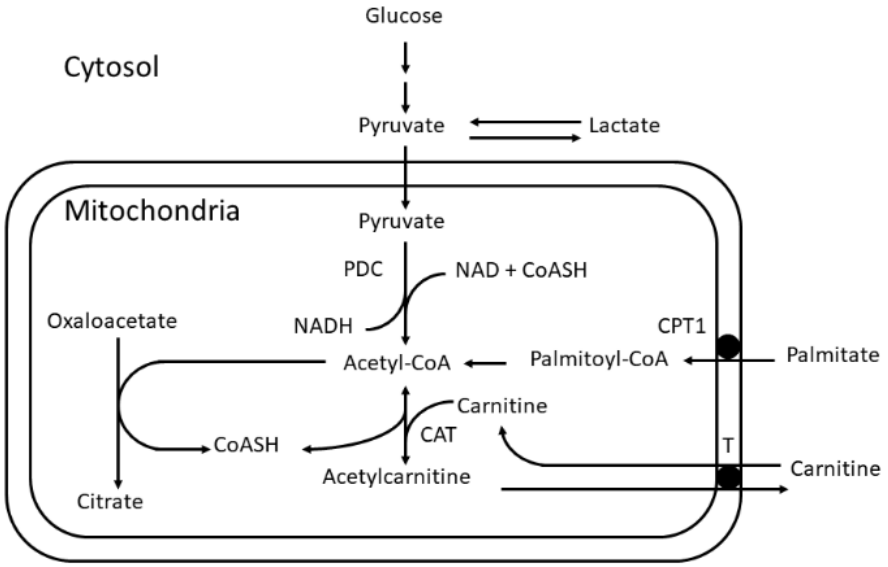


Figure 1-10. A schematic illustration of metabolic pathways involved in the acetylcarnitine formation and accumulation during muscle contraction.

1-4-5. The role of pyruvate dehydrogenase kinase 4 (PDK4) in the regulation of PDC and glucose homeostasis in skeletal muscle

The activity of PDC in muscle appears to decrease whenever muscle PDK4 expression is selectively upregulated. In starvation and diabetes, PDC activity is significantly declined in most tissues, while PDK4 expression is enhanced. High-fat diets can also up-regulate PDK4 protein expression levels (Rinnankoski-Tuikka, Silvennoinen *et al.* 2012).

Conversely, PDK4 deficient mice have lower glucose levels, improved glucose tolerance, and are protected from high-fat diet-induced skeletal muscle insulin resistance (Jeoung and Harris 2008, Thoudam, Ha *et al.* 2019). Also, PDK4 deficiency ameliorates hepatic steatosis in mice (Zhang, Zhao *et al.* 2018). These studies show that PDK4 might play an essential role in insulin-resistant states, like diabetes. Systemic inflammation-induced by continuous LPS (lipopolysaccharide) infusion also increases PDK4 mRNA expression, with the PDC activity decreasing by about 65% (Alamdari, Constantin-Teodosiu *et al.* 2008). These changes are associated with increased tumour necrosis factor-α and interleukin-6 mRNA expressions. Cytokines may be therefore considered as essential factors in the induction of insulin resistance/diabetes. It is therefore not unreasonable to suggest that PDK4 could make the

connection between these two entities: declined PDC activity and impaired glucose handling. There is also evidence to suggest that a pharmacological intervention using dichloroacetate (DCA) compound declines muscle PDK4 mRNA expression and increases CHO oxidation and reduces blood glucose and muscle lactate accumulation (Stacpoole, Moore *et al.* 1978). DCA, which is a potent PDK4 inhibitor, has also been shown to rescue high-fat diet-induced PDC activity inhibition (Constantin-Teodosiu, Constantin *et al.* 2012). DCA administration can also decrease lactate accumulation in mouse skeletal muscle during regular exercise suggestive of increased PDC activity (Hoshino, Tamura *et al.* 2015). Overall, these findings support the contention that PDK4 plays an essential role in the regulation of glucose metabolism in skeletal muscle via the regulation of PDC.

1-5. Methods to assess mitochondrial function

Why are measurements of mitochondrial function important? Any illness that has an energy problem might be related to mitochondria. A relationship between mitochondrial dysfunction and a wide range of disease states (aging, myopathies, obesity, diabetes, cardiovascular disease, and stroke, Alzheimer's Dementia, Parkinson's disease, Huntington Disease, mental retardation, deafness and blindness) is known to exist, but whether the mitochondrial dysfunction is responsible for some particular diseases is still in question.

1-5-1. Mitochondrial Metabolic Function Assessed *In Vivo* and *In Vitro*

1-5-1.1. *In vivo* non-invasive methods

Non-invasive *in vivo* assessments of mitochondrial function methods that mainly ground on magnetic resonance spectroscopy (MRS) and near-infrared spectroscopy (NIRS) techniques are becoming accessible to many investigators. Likewise, it is now possible to gather information on the regulation of mitochondrial content by measuring the *in vivo* synthesis rate of individual mitochondrial proteins. The cost for these types of assessments is, however, prohibitively too high. Furthermore, the main endpoint of these type of measurements is connected to the recording of the variation in the NMR peak of either ATP or PCr, which provides a limited scope for fuel type use identification.

1-5-1.2 *In vitro* invasive methods

Several techniques have been described for monitoring cell respiration in a multi-well format using oxygen-dependent fluorescence quenching to monitor the oxygen concentration in the medium. These techniques either operate at steady-state, where oxygen consumption is balanced by inward diffusion of oxygen or measure oxygen depletion in a closed chamber (Brand and Nicholls 2011). Although these can be used to monitor or compare basal cell respiration rates, their low time resolution and the limited ability to make additions during the

run preclude them from being used for sophisticated respiration assays. A recent novel added method is based on measurements of rates of mitochondrial ATP production.

In summary, the mitochondrial function could be directly assessed using:

1. Measurements of respiration

- direct oxygen consumption (Clark electrode, Seahorse)

- oxygen-dependent fluorescence quenching

2. Measurements of membrane potential (Fluorescent probes)

3. MTT/XTT assays (Tetrazolium dye reduction)

4. Measurements of rates of ATP production

The Seahorse XF24 and XF96 extracellular flux analysers refine the respiration technology by using a piston to enclose reversibly a small volume (7 µl in the XF-24) above the cells, monitoring oxygen uptake in that volume for 2–5 min, then raising the piston, allowing the bulk incubation medium (~1 ml) to re-equilibrate (Wu, Neilson et al. 2007). The ability to make several additions during the experiment allows respiratory cell control to be measured, with the possibility of further additions of a compound to screen its effects. The Seahorse instrument can be used to monitor the respiration of cells and organelles that can be attached to the multiwell plate, either spontaneously in the case of cultured cells (Wu, Neilson et al. 2007) or by centrifugation of isolated mitochondria.

There are also a couple of indirect methods to assess mitochondrial function, and these include assaying individual enzymes activity of the ETC (Wibom, Hagenfeldt et al. 2002) or total ATP content (Harris, Hultman et al. 1974), or determining the content of mitochondrial proteins using Western blotting (Picard 2011).

1-5-2. High-resolution respirometry versus bio-luminometric measurements of the ATP formation rates

It appears that related to assessing mitochondrial function two contenders meet most of the technical and scientific challenges: high-resolution respirometry and bio-luminometric measurements of the ATP formation rates. However, the latter method is superior to the former as using bioluminescence over oxygen consumption in cells, isolated mitochondria, or skinned single fibers because:

- It measures solely the rate of ATP production rather than the rate of oxygen consumption.

In normally functioning mitochondria, the transport of electrons to oxygen is coupled to the formation of ATP, which means that the one process does not occur without the other. Electron transport results in the retaining of about half of the released energy as new ATP molecules. The rest of the energy is released as heat. If electron transport is uncoupled from ATP synthesis, then electron transport can occur without ATP synthesis - and all the released energy will be

heat. Yet, the oxygen is still consumed. Since in few scenarios the mitochondrial uncoupling has been linked to some pathological states i.e. diabetes aging, chronic muscle weakness, it would be important to isolate normality from mitochondrial dysfunction using the maximal rate of mitochondrial ATP production (MAPR) for a given condition. Details of how the uncoupling affects the mitochondrial function will be provided later.

MAPR is a sensitive method that requires low amounts of tissue or cell number, with a high degree of reproducibility (its coefficient of variation (CV) between duplicate samples is far more reliable (CV = 2.2 - 3.9 %, our laboratory data) in comparison to the 10-20 % in Oroboros O2K respirometry (reported in the manufacturer's factsheets). MAPR can easily be corrected for markers of mitochondrial protein content with high consistency (protein CV = 1.6 %).

There is also a significant cost association of high-resolution respirometry (e.g. Oroboros O2K and Seahorse XF analyzers). In contrast, the highly reproducible MAPR assay is relatively inexpensive given that many laboratories typically own a luminometer. Taking these factors into account, MAPR, may, therefore, appear a superior method for assessing mitochondrial function.

1-5-3. Mitochondrial electron and proton uncoupling

Mitochondria can present proton and electron leaks, which can have a major impact on mitochondrial coupling efficiency (lower percentage of the proton gradient is used to generate

ATP) and production of reactive oxygen species. The basal leak is unregulated, and a major loss can be attributed to mitochondrial anion transporters. In contrast, the proton leak through the lipid bilayer appears to be minor (Jastroch, Divakaruni et al. 2010). The basal proton leak is cell-type specific and correlates with the metabolic rate. The inducible leak through the ANT (adenine nucleotide translocase) and UCP1-3 (uncoupling proteins) can be activated by fatty acids, superoxide, or lipid peroxidation products. The physiological role of inducible leak through UCP1 in mammalian brown adipose tissue is heat production.

The exit of electrons prior to the reduction of oxygen to water at cytochrome *c* oxidase causes superoxide production. As the mechanisms of electron leak are central to understanding their physiological relevance, the summarised mechanisms and topology of electron leak from complexes I and III in studies using isolated mitochondria are displayed in Figure 1-11. It is also important to emphasise that proton and electron leak as therapeutic goals have become targets of study in body mass regulation and insulin secretion (Jastroch, Divakaruni et al. 2010).

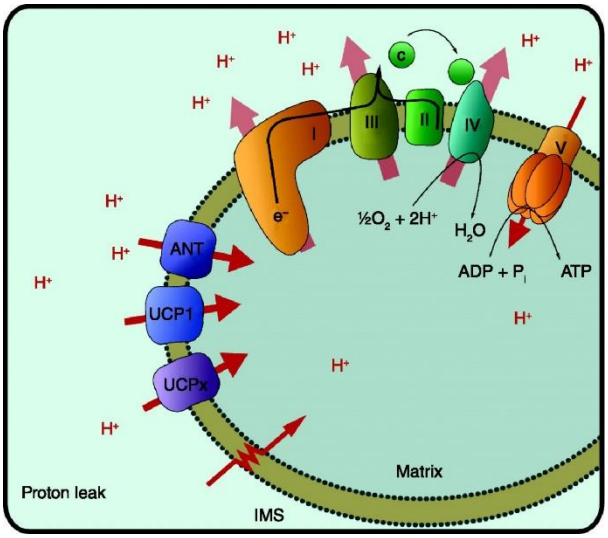


Figure 1-11. Mitochondrial proton and electron leaks (Zhang and Avalos 2017)

1-5-4. Modulating the insulin response through mild mitochondrial uncoupling

Glucose-stimulated insulin secretion (GSIS) by pancreatic β cells is vital for preserving euglycemia. Glucose stimulates the respiration of β-cell mitochondria, generating a high ATP/ADP ratio, which triggers the release of insulin-containing vesicles. Upon observation that UCP2 silencing increases GSIS, UCP2 was described as a negative regulator of insulin secretion, apparently by lowering the ATP/ADP ratio (Zhang, Baffy et al. 2001). Although uncoupling mediated by UCP2 can explain the modulation of GSIS, this view of UCP2 activity in regulating glucose homeostasis was complicated by the role the protein may play in ROS attenuation. A recent investigation of UCP2 gene silencing in mice revealed chronic oxidative stress, as determined by a low proportion of reduced glutathione, and increased expression of other antioxidants (Pi, Bai et al. 2009). To conclude, in order to clarify the role of UCP2 in β cells, the determination of its function is required.

1-6. Mechanism of PDK4 activation by PPARs/FOXO1

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors regulating the expression of several genes. Thus, PPARs have been shown to control metabolism, cellular differentiation, tumorigenesis, to name a few. PPARs, which are expressed in many tissues such as adipose tissue, muscle, liver, heart, and

kidney can be classified into three types: α, β/δ, and γ. Natural (endogenous) ligands for the PPARs include free fatty acids and eicosanoids. PPARγ can be activated by prostaglandin and PPARα can be activated by leukotriene. With this background, PPARs are targets for therapeutic type 2 diabetes. Previously, PPARα has been considered as a PDC regulator based on the observation that parallel rises in muscle PPARα and PDK4 gene expressions were reported during conditions of chronic high levels of circulating FFAs induced by starvation (Huang, Wu et al. 2002).

Conversely, other studies were not able to replicate a relationship between PPARα and PDK4 as Holness *et al.* (2002) showed that expression of PDK4 gene occurs independently from PPARα signalling in oxidative skeletal muscle (Holness, Bulmer *et al.* 2002). Also, it has been demonstrated that the enhancement of PDK4 mRNA expression is much faster than PPARα mRNA expression after administration of PPARα receptor agonist (Motojima 2002). Therefore, whether PPARα is involved in the regulation of PDK4 gene is still debatable. On the other hand, *in vivo* and hind-limb rodent models have shown that PPARδ agonism (GW610742) increases muscle PDK4 protein expression (Constantin, Constantin-Teodosiu *et al.* 2007), which subsequently attenuated the exercise-induced activation of PDC and mitochondrial ATP production (Constantin-Teodosiu, Baker *et al.* 2009). Keeping in line with the latter findings, expression of PDK4 mRNA was markedly reduced in a PPARδ knock-out mice model (Nahle, Hsieh *et al.* 2008). However, a mechanistic evidence at the protein level

regarding the relative contribution (if any) of PPARδ to the reported increase in muscle PDK4 expression and associated changes in CHO metabolism is still missing.

Clear evidence shows that FOXO1 can up-regulates PDK4 expression since FOXO1 can directly bind the promoter region of the PDK4 gene. It is reasonable to suggest that FOXO1 could also play an essential role in promoting the up-regulation of PDK4 mRNA in response to increased FFA availability. Indeed, the FOXO family of transcription factors (including FOXO1) have been implicated in the induction of muscle insulin resistance in vivo following the upstream dysregulation of insulin-mediated stimulation of phosphoinositol 3-kinase and Akt1 (Kim, Lee et al. 2006, Wang, Hu et al. 2006). This is a pathway known to be sensitive to circulating insulin and FFA availability in humans (Alamdari, Constantin-Teodosiu et al. 2008). Since FOXO1 can sense changes in the availability of FFAs or insulin and relay the message downstream by modulating transcription of many skeletal muscle genes, including PDK4 (Furuyama, Kitayama et al. 2003, Kim, Lee et al. 2006), it could in that way inhibit the PDC controlled CHO oxidation (Figure 1-12).

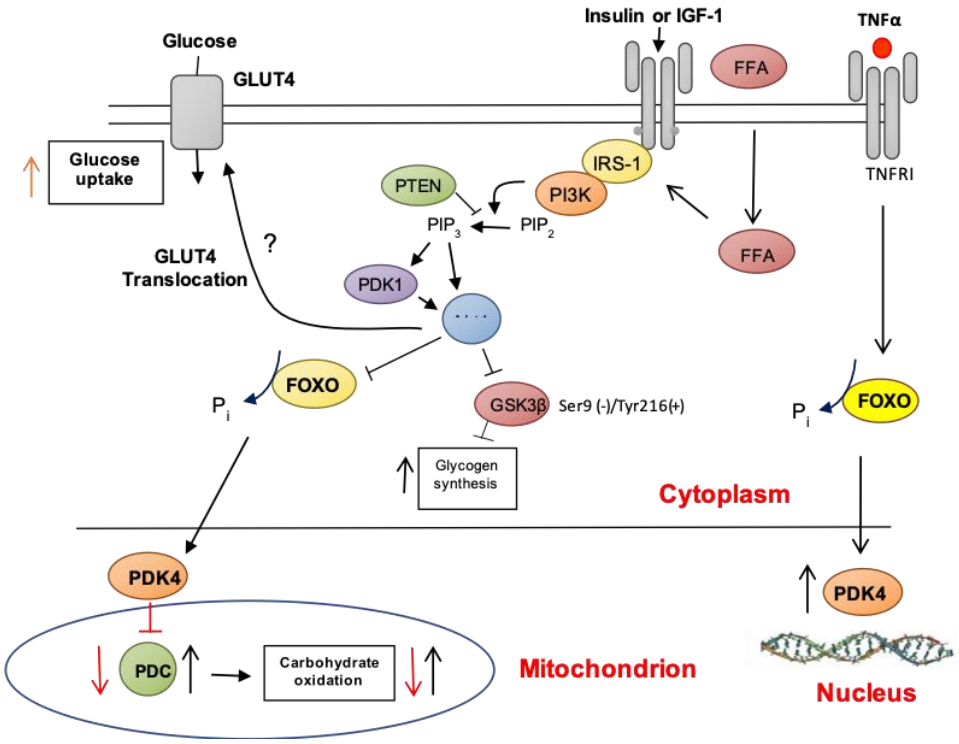


Figure 1-12. Mechanism of forkhead class O (FOXO) 1 mediated pyruvate dehydrogenase kinase isoform (PDK) 4 up-regulation and thereby pyruvate dehydrogenase complex (PDC)-mediated inhibition of carbohydrate oxidation. GLUT4, glucose transporter isoform 4; IGF, insulin-like growth factor; FFA, free fatty acid; TNF, tumour necrosis factor; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositol 3-kinase; GSK, glycogen synthase; IRS-1, insulin receptor substrate 1.

1-7. Physiological and pharmacological interventions aimed at increasing activity of PDC.

Following a meal, approximately two-thirds of the ingested glucose by lean, healthy normal glucose tolerant individuals is taken up by the peripheral tissues, primarily skeletal muscle, via an insulin-dependent mechanism. Since the activity of PDC is the primary biochemical drive for the glucose oxidation, this assigns to the activity of muscle PDC a significant role in the overall whole-body glucose oxidative disposal.

When insulin fails to activate muscle PDC, several interventions may contribute to an acute/chronic activation of muscle PDC (Fig. 1-13). They could go classified into physiological (e.g. modulation of muscle mass, exercise) and pharmacological interventions. This thesis will focus on the most important physiological activator (exercise) and on two pharmacological interventions (dichloroacetate and R-alpha-lipoic acid), which have shown usefulness at activating PDC during conditions presented with chronically elevated circulating FFA levels.

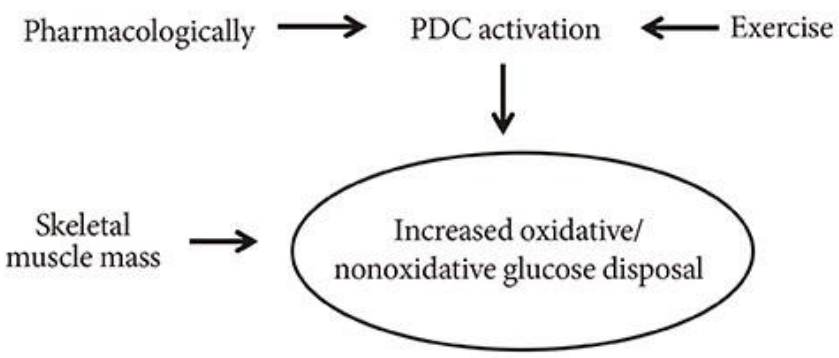


Figure 1-13. Interventions aimed at improving muscle glucose disposal through activating pyruvate dehydrogenase complex.

1-7-1. Dichloroacetate (DCA)

Dichloroacetate (DCA) is a pyruvate structural analogue that has been shown to inhibit all of the PDK isoforms, and it has the greatest effect on PDK2. In a previous study, DCA increased CHO oxidation and reduces blood glucose and lactate accumulation, in parallel with a decline in the PDK4 mRNA expression (Stacpoole, Moore *et al.* 1978). Also, DCA has been shown to rescue the high-fat diet-induced PDC activity inhibition in humans (Constantin-Teodosiu, Constantin *et al.* 2012). The latter study also showed that DCA administration can rescue the negative effects of 3-day of high-fat diet-mediated muscle PDC activation reduction both at rest and during subsequent exercise. However, so far no study investigating whether DCA could improve the palmitate-induced CHO oxidation impairment in a metabolic inflexibility condition has been made available.

1-7-2. R-α-lipoic acid

Alpha-lipoic acid (ALA) is an organosulfur compound derived from octanoic acid and RALA is the natural form. Several clinical trials have found that ALA may increase insulin sensitivity and help reduce blood sugar and lipids, mainly through improving markers of oxidative stress and inflammation. A study reported by Korotchkina LG *et al.* demonstrated that ALA also decreases the PDK4 activity, thereby enhancing the PDC activity (Korotchkina, Sidhu *et al.* 2004). However, a study showed ALA increased PDC activity, which is controversial to Korotchkina LG's article (Hong, Jacobia et al. 1999). Therefore, the role of RALA in the

regulation of PDC and PDK4 activity in palmitate-induced metabolic inflexibility is still unclear.

1-7-3. Exercise

The muscle fuel use increases several folds with voluntary muscle contraction, aka exercise, above resting muscle. During exercise, fat and carbohydrate are the primary substrates that fuel aerobic ATP synthesis in human skeletal muscle. The relative utilisation of fat and carbohydrate during exercise can vary significantly and depends broadly on exercise intensity (van Loon, Greenhaff et al. 2001). Electrical pulse stimulation (EPS) is an effective model to standardise the contraction and to avoid the central fatigue in humans. It can be also applied and sustained for lengthy times in *in vitro* models (Nikolic, Bakke *et al.* 2012). Muscle contraction activates PDC activity by increasing the amount of PDC in a dephosphorylated form (PDCa). It is worth noting that isometric muscle contraction induced by electric stimulation leads to full PDC transformation to PDCa with NADH/NAD^+ and acetyl-CoA/CoASH ratios markedly increased (Constantin-Teodosiu, Cederblad et al. 1993, Constantin-Teodosiu, Baker et al. 2009). Also, previous studies showed that electric stimulation affects the expression of muscle PDK4 mRNA. However, while PDK4 gene expression increases in the human muscle cell and tissue during acute stimulation, PDK4 mRNA was unchanged after >1 year of chronic stimulation (Nikolic, Bakke *et al.* 2012, Petrie, Suneja *et al.* 2014).

Despite all this evidence, no one has so far investigated whether EPS can rescue the CHO oxidation in a metabolic inflexible C2C12 myotube model induced by palmitate supplementation.

1-8. Hypothesis & Aims

1. As the underlying mechanism regulating the CHO oxidation during conditions of palmitate-induced metabolic inflexibility state is not yet fully understood, we hypothesised that FOXO1 and/or PPARs would be involved in the regulation of pyruvate dehydrogenase, the gatekeeper of cellular carbohydrate oxidation in skeletal muscle, via controlling the regulation of PDK4 expression. This thesis would provide extensive evidence of the extent of FOXO1 and PPARs PDK4 gene and protein expressions, PDC activity, intermediary metabolism, and rates of mitochondrial ATP production in the presence of glucose-derived pyruvate responses to conditions of palmitate-induced metabolic inflexibility in a C2C12 skeletal muscle myotube model.
2. The thesis will also aim to rank the importance of PPARs and FOXO1 transcription factors to the regulation of PDK4, and thereby to that of CHO oxidation. This would be achieved by investigating the consequences of selective siRNA FOXO1, PPARα, and PPARδ gene knock-down (silencing) on the same endpoint measurements described at aim 1 under conditions of palmitate-induced metabolic inflexibility.

3. Once the ranking of these transcription factors was established, the extent to which administration of DCA, RALA, and cell contraction using electrical pulse stimulation could protect muscle PDC activity and carbohydrate oxidation under conditions of palmitate-induced metabolic inflexibility would also be investigated.

Chapter 2
Materials and general methods

2-1. Materials.

All cell culture media (Dulbecco's modified Eagle's medium, phosphate-buffered saline, and penicillin-streptomycin), sodium palmitate, sodium pyruvate, 2-deoxy-D-glucose, dichloroacetate (DCA), and (R)-lipoic acid (RALA) were purchased from Sigma Aldrich (St. Louis, MO). Foetal bovine serum (3), horse serum, cell extraction buffer and cocktail inhibitor were obtained from Life Technologies (UK). Tritiated glucose, D-[3-^{3}H] was obtained from Perkin Elmer (Boston, MA). Non-specific, PPARα, PPARδ, and FOXO1 small interfering Ribonucleic Acid (siRNA) were purchased from Fisher Scientific, Thermo Fisher Scientific, Inc. (Loughborough UK). Lipofectamine 2000 reagent was obtained from Invitrogen, Thermo Fisher Scientific, Inc. (UK). Mitochondrial ATP monitoring reagent was purchased from Biothema (Handen, Sweden). Protein assay reagents were purchased from BioRad (Copenhagen, Denmark). Anti-PDK4 (ab214938), anti-PPAR alpha (ab24509), Anti-PPAR delta (ab23673), Goat anti-Mouse (IRDye® 800CW) (ab216772), Goat Anti-Rabbit (IRDye® 680RD) (ab216777) and anti-alpha actin (ab28052) antibodies were purchased from Abcam (Cambridge, UK). Anti-Phospho-FoxO1 (Ser256) (#9461) and Anti-FoxO1 (#2880) antibodies were purchased from Cell Signaling Technology. Levels of cytosolic and nuclear fractions of p- and t-total FOXO1 were identified using the same antibodies that were used for total cellular lysate measurements, which were also applied to isolated cellular fractions using a nuclear protein extraction kit (Active Motif Europe, La Hulpe, Belgium).

2-2. Cell culture.

C2C12 skeletal muscle cells were purchased from ATCC, cultured in collagen-coated 6-well plates, maintained in a growth media containing High Glucose Dulbecco's Modified Eagle Media (DMEM), 10% Foetal Bovine Serum (FBS) and a 1% Pen/Strep. Once 80-100% confluent, the cells were differentiated using a differentiating media containing high glucose DMEM, 2% Donor Horse Serum and 1% Pen/Strep. Cells were ready for stimulation after C2C12 myotubes were fully differentiated into contractile myotubes (4 to 5 days).

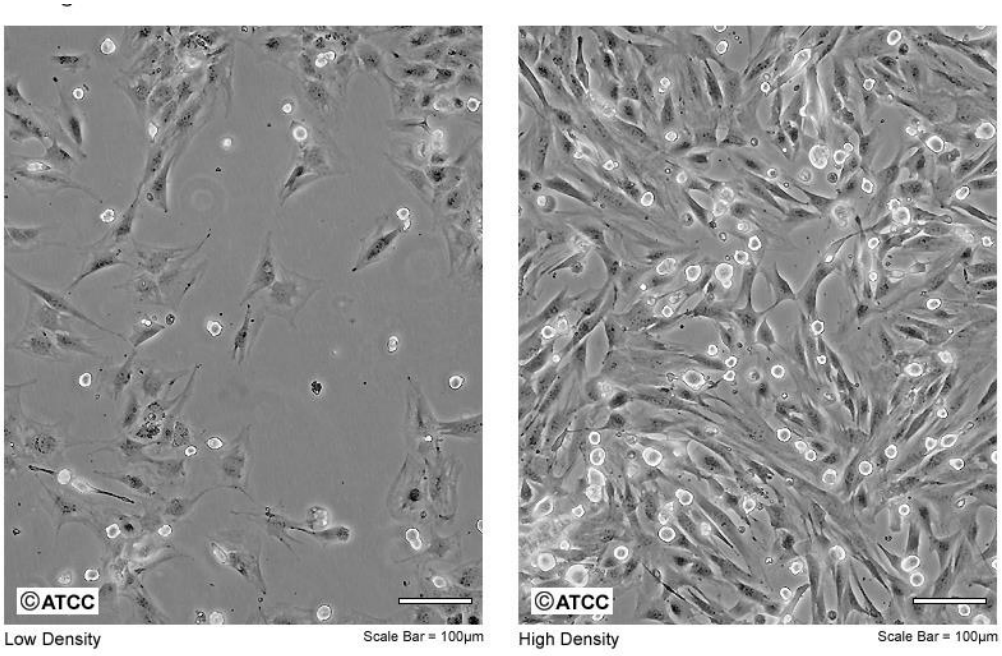


Figure 2-1. Low and high densityC2C12 skeletal muscle cells

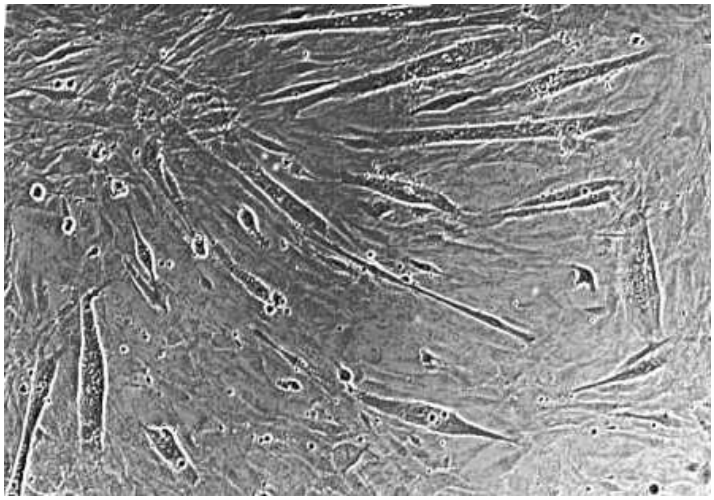


Figure 2-2. Horse serum differentiated C2C12 skeletal muscle cells

2-3. Stock palmitate preparation

The utilisation of palmitate in cell-based assays is challenging due to its low solubility in aqueous solutions. So, we use bovine serum albumin (BSA) as a carrier to promote palmitate dissolve as palmitate conjugated to BSA, by creating an aqueous-soluble compound that can be then absorbed and utilised by cells. Briefly, I weighed out the quantity of BSA required for sufficient volume of 2 mM solution. The formula weight of BSA is 66,000. Then, I added BSA corresponding to ~80% of the target volume. NB: the quantity of BSA needed to make a 2 mM solution makes a significant contribution to the final volume. I kept the BSA solution at 4^0C fridge overnight to make BSA dissolve. Once in solution, I sterilised the BSA solution in the culture hood and stored it at 4°C, away from light. Next, I prepared a 20 mM sodium palmitate by mixing a suitable volume of 0.01 NaOH and powder of sodium palmitate. Then, I placed

the mixtures in a 70-80°C water bath to make Na-palmitate in NaOH dissolve. Lastly, I processed the coupling of Na-palmitate with BSA as follows: I set up two water baths, one at 70°C (for the Na-palmitate) and the other at 50°C (for the BSA and coupling). Then, I dissolved the 20 mM stock of Na-palmitate in the 70°C water bath and quickly moved 250 μl of Na-palmitate into 750 μl 2 mM BSA, which was incubated in the water bath at 50°C. I pipetted up and down gently, several times, capped the tube, followed by a gentle vortex for a few seconds. I incubated the mix for at least 1 hr to make palmitate and BSA conjugate. During incubation, I gently vortexed the mix every 10-15 min. Finally, the palmitate-BSA conjugate solution was aliquoted and stored -30ºC for further use.

2-4. MTT assay

Cell viability was determined by MTT assay. Briefly, various concentrations of palmitate were used on day four after cell differentiation. Twenty-four hrs later, the medium was discarded from cell culture. An MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which is a tetrazolium salt commonly used to detect reductive metabolism in cells for viability, proliferation and cytotoxicity assays, and serum-free medium were added to each well and incubated for 3 hrs at 37°C. After incubation, MTT solvent such as ethanol was added into each well to dissolve the solubilised formazan. The plate was shaken for 15 min and absorbance at OD at 590 nm recorded.

2-5. Glucose uptake assay

Glucose uptake was determined, as previously reported (Chien, Chan et al. 2018). Briefly, C2C12 myotubes attached to the plate were incubated in 1 ml of media buffer supplemented with 138 mM NaCl, 1.85 mM CaCl2 1.3 mM MgSO4, 4.8 mM KCl, 50 mM HEPES pH 7.4 and 0.2% (W/V) BSA for 2 hrs. Then 250 ml of 2-deoxy-H^3-glucose (1 µCi/ml) were added to the well, and the cells were incubated for 15 min. After incubation, the media was removed, and the well was washed three times with cold PBS buffer. After the removal of the last wash content, 500 ml of NaOH 50 mM and SDS 0.1% were added to solubilise the cells. An aliquot of the solubilized cell mixture was removed and pipetted into a vial containing 5 ml of scintillation liquid, and the radioactivity was measured with a scintillation counter".

2-6. Media lactate level measurement

At the end of experiments, cell media supernatants were individually collected from the wells and transferred to corresponding 1.5 ml tubes. From each sample, 100 µl of the medium was removed and incubated in a reagent buffer (2-Amino-2-methyl-1-propanol buffer 1 M, hydrazine 1 M and NAD^+ 100 mM). After a thorough mix and incubation at RT for 5 min, the initial fluorescence of the sample (F1) read in a Greiner 96 well plate at Ex-340 nm/Em-460 nm was recorded. Five µl of LDH beef heart 550 U/ml were added to each well. After 30 min of storing the plate in the dark, a second read F2 was recorded.

2-7. Pyruvate dehydrogenase complex activity

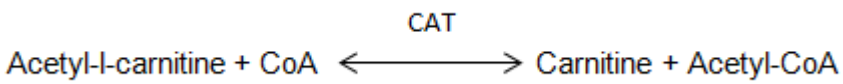
Pyruvate dehydrogenase complex (PDC) activity was measured by the method previously described (Constantin-Teodosiu, Cederblad *et al.* 1991). The assay is based on the measurement of the PDC active form (PDCa) at the time of extraction from the muscle cell. Muscle cells were grown and homogenized in a tube on ice for 1 min in the cold sucrose extraction buffer (pH 7.8) containing sodium fluoride (NaF) and DCA. The NaF and DCA were used to inhibit the pyruvate dehydrogenase phosphatases (PDPs) and pyruvate dehydrogenase kinases (PDKs), respectively, ensuring that there was no interconversion between its active and inactive form during extraction protocol. Thirty µl of cell homogenate was added into pre-warmed incubation buffer (pH 7.8) containing NAD^+ and CoASH in duplicate, and the formation of acetyl-CoA was then triggered by the addition of 30 µl of 26 mM pyruvate solution.

Aliquots of the reaction mixture were collected at 1, 2 and 3 min of the incubation, by removing sequentially 210 µl of incubation mix to tubes containing 40 µl of 0.5 M PCA. The amount of acetyl-CoA in each aliquot was determined using a ^{14}C based radioenzymatic method (Constantin-Teodosiu, Cederblad *et al.* 1991). Briefly, 100 µl of the sample containing acetyl-CoA was incubated with 30 µl N-ethylmaleimid, which reacts with CoASH to remove it, and freshly prepared [^{14}C] oxaloacetate and then citrate synthase were added. After 20 min, a

transamination mix (glutamate + glutamate transaminase) was added to remove excess [14 C] oxaloacetate, by converting it back to [^{14}C] aspartate. One ml of an H^+ ion exchange resin suspension was pipetted to each tube to bind the [^{14}C] citrate (negatively charged) from the [^{14}C] aspartate (positively charged). Scintillation fluid was added, and the number of disintegrations per minute (DPM) was quantified. Initial rates of acetyl-CoA production were calculated using the slope of a standard curve and expressed as DPM per pmol of acetyl-CoA/min, and finally were calculated as the rate of mmol acetyl-CoA/min/mg protein based upon protein concentrations.

2-8. Muscle cell acetylcarnitine content

Skeletal muscle C2C12 cells acetylcarnitine content was determined by the radioisotopic method described previously (Cederblad, Carlin *et al.* 1990). Muscle cell extracts and acetyl-L-carnitine standards (0-125 pmol) were incubated for 30 min in a HEPES buffer. Then carnitine acetyltransferase (CAT) enzyme was added to catalyse the transfer of the acetyl group, as shown in the following reaction:



Then, samples were incubated in a 95°C water bath for 3 min to denature the CAT enzyme to stop the reaction. The acetyl-CoA formed mirrored the content of muscle cell acetylcarnitine content stoichiometrically. Subsequent steps were the same for the acetyl-CoA assay as

described for the pyruvate dehydrogenase activity assay. The acetylcarnitine content was then calculated from the regression line slope of the standard curve and expressed as DPM per pmol of acetylcarnitine/protein content.

2-9. Measurement of maximal rates of mitochondrial ATP production (MAPR) in C2C12 myotubes.

The measurement of maximal rates of ATP production (MAPR) in isolated mitochondria was based on the previously described MAPR method (Wibom, Hagenfeldt *et al.* 2002). It will be used as a measurement of mitochondrial function throughout this thesis. MAPR has previously been shown to be a sensitive index to test mitochondrial function. MAPR is determined luminometrically at 25°C. Briefly, C2C12 myotubes were incubated with trypsin for 5 min to detach the cells and then transferred to 1.5 ml tubes. After centrifuge, cell lysates were permeabilised in the buffer containing digitonin (0.33 mg/ml). Ten μl permeabilized cell suspension was added to each well of the FLUROstar OPTIMA luminometer plate reader (Fig. 2-3)

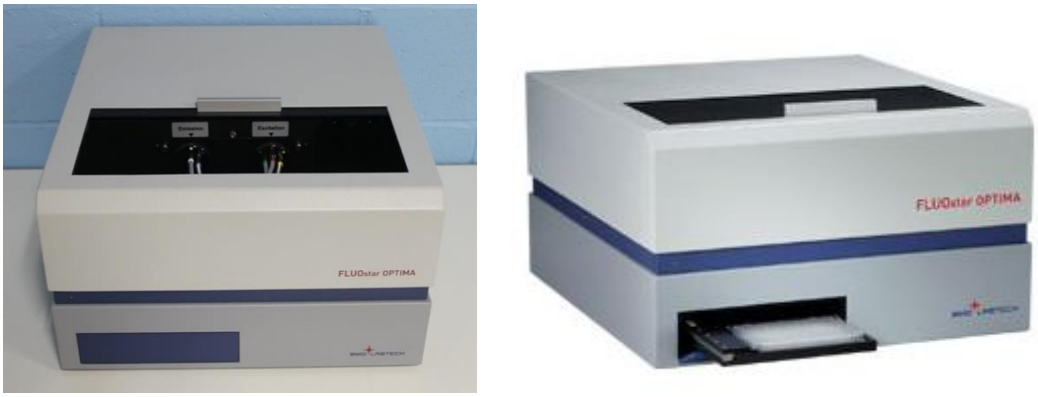


Figure 2-3. FLUOROstar OPTIMA plate reader

Each well contained 200 µl ATP monitoring reagent (firefly luciferase, 357 µM D-luciferin, 14.3 µM L-luciferin, 15 mM BSA, 1 µM sodium pyrophosphate decahydrate, 186 mM sucrose, 18.8 mM monopotassium phosphate, 2.5 mM magnesium acetate tetrahydrate, 677 µM EDTA (K salt) pH 7.0), 12.5 µl of 12 mM ADP and 35 µl of the following pyruvate (50 mM) and malate (22 mM) as a substrate. Luminescence was continuously recorded for 10 min (BMG LABTECH, Ortenberg, Germany) and an injection of 150 pM ATP standard took place at 4.6 min (Figure 2-4). The change in luminescence elicited by the ATP standard was used to calculate MAPR. Also, MAPR values were corrected for the mitochondrial protein content (Bradford).

Reaction:

$$\text{Luciferin} + \text{ATP} \rightarrow \text{Luciferyl Adenylate} + \text{PP}_\text{i}$$

$$\text{Luciferyl Adenylate} + \text{O}_2 \rightarrow \text{Oxyluciferin} + \text{AMP} + \text{Light}$$

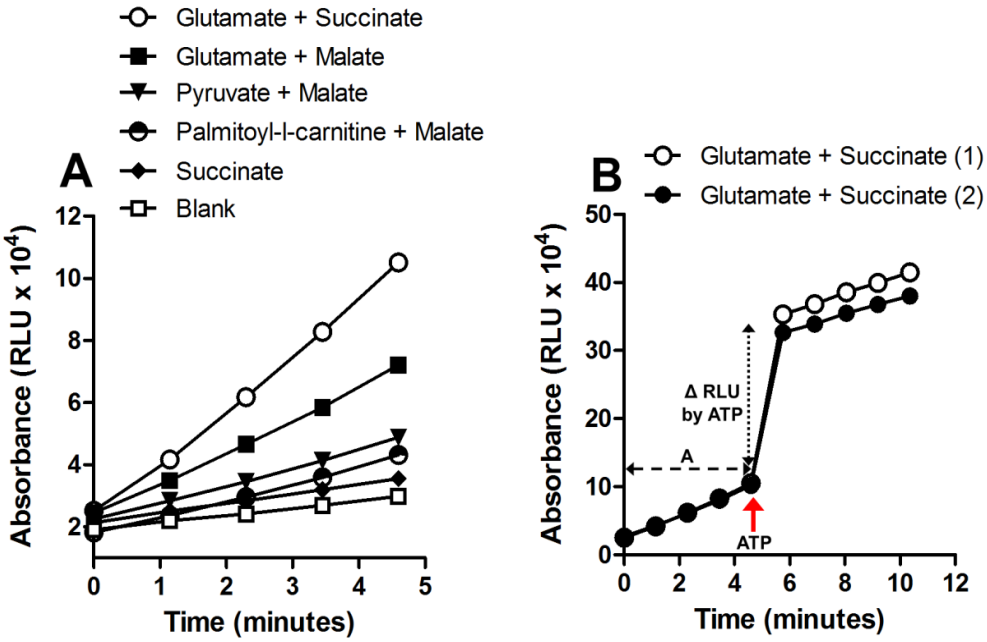


Figure 2-4: Kinetics of maximal mitochondrial ATP production. (A) Following the addition of mitochondria, the change in luminescence (RLU) is measured for 4.6 min. Glutamate + Succinate have the greatest kinetics followed by glutamate + malate, pyruvate + malate, palmitoyl L-carnitine + malate and then succinate. Note the reaction rate with succinate is just above the blank. (B) After ATP injection (red arrow), there is a rapid change in luminescence (Δ RLU by ATP). MAPR is calculated using the equation below.

Calculation:

$$\text{MAPR} = \frac{\Delta \text{ RLU by mitochondria and substrate}/\ \Delta \text{ RLU by ATP standard}}{\text{Time (start until ATP injection)}}$$

2-10. Making ATP standard

ATP standard was injected during the measurement of ATP production to act as an internal standard from which ATP production can be calculated. One to 2 mg of ATP was weighed and was dissolved in 10 ml ddH_2O. Having read a blank of 150 µl ddH_2O at 259 nm, the cuvette with 150 µl ATP solution was added, and the initial absorbance was measured. The solution was then diluted with ddH2O to obtain the desired ATP concentration (50 µM).

2-11. RNA extraction and Real-time PCR

The primers and the MGB (minor groove binding) probes used for the PCR analysis were designed by the manufacturer (Applied Biosystems) and purchased as shelf-inventoried assays. RNA was extracted from C2C12 cells using Tri-reagent (Applied Biosystems, UK) following standard protocols. Briefly, 1 ml of Tri reagent was added to each well containing 5-10 x 10^6 cells, or per 10 cm^2 culture dish area, after which all content was transferred to a 1.5 ml Eppendorf tube. Then 200 µl chloroform was added and the tube was shaken vigorously for 15 s. After centrifugation of the tube, the upper layer was carefully removed and transferred to a new test tube to which 500 µl of isopropanol was added per 1 mL of TRI Reagent solution, vortexed for 5-10 s, and incubated at room temp for another 5-10 min. After centrifugation at 12,000 g for 5 min, the total RNA was pelleted in the test tube. The isopropanol was carefully removed and 1 mL of 75% ethanol for each 1 mL of initially added TRI Reagent solution was

added to the RNA. The RNA was pelleted again, and the ethanol removed. The RNA pellet was briefly allowed to dry at room temperature and then solubilised in 30 µl of molecular grade water. The quantity and quality of the isolated mRNA were determined by measurements at 260 nm and the ratio 260/280 nm ratio, respectively. The ratio of high-quality RNA should be between 1.8-2.0.

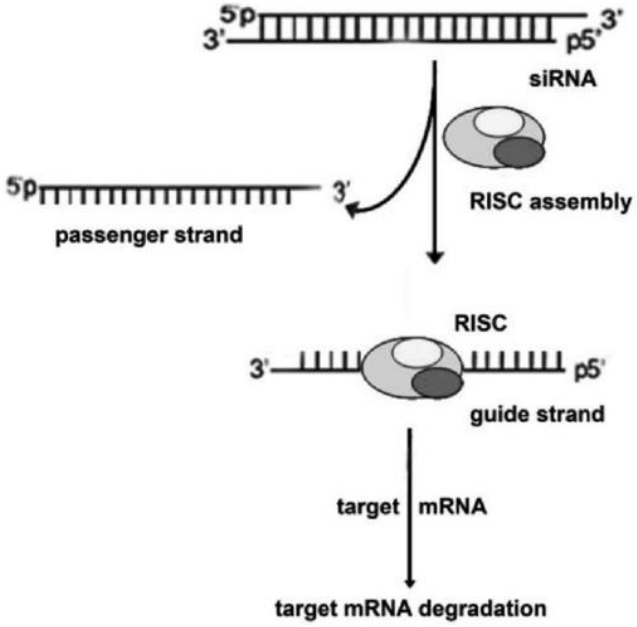
For total cDNA conversion, 1 µg of total RNA was reverse transcribed with buffer, reverse transcriptase (Superscript III), dNTPs and RNAase inhibitor combined in a final volume of 20 µl. Reactions were incubated for 60 min at 42^0C with a final termination step of 70^0C for 5 min. cDNA was stored at -20°C before use. Amplification of gene products was carried out using the TaqMan® Universal PCR Master Mix kit (Applied Biosystems, UK). Briefly, 5 µl of cDNA was combined with 25 µl supplied TaqMan Universal PCR Master Mix, 5 µl of forward and reverse primer each at 10 µM, 5 µl of the probe and 5 µl ddH2O. The reaction conditions are 95ºC for 10 min, 40 cycles of 95ºC for 15 s, 60ºC for 60 s and 72ºC for 60 s followed by a final 5 min at 72ºC.

2-12. Small interfering RNA (siRNA) transfection.

Small interfering RNA (siRNA) transfection assay was performed to downregulate specific genes of interest. Small interfering RNA (siRNA), known also as short interfering RNA or silencing RNA, is a class of double-stranded RNA non-coding RNA, 20-25 base pairs in length,

similar to miRNA, and operating within the RNA interference (RNAi) pathway. It interferes with the expression of specific genes with complementary nucleotide sequences by degrading mRNA after transcription, thereby preventing translation (Gualerzi and Pon 1990).

The mechanism by which siRNA causes gene silencing through repression of translation occurs as follows:



1. Once siRNA enters the cell, it gets incorporated into multiple protein complexes to form the RNA-Induced Silencing Complex (RISC)
2. Once the siRNA is part of the RISC complex, the siRNA is unwrapped to form single-stranded siRNA.
3. The strand that is energy like less stable due to its base pairing at the 5´end is chosen to remain part of the RISC-complex

4. The single-stranded siRNA which is part of the RISC complex can now scan and find a complementary mRNA
5. Once the single-stranded siRNA (part of the RISC complex) binds to its target mRNA, it induces mRNA cleavage.
6. The mRNA is now cut and recognised as abnormal by the cell. This causes degradation of the mRNA and in turn no translation of the mRNA into amino acids, and then proteins, can occur. Thus silencing the gene that encodes that mRNA.

siRNAs typically work by cleaving the mRNA before translation, and have 100% complementarity, thus very tight target specificity.

2-12-1. siRNA off-targeting

Off-targeting is a challenge to the use of siRNAs as a gene knockdown tool. Here, genes with incomplete complementarity are accidentally downregulated by the siRNA, leading to problems in data interpretation and potential toxicity. This, however, can be addressed by designing appropriate control experiments, and siRNA design algorithms are currently being developed to produce siRNAs free from off-targeting. Validated siRNA duplex oligonucleotides targeting constructs for the specific knockdown of the proteins of our interest (PPARα, PPRAδ and FOXO1) and the constructs and negative control siRNA along with all buffers of interest were presently purchased from Applied Biosystems (UK). Besides, negative

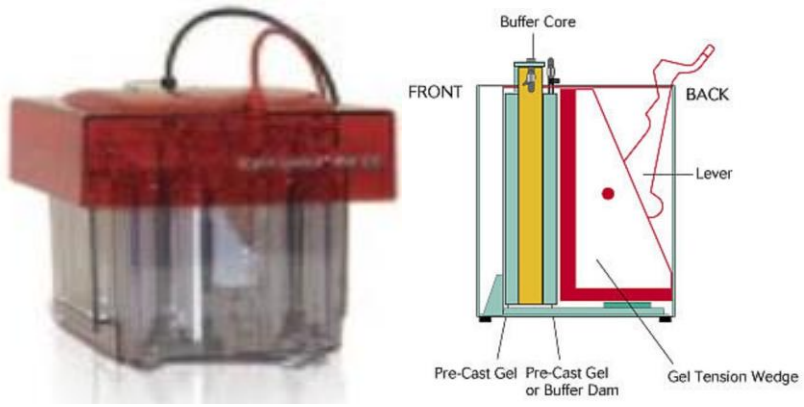
controls (scrambles - which involves taking the siRNA sequence and randomly rearranging its nucleotide sequence) were run for each experiment, which was presently reported.

2-12-2. Our experimental siRNA transfection protocol:

After cell differentiation, the C2C12 myotubes were treated 100 nM siRNA with 3 μl of Lipofectamine in an antibiotic-free medium in a well for 48 hrs before the experiment according to the manufacturer's instructions. The same concentrations of non-specific siRNA (scramble) with Lipofectamine were transfected in parallel plates as controls. Cells were incubated at 37°C in 5% CO 2 for 48 hrs, and then, the mRNA and protein expression were measured by using real-time PCR and Western blotting, respectively.

2-13. Western blotting.

Near-Infrared (NIR) Western Blot Detection was employed, which is more dynamic, covers a wider linear range and has a greater sensitivity than traditional chemiluminescent Western blotting. Briefly, C2C12 myotubes were lysed with a buffer containing a cocktail of protease inhibitors. The total protein concentration was determined using a protein assay (Bio-Rad). In total, 25 μg of protein from each lysate was denatured, dissolved and separated with sodium dodecyl sulfate-polyacrylamide using gel electrophoresis (SDS-PAGE) in an XCell4 SureLock™ Midi-Cell system (Invitrogen, USA; see below).



The separated proteins were transferred to a polyvinylidene fluoride (PVDF) or nitrocellulose membrane and blocked with 5% milk in Tris-buffered saline with Tween-20 (TBST) for 1 hr at room temperature. After being washed three times with TBST, the membrane was probed with the primary antibodies corresponding to the proteins of interest in TBST overnight at 4^0C. The following day, the fluorescent secondary antibody was applied in TBST for 1 hr at room temperature. The proteins were detected by using a near infra-red Licor system (see below).



Figure 2-5. near infra-red Licor system

2-14. Electrical pulse stimulation.

Fully differentiated C2C12 myotubes were induced to contract by EPS. The stimulation pulse was set at 2 ms, delivering 11.5 V at a frequency of 1 Hz for 16 hrs using C-Dish combined with a pulse generator (C-Pace 100; IonOptix, Milton, MA, USA) (figure 2-6). This instrument delivers electrical stimuli via the carbon electrodes of the C-dish, which were placed into the culture medium. Cell extracts from each dish were prepared immediately after the termination of EPS.



Figure 2-6. ION Optix waveform generator

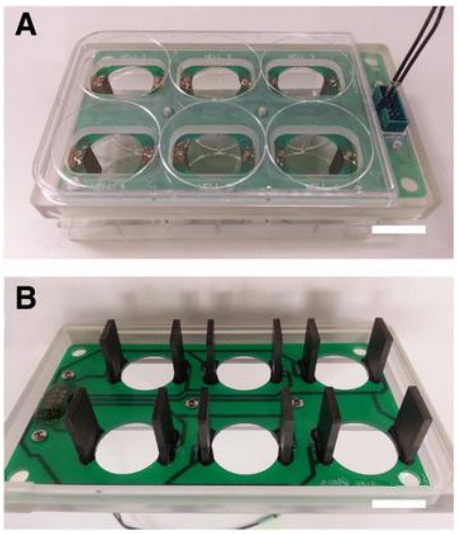


Figure 2-7. C-Dish™ electrode board used for use in the cell stimulation. A. Top view of board on 6-well plate and pin connectors to external waveform generator for single chamber stimulation, and B. Bottom view of the board with carbon electrode pair for each cell chamber and underlying circuit pathways.

2-15. Bradford protein assay

Protein content was determined using the Bradford method (Bradford, 1976). A Bovine Serum Albumin (BSA) stock solution of 1 mg/ml was diluted ten-fold to 0.1 µg/µl, and protein standards were made ranging from 0 µg protein (400 µl dH2O, 0 µl of 0.1 µg/µl BSA) to 10 µg protein (300 µl dH2O, 10 µl of 0.1 µg/µl BSA). To prepare the samples, 5 µl of the sample was added to 395 µl dH2O. One hundred µl Bradford reagent (Coomassie Blue 0.01 %, Ethanol 4.7 %, H3PO4 8.5 %) was added to both standards and samples, they were vortexed, and incubated at room temperature for 5 min to allow the colour to develop. Once the colour is developed,

this is stable for ≤ 1 hr. Two hundred µl from the reaction mixture of the standards and samples were transferred in duplicate to an optical permissive microtitre plate and the absorbance of the solutions was measured spectrophotometrically at 595 nm. The protein standards created a standard curve (Figure 2-8) which was used to determine the protein content of samples. The absolute protein values were multiplied by 2.1 because both unpublished in-house lab observations (Constantin-Teodosiu, personal communication) and some manufacturer's instructions (e.g. AppliChem GmbH, Darmstadt, Germany) suggest BSA is a relatively poor standard in comparison with chicken ovalbumin. This correction factor gives a more accurate determination of protein content comparable to that of ovalbumin, lysozyme or catalase (AppliChem GmbH, Darmstadt, Germany).

Bradford reaction

Protein + Coomassie blue dye → Protein blue complex which absorbs at 595 nm

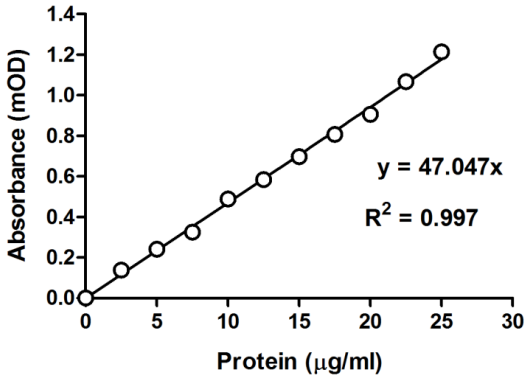


Figure 2-8. Typical Bradford Assay Standard Curve.

2-16. Statistical analyses

All data reported in the present thesis represent means ± SEM. Statistical differences between group means were tested using one-way ANOVA's (one independent variable) with Newman-Keuls correction to allow for post-hoc comparisons.

In every experiment, six independent groups in Chapter 4 and four independent groups in Chapters 5 and 6 were investigated. Since the means of more than three groups were compared in each experiment, one-way ANOVA would have been the appropriate statistical test to localise multiple significances across several means differences in order to control for false discovery rate (type I errors). The Newman-Keuls post-hoc analysis was conducted to uncover specific differences between three or more group means. All data were also assessed for normal distributions using Shapiro-Wilk tests (SPSS v 26, IBM).

Chapter 3

Metabolic inflexibility in an *in vitro* palmitate-induced C2C12 skeletal muscle cell model：Molecular responses associated with impairment of mitochondrial CHO oxidation

3-1 Introduction

3-1-1. Metabolic inflexibility

Metabolic flexibility is defined as the ability to adapt substrate oxidation rates in response to changes in fuel availability or adapt to conditional changes in the metabolic request. Conversely, metabolic inflexibility is the inability to switch between oxidation of lipid and carbohydrate, and this appears to be a wide-spread feature of chronic disorders such as obesity and type 2 diabetes.

Much of the attention around metabolic flexibility was because of its implication in insulin resistance, a concept first advanced by Wilhelm Falta and published in Vienna in 1931, as a possible underlying cause of type 2 diabetes. Over time, the insulin resistance term has progressed to become generally accepted as the foremost factor leading to type 2 diabetes, and the most probable single link among a collection of cardiometabolic risk factors known as the metabolic syndrome linking obesity, type 2 diabetes and cardiovascular disease (Reaven 1988).

The typical modern concept of metabolic inflexibility has been circulated in the context of cellular fuel selection in the conversion from fasting to fed states or fasting to insulin stimulation to explain metabolic inflexibility/insulin resistance (Goodpaster, Wolfe et al. 2002)

In the fasted state, activation of lipolysis delivers tissues with fatty acids, which become the chosen fuel for respiration. In the liver, β-oxidation of fatty acids satisfies the local energy needs and may lead to ketogenesis. As the lowered C-chained fatty acids, ketone bodies are

then preferentially oxidised in extrahepatic tissues, especially in the brain and heart. By inhibiting glucose oxidation, fatty acids and ketone bodies thus contribute to a glucose-sparing effect, an essential survival mechanism for the brain during starvation. In addition, inhibition of glucose oxidation at the level of PDC preserves pyruvate and lactate, both of which are gluconeogenic precursors. However, the inhibition of glucose oxidation by fatty acids is not restricted to the fasted state. It also happens under feeding conditions (quite often after high dietary fat intake) and during low-intensity exercise when fatty acids and ketones are oxidised as well.

The original Randle Cycle or glucose-fatty acid cycle (Randle, Garland et al. 1963), which described the biochemical mechanism behind the inhibition of glucose oxidation by fatty acid in muscle and adipose tissues was put forth as a rule to portray that the reduction of glucose oxidation by elevated fatty acid oxidation, is a critical factor in the pathogenesis of obesity (lipid)-associated skeletal muscle metabolic inflexibility. The mechanisms involved in the Randle Cycle included allosteric control, reversible phosphorylation and the expression of key enzymes, where the mitochondrial pyruvate dehydrogenase complex seemed to be a major player (Hue and Taegtmeyer 2009).

Under normal physiological conditions, the glucose fatty acid cycle should be seen, however, as a homeostatic mechanism to control unabatedly the circulating levels of glucose and fatty

acids, i.e. the cycle should describe a **reciprocal control** between glucose and fatty acid metabolism as depicted in Fig. 3-1-1.

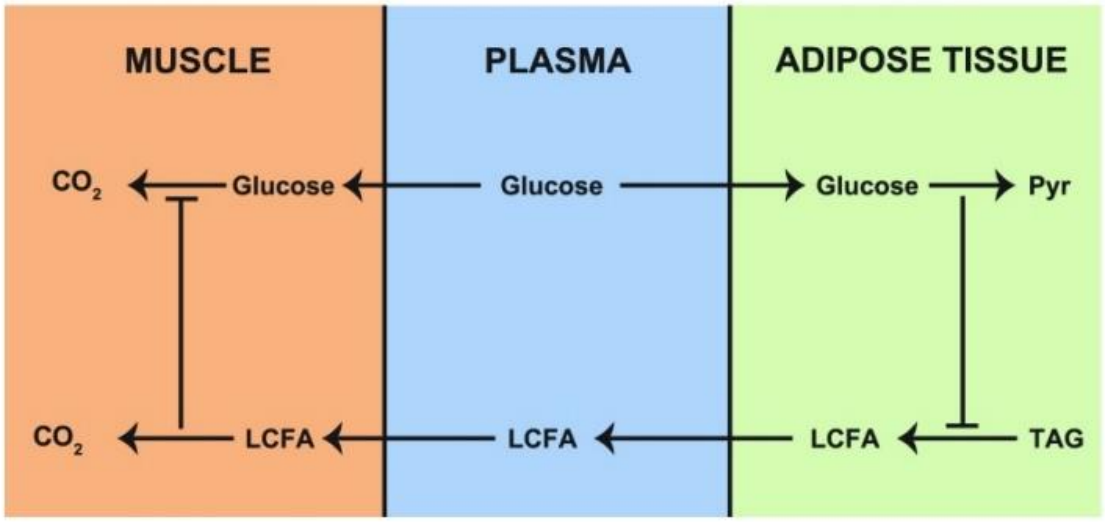


Fig. 3-1-1. A steady-state/ongoing loop of the Randle cycle where the effect of glucose is mediated by insulin. Also, LCFA, long-chain fatty acids, TAG is triacylglycerol and Pyr is pyruvate.

However, one of the major points in the Randle Cycle was that, under normal conditions, only one of the fuels - fat or CHO - might be predominantly oxidised. Thus, when fat oxidation prevailed over CHO oxidation, the details of the Randle cycle would look like this

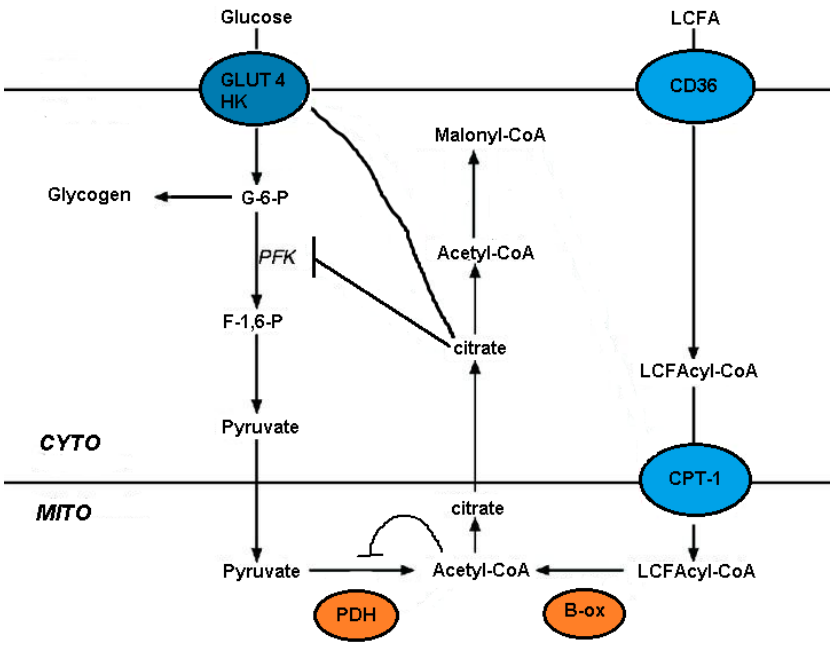
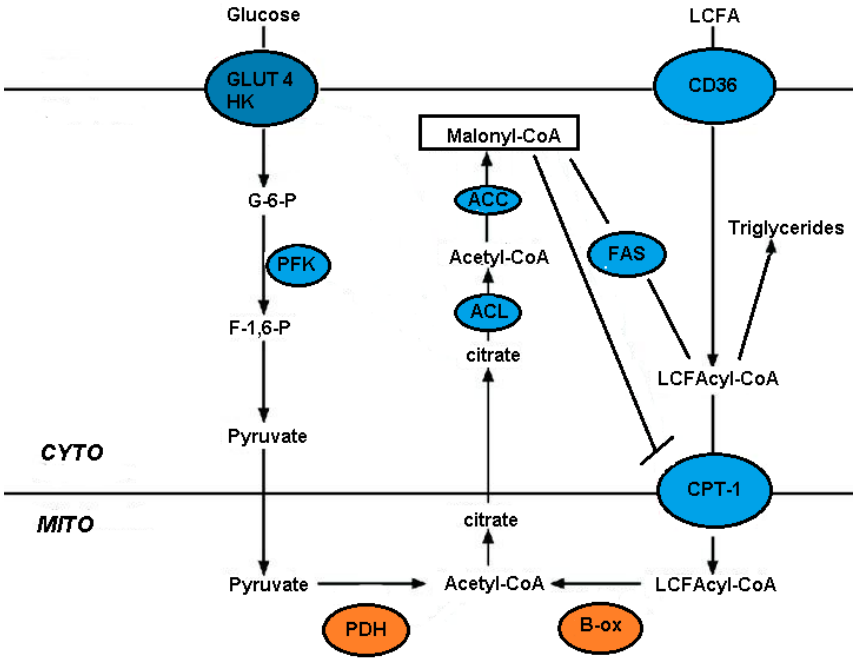


Fig. 3-1-2. The fat oxidation mediated metabolic inhibitory effect on CHO oxidation

Conversely, when CHO oxidation prevailed over fat oxidation, the details of the Randle cycle would look like this (Fig. 3-1-3). The mechanism was mediated by malonyl-CoA, the concentration of which depends on acetyl-CoA carboxylase (ACC) activity and which inhibits the entry of long-chain acyl groups LC acyl-CoA into mitochondria. This effect re-routes fatty acids towards esterification. Additional information: FAS fatty acid synthase ACL, ATP-citrate lyase. While much literature describing the metabolic and enzymatic phenotypes of the metabolic inflexibility with increased fat availability/oxidation has been made available, however, the molecular mechanisms underlying the instauration of the metabolic inflexibility are still scarce.



.

Fig. 3-1-3. The CHO oxidation mediated metabolic inhibitory effect on fat oxidation

3-1-2. Fatty acid and PPARs

Certain fatty acids can bind to peroxisome proliferator-activated receptors (PPARs), a class of transcription factors capable of hypolipidaemic action, which regulate lipid metabolism through their long-term transcriptional effects. There are three distinct PPAR subtypes that are commonly designated as PPARα, PPARδ (also known as PPARβ) and PPARγ (Lee, Olson et al. 2003). PPARα (fibrates) and PPARγ agonists are now used frequently in the treatment of insulin resistance and dyslipidemia. Furthermore, PPARδ agonists have been also identified as being a viable treatment strategy since they can reduce body fat content and insulin resistance in obese mice by apparently increasing fatty acid oxidation in skeletal muscle (Tanaka,

Yamamoto et al. 2003). However, according to Randle cycle, an upregulation of fat oxidation could impinge negatively on the rate of CHO oxidation. Indeed, we have previously shown in *vivo* that human whole-body or isolated rodent tissue presented lower rates of CHO oxidation following either an increase in fat availability achieved through dietary manipulation or via PPARδ agonism (Constantin, Constantin-Teodosiu et al. 2007, Constantin-Teodosiu, Constantin et al. 2012). These findings were associated with marked increases in all muscle PPARs, PDK4 and FOXO1 expressions and reductions in muscle PDC activity.

3-1-3. PPARs activity and mitochondrial function

Cell oxygen consumption is enhanced when fatty acids are used. The $NADH/NAD^+$ ratio increases significantly. Mitochondrial membrane potential ($\Delta\Psi$) also increases. When fatty acids are oxidised over glucose, more electrons are being transferred to complex II (C2) of the respiratory chain, rather than to complex I (C1). This difference is echoed by a less efficient oxidative phosphorylation (ATP/O), because electrons entering complex I result in a higher ATP/O ratio than those entering complex II, being equivalent to a form of "intrinsic uncoupling." To maintain $\Delta\Psi$ and the rate of ATP synthesis, mitochondria would have no other alternative, but to increase respiration (oxygen consumption). Furthermore, high values of $\Delta\Psi$ can prevent the normal electron flow and lead to reversed electron flow and eventually enhance the production of reactive oxygen species (ROS) (Fig. 3-1-4). Therefore, mitochondria increase

their respiration, their membrane potential, and the production of ROS when oxidising fatty acids forced by PPAR agonism.

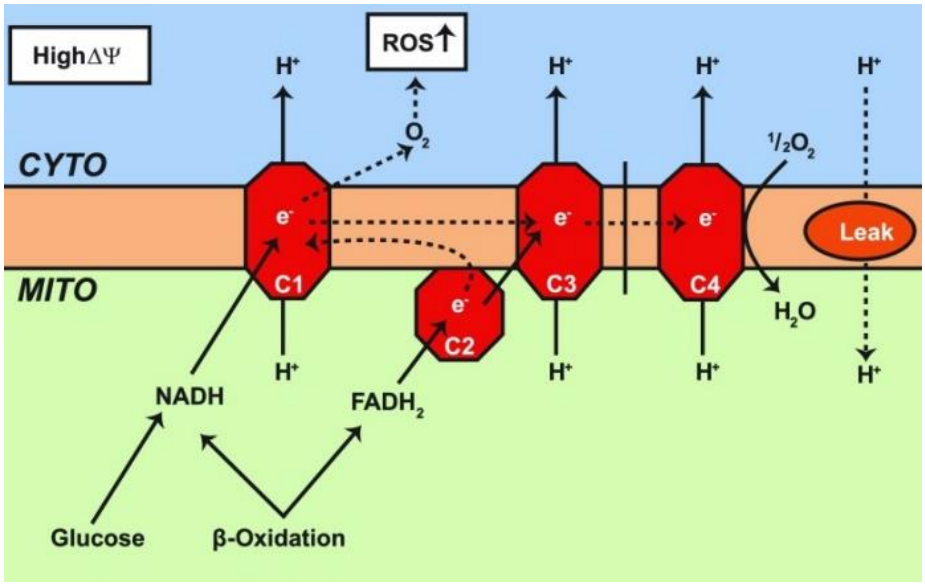


Figure 3-1-4. ROS production in mitochondria

Indeed, PPARα agonism has been reported to impair mitochondrial function, and on the above earlier described mitochondrial events, several different mechanisms have been proposed to explain this phenomenon, including membrane depolarisation, uncoupling of respiration, induction of mitochondrial permeability transition, and direct inhibition of mitochondrial respiration (Zhou and Wallace 1999, Qu and Lin 2001)

3-1-4. Aims of this chapter

This chapter will describe whether it was possible to create a state of metabolic inflexibility in an *in vitro* model of C2C12 muscle cells by treating myotubes with fat (palmitate). Evidence

in support of this model would be provided by lower than control (no palmitate) values for the cellular glucose uptake, PDC activity and its flux (as reflected by lower rates of acetyl group accumulation), lower rates of aerobic ATP production from glucose-derived pyruvate and higher levels of media lactate accumulation. Also, it was necessary to confirm at the molecular levels whether the above anticipated metabolic responses would be matched, similar to previous *in vivo* findings, by upregulation of muscle PDK4, PPARα, PPARδ, and FOXO1 gene and protein expression in response to chronic high-fat dietary intake.

3-2. Methods

A murine skeletal muscle cell line C2C12 was used in this experiment. On day four, after FBS differentiation, palmitate 500 µM was provided to the cells and let them grow for another 24 hr. Then, the cells were collected and underwent different experimental measurements as will be further described. Six independent experiments was undertaken for each intervention group.

3-2-1. MTT assay

Cell viability in the presence of palmitate was determined using a MTT assay. Briefly, various concentrations of palmitate were used on day four after cell differentiation. For further details please Chapter 2: Materials and General Methods.

3-2-2. Glucose uptake

Cellular glucose uptake was measured as previously described in Chapter 2: Materials and General Methods.

3-2-3. Cellular total RNA extraction and Real-time qPCR

The primers and the MGB (minor groove binding) probes used for the PCR analysis were designed by the manufacturer (Applied Biosystems) and purchased as shelf-inventoried assays. RNA was extracted from C2C12 cells using Tri-reagent (Applied Biosystems, UK) following standard protocols. For further details, please see the details described in Chapter 2: General Methods.

3-2-4. MAPR

Maximal mitochondrial ATP production rates (MAPR) were measured as previously described in Chapter 2: Materials and General Methods. MAPR values were corrected for total protein content of the cellular extract, measured using the Bradford method (Bradford, 1976) as described in Chapter 2: General Methods.

3-2-5. Cellular acetyl group and media lactate accumulation

Cellular acetyl group and media lactate accumulation were measured as previously described in Chapter 2: Materials and General Methods.

3-2-6. Cellular PDC activity measurements:

Cellular PDC activity measurements were measured as previously described in Chapter 2: Materials and General Methods.

3-2-7. Western blotting measurements

Expression of PDK4, PPARα a, δ and FOXO1 proteins were measured as previously described in Chapter 2: Materials and General Methods.

3-2-8. Statistical Analyses

The reported data represent means ± SEM. Statistical differences between group means were tested using one-way ANOVA's (one independent variable) with Newman-Keuls correction to allow for post-hoc comparisons. Data were assessed for normal distributions using Shapiro-Wilk tests (SPSS v 26, IBM).

3-3. Results

3-3-1. Cell viability in C2C12 myotubes treated with increasing levels of palmitate

In order to identify the suitability of palmitate concentrations that would allow cell survival, the viability of C2C12 myotubes measured by MTT was assessed at increasing palmitate concentrations. In Fig. 3-3-1, it was found that there was no significant change in the viability

between 0-500 µM palmitate administrations. C2C12 myotubes treated with 750 µM palmitate showed to have a decreased viability, suggesting that 750 µM palmitate was harmful to C2C12 myotubes. Since the human average circulating plasma FFA concentration is similar to our highest FFA concentration viable to our cell model, i.e. 500 µM, I choose this level as the working concentration. I also undertook further experiments to establish whether the treatment of C2C12 cells with the selected palmitate concentration was able to elicit responses associate with instauration of a cellular metabolic inflexibility, i.e. cellular glucose uptake and lactate concentration, PDC activity, acetyl-CoA, and acetylcarnitine concentrations, glucose-derived pyruvate maximal rates of ATP production, and PDK4, PPARα, PPARδ, and FOXO1 gene and proteins expressions.

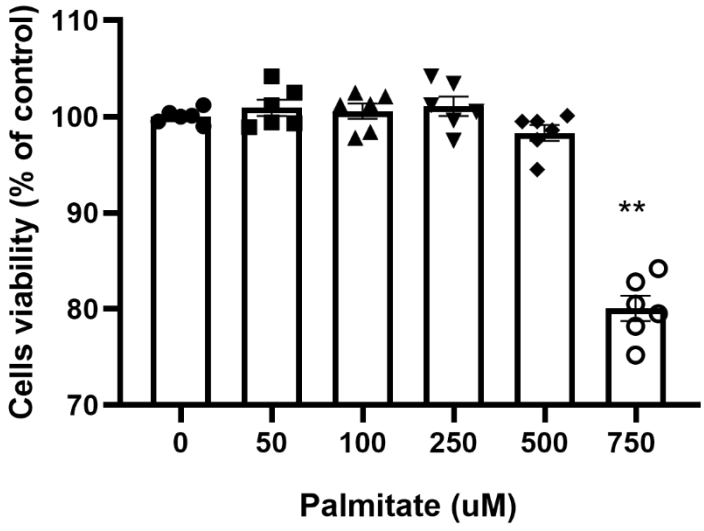


Figure 3-3-1. C2C12 myotubes viability in the presence of different concentrations of palmitate

C2C12 myotubes were treated with 500 μM palmitate (*n*=6)**.** **Significantly different from the corresponding control group (*P*<0.01).

3-3-2. Glucose uptake and lactate concentration in the presence or absence of palmitate in C2C12 myotubes

Skeletal muscle cell C2C12 glucose uptake after treating the cells with palmitate was shown in Fig. 3-3-2. Palmitate significantly decreased glucose uptake of the muscle cell (100.0±1.7 vs 85.6±2.0%, *P*<0.01) and increased medium lactate concentration (7.5±0.1 vs 8.5±0.1 mmol/L, *P*<0.01) when compared to the control.

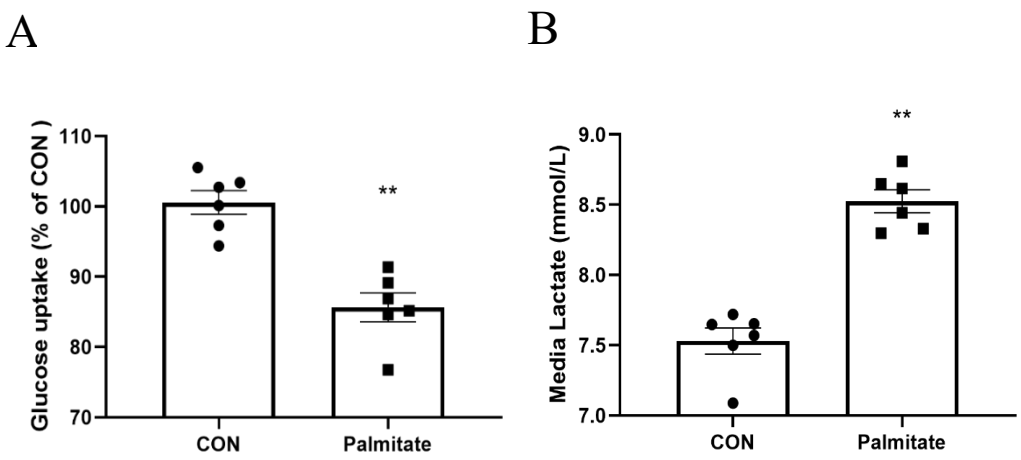


Figure 3-3-2. C2C12 myotubes glucose uptake and medium lactate concentration in the presence or the absence of palmitate.

C2C12 myotubes were treated with 500 μM palmitate (*n*=6)**.** **Significantly different from the control group (*P*< 0.01).

3-3-3. PDC activity, acetyl-CoA, and acetylcarnitine content in C2C12 myotubes in the presence or the absence of palmitate.

The PDC activity, acetyl-CoA, acetylcarnitine and free carnitine concentration of skeletal muscle cell C2C12 after palmitate treatment are presented in Fig. 3-3-3. Palmitate significantly decreased PDC activity compared to control (3.8±0.1vs 2.9±0.1, pmol acetyl-CoA/mg/min, *P*<0.01). Also, palmitate significantly decreased acetylcarnitine (101.4±5.8 vs 82.5±3.9 pmol/mg protein, *P*<0.05). On the other hand, palmitate significantly increased the cells' acetyl-CoA content from 23.3±0.9 to 29.9±0.8 pmol/mg protein, *P*<0.01.

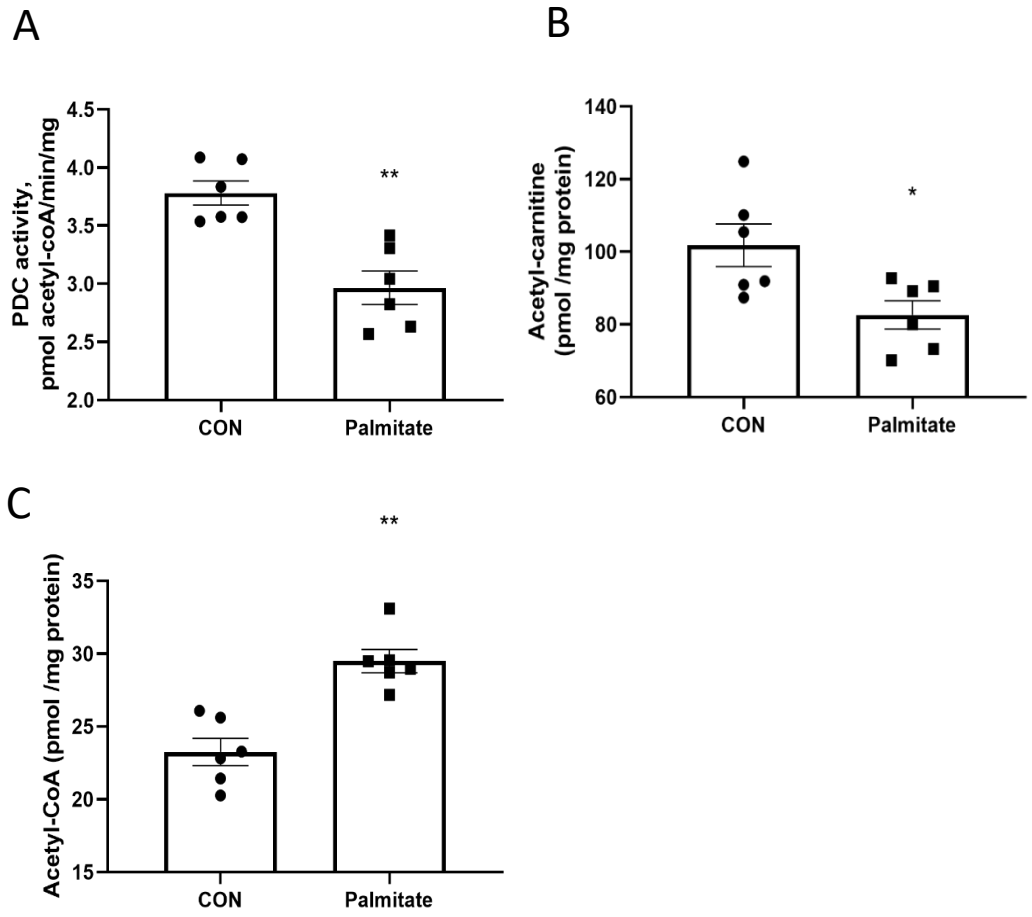


Figure 3-3-3. C2C12 myotubes PDC activity, and acetylcarnitine, and acetyl-CoA levels in the presence or the absence of palmitate. C2C12 myotubes were treated with 500 μM palmitate (n=6)**.** **Significantly different from the corresponding control group (P<0.01).

3-3-4. Pyruvate based maximal generated rates of ATP production in C2C12 myotubes in the presence or the absence of palmitate

In Fig. 3-3-4, administration of palmitate significantly decreased total mitochondrial ATP production rate in C2C12 cells (10.5±0.6 vs 7.7±0.7 nmol ATP /min/mg protein, $P<0.01$).

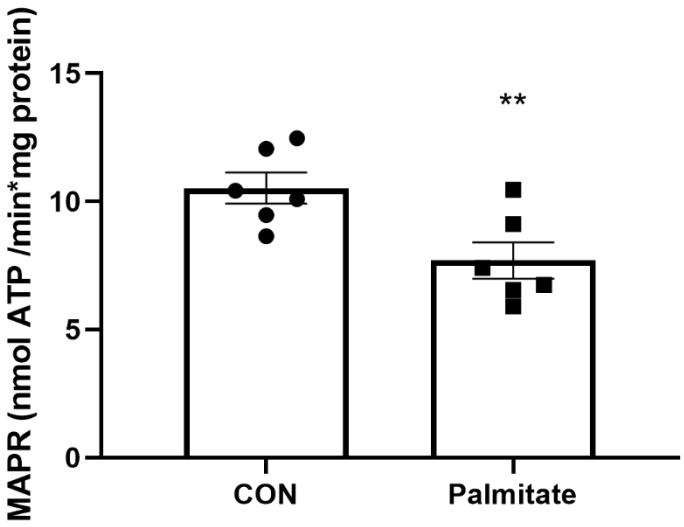


Figure 3-3-4. C2C12 myotubes pyruvate based maximum ATP production rate in the presence or the absence of palmitate. C2C12 myotubes were treated with 500 μM palmitate (PAL) (n=6)**.** **Significantly different from the control group ($P<0.01$).

3-3-5. mRNA gene expression in C2C12 myotubes in the presence or the absence of palmitate.

The effect of palmitate on PDK4, FOXO1, PPARα and PPARδ gene expression are presented in Fig. 3-3-5. Palmitate significantly increased PDK4 (1.0±0.1 vs 2.2±0.2, $P<0.01$), PPARα (1.0±0.1 vs 1.5±0.1, $P<0.01$), PPARδ (1.0±0.1 vs 1.6±0.1, $P<0.01$) and FOXO1 (1.0±0.2 vs 1.5±0.1, $P<0.01$) gene expression compared to control group.

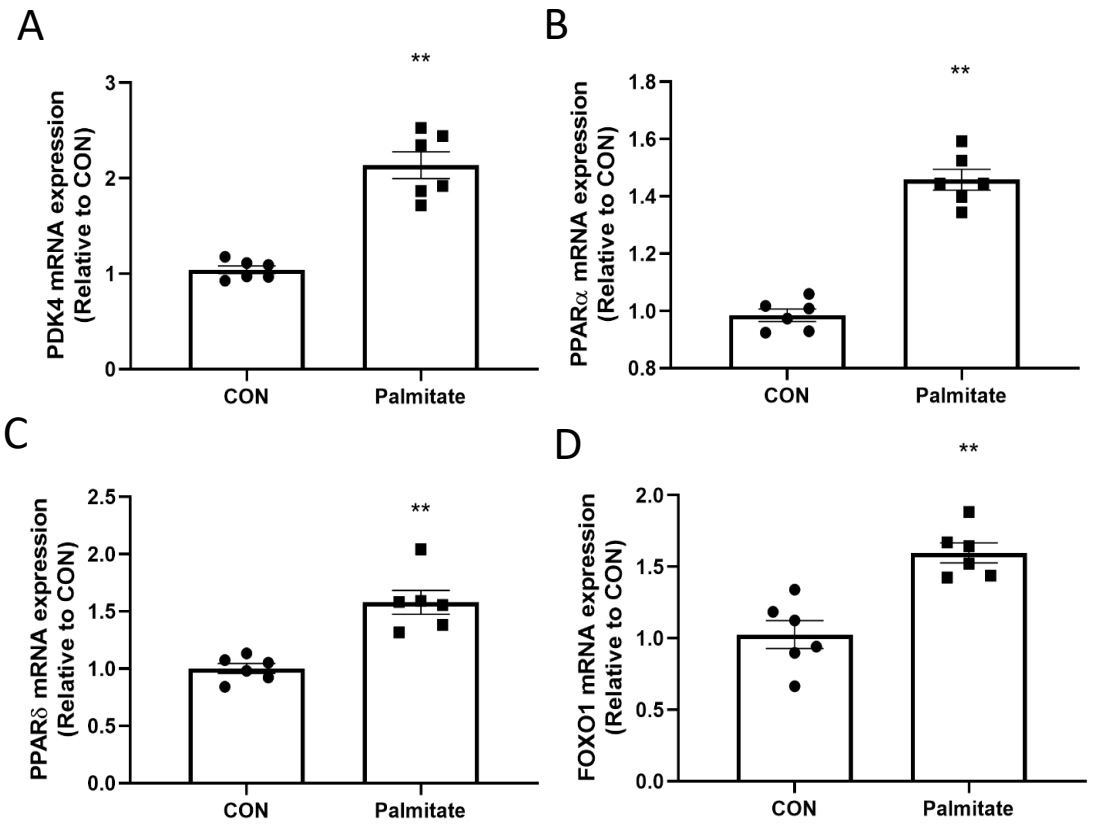


Figure 3-3-5. PDK4, PPARα, PPARδ, and FOXO1 gene expressions in C2C12 myotubes in the presence or the absence of palmitate. C2C12 myotubes were treated with 500 μM palmitate (n=6)**.** **Significantly different from the control group (P<0.01).

3-3-6. Protein expression in C2C12 myotubes in the presence or the absence of palmitate.

The effect of palmitate on PDK4, FOXO1, PPARα, and PPARδ protein expression are presented in Fig. 3-3-6. Palmitate significantly increased PDK4 (1.0±0.1 vs 2.5±0.2 relative to control, P<0.01), PPARα (1.0±0.1 vs 1.6±0.1, P<0.01), PPARδ (1.0±0.1 vs 1.8±0.2, P<0.01), and FOXO1 (1.0±0.2 vs 1.5±0.1, P<0.01) protein expression and decreased phospho-FOXO1 (1.0±0.1 vs 0.8±0, P<0.01) protein expression compared to control group. The ratio of phospho-total FOXO1 also decreased (1.0±0.1 vs 0.4±0.1 relative to control, P<0.01).

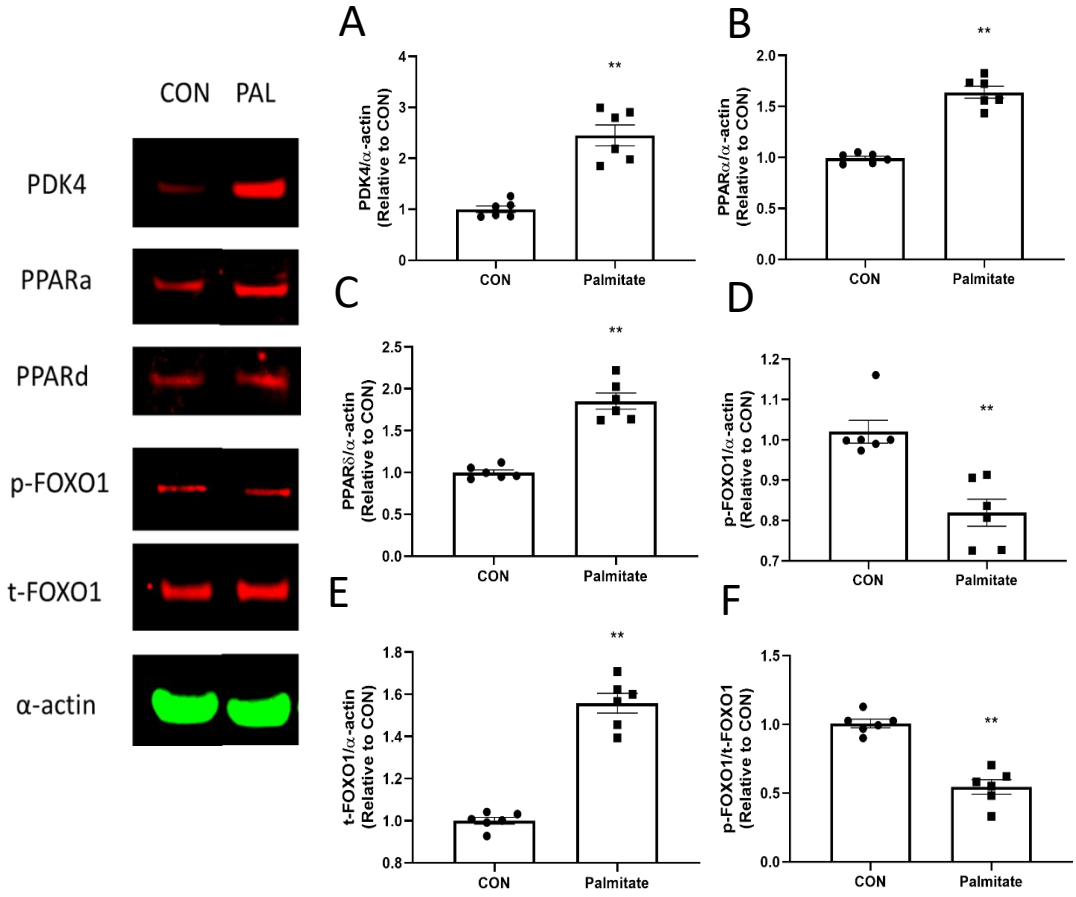


Figure 3-3-6. C2C12 myotubes PDK4, PPARα, PPARδ, phosphorylation of FOXO1 and total FOXO1 protein expression in the presence or the absence of palmitate. C2C12 myotubes were treated with 500 μM palmitate (PAL; *n*=6)**.** **Significantly different from the control group ($P<0.01$).

3-4. Discussion

In this chapter, it was established what palmitate concentration could be tolerated by the *in vitro* skeletal muscle cell model without affecting the cells' viability, and whether this palmitate level would be sufficient to induce an inflexible metabolic state as reflected by outcomes of accepted markers cited in the literature as they will be discussed later. Thus, it was found that to the cell viable identified palmitate concentration not only resulted in decreased glucose uptake, lower pyruvate mediated mitochondrial ATP production rate, and increased media lactate concentration, but it also decreased cellular PDC activity, which is the rate-limiting step in CHO oxidation. It was also demonstrated that palmitate increased both PDK4, PPARα, PPARδ, and FOXO1 mRNA gene expression and protein expressions significantly.

FFAs, and in particular palmitic acid, are a significant source of energy. However, when circulating FFAs are chronically elevated, they lead to metabolic inflexibility/insulin resistance. This state is reflected by lower rates of carbohydrate oxidation, lower rates of muscle glucose uptake, and lower muscle PDC activity. In the translation from an *in vivo* chronic exposure of high dietary fat intake model to an *in vitro* model, the use of palmitate and oleic acid, which are the most prevalent saturated long-chain FFAs in the diet, has been widely used. This has allowed exploring the effects and underlying molecular mechanisms of a large plethora of metabolic disturbances all covered under the umbrella of hyperlipidaemia without the influence of any hormonal or any other exocrine intervention. Palmitate alone is widely

used to induce insulin resistance in skeletal muscle cells, liver cells, and adipose cells, which has been shown to decrease glucose uptake, lower utilisation of glucose, induce oxidative damage, and impair mitochondrial function in cells (Nieuwoudt, Mulya *et al.* 2017, Chen, Yi *et al.* 2018). However, since there was so far no study where the effect of palmitate on PDC in a skeletal muscle cell-based model available, I wished to address this shortcoming.

The results showed that palmitate decreased C2C12 myotubes PDC activity and acetylcarnitine content in parallel with increases in acetyl-CoA content. Carnitine helps to maintain a status quo of acetyl-CoA levels when its rate of condensation with oxaloacetate is less than its rate of formation from PDC mediated pyruvate decarboxylation. The increase of cellular acetylcarnitine also demonstrates that the rate of acetyl group formation is higher than the capacity of the tricarboxylic acid cycle to oxidise acetyl-CoA to CoASH. Palmitate oxidation increases acetyl-CoA concentration more than CHO oxidation does as one molecule of palmitate generates eight acetyl-CoA molecules, while one glucose molecule generates two acetyl-CoA molecules. Accumulation of acetyl-CoA results in a reduction of PDC activity which leads to CHO metabolism switch from CHO to FFA. However, it is safe to state that accumulation in the cellular acetyl-CoA is stoichiometrically matched by an accumulation in the cellular NADH as both of the products of palmitate oxidation. The inhibition of PDC activity by palmitate/increased levels of acetyl-CoA and NADH occurs mainly via two mechanisms:

1. Direct inhibition of PDC activity via a covalent (phosphorylation/dephosphorylation cycle of PDC complex) and allosteric inhibition of a given level of activated/dephosphorylated amount of PDC by increases in the acetyl-CoA/CoASH and NADH/NAD^+ ratios. *In vitro* experiments have shown that an increase in the acetyl-CoA/CoASH ratio from 0.1 to 10 (Cooper *et al.* 1975, Pettit *et al.* 1975) can stimulate PDK4 kinase and thus induce a decrease in PDCa activity. It has also been suggested that the transformation of PDC to PDCa may be influenced more by changes in the mitochondrial redox state than by changes in the acetyl-CoA/CoASH ratio (Roche & Cate 1977, Hansford & Cohen 1978). Thus, an increase in the NADH/NAD^+ ratio has been shown to inhibit PDC transformation in vitro by inhibiting the PDC phosphatase (Pettit et 1975) and by simultaneously activating the PDC kinase (Copper *et al.* 1975, Red *et al.* 1980). And in keeping with the above, it was supportive to know that an increase in the cellular NADH concentration in human muscle reflects changes in the redox state within the mitochondria (Sahlin 1983, Henriksson *et al.* 1985).
2. Indirectly, by activation transcription factors PPARs and FOXO1 following the increase in palmitate/FFAs availability.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors that interact with several endogenous lipids and drugs normally used to treat metabolic diseases in humans (Berger &

Wagner, 2002). There are three distinct PPAR subtypes that are commonly designated PPARα, PPARδ (also known as PPARβ) and PPARγ (Lee, Olson et al. 2003). PPARα (fibrates) and PPARγ agonists are now used widely in the treatment of insulin resistance and dyslipidaemia. Furthermore, PPARδ agonists have also been identified as being a viable treatment strategy since they can reduce body fat content and insulin resistance in obese mice by apparently increasing fatty acid oxidation in skeletal muscle (Tanaka, Yamamoto et al. 2003). PPARα agonist administration has been reported to impair mitochondrial function, at least in *vitro*. Several different mechanisms have been proposed to explain this phenomenon, including membrane depolarisation, uncoupling of respiration, induction of mitochondrial permeability transition, and direct inhibition of mitochondrial respiration (Zhou and Wallace 1999, Qu and Lin 2001). Evidence that PPARδ agonist administration impairs mitochondrial function was less clear.

An increase in fatty acid oxidation induced by PPARα and δ agonism (Motojima 2002) and starvation (Wu, Peters et al. 2001) has been shown to result in the transcriptional activation of muscle pyruvate dehydrogenase kinase isoform 4 (PDK4), the enzyme that phosphorylates, and therefore inactivates pyruvate dehydrogenase complex (PDC), the rate-limiting step in muscle carbohydrate oxidation. The mechanism of PDK4-induced PDC inhibition in starvation has been attributed to either increased fatty acid-mediated activation of the forkhead transcription factor FOXO1, followed by direct binding to the promoter region of the PDK4

gene (Furuyama, Kitayama et al. 2003), or through free fatty acid-mediated activation of the PPARα receptor (Wu, Peters et al. 2001). Irrespective of the mechanism, PDC inhibition could alter CHO selection in skeletal muscle towards fat oxidation, when free fatty acid availability is increased, and thereby explain some of the reported effects of PPAR agonists on muscle CHO utilisation. Our laboratory has also shown that GW610742, a PPARδ agonist, switches muscle CHO metabolism towards decreased carbohydrate use and enhanced lipid utilisation, by increasing expression of PDK2 and PDK4 gene and protein isoforms (Constantin, Constantin-Teodosiu et al. 2007).

FOXO1 is also an important regulator in CHO metabolism since FFAs can indirectly induce the translocation of FOXO1 to the nucleus where by binding to the PDK4 gene promoter can initiate PDK4 gene expression (Furuyama, Kitayama *et al.* 2003). Additionally, a rodent model has been shown that high-fat diet can also control PDK4 gene expression via an allosteric mechanism by lowering phosphorylated (inactive form) of FOXO1 (Pandey, Raj *et al.* 2017). Similar findings such as PDK4 protein being up-regulated by high fed diet feeding and increased plasma free fatty acid has also been shown in human (Yamaguchi, Moseley et al. 2018). However, the evidence of the effects of high-fat diet mediated rise in PPARα, PPARδ, and FOXO1 gene and protein expression on PDK4 expression, and thereby inhibition of carbohydrate oxidation, has been so far derivative (not directly causative), isolated (not all factors simultaneously investigated), or mostly limited to the gene level. Therefore, we aimed

to test on a comprehensive basis how changes in PPARα, PPARδ, and FOXO1 gene and protein expression induced by increased in palmitate availability would alter PDK4 gene and protein expression under the same experimental conditions by using advanced tools such real-time TaqMan probe real-time PCR and infra-red western blot in a skeletal cell model devoided from any hormonal or any other endocrine influence.

It was found that palmitate significantly increased PDK4 gene and protein expression by 2.2 and 2.6 times, respectively. Also, palmitate significantly increased PPARα, PPARδ, and FOXO1 gene expression by 1.5, 1.6, and 1.5-fold, respectively, and that of protein expression by 1.5, 1.6, and 1.4-fold, respectively. Collectively, the results showed that palmitate regulates PDK4 by modulating PPARα, PPARδ, and FOXO1 gene and protein expression. Nevertheless, there is still not enough evidence to attribute an individual contribution of PPARα, PPARδ, and FOXO1 to the regulation of PDK4. Therefore, in the next chapter, I will use an intervention at molecular level by using siRNA silencing aimed to knockdown PPARα, PPARδ, and FOXO1, on a one-knock at the time basis, to allow individual responses to be uncovered.

In conclusion, a novel palmitate-induced metabolic inflexibility in a skeletal muscle cell model was successfully established. The model showed marked decreases in cellular glucose uptake, PDC activity, acetylcarnitine content, ATP production rate in parallel with increased media lactate and acetyl-CoA concentrations. The results also showed that palmitate increased PDK4, PPARα, PPARδ, and FOXO1 gene and protein expressions. This chapter set up the

basis for further explorations of the individual role of PPARα, PPARδ, and FOXO1 on the PDK4 regulation of PDC-controlled CHO oxidation.

Chapter 4

The impact of siRNA PPARα, PPARδ and FOXO1 gene silencing on palmitate-induced impairment of mitochondrial CHO oxidation

4-1. Introduction

4-1-1. RNA interference

RNA interference (RNAi) is a conserved mechanism found in most eukaryotes. RNA interference (RNAi) or "gene silencing" is an attractive mechanism in which a cell utilises a gene's RNA sequence to end the expression of that gene. This process can be exploited to analyse the role of genes in disease and to design therapeutic approaches involving gene silencing (Wang, Lu et al. 2010, Ozcan, Ozpolat et al. 2015). RNAi uses short double-stranded RNAs (dsRNAs), typically 21-24 nucleotides long, and RNA-induced Silencing Complex (RISC) machinery to silence expression of genes with complementary sequences to the dsRNA. While both strands of siRNA could potentially be used for silencing, only one strand in the duplex, referred to as the guide strand or silencing strand, is fused into RISC. Directed to its mRNA target by the guide siRNA strand, the RISC complex then destroys the mRNA target, effectively "silencing" the RNA.

4-1-2. *In vitro* use of siRNAs

Synthetic siRNAs have been used broadly as a research tool for investigating and validating gene function and functional pathways, and have also been used as therapeutic agents in clinical applications. While siRNA specificity can be validated *in vitro*, several points need to be taken into consideration for *in vivo* incorporation: limiting off-target effect (which can arise when the

siRNA has a sequence that can pair and reduce the expression of multiple genes at a time), determining the optimal route of administration and delivery method, and facilitating cellular uptake while minimising the immune response. To circumvent the transient nature of siRNA regulation, lentiviral systems have been used as part of the gene therapy approach to allow stable and permanent integration of siRNA sequences into the genome.

4-1-3. PPARα

Peroxisome proliferator-activated receptor alpha (PPARα) is a major regulator of lipid metabolism in the liver, heart muscle, kidney, skeletal muscle, retina, and brown adipose tissues. PPARα is activated under conditions of energy deprivation or increased fatty acid availability. Activation of PPARα increases the uptake, utilisation, and catabolism of fatty acids by upregulation of genes involved in fatty acid transport, fatty acid-binding and activation (Kersten 2014). PPARα has been shown to control β-oxidation related gene expressions such as ACOX1 and EHHADH in rodents (Rakhshandehroo, Hooiveld *et al.* 2009). Also, fatty acid transport CPT1 and CPT2, which are enzymes located in the outer and inner mitochondrial membranes, respectively, seem to be regulated by PPARα (Mascaro, Acosta *et al.* 1998). PPARα agonism can increase hepatic mitochondria activity and suppress the lipogenic pathway in diet-induced obese mice (Veiga, Graus-Nunes *et al.* 2017). Some studies showed that PPARα activation might also regulate glucose metabolism. Thus, WY14643, a PPARα agonist,

decreased glucose level and improved insulin resistance in rodents (Chou, Haluzik *et al.* 2002, Kim, Haluzik *et al.* 2003).

Previously, PPARα has been considered as a PDC regulator based on the observation that parallel rises in muscle PPARα and PDK4 gene expressions were reported during conditions of chronic high levels of circulating FFAs induced by starvation (Huang et al**.** Diabetes 2002 Feb; 51(2): 276-283). Conversely, other studies were not able to replicate a relationship between PPARα and PDK4 as Holness *et al.* (2002) showed that expression of PDK4 gene occurs independently from PPARα signalling in oxidative skeletal muscle (Holness, Bulmer *et al.* 2002). Also, it has been demonstrated that the enhancement of PDK4 mRNA expression is much faster than PPARα mRNA expression after administration of PPARα receptor agonist (Motojima 2002). Therefore, whether PPARα is involved in the regulation of PDK4 gene is still debatable.

4-1-4. PPARδ

PPARδ is a nuclear hormone receptor that governs a variety of biological processes and may be involved in the development of several chronic diseases, including diabetes, obesity, atherosclerosis, and cancer(Berger and Moller 2002). PPARδ is known to modulate cellular energy consumption. In muscle cell, PPARδ activation switches production of energy from glycolysis to fat oxidation mimicking the effects of running endurance (Fan, Waizenegger *et al.* 2017). Besides, PPARδ activation increases β-oxidation after promoting fatty acid uptake

following upregulation of fatty acid translocase/CD36 (FAT/CD36) and heart fatty acid-binding protein (H-FABP) (Holst, Luquet *et al.* 2003). PPARδ agonists can also reduce body fat content in obese mice by apparently increasing fatty acid oxidation in skeletal muscle (Tanaka, Yamamoto *et al.* 2003). Overexpression of PPARδ in white adipose tissue decreases adiposity and induces expression of genes required for fatty acid oxidation (Wang, Lee *et al.* 2003). In addition to the impact on fatty acid oxidation, PPARδ activation also improves blood glucose homeostasis. Therefore, PPARδ agonism has been trumpeted as being a viable treatment strategy in hyperlipidaemia.

Activation of PPARδ enhances mitochondrial oxidation and insulin secretion in islet cells (Iglesias, Barg *et al.* 2012) and protects type 2 Diabetic mice from lipoapoptosis (Li, Xu *et al.* 2019). PPARδ activation is also protective against insulin resistance as PPARδ knockout mice are glucose-intolerant, while PPARδ activation improved insulin sensitivity in db/db mice (Lee, Olson *et al.* 2006). Activation of PPARδ reduces also fasting glucose levels in mice. Thus, GW50516, a PPARδ agonist, attenuated high-fat diet-induced obesity and insulin resistance and improved diabetes (Tanaka, Yamamoto *et al.* 2003). Also, PPARδ agonist attenuates hepatic endoplasmic reticulum stress by improving hepatic energy metabolism in high-fat diet-fed mice (Silva-Veiga, Rachid *et al.* 2018). However, concerning carbohydrate metabolism, especially oxidation, PPARδ has an ambivalent role as it has been shown *in vivo* and hindlimb rodent models that PPARδ agonism (GW610742) increases muscle PDK4 protein

expression (Constantin, Constantin-Teodosiu *et al.* 2007) and subsequently attenuated the exercise-induced activation of PDC and mitochondrial ATP production (Constantin-Teodosiu, Baker *et al.* 2009). Conversely, expression of PDK4 mRNA was markedly reduced in a PPARδ knock-out mice model (Nahle, Hsieh *et al.* 2008). Moreover, the lysophosphatidyl cholines-induced PPARδ activation up-regulated PDK4 gene expression, which was abolished by the PPARδ antagonist GSK0660 or GSK3787 (Klingler, Zhao *et al.* 2016). All these studies showed that PPARδ has a strong impact on PDK4 expression, which thereby attributes PPARδ agonism an important role in downregulating PDC activity. However, any mechanistic evidence at translational level regarding the relative contribution (if any) of PPARδ to the reported increase in muscle PDK4 expression and associated changes in CHO metabolism is still missing.

4-1-5. FOXO1

FOXO1 is an important insulin signalling downstream negative regulator expressed mostly in skeletal muscle, liver and adipose tissue (Rena, Guo *et al.* 1999). During normal physiological conditions, FOXO1 activates gluconeogenesis and glycogenolysis by inducing glucose-6-phosphatase (G-6-Pase) and phosphoenolpyruvate carboxylase (PEPCK) expression. At the same time, starvation reduces blood glucose and insulin concentrations, decreases insulin's actions and results in the nuclear accumulation of FOXO factors. Insulin inhibits FOXO1 function by activating PI3K/AKT pathway, which phosphorylates FOXO1 at

Thr^{24}, Ser^{256} or Ser^{319}, thereby resulting in FOXO1 exclusion from the nucleus. It has been proposed that FOXO1 regulation may have a protective effect on hyperglycaemia. Genetic ablation of FOXO1 has shown to enhance glucose uptake in the hypothalamus. Besides, FOXO1 mutant mice showed improvement of glucose tolerance and insulin sensitivity accompanied by smaller-sized adipocyte (Nakae, Cao *et al.* 2008), suggesting that FOXO1 regulates energy storage and expenditure. Alteration of FOXO1 also has been implicated in mitochondria adaption. Thus, adenosine triphosphate (ATP) production and oxygen consumption were reduced when FOXO1 was inhibited in hepatocyte treated with berberine (Xia, Yan *et al.* 2011). Besides, up-regulation of peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1α), which is a central metabolism regulator, decreased phosphorylation of FOXO1 at Ser^{256} in a mouse of hepatitis C infection (Bernsmeier, Calabrese *et al.* 2014). Other evidence has shown that FOXO1 up-regulates PDK4 expression since FOXO1 can directly bind the promoter region of the PDK4 gene. Also, after exercise, the gene expression of FOXO1 and PDK4 are positively upregulated (Constantin-Teodosiu, Constantin *et al.* 2012). In addition, FOXO1 protein expression significantly decreases in heart after treating with the PDK4 inhibitor DCA (Piao, Sidhu *et al.* 2013). Moreover, insulin suppression of PDK4 gene expression is impaired in acute insulin resistance, and this is accompanied by increased levels of FOXO1 gene expression (Kim, Lee *et al.* 2006). Several nuclear receptors and transcription factors including the oestrogen-related receptor (ERR), glucocorticoid

receptor (GR), and hepatic nuclear factor 4 (HNF4) have been found to regulate PDK4 gene expression by regulating FOXO1 (Ma, Zhang *et al.* 2005, Zhang, Ma *et al.* 2006). Dexamethasone, which is a glucocorticoid, enhances gene expression of both PDK4 and FOXO1, while insulin suppresses the induction (Kwon, Huang *et al.* 2004, Connaughton, Chowdhury *et al.* 2010). Collectively, this evidence suggests that an association between PDK4 and FOXO1 exists. However, the cause-effect hierarchy between the two entities (FOXO1 and PDK4) in an inflexible metabolic model and the underlying mechanism is still unclear.

4-1-6. Aim of this chapter

In this chapter, it was hypothesised that if PPARα, PPARδ and FOXO1 were instrumental to the upregulation of PDK4 and inhibition of PDC mediated CHO oxidation in our metabolic inflexible C2C12 myotube model, then this evidence should become apparent in the outcomes of well-defined markers of the level of CHO oxidation (e.g. glucose uptake, acetyl-group and lactate accumulation, glucose-derived pyruvate mediated ATP production, PDC activity) after individual gene silencing. Additionally, the rate of success of the gene silencing intervention for each target will be monitored at the gene and protein levels. The impact of individual PPARα, PPARδ and FOXO1 silencing on the other still active genes will also be investigated at transcriptional, translational and post-translational (phosphorylation) level. Additionally, this chapter will aim to rank the importance of PPARs and FOXO1 transcription factors to the regulation of PDK4, and thereby to that of CHO oxidation.

4-2. Methods

A murine skeletal muscle cell line C2C12 was used in this experiment. On day four, after FBS differentiation, palmitate 500 µM was provided to the cells and let them grow for another 24 hr. Then, the cells were collected and underwent different experimental measurements as will be further described. siRNA was given on day two after differentiation. Six individual experiments for each intervention group was undertaken.

4-2-1. Gene silencing (siRNA transfection)

siRNA transfection was undertaken as previously described in Chapter 2: Materials and General Methods.

4-2-2. Glucose uptake

Cellular glucose uptake was measured as previously described in Chapter 2: Materials and General Methods.

4-2-3. Cellular total RNA extraction and Real-time qPCR

The primers and the MGB (minor groove binding) probes used for the PCR analysis were designed by the manufacturer (Applied Biosystems) and purchased as shelf-inventoried assays. RNA was extracted from C2C12 cells using Tri-reagent (Applied Biosystems, UK) following standard protocols. For further details, please see the details described in Chapter 2: General

4-2-4. MAPR

Maximal mitochondrial ATP production rates (MAPR) were measured as previously described in Chapter 2: Materials and General Methods. MAPR values were corrected for total protein content of the cellular extract, measured using the Bradford method (Bradford, 1976) as described in Chapter 2: General Methods.

4-2-5. Cellular acetyl group and media lactate accumulation

Cellular acetyl group and media lactate accumulation were measured as previously described in Chapter 2: Materials and General Methods.

4-2-6. Cellular PDC activity measurements:

Cellular PDC activity measurements were measured as previously described in Chapter 2: Materials and General Methods.

4-2-7. Western blotting measurements

Expression of PDK4, PPARα, δ and FOXO1 proteins was measured as previously described in Chapter 2: Materials and General Methods.

4-2-8. Statistical Analyses

The reported data represent means ± SEM. Statistical differences between group means were tested using one-way ANOVA's (one independent variable) with Newman-Keuls correction to allow for

post-hoc comparisons. Data were assessed for normal distributions using Shapiro-Wilk tests (SPSS v 26, IBM).

4-3. Results

4-3-1. Study 1: The effect of siRNA PPARα gene silencing on the palmitate-induced impairment of CHO oxidation in C2C12 myotubes.

4-3-1.1. Validation of siRNA PPARα gene silencing in C2C12 myotubes in the presence or the absence of palmitate.

Firstly, I established whether the transfection with siRNA was successful in knocking down the PPARα gene and protein by using real-time PCR and Western blotting, respectively. The results displayed in Fig. 4-3-1.1A show that PPARα mRNA expression was significantly reduced after siRNA treatment in the presence and the absence of palmitate ($P<0.01$). The result of Western blot also showed that siRNA intervention resulted in PPARα protein

expression being significantly decreased in the absence and the presence of palmitate (P<0.01). The result demonstrated that the transfection was successful.

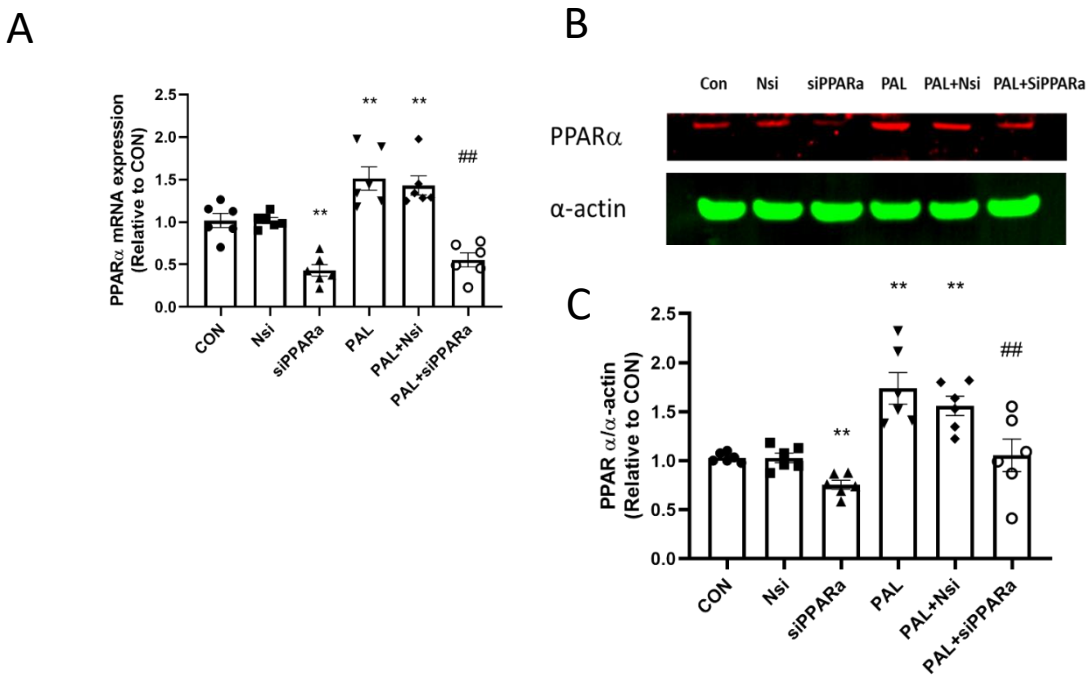


Figure 4-3-1.1. The effects of siRNA PPARα gene silencing on selective gene and protein expression in palmitate treated C2C12 myotubes.

C2C12 myotubes were treated with PPARα siRNA, 500 μM palmitate (PAL) and the combination of both interventions. **Significantly different from control (CON; P<0.01). ##Significantly different from palmitate (PAL; P<0.01). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siPPARa: PPARα siRNA.

4-3-1.2. The siRNA PPARα gene silencing reversed the palmitate-induced impairment of cellular glucose uptake in C2C12 myotubes.

Fig. 4-3-1.2A shows that siRNA PPARα gene silencing and palmitate reduced the C2C12 myotubes glucose uptake compared to control, but not in the non-specific siRNA (Nsi) group. The knockdown of PPARα *per se* increased C2C12 myotube glucose uptake (106.3±4.1% of control; $P<0.01$). Also, the knockdown of PPARα rescued the palmitate-induced glucose uptake reduction in C2C12 myotubes (85.7±2.4 vs 112.6±2.8% of control; $P<0.01$). Palmitate only intervention increased the C2C12 myotubes media lactate concentration compared to the control group (7.5±0.1 and 8.5±0.1 mmol/L, respectively; $P<0.01$). Furthermore, the knockdown of PPARα did not affect the media's lactate the absence or the presence of palmitate (Fig. 4-3-1.2B).

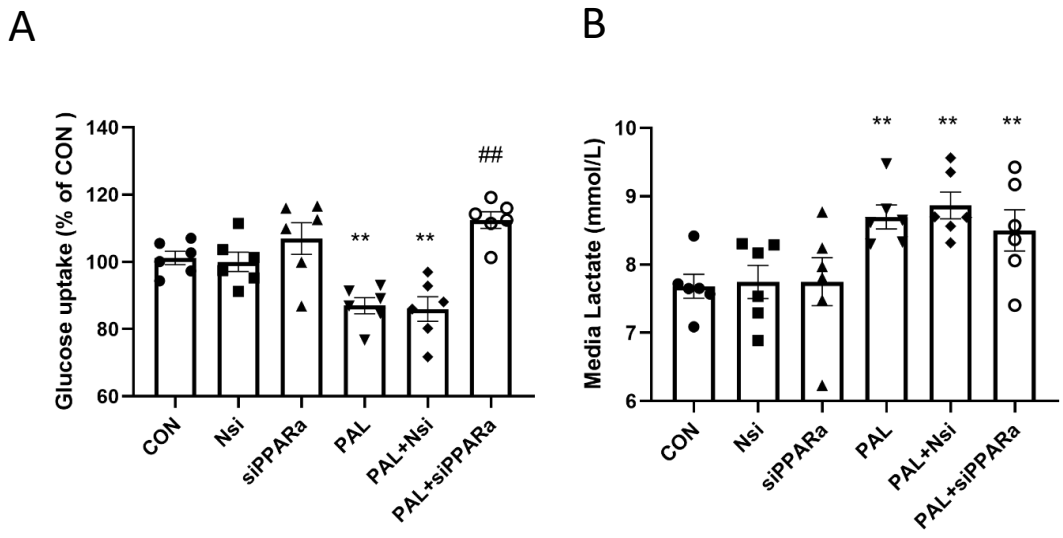


Figure 4-3-1.2. The effect of siRNA PPARα gene silencing on glucose uptake and cell media lactate concentration in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with PPARα siRNA, 500 μM palmitate (PAL) and the combination of both interventions. (A) glucose uptake (B) cell media lactate concentration. **Significantly different from control (CON; $P<0.01$). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siPPARa: PPARα siRNA.

4-3-1.3. The siRNA PPARα gene silencing reversed the palmitate-induced inhibition of PDC activity in C2C12 myotubes

The results in Fig. 4-3-1.3 show that palmitate decreased PDC activity compared to control (3.4±0.1 and 2.2±0.2 pmol acetyl-CoA/min/mg protein; $P<0.01$). Furthermore, the knockdown of PPARα significantly reversed the palmitate-induced PDC activity impairment (2.9±0.1 pmol acetyl-CoA/min/mg protein, $P<0.01$).

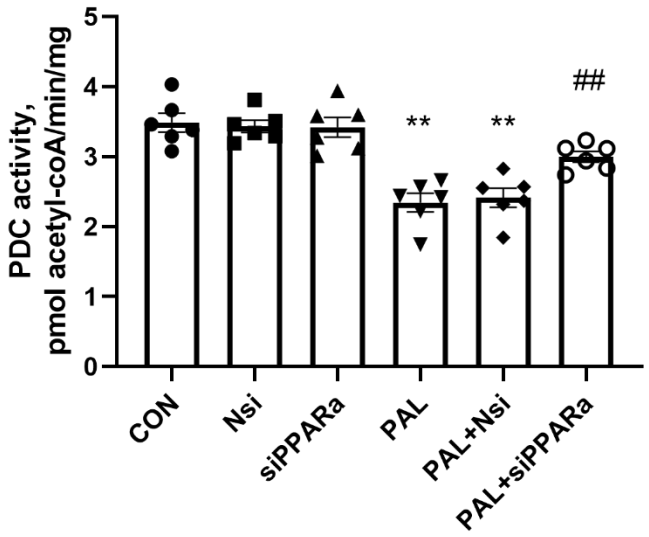


Figure 4-3-1.3. The effect of siRNA PPARα gene silencing on PDC activity in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either PPARα siRNA, 500 μM palmitate (PAL) or the combination of both interventions. **Significantly different from control (CON; $P<0.01$). ##Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siPPARa: PPARα siRNA.

4-3-1.4. The siRNA PPARα gene silencing did not change the acetylcarnitine, and acetyl-CoA content in the palmitate treated C2C12 myotubes

The content of acetylcarnitine and acetyl-CoA was measured to allow an estimation of the flux of PDC. These results are presented in Fig. 4-3-1.4A. The results show that knockdown of PPARα significantly decreased acetylcarnitine content *per se* (90.5±2.5 vs 72.6±3.6 pmol/mg protein, $P<0.01$). However, there was no change in acetylcarnitine content in the PPARα knockdown group in the presence of palmitate. The acetyl-CoA levels that are presented in Fig. 4-3-1.4B. The graphs show that acetyl-CoA concentrations in the palmitate treated groups were significantly higher than any group without palmitate. There were no differences in acetyl-CoA levels across the palmitate groups with and without PPARα knockout.

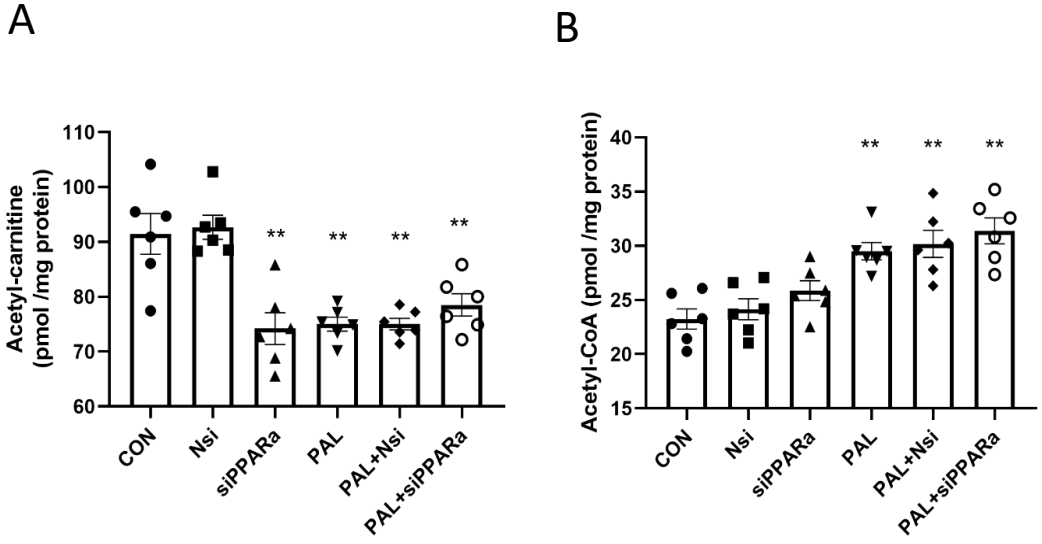


Figure 4-3-1.4. The effect of siRNA PPARα gene silencing on acetylcarnitine and acetyl-CoA content in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either PPARα siRNA, 500 μM palmitate (PAL) or with the combination of both interventions. **Significantly different from control (CON; $P<0.01$). ##Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siPPARa: PPARα siRNA.

4-3-1.5. The siRNA PPARα gene silencing restored the palmitate-induced reduction in the pyruvate derived maximal mitochondrial ATP production rates in C2C12 myotubes.

The results in Fig. 4-3-1.5 show that palmitate alone reduced MAPR compared to control (10.5±0.3 vs 7.7±0.4 nmol ATP/min/mg protein, $P<0.01$), while PPARα silencing in the absence or the presence of palmitate increased it compared to control and palmitate alone groups (12.5±0.1 and 10.1±0.2 nmol ATP/min/mg protein, respectively; $P<0.01$ and $P<0.01$).

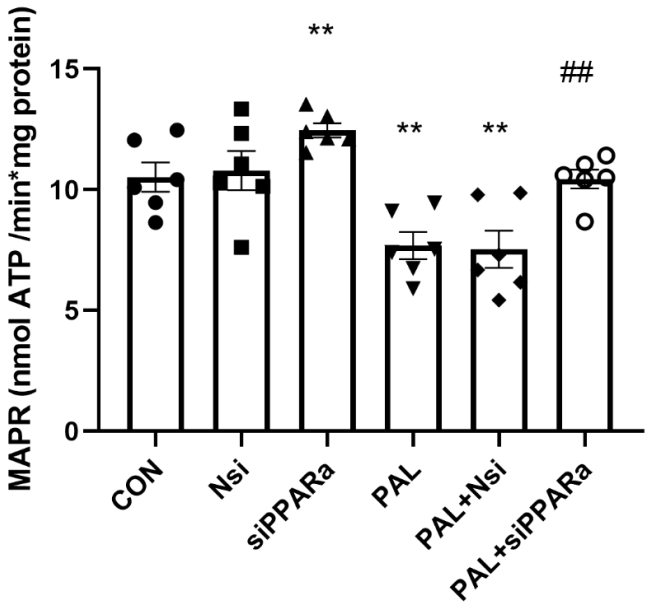


Figure 4-3-1.5. The effect of siRNA PPARα gene silencing on the pyruvate derived maximal mitochondrial ATP production rates in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either PPARα siRNA, 500 μM palmitate (PAL) or the combination of both interventions. **Significantly different from control (CON; $P<0.01$). ##Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siPPARa: PPARα siRNA.

4-3-1.6. The siRNA PPARα gene silencing did not change the PDK4 protein expression in the presence of palmitate in C2C12 myotubes.

The effect of PPARα siRNA treatment on PDK4 protein expression is shown in Fig 4-3-1.6. The PDK4 protein expressions in C2C12 myotubes in the palmitate treated groups were significantly higher than any group without palmitate. There were no differences in PDK4 protein levels across the palmitate groups with and without PPARα knockout.

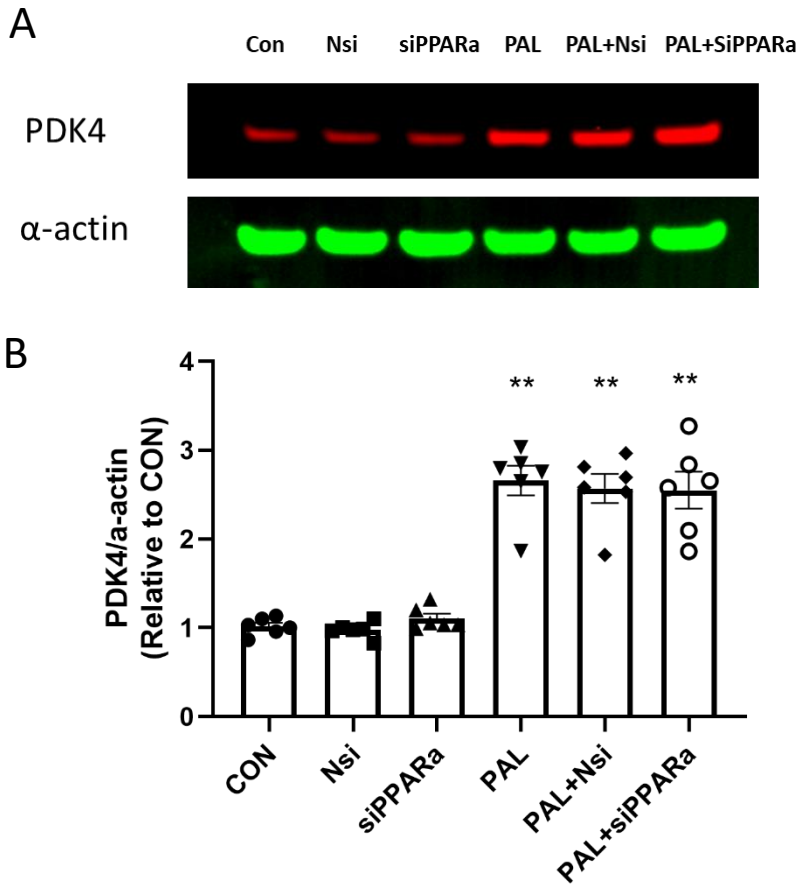


Figure 4-3-1.6. The effect of siRNA PPARα gene silencing on the PDK4 protein expression in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either PPARα siRNA, 500 μM palmitate (PAL) or the combination of both interventions. **Significantly different from control (CON; $P<0.01$). ##Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siPPARa: PPARα siRNA.

4-3-1.7. The siRNA PPARα gene silencing decreased phosphorylated FOXO1 and total FOXO1 protein expressions in the presence of palmitate in C2C12 myotubes.

I explored further whether PPARα silencing could interact with the expression of PPARδ and FOXO1 proteins. The results in Fig. 4-3-1.7B show that siRNA PPARα silencing had no significant effect on PPARδ protein expression. However, the knockdown of PPARα in the presence of palmitate reduced the phosphorylated FOXO1 (0.8±0.1 vs 0.55±0.1 relative to CON, $P<0.05$, Fig. 4-3-1.7 C) and total-FOXO1 (1.4±0.1 vs 0.8±0.2 relative to CON, $P<0.01$: Fig. 4-3-1.7 D) protein expression, while the p-FOXO1/t-FOXO1 ratio increased (0.7±0.1 vs 1.0±0.1 relative to CON, $P<0.01$: Fig. 4-3-1.7 E).

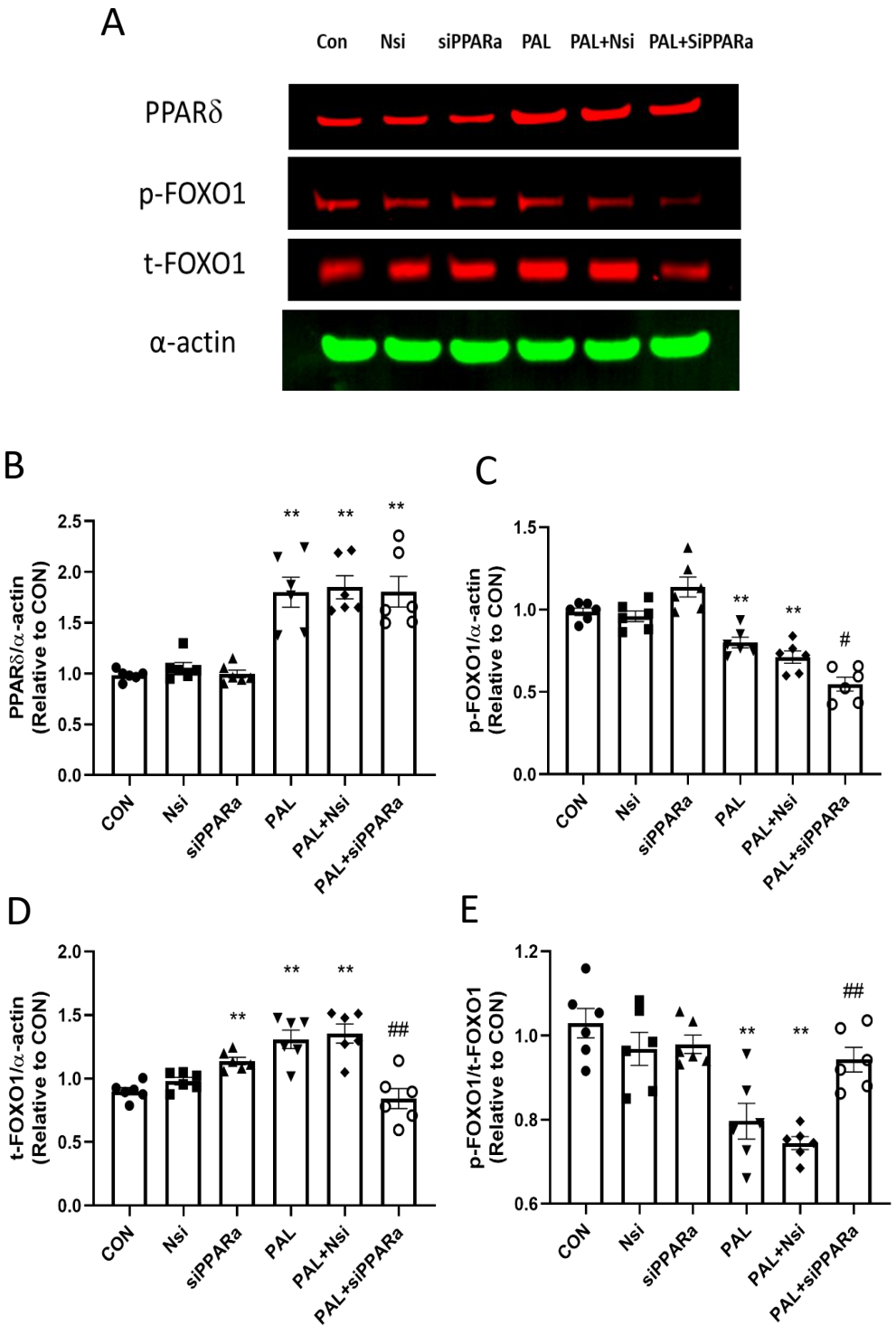

A
Con
Nsi
siPPARa
PAL
PAL+Nsi
PAL+SiPPARa
PPARδ
p-FOXO1
t-FOXO1
α-actin
B
PPARδ/α-actin (Relative to CON)
C
p-FOXO1/α-actin (Relative to CON)
D
t-FOXO1/α-actin (Relative to CON)
E
p-FOXO1/t-FOXO1 (Relative to CON)
CON
Nsi
siPPARa
PAL
PAL+Nsi
PAL+siPPARa

Figure 4-3-1.7. The effect of PPARα silencing on PPARδ, phosphorylated FOXO1, and total FOXO1 protein expression in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either PPARα siRNA, 500 μM palmitate (PAL) or the combination of both interventions. **Significantly different from control (CON; $P<0.01$). #, ## Significantly different from palmitate (PAL; $P<0.05$ and $P<0.01$, respectively). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siPPARa: PPARα siRNA.

4-3-2 Study 2: the effects of siRNA PPARδ gene silencing on the palmitate-induced impairment of CHO oxidation

4-3-2.1. Validation of siRNA PPARδ gene silencing in C2C12 myotubes in the presence or the absence of palmitate.

In an early stage, I established whether the transfection of the PPARδ gene with siRNA was successful. This was verified at transcriptional and translational levels by real-time PCR and Western blotting techniques, respectively. The results showed in Fig. 4-3-2.1 presents that PPARδ mRNA expression was significantly reduced after siRNA treatment in the absence and the presence of palmitate ($P<0.01$). The result of Western blot also showed that the administration of siRNA resulted in PPARδ protein expression significantly decreased in the absence and presence of palmitate ($P<0.01$). The result demonstrated that we successfully performed the transfection of PPARδ.

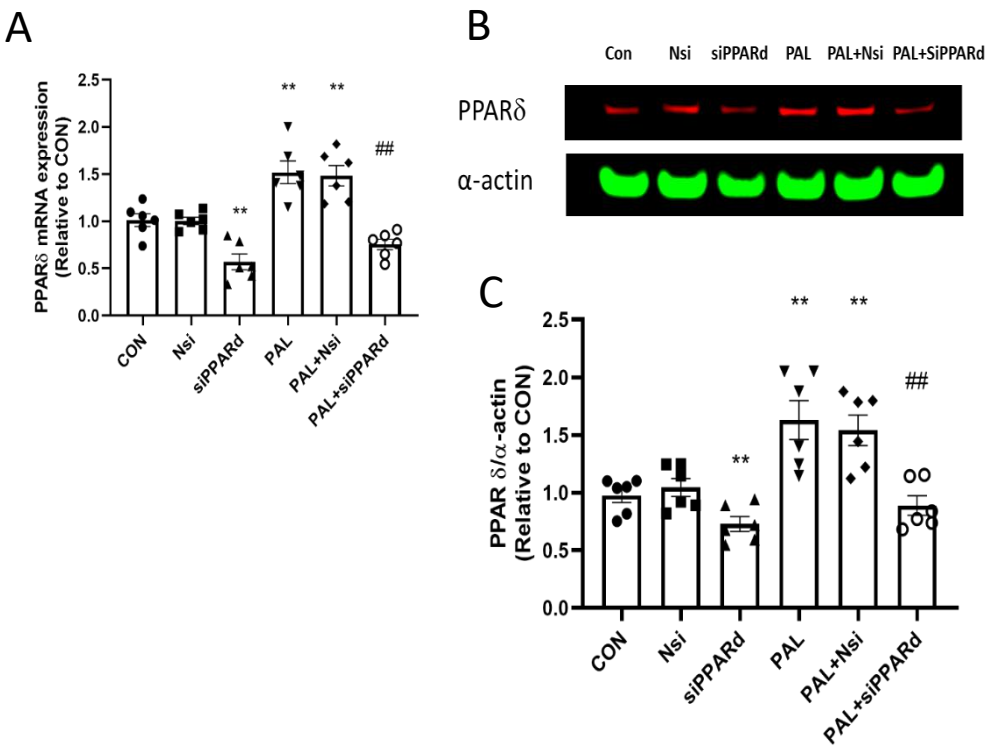


Figure 4-3-2.1. The effects of siRNA PPARδ silencing on selective gene and protein expression in palmitate treated C2C12 myotubes.

C2C12 myotubes were treated with PPARδ siRNA, 500 μM palmitate (PAL) and the combination of both interventions. **Significantly different from control (CON; $P<0.01$). ##Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siPPARd: PPARδ siRNA.

4-3-2.2. The siRNA PPARδ gene silencing reversed the palmitate-induced reduction in the cellular glucose uptake and the increase in media lactate levels in C2C12 myotubes.

Fig. 4-3-2.2A shows that palmitate reduced C2C12 myotubes glucose uptake compared to control, but the non-specific siRNA (Nsi) did not affect it. Knockdown of PPARδ *per se* increased C2C12 myotube glucose uptake ($P<0.05$; 107.3±4.1%, of control). Also, PPARδ silencing rescued palmitate-induced glucose uptake reduction in C2C12 myotubes ($P<0.01$; 85.7±2.4 vs 102.6±4.0%, of control). Palmitate alone increased C2C12 myotubes media lactate concentration compared to control ($P<0.01$, 7.5±0.1 and 8.5±0.1 mmol/L, respectively) and non-specific siRNA (Nsi) did not affect it. The knockdown of PPARδ gene alone decreased the media lactate concentration ($P<0.01$, 6.9±0.2 mmol/L). PPARδ silencing could also reverse the palmitate-induced raise in media lactate concentration ($P<0.01$, 7.4±0.3 mmol/L). (Fig. 4-3-2.2B).

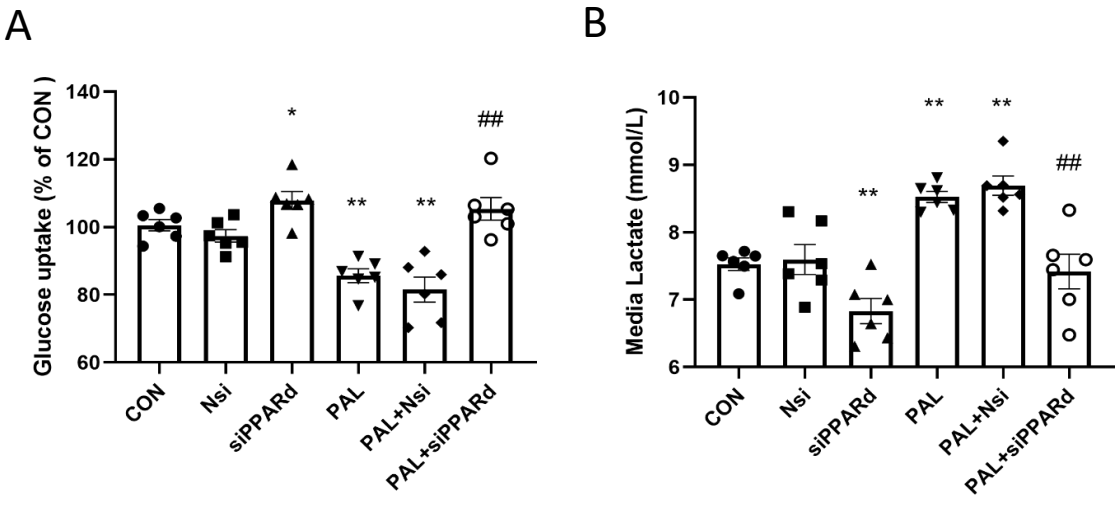


Figure 4-3-2.2. The effect of PPARδ silencing on glucose uptake and cell media lactate concentration in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with PPARδ siRNA, 500 μM palmitate (PAL) and the combination of both interventions. (A) glucose uptake (B) cell media lactate concentration. **Significantly different from control (CON; $P<0.01$). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siPPARd: PPARδ siRNA.

4-3-2.3. The siRNA PPARδ gene silencing reversed the palmitate-induced reduction in PDC activity in C2C12 myotubes.

The results in Fig. 4-3-2.3 show that palmitate alone decreased PDC activity compared to control ($P<0.01$; 3.4±0.1 and 2.2±0.2 pmol acetyl-CoA/min/mg protein, respectively) and that non-specific siRNA (Nsi) did not affect it. The knockdown of PPARδ alone increased PDC activity above control (4.0±0.2 pmol acetyl-CoA/min/mg protein; $P<0.01$). Also, PPARδ silencing significantly reversed palmitate-induced PDC activity impairment ($P<0.01$; 3.2±0.2 pmol acetyl-CoA/min/mg protein).

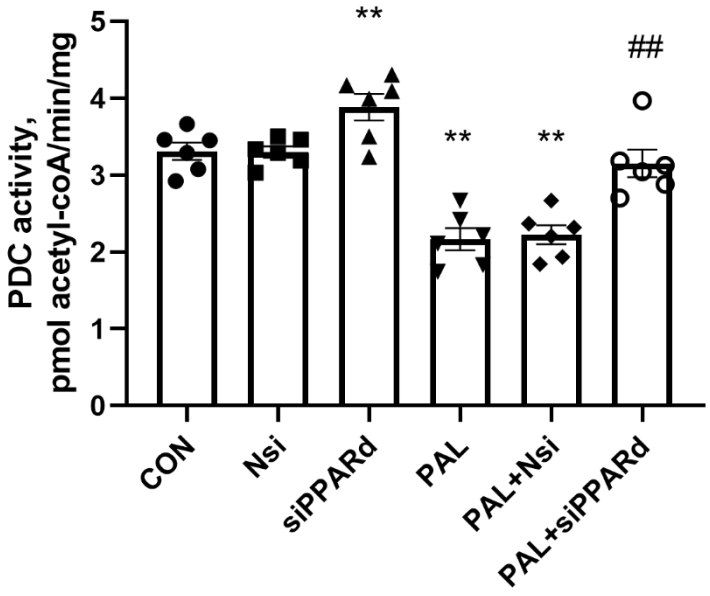


Figure 4-3-2.3. The effect of siRNA PPARδ gene silencing on PDC activity in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with PPARδ siRNA, 500 μM palmitate (PAL) and the combination of both interventions. **Significantly different from control (CON; $P<0.01$). ##Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siPPARd: PPARδ siRNA.

4-3-2.4. The siRNA PPARδ gene silencing reversed the palmitate-induced reduction in acetylcarnitine content in palmitate treated C2C12 myotubes.

Next, the content of cellular acetylcarnitine was measured. The results presented in Fig. 4-3-2.4A show that palmitate alone significantly decreased the acetylcarnitine content (90.5±2.5 and 73.4±1.7 pmol/mg protein, $P<0.01$), while PPARδ silencing with palmitate rescued the reduction (84.4±3.7 pmol/mg protein, $P<0.05$). Palmitate alone significantly increased acetyl-CoA levels (23.3±1.1 vs 29.5±0.8 pmol/mg protein, $P<0.01$). PPARδ silencing further increased the acetyl-CoA levels in the presence of palmitate (35.4±1.3 pmol/mg protein, $P<0.01$; Fig. 4-3-2.4B).

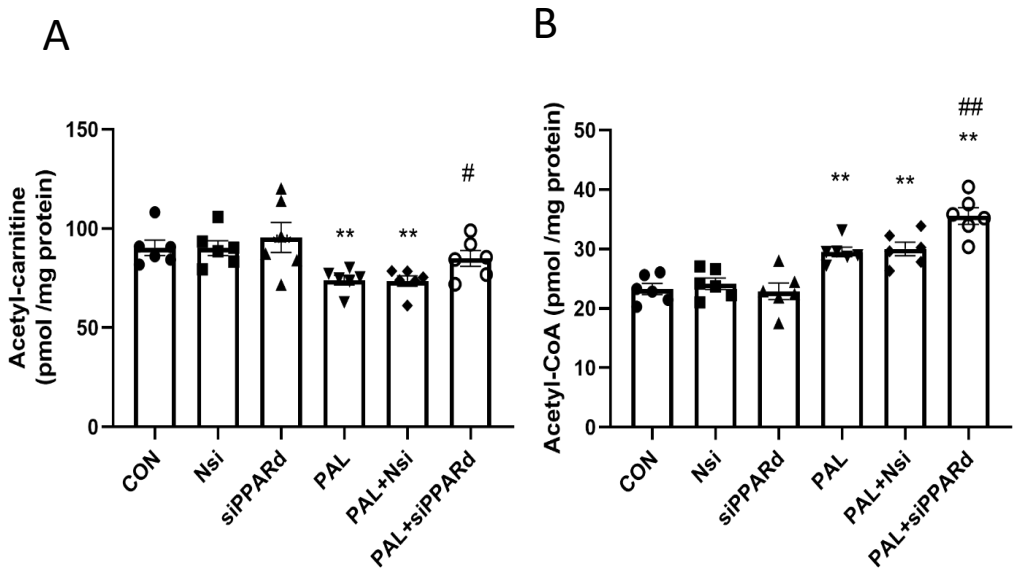


Figure 4-3-2.4. The effect of siRNA PPARδ gene silencing on acetylcarnitine and acetyl-CoA content in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with PPARδ siRNA, 500 μM palmitate (PAL) and the combination of both interventions. **Significantly different from control (CON; $P<0.01$). #, ##Significantly different from palmitate (PAL; $P<0.05$ and $P<0.01$, respectively). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siPPARd: PPARδ.

4-3-2.5. The siRNA PPARδ gene silencing reversed the palmitate-mediated reduction in pyruvate derived maximal mitochondrial ATP production in C2C12 myotubes

Fig. 4-3-2.5 shows that palmitate reduced MAPR compared to control (10.5±0.3 vs 7.7±0.4 nmol ATP/min/mg protein, $P<0.01$). PPARδ gene silencing decreased MAPR in the absence of palmitate (8.9±0.1, $P<0.01$) and the presence of palmitate (4.7±0.2 nmol ATP/min/mg protein, $P<0.01$).

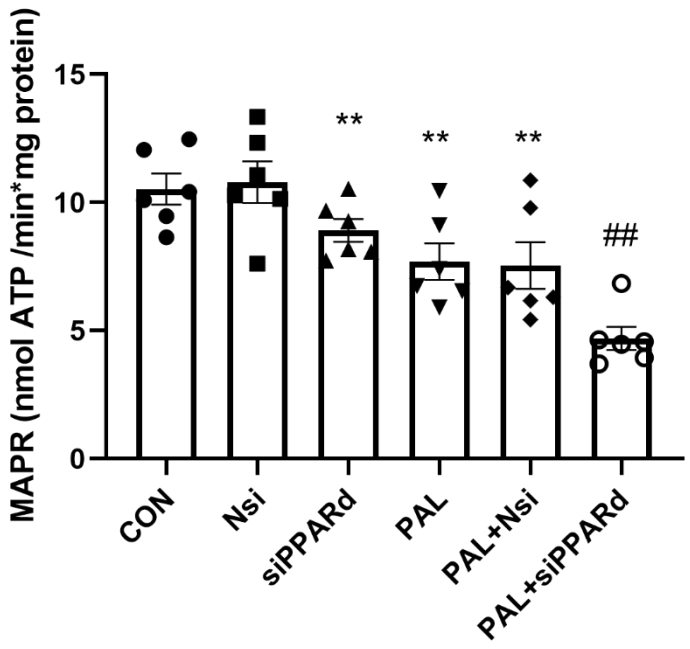


Figure 4-3-2.5. The effect of siRNA PPARδ gene silencing on the pyruvate based maximal mitochondrial ATP production rates in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with PPARδ siRNA, 500 μM palmitate (PAL) or the combination of both interventions **Significantly different from control (CON; $P<0.01$). ##Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siPPARd: PPARδ.

4-3-2.6. The siRNA PPARδ gene silencing reversed the palmitate-mediated increase in PDK4 protein expression in C2C12 myotubes

The effect of PPARδ siRNA treatment on PDK4 protein expression is shown in Fig 4-3-2.6. Palmitate increased the C2C12 myotubes PDK4 protein expression compared to control (P<0.01). However, the knockdown of PPARδ decreased the PDK4 protein expression both in the absence of palmitate (1.0±0.1 vs 0.7±0.1 relative to CON, P<0.05) and the presence of palmitate (2.2±0.2 vs 1.5±0.2 relative to palmitate, P<0.01).

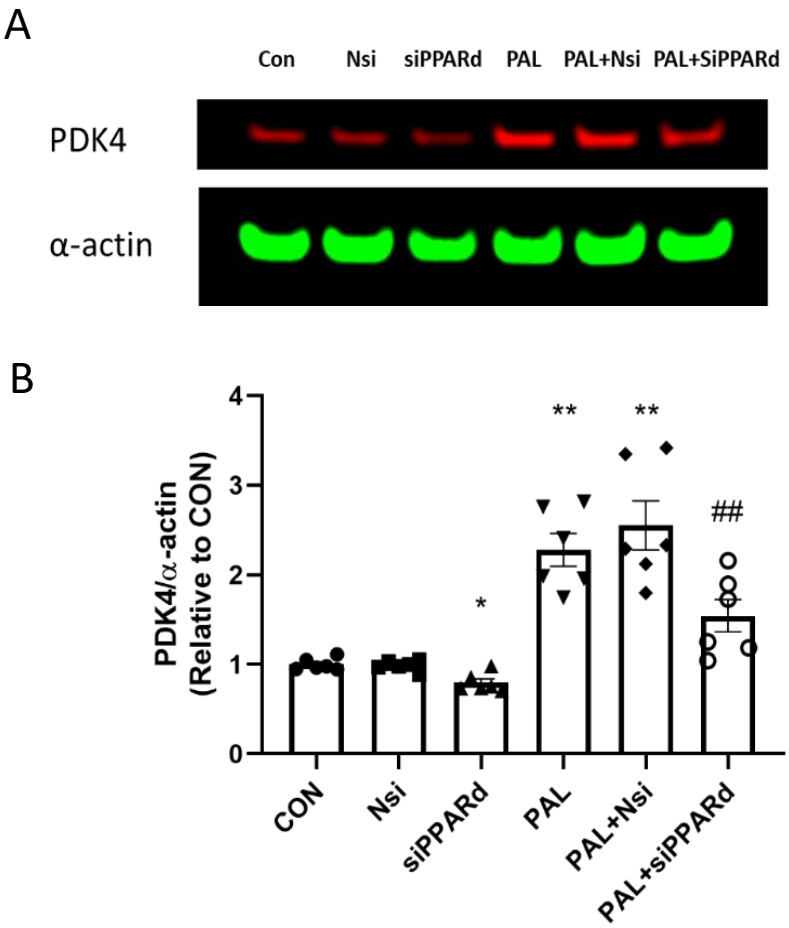


Figure 4-3-2.6. The effect of PPARδ silencing on PDK4 protein expression in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either PPARδ siRNA, 500 μM palmitate (PAL) or a combination of both interventions. *,**Significantly different from control (CON; $P<0.05$, and $P<0.01$, respectively). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siPPARd: PPARδ siRNA.

4-3-2.7. The siRNA PPARδ gene silencing reversed the palmitate-mediated increase in PPARα, phosphorylated FOXO1 and total-FOXO1 protein expression in C2C12 myotubes

The results of the siRNA PPARδ gene knockdown are shown in Fig. 4-3-2.7. PPARδ silencing decreased PPARα protein expression (1.7±0.1 vs 1.4±0.1 relative to CON, $P<0.01$) in the presence of palmitate. Also, the knockdown of PPARδ reduced phosphorylated FOXO1 (0.6±0.1 vs 0.4±0.1 relative to CON, $P<0.05$) and total FOXO1 (1.5±0.1 vs 1.1±0.1 relative to CON, $P<0.01$) protein expression in the presence of palmitate. However, there was no change in the p-FOXO1/t-FOXO1 ratio.

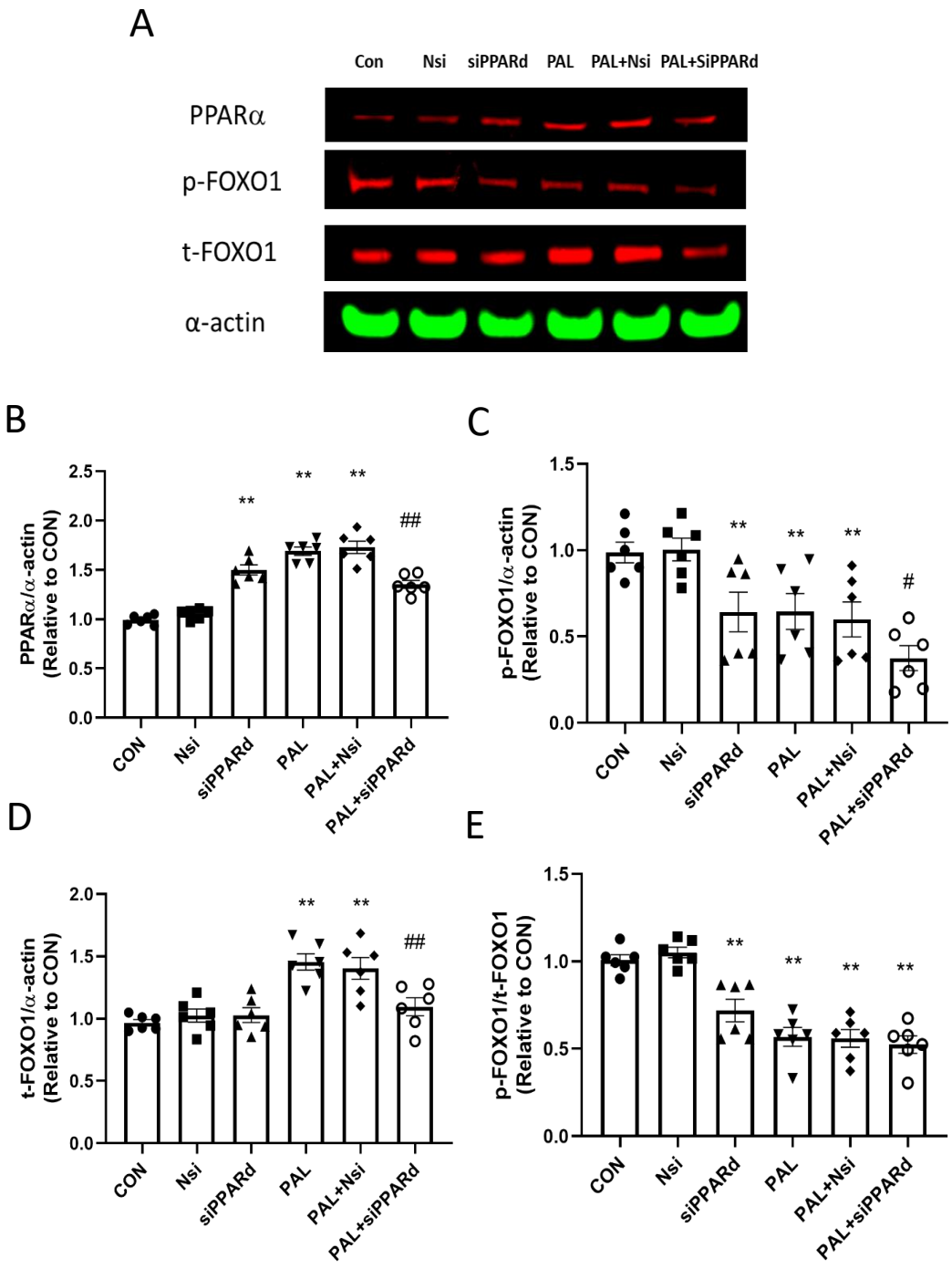

A
Con
Nsi
siPPARd
PAL
PAL+Nsi
PAL+SiPPARd
PPARα
p-FOXO1
t-FOXO1
α-actin
B
PPARα/α-actin (Relative to CON)
C
p-FOXO1/α-actin (Relative to CON)
D
t-FOXO1/α-actin (Relative to CON)
E
p-FOXO1/t-FOXO1 (Relative to CON)
CON
Nsi
siPPARd
PAL
PAL+Nsi
PAL+siPPARd

Figure 4-3-2.7. The effect of siRNA PPARδ silencing on PPARα, phosphorylated FOXO1, and total FOXO1 protein expression in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either PPARδ siRNA, 500 μM palmitate (PAL) or the combination of both interventions. **Significantly different from control (CON; $P<0.01$). #, ##Significantly different from palmitate (PAL; $P<0.05$ and $P<0.01$, respectively). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siPPARd: PPARδ siRNA.

4-3-3 Study 3: the effect of siRNA FOXO1 gene silencing on the palmitate-induced impairment of CHO oxidation in C2C12 myotubes in the presence or the absence of palmitate.

4-3-3.1. Validation of the siRNA FOXO1 gene silencing in C2C12 myotubes in the presence or the absence of palmitate.

In an early stage, I established whether the siRNA transfection of FOXO1 gene was successful. This was verified at the transcriptional and translational levels using real-time PCR and Western blotting techniques, respectively. The results showed in Fig. 4-3-3.1 shows that transfection was successful as that FOXO1 mRNA expression was significantly reduced after siRNA treatment in the absence and presence of palmitate ($P<0.01$). Additionally, the Western blot results also confirmed the silencing of both FOXO1 gene and protein in the absence and presence of palmitate ($P<0.01$). Collectively, the results confirmed we successfully performed the knockdown of FOXO1 gene through siRNA transfection.

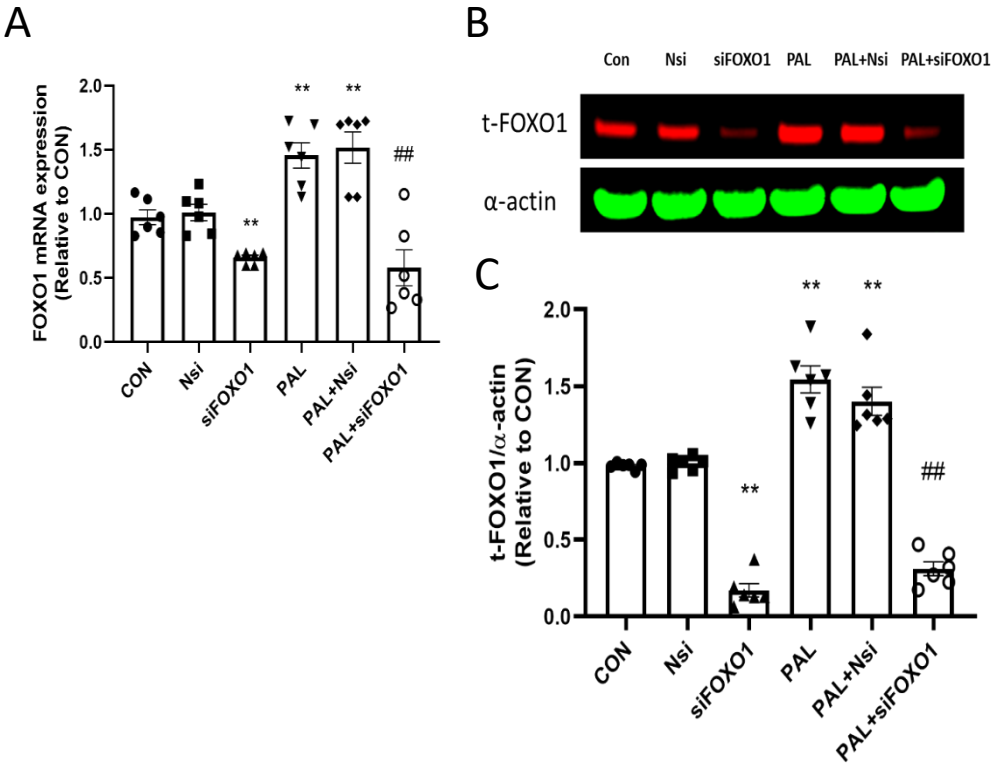


Figure 4-3-3.1. FOXO1 gene and protein expression after its silencing in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either FOXO1 siRNA, 500 µM palmitate (PAL) or a combination of both interventions. **Significantly different from control (CON; $P<0.01$). ##Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siFOXO1: FOXO1 siRNA.

4-3-3.2. The siRNA FOXO1 gene silencing reversed the palmitate-induced impairment of the cellular glucose uptake and increase in media lactate level in C2C12 myotubes.

Fig. 4-3-3.2A shows that compared to control, palmitate reduced C2C12 myotubes glucose uptake with and without FOXO1 gene silencing, and that non-specific siRNA (Nsi) had no effect on it. FOXO1 silencing rescued the palmitate-induced glucose uptake reduction in C2C12 myotubes (P<0.01; 85.7±2.4 vs 114.3±2.2%, of control). Palmitate alone increased C2C12 myotubes media lactate concentration (7.5±0.1 and 8.5±0.1 mmol/L, respectively P<0.01) compared to control. The non-specific siRNA (Nsi) did not affect it. Also, the FOXO1 gene silencing reversed the palmitate-induced increase in media lactate concentration (P<0.05, 8.0±0.2 mmol/L). (Fig. 4-3-3.2B).

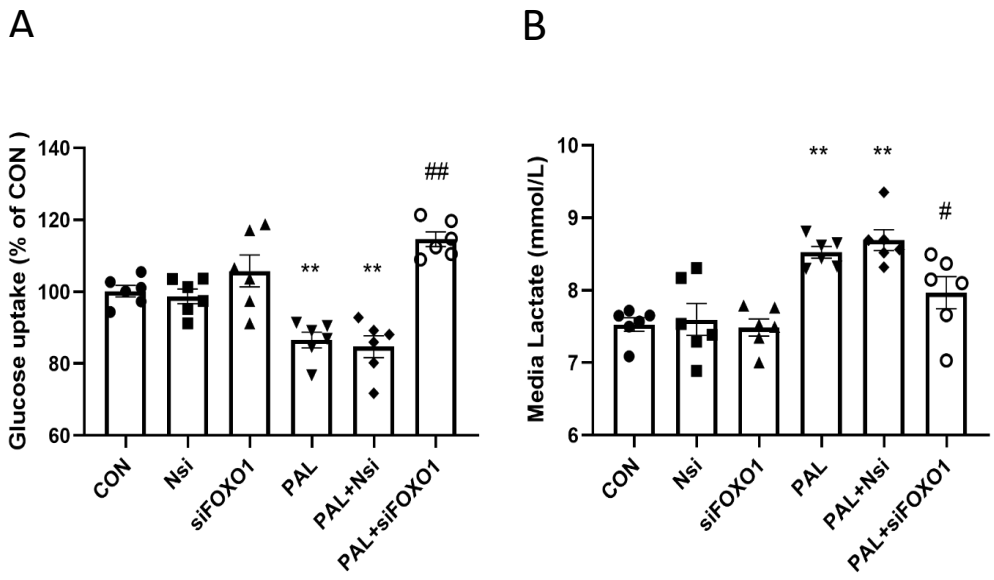


Figure 4-3-3.2. The effect of FOXO1 gene silencing on glucose uptake and cell media lactate concentration in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either FOXO1 siRNA, 500 µM palmitate (PAL) or a combination of both interventions. (A) glucose uptake (B) cell media lactate concentration. **Significantly different from control (CON; $P<0.01$). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siFOXO1: FOXO1 siRNA.

4-3-3.3. The siRNA FOXO1 gene silencing reversed the palmitate-induced reduction in PDC activity in C2C12 myotubes

The results in Fig. 4-3-3.3 show that palmitate decreased PDC activity compared to control ($P<0.01$; 3.4±0.1 and 2.2±0.2 pmol acetyl-CoA/min/mg protein, respectively). The non-specific siRNA (Nsi) did not affect it. However, we found that FOXO1 silencing reversed significantly the palmitate-induced impairment of PDC activity (2.9±0.3 pmol acetyl-CoA/min/mg protein; $P<0.01$).

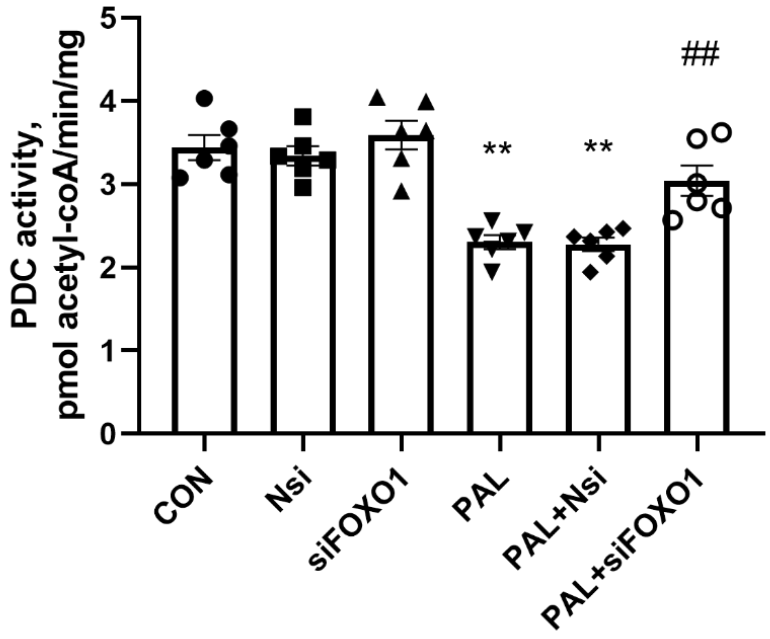


Figure 4-3-3.3. The effect of FOXO1 gene silencing on PDC activity in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either FOXO1 siRNA, 500 µM palmitate (PAL) or the combination of both interventions **Significantly different from control (CON; $P<0.01$). ##Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siFOXO1: FOXO1 siRNA.

4-3-3.4. The siRNA FOXO1 gene silencing reversed the palmitate-induced reduction in the acetylcarnitine content in C2C12 myotubes.

The content of acetylcarnitine was measured to allow an estimation of the flux through the PDC reaction. The results are shown in Fig. 4-3-3.4A. Palmitate alone decreased significantly the cellular acetylcarnitine content, which was reversed by FOXO1 silencing (90.5±2.5, 73.4±1.7 and 82.7±4.0 pmol/mg protein, $P<0.01$ and $P<0.05$). On the other hand, palmitate significantly increased acetyl-CoA levels (23.3±1.1 vs 29.5±0.8 pmol/mg protein, $P<0.01$). Also, FOXO1 silencing increased acetyl-CoA levels in the presence of palmitate (33.4±1.1 pmol/mg protein, $P<0.05$; Fig. 4-3-3.4B).

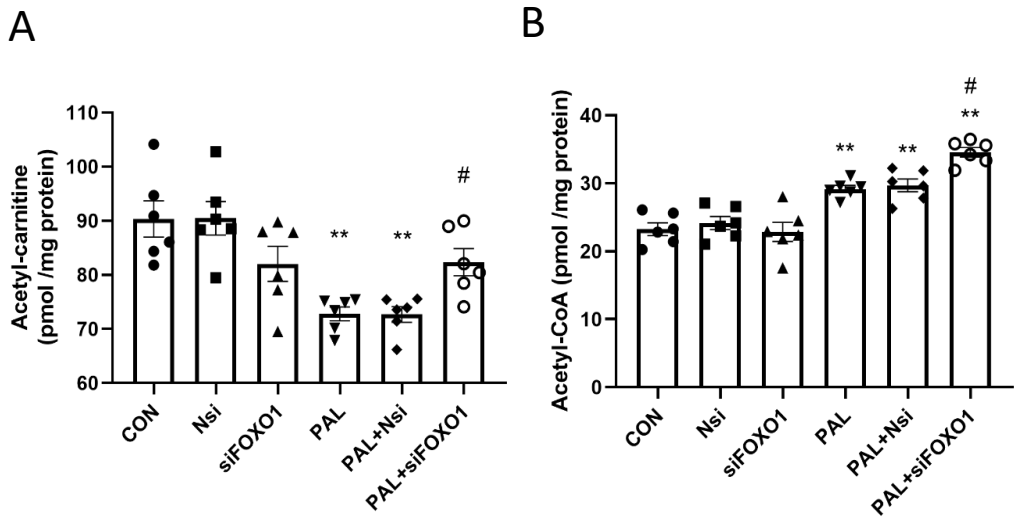


Figure 4-3-3.4. The effect of FOXO1 gene silencing on acetylcarnitine and acetyl-CoA content in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either FOXO1 siRNA, 500 µM palmitate (PAL) or a combination of both interventions. **Significantly different from control (CON; $P<0.01$). #, ##Significantly different from palmitate (PAL; $P<0.05$ and $P<0.01$, respectively). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siFOXO1: FOXO1 siRNA.

4-3-3.5. The siRNA FOXO1 gene silencing reversed the palmitate-induced reduction in the pyruvate based maximal mitochondrial ATP production rates in C2C12 myotubes

Fig. 4-3-3.5 shows that palmitate reduced MAPR compared to control (10.5±0.3 vs 7.1±0.2 nmol ATP/min/mg protein, $P<0.01$). However, FOXO1 gene silencing reversed the palmitate-induced reduction in MAPR (9.3±0.2 nmol ATP/min/mg protein, $P<0.05$).

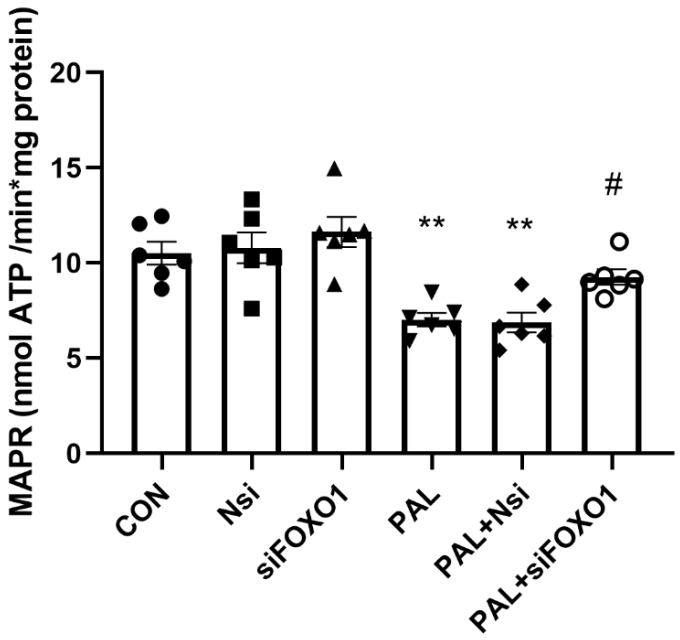


Figure 4-3-3.5. The effect of FOXO1 gene silencing on the pyruvate derived maximal mitochondrial ATP production rates in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either FOXO1 siRNA, 500 µM palmitate (PAL) or a combination of both interventions **Significantly different from control (CON; $P<0.01$). #, ##Significantly different from palmitate (PAL; $P<0.05$ and $P<0.01$, respectively). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siFOXO1: FOXO1 siRNA.

4-3-3.6. The siRNA FOXO1 gene silencing reversed the palmitate mediated increase in PDK4 mRNA and protein expression in C2C12 myotubes

The effect of FOXO1 siRNA treatment on PDK4 protein expression is shown in Fig 4-3-3.6. Palmitate alone increased the C2C12 myotubes PDK4 protein expression (P<0.01). Knockdown of FOXO1 gene was found not only to decrease the PDK4 protein expression in the absence of palmitate (1.0±0.1 vs 0.6±0.1 relative to CON, P<0.01), but it also reversed the increase observed in the presence of palmitate (3.2±0.2 vs 2.0±0.3 relative to CON, P<0.01).

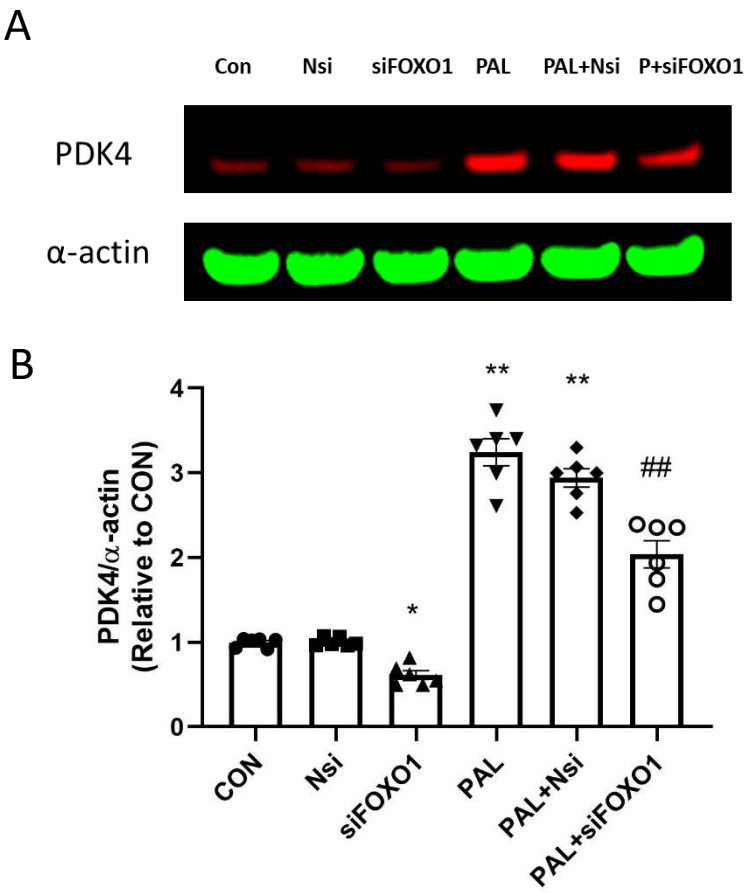


Figure 4-3-3.6. The effect of FOXO1 gene silencing on PDK4 protein expression in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either FOXO1 siRNA, 500 μM palmitate (PAL) or a combination of both interventions. **Significantly different from control (CON; $P<0.01$). ##Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siFOXO1: FOXO1 siRNA.

4-3-3.7. The siRNA FOXO1 gene silencing reversed the palmitate-induced decrease in PPARα, but not PPARδ, gene and protein expression.

The effect of FOXO1 siRNA treatment on protein expression is presented in Fig. 4-3-3.7. The FOXO1 gene silencing decreased the PPARα protein expression (1.7±0.2 vs 1.0±0.1 relative to CON, $P<0.01$) in the presence of palmitate. However, the FOXO1 gene silencing did not change the PPARδ protein expression with palmitate treatment.

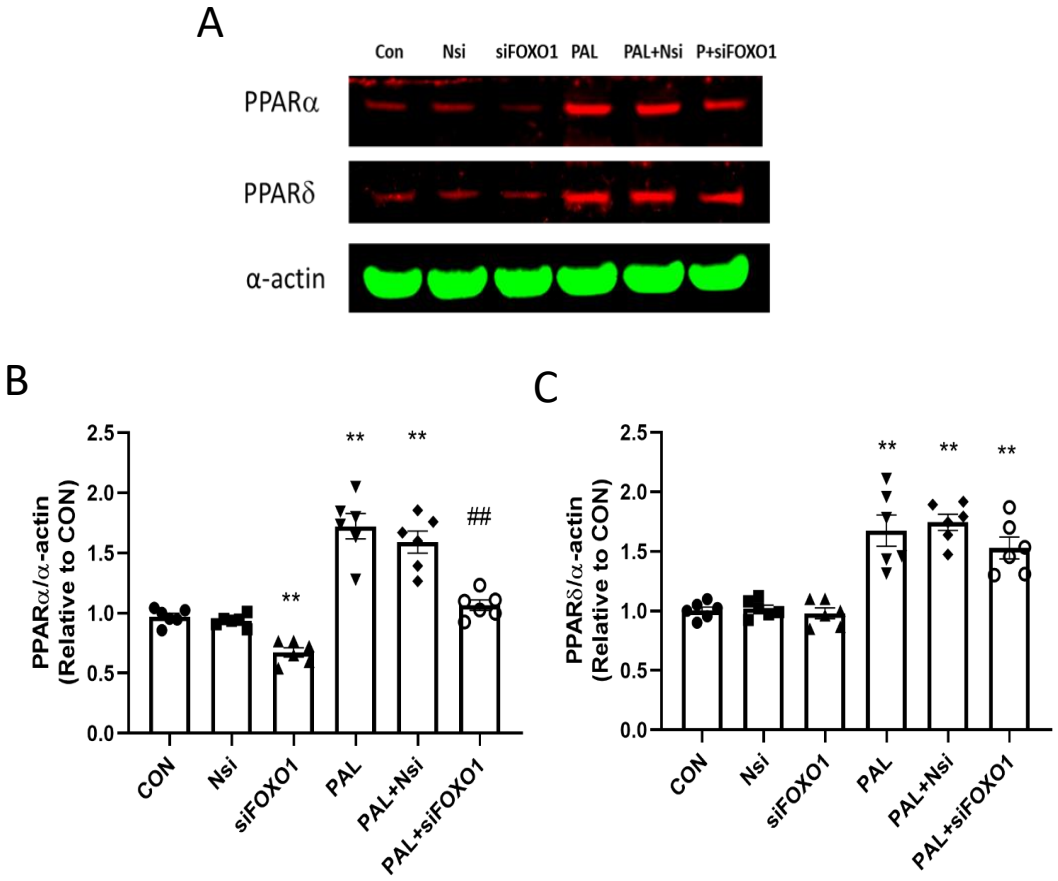


Figure 4-3-3.7. The effect of siRNA FOXO1 gene silencing on PPARα and PPARδ protein expression in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either FOXO1 siRNA, 500 µM palmitate (PAL) or a combination of both interventions. **Significantly different from control (CON; $P<0.01$). ##Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group. Nsi: Non-specific siRNA, siFOXO1: FOXO1 siRNA.

4-4. Discussion

The main findings of chapter 4 were that palmitate-induced reduction in PDC activity was rescued by PPARδ and FOXO1 silencing, and these were matched by a decline in the PDK4 protein expression. PPARα gene silencing also restored the palmitate-induced reduction in PDC activity, but this occurred independently from changes in the PDK4 protein levels Besides, PPARα, PPARδ, and FOXO1 silencing rescued the palmitate-induced reduction in the cellular glucose uptake. In contrast, the restoration of the palmitate-induced increase and decrease in media lactate and in acetylcarnitine concentrations, respectively, were observed only after PPARδ and FOXO1 gene silencing. Concerning the MAPR values, PPARα and FOXO1 silencing restored the palmitate-induced reduction in MAPR, while PPARδ silencing could not rescue the palmitate-induced decline in MAPR.

It was found that while PPARδ gene silencing decreased FOXO1 protein expression, the FOXO1 gene silencing did not change PPARδ protein expression. The finding suggests that FOXO1 may be a PPARδ downstream target that regulates PDK4 protein expression. Overall, the findings of this chapter suggest that PPARα, PPARδ, and FOXO1 play important roles in the downregulation of the PDC activity, via upregulating muscle PDK4 protein and thereby downregulating CHO oxidation. However, this mechanism is not valid for PPARα, which appears to regulate the CHO oxidation independently from the action of PDK4 protein.

A consistent finding in the literature is that muscle PDC activity appears to decline with increased muscle PDK4 protein expression. For example, when the muscle is exposed to changes in FFA and insulin availability during fasting and under pathophysiological conditions such as insulin resistance and Type 2 diabetes, trauma and inflammation, or following pharmacological intervention with peroxisome proliferator-activated receptor (PPAR) agonists or statins (Wu, Peters *et al.* 2001, Degenhardt, Saramaki *et al.* 2007, Alamdari, Constantin-Teodosiu *et al.* 2008, Constantin-Teodosiu, Baker *et al.* 2009, Mallinson, Constantin-Teodosiu *et al.* 2012, Constantin-Teodosiu 2013, Klingler, Zhao *et al.* 2016). Besides, another study reported that treatment with the PPARδ agonist compound GW610742 increased muscle PDK4 protein expression *in vivo* and *ex-vivo* rat model (Constantin, Constantin-Teodosiu *et al.* 2007) and attenuated the activation of muscle PDC activity during a subsequent exercise.

It has been suggested that changes in PDK4 gene expression may be follow that of PPARα as expressions of both genes are increased during starvation. There is some evidence, although moderate, of the existance of a relationship between PPARα and PDK4 gene expression after a bout of exercise preceded by a 3-day high fat dietary intake (Constantin-Teodosiu, Constantin *et al.* 2012). Equally, FOXO1 can also up-regulate PDK4 expression since FOXO1 can directly bind the promoter region of the PDK4 gene.

Present and previous results show that overexpressing or the knockdown of PPARα, PPARδ, and FOXO1 can modulate the PDK4 gene or protein expression. Presently, it was also found

that PPARα, PPARδ, and FOXO1 silencing can rescue the palmitate-induced reduction in PDC activity. Among the three silenced genes investigated, it was found that PPARδ silencing had the highest potency of all three to rescue the activity of PDC (45%), suggesting that along with the well-established role of PPARδ in mobilising fat oxidation it has also, although no uniquely, the ability to reduce CHO oxidation via PDK4-mediated inhibition of PDC activity. The recorded responses of PDK4 protein after each gene silencing showed that PPARδ and FOXO1 genes, rather than PPARα, mediate the palmitate-induced upregulation of PDK4 protein, confirming thereby that PPARδ and FOXO1 transcription factors are de facto modulators controlling PDC activity via changes in PDK4 protein expression. PPARα may still regulate PDC activity, but no evidence suggests that it would occur via the PDK4 pathway. Interestingly, in one of our previous studies in humans where we investigated the transcriptional responses of these transcription factors along PDK4 after three days of high fat dietary intake, we found that the relationship between PPARα and PDK4 was the weakest of all (Constantin-Teodosiu, Constantin *et al.* 2012). Putting the roles of PPARδ and FOXO1 in the PDK4 modulation on the same page, we would like to contemplate that although both PPARδ and FOXO1 silencing resulted in reductions in the PDK4 protein, however, since the PPARδ silencing had nevertheless a greater recovery effect on the PDC activity in the presence of palmitate, this may suggest that overall the PPARδ might have a greater role in the modulation of CHO oxidation.

The results of this chapter show that PPARα, PPARδ, and FOXO1 silencing collectively rescued the palmitate-induced glucose uptake impairment. With this respect, silencing PPARα and FOXO1 genes induced a greater response compared to the silencing PPARδ gene. The results also showed that PPARδ and FOXO1 silencing, rather than PPARα silencing, decreased the rise in palmitate-induced cell media lactate levels. In the case of PPARδ and FOXO1 silencing, the higher recordings of PDC activity and glucose uptake matched with lower lactate levels suggest that the acetyl group formation feeding into the Krebs cycle was higher than in after PPARα silencing. Generally, there is a positive relationship between the flux through the PDC reaction and the intracellular levels acetylcarnitine accumulation, as when the rate of PDC mediated mitochondrial acetyl-CoA formation exceeds the rate of acetyl-CoA entry into the Krebs cycle, the excess acetyl group is buffered by carnitine making thereby the cellular acetylcarnitine content increase. Our results showed that PPARδ and FOXO1 silencing increased both acetyl-CoA and acetyl-carnitine levels in the presence of palmitate better than after PPARα silencing, suggestive of a higher PDC flux in the former two than in the latter case. Additionally, PPARδ and FOXO1 silencing presented lower lactate levels while PPARα silencing, despite increased glucose uptake and PDC activity, presented no change in lactate levels in the presence of palmitate. Collectively, these findings suggest that is safe to suggest that although the activity of PDC or the amount of PDC in the dephosphorylated form (PDCa) appeared to have been similar across PPARα, δ, and FOXO1 silencing the flux through the

PDC reaction after PPARδ and FOXO1 gene silencing were significantly greater than after PPARα silencing.

Interestingly, a similarity in the pattern of PDC activity modulation and its relative function after PPARδ and FOXO1 silencing was noticed. Therefore, we explored whether an interaction between PPARδ and FOXO1 may exist. The protein expression results showed that PPARδ silencing decreased FOXO1 protein expression, but FOXO1 silencing did not change the PPARδ protein expression. The results suggest that PPARδ may be an upstream modulator of FOXO1.

From a mitochondrial perspective, the MAPR responses to PPARδ silencing seemed to show a different pattern from that displayed after PPARα and FOXO1 silencing, which was that to depress further the MAPR in the presence of palmitate. In the initial instance, this finding may prove to be unexplained given that a previous *in vivo* study reported by our laboratory showed that PPARδ agonism inhibits mitochondria ATP production during 30 min of submaximal intensity electrically evoked isometric contraction (Constantin-Teodosiu, Baker *et al.* 2009). However, expression of PGC-1*α* (peroxisome-proliferator-activated receptor γ co-activator-1α) in skeletal muscle is under the control of PPAR*δ* (Hondares, Pineda-Torra et al. 2007). PGC-1α is a co-transcriptional regulation factor that induces mitochondrial biogenesis by activating different transcription factors, including nuclear respiratory factor 1 and nuclear respiratory factor 2, which activate mitochondrial transcription factor A. The latter drives

transcription and replication of mitochondrial DNA (mtDNA) (Jornayvaz and Shulman 2010). In summary, it cannot be therefore ruled out that a decline of the directly mtDNA encoded mitochondrial proteins (n=13, e.g. the cytochrome oxidase subunits 1-3, components of complex I (ND1 and ND2)) might have occurred with PPARδ silencing, which might have thereby reduced the mitochondrial function (e.g. MAPR), but not necessarily the activity of the constitutive PDC proteins, which are entirely encoded by genomic DNA.

In summary, the results in this chapter showed that PPARα, PPARδ, and FOXO1 silencing all rescued palmitate-induced glucose uptake and PDC activity. Also, PPARδ and FOXO1 silencing reversed the palmitate-induced increase in PDK4 protein. However, PPARδ gene silencing further depressed MAPR in the presence of palmitate, while PPARα and FOXO1 silencing rescued it. The results of this chapter suggest that PPARδ, FOXO1, and to a lesser extent, PPARα have significant roles in the palmitate-induced impairment of CHO metabolism.

Chapter 5
The impact of the pharmacological intervention on the palmitate-induced impairment of mitochondrial CHO oxidation

5-1. Introduction

Lifestyle intervention such as exercise, which is the most potent physiological activator of muscle PDC, along with pharmacological intervention such as administration of dichloroacetate (DCA) or R-alpha lipoic acid (RALA) as we presently have tested can prove to be viable strategies for treating muscle metabolic inflexibility as they can potentially restore cellular CHO oxidation rates through activation of PDC.

5-1-1. Dichloroacetate (DCA)

Dichloroacetate (DCA) is a pyruvate structural analogue that has been shown to inhibit all of the PDK isoforms, and it has the most significant effect on PDK2. DCA is taken up by cells by the monocarboxylate transporter (James, Jahn *et al.* 2017) and solute carrier family-5 member 8 (SLC5A8) transporter (Babu, Ramachandran *et al.* 2011) and then binds to the pyruvate binding site in the PDK N-terminal regulatory R domain, resulting in PDK activity inhibition. DCA has been used to treat mitochondrial syndromes that result in lactic acidosis, as well as pulmonary hypertension (Dromparis, Sutendra *et al.* 2010). Also, DCA is used as an anti-cancer drug by increasing mitochondrial reactive oxygen species and inducing apoptosis in glioblastoma cells (Michelakis, Sutendra *et al.* 2010). In addition, DCA has shown metabolic improvements in cardiac tissue with a hypoxic component (Handzlik, Constantin-Teodosiu *et*

al. 2018) and is effective on reverting systemic lactic acidosis related to metabolic diseases (Stacpoole, Wright *et al.* 1992).

Administration of DCA has been shown to result in the acetylation of cellular free carnitine, and coenzyme-A pools at rest to level seen during maximal exercise. DCA can also increase pyruvate flow through the PDC reaction leading to an increase in the regeneration of ATP via the oxygen-dependent pathways (Howlett, Heigenhauser *et al.* 1999). It is also worth noting that DCA can markedly reduce the level of ATP re-synthesis from oxygen-independent routes during the rest-to-work transition period (Greenhaff, Campbell-O'Sullivan *et al.* 2002). Also, DCA increases CHO oxidation and reduces blood glucose and lactate accumulation, in parallel with a decline in the PDK4 mRNA expression (Stacpoole, Moore *et al.* 1978). In patients with Type 2 diabetes and hyperlipidemia, DCA can reduce blood triglycerides, cholesterol, and VLDL levels and reduced hepatic triglyceride synthesis (Stacpoole, Moore *et al.* 1978). When DCA markedly inhibited oleate conversion to carbon dioxide in diabetic rat, the authors suggested that this occurred through DCA lowering the tissue levels of citrate in the muscles of diabetic rats (Stacpoole and Felts 1970). In humans, DCA has been shown to rescue the high-fat diet-induced PDC activity inhibition (Constantin-Teodosiu, Constantin *et al.* 2012). This study showed that DCA administration can rescue the negative effects of 3-day of high-fat diet-mediated decline in muscle PDC activation both at rest and during subsequent exercise.

DCA administration can also decrease lactate accumulation in mouse skeletal muscle during regular exercise suggestive of increased PDC activity (Hoshino, Tamura *et al.* 2015).

Collectively, these findings would highlight the inhibition of muscle-specific PDK4 by the synthetic DCA as a therapeutic target for the muscle metabolic inflexibility. However, there is presently no evidence to advocate whether DCA can increase the mitochondrial ATP turnover using glucose-derived pyruvate as a substrate during conditions of sustained high levels of FFA availability like in our present palmitate-induced C2C12 skeletal myotubes model.

5-1-2. R-Alpha Lipoic acid (RALA)

Alpha-lipoic acid (ALA) is an organosulfur compound derived from octanoic acid. ALA exists as two enantiomers (R)- (+)-lipoic acid (RLA) and (S)- (-)-lipoic acid (SLA) and RALA is the natural form. Lipoic acid is a cofactor for several mitochondria enzymes, such as pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase and thus could facilitate important mitochondrial functions related to energy delivery. Although ALA can be endogenously synthesized, it can also be acquired from dietary sources and more recently has become a common ingredient in multivitamin formulas and anti-ageing supplements. ALA stimulates glucose uptake via GLU4 activation and translocation by regulating the p38 MAPK pathway (Konrad, Somwar *et al.* 2001). Similar to insulin, ALA administration led to increased tyrosine phosphorylation of the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) (Yaworsky, Somwar *et al.* 2000). Also, ALA has been shown to improve ageing-related

mitochondrial dysfunction and to increase glucose utilisation in type 2 diabetes mellitus (Jacob, Ruus *et al.* 1999, Liu 2008). Besides, ALA has been suggested to prevent endotoxemia-induced hepatic inflammation, β-cell dysfunction, and steatohepatitis and pancreatic inflammation in rats (Tian, Hsieh *et al.* 2012, Tian, He *et al.* 2013).

Collectively, all these results demonstrate a positive effect of ALA on intermediary metabolism. Pertinent to the present investigations, ALA has also shown to regulate PDC activity. Thus, in a sporadic study of this nature, Hong YS *et al.* showed that ALA inhibits PDC activity in heart, which was mainly achieved by lowering the activity of E3, one of the PDC constitutive components, activity (Hong, Jacobia *et al.* 1999). Other studies like that reported by Korotchkina LG *et al.* demonstrated that ALA also decreases the PDK4 activity directly, thereby enhancing the PDC activity (Korotchkina, Sidhu *et al.* 2004). Based on *in vitro* studies, it has also been hypothesised that the mechanism of ALA's action may involve targets and pathways related to the activation of skeletal muscle AMP-activated protein kinase (AMPK) (Shen, Zhu *et al.* 2007). At the metabolic level, ALA increased ATP production and improved the function of mitochondria in the liver cell (Lei, Zhu *et al.* 2016). However, there is no evidence to suggest whether RALA, rather than the racemic mix of ALA, can increase the mitochondrial ATP turnover using glucose-derived pyruvate in a palmitate-induced insulin resistance model.

5-1-3 Aim of this chapter

In this chapter, the effects of the pharmacological intervention with DCA or RALA targeting the mitochondrial PDC during conditions of palmitate-induced inhibition of PDC in C2C12 skeletal muscle cells were investigated. Additionally, the effects of DCA or RALA intervention on palmitate-induced glucose uptake inhibition, acetyl-group levels, glucose-derived pyruvate mediated ATP production, and PDK4, PPARα, δ, and FOXO1 protein expression were measured.

5-2. Methods

A murine skeletal muscle cell line C2C12 was used in this experiment. On day four, after FBS differentiation, palmitate 500 µM was provided to the cells and let them grow for another 24 hr. Then, the cells were collected and underwent different experimental measurements as will be further described. DCA or RALA was given with palmitate simultaneously. Six individual experiments for each intervention group was undertaken.

5-2-1. Glucose uptake

Cellular glucose uptake was measured as previously described in Chapter 2: Materials and General Methods.

5-2-2. Cellular PDC activity measurements:

Cellular PDC activity measurements were measured as previously described in Chapter 2: Materials and General Methods.

5-2-3. Cellular acetyl group and media lactate accumulation

Cellular acetyl group and media lactate accumulation were measured as previously described in Chapter 2: Materials and General Methods.

5-2-4. MAPR

Maximal mitochondrial ATP production rates (MAPR) were measured as previously described in Chapter 2: Materials and General Methods. MAPR values were corrected for total protein content of the cellular extract, measured using the Bradford method (Bradford, 1976) as described in Chapter 2: General Methods.

5-2-5. Western blotting measurements

Expressions of PDK4, PPARα, δ and FOXO1 proteins were measured as previously described in Chapter 2: Materials and General Methods.

5-2-6. Statistical Analyses

The reported data represent means ± SEM. Statistical differences between group means were tested using one-way ANOVA's (one independent variable) with Newman-Keuls correction to allow for post-hoc comparisons. Data were assessed for normal distributions using Shapiro-Wilk tests (SPSS v 26, IBM).

5-3. Results

5-3-1. Study 4: The effect of dichloroacetate (DCA) treatment on the palmitate-induced impairment of CHO oxidation

5-3-1.1. Effect of DCA titration curve on the activity of PDC. DCA treatment rescued the palmitate-induced reduction in PDC activity in C2C12 myotubes

DCA is the sodium salt of dichloroacetic acid ($CHCl_2COOH$). The compound is well known and has a long-standing clinical career. DCA has been studied as a potential drug because the DCA stimulates pyruvate dehydrogenase complex activity by inhibiting PDK (Stacpoole 1989). Of all four PDK isoforms, DCA is a more potent pharmacological inhibitor of PDK2 and 4 protein than pyruvate (Stacpoole and Greene 1992). It can fully activate muscle PDC at rest in humans (Timmons, Gustafsson et al. 1998), thereby decreasing lactate production via shifting the metabolism of pyruvate from anaerobic towards aerobic oxidation in the mitochondria. Our laboratory has shown that infusion of the PDK4 inhibitor DCA in human

volunteers abolished the inhibitory effects of three-day high fat dietary intake on PDC activity at rest and during exercise (Constantin-Teodosiu, Constantin et al. 2012). Collectively, these findings would highlight the potential of muscle-specific PDK4 inhibition by DCA as a therapeutic target for muscle insulin-resistant states.

As there was a clear causative effect relationship between DCA administration and activity of PDC, there was however in an early phase a need to identify what concentration of DCA is required to elicit the highest PDC activity while maintaining the cell viability. Therefore, we conducted a dose-response of PDC activity experiments with C1C12 myotubes with increasing DCA concentrations. The results of this experiment are displayed in Fig. 5-3-1.1A. DCA significantly increased the C2C12 myotubes PDC activity in a dose-dependent manner up to 30 mM (3.6±0.1 (CON) vs 4.9±0.3 pmol acetyl-CoA/mg/min at 5 mM, 5.2±0.2 at 10 mM, 5.5±0.2 at 30 mM, $P<0.01$). Furthermore, DCA reversed the palmitate-induced inhibition of PDC activity (3.0±0.2 vs 4.2±0.4, pmol acetyl-CoA/mg/min, $P<0.01$; Fig. 5-3-1.1B). All subsequent experiments with DCA were executed with concentrations of DCA setup at 30 mM.

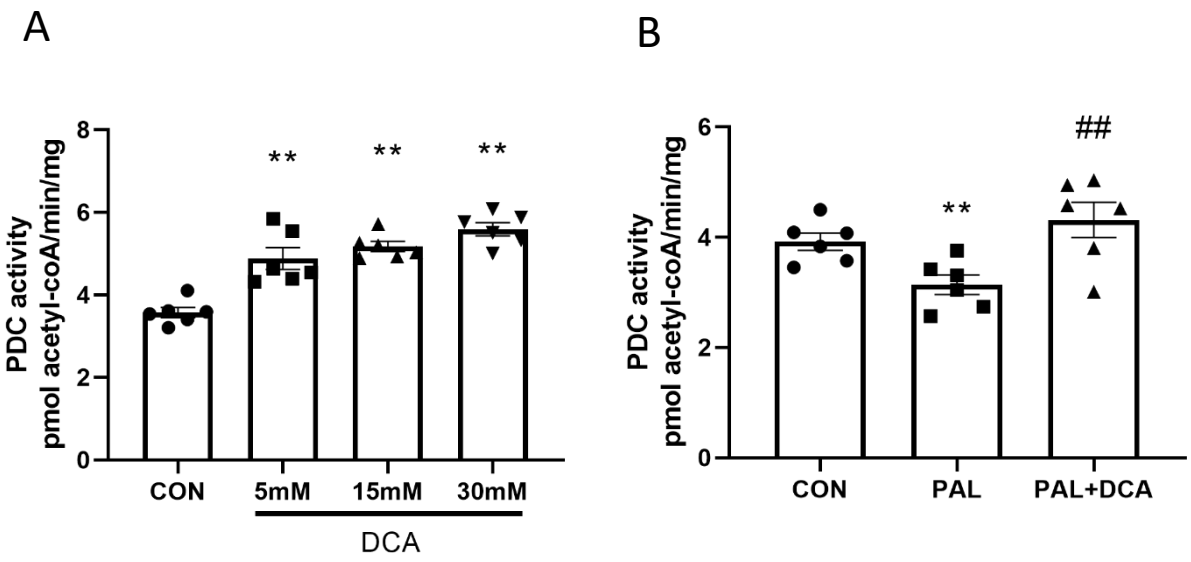


Figure 5-3-1.1. The effect of increasing levels of DCA on PDC activity in C2C12 myotubes

(A) C2C12 myotubes were treated with media only, 5, 15, and 30 mM dichloroacetate (DCA) (B) C2C12 myotubes were treated with either 30 mM DCA and 500 µM palmitate (PAL) or the combination of both interventions. **Significantly different from control (CON; $P<0.01$). ##Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group

5-3-1.2. DCA did not change the cellular glucose uptake with or without palmitate, but reversed the palmitate-mediated increase in media lactate concentration in C2C12 myotubes

The C2C12 myotubes glucose uptake after treatment with palmitate, with or without DCA, is presented below (Fig. 5-3-1.2A). Palmitate alone significantly decreased cellular glucose uptake compared to control (80.4%±2.8 of control; $P<0.01$). Addition of DCA did not show any improvement in the glucose uptake compared to palmitate alone. Palmitate alone increased C2C12 myotubes media lactate concentration (7.1±0.2 and 8.4±0.2 mmol/L, respectively; $P<0.01$). Treatment with DCA significantly decreased the cell media lactate concentrations in the presence or absence of palmitate compared to palmitate alone (6.9±0.2 mmol/L, $P<0.01$) or control group (5.8±0.2 mmol/L $P<0.01$), respectively; Fig. 5-3-1.2B.

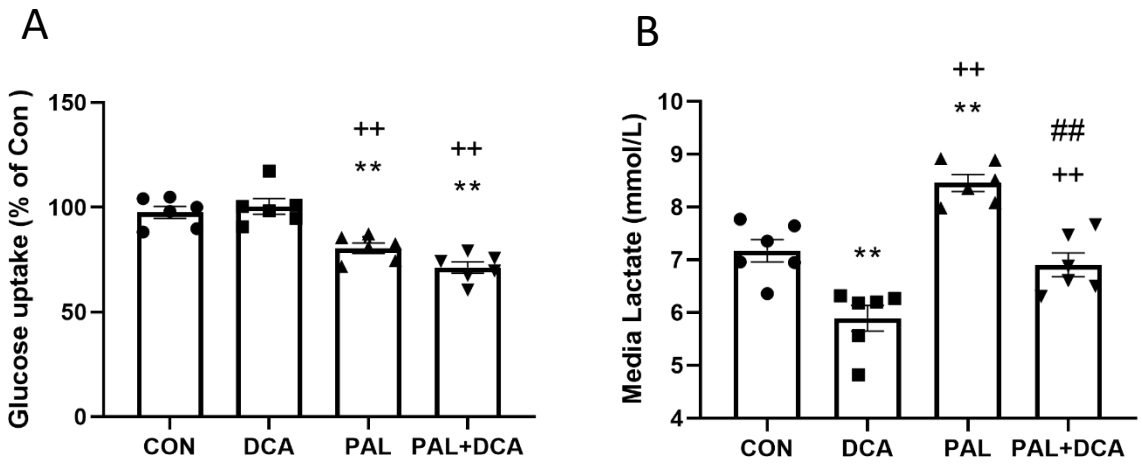


Figure 5-3-1.2. The effect of DCA on glucose uptake and cell media lactate concentration in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either 500 μM palmitate (PAL) or 30 mM dichloroacetate (DCA), or the combination of both interventions. (A) glucose uptake (B) cell media lactate concentration. **Significantly different from control (CON; $P<0.01$). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). $^{++}$Significantly different from the corresponding drug only group (DCA; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

5-3-1.3. DCA reversed the palmitate-induced reduction in the cellular acetylcarnitine and acetyl-CoA content in C2C12 myotubes.

Acetylcarnitine contents in C2C12 myotubes after palmitate administration with or without DCA administration are presented below (Fig. 5-3-1.3A). Palmitate alone decreased acetylcarnitine content (94.4±3.0 vs 82.5±3.5 pmol/mg protein, $P<0.01$) and DCA alone increased acetylcarnitine content (111.4±3.5 pmol/mg protein, $P<0.01$). Besides, DCA reversed the palmitate-induce decline in acetylcarnitine (104.7±2.9 pmol/mg protein, $P<0.01$). Also, we found that palmitate increased the intracellular acetyl-CoA in C2C12 myotubes (20.5±1.1 vs 25.3±0.7 pmol/mg protein, $P<0.01$) and that DCA further increased the amount

of acetyl-CoA in the absence or presence of palmitate (29.5±0.9 and 36.2±1.0, pmol/mg protein, $P<0.01$) (Fig. 5-3-1.3B).

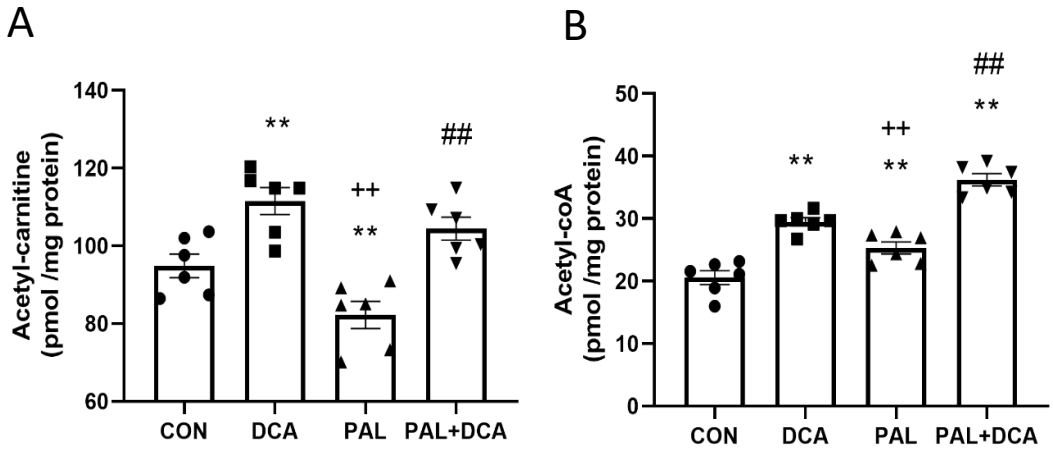


Figure 5-3-1.3. The effect of DCA on acetylcarnitine and acetyl-CoA content in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either 30 mM dichloroacetate (DCA), 500 µM palmitate (PAL) or the combination of both interventions. $^{*, **}$Significantly different from control (CON; $P<0.05$ and $P<0.01$, respectively). $^{\#, \#\#}$Significantly different from palmitate (PAL; $P<0.05$ and $P<0.0$, respectively). $^{++}$Significantly different from the corresponding drug only group (DCA; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

5-3-1.4. DCA decreased the pyruvate based maximal mitochondrial ATP production rates in the presence of palmitate in C2C12 myotube.

Fig. 5-3-1.4 shows that palmitate reduced MAPR compared to control (10.3±0.4 vs 7.2±0.4 nmol ATP/min/mg protein, $P<0.01$). We found that DCA only did not change MAPR (9.9±0.9 nmol ATP/min/mg protein, $P<0.01$). However, DCA further decreased MAPR in the presence of palmitate compared to palmitate alone (5.4±0.4 nmol ATP/min/mg protein, $P<0.01$).

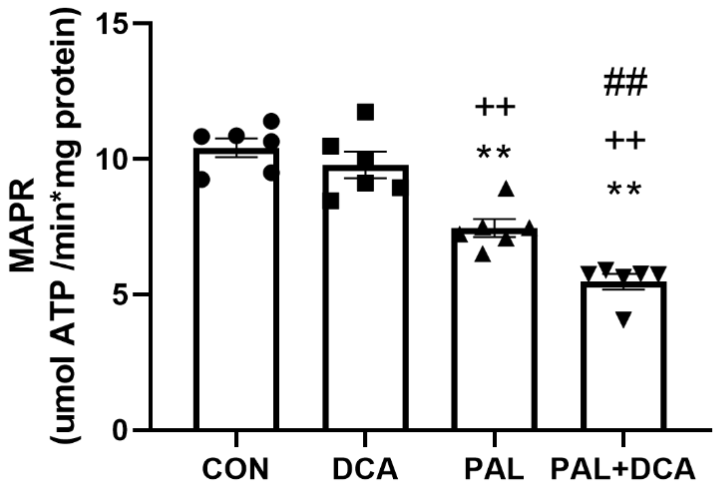


Figure 5-3-1.4. The effect of DCA treatment on maximal mitochondrial ATP production rates from pyruvate in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either 30 mM dichloroacetate (DCA), 500 μM palmitate (PAL) or the combination of both interventions. **Significantly different from control (CON; $P<0.01$). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). $^{++}$Significantly different

from the corresponding drug only group (DCA; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

5-3-1.5. DCA treatment reversed the palmitate-induced increase in PDK4 protein expression in C2C12 myotubes

The effect of DCA treatment on PDK4 protein expression is shown in Fig 5-3-1.5. Palmitate increased the C2C12 myotubes PDK4 protein expression compared to control ($P<0.01$). Conversely, DCA treatment not only reduced PDK4 protein expression in the absence of palmitate compared to control (1.0±0.1 vs 0.2±0.1 relative to CON, $P<0.01$), but also reversed the marked increase recorded with palmitate alone (2.6±0.1 vs 0.5±0.1 relative to CON, $P<0.01$).

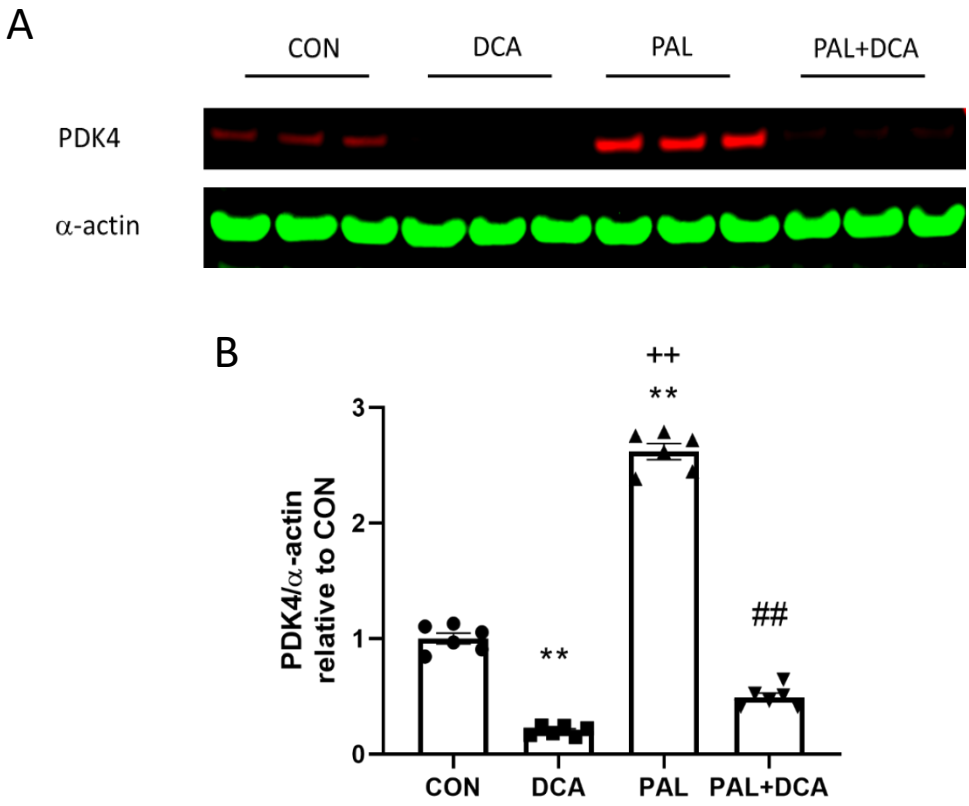


Figure 5-3-1.5. Effect of DCA on PDK4 protein expression in C2C12 myotubes in the presence or absence of palmitate.

C2C12 myotubes were treated with either 30 mM dichloroacetate (DCA), 500 µM palmitate (PAL) or the combination of both interventions. **Significantly different from control (CON; $P<0.01$). ##Significantly different from palmitate (PAL; $P<0.01$). ++Significantly different from the corresponding drug only group (DCA; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

5-3-1.6. DCA treatment decreased the palmitate-induce increase in PPARα and PPARδ protein expression

The effect of DCA in the presence and the absence of palmitate on PPARα and PPARδ protein expression are presented in Fig. 5-3-1.6. Palmitate alone significantly increased PPARα and PPARδ protein expression compared to control (P<0.01). Also, DCA significantly reversed the palmitate-induced increase in PPARα protein expression (1.5±0.1 vs 1.1±0.1 relative to CON, P<0.01). Besides, DCA rescued the palmitate-induced increase in PPARδ protein expression (2.0±0.1 vs 1.2±0.1 relative to CON, P<0.01).

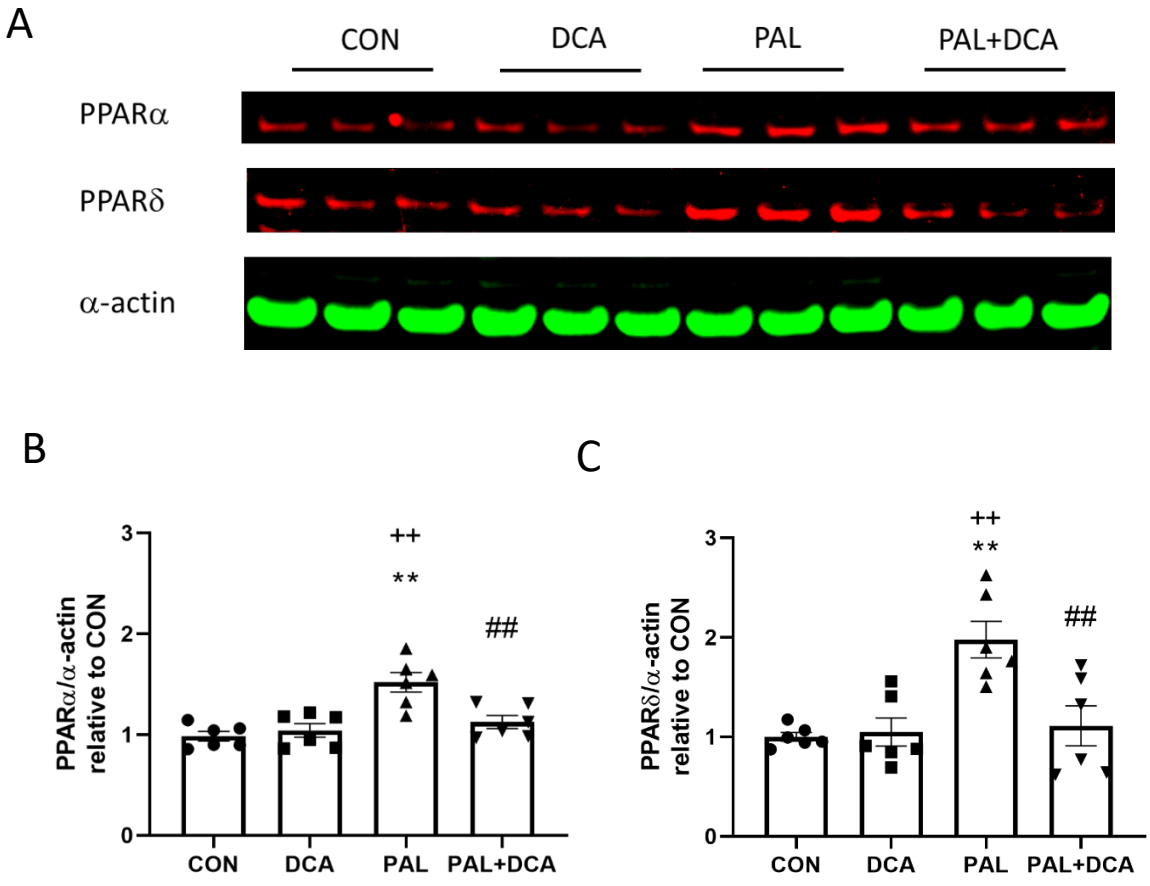


Figure 5-3-1.6. The effect of DCA treatment on PPARα and PPARδ protein expression in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either 30 mM dichloroacetate (DCA) or 500 µM palmitate (PAL) only, or with the combination of both interventions. **Significantly different from control (CON; $P<0.01$). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). $^{++}$Significantly different from the corresponding drug only group (DCA; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

5-3-1.7. DCA decreased total FOXO1 protein expression in the presence of palmitate

Lastly, to expand the knowledge on what can be achieved with DCA intervention during conditions of metabolic inflexibility we investigated whether or not DCA can reverse the palmitate mediated decline in expression of phospho-FOXO1 protein. Fig. 5-3-1.7 shows that in line with the expectation, palmitate significantly decreased phospho-FOXO1, when compared to the control group (0.9±0.1 vs 0.7±0.1, $P<0.01$). However, the treatment with DCA did not revert the effect of palmitate on phospho-FOXO1. It was also found that palmitate alone significantly increased t-FOXO1 compared to control and DCA alone groups. (1.6±0.1 and 1.2±0.1, relative to CON $P<0.01$). Collectively, the observed changes in phospho- and total FOXO1 contributed to reductions in p-FOXO1/t-FOXO1 ratio with palmitate, but not in the presence of DCA (0.6±0.1 and 0.9±0.1 relative to CON, $P<0.01$; Figure 5-3-1.7D).

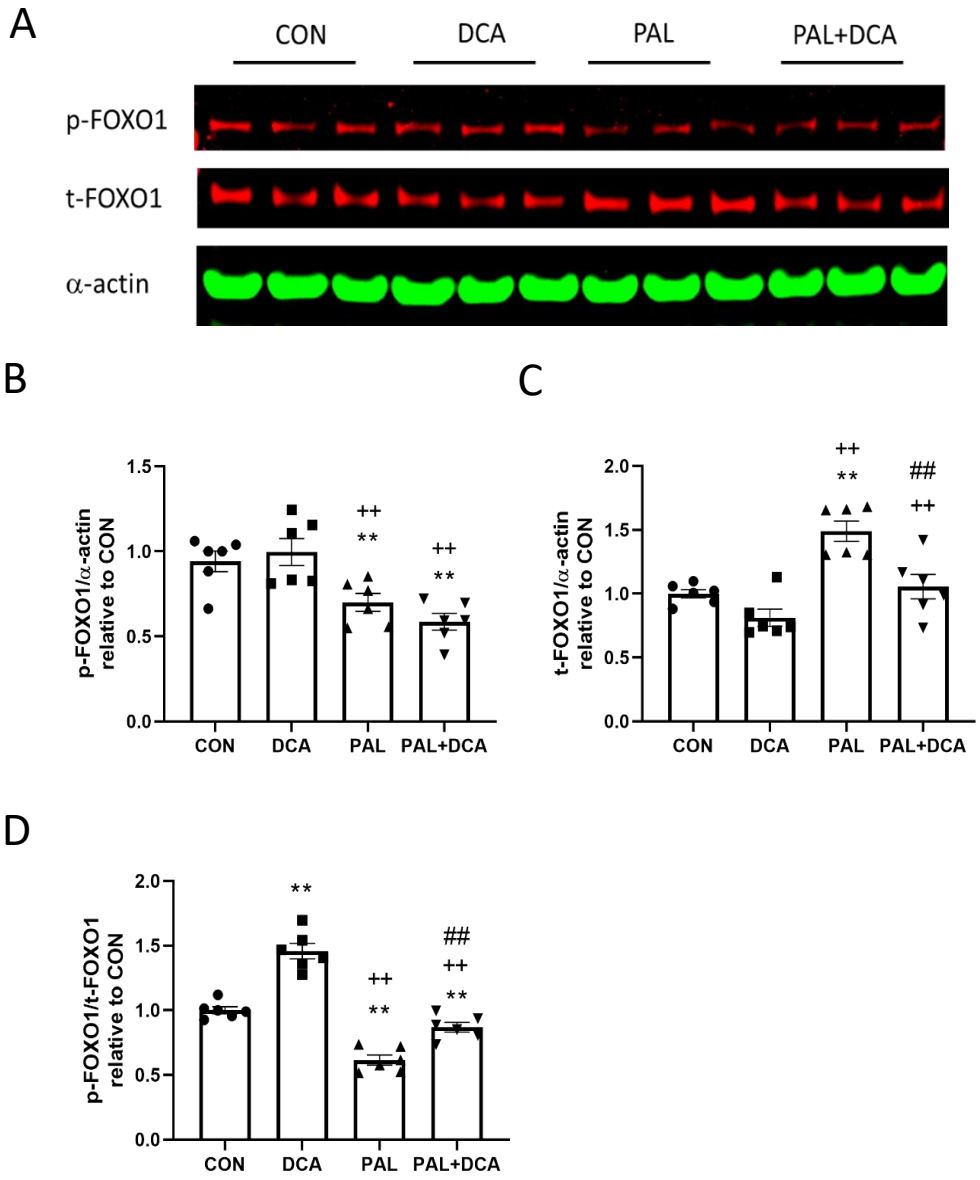


Figure 5-3-1.7. Effect of DCA on FOXO1 protein expression in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated either 30 mM dichloroacetate (DCA), 500 µM palmitate (PAL) or a combination of both interventions. **Significantly different from control (CON; $P<0.01$).

[##]Significantly different from palmitate (PAL; $P<0.01$). [++]Significantly different from the corresponding drug only group (DCA; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

5-3-2. Study 5: The effect of R-α-lipoic acid (RALA) treatment on the palmitate-induced impairment of CHO oxidation

5-3-2.1. RALA intervention reversed the palmitate-induced reduction in PDC activity in C2C12 myotubes.

Before the proposed metabolic investigations were undertaken, there was a need to demonstrate that RALA had efficacy on the activity of PDC, and if there was a clear causative relationship between RALA and activity of PDC, then there was a need to indentify what concentration of RALA would elicit the highest PDC activity. Therefore, a dose-response curve of PDC activity with increasing RALA concentrations in C1C12 myotubes was conducted. The effect of RALA administration on PDC activity of C2C12 myotubes is presented below (Fig. 5-3-2.1A). RALA enhanced PDC activity (3.8±0.1 vs 4.5±0.2 at 50 μM, 4.8±0.1 at 200 μM, 4.5±0.2 at 500 μM, pmol acetyl-CoA/mg/min, $P<0.01$). As a second endpoint of the experiment, evidence that RALA could revert the palmitate-induced inhibition of PDC activity (2.8±0.1 vs 3.2±0.1, pmol acetyl-CoA/mg/min, $P<0.01$; Fig. 5-3-2.1B) was also provided. Overall, it was clear that the activity of PDC responded linearly to increasing concentrations of RALA up to 200 μM, which concentration became the default working concentration for all subsequent experiments.

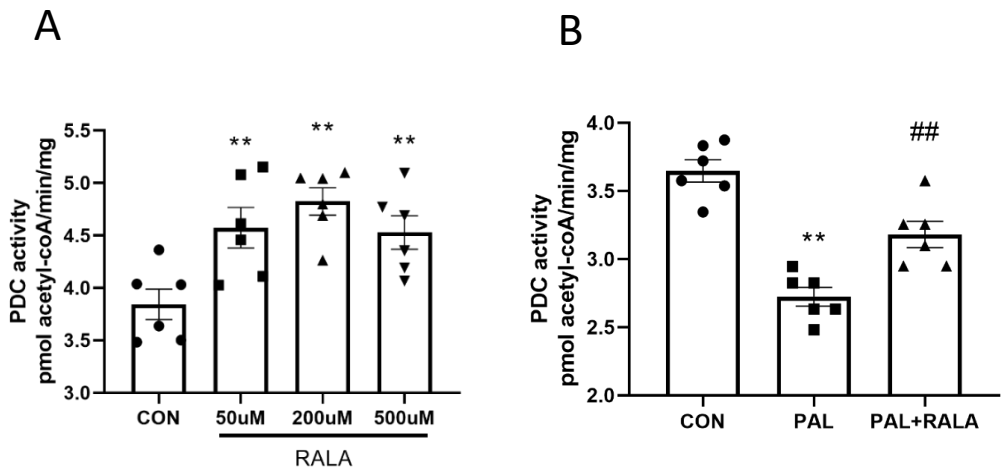


Figure 5-3-2.1. Effect of increasing RALA concentrations on PDC activity in C2C12 myotubes in the presence or the absence of palmitate.

Activity (A) C2C12 myotubes were treated with 50, 200, and 500 µM R-α-lipoic acid (RALA) (B) C2C12 myotubes were treated with 200 µM R-α-lipoic acid (RALA), 500 µM palmitate (PAL) and the combination of both interventions. **Significantly different from control (CON; $P<0.01$). ##Significantly different from palmitate (PAL; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

5-3-2.2. RALA intervention reversed the palmitate-induced inhibition of glucose uptake and increase in media lactate concentration

The mean values of the C2C12 myotubes glucose uptake recorded after treating C2C12 myotubes with palmitate with or without RALA are presented in Fig. 5-3-2.2A. Palmitate significantly decreased glucose uptake of the myotubes compared to CON (78.4%±2.0 of control; $P<0.01$). RALA only enhanced glucose uptake of C2C12 (144.0±6.5% compared to CON, $P<0.01$). Also, RALA significantly reversed the palmitate-induced inhibition of cellular glucose uptake (78.0±2.0 vs 103.0±4.0% compared to CON, $P<0.01$). Conversely, palmitate increased the C2C12 myotubes media lactate concentration ($P<0.01$, 7.0±0.1 and 8.4±0.1 mmol/L) compared to CON. It was found that RALA not only decreased cell media lactate standalone ($P<0.05$, 6.5±0.1 mmol/L), but also reversed the palmitate-induced increase in media lactate concentration (7.5±0.2 mmol/L; $P<0.01$, Fig. 5-3-2.2B).

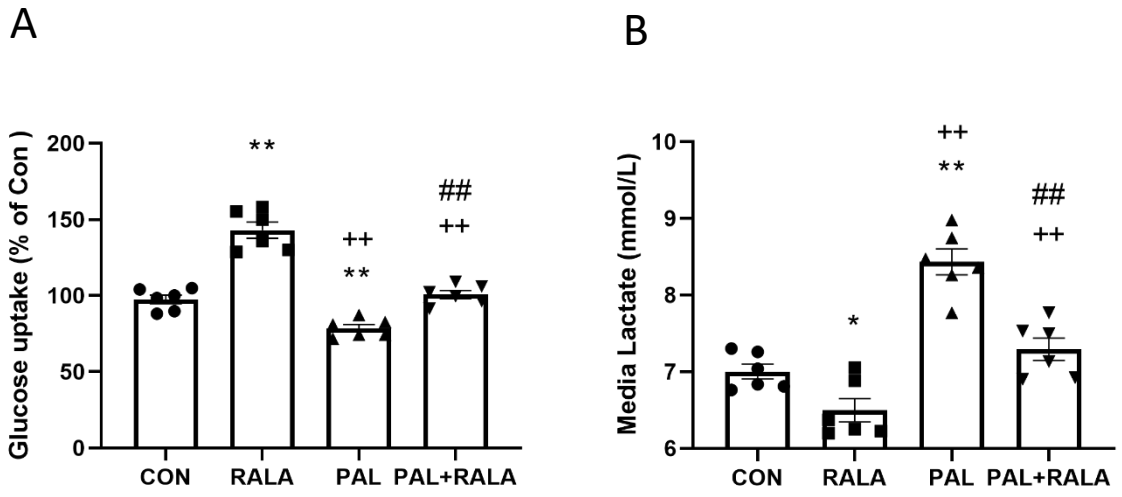


Figure 5-3-2.2. The effect of RALA treatment on glucose uptake and cell media lactate concentration in C2C12 in the presence or the absence of palmitate.

C2C12 myotubes were treated with 200 µM R-α-lipoic acid (RALA), 500 µM palmitate (PAL) or the combination of both interventions. (A) glucose uptake (B) cell media lactate concentration. *,**Significantly different from control (CON; $P<0.05$ and $P<0.01$, respectively). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). $^{++}$Significantly different from the corresponding RALA only group (RALA; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

5-3-2.3. There was no change in the acetylcarnitine, and acetyl-CoA levels in the palmitate treated C2C12 myotubes with RALA as a co-treatment.

Acetylcarnitine concentrations after palmitate administration with or without RALA administration in C2C12 myotubes are presented in Fig. 5-3-2.3. Treatment with palmitate alone decreased acetylcarnitine concentrations (93.7±4.0 vs 76.2±2.6 pmol/mg protein, $P<0.01$). The addition of RALA could not recover the palmitate-induced reduction in the cellular acetylcarnitine as there were no significant mean differences across RALA with or without palmitate (76.2±2.6 vs 78.4±2.4 pmol/mg protein, $P<0.01$). On the other hand, palmitate increased the intracellular acetyl-CoA in C2C12 myotubes (21.3±1.1 vs 24.9±1.9 pmol/mg protein, $P<0.01$). RALA treatment alone increased marginally, albeit significantly,

the acetyl-CoA content in C2C12 cells (21.3±1.1 vs 24.9±1.3 pmol/mg protein, $P<0.01$). However, there was no significant change after RALA administration with palmitate (25.5±1.6 vs 25.4±1.2 pmol/mg protein).

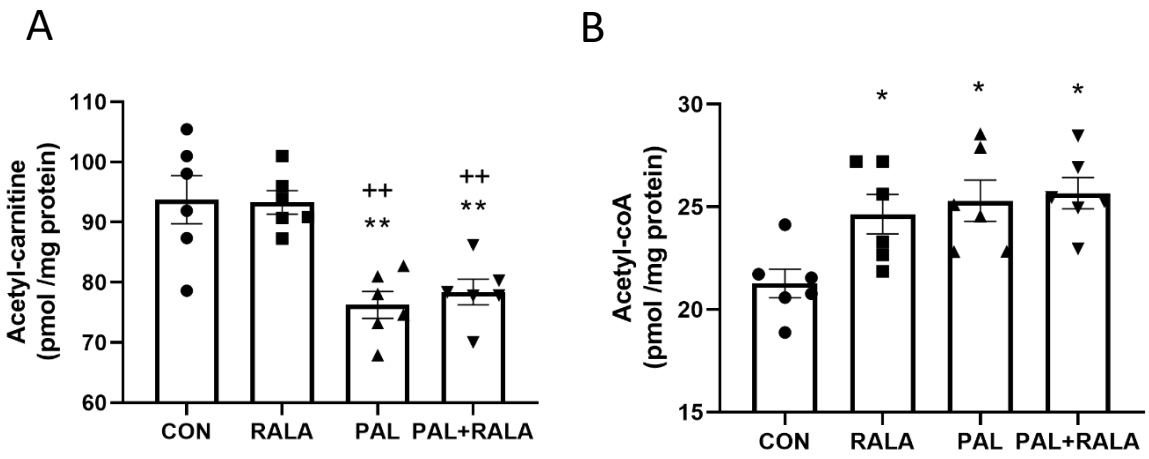


Figure 5-3-2.3. Effect of RALA on acetylcarnitine and acetyl-CoA content in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with 200 µM R-α-lipoic acid (RALA), 500 µM palmitate (PAL) and the combination of both interventions. *,**Significantly different from control (CON; $P<0.05$ and $P<0.01$, respectively). $^{++}$Significantly different from the corresponding RALA group only (RALA; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

5-3-2.4. RALA reversed the palmitate-induced reduction in the pyruvate fuelled maximal mitochondrial ATP production rates in C2C12 myotubes

It was found that palmitate reduced MAPR compared to control (10.3±0.2 vs 7.3±0.5 nmol ATP/min/mg protein, $P<0.01$; Fig. 5-4-4), while RALA only increased it (11.4±0.5, $P<0.05$). RALA could also rescue the palmitate-induced reduction in MAPR (9.5±0.4 nmol ATP/min/mg protein, $P<0.01$).

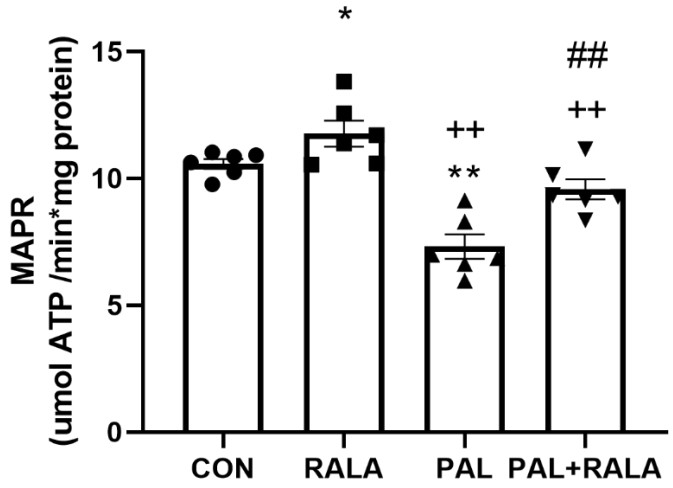


Figure 5-3-2.4. The effect of RALA on C2C12 myotubes maximal mitochondrial ATP production rates from pyruvate in the presence or the absence of palmitate.

C2C12 myotubes were treated with 200 µM R-α-lipoic acid (RALA), 500 µM palmitate (PAL) or the combination of both interventions. **Significantly different from control (CON; $P<0.01$). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). $^{++}$Significantly different from the corresponding drug only group (RALA; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

5-3-2.5. RALA increased the PDK4 protein expression in the presence of palmitate in C2C12 myotubes

The effect of RALA treatment on PDK4 protein expression is shown in Fig 5-3-2.5. Palmitate alone increased the C2C12 myotubes PDK4 protein expression compared to CON (P<0.01). The recorded results also showed that RALA treatment increased further expression of PDK4 protein in the presence of palmitate compared to palmitate alone (2.5±0.2 vs 3.3±0.2 relative to CON, P<0.01)

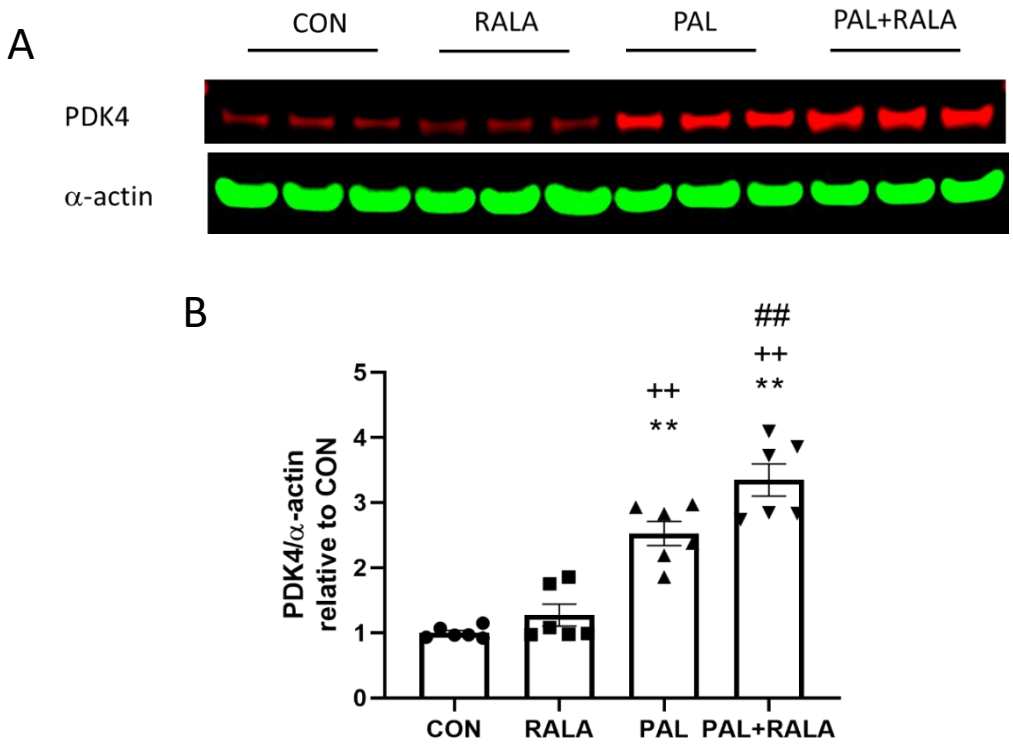


Figure 5-3-2.5. Effect of RALA intervention on PDK4 protein expression in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with 200 μM R-α-lipoic acid (RALA), 500 μM palmitate (PAL) or the combination of both interventions. **Significantly different from control (CON; $P<0.01$). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). $^{++}$Significantly different from the corresponding drug only group (RALA; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

5-3-2.6. RALA decreased the PPARα and PPARδ protein expression in C2C12 myotubes in the presence of palmitate

The effect of RALA with palmitate on PPARα and PPARδ protein expression is presented in Fig. 5-3-2.6. Palmitate significantly increased PPARα and PPARδ protein expression compared to CON ($P<0.01$). RALA alone decreased PPARα and PPARδ protein expression (1.0±0.1 vs 0.7±0.2 and 0.7±0.1 relative to CON, $P<0.01$). It was also found that RALA could rescue the palmitate-induced increase in PPARα (1.4±0.1 vs 0.9±0.1 relative to CON, $P<0.01$) and PPARδ protein expressions (1.9±0.1 vs 1.5±0.1 relative to CON, $P<0.01$).

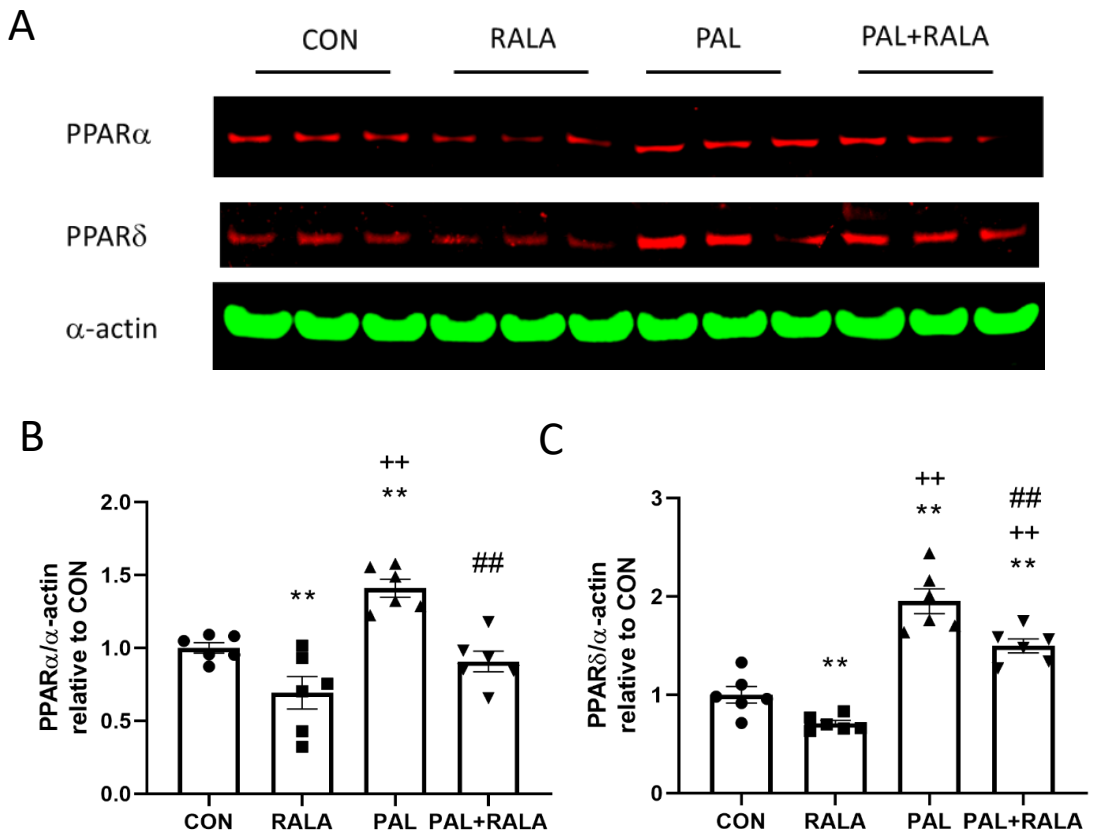
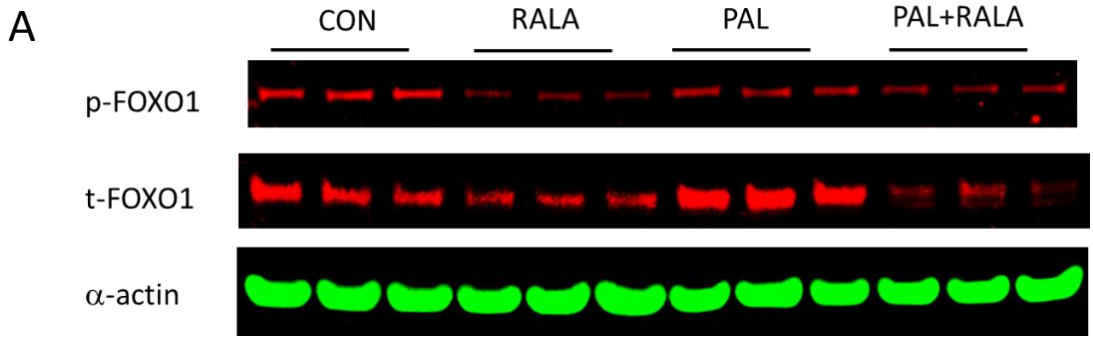


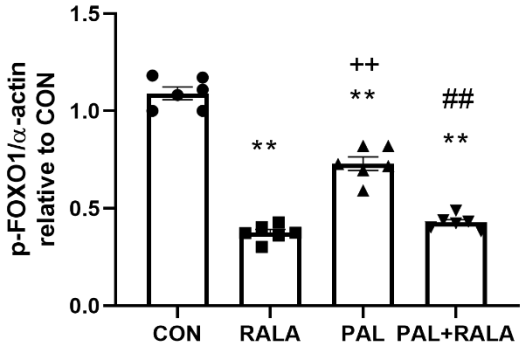
Figure 5-3-2.6. The effect of RALA treatment on PPARα and PPARδ protein expression in C2C12 myotubes in the presence or the absence of palmitate.

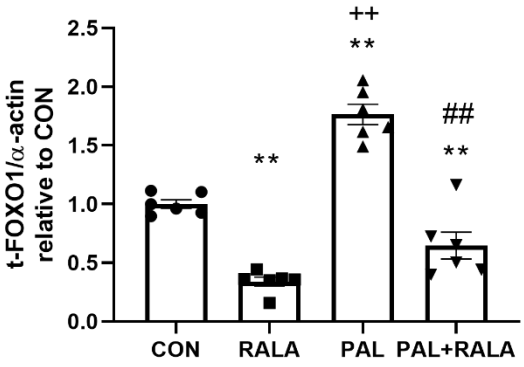
C2C12 myotubes were treated with 200 µM R-α-lipoic acid (RALA), 500 µM palmitate (PAL) and the combination of both interventions. **Significantly different from control (CON; $P<0.01$). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). $^{++}$Significantly different from the corresponding drug only group (RALA; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

5-3-2.7. RALA decreased phosphorylated and total FOXO1 protein expression in C2C12 myotubes in the presence of palmitate.

The result of FOXO1 protein expression is shown in Fig. 5-3-2.7. Palmitate alone significantly increased FOXO1 protein expression compared to the control group ($P<0.01$). RALA alone treatment decreased the phosphorylated and total-FOXO1 protein expression (1.0±0.1 vs 0.4±0.1 and 0.3±0.1 relative to CON, $P<0.01$). Besides, RALA decreased the phosphorylated FOXO1 (0.7±0.1 vs 0.4±0.1 relative to CON, $P<0.01$), and total-FOXO1 protein expression (1.7±0.1 vs 0.7±0.1 relative to CON, $P<0.01$), while the p-FOXO1/t-FOXO1 ratio increased (0.3±0.1 vs 0.6±0.1 relative to CON, $P<0.01$) in the presence of palmitate.


A
CON
RALA
PAL
PAL+RALA
p-FOXO1
t-FOXO1
α-actin


B
p-FOXO1/α-actin relative to CON
1.5
1.0
0.5
0.0
**
++
**
##
**
CON
RALA
PAL
PAL+RALA


C
t-FOXO1/α-actin relative to CON
2.5
2.0
1.5
1.0
0.5
0.0
**
++
**
##
**
CON
RALA
PAL
PAL+RALA

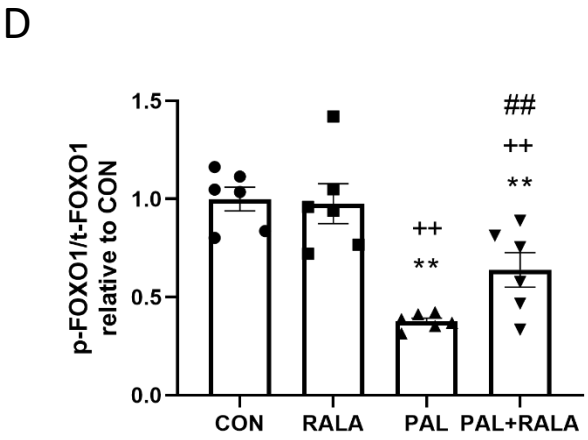

D
p-FOXO1/t-FOXO1 relative to CON
1.5
1.0
0.5
0.0
++
**
##
++
**
CON
RALA
PAL
PAL+RALA

Figure 5-3-2.7. The effect of RALA treatment on FOXO1 protein expression in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with 200 µM R-α-lipoic acid (RALA), 500 µM palmitate (PAL) or the combination of both interventions. **Significantly different from control (CON; $P<0.01$). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). $^{++}$Significantly different from the corresponding RALA only group ($P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group

5-4. Discussion

In chapter 5, evidence was provided to show the administration of RALA or DCA could revert the palmitate-induced inhibition of PDC activity and the rise in media lactate levels. The results also showed that RALA could improve glucose uptake and rescue the reduction in the mitochondrial ATP production rate mediated by palmitate. At the same time, it was found that DCA reduced PDK4 protein expression. As RALA unexpectedly increased the PDK4 protein expression in the presence of palmitate, this would suggest that PDK4 was not involved in the RALA-mediated recovery of PDC activity. I will later present a possible underlying mechanism behind these circumstances. It was also found that DCA and RALA reversed the palmitate-induced rise in PPARα and PPARδ protein expression. Moreover, the administration of DCA and RALA rescued the palmitate-induced increase in total-FOXO1 protein expression, which lead to a rise in the phospho-FOXO1 to total-FOXO1 ratio, which was equivalent to an inhibition of FOXO1 protein.

It has been known that PDC activity would be reduced when muscle PDK4 was up-regulated by enhanced FFA availability, insulin resistance, or the presence of Type 2 diabetes (Alamdari, Constantin-Teodosiu *et al.* 2008, Constantin-Teodosiu, Baker *et al.* 2009, Constantin-Teodosiu 2013, Constantin-Teodosiu, Cederblad *et al.* 2018). DCA has been shown to enhance PDC activity by inhibiting PDK2 and 4 expressions. However, the previous studies, where DCA was used to improve PDC activity, presented with normal physiology rather than with

pathophysiology conditions, i.e. chronic high level of palmitate availability. Furthermore, no underlying mechanism behind the effects of DCA on PDK4 was made available. In study 4, It was demonstrated that DCA improved palmitate-induced reduction in PDC activity by down-regulating the PDK4 gene and protein expression. Also, it was the first study to show that palmitate-induced increases in PPARα, PPARδ, and FOXO1 protein expression were reversed by DCA treatment. These findings provide further evidence that PPARδ and FOXO1, but not PPARα, transcription factors are involved in the regulation of PDK4 expression.

Supplementation with RALA, which is the natural enantiomer of alpha-lipoic acid, has been shown to improve diabetic hypertension polyneuropathies and atherosclerosis (Rousseau, Sibille *et al.* 2016). However, whether RALA could reverse the FFA mediated inhibition of PDC activity, and if this was the case, whether RALA could regulate PDK4 expression as an underlying mechanism, were unknown. Earlier, Korotchkina LG *et al.* demonstrated in an out of a living cell purified scaffold protein system that alpha-ALA, which consists of a 50/50 mixture of the R- (natural) and S- (unnatural) forms (enantiomers) decreased the PDK4 activity, albeit at a rate lower than PDK1, which could, therefore theoretically, result in an improvement of PDC activity (Korotchkina, Sidhu, Patel 2004).

It was reassuring, however, that in line with our protein PDK4 results, Anne-Sophie Rousseau *et al.* showed previously in a cell model similar to ours that 250 μM alpha-ALA increases PDK4 gene expression. Furthermore, this effect was completely obliterated by the treatment

with the irreversible and selective PPARδ antagonist GSK3787 (Korotchkina, Sidhu et al. 2004, Rousseau, Sibille et al. 2016). However, it was presently demonstrated that RALA could rescue the palmitate-induced inhibition of PDC activity independently of that of PDK4 pathway as expression of PDK4 protein further increased, rather than decrease with RALA. A possible explanation for this finding is that RALA presently increased glucose uptake and decreased medium lactate, suggestive of increased intracellular concentration of pyruvate. High levels of pyruvate, as well as its synthetic analogue DCA, is a powerful natural inhibitor of PDK4 protein (Wieland 1972, Denton 1975, Stacpoole 1989), which promoted thereby the activation of PDC activity. Alternatively, RALA might have indirectly counteracted the deleterious effect of PDK4 protein on PDC activity via activating pathways such as PI3K/AKT (Hong, Son *et al.* 2018) and AMPK (Shen, Zhu *et al.* 2007). However, since we presently did not measure any of these pathways, further experiments in this area are warrant.

Pyruvate is converted into lactate by lactate dehydrogenase (LDH) or converted into acetyl-CoA by PDC. Increased availability and delivery of saturated free fatty acids into skeletal muscle has been shown to promote intracellular glycolysis and impairment of PDC activity, thereby increasing the lactate levels and decreasing ATP production rates (Constantin-Teodosiu, Baker *et al.* 2009, Chen, Yi *et al.* 2018). In previous studies, DCA reduced exercised induced lactate accumulation in mouse skeletal muscle (Hoshino, Tamura *et al.* 2015). RALA has also provided efficacy at reducing lactate levels in cultured hepatoma cells (Ikuta,

Chikamoto *et al.* 2017). However, no studies have addressed the question of whether DCA and RALA could rescue the mitochondrial ATP production rates in the presence of excess fatty acids. The results reported in study 5 showed that RALA rescued palmitate-induced MAPR inhibition in parallel with decreased lactate levels in C2C12 myotubes. Overall, the results indicate that RALA treatment leads to an increase in the oxidative route of glucose disposal. Based on the present data, it seems reasonable to suggest that mechanistically RALA increased glucose uptake by rescuing the PDC activity from the palmitate-induced inhibition.

Alpha-lipoic acid has been shown to improve mitochondrial dysfunction related to excess fatty acid such as increased production of reactive oxygen species (ROS) and decreased mitochondrial membrane potential (MMP) while increasing the citrate synthase activity (Yang, Li *et al.* 2014, Chen, Yi *et al.* 2018). These pieces of evidence lean support to possible alternative pathways that may explain the improvement of MAPR resulting from the treatment with RALA. Although DCA treatment decreased the lactate levels and improved PDC activity in the C2C12 muscle cells model, surprisingly, it could not improve the palmitate-induced reduction in MAPR. Although not validated, there is a possible explanation that chronic, rather than casual DCA administration in the presence of palmitate can synergically enable an increase in the rates of reactive oxygen species (ROS) production (Haugrud, Zhuang *et al.* 2014, Lee, Lim *et al.* 2017, Ward, Poff *et al.* 2017). It has been repeatedly demonstrated, in different experimental models, that a strong positive correlation between ROS production and

mitochondrial membrane potential (ΔΨ) exists (Turrens 2003), thereby disrupting the rates of mitochondrial ATP production. Besides, sustained/chronic DCA supplementation has also been shown to decrease cytochrome c oxidase subunit IV (COX IV) mRNA expression, which is a critical enzyme of the mitochondrial respiratory chain (Li, Park *et al.* 2006, Hoshino, Tamura *et al.* 2015).

Chapter 4 showed that PPARδ, FOXO1 and PPARα and are involved to different degrees and under different mechanisms in the regulation of PDC activity. Therefore, whether the effects of DCA and RALA on the regulation of PDC activity could have occurred via their involvement on PPARα and PPARδ gene and/or protein expression were investigated.

Previous studies provided evidence implying that DCA could at least regulate PPARα and PPARδ gene expression (Constantin-Teodosiu, Constantin *et al.* 2012, Morales-Cano, Moreno *et al.* 2016). Further evidence of a role for DCA in the PPARδ regulation came from another study that documented that the beneficial effects of PPARδ agonists GW0742 and L165041 on the restoration of the high glucose-mediated impairment of insulin signalling were suppressed by the co-treatment with DCA (Quintela, Jimenez *et al.* 2014). Collectively, these results lean support towards the contention that DCA may regulate PPARα and PPARδ expression. Indeed, and for the first time, it was presently shown that DCA treatment reversed the palmitate-induced increase in PPARα and PPARδ protein expression in parallel with a

noticeable reduction in the PDK4 protein expression (Fig. 5-3-1.5). Furthermore, the decline in PPARδ protein expression was greater than that of PPARα. Chapter 4 showed also that PPARα and PPARδ silencing rescued the palmitate-induced impairment of PDC activity, although only the PPARδ silencing decreased the expression of PDK4 protein. Since in the same chapter, PPARδ silencing decreased also the expression of PPARα protein, this may assign to PPARδ a more important role in the DCA-mediated recovery of PDC activity. Although several past studies showed that RALA could regulate the PPARs gene expression (Wang, Tsai *et al.* 2013, Li, Dungan *et al.* 2014, Rousseau, Sibille *et al.* 2016), the present results have provided evidence that the impact of RALA on the regulation PPARα and PPARδ protein expression has a more lasting effect than the often short-lived gene expression switch on/off.

FOXO1 up-regulates straight PDK4 expression as FOXO1 can directly bind the promoter region of the PDK4 gene; (Constantin-Teodosiu, Constantin et al. 2012). The present results demonstrated that palmitate treatment increased FOXO1 protein expression in C2C12 myotubes, which was nevertheless reversed by DCA and RALA. In line with our findings, but in different tissue (heart), a previous study showed a similar finding that DCA decreases FOXO1 protein expression (Piao, Sidhu *et al.* 2013). However, RALA reversed the palmitate-induced decline in the ratio of phospho- to total FOXO1 protein expression (aka FOXO1 inactivation) in parallel with increased PDK4 protein expression, which contradicts the finding

that FOXO1 silencing decreased PDK4 protein expression. The possible explanation for this inconsistency may be that RALA could not directly regulate FOXO1 expression. Interestingly, RALA decreased phospho- and total FOXO1 protein expression simultaneously, while DCA decreased total FOXO1 protein expression only. This may account for the differences in the PDK4 protein expression between DCA and RALA treatment. However, in chapter 4 PPARα and PPARδ silencing all decreased phospho- and total FOXO1 protein expression, while PPARδ, but not PPARα, silencing reversed the palmitate-induced rise in the level of PDK4 protein expression. Collectively, these results suggest that not only phospho-FOXO1, but also phospho to the total FOXO1 ratio may regulate PDK4 protein expression.

In conclusion, in this chapter, it was proven that DCA improved PDC activity in the presence of palmitate by decreasing PPARα, PPARδ and FOXO1 protein expression compared to palmitate alone. However, DCA could not rescue the palmitate-induced MAPR inhibition. Additionally, the treatment with RALA reversed the palmitate-induced reduction in glucose uptake, pyruvate dehydrogenase complex activity, and mitochondrial ATP production via a PDK4-independent pathway. These results suggest the treatment with RALA can protect against chronically increased FFA availability by providing support to PDC activity and mitochondrial ATP production.

Chapter 6
The impact of chronic low-frequency electrical pulse stimulation (EPS) on palmitate-induced impairment of mitochondrial CHO oxidation

6-1. Introduction

6-1-1. Electrical pulse stimulation (EPS)

Voluntary or involuntary muscle contraction is beneficial to health and related to the latter electrical pulse stimulation (EPS) is a useful model to standardise the contraction and to avoid the central fatigue in humans. It can also be applied and sustained for lengthy times in *vitro* models (Nikolic, Bakke *et al.* 2012). Nedachi *et al.* showed that EPS applied for 24 hrs induced contraction of myotubes and elicited exercise-like effects like AMPK and MAPK activation, increased glucose uptake and increased insulin sensitivity (Nedachi, Fujita *et al.* 2008), providing thereby evidence that electrical stimulation is a good model to mimic exercise. EPS can be classified into acute high-frequency stimulation, which might resemble an acute high-intensity bout of exercise and chronic low-frequency stimulation, which mimics regular submaximal physical activity (Hawley, Hargreaves *et al.* 2014).

Increasing evidence suggests that electrical stimulation of muscle improves glucose metabolism and insulin sensitivity (Manneras, Jonsdottir *et al.* 2008, Tominaga, Ishizaki *et al.* 2011). After electric stimuli on skeletal muscle cells, GLUT4 translocation and glucose uptake were enhanced via increasing phosphorylation of PI3K/AKT pathway (Yano, Morino-Koga *et al.* 2011, Osorio-Fuentealba, Contreras-Ferrat *et al.* 2013). Even low-intensity electrical current (or mild electrical stimulation; MES), but in conjunction with heat shock can lower blood glucose and insulin levels, improving thereby insulin sensitivity (Morino, Kondo *et al.*

2008). Electrical stimulation can also mitigate the doxorubicin-induced atrophy and mitochondrial loss in cultured C2C12 cells and upregulates expression of metabolic genes in an otherwise paralysed muscle (Petrie, Suneja *et al.* 2015, Guigni, Fix *et al.* 2019). Besides, electrical stimulation reduces electrochemical Na^+ and K^+ gradients in C2C12 myotubes (Danilov, Sidorenko *et al.* 2017). It also increases the abundance of the protein complexes II, IV, and V of the electron transport chain (Lambernd, Taube *et al.* 2012).

Collectively, these findings suggest that electric stimuli represent a likely therapy to treat type 2 diabetes and its related traits as EPS has a proved beheficial impact on the rate of carbohydrate oxidation in man. Thus, Constantin-Teodosiu *et al.* have shown that isolated bouts of voluntary or electrical evoked muscle contraction can fully activate muscle PDC in humans (Constantin-Teodosiu, Cederblad *et al.* 1991, Constantin-Teodosiu, Cederblad *et al.* 1992, Constantin-Teodosiu, Cederblad *et al.* 1993). This response was nevertheless markedly reduced irrespective of the type or intensity of muscle contraction performed when exercise was preceded by three days of high-fat dietary intake, (Constantin-Teodosiu, Constantin *et al.* 2012, Constantin-Teodosiu, Cederblad *et al.* 2018). It is worth noting that isometric muscle contraction induced by electric stimulation leads to full PDC transformation to PDCa despite the $NADH/NAD^+$ and acetyl-CoA/CoASH ratios being markedly increased (Constantin-Teodosiu, Cederblad *et al.* 1993, Constantin-Teodosiu, Baker *et al.* 2009). Electric stimulation also affects the expression of muscle PDK4 mRNA. While PDK4 gene expression increases in

human muscle cell and tissue during acute stimulation, after >1 year of chronic stimulation PDK4 mRNA was found unchanged (Nikolic, Bakke *et al.* 2012, Petrie, Suneja *et al.* 2014). Overall, there is so far no evidence to suggest chronic low-intensity electrical stimulation can increase the mitochondrial ATP turnover using glucose-derived pyruvate in a palmitate-induced metabolic inflexible model.

6-1-2. Aim of this chapter

In this chapter, we hypothesised that chronic low-frequency EPS would improve the palmitate-induced inhibition of PDC activity in C2C12 skeletal muscle cell as a primary endpoint. Additionally, we investigated whether EPS intervention could also reverse other metabolic outcomes to the palmitate-induced impairment of CHO oxidation such as declines in glucose uptake, acetyl groups levels, glucose-derived pyruvate mediated ATP production, and increases in PDK4, PPARα, δ, and FOXO1 protein expression.

6-2. Methods

A murine skeletal muscle cell line C2C12 was used in this experiment. On day four, after FBS differentiation, palmitate 300 µM was provided to the cells and let them grow for another 24 hr. Then, the cells were collected and underwent different experimental measurements as will be further described. EPS was conducted on day four for 16 hours. 6 individual experiments for each intervention group was undertaken.

6-2-1. Electrical pulse stimulation (EPS).

EPS was conducted as previously described in Chapter 2: Materials and General Methods.

6-2-2. Glucose uptake

Cellular glucose uptake was measured as previously described in Chapter 2: Materials and General Methods.

6-2-3. Cellular acetyl group and media lactate accumulation

Cellular acetyl group and media lactate accumulation were measured as previously described in Chapter 2: Materials and General Methods.

6-2-4. Cellular PDC activity measurements:

Cellular PDC activity measurements were measured as previously described in Chapter 2: Materials and General Methods.

6-2-5. MAPR

Maximal mitochondrial ATP production rates (MAPR) were measured as previously described in Chapter 2: Materials and General Methods. MAPR values were corrected for total protein

content of the cellular extract, measured using the Bradford method (Bradford, 1976) as described in Chapter 2: General Methods.

6-2-6. Western blotting measurements

Expressions of PDK4, PPARα, δ and FOXO1 proteins were measured as previously described in Chapter 2: Materials and General Methods.

6-2-7. Statistical Analyses

The reported data represent means ± SEM. Statistical differences between group means were tested using one-way ANOVA's (one independent variable) with Newman-Keuls correction to allow for post-hoc comparisons. Data were assessed for normal distributions using Shapiro-Wilk tests (SPSS v 26, IBM).

6-3. Results

6-3-1. Palmitate reduced the C2C12 myotube glucose uptake, which was restored by the low-frequency electrical pulse stimulation, but with no change in the media lactate concentration

Palmitate alone reduced C2C12 myotube glucose uptake compared to control, (P<0.05), while EPS alone increased it (88.0±3.6 % and 139.0±6.3 % of control, respectively, P<0.01; Fig. 6-3-1A). Electrical pulse stimulation rescued the palmitate-induced inhibition of cellular glucose

uptake (119.0±7.7 % of control, $P<0.01$; Fig. 6-1A)**.** For the same experimental conditions as above, cell media lactate concentrations are presented in Fig. 6-3-1B. Administration of isolated EPS and palmitate increased medium lactate concentration compared to control (both $P<0.01$). The combination of palmitate and EPS further increased the medium lactate concentration over control ($P<0.01$), and above EPS and palmitate (both $P<0.05$).

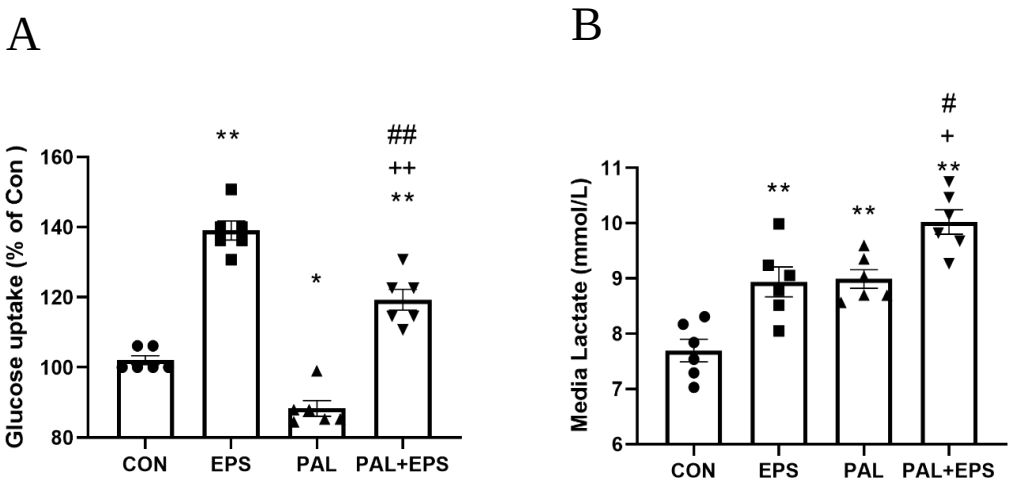


Figure 6-3-1. The effect of EPS on glucose uptake and media lactate concentration in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were exposed to standard growth media (CON), 16 hrs of electrical pulse stimulation (EPS; 11.5V, 1Hz, 2ms), 300 µM palmitate (PAL) or the combination of both palmitate and EPS (PAL+EPS). $^{*,\ **}$Significantly different from CON ($P<0.05$, $P<0.01$). $^{\#\#}$Significantly different from PAL ($P<0.01$). $^{+,\ ++}$Significantly different from EPS ($P<0.05$,

P<0.01). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

6-3-2. Palmitate reduced the C2C12 myotube's PDC activity, which was rescued by EPS

Palmitate alone reduced PDC activity compared to control (3.0±0.2 and 3.7±0.2 pmol acetyl-CoA/min/mg protein, *P*<0.01; Fig. 6-3-2), while EPS alone increased it above control (4.6±0.2 pmol acetyl-CoA/min/mg protein, *P*<0.01). Nevertheless, the palmitate-induced inhibition of PDC activity was rescued by EPS (3.6±0.2 pmol acetyl-CoA/ min/mg protein; Fig. 6-2A), such that there was no difference from control, although this activity was lower than EPS alone (*P*<0.01).

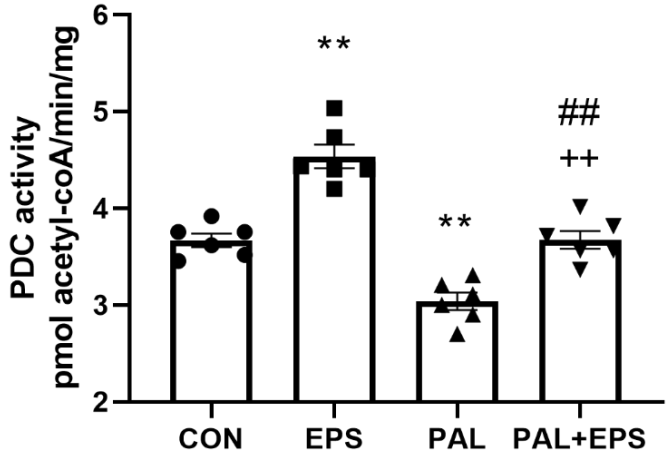


Figure 6-3-2. The effect of EPS on PDC activity in C2C12 myotube in the presence or the absence of palmitate.

C2C12 myotubes were treated with 16 hrs of electrical pulse stimulation (EPS; 11.5 V, 1 Hz, 2 ms), 300 μM palmitate (PAL) and the combination of both interventions (PAL+EPS). *,**Significantly different from control (CON; $P<0.05$, $P<0.01$). $^{\#\#}$Significantly different Palmitate (PAL; $P<0.01$). $^{++}$Significantly different from EPS ($P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

6-3-3. EPS reversed the palmitate-induced reduction in the cellular acetylcarnitine content in C2C12 myotubes.

Myotube acetylcarnitine content (Fig. 6-3-3A) was used as an index of PDC flux. Palmitate alone reduced acetylcarnitine content compared to control (105±11 vs 83±4 pmol/mg protein, $P<0.05$), while EPS alone increased it (172 ± 30 pmol/mg protein, $P<0.01$). Moreover, EPS rescued the palmitate-induced reduction in acetylcarnitine content (142±3 pmol/mg protein, $P<0.01$). Skeletal muscle cell C2C12 acetyl-CoA levels after treating with palmitate with or without EPS are presented in Fig. 6-3-3B. There was no significant difference between control and EPS administration alone, but when palmitate was added with and without EPS acetyl-CoA significantly increased above the control ($P<0.05$ and $P<0.01$, 18.2±1.5 vs 17.2±1.4 pmol/mg protein and 24.8±1.4 vs 26.7±2.7 pmol/mg protein, respectively).

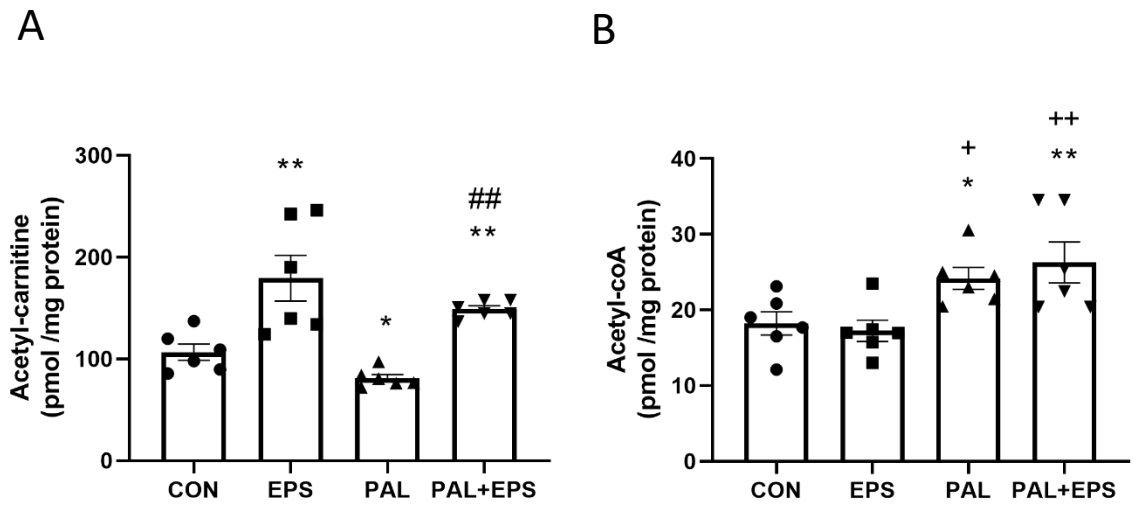


Figure 6-3-3. The effect of EPS on acetylcarnitine and acetyl-CoA content in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with 16 hrs of low-frequency electrical pulse stimulation (EPS; 11.5 V, 1 Hz, 2 ms), 300 μM palmitate (PAL) or the combination of both interventions (PAL+EPS). *,**Significantly different from control (CON; $P<0.05$, $P<0.01$). $^{\#\#}$Significantly different palmitate (PAL; $P<0.01$). $^{++}$Significantly different from EPS ($P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

6-3-4. Palmitate reduced the C2C12 myotube' s maximal mitochondrial ATP production rates from pyruvate, which was restored by electrical pulse stimulation.

Palmitate reduced MAPR compared to control (9.8±0.3 vs 10.8±0.2 nmol ATP/min/mg protein, $P<0.01$; Fig 6-3-4), while EPS increased it (12.0±0.3, $P<0.01$). Electrical pulse

stimulation rescued the palmitate-induced reduction in MAPR (10.8±0.3 nmol ATP/min/mg protein, $P<0.01$).

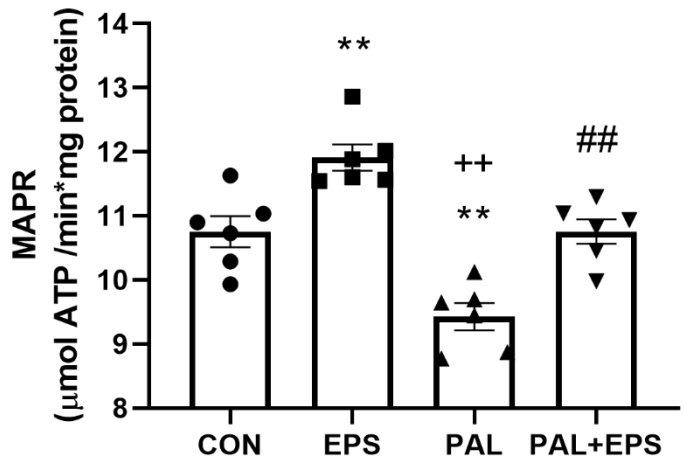


Figure 6-3-4. The effect of EPS on maximal mitochondrial ATP production rates from pyruvate in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with 16 hrs of electrical pulse stimulation (EPS; 11.5 V, 1 Hz, 2 ms), 300 μM palmitate (PAL) and the combination of both interventions (PAL+EPS). *,**Significantly different from control (CON; $P<0.05$, $P<0.01$). $^{\#\#}$Significantly different Palmitate (PAL; $P<0.01$). $^{++}$Significantly different from EPS ($P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

6-3-5. Palmitate increased the C2C12 myotube PDK4 protein expression, which was reversed by EPS

Palmitate increased the C2C12 myotube's PDK4 protein expression 2.5-fold relative to control (P<0.01; Fig. 6-3-5). Electrical pulse stimulation alone did not affect myotube PDK4 protein expression compared to control, but reversed the increase in PDK4 protein observed with palmitate alone (1.4±0.1 vs 2.5±0.1, P<0.01), such that protein expression following combined administration of palmitate and EPS was no different from control.

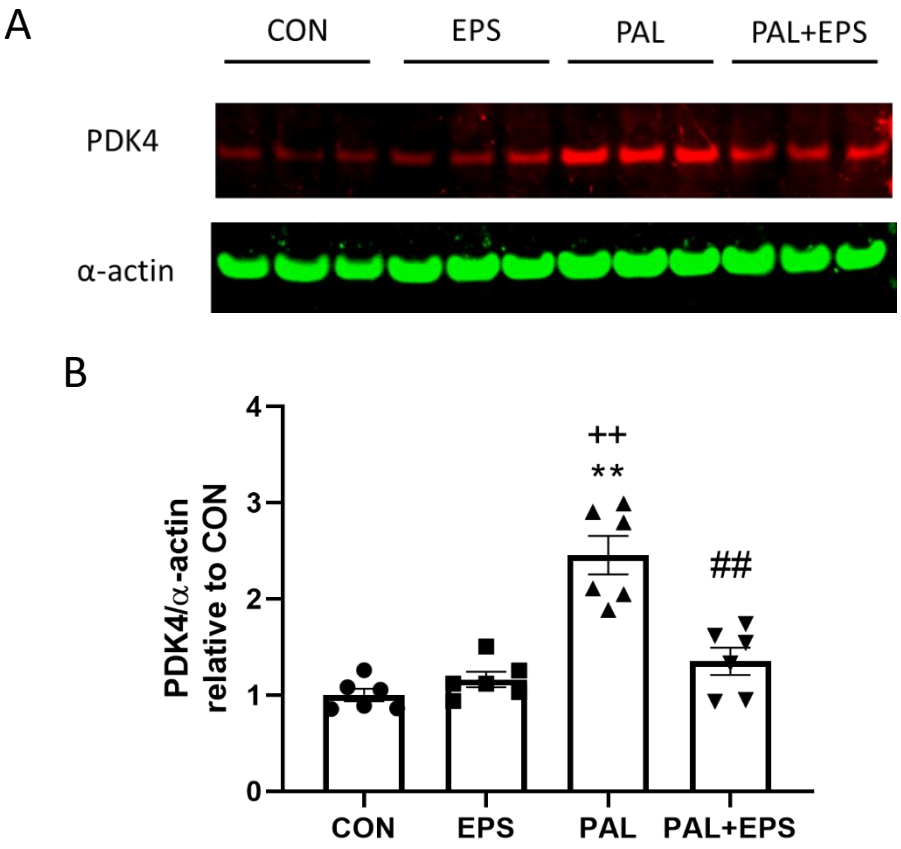


Figure 6-3-5. The effect of EPS on PDK4 protein expression in C2C12 myotubes in the presence or the absence of palmitate

C2C12 myotubes were treated with 16 hrs of electrical pulse stimulation (EPS; 11.5 V, 1 Hz, 2 ms), 300 μM palmitate (PAL) and the combination of both interventions (PAL+EPS). **Significantly different from control (CON; $P<0.01$). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). $^{++}$Significantly different from the corresponding drug only group (DCA; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

6-3-6. Palmitate increased the PPARδ protein expression in C2C12 myotubes, and this was restored by electrical pulse stimulation

The effect of EPS on PPARα and PPARδ protein expressions is presented in Fig. 6-3-6. Palmitate alone increased myotube PPARα and PPARδ protein expression (1.5±0.1 and 1.9±0.1 fold over control, $P<0.01$ and $P<0.01$; respectively). We also found that EPS reversed the palmitate-induced increase in PPARδ protein expression (1.2±0.2 relative to control, $P<0.01$). However, there was no significant change in PPARα protein expression with EPS treatment in the presence of palmitate (1.5±0.2 relative to control).

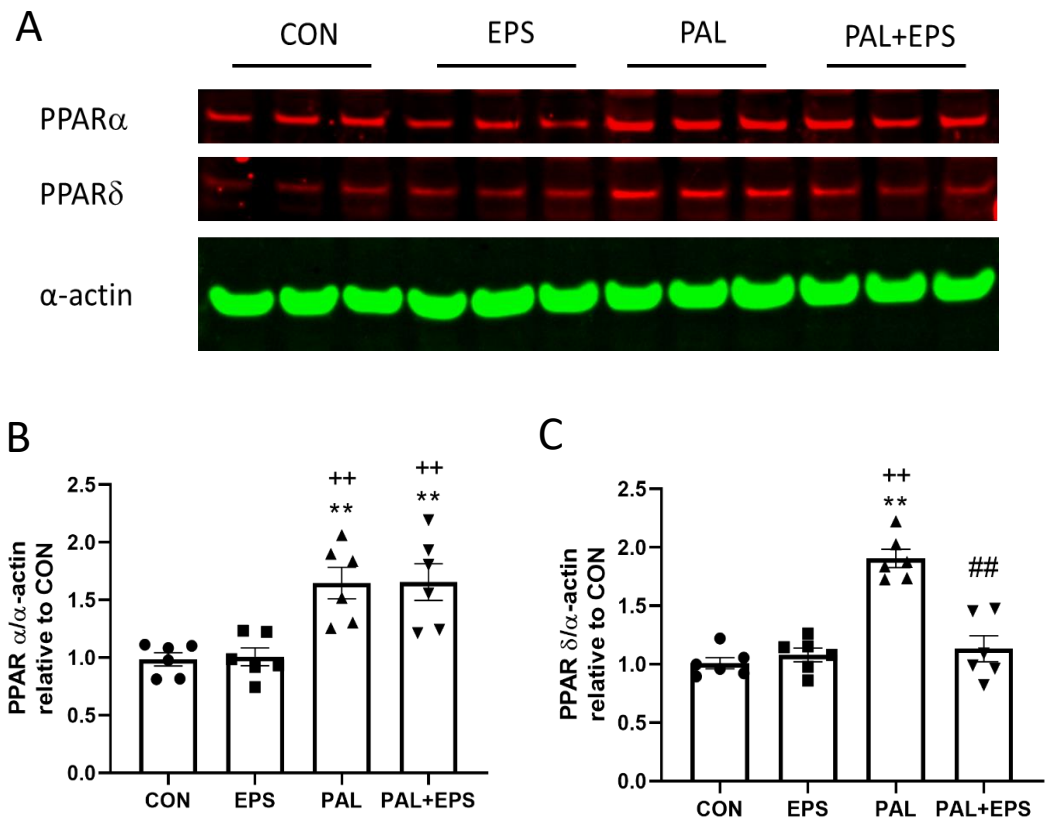


Figure 6-3-6. The effect of EPS on PPARα and PPARδ protein expression in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with 16 hrs of electrical pulse stimulation (EPS; 11.5 V, 1 Hz, 2 ms), 300 μM palmitate (PAL) or the combination of both interventions (PAL+EPS). **Significantly different from control (CON; $P<0.01$). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). $^{++}$Significantly different from the corresponding drug only group (DCA; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

6-3-7. EPS decreased the phosphorylated and total FOXO1 protein expression in C2C12 myotubes in the presence of palmitate

Fig. 6-3-7 shows that palmitate significantly increased FOXO1 protein expression compared to control (P<0.01). EPS decreased phospho-FOXO1 (0.8±0.1 vs 0.5±0.1 relative to CON, P< 0.01) and total-FOXO1 protein expression (1.6±0.1 vs 1.2±0.1 relative to CON, P< 0.01), while the p-FOXO1/t-FOXO1 ratio did not change in the presence of palmitate. Palmitate had no impact on the p-FOXO1/t-FOXO1 ratio normalised to that of nuclear to cytoplasmic factor, but EPS reduced this ratio in the absence (57%, P<0.01) or presence (54%, P<0.01) of palmitate (Fig. 6-3-8).

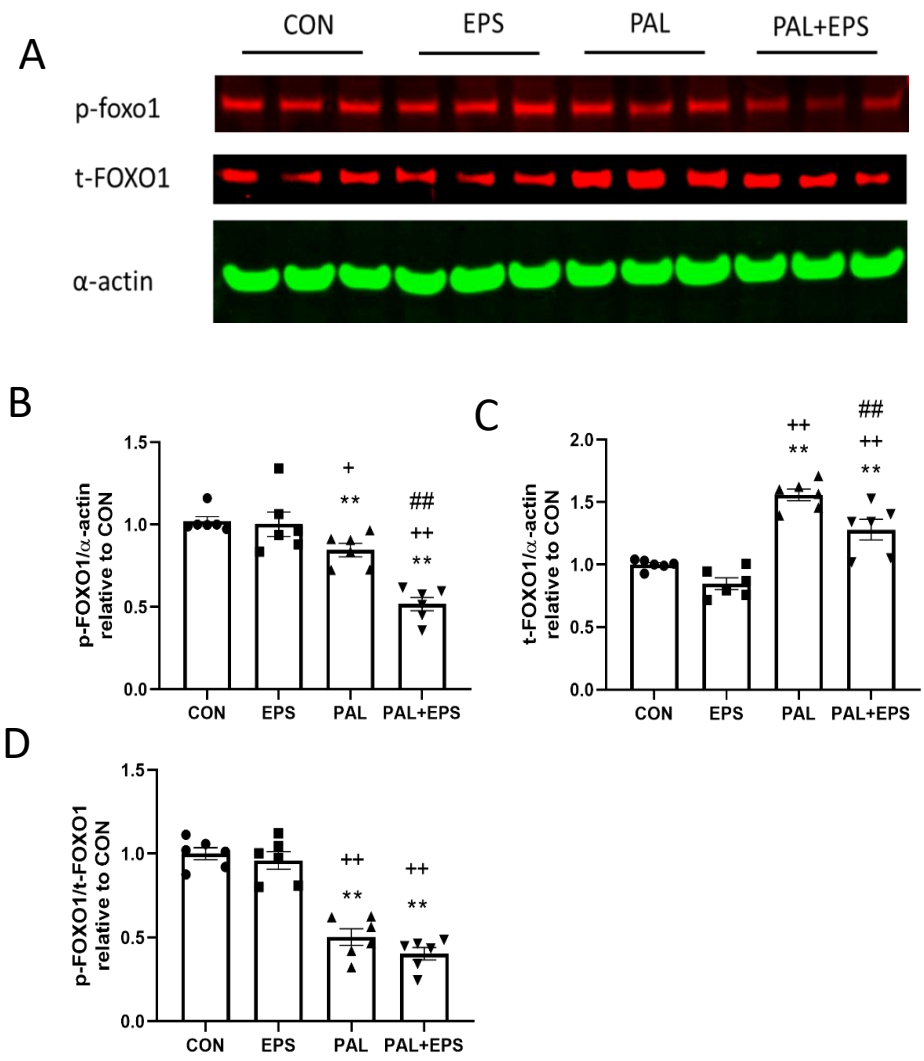
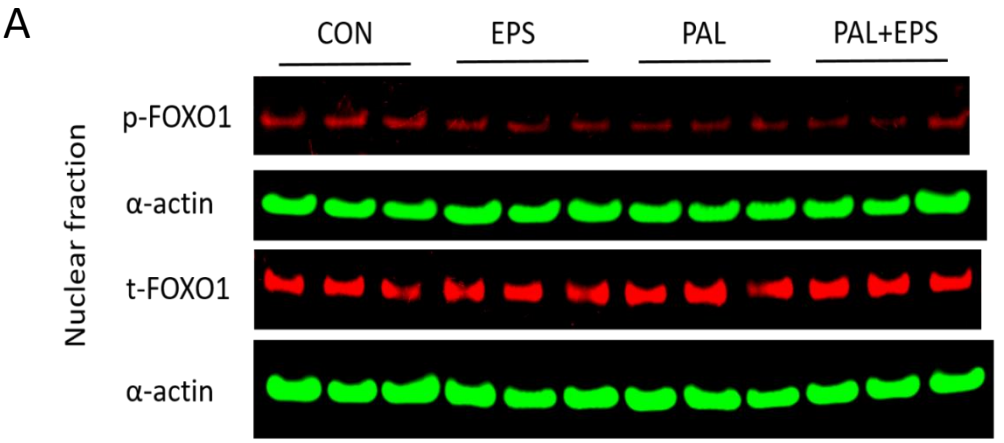
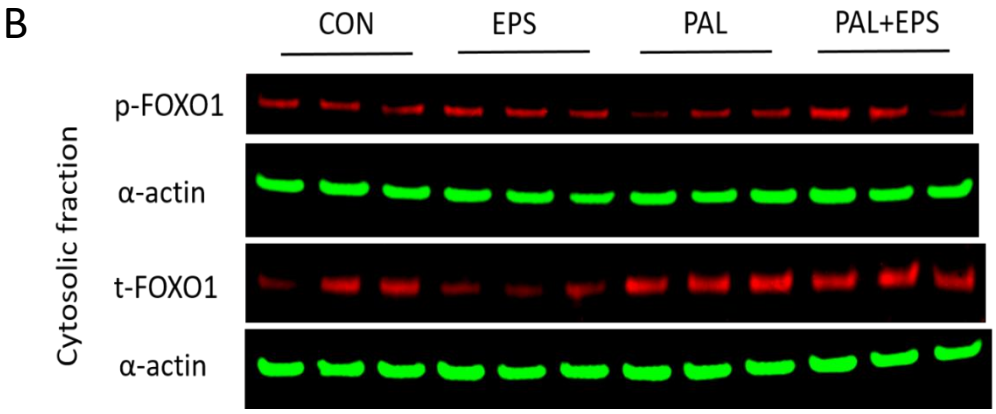


Figure 6-3-7. The effect of EPS on FOXO1 protein expression in C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with 16 hrs of electrical pulse stimulation (EPS; 11.5 V, 1 Hz, 2 ms), 300 μM palmitate (PAL) or the combination of both interventions (PAL+EPS). **Significantly different from control (CON; $P<0.01$). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). $^{++}$Significantly different from DCA treated group (DCA; $P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.


A
CON
EPS
PAL
PAL+EPS
Nuclear fraction
p-FOXO1
α-actin
t-FOXO1
α-actin


B
CON
EPS
PAL
PAL+EPS
Cytosolic fraction
p-FOXO1
α-actin
t-FOXO1
α-actin

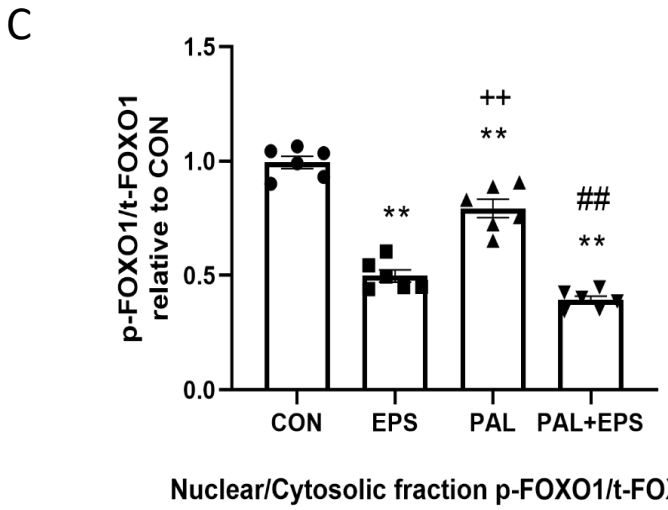

C
p-FOXO1/t-FOXO1 relative to CON
1.5
1.0
0.5
0.0
++
**
**
**
##
CON
EPS
PAL
PAL+EPS
Nuclear/Cytosolic fraction p-FOXO1/t-FOXO1

Figure 6-3-8. Phospho- and total-FOXO1 protein expression in the cytosolic and nuclear cell fractions of C2C12 myotubes in the presence or the absence of palmitate.

C2C12 myotubes were treated with either16 hrs of electrical pulse stimulation (EPS; 11.5 V, 1 Hz, 2 ms), 300 µM palmitate (PAL) or the combination of both interventions (PAL+EPS). **Significantly different from control (CON; $P<0.01$). $^{\#\#}$Significantly different from palmitate (PAL; $P<0.01$). $^{++}$Significantly different from EPS ($P<0.01$). Data represent mean +/- SEM of 6 individual experiments for each intervention group.

6-4. Discussion

The present study demonstrated that exposure of palmitate-treated cells to chronic low-frequency EPS rescued the palmitate-induced dysregulation of CHO oxidative oxidation, as reflected by the restoration of deficits in cellular glucose uptake, PDC activity, and its flux, and the pyruvate based MAPR. Furthermore, these events were accompanied by a restoration of the protein expression levels of PDK4, PPARδ, but not PPARα, and the p-FOXO1/t-FOXO1 ratio normalised to that of nuclear to cytoplasmic factor. Overall, these novel data suggest once again that palmitate-induced increases in the expression of PPARδ and FOXO1 transcription factors underpin the downstream increase in myotube PDK4 protein expression, which then limits PDC activity and flux, and reduces the mitochondrial CHO oxidation. Furthermore, the application of EPS dampens this palmitate-induced dysregulation of CHO oxidation via modulation of the same molecular and cellular events.

Electric stimulation has been shown to improve glucose metabolism and insulin sensitivity and was recently reported to improve the palmitate-induced impairment of glucose uptake (Nieuwoudt, Mulya *et al.* 2017). This study demonstrated that application of chronic low-frequency (1 Hz) EPS to the cells for 16 hrs in the presence or absence of palmitate increased glucose uptake by 20% and 40%, respectively, compared to control (Fig. 6-3-1). Further increases increase in the cellular glucose uptake can be achieved (70%) acutely with high-frequency EPS (up to 10 Hz). However, this stimulation frequency cannot be sustained by the

muscle cells beyond 60 min probably due to the high lactate accumulation generated by the acceleration of glycolytic pathway (Nikolic, Bakke *et al.* 2012). It is known that during muscle contraction pyruvate generation is increased due to an enhancement of muscle glycogen degradation. The pyruvate formed can either converted to lactate or oxidised to acetyl-CoA by pyruvate dehydrogenase complex (PDC). When the rate of pyruvate formation overcomes the rate of acetyl-CoA formation, then the excess of pyruvate is reduced to lactate. The result showed presently that cell media lactate accumulation further increased with EPS treatment compared to control, but these values were yet half of those recorded with high-frequency EPS (Nikolic, Bakke et al. 2012).

Previous studies have shown that exercise increases muscle PDC activity. For example, human studies have shown a direct relationship between the intensity of muscle contraction and the magnitude of PDC activation, and thereby the rate of muscle glucose oxidation (Constantin-Teodosiu, Cederblad *et al.* 1993, Constantin-Teodosiu, Baker *et al.* 2009, Constantin-Teodosiu, Cederblad *et al.* 2019). However, there is still much debate around the blunted responses of PDC activity to exercise in the presence of chronic high fatty acid excess and the underlying mechanism behind this deregulation. The present study was able to show that *in vitro* 16 hrs of chronic low-frequency EPS rescued the reduction of PDC activity and its flux, normally observed in the presence of palmitate, most likely due to the sharp decline in the PDK4 protein expression. Chapter 4 provided evidence that PPARα, PPARδ and FOXO1

individually can modulate to different ranges the activity of PDC. Therefore, whether the metabolic improvements related to the activity of PDC in response to the concurrent palmitate and EPS treatment were enabled by changes in the same transcription factor proteins was to be confirmed. Hence, we provided detailed evidence that the normalisation of the palmitate-induced deficits in the activity of PDC and its flux after 16 hrs of EPS was indeed matched by reductions in PDK4, PPARδ, and the nuclear to cytosolic p-FOXO1/t-FOXO1 ratio, protein expression, but not by a reduction in PPARα protein expression. In chapter 4, it was also identified that while PPARδ and FOXO1 silencing decreased PDK4 protein expression, PPARα silencing did not change it. The results of the EPS treatment replicated the findings reported in Chapter 4, as EPS decreased PPARδ protein expression and the translocation of FOXO1 to the nucleus, contributing thereby to the decline in the PDK4 protein expression. In the case of FOXO1, dephosphorylation results in its translocation from the cytosol to the nucleus, where it can bind directly to the promoter region of the PDK4 gene enabling an increase in the transcriptional levels of PDK4 gene (Furuyama, Kitayama *et al.* 2003). Moreover, FOXO1 dephosphorylation appears to be coupled via a signalling cascade to changes in circulatory FFA and/or insulin availability (Kim, Lee *et al.* 2006), tying thereby muscle PDK4 gene transcription (amongst others) to substrate availability. Additionally, so far no study could establish a direct relationship between EPS and PPARδ protein expression. Incidental past evidence showed in a rodent model that seven days of PPARδ agonism with the

compound GW610742 increased muscle PDK4 protein expression (Constantin, Constantin-Teodosiu *et al.* 2007), which later attenuated activation of muscle PDC and the ATP aerobic formation during a single bout of exercise (Constantin-Teodosiu, Baker *et al.* 2009). This thesis showed for the first time that the concurrent EPS intervention with palmitate supplementation could moderate the expression of PPARδ protein, and thereby lower PDK4 protein expression and increase activity of PDC. Collectively, these data underpin once again the paradigm that PPARδ plays an important role in the regulation of PDC activity. Concerning PPARα, EPS did not change the PPARα protein expression, which was reassuringly in line with the previous finding described in Chapter 4 that silencing PPARα decreased PDC activity independently from PDK4 signalling. Overall, the results recorded after EPS treatment suggests that the effect of PPARδ and PPARα, albeit of a lower magnitude, on PDC activity occur through different pathways.

The results showed also that EPS rescued palmitate-induced reduction in MAPR when using the glucose-derived pyruvate as a substrate. A previous study reported low cellular ATP and high lactate levels resulted from acute high-frequency EPS (Nikolic, Bakke *et al.* 2012), which may primarily contravene with the results. However, application high-frequency EPS like in the latter study requires a higher muscle energy provision that will bring about higher rates of glycogenolysis and glycolysis, which despite a simultaneous increase in the PDC mediated CHO oxidation, will be met by an even higher level of anaerobic metabolism derived lactate

accumulation. Hence, it is important to highlight that PDC controls not only the rate of aerobic CHO respiration as the activation of PDC has been shown to increase with aerobic respiration (Yang, Wang *et al.* 2018), but it also links via its reaction glycolysis to tricarboxylic acid cycle.

A previous study showed that PPARδ agonism decreased aerobic ATP production in a rat model (Constantin-Teodosiu, Baker *et al.* 2009). Since EPS reversed the palmitate-induced increase in PPARδ protein expression, it would be tempting to suggest that the EPS mediated upregulation of MAPR may have occurred via the PPARδ signalling pathway. However, in chapter 4, we showed that PPARδ silencing decreased MAPR in the presence of palmitate, while FOXO1 and PPARα silencing reversed the palmitate-induced decline in MAPR. Furthermore, PPARα protein expression has presently not responded to the EPS treatment. Therefore, the most likely pathway through which EPS upregulated MAPR was via FOXO1 pathway as exemplified by the decline in the nuclear to cytosolic p-FOXO1/t-FOXO1 ratio protein expression (Fig. 6-3-8C).

In conclusion, the present results point to a central role for PPARδ and FOXO1 in the palmitate-induced increase in myotube PDK4 expression and linked reductions in cellular glucose uptake, PDC activity and flux, and rates of CHO derived MAPR. Furthermore, the finding that 16 hrs of EPS rescued this palmitate-induced dysregulation of cellular CHO metabolism via modulation of FOXO1 pathway alludes to the mechanism by which chronic

physical activity will protect skeletal muscle against lipid-induced muscle metabolic inflexibility and IR.

Chapter 7

General discussion

Foreword

This thesis has investigated the impact of PPARα, PPARδ, and FOXO1 silencing (chapter 4), DCA and RALA (chapter 5) and EPS (chapter 6) on a metabolic inflexibility condition in an *in vitro* skeletal muscle cell-based model by treating C2C12 cells with a prior identified optimal palmitate concentration (500 μM throughout and 300 μM in study 6). This palmitate concentration allowed the maintenance of the cell's viability. However, it was of a physiological concentration sufficient to elicit in *in vitro* changes of accepted clinical markers of *in vivo* metabolic inflexibility/insulin resistance condition. This chapter will discuss the broader implications of the results of this thesis.

7-1. Primary findings and discussion

7-1-1 Chapter 3

In this chapter, it was established what palmitate concentration could be tolerated by an *in vitro* skeletal muscle cell model without affecting the cells' viability, and whether this palmitate level would be sufficient to induce an inflexible metabolic state as reflected by responses of accepted markers cited in the literature as they will be discussed later. After the establishment of a palmitate-induced metabolic inflexibility condition, we explored the impact of palmitate supplementation on markers of CHO oxidation, and PPARs and FOXO1 gene and protein expression in C2C12 skeletal muscle cells. The results showed that the identified palmitate

concentration not only resulted in decreased glucose uptake, lower pyruvate mediated mitochondrial ATP production rate, and increased media lactate concentration, but it also decreased the mitochondrial PDC activity. The skeletal muscle cell-based model also presented significant changes in gene and protein expressions of downstream insulin signalling factors like PDK4, PPARα, PPARδ, and FOXO1 mRNA.

At the start of the investigation, it was clear that an appropriate working concentration of palmitate had to be established. This is important since excess of long-chain acyl groups, as palmitate, are cytotoxic as they behave as a detergent, thereby disrupting cellular membrane integrity, increase cytosolic and mitochondrial reactive oxygen species, depolarise the mitochondrial membrane potential, impair ATP synthesis and elicit apoptotic cell death (S Xu *et al.* 2015, Cell Death & Disease). (Cnop, Hannaert *et al.* 2001). The concentration of palmitate used in previous studies varied from 250 (Zhang, Kong *et al.* 2019) to 750 µM (Abu Bakar and Tan 2017).

We have tested for a suitable palmitate working concentration within the range identified in the literature search. Myotubes were therefore cultured with increasing palmitate concentrations and tested for cell viability with the MTT assay. I have used in my thesis to cover the below and above the concentration of 500 µM, which would be considered by many as an acceptable extracellular physiological concentration. Indeed, the 500 µM mark is the commonest cited concentration in countless published studies, which involved treatment of

various cell cultures with palmitate. Furthermore, and more importantly, these studies did not report any loss of cell viability. Yet in an effort to test whether this concentration would maintain cell viability in my C2C12 myotube model, I tested several concentrations below the 500 µM mark: 0 (control) 50, 100, and 250 µM. Separately, I have also investigated the 300 µM mark in Chapter 6. Therefore, in practice, I tested six levels of palmitate concentrations between 0 to 500 µM. In retrospection, since no decline in cell viability between 300 and 500 µM was observed, the omission of a prospective 400 µM mark from the titration curve is unlikely to have contributed to the final validation of myotube's viability with working palmitate concentration between 0 to 500 µM.

With respect to the upper range of palmitate between 500 to 750 µM, the selection of upper 750 µM was merely a challenge to our model rather than an attempt to fine-tune a workable palmitate concentration above what many might have considered as a supraphysiological level. Indeed, C2C12 myotubes treated with 750 µM palmitate showed clear signs of decline in their viability. Therefore, 500 µM was chosen as the working concentration, as this is also the FFAs concentration typically found in human circulating plasma.

The activity of the mammalian pyruvate dehydrogenase complex is regulated by a reversible phosphorylation-dephosphorylation cycle of three specific serine residues (site 1, Ser-264; site 2, Ser-271; site 3, Ser-203) of the alpha subunit of the pyruvate dehydrogenase (E1) component. Phosphorylation is carried out by four pyruvate dehydrogenase kinase (PDK)

isoenzymes. The specificity of the four mammalian PDKs toward the three phosphorylation sites of E1 was investigated using the recombinant E1 mutant proteins with only one functional phosphorylation site present at a time (Korotchkina and Patel 2001). The results showed that: all four PDKs phosphorylated site 1 and site 2 (Ser-264-α and Ser-271-α), however, with different rates in phosphate buffer (for site 1, PDK2 > PDK4 approximately PDK1 > PDK3; for site 2, PDK3 > PDK4 > PDK2 > PDK1). Site 3 (Ser-203-α) was phosphorylated by PDK1 only. The maximum activation by dihydrolipoamide acetyltransferase was demonstrated by PDK3 (Patel, Nemeria et al. 2014). In contrast, a similar action on all the three sites was observed for the two phosphatases.

PDK2 and PDK4 mRNA are expressed in most tissues, but their prevalence is highest in skeletal and heart muscles. Since the specific activity of PDK4 is 8-fold greater than that of PDK2, this would assign to PDK4 a greater regulatory significance on PDC activity than PDK2. Conversaly, PDK1 and PDK3 appear to be limited to heart, pancreatic islets and kidney (Bowker-Kinley, Davis et al. 1998).

The principal physiological modulators that regulate PDK4 expression in resting skeletal muscle are changes in FFA and insulin availability, which explains why PDK4 mRNA is selectively up-regulated in response to starvation and hormonal and substrate changes, and in pathologies such as insulin resistance and type 2 diabetes (Wu, Inskeep et al. 1999, Sugden, Kraus et al. 2000, Huang, Wu et al. 2002). It is worth to note that the activity of each isoenzyme

responds not only to the transcriptional and translational requests but they also respond allosterically to the levels of the FFAs degradation products, i.e. acetyl-CoA and NADH, albeit slightly differently (Bowker-Kinley, Davis et al. 1998). Thus, NADH stimulates PDK1 activity by 20% and PDK2 activity by 30%. NADH with acetyl-CoA increases activity in these enzymes by 200% and 300% respectively. For similar conditions, PDK3 is insensitive to NADH but surprisingly inhibited by NADH with acetyl-CoA. PDK4 present a 200% activity increase with NADH, but the addition of acetyl-CoA does not increase activity further.

Past experimental investigations indicated that the levels of PDC phosphorylation are significantly high in diffused diseases like type II diabetes, which is often related to high fat dietary intake (Saunier, Benelli et al. 2016, Stacpoole 2017). Lengthy exposure of PDC to high levels of cellular PDK4 protein mediated by chronic high fat dietary intake may appear to affect the location of the phosphorylation site, and thereby the enzymatic function of PDC protein. Although we do not have any personal or published data related to the PDK4 in particular, *in vitro* studies demonstrated that PDK1 isoform, which is the only kinase that can phosphorylate all the three sites, has different preferences and kinetics for each serine site (Korotchkina and Patel 2001). Additional experimental data indicate that the phosphorylation of specific serine residues (Ser-264-α, Ser-271-α, and Ser-203-α in the human sequence) results in different degrees of PDC enzyme inhibition, with the most potent effect observed with Ser-264-α-P (Patel, Nemeria et al. 2014). Conversely, other evidence suggests that upregulation of PDK4

in muscle with high fat dietary intake is rapid and does not change as the consumption of the high-fat diet intake continues. However, the PDC activity will continue to decline (Crewe, Kinter et al. 2013). This finding may suggest that is the length of exposure to PDK4 that causes higher levels of PDC phosphorylation.

7-1-2. Chapter 4

In this chapter, we undertook a series of experiments aimed to provide conclusive evidence of the involvement of our proposed transcription factors in instauration of a cellular condition presented with a palmitate-induced dysregulation of CHO metabolism aka metabolic inflexibility. This was achieved by successive individual siRNA silencing of PPARα, PPARδ, and FOXO1 gene experiments validated at transcriptional and translation levels matched by parallel measurements of cellular glucose uptake, PDC activity, selective metabolite measurements reflective of the flux through the PDC reaction (e.g. acetyl-CoA and acetylcarnitine) and MAPR. It was found that palmitate-induced reduction in PDC activity could be reversed by siRNA PPARδ and FOXO1 gene silencing in parallel with a reduction in PDK4 protein expression. It appeared, however, that although PPARα gene silencing could also restore the palmitate-induced reduction in PDC activity, this occurred independently from changes in PDK4 protein expression. Communal to all transcription factors tested, the siRNA PPARα, PPARδ, and FOXO1 gene silencing rescued the palmitate-induced reduction in the cellular glucose uptake, whereas only PPARδ and FOXO1 gene silencing reversed the

palmitate-induced increase and decrease in media lactate and acetylcarnitine concentrations, respectively. The latter findings, which are in line with the contention these transcription factors are targeting PDC, also provide further evidence that the effects of PPARα on PDC activity occur independently of that PDK4 protein. Additionally, siRNA PPARα and FOXO1 silencing restored the palmitate-induced reduction in MAPR, while PPARδ silencing could surprisingly not rescue the palmitate-induced decline in MAPR. It was also found that the siRNA PPARδ gene silencing decreased FOXO1 protein expression. In contrast, the siRNA FOXO1 gene silencing did not change PPARδ protein expression, suggesting that FOXO1 may be a PPARδ downstream target thereby modulating PDK4 protein expression.

Considering the role of PPARδ in the context of the current findings, our laboratory has previously reported in *in vivo* and *ex-vivo* rat model studies that pre-treatment with the PPARδ agonist compound (GW610742) increased muscle PDK4 protein expression (Constantin, Constantin-Teodosiu et al. 2007) and attenuate exercise-induced activation of muscle PDC and MAPR (Constantin-Teodosiu, Baker et al. 2009). Conversely, work by others revealed that PPARδ knock-out in mice was associated with a decline in PDK4 mRNA expression (Nahle, Hsieh et al. 2008). Collectively, these observations point to an important role for PPARδ in the regulation of muscle PDC activation and flux and mitochondrial CHO oxidation. Although PPARα, alone or in association with PPARδ, was previously also considered as a regulator of muscle PDC activity during conditions associated with increased FFA availability (e.g.

starvation or dietary high-fat intake) (Wu, Peters et al. 2001, Jucker, Yang et al. 2007), the present study revealed no clear association between changes in cellular fuel metabolism and PPARα protein expression. Our results bear similarity with other data that indicated that there was no relationship between PPARα and PDK4, as it was demonstrated that expression of PDK4 gene occurs independently from PPARα signalling in oxidative skeletal muscle (Holness, Bulmer et al. 2002).

Figure 7-1 would summarise schematically the underlying mechanism by which chronic excess of fatty acid availability may affect CHO oxidation. Excess fatty acid does not only affect the insulin signalling pathway, which regulates transcription factor FOXO1, but also PPARα and PPARδ in the nucleus. To elucidate the role of PPARα, PPARδ, and FOXO1 in palmitate-induced CHO oxidation-reduction, we silenced the three genes. In chapter 4, it was found PPARδ silencing had a greater recovery of the palmitate-induced reduction in PDC activity compared to PPARα and FOXO1 silencing and this was matched by decreased PDK4 expression, lower media lactate concentration, and decreased MAPR. Interestingly, we found that DCA treatment generated similar results to those after PPARδ silencing, e.g. both interventions lowered PDK4 protein expression and media lactate concentration, and MAPR in the presence of palmitate. This suggests that the effects of DCA may be exerted through modulation of the PPARδ signalling pathway. Also, as we commented in chapter 5, chronic DCA administration in the presence of palmitate may contribute to a decline in MAPR by

enabling increases in the rates of ROS production (Haugrud, Zhuang *et al.* 2014, Lee, Lim *et al.* 2017, Ward, Poff *et al.* 2017) and/or declines in cytochrome c oxidase subunit IV (Li, Park *et al.* 2006, Hoshino, Tamura *et al.* 2015).

In the case of FOXO1, dephosphorylation results in its translocation from the cytosol to the nucleus, where it can bind directly to the promoter region of the PDK4 gene (Furuyama, Kitayama et al. 2003). Moreover, FOXO1 dephosphorylation appears to be coupled via a signalling cascade to changes in circulatory FFA and/or insulin availability (Kim, Lee et al. 2006), thereby tying muscle PDK4 gene transcription (amongst others) to substrate availability. Indeed, we have previously highlighted the role of FOXO1 in the up-regulation of muscle PDK4 mRNA expression, and reduction in muscle PDC activity, in the resting state and during 60 min of submaximal exercise following three days of dietary high-fat feeding in healthy volunteers (Constantin-Teodosiu, Constantin et al. 2012).

7-1-3. Chapter 5

In Chapter 5, it was shown that the individual administration of RALA or DCA rescued the palmitate-induced PDC activity inhibition and the rise in media lactate concentration. However, only RALA could improve the deleterious effects of palmitate supplementation on glucose uptake and mitochondrial ATP production rate. At the molecular level, it was shown that DCA, but not RALA, reduced PDK4 protein expression in the presence of palmitate. Also,

DCA and RALA reversed the palmitate-induced upregulation of PPARα and PPARδ and total-FOXO1 protein expression.

PPARα silencing and RALA were the only treatments, which rescued the reduction in PDC activity in the presence of palmitate without decreasing PDK4 protein expression. Both could, however, increase glucose uptake and ATP production rate. Increased glucose influx increases the pyruvate content, which is a strong activator PDC activity by inhibiting the activity of PDK4 protein (Denton, Randle et al. 1975). However, we could not find any accumulation of acetyl groups in PPARα silencing and RALA treatment, which may suggest a near steady-state equilibrium between the acetyl-CoA formation via the enhanced PDC reaction and the rate of its entry into Krebs cycle with these two interventions. On this basis, both PPARα silencing and RALA treatments generated greater MAPR in the presence of palmitate than palmitate alone. Overall, these results provide evidence that RALA treatment may be suitable for patients who present insulin resistance and fatty acid dysregulation.

On a parity with the similarity between PPARδ and DCA interventions, it was found that the cellular responses to FOXO1 silencing and EPS were similar: both rescued glucose uptake, PDC activity, and MAPR in the presence of palmitate in parallel with decreased PDK4 protein expression and lower lactate content. In line with our findings, FOXO1 inhibitor 5-amino-7-(cyclohexylamino)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (AS1842856) has been shown to increase glucose oxidation, improve fasting glycaemia

(Nagashima, Shigematsu et al. 2010) and suppress adipogenesis (Zou, Liu et al. 2014), being thereby considered as a potential agent of anti-diabetes. Our results provided further insight by showing uniquely that low-frequency EPS treatment, rather than a pharmacological intervention, reversed the palmitate-induced FOXO1 increased and decreased nuclear/cytosolic fraction of p-FOXO1/t-FOXO1. This provides evidence to suggest that EPS modulates CHO oxidation by regulating FOXO1 pathway, and this mechanism may account for the similarity of the cellular responses between FOXO1 silencing and EPS interventions reported in this thesis.

DCA was previously known as a potent inhibitor of the PDK4 activity, but the present thesis went further by providing evidence that DCA treatment can also modulate/decrease the PDK4 protein expression. However, the documented enhancement in PDC activity with the DCA treatment in the presence of palmitate could not be matched by an improvement in the cellular glucose uptake, reducing thereby any benefits to the MAPR that might have been provided by the improvement in the PDC activity. The result suggested that DCA administration may not be suitable for treating insulin resistance states. Interestingly, RALA intervention generated opposite cellular responses from those that came from DCA in terms of glucose uptake, MAPR, acetyl group accumulation, and PDK4 protein expression. Since the effects of RALA and DCA seem to be complementary, it may be therefore tempting to consider a combination of the DCA and RALA for the treatment on palmitate-induced CHO oxidation impairment.

In chapters 5 and 6, the results suggest that DCA, RALA, and EPS interventions modulate CHO oxidation through different pathways. Thus, DCA decreased PPARs protein expression matched by a reduction in the PDK4 expression, which consequently improved PDC activity. DCA could not, however, modulate either glucose uptake or MAPR. While similar to DCA, RALA treatment could reverse the reduction of PDC activity induced by palmitate, but this occurred, however, without changing the PDK4 protein expression. RALA could also increase the glucose uptake and ATP production rate in the presence of palmitate compared to palmitate alone. On the other side, low-frequency EPS intervention not only decreased PDK4 protein expression, but it also increased glucose uptake and MAPR in the presence of palmitate compared to palmitate alone. Overall, the results may predictably suggest that low-frequency EPS, which is a good model to mimic exercise, is better than DCA and RALA interventions at improving the palmitate-mediated impairment of CHO oxidation. In addition, and similar to exercise, chronic application of low-frequency EPS can also promote fatty acid oxidation and reduction in lipid accumulation in a skeletal muscle cell model (Li, Ma et al. 2019). With larger implication to the overall cellular glucose and other metabolic pathways, low-frequency EPS has been shown to activate AMPK expression (Dong, Wu et al. 2018).

7-1-4. Chapter 6

In Chapter 6, similar to Chapter 3, it was demonstrated that incubation of C2C12 myotubes in a physiological concentration of palmitate (300 μM) reduced cellular glucose uptake, PDC activity, and flux, and rates of CHO derived mitochondrial ATP production compared to control conditions. Furthermore, these palmitate-induced cellular events were accompanied by increased PPARα (50%) and PPARδ (100%) protein expression and a reduction in the p-FOXO/t-FOXO protein ratio (60%) in total cell lysates, alongside greater PDK4 protein expression relative to control.

In Chapter 6, it was also demonstrated that 16 hrs of exposure of palmitate-treated cells to EPS rescued the palmitate-induced dysregulation of CHO metabolism, as reflected by the restoration of deficits in cellular glucose uptake, PDC activity, and its flux, and the mitochondrial glucose-derived pyruvate ATP production. Furthermore, these events were accompanied by normalization of the protein expression levels of PDK4, PPARδ, but not PPARα, and the p-FOXO1/t-FOXO1 ratio normalised to that of nuclear to cytoplasmic factor. Overall, the present results point to a central role for PPARδ and FOXO1 in the palmitate-induced increase in myotube PDK4 expression and linked reductions in cellular glucose uptake, PDC activity, and flux, and rates of CHO derived oxidative ATP generation. Furthermore, the finding that 16 hrs of low-frequency EPS rescued this palmitate-induced dysregulation of cellular CHO metabolism via modulation of the same molecular and cellular events alludes to

the mechanisms by which regular physical activity will protect skeletal muscle against lipid-induced muscle metabolic inflexibility and IR.

It is worth considering in more detail that the normalization of palmitate-induced deficits in cellular glucose uptake, PDC activity and flux, and rates of CHO derived MAPR with 16 hrs of low-frequency EPS were matched by reductions in PDK4, PPARδ, and the nuclear to cytosolic p-FOXO1/t-FOXO1 ratio, protein expression, but not by a reduction in PPARα protein expression. These findings suggest that FOXO1 and PPARδ transcription factors by *de facto* are controlling the expression of PDK4 protein. Our laboratory carried out in the past several *in vivo* studies (Constantin et al. 1991, 1992, and more), which clearly showed that in the isolated heart tissue (Randle's cycle) proposed covalent and allosteric control of the active form of PDC, PDCa, by direct (PDC phosphorylation-dephosphorylation) and indirect (product inhibition and activation control of PDC and PDK4 reactions, respectively; for review see Otto Wieland 1972), are not fit for purpose during muscle contraction, albeit under the caveat that the exercise has not been preceded by a high-fat dietary intake intervention. More explicitly, during exercise models like voluntary incremental or exhaustive voluntary and involuntary exercise induced by high-intensity electrical stimulation, near-maximal activation/transformation of PDC to active form matched by marked increases in the cellular NADH/NAD^+ and acetyl-CoA/CoASH ratio were reported. Full activation of PDC by submaximal/maximal intensity exercise can occur in human skeletal muscle even during the

condition of glycogen depleted, and therefore low pyruvate availability, or near full reduction of cellular NAD^+ to NADH by prolonged occlusion of the blood supply the lower limb. Collectively, these studies strongly advocate for the exercise-mediated calcium ions and pyruvate as the main drivers of PDC activation when the cellular ratios of acetyl-CoA/CoASH and NADH/NAD^+ are high via activation and inhibiting the activities of PDC phosphatase and PDK4, respectively.

Conversely, when exercise, including maximal isometric electrically evoked stimulation, has been preceded by three days of high fat dietary intake the calcium stimulus is not enough to fully activate PDC (Constantin-Teodosiu et al. 2019). The difference in the outcome of PDC activation in the two scenarios, i.e. when exercise is applied after a normal diet or after a chronic high fat dietary intake, is most likely due to the longer exposure of phosphorylation (inactivation) sites on PDC protein to the fat-mediated elevation of PDK4 protein before exercise, as it will be explained further on.

As an experimental message, in chapters 3, 4, and 5 we used 500 µM palmitate to induce the traits of cellular insulin resistance/metabolic dysfunction. However, in testing the impact of EPS on palmitate-induced impairment of CHO oxidation, we reached our conclusions by using a concentration of 300 µM palmitate. Initially, when we applied the low-frequency EPS on the cells treated with 500 µM palmitate, we found that some C2C12 cells became unviable. Fatty acids (like palmitate) can form micelles in solution and act as detergents in the presence of lipid

membranes. This process can be classified as a cytotoxic (Henriksen, Andresen et al. 2010). The toxicity is reflected by disruptions of the cellular membrane integrity, increase cytosolic and mitochondrial reactive oxygen species, depolarising of the mitochondrial membrane potential, impairment of ATP synthesis and induction of apoptotic cell death (Lambertucci, Hirabara et al. 2008, Zhang, Xia et al. 2017, Li, Xiao et al. 2018). However, we did not record any of these cytotoxic phenotypes for those experiments conducted with 500 μM palmitate alone. However, addition of low-frequency EPS tipped off the balance and caused some cell death to occur. In its right, low-frequency EPS has been shown to induce reactive oxygen species and reactive nitrogen species (ROS/RNS) production in skeletal muscle, which results in additional oxidative damage to the cellular constituents (Horie, Warabi et al. 2015). EPS treatment per se increased medium lactate concentrations that may cause an additional acid-base imbalance, and thereby add more volume to the overall cellular myotoxicity burden (Taha, De Moor et al. 2016). Therefore, we tested a lower working palmitate concentration in the presence of low-frequency EPS. We found that the nearest to 500 μM acceptable concentration to maintain cell viability was 300 μM. We then validated the 300 μM palmitate mediated insulin-resistant model, which similar to the 500 μM resulted in decreased glucose uptake, lower pyruvate mediated mitochondrial ATP production rate, increased media lactate concentration and decreased cellular PDC activity. We also demonstrated that 300 μM

palmitate increased both PDK4, PPARα, PPARδ, and FOXO1 mRNA gene expression and protein expressions significantly.

7-2. The advantages and limitations of the *in vitro* cell model

There are advantages in working with cell culture vs *in vivo* experimental models. Firstly, the cells are depleted of any extracellular hormonal and humoral immune responses, which will thereby limit the effects of confounding factors. In cell cultures, the maintenance of the extracellular environment constraints such as pH value, gas composition, temperature, and osmolarity are easily controlled. Secondly, although the cells grow fast, it is easy to standardize their growth so that the variation in their intrinsic biological responses is minimized. Thirdly, the progress of culture cell growth can be straightforwardly characterized by immune-staining techniques. Fourth, the concentration and time effect of compounds such as drugs and physiological activators or inhibitors can be easily monitored in cell cultures. Fifth, genes and protein expressions in a cell model are much easier to be manipulated than *in vivo* by using siRNA or shRNA silencing. Last, but not least important, the use of cell culture allows a decrease in the number of animals used in scientific research.

Conversely, there are some disadvantages and limitations such as there is no circulation in *vitro* model, which means it cannot fully mimic the environment of the whole body. Also, it is not easy to keep a sterile environment of cell culture if people are not trained sufficiently before

starting the experiments. Also, due to a rapid growth rate, there is a high chance of genetic divergence from the original genotype. To prevent these limitations, we did the experiments in a strictly sterile environment and kept the growing cells in sterile incubators equipped with advanced HEPA filters. Equally important, we collected our data from a low cell generation (4 to 5) to prevent the cells from entering the genetic divergence phase.

The skeletal muscle cells used in this thesis were C2C12, which are immortalised mouse myoblasts. C2C12 cells are useful in biomedical research. These cells are capable of rapid proliferation under high serum conditions. They can easily differentiate into myoblasts under low serum conditions, which can then transform from mononucleated myoblasts to multinucleated myotubes. Closed and crucial to the heart of this thesis was that the C2C12 cells are suitable for stable transfection experiments (McMahon, Anderson et al. 1994). C2C12 cells have been also used in the past in electrical pulse stimulation experiments as an *in vitro* exercise models (Evers-van Gogh, Alex et al. 2015, Danilov, Sidorenko et al. 2017, Li, Ma et al. 2019). To add more support to the suitability of our selected cell culture, there are several published studies, whose interests were not far from ours, where the exercise-induced hormone secretion (Nedachi, Hatakeyama et al. 2009), substrate utilisation and metabolic signalling (Nedachi, Fujita et al. 2008), or gene expression (Burch, Arnold et al. 2010) were investigated. The choice of C2C12 cell culture proved to be invaluable. However, there is a need for cautious in the interpretation of the present results as our findings of CHO regulation in a murine C2C12

palmitate-induced metabolic inflexible skeletal muscle cell line model may be different from what may be occurring in human skeletal muscle, as the rodents' skeletal muscle CHO metabolism differs from that of humans. Although the mouse is a tractable metabolic system, it is not, however, a human. Overall, the results in the cell models cannot fully represent the effect of treatment/intervention in the body. Therefore, an animal model closer to human is further warrant for use to validate what we have found at the cell level.

7-3. Conclusion

The present results clearly show that palmitate decreased cellular glucose uptake, PDC activity, and maximal ATP production rates from glucose-derived pyruvate as a substrate, which are all hallmarks of insulin resistance/metabolic dysfunction. DCA improved PDC activity by decreasing PPARδ and FOXO1 mRNA expression. However, DCA could not rescue the palmitate-induced MAPR inhibition. Besides, RALA reversed the palmitate-induced glucose uptake, pyruvate dehydrogenase complex activity, and mitochondrial ATP production reduced via a PDK4-independent pathway. These results suggest that RALA protects against chronic FFA excess mediated inhibition of PDC activity and mitochondrial ATP production. However, our molecular findings indicate that the best route for the preservation of CHO oxidation in the presence of palmitate is achieved by undertaking regular exercise.

Overall, this thesis provides compelling evidence that the underlying mechanism by which fat/palmitate downregulates the PDC-mediated reduction in CHO oxidation in muscle consists of the following series of events:

Palmitate ⟶ PPARδ ⟶ FOXO1 ⟶ PDK4 ⟶ PDC ⟶ CHO oxidation.

Of a note, the action of PPARα on the rate of CHO oxidation is weak, and it does not seem to involve upregulation of PDK4 protein pathway. This thesis provides also evidence that in addition to exercise, DCA and RALA alone or in combination can rescue the palmitate-induced reduction in CHO oxidation in conditions that exercise cannot be served due to certain medical constrains (e.g. morbid obese patients). Overall, these results are of clinical importance as they open the path for new potential therapies to insulin resistance mediated non-transmissible illnesses stemmed from chronic circulating free fatty acid excess.

7-4. Future directions

C2C12 is a murine skeletal muscle cell line lacking any extracellular natural environment restrictions. Therefore, the present results may be different in a human model where the presence of cofounding factors like different-than-insulin hormonal and humoral stimuli in response to other than high-fat diet lifestyle related factors may affect the modulation of PDK4 gene and protein expression. Thus, PDK4 gene expression is stimulated by thyroid hormone

(T_3), glucocorticoids, hormones which are all associated with stress (Attia, Connnaughton et al. 2010).

Besides, the results gathered in the present metabolic inflexible cell model after DCA and RALA interventions might not be fully reproducible in the whole-body as their circulating level and action might be affected by them being metabolised/converted in the liver. Additionally, DCA has the remarkable property of obstructing its own metabolism upon repeat treating, resulting in modification of its pharmacokinetics (James, Jahn et al. 2017). The first step in DCA metabolism is conversion to glyoxylate catalysed by glutathione transferase zeta 1 (GSTZ1), for which DCA is a mechanism-based inactivator. The rate of GSTZ1 inactivation by DCA is also affected by ageing. Therefore, the effect of DCA on its metabolism obscures the choice of an operative dose with minimal side outcomes.

Due to these shortcomings, the researcher needs, therefore, to move away from the cell-based model, and since a direct clinical translation might be too early due to ethical and safety reasons, a similar-to-us investigation, but in an animal model could offer a helping hand in the transition cell-to-man translation. For instance, insulin resistance can be straightforwardly induced in rodent models with high-fat diet feeding, followed by an increase in body weight and diabetes (Johnson, Greenwood et al. 1991). In future studies, one could, therefore, administer PPARα, PPARδ, FOXO1 inhibitors, or DCA and RALA to mice exposed to high-

fat diet and explore their efficacy to recover muscle PDC activity and the rates of CHO oxidation *in vivo*.

Although EPS is an excellent exercise model for *in vitro* cell models, it has been successfully used *in vivo* in rodent models. Treadmill exercise and forced swimming, which are forced exercise models, and wheel running, which is a voluntary exercise model, can be also used in mice (Svensson, Rosvall et al. 2016, Manzanares, Brito-da-Silva et al. 2018). Treadmill training provides the investigator with the advantage of controlling the amount of work performed in each training session (Gibb, McNally et al. 2016), while the voluntary wheel running is more similar to the natural running pattern. Voluntary wheel running would be ideal as is completed under non-stress conditions as the negative stimulus such electrical shock or touching the animals, which are used in forced treadmill running, is not required (Manzanares, Brito-da-Silva et al. 2018). Harvesting muscle tissues from these exercised animals could provide then evidence of whether the improved CHO oxidation with exercise in the fat overfed animals displays similar molecular and biochemical responses to those identified in the present thesis.

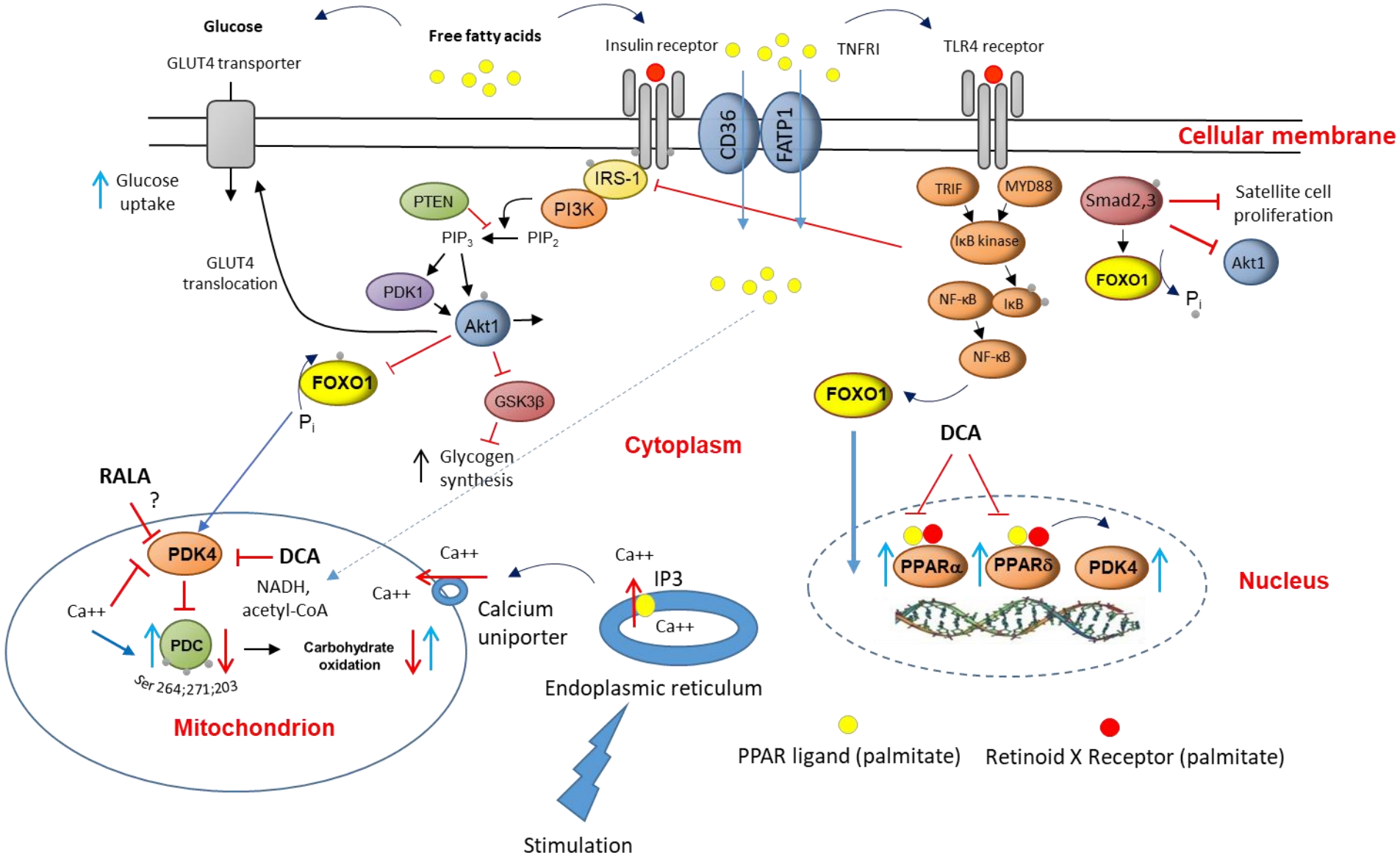


Figure 7-1 Proposed underlying mechanism by which chronic fatty acid excess may affect CHO oxidation: Responses to interventions.

References

Abbot, E. L., J. G. McCormack, C. Reynet, D. G. Hassall, K. W. Buchan and S. J. Yeaman (2005). "Diverging regulation of pyruvate dehydrogenase kinase isoform gene expression in cultured human muscle cells." Febs j **272**(12): 3004-3014.

Abu Bakar, M. H. and J. S. Tan (2017). "Improvement of mitochondrial function by celastrol in palmitate-treated C2C12 myotubes via activation of PI3K-Akt signaling pathway." Biomed Pharmacother **93**: 903-912.

Alamdari, N., D. Constantin-Teodosiu, A. J. Murton, S. M. Gardiner, T. Bennett, R. Layfield and P. L. Greenhaff (2008). "Temporal changes in the involvement of pyruvate dehydrogenase complex in muscle lactate accumulation during lipopolysaccharide infusion in rats." J Physiol **586**(6): 1767-1775.

Amati, F., J. J. Dube, E. Alvarez-Carnero, M. M. Edreira, P. Chomentowski, P. M. Coen, G. E. Switzer, P. E. Bickel, M. Stefanovic-Racic, F. G. Toledo and B. H. Goodpaster (2011). "Skeletal muscle triglycerides, diacylglycerols, and ceramides in insulin resistance: another paradox in endurance-trained athletes?" Diabetes **60**(10): 2588-2597.

Attia, R. R., S. Connnaughton, L. R. Boone, F. Wang, M. B. Elam, G. C. Ness, G. A. Cook and E. A. Park (2010). "Regulation of pyruvate dehydrogenase kinase 4 (PDK4) by thyroid hormone: role of the peroxisome proliferator-activated receptor gamma coactivator (PGC-1 alpha)." J Biol Chem **285**(4): 2375-2385.

Babu, E., S. Ramachandran, V. CoothanKandaswamy, S. Elangovan, P. D. Prasad, V. Ganapathy and M. Thangaraju (2011). "Role of SLC5A8, a plasma membrane transporter and a tumor suppressor, in the antitumor activity of dichloroacetate." Oncogene **30**(38): 4026-4037.

Berger, J. and D. E. Moller (2002). "The mechanisms of action of PPARs." Annu Rev Med **53**: 409-435.

Bernsmeier, C., D. Calabrese, M. H. Heim and H. T. Duong (2014). "Hepatitis C virus dysregulates glucose homeostasis by a dual mechanism involving induction of PGC1alpha and dephosphorylation of FoxO1." J Viral Hepat **21**(1): 9-18.

Boden, G., B. Lebed, M. Schatz, C. Homko and S. Lemieux (2001). "Effects of acute changes of plasma free fatty acids on intramyocellular fat content and insulin resistance in healthy subjects." Diabetes **50**(7): 1612-1617.

Bowker-Kinley, M. M., W. I. Davis, P. Wu, R. A. Harris and K. M. Popov (1998). "Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex." Biochem J **329 (Pt 1)**: 191-196.

Brand, M. D. and D. G. Nicholls (2011). "Assessing mitochondrial dysfunction in cells." Biochem J **435**(2): 297-312.

Burch, N., A. S. Arnold, F. Item, S. Summermatter, G. Brochmann Santana Santos, M. Christe, U. Boutellier, M. Toigo and C. Handschin (2010). "Electric pulse stimulation of cultured murine muscle cells reproduces gene expression changes of trained mouse muscle." PLoS One **5**(6): e10970.

Cadoudal, T., E. Distel, S. Durant, F. Fouque, J. M. Blouin, M. Collinet, S. Bortoli, C. Forest and C. Benelli (2008). "Pyruvate dehydrogenase kinase 4: regulation by thiazolidinediones and implication in glyceroneogenesis in adipose tissue." Diabetes **57**(9): 2272-2279.

Capel, F., N. Cheraiti, C. Acquaviva, C. Henique, J. Bertrand-Michel, C. Vianey-Saban, C. Prip-Buus and B. Morio (2016). "Oleate dose-dependently regulates palmitate metabolism and insulin signaling in C2C12 myotubes." Biochim Biophys Acta **1861**(12 Pt A): 2000-2010.

Cederblad, G., J. I. Carlin, D. Constantin-Teodosiu, P. Harper and E. Hultman (1990). "Radioisotopic assays of CoASH and carnitine and their acetylated forms in human skeletal muscle." Anal Biochem **185**(2): 274-278.

Chen, X., L. Yi, S. Song, L. Wang, Q. Liang, Y. Wang, Y. Wu and Q. Gao (2018). "Puerarin attenuates palmitate-induced mitochondrial dysfunction, impaired mitophagy and inflammation in L6 myotubes." Life Sci **206**: 84-92.

Chien, H. C., P. C. Chan, C. C. Tu, Y. J. Day, L. M. Hung, C. C. Juan, Y. F. Tian and P. S. Hsieh (2018). "Importance of PLC-Dependent PI3K/AKT and AMPK Signaling in RANTES/CCR5 Mediated Macrophage Chemotaxis." Chin J Physiol **61**(5): 266-279.

Chou, C. J., M. Haluzik, C. Gregory, K. R. Dietz, C. Vinson, O. Gavrilova and M. L. Reitman (2002). "WY14,643, a peroxisome proliferator-activated receptor alpha (PPARalpha) agonist, improves hepatic and muscle steatosis and reverses insulin resistance in lipoatrophic A-ZIP/F-1 mice." J Biol Chem **277**(27): 24484-24489.

Cnop, M., J. C. Hannaert, A. Hoorens, D. L. Eizirik and D. G. Pipeleers (2001). "Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation." Diabetes **50**(8): 1771-1777.

Coll, T., X. Palomer, F. Blanco-Vaca, J. C. Escola-Gil, R. M. Sanchez, J. C. Laguna and M. Vazquez-Carrera (2010). "Cyclooxygenase 2 inhibition exacerbates palmitate-induced inflammation and insulin resistance in skeletal muscle cells." Endocrinology **151**(2): 537-548.

Connaughton, S., F. Chowdhury, R. R. Attia, S. Song, Y. Zhang, M. B. Elam, G. A. Cook and E. A. Park (2010). "Regulation of pyruvate dehydrogenase kinase isoform 4 (PDK4) gene expression by glucocorticoids and insulin." Mol Cell Endocrinol **315**(1-2): 159-167.

Constantin-Teodosiu, D. (2013). "Regulation of muscle pyruvate dehydrogenase complex in insulin resistance: effects of exercise and dichloroacetate." Diabetes Metab J **37**(5): 301-314.

Constantin-Teodosiu, D., D. J. Baker, D. Constantin and P. L. Greenhaff (2009). "PPARdelta agonism inhibits skeletal muscle PDC activity, mitochondrial ATP production and force generation during prolonged contraction." J Physiol **587**(1): 231-239.

Constantin-Teodosiu, D., J. I. Carlin, G. Cederblad, R. C. Harris and E. Hultman (1991). "Acetyl group accumulation and pyruvate dehydrogenase activity in human muscle during incremental exercise." Acta Physiol Scand **143**(4): 367-372.

Constantin-Teodosiu, D., G. Cederblad, M. Bergstrom and P. L. Greenhaff (2018). "Maximal-intensity exercise does not fully restore muscle pyruvate dehydrogenase complex activation after 3 days of high-fat dietary intake." Clin Nutr.

Constantin-Teodosiu, D., G. Cederblad, M. Bergstrom and P. L. Greenhaff (2019). "Maximal-intensity exercise does not fully restore muscle pyruvate dehydrogenase complex activation after 3 days of high-fat dietary intake." Clin Nutr **38**(2): 948-953.

Constantin-Teodosiu, D., G. Cederblad and E. Hultman (1991). "A sensitive radioisotopic assay of pyruvate dehydrogenase complex in human muscle tissue." Anal Biochem **198**(2): 347-351.

Constantin-Teodosiu, D., G. Cederblad and E. Hultman (1992). "PDC activity and acetyl group accumulation in skeletal muscle during prolonged exercise." J Appl Physiol (1985) **73**(6): 2403-2407.

Constantin-Teodosiu, D., G. Cederblad and E. Hultman (1993). "PDC activity and acetyl group accumulation in skeletal muscle during isometric contraction." J Appl Physiol (1985) **74**(4): 1712-1718.

Constantin-Teodosiu, D., D. Constantin, F. Stephens, D. Laithwaite and P. L. Greenhaff (2012). "The role of FOXO and PPAR transcription factors in diet-mediated inhibition of PDC activation and carbohydrate oxidation during exercise in humans and the role of pharmacological activation of PDC in overriding these changes." Diabetes **61**(5): 1017-1024.

Constantin-Teodosiu, D., S. Howell and P. L. Greenhaff (1996). "Carnitine metabolism in human muscle fiber types during submaximal dynamic exercise." J Appl Physiol (1985) **80**(3): 1061-1064.

Constantin-Teodosiu, D., N. S. Peirce, J. Fox and P. L. Greenhaff (2004). "Muscle pyruvate availability can limit the flux, but not activation, of the pyruvate dehydrogenase complex during submaximal exercise in humans." J Physiol **561**(Pt 2): 647-655.

Constantin, D., D. Constantin-Teodosiu, R. Layfield, K. Tsintzas, A. J. Bennett and P. L. Greenhaff (2007). "PPARdelta agonism induces a change in fuel metabolism and activation of an atrophy programme, but does not impair mitochondrial function in rat skeletal muscle." J Physiol **583**(Pt 1): 381-390.

Corpeleijn, E., W. H. Saris and E. E. Blaak (2009). "Metabolic flexibility in the development of insulin resistance and type 2 diabetes: effects of lifestyle." Obes Rev **10**(2): 178-193.

Crewe, C., M. Kinter and L. I. Szweda (2013). "Rapid inhibition of pyruvate dehydrogenase: an initiating event in high dietary fat-induced loss of metabolic flexibility in the heart." PLoS One **8**(10): e77280.

Danilov, K., S. Sidorenko, K. Milovanova, E. Klimanova, L. V. Kapilevich and S. N. Orlov (2017). "Electrical pulse stimulation decreases electrochemical Na(+) and K(+) gradients in C2C12 myotubes." Biochem Biophys Res Commun **493**(2): 875-878.

Degenhardt, T., A. Saramaki, M. Malinen, M. Rieck, S. Vaisanen, A. Huotari, K. H. Herzig, R. Muller and C. Carlberg (2007). "Three members of the human pyruvate dehydrogenase kinase gene family are direct targets of the peroxisome proliferator-activated receptor beta/delta." J Mol Biol **372**(2): 341-355.

Denton, R. M., P. J. Randle, B. J. Bridges, R. H. Cooper, A. L. Kerbey, H. T. Pask, D. L. Severson, D. Stansbie and S. Whitehouse (1975). "Regulation of mammalian pyruvate dehydrogenase." Mol Cell Biochem **9**(1): 27-53.

Dong, H. L., H. Y. Wu, Y. Tang, Y. W. Huang, R. Z. Lin, J. Zhao and X. Y. Xu (2018). "[AMPK regulates mitochondrial oxidative stress in C2C12 myotubes induced by electrical stimulations of different intensities]." Nan Fang Yi Ke Da Xue Xue Bao **38**(6): 742-747.

Dromparis, P., G. Sutendra and E. D. Michelakis (2010). "The role of mitochondria in pulmonary vascular remodeling." J Mol Med (Berl) **88**(10): 1003-1010.

Eckardt, K., A. Taube and J. Eckel (2011). "Obesity-associated insulin resistance in skeletal muscle: role of lipid accumulation and physical inactivity." Rev Endocr Metab Disord **12**(3): 163-172.

Evers-van Gogh, I. J., S. Alex, R. Stienstra, A. B. Brenkman, S. Kersten and E. Kalkhoven (2015). "Electric Pulse Stimulation of Myotubes as an In Vitro Exercise Model: Cell-Mediated and Non-Cell-Mediated Effects." Sci Rep **5**: 10944.

Fan, W., W. Waizenegger, C. S. Lin, V. Sorrentino, M. X. He, C. E. Wall, H. Li, C. Liddle, R. T. Yu, A. R. Atkins, J. Auwerx, M. Downes and R. M. Evans (2017). "PPARdelta Promotes Running Endurance by Preserving Glucose." Cell Metab **25**(5): 1186-1193.e1184.

Foster, C. V. and R. C. Harris (1987). "Formation of acetylcarnitine in muscle of horse during high intensity exercise." Eur J Appl Physiol Occup Physiol **56**(6): 639-642.

Furuyama, T., K. Kitayama, H. Yamashita and N. Mori (2003). "Forkhead transcription factor FOXO1 (FKHR)-dependent induction of PDK4 gene expression in skeletal muscle during energy deprivation." Biochem J **375**(Pt 2): 365-371.

Gibb, A. A., L. A. McNally, D. W. Riggs, D. J. Conklin, A. Bhatnagar and B. G. Hill (2016). "FVB/NJ Mice Are a Useful Model for Examining Cardiac Adaptations to Treadmill Exercise." Front Physiol **7**: 636.

Goodpaster, B. H., R. R. Wolfe and D. E. Kelley (2002). "Effects of obesity on substrate utilization during exercise." Obes Res **10**(7): 575-584.

Gorgani-Firuzjaee, S., S. Ahmadi and R. Meshkani (2014). "Palmitate induces SHIP2 expression via the ceramide-mediated activation of NF-kappaB, and JNK in skeletal muscle cells." Biochem Biophys Res Commun **450**(1): 494-499.

Greenhaff, P. L., S. P. Campbell-O'Sullivan, D. Constantin-Teodosiu, S. M. Poucher, P. A. Roberts and J. A. Timmons (2002). "An acetyl group deficit limits mitochondrial ATP production at the onset of exercise." Biochem Soc Trans **30**(2): 275-280.

Gualerzi, C. O. and C. L. Pon (1990). "Initiation of mRNA translation in prokaryotes." Biochemistry **29**(25): 5881-5889.

Guigni, B. A., D. K. Fix, J. J. Bivona, 3rd, B. M. Palmer, J. A. Carson and M. J. Toth (2019). "Electrical stimulation prevents doxorubicin-induced atrophy and mitochondrial loss in cultured myotubes." Am J Physiol Cell Physiol.

Handzlik, M. K., D. Constantin-Teodosiu, P. L. Greenhaff and M. A. Cole (2018). "Increasing cardiac pyruvate dehydrogenase flux during chronic hypoxia improves acute hypoxic tolerance." J Physiol **596**(15): 3357-3369.

Harris, R. A., M. M. Bowker-Kinley, B. Huang and P. Wu (2002). "Regulation of the activity of the pyruvate dehydrogenase complex." Adv Enzyme Regul **42**: 249-259.

Harris, R. C., E. Hultman and L. O. Nordesjo (1974). "Glycogen, glycolytic intermediates and high-energy phosphates determined in biopsy samples of musculus quadriceps femoris of man at rest. Methods and variance of values." Scand J Clin Lab Invest **33**(2): 109-120.

Haugrud, A. B., Y. Zhuang, J. D. Coppock and W. K. Miskimins (2014). "Dichloroacetate enhances apoptotic cell death via oxidative damage and attenuates lactate production in metformin-treated breast cancer cells." Breast Cancer Res Treat **147**(3): 539-550.

Hawley, J. A., M. Hargreaves, M. J. Joyner and J. R. Zierath (2014). "Integrative biology of exercise." Cell **159**(4): 738-749.

Henriksen, J. R., T. L. Andresen, L. N. Feldborg, L. Duelund and J. H. Ipsen (2010). "Understanding detergent effects on lipid membranes: a model study of lysolipids." Biophys J **98**(10): 2199-2205.

Hirabara, S. M., R. Curi and P. Maechler (2010). "Saturated fatty acid-induced insulin resistance is associated with mitochondrial dysfunction in skeletal muscle cells." J Cell Physiol **222**(1): 187-194.

Holness, M. J., K. Bulmer, G. F. Gibbons and M. C. Sugden (2002). "Up-regulation of pyruvate dehydrogenase kinase isoform 4 (PDK4) protein expression in oxidative skeletal muscle does not require the obligatory participation of peroxisome-proliferator-activated receptor alpha (PPARalpha)." Biochem J **366**(Pt 3): 839-846.

Holst, D., S. Luquet, V. Nogueira, K. Kristiansen, X. Leverve and P. A. Grimaldi (2003). "Nutritional regulation and role of peroxisome proliferator-activated receptor delta in fatty acid catabolism in skeletal muscle." Biochim Biophys Acta **1633**(1): 43-50.

Hondares, E., I. Pineda-Torra, R. Iglesias, B. Staels, F. Villarroya and M. Giralt (2007). "PPARdelta, but not PPARalpha, activates PGC-1alpha gene transcription in muscle." Biochem Biophys Res Commun **354**(4): 1021-1027.

Hong, O. K., J. W. Son, H. S. Kwon, S. S. Lee, S. R. Kim and S. J. Yoo (2018). "Alpha-lipoic acid preserves skeletal muscle mass in type 2 diabetic OLETF rats." Nutr Metab (Lond) **15**: 66.

Hong, Y. S., S. J. Jacobia, L. Packer and M. S. Patel (1999). "The inhibitory effects of lipoic compounds on mammalian pyruvate dehydrogenase complex and its catalytic components." Free Radic Biol Med **26**(5-6): 685-694.

Horie, M., E. Warabi, S. Komine, S. Oh and J. Shoda (2015). "Cytoprotective Role of Nrf2 in Electrical Pulse Stimulated C2C12 Myotube." PLoS One **10**(12): e0144835.

Hoshino, D., Y. Tamura, H. Masuda, Y. Matsunaga and H. Hatta (2015). "Effects of decreased lactate accumulation after dichloroacetate administration on exercise training-induced mitochondrial adaptations in mouse skeletal muscle." Physiol Rep **3**(9).

Howlett, R. A., G. J. Heigenhauser, E. Hultman, M. G. Hollidge-Horvat and L. L. Spriet (1999). "Effects of dichloroacetate infusion on human skeletal muscle metabolism at the onset of exercise." Am J Physiol **277**(1): E18-25.

Huang, B., P. Wu, M. M. Bowker-Kinley and R. A. Harris (2002). "Regulation of pyruvate dehydrogenase kinase expression by peroxisome proliferator-activated receptor-alpha ligands, glucocorticoids, and insulin." Diabetes **51**(2): 276-283.

Hue, L. and H. Taegtmeyer (2009). "The Randle cycle revisited: a new head for an old hat." Am J Physiol Endocrinol Metab **297**(3): E578-591.

Iglesias, J., S. Barg, D. Vallois, S. Lahiri, C. Roger, A. Yessoufou, S. Pradevand, A. McDonald, C. Bonal, F. Reimann, F. Gribble, M. B. Debril, D. Metzger, P. Chambon, P. Herrera, G. A. Rutter, M. Prentki, B. Thorens and W. Wahli (2012). "PPARbeta/delta affects pancreatic beta cell mass and insulin secretion in mice." J Clin Invest **122**(11): 4105-4117.

Ikuta, N., K. Chikamoto, Y. Asano, Y. Yasui, H. Yokokawa, K. Terao, G. Rimbach and S. Matsugo (2017). "Time Course Effect of R-Alpha-Lipoic Acid on Cellular Metabolomics in Cultured Hepatoma Cells." J Med Food **20**(3): 211-222.

Jacob, S., P. Ruus, R. Hermann, H. J. Tritschler, E. Maerker, W. Renn, H. J. Augustin, G. J. Dietze and K. Rett (1999). "Oral administration of RAC-alpha-lipoic acid modulates insulin sensitivity in patients with type-2 diabetes mellitus: a placebo-controlled pilot trial." Free Radic Biol Med **27**(3-4): 309-314.

James, M. O., S. C. Jahn, G. Zhong, M. G. Smeltz, Z. Hu and P. W. Stacpoole (2017). "Therapeutic applications of dichloroacetate and the role of glutathione transferase zeta-1." Pharmacol Ther **170**: 166-180.

Jastroch, M., A. S. Divakaruni, S. Mookerjee, J. R. Treberg and M. D. Brand (2010). "Mitochondrial proton and electron leaks." Essays Biochem **47**: 53-67.

Jeoung, N. H. and R. A. Harris (2008). "Pyruvate dehydrogenase kinase-4 deficiency lowers blood glucose and improves glucose tolerance in diet-induced obese mice." Am J Physiol Endocrinol Metab **295**(1): E46-54.

Johnson, P. R., M. R. Greenwood, B. A. Horwitz and J. S. Stern (1991). "Animal models of obesity: genetic aspects." Annu Rev Nutr **11**: 325-353.

Jornayvaz, F. R. and G. I. Shulman (2010). "Regulation of mitochondrial biogenesis." Essays Biochem **47**: 69-84.

Joza, N., S. A. Susin, E. Daugas, W. L. Stanford, S. K. Cho, C. Y. Li, T. Sasaki, A. J. Elia, H. Y. Cheng, L. Ravagnan, K. F. Ferri, N. Zamzami, A. Wakeham, R. Hakem, H. Yoshida, Y. Y. Kong, T. W. Mak, J. C. Zuniga-Pflucker, G. Kroemer and J. M. Penninger (2001). "Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death." Nature **410**(6828): 549-554.

Jucker, B. M., D. Yang, W. M. Casey, A. R. Olzinski, C. Williams, S. C. Lenhard, J. J. Legos, C. T. Hawk, S. K. Sarkar and S. J. Newsholme (2007). "Selective PPARdelta agonist treatment increases skeletal muscle lipid metabolism without altering mitochondrial energy coupling: an in vivo magnetic resonance spectroscopy study." Am J Physiol Endocrinol Metab **293**(5): E1256-1264.

Jue, T., D. L. Rothman, G. I. Shulman, B. A. Tavitian, R. A. DeFronzo and R. G. Shulman (1989). "Direct observation of glycogen synthesis in human muscle with 13C NMR." Proc Natl Acad Sci U S A **86**(12): 4489-4491.

Kersten, S. (2014). "Integrated physiology and systems biology of PPARalpha." Mol Metab **3**(4): 354-371.

Khodabandehloo, H., S. Gorgani-Firuzjaee, G. Panahi and R. Meshkani (2016). "Molecular and cellular mechanisms linking inflammation to insulin resistance and beta-cell dysfunction." Transl Res **167**(1): 228-256.

Kim, H., M. Haluzik, Z. Asghar, D. Yau, J. W. Joseph, A. M. Fernandez, M. L. Reitman, S. Yakar, B. Stannard, L. Heron-Milhavet, M. B. Wheeler and D. LeRoith (2003). "Peroxisome proliferator-activated receptor-alpha agonist treatment in a transgenic model of type 2 diabetes reverses the lipotoxic state and improves glucose homeostasis." Diabetes **52**(7): 1770-1778.

Kim, Y. I., F. N. Lee, W. S. Choi, S. Lee and J. H. Youn (2006). "Insulin regulation of skeletal muscle PDK4 mRNA expression is impaired in acute insulin-resistant states." Diabetes **55**(8): 2311-2317.

Klingler, C., X. Zhao, T. Adhikary, J. Li, G. Xu, H. U. Haring, E. Schleicher, R. Lehmann and C. Weigert (2016). "Lysophosphatidylcholines activate PPARdelta and protect human skeletal muscle cells from lipotoxicity." Biochim Biophys Acta **1861**(12 Pt A): 1980-1992.

Konrad, D., R. Somwar, G. Sweeney, K. Yaworsky, M. Hayashi, T. Ramlal and A. Klip (2001). "The antihyperglycemic drug alpha-lipoic acid stimulates glucose uptake via both GLUT4 translocation and GLUT4 activation: potential role of p38 mitogen-activated protein kinase in GLUT4 activation." Diabetes **50**(6): 1464-1471.

Korotchkina, L. G. and M. S. Patel (2001). "Site specificity of four pyruvate dehydrogenase kinase isoenzymes toward the three phosphorylation sites of human pyruvate dehydrogenase." J Biol Chem **276**(40): 37223-37229.

Korotchkina, L. G., S. Sidhu and M. S. Patel (2004). "R-lipoic acid inhibits mammalian pyruvate dehydrogenase kinase." Free Radic Res **38**(10): 1083-1092.

Kraegen, E. W., G. J. Cooney and N. Turner (2008). "Muscle insulin resistance: a case of fat overconsumption, not mitochondrial dysfunction." Proc Natl Acad Sci U S A **105**(22): 7627-7628.

Kukimoto-Niino, M., A. Tokmakov, T. Terada, N. Ohbayashi, T. Fujimoto, S. Gomi, I. Shiromizu, M. Kawamoto, T. Matsusue, M. Shirouzu and S. Yokoyama (2011). "Inhibitor-

bound structures of human pyruvate dehydrogenase kinase 4." Acta Crystallogr D Biol Crystallogr **67**(Pt 9): 763-773.

Kwon, H. S., B. Huang, T. G. Unterman and R. A. Harris (2004). "Protein kinase B-alpha inhibits human pyruvate dehydrogenase kinase-4 gene induction by dexamethasone through inactivation of FOXO transcription factors." Diabetes **53**(4): 899-910.

Lambernd, S., A. Taube, A. Schober, B. Platzbecker, S. W. Gorgens, R. Schlich, K. Jeruschke, J. Weiss, K. Eckardt and J. Eckel (2012). "Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways." Diabetologia **55**(4): 1128-1139.

Lambertucci, R. H., S. M. Hirabara, R. Silveira Ldos, A. C. Levada-Pires, R. Curi and T. C. Pithon-Curi (2008). "Palmitate increases superoxide production through mitochondrial electron transport chain and NADPH oxidase activity in skeletal muscle cells." J Cell Physiol **216**(3): 796-804.

Lee, C. H., P. Olson and R. M. Evans (2003). "Minireview: lipid metabolism, metabolic diseases, and peroxisome proliferator-activated receptors." Endocrinology **144**(6): 2201-2207.

Lee, C. H., P. Olson, A. Hevener, I. Mehl, L. W. Chong, J. M. Olefsky, F. J. Gonzalez, J. Ham, H. Kang, J. M. Peters and R. M. Evans (2006). "PPARdelta regulates glucose metabolism and insulin sensitivity." Proc Natl Acad Sci U S A **103**(9): 3444-3449.

Lee, H., J. Y. Lim and S. J. Choi (2017). "Oleate Prevents Palmitate-Induced Atrophy via Modulation of Mitochondrial ROS Production in Skeletal Myotubes." Oxid Med Cell Longev **2017**: 2739721.

Lei, L., Y. Zhu, W. Gao, X. Du, M. Zhang, Z. Peng, S. Fu, X. Li, W. Zhe, X. Li and G. Liu (2016). "Alpha-lipoic acid attenuates endoplasmic reticulum stress-induced insulin resistance by improving mitochondrial function in HepG2 cells." Cell Signal **28**(10): 1441-1450.

Li, H., Y. Xiao, L. Tang, F. Zhong, G. Huang, J. M. Xu, A. M. Xu, R. P. Dai and Z. G. Zhou (2018). "Adipocyte Fatty Acid-Binding Protein Promotes Palmitate-Induced Mitochondrial Dysfunction and Apoptosis in Macrophages." Front Immunol **9**: 81.

Li, J., S. Xu, Y. Liu, Z. Yan, F. Zhang, Q. Lv and N. Tong (2019). "Activated PPARbeta/delta Protects Pancreatic beta Cells in Type 2 Diabetic Goto-Kakizaki Rats from Lipoapoptosis via GPR40." Lipids **54**(10): 603-616.

Li, L. J., J. Ma, S. B. Li, X. F. Chen and J. Zhang (2019). "Electric pulse stimulation inhibited lipid accumulation on C2C12 myotubes incubated with oleic acid and palmitic acid." Arch Physiol Biochem: 1-7.

Li, Y., J. S. Park, J. H. Deng and Y. Bai (2006). "Cytochrome c oxidase subunit IV is essential for assembly and respiratory function of the enzyme complex." J Bioenerg Biomembr **38**(5-6): 283-291.

Li, Z., C. M. Dungan, B. Carrier, T. C. Rideout and D. L. Williamson (2014). "Alpha-lipoic acid supplementation reduces mTORC1 signaling in skeletal muscle from high fat fed, obese Zucker rats." Lipids **49**(12): 1193-1201.

Liu, J. (2008). "The effects and mechanisms of mitochondrial nutrient alpha-lipoic acid on improving age-associated mitochondrial and cognitive dysfunction: an overview." Neurochem Res **33**(1): 194-203.

Ma, K., Y. Zhang, M. B. Elam, G. A. Cook and E. A. Park (2005). "Cloning of the rat pyruvate dehydrogenase kinase 4 gene promoter: activation of pyruvate dehydrogenase kinase 4 by the peroxisome proliferator-activated receptor gamma coactivator." J Biol Chem **280**(33): 29525-29532.

Mallinson, J. E., D. Constantin-Teodosiu, P. D. Glaves, E. A. Martin, W. J. Davies, F. R. Westwood, J. E. Sidaway and P. L. Greenhaff (2012). "Pharmacological activation of the

pyruvate dehydrogenase complex reduces statin-mediated upregulation of FOXO gene targets and protects against statin myopathy in rodents." J Physiol **590**(24): 6389-6402.

Manneras, L., I. H. Jonsdottir, A. Holmang, M. Lonn and E. Stener-Victorin (2008). "Low-frequency electro-acupuncture and physical exercise improve metabolic disturbances and modulate gene expression in adipose tissue in rats with dihydrotestosterone-induced polycystic ovary syndrome." Endocrinology **149**(7): 3559-3568.

Manzanares, G., G. Brito-da-Silva and P. G. Gandra (2018). "Voluntary wheel running: patterns and physiological effects in mice." Braz J Med Biol Res **52**(1): e7830.

Mascaro, C., E. Acosta, J. A. Ortiz, P. F. Marrero, F. G. Hegardt and D. Haro (1998). "Control of human muscle-type carnitine palmitoyltransferase I gene transcription by peroxisome proliferator-activated receptor." J Biol Chem **273**(15): 8560-8563.

McBride, H. M., M. Neuspiel and S. Wasiak (2006). "Mitochondria: more than just a powerhouse." Curr Biol **16**(14): R551-560.

McMahon, D. K., P. A. Anderson, R. Nassar, J. B. Bunting, Z. Saba, A. E. Oakeley and N. N. Malouf (1994). "C2C12 cells: biophysical, biochemical, and immunocytochemical properties." Am J Physiol **266**(6 Pt 1): C1795-1802.

Michelakis, E. D., G. Sutendra, P. Dromparis, L. Webster, A. Haromy, E. Niven, C. Maguire, T. L. Gammer, J. R. Mackey, D. Fulton, B. Abdulkarim, M. S. McMurtry and K. C. Petruk (2010). "Metabolic modulation of glioblastoma with dichloroacetate." Sci Transl Med **2**(31): 31ra34.

Morales-Cano, D., L. Moreno, B. Barreira, A. M. Briones, R. Pandolfi, J. Moral-Sanz, M. Callejo, G. Mondejar-Parreno, J. Cortijo, M. Salaices, J. Duarte, F. Perez-Vizcaino and A. Cogolludo (2016). "Activation of PPARbeta/delta prevents hyperglycaemia-induced impairment of Kv7 channels and cAMP-mediated relaxation in rat coronary arteries." Clin Sci (Lond) **130**(20): 1823-1836.

Morino, S., T. Kondo, K. Sasaki, H. Adachi, M. A. Suico, E. Sekimoto, T. Matsuda, T. Shuto, E. Araki and H. Kai (2008). "Mild electrical stimulation with heat shock ameliorates insulin resistance via enhanced insulin signaling." PLoS One **3**(12): e4068.

Motojima, K. (2002). "A metabolic switching hypothesis for the first step in the hypolipidemic effects of fibrates." Biol Pharm Bull **25**(11): 1509-1511.

Muoio, D. M. (2014). "Metabolic inflexibility: when mitochondrial indecision leads to metabolic gridlock." Cell **159**(6): 1253-1262.

Nagashima, T., N. Shigematsu, R. Maruki, Y. Urano, H. Tanaka, A. Shimaya, T. Shimokawa and M. Shibasaki (2010). "Discovery of novel forkhead box O1 inhibitors for treating type 2 diabetes: improvement of fasting glycemia in diabetic db/db mice." Mol Pharmacol **78**(5): 961-970.

Nahle, Z., M. Hsieh, T. Pietka, C. T. Coburn, P. A. Grimaldi, M. Q. Zhang, D. Das and N. A. Abumrad (2008). "CD36-dependent regulation of muscle FoxO1 and PDK4 in the PPAR delta/beta-mediated adaptation to metabolic stress." J Biol Chem **283**(21): 14317-14326.

Nakae, J., Y. Cao, M. Oki, Y. Orba, H. Sawa, H. Kiyonari, K. Iskandar, K. Suga, M. Lombes and Y. Hayashi (2008). "Forkhead transcription factor FoxO1 in adipose tissue regulates energy storage and expenditure." Diabetes **57**(3): 563-576.

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

Nedachi, T., H. Hatakeyama, T. Kono, M. Sato and M. Kanzaki (2009). "Characterization of contraction-inducible CXC chemokines and their roles in C2C12 myocytes." Am J Physiol Endocrinol Metab **297**(4): E866-878.

Nieuwoudt, S., A. Mulya, C. E. Fealy, E. Martelli, S. Dasarathy, S. V. Naga Prasad and J. P. Kirwan (2017). "In vitro contraction protects against palmitate-induced insulin resistance in C2C12 myotubes." Am J Physiol Cell Physiol **313**(5): C575-C583.

Nikolic, N., S. S. Bakke, E. T. Kase, I. Rudberg, I. Flo Halle, A. C. Rustan, G. H. Thoresen and V. Aas (2012). "Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise." PLoS One **7**(3): e33203.

Osorio-Fuentealba, C., A. E. Contreras-Ferrat, F. Altamirano, A. Espinosa, Q. Li, W. Niu, S. Lavandero, A. Klip and E. Jaimovich (2013). "Electrical stimuli release ATP to increase GLUT4 translocation and glucose uptake via PI3Kgamma-Akt-AS160 in skeletal muscle cells." Diabetes **62**(5): 1519-1526.

Ozcan, G., B. Ozpolat, R. L. Coleman, A. K. Sood and G. Lopez-Berestein (2015). "Preclinical and clinical development of siRNA-based therapeutics." Adv Drug Deliv Rev **87**: 108-119.

Pandey, A., P. Raj, S. K. Goru, A. Kadakol, V. Malek, N. Sharma and A. B. Gaikwad (2017). "Esculetin ameliorates hepatic fibrosis in high fat diet induced non-alcoholic fatty liver disease by regulation of FoxO1 mediated pathway." Pharmacol Rep **69**(4): 666-672.

Patel, M. S., N. S. Nemeria, W. Furey and F. Jordan (2014). "The pyruvate dehydrogenase complexes: structure-based function and regulation." J Biol Chem **289**(24): 16615-16623.

Petrie, M., M. Suneja and R. K. Shields (2015). "Low-frequency stimulation regulates metabolic gene expression in paralyzed muscle." J Appl Physiol (1985) **118**(6): 723-731.

Petrie, M. A., M. Suneja, E. Faidley and R. K. Shields (2014). "A minimal dose of electrically induced muscle activity regulates distinct gene signaling pathways in humans with spinal cord injury." PLoS One **9**(12): e115791.

Pi, J., Y. Bai, K. W. Daniel, D. Liu, O. Lyght, D. Edelstein, M. Brownlee, B. E. Corkey and S. Collins (2009). "Persistent oxidative stress due to absence of uncoupling protein 2 associated with impaired pancreatic beta-cell function." Endocrinology **150**(7): 3040-3048.

Piao, L., V. K. Sidhu, Y. H. Fang, J. J. Ryan, K. S. Parikh, Z. Hong, P. T. Toth, E. Morrow, S. Kutty, G. D. Lopaschuk and S. L. Archer (2013). "FOXO1-mediated upregulation of pyruvate dehydrogenase kinase-4 (PDK4) decreases glucose oxidation and impairs right ventricular function in pulmonary hypertension: therapeutic benefits of dichloroacetate." J Mol Med (Berl) **91**(3): 333-346.

Picard, M. (2011). "Pathways to aging: the mitochondrion at the intersection of biological and psychosocial sciences." J Aging Res **2011**: 814096.

Putman, C. T., L. L. Spriet, E. Hultman, M. I. Lindinger, L. C. Lands, R. S. McKelvie, G. Cederblad, N. L. Jones and G. J. Heigenhauser (1993). "Pyruvate dehydrogenase activity and acetyl group accumulation during exercise after different diets." Am J Physiol **265**(5 Pt 1): E752-760.

Qu, F. and J. M. Lin (2001). "[Determination of coenzyme Q and alpha-tocopherol in mouse plasma and tissue mitochondria by liquid chromatography with electrochemical detection]." Se Pu **19**(6): 489-492.

Quintela, A. M., R. Jimenez, L. Piqueras, M. Gomez-Guzman, J. Haro, M. J. Zarzuelo, A. Cogolludo, M. J. Sanz, M. Toral, M. Romero, F. Perez-Vizcaino and J. Duarte (2014). "PPARbeta activation restores the high glucose-induced impairment of insulin signalling in endothelial cells." Br J Pharmacol **171**(12): 3089-3102.

Rakhshandehroo, M., G. Hooiveld, M. Muller and S. Kersten (2009). "Comparative analysis of gene regulation by the transcription factor PPARalpha between mouse and human." PLoS One **4**(8): e6796.

Randle, P. J., P. B. Garland, C. N. Hales and E. A. Newsholme (1963). "The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus." Lancet **1**(7285): 785-789.

Reaven, G. M. (1988). "Banting lecture 1988. Role of insulin resistance in human disease." Diabetes **37**(12): 1595-1607.

Rena, G., S. Guo, S. C. Cichy, T. G. Unterman and P. Cohen (1999). "Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B." J Biol Chem **274**(24): 17179-17183.

Rinnankoski-Tuikka, R., M. Silvennoinen, S. Torvinen, J. J. Hulmi, M. Lehti, R. Kivela, H. Reunanen and H. Kainulainen (2012). "Effects of high-fat diet and physical activity on pyruvate dehydrogenase kinase-4 in mouse skeletal muscle." Nutr Metab (Lond) **9**(1): 53.

Rousseau, A. S., B. Sibille, J. Murdaca, I. Mothe-Satney, P. A. Grimaldi and J. G. Neels (2016). "alpha-Lipoic acid up-regulates expression of peroxisome proliferator-activated receptor beta in skeletal muscle: involvement of the JNK signaling pathway." FASEB J **30**(3): 1287-1299.

Saunier, E., C. Benelli and S. Bortoli (2016). "The pyruvate dehydrogenase complex in cancer: An old metabolic gatekeeper regulated by new pathways and pharmacological agents." Int J Cancer **138**(4): 809-817.

Savkur, R. S., K. S. Bramlett, L. F. Michael and T. P. Burris (2005). "Regulation of pyruvate dehydrogenase kinase expression by the farnesoid X receptor." Biochem Biophys Res Commun **329**(1): 391-396.

Shen, Q. W., M. J. Zhu, J. Tong, J. Ren and M. Du (2007). "Ca2+/calmodulin-dependent protein kinase kinase is involved in AMP-activated protein kinase activation by alpha-lipoic acid in C2C12 myotubes." Am J Physiol Cell Physiol **293**(4): C1395-1403.

Shulman, G. I. (2000). "Cellular mechanisms of insulin resistance." J Clin Invest **106**(2): 171-176.

Shulman, G. I., D. L. Rothman, T. Jue, P. Stein, R. A. DeFronzo and R. G. Shulman (1990). "Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-

dependent diabetes by 13C nuclear magnetic resonance spectroscopy." N Engl J Med **322**(4): 223-228.

Silva-Veiga, F. M., T. L. Rachid, L. de Oliveira, F. Graus-Nunes, C. A. Mandarim-de-Lacerda and V. Souza-Mello (2018). "GW0742 (PPAR-beta agonist) attenuates hepatic endoplasmic reticulum stress by improving hepatic energy metabolism in high-fat diet fed mice." Mol Cell Endocrinol **474**: 227-237.

Sparks, L. M., H. Xie, R. A. Koza, R. Mynatt, M. W. Hulver, G. A. Bray and S. R. Smith (2005). "A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle." Diabetes **54**(7): 1926-1933.

Stacpoole, P. W. (1989). "The pharmacology of dichloroacetate." Metabolism **38**(11): 1124-1144.

Stacpoole, P. W. (2017). "Therapeutic Targeting of the Pyruvate Dehydrogenase Complex/Pyruvate Dehydrogenase Kinase (PDC/PDK) Axis in Cancer." J Natl Cancer Inst **109**(11).

Stacpoole, P. W. and J. M. Felts (1970). "Diisopropylammonium dichloroacetate (DIPA) and sodium dichloracetate (DCA): effect on glucose and fat metabolism in normal and diabetic tissue." Metabolism **19**(1): 71-78.

Stacpoole, P. W. and Y. J. Greene (1992). "Dichloroacetate." Diabetes Care **15**(6): 785-791.

Stacpoole, P. W., G. W. Moore and D. M. Kornhauser (1978). "Metabolic effects of dichloroacetate in patients with diabetes mellitus and hyperlipoproteinemia." N Engl J Med **298**(10): 526-530.

Stacpoole, P. W., E. C. Wright, T. G. Baumgartner, R. M. Bersin, S. Buchalter, S. H. Curry, C. A. Duncan, E. M. Harman, G. N. Henderson, S. Jenkinson and et al. (1992). "A controlled clinical trial of dichloroacetate for treatment of lactic acidosis in adults. The Dichloroacetate-Lactic Acidosis Study Group." N Engl J Med **327**(22): 1564-1569.

Sugden, M. C., A. Kraus, R. A. Harris and M. J. Holness (2000). "Fibre-type specific modification of the activity and regulation of skeletal muscle pyruvate dehydrogenase kinase (PDK) by prolonged starvation and refeeding is associated with targeted regulation of PDK isoenzyme 4 expression." Biochem J **346 Pt 3**: 651-657.

Svensson, M., P. Rosvall, A. Boza-Serrano, E. Andersson, J. Lexell and T. Deierborg (2016). "Forced treadmill exercise can induce stress and increase neuronal damage in a mouse model of global cerebral ischemia." Neurobiol Stress **5**: 8-18.

Taha, D. A., C. H. De Moor, D. A. Barrett, J. B. Lee, R. D. Gandhi, C. W. Hoo and P. Gershkovich (2016). "The role of acid-base imbalance in statin-induced myotoxicity." Transl Res **174**: 140-160.e114.

Tanaka, T., J. Yamamoto, S. Iwasaki, H. Asaba, H. Hamura, Y. Ikeda, M. Watanabe, K. Magoori, R. X. Ioka, K. Tachibana, Y. Watanabe, Y. Uchiyama, K. Sumi, H. Iguchi, S. Ito, T. Doi, T. Hamakubo, M. Naito, J. Auwerx, M. Yanagisawa, T. Kodama and J. Sakai (2003). "Activation of peroxisome proliferator-activated receptor delta induces fatty acid beta-oxidation in skeletal muscle and attenuates metabolic syndrome." Proc Natl Acad Sci U S A **100**(26): 15924-15929.

Thoudam, T., C. M. Ha, J. Leem, D. Chanda, J. S. Park, H. J. Kim, J. H. Jeon, Y. K. Choi, S. Liangpunsakul, Y. H. Huh, T. H. Kwon, K. G. Park, R. A. Harris, K. S. Park, H. W. Rhee and I. K. Lee (2019). "PDK4 Augments ER-Mitochondria Contact to Dampen Skeletal Muscle Insulin Signaling During Obesity." Diabetes **68**(3): 571-586.

Tian, Y. F., C. T. He, Y. T. Chen and P. S. Hsieh (2013). "Lipoic acid suppresses portal endotoxemia-induced steatohepatitis and pancreatic inflammation in rats." World J Gastroenterol **19**(18): 2761-2771.

Tian, Y. F., C. H. Hsieh, Y. J. Hsieh, Y. T. Chen, Y. J. Peng and P. S. Hsieh (2012). "alpha-Lipoic acid prevents mild portal endotoxaemia-induced hepatic inflammation and beta cell dysfunction." Eur J Clin Invest **42**(6): 637-648.

Timmons, J. A., T. Gustafsson, C. J. Sundberg, E. Jansson, E. Hultman, L. Kaijser, J. Chwalbinska-Moneta, D. Constantin-Teodosiu, I. A. Macdonald and P. L. Greenhaff (1998). "Substrate availability limits human skeletal muscle oxidative ATP regeneration at the onset of ischemic exercise." J Clin Invest **101**(1): 79-85.

Tominaga, A., N. Ishizaki, Y. Naruse, H. Kitakoji and Y. Yamamura (2011). "Repeated application of low-frequency electroacupuncture improves high-fructose diet-induced insulin resistance in rats." Acupunct Med **29**(4): 276-283.

Tsintzas, K., K. Chokkalingam, K. Jewell, L. Norton, I. A. Macdonald and D. Constantin-Teodosiu (2007). "Elevated free fatty acids attenuate the insulin-induced suppression of PDK4 gene expression in human skeletal muscle: potential role of intramuscular long-chain acyl-coenzyme A." J Clin Endocrinol Metab **92**(10): 3967-3972.

Turner, N., G. M. Kowalski, S. J. Leslie, S. Risis, C. Yang, R. S. Lee-Young, J. R. Babb, P. J. Meikle, G. I. Lancaster, D. C. Henstridge, P. J. White, E. W. Kraegen, A. Marette, G. J. Cooney, M. A. Febbraio and C. R. Bruce (2013). "Distinct patterns of tissue-specific lipid accumulation during the induction of insulin resistance in mice by high-fat feeding." Diabetologia **56**(7): 1638-1648.

Turrens, J. F. (2003). "Mitochondrial formation of reactive oxygen species." J Physiol **552**(Pt 2): 335-344.

van Loon, L. J., P. L. Greenhaff, D. Constantin-Teodosiu, W. H. Saris and A. J. Wagenmakers (2001). "The effects of increasing exercise intensity on muscle fuel utilisation in humans." J Physiol **536**(Pt 1): 295-304.

Veiga, F. M. S., F. Graus-Nunes, T. L. Rachid, A. B. Barreto, C. A. Mandarim-de-Lacerda and V. Souza-Mello (2017). "Anti-obesogenic effects of WY14643 (PPAR-alpha agonist): Hepatic mitochondrial enhancement and suppressed lipogenic pathway in diet-induced obese mice." Biochimie **140**: 106-116.

Wang, J., Z. Lu, M. G. Wientjes and J. L. Au (2010). "Delivery of siRNA therapeutics: barriers and carriers." Aaps j **12**(4): 492-503.

Wang, K. C., C. P. Tsai, C. L. Lee, S. Y. Chen, G. J. Lin, M. H. Yen, H. K. Sytwu and S. J. Chen (2013). "alpha-Lipoic acid enhances endogenous peroxisome-proliferator-activated receptor-gamma to ameliorate experimental autoimmune encephalomyelitis in mice." Clin Sci (Lond) **125**(7): 329-340.

Wang, X., Z. Hu, J. Hu, J. Du and W. E. Mitch (2006). "Insulin resistance accelerates muscle protein degradation: Activation of the ubiquitin-proteasome pathway by defects in muscle cell signaling." Endocrinology **147**(9): 4160-4168.

Wang, Y. X., C. H. Lee, S. Tiep, R. T. Yu, J. Ham, H. Kang and R. M. Evans (2003). "Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity." Cell **113**(2): 159-170.

Ward, N. P., A. M. Poff, A. P. Koutnik and D. P. D'Agostino (2017). "Complex I inhibition augments dichloroacetate cytotoxicity through enhancing oxidative stress in VM-M3 glioblastoma cells." PLoS One **12**(6): e0180061.

Wibom, R., L. Hagenfeldt and U. von Döbeln (2002). "Measurement of ATP production and respiratory chain enzyme activities in mitochondria isolated from small muscle biopsy samples." Analytical Biochemistry **311**(2): 139-151.

Wibom, R., L. Hagenfeldt and U. von Dobeln (2002). "Measurement of ATP production and respiratory chain enzyme activities in mitochondria isolated from small muscle biopsy samples." Anal Biochem **311**(2): 139-151.

Wu, M., A. Neilson, A. L. Swift, R. Moran, J. Tamagnine, D. Parslow, S. Armistead, K. Lemire, J. Orrell, J. Teich, S. Chomicz and D. A. Ferrick (2007). "Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells." Am J Physiol Cell Physiol **292**(1): C125-136.

Wu, P., K. Inskeep, M. M. Bowker-Kinley, K. M. Popov and R. A. Harris (1999). "Mechanism responsible for inactivation of skeletal muscle pyruvate dehydrogenase complex in starvation and diabetes." Diabetes **48**(8): 1593-1599.

Wu, P., J. M. Peters and R. A. Harris (2001). "Adaptive increase in pyruvate dehydrogenase kinase 4 during starvation is mediated by peroxisome proliferator-activated receptor alpha." Biochem Biophys Res Commun **287**(2): 391-396.

Xia, X., J. Yan, Y. Shen, K. Tang, J. Yin, Y. Zhang, D. Yang, H. Liang, J. Ye and J. Weng (2011). "Berberine improves glucose metabolism in diabetic rats by inhibition of hepatic gluconeogenesis." PLoS One **6**(2): e16556.

Yamaguchi, S., A. C. Moseley, P. Almeda-Valdes, K. L. Stromsdorfer, M. P. Franczyk, A. L. Okunade, B. W. Patterson, S. Klein and J. Yoshino (2018). "Diurnal Variation in PDK4 Expression Is Associated With Plasma Free Fatty Acid Availability in People." J Clin Endocrinol Metab **103**(3): 1068-1076.

Yang, Y., W. Li, Y. Liu, Y. Sun, Y. Li, Q. Yao, J. Li, Q. Zhang, Y. Gao, L. Gao and J. Zhao (2014). "Alpha-lipoic acid improves high-fat diet-induced hepatic steatosis by modulating the transcription factors SREBP-1, FoxO1 and Nrf2 via the SIRT1/LKB1/AMPK pathway." J Nutr Biochem **25**(11): 1207-1217.

Yang, Z., Y. Wang, Y. Zhang, X. He, C. Q. Zhong, H. Ni, X. Chen, Y. Liang, J. Wu, S. Zhao, D. Zhou and J. Han (2018). "RIP3 targets pyruvate dehydrogenase complex to increase aerobic respiration in TNF-induced necroptosis." Nat Cell Biol **20**(2): 186-197.

Yano, S., S. Morino-Koga, T. Kondo, M. A. Suico, T. Koga, Y. Shimauchi, S. Matsuyama, T. Shuto, T. Sato, E. Araki and H. Kai (2011). "Glucose uptake in rat skeletal muscle L6 cells is increased by low-intensity electrical current through the activation of the phosphatidylinositol-3-OH kinase (PI-3K) / Akt pathway." J Pharmacol Sci **115**(1): 94-98.

Yaworsky, K., R. Somwar, T. Ramlal, H. J. Tritschler and A. Klip (2000). "Engagement of the insulin-sensitive pathway in the stimulation of glucose transport by alpha-lipoic acid in 3T3-L1 adipocytes." Diabetologia **43**(3): 294-303.

Yeaman, S. J., E. T. Hutcheson, T. E. Roche, F. H. Pettit, J. R. Brown, L. J. Reed, D. C. Watson and G. H. Dixon (1978). "Sites of phosphorylation on pyruvate dehydrogenase from bovine kidney and heart." Biochemistry **17**(12): 2364-2370.

Zhang, C. Y., G. Baffy, P. Perret, S. Krauss, O. Peroni, D. Grujic, T. Hagen, A. J. Vidal-Puig, O. Boss, Y. B. Kim, X. X. Zheng, M. B. Wheeler, G. I. Shulman, C. B. Chan and B. B. Lowell (2001). "Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes." Cell **105**(6): 745-755.

Zhang, M., Y. Zhao, Z. Li and C. Wang (2018). "Pyruvate dehydrogenase kinase 4 mediates lipogenesis and contributes to the pathogenesis of nonalcoholic steatohepatitis." Biochem Biophys Res Commun **495**(1): 582-586.

Zhang, Q., X. Kong, H. Yuan, H. Guan, Y. Li and Y. Niu (2019). "Mangiferin Improved Palmitate-Induced-Insulin Resistance by Promoting Free Fatty Acid Metabolism in HepG2 and C2C12 Cells via PPARalpha: Mangiferin Improved Insulin Resistance." J Diabetes Res **2019**: 2052675.

Zhang, Y. and J. L. Avalos (2017). "Traditional and novel tools to probe the mitochondrial metabolism in health and disease." Wiley Interdiscip Rev Syst Biol Med **9**(2).

Zhang, Y., K. Ma, P. Sadana, F. Chowdhury, S. Gaillard, F. Wang, D. P. McDonnell, T. G. Unterman, M. B. Elam and E. A. Park (2006). "Estrogen-related receptors stimulate pyruvate dehydrogenase kinase isoform 4 gene expression." J Biol Chem **281**(52): 39897-39906.

Zhang, Y., G. Xia, Y. Zhang, J. Liu, X. Liu, W. Li, Y. Lv, S. Wei, J. Liu and J. Quan (2017). "Palmitate induces VSMC apoptosis via toll like receptor (TLR)4/ROS/p53 pathway." Atherosclerosis **263**: 74-81.

Zhou, S. and K. B. Wallace (1999). "The effect of peroxisome proliferators on mitochondrial bioenergetics." Toxicol Sci **48**(1): 82-89.

Zou, P., L. Liu, L. Zheng, L. Liu, R. E. Stoneman, A. Cho, A. Emery, E. R. Gilbert and Z. Cheng (2014). "Targeting FoxO1 with AS1842856 suppresses adipogenesis." Cell Cycle **13**(23): 3759-3767.